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

Trusha Parekh, Marcus Tsai and Stephen Spiro[#]

Department of Biological Sciences, The University of Texas at Dallas, Richardson, Texas 75080, USA.

[#]Correspondent footnote: stephen.spiro@utdallas.edu

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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. This induction involves a histidine kinase response regulator pair (FlhSR) that is likely triggered by formaldehyde. Catabolism of some complex organic substrates (for example choline and L-proline betaine) also generates formaldehyde. Thus, *flhS* and *flhR* mutants that fail to induce expression of the formaldehyde catabolic enzymes cannot grow on methanol, methylamine and choline. Choline is oxidized to glycine via glycine betaine, dimethylglycine and sarcosine. By exploring *flhSR* growth phenotypes and the activities of a promoter and enzyme known to be up-regulated by formaldehyde, we identify the oxidative demethylations of glycine betaine, dimethylglycine and sarcosine as sources of formaldehyde. Growth on glycine betaine, dimethylglycine and sarcosine is accompanied by the production of up to three, two and one equivalents of formaldehyde, respectively. 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 infer that oxidation of choline to glycine generates up to three
53 equivalents of formaldehyde and we identify the three steps in the catabolic pathway that are
54 responsible. Our work sheds further light on metabolic pathways that are likely important in a
55 variety of 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, probably by a specific transport system (8), and is oxidized to formate by a
65 cytoplasmic glutathione (GSH) dependent formaldehyde dehydrogenase, GD-FALDH (7, 9). *P.*
66 *denitrificans* also has a gene (Pden_1186, *hpbM*) encoding a predicted GSH-independent
67 formaldehyde dehydrogenase (10, 11). Formate is oxidized to carbon dioxide, which is

68 assimilated by the Calvin Cycle (11, 12), although other species in the *Paracoccus* genus may
69 use the serine cycle (13).

70 Transcription of the genes required for the oxidation of methanol, methylamine and
71 formaldehyde is regulated by two two-component systems comprising histidine kinases (FlhS
72 and MoxY) and their cognate response regulators (FlhR and MoxX, respectively). MoxXY (also
73 called MxaXY) are required for transcription of the genes encoding methanol dehydrogenase and
74 associated proteins and *moxXY* mutants have a growth defect on methanol but not methylamine
75 (14). FlhSR-deficient mutants are unable to grow on both methanol and methylamine (15) and
76 the FlhSR proteins are believed to activate transcription of the operons encoding the methanol,
77 methylamine and formaldehyde dehydrogenases, and MoxXY (7). The signal that triggers the
78 FlhSR signal transduction pathway has not been identified biochemically, but it is suggested that
79 both FlhS and MoxX respond to formaldehyde (7). The DeepTMHMM algorithm (16) predicts
80 FlhS to be located in the cytoplasm, which is therefore the likely site of formaldehyde sensing.

81 FlhSR-deficient mutants are also unable to grow on choline as a source of carbon and
82 energy (15). The explanation for this phenotype is that the oxidation of choline to glycine is
83 accompanied by the production of formaldehyde and the *flhSR* mutants are poisoned because
84 they are unable to up-regulate expression of the formaldehyde dehydrogenase. Thus, ectopic
85 expression of the GD-FALDH rescues the choline growth defect of *flhS* and *flhR* mutants (15).
86 Choline catabolism in *P. denitrificans* has been variously suggested to be accompanied by the
87 production of 'several' (17), two (18) or three (19) equivalents of formaldehyde.

88 Under aerobic conditions, choline is degraded in the cytoplasm through betaine aldehyde,
89 glycine betaine, dimethylglycine and sarcosine to glycine (Fig. 1; 20). Each of the three steps
90 from glycine betaine to glycine involves the removal of one methyl group and is a potential

91 source of formaldehyde. Glycine betaine can be oxidatively demethylated by a glycine betaine
92 monooxygenase that generates dimethylglycine and formaldehyde (21, 22). In other species, a
93 glycine betaine:homocysteine methyl transferase donates the methyl group to homocysteine and
94 so makes methionine rather than formaldehyde (23, 24). Dimethylglycine can be oxidatively
95 demethylated by a dimethylglycine dehydrogenase (or oxidase). These are tetrahydrofolate
96 (THF) linked enzymes that generate methylene-THF (rather than formaldehyde) as the reaction
97 product (25). There is also a dimethylglycine demethylase that has not been characterized
98 biochemically but is a potential source of formaldehyde (20, 21). Sarcosine is oxidized either by
99 a monomeric or a tetrameric sarcosine oxidase. The former generates formaldehyde as the
100 reaction product, while the tetrameric sarcosine oxidase is THF-dependent and so makes
101 methylene-THF (26). The THF-dependent dimethylglycine and sarcosine oxidases can make
102 formaldehyde as a reaction product in vitro in the absence of THF (25, 26); whether these
103 enzymes are a source of formaldehyde in vivo is not known. Depending upon the combination of
104 enzymes used and whether or not the THF-dependent enzymes are sources of formaldehyde in
105 vivo, the conversion of choline to glycine may be accompanied by the formation of between zero
106 and three equivalents of formaldehyde (20, 24). *P. denitrificans* also has a characterized pathway
107 for the degradation of proline betaine which is predicted to generate two equivalents of
108 formaldehyde through the action of proline betaine and methyl proline demethylases (10).
109 While studying the regulation of formaldehyde metabolism in *P. denitrificans*, we
110 became interested to identify the source(s) of the formaldehyde generated during choline
111 catabolism. Reconstructing the pathway from the genome sequence is challenging for several
112 reasons: (1) for some steps there are multiple candidate enzymes encoded in the genome; (2)
113 some potentially relevant genes are duplicated, and (3) definitively assigning biochemical

114 functions to gene products is not always possible. Therefore, we have adopted an empirical
115 approach (informed by the genome sequence) to determine the likely pathway for choline
116 degradation in *P. denitrificans* and the source(s) of formaldehyde. To do so, we have taken
117 advantage of the fact that *flhSR* mutants have a defect for growth on substrates that generate
118 formaldehyde. We present evidence to indicate that the major sources of formaldehyde are two
119 orthologous monooxygenases that oxidatively demethylate glycine betaine to form
120 dimethylglycine and the subsequent oxidative demethylations of both dimethylglycine and
121 sarcosine.
122

123 **RESULTS**

124 **Formaldehyde is a by-product of the oxidation of glycine betaine and dimethylglycine.**
125 It has been reported previously that *flhS* and *flhR* mutants are unable to grow on solid media
126 containing choline as the sole source of carbon and energy (15). We have reconstructed
127 unmarked deletions in *flhS* and *flhR* and confirmed that these mutants are unable to grow on solid
128 minimal medium containing choline (Fig. S4). In liquid media, the *flhS* and *flhR* mutants grow
129 normally to an optical density of around 0.3 before arresting (Fig. 2). This pattern is consistent
130 with the accumulation of a toxic intermediate (likely to be formaldehyde).

131 We established that *P. denitrificans* grows well on intermediates of the choline
132 degradation pathway: glycine betaine, dimethylglycine and sarcosine (Fig. 2, Table 1). In liquid
133 media, the *flhS* and *flhR* mutants show growth defects on glycine betaine similar to the choline
134 phenotypes (Fig. 2), whereas both mutants grow normally on dimethylglycine and sarcosine. The
135 patterns are similar on solid media, except that the *flhS* and *flhR* mutants exhibit a partial defect
136 for growth with dimethylglycine as the substrate (Figs. S5-7). We could detect significant

137 concentrations of formaldehyde in the supernatants of *flhS* and *flhR* mutants grown on choline,
138 glycine betaine and dimethylglycine (Table 1). Thus far, we conclude that formaldehyde is
139 generated as a product of the oxidation of both glycine betaine and dimethylglycine, but that the
140 amount of formaldehyde accumulated in dimethylglycine grown cells is insufficient to impose a
141 phenotype on the *flhS* and *flhR* mutants.

142 All growth phenotypes on solid (Figs. S4-S7) and liquid (not shown) media associated
143 with the *flhS* and *flhR* deletions could be complemented by expression of the corresponding
144 genes *in trans* from recombinant plasmids.

145 **Formaldehyde generated from choline degradation activates the FlhSR signaling pathway.**
146 It has been proposed that formaldehyde generated during choline degradation activates
147 transcription of genes required for formaldehyde oxidation (15). To confirm that choline-derived
148 formaldehyde activates FlhSR and to determine the source(s) of formaldehyde, we took
149 advantage of the fact that the promoter of the structural gene (*mxaF*) encoding the methanol
150 dehydrogenase is activated by formaldehyde, by a mechanism that requires FlhSR (7, 15). We
151 constructed an *mxaF-lacZ* promoter-reporter fusion and confirmed the expected low activity in
152 cells grown on succinate and >10-fold higher activity in cells grown on choline (Table 2). The
153 promoter fusion also had >10-fold higher activities in cells grown on glycine betaine and
154 dimethylglycine, confirming that degradation of these substrates generates formaldehyde (Table
155 2). In sarcosine grown cells, *mxaF* promoter activity was intermediate between that measured in
156 succinate and dimethylglycine grown cells, suggesting that sarcosine oxidation is also a source of
157 formaldehyde. In all cases, induction of the *mxaF* promoter was eliminated in *flhS* and *flhR*
158 mutants, and introduction of the corresponding genes on plasmids complemented or partially
159 complemented the mutant phenotypes. These patterns of *mxaF* promoter activity correlate well

160 with the formaldehyde accumulation data and mutant growth phenotypes and further suggest that
161 degradation of glycine betaine, dimethylglycine and sarcosine produces sufficient formaldehyde
162 to switch on an FlhSR-activated promoter.

163 We also measured activity of the GSH-dependent formaldehyde dehydrogenase,
164 expression of which is believed to be activated by FlhSR (15). Similar to the pattern of *mxaF-*
165 *lacZ* activity, GD-FALDH activity was low in extracts of cells grown on succinate, high in cells
166 grown on choline, glycine betaine and dimethylglycine, and intermediate in cells grown on
167 sarcosine (Table 3). Note that, if each oxidative demethylation generates one equivalent of
168 formaldehyde, then growth on glycine betaine, dimethylglycine and sarcosine would be
169 accompanied by production of three, two and one equivalents of formaldehyde, respectively.
170 Accumulation of less formaldehyde is consistent with the absence of *flhSR* mutant phenotypes on
171 dimethylglycine and sarcosine and the evidence for reduced FlhSR activity in cells grown on
172 these substrates, compared to glycine betaine and choline. Induction of GD-FALDH activity was
173 lost in *flhS* and *flhR* mutants (Table 3) and mutant phenotypes could be complemented by
174 expression of the corresponding genes from a plasmid (data not shown).

175 Our measurements of *mxaF* promoter activity and GD-FALDH enzyme activity both
176 implicate the oxidation of glycine betaine, dimethylglycine and sarcosine as the sources of
177 formaldehyde. The predicted dimethylglycine dehydrogenase encoded by Pden_1940 is likely to
178 be required for dimethylglycine catabolism, and there are at least two candidate sarcosine
179 oxidases encoded in the genome (Fig. S1). We focused on identifying the enzyme(s) required
180 for growth on glycine betaine.

181

182

183 **Overlapping functions of enzymes processing glycine betaine.**

184 In *Pseudomonas aeruginosa* and *P. syringae*, a two gene operon required for growth on glycine
185 betaine encodes a predicted redox enzyme, GbcAB (21, 27). In *Chromohalobacter salexigens*,
186 this enzyme has been characterized as a formaldehyde-generating glycine betaine
187 monooxygenase (named BmoAB) comprising a Rieske non-heme iron oxygenase (BmoA) and
188 an NADH-dependent flavin reductase, BmoB (22). In *P. denitrificans*, the predicted proteins
189 most similar to GbcAB are encoded by Pden_4896 and 4897. We constructed a deletion mutant
190 lacking these two genes and found only a modest defect for growth on choline and glycine
191 betaine (Fig. 3).

192 A previous study of stachydrine catabolism in *P. denitrificans* characterized two
193 additional members of the GbcAB family (10). HpbC1B1 (encoded by Pden_1188-89) and
194 HpbC2B2 (Pden_2831-32) are both annotated as stachydrine (L-proline betaine) demethylases
195 (or monooxygenases). Deletion of both pairs of genes was required to eliminate growth on
196 stachydrine, the implication being that both enzymes are able to oxidize stachydrine to methyl-L-
197 proline (10). Since HpbC2B2 are more similar to GbcAB than HpbC1B1, we deleted the
198 *hpbC2B2* (Pden_2831-32) genes and found that the mutant exhibits markedly biphasic growth
199 when cultured on choline and glycine betaine. When subcultured into fresh medium, biphasic
200 growth of the *hpbC2B2* mutant repeated, from which we infer that the extended lag phase and
201 biphasic pattern is not due to the accumulation of mutations. The double mutant lacking both
202 enzymes has a null phenotype for growth on choline and glycine betaine, implying that both
203 GbcAB and HpbC2B2 can oxidize glycine betaine. This further suggests that HpbC2B2 has a
204 relaxed substrate specificity being able to act on both glycine betaine (this work) and stachydrine
205 (10).

206 Catabolism of stachydrine is predicted to generate two equivalents of formaldehyde (10).
207 We found that *flhS* and *flhR* mutants have no growth defect on stachydrine (Figs. S2 and S8), as
208 is the case for growth on dimethylglycine (Fig. 2), which we suggest also generates two
209 equivalents of formaldehyde. From both these growth substrates, sufficient formaldehyde is
210 made to activate the *mxaF* promoter (Table 2), but insufficient to impose a phenotype on the
211 *flhSR* mutants (Figs. 2 and S2).
212 The predicted GSH-independent formaldehyde dehydrogenase is encoded by a gene
213 (Pden_1186, *hpbM*) immediately downstream of the genes encoding the stachydrine demethylase
214 HpbC1B1 and a proline betaine epimerase (10). We therefore speculated that expression of
215 HpbM might be specifically induced by growth on stachydrine. We measured only background
216 levels of GD-FALDH in extracts of cells grown on stachydrine but increased activities of a
217 GSH-independent formaldehyde dehydrogenase compared to cells grown on all other substrates.
218 Our results are consistent with transcriptomics data that show upregulation of *hbpM* in cells
219 grown on stachydrine versus cells grown on methanol (10). However, the same transcriptomics
220 data also suggest up-regulation of genes encoding the GD-FALDH by growth on stachydrine
221 (10), which we do not see reflected in the enzyme activity.
222
223 **DISCUSSION**
224 Considering the literature, genome annotations and metabolic predictions in the KEGG database
225 (28), and our data, we propose a choline metabolic pathway shown in Figure 1, comprising
226 enzymes that are the products of the genes and operons in Figure S1. Choline degradation is
227 initiated with choline oxidation to betaine aldehyde by a choline dehydrogenase (BetA), and then
228 to glycine betaine by a betaine aldehyde dehydrogenase (BetB). In *P. denitrificans*, these

229 enzymes are likely encoded by Pden_1896 and Pden_1897, respectively. Upstream of these
230 genes is Pden_1898 which is annotated to encode BetI, a repressor that likely mediates choline
231 regulation of the *betAB* genes (29).

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

237 Conversion of dimethylglycine to sarcosine is likely catalyzed by a THF-dependent
238 dimethylglycine dehydrogenase (encoded by Pden_1940). This enzyme can generate
239 formaldehyde in vitro (25) and, our evidence suggests, also in vivo. In either case, our evidence
240 indicates that this reaction does not liberate sufficient formaldehyde to inhibit growth of the
241 *flhSR* mutants.

242 The *P. denitrificans* genome does not encode a predicted monomeric sarcosine oxidase
243 but has two sets of genes annotated to encode the subunits of a tetrameric enzyme (Pden_0512,
244 0514-0516, and Pden_4909-4906), with the latter likely being co-expressed with a 5,10-
245 methylene THF dehydrogenase (Pden_4905) from a shared promoter. Although the tetrameric
246 sarcosine oxidase is THF-linked, it can make formaldehyde as a reaction product in vitro (26),
247 our evidence suggests that this enzyme is also a source of formaldehyde in vivo.

248 In this work, we have successfully used mutant phenotypes and promoter and enzyme
249 activities as probes for the endogenous generation of formaldehyde. We infer that up to three
250 equivalents of formaldehyde may be produced during choline degradation in *P. denitrificans*.
251 Direct quantitation of this stoichiometry would require measurement of formaldehyde

252 production. This is challenging in part because of the pathways for the onward catabolism of
253 formaldehyde. Ultimately, isotope-based metabolic flux analysis and metabolic modeling (11)
254 could be used to provide a complete description of the metabolic fate of the methyl groups
255 abstracted from quaternary amines such as choline and stachydrine.
256

257 **METHODS**

258 **Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids used in this
259 study are detailed in Supplementary Table S1. *Paracoccus denitrificans* Pd1222 (30) was used as
260 the wild type strain. *Escherichia coli* S17-1 (31) was used as the host for the introduction of
261 plasmids into *P. denitrificans* by conjugation (32). L broth (10 g tryptone.liter^{-1}, 5 g yeast
262 extract.liter^{-1}, 5 g NaCl.liter^{-1}) was used for the routine propagation of *E. coli* and *P. denitrificans*
263 at 37 °C or 30 °C, respectively.

264 For phenotypic assays, cultures were grown in a defined minimal medium (33)
265 containing sodium succinate (20 mM), methanol (50 mM), choline chloride (32 mM), glycine
266 betaine (32 mM), dimethylglycine (40 mM), sarcosine (54.3 mM), stachydrine hydrochloride (12
267 mM), L-proline (32 mM). Aerobic cultures were grown in 200 µl minimal medium in 96-well
268 plates shaken at 420 rpm. Growth was assayed every 2-4 hours by measurement of light scatter
269 at 650 nm (OD650). Growth was also assessed on minimal medium agar streak plates.

270 **Genetic manipulations.** Plasmid pSTBlue-1 (Novagen) was used as the vector for routine DNA
271 manipulations. Triparental matings were performed by mixing log-phase *P. denitrificans*, *E. coli*
272 S17-1 transformed with recombinant plasmid derivatives, and *E. coli* DH5α (pRK2013) in a
273 2:1:1 ratio and incubating on L agar for 24 h at 30 °C. *P. denitrificans* exconjugants were

274 selected by rifampicin and indicated antibiotic(s). For conjugation and subsequent selection salt-
275 free L broth and a low-salt (4 g sodium chloride.liter^{-1}) L agar were used.
276 Unmarked in-frame deletions were made by allelic replacement using pK18*mobsacB*
277 (34). Briefly, ~500 bp regions flanking the gene(s) to be deleted were amplified separately and
278 then ligated together by PCR using overlapping regions added to the primers. The ligated
279 fragment was cloned into pK18*mobsacB* then mobilized into *P. denitrificans* as described above.
280 Selection for kanamycin resistance yielded strains in which the plasmid was integrated into the
281 chromosome by a single homologous recombination event. Recombinants with double
282 crossovers were then isolated on the basis of sucrose resistance and kanamycin sensitivity;
283 deletion mutations were confirmed by PCR.
284 For complementation tests, genes were cloned into the broad host-range expression
285 vector pIND4 which allows induction of expression by IPTG (35). A promoter-*lacZ* reporter
286 fusion was constructed by cloning 300 bp upstream of the start codon of the methanol
287 dehydrogenase structural gene *mxaF* into pMP220 (36). After triparental mating into *P.*
288 *denitrificans*, selection for recombinant pIND4 and pMP220 plasmids was done using 25 µg/ml
289 kanamycin or 4 µg/ml tetracycline respectively. PCR confirmation was done with isolated
290 plasmids.
291 **Formaldehyde measurement.** Formaldehyde accumulation in supernatants collected from
292 stationary phase cultures was measured using a modified NASH assay (37). 96-well plates were
293 centrifuged at 5,000 *g* for 5 min at 4 °C after the completion of growth measurements. Triplicate
294 125 µl aliquots of each supernatant were mixed with 125 µl of NASH reagent (0.3% acetic acid
295 and 0.2 % acetylacetone added freshly to a 3% ammonium acetate solution) in a clear round
296 bottom 96 well plate and incubated at 37 °C for 50 minutes for color development.

297 Formaldehyde standards ranged from 0.01 mM to 1 mM. The plate was equilibrated to room
298 temperature and the absorbance at 412 nm was recorded. Concentrations are reported after
299 normalization to the final culture densities.

300 **Enzyme assays**. β-galactosidase expressed from the *mxaF* promoter-*lacZ* fusion was assayed as
301 described by Miller (38). Cultures (three independent replicates) were grown in 96 well plates,
302 0.05 ml samples in 0.9 ml reaction mix were made permeable with chloroform and SDS and
303 assayed according to the standard protocol (38), the absorbance at 420 nm was measured in 96-
304 well plates.

305 Glutathione-dependent formaldehyde dehydrogenase and GSH-independent
306 formaldehyde dehydrogenase were assayed in extracts prepared from 5-50 ml cultures. Mid- to
307 late-log phase cultures were harvested at 6,000 *g* at 4 °C for 20 min and cells were washed twice
308 with sodium phosphate buffer (pH 7.4). The wet cell pellet was resuspended in approximately
309 three volumes of phosphate buffer along with 2 mM DTT and 2 mM PMSF. The cell suspension
310 was sonicated with a cycle of 20s on/20s off for 2-5 min in an ice-water bucket. The sonicated
311 suspension was clarified by centrifugation at 16,000 g at 4 °C for 20 min.

312 Enzyme activity was measured at 30 °C using a Cary UV spectrophotometer. The 3 ml
313 reaction mixture contained 50-200 µl of cell lysate in 100 mM sodium phosphate buffer (pH 7.4)
314 and 1 mM NAD^+. For the GSH-independent formaldehyde dehydrogenase, assays were started
315 by the addition of 10 mM formaldehyde. For the GD-FALDH, formaldehyde addition was
316 followed by 20 mM GSH. Specific activity was calculated as previously described using 6.22 as
317 the millimolar extinction coefficient of NADH at 340 nm (39). Enzyme activities were assayed
318 in triplicate in extracts of three independently grown cultures. Protein concentrations in cell
319 lysates were measured with the Pierce BCA assay kit.

320

321 **FIGURE LEGENDS**

322

323 **FIGURE 1.**

324 **Pathways for catabolism of choline, stachydrine (L-proline betaine) and C1 substrates in**

325 ***Paracoccus denitrificans*.**

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

327 KEGG database (28) and results presented in this paper. Enzymes that are known or likely

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

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

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

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

332 oxidizes formaldehyde generated from L-proline betaine and methyl-L-proline. The genes

333 encoding the enzymes shown in this diagram are depicted in Figure S1.

334

335 **FIGURE 2.**

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

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

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

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

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

341 standard deviations.

342

343

344

345 **FIGURE 3.**

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

347 Wild type *P. denitrificans* and Δ*gbcAB*, Δ*hpbC2B2* and Δ*gbcAB* Δ*hpbC2B2* mutants were grown
348 in minimal medium containing (A) choline, (B) glycine betaine, (C) dimethylglycine, and (D)
349 sarcosine as sole sources of carbon and energy (substrate concentrations were adjusted such that
350 carbon was equimolar). Growth data are the mean of three biological replicates and the error
351 bars represent standard deviations.

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

353

	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	+++	+++	+++	+++	+++	+++	-	-	-

354

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

356 are in Figures S4-S8.

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

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

359

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

361

	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

362

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

366 b. ND: not done.

367

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

369

	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

370

371 a. GSH-dependent formaldehyde dehydrogenase activities are the means of assays from three independently grown cultures with
372 the standard deviation in parentheses.
373 b. ND: not done.

374

375

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

377

378

	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)
Choline	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)

379

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

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

382

383

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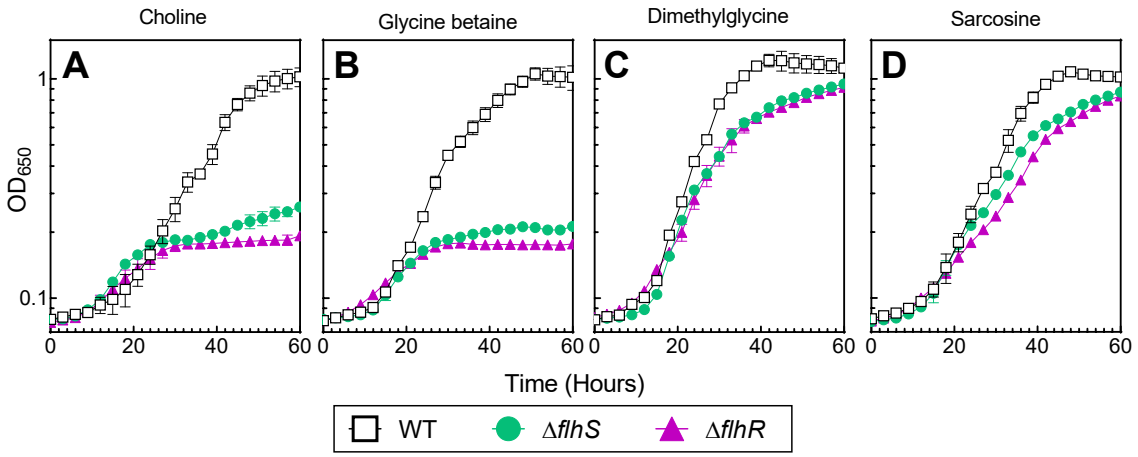
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Glycine betaine
Dimethylglycine
Sarcosine
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B
C
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20
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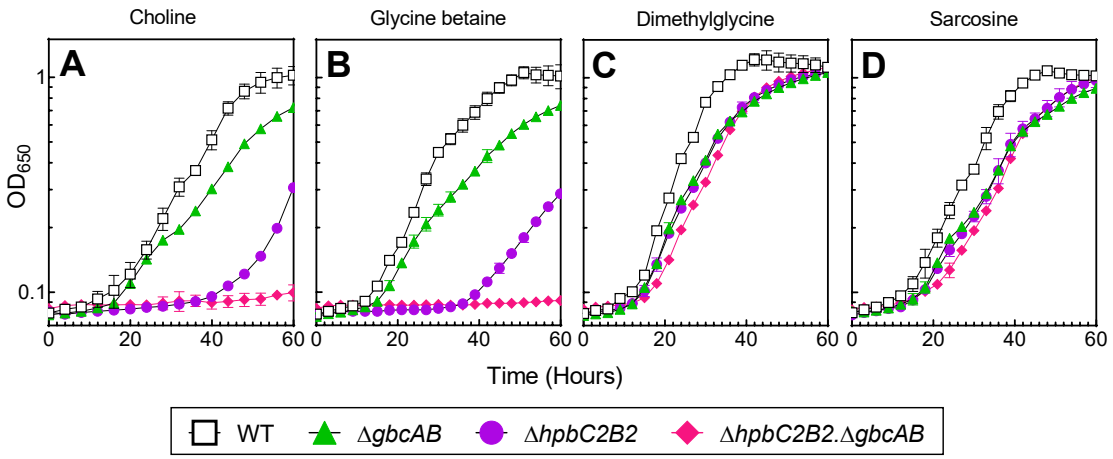
60
Time (Hours)
WT
ΔflhS
ΔflhR

FIGURE 2.


Choline
Glycine betaine
Dimethylglycine
Sarcosine
A
B
C
D
OD650
1
0.1
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20
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Time (Hours)
WT
ΔgbcAB
ΔhpbC2B2
ΔhpbC2B2.ΔgbcAB

FIGURE 3.

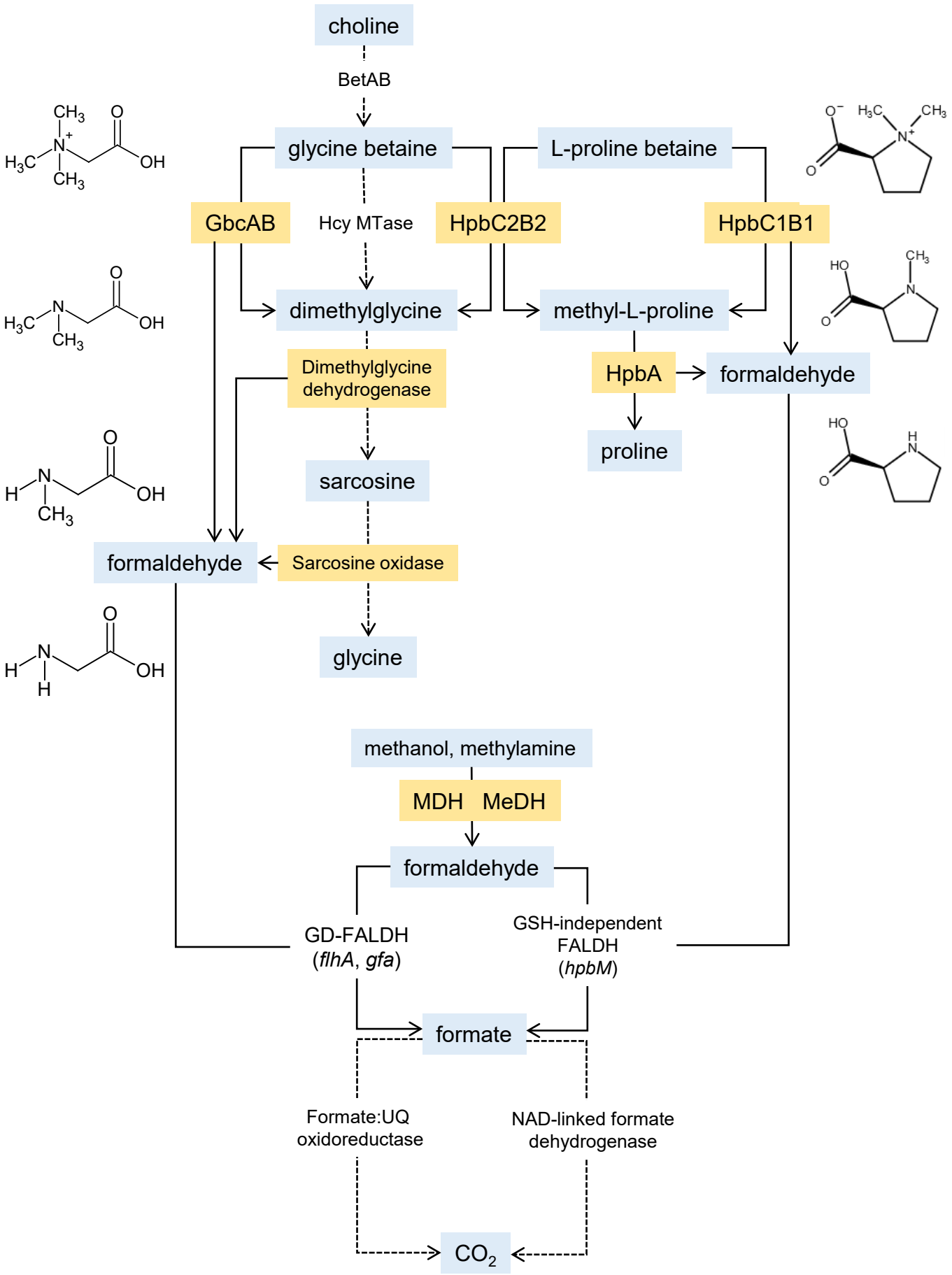
choline
BetAB
glycine betaine
L-proline betaine
GbcAB
Hcy MTase
HpbC2B2
HpbC1B1
dimethylglycine
methyl-L-proline
Dimethylglycine dehydrogenase
HpbA
formaldehyde
proline
sarcosine
formaldehyde
Sarcosine oxidase
glycine
methanol, methylamine
MDH MeDH
formaldehyde
GD-FALDH (flhA, gfa)
GSH-independent FALDH (hpbM)
formate
Formate:UQ oxidoreductase
NAD-linked formate dehydrogenase
CO2

FIGURE 1