DksA homologs, ppGpp and RegAB regulate dissimilatory nitrogen metabolism in *Paracoccus denitrificans*

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ABSTRACT

The *Paracoccus denitrificans* genome encodes three members of the DksA/TraR family of transcription factors, two of which appear to be *bona fide* DksA proteins (hence designated $DksA1_{Pd}$ and $DksA2_{Pd}$). There is a single *relA/spoT* gene in the genome encoding a predicted bifunctional ppGpp synthetase and hydrolase (designated RSH, for RelASpoT Homolog). Both $DksA1_{Pd}$ and $DksA2_{Pd}$ can rescue the amino acid auxotrophy of an *Escherichia coli dksA* mutant. Deletion of either *dksA1* or *relA/spoT* eliminates the upregulation of expression of the periplasmic nitrate reductase (NAP) that is seen when cultures are grown on a reduced substrate such as butyrate. Thus, we conclude that $DksA1_{Pd}$ and ppGpp activate *nap* transcription in response to growth on a reduced substrate and that these proteins therefore help to maintain redox homeostasis by activating a mechanism for the disposal of excess reductant. We also show that the redox-sensing RegAB two-component pair acts as a negative regulator of *nap* expression under anaerobic growth conditions. Under the same conditions, RegAB acts as a positive regulator of expression of the membrane-associated nitrate reductase Nar and thus mediates reciprocal regulation of the two nitrate reductases that have distinct physiological roles. The *dksA1* and *relA* genes are conditionally synthetically lethal; the double mutant has a null phenotype for growth on butyrate and other reduced substrates while growing normally on succinate and citrate. The flavohemoglobin gene *hmp* is modestly upregulated in *dksA2* and *relA* mutants, which are also defective for biofilm formation (possibly because of enhanced scavenging of nitric oxide).

IMPORTANCE

Paracoccus denitrificans is a metabolically versatile Gram-negative bacterium that is used as a model for studies of respiratory metabolism. The organism can utilize nitrate as an electron

46 acceptor for anaerobic respiration, reducing it to dinitrogen via nitrite, nitric oxide, and nitrous
47 oxide. This pathway (known as denitrification) is important as a route for loss of fixed nitrogen
48 from soil and as a source of the greenhouse gas nitrous oxide. Thus, it is important to understand
49 those environmental and genetic factors that govern flux through the denitrification pathway. Here
50 we identify novel genes, the protein products of which function as regulators of expression of
51 enzymes that reduce nitrate and nitric oxide.
52

53 **INTRODUCTION**

54 *Paracoccus denitrificans* is a gram-negative α-proteobacterium and a facultative anaerobe that can
55 respire using nitrogen oxyanions and oxides as terminal electron acceptors in a process known as
56 denitrification (1). In the denitrification pathway, the toxic intermediate nitric oxide (NO) is
57 generated from nitrite by the respiratory nitrite reductase and is subsequently reduced to nitrous
58 oxide (N_2O) by the respiratory NO reductase. *P. denitrificans* also has an *hmp* gene encoding a
59 flavohemoglobin (Hmp) that potentially contributes to the metabolism of NO (2, 3). NO acts in
60 conjunction with transcriptional regulators as an important signal molecule to regulate gene
61 expression during the transition from aerobic respiration to denitrification (4, 5). Pathogenic
62 strains of *Salmonella* and *E. coli* have several transcriptional regulators which control the
63 expression of genes encoding enzymes that scavenge NO. These regulators and their target genes
64 may help to protect the bacteria against the NO that is made by host phagocytes (6–8). One such
65 transcriptional regulator is DksA, which acts as a thiol based NO sensor in *Salmonella* and
66 regulates detoxification of NO through Hmp in *E. coli* (9–11).

67 DksA, first described in *E. coli* as DnaK suppressor A (12) is a founder member of
68 TraR/DksA family of transcriptional regulators (13, 14). $DksA_{Ec}$ (*E. coli* DksA) is 151 amino acids

69 long and consists of three major domains (Fig. S1): a coiled-coil (CC) domain with a conserved
70 DXXDXA motif at its tip, a globular domain with a conserved four-cysteine Zn-finger motif, and
71 a C-terminal α-helical domain (15). The conserved acidic residues located at the tip of the CC
72 domain in DksA$_{Ec}$ are critical for binding to RNA polymerase (RNAP) and for DksA$_{Ec}$ to function
73 as a transcriptional regulator (16, 17). The globular domain has been shown to interact with the
74 rim of the secondary channel of RNAP (18), and the conserved redox-sensitive cysteines act as a
75 sensor for NO in *Salmonella* (9). Some bacterial genomes encode multiple homologs of DksA,
76 which differ in the presence or absence of a complete DXXDXA motif or the presence of one, two,
77 or four conserved cysteines in the Zn-finger motif (19). For instance, *Pseudomonas aeruginosa*
78 has two functional homologs, DksA1 and DksA2 (20). DksA1 has the conserved four cysteine Zn-
79 finger motif whereas DksA2 does not (21, 22). In α-proteobacteria more closely related to *P.*
80 *denitrificans*, such as *Sinorhizobium meliloti* and *Rhodobacter sphaeroides*, the assigned *dksA*
81 gene encodes a protein with a complete DXXDXA motif and only one cysteine in the Zn-finger
82 motif (23, 24). Deletion or overexpression of *dksA* in various species has pleiotropic effects on
83 multiple physiological processes. These include amino acid biosynthesis (25), motility (26),
84 biofilm formation (26), plant symbiosis (23), DNA repair (27), quorum sensing and virulence (28)
85 and oxidative and nitrosative damage (9, 11).

86 DksA, along with the alarmone guanosine-5', 3'-tetraphosphate (ppGpp) is known to alter
87 global gene expression to adjust cellular metabolism in response to nutritional stress, a mechanism
88 known as the stringent response (when triggered by amino acid starvation) in *E. coli* and other
89 related γ-proteobacteria (29–31). ppGpp is found throughout the bacterial kingdom as a second
90 messenger and is associated with various stress response mechanisms and growth rate regulation
91 (29). The metabolism of ppGpp is controlled by two enzymes encoded by *relA* and *spoT* (32, 33).

92 RelA is a ribosome-associated ppGpp synthetase, whereas SpoT is a bifunctional enzyme
93 responsible for both synthesis and hydrolysis of ppGpp. The presence of both *relA* and *spoT* is
94 characteristic of γ-proteobacteria, while only one RelA/SpoT bifunctional homolog (RSH)
95 possessing both conserved ppGpp synthase and hydrolase domains is typically present in α-
96 proteobacteria (34, 35). While DksA and ppGpp coregulate many genes, some phenotypic and
97 transcriptomic studies report independent effects of both DksA and ppGpp (36, 37). The
98 independent effects of DksA and ppGpp might be attributed to their direct binding to RNAP;
99 ppGpp is also a direct effector of a number of metabolic enzymes (38–40)

100 In *P. denitrificans* Pd1222, three gene products have been annotated as members of the
101 TraR/DksA family: Pden_0547 (which we designate $DksA1_{Pd}$), Pden_0916 ($DksA2_{Pd}$), and
102 Pden_1862, which are 45%, 26% and 30% identical to *E. coli* DksA, respectively. The *P.*
103 *denitrificans* proteins are more similar to their homologs from related α-proteobacteria. $DksA1_{Pd}$,
104 $DksA2_{Pd}$, and Pden_1862 are 90%, 64%, and 33% identical to $DksA_{Rsp}$, respectively, while they
105 are 64%, 47%, and 36% identical to $DksA_{Sm}$, respectively. *P. denitrificans* also has a single
106 *relA*/*spoT* gene: Pden_1400 (henceforward called *relA*) which encodes for a RelA/SpoT
107 bifunctional homolog (RSH). The predicted RSH is 705 amino acids long and possesses a
108 phosphohydrolase HD domain with a conserved HDXXED motif in the N-terminal region, a RelA-
109 SpoT region, and conserved TGS and ACT domains in the C-terminal domain (Fig. S2). On the
110 basis of sequence similarity, Pden_1400 is predicted to have both ppGpp synthetase and hydrolase
111 activities. None of these *P. denitrificans* genes and proteins has been functionally characterized.
112 A proteomic analysis addressing aerobic to anerobic growth adaptation in *P. denitrificans*
113 demonstrated a significant increase in expression of $DksA1_{Pd}$ (A1AZG5) under anaerobic

114 conditions. Another study using microarray analysis showed a 3.4-fold higher expression of *dksA2*
115 during anaerobic growth (41, 42).

116 In this work, we report distinct phenotypes associated with deletion of two of the *dksA*-like
117 genes and the *relA* homolog in *P. denitrificans* Pd1222. We show that deletion of *dksA1* causes an
118 aerobic growth defect on reduced carbon substrates such as butyrate and that *dksA1* and *relA* null
119 strains fail to up-regulate expression and activity of the periplasmic nitrate reductase NAP under
120 these conditions. Furthermore, we demonstrate that ppGpp is involved in the stringent response in
121 *P. denitrificans* and that a *dksA1 relA* double mutant is unable to grow on reduced carbon
122 substrates. In addition, we show that *dksA2* is involved in biofilm production, possibly indirectly
123 via negative regulation of *hmp* transcription.

124

125 **RESULTS**

126 ***P. denitrificans dksA1* and *dksA2* complement Δ*dksA*$_{Ec}$ strains.**

127 Sequence alignment of the three DksA/TraR proteins revealed that two (Pden_0547 and
128 Pden_0916) have a complete DXXDXA motif which is required for interaction with RNA
129 polymerase (16, 17) while the third (Pden_1862) does not. Since we have also not found any
130 phenotype associated with deletion of Pden_1862 (data not shown) our attention has focused on
131 Pden_0547 and Pden_0916, which we have designated *dksA1* and *dksA2*, respectively. Unlike the
132 canonical DksA of *E. coli*, DksA1$_{Pd}$ has only one cysteine conserved in the zinc finger motif
133 whereas DksA2$_{Pd}$ has all four cysteines (Fig. S1).

134 *E. coli* MG1655 deleted for *dksA* was complemented with either *dksA1* or *dksA2* cloned in
135 pIND4 and expressed from their native promoter. *E. coli* Δ*dksA* is auxotrophic for multiple amino
136 acids and fails to grow on M9-glucose medium without amino acid supplementation (11).

137 Expression of either *dksA1* or *dksA2* rescued growth of Δ*dksA*$_{EC}$ on M9-glucose plates suggesting
138 that either gene may complement the *E. coli dksA* mutation (Fig. 1).

139 **Deletion of *dksA1*$_{Pd}$ impairs nitrate stimulation of aerobic growth on butyrate**

140 To characterize the *dksA*-like genes of *P. denitrificans*, we constructed and tested the growth of
141 deletion strains lacking *dksA1* and *dksA2*. The mutants grew as well as the parent strain on rich
142 (LB) medium under both aerobic and anaerobic conditions (Fig. S3). We also did not observe
143 amino acid auxotrophy when the mutants were grown in succinate or glucose minimal medium
144 (data not shown). In *Salmonella enterica* sv. *typhimurium*, DksA is involved in redox homeostasis
145 since it regulates the expression of enzymes from central metabolic pathways that generate reduced
146 pyridine nucleotides (43). *P. denitrificans* may experience redox imbalance during growth on
147 reduced carbon substrates due to the generation of excess reducing equivalents (3). Therefore, we
148 assayed aerobic growth in minimal medium supplemented with succinate (MM-S) or butyrate
149 (MM-B) as sole carbon and energy sources that are relatively oxidized and reduced, respectively.
150 Growth of the wild-type strain in MM-B was significantly enhanced by the addition of nitrate to
151 the medium. Reduction of nitrate by the periplasmic nitrate reductase NAP provides a mechanism
152 to dispose of excess reducing equivalents during growth on reduced carbon substrates (44). The
153 Δ*dksA1* mutant grew normally on MM-S but showed impaired growth on MM-B that was not
154 improved by addition of nitrate to the medium (Fig. 2A and B). Expression of *dksA1* from a
155 recombinant plasmid in the *dksA1* mutant restored a wild type phenotype for growth on MM-B
156 and enhanced in the presence of nitrate (Fig. S4). These phenotypes suggest that DksA1$_{Pd}$ may
157 regulate aerobic nitrate reduction by NAP during growth on reduced carbon substrates.

158 To determine if the Δ*dksA1* associated phenotype is ppGpp-dependent, we measured
159 growth of the Δ*relA* strain under similar conditions. The Δ*relA* mutant showed significantly

160 reduced growth in MM-B, however, unlike for Δ*dksA1*, growth was improved by addition of
161 nitrate to the medium (Fig. 3A,B and S5), though the magnitude of nitrate stimulation was less
162 than was observed in the wild-type strain. Complementation of the Δ*relA* mutant with the *relA*
163 gene cloned in a plasmid restored the wild type growth phenotypes on MM-B (Fig. S6).

164 **Δ*dksA1* and Δ*relA* mutants have reduced *napE* promoter activity and NAP enzyme activity**

165 It has been shown previously that the periplasmic nitrate reductase, NAP, has increased expression
166 and activity in cells grown aerobically on reduced energy sources (45, 46). Our results suggest that
167 the absence of *dksA1* impairs nitrate reduction in *P. denitrificans* during aerobic growth. We
168 hypothesized that $DksA1_{Pd}$ regulates expression of NAP during aerobic growth on reduced energy
169 sources. To test this hypothesis, we constructed a *napE-lacZ* promoter transcriptional fusion and
170 measured *napE* promoter activity using β-galactosidase as the reporter. Consistent with previous
171 reports (5), we observed an increase in *napE* promoter activity in cells grown aerobically in MM-
172 B compared to those grown in MM-S (Fig. 4A). The increase in *napE* promoter activity caused by
173 growth on butyrate was eliminated in the Δ*dksA1* mutant. The increase in *napE* promoter activity
174 in MM-B versus MM-S also disappeared when the cultures were grown anaerobically (Fig. 4A).
175 To confirm that these observations were due to loss of *dksA1,* we measured *napE* promotor activity
176 in the complemented Δ*dksA1* strains. Complementation with the *dksA1* gene restored the butyrate
177 stimulation of *napE* promotor activity (Fig. S7)

178 In parallel with the *napE* promoter activity assays we also measured the activity of NAP in
179 periplasmic fractions using reduced methylviologen as the electron donor to the enzyme. As
180 previously reported (45), and consistent with our promoter activity data, NAP activity in the wild-
181 type was significantly higher during aerobic growth in MM-B compared to MM-S (Fig. 4B). The
182 stimulation of activity by growth on butyrate disappeared in the Δ*dksA1* mutants and in cells grown

183 anaerobically in either MM-S or MM-B (Fig. 4B). Our results suggest that DksA1$_{Pd}$ upregulates
184 (either directly or indirectly) the *napE* promoter during aerobic growth on reduced carbon
185 substrates such as butyrate.

186 The *relA* mutant was found to have the same phenotype as the *dksA1* mutant with respect
187 to *nap* promoter activity and NAP enzyme activity. That is, the butyrate-dependent stimulation
188 was also lost in the *relA* mutant (Figs. 4A, B and S7). Since DksA and ppGpp frequently act in
189 concert by binding together to a single site on RNA polymerase (30), the likely explanation that
190 DksA-dependent regulation of *nap* requires ppGpp.

191 **The RegAB system regulates anaerobic *napE* promoter activity and NAP enzyme activity**

192 In *P. pantotrophus*, regulation of the *nap* operon in response to carbon substrate oxidation state
193 involves *cis*-acting regulatory sequences in the *napE* promoter region (48). Specifically, a
194 heptameric imperfect inverted repeat sequence (TGAGACAttTGTCGCA) was suggested to be the
195 binding site for a negatively acting redox-responsive regulator (48). The inverted repeat is a partial
196 match to the binding site (GCGGC-N_5-GTCGC) for the response regulator RegR (RegA) in
197 *Bradyrhizobium japonicum* (49). We found a similar sequence (5′-**GAGGC**GTTTT**GACGC**-3′)
198 in the promoter region of the *nap* operon in *P. denitrificans* Pd1222. Therefore, we examined *napE*
199 promoter activity in Δ*regA* and Δ*regB* strains to determine if *nap* is regulated by *dksA1* alongside
200 the RegAB system. In the Δ*regA* mutant, we observed ~2-fold increases in *napE* promoter activity
201 in cells grown aerobically on succinate (Fig. 5A). In cells grown aerobically on butyrate, *napE*
202 promoter activity was similar in both *regA* and *regB* mutants and the wild-type strain. Interestingly,
203 there were significant increases in promoter activities in *regA* and *regB* mutants grown
204 anaerobically on either succinate or butyrate (Fig. 5A). Expression of *regA* and *regB* in the Δ*regA*

205 and Δ*regB* strains respectively reduced *napE* promoter activity to levels below those seen in the
206 wild type strain, confirming that RegAB acts negatively on the napE promoter.

207 Our data suggest that RegA is the negatively acting regulator that Ellington et al. suggested
208 (48) binds to the inverted repeat sequence in the *napE* promoter, and that the RegAB system acts
209 to down-regulate this promoter principally under anaerobic growth conditions (but also to a lesser
210 extent) during aerobic growth on succinate. The less severe phenotype of the *regB* mutant under
211 some conditions suggests the possibility that RegA is phosphorylated by another histidine kinase
212 in the absence of RegB. Assays of NAP activity in periplasmic fractions prepared from
213 anaerobically grown cells confirm that RegAB functions as a negative regulator under these
214 conditions (Fig. 5B).

215 The membrane-associated nitrate reductase, Nar, is upregulated in *P. denitrificans* in
216 oxygen-limiting conditions (1, 50). Known regulators of the *nar* promoter are NarR (50) and,
217 probably, FnrP (51). Assays of Nar activity in membrane fractions revealed a reduced activity in
218 both Δ*regA* and Δ*regB* mutants suggesting a positive regulation of the *nar* operon by RegAB under
219 these conditions (Fig. 5C). Addition of 20 μM sodium azide (a selective inhibitor of Nar) to the
220 reaction mixture inhibited nitrate reduction confirming that the reductase activity in the membrane
221 fraction was due to Nar (data not shown). Reduced Nar activity also coincided with reduced growth
222 yield for the Δ*regA* mutant when grown anaerobically (Fig. S9). Thus, the RegAB system mediates
223 reciprocal regulation of the two nitrate reductases under anaerobic conditions, being a negative
224 regulator of NAP expression and a positive regulator of Nar expression.

225 **The *dksA1* and *relA* genes are synthetically lethal for growth on reduced substrates**

226 The DksA protein interacts with ppGpp to regulate several functions and deletion of either *dksA*
227 or *relA* gives rise to overlapping pleiotropic phenotypes (36). The similar phenotypes of Δ*dksA1*

228 and *relA* mutants with respect to loss of butyrate stimulation of *nap* promoter activity suggests that
229 DksA1$_{Pd}$ and ppGpp act in concert to regulate expression of the periplasmic nitrate reductase. A
230 Δ*dksA1*Δ*relA* double mutant exhibited a complete growth defect on reduced carbon substrates such
231 as butyrate, caproate and choline (Fig. 3, Fig. S10), while growth on the relatively oxidized
232 substrates succinate and citrate was normal (Fig. S11). Expression of either *dksA1* or *relA* on a
233 broad host range plasmid from their native promoters restored growth of the Δ*dksA1*Δ*relA* mutant,
234 confirming that both mutations are required for the null growth phenotype (Fig. 3). Thus, *dksA1*
235 and *relA* exhibit synthetic lethality with respect to growth on reduced substrates. The likely
236 explanation is that two (or more) genes that are independently regulated by DksA1$_{Pd}$ and ppGpp
237 are together required for growth on reduced substrates.

238 **RSH is essential for initiating the stringent response in *P. denitrificans*.**

239 The *relA/spoT* gene product is a bifunctional ppGpp synthetase/hydrolase (RSH) that synthesizes
240 ppGpp in response to nutrient (for example, amino acid) starvation in many bacteria (29, 52). To
241 determine if the RSH of *P. denitrificans* is involved in the stringent response to amino acid
242 starvation, wild-type strains were treated with serine hydroxamate (SHX) during growth in MM-
243 S. SHX, a structural analog of serine is known to cause amino acid starvation in a concentration
244 dependent manner and has been used to induce the stringent response in *E. coli*, which is
245 characterized by accumulation of ppGpp (12, 31). When strain Pd1222 was treated with SHX, we
246 observed that, unlike *E. coli* (53), there was no inhibition of growth at concentrations below 3 mM
247 (data not shown). On treatment with 3 mM SHX the wild-type strain exhibited an unusually long
248 lag phase but recovered growth while the Δ*relA* strain was completely inhibited (Fig 6B). Addition
249 of casamino acids reversed the SHX-induced growth inhibition in the wild-type and Δ*relA* strains
250 (Fig. 6C). Constitutive expression of *relA*$_{Pd}$ in the deletion strain reversed the observed phenotypes

251 and the strains grow as well as the wild-type strain (Fig. 6B and C) suggesting that RSH is required
252 for initiating the stringent response following amino acid starvation.

253 **DksA2$_{Pd}$ is a positive regulator of the *hmp* promoter**

254 In *E. coli*, *dksA* regulates the expression and activity of Hmp, a flavohemoprotein. Hmp is a nitric
255 oxide dioxygenase or denitrosylase which oxidizes nitric oxide to nitrate, and is known to be the
256 dominant NO defense mechanism under aerobic conditions (11). *P. denitrificans* has an *hmp* gene,
257 Pden_1689 (2, 3) and we have shown that the Hmp is functional for NO scavenging (unpublished
258 data). To determine if regulation of *hmp* is mediated by DksA in this organism, we assayed *hmp*
259 promoter activity in *P. denitrificans* and the Δ*dksA* mutants. Our results indicate that *hmp* promoter
260 activity is modestly but significantly elevated in the Δ*dksA2* mutant but is unaffected by the
261 Δ*dksA1* deletion (Fig. 7A). The Δ*relA* mutant also shows higher *hmp* promoter activity similar to
262 the Δ*dksA2* strain suggesting that the regulation of *hmp* by DksA2 is ppGpp-dependent (Fig. 7A).

263 **Both DksA2$_{Pd}$ and RSH regulate biofilm formation in *P. denitrificans***

264 Our previous work has shown that deletion of *hmp* increases biofilm formation in *P. denitrificans*
265 Pd1222 possibly due to accumulation of NO (2). Given the evidence that DksA2$_{Pd}$ regulates *hmp*
266 expression in *P. denitrificans*, we assayed attached growth to determine if DksA2$_{Pd}$ also affects
267 biofilm formation. We observed that Δ*dksA2* strains had greatly reduced levels of attached growth
268 (Fig. 7B and C). Addition of an *hmp* mutation to the *dksA2* mutant rescued biofilm formation to
269 wild-type levels (Fig. 7B and C) suggesting that the *dksA2* phenotype requires Hmp. Deletion of
270 *nsrR*, which encodes a transcriptional repressor of *hmp* (2, and unpublished data), led to
271 significantly reduced biofilm formation similar to Δ*dksA2* (Fig. 7B and C). We also observed
272 reduced biofilm in Δ*relA* strains suggesting that *dksA2* and *relA* could be jointly involved in
273 regulating biofilm formation in *P. denitrificans* via negative regulation of *hmp* (Fig. 7B and C).

274

275 **DISCUSSION**

276 Expression of the periplasmic nitrate reductase NAP is upregulated by aerobic growth on reduced
277 carbon and energy sources, the likely explanation being that nitrate reduction by NAP provides a
278 means to dispose of excess reducing equivalents (44–48). We have shown that DksA1$_{Pd}$ and RSH
279 are required for the butyrate-stimulation of *napE* promoter and NAP enzyme activity. RSH is the
280 only protein encoded in the *P. denitrificans* genome with predicted ppGpp synthetase activity and
281 that it is enzymatically functional is supported by the detection of ppGpp in slow-growing cultures
282 of this organism (54). It is likely that DksA1$_{Pd}$ and ppGpp directly activate the *napE* promoter,
283 although indirect effects cannot be excluded. In either case, the implication is that growth on
284 reduced substrates somehow triggers the synthesis of ppGpp by RSH. Data from continuous
285 cultures show that the *napE* promoter is regulated both by growth rate and by carbon substrate,
286 leading to the conclusion that "…*nap* regulation is affected by a physiological signal which
287 integrates aspects of growth rate and carbon substrate utilization" (55). Our work suggests that
288 ppGpp (together with DksA1$_{Pd}$) is the integrating signal, which is consistent with the well-known
289 role of ppGpp in growth rate control (29).

290 One difference between the phenotypes of the *dksA1* and *relA* mutants is that nitrate does
291 not improve aerobic growth of the *dksA1* mutant on butyrate (as it does in the case of the wild-
292 type strain) but does modestly improve growth of the *relA* mutant. This improvement of growth
293 cannot be attributed to butyrate stimulation of *nap* expression, since that is lost in the *relA* mutant.
294 It is also the case that the *relA* mutant grows less well on butyrate (in the absence of nitrate) than
295 the wild-type strain and the *dksA1* mutant, and that the double mutant does not grow at all on

296 reduced substrates. It is likely that there are DksA1$_{Pd}$ and ppGpp regulated genes that are involved
297 in redox homeostasis that are yet to be identified.

298 We showed that regulation of *nap* expression also involves the RegAB two-component
299 system, which is redox-sensing and involved in redox homeostasis (56). Our results show that
300 RegAB is not a major player in mediating up-regulation of the *nap* promoter by growth on butyrate,
301 but instead negatively regulates *nap* expression during anaerobic growth. The response regulator
302 RegA very likely binds to an inverted repeat in the *napE* promoter that was initially characterized
303 in the closely related organism *P. pantotrophus* and suggested to be the binding site for a
304 negatively acting regulator (48). Evidently, regulation of the *napE* promoter by carbon substrate
305 and by oxygen availability can be separated, with the former requiring DksA1$_{Pd}$ and ppGpp and
306 the latter RegAB. Interestingly, RegAB also contribute (either directly or indirectly) to the
307 upregulation of the membrane associated nitrate reductase under anaerobic growth conditions.
308 Thus, NAP and Nar are reciprocally regulated by oxygen availability to be the dominant nitrate
309 reductases under aerobic and anaerobic conditions, respectively.

310 A recent study has shown that RegAB acts both positively and negatively under hypoxic
311 conditions in *Burkholderia pseudomallei*, an α-proteobacterium quite closely related to *P.*
312 *denitrificans* (57). Specifically, RegAB are required for upregulation of Nar under hypoxic
313 conditions in this organism, though this may involve both direct regulation by RegA and indirect
314 regulation, via FNR (57). Indirect effects of RegAB via NarR and/or FnrP are also possible in *P.*
315 *denitrificans*; we are able to discern a potential RegA binding site in the *narR-narK* intergenic
316 region that controls expression of the *narGHJI* genes encoding Nar (50).

317 In *E. coli*, DksA and ppGpp contribute to regulation of the *hmp* gene encoding the
318 flavohemoglobin, Hmp, which is responsible for detoxifying nitric oxide (11, 58, 59). In *P.*

319 *denitrificans*, we find that deletion of *dksA2* or *relA* leads to modestly increased *hmp* promoter
320 activity and dramatically reduced ability to form biofilm. Since the biofilm-defective phenotype
321 requires Hmp, we suggest that reduced expression of *hmp* in the mutants causes a reduced
322 accumulation of nitric oxide, which stimulates biofilm formation in this organism (2). Reduced
323 growth as biofilm in *dksA* mutants has been reported for other organisms, though the regulatory
324 mechanisms are likely different (26, 60).

325 Our study has identified two functional DksA proteins in *P. denitrificans* that have distinct
326 roles in growth on reduced carbon substrates, aerobic and anaerobic nitrate respiration, expression
327 of flavohemoglobin, and biofilm formation. In other α-proteobacteria which have at least two
328 DksA homologs, physiological roles have so far been ascribed to only one of them (23, 24). Further
329 functional analysis of the *P. denitrificans* DksA-like proteins and of the role of ppGpp will advance
330 our broader understanding of DksA functions in α-proteobacteria.

331

332 **MATERIALS AND METHODS**

333 **Bacterial strains and growth conditions**. The bacterial strains and plasmids used are listed in
334 Table S1. All *Paracoccus denitrificans* strains were derived from the wild-type Pd1222 (61).
335 *Escherichia coli* S.17-1 was used as the donor and *E. coli* DH5α (pRK2013) as the helper strain
336 for the introduction of plasmids into *P. denitrificans* by conjugation (62, 63). *E. coli* Novablue
337 competent cells (Novagen) were used as the host for all genetic manipulations. The rich medium
338 used for routine growth was LB (Miller) Broth (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl),
339 and the defined minimal medium (MM) for *P. denitrificans* was as previously described (64),
340 solidified with 1.5 % agar (w/v) as needed and supplemented with carbon and energy sources and
341 with 20 mM nitrate as indicated. For growth assays, *P. denitrificans* strains were first streaked on

342 LB agar plates, single colonies were inoculated into LB broth and grown overnight in 15 ml tubes
343 at 250 rpm. These cells were washed with MM, inoculated into succinate-supplemented MM, and
344 grown overnight in 15 ml tubes at 250 rpm. Cells were pelleted, washed, and finally inoculated
345 into MM supplemented with different carbon sources with or without nitrate. For aerobic growth
346 assays, cultures were grown in triplicate wells in a BioTek plate reader with horizontal orbital
347 shaking at 280 rpm and a volume of 0.2 ml (<50% of total volume) to ensure proper aeration while
348 preventing cross contamination. The optical density was measured at 600 nm and adjusted for path
349 length variation. Serine hydroxamate was added directly from a freshly made 100 mM stock to the
350 growth medium to a final concentration of 3 mM at the time of inoculation, where indicated.

351 Anaerobic cultures for β-galactosidase assays were grown in filled glass cuvettes sealed
352 with a cap and septum, with medium supplemented with 20 mM nitrate. The cuvettes were gassed
353 with N_2 and incubated statically. Aerobic cultures were grown in 250 ml flasks shaking at 250 rpm
354 in MM supplemented with the indicated carbon substrate and with or without 20 mM nitrate. For
355 *E. coli* complementation assays, solid M9 medium (65) with 10 mM glucose was used, with or
356 without 0.2 % (w/v) casamino acids.

357 Plasmids were maintained by addition of antibiotics at the following concentrations:
358 ampicillin 100 µg/ml, kanamycin 25 µg/ml, rifampicin 100 µg/ml, tetracycline 2 µg/ml for *P.*
359 *denitrificans* and 15 µg/ml for *E. coli*. All *P. denitrificans* strains were grown at 30 ºC and *E. coli*
360 at 37 ºC.

361 **Genetic manipulations.** All primers used are listed in Table S2. Unmarked deletions were made
362 by allelic replacement using pK18*mobsacB* as described by Sullivan et al. with minor
363 modifications (66, 67). Briefly, in-frame marker-less deletions were constructed by PCR
364 amplification of ~500-1000 bp fragments from either side of the gene of interest. The two

365 amplicons were ligated together either by PCR using overlapping regions added to the primers or
366 by digestion and ligation using a restriction site incorporated in the primers (68). The ligated
367 fragment was first cloned into pSTBlue-1 (Novagen), then sub-cloned into pK18*mobsacB*,
368 transformed into *E. coli* S.17-1 and mobilized into *P. denitrificans* by conjugation. Triparental
369 mating was performed by mixing *P. denitrificans*, *E. coli* S.17-1 harboring recombinant
370 pK18*mobsacB* or pMP220 or pIND4 derivatives, and *E. coli* DH5α (pRK2013) cells grown to log
371 phase, in a 2:1:1 ratio and by incubation on LB agar plate for 2-3 days at 30 ºC. Single-crossover
372 recombination events were selected on rifampicin and kanamycin plates, and then recombinants
373 with double crossovers were isolated by growth on 6 % (w/v) sucrose plates and kanamycin
374 sensitivity as described by Schafer et al. (66). All mutations were designed to be in-frame deletions
375 to prevent polarity. Promoter-*lacZ* reporter fusions were constructed by amplifying ~300-400 bp
376 upstream of the start codon of genes of interest and first cloning into pSTBlue-1. The sequence
377 was verified and the fragment cloned into the low copy number vector pMP220 between *Eco*RI
378 and *Pst*I restriction sites (69). Plasmids were introduced into *P. denitrificans* by conjugation from
379 *E. coli* S.17-1. *P. denitrificans* exconjugants were selected by rifampicin and tetracycline
380 resistance. For complementation tests in *P. denitrificans*, the gene of interest with its native
381 promoter was amplified and cloned directly into pIND4 (70) and the sequence verified. The
382 cloning was done using the NEBuilder HiFi DNA assembly cloning kit (NEB). Plasmids were
383 transformed into *E. coli* S.17-1 and mobilized into *P. denitrificans* strains by conjugation as
384 described above.

385 **β-galactosidase assay:** Liquid cultures were grown aerobically and anaerobically in minimal
386 medium with the indicated carbon sources and with or without 20 mM nitrate. Cells were harvested
387 at the mid-log phase of growth and the assay was performed as described by Miller (71). B-

388 galactosidase activity was measured using 0.1 ml of cells made permeable by treatment with
389 sodium dodecyl sulfate and chloroform. In the case of the *hmp-lacZ* fusion, strains were grown to
390 mid-log phase in the same medium and growth conditions as used for the biofilm assays.

391 **Biofilm assays:** Attached growth was assayed in 60 mm x 15 mm polystyrene petri dishes (2). A
392 70 µl aliquot of overnight culture was inoculated into 7 ml of LB broth supplemented with 10 mM
393 calcium chloride. The petri dishes were incubated statically at 30 ºC for 72 hours. The plates were
394 then washed thrice under gently running tap water and stained with crystal violet (0.1 % in water)
395 for 20 minutes, washed three times with water, and air dried. For quantitation, the crystal violet
396 stain was extracted into 3 ml 95 % ethanol, diluted as necessary, and absorbance measured at 595
397 nm (2, 72).

398 **Cell fractionation:** *P. denitrificans* strains were grown aerobically and anaerobically in MM with
399 either succinate (10 mM) or butyrate (10 mM) as the sole carbon source; nitrate (20 mM) was
400 added as indicated. Aerobic cultures (50 ml) were grown at 30 ºC in 250 ml conical flasks shaken
401 at 250 rpm. Anaerobic cultures were grown in 100 ml bottles filled to the neck with medium and
402 incubated at 30 ºC without shaking. Periplasmic fractions for the nitrate reductase assay were
403 prepared as described in Quan et al (73) . In short, mid-exponential phase cells were harvested by
404 centrifugation at 3,000 *g* for 20 minutes at 4 ºC. Cells were then gently resuspended in TSE buffer
405 (200 mM Tris–HCl, pH 8.0, 500 mM sucrose, 1 mM EDTA without any protease inhibitor) and
406 incubated on ice for 30 minutes, and centrifuged at 16,000 *g* for 30 minutes at 4 ºC. The supernatant
407 was transferred to a fresh tube and used as the periplasmic fraction. Membrane fractions were
408 prepared according to Dunn et al. with some modifications (74). Briefly, cultures were harvested
409 in the mid-exponential phase of growth by centrifugation at 8,000 *g* for 5 minutes at 4 ºC and
410 washed twice in 10 mM potassium phosphate buffer (pH 7.5) in 50 ml Falcon tubes. Cells were

411 resuspended in 2.5 ml of ice cold 10 mM phosphate buffer, 10 mM $MgSO_4$, 0.2 M NaCl. Samples
412 were sonicated on ice using a Fisher Scientific Sonic Dismembrator model 500 outfitted with a 3
413 mm diameter tip at 20 % amplitude (10 W) for five 20 second bursts at 1 minute intervals. To
414 clarify the lysate, samples were centrifuged at 16,000 *g* for 20 minutes at 4 ºC. The clarified total
415 lysate was further centrifuged at 100,000 *g* for 2 hours at 4 ºC. The pellet was suspended in 0.2 ml
416 of phosphate buffer and retained as the membrane fraction for the enzyme assay. Fractions were
417 kept on ice and used within hours of preparation.

418 **Enzyme activity:** Nitrate reductase activity was determined spectrophotometrically using reduced
419 methylviologen (MV^+) as the electron donor and 10 mM sodium nitrate as the electron acceptor
420 (45). The extinction coefficient of reduced methylviologen used was 8.25 mM^{-1} cm^{-1} at 600 nm
421 (75). The protein content of the periplasmic and membrane fractions was measured by the protein
422 estimation method of Lowry et al., using bovine serum albumin as the standard (76).

423

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427

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652

653 **FIGURE LEGENDS**

654

655 **FIGURE 1.**

656 *P. denitrificans* $dksA1_{Pd}$ and $dksA2_{Pd}$ complement an *E. coli* Δ*dksA* mutant (Δ$dksA_{EC}$). Strains
657 were streaked on M9 minimal (M9) agar plates containing 10 mM glucose (G) with or without
658 0.2% (w/v) casamino acids (CAA). Plates were incubated overnight at 37 ºC. 1 - *E. coli*
659 MG1655; 2 - Δ$dksA_{EC}$ complemented with $dksA1_{Pd}$; 3 - Δ$dksA_{EC}$ complemented with $dksA2_{Pd}$; 4 -
660 Δ$dksA_{EC}$ carrying empty pIND4.

661

662 **FIGURE 2.**

663 Deletion of *dksA1* impairs nitrate utilization during aerobic growth. Aerobic growth of the
664 indicated strains in: (A) minimal medium containing succinate or (B) minimal medium
665 containing butyrate, with (+ NO_3^-) or without (- NO_3^-) 20 mM nitrate. Optical density was
666 measured (at 600 nm) at the indicated time points (the means from three independent
667 experiments).

668

669 **FIGURE 3.**

670 Loss of both *dksA1* and *relA* leads to failure to grow on reduced carbon substrates. Aerobic growth
671 of indicated strains in liquid minimal medium with butyrate (A) without (- NO_3^-) or (B) with 20

672 mM nitrate (+ NO_3^-). Optical density was measured (at 600 nm) at the indicated time points (the
673 means from three independent experiments).

674

675 **FIGURE 4.**

676 The Δ*dksA1* mutant has reduced *nap* promoter and NAP enzyme activity. (A) β-galactosidase
677 activity in *P. denitrificans* wild-type, Δ*dksA1* and Δ*relA* carrying plasmid p*nap-lacZ*. (B) NAP
678 enzyme activity in the periplasmic fractions of *P. denitrificans* wild-type, Δ*dksA1* and Δ*relA* cells.
679 Cultures were grown in minimal medium containing 10 mM succinate (S) or butyrate (B) as sole
680 carbon and energy source with or without 20 mM nitrate (N). Aerobic (+O_2) cultures were grown
681 in shake flasks at 250 rpm. Anaerobic (-O_2) cultures were grown statically in filled closed cap
682 bottles. The bars represent the mean of at least three separate experiments and the error bars
683 represent the standard deviation of the mean. Asterisks on the bars indicate a significant difference
684 compared to the wild-type within the group according to Student's t-test (*$P \leq 0.05$, **$P \leq 0.01$,
685 ***$P \leq 0.001$).

686

687 **FIGURE 5.**

688 The RegAB system regulates anaerobic *nap* promoter and NAP activity. (A) β-galactosidase
689 activity in *P. denitrificans* wild-type, Δ*regA* and Δ*regB* carrying plasmid p*nap-lacZ*. (B) NAP
690 enzyme activity in the periplasmic fractions of anaerobically grown *P. denitrificans* wild-type,
691 Δ*regA* and Δ*regB* cells. (C) NAR enzyme activity in the membrane fractions of *P. denitrificans*
692 wild-type, Δ*regA* and Δ*regB* cells. Cultures were grown in minimal medium containing 10 mM
693 succinate (S) or butyrate (B) as sole carbon source with or without 20 mM nitrate (N). Aerobic

694 (+O_2) cultures were grown in shake flasks at 250 rpm. Anaerobic (-O_2) cultures were grown in
695 filled closed cap bottles statically. The bars represent the mean of at least three separate
696 experiments and the error bars represent the standard deviation of the mean. Asterisks on the bars
697 indicate a significant difference compared to the wild-type within the group according to Student's
698 t-test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

699

700 **FIGURE 6.**

701 The *relA* gene is required for the response to amino acid starvation in *P. denitrificans.* Aerobic
702 growth of indicated strains in minimal medium containing: (A) succinate only, (B) succinate and
703 3 mM serine hydroxamate, and (C) succinate, 3 mM serine hydroxamate and 0.2% (w/v) casamino
704 acids. Optical density was measured (at 600 nm) at the indicated time points (the means from three
705 independent experiments).

706

707 **FIGURE 7.**

708 DksA2$_{Pd}$ and RSH regulate *hmp* promoter activity and biofilm formation in *P. denitrificans*. (A)
709 β-galactosidase activities in *P. denitrificans* wild-type, Δ*dksA2* and Δ*relA* carrying plasmid p*hmp-*
710 *lacZ*. (B) Assays of biofilm formation by the wild-type, deletion, and complemented strains.
711 Dishes were washed, stained with crystal violet, and photographed from above. (C) The crystal
712 violet was extracted with 95% ethanol, and the absorbance measured at 595 nm. Means and
713 standard deviations were calculated from results of three separate assays. Cultures were grown in
714 6-cm-diameter petri dishes in liquid L broth (Miller), supplemented with 10 mM $CaCl_2$ and
715 incubated without shaking at 30 ºC. Cells were harvested at mid-log phase for the β-galactosidase

716 assay or left undisturbed for 72 hours for the biofilm assay. Asterisks on the bars indicate a
717 significant difference compared to the wild-type according to Student's t-test (**P $\leq$ 0.01, ***P
718 $\leq$ 0.001).

Fig. 1

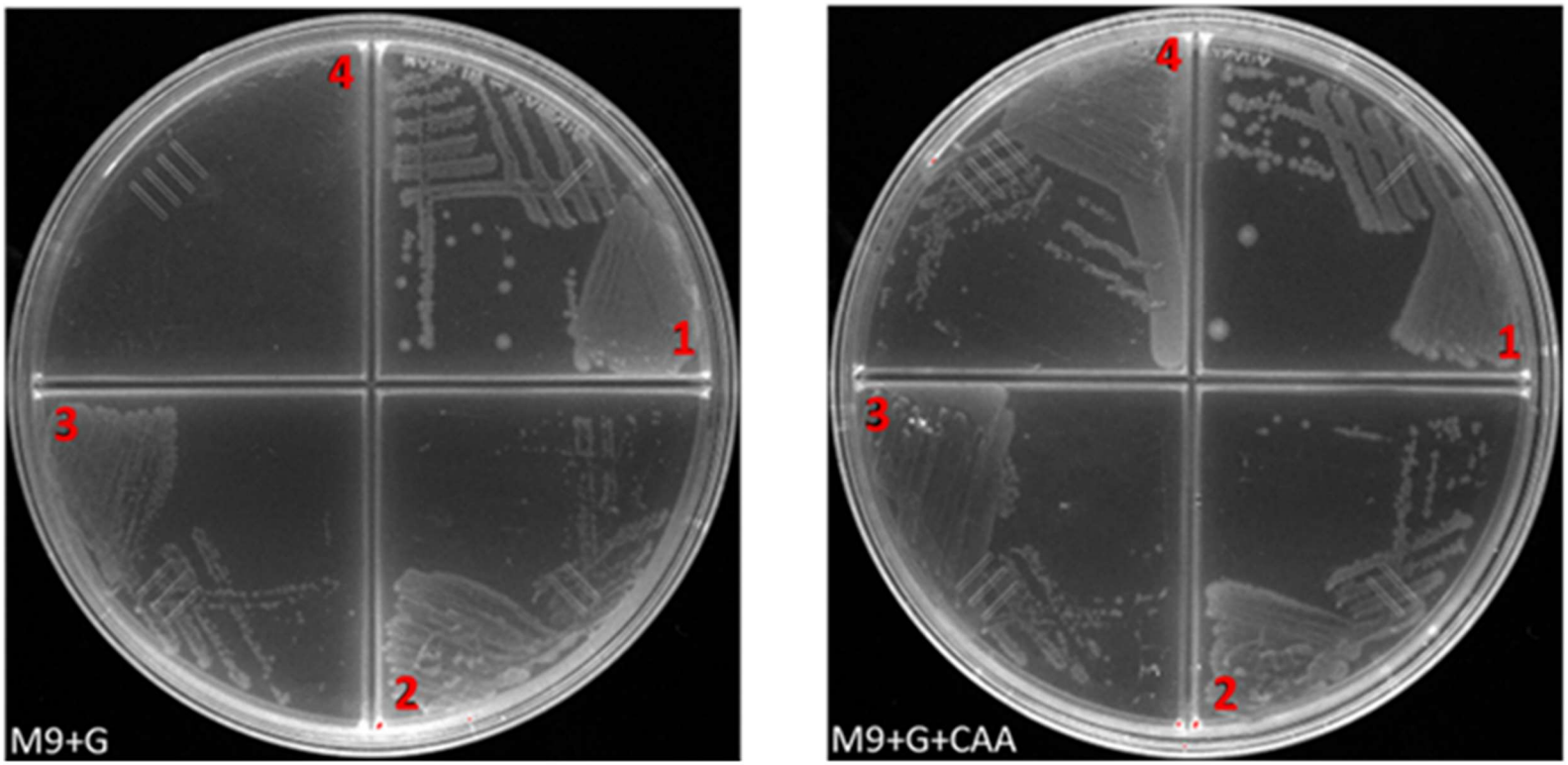


Figure 1. *P. denitrificans dksA1*$_{Pd}$ and *dksA2*$_{Pd}$ complement an *E. coli* Δ*dksA* mutant (Δ*dksA*$_{EC}$). Strains were streaked on M9 minimal (M9) agar plates containing 10 mM glucose (G) with or without 0.2% (w/v) casamino acids (CAA). Plates were incubated overnight at 37 ºC. 1 - *E. coli* MG1655; 2 - Δ*dksA*$_{EC}$ complemented with *dksA1*$_{Pd}$; 3 - Δ*dksA*$_{EC}$ complemented with *dksA2*$_{Pd}$; 4 - Δ*dksA*$_{EC}$ carrying empty pIND4.

Fig. 2

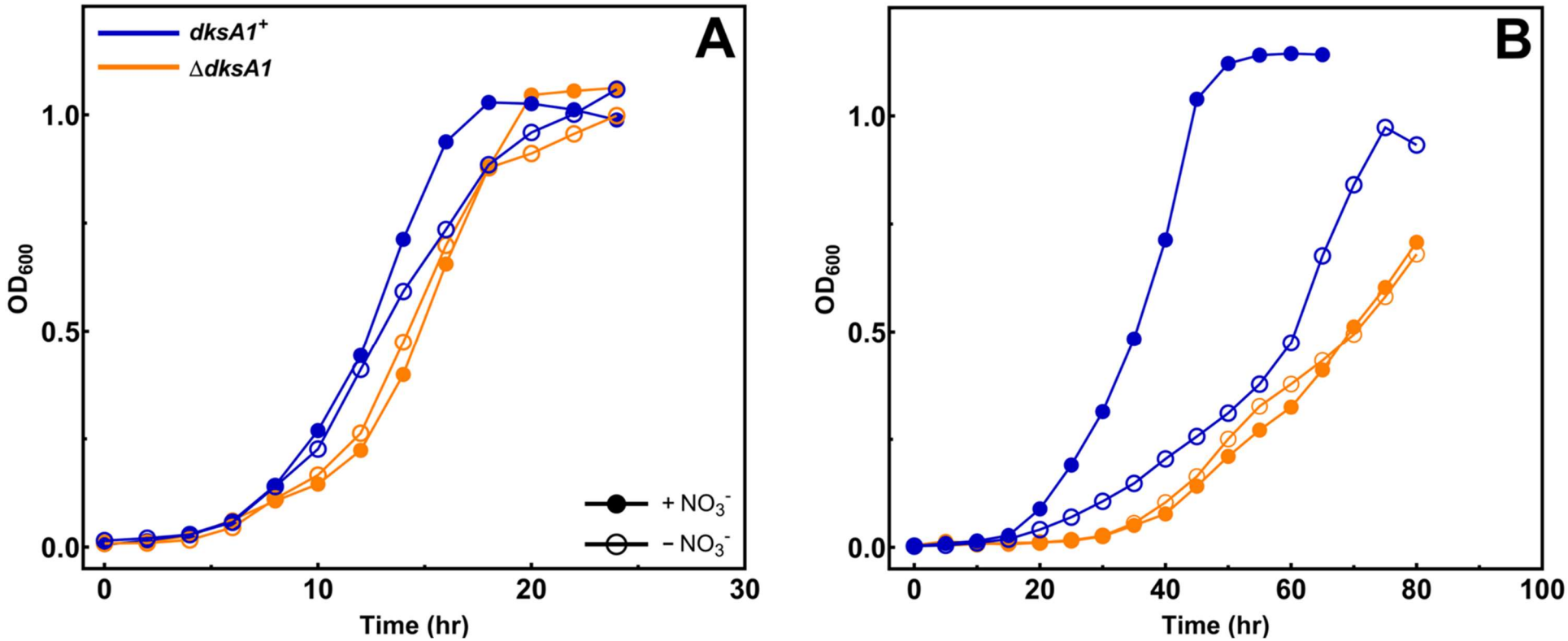


Figure 2. Deletion of *dksA1* impairs nitrate utilization during aerobic growth. Aerobic growth of indicated strains in, **(A)** minimal medium containing succinate or **(B)** minimal medium containing butyrate, with (+ NO_3^-) or without (- NO_3^-) 20 mM nitrate. Optical density was measured (at 600 nm) at the indicated time points (the means from three independent experiments).

Fig. 3

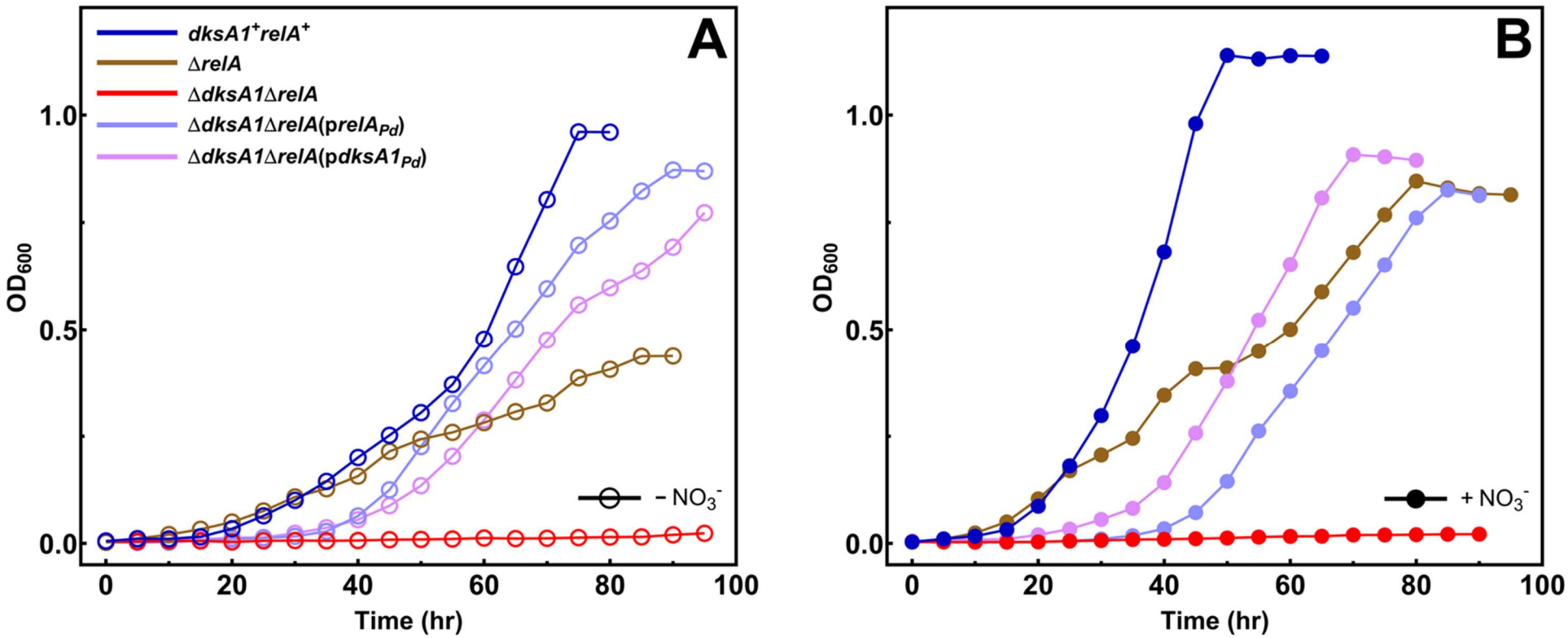


Figure 3. Loss of both *dksA1* and *relA* leads to failure to grow on reduced carbon substrates. Aerobic growth of indicated strains in liquid minimal medium with butyrate **(A)** without (- NO_3^-) or **(B)** with 20 mM nitrate (+ NO_3^-). Optical density was measured (at 600 nm) at the indicated time points (the means from three independent experiments).

Fig. 4

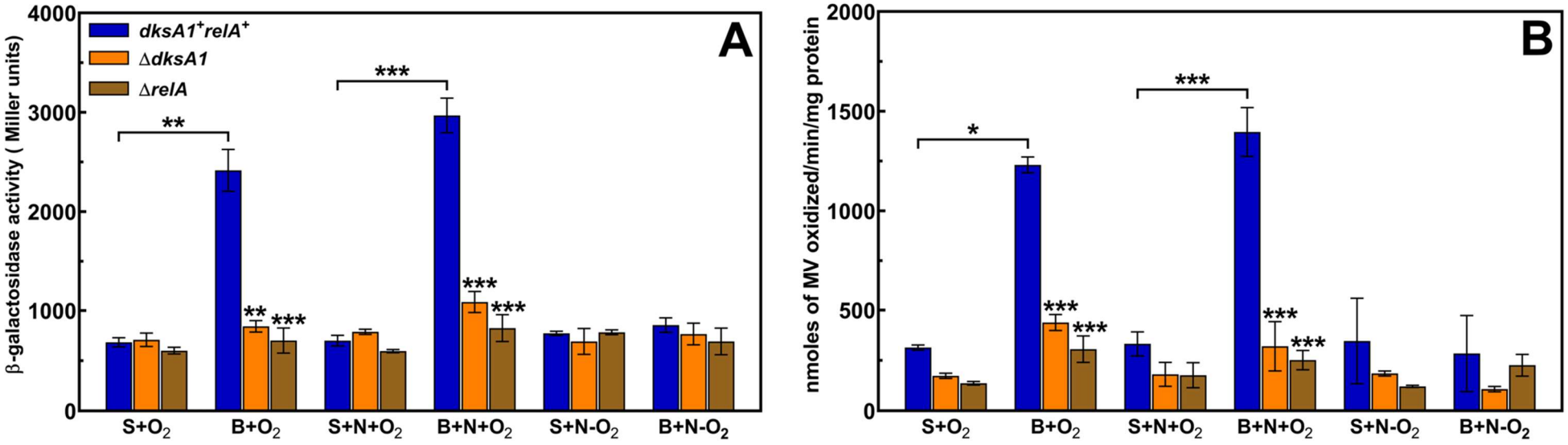


Figure 4. The Δ*dksA1* mutant has reduced *nap* promoter and NAP enzyme activity. (A) β-galactosidase activity in *P. denitrificans* wild-type, Δ*dksA1* and Δ*relA* carrying plasmid p*nap-lacZ*. **(B)** NAP enzyme activity in the periplasmic fraction of *P. denitrificans* wild-type, Δ*dksA1* and Δ*relA* cells. Cultures were grown in minimal medium containing 10 mM succinate (S) or butyrate (B) as sole carbon and energy source with or without 20 mM nitrate (N). Aerobic ($+O_2$) cultures were grown in shake flasks at 250 rpm. Anaerobic ($-O_2$) cultures were grown statically in filled closed cap bottles. The bars represent the mean of at least three separate experiments and the error bars represent the standard deviation of the mean. Asterisks on the bars indicate a significant difference compared to the wild-type within the group according to Student's t-test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

Main Figures

Fig. 5

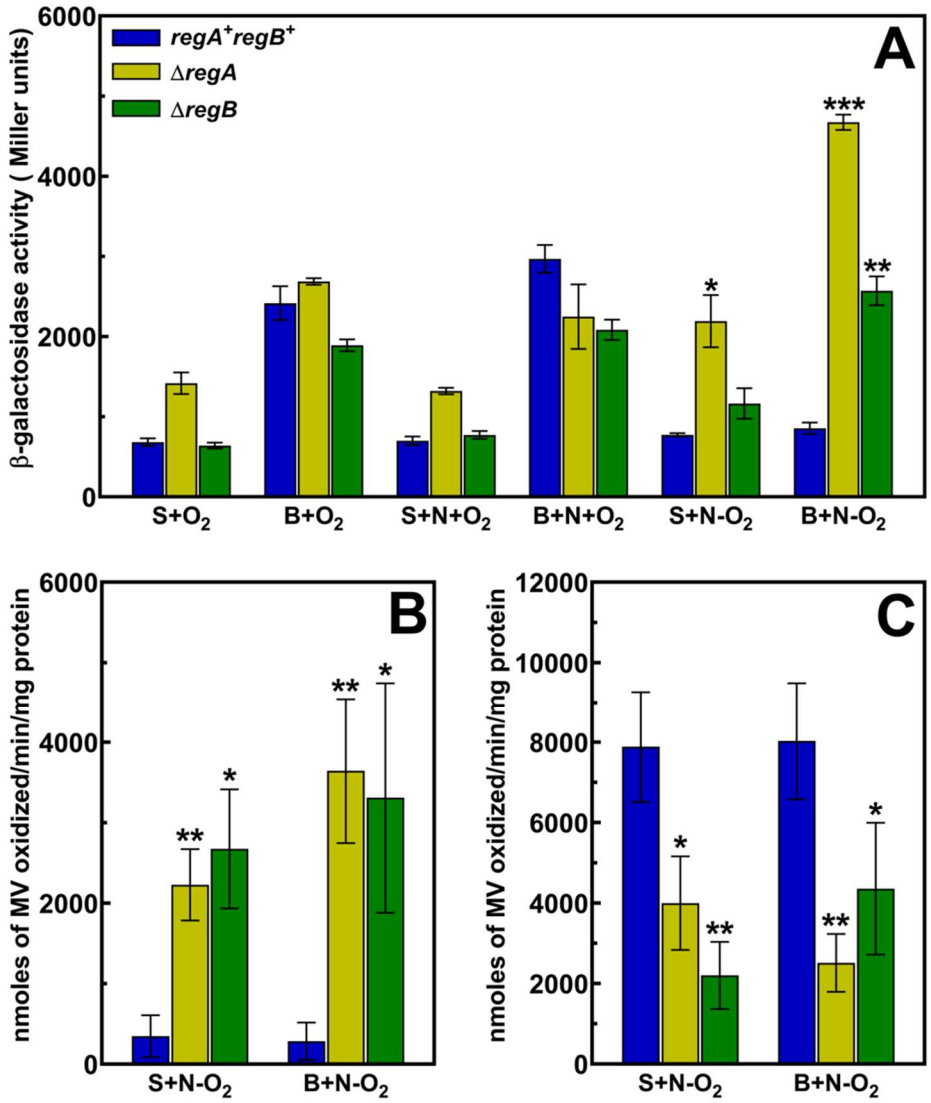


Figure 5. The RegAB system regulates anaerobic *nap* promoter and NAP activity. (A) β-galactosidase activity in *P. denitrificans* wild-type, Δ*regA* and Δ*regB* carrying plasmid p*nap-lacZ*. **(B)** NAP enzyme activity in the periplasmic fractions of anaerobically grown *P. denitrificans* wild-type, Δ*regA* and Δ*regB* cells. **(C)** NAR enzyme activity in the membrane fractions of *P. denitrificans* wild-type, Δ*regA* and Δ*regB* cells. Cultures were grown in minimal medium containing 10 mM succinate (S) or butyrate (B) as sole carbon source with or without 20 mM nitrate (N). Aerobic (+O_2) cultures were grown in shake flasks at 250 rpm. Anaerobic (-O_2) cultures were grown in filled closed cap bottles statically. The bars represent the mean of at least three separate experiments and the error bars represent the standard deviation of the mean. Asterisks on the bars indicate a significant difference compared to the wild-type within the group according to Student's t-test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

Fig. 6

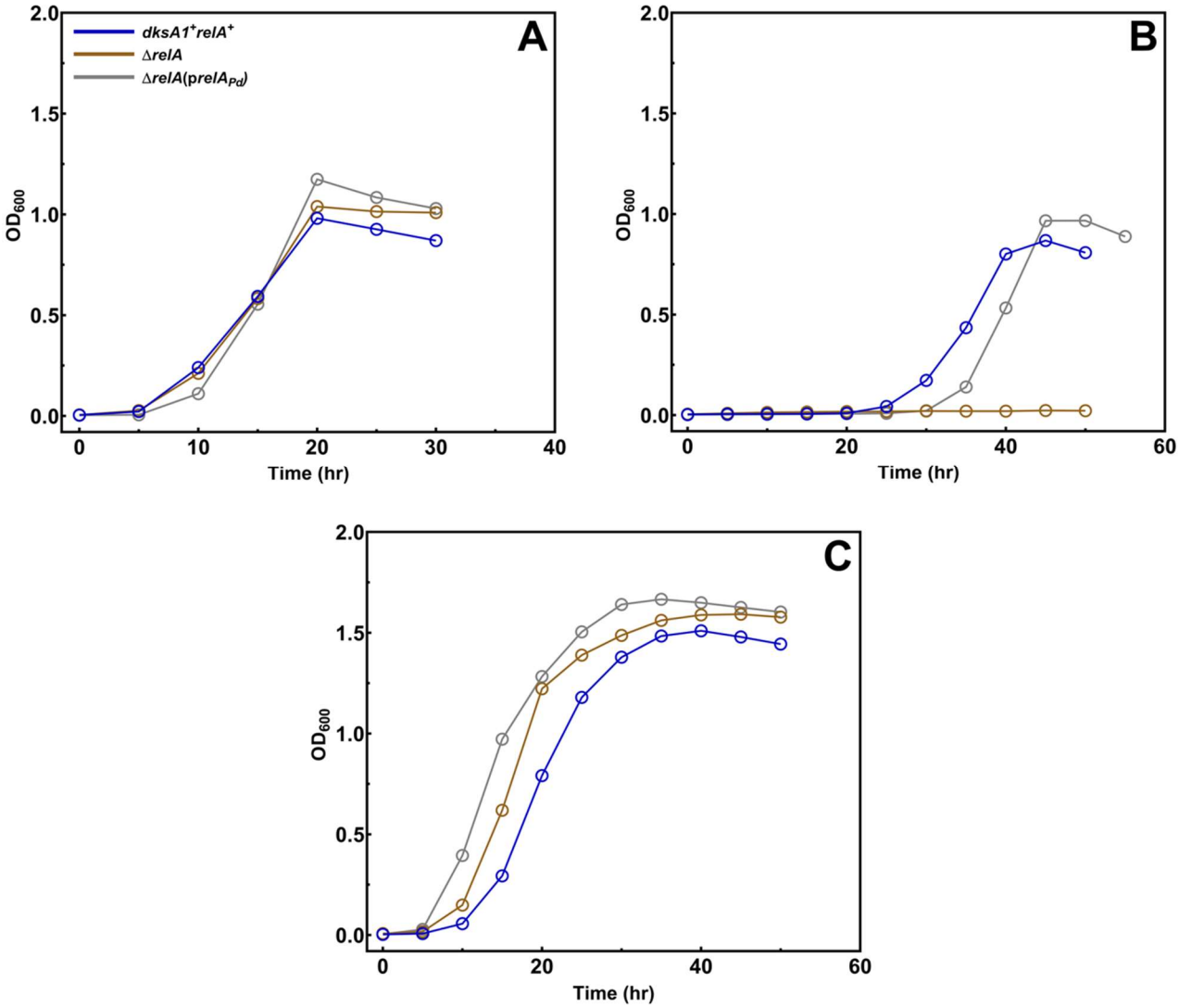
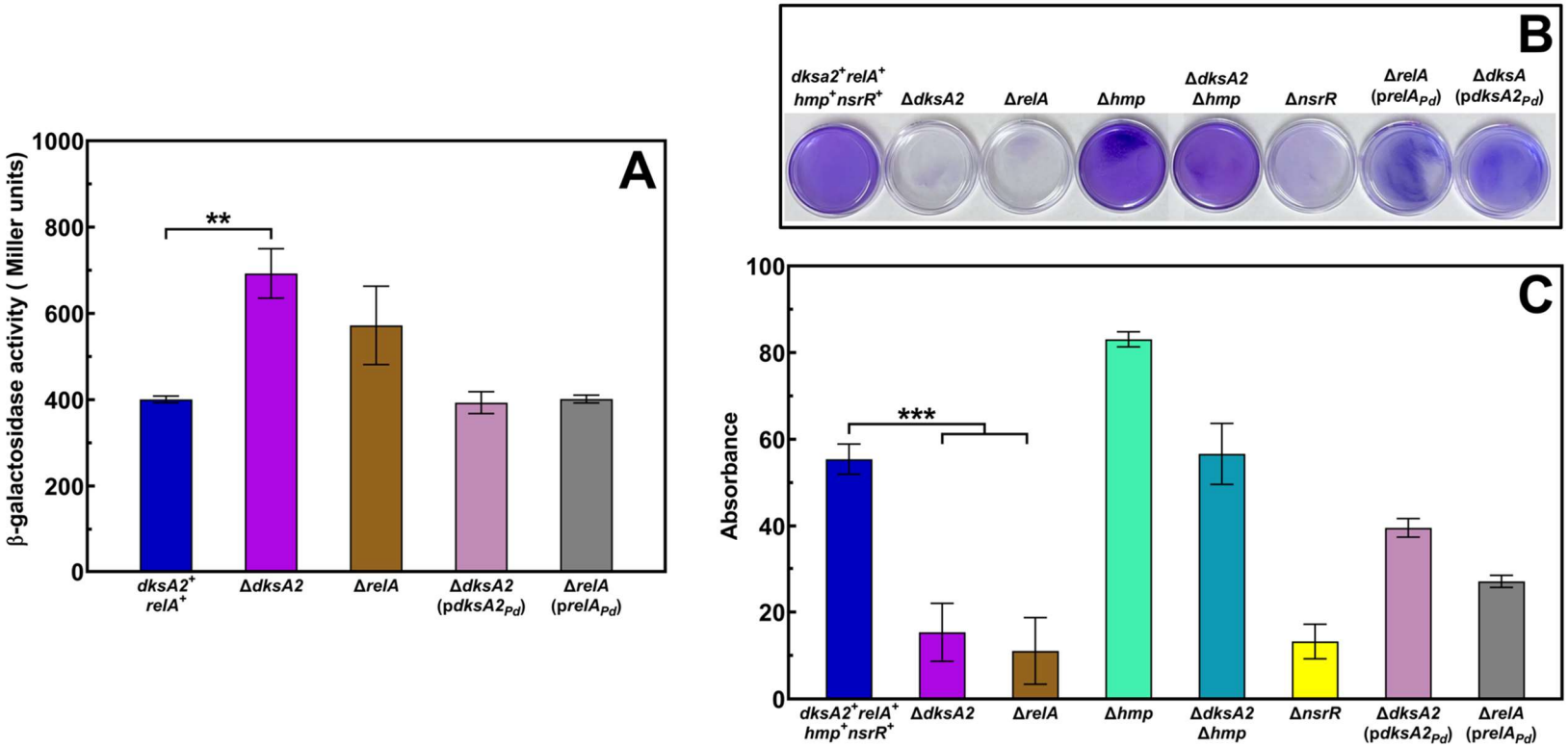


Figure 6. The *relA* gene is required for the response to amino acid starvation in *P. denitrificans.* Aerobic growth of indicated strains in minimal medium containing: **(A)** succinate only, **(B)** succinate and 3 mM serine hydroxamate, and **(C)** succinate, 3 mM serine hydroxamate and 0.2% (w/v) casamino acids. Optical density was measured (at 600 nm) at the indicated time points (the means from three independent experiments).

Main Figures

Fig. 7



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