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). These phenotypes suggest that
155 DksA1$_{Pd}$ may regulate aerobic nitrate reduction by NAP during growth on reduced carbon
156 substrates.

157 To determine if this Δ*dksA1* associated phenotype is ppGpp-dependent, we measured
158 growth of the Δ*relA* strain under similar conditions. The Δ*relA* mutant showed significantly
159 reduced growth in MM-B, however, unlike for Δ*dksA1*, growth was improved by addition of

160 nitrate to the medium (Fig. 3A and B), though the magnitude of nitrate stimulation was less than
161 was observed in the wild-type strain.

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

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

173 In parallel with the *napE* promoter activity assays we also measured the activity of NAP in
174 periplasmic fractions using reduced methylviologen as the electron donor to the enzyme. As
175 previously reported (45), and consistent with our promoter activity data, NAP activity in the wild-
176 type was significantly higher during aerobic growth in MM-B compared to MM-S (Fig. 4B). The
177 stimulation of activity by growth on butyrate disappeared in the Δ*dksA1* mutants and in cells grown
178 anaerobically in either MM-S or MM-B (Fig. 4B). Our results suggest that $DksA1_{Pd}$ upregulates
179 (either directly or indirectly) the *napE* promoter during aerobic growth on reduced carbon
180 substrates such as butyrate.

181 The *relA* mutant was found to have the same phenotype as the *dksA1* mutant with respect
182 to *nap* promoter activity and NAP enzyme activity. That is, the butyrate-dependent stimulation

183 was also lost in the *relA* mutant. Since DksA and ppGpp frequently act in concert by binding
184 together to a single site on RNA polymerase (30), the likely explanation that DksA-dependent
185 regulation of *nap* requires ppGpp.

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

187 In *P. pantotrophus*, regulation of the *nap* operon in response to carbon substrate oxidation state
188 involves *cis*-acting regulatory sequences in the *napE* promoter region (48). Specifically, a
189 heptameric imperfect inverted repeat sequence (TGAGACAttTGTCGCA) was suggested to be the
190 binding site for a negatively acting redox-responsive regulator (48). The inverted repeat is a partial
191 match to the binding site (GCGGC-N_5-GTCGC) for the response regulator RegR (RegA) in
192 *Bradyrhizobium japonicum* (49). We found a similar sequence (5′-**GAGGC**GTTTT**GACGC**-3′)
193 in the promoter region of the *nap* operon in *P. denitrificans* Pd1222. Therefore, we examined *napE*
194 promoter activity in Δ*regA* and Δ*regB* strains to determine if *nap* is regulated by *dksA1* alongside
195 the RegAB system. In the Δ*regA* mutant, we observed ~2-fold increases in *napE* promoter activity
196 in cells grown aerobically on succinate (Fig. 5A). In cells grown aerobically on butyrate, *napE*
197 promoter activity was similar in both *regA* and *regB* mutants and the wild-type strain. Interestingly,
198 there were significant increases in promoter activities in *regA* and *regB* mutants grown
199 anaerobically on either succinate or butyrate (Fig. 5A). These data suggest that RegA is the
200 negatively acting regulator that Ellington et al. suggested (48) binds to the inverted repeat sequence
201 in the *napE* promoter, and that the RegAB system acts to down-regulate this promoter principally
202 under anaerobic growth conditions (but also to a lesser extent) during aerobic growth on succinate.
203 The less severe phenotype of the *regB* mutant under some conditions suggests the possibility that
204 RegA is phosphorylated by another histidine kinase in the absence of RegB. Assays of NAP

205 activity in periplasmic fractions prepared from anaerobically grown cells confirm that RegAB
206 functions as a negative regulator under these conditions (Fig. 5B).

207 The membrane-associated nitrate reductase, Nar, is upregulated in *P. denitrificans* in
208 oxygen-limiting conditions (1, 50). Known regulators of the *nar* promoter are NarR (50) and,
209 probably, FnrP (51). Assays of Nar activity in membrane fractions revealed a reduced activity in
210 both Δ*regA* and Δ*regB* mutants suggesting a positive regulation of the *nar* operon by RegAB under
211 these conditions (Fig. 5C). Addition of 20 μM sodium azide (a selective inhibitor of Nar) to the
212 reaction mixture inhibited nitrate reduction confirming that the reductase activity in the membrane
213 fraction was due to Nar (data not shown). Reduced Nar activity also coincided with reduced growth
214 yield for the Δ*regA* mutant when grown anaerobically (Fig. S4). Thus, the RegAB system mediates
215 reciprocal regulation of the two nitrate reductases under anaerobic conditions, being a negative
216 regulator of NAP expression and a positive regulator of Nar expression.

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

218 The DksA protein interacts with ppGpp to regulate several functions and deletion of either *dksA*
219 or *relA* gives rise to overlapping pleiotropic phenotypes (36). The similar phenotypes of Δ*dksA1*
220 and *relA* mutants with respect to loss of butyrate stimulation of *nap* promoter activity suggests that
221 $DksA1_{Pd}$ and ppGpp act in concert to regulate expression of the periplasmic nitrate reductase. A
222 Δ*dksA1*Δ*relA* double mutant exhibited a complete growth defect on reduced carbon substrates such
223 as butyrate, caproate and choline (Fig. 3, Fig. S5), while growth on the relatively oxidized
224 substrates succinate and citrate was normal (Fig. S6). Expression of either *dksA1* or *relA* on a broad
225 host range plasmid from their native promoters restored growth of the Δ*dksA1*Δ*relA* mutant,
226 confirming that both mutations are required for the null growth phenotype (Fig. 3). Thus, *dksA1*
227 and *relA* exhibit synthetic lethality with respect to growth on reduced substrates. The likely

228 explanation is that two (or more) genes that are independently regulated by DksA1$_{Pd}$ and ppGpp
229 are together required for growth on reduced substrates.

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

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

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

246 In *E. coli*, *dksA* regulates the expression and activity of Hmp, a flavohemoprotein. Hmp is a nitric
247 oxide dioxygenase or denitrosylase which oxidizes nitric oxide to nitrate, and is known to be the
248 dominant NO defense mechanism under aerobic conditions (11). *P. denitrificans* has an *hmp* gene,
249 Pden_1689 (2, 3) and we have shown that the Hmp is functional for NO scavenging (unpublished
250 data). To determine if regulation of *hmp* is mediated by DksA in this organism, we assayed *hmp*

251 promoter activity in *P. denitrificans* and the Δ*dksA* mutants. Our results indicate that *hmp* promoter
252 activity is modestly but significantly elevated in the Δ*dksA2* mutant but is unaffected by the
253 Δ*dksA1* deletion (Fig. 7A). The Δ*relA* mutant also shows higher *hmp* promoter activity similar to
254 the Δ*dksA2* strain suggesting that the regulation of *hmp* by DksA2 is ppGpp-dependent (Fig. 7A).

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

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

266

267 **DISCUSSION**

268 Expression of the periplasmic nitrate reductase NAP is upregulated by aerobic growth on reduced
269 carbon and energy sources, the likely explanation being that nitrate reduction by NAP provides a
270 means to dispose of excess reducing equivalents (44–48). We have shown that DksA1$_{Pd}$ and RSH
271 are required for the butyrate-stimulation of *napE* promoter and NAP enzyme activity. RSH is the
272 only protein encoded in the *P. denitrificans* genome with predicted ppGpp synthetase activity and
273 that it is enzymatically functional is supported by the detection of ppGpp in slow-growing cultures

274 of this organism (54). It is likely that DksA1$_{Pd}$ and ppGpp directly activate the *napE* promoter,
275 although indirect effects cannot be excluded. In either case, the implication is that growth on
276 reduced substrates somehow triggers the synthesis of ppGpp by RSH. Data from continuous
277 cultures show that the *napE* promoter is regulated both by growth rate and by carbon substrate,
278 leading to the conclusion that "…*nap* regulation is affected by a physiological signal which
279 integrates aspects of growth rate and carbon substrate utilization" (55). Our work suggests that
280 ppGpp (together with DksA1$_{Pd}$) is the integrating signal, which is consistent with the well-known
281 role of ppGpp in growth rate control (29).

282 One difference between the phenotypes of the *dksA1* and *relA* mutants is that nitrate does
283 not improve aerobic growth of the *dksA1* mutant on butyrate (as it does in the case of the wild-
284 type strain) but does modestly improve growth of the *relA* mutant. This improvement of growth
285 cannot be attributed to butyrate stimulation of *nap* expression, since that is lost in the *relA* mutant.
286 It is also the case that the *relA* mutant grows less well on butyrate (in the absence of nitrate) than
287 the wild-type strain and the *dksA1* mutant, and that the double mutant does not grow at all on
288 reduced substrates. It is likely that there are DksA1$_{Pd}$ and ppGpp regulated genes that are involved
289 in redox homeostasis that are yet to be identified.

290 We showed that regulation of *nap* expression also involves the RegAB two-component
291 system, which is redox-sensing and involved in redox homeostasis (56). Our results show that
292 RegAB is not a major player in mediating up-regulation of the *nap* promoter by growth on butyrate,
293 but instead negatively regulates *nap* expression during anaerobic growth. The response regulator
294 RegA very likely binds to an inverted repeat in the *napE* promoter that was initially characterized
295 in the closely related organism *P. pantotrophus* and suggested to be the binding site for a
296 negatively acting regulator (48). Evidently, regulation of the *napE* promoter by carbon substrate

297 and by oxygen availability can be separated, with the former requiring DksA1$_{Pd}$ and ppGpp and
298 the latter RegAB. Interestingly, RegAB also contribute (either directly or indirectly) to the
299 upregulation of the membrane associated nitrate reductase under anaerobic growth conditions.
300 Thus, NAP and Nar are reciprocally regulated by oxygen availability to be the dominant nitrate
301 reductases under aerobic and anaerobic conditions, respectively.

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

309 In *E. coli*, DksA and ppGpp contribute to regulation of the *hmp* gene encoding the
310 flavohemoglobin, Hmp, which is responsible for detoxifying nitric oxide (11, 58, 59). In *P.*
311 *denitrificans*, we find that deletion of *dksA2* or *relA* leads to modestly increased *hmp* promoter
312 activity and dramatically reduced ability to form biofilm. Since the biofilm-defective phenotype
313 requires Hmp, we suggest that reduced expression of *hmp* in the mutants causes a reduced
314 accumulation of nitric oxide, which stimulates biofilm formation in this organism (2). Reduced
315 growth as biofilm in *dksA* mutants has been reported for other organisms, though the regulatory
316 mechanisms are likely different (26, 60).

317 Our study has identified two functional DksA proteins in *P. denitrificans* that have distinct
318 roles in growth on reduced carbon substrates, aerobic and anaerobic nitrate respiration, expression
319 of flavohemoglobin, and biofilm formation. In other α-proteobacteria which have at least two

320 DksA homologs, physiological roles have so far been ascribed to only one of them (23, 24). Further
321 functional analysis of the *P. denitrificans* DksA-like proteins and of the role of ppGpp will advance
322 our broader understanding of DksA functions in α-proteobacteria.
323

324 **MATERIALS AND METHODS**

325 **Bacterial strains and growth conditions**. The bacterial strains and plasmids used are listed in
326 Table S1. All *Paracoccus denitrificans* strains were derived from the wild-type Pd1222 (61).
327 *Escherichia coli* S.17-1 was used as the donor and *E. coli* DH5α (pRK2013) as the helper strain
328 for the introduction of plasmids into *P. denitrificans* by conjugation (62, 63). *E. coli* Novablue
329 competent cells (Novagen) were used as the host for all genetic manipulations. The rich medium
330 used for routine growth was LB (Miller) Broth (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl),
331 and the defined minimal medium (MM) for *P. denitrificans* was as previously described (64),
332 solidified with 1.5 % agar (w/v) as needed and supplemented with carbon and energy sources and
333 with 20 mM nitrate as indicated. For growth assays, *P. denitrificans* strains were first streaked on
334 LB agar plates, single colonies were inoculated into LB broth and grown overnight in 15 ml tubes
335 at 250 rpm. These cells were washed with MM, inoculated into succinate-supplemented MM, and
336 grown overnight in 15 ml tubes at 250 rpm. Cells were pelleted, washed, and finally inoculated
337 into MM supplemented with different carbon sources with or without nitrate. For aerobic growth
338 assays, cultures were grown in triplicate wells in a BioTek plate reader with horizontal orbital
339 shaking at 280 rpm and a volume of 0.2 ml (<50% of total volume) to ensure proper aeration while
340 preventing cross contamination. The optical density was measured at 600 nm and adjusted for path
341 length variation. Serine hydroxamate was added directly from a freshly made 100 mM stock to the
342 growth medium to a final concentration of 3 mM at the time of inoculation, where indicated.

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

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

353 **Genetic manipulations.** All primers used are listed in Table S2. Unmarked deletions were made
354 by allelic replacement using pK18*mobsacB* as described by Sullivan et al. with minor
355 modifications (66, 67). Briefly, in-frame marker-less deletions were constructed by PCR
356 amplification of ~500-1000 bp fragments from either side of the gene of interest. The two
357 amplicons were ligated together either by PCR using overlapping regions added to the primers or
358 by digestion and ligation using a restriction site incorporated in the primers (68). The ligated
359 fragment was first cloned into pSTBlue-1 (Novagen), then sub-cloned into pK18*mobsacB*,
360 transformed into *E. coli* S.17-1 and mobilized into *P. denitrificans* by conjugation. Triparental
361 mating was performed by mixing *P. denitrificans*, *E. coli* S.17-1 harboring recombinant
362 pK18*mobsacB* or pMP220 or pIND4 derivatives, and *E. coli* DH5α (pRK2013) cells grown to log
363 phase, in a 2:1:1 ratio and by incubation on LB agar plate for 2-3 days at 30 ºC. Single-crossover
364 recombination events were selected on rifampicin and kanamycin plates, and then recombinants
365 with double crossovers were isolated by growth on 6 % (w/v) sucrose plates and kanamycin

366 sensitivity as described by Schafer et al. (66). All mutations were designed to be in-frame deletions
367 to prevent polarity. Promoter-*lacZ* reporter fusions were constructed by amplifying ~300-400 bp
368 upstream of the start codon of genes of interest and first cloning into pSTBlue-1. The sequence
369 was verified and the fragment cloned into the low copy number vector pMP220 between *Eco*RI
370 and *Pst*I restriction sites (69). Plasmids were introduced into *P. denitrificans* by conjugation from
371 *E. coli* S.17-1. *P. denitrificans* exconjugants were selected by rifampicin and tetracycline
372 resistance. For complementation tests in *P. denitrificans*, the gene of interest with its native
373 promoter was amplified and cloned directly into pIND4 (70) and the sequence verified. The
374 cloning was done using the NEBuilder HiFi DNA assembly cloning kit (NEB). Plasmids were
375 transformed into *E. coli* S.17-1 and mobilized into *P. denitrificans* strains by conjugation as
376 described above.
377 **β-galactosidase assay:** Liquid cultures were grown aerobically and anaerobically in minimal
378 medium with the indicated carbon sources and with or without 20 mM nitrate. Cells were harvested
379 at the mid-log phase of growth and the assay was performed as described by Miller (71). B-
380 galactosidase activity was measured using 0.1 ml of cells made permeable by treatment with
381 sodium dodecyl sulfate and chloroform. In the case of the *hmp-lacZ* fusion, strains were grown to
382 mid-log phase in the same medium and growth conditions as used for the biofilm assays.
383 **Biofilm assays:** Attached growth was assayed in 60 mm x 15 mm polystyrene petri dishes (2). A
384 70 µl aliquot of overnight culture was inoculated into 7 ml of LB broth supplemented with 10 mM
385 calcium chloride. The petri dishes were incubated statically at 30 ºC for 72 hours. The plates were
386 then washed thrice under gently running tap water and stained with crystal violet (0.1 % in water)
387 for 20 minutes, washed three times with water, and air dried. For quantitation, the crystal violet

388 stain was extracted into 3 ml 95 % ethanol, diluted as necessary, and absorbance measured at 595
389 nm (2, 72).

390 **Cell fractionation:** *P. denitrificans* strains were grown aerobically and anaerobically in MM with
391 either succinate (10 mM) or butyrate (10 mM) as the sole carbon source; nitrate (20 mM) was
392 added as indicated. Aerobic cultures (50 ml) were grown at 30 ºC in 250 ml conical flasks shaken
393 at 250 rpm. Anaerobic cultures were grown in 100 ml bottles filled to the neck with medium and
394 incubated at 30 ºC without shaking. Periplasmic fractions for the nitrate reductase assay were
395 prepared as described in Quan et al (73) . In short, mid-exponential phase cells were harvested by
396 centrifugation at 3,000 *g* for 20 minutes at 4 ºC. Cells were then gently resuspended in TSE buffer
397 (200 mM Tris–HCl, pH 8.0, 500 mM sucrose, 1 mM EDTA without any protease inhibitor) and
398 incubated on ice for 30 minutes, and centrifuged at 16,000 *g* for 30 minutes at 4 ºC. The supernatant
399 was transferred to a fresh tube and used as the periplasmic fraction. Membrane fractions were
400 prepared according to Dunn et al. with some modifications (74). Briefly, cultures were harvested
401 in the mid-exponential phase of growth by centrifugation at 8,000 *g* for 5 minutes at 4 ºC and
402 washed twice in 10 mM potassium phosphate buffer (pH 7.5) in 50 ml Falcon tubes. Cells were
403 resuspended in 2.5 ml of ice cold 10 mM phosphate buffer, 10 mM $MgSO_4$, 0.2 M NaCl. Samples
404 were sonicated on ice using a Fisher Scientific Sonic Dismembrator model 500 outfitted with a 3
405 mm diameter tip at 20 % amplitude (10 W) for five 20 second bursts at 1 minute intervals. To
406 clarify the lysate, samples were centrifuged at 16,000 *g* for 20 minutes at 4 ºC. The clarified total
407 lysate was further centrifuged at 100,000 *g* for 2 hours at 4 ºC. The pellet was suspended in 0.2 ml
408 of phosphate buffer and retained as the membrane fraction for the enzyme assay. Fractions were
409 kept on ice and used within hours of preparation.

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

415

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419

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619

620 **FIGURE LEGENDS**

621

622 **FIGURE 1.**

623 *P. denitrificans* $dksA1_{Pd}$ and $dksA2_{Pd}$ complement an *E. coli* Δ*dksA* mutant (Δ$dksA_{EC}$). Strains
624 were streaked on M9 minimal (M9) agar plates containing 10 mM glucose (G) with or without
625 0.2% (w/v) casamino acids (CAA). Plates were incubated overnight at 37 ºC. 1 - *E. coli*
626 MG1655; 2 - Δ$dksA_{EC}$ complemented with $dksA1_{Pd}$; 3 - Δ$dksA_{EC}$ complemented with $dksA2_{Pd}$; 4 -
627 Δ$dksA_{EC}$ carrying empty pIND4.

628

629 **FIGURE 2.**

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

635

636 **FIGURE 3.**

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

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

641

642 **FIGURE 4.**

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

653

654 **FIGURE 5.**

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

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

666

667 **FIGURE 6.**

668 The *relA* gene is required for the response to amino acid starvation in *P. denitrificans.* Aerobic
669 growth of indicated strains in minimal medium containing: (A) succinate only, (B) succinate and
670 3 mM serine hydroxamate, and (C) succinate, 3 mM serine hydroxamate and 0.2% (w/v) casamino
671 acids. Optical density was measured (at 600 nm) at the indicated time points (the means from three
672 independent experiments).

673

674 **FIGURE 7.**

675 DksA2$_{Pd}$ and RSH regulate *hmp* promoter activity and biofilm formation in *P. denitrificans*. (A)
676 β-galactosidase activities in *P. denitrificans* wild-type, Δ*dksA2* and Δ*relA* carrying plasmid p*hmp-*
677 *lacZ*. (B) Assays of biofilm formation by the wild-type, deletion, and complemented strains.
678 Dishes were washed, stained with crystal violet, and photographed from above. (C) The crystal
679 violet was extracted with 95% ethanol, and the absorbance measured at 595 nm. Means and
680 standard deviations were calculated from results of three separate assays. Cultures were grown in
681 6-cm-diameter petri dishes in liquid L broth (Miller), supplemented with 10 mM $CaCl_2$ and
682 incubated without shaking at 30 ºC. Cells were harvested at mid-log phase for the β-galactosidase

683 assay or left undisturbed for 72 hours for the biofilm assay. Asterisks on the bars indicate a
684 significant difference compared to the wild-type according to Student's t-test (**P $\leq$ 0.01, ***P
685 $\leq$ 0.001).

Fig. 1

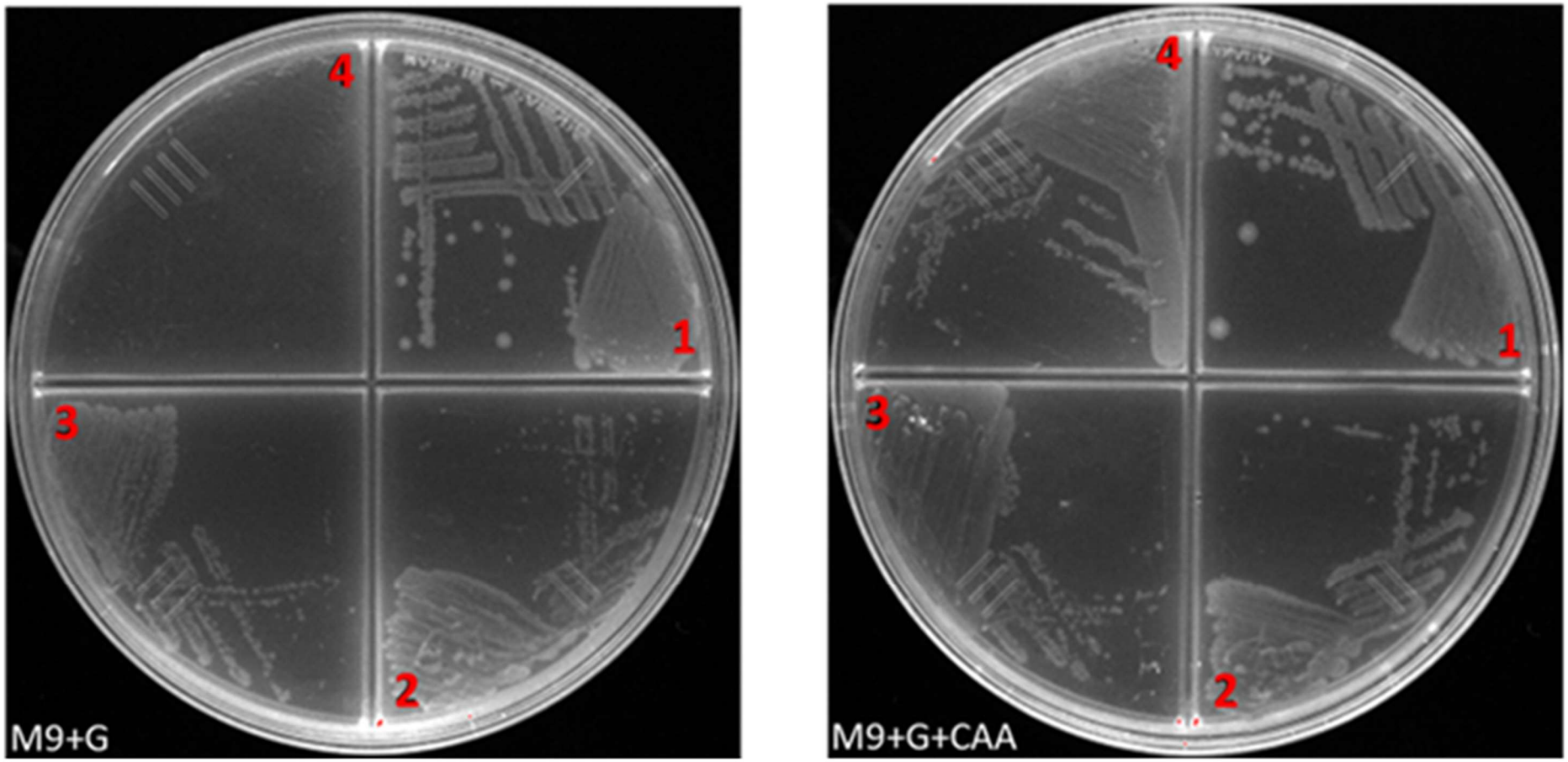


Fig. 2

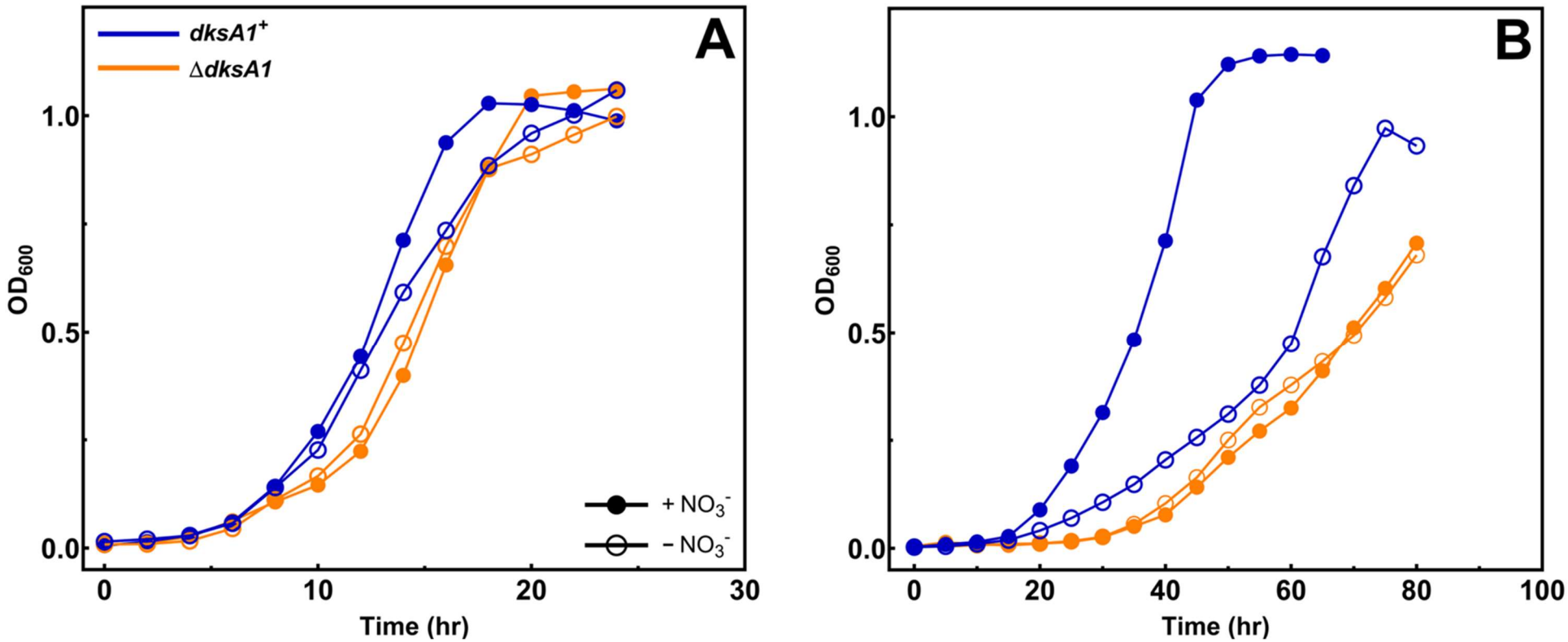

A
dksA1+
ΔdksA1
+ NO3-
− NO3-
OD600
Time (hr)
0.0
0.5
1.0
0
10
20
30
B
OD600
Time (hr)
0.0
0.5
1.0
0
20
40
60
80
100

Fig. 3

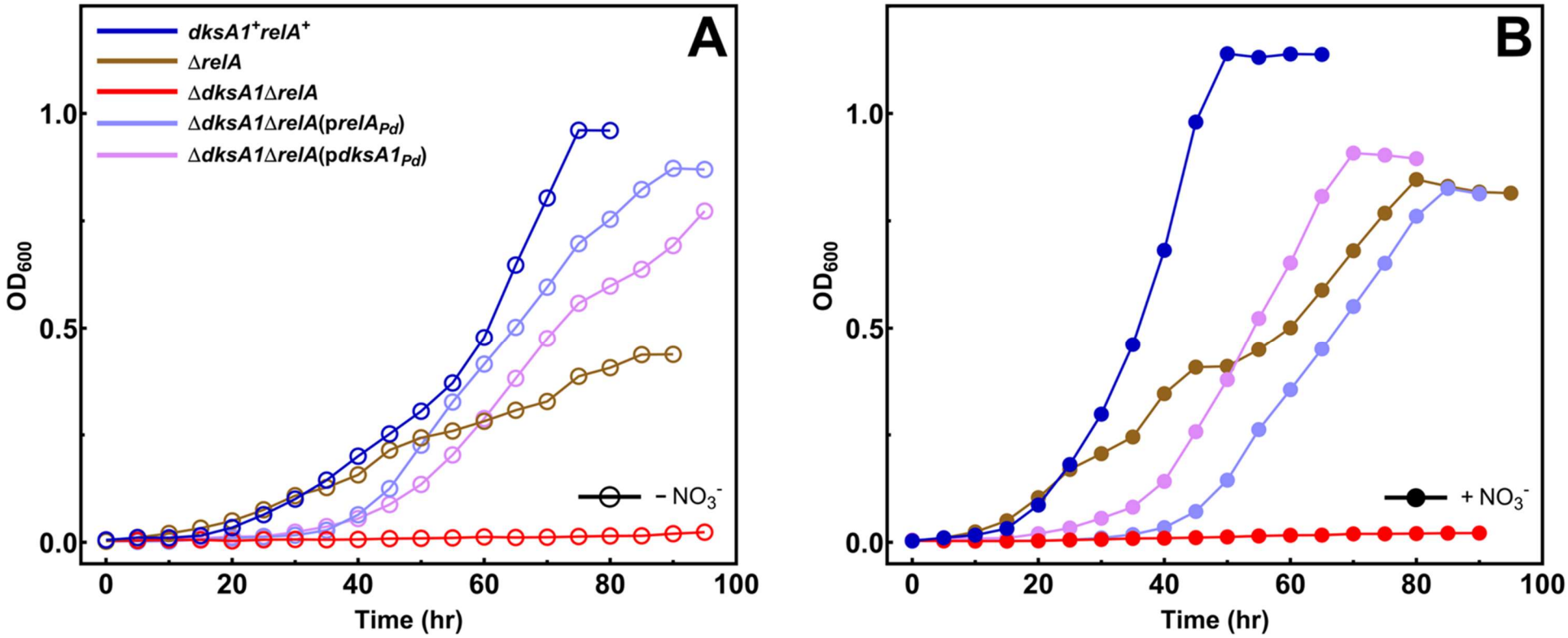
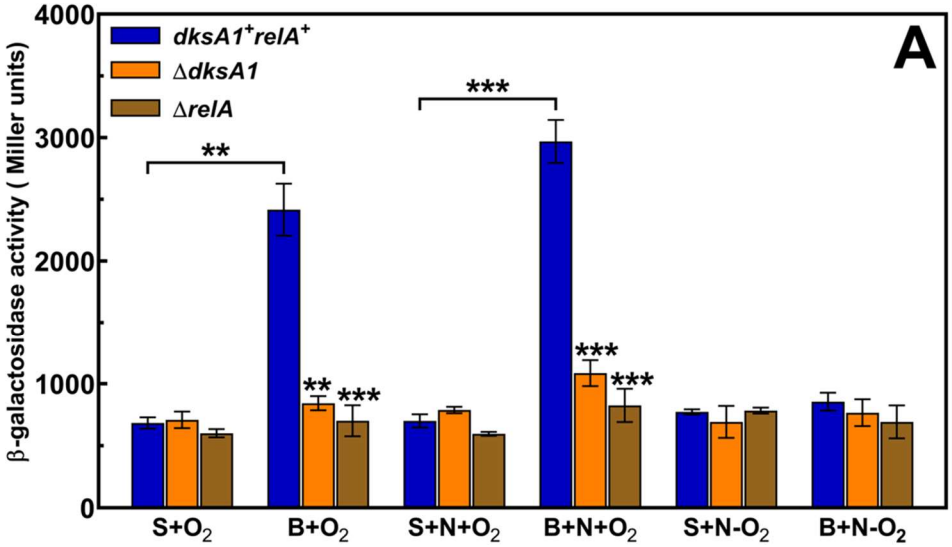
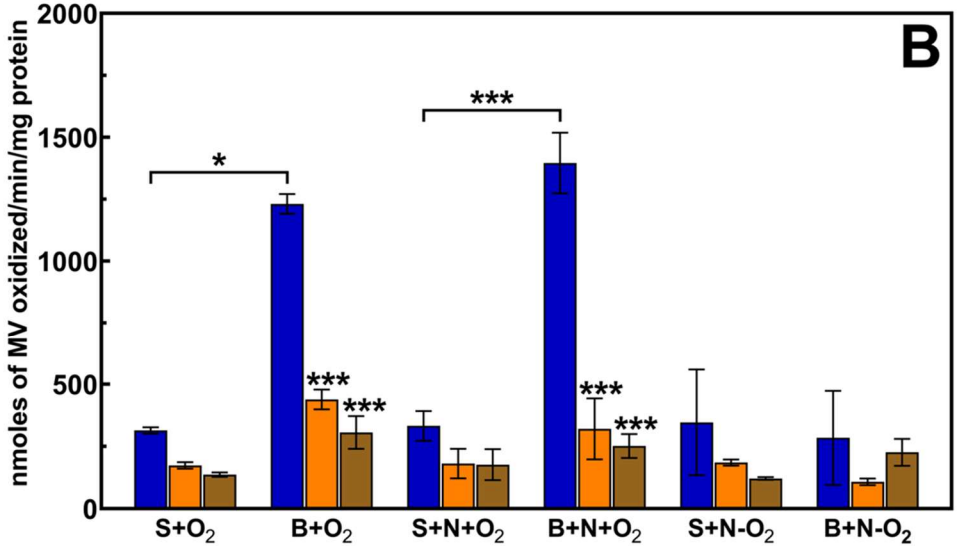

A
B
dksA1+relA+
ΔrelA
ΔdksA1ΔrelA
ΔdksA1ΔrelA(prelAPd)
ΔdksA1ΔrelA(pdksA1Pd)
– NO3-
+ NO3-
OD600
Time (hr)
0.0
0.5
1.0
0
20
40
60
80
100

Fig. 4


A
dksA1+relA+
ΔdksA1
ΔrelA
β-galactosidase activity (Miller units)
4000
3000
2000
1000
0
**
***
**
***
***
***
S+O2
B+O2
S+N+O2
B+N+O2
S+N-O2
B+N-O2


B
nmoles of MV oxidized/min/mg protein
2000
1500
1000
500
0
*
***
***
***
***
***
S+O2
B+O2
S+N+O2
B+N+O2
S+N-O2
B+N-O2

Main Figures

Fig. 5

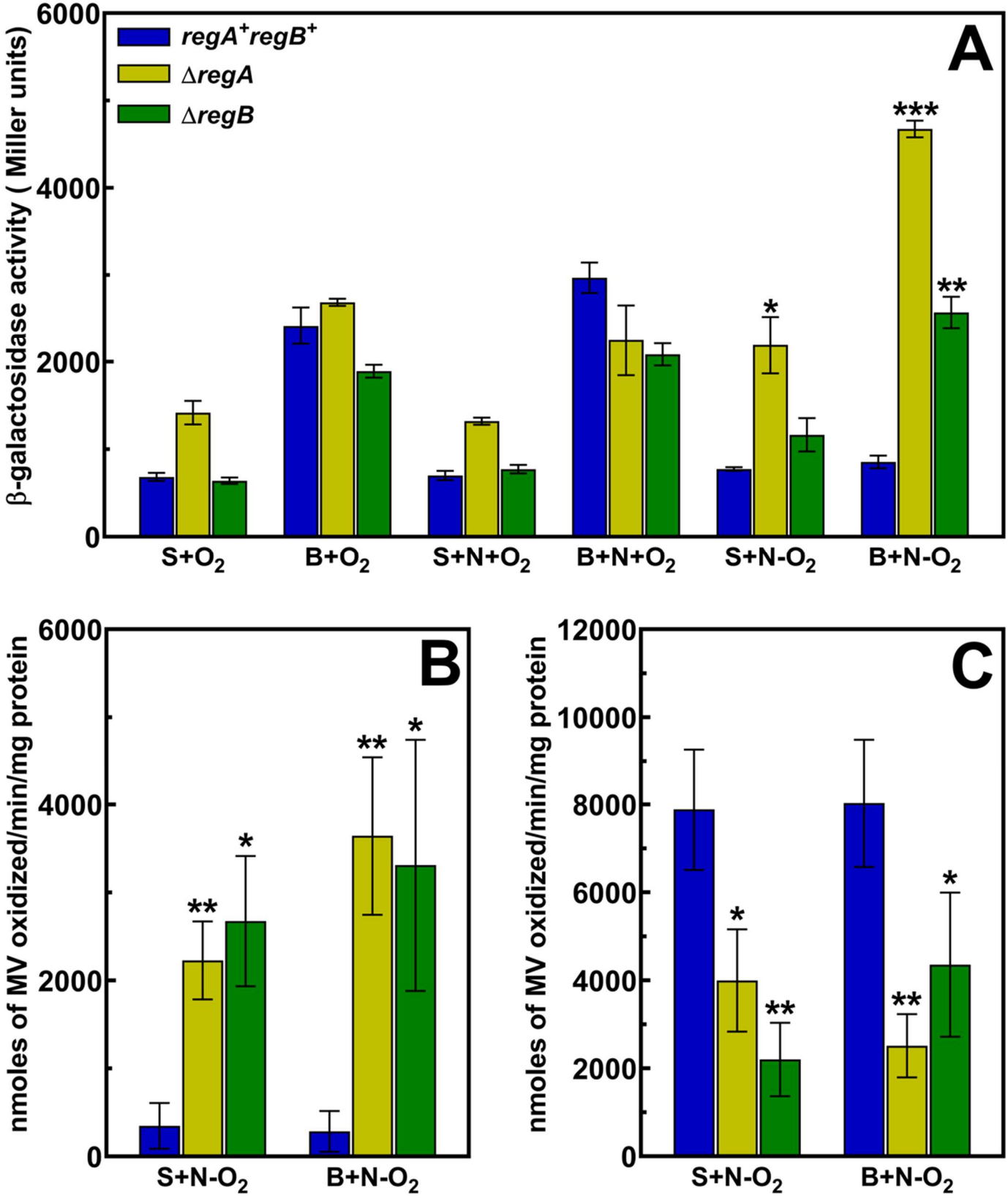


Fig. 6

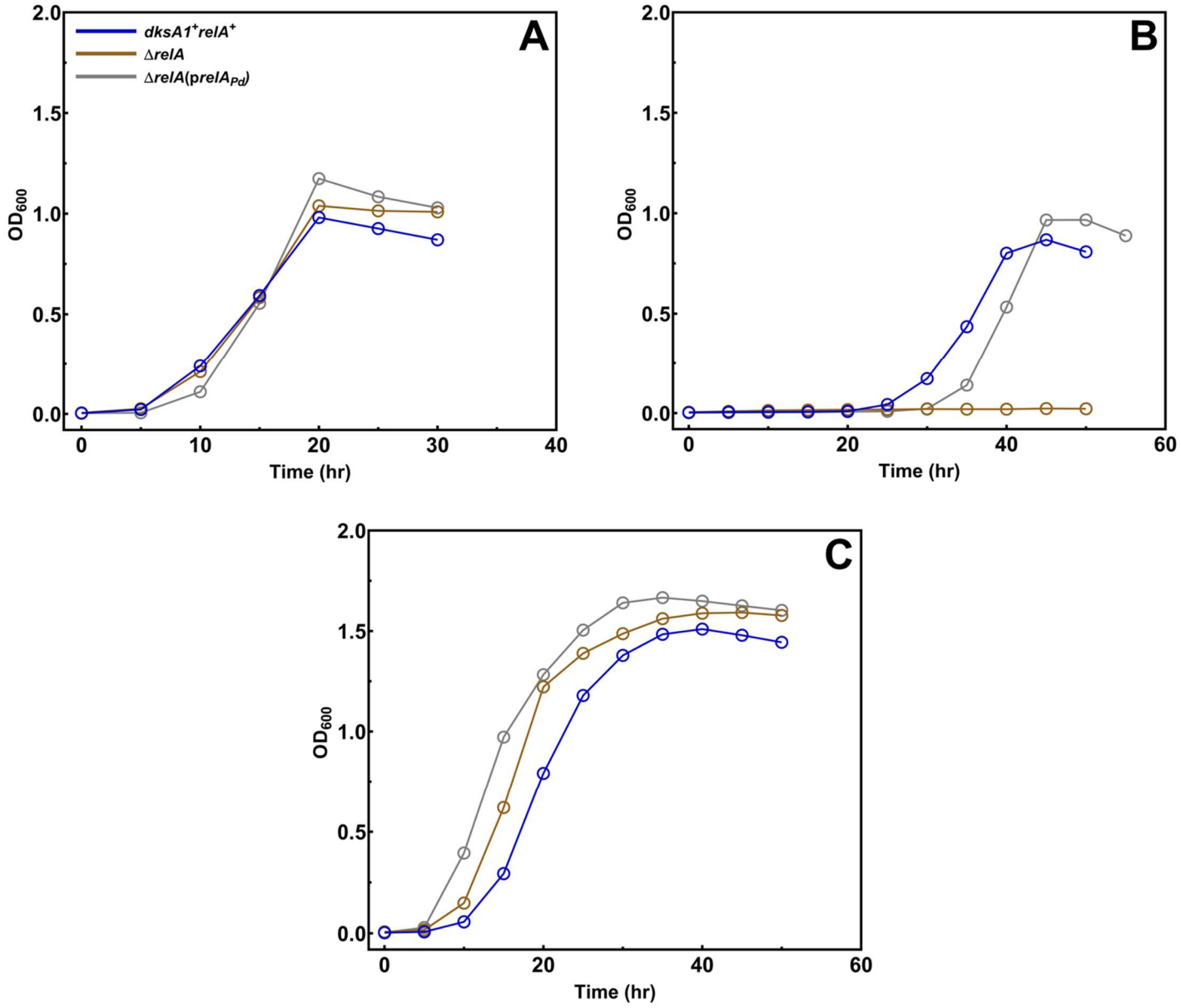
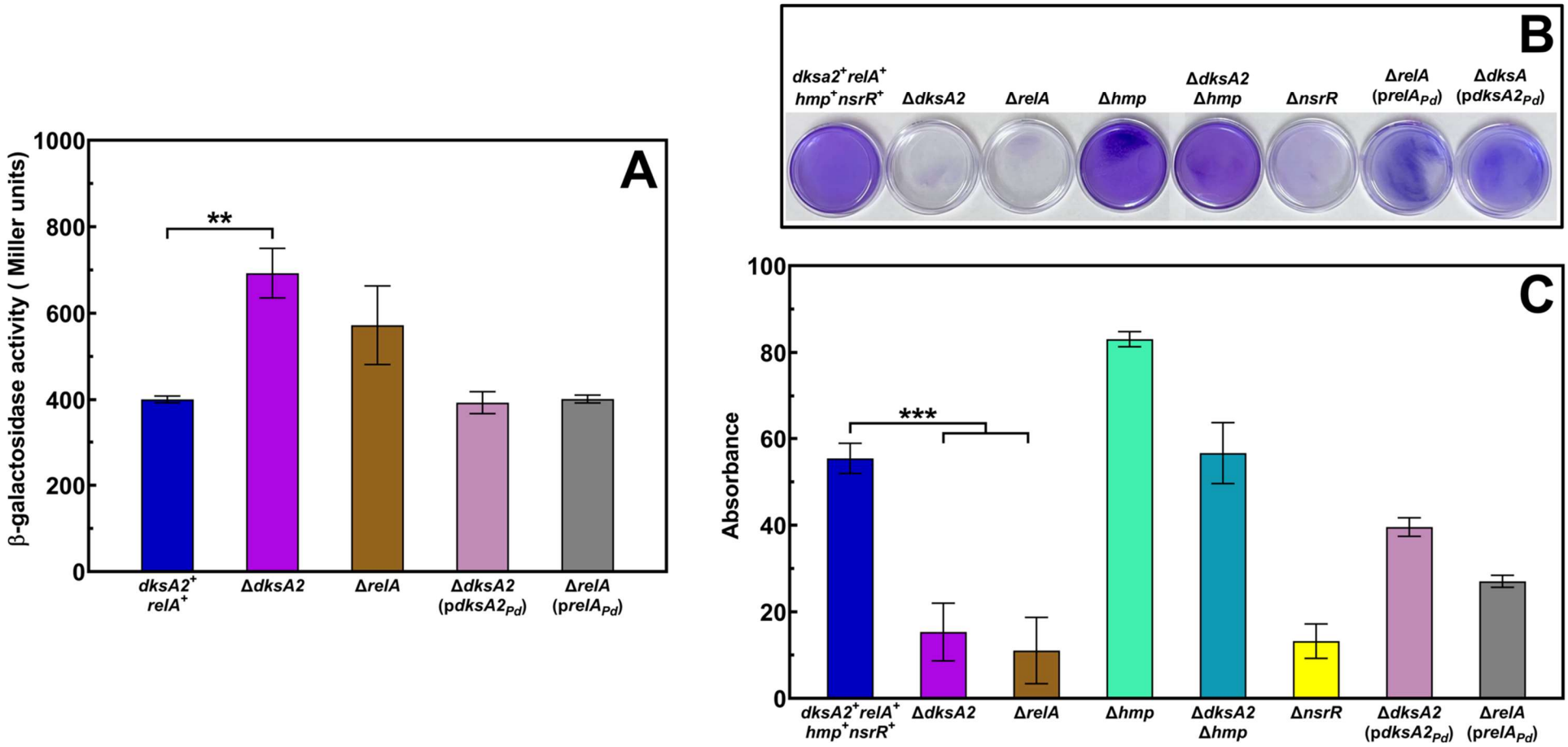

A
B
C
dksA1+relA+
ΔrelA
ΔrelA(prelAPd)
OD600
Time (hr)
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
60

Fig. 7


A
β-galactosidase activity (Miller units)
1000
800
600
400
200
0
**
dksA2+ relA+
ΔdksA2
ΔrelA
ΔdksA2 (pdksA2Pd)
ΔrelA (prelAPd)
B
dksa2+relA+ hmp+nsrR+
ΔdksA2
ΔrelA
Δhmp
ΔdksA2 Δhmp
ΔnsrR
ΔrelA (prelAPd)
ΔdksA (pdksA2Pd)
C
Absorbance
100
80
60
40
20
0
***
dksA2+relA+ hmp+nsrR+
ΔdksA2
ΔrelA
Δhmp
ΔdksA2 Δhmp
ΔnsrR
ΔdksA2 (pdksA2Pd)
ΔrelA (prelAPd)