

1 **Nitrous oxide reduction by two partial denitrifying bacteria requires denitrification**
2 **intermediates that cannot be respired**

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16
17 **Abstract**

18
19 Denitrification is a form of anaerobic respiration wherein nitrate (NO_3^-) is sequentially reduced
20 via nitrite (NO_2^-), nitric oxide, and nitrous oxide (N_2O) to dinitrogen gas (N_2) by four reductase
21 enzymes. Partial denitrifying bacteria possess only one, or some, of these four reductases and use
22 them as independent respiratory modules. However, it is unclear if partial denitrifiers sense and
23 respond to denitrification intermediates outside of their reductase repertoire. Here we tested the
24 denitrifying capabilities of two purple nonsulfur bacteria, *Rhodopseudomonas palustris*
25 CGA0092 and *Rhodobacter capsulatus* SB1003. Each had denitrifying capabilities that matched
26 their genome annotation; CGA0092 reduced NO_2^- to N_2 and SB1003 reduced N_2O to N_2 . For
27 each bacterium, N_2O reduction could be used for both electron balance during growth on
28 electron-rich organic compounds in light and for energy transformation via respiration in the
29 dark. However, N_2O reduction required supplementation with a denitrification intermediate,
30 including those for which there was no associated denitrification enzyme. For CGA0092, NO_3^-
31 served as a stable, non-catalyzable molecule that was sufficient to activate N_2O reduction. Using
32 a β -galactosidase reporter we found that NO_3^- acted, at least in part, by stimulating N_2O
33 reductase gene expression. In SB1003, NO_2^- , but not NO_3^- , activated N_2O reduction but NO_2^-
34 was slowly removed, likely by a promiscuous enzyme activity. Our findings reveal that partial
35 denitrifiers can still be subject to regulation by denitrification intermediates that they cannot use.

36
37 **Importance.** Denitrification is a form of microbial respiration wherein nitrate is converted via
38 several nitrogen oxide intermediates into harmless dinitrogen gas. Partial denitrifying bacteria,
39 which individually have some but not all denitrifying enzymes, can achieve complete
40 denitrification as a community by cross-feeding nitrogen oxide intermediates. However, the last
41 intermediate, nitrous oxide (N_2O), is a potent greenhouse gas that often escapes, motivating
42 efforts to understand and improve the efficiency of denitrification. Here we found that at least
43 some partial denitrifying N_2O reducers can sense and respond to nitrogen oxide intermediates
44 that they cannot otherwise use. The regulatory effects of nitrogen oxides on partial denitrifiers
45 are thus an important consideration in understanding and applying denitrifying bacterial
46 communities to combat greenhouse gas emissions.

47 **Introduction**

48
49 Denitrification is a multistep respiratory pathway that sequentially reduces nitrate (NO_3^-) via
50 nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) to dinitrogen gas (N_2) (1, 2) (Fig. 1A).
51 Denitrifying bacteria are important in several contexts. Denitrifiers in the human gut help fight
52 pathogens and maintain vascular homeostasis through the generation of NO_2^- and NO (3).
53 Denitrification is also important to the global nitrogen cycle, returning nitrogen to the
54 atmosphere as N_2 . However, N_2O often escapes denitrifying communities before it can be
55 reduced to N_2 . N_2O is a potent greenhouse gas that damages the ozone layer (4). N_2O emissions
56 have increased to concerning levels, primarily due to transformation of NO_3^- in agricultural
57 fertilizers to N_2O by naturally occurring denitrifying bacteria in the soil (5, 6). Thus, there is a
58 need to better understand and improve the efficiency of denitrification.

59
60 Many bacteria lack a complete denitrification pathway and are thus called partial or truncated
61 denitrifiers (7-11). Partial denitrifiers use single or multiple steps of the pathway as independent
62 respiratory modules (1, 2). Although incapable of reducing NO_3^- to N_2 on their own, partial
63 denitrifiers are important contributors to complete denitrification as a community process, with
64 intermediates cross-fed between community members that have different segments of the
65 pathway (7-12). Notably, nitrogen oxides (NO_3^- , NO_2^- , NO, or N_2O) not only serve as substrates
66 for denitrification reductases can also act as regulators of denitrification. Although regulatory
67 roles have been well-characterized in bacteria capable of complete denitrification, regulatory
68 roles in partial denitrifiers have received comparatively less attention. In particular, it is unclear
69 if the regulatory effects of nitrogen oxides in partial denitrifiers matches their reductase
70 repertoire.

71
72 Here we characterized the ability of two purple non-sulfur bacteria (PNSB) that are putative
73 partial denitrifiers, *Rhodopseudomonas palustris* CGA0092 and *Rhodobacter capsulatus*
74 SB1003, to carry out denitrification under phototrophic and chemoheterotrophic
75 conditions. Under phototrophic conditions, where light is the energy source, we tested if nitrogen
76 oxides can serve as an essential electron acceptor to maintain electron balance during growth on
77 the electron-rich substrate butyrate. Under chemotrophic conditions, we tested if nitrogen oxides
78 can serve as an essential electron acceptor to generate energy via oxidative phosphorylation. As
79 expected, each bacterium was only able to grow using the nitrogen oxides for which
80 corresponding reductases were annotated in their genomes. However, N_2O utilization required
81 supplementation with additional nitrogen oxides other than N_2O , including nitrogen oxides for
82 which there was no predicted reductase and which did not support growth on their own. Our
83 results indicate that at least some partial denitrifiers require nitrogen oxides that they cannot
84 respire to reduce N_2O .

85
86 **Results**

87
88 ***R. palustris* CGA0092 has a partial denitrification pathway.** *R. palustris* is one of the most
89 metabolically versatile PNSB (13), yet little is known about its ability to respire anaerobically.
90 Unlike some other model PNSB, CGA009, and its derivative CGA0092 (14) used herein, cannot
91 grow via respiration with dimethylsulfoxide (15-17). However, according to its genome
92 sequence, it should be capable of partial denitrification, as it has putative enzymes for converting

93 NO_3^- to N_2 (10, 13) (Fig. 1B). Expanding on past analyses by others (10, 13), we used PSI-
94 BLAST to verify that there are no genes with significant similarity to *nar*, *nap*, or *nas* nitrate
95 reductase genes nor to *nasB*, *nirB*, *nirD*, *nirA*, *nrf*, or eight-heme nitrite reductase genes (Table
96 S1) (18).

97

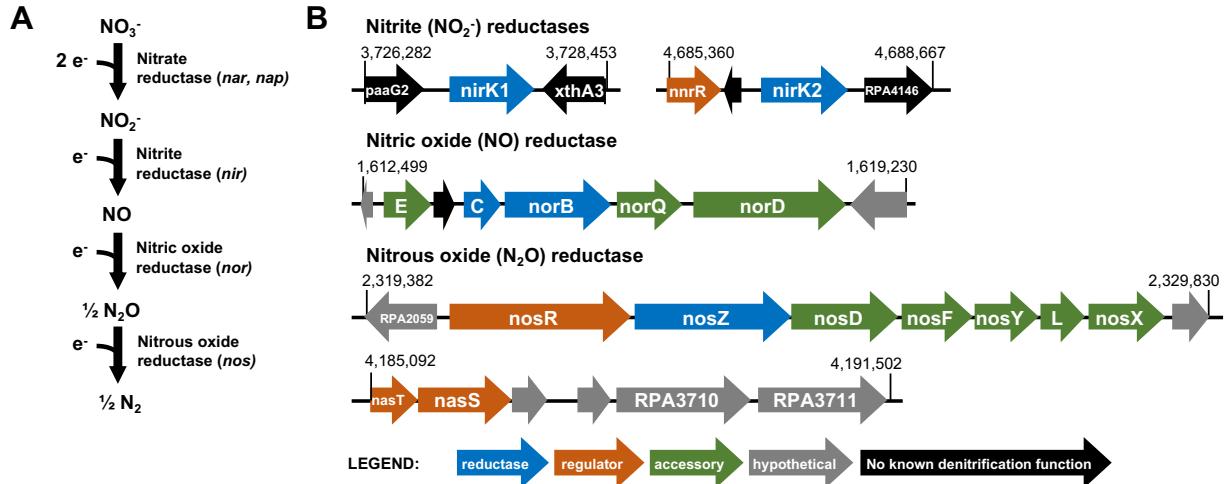


Fig. 1. General denitrification pathway (A) and denitrification genes annotated in *R. palustris* CGA0092 (B). Numbers indicate the chromosome nucleotide positions. Several CRP/Fnr-family transcriptional regulators with >25% sequence identity to known denitrification regulators are not shown.

98 When incubated anaerobically in darkness, PNSB typically use electron acceptors to establish a
99 proton motive force and generate ATP. When incubated in light, PNSB generate ATP by
100 photophosphorylation but electron acceptors, such as CO_2 or NaHCO_3 , are required for growth
101 on electron-rich compounds like butyrate to prevent an accumulation of reduced electron carriers
102 that halts metabolism; butyrate contains more electrons than can be incorporated into biomass
103 and so the excess electrons must be deposited on an electron acceptor or released as H_2 (19, 20).
104 Given that *R. palustris* grows best in light, we first examined if it could use denitrification
105 intermediates as electron acceptors during growth with butyrate.

106

107 In agreement with the apparent lack of NO_3^- reductase in the CGA0092 genome (Fig. 1B),
108 phototrophic growth on butyrate was not supported when supplemented with a wide range of
109 NaNO_3 concentrations (Fig. 2A). However, growth was observed when CGA0092 was provided
110 with 1mM NaNO_2 (Fig. 2A). We determined that this concentration was near the toxicity limit
111 because it caused a lag in phototrophic growth on succinate, which does not require
112 supplementation with an electron acceptor (Fig. 2B). We verified NO_2^- utilization using the
113 colorimetric Griess assay. All of the NO_2^- removed could be accounted for in the accumulated N_2
114 and N_2O , as measured by gas chromatography (Fig. 2C). N_2 and N_2O levels were, in fact, higher
115 than what could be explained by conversion of the supplied NO_2^- , although not significantly
116 different from the expected 1:1 correspondence. If real, the excess nitrogen was likely due to
117 contamination with atmospheric N_2 during sampling and an inability to distinguish N_2O from
118 CO_2 produced by other metabolic reactions (CO_2 and N_2O coeluted in our gas chromatography
119 method).

120

121 Generation of N_2 from NO_2^- indicated that the latter three reductases for denitrification are active
 122 in CGA0092. We did not directly address reduction of exogenously added NO because it is
 123 highly toxic and would likely be impossible to add in amounts that would be practical to yield
 124 observable growth. We directly addressed N_2O reduction by providing CGA0092 with a
 125 headspace of 100% N_2O . However, no growth was observed within 15 days (Fig. 2A),
 126 suggesting that N_2O alone cannot activate N_2O reduction.
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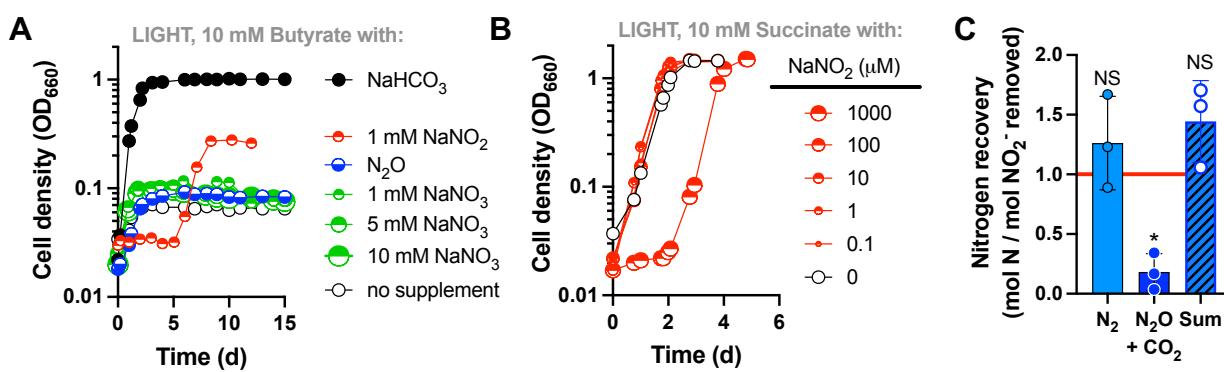


Fig. 2. NaNO_2 supports phototrophic growth of CGA0092 on butyrate within toxicity limits. **A.** Phototrophic growth with butyrate and various potential electron acceptors. Single representatives are shown. Similar trends were observed for three biological replicates except for conditions exploring different NaNO_3 concentrations where only single representatives were used. **B.** Phototrophic growth with succinate, a condition that readily supports growth without supplementation with an electron acceptor, with various concentrations of NaNO_2 to identify the toxicity limit. Cultures had a 100% Ar headspace unless N_2O is indicated (100% N_2O). **C.** Proportion of NO_2^- recovered as N_2 and N_2O . All of the supplied NO_2^- was removed. N_2O peak area includes a minor contribution of CO_2 due to coelution during gas chromatography. Each point represents an independent biological replicate. *, significantly different from 1 ($p < 0.05$); NS, not significantly different from 1.0 ($p > 0.05$), determined by a one sample t and Wilcoxon test.

128 **Photoheterotrophic N_2O reduction by CGA0092 requires NaNO_2 or NaNO_3 .** In some
 129 bacteria, denitrification intermediates other than N_2O enhance, or are required for, N_2O reduction
 130 (1, 2, 21-23). For example, NO_2^- induces N_2O reductase at the transcriptional level via the
 131 regulatory protein NnrR , though NO_2^- might first need to be converted to NO (23). NO_3^- induces
 132 N_2O reductase via NasTS regulatory proteins and an anti-terminator mechanism that affects
 133 transcription of nos genes (24, 25). CGA0092 encodes NnrR upstream of NirK2 and NasTS
 134 homologs upstream of a gene cluster encoding a potential nitrite/sulfite reductase (RPA3710-11;
 135 Fig. 1B). We thus tested if NaNO_2 or NaNO_3 could enable growth with N_2O . In agreement with
 136 our hypothesis, micromolar amounts of NaNO_2 as low as 1 μM stimulated growth with N_2O ,
 137 with final cell densities increasing in accordance with the amount of NaNO_2 added (Fig. 3A).
 138 NaNO_2 at 100 μM caused a 4-day lag in growth (Fig. 3A), suggesting that this level of NO_2^- was
 139 slightly toxic under these conditions. Notably, the increase in final cell density afforded by high
 140 amounts of NaNO_2 depended on the presence of N_2O , as growth with 100 μM NaNO_2 alone was
 141 much lower (Fig. 3A). This indicated that N_2O was being used as the primary electron acceptor
 142 in the presence of NO_2^- , despite that N_2O could not serve as an electron acceptor when provided
 143 alone (Fig. 2A). We speculate that exhaustion of NO_2^- eliminated the activation of N_2O

144 reduction, thereby eliminating the ability to use N_2O as an electron acceptor; consequently,
 145 growth on butyrate with N_2O lasts only as long as the pool of NaNO_2 .
 146
 147 Despite lacking NO_3^- reductase, NaNO_3 was also sufficient to stimulate phototrophic growth on
 148 butyrate with N_2O (Fig. 3B). Similar growth trends were observed between 1 μM to 10 mM
 149 NaNO_3 , indicating that NaNO_3 is relatively non-toxic and suggesting that NO_3^- was not being
 150 reduced. Indeed, NO_3^- levels were stable when we used 0.1 mM NaNO_3 to stimulate
 151 photoheterotrophic N_2O reduction (Fig. 3C). The amount of N_2O provided, all of which was
 152 ultimately removed, was linearly correlated with N_2 generated (Fig. 3C), although we again
 153 observed more N_2 generated than should be possible from the N_2O provided, likely due to
 154 contamination with atmospheric N_2 . Culture growth and butyrate consumed were also linearly
 155 correlated with N_2O supplied (and removed), further demonstrating the use of N_2O as an electron
 156 acceptor (e.g., in place of NaHCO_3 ; Fig. 2A) for phototrophic growth with butyrate.
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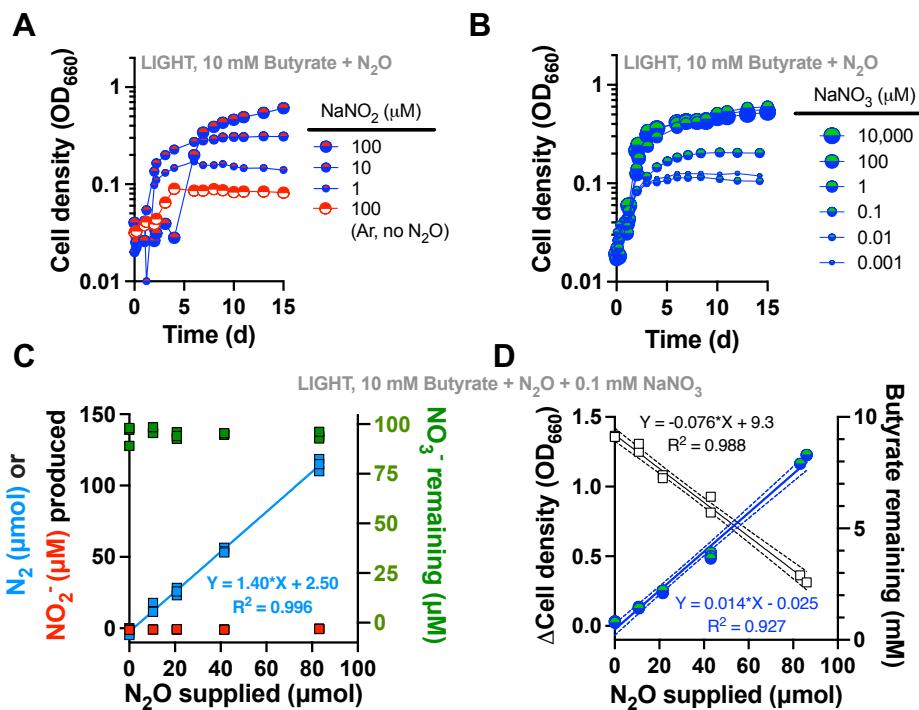


Fig. 3. NaNO_2 or NaNO_3 is required for phototrophic N_2O utilization by CGA0092. **A.** Phototrophic growth with butyrate +/- N_2O and different concentrations of NaNO_2 . The 'Ar, no N_2O ' control had a 100% argon headspace. **B.** Phototrophic growth with butyrate + N_2O and different concentrations of NaNO_3 . Single representatives were surveyed. **C, D.** Changes in N_2 , NO_3^- , NO_2^- , cell density, and butyrate in N_2O -limited cultures. Measurements were taken at inoculation and when the maximum cell density was reached. Each point represents a single independent culture. All N_2O was removed by stationary phase. Linear regression for NO_3^- (C) gave a slope that was not significantly different from zero ($p = 0.57$).

158 To determine if the requirement of NaNO_3 or NaNO_2 for N_2O reduction is manifested at the level
 159 of N_2O reductase expression (the combination of transcription and translation), we created a
 160 reporter that fused the region upstream of *nosR*, which should contain both a transcriptional
 161 promoter and a ribosomal binding site, to the *lacZ* gene at the start codon and integrated the

162 reporter into the CGA0092 chromosome. We then grew the resulting reporter strain (CGA4070)
163 under phototrophic conditions with acetate, a condition where an electron acceptor supplement is
164 not required, and added either NaCl (negative control), NaNO₃, NaNO₂, or N₂O as possible
165 inducers of expression. In agreement with growth trends (Fig. 3), NaNO₃ and NaNO₂, but not
166 N₂O, led to a significant, albeit low (1.6 – 2.4-fold), increase in LacZ activity over the NaCl
167 control (Fig. 4). Thus, NaNO₃ and NaNO₂ are inducers of *nos* gene expression, although the
168 relatively small effect suggests that there might be additional levels of regulatory control.

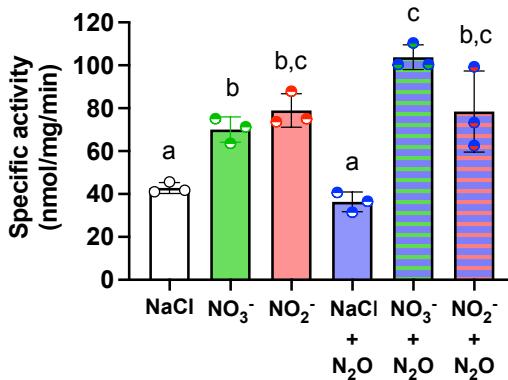


Fig. 4. NaNO₃ and NaNO₂, but not N₂O, positively affect *nosR* expression. β -galactosidase measurements were made in cell extracts of CGA4070, which harbors a chromosomally integrated *nosR* promoter–*lacZ* fusion. CGA4070 was grown phototrophically with 23 mM acetate and 0.1 mM NaCl, NaNO₃ or NaNO₂. Cultures received 4 ml N₂O, where indicated. Each point represents a single independent culture. All values were corrected for a background *o*-nitrophenol production rate of 8.7 nmol/mg/min as measured in cell extracts from CGA0092 grown under identical conditions with 0.1 mM NaCl or NaNO₃; activity was not significantly different between conditions with NaCl or NaNO₃. Floating letters indicate significant differences between strains (One-way ANOVA with Tukey post-test) $p < 0.5$.

169 **N₂O plus NaNO₃ can rescue photoheterotrophic growth of *R. palustris* Calvin cycle
170 mutants.** Under most photoheterotrophic growth conditions, the CO₂-fixing Calvin cycle is
171 essential to maintain electron balance, even on relatively oxidized substrates like succinate (19,
172 26, 27). To distinguish this essential electron balancing role from the Calvin cycle's better
173 known role in carbon assimilation, alternative electron acceptors are a useful tool because they
174 permit growth of Calvin cycle mutants (28, 29). Thus far, the only method known to allow
175 growth of *R. palustris* Calvin cycle mutants under conditions where the cycle is normally
176 essential was via NifA* mutations that result in constitutive nitrogenase activity (19, 26, 27).
177 NifA* mutants dispose of excess electrons as H₂, an obligate product of the nitrogenase reaction.
178 However, our results suggested that N₂O could be used as an electron acceptor to grow *R.*
179 *palustris* Calvin cycle mutants without additional genetic intervention. Indeed, N₂O with NaNO₃
180 rescued an *R. palustris* Calvin cycle mutant (Δ Calvin) during phototrophic growth on succinate
181 (Fig. 5). N₂O reduction resulted in more immediate growth than a NifA* mutation. However,
182 growth eventually slowed and the culture reached a lower final cell density than the Δ Calvin
183 NifA* mutant (Fig. 5). Gas chromatographic analysis of headspace samples confirmed that
184 growth of cultures with N₂O plus NaNO₃ was not due to spontaneous mutations that enabled H₂
185 production (i.e., no H₂ was detected; data not shown).

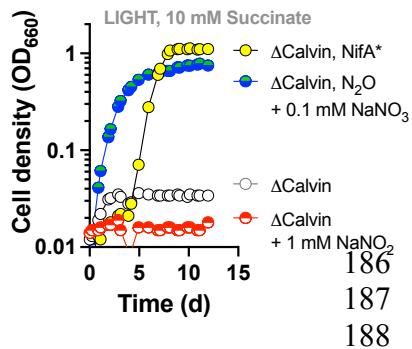


Fig. 5. N₂O plus NaNO₃ supports phototrophic growth of an *R. palustris* Calvin cycle deletion mutant. Cultures had a 100% Ar headspace unless N₂O is indicated (100% N₂O). Single representatives are shown. Similar trends were observed for three biological replicates. ΔCalvin is strain CGA4008; ΔCalvin, NifA* is strain CGA4011.

189 **NaNO₃ does not improve *R. palustris* photoheterotrophic growth with NaNO₂.** We wondered
190 if NaNO₃ might also improve growth with NO₂⁻, perhaps by stimulating NO₂⁻ reductase activity.
191 However, supplementation with NaNO₃ did not affect photoheterotrophic growth trends on
192 butyrate with 1 mM NaNO₂, even when NaNO₃ was also added to starter cultures as a possible
193 'pre-inducing' condition (Fig. 6A). The same strategy also did not decrease the lag phase during
194 phototrophic growth on succinate with 1 mM NaNO₂ (Fig. 6B).
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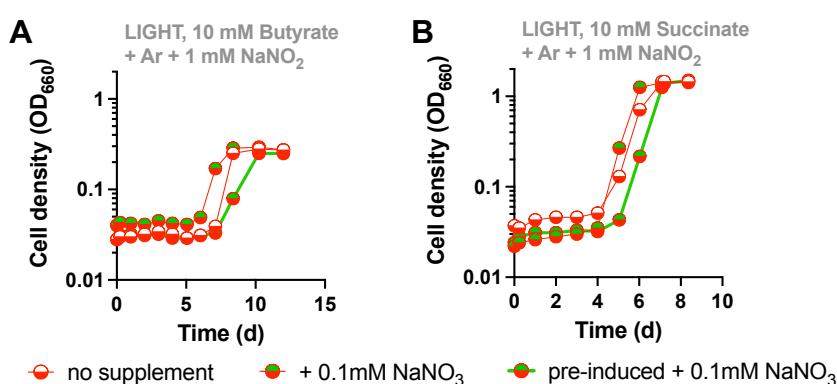


Fig. 6. NaNO₃ does not improve growth trends when NaNO₂ is present as an essential electron sink (A) or as a toxic compound (B). Single representatives are shown. Similar trends were observed for three biological replicates.

196 **NaNO₃ is required for anaerobic respiration with N₂O by CGA0092 in the dark.** Without
197 access to light, many PNSB can grow chemoheterotrophically via anaerobic respiration. We
198 tested if NaNO₂ or N₂O could support chemoheterotrophic growth by CGA0092 in the dark.
199 Acetate and butyrate were chosen as two carbon sources that are metabolized via similar
200 pathways but contain different amounts of electrons (19). Unlike phototrophic conditions (Fig.
201 2), supplementation with either 0.3 or 1 mM NaNO₂ did not support observable growth in the
202 dark with either acetate or butyrate within 15 days. In contrast, N₂O supported growth with either
203 acetate or butyrate but only when NaNO₃ was also provided (Fig. 7). Because growth was slower
204 with acetate than with butyrate (doubling time \pm SD = 88 \pm 2 h vs 51 \pm 3, respectively), we
205 performed further analyses with butyrate. As during phototrophy (Fig. 3), chemotrophic N₂
206 production, culture growth, and butyrate consumption were linearly correlated with the amount
207 of N₂O provided (Fig. 7C, D). All or nearly all N₂O was removed while NO₃⁻ levels remained
208 stable, without NO₂⁻ production (Fig. 7C).

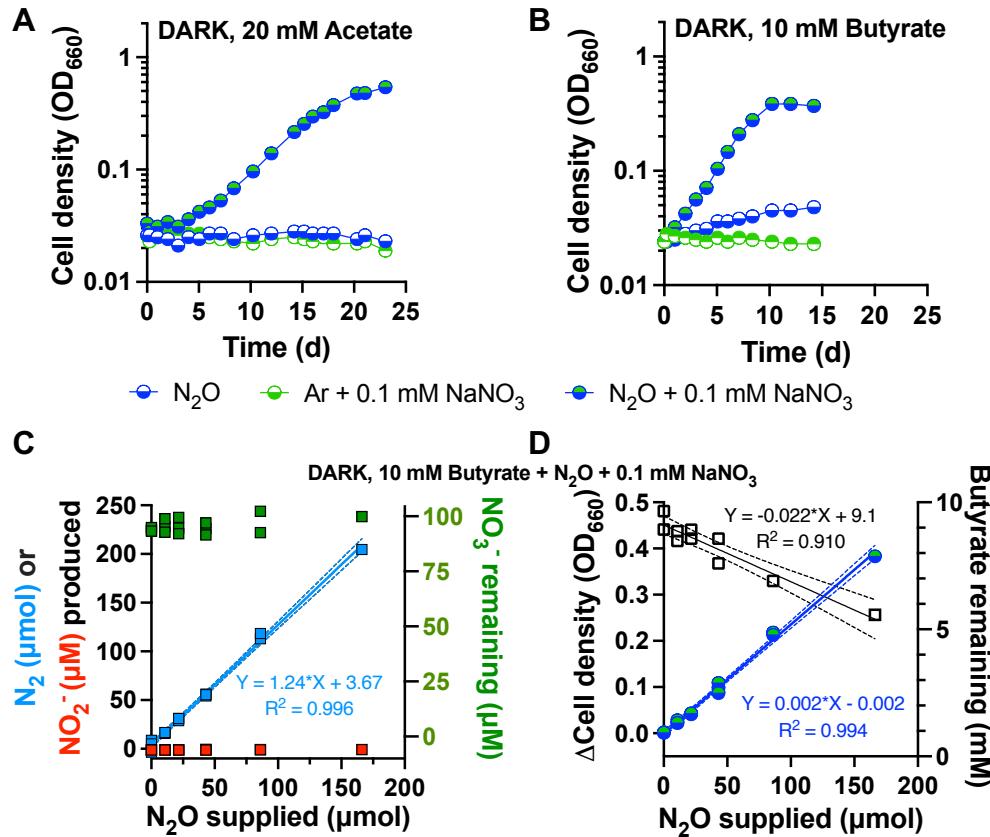


Fig. 7. NaNO₃ is required for N₂O respiration by CGA0092 in the dark with acetate (A) or butyrate (B). Single representatives are shown. Similar trends were observed for three biological replicates. Cultures had a 100% Ar headspace or 100% N₂O headspace as indicated. **C, D.** Changes in N₂, NO₃⁻, cell density, and butyrate in N₂O-limited cultures. Measurements were taken at inoculation and when the maximum cell density was reached. Each point represents a single independent culture. Most or all N₂O was removed by stationary phase. Linear regression for NO₃⁻ (C) gave a slope that was not significantly different from zero ($p = 0.11$).

210 The chemotrophic growth rate and growth yield with butyrate was 24% and 17%, respectively,
 211 of those observed under phototrophic N₂O-reducing conditions (Table 1). However, the specific
 212 rate of N₂O reduction was 1.4-fold higher under chemotrophic conditions (Table 1), suggesting
 213 that the rate of N₂O reduction needed to support electron balance under phototrophic conditions
 214 is less than that possible when N₂O reduction is needed for energy transformation. In agreement
 215 with the lower growth yield, the N₂O product yield was 3.3-fold higher under chemotrophic
 216 conditions (Table 1), indicating that more electrons from butyrate were directed to energy
 217 transformation compared to biosynthesis during chemotrophic growth.
 218

219 **NaNO₂ is required for phototrophic N₂O reduction by *R. capsulatus* SB1003.** We wondered
 220 if the requirement for non-catalyzable denitrification intermediates for N₂O utilization was
 221 specific to *R. palustris* or was also true in other partial denitrifiers. To examine this possibility,
 222 we turned to *R. capsulatus* SB1003, which stood out as an easily cultivatable and
 223 phylogenetically distant PNSB that is annotated to only have N₂O reductase (10) (Fig. 8A).

224 Using PSI-BLAST, we built upon past analyses (10) and confirmed that SB1003 does not have
225 genes with significant similarity to known assimilatory and dissimilatory NO_3^- and NO_2^- reductase
226 genes (Table S1). As predicted, phototrophic growth of SB1003 on butyrate was not supported
227 by NaNO_3 or NaNO_2 (Fig. 8B), although our ability to assess the latter was limited by the
228 sensitivity of SB1003 to NaNO_2 concentrations > 0.5 mM (Fig. 8C). Similar to what was
229 observed for *R. palustris*, N_2O alone did not support phototrophic growth of SB1003 (Fig. 8B).
230 However, supplementation with 0.1 mM NaNO_2 , but not NaNO_3 , led to phototrophic growth on
231 butyrate with N_2O (Fig. 8B). Also, N_2 production, growth, and butyrate production were linearly
232 correlated with the amount of N_2O provided (Fig. 8D, E), with all or nearly all N_2O removed.
233 However, unlike with *R. palustris*, levels of the stimulating compound, in this case NO_2^- , were
234 not stable. NO_2^- concentration declined with a roughly linear correlation to the amount of N_2O
235 provided (Fig. 8D). NO_3^- concentrations remained close to zero (Fig. 8D), suggesting that NO_2^-
236 was reduced, rather than oxidized. The specific rate of N_2O reduction was 300-times higher than
237 that of NO_2^- reduction (Table 1). This disparity suggests that NO_2^- removal was likely due to a
238 promiscuous enzyme activity or a growth-correlated abiotic factor rather than due to an
239 unannotated bona fide NO_2^- reductase.

240
241

Table 1. Growth and metabolic parameters from N_2O -reducing conditions with butyrate.

Strain, growth condition	Sp. growth rate (d^{-1})	Doubling time (d)	Sp. N_2O reduction rate (fmol/CFU/d)	Sp. NO_2^- reduction rate (fmol/CFU/d)	Growth yield (CFU/pmol N_2O)	Growth yield (CFU/pmol butyrate)	N_2O product yield (mol/mol butyrate)
CGA0092, light	1.36 ± 0.09	0.51 ± 0.04	1877 ± 182	ND	70 ± 5	90 ± 10	1.3 ± 0.1
CGA0092, dark	0.32 ± 0.02	2.20 ± 0.16	2645 ± 221	ND	12 ± 0	50 ± 10	4.3 ± 0.8
SB1003, light	1.32 ± 0.18	0.53 ± 0.07	1503 ± 229	5 ± 3	87 ± 6	105 ± 5	1.6 ± 0.3
SB1003, dark	0.79 ± 0.16	0.87 ± 0.02	6332 ± 1348	5 ± 4	13 ± 0	75 ± 10	5.9 ± 0.8

242 Values show averages \pm 95% CI. Doubling time = $\ln 2$ /growth rate. Specific (Sp) reduction rates
243 were determined by multiplying the growth rate by the slope of a linear regression of product vs
244 cell density (30, 31) from N_2O -limited cultures. Colony forming units (CFU) were obtained
245 using a conversion factor of $1 \text{ OD}_{660} = 5 \times 10^8 \text{ CFU/ml}$ (32). Product yields were determined by
246 linear regression using measurements of N_2O , NO_2^- , and butyrate from N_2O -limited cultures.
247

248 **NaNO₂ is required for anaerobic respiration with N₂O by SB1003 in the dark.** SB1003
 249 could also respire N₂O in the dark with butyrate, but again only when NaNO₂ was also present
 250 (Fig. 9A). Most or all N₂O was converted to N₂ when limiting amounts of N₂O was provided in
 251 the presence of 0.1 mM NaNO₂ (Fig. 9B). N₂O supplied also showed linear correlation with
 252 culture growth and butyrate consumption (Fig. 9C). Although only a small amount, some NO₂⁻
 253 was likely removed during N₂O reduction because the linear correlation of NO₂⁻ levels with N₂O
 254 supplied was negative and significantly different from zero (Fig. 9B). The specific NO₂⁻
 255 reduction rate was 3-orders of magnitude slower than the specific N₂O reduction rate (Table 1),

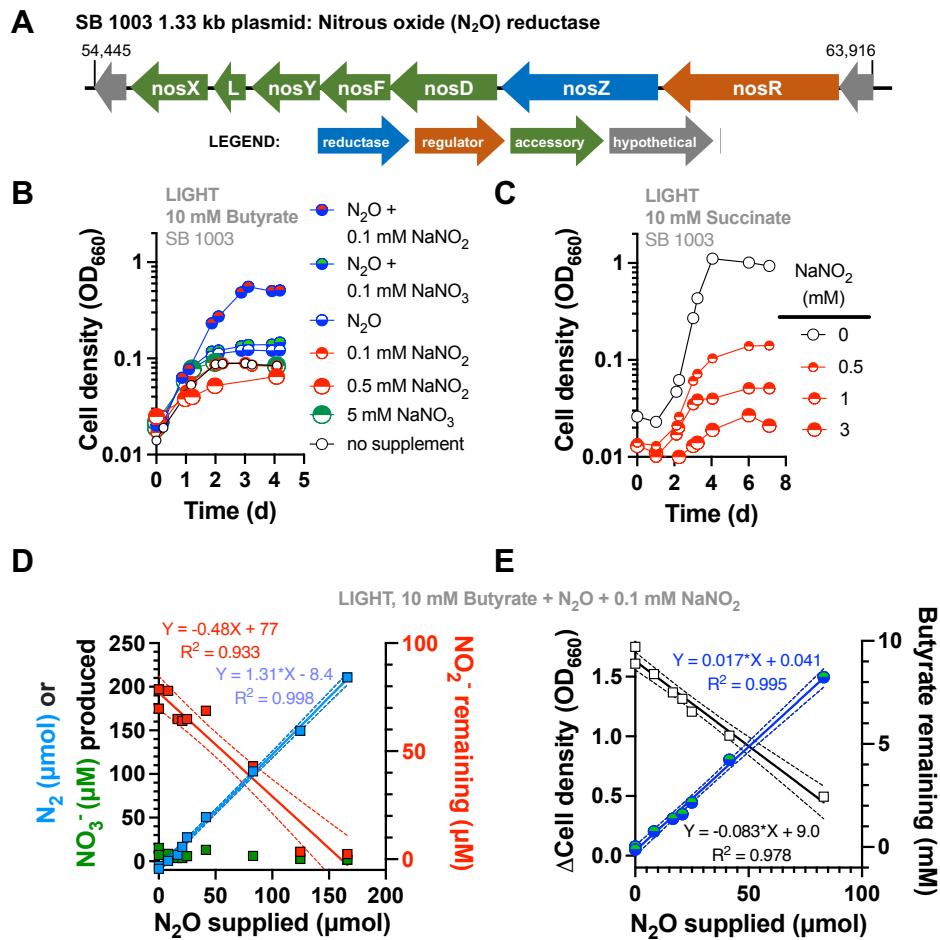


Fig. 8. NaNO₂ is required for phototrophic N₂O reduction by *R. capsulatus* SB1003.

A. Plasmid location of predicted N₂O reductase genes (*nos*). Numbers indicate nucleotide positions. **B.** Phototrophic growth with butyrate and various denitrification intermediates. Single representatives are shown. Similar trends observed for three biological replicates. Cultures had a 100% Ar headspace unless N₂O is indicated (100% N₂O). **C.** Phototrophic growth with succinate and various NaNO₂ concentrations to determine the toxicity limit. Single representatives were surveyed. **D, E.** Changes in N₂, NO₃⁻, cell density, and butyrate in N₂O-limited cultures. Measurements were taken at inoculation and when the maximum cell density was reached. Each point represents a single independent culture. Samples were diluted in cuvettes where necessary to ensure linear correlation between OD and cell density. Most or all N₂O was removed by stationary phase. Linear regression for NO₃⁻ (**D**) gave a slope that was significantly different from zero (p-value = 0.0002).

256 again suggesting that the activity was not associated with a canonical denitrification reaction.
 257 Similar to *R. palustris*, the SB1003 chemotrophic growth rate and growth yield were lower than
 258 those in phototrophic conditions, and more electrons in butyrate were diverted to N₂O reduction
 259 compared to biosynthesis (Table 1). However, SB1003 appears to be capable of a 2.4-fold higher
 260 specific N₂O reduction rate, which is likely behind the proportionately higher chemotrophic
 261 growth rate compared to *R. palustris* CGA0092 (Table 1).
 262

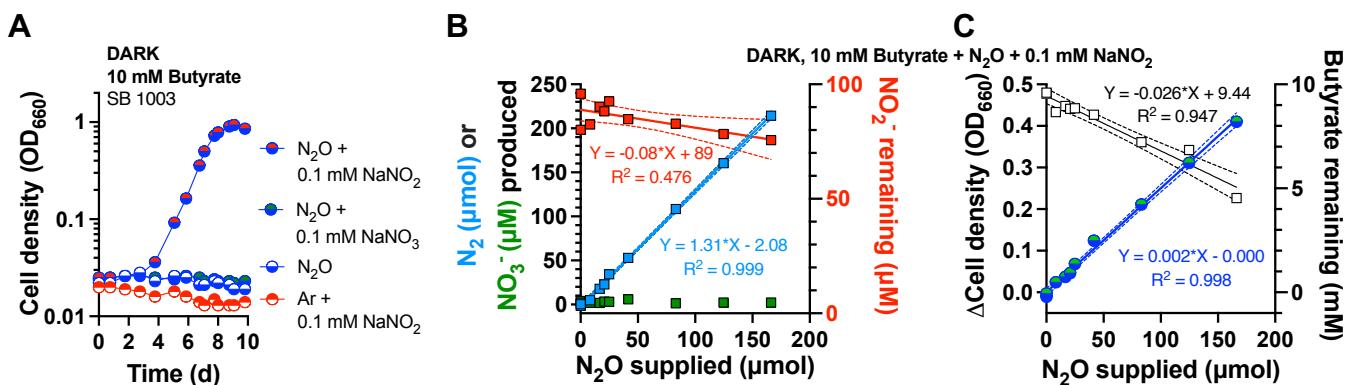


Fig. 9. NaNO₂ is required for N₂O respiration by SB1003 in the dark. A. Chemotrophic growth of SB1003 on butyrate with N₂O as an electron acceptor. Single representatives are shown. Similar trends were observed for three biological replicates. Cultures had a 100% Ar headspace unless N₂O is indicated (100% N₂O). **B, C.** Changes in N₂, NO₃⁻, cell density, and butyrate in N₂O-limited cultures. Measurements were taken at inoculation and when the maximum cell density was reached. Each point represents a single independent culture. Most or all N₂O was removed by stationary phase. Linear regression for NO₂⁻ (**B**) gave a slope that was significantly different from zero (p-value = 0.0097).

263

264 Discussion

265

266 Here we verified that *R. palustris* CGA0092 and *R. capsulatus* SB1003 are partial denitrifiers,
 267 with each being capable of respiration the nitric oxides predicted from their genome annotations.
 268 We observed that these bacteria can only reduce N₂O when supplied with other denitrification
 269 intermediates. Most importantly, nitrogen oxides required for N₂O reduction include those
 270 outside of each organism's partial denitrification repertoire, that is, NO₃⁻ in the case of CGA0092
 271 and NO₂⁻ in the case of SB1003.

272

273 **NO₃⁻ as a non-catalyzable inducer of nos gene expression.** Using a LacZ reporter under
 274 control of an *R. palustris* nosR promoter, we found that N₂O reduction is induced by NO₃⁻ and
 275 NO₂⁻, at least in part, at the level of gene expression (our construct likely captures transcriptional
 276 and translational regulatory features). The level of induction was low compared to the 10-fold
 277 increase seen with a *B. diazoeficiens* nosZ-lacZ reporter (24, 33). One possible explanation for
 278 the discrepancy is our use of a nosR promoter-lacZ fusion. NosR is a required regulatory protein
 279 for nitrous oxide reductase activity (34, 35) that is typically encoded with little to no intergenic
 280 region between nosR and nosZ (Fig. 1). While the nosR promoter drives nosZ expression in some
 281 bacteria (23), in other bacteria nosZ expression can occur from separate, and sometimes multiple,
 282 transcriptional start sites (25, 36). Substantial work beyond the current study will be necessary to
 283 decipher the transcriptional and post-transcriptional regulatory mechanisms governing *R.*

284 *palustris nos* genes. However, our findings clearly indicate that NO_3^- or NO_2^- are required for
285 N_2O reduction by *R. palustris* CGA0092, and that they play a role as inducers of *nos* gene
286 expression.

287
288 **Modification of NO_2^- and NO_3^- ?** A caveat to the observed activation of N_2O reduction activity
289 by NO_2^- and NO_3^- is that some promiscuous biotic or abiotic transformation could be necessary
290 to generate the inducing/activating molecule. We hypothesize that a promiscuous enzyme
291 activity led to the slow NO_2^- removal in SB1003 cultures (Fig. 8, 9). One possible candidate is
292 sulfite reductase (CysIJ; RCAP_rcc01594 and 03007), which bears homology to assimilatory
293 nitrite reductase. In *E. coli*, CysIJ can convert NO_2^- to NH_4^+ 1.7-times faster than sulfite
294 reduction but with a 200-fold lower affinity for NO_2^- compared to sulfite ($k_m = 0.8 \text{ mM } \text{NO}_2^-$, 8-
295 times above the concentration used in our SB1003 experiments) (37). If CysIJ was responsible
296 for NO_2^- removal, then NO_2^- was likely the molecule activating N_2O reduction because CysIJ
297 would convert NO_2^- to NH_4^+ , which was already present at mM concentrations in the growth
298 medium. However, there could be another enzymatic or spontaneous activity reducing NO_2^- to
299 NO as a separate inducing molecule. For CGA0092, levels of N_2O reductase-inducing NO_3^- were
300 stable. However, it is still possible that some of the 100 $\mu\text{M } \text{NO}_3^-$ was converted below our
301 detection limit to NO_2^- and/or NO . These molecules can induce N_2O reductase in other
302 organisms like *P. aeruginosa*, although they are typically applied at μM or mM levels (23).
303

304 **Possible regulators of N_2O reduction.** Our work calls for future investigation into the
305 regulatory mechanisms controlling N_2O reduction in CGA0092 and SB1003. However, we can
306 speculate on the regulatory proteins involved based on genome annotations. In considering NO_3^-
307 as an inducing molecule in CGA0092, NasTS stands out as a candidate. In *Bradyrhizobium*
308 *diazoefficiens*, NasTS controls the transcriptional activation of *nos* genes (24, 25). CGA0092 has
309 genes with significant sequence identity to *B. diazoefficiens* NasTS (77 and 66%, respectively;
310 Fig. 1B). NnrR (Fig. 1B) could also be involved in regulating N_2O reductase, though more likely
311 in response to NO (1, 23, 38-40).
312

313 NO_2^- could also activate N_2O reduction in SB1003 via an NnrR-like regulator. SB1003 has
314 several CRP/Fnr-family transcriptional regulator genes encoded in its chromosome with >25%
315 amino acid identity to denitrification regulators like *P. denitrificans* FnrP (RCAP_rcc02493;
316 74% identity) and *Pseudomonas aeruginosa* Dnr/NnrR (e.g., RCAP_rcc00107; 36% identity).
317 One or more of these regulators could be involved in regulating N_2O reductase, though more
318 likely in response to NO generated biotically or abiotically from NO_2^- (23).
319

320 **Denitrification inventories should consider both reductases and regulators.** Denitrification
321 gene inventories are notoriously inconsistent with organismal phylogeny; it is common for one
322 species to carry more or less denitrification genes than a close relative (8-10, 12, 41, 42). This
323 inconsistency is also true for strains of *R. palustris* and *R. capsulatus* (20, 43-46). In some cases,
324 horizontal gene transfer (HGT) could explain the phylogenetic discrepancies. The location of the
325 *nos* operon on a plasmid in SB1003 is a straight-forward example of HGT (Fig. 8A). However,
326 HGT cannot explain many of the chromosomal phylogenetic discrepancies. Phylogenetic
327 analyses have suggested the involvement of other factors like gene duplication and divergence,
328 lineage sorting (41), and gene loss (12). Gene loss could be advantageous in communities where
329 there are redundant denitrification functions. As proposed in the Black Queen Hypothesis (47),

330 gene loss can occur when the cost of producing something outweighs the benefit of obtaining it
331 from a neighbor. In this case, the cost of a full denitrification pathway could drive loss of
332 denitrification genes if sufficient energy can be obtained by using a denitrification intermediate
333 released by a neighbor (12). Benefits of partial denitrification pathways have been demonstrated
334 using a synthetic community, though the benefits stemmed more from NO_2^- detoxification than
335 energy savings (48).

336
337 Inventories of denitrification regulators have not received the same level of phylogenetic scrutiny
338 as the reductases. Such analyses would be complicated by the fact that phylogenetically similar
339 regulators can regulate different genes (49). However, regulator inventories are likely an
340 important determinant of reductase inventories because improper regulation could influence
341 maintenance or loss of a reductase gene. Regulator inventories also raise questions about the
342 evolutionary histories of denitrification genes. For example, if NasTS is required for expression
343 of the *nos* operon in *R. palustris* CGA0092, were both regulator and reductase genes
344 serendipitously acquired at the same time as separate DNA molecules by HGT or were they
345 acquired together as a single DNA molecule and then physically separated through genome
346 rearrangements (Fig. 1B)? Alternatively, perhaps the common ancestor to CGA0092 and *B.*
347 *diazoefficiens* USDA110 was capable of complete denitrification and CGA0092 lost nitrate
348 reductase genes but retained the native regulatory network that was responsive to NO_3^- . Both
349 genera clade together within the Nitrobacteraceae family but the CGA0092 genome is 3.4 Mb
350 smaller than that of USDA110 and in each case the NasTS and the *nos* genes are separated by
351 large stretches of chromosome (~2Mb in CGA0092 and ~5Mb in USDA110). Regulator
352 inventory might also support a community role for partial denitrifiers. The regulation of N_2O
353 reductase by NO_3^- and NO_2^- in this study could suggest that CGA0092 and SB1003 are primed to
354 sense signals by denitrifying partners.

355
356 Our findings suggest that within communities of partial denitrifiers, nitric oxides are not only
357 cross-fed metabolites but also important regulatory molecules. The requirement of these
358 molecules for N_2O reduction is an important consideration in efforts to mitigate greenhouse gas
359 emissions from agricultural soils, which is the largest source of N_2O emissions (6). Given that
360 agricultural soils are fertilized with NO_3^- , we do not anticipate a shortage of NO_3^- in those
361 environments. However, our findings expose a potential pitfall in overlooking the capacity of
362 *nosZ*-harboring bacteria to reduce N_2O , if unanticipated inducing molecules are omitted from lab
363 cultures.

364
365 **Methods**

366
367 **Strains.** *R. palustris* CGA0092 is a chloramphenicol-resistant type strain derived from CGA001
368 and differs from CGA009 by a single nucleotide polymorphism (13, 14). The Calvin cycle
369 mutant $\Delta cbbLSMP:\text{km}^R$ (Δ Calvin, CGA4008) was constructed by deleting *cbbLS*, encoding
370 ribulose-1,5-bisphosphate carboxylase (Rubisco) form I, in a previously described mutant
371 lacking Rubisco form II ($\Delta cbbM$; CGA668; (26)) via introduction of the suicide vector
372 pJQ $\Delta cbbLS$ (50) by conjugation with *E. coli* S17 as described (50, 51). The gene encoding
373 phosphoribulokinase, *cbbP*, was then deleted in the resulting strain ($\Delta cbbLSM$; CGA4006) by
374 introducing the suicide vector pJQ $\Delta cbbP:\text{km}^R$ (50), as above, to generate the $\Delta cbbLSMP:\text{km}^R$
375 strain, CGA4008. The elimination of three genes unique to the Calvin cycle greatly decreases the

376 odds of enriching for suppressor mutations. All strain genotypes were verified by PCR and
377 Sanger sequencing. CGA4011 is a NifA* derivative of CGA4008 that has constitutive
378 nitrogenase activity/H₂ production (50). *R. capsulatus* SB1003 was provided courtesy of Carl
379 Bauer (Indiana University).

380
381 CGA4070 was derived from CGA0092 for assaying *nosR* promoter activity using a *lacZ* reporter
382 chromosomally integrated upstream of the *nos* gene cluster, a similar strategy as that used in *B.*
383 *diazoefficiens* (24, 52). Briefly, a 398-nt region upstream of the *nosR* start codon was synthesized
384 in front of *lacZ* and incorporated into pTwist Kan High Copy plasmid by Twist Bioscience
385 (twistdnna.com) to create pTwist_PNos-LacZ. CGA0092 was transformed with pTwist_PNos-
386 LacZ by electroporation and plated on photosynthetic medium (PM) agar with 10 mM succinate
387 and 100 µg/ml kanamycin. Colonies were screened for integration by both PCR and by the
388 appearance of blue color when patched to identical agar that also contained 5-bromo-4-chloro-3-
389 indolyl-β-D-galactoside.

390
391 **Growth conditions.** Strains were routinely cultivated in 10 ml PM in 27-ml anaerobic test tubes.
392 PM is based on described media compositions (53, 54) and contains (final concentrations): 12.5
393 mM Na₂HPO₄, 12.5 mM KH₂PO₄, 7.5 mM (NH₄)₂SO₄, 0.1 mM Na₂S₂O₃, 15 µM p-
394 aminobenzoic acid, and 1 ml/L concentrated base (54). Concentrated base contains: 20 g/L
395 nitriloacetic acid, 28.9 g/L MgSO₄, 6.67 g/L CaCl₂·2H₂O, 0.019 g/L (NH₄)₆Mo₇O₂₄·4H₂O, 0.198
396 g/L FeSO₄·7H₂O, and 100 ml/L Metals 44 (55). Metals 44 contains: 2.5 g/L
397 ethylenediaminetetraacetic acid, 10.95 g/L ZnSO₄·7H₂O, 5 g/L FeSO₄·7H₂O, 1.54 g/L
398 MnSO₄·H₂O, 0.392 g/L CuSO₄·5H₂O, 0.25 g/L Co(NO₃)₂·6H₂O, 0.177 g/L Na₂B₄O₇·10H₂O.
399 PM was made anaerobic by bubbling tubes with 100% Ar then sealing with rubber stoppers and
400 aluminum crimps prior to autoclaving. After autoclaving, tubes were supplemented with either
401 20 mM sodium acetate, 10 mM sodium butyrate, or 10 mM disodium succinate from 100X
402 anaerobic stock solutions. Where indicated, cultures were additionally supplemented with 20
403 mM NaHCO₃. SB1003 cultures were also supplemented with 0.1 µg/ml nicotinic acid, 0.2 µg/ml
404 riboflavin, and 1.3 µg/ml thiamine-HCl. NaNO₂ or NaNO₃ were added from anaerobic stock
405 solutions to the final concentrations indicated in the text. For conditions with N₂O, tubes were
406 flushed with 100% N₂O through a 0.45 µm syringe filter and needle after all liquid supplements
407 were added. A second needle was used for off-gassing. For N₂O-limited cultures, the indicated
408 volume of filtered gas was added via syringe. Cultures were inoculated with a 1% inoculum from
409 starter cultures grown phototrophically in anaerobic PM with succinate, except for the
410 experiment testing Calvin cycle mutants (Fig. 3C) in which all starter cultures were grown
411 aerobically in 3 ml PM with succinate in the dark. These aerobic conditions were used to
412 accommodate the $\Delta cbbLSMP::km^R$ mutant (CGA4008) that requires an electron sink to grow.

413
414 **Analytical procedures.** Culture growth was monitored via optical density at 660 nm (OD₆₆₀)
415 using a Genesys 20 spectrophotometer (Thermo-Fisher, Waltham, MA, USA) directly in culture
416 tubes without sampling. For N₂O-limited cultures, samples were diluted in cuvettes where
417 specified. Specific growth rates were calculated using OD₆₆₀ values between 0.1
418 and 1.0 where cell density and OD are linearly correlated. N₂, N₂O, and H₂ were sampled from
419 culture headspace using a gas-tight syringe and analyzed using a Shimadzu GC-2014 gas
420 chromatograph (GC) equipped with a thermal conductivity detector. GC conditions for H₂ were
421 described previously (56). GC conditions for N₂ and N₂O used He as a carrier gas at 20 ml/min, a

422 80/100 Porapak N column (6' x 1/8" x 2.1 mm; Supelco) at 170 °C, an inlet temperature of
423 120°C, and a detector temperature of 155°C with a current of 150 mA. Gas standards were
424 prepared by injecting specific volumes of 1 ATM of pure gasses (41.6 mM based on the ideal gas
425 law and a temperature of 293 K) into a stopper-sealed serum vial of known volume, containing
426 with a few glass beads to aid in mixing. Gas standards were mixed by shaking, sampled with a
427 gas-tight syringe, and then injected at 1 ATM by releasing pressure prior to injection. Pressure
428 was not released prior to injection for culture headspace samples. Syringes were flushed with He
429 prior to each standard and culture injection to minimize contamination with atmospheric N₂.
430 NO₃⁻ and NO₂⁻ were measured using a colorimetric Griess assay kit according to the
431 manufacturer's instructions (Cayman Chemical). Conversion of NO₃⁻ to NO₂⁻ was accomplished
432 via NO₃⁻ reductase provided with the kit. N₂, N₂O, NO₃⁻ and NO₂⁻ were measured at the time of
433 inoculation and at stationary phase.

434

435 **β-galactosidase reporter assays.** *R. palustris* strains were grown to mid-late exponential phase
436 (0.4 - 1.1 OD₆₆₀) with 23 mM sodium acetate and either 0.1 mM NaCl, NaNO₃, or NaNO₂, with
437 or without 4 ml N₂O. Cultures were then chilled on ice and all subsequent processing was carried
438 out between 0 - 4 °C. Cells were harvested by centrifugation, supernatants were discarded, and
439 cells were resuspended in 0.5 ml Z-buffer. Cells were lysed by five 20-s rounds of bead beating
440 at maximum speed using a FastPrep®-24 benchtop homogenizer (MP Biomedical), with 5 min on
441 ice between rounds. Cell debris was pelleted by centrifugation and supernatant protein was
442 quantified using Bio-Rad's Bradford assay kit. Cell lysate (50 µl supernatant) was mixed with
443 100 µl Z-buffer in wells of a 96-well plate. Reactions were started with the addition of 30 µl of 4
444 mg/ml ortho-nitrophenyl-β-galactoside. Formation of *o*-nitrophenol was monitored at 420 nm
445 over time at 30°C using a BioTek Synergy plate reader. Specific activity was determined by
446 linear regression of the initial velocity and normalized for protein concentration.

447

448 **Bioinformatics.** PSI-BLAST used default parameters except for 500 targets, an expect threshold
449 of 10, a word size of 3, and a PSI-BLAST threshold of 0.005 using the refseq_protein database
450 for bacteria. At least five iterations were run or until no further sequences were found. Accession
451 numbers for the query sequences are in Table S1.

452

453 **Statistical analyses.** Graphpad Prism v10 was used for all statistical analyses.

454

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456

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465

466

467

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