DksA, ppGpp and RegAB regulate nitrate respiration in *Paracoccus denitrificans*

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ABSTRACT

The periplasmic (NAP) and membrane-associated (Nar) nitrate reductases of *Paracoccus denitrificans* are responsible for nitrate reduction under aerobic and anaerobic conditions, respectively. Expression of NAP is elevated in cells grown on a relatively reduced carbon and energy source (such as butyrate); it is believed that NAP contributes to redox homeostasis by coupling nitrate reduction to the disposal of excess reducing equivalents. Here we show that deletion of either *dksA1* (encoding one of two *dksA* homologs in the *P. denitrificans* genome) or *relA*/*spoT* (encoding a bifunctional ppGpp synthetase and hydrolase) eliminates the butyrate-dependent increase in *nap* promoter and NAP enzyme activity. We conclude that ppGpp likely signals growth on a reduced substrate and, together with DksA1, mediates increased expression of the genes encoding NAP. Support for this model comes from the observation that *nap* promoter activity is increased in cultures exposed to a protein synthesis inhibitor that is known to trigger ppGpp synthesis in other organisms. We also show that, under anaerobic growth conditions, the redox-sensing RegAB two-component pair acts as a negative regulator of NAP expression and as a positive regulator of expression of the membrane-associated nitrate reductase Nar. The *dksA1* and *relA*/*spoT* 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. We also show that the second *dksA* homolog (*dksA2*) and *relA*/*spoT* have roles in regulation of expression of the flavohemoglobin Hmp and in biofilm formation.

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 acceptor for anaerobic respiration, reducing it to dinitrogen via nitrite, nitric oxide, and nitrous oxide. This pathway (known as denitrification) is important as a route for loss of fixed nitrogen from soil and as a source of the greenhouse gas nitrous oxide. Thus, it is important to understand

48 those environmental and genetic factors that govern flux through the denitrification pathway. Here
49 we identify four proteins and a small molecule (ppGpp) which function as previously unknown
50 regulators of expression of enzymes that reduce nitrate and oxidize nitric oxide.
51

52 **INTRODUCTION**

53 *Paracoccus denitrificans* is a Gram-negative α-proteobacterium and a facultative anaerobe that
54 can respire using nitrogen oxyanions and oxides as terminal electron acceptors in an anaerobic
55 process known as denitrification (1). In the first step of denitrification, nitrate is reduced to nitrite
56 by the membrane-associated nitrate reductase, Nar (2). *P. denitrificans* also expresses a
57 periplasmic nitrate reductase, NAP, which makes a smaller contribution to the generation of a
58 proton motive force than does Nar (2). Maximal expression of NAP occurs during aerobic growth
59 on relatively reduced substrates, and it is thought that NAP facilitates redox balancing by allowing
60 for the disposal of excess reducing equivalents (3–5).

61 In the denitrification pathway, the toxic intermediate nitric oxide (NO) is generated from
62 nitrite by the respiratory nitrite reductase and is subsequently reduced to nitrous oxide (N_2O) by
63 the respiratory NO reductase. *P. denitrificans* also has an *hmp* gene encoding a flavohemoglobin
64 (Hmp) that potentially contributes to the metabolism of NO (6, 7). NO acts in conjunction with
65 transcriptional regulators as an important signal molecule to regulate gene expression during the
66 transition from aerobic respiration to denitrification (8, 9). Pathogenic strains of *Salmonella* and
67 *E. coli* have several transcriptional regulators which control the expression of genes encoding
68 enzymes that scavenge NO. These regulators and their target genes may help to protect the bacteria
69 against the NO that is made by host phagocytes (10–12). One such transcriptional regulator is

70 DksA, which acts as a thiol based NO sensor in *Salmonella* and regulates detoxification of NO
71 through Hmp in *E. coli* (13–15).

72 DksA, first described in *E. coli* as DnaK suppressor A (16) is a founder member of
73 TraR/DksA family of transcriptional regulators (17, 18). $DksA_{Ec}$ (*E. coli* DksA) is 151 amino acids
74 long and consists of three major domains (Fig. S1): a coiled-coil (CC) domain with a conserved
75 DXXDXA motif at its tip, a globular domain with a conserved four-cysteine Zn-finger motif, and
76 a C-terminal α-helical domain (19). The conserved acidic residues located at the tip of the CC
77 domain in $DksA_{Ec}$ are critical for binding to RNA polymerase (RNAP) and for $DksA_{Ec}$ to function
78 as a transcriptional regulator (20, 21). The globular domain has been shown to interact with the
79 rim of the secondary channel of RNAP (22), and the conserved redox-sensitive cysteines act as a
80 sensor for NO in *Salmonella* (13). Some bacterial genomes encode multiple homologs of DksA,
81 which differ in the presence or absence of a complete DXXDXA motif or the presence of one, two,
82 or four conserved cysteines in the Zn-finger motif (23). For instance, *Pseudomonas aeruginosa*
83 has two functional homologs, DksA1 and DksA2 (24). DksA1 has the conserved four cysteine Zn-
84 finger motif whereas DksA2 does not (25, 26). In α-proteobacteria more closely related to *P.*
85 *denitrificans*, such as *Sinorhizobium meliloti* and *Rhodobacter sphaeroides*, the assigned *dksA*
86 gene encodes a protein with a complete DXXDXA motif and only one cysteine in the Zn-finger
87 motif (27, 28). Deletion or overexpression of *dksA* in various species has pleiotropic effects on
88 multiple physiological processes. These include amino acid biosynthesis (29), motility (30),
89 biofilm formation (30), plant symbiosis (27), DNA repair (31), quorum sensing and virulence (32)
90 and oxidative and nitrosative damage (13, 15).

91 DksA, along with the alarmone guanosine-5', 3'-tetraphosphate (ppGpp) is known to alter
92 global gene expression to adjust cellular metabolism in response to nutritional stress, a mechanism

93 known as the stringent response (when triggered by amino acid starvation) in *E. coli* and other
94 related γ-proteobacteria (33–35). ppGpp is found throughout the bacterial kingdom as a second
95 messenger and is associated with various stress response mechanisms and with growth rate
96 regulation (33). The metabolism of ppGpp is controlled by two enzymes encoded by *relA* and *spoT*
97 (36, 37). RelA is a ribosome-associated ppGpp synthetase, whereas SpoT is a bifunctional enzyme
98 responsible for both synthesis and hydrolysis of ppGpp. The presence of both *relA* and *spoT* is
99 characteristic of γ-proteobacteria, while only one RelA/SpoT bifunctional homolog (RSH)
100 possessing both conserved ppGpp synthetase and hydrolase domains is typically present in α-
101 proteobacteria (2, 38). While DksA and ppGpp coregulate many genes, some phenotypic and
102 transcriptomic studies report independent effects of both DksA and ppGpp (39, 40). The
103 independent effects of DksA and ppGpp might be attributed to their direct binding to RNAP;
104 ppGpp is also a direct effector of a number of metabolic enzymes (41–43)

105 In *P. denitrificans* Pd1222, three gene products have been annotated as members of the
106 TraR/DksA family: Pden_0547 (which we designate $DksA1_{Pd}$), Pden_0916 ($DksA2_{Pd}$), and
107 Pden_1862, which are 45%, 26% and 30% identical to *E. coli* DksA, respectively. The *P.*
108 *denitrificans* proteins are more similar to their homologs from related α-proteobacteria. $DksA1_{Pd}$,
109 $DksA2_{Pd}$, and Pden_1862 are 90%, 64%, and 33% identical to $DksA_{Rsp}$ (*R. sphaeroides* DksA),
110 respectively, while they are 64%, 47%, and 36% identical to $DksA_{Sm}$ (*S. meliloti* DksA),
111 respectively. *P. denitrificans* also has a single *relA*/*spoT* gene: Pden_1400 (henceforward called
112 *rel*) which encodes for a RelA/SpoT bifunctional homolog (RSH). The predicted RSH is 705
113 amino acids long and possesses a phosphohydrolase HD domain with a conserved HDXXED motif
114 in the N-terminal region, a RelA-SpoT region, and conserved TGS and ACT domains in the C-
115 terminal domain (Fig. S2). On the basis of sequence similarity, Pden_1400 is predicted to have

116 both ppGpp synthetase and hydrolase activities. None of these *P. denitrificans* genes and proteins
117 has been functionally characterized. A proteomic analysis addressing aerobic to anaerobic growth
118 adaptation in *P. denitrificans* demonstrated a significant increase in expression of $DksA1_{Pd}$
119 (A1AZG5) under anaerobic conditions. Another study using microarray analysis showed a 3.4-
120 fold higher expression of *dksA2* during anaerobic growth (44, 45). Our attention was drawn to the
121 DksA homologs of *P. denitrificans* because they are differentially expressed under denitrifying
122 conditions, and by the report that DksA regulates *hmp* expression in *E. coli* (15). Our goal for the
123 work described here was therefore to use a reverse-genetics approach in an effort to identify
124 physiological roles for the two DksA proteins and for RSH.

125 In this work, we report distinct phenotypes associated with deletion of two of the *dksA*-like
126 genes and the *relA/spoT* homolog in *P. denitrificans* Pd1222. We show that deletion of *dksA1*
127 causes an aerobic growth defect on reduced carbon substrates such as butyrate and that *dksA1* and
128 *rel* null strains fail to up-regulate expression and activity of the periplasmic nitrate reductase NAP
129 under these conditions. Furthermore, we demonstrate that a *dksA1 rel* double mutant is unable to
130 grow on reduced carbon substrates. In addition, we show that *dksA2* is involved in biofilm
131 production, possibly indirectly via negative regulation of *hmp* transcription.

132

133 **RESULTS**

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

135 Sequence alignment of the three DksA/TraR proteins revealed that two (Pden_0547 and
136 Pden_0916) have a complete DXXDXA motif which is required for interaction with RNA
137 polymerase (20, 21) while the third (Pden_1862) does not. For this reason, our attention has
138 focused on Pden_0547 and Pden_0916, which we have designated *dksA1* and *dksA2*, respectively.

139 Unlike the canonical DksA of *E. coli*, $DksA1_{Pd}$ has only one cysteine conserved in the zinc finger
140 motif whereas $DksA2_{Pd}$ has all four cysteines (Fig. S1).

141 *E. coli* MG1655 deleted for *dksA* was complemented with either *dksA1* or *dksA2* cloned in
142 pIND4 and expressed from their native promoter. *E. coli* Δ*dksA* is auxotrophic for multiple amino
143 acids and fails to grow on M9-glucose medium without amino acid supplementation (15).
144 Expression of either *dksA1* or *dksA2* rescued growth of $\Delta dksA_{EC}$ on M9-glucose plates suggesting
145 that either gene may complement the *E. coli dksA* mutation (Fig. 1).

146 **Deletion of $dksA1_{Pd}$ impairs nitrate stimulation of aerobic growth on butyrate**

147 To characterize the *dksA*-like genes of *P. denitrificans*, we constructed and tested the growth of
148 deletion strains lacking *dksA1* or *dksA2*. Each mutant grew as well as the parent strain on rich (LB)
149 medium under both aerobic and anaerobic conditions (Fig. S3). We also did not observe amino
150 acid auxotrophy when the mutants were grown in succinate or glucose minimal medium (data not
151 shown). In *Salmonella enterica* sv. *typhimurium*, DksA is involved in redox homeostasis since it
152 regulates the expression of enzymes from central metabolic pathways that generate reduced
153 pyridine nucleotides (46). *P. denitrificans* may experience redox imbalance during growth on
154 reduced carbon substrates due to the generation of excess reducing equivalents (3). Therefore, we
155 assayed aerobic growth in minimal medium supplemented with succinate (MM-S) or butyrate
156 (MM-B) as sole carbon and energy sources that are relatively oxidized and reduced, respectively.
157 Growth of the wild-type strain in MM-B was significantly enhanced by the addition of nitrate to
158 the medium. Reduction of nitrate by the periplasmic nitrate reductase NAP provides a mechanism
159 to dispose of excess reducing equivalents during growth on reduced carbon substrates (4). The
160 Δ*dksA1* mutant grew normally on MM-S but showed slightly impaired growth on MM-B relative
161 to the wild type and growth on MM-B was not improved by addition of nitrate to the medium

162 (Fig. 2A and B). Expression of *dksA1* from a recombinant plasmid in the *dksA1* mutant restored a
163 wild type phenotype for growth on MM-B both in the presence and absence of nitrate (Fig. S4).
164 These phenotypes suggest that $DksA1_{Pd}$ may regulate aerobic nitrate reduction by NAP during
165 growth on reduced carbon substrates.

166 To determine if the Δ*dksA1* associated phenotype is ppGpp-dependent, we measured
167 growth of the Δ*rel* strain under similar conditions. The Δ*rel* mutant showed significantly reduced
168 growth in MM-B compared to wild type, however, unlike for Δ*dksA1*, growth was improved by
169 addition of nitrate to the medium (Fig. 3 and S5), though the magnitude of nitrate stimulation was
170 less than was observed in the wild-type strain. Complementation of the Δ*rel* mutant with the *rel*
171 gene cloned in a plasmid restored the wild type growth phenotypes on MM-B (Fig. S6).

172 **Δ*dksA1* and Δ*rel* mutants have reduced *napE* promoter activity and NAP enzyme activity**

173 It has been shown previously that the periplasmic nitrate reductase, NAP, has increased expression
174 and activity in cells grown aerobically on reduced energy sources (3, 5). Our results suggest that
175 the absence of *dksA1* impairs nitrate reduction in *P. denitrificans* during aerobic growth. We
176 hypothesized that $DksA1_{Pd}$ regulates expression of NAP during aerobic growth on reduced energy
177 sources. To test this hypothesis, we constructed a *napE-lacZ* promoter transcriptional fusion and
178 measured *napE* promoter activity using β-galactosidase as the reporter. Consistent with previous
179 reports (5), we observed a ~3-fold increase in *napE* promoter activity in cells grown aerobically in
180 MM-B compared to those grown in MM-S (Fig. 4A). The increase in *napE* promoter activity
181 caused by growth on butyrate was eliminated in the Δ*dksA1* mutant. The increase in *napE* promoter
182 activity in MM-B versus MM-S also disappeared when the cultures were grown anaerobically
183 (Fig. 4A). To confirm that these observations were due to loss of *dksA1,* we measured *napE*

184 promotor activity in the complemented Δ*dksA1* strains. Complementation with the *dksA1* gene
185 restored the butyrate stimulation of *napE* promotor activity (Fig. S7)

186 In parallel with the *napE* promoter activity assays we also measured the activity of NAP in
187 periplasmic fractions using reduced methylviologen as the electron donor to the enzyme. As
188 previously reported (3), and consistent with our promoter activity data, NAP activity in the wild-
189 type was significantly (~4-fold) higher during aerobic growth in MM-B compared to MM-S (Fig.
190 4B). The stimulation of activity by growth on butyrate disappeared in the Δ*dksA1* mutants and in
191 cells grown anaerobically in either MM-S or MM-B (Fig. 4B). Our results suggest that $DksA1_{Pd}$
192 upregulates (either directly or indirectly) the *napE* promoter during aerobic growth on reduced
193 carbon substrates such as butyrate.

194 The *rel* mutant was found to have the same phenotype as the *dksA1* mutant with respect to
195 *nap* promoter activity and NAP enzyme activity. That is, the butyrate-dependent stimulation was
196 also lost in the *rel* mutant (Fig. 4 and S7). Since DksA and ppGpp frequently act in concert by
197 binding together to a single site on RNA polymerase (34), the likely explanation that DksA-
198 dependent regulation of *nap* requires ppGpp.

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

200 In *P. pantotrophus*, regulation of the *nap* operon in response to carbon substrate oxidation state
201 involves *cis*-acting regulatory sequences in the *napE* promoter region (48). Specifically, a
202 heptameric imperfect inverted repeat sequence (TGAGACAtttTGTCGCA) was suggested to be
203 the binding site for a negatively acting redox-responsive regulator (48). The inverted repeat is a
204 partial match to the binding site (GCGGC-N_5-GTCGC) for the response regulator RegR (RegA)
205 from *Bradyrhizobium japonicum* (49). We found a similar sequence (5′-GAGGCgttttGACGC-3′)
206 in the promoter region of the *nap* operon in *P. denitrificans* Pd1222 (matches to the

207 experimentally-determined sequence from *B. japonicum* are underlined). Therefore, we examined
208 *napE* promoter activity in Δ*regA* and Δ*regB* strains to determine if *nap* is regulated by RegAB
209 alongside *dksA1*. In the Δ*regA* mutant, we observed ~2-fold increases in *napE* promoter activity
210 in cells grown aerobically on succinate (Fig. 5A). In cells grown aerobically on butyrate, *napE*
211 promoter activity was similar in both *regA* and *regB* mutants and the wild-type strain. Interestingly,
212 there were significant increases in promoter activities in *regA* and *regB* mutants grown
213 anaerobically on either succinate or butyrate, compared to the wild type under the same conditions
214 (Fig. 5A). Expression of *regA* and *regB* in the Δ*regA* and Δ*regB* strains respectively reduced *napE*
215 promoter activity to levels below those seen in the wild type strain, confirming that RegAB acts
216 negatively on the *napE* promoter (Fig. S8).

217 Our data suggest that RegA is the negatively acting regulator that Ellington et al. suggested
218 (48) binds to the inverted repeat sequence in the *napE* promoter, and that the RegAB system acts
219 to down-regulate this promoter principally under anaerobic growth conditions (but also to a lesser
220 extent during aerobic growth on succinate). The less severe phenotype of the *regB* mutant under
221 some conditions suggests the possibility that RegA is phosphorylated by another histidine kinase
222 (or by a low molecular weight phospho-donor) in the absence of RegB. Assays of NAP activity in
223 periplasmic fractions prepared from anaerobically grown cells confirm that RegAB functions as a
224 negative regulator under these conditions (Fig. 5B).

225 The membrane-associated nitrate reductase, Nar, is upregulated in *P. denitrificans* in
226 oxygen-limiting conditions (1, 50). Known regulators of the *nar* promoter are NarR (50) and,
227 probably, FnrP (51). Assays of Nar activity in membrane fractions revealed a reduced activity in
228 both Δ*regA* and Δ*regB* mutants suggesting positive regulation of the *nar* operon by RegAB under
229 these conditions (Fig. 5C). Addition of 20 μM sodium azide (a selective inhibitor of Nar) to the

230 reaction mixture inhibited nitrate reduction confirming that the reductase activity in the membrane
231 fraction was due to Nar (data not shown). Reduced Nar activity also coincided with reduced growth
232 yield for the Δ*regA* mutant when grown anaerobically (Fig. S9). Thus, the RegAB system mediates
233 reciprocal regulation of the two nitrate reductases under anaerobic conditions, being a negative
234 regulator of NAP expression and a positive regulator of Nar expression.

235 **The *dksA1* and *rel* genes are synthetically lethal for growth on reduced substrates**

236 The DksA protein interacts with ppGpp to regulate several functions and deletion of either *dksA*
237 or *rel* gives rise to overlapping pleiotropic phenotypes (39). The similar phenotypes of Δ*dksA1* and
238 *rel* mutants with respect to loss of butyrate stimulation of *nap* promoter activity suggests that
239 $DksA1_{Pd}$ and ppGpp act in concert to regulate expression of the periplasmic nitrate reductase. A
240 Δ*dksA1*Δ*rel* double mutant exhibited a complete growth defect on reduced carbon substrates such
241 as butyrate, caproate and choline (Fig. 3, Fig. S10), while growth on the relatively oxidized
242 substrates succinate and citrate was normal (Fig. S11). Expression of either *dksA1* or *rel* on a broad
243 host range plasmid from their native promoters restored growth of the Δ*dksA1*Δ*rel* mutant,
244 confirming that both mutations are required for the null growth phenotype (Fig. S12). Thus, *dksA1*
245 and *rel* exhibit synthetic lethality with respect to growth on reduced substrates. The likely
246 explanation is that two (or more) genes that are independently regulated by $DksA1_{Pd}$ and ppGpp
247 are together required for growth on reduced substrates.

248 **Partial activation of the *napE* promoter by a protein synthesis inhibitor**

249 In *E. coli*, ppGpp synthesis by RelA can be stimulated by manipulations that reduce the extent of
250 aminoacyl tRNA charging, including amino acid limitation or inhibition of an amino acyl tRNA
251 synthetase (52). For example, serine hydroxamate (SHX) is a seryl-tRNA synthetase inhibitor that
252 triggers ppGpp synthesis (52–54). SHX (1.28 mM) inhibits *E. coli* growth, and inhibition can be

253 reversed by exogenous serine, presumably because serine overcomes competitive inhibition
254 mediated by SHX (53). Addition of 3 mM SHX to MM-S caused a prolonged growth inhibition
255 of wild type *P. denitrificans* that was reversed by the addition of casamino acids to the medium
256 (Fig. 6). The physiological basis for the eventual recovery of the culture is not known but it may
257 be ppGpp-dependent since the *rel* mutant did not recover from the SHX treatment (Fig. 6). The
258 simplest interpretation of these observations is that SHX treatment triggers ppGpp synthesis by
259 RSH and that ppGpp is required for the recovery from growth arrest. A prediction that follows is
260 that treatment of a culture with a sub-inhibitory concentration of SHX will stimulate a promoter
261 activated by ppGpp. Therefore, we measured activity of the *napE-lacZ* fusion in cultures grown
262 aerobically in MM-S and treated with 2 mM SHX while in the logarithmic phase of growth. Ninety
263 minutes after SHX addition, promoter activity in the treated cultures was ~2-fold higher than in
264 the untreated cultures (1101 ± 96 versus 609 ± 60 units of β-galactosidase activity). In the *rel*
265 mutant, we saw no induction of the *napE* promoter in response to 2 mM SHX. Partial induction of
266 the *napE* promoter by a treatment known to trigger ppGpp synthesis in other organisms is
267 consistent with the idea that the promoter is regulated by ppGpp and DksA1.

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

269 In *E. coli*, *dksA* regulates the expression and activity of Hmp, a flavohemoprotein. Hmp is a nitric
270 oxide dioxygenase or denitrosylase which oxidizes nitric oxide to nitrate, and is known to be the
271 dominant NO defense mechanism under aerobic conditions (15). *P. denitrificans* has an *hmp* gene,
272 Pden_1689 (6, 7) and we have shown that the Hmp is functional for NO scavenging (unpublished
273 data). To determine if regulation of *hmp* is mediated by DksA in this organism, we assayed *hmp*
274 promoter activity in *P. denitrificans* and the Δ*dksA* mutants. Our results indicate that *hmp* promoter
275 activity is modestly but significantly elevated in the Δ*dksA2* mutant but is unaffected by the

276 Δ*dksA1* deletion (Fig. 7A). The Δ*rel* mutant also shows higher *hmp* promoter activity similar to
277 the Δ*dksA2* strain suggesting that the regulation of *hmp* by DksA2 is ppGpp-dependent (Fig. 7A).

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

279 Our previous work has shown that deletion of *hmp* increases biofilm formation in *P. denitrificans*
280 Pd1222 possibly due to accumulation of NO (6). Given the evidence that DksA2$_{Pd}$ regulates *hmp*
281 expression in *P. denitrificans*, we assayed attached growth to determine if DksA2$_{Pd}$ also affects
282 biofilm formation. We observed that Δ*dksA2* strains had greatly reduced levels of attached growth
283 (Fig. 7B and C). Addition of an *hmp* mutation to the *dksA2* mutant rescued biofilm formation to
284 wild-type levels (Fig. 7B and C) suggesting that the *dksA2* phenotype requires Hmp. Deletion of
285 *nsrR*, which encodes a transcriptional repressor of *hmp* (6), led to significantly reduced biofilm
286 formation similar to Δ*dksA2* (Fig. 7B and C). We also observed reduced biofilm in a Δ*rel* strain
287 (and in a Δ*dksA2* Δ*rel* double mutant, not shown) suggesting that *dksA2* and *rel* could be jointly
288 involved in regulating biofilm formation in *P. denitrificans* via negative regulation of *hmp* (Fig.
289 7B and C).

290

291 **DISCUSSION**

292 Expression of the periplasmic nitrate reductase NAP is upregulated by aerobic growth on reduced
293 carbon and energy sources, the likely explanation being that nitrate reduction by NAP provides a
294 means to dispose of excess reducing equivalents (3–5, 47, 48). We have shown that DksA1$_{Pd}$ and
295 RSH are required for the butyrate-stimulation of *napE* promoter and NAP enzyme activity. RSH
296 is the only protein encoded in the *P. denitrificans* genome with predicted ppGpp synthetase activity
297 and that it is enzymatically functional is supported by the detection of ppGpp in slow-growing
298 cultures of this organism (55). It is likely that DksA1$_{Pd}$ and ppGpp directly activate the *napE*

299 promoter, although indirect effects cannot be excluded. In either case, the implication is that
300 growth on reduced substrates somehow triggers the synthesis of ppGpp by RSH. Data from
301 continuous cultures show that the *napE* promoter is regulated both by growth rate and by carbon
302 substrate, leading to the conclusion that "…*nap* regulation is affected by a physiological signal
303 which integrates aspects of growth rate and carbon substrate utilization" (56). Our work suggests
304 that ppGpp (together with $DksA1_{Pd}$) is the integrating signal, which is consistent with the well-
305 known role of ppGpp in growth rate control (33).

306 One difference between the phenotypes of the *dksA1* and *rel* mutants is that nitrate does
307 not improve aerobic growth of the *dksA1* mutant on butyrate (as it does in the case of the wild-
308 type strain) but does modestly improve growth of the *rel* mutant. This improvement of growth
309 cannot be attributed to butyrate stimulation of *nap* expression, since that is lost in the *rel* mutant.
310 It is also the case that the *rel* mutant grows less well on butyrate (in the absence of nitrate) than
311 the wild-type strain and the *dksA1* mutant, and that the double mutant does not grow at all on
312 reduced substrates. It is likely that there are $DksA1_{Pd}$ and ppGpp regulated genes that are involved
313 in redox homeostasis that are yet to be identified.

314 We showed that regulation of *nap* expression also involves the RegAB two-component
315 system, which is redox-sensing and involved in redox homeostasis (57). Our results show that
316 RegAB is not a major player in mediating up-regulation of the *nap* promoter by growth on butyrate,
317 but instead negatively regulates *nap* expression during anaerobic growth. The response regulator
318 RegA very likely binds to an inverted repeat in the *napE* promoter that was initially characterized
319 in the closely related organism *P. pantotrophus* and suggested to be the binding site for a
320 negatively acting regulator (48). Evidently, regulation of the *napE* promoter by carbon substrate
321 and by oxygen availability can be separated, with the former requiring $DksA1_{Pd}$ and ppGpp and

322 the latter RegAB. Interestingly, RegAB also contribute (either directly or indirectly) to the
323 upregulation of the membrane associated nitrate reductase under anaerobic growth conditions.
324 Thus, NAP and Nar are reciprocally regulated by oxygen availability to be the dominant nitrate
325 reductases under aerobic and anaerobic conditions, respectively.

326 A recent study has shown that RegAB acts both positively and negatively under hypoxic
327 conditions in *Burkholderia pseudomallei*, an α-proteobacterium quite closely related to *P.*
328 *denitrificans* (58). Specifically, RegAB are required for upregulation of Nar under hypoxic
329 conditions in this organism, though this may involve both direct regulation by RegA and indirect
330 regulation, via FNR (58). Indirect effects of RegAB via NarR and/or FnrP are also possible in *P.*
331 *denitrificans*; we are able to discern a potential RegA binding site in the *narR-narK* intergenic
332 region that controls expression of the *narGHJI* genes encoding Nar (50).

333 In *E. coli*, DksA and ppGpp contribute to regulation of the *hmp* gene encoding the
334 flavohemoglobin, Hmp, which is responsible for detoxifying nitric oxide (15, 59, 60). In *P.*
335 *denitrificans*, we find that deletion of *dksA2* or *rel* leads to modestly increased *hmp* promoter
336 activity and dramatically reduced ability to form biofilm. Since the biofilm-defective phenotype
337 requires Hmp, we suggest that reduced expression of *hmp* in the mutants causes a reduced
338 accumulation of nitric oxide, which stimulates biofilm formation in this organism (6). Reduced
339 growth as biofilm in *dksA* mutants has been reported for other organisms, though the regulatory
340 mechanisms are likely different (30, 61).

341 Our study has identified two functional DksA proteins in *P. denitrificans* that have distinct
342 roles in growth on reduced carbon substrates, aerobic and anaerobic nitrate respiration, expression
343 of flavohemoglobin, and biofilm formation. In other α-proteobacteria which have at least two
344 DksA homologs, physiological roles have so far been ascribed to only one of them (27, 28). Further

345 functional analysis of the *P. denitrificans* DksA-like proteins and of the role of ppGpp will advance
346 our broader understanding of DksA functions in α-proteobacteria.
347

348 **MATERIALS AND METHODS**

349 **Bacterial strains and growth conditions**. The bacterial strains and plasmids used are listed in
350 Table S1. All *Paracoccus denitrificans* strains were derived from the wild-type Pd1222 (62).
351 *Escherichia coli* S.17-1 was used as the donor and *E. coli* DH5α (pRK2013) as the helper strain
352 for the introduction of plasmids into *P. denitrificans* by conjugation (63, 64). *E. coli* Novablue
353 competent cells (Novagen) were used as the host for all genetic manipulations. The rich medium
354 used for routine growth was LB (Miller) Broth (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl),
355 and the defined minimal medium (MM) for *P. denitrificans* was as previously described (65),
356 solidified with 1.5 % agar (w/v) as needed and supplemented with carbon and energy sources and
357 with 20 mM nitrate as indicated. For growth assays, *P. denitrificans* strains were first streaked on
358 LB agar plates, single colonies were inoculated into LB broth and grown overnight in 15 ml tubes
359 at 250 rpm. These cells were washed with MM, inoculated into succinate-supplemented MM, and
360 grown overnight in 15 ml tubes at 250 rpm. Cells were pelleted, washed, and finally inoculated
361 into MM supplemented with different carbon sources with or without nitrate. For aerobic growth
362 assays, cultures were grown in triplicate wells in a BioTek plate reader with horizontal orbital
363 shaking at 280 rpm and a volume of 0.2 ml (<50% of total volume) to ensure proper aeration while
364 preventing cross contamination. The optical density was measured at 600 nm and adjusted for path
365 length variation. Serine hydroxamate was added directly from a freshly made 100 mM stock to the
366 growth medium to a final concentration of 2-3 mM either at the time of inoculation or to a growing
367 culture, as indicated.

368 Anaerobic cultures for β-galactosidase assays were grown in filled glass cuvettes sealed
369 with a cap and septum, with medium supplemented with 20 mM nitrate. The cuvettes were gassed
370 with N_2 and incubated statically. Aerobic cultures were grown in 250 ml flasks shaking at 250 rpm
371 in MM supplemented with the indicated carbon substrate and with or without 20 mM nitrate. For
372 *E. coli* complementation assays, solid M9 medium (66) with 10 mM glucose was used, with or
373 without 0.2 % (w/v) casamino acids.

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

378 **Genetic manipulations.** All primers used are listed in Table S2. Unmarked deletions were made
379 by allelic replacement using pK18*mobsacB* as described by Sullivan et al. with minor
380 modifications (67, 68). Briefly, in-frame marker-less deletions were constructed by PCR
381 amplification of ~500-1000 bp fragments from either side of the gene of interest. The two
382 amplicons were ligated together either by PCR using overlapping regions added to the primers or
383 by digestion and ligation using a restriction site incorporated in the primers (69). The ligated
384 fragment was first cloned into pSTBlue-1 (Novagen), then sub-cloned into pK18*mobsacB*,
385 transformed into *E. coli* S.17-1 and mobilized into *P. denitrificans* by conjugation. Triparental
386 mating was performed by mixing *P. denitrificans*, *E. coli* S.17-1 harboring recombinant
387 pK18*mobsacB* or pMP220 or pIND4 derivatives, and *E. coli* DH5α (pRK2013) cells grown to log
388 phase, in a 2:1:1 ratio and by incubation on LB agar plate for 2-3 days at 30 ºC. Single-crossover
389 recombination events were selected on rifampicin and kanamycin plates, and then recombinants
390 with double crossovers were isolated by growth on 6 % (w/v) sucrose plates and kanamycin

391 sensitivity as described by Schafer et al. (67). All mutations were designed to be in-frame deletions
392 to prevent polarity. Promoter-*lacZ* reporter fusions were constructed by amplifying ~300-400 bp
393 upstream of the start codon of genes of interest and first cloning into pSTBlue-1. The sequence
394 was verified and the fragment cloned into the low copy number vector pMP220 between *Eco*RI
395 and *Pst*I restriction sites (70). Plasmids were introduced into *P. denitrificans* by conjugation from
396 *E. coli* S.17-1. *P. denitrificans* exconjugants were selected by rifampicin and tetracycline
397 resistance. For complementation tests in *P. denitrificans*, the gene of interest with its native
398 promoter was amplified and cloned directly into pIND4 (71) and the sequence verified. The
399 cloning was done using the NEBuilder HiFi DNA assembly cloning kit (NEB). Plasmids were
400 transformed into *E. coli* S.17-1 and mobilized into *P. denitrificans* strains by conjugation as
401 described above.
402 **β-galactosidase assay:** Liquid cultures were grown aerobically and anaerobically in minimal
403 medium with the indicated carbon sources and with or without 20 mM nitrate. Cells were harvested
404 at the mid-log phase of growth and the assay was performed as described by Miller (66). β-
405 galactosidase activity was measured using 0.1 ml of cells made permeable by treatment with
406 sodium dodecyl sulfate and chloroform. In the case of the *hmp-lacZ* fusion, strains were grown to
407 mid-log phase in the same medium and growth conditions as used for the biofilm assays.
408 **Biofilm assays:** Attached growth was assayed in 60 mm x 15 mm polystyrene petri dishes (6). A
409 70 µl aliquot of overnight culture was inoculated into 7 ml of LB broth supplemented with 10 mM
410 calcium chloride. The Petri dishes were incubated statically at 30 ºC for 72 hours. The plates were
411 then washed thrice under gently running tap water and stained with crystal violet (0.1 % in water)
412 for 20 minutes, washed three times with water, and air dried. For quantitation, the crystal violet

413 stain was extracted into 3 ml 95 % ethanol, diluted as necessary, and absorbance measured at 595
414 nm (6, 72).

415 **Cell fractionation:** *P. denitrificans* strains were grown aerobically and anaerobically in MM with
416 either succinate (10 mM) or butyrate (10 mM) as the sole carbon source; nitrate (20 mM) was
417 added as indicated. Aerobic cultures (50 ml) were grown at 30 ºC in 250 ml conical flasks shaken
418 at 250 rpm. Anaerobic cultures were grown in 100 ml bottles filled to the neck with medium and
419 incubated at 30 ºC without shaking. Periplasmic fractions for the nitrate reductase assay were
420 prepared as described in Quan et al (73) . In short, mid-exponential phase cells were harvested by
421 centrifugation at 3,000 *g* for 20 minutes at 4 ºC. Cells were then gently resuspended in TSE buffer
422 (200 mM Tris–HCl, pH 8.0, 500 mM sucrose, 1 mM EDTA without any protease inhibitor) and
423 incubated on ice for 30 minutes, and centrifuged at 16,000 *g* for 30 minutes at 4 ºC. The supernatant
424 was transferred to a fresh tube and used as the periplasmic fraction. Membrane fractions were
425 prepared according to Dunn et al. with some modifications (74). Briefly, cultures were harvested
426 in the mid-exponential phase of growth by centrifugation at 8,000 *g* for 5 minutes at 4 ºC and
427 washed twice in 10 mM potassium phosphate buffer (pH 7.5) in 50 ml Falcon tubes. Cells were
428 resuspended in 2.5 ml of ice cold 10 mM phosphate buffer, 10 mM $MgSO_4$, 0.2 M NaCl. Samples
429 were sonicated on ice using a Fisher Scientific Sonic Dismembrator model 500 outfitted with a 3
430 mm diameter tip at 20 % amplitude (10 W) for five 20 second bursts at 1 minute intervals. To
431 clarify the lysate, samples were centrifuged at 16,000 *g* for 20 minutes at 4 ºC. The clarified total
432 lysate was further centrifuged at 100,000 *g* for 2 hours at 4 ºC. The pellet was suspended in 0.2 ml
433 of phosphate buffer and retained as the membrane fraction for the enzyme assay. Fractions were
434 kept on ice and used within hours of preparation.

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

440

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444

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660

661

662 **FIGURE LEGENDS**

663

664 **FIGURE 1.**

665 *P. denitrificans* $dksA1_{Pd}$ and $dksA2_{Pd}$ complement an *E. coli* Δ*dksA* mutant (Δ$dksA_{EC}$). Strains
666 were streaked on M9 minimal (M9) agar plates containing 10 mM glucose (G) with or without
667 0.2% (w/v) casamino acids (CAA). Plates were incubated overnight at 37 ºC. 1 - *E. coli*
668 MG1655; 2 - Δ$dksA_{EC}$ complemented with $dksA1_{Pd}$; 3 - Δ$dksA_{EC}$ complemented with $dksA2_{Pd}$; 4 -
669 Δ$dksA_{EC}$ carrying empty pIND4.

670

671 **FIGURE 2.**

672 Deletion of *dksA1* impairs nitrate utilization during aerobic growth. Aerobic growth of the
673 indicated strains in: (A) minimal medium containing succinate or (B) minimal medium
674 containing butyrate, with (+ NO_3^-) or without (- NO_3^-) 20 mM nitrate. Optical density was
675 measured (at 600 nm) at the indicated time points (the means from three independent
676 experiments). The estimated doubling times for growth assays are reported in Table S3.

677

678 **FIGURE 3.**

679 Loss of both *dksA1* and *rel* leads to failure to grow on reduced carbon substrates. Aerobic growth
680 of indicated strains in liquid minimal medium with butyrate (A) without (- NO_3^-) or (B) with 20
681 mM nitrate (+ NO_3^-). Optical density was measured (at 600 nm) at the indicated time points (the
682 means from three independent experiments). The estimated doubling times for growth assays are
683 reported in Table S3.

684

685

686 **FIGURE 4.**

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

697

698 **FIGURE 5.**

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

710

711 **FIGURE 6.**

712 The *rel* gene is required for recovery from serine hydroxamate treatment. Aerobic growth of
713 indicated strains in minimal medium containing: (A) succinate only, (B) succinate and 3 mM
714 serine hydroxamate, and (C) succinate, 3 mM serine hydroxamate and 0.2% (w/v) casamino
715 acids. Optical density was measured (at 600 nm) at the indicated time points (the means from
716 three independent experiments). The estimated doubling times for growth assays are reported in
717 Table S4.

718

719

720 **FIGURE 7.**

721 $DksA2_{Pd}$ and RSH regulate *hmp* promoter activity and biofilm formation in *P. denitrificans*. (A)
722 β-galactosidase activities in *P. denitrificans* wild-type, Δ*dksA2* and Δ*rel* carrying plasmid p*hmp-*
723 *lacZ*. (B) Assays of biofilm formation by the wild-type, deletion, and complemented strains.
724 Dishes were washed, stained with crystal violet, and photographed from above. (C) The crystal
725 violet was extracted with 95% ethanol, and the absorbance measured at 595 nm. Means and
726 standard deviations were calculated from results of three separate assays. Cultures were grown in
727 6-cm-diameter petri dishes in liquid L broth (Miller), supplemented with 10 mM $CaCl_2$ and
728 incubated without shaking at 30 ºC. Cells were harvested at mid-log phase for the β-galactosidase
729 assay or left undisturbed for 72 hours for the biofilm assay. Asterisks on the bars indicate a
730 significant difference compared to the wild-type according to Student's t-test (**$P \leq 0.01$, ***P
731 ≤ 0.001).

Fig. 1

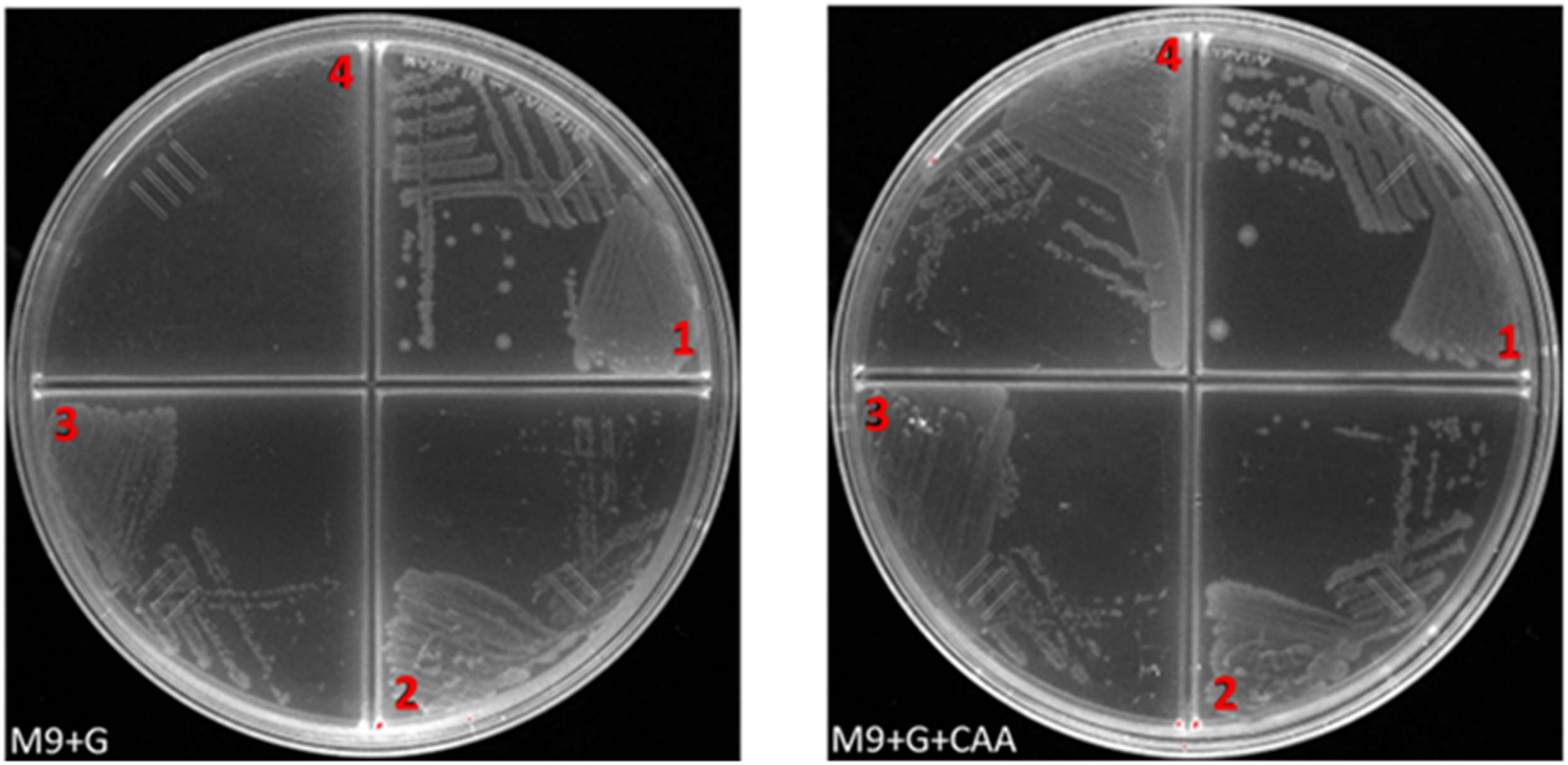

Figure 1. *P. denitrificans dksA1*$_{Pd}$ and *dksA2*$_{Pd}$ complement an *E. coli* Δ*dksA* mutant (Δ*dksA*$_{EC}$). Strains were streaked on M9 minimal (M9) agar plates containing 10 mM glucose (G) with or without 0.2% (w/v) casamino acids (CAA). Plates were incubated overnight at 37 ºC. 1 - *E. coli*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

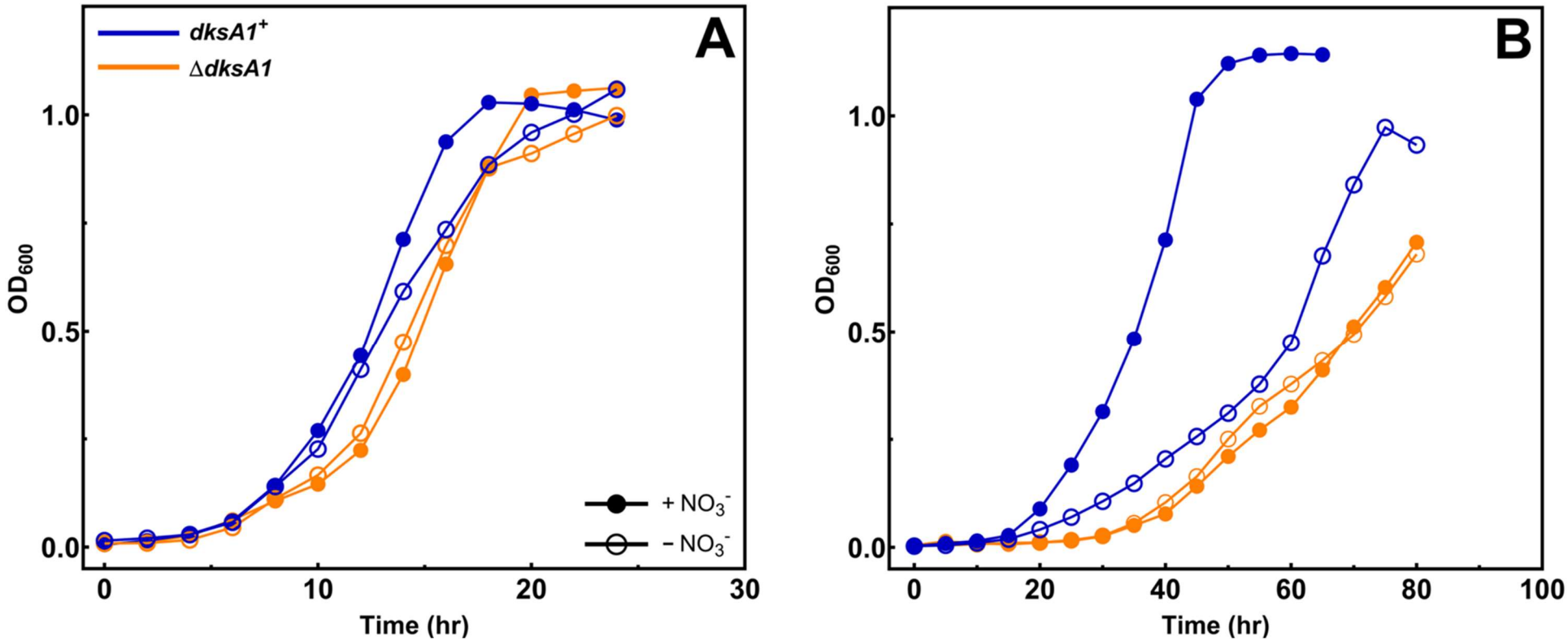

Figure 2. Deletion of *dksA1* impairs nitrate utilization during aerobic growth. Aerobic growth of indicated strains in, **(A)** minimal medium containing succinate or **(B)** minimal medium containing butyrate, with (+ NO_3^-) or without (- NO_3^-) 20 mM nitrate. Optical density was measured (at 600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

Fig. 3

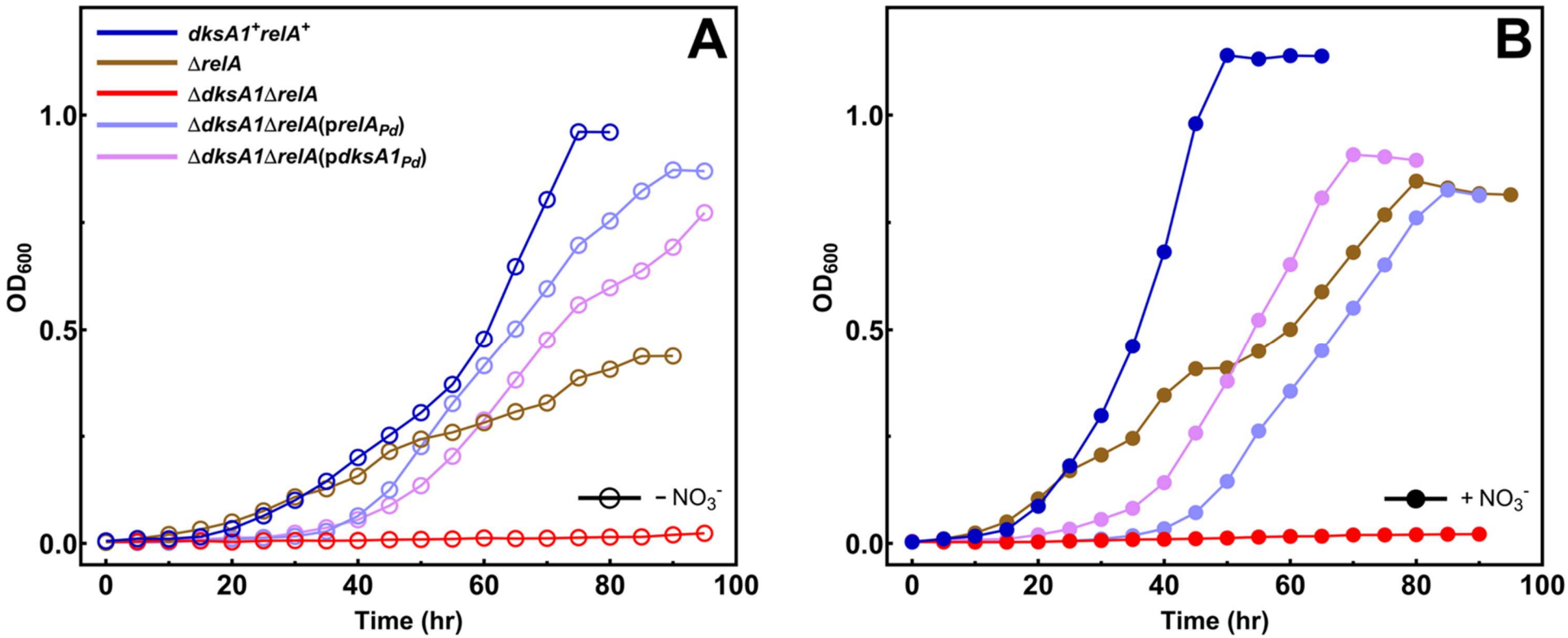

Figure 3. Loss of both *dksA1* and *relA* leads to failure to grow on reduced carbon substrates. Aerobic growth of indicated strains in liquid minimal medium with butyrate **(A)** without (- NO_3^-) or **(B)** with 20 mM nitrate (+ NO_3^-). Optical density was measured (at 600 nm) at the indicated time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

Fig. 4

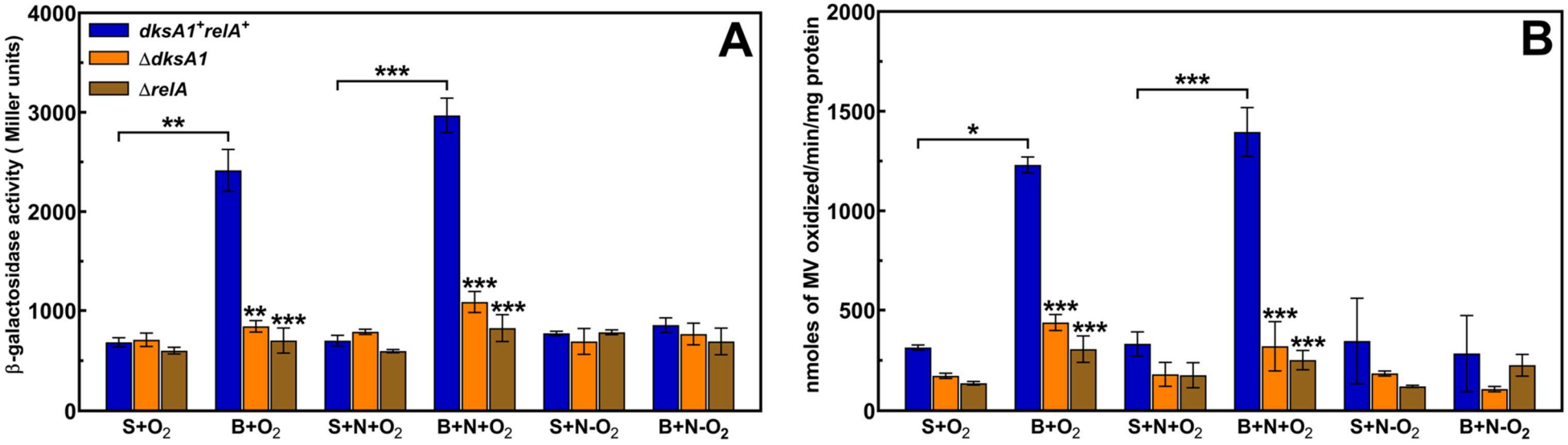

Figure 4. The Δ*dksA1* mutant has reduced *nap* promoter and NAP enzyme activity. (A) β-galactosidase activity in *P. denitrificans* wild-type, Δ*dksA1* and Δ*relA* carrying plasmid p*nap-lacZ*. **(B)** NAP enzyme activity in the periplasmic fraction of *P. denitrificans* wild-type, Δ*dksA1* and Δ*relA* 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

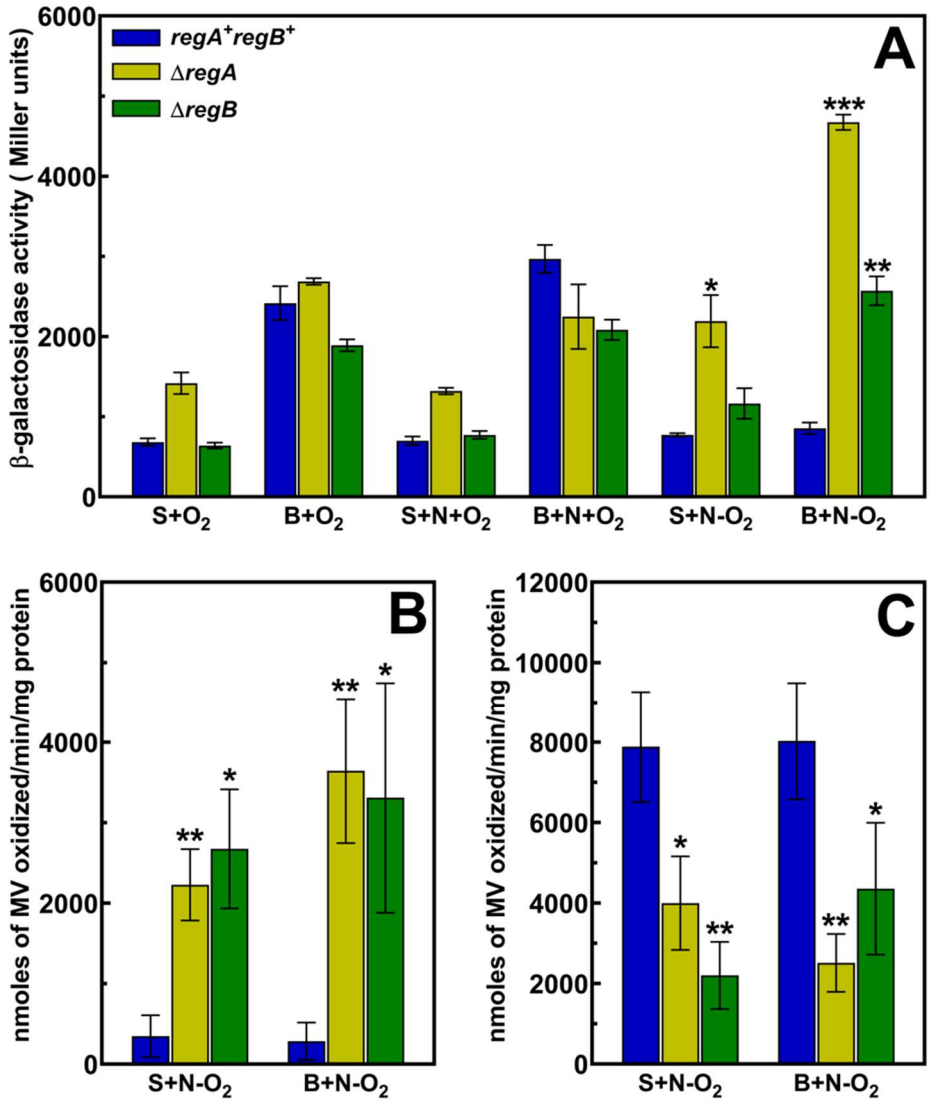

Figure 5. The RegAB system regulates anaerobic *nap* promoter and NAP activity. (A) β-galactosidase activity in *P. denitrificans* wild-type, Δ*regA* and Δ*regB* carrying plasmid p*nap-lacZ*. **(B)** NAP enzyme activity in the periplasmic fractions of anaerobically grown *P. denitrificans* wild-type, 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

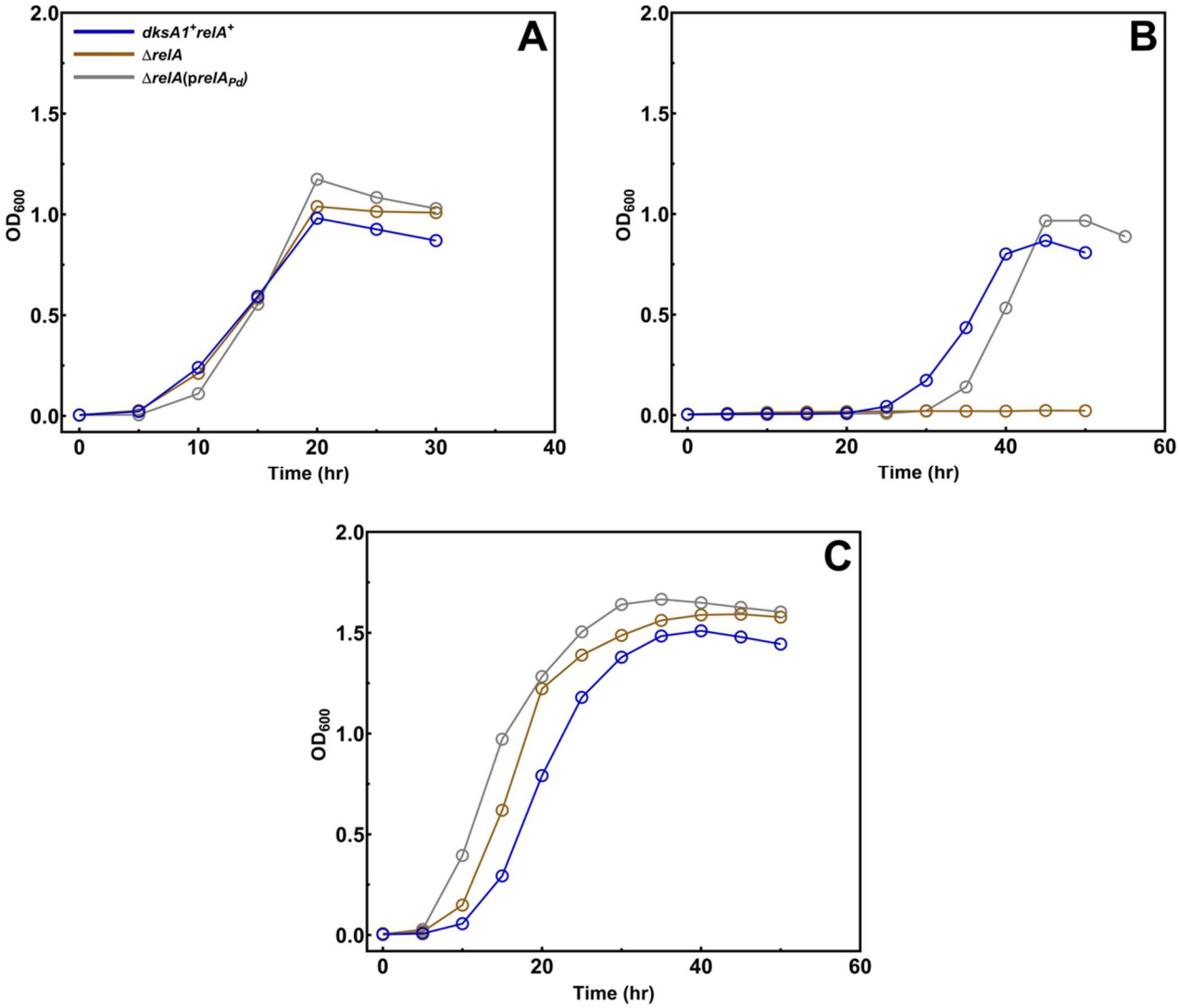

Figure 6. The *relA* gene is required for the response to amino acid starvation in *P. denitrificans.* Aerobic growth of indicated strains in minimal medium containing: **(A)** succinate only, **(B)** succinate and 3 mM serine hydroxamate, and **(C)** succinate, 3 mM serine hydroxamate and 0.2% (w/v)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

Main Figures

Fig. 7

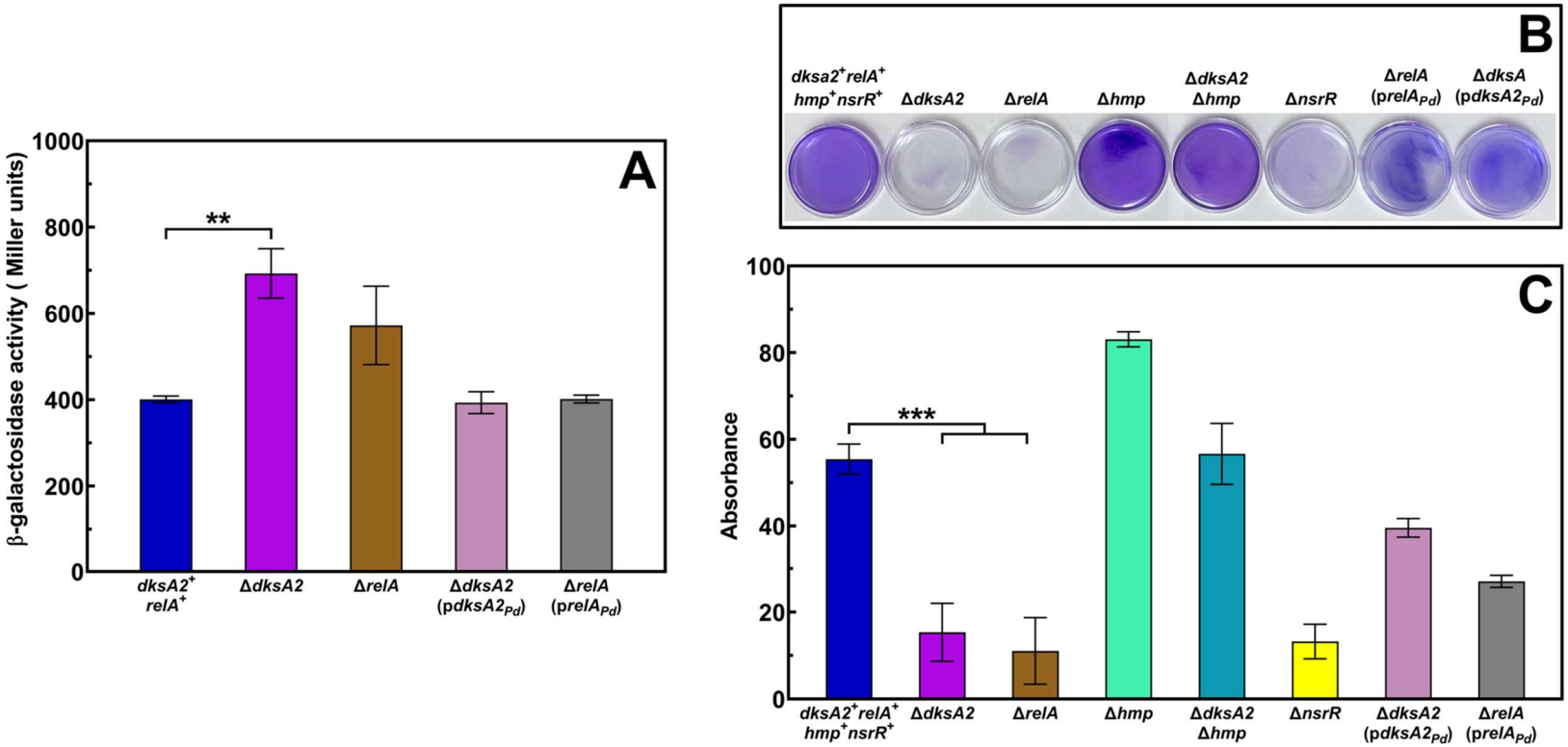

Figure 7. DksA2$_{Pd}$ and RSH regulate *hmp* promoter activity and biofilm formation in *P. denitrificans*. (A) β-galactosidase activities in *P. denitrificans* wild-type, Δ*dksA2* and Δ*relA* carrying plasmid p*hmp-lacZ*. **(B)** Assays of biofilm formation by the wild-type, deletion, and complemented strains. Dishes were washed, stained with crystal violet, and photographed from above. **(C)** The crystal violet was extracted with 95% ethanol, and the absorbance measured at 595 nm. Means and standard deviations were calculated from results of three separate assays. Cultures were grown in 6-cm-diameter petri dishes in liquid L broth (Miller), supplemented with 10 mM $CaCl_2$ and incubated without shaking at 30 ºC. Cells were harvested at mid-log phase for the β-galactosidase assay or left undisturbed for 72 hours for the biofilm assay. Asterisks on the bars indicate a significant difference compared to the wild-type according to Student's t-test (**$P \leq 0.01$, ***$P \leq 0.001$).