Non-catalyzable denitrification intermediates induce nitrous oxide reduction in two purple nonsulfur bacteria

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Abstract

Denitrification is a form of anaerobic respiration wherein nitrate is sequentially reduced via nitrite, nitric oxide, and nitrous oxide to dinitrogen gas. The pathway has mostly been characterized in bacteria that have the complete denitrification pathway. However, each individual step can serve as a form of anaerobic respiration and many bacteria only have partial denitrification pathways. The conditions under which these partial denitrification pathways are activated have received comparatively little attention. Here we report the activity of partial denitrification pathways in two purple nonsulfur bacteria, *Rhodopseudomonas palustris* CGA009 and *Rhodobacter capsulatus* SB1003. These bacteria can use one or more denitrification steps as an electron sink during phototrophic growth or to transform energy during anaerobic respiration in the dark. In each case, nitrous oxide reduction required supplementation with a non-catalyzable denitrification intermediate. Thus, bacteria that maintain partial denitrification pathways are still subject to regulation by denitrification intermediates that they cannot use.

Importance. Denitrification is a form of microbial respiration wherein nitrate is converted into dinitrogen gas, a major component of Earth's atmosphere, via several nitrogen oxide intermediates. Unfortunately, denitrification of nitrate in agricultural fertilizers is often incomplete, resulting in the emission of the potent greenhouse gas, nitrous oxide. Some bacteria do not have all the steps for denitrification, but many can still reduce nitrous oxide into harmless dinitrogen gas. These partial denitrifying bacteria have received little attention. Here we characterized two partial denitrifying bacteria that are capable of nitrous oxide reduction. Surprisingly, nitrous oxide reduction was induced by denitrification intermediates that the bacteria could not respire, suggesting that regulation of nitrous oxide reduction was subject to the same cues as in complete denitrifiers. This work thus informs on how partial denitrifying bacteria can contribute to, and potentially combat, greenhouse gas emissions.

Introduction

Denitrification is a multistep respiratory pathway that sequentially reduces nitrate (NO_3^-) via nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) to dinitrogen gas (N_2) (1, 2) (Fig. 1A). While many bacteria have the complete denitrification pathway, the entire pathway is not required; any single denitrification intermediate can potentially support growth via anaerobic respiration (1, 2). Denitrification impacts the health of both humans and the planet (2). Denitrifiers in the human gut help fight pathogens and maintain vascular homeostasis through the generation of NO_2^- and NO (3). Denitrification is also important to the global nitrogen cycle, returning nitrogen to the atmosphere as N_2. Denitrifiers also play important roles in anthropogenic pollution and climate change. Although denitrifiers have the potential to convert NO_3^- in runoff from agricultural fertilizers to harmless N_2 before it can lead to eutrophication of water resources (4), this process often fails to run to completion, even in bacteria capable of complete denitrification. Currently, denitrification is a net generator of the potent greenhouse gas N_2O, driven largely by the wide use of agricultural fertilizers (5).

Denitrifiers can be phototrophs or chemotrophs. Among the bacteria that may be capable of both phototrophic and chemotrophic denitrification are purple nonsulfur bacteria (PNSB). PNSB are perhaps the most versatile organisms ever described. PNSB can grow with either organic or inorganic electron and carbon sources, employing either phototrophy in light or aerobic or anaerobic respiration in the dark. In the dark, anaerobic respiratory pathways serve a critical role in energy transformation (i.e., oxidative phosphorylation). However, during photoheterotrophic growth, wherein light is used for energy and organic substrates are used for carbon and electrons, anaerobic respiratory pathways can help maintain electron balance (6, 7). Electron balance is a challenge to photoheterotrophic growth because more electrons are liberated from many organic substrates than can be incorporated into biomass. Excess electrons must be deposited onto an electron acceptor. CO_2 is commonly used as an electron acceptor under photoheterotrophic conditions, but electron acceptors for anaerobic respiration can also serve this role.

Here we characterized the ability of the PNSB *Rhodopseudomonas palustris* CGA009 and *Rhodobacter capsulatus* SB1003, to carry out denitrification under photoheterotrophic and chemoheterotrophic conditions. In agreement with the genome sequences, we found that CGA009 could use all denitrification intermediates except for NO_3^- and SB1003 could only use N_2O. However, in each case, N_2O utilization required supplementation with other denitrification intermediates, including intermediates that each organism could not reduce. Thus, although each organism only has a partial denitrification pathway, the regulatory schemes resemble those of a complete denitrifier.

Results

***R. palustris* CGA009 has a partial denitrification pathway.** One of the most intensively studied PNSB is *R. palustris* CGA009 (8), yet little is known about its ability to respire anaerobically. Unlike some other model PNSB, CGA009 cannot grow via respiration with dimethylsulfoxide (9-11). However, a partial denitrification pathway was identified in the CGA009 genome (8).

We verified this partial denitrification pathway by aligning query sequences for reductases from *Paracoccus denitrificans* PD122 against the CGA009 genome using BlastP (12). We found no significant similarity to *nar* and *nap* nitrate reductase genes nor to *nirS* nitrite reductase. However, we found genes with significant sequence identity to those responsible for the last three steps of denitrification: NO_2^- reduction, NO reduction, and N_2O reduction. The gene clusters for each of these steps were located at distinct regions in the genome (Fig. 1B). For the first step of NO_2^- reduction, *R. palustris* CGA009 appears to have two, possibly redundant, copper-containing NirK NO_2^- reductases encoded far apart on the genome. The latter two steps of NO reduction and N_2O reduction are each encoded by single gene clusters (Fig. 1B).

Several possible regulatory genes were also identified. A gene that likely encodes NnrR, a protein that typically regulates NO_2^-, NO, and N_2O reductases in other bacteria in response to NO (1, 13, 14), was found near the *nirK2* gene (Fig. 1B). Genes with significant sequence identity to NasTS, which can regulate N_2O reductase in response to NO_3^- (15, 16), were found far from the other gene clusters, upstream of a NirA-family reductase (RPA3710), which could be involved in either nitrite or sulfite reduction.

PNSB typically use electron acceptors anaerobically to establish a proton motive force and generate ATP when incubated in dark. When incubated in light, cells generate ATP by photophosphorylation and use electron acceptors to enable growth on relatively reduced organic compounds . Since *R. palustris* grows best in light, we first examined if it could use denitrification electron acceptors when provided with butyrate, an electron-rich carbon source that requires an electron sink for photoheterotrophic growth (7, 17).

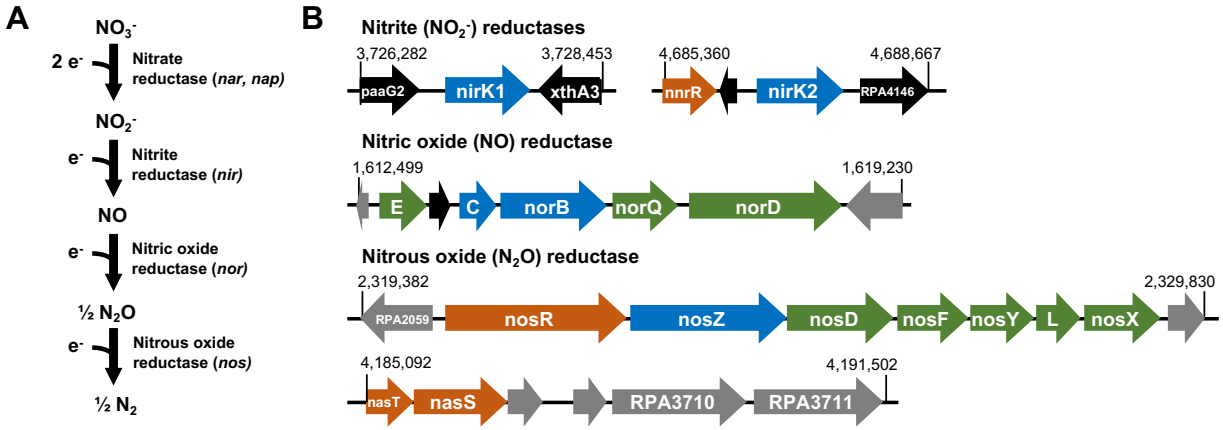

Fig. 1. General denitrification pathway (A) and the denitrification genes found in the *R. palustris* CGA009 genome (B). Gene products: blue, nitrogen oxide reductases; green, accessory proteins for electron transport, enzyme cofactor assembly, etc.; orange, denitrification regulators; gray, unknown function; black, likely unrelated to denitrification. Numbers indicate the chromosome nucleotide positions. Several CRP/Fnr-family transcriptional regulators with >25% sequence identity to known denitrification regulators are not shown.

In agreement with the genome annotation (Fig. 1B), phototrophic growth on butyrate was not supported when supplemented with a wide range of $NaNO_3$ concentrations (Fig. 2A). However, growth on butyrate was observed when CGA009 was provided with 1mM $NaNO_2$, a

concentration that we determined to be near the toxicity limit because it caused a lag in phototrophic growth on succinate, which does not require supplementation with an electron acceptor (Fig. 2B). We did not test exogenously-added NO because it is highly toxic and would likely be impossible to add in amounts that would be practical as an electron sink to yield observable growth.

Based on the amount of phototrophic growth observed on butyrate with $NaNO_2$, we estimated whether NO_2^- was reduced completely to N_2. From our previous work, we estimated that ~6 mM bicarbonate (HCO_3^-) is consumed for an increase in cell density of ~1 OD_{660} during phototrophic growth on butyrate with $NaHCO_3$ (17). Whereas HCO_3^- can serve as a sink for two electrons, each molecule of NO_2^- could serve as a sink for three electrons if NO_2^- is reduced sequentially to N_2 but only one electron if NO_2^- is only reduced to NO (Fig. 1A). Based on this stoichiometry, we predicted a cell density of ~ 0.25 OD_{660} during phototrophic growth with butyrate plus 1 mM $NaNO_2$ assuming that all the $NaNO_2$ is reduced to N_2. Close to our prediction, we observed a final OD_{660} of 0.28 ± 0.00 (n=3) (Fig. 2A). Thus, we deduce that the latter three reductases for denitrification (Fig. 1A) are all active in CGA009. However, when CGA009 was provided with a 100% headspace of N_2O, no growth was observed within 15 days (Fig. 2A), suggesting that N_2O alone cannot activate N_2O reductase.

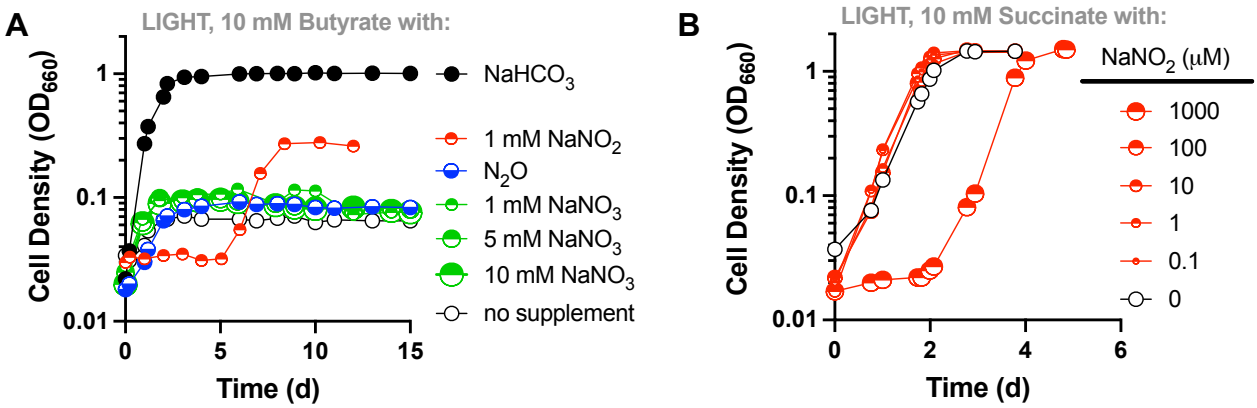

Fig. 2. $NaNO_2$ supports phototrophic growth of CGA009 on butyrate within toxicity limits. A. Phototrophic growth curves with butyrate and various potential electron acceptors. **B.** Phototrophic growth curves on succinate, a condition that readily supports growth without supplementation with an electron acceptor, with various concentrations of $NaNO_2$ to identify the toxicity limit. **A, B.** Plots are single representatives of similar trends observed for three biological replicates except for those exploring different $NaNO_2$ and $NaNO_3$ concentrations. Cultures had a 100% Ar headspace unless N_2O is indicated (100% N_2O).

N_2O reduction by CGA009 is induced by NO_2^- and NO_3^- during photoheterotrophic growth. In some bacteria, denitrification intermediates other than N_2O enhance N_2O reduction or are required to induce N_2O reduction (1, 2, 14, 18, 19). NO_2^--induction of N_2O reductase can be mediated by NnrR, though NO_2^- might first need to be converted to NO (14) (Fig. 1B). NO_3^--induction of N_2O reductase can be mediated by NasTS (15, 16) (Fig. 1B). We thus tested whether $NaNO_2$ or $NaNO_3$ could induce growth with N_2O. Micromolar amounts of $NaNO_2$

stimulated growth with N_2O. $NaNO_2$ at 100 µM caused a 4-day lag in growth, probably because it was toxic at this concentration (Fig. 3A). Final cell densities increased with the amount of $NaNO_2$ added (Fig. 3A). We conclude that the higher final cell density when N_2O was provided along with 100 µM $NaNO_2$ is due to the use of N_2O as an electron acceptor rather than NO_2^- because growth trends with 100 µM $NaNO_2$ alone were much lower (Fig. 3A vs Fig. 2A). Thus, we speculate that exhaustion of NO_2^- as an electron acceptor eliminates induction of N_2O reductase, and thus growth on N_2O lasts only as long as the pool of $NaNO_2$.

Despite lacking NO_3^- reductase, $NaNO_3$ also induced phototrophic growth on butyrate with N_2O (Fig. 3B). Similar growth trends were observed between 1 µM to 10 mM $NaNO_3$. Slower growth and a lower final cell density was observed at $NaNO_3$ concentrations of 0.1 µM or lower (Fig. 3B). The fact that 1 µM $NaNO_3$ resulted in similar growth trends as 10 mM $NaNO_3$ suggests that NO_3^- is not being reduced and that $NaNO_3$ is relatively non-toxic. Thus, $NaNO_3$ is potentially useful as a stable inducer of N_2O reductase compared to $NaNO_2$.

Initial phototrophic growth on butyrate with $NaNO_3$ was faster than that with bicarbonate (doubling time ± SD = 13 ± 3 vs 17 ± 2 h, respectively). However, growth with $NaNO_3$ transitioned to slow growth at a cell density of ~0.2 – 0.3 OD_{660} and reached a lower final cell density than with bicarbonate (ΔOD_{660} ± SD = 0.72 ± 0.05 vs 0.99 ± 0.02, respectively). Flushing the headspace with N_2O after the transition did not restore the initial growth rate (data not shown).

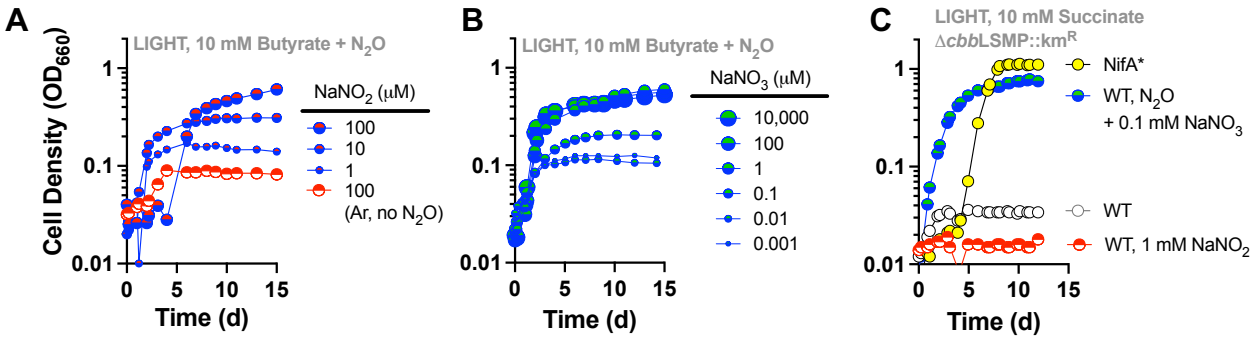

Fig. 3. $NaNO_2$ and $NaNO_3$ induce phototrophic N_2O utilization by CGA009. A. Phototrophic growth curves with butyrate +/- N_2O and different concentrations of $NaNO_2$. The Ar, no N_2O control had a 100% argon headspace in place of N_2O.**B.** Phototrophic growth curves with butyrate + N_2O and different concentrations of $NaNO_3$. **C.** Phototrophic growth curves of Calvin cycle deletion mutants with succinate. Cultures had a 100% Ar headspace unless N_2O is indicated (100% N_2O). **A-C.** Single representatives of similar trends observed for three biological replicates are shown for each condition except for those exploring different

We also tested whether N_2O reduction could serve as an electron sink to rescue *R. palustris* growth when the CO_2-fixing Calvin cycle is genetically disrupted. *R. palustris* Calvin cycle mutants cannot grow phototrophically even on relatively oxidized carbon sources like succinate unless an alternative electron sink is provided (6). For example, NifA* mutations rescue Calvin cycle mutants by disposing of excess electrons as H_2 gas via constitutively active nitrogenase (6, 17, 20). Here we found that $NaNO_3$ plus N_2O could also rescue an *R. palustris* Calvin cycle

mutant during phototrophic growth on succinate (Fig. 3C). N_2O reduction resulted in more immediate growth than a NifA* positive control. However, growth eventually slowed and the culture reached a lower final cell density that the NifA* control, similar to what was observed during growth on butyrate compared to a bicarbonate-containing control (Fig. 3C vs 2A). Gas chromatographic analysis of headspace samples confirmed that growth of all cultures with $NaNO_3$ plus N_2O was not due to spontaneous mutations enabling H_2 production (data not shown).

We wondered whether $NaNO_3$ might also improve growth with NO_2^-, perhaps by stimulating NO_2^- reductase activity. However, adding $NaNO_3$ did not affect photoheterotrophic growth trends on butyrate with 1 mM $NaNO_2$, even when $NaNO_3$ was also added to starter cultures as a possible 'pre-inducing' condition (Fig. 4A). The same strategy also did not decrease the lag phase during phototrophic growth on succinate with 1 mM $NaNO_2$.

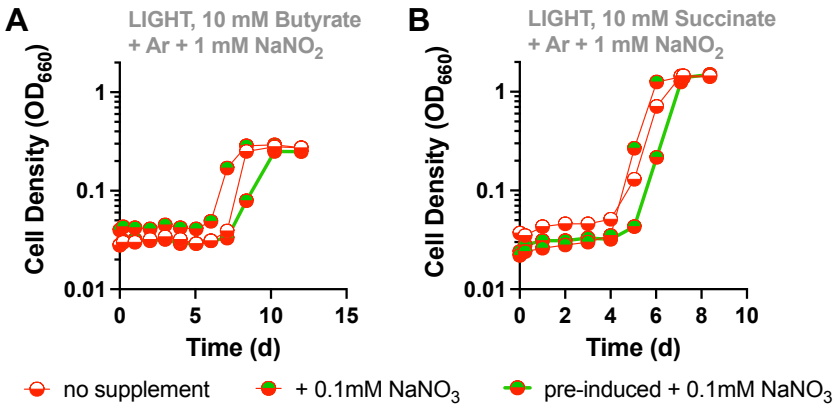

Fig. 4. $NaNO_3$ does not improve growth trends when NO_2^- is present as an essential electron sink (A) or as a toxic compound (B). Single representatives of similar trends observed for three biological replicates are shown for each condition.

N_2O supports anaerobic respiration by CGA009 in the dark when induced with $NaNO_3$. Without access to light, many PNSB can transform energy via anaerobic respiration. We tested whether $NaNO_2$ or N_2O could support anaerobic respiration by CGA009 in the dark. Acetate and butyrate were chosen as two carbon sources that are metabolized via similar pathways but present the cell with different amounts of electrons (17). Unlike phototrophic conditions, supplementation with either 0.3 or 1 mM $NaNO_2$ did not lead to observable growth in the dark with either acetate or butyrate within at least 15 days. However, N_2O plus $NaNO_3$ supported growth with either acetate or butyrate (Fig. 5). Growth was slower with acetate than with butyrate (doubling time ± SD = 88 ± 2 h vs 51 ± 3, respectively).

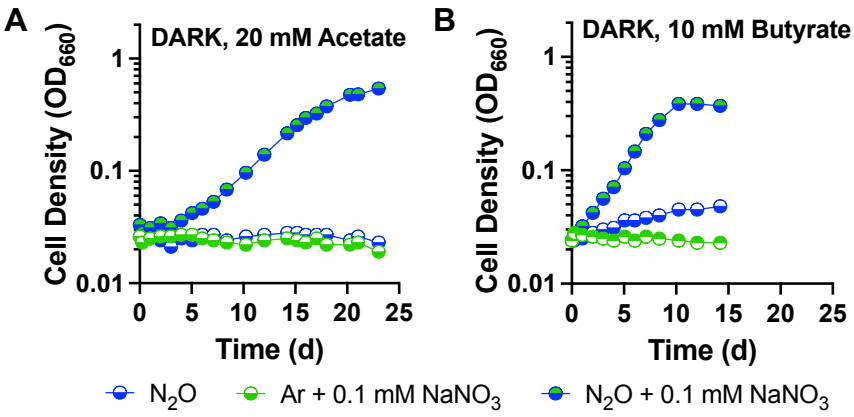

Fig. 5. Anaerobic respiration by CGA009 with N_2O in the dark with acetate (A) or butyrate (B). Single representatives of similar trends observed for three biological replicates are shown for each condition. Cultures had a 100% Ar headspace or 100% N_2O headspace as indicated.

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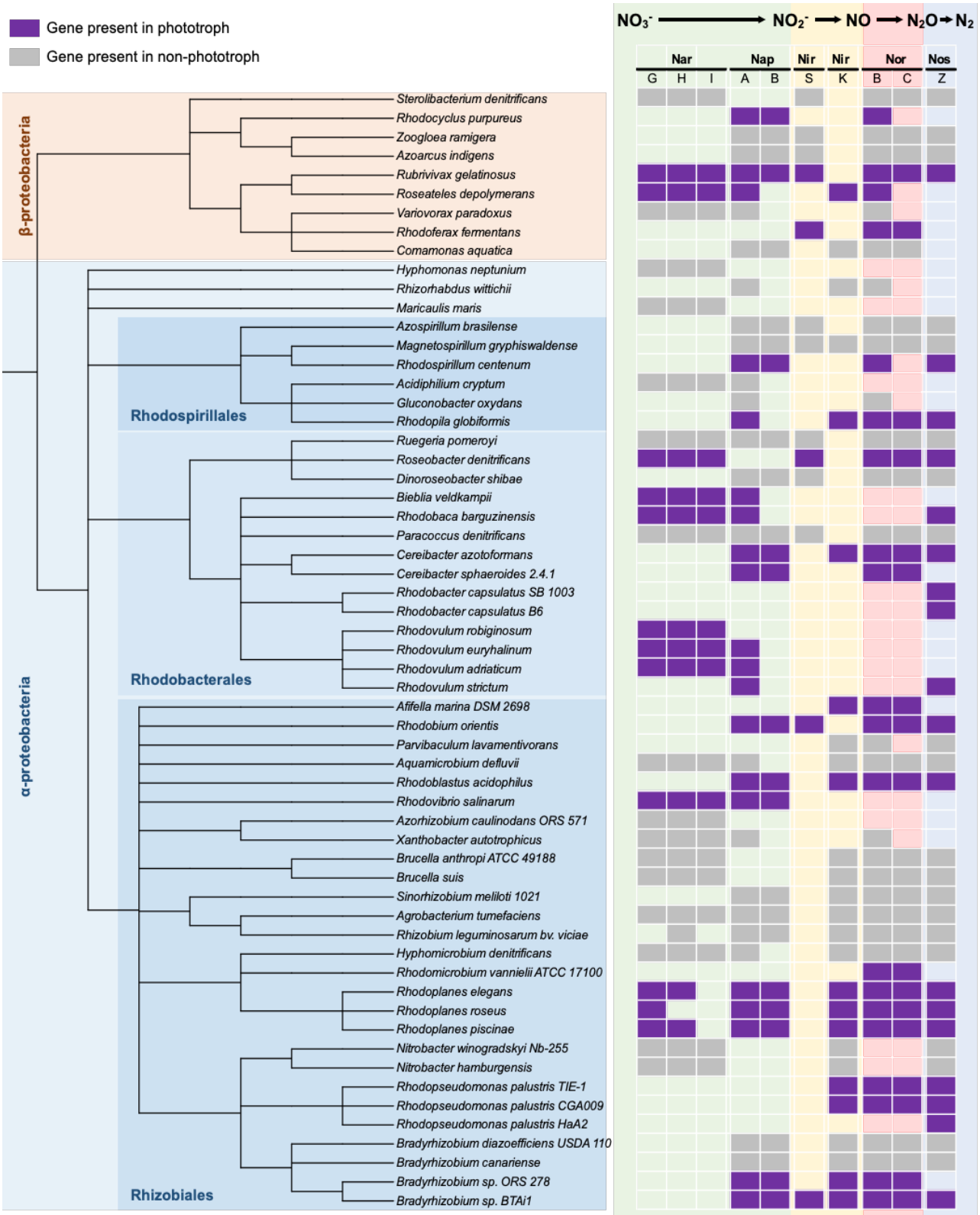

Fig. 6. Denitrification reductase genes in PNSB and related non-phototrophs. A gene was considered present if a predicted gene product exhibited >25% amino acid identity over 50% of its length relative to the query sequence. All subunits for a given enzyme are needed for the activity of a given reductase. The phylogenetic tree was built using PhyloT v2 (phylot.biobyte.de) based on NCBI taxonomy.

***R. capsulatus* SB 1003 requires NO_2^- to induce growth on N_2O.** We wondered if induction of N_2O reductase by non-catalyzable denitrification intermediates occurs in other bacteria with partial denitrification pathways. We thus surveyed the genomes of various PNSB and non-phototrophic relatives using BlastP analysis with the same query denitrification reductase genes as for *R. palustris* in Figure 1 (Fig. 6). *R. capsulatus* SB 1003 stood out as an easily cultivatable and phylogenetically distant PNSB that has N_2O reductase but no other denitrification genes (Fig. 6 and 7A). In agreement with the genome annotation, SB 1003 phototrophic growth on butyrate was not supported by $NaNO_3$ (Fig. 7B). $NaNO_2$ also did not support growth, though SB 1003 was sensitive to $NaNO_2$ concentrations > 0.5 mM (Fig. 7C), which would likely be needed to see a clear OD increase. Similar to CGA009, N_2O alone did not lead to phototrophic growth on butyrate. However, supplementation with 100 µM $NaNO_2$, but not $NaNO_3$, stimulated phototrophic growth on butyrate with N_2O (Fig. 7B). The same was true of growth in the dark on succinate (Fig. 7D), a condition chosen to test whether the same regulatory trend held true during anaerobic N_2O respiration in the dark.

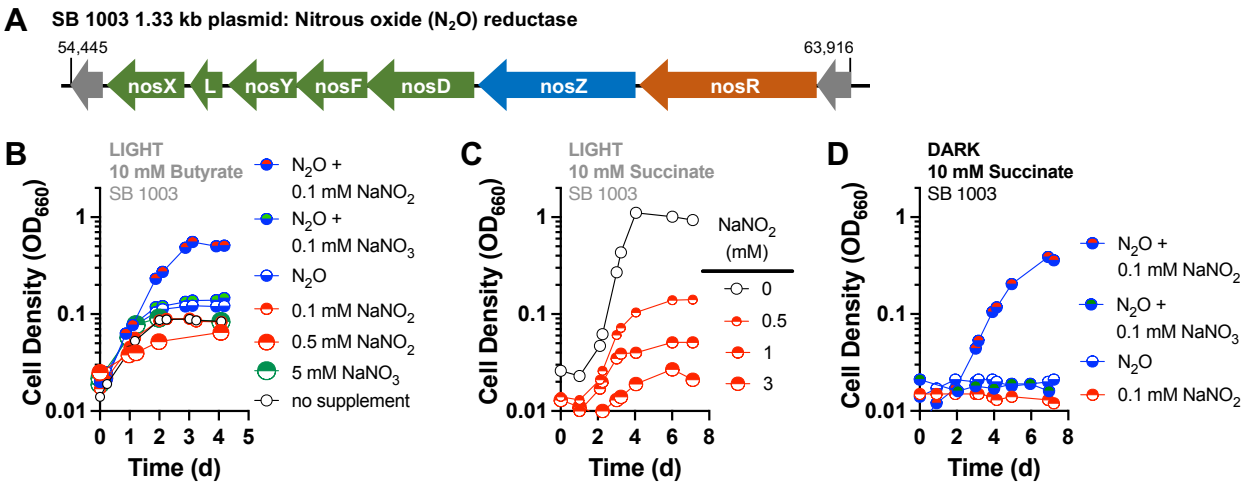

Fig. 7. Activation of the single *R. capsulatus* SB 1003 denitrification step, N_2O reduction, requires NO_2^-. A. SB 1003 N_2O reductase genes (*nos*) are on a plasmid. A possible regulator, NnrR, is encoded on the chromosome. Gene products: blue, N_2O reductase; green, accessory proteins for electron transport, enzyme cofactor assembly, etc.; orange, denitrification regulators; gray, unknown function. Numbers indicate nucleotide positions. **B.** Phototrophic growth with butyrate and various denitrification intermediates. **C.** Phototrophic growth with succinate and various $NaNO_2$ concentrations to determine the toxicity limit. **D.** Chemotrophic growth with succinate with N_2O as an electron acceptor for anaerobic respiration. **B-D.** Single representatives of similar trends observed for three biological replicates are shown for each condition except for the test of $NaNO_2$ toxicity in panel C. Cultures had a 100% Ar headspace unless N_2O is indicated (100% N_2O).

Discussion

We determined that non-catalyzable denitrification intermediates were required to induce the final denitrification step of N_2O reduction in two phylogenetically distant PNSB. This regulatory response to non-catalyzable intermediates could be the result of gene loss from an ancestor that had a complete denitrification pathway; that is, reductase genes have been lost but the regulatory network remains. For CGA009, gene loss seems more plausible than an alternative scenario of gene gain through horizontal gene transfer given the number of genes present for both catalysis and regulation (Fig. 1B). The spectrum of denitrification activities and gene content also varies for different *R. palustris* strains (21-23) (Fig. 6). However, a combination of gene loss and gene gain, or even gene duplication could have shaped the CGA009 denitrification inventory. Gene duplication could explain the existence of two nitrite reductase genes (Fig. 1B), that share 82% nucleotide sequence identity.

SB 1003 could be an example of gene gain given that the N_2O reductase gene cluster is encoded on a plasmid. How this cluster is regulated in response to NO_2^- remains unclear. Others have reported that unsequenced *R. capsulatus* strains reduce NO_3^- (7), and one isolate carried out complete denitrification (24). If these strains are truly close relatives of SB 1003, then perhaps a common ancestor was capable of denitrification and some regulatory genes were maintained in SB 1003. Alternatively, an unrelated regulatory feature might have evolved to control N_2O reductase activity (25).

The requirement of NO_2^-, but not NO_3^-, for SB 1003 growth with N_2O is unexpected. To the best of our knowledge, there are no clear examples of NO_2^- regulation of N_2O reductase. SB 1003 has several CRP/Fnr-family transcriptional regulator genes encoded in its chromosome with >25% amino acid identity to denitrification regulators like *P. denitrificans* FnrP (RCAP_rcc02493; 74% identity) and *Pseudomonas aeruginosa* Dnr/NnrR (RCAP_rcc00107; 36% identity) (Fig. 7A). However, these regulators respond to NO. It is possible that some of the NO_2^- we added was converted to NO by an unknown, and perhaps non-specific, enzyme activity and then NO activated N_2O reductase via the NnrR regulator (26).

One can also question why bacteria would lack a complete denitrification pathway. Denitrification genes could be lost if their activity is neutral or detrimental to fitness. NO_3^- reduction by Nar could be detrimental as the only denitrification enzyme with a cytoplasmic active site; the rest of the enzymes operate in the periplasm (1, 2, 27). As such, generation of toxic NO_2^- in the cytoplasm by Nar could lead to a selective disadvantage. Operating Nar during phototrophic generation of a proton motive force could also be more susceptible to backpressure (22) than the periplasmic denitrification steps. Nar eliminates protons in the cytoplasm, thus contributing to the proton gradient, whereas the other denitrification steps eliminate protons in the periplasm, detracting from the gradient (27). Thus, light-driven proton translocation could impose a thermodynamic impediment on Nar and thereby slow growth if, for example, the organism is using denitrification to dispose of electrons. However, these explanations do not explain why a phototroph would not have Nap, which reduces NO_3^- in the periplasm. Our survey revealed several examples of Nar or Nap in PNSB (Fig. 6).

Loss of denitrification genes could also be neutral or advantageous when within a community of denitrifiers. Such scenarios are predicted by the Black Queen Hypothesis, wherein a gene function that benefits other community members leads to loss of that function in a subset of beneficiary community members (28). For example, if denitrification intermediates are reliably generated by a neighbor and not all steps are needed for energy transformation and/or electron balance, then it could be beneficial to lose other steps rather than incur the cost of synthesizing the associated enzymes. Dispensing of NO_2^- and NO reductases could also be beneficial if NO_2^- and NO are reliably removed by other community members. Membership within a denitrifying community could also explain why non-catalyzable intermediates stimulate N_2O reduction, as there would be little pressure to evolve responses to N_2O alone if N_2O is always found with other intermediates of denitrification.

Partial denitrification pathways are both variable and widespread in PNSB (Fig. 6). This trend could very well be true outside of PNSB and their close relatives. Despite decades of investigation into the activity and regulation of denitrification, there has been comparatively little research on partial denitrification pathways (2). Further investigation into the regulation of partial denitrification pathways is needed, as is their potential impact on N_2O greenhouse gas emissions from agricultural fertilizers.

Methods.

Strains. *R. palustris* CGA009 is a chloramphenicol-resistant type strain derived from CGA001 (8). The Calvin cycle mutant Δ*cbbLSMP*::km^R (CGA4008) was constructed by deleting *cbbLS*, encoding ribulose-1,5-bisphosphate carboxylase (Rubisco) form I, in a previously described mutant lacking Rubisco form II (Δ*cbbM*; CGA668; (20)) via introduction of the suicide vector pJQΔ*cbbLS* (29) by conjugation with *E. coli* S17 as described (29, 30). The gene encoding phosphoribulokinase, *cbbP*, was then deleted in the resulting strain (Δ*cbbLSM*; CGA4006) by introducing the suicide vector pJQΔ*cbbP*::km^r (29), as above, to generate the Δ*cbbLSMP*::km^R strain, CGA4008. All strain genotypes were verified by PCR and Sanger sequencing. The NifA* derivative of CGA4008 with constitutive nitrogenase activity/H_2 production (CGA4011) was described previously (29). The elimination of three genes unique to the Calvin cycle greatly decreases the odds of enriching for suppressor mutations. *R. capsulatus* SB1003 was provided courtesy of Carl Bauer (Indiana University).

Growth conditions. Strains were routinely cultivated in 10 ml photosynthetic medium (PM) (31) in 27-ml anaerobic test tubes. PM was made anaerobic by bubbling tubes with 100% Ar then sealing with rubber stoppers and aluminum crimps prior to autoclaving. After autoclaving, tubes were supplemented with either 20 mM sodium acetate, 10 mM sodium butyrate, or 10 mM sodium succinate from 100X anaerobic stock solutions. SB1003 cultures were also supplemented with 0.1 µg/ml nicotinic acid, 0.2 µg/ml riboflavin, and 1.3 µg/ml thiamine-HCl. $NaNO_2$ or $NaNO_3$ were added as indicated in the text. For conditions with N_2O, tubes were flushed with 100% N_2O through a 0.45 µm syringe filter and needle after all liquid supplements were added. A second needle was used for off-gassing. Cultures were inoculated with a 1% inoculum from starter cultures grown phototrophically in anaerobic PM with succinate, except for the experiment testing Calvin cycle mutants (Fig. 3C) in which all starter cultures were grown aerobically in 3 ml PM with succinate in the dark. Aerobic conditions were chosen for these

starter cultures to accommodate the Δ*cbbLSMP*::km^R mutant (CGA4008) that requires an electron sink to grow.

Analytical procedures. Culture growth was monitored via optical density at 660 nm (OD_{660}) using a Genesys 20 spectrophotometer (Thermo-Fisher, Waltham, MA, USA) directly in culture tubes without sampling. Specific growth rates were calculated using OD_{660} values between 0.1 and 1.0 where cell density and OD are linearly correlated. H_2 was sampled from culture headspace using a gas-tight syringe and analysed using a Shimadzu GC-2014 gas chromatograph as described (32).

Bioinformatics. Denitrification reductase gene inventories were determined using NCBI's protein BLAST (12) to search for query sequences from *P. denitrificans* PD122 (Accession numbers) NarG (GEK69895), NarH (CAA81215), NarI (CAA81217), NapA (CAA85346), NapB (GEK66554), NirS (GEK68816), NirK (GEC49504), NorB (BAA32546), NorC (BAA32545), and NosZ (GEK69878). Denitrification regulatory genes inventories were similarly determined using query sequences from *P. denitrificans* PD122 FnrP (WP_041529894 (33)) and Nnr (WP_041529894 (33)), *B. japonicum* USDA110 NasT (BAC49838) and NasS (BAC49837), and *P. aeruginosa* PAO1 Dnr/NnrR (BAA08744). The phylogenetic tree was built using PhyloT v2 (phylot.biobyte.de) based on NCBI taxonomy and visualized using iTOL v6 (itol.embl.de).

Acknowledgements

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