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

Breah LaSarre[1,a], Ryan Morlen[2], Gina C. Neumann[1],
Caroline S. Harwood[2], and James B. McKinlay[1]*

[1] Department of Biology, Indiana University, Bloomington, IN
[2] Department of Microbiology, University of Washington, Seattle, WA

*Corresponding author: jmckinla@iu.edu

Current address:
[a] Department of Plant Pathology and Microbiology, Iowa State University, Ames, Iowa, USA
[b] Benson Hill, St. Louis MO, USA

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).

91 We verified this partial denitrification pathway by aligning query sequences for reductases from
92 *Paracoccus denitrificans* PD122 against the CGA009 genome using BlastP (12). We found no
93 significant similarity to *nar* and *nap* nitrate reductase genes nor to *nirS* nitrite reductase.
94 However, we found genes with significant sequence identity to those responsible for the last
95 three steps of denitrification: NO_2^- reduction, NO reduction, and N_2O reduction. The gene
96 clusters for each of these steps were located at distinct regions in the genome (Fig. 1B). For the
97 first step of NO_2^- reduction, *R. palustris* CGA009 appears to have two, possibly redundant,
98 copper-containing NirK NO_2^- reductases encoded far apart on the genome. The latter two steps
99 of NO reduction and N_2O reduction are each encoded by single gene clusters (Fig. 1B).
100
101 Several possible regulatory genes were also identified. A gene that likely encodes NnrR, a
102 protein that typically regulates NO_2^-, NO, and N_2O reductases in other bacteria in response to
103 NO (1, 13, 14), was found near the *nirK2* gene (Fig. 1B). Genes with significant sequence
104 identity to NasTS, which can regulate N_2O reductase in response to NO_3^- (15, 16), were found
105 far from the other gene clusters, upstream of a NirA-family reductase (RPA3710), which could
106 be involved in either nitrite or sulfite reduction.
107
108 PNSB typically use electron acceptors anaerobically to establish a proton motive force and
109 generate ATP when incubated in dark. When incubated in light, cells generate ATP by
110 photophosphorylation and use electron acceptors to enable growth on relatively reduced organic
111 compounds . Since *R. palustris* grows best in light, we first examined if it could use
112 denitrification electron acceptors when provided with butyrate, an electron-rich carbon source
113 that requires an electron sink for photoheterotrophic growth (7, 17).
114

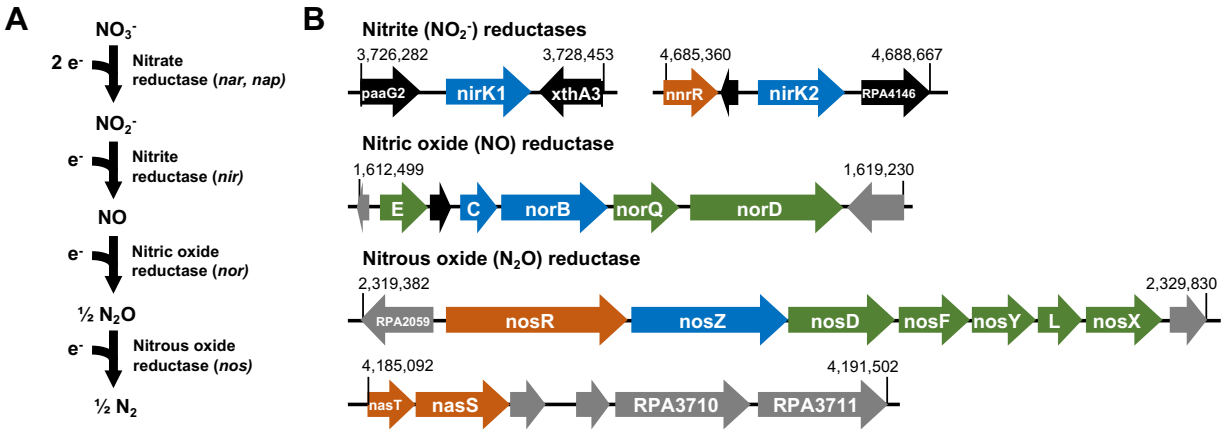

Fig. 1. General denitrification pathway (A) and the denitrification genes found in the *R. palustris* CGA009 genome (B). Gene products: blue, nitrogen oxide reductases; green, accessory proteins for electron transport, enzyme cofactor assembly, etc.; orange, denitrification regulators; gray, unknown 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.

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

118 concentration that we determined to be near the toxicity limit because it caused a lag in
119 phototrophic growth on succinate, which does not require supplementation with an electron
120 acceptor (Fig. 2B). We did not test exogenously-added NO because it is highly toxic and would
121 likely be impossible to add in amounts that would be practical as an electron sink to yield
122 observable growth.
123
124 Based on the amount of phototrophic growth observed on butyrate with $NaNO_2$, we estimated
125 whether NO_2^- was reduced completely to N_2. From our previous work, we estimated that ~6 mM
126 bicarbonate (HCO_3^-) is consumed for an increase in cell density of ~1 OD_{660} during phototrophic
127 growth on butyrate with $NaHCO_3$ (17). Whereas HCO_3^- can serve as a sink for two electrons,
128 each molecule of NO_2^- could serve as a sink for three electrons if NO_2^- is reduced sequentially to
129 N_2 but only one electron if NO_2^- is only reduced to NO (Fig. 1A). Based on this stoichiometry,
130 we predicted a cell density of ~ 0.25 OD_{660} during phototrophic growth with butyrate plus 1 mM
131 $NaNO_2$ assuming that all the $NaNO_2$ is reduced to N_2. Close to our prediction, we observed a
132 final OD_{660} of 0.28 ± 0.00 (n=3) (Fig. 2A). Thus, we deduce that the latter three reductases for
133 denitrification (Fig. 1A) are all active in CGA009. However, when CGA009 was provided with a
134 100% headspace of N_2O, no growth was observed within 15 days (Fig. 2A), suggesting that N_2O
135 alone cannot activate N_2O reductase.

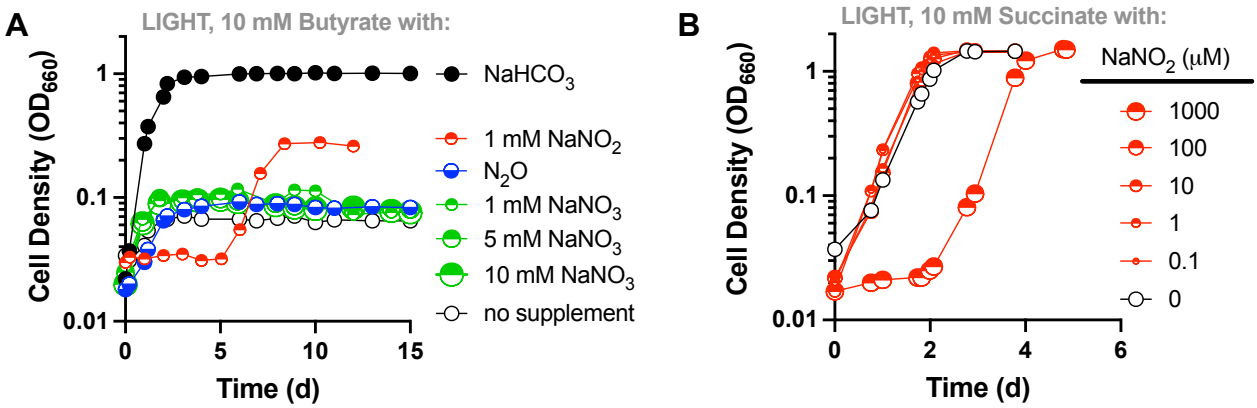

Fig. 2. $NaNO_2$ supports phototrophic growth of CGA009 on butyrate within toxicity limits. A. Phototrophic growth curves with butyrate and various potential electron acceptors. **B.** Phototrophic growth curves on succinate, a condition that readily supports growth without supplementation with an electron 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).

136
137 **N_2O reduction by CGA009 is induced by NO_2^- and NO_3^- during photoheterotrophic growth.**
138 In some bacteria, denitrification intermediates other than N_2O enhance N_2O reduction or are
139 required to induce N_2O reduction (1, 2, 14, 18, 19). NO_2^--induction of N_2O reductase can be
140 mediated by NnrR, though NO_2^- might first need to be converted to NO (14) (Fig. 1B). NO_3^--
141 induction of N_2O reductase can be mediated by NasTS (15, 16) (Fig. 1B). We thus tested
142 whether $NaNO_2$ or $NaNO_3$ could induce growth with N_2O. Micromolar amounts of $NaNO_2$

143 stimulated growth with N_2O. $NaNO_2$ at 100 µM caused a 4-day lag in growth, probably because
144 it was toxic at this concentration (Fig. 3A). Final cell densities increased with the amount of
145 $NaNO_2$ added (Fig. 3A). We conclude that the higher final cell density when N_2O was provided
146 along with 100 µM $NaNO_2$ is due to the use of N_2O as an electron acceptor rather than NO_2^-
147 because growth trends with 100 µM $NaNO_2$ alone were much lower (Fig. 3A vs Fig. 2A). Thus,
148 we speculate that exhaustion of NO_2^- as an electron acceptor eliminates induction of N_2O
149 reductase, and thus growth on N_2O lasts only as long as the pool of $NaNO_2$.
150
151 Despite lacking NO_3^- reductase, $NaNO_3$ also induced phototrophic growth on butyrate with N_2O
152 (Fig. 3B). Similar growth trends were observed between 1 µM to 10 mM $NaNO_3$. Slower growth
153 and a lower final cell density was observed at $NaNO_3$ concentrations of 0.1 µM or lower (Fig.
154 3B). The fact that 1 µM $NaNO_3$ resulted in similar growth trends as 10 mM $NaNO_3$ suggests that
155 NO_3^- is not being reduced and that $NaNO_3$ is relatively non-toxic. Thus, $NaNO_3$ is potentially
156 useful as a stable inducer of N_2O reductase compared to $NaNO_2$.
157
158 Initial phototrophic growth on butyrate with $NaNO_3$ was faster than that with bicarbonate
159 (doubling time ± SD = 13 ± 3 vs 17 ± 2 h, respectively). However, growth with $NaNO_3$
160 transitioned to slow growth at a cell density of ~0.2 – 0.3 OD_{660} and reached a lower final cell
161 density than with bicarbonate (ΔOD_{660} ± SD = 0.72 ± 0.05 vs 0.99 ± 0.02, respectively). Flushing
162 the headspace with N_2O after the transition did not restore the initial growth rate (data not
163 shown).
164

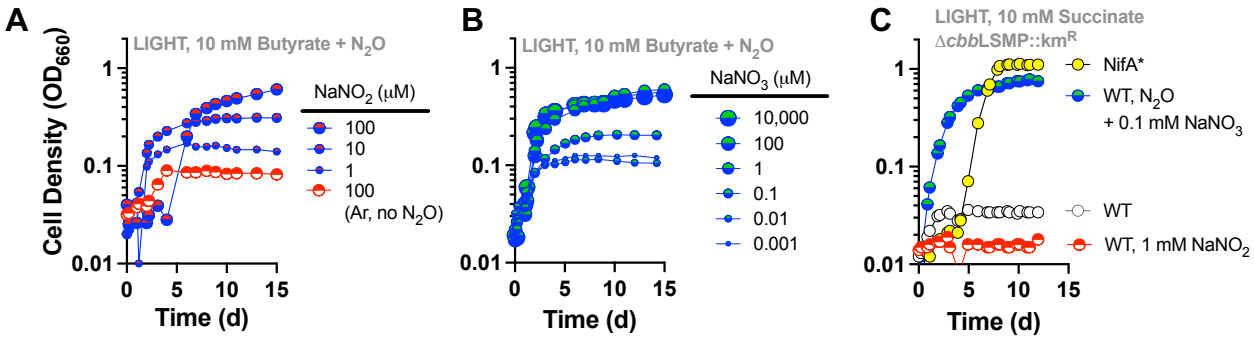

Fig. 3. $NaNO_2$ and $NaNO_3$ induce phototrophic N_2O utilization by CGA009. A. Phototrophic growth curves with butyrate +/- N_2O and different concentrations of $NaNO_2$. The Ar, no N_2O control had a 100% argon headspace in place of N_2O.**B.** Phototrophic growth curves with butyrate + N_2O and different 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

165
166 We also tested whether N_2O reduction could serve as an electron sink to rescue *R. palustris*
167 growth when the CO_2-fixing Calvin cycle is genetically disrupted. *R. palustris* Calvin cycle
168 mutants cannot grow phototrophically even on relatively oxidized carbon sources like succinate
169 unless an alternative electron sink is provided (6). For example, NifA* mutations rescue Calvin
170 cycle mutants by disposing of excess electrons as H_2 gas via constitutively active nitrogenase (6,
171 17, 20). Here we found that $NaNO_3$ plus N_2O could also rescue an *R. palustris* Calvin cycle

172 mutant during phototrophic growth on succinate (Fig. 3C). N_2O reduction resulted in more
173 immediate growth than a NifA* positive control. However, growth eventually slowed and the
174 culture reached a lower final cell density that the NifA* control, similar to what was observed
175 during growth on butyrate compared to a bicarbonate-containing control (Fig. 3C vs 2A). Gas
176 chromatographic analysis of headspace samples confirmed that growth of all cultures with
177 $NaNO_3$ plus N_2O was not due to spontaneous mutations enabling H_2 production (data not
178 shown).
179
180 We wondered whether $NaNO_3$ might also improve growth with NO_2^-, perhaps by stimulating
181 NO_2^- reductase activity. However, adding $NaNO_3$ did not affect photoheterotrophic growth
182 trends on butyrate with 1 mM $NaNO_2$, even when $NaNO_3$ was also added to starter cultures as a
183 possible 'pre-inducing' condition (Fig. 4A). The same strategy also did not decrease the lag
184 phase during phototrophic growth on succinate with 1 mM $NaNO_2$.

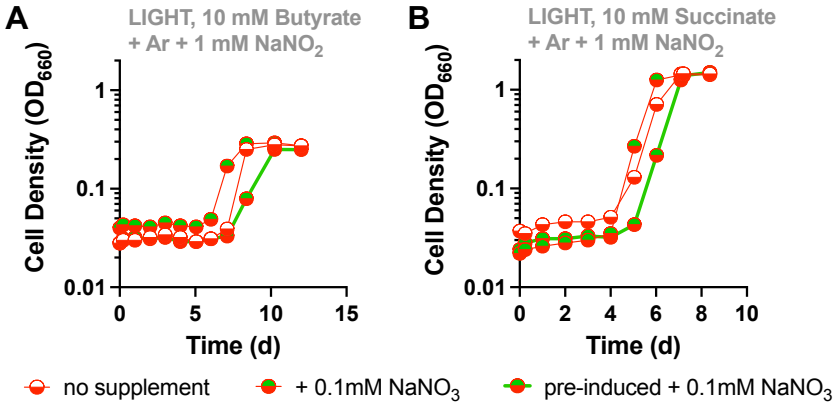

Fig. 4. $NaNO_3$ does not improve growth trends when NO_2^- is present as an essential electron sink (A) or as a toxic compound (B). Single representatives of similar trends observed for three biological replicates are shown for each condition.

185
186 **N_2O supports anaerobic respiration by CGA009 in the dark when induced with $NaNO_3$.**
187 Without access to light, many PNSB can transform energy via anaerobic respiration. We tested
188 whether $NaNO_2$ or N_2O could support anaerobic respiration by CGA009 in the dark. Acetate and
189 butyrate were chosen as two carbon sources that are metabolized via similar pathways but
190 present the cell with different amounts of electrons (17). Unlike phototrophic conditions,
191 supplementation with either 0.3 or 1 mM $NaNO_2$ did not lead to observable growth in the dark
192 with either acetate or butyrate within at least 15 days. However, N_2O plus $NaNO_3$ supported
193 growth with either acetate or butyrate (Fig. 5). Growth was slower with acetate than with
194 butyrate (doubling time ± SD = 88 ± 2 h vs 51 ± 3, respectively).

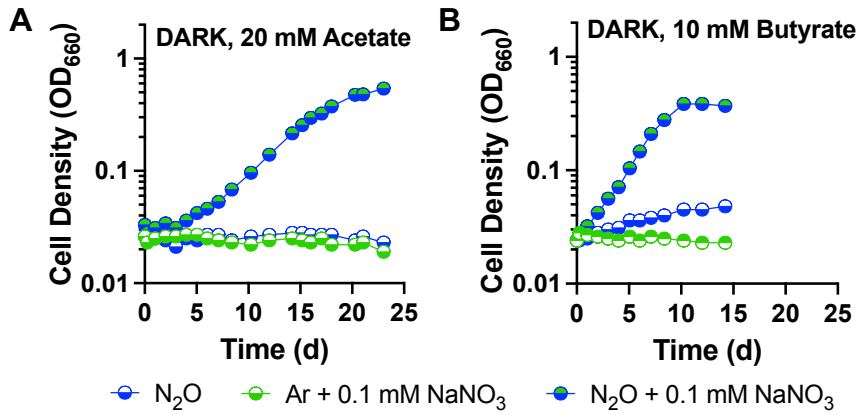

Fig. 5. Anaerobic respiration by CGA009 with N_2O in the dark with acetate (A) or butyrate (B). Single representatives of similar trends observed for three biological replicates are shown for each condition. Cultures had a 100% Ar headspace or 100% N_2O headspace as indicated.

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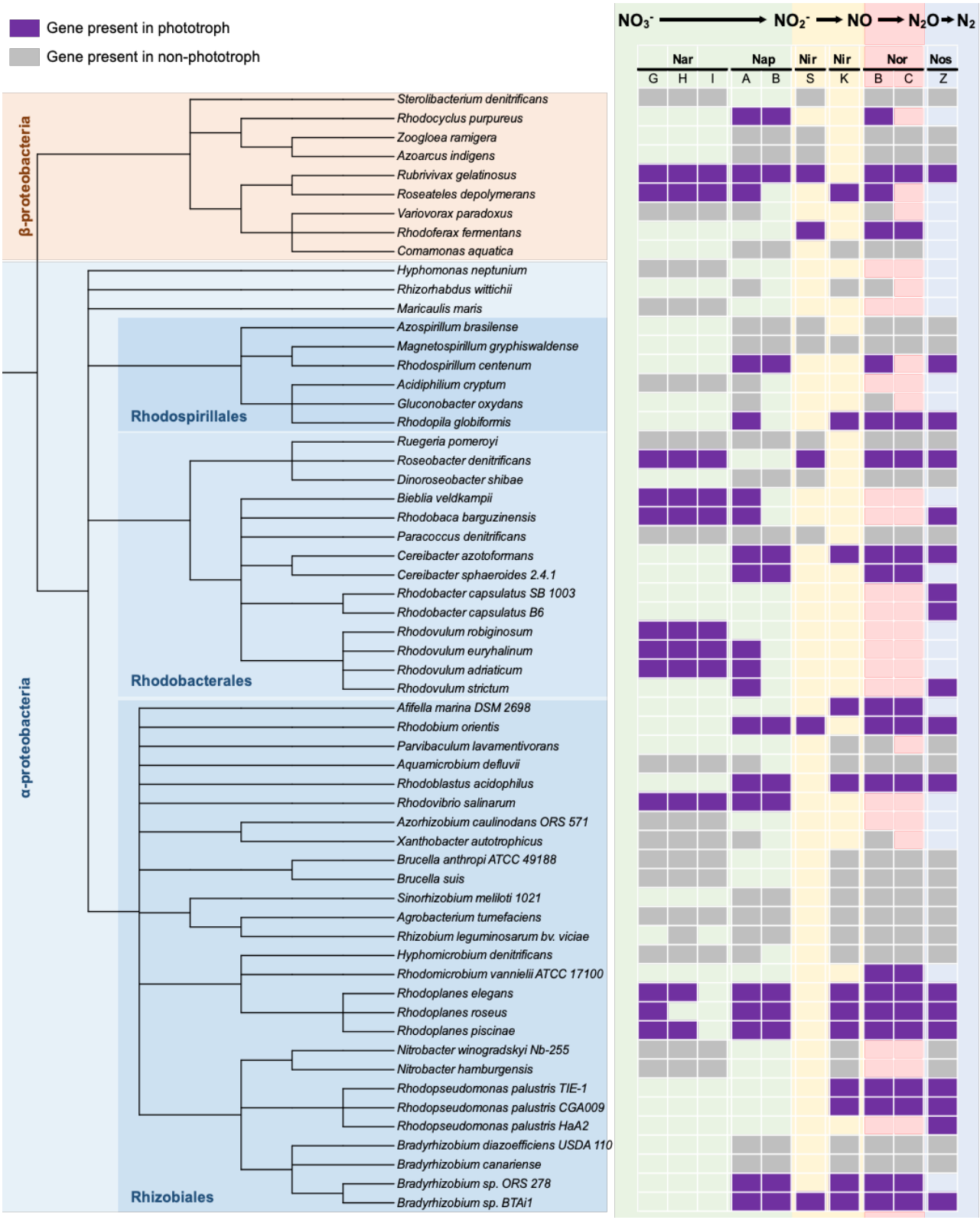

Fig. 6. Denitrification reductase genes in PNSB and related non-phototrophs. A gene was considered present if a predicted gene product exhibited >25% amino acid identity over 50% of its length relative to the query sequence. All subunits for a given enzyme are needed for the activity of a given reductase.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

197 ***R. capsulatus* SB 1003 requires NO_2^- to induce growth on N_2O.** We wondered if induction of
198 N_2O reductase by non-catalyzable denitrification intermediates occurs in other bacteria with
199 partial denitrification pathways. We thus surveyed the genomes of various PNSB and non-
200 phototrophic relatives using BlastP analysis with the same query denitrification reductase genes
201 as for *R. palustris* in Figure 1 (Fig. 6). *R. capsulatus* SB 1003 stood out as an easily cultivatable
202 and phylogenetically distant PNSB that has N_2O reductase but no other denitrification genes
203 (Fig. 6 and 7A). In agreement with the genome annotation, SB 1003 phototrophic growth on
204 butyrate was not supported by $NaNO_3$ (Fig. 7B). $NaNO_2$ also did not support growth, though SB
205 1003 was sensitive to $NaNO_2$ concentrations > 0.5 mM (Fig. 7C), which would likely be needed
206 to see a clear OD increase. Similar to CGA009, N_2O alone did not lead to phototrophic growth
207 on butyrate. However, supplementation with 100 µM $NaNO_2$, but not $NaNO_3$, stimulated
208 phototrophic growth on butyrate with N_2O (Fig. 7B). The same was true of growth in the dark on
209 succinate (Fig. 7D), a condition chosen to test whether the same regulatory trend held true during
210 anaerobic N_2O respiration in the dark.
211

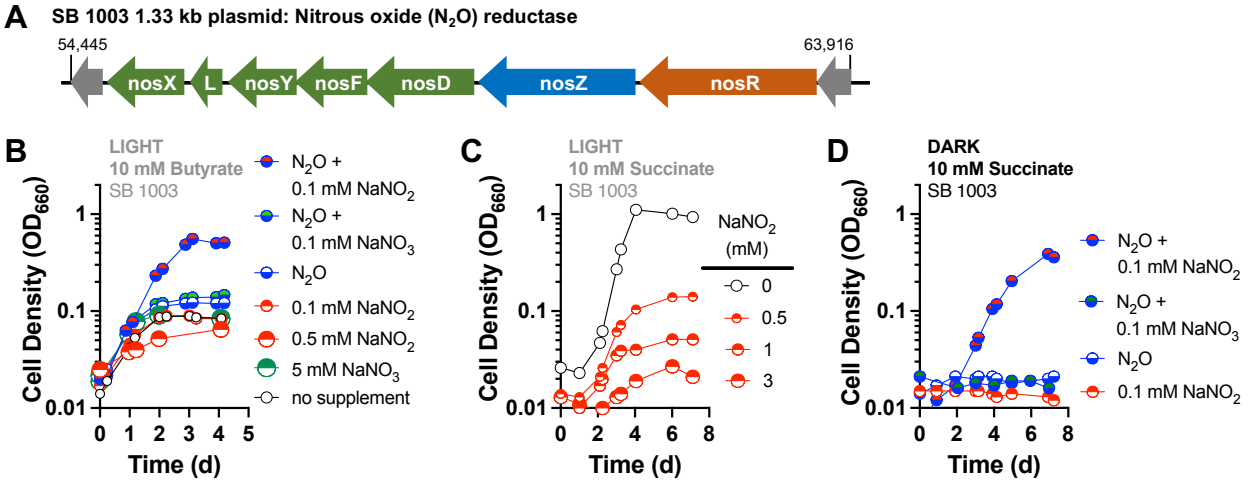

Fig. 7. Activation of the single *R. capsulatus* SB 1003 denitrification step, N_2O reduction, requires NO_2^-. A. SB 1003 N_2O reductase genes (*nos*) are on a plasmid. A possible regulator, NnrR, is encoded on the chromosome. Gene products: blue, N_2O reductase; green, accessory proteins for electron 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).

212

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).

258 Loss of denitrification genes could also be neutral or advantageous when within a community of
259 denitrifiers. Such scenarios are predicted by the Black Queen Hypothesis, wherein a gene
260 function that benefits other community members leads to loss of that function in a subset of
261 beneficiary community members (28). For example, if denitrification intermediates are reliably
262 generated by a neighbor and not all steps are needed for energy transformation and/or electron
263 balance, then it could be beneficial to lose other steps rather than incur the cost of synthesizing
264 the associated enzymes. Dispensing of NO_2^- and NO reductases could also be beneficial if NO_2^-
265 and NO are reliably removed by other community members. Membership within a denitrifying
266 community could also explain why non-catalyzable intermediates stimulate N_2O reduction, as
267 there would be little pressure to evolve responses to N_2O alone if N_2O is always found with other
268 intermediates of denitrification.
269
270 Partial denitrification pathways are both variable and widespread in PNSB (Fig. 6). This trend
271 could very well be true outside of PNSB and their close relatives. Despite decades of
272 investigation into the activity and regulation of denitrification, there has been comparatively little
273 research on partial denitrification pathways (2). Further investigation into the regulation of
274 partial denitrification pathways is needed, as is their potential impact on N_2O greenhouse gas
275 emissions from agricultural fertilizers.
276
277 **Methods.**
278
279 **Strains.** *R. palustris* CGA009 is a chloramphenicol-resistant type strain derived from CGA001
280 (8). The Calvin cycle mutant Δ*cbbLSMP*::km^R (CGA4008) was constructed by deleting *cbbLS*,
281 encoding ribulose-1,5-bisphosphate carboxylase (Rubisco) form I, in a previously described
282 mutant lacking Rubisco form II (Δ*cbbM*; CGA668; (20)) via introduction of the suicide vector
283 pJQΔ*cbbLS* (29) by conjugation with *E. coli* S17 as described (29, 30). The gene encoding
284 phosphoribulokinase*, cbbP*, was then deleted in the resulting strain (Δ*cbbLSM*; CGA4006) by
285 introducing the suicide vector pJQΔ*cbbP*::km^r (29), as above, to generate the Δ*cbbLSMP*::km^R
286 strain, CGA4008. All strain genotypes were verified by PCR and Sanger sequencing. The NifA*
287 derivative of CGA4008 with constitutive nitrogenase activity/H_2 production (CGA4011) was
288 described previously (29). The elimination of three genes unique to the Calvin cycle greatly
289 decreases the odds of enriching for suppressor mutations. *R. capsulatus* SB1003 was provided
290 courtesy of Carl Bauer (Indiana University).
291
292 **Growth conditions.** Strains were routinely cultivated in 10 ml photosynthetic medium (PM)
293 (31) in 27-ml anaerobic test tubes. PM was made anaerobic by bubbling tubes with 100% Ar
294 then sealing with rubber stoppers and aluminum crimps prior to autoclaving. After autoclaving,
295 tubes were supplemented with either 20 mM sodium acetate, 10 mM sodium butyrate, or 10 mM
296 sodium succinate from 100X anaerobic stock solutions. SB1003 cultures were also supplemented
297 with 0.1 µg/ml nicotinic acid, 0.2 µg/ml riboflavin, and 1.3 µg/ml thiamine-HCl. $NaNO_2$ or
298 $NaNO_3$ were added as indicated in the text. For conditions with N_2O, tubes were flushed with
299 100% N_2O through a 0.45 µm syringe filter and needle after all liquid supplements were added.
300 A second needle was used for off-gassing. Cultures were inoculated with a 1% inoculum from
301 starter cultures grown phototrophically in anaerobic PM with succinate, except for the
302 experiment testing Calvin cycle mutants (Fig. 3C) in which all starter cultures were grown
303 aerobically in 3 ml PM with succinate in the dark. Aerobic conditions were chosen for these

304 starter cultures to accommodate the Δ*cbbLSMP*::km^R mutant (CGA4008) that requires an
305 electron sink to grow.
306
307 **Analytical procedures.** Culture growth was monitored via optical density at 660 nm (OD_{660})
308 using a Genesys 20 spectrophotometer (Thermo-Fisher, Waltham, MA, USA) directly in culture
309 tubes without sampling. Specific growth rates were calculated using OD_{660} values between 0.1
310 and 1.0 where cell density and OD are linearly correlated. H_2 was sampled from culture
311 headspace using a gas-tight syringe and analysed using a Shimadzu GC-2014 gas chromatograph
312 as described (32).
313
314 **Bioinformatics.** Denitrification reductase gene inventories were determined using NCBI's
315 protein BLAST (12) to search for query sequences from *P. denitrificans* PD122 (Accession
316 numbers) NarG (GEK69895), NarH (CAA81215), NarI (CAA81217), NapA (CAA85346),
317 NapB (GEK66554), NirS (GEK68816), NirK (GEC49504), NorB (BAA32546), NorC
318 (BAA32545), and NosZ (GEK69878). Denitrification regulatory genes inventories were
319 similarly determined using query sequences from *P. denitrificans* PD122 FnrP (WP_041529894
320 (33)) and Nnr (WP_041529894 (33)), *B. japonicum* USDA110 NasT (BAC49838) and NasS
321 (BAC49837), and *P. aeruginosa* PAO1 Dnr/NnrR (BAA08744). The phylogenetic tree was built
322 using PhyloT v2 (phylot.biobyte.de) based on NCBI taxonomy and visualized using iTOL v6
323 (itol.embl.de).
324
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