Choline degradation in *Paracoccus denitrificans*: identification of sources of formaldehyde

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ABSTRACT

Paracoccus denitrificans is a facultative methylotroph that can grow on methanol and methylamine as sole sources of carbon and energy. Both are oxidized to formaldehyde and then to formate, so growth on C1 substrates induces the expression of genes encoding enzymes required for the oxidation of formaldehyde and formate. Catabolism of some complex organic substrates (for example choline and L-proline betaine) generates formaldehyde, which is oxidized to CO_2, likely by the same enzymes that oxidize formaldehyde generated from C1 substrates. Thus, regulatory mutants that fail to induce expression of the formaldehyde catabolic enzymes cannot grow on methanol, methylamine and choline. The pathway for the oxidation of choline to glycine includes three steps that may be oxidative demethylations that generate formaldehyde. However, among characterized choline degradation pathways in several organisms there are variants of these reactions that do not generate formaldehyde. Therefore, the number of equivalents and the sources of formaldehyde made during choline degradation in *P. denitrificans* are not known. By exploring mutant phenotypes and the activities of a promoter and enzyme known to be up-regulated by formaldehyde, we identify the oxidations of glycine betaine and dimethylglycine as sources of formaldehyde. Genetic evidence implicates two orthologous monooxygenases in the oxidation of glycine betaine. Interestingly, one of these appears to be a bifunctional enzyme that also oxidizes L-proline betaine (stachydrine). We present preliminary evidence to suggest that growth on L-proline betaine induces expression of a formaldehyde dehydrogenase distinct from the enzyme induced during growth on other formaldehyde-generating substrates.

46 **IMPORTANCE**

47 The bacterial degradation of one carbon compounds (methanol and methylamine) and of some
48 complex multi-carbon compounds (for example, choline) generates formaldehyde. Formaldehyde
49 is toxic and must be removed, which can be done by oxidation to formate and then to carbon
50 dioxide. These oxidations provide a source of energy, in some species the CO_2 thus generated
51 can be assimilated into biomass. Using the Gram-negative bacterium *Paracoccus denitrificans* as
52 the experimental model, we show that oxidation of choline to glycine generates two equivalents
53 of formaldehyde and we identify the two steps in the catabolic pathway that are responsible. Our
54 work sheds further light on metabolic pathways that are likely important in a variety of
55 environmental contexts.

56

57 **INTRODUCTION**

58 *Paracoccus denitrificans* is a metabolically versatile Alphaproteobacterium that is frequently
59 detected in terrestrial and aquatic environments, in waste treatment reactors, and in association
60 with animals and plants (1–5). The organism can grow on C1 compounds (methanol and
61 methylamine) as sole sources of carbon and energy and has been used as a model for studies of
62 the genetics and biochemistry of methylotrophy (6, 7). Both methanol and methylamine are
63 oxidized to formaldehyde by PQQ-dependent periplasmic enzymes. The formaldehyde is
64 internalized and oxidized to formate by a glutathione (GSH) dependent formaldehyde
65 dehydrogenase, GD-FALDH (7, 8). *P. denitrificans* also has a gene (Pden_1186, *hpbM*)
66 encoding a predicted GSH-independent formaldehyde dehydrogenase (9). Formate is oxidized to
67 carbon dioxide, which can be assimilated by the Calvin Cycle, although other species in the
68 *Paracoccus* genus may use the serine cycle (10).

69 Transcription of the genes required for the oxidation of methanol, methylamine and
70 formaldehyde is regulated by two two-component systems comprising histidine kinases (FlhS
71 and MoxY) and their cognate response regulators (FlhR and MoxX, respectively). MoxXY (also
72 called MxaXY) are required for transcription of the genes encoding methanol dehydrogenase and
73 associated proteins and *moxXY* mutants have a growth defect on methanol but not methylamine
74 (11). FlhSR-deficient mutants are unable to grow on both methanol and methylamine (12) and
75 the FlhSR proteins are believed to activate transcription of the operons encoding the methanol,
76 methylamine and formaldehyde dehydrogenases, and MoxXY (7). The signal that triggers the
77 FlhSR signal transduction pathway has not been identified biochemically, but it is suggested that
78 both FlhS and MoxX respond to formaldehyde (7). Thus, expression of the methanol
79 dehydrogenase is induced not by the substrate methanol but rather by the product, formaldehyde.
80 FlhSR-deficient mutants are also unable to grow on choline as a source of carbon and
81 energy (12). The explanation for this phenotype is that the oxidation of choline to glycine is
82 accompanied by the production of formaldehyde and the *flhSR* mutants are poisoned because
83 they are unable to up-regulate expression of the formaldehyde dehydrogenase. Thus, ectopic
84 expression of the GD-FALDH rescues the choline growth defect of *flhS* and *flhR* mutants (12).
85 Choline catabolism in *P. denitrificans* has been variously suggested to be accompanied by the
86 production of 'several' (13), two (14) or three (15) equivalents of formaldehyde.
87 The aerobic pathway for choline degradation (Fig. 1) proceeds through betaine aldehyde,
88 glycine betaine, dimethylglycine and sarcosine to glycine (16). Each of the three steps from
89 glycine betaine to glycine involves the removal of one methyl group and is a potential source of
90 formaldehyde. Glycine betaine can be oxidatively demethylated by a glycine betaine
91 monooxygenase that generates dimethylglycine and formaldehyde (17, 18). In other species, a

92 glycine betaine:homocysteine methyl transferase donates the methyl group to homocysteine and
93 so makes methionine rather than formaldehyde (19, 20). Dimethylglycine can be oxidatively
94 demethylated by a dimethylglycine dehydrogenase (or oxidase). These are tetrahydrofolate
95 (THF) linked enzymes that generate methylene-THF (rather than formaldehyde) as the reaction
96 product (21). There is also a dimethylglycine demethylase that has not been characterized
97 biochemically but is a potential source of formaldehyde (16, 17). Sarcosine is oxidized either by
98 a monomeric or a tetrameric sarcosine oxidase. The former generates formaldehyde as the
99 reaction product, while the tetrameric sarcosine oxidase is THF-dependent and so makes
100 methylene-THF (22). The THF-dependent dimethylglycine and sarcosine oxidases can make
101 formaldehyde as a reaction product in vitro in the absence of THF (21, 22); whether these
102 enzymes are a source of formaldehyde in vivo is not known. Depending upon the combination of
103 enzymes used and whether or not the THF-dependent enzymes are sources of formaldehyde in
104 vivo, the conversion of choline to glycine may be accompanied by the formation of between zero
105 and three equivalents of formaldehyde (16, 20). *P. denitrificans* also has a characterized pathway
106 for the degradation of proline betaine which is predicted to generate two equivalents of
107 formaldehyde through the action of proline betaine and methyl proline demethylases (9).
108 While studying the regulation of formaldehyde metabolism in *P. denitrificans*, we
109 became interested to identify the source(s) of the formaldehyde generated during choline
110 catabolism. Reconstructing the pathway from the genome sequence is challenging for several
111 reasons: (1) for some steps there are multiple candidate enzymes encoded in the genome; (2)
112 some potentially relevant genes are duplicated, and (3) definitively assigning biochemical
113 functions to gene products is not always possible. Therefore, we have adopted an empirical
114 approach (informed by the genome sequence) to determine the likely pathway for choline

115 degradation in *P. denitrificans* and the source(s) of formaldehyde. To do so, we have taken
116 advantage of the fact that *flhSR* mutants have a defect for growth on substrates that generate
117 formaldehyde. We present evidence to indicate that the major sources of formaldehyde are two
118 orthologous monooxygenases that oxidatively demethylate glycine betaine to form
119 dimethylglycine and the subsequent oxidative demethylation of dimethylglycine.
120

121 **RESULTS**

122 **Formaldehyde is a by-product of the oxidation of glycine betaine and dimethylglycine.**
123 It has been reported previously that *flhS* and *flhR* mutants are unable to grow on solid media
124 containing choline as the sole source of carbon and energy (12). We have reconstructed
125 unmarked deletions in *flhS* and *flhR* and confirmed that these mutants are unable to grow on solid
126 minimal medium containing choline (Figure S1). In liquid media, the *flhS* and *flhR* mutants grow
127 normally to an optical density of around 0.3 before arresting (Fig. 2). This pattern is consistent
128 with the accumulation of a toxic intermediate (likely to be formaldehyde).
129 We established that *P. denitrificans* grows well on intermediates of the choline
130 degradation pathway: glycine betaine, dimethylglycine and sarcosine (Fig. 2, Table 1). In liquid
131 media, the *flhS* and *flhR* mutants show growth defects on glycine betaine similar to the choline
132 phenotypes (Fig. 2), whereas both mutants grow normally on dimethylglycine and sarcosine. The
133 patterns are similar on solid media, except that the *flhS* and *flhR* mutants exhibit a partial defect
134 for growth with dimethylglycine as the substrate (Figs. S5-7). We could detect significant
135 concentrations of formaldehyde in the supernatants of *flhS* and *flhR* mutants grown on choline,
136 glycine betaine and dimethylglycine (Table 1). We conclude that formaldehyde is generated as a
137 product of the oxidation of both glycine betaine and dimethylglycine, but that the amount of

138 formaldehyde accumulated in dimethylglycine grown cells is insufficient to impose a phenotype
139 on the *flhS* and *flhR* mutants. If one equivalent of formaldehyde is generated during the oxidation
140 of glycine betaine and of dimethylglycine, then growth on choline and glycine betaine is
141 accompanied by the formation of two equivalents of formaldehyde, while growth on
142 dimethylglycine generates just one equivalent.

143 All growth phenotypes on solid (Figs. S1-S7) and liquid (not shown) media associated
144 with the *flhS* and *flhR* deletions could be complemented by expression of the corresponding
145 genes *in trans* from recombinant plasmids.

146 **Formaldehyde generated from choline degradation activates the FlhSR signaling pathway.**
147 It has been proposed that formaldehyde generated during choline degradation activates
148 transcription of genes required for formaldehyde oxidation (12). To confirm that choline-
149 derived formaldehyde activates FlhSR and to determine the source(s) of formaldehyde, we took
150 advantage of the fact that the promoter of the structural gene (*mxaF*) encoding the methanol
151 dehydrogenase is activated by formaldehyde, by a mechanism that requires FlhSR (7, 12). We
152 constructed an *mxaF-lacZ* promoter-reporter fusion and confirmed the expected low activity in
153 cells grown on succinate and >10-fold higher activity in cells grown on choline (Table 1). The
154 promoter fusion also had >10-fold higher activities in cells grown on glycine betaine, and
155 dimethylglycine, confirming that degradation of these substrates generates formaldehyde (Table
156 2). In all cases, induction of the *mxaF* promoter was eliminated in *flhS* and *flhR* mutants, and
157 introduction of the corresponding genes on plasmids complemented or partially complemented
158 the mutant phenotypes. These patterns of *mxaF* promoter activity correlate well with the
159 formaldehyde accumulation data and mutant growth phenotypes and further suggest that

160 degradation of glycine betaine and dimethylglycine produces formaldehyde that can switch on an
161 FlhSR-activated promoter, while sarcosine catabolism does not.
162 We also measured activity of the GSH-dependent formaldehyde dehydrogenase,
163 expression of which is believed to be activated by FlhSR (12). Similar to the pattern of *mxaF-*
164 *lacZ* activity, GD-FALDH activity was low in extracts of cells grown on succinate and sarcosine,
165 and high in cells grown on choline, glycine betaine and dimethylglycine (Table 3). Induction of
166 GD-FALDH activity was lost in *flhS* and *flhR* mutants (Table 3) and mutant phenotypes could be
167 complemented by expression of the corresponding genes from a plasmid (data not shown).
168 Our measurements of *mxaF* promoter activity and GD-FALDH enzyme activity both
169 implicate the oxidation of glycine betaine and dimethylglycine as the major sources of
170 formaldehyde. The predicted dimethylglycine dehydrogenase encoded by Pden_1940 is likely to
171 be required for dimethylglycine catabolism. We focused on identifying the enzyme(s) required
172 for growth on glycine betaine.
173 **Redundancy of enzymes processing glycine betaine.**
174 In *Pseudomonas aeruginosa* and *P. syringae*, a two gene operon required for growth on glycine
175 betaine encodes a predicted redox enzyme, GbcAB (17, 23). In *Chromohalobacter salexigens*,
176 this enzyme has been characterized as a formaldehyde-generating glycine betaine
177 monooxygenase (named BmoAB) comprising a Rieske non-heme iron oxygenase (BmoA) and
178 an NADH-dependent flavin reductase, BmoB (18). In *P. denitrificans*, the predicted proteins
179 most similar to GbcAB are encoded by Pden_4896 and 4897. We constructed a deletion mutant
180 lacking these two genes and found only a modest defect for growth on choline and glycine
181 betaine (Fig. 3).

182 A previous study of proline betaine catabolism in *P. denitrificans* characterized two
183 additional members of the GbcAB family (9). HpbC1B1 (encoded by Pden_1188-89) and
184 HpbC2B2 (Pden_2831-32) are both annotated as stachydrine (L-proline betaine) demethylases
185 (or monooxygenases). Deletion of both pairs of genes was required to eliminate growth on
186 stachydrine, the implication being that both enzymes are able to oxidize stachydrine to
187 methylproline (9). Since HpbC2B2 are more similar to GbcAB than HpbC1B1, we deleted the
188 *hpbC2B2* (Pden_2831-32) genes and found that the mutant exhibits a greatly extended lag phase
189 when cultured on glycine betaine. The double mutant lacking both enzymes has a null
190 phenotype for growth on choline and glycine betaine, implying that both GbcAB and HpbC2B2
191 can oxidize glycine betaine. This further suggests that HpbC2B2 has a relaxed substrate
192 specificity being able to act on both glycine betaine (this work) and stachydrine (9).
193 Catabolism of stachydrine is predicted to generate two equivalents of formaldehyde (9)
194 yet we found that *flhS* and *flhR* mutants have no growth defect on stachydrine (Fig. S8), in
195 contrast to growth on glycine betaine (Fig. 2), which also generates two equivalents of
196 formaldehyde. The predicted GSH-independent formaldehyde dehydrogenase is encoded by a
197 gene (Pden_1186, *hpbM*) immediately downstream of the genes encoding the stachydrine
198 demethylase HpbC1B1 and a proline betaine epimerase (9). We therefore speculated that
199 expression of HpbM might be specifically induced by growth on stachydrine. We measured only
200 background levels of GD-FALDH in extracts of cells grown on stachydrine but increased
201 activities of a GSH-independent formaldehyde dehydrogenase compared to cells grown on all
202 other substrates. This induction by growth on stachydrine is dependent on FlhSR, thus induction
203 of the GSH-independent formaldehyde dehydrogenase by growth on stachydrine does not
204 explain the ability of *flhS* and *flhR* mutants to grow on this substrate. Perhaps the poorly

205 characterized methylproline demethylase is not a source of formaldehyde (for example, if it is
206 H_4F-linked) such that growth on stachydrine generates only one equivalent of formaldehyde that
207 is insufficient to impose a growth defect on the regulatory mutants. Assay of *mxaF-lacZ* reporter
208 activity confirmed that sufficient formaldehyde is generated during growth on stachydrine to
209 induce the promoter (Table 2). The phenotypes exhibited during growth on stachydrine resemble
210 those observed during growth on dimethylglycine, which we suggest generates just one
211 equivalent of formaldehyde.

212 **The pathway for choline degradation in *Paracoccus denitrificans.***

213 Considering the literature, genome annotations and metabolic predictions in the KEGG database
214 (24), and our data, we propose a choline metabolic pathway shown in Figure 1, comprising
215 enzymes that are the products of the genes and operons in Figure S1. Choline degradation is
216 initiated with choline oxidation to betaine aldehyde by a choline dehydrogenase (BetA), and then
217 to glycine betaine by a betaine aldehyde dehydrogenase (BetB). In *P. denitrificans*, these
218 enzymes are likely encoded by Pden_1896 and Pden_1897, respectively. Upstream of these
219 genes is Pden_1898 which is annotated to encode BetI, a repressor that likely mediates choline
220 regulation of the *betAB* genes (25).

221 Our results indicate that the conversion of glycine betaine to dimethylglycine can be
222 catalyzed primarily by HpbC2B2 (encoded by Pden_2832-2831), and in its absence, by the
223 GbcAB (Pden_4897-4896). This step is a major source of formaldehyde in the choline
224 degradation pathway and FlhSR signaling is required for expression of the enzyme(s) responsible
225 for further catabolism of formaldehyde.

226 Conversion of dimethylglycine to sarcosine is likely catalyzed by a THF-dependent
227 dimethylglycine dehydrogenase (encoded by Pden_1940). This enzyme can generate

228 formaldehyde in vitro (21) and, our evidence suggests, also in vivo. In either case, our evidence
229 indicates that this reaction does not liberate sufficient formaldehyde to inhibit growth of the
230 *flhSR* mutants.

231 The *P. denitrificans* genome does not encode a predicted monomeric sarcosine oxidase
232 but has two sets of genes annotated to encode the subunits of a tetrameric enzyme (Pden_0512,
233 0514-0516, and Pden_4909-4906), with the latter likely being co-expressed with a 5,10-
234 methylene THF dehydrogenase (Pden_4905) from a shared promoter. Our data strongly suggest
235 that sarcosine oxidation does not generate formaldehyde, which is consistent with catabolism by
236 a THF-dependent sarcosine oxidase.

237

238 **DISCUSSION**

239 The quaternary amine choline is ubiquitously present in eukaryotes, being a component of, for
240 example, choline phospholipids. Bacterial degradation of choline by the aerobic pathway
241 generates glycine betaine as an intermediate (16). The anaerobic degradation of choline is not
242 considered here since it requires a glycyl radical enzyme (26) that is not encoded in the *P.*
243 *denitrificans* genome. Glycine betaine and proline betaine are quaternary amines that function as
244 compatible solutes, accumulating to high concentrations in bacteria exposed to osmotic stress
245 (27, 28). These and other compatible solutes may also protect against other environmental
246 stresses, for example cold stress (29). Glycine and proline betaine are also osmoprotective in
247 plants and marine algae (30–32). Thus, betaines and choline are potentially abundant in the
248 biosphere and pathways for the aerobic degradation of choline, glycine betaine and proline
249 betaine have been characterized in a variety of environmental and plant- and animal-associated
250 bacteria (9, 16).

251 The catabolic degradation of quaternary amines involves the removal of methyl groups
252 that may be released as formaldehyde, conjugated with tetrahydrofolate (H_4F) to generate
253 methylene-H_4F, or transferred to a suitable acceptor molecule by a methyltransferase (for
254 example, to homocysteine, making methionine). Thus, while degradation of choline and glycine
255 betaine is associated with the production of formaldehyde, the number of equivalents of
256 formaldehyde and their source(s) have typically not been explicitly defined (16, 20). We have
257 shown that the formaldehyde that is made in vivo from glycine betaine in *Paracoccus*
258 *denitrificans* originates predominantly from the oxidations of glycine betaine and
259 dimethylglycine. This sets an upper limit on two equivalents of formaldehyde made from choline
260 and glycine, and the number may be lower than that if the H_4F-linked dimethylgycine
261 dehydrogenase conserves some methyl carbon in the form of methylene-H_4F (20, 21, 33).
262 Carnitine is another quaternary amine that can be degraded by a pathway that generates
263 glycine betaine as an intermediate (34). It was therefore unexpected that our mutant lacking both
264 GbcAB and HpbC2B2 (which cannot grow on glycine betaine) retains some ability to grow on
265 carnitine, albeit significantly less well than wild type. One possible explanation is that *P.*
266 *denitrificans* has an alternative pathway for carnitine degradation that does not generate glycine
267 betaine, as described in *Acinetobacter baumanii* (35). Some of the enzymes of this pathway
268 appear to be conserved in *P. denitrificans*, however there is no indication that this strain can
269 catabolize the trimethylamine by-product of carnitine metabolism by the *A. baumanii* pathway
270 (10, 35).
271 In this work, we have successfully used mutant phenotypes and promoter and enzyme
272 activities as probes for the endogenous generation of formaldehyde. In addition to the C1
273 substrates methanol and methylamine, a variety of quaternary amines can be sources of

274 formaldehyde when degraded, requiring the induction of mechanisms to remove the
275 formaldehyde which is otherwise toxic.
276
277 **METHODS**
278 **Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids used in this
279 study are detailed in Supplementary Table S1. *Paracoccus denitrificans* Pd1222 (36) was used as
280 the wild type strain. *Escherichia coli* S17-1 (37) was used as the host for the introduction of
281 plasmids into *P. denitrificans* by conjugation (38). L broth (10 g tryptone.liter^{-1}, 5 g yeast
282 extract.liter^{-1}, 5 g NaCl.liter^{-1}) was used for the routine propagation of *E. coli* and *P. denitrificans*
283 at 37 °C or 30 °C, respectively.
284 For phenotypic assays, cultures were grown in a defined minimal medium (39)
285 containing sodium succinate (20 mM), methanol (50 mM), choline chloride (32 mM), glycine
286 betaine (32 mM), dimethylglycine (40 mM), sarcosine (54.3 mM), stachydrine hydrochloride (12
287 mM), L-proline (32 mM) or carnitine hydrochloride (12 mM). Aerobic cultures were grown in
288 200 µl minimal medium in 96-well plates shaken at 420 rpm. Growth was assayed every 2-4
289 hours by measurement of light scatter at 650 nm (OD650). Growth was also assessed on minimal
290 medium agar streak plates.
291 **Genetic manipulations.** Plasmid pSTBlue-1 (Novagen) was used as the vector for routine DNA
292 manipulations. Triparental matings were performed by mixing log-phase *P. denitrificans*, *E. coli*
293 S17-1 transformed with recombinant plasmid derivatives, and *E. coli* DH5α (pRK2013) in a
294 2:1:1 ratio and incubating on L agar for 24 h at 30 °C. *P. denitrificans* exconjugants were
295 selected by rifampicin and indicated antibiotic(s). For conjugation and subsequent selection salt-
296 free L broth and a low-salt (4 g sodium chloride.liter^{-1}) L agar were used.

297 Unmarked in-frame deletions were made by allelic replacement using pK18*mobsacB*
298 (40). Briefly, ~500 bp regions flanking the gene(s) to be deleted were amplified separately and
299 then ligated together by PCR using overlapping regions added to the primers. The ligated
300 fragment was cloned into pK18*mobsacB* then mobilized into *P. denitrificans* as described above.
301 Selection for kanamycin resistance yielded strains in which the plasmid was integrated into the
302 chromosome by a single homologous recombination event. Recombinants with double
303 crossovers were then isolated on the basis of sucrose resistance and kanamycin sensitivity;
304 deletion mutations were confirmed by PCR.

305 For complementation tests, genes were cloned into the broad host-range expression
306 vector pIND4 which allows induction of expression by IPTG (41). A promoter-*lacZ* reporter
307 fusion was constructed by cloning 300 bp upstream of the start codon of the methanol
308 dehydrogenase structural gene *mxaF* into pMP220 (42). After triparental mating into *P.*
309 *denitrificans*, selection for recombinant pIND4 and pMP220 plasmids was done using 25 µg/ml
310 kanamycin or 4 µg/ml tetracycline respectively. PCR confirmation was done with isolated
311 plasmids.

312 **Formaldehyde measurement.** Formaldehyde accumulation in supernatants collected from
313 stationary phase cultures was measured using a modified NASH assay (43). 96-well plates were
314 centrifuged at 5,000 *g* for 5 min at 4 °C after the completion of growth measurements. Triplicate
315 125 µl aliquots of each supernatant were mixed with 125 µl of NASH reagent (0.3% acetic acid
316 and 0.2 % acetylacetone added freshly to a 3% ammonium acetate solution) in a clear round
317 bottom 96 well plate and incubated at 37 °C for 50 minutes for color development.
318 Formaldehyde standards ranged from 0.01 mM to 1 mM. The plate was equilibrated to room

319 temperature and the absorbance at 412 nm was recorded. Concentrations are reported after
320 normalization to the final culture densities.
321 **Enzyme assays**. β-galactosidase expressed from the *mxaF* promoter-*lacZ* fusion was assayed as
322 described by Miller (44). Cultures (three independent replicates) were grown in 96 well plates,
323 0.05 ml samples in 0.9 ml reaction mix were made permeable with chloroform and SDS and
324 assayed according to the standard protocol (44), the absorbance at 420 nm was measured in 96-
325 well plates.
326 Glutathione-dependent formaldehyde dehydrogenase and GSH-independent
327 formaldehyde dehydrogenase were assayed in extracts prepared from 5-50 ml cultures. Mid- to
328 late-log phase cultures were harvested at 6,000 *g* at 4 °C for 20 min and cells were washed twice
329 with sodium phosphate buffer (pH 7.4). The wet cell pellet was resuspended in approximately
330 three volumes of phosphate buffer along with 2 mM DTT and 2 mM PMSF. The cell suspension
331 was sonicated with a cycle of 20s on/20s off for 2-5 min in an ice-water bucket. The sonicated
332 suspension was clarified by centrifugation at 16,000 g at 4 °C for 20 min.
333 Enzyme activity was measured at 30 °C using a Cary UV spectrophotometer. The 3 ml
334 reaction mixture contained 50-200 µl of cell lysate in 100 mM sodium phosphate buffer (pH 7.4)
335 and 1 mM NAD^+. For the GSH-independent formaldehyde dehydrogenase, assays were started
336 by the addition of 10 mM formaldehyde. For the GD-FALDH, formaldehyde addition was
337 followed by 20 mM GSH. Specific activity was calculated as previously described using 6.22 as
338 the millimolar extinction coefficient of NADH at 340 nm (45). Enzyme activities were assayed
339 in triplicate in extracts of three independently grown cultures. Protein concentrations in cell
340 lysates were measured with the Pierce BCA assay kit.
341

342 **FIGURE LEGENDS**

343

344 **FIGURE 1.**

345 **Pathways for catabolism of choline, carnitine and L-proline betaine and C1 substrates in**

346 ***Paracoccus denitrificans*.**

347 Metabolic pathways are proposed based on previous literature (see citations in the text), the

348 KEGG database (24) and results presented in this paper. Enzymes that are known or likely

349 sources of formaldehyde are highlighted in orange. Solid arrows denote experimentally

350 supported reactions, dashed lines are predictions based on the genome annotation. Based on the

351 data presented in this paper, we suggest that formaldehyde generated from glycine betaine and

352 dimethylglycine is oxidized predominantly by GD-FALDH, while the GSH-independent enzyme

353 oxidizes formaldehyde generated from L-proline betaine and methylpoline. The genes encoding

354 the enzymes shown in this diagram are depicted in Figure S1.

355

356 **FIGURE 2.**

357 **Growth phenotypes of Δ*flhS* and Δ*flhR* mutants.**

358 Wild type *P. denitrificans* and Δ*flhS* and Δ*flhR* deletion mutants were grown in minimal media

359 containing either (A) choline, (B) glycine betaine, (C) dimethylglycine or (D) sarcosine as sole

360 sources of carbon and energy (substrate concentrations were adjusted such that carbon was

361 equimolar). Growth data are the mean of three biological replicates and the error bars represent

362 standard deviations.

363

364

365

366 **FIGURE 3.**

367 **Growth phenotypes of Δ*gbcAB*, Δ*hpbC2B2* and Δ*gbcAB* Δ*hpbC2B2* mutants.**

368 Wild type *P. denitrificans* and Δ*gbcAB*, Δ*hpbC2B2* and Δ*gbcAB* Δ*hpbC2B2* mutants were grown
369 in minimal media containing (A) choline, (B) glycine betaine, (C) dimethylglycine, and (D)
370 stachydrine as sole sources of carbon and energy (substrate concentrations were adjusted such
371 that carbon was equimolar). Growth data are the mean of three biological replicates and the error
372 bars represent standard deviations.

373 **TABLE 1**. Growth phenotypes on solid media and formaldehyde accumulation in liquid culture supernatants.

374

	Growth phenotype[a]						Formaldehyde accumulation[b]		
	Wild type	Δ*flhS*	Δ*flhR*	Δ*gbcAB*	Δ*hpbC2B2*	Δ*gbcAB* Δ*hpbC2B2*	Wild type	Δ*flhS*	Δ*flhR*
Succinate	+++	+++	+++	+++	+++	+++	-	-	-
Choline	+++	-	+	+++	+	-	-	++	++
Glycine betaine	+++	-	+	+++	-	-	-	++	++
Dimethylglycine	+++	++	++	+++	+++	+++	-	+	+
Sarcosine	+++	+++	++	+++	+++	+++	-	-	-
Stachydrine	+++	+++	+++	+++	+++	+++	-	-	-
Carnitine	+++	+	-	++	++	+++	-	+	++

375

376 a. Streak plates were incubated for approximately 72 hours at 30 ºC. Images of representative plates and of complementation tests

377 are in Figures S4-S7.

378 b. -: not detectable, below the detection limit of the assay (0.6 μM/OD unit); +: detectable in the range 10-13 μM/OD unit; ++:

379 detectable in the range 13-28 μM/OD unit.

380

381 **TABLE 2**. β-Galactosidase activities expressed from the *mxaF-lacZ* reporter fusion.

382

	β-Galactosidase[a]					
	Wild type	Δ*flhS*	Δ*flhR*	Δ*gbcAB*	Δ*hpbC2B2*	Δ*gbcAB* Δ*hpbC2B2*
Succinate	41 (2)	39 (3)	40 (1)	ND[b]	ND	ND
Choline	574 (84)	20 (17)	30 (36)	438 (41)	134 (16)	65 (9)
Glycine betaine	646 (61)	11 (9)	2 (1)	258 (49)	118 (4)	83 (5)
Dimethylglycine	413 (115)	22 (4)	44 (33)	319 (102)	77 (20)	73 (7)
Sarcosine	200 (4)	30 (25)	51 (23)	81 (18)	104 (4)	107 (2)
Stachydrine	434 (101)	16 (5)	28 (18)	ND	ND	ND

383

384 a. β-Galactosidase activity was measured in cells grown on the indicated carbon sources. Units of activity are as defined by Miller
385 (44). β-Galactosidase activities are the means of triplicate assays from three independently grown cultures with the standard
386 deviation in parentheses.

387 b. ND: not done.

388

389 **TABLE 3**. GSH-dependent formaldehyde dehydrogenase activities.

390

	GSH-dependent formaldehyde dehydrogenase (μ mol NADH/min/mg protein)[a]					
	Wild type	Δ*flhS*	Δ*flhR*	Δ*gbcAB*	Δ*hpbC2B2*	Δ*gbcAB* Δ*hpbC2B2*
Succinate	75 (8)	6 (2)	27 (13)	ND[b]	ND	ND
Choline	361 (107)	53 (7)	39 (7)	95 (35)	264 (60)	25 (12)
Glycine betaine	539 (73)	9 (8)	21 (12)	82 (36)	207 (99)	26 (13)
Dimethylglycine	313 (62)	37 (18)	29 (3)	244 (106)	145 (49)	53 (31)
Sarcosine	135 (39)	16 (10)	14 (6)	140 (90)	32 (18)	20 (7)
Stachydrine	63 (23)	34 (5)	11 (7)	ND[b]	ND	ND

391

392 a. GSH-dependent formaldehyde dehydrogenase activities are the means of assays from three independently grown cultures with

393 the standard deviation in parentheses.

394 b. ND: not done.

395

396

397 **TABLE 4**. GSH-independent formaldehyde dehydrogenase activities.

398

399

	GSH-independent formaldehyde dehydrogenase (μ mol NADH/min/mg protein)[a]		
	Wild type	Δ*flhS*	Δ*flhR*
Succinate	4 (0.1)	2 (0.6)	3 (0.9)
Methanol	5 (2)	1 (0.6)	2(0.2)
Glycine betaine	6 (2)	5 (1)	4 (0.4)
Dimethylglycine	20 (3)	7 (4)	9 (1)
Sarcosine	13 (5)	6 (4)	14 (7)
Stachydrine	74 (21)	8 (3)	28 (4)

400

401 a. GSH-independent formaldehyde dehydrogenase activities are the means of assays from

402 three independently grown cultures with the standard deviation in parentheses.

403

404

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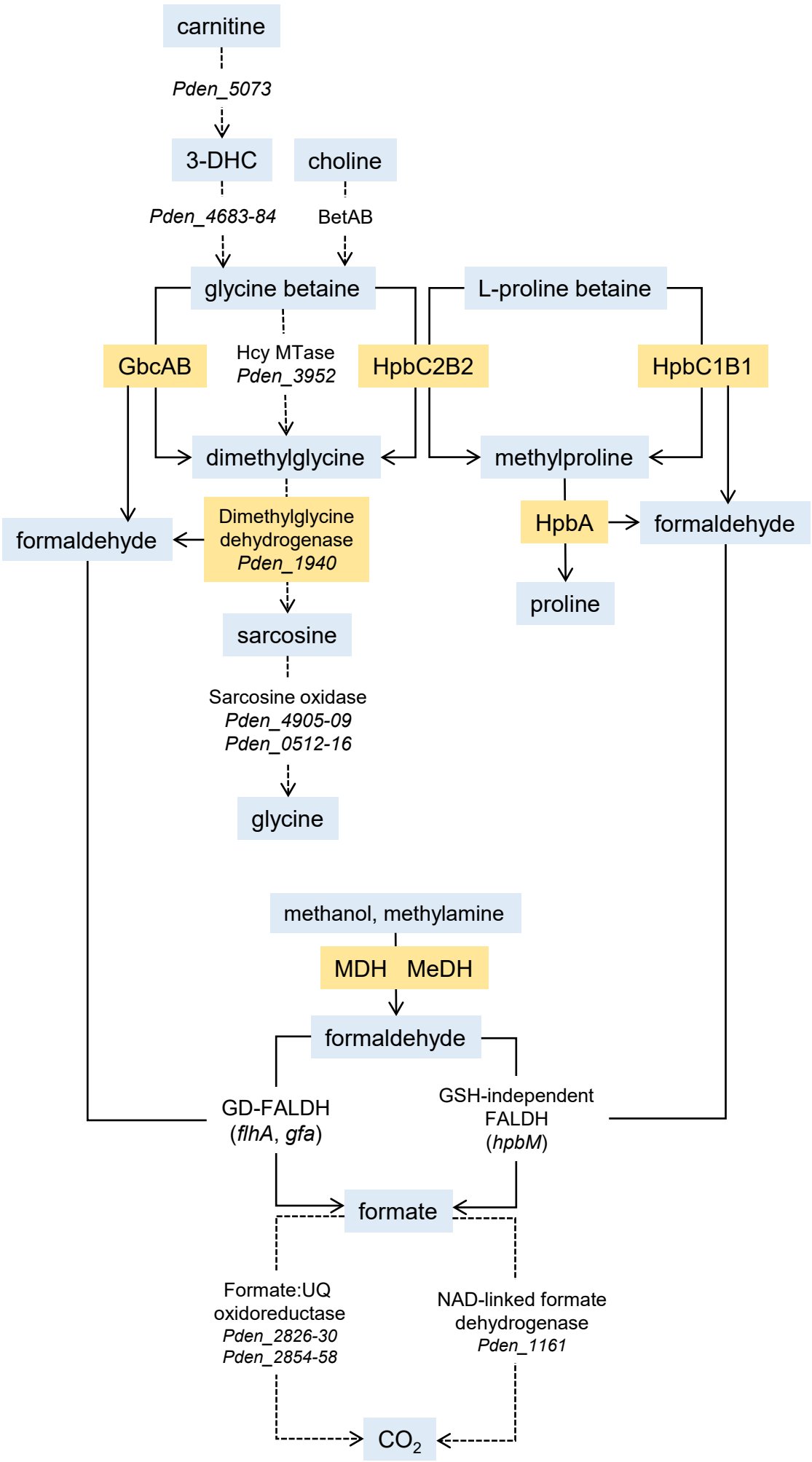
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formaldehyde
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formate
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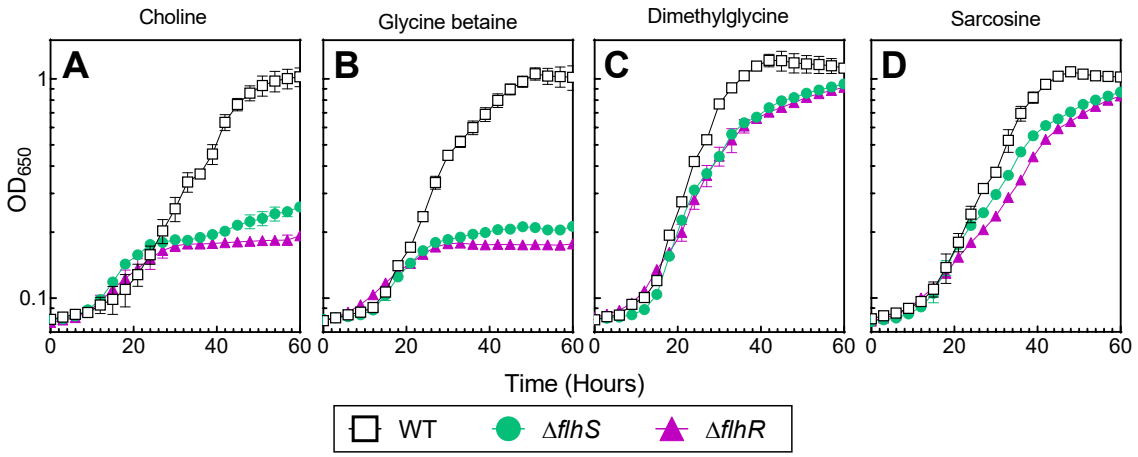
FIGURE 1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WT
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ΔflhR

FIGURE 2.

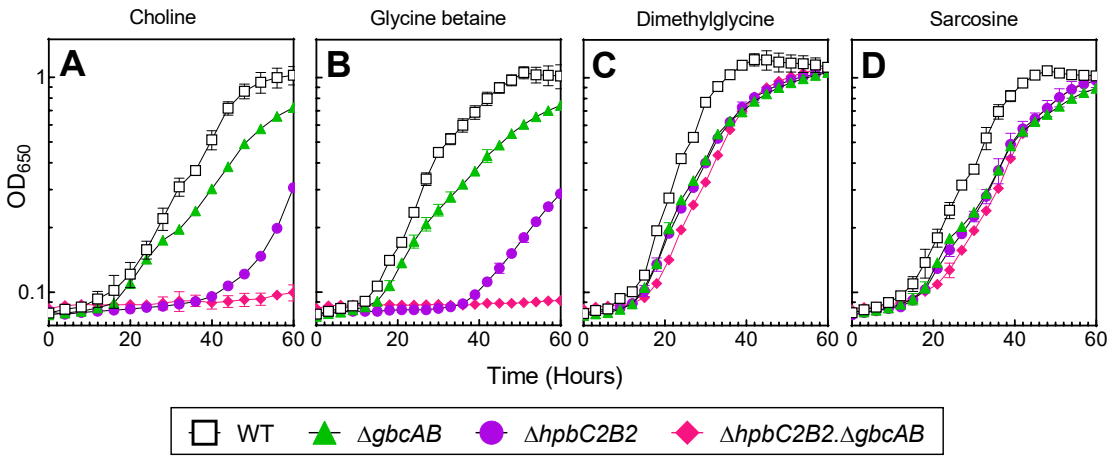
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FIGURE 3.