DTU Library

Discovery of enzymes for toluene synthesis from anoxic microbial communities

Beller, Harry R.; Rodrigues, Andria V.; Zargar, Kamrun; Wu, Yu-Wei; Saini, Avneesh K.; Saville, Renee M.; Pereira, Jose H.; Adams, Paul D.; Tringe, Susannah G.; Petzold, Christopher J.

[Link back to DTU Orbit](#)

Title
Discovery of enzymes for toluene synthesis from anoxic microbial communities

Permalink
https://escholarship.org/uc/item/1cg4v7qr

Journal
Nature Chemical Biology, 14(5)

ISSN
1552-4450

Authors
Beller, HR
Rodrigues, AV
Zargar, K
et al.

Publication Date
2018-05-01

DOI
10.1038/s41589-018-0017-4

Peer reviewed

1 Enzyme discovery for toluene synthesis in anoxic microbial communities
2

3 H. R. Beller†*[1,2], A. V. Rodrigues†[1], K. Zargar†[1], Y.-W. Wu[3], A. K. Saini[1], R. M. Saville[1], J. H.
4 Pereira[1], P. D. Adams[1], S. G. Tringe[4], C. J. Petzold[1,5], J. D. Keasling[1,5,6,7]

5

6

7 Joint BioEnergy Institute, 5885 Hollis Avenue, Emeryville, CA, USA[1]; Earth and Environmental
8 Sciences, LBNL, Berkeley, CA, USA[2]; Graduate Institute of Biomedical Informatics, College of
9 Medical Science and Technology, Taipei Medical University, Taipei, Taiwan[3]; Joint Genome
10 Institute, 2800 Mitchell Drive, Walnut Creek, CA, USA[4]; Biological Systems and Engineering,
11 Lawrence Berkeley National Laboratory (LBNL), Berkeley, CA, USA[5]; Departments of
12 Chemical & Biomolecular Engineering and of Bioengineering, University of California,
13 Berkeley, CA, USA[6]; Novo Nordisk Foundation Center for Biosustainability, Technical
14 University of Denmark, Kogle Allé, DK2970-Hørsholm, Denmark[7]

15
16
17

18

19

20

21 †These authors contributed equally to this work.

22 * Corresponding author: HRBeller@lbl.gov

23

24

Microbial toluene biosynthesis was reported in anoxic lake sediments more than 3 decades ago, however the enzyme(s) catalyzing this biochemically challenging reaction have never been elucidated. Here we report the first toluene synthase, a glycyl radical enzyme of bacterial origin that catalyzes phenylacetic acid decarboxylation (PhdB), and its cognate activating enzyme (PhdA, a radical *S*-adenosylmethionine enzyme), discovered in two distinct anoxic microbial communities that produced toluene. The unconventional process of enzyme discovery from a complex microbial community (>300,000 genes) rather than from a microbial isolate, involved metagenomics- and metaproteomics-enabled biochemistry, as well as *in-vitro* confirmation of activity with recombinant enzymes. This work expands the known catalytic range of glycyl radical enzymes (only seven reaction types had been characterized previously) and aromatic hydrocarbon-producing enzymes (only one reaction type characterized previously), and will enable first-time biochemical synthesis of an aromatic fuel hydrocarbon from renewable resources, such as lignocellulosic biomass, rather than petroleum.

The extraordinary metabolic diversity of microorganisms in combination with ready access to increasingly rapid and less expensive DNA sequencing technologies has revealed a well-recognized challenge in modern biology: the dearth of experimental evidence to support functional annotation of a large fraction of genes/proteins in public data repositories[1-3]. A related challenge, termed "orphan enzymes"[4], is the abundance of unambiguously defined enzymatic activities that are not linked with specific amino acid sequences; in 2014, 22% of defined EC (Enzyme Commission) numbers were orphan enzymes[5]. To the extent that specific enzymes can be better linked to a broad range of chemically diverse reactions, the scope and versatility of

1

48biochemical transformations harnessed for biotechnological applications will be enhanced. One

49area in which knowledge of enzymes is very limited is biosynthesis of aromatic hydrocarbons,

50which could be useful as renewable fuels or chemicals made from non-petroleum feedstocks. To

51our knowledge, the only aromatic hydrocarbon that can currently be synthesized wholly from

52known enzymes is styrene, which can be produced from phenylalanine-derived *trans*-cinnamic

53acid by enzymes displaying phenylacrylate decarboxylase activity, such as ~~PAL2 from~~

54*~~Arabidopsis thaliana~~* ~~or~~ FDC1 from *Saccharomyces cerevisiae*[6].

55   We targeted the aromatic hydrocarbon toluene for enzyme discovery, as it is an important

56petrochemical with a global market of 29 million tons per year whose uses include synthesis of

57other aromatic feedstocks and serving as an effective octane booster in gasoline (octane number,

58114). Microbial sources of biogenic toluene were reported more than three decades ago,

59however, the underlying biochemistry and specific enzymes catalyzing toluene biosynthesis have

60never been elucidated. Biogenic toluene was observed in anoxic lake sediments / hypolimnion[7],

61in anoxic enrichment cultures derived from municipal sewage sludge[8], and in two bacterial

62isolates, *Tolumonas auensis*[9] and *Clostridium aerofoetidum*[10], which were reported to synthesize

63toluene from phenylacetate and L-phenylalanine (however, recent attempts to reproduce toluene

64biosynthesis by these two isolates were unsuccessful[8]). Although a toluene synthase has not been

65specifically identified, *in vitro* studies with cell-free extracts from a toluene-producing culture

66suggest catalysis by a glycyl radical enzyme (GRE)[8]. Evidence supporting the hypothesized role

67of a GRE in toluene biosynthesis included (a) irreversible inactivation by O_2 (a characteristic of

68GREs), (b) the ruling out of a mechanism involving successive reduction (phenylacetate to

69phenylacetaldehyde) and decarbonylation/deformylation (phenylacetaldehyde to toluene), which

70would not be expected to be catalyzed by GREs[11,12], and (c) the observation that the known

2

71enzyme with the greatest functional similarity to phenylacetate decarboxylase, namely *p*-
72hydroxyphenylacetate decarboxylase (HpdBC or CsdBC), is a GRE[13,14]. Although a GRE has
73been implicated in toluene biosynthesis, even the most detailed *in vitro* studies conducted to date
74have not identified any specific gene candidates[8].

75Identification of toluene synthase candidates

76   Studies to identify a toluene synthase (phenylacetate decarboxylase) were conducted with
77anaerobic, toluene-producing microbial cultures that derived from two different inocula:
78municipal sewage sludge[8] and lake sediments from Berkeley, CA (Extended Data Fig. 1). The
79sewage culture, which was more amenable to cultivation and *in vitro* studies, served as the basis
80for most of the experimental discovery studies, whereas the lake sediment culture was used
81primarily for metagenome sequencing. We employed a metagenomics- and metaproteomics-
82enabled protein purification approach for enzyme discovery from these microbial communities.
83Toluene synthase activity was monitored in chromatographically separated fractions of cell-free
84extracts from the sewage culture using *in vitro* assays that measured phenylacetic acid-2-^{13}C
85conversion to [*methyl*-^{13}C]toluene. All experimental procedures, including cultivation, cell lysis,
86protein purification by FPLC (fast protein liquid chromatography), and *in vitro* assays, were
87performed under strictly anaerobic conditions to protect the organisms and enzymes from
88molecular oxygen. Proteomic profiles of active FPLC fractions were compared to those of
89adjacent inactive (or much less active) fractions to identify toluene synthase candidates (i.e.,
90those proteins enriched in, and ideally unique to, active fractions). An unknown GRE (hereafter
91referred to as PhdB) co-eluted with the maximal toluene synthase activity (Extended Data Fig.
922). Although more than 650 proteins co-eluted with PhdB in these fractions (Supplementary
93Data File 1), this protein was initially of interest because the toluene synthase in this sewage-

3

94derived culture had been postulated to be a GRE based upon *in vitro* studies with cell-free

95extracts[8]. Notably, PhdB was one of the few glycyl radical enzymes detected in active fractions

96among the many glycyl radical enzymes encoded in the sewage community metagenome (Fig.

971). As shown in Fig. 1, only three glycyl radical enzymes were detected in the active FPLC

98fractions: (1) PhdB, (2) pyruvate formate-lyase (PflB; JGI2065J20421_100036324; IMG Taxon

99ID 3300001865), which had 99% sequence identity to known *Enterobacter* PflB copies], and (3)

100an unknown glycyl radical enzyme (JGI2065J20421_10067673; IMG Taxon ID 3300001865) –

101this protein shares ca. 47% sequence identity and key conserved residues with a known glycerol

102dehydratase (PDB 1R8W). Of these three proteins, only PhdB and the PflB had greater

103abundance in active than in flanking inactive fractions (Fig. 1), and PflB was among the most

104abundant proteins in both active *and* inactive fractions (Supplementary Data File 1), which,

105along with its well-characterized function, reduced its plausibility as a toluene synthase

106candidate.

107 The strength of *phdB* as a candidate toluene synthase gene was enhanced by its

108identification in metagenomes of both the anoxic, toluene-producing sewage and lake sediment

109cultures, despite the fact that these cultures had disparate inocula and phylogenetic compositions

110(a comparison of dominant taxa in these two cultures is shown in Extended Data Fig. 3 and

111Supplementary Data Files 2 and 3). In sewage culture metagenomes, *phdB* occurred in a three-

112gene cluster consisting of a putative transcription factor (Sequence 11, Supplementary Data File

1134), *phdB* (Sequence 6, Supplementary Data File 4), and a glycyl radical activating enzyme

114(hereafter referred to as *phdA*; Sequence 1, Supplementary Data File 4) (Fig. 2). Such adjacent

115positioning in genomes of genes encoding glycyl radical enzymes and their cognate activating

116enzymes is very common[15], as indicated in Fig. 2. Although assembled contigs from the lake

4

117sediment metagenomes (e.g., IMG Taxon ID 2100351000) were not observed to harbor the
118complete three-gene cluster detected in the sewage metagenome, the quality of these assemblies
119was suboptimal as a result of older sequencing methods used. Indeed, PCR amplification and
120Sanger sequencing of this cluster from genomic DNA of the lake culture revealed an intact three-
121gene cluster (Sequence 13-Sequence 9-Sequence 4; Supplementary Data File 4) with identical
122length (6065 bp) and strikingly similar coding and intergenic sequences compared to the sewage
123culture (Fig. 2). As shown in Fig. 2, the three genes share from ca. 87 to 96% sequence identity
124(and 86 to 97% translated sequence identity) in the sewage and lake cultures and the intergenic
125regions are ca. 82-85% identical (Sequences 15 and 16; Supplementary Data File 4).

126***In vitro* confirmation of PhdB and PhdA activity**

127 Recombinant versions of PhdA and PhdB were assayed for *in vitro* activity to confirm
128their role in catalyzing toluene biosynthesis from phenylacetate. The expected activity for PhdA
129was based on characterization of other glycyl radical activating enzymes[16]. In glycyl radical
130systems, the reduced $[4Fe-4S]^{+1}$ cluster of the activase, a radical *S*-adenosylmethionine (SAM)
131enzyme, transfers an electron to SAM, resulting in homolytic cleavage of SAM to form
132methionine and a 5'-deoxyadenosyl radical (Fig. 3a). The 5'-deoxyadenosyl radical activates the
133GRE by stereospecific abstraction of a C-2 *pro-S* H atom from a highly conserved glycine
134residue, which in turn abstracts an H atom from a conserved cysteine residue in the GRE to form
135a thiyl radical. A substrate radical is formed when the thiyl radical abstracts an H atom from the
136substrate (phenylacetic acid, in the case of PhdB; Fig. 3b).

137 *In vitro* reconstitution of the [4Fe-4S] cluster of PhdA was performed before final
138purification (all under strictly anaerobic conditions), and the [4Fe-4S] cluster was reduced with
139dithionite in an ~~anaerobic~~ anoxic assay measuring methionine production from SAM using liquid

5

140chromatography-mass spectrometry (LC/MS). Observed methionine production in the presence

141of PhdA, but not in its absence (Fig. 3a), demonstrated the expected activity of a glycyl radical

142activating enzyme.

143 The ability of activated (enzyme-radical) PhdB to catalyze decarboxylation of

144phenylacetic acid-2-^{13}C to [*methyl*-^{13}C]toluene was tested in an ~~anaerobic~~anoxic, *in vitro* assay in

145the presence of dithionite-reduced PhdA and SAM (Fig. 3b). Labeled toluene was detected by

146gas chromatography-mass spectrometry (GC/MS) in the presence of SAM but not in its absence,

147confirming the role of PhdB in catalyzing toluene biosynthesis *via* a radical mechanism. A series

148of other negative control assays also displayed negligible activity, including the following: (1)

149assays lacking PhdB but containing dithionite-reduced PhdA and SAM, (2) assays conducted

150with a mutant version of PhdB (G815A) in which the putative site of the glycyl radical was

151modified to alanine, and (3) assays in which the assay mixture was briefly exposed to air before

152the substrate was added, demonstrating O_2 sensitivity that is characteristic of GREs (Extended

153Figure 4). Specific activities observed in SAM-containing assays represented in Figure 3b were

154relatively low (in the pmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1} range) compared to reported values for most other

155GREs, which range broadly from pmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1} (benzylsuccinate synthase[17]) to mmol

156 $\cdot$ min^{-1} $\cdot$ mg protein^{-1} (glycerol dehydratase[18]). In part, low PhdB activity may reflect the generally

157sensitive nature of GREs when purified and manipulated *in vitro*. For example, even for a given

158enzyme, reported specific activities have differed by orders of magnitude in various studies [e.g.,

159for benzylsuccinate synthase, from 0.02[17] to 72 nmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1} [19]; for *p*-

160hydroxyphenylacetate decarboxylase, from 0.034[13] to 18.45 µmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1} [14]]. In the

161present study, a likely factor affecting PhdB activity was the poor solubility of the recombinant

162protein when expressed in *E. coli* (Extended Figure 5); a maltose-binding protein (MBP) tag was

6

163used to enhance solubility but may not have fully ameliorated suboptimal folding. For
164biotechnological application of PhdB, enhanced solubility (e.g., through protein engineering)
165will be required.

166   While PhdB displays phenylacetate decarboxylase activity, it does not display
167comparable *p*-hydroxyphenylacetate decarboxylase activity (characteristic of the GRE
168HpdBC/CsdBC). During assays in which equimolar amounts of phenylacetate and *p*-
169hydroxyphenylacetate were amended to a mixture containing PhdA, PhdB, and SAM, labeled
170toluene production was readily observed, however, *p*-cresol (the product of *p*-
171hydroxyphenylacetate decarboxylation) was detected at levels approximately 100-fold lower
172than those expected if PhdB activity were comparable for phenylacetate and *p*-
173hydroxyphenylacetate (Extended Data Fig. 46).   Analogous assays with *o*- and *m*-
174hydroxyphenylacetate similarly indicated very low (in this case, undetectable) PhdB activity for
175these hydroxyphenylacetate isomers, whereas labeled toluene was easily detected.

176**Comparison of PhdB-PhdA to other glycyl radical systems**

177   The demonstration of PhdB as a phenylacetate decarboxylase adds it to the group of
178seven characterized GREs (Fig. 4), which includes pyruvate formate-lyase (EC 2.3.1.54[20]),
179anaerobic ribonucleotide reductase (EC 1.17.4.1[21]), benzylsuccinate synthase (EC
1804.1.99.11[17,19,22]), *p*-hydroxyphenylacetate decarboxylase (EC 4.1.1.82[13,14,23]), B$_{12}$-independent
181glycerol (and 1,2-propanediol) dehydratase (EC 4.2.1.30[18]), choline trimethylamine-lyase (EC
1824.3.99.4[24,25]), and the very recently discovered *trans*-4-hydroxy-L-proline dehydratase[26]. Note
183that benzylsuccinate synthase, which catalyzes the first step of anaerobic toluene degradation, is
184the best characterized representative of a larger group of aromatic- and alkylsuccinate synthase

7

185enzymes that activate substrates including 2-methylnaphthalene, *p*-cresol, and *n*-hexane by

186fumarate addition and have been collectively termed "X-succinate synthases"[27].

187   PhdB shares important features characteristic of all known GREs, including the

188following: (1) a conserved glycyl radical motif (RVx**G**[FWY]x$_{6-8}$[*I*L]x$_4$Qx$_2$[IV]x$_2$R —

189modification from Selmer et al.[15] indicated in italics) near the C-terminus of the protein (Fig. 5a),

190(2) a conserved cysteine residue near the middle of the protein sequence (the site of the thiyl

191radical in the active site that initiates H atom abstraction from the substrate) (Fig. 5b), and (3) a

192cognate activating enzyme that belongs to the radical SAM superfamily[15]. However, PhdB is

193clearly distinct from the other known glycyl radical enzymes in a number of ways. For example,

194the sequence identity of PhdB (from the sewage and lake cultures) to other GREs is relatively

195low, ranging from ca. 14 to 31% (Extended Data Fig. 75). Further, PhdB does not share all of the

196conserved residues that have been assigned for other GREs. To illustrate, in the region near the

197conserved active-site C residue (Fig. 5b), some conserved residues not shared by PhdB include

198an additional C adjacent to the strictly conserved active-site C (PflB[20]), an E located two residues

199downstream of the active-site C (CsdB[23], Gdh[18], CutC[24], HypD[26]), and M-S-P residues

200immediately downstream of the active-site C (BssA[27]).

201   With respect to *p*-hydroxyphenylacetate decarboxylase in particular, differences from

202PhdB are noteworthy, since these proteins might be expected to be very similar based on the

203seemingly analogous reactions that they catalyze (Fig. 4). Phenylacetate decarboxylase (PhdB)

204has only one subunit type, in contrast to *p*-hydroxyphenylacetate decarboxylase (CsdBC or

205HpdBC), which has two (Fig. 2), and does not share conserved CsdB residues postulated to

206interact with the *para*-hydroxy group (e.g., active-site residue E637 of CsdB[23]). Furthermore, *p*-

207hydroxyphenylacetate decarboxylase (CsdBC) does not act on phenylacetate[8], and conversely,

8

208PhdB has far lower activity on *p*-hydroxyphenylacetate than on phenylacetate (Extended Data

209Fig. 64). Based upon the sole structural feature that differentiates the substrates of PhdB and *p*-

210hydroxyphenylacetate decarboxylase (CsdBC/HpdBC), namely a *para*-hydroxy group, and its

211essential role in the proposed mechanism of the latter enzyme, it is likely that PhdB and

212CsdBC/HpdBC differ mechanistically. The Kolbe-type decarboxylation proposed for CsdBC[23,28]

213involves an unprecedented mechanism for *p*-hydroxyphenylacetate activation: a concerted

214abstraction of a proton from the *para*-hydroxy group by E637 and abstraction of an electron from

215the carboxyl group by C503[23]. Together, the proton and electron abstraction constitute a *de facto*

216H-atom abstraction, although the abstraction occurs in two distinct locations on the substrate

217molecule. Molecular modeling of the substrate-bound active sites of PhdB (based on homology

218modeling) and CsdBC (based on crystallographic data) indicates important conserved residues,

219such as the sites of the thiyl radical (C482 in PhdB and C503 in CsdB) and glycyl radical (G815

220in PhdB and G873 in CsdB), but also important differences, such as a hydrophobic pocket in

221PhdB (including W495, Y691, and V693) accommodating the unsubstituted ring of

222phenylacetate and lacking the H536 and E637 residues in CsdB that are proposed to interact with

223the *para*-hydroxy group of *p*-hydroxyphenylacetate (Extended Figure 8).

224   Just as PhdB represents a novel glycyl radical enzyme, PhdA represents a new glycyl

225radical activating enzyme. Whereas PhdA shares some characteristics of the cognate activating

226enzymes for the seven GREs described above, such as a conserved CxxxCxxC [4Fe-4S]-binding

227motif near the N-terminus of the protein (Fig. 5c), its sequence identity to these activating

228enzymes is relatively low (from ca. 23 to 42% for both the sewage and lake culture versions of

229PhdA; Extended Data Fig. 75). To date, studies have indicated that glycyl radical activating

230enzymes are not interchangeable but rather are specific to their cognate glycyl radical enzymes[16].

9

231 **Identity of toluene-producing bacterium**

232   As toluene synthase discovery was conducted with the proteome of a complex microbial

233 community rather than that of a microbial isolate, the task of identifying the microbe whose

234 genome encodes *phdA* and *phdB* was challenging. Nonetheless, we were able to recover the

235 draft genome of the bacterium in the sewage community that putatively expressed *phdA* and

236 *phdB* (Fig. 6a). This 3.61-Mbp genome (Fig. 6a, Supplementary Data File 5), which resulted

237 from co-assembly of Illumina reads from multiple metagenome sequences produced from the

238 sewage culture, is estimated to be 96.35% complete and contains a 51.8-kb contig including the

239 three-gene *phd* cluster (Fig. 2) relevant to toluene biosynthesis. In addition to *phdA* and *phdB*,

240 the genome encodes other putative radical-related enzymes (Fig. 6a), including a GRE of

241 unknown function (TOLSYN_01027) and seven putative radical SAM enzymes that contain the

242 CxxxCxxC motif near the N terminus (TOLSYN_00781, TOLSYN_01308, TOLSYN_01024,

243 TOLSYN_00072, TOLSYN_00941, TOLSYN_02430, and TOLSYN_01488).

244   The recovered genome contained a partial 16S rRNA gene indicating that the toluene-

245 producing bacterium (hereafter referred to as *Acidobacteria* strain Tolsyn) belongs to the

246 *Acidobacteria* phylum (Extended Data Fig. 96). The closest match among bacterial isolates is to

247 *Candidatus* Koribacter versatilis (95% identity), which is classified in Subdivision 1 of the

248 *Acidobacteria* but is not well characterized with respect to its physiology and metabolism[29].

249 Evaluation of the recovered genome against the available *Acidobacteria* isolate genomes using

250 129 concatenated proteins (including 33 ribosomal proteins) indicated, as did the 16S rRNA

251 analysis, that the closest isolated relative is *Ca.* Koribacter versatilis (Fig. 6b). However, the

252 genomes of *Acidobacteria* strain Tolsyn and *Ca.* Koribacter versatilis are much less similar than

253 the 16S rRNA comparison would suggest: average sequence identity for the proteins in these two

10

254genomes was only ca. 56%. Admittedly, there are few *Acidobacteria* isolates for comparison to

255strain Tolsyn, as *Acidobacteria* are notoriously difficult to isolate[29,30]. Notably, BLASTP[31]

256searches of the *Ca.* Koribacter versatilis genome did not yield any hits to PhdA or PhdB.

257   From an ecological perspective, the selective advantage conferred by toluene production

258in strain Tolsyn is currently unknown. The metabolic advantages rendered by phenylacetate

259conversion to toluene are not obvious, as the reaction yields only CO_2, which is unlikely to be

260limiting in environments like anoxic lake sediments or sewage sludge, and toluene, which is

261likely lost from the cell by diffusion and not further metabolized [e.g., benzylsuccinate synthase[22]

262was not found in the genome nor, indeed, in the entire sewage metagenome (IMG Taxon ID

2633300001865)]._ ~~Further, the PhdB reaction will not provide reducing equivalents to the host~~

264~~because it is not an oxidation-reduction reaction.~~ Here, we present two possible explanations for

265the selective advantage offered by toluene biosynthesis.   ~~By~~ First, by analogy to *p*-

266hydroxyphenylacetate decarboxylation to *p*-cresol, as catalyzed by the nocosomial pathogen

267*Peptoclostridium difficile* (formerly *Clostridium difficile*), it is possible that toluene production

268represents a form of negative allelopathy. In *P. difficile*, production of the bacteriostatic agent *p*-

269cresol is thought to provide a competitive advantage to the producing strain and has been

270proposed as a virulence factor[32]. Just as the ultimate source of *p*-hydroxyphenylacetate to *P.*

271*difficile* is tyrosine metabolism, the source of phenylacetate to strain Tolsyn is likely

272phenylalanine metabolism[8], potentially involving transamination of phenylalanine to

273phenylpyruvate (e.g., *via* phenylalanine transaminase; EC 2.6.1.57), decarboxylation to

274phenylacetaldehyde (e.g., *via* phenylpyruvate decarboxylase; EC 4.1.1.43), and oxidation to

275phenylacetate (e.g., *via* phenylacetaldehyde dehydrogenase; EC 1.2.1.39)[33], although other

276pathways are possible[34]. Notably, BLASTP searches of the *Acidobacteria* strain Tolsyn genome

277 did not reveal definitive copies of genes encoding any of these enzymes, suggesting that the

278 conversion of phenylalanine to phenylacetate may not occur within strain Tolsyn, but rather that

279 phenylacetate may be imported from its environment. Regardless of which microorganisms are

280 converting phenylalanine to phenylacetate, previous studies have documented that the

281 conversion of labeled phenylalanine (L-phenylalanine-β-^{13}C) to labeled toluene ([*methyl*-

282 ^{13}C]toluene) definitively occurs in this sewage culture[8].

283   The prospect of phenylacetate import into *Acidobacteria* strain Tolsyn introduces a

284 second possible explanation for the selective advantage offered by toluene biosynthesis:

285 intracellular pH homeostasis and/or development of a proton motive force (pmf). If the anion

286 phenylacetate were imported into the cell, the PhdB-catalyzed decarboxylation to toluene

287 consumed a proton from the cytoplasm (consistent with the balanced reaction of $C_8H_7O_2^- + H^+ \rightleftharpoons$

288 $C_7H_8 + CO_2$), and the neutral reaction products toluene and CO_2 (or H_2CO_3) exited the cell (e.g.,

289 by diffusion), the result would be alkalinization of the cytoplasm and indirect development of a

290 pmf (by depletion of protons from the cytoplasm rather than the canonical pumping of protons

291 across the cytoplasmic membrane). Studies of tyrosine and histidine decarboxylation in

292 *Enterococcus* and *Lactobacillus* spp. have experimentally supported analogous mechanisms for

293 pmf development and intracellular pH regulation[35,36]. Thus, alkalinization of the cytoplasm *via*

294 phenylacetate decarboxylation could promote tolerance to the moderately acidic conditions

295 characteristic of some fermentative environments (such as those used to cultivate the sewage and

296 lake sediment cultures and likely representative of their native habitats) and could also provide a

297 source of energy to the bacterium (as pmf), even though the PhdB reaction would not provide

298 reducing equivalents to the host because it is not an oxidation-reduction reaction.

299

12

300

301 Conclusion

302   We have discovered a GRE that catalyzes an activity heretofore unavailable to 303 biotechnology, enabling biochemical synthesis of toluene (and potentially other products of 304 aromatic acid decarboxylation) from renewable feedstocks. Furthermore, this study, like the 305 recent discovery of another GRE (*trans*-4-hydroxy-L-proline dehydratase[26]), provides a glimpse 306 into the untapped catalytic potential of GREs. It is likely that the catalytic diversity of GREs has 307 been widely underestimated because automated annotation pipelines routinely misidentify 308 diverse GREs as pyruvate formate-lyase (as was the case for PhdB), and there is a dearth of 309 experimental data to correct such misannotation. To illustrate the unexplored diversity of GREs, 310 consider the sewage-derived microbial community investigated in this study. In addition to 311 PhdB, we conservatively estimate that there are at least four other novel GREs represented in the 312 sewage culture metagenome (Fig. 1), as detailed in Extended Data Fig. 10~~7~~. These GREs deviate 313 from known GREs with respect to at least one conserved residue, and share only ca. 16 to 38% 314 protein sequence identity with known GREs and each other. All four of these putatively novel 315 GREs were misannotated as pyruvate formate-lyase by an automated pipeline.   Further 316 experimental characterization of the catalytic range of GREs promises to expand our 317 understanding of the metabolic diversity of anaerobic bacteria and the reach of biotechnology to 318 catalyze challenging reactions.

319 METHODS

320   Unless stated otherwise, all cultivation and biochemical processes were conducted under 321 strictly anaerobic conditions[37] in an anaerobic glove box (Type B, Coy Laboratory Products, Inc., 322 Grass Lake, MI) with a nominal gas composition of 85% N_2 − 10% CO_2 − 5% H_2 (ultra-high

13

323purity, anaerobic mixture) maintained at ambient temperature (~22°C). Glass, plastic, and

324stainless steel materials used to manipulate microbial cells, cell-free extracts, and purified

325enzymes in the glove box were allowed to degas in the anaerobic glove box for at least one day

326before use, as were heat-labile solids that could not be prepared in autoclaved and purged

327solutions. Highly purified water (18 MΩ resistance) obtained from a Barnstead Nanopure

328system (Thermo Scientific, Waltham, MA) was used to prepare all aqueous solutions described

329in this article. Chemicals used in this study were of the highest purity available and were used as

330received.

331Cultivation of anaerobic sewage and lake sediment cultures

332   Anaerobic cultivation of sewage-derived cultures has been described previously[8]. In a

333similar fashion, reducing sediments from a lake in Berkeley, California, were used to inoculate

334cultures under anaerobic conditions using TP[9] or modified TP[8] growth medium in an anaerobic

335glove box. Amended phenylacetate (typically 200 µM) and evolved toluene were monitored by

336LC/MS and GC/MS, respectively, using methods described previously[8].

337Partial purification of phenylacetate decarboxylase activity in sewage cultures with FPLC

338   As described in detail elsewhere[8], cell-free extracts from the sewage-derived culture were

339generated under strictly anaerobic conditions with a French pressure cell[19] (138 MPa) and

340clarified by ultracentrifugation, before being subjected to FPLC fractionation in an anaerobic

341glove box with a Bio-Scale Mini CHT-II ceramic hydroxyapatite column (5-mL bed volume, 40-

342µm particle diameter; Bio-Rad, Hercules, CA) and Bio-Rad Econo Gradient Pump. ~~Toluene~~

343~~synthase~~Phenylacetate decarboxylase activity in FPLC fractions was determined with a GC/MS

344static headspace assay that measured conversion of phenylacetic acid-2-[13]C (Icon Isotopes,

345Summit, NJ; 99 atom% [13]C) to [*methyl*-^{13}C]toluene[8].

14

346

Proteomic analysis of FPLC fractions by LC/MS/MS

Details on proteomic analysis of selected FPLC fractions, including data processing, were provided by Zargar et al.[8]. Briefly, proteomic LC/MS/MS analysis was performed with a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) in conjunction with a Proxeon Easy-nLC II HPLC (Thermo Scientific) and Proxeon nanospray source.

Characterization of sewage and lake cultures by next-generation sequencing of metagenomes and PCR-amplified 16S rRNA genes

Extraction of genomic DNA from toluene-producing cultures was performed with a bead-beating method involving hexadecyltrimethylammonium bromide (CTAB) extraction buffer described elsewhere[8]. Genomic DNA was purified with Allprep DNA/RNA kits (Qiagen, Valencia, CA). Methods used by the Joint Genome Institute (JGI) for metagenome library construction, next-generation sequencing (Illumina and PacBio), and assembly for sewage- and lake-derived cultures are summarized in Supplementary Data File 6, along with accession numbers for NCBI's SRA (Sequence Read Archive; https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi). The automated annotation pipeline for metagenome sequences was described previously[38].

Composition of the sewage-derived community was analyzed at the JGI by Illumina sequencing of 16S rRNA genes amplified from the V4 region (primers 515F and 806R). Library construction and sequencing methods are described in Supplementary Data File 6, and data analysis with iTagger v. 1.1 was performed as described previously[8].

Composition of the lake sediment-derived community was also assessed by Illumina sequencing of 16S rRNA genes amplified from the V4 region (primers 515F and 806R). Library

15

369construction was performed according to the Earth Microbiome Project standard protocol 370(http://www.earthmicrobiome.org/protocols-and-standards/16s/). Sequencing was conducted by 371the QB3-Berkeley Core Research Facility at UC Berkeley on the Illumina MiSeq platform (San 372Diego, CA) with paired-end, 300-bp reads (MiSeq Reagent Kit v3, 600 cycle). The UPARSE 373method was used for sequence processing and operational taxonomic unit (OTU) clustering at 37497% identity to process raw sequences (fastq_maxdiffs=3, fastq_trunclen=250, 375fastq_maxee=0.1). A set of 217 OTUs from a total of 108,041 filtered sequences were identified. 376For each OTU, a representative sequence was selected as described by Edgar[39]. Taxonomic 377assignments were made with a Naïve Bayes Classifier using the V4 region of the SILVA[40] SEED 378sequences and their taxonomic identities as a training set.

379**Cloning, expression, *in vitro* reconstitution, and purification of PhdA and PhdB**

380 Bacterial strains and plasmids used in this study are listed in Extended Data Table 1. 381Strains and plasmids along with their associated information (annotated GenBank-format 382sequence files) have been deposited in the public version of the JBEI Registry (https://public-383registry.jbei.org; entries JPUB_xxx to JPUB_xxx) and are physically available from the authors 384and/or addgene (http://www.addgene.org) upon request [Note to editor: JPUB names will be 385generated and made public upon publication of the manuscript]. Restriction enzymes were 386purchased from Thermo Scientific (Waltham, MA), and Phusion DNA polymerase and T4 ligase 387were from New England Biolabs (Ipswich, MA). Plasmid extractions were carried out using 388Qiagen (Valencia, CA) miniprep kits. Oligonucleotide primers were designed using the web-389based PrimerBlast program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi? 390LINK_LOC=BlastHomeAd) and synthesized by Integrated DNA Technologies (IDT), Inc. (San 391Diego, CA) or Eurofins MWG Operon (Huntsville, AL).

16

392   *phdA* and *phdB* were codon optimized (GenScript, Piscataway, NJ) for expression in

393*E.coli* BL21(DE3) (listed as Sequences 3 and 8 in Supplementary Data File 4). Each codon-

394optimized gene was individually cloned into plasmid pET28b (Novagen, Madison, WI). *phdA*

395was cloned between NdeI and BamHI restriction sites (primers listed in Extended Data Table 2),

396resulting in a construct that encodes an N-terminal His_6-PhdA protein (pAS004; Extended Data

397Table 1). *phdB* was cloned between NdeI and XhoI restriction sites (primers listed in Extended

398Data Table 2). To enhance soluble PhdB yield, the construct also included the gene encoding

399maltose-binding protein (MBP) and a sequence encoding the tobacco etch virus (TEV) protease

400recognition site, which were inserted downstream of the N-terminal His_6 sequence and upstream

401of the *phdB* start codon, resulting in a construct that encodes a His_6-MBP-PhdB fusion protein

402with a TEV protease-cleavable His_6-MBP tag (pAS010, Extended Data Table 1). Plasmids were

403transformed into chemically competent *E.coli* DH10B cells grown on lysogeny broth (LB) agar

404plates under 50 µg/mL kanamycin selection (LB Kan-50 plates; Teknova, Hollister, CA).

405Plasmids were sequence-confirmed (Genewiz, South San Francisco, CA). Plasmids pAS006

406(with *phdA*) and pAS010 (with *phdB*) were separately transformed into chemically competent

407*E.coli* BL21(DE3) cells (New England Biolabs) on LB Kan-50 plates. Transformants were

408grown in LB broth and stored as 100 µL glycerol stock aliquots at -80°C.

409   For overexpression of PhdA, a frozen glycerol stock of strain AS013 (Extended Data

410Table 1) was used to inoculate 50 mL LB broth containing 50 µg/mL kanamycin (Teknova) in a

411250-mL shake flask. The starter culture was incubated overnight at 30°C with constant shaking at

412200 rpm. For larger scale growth, the starter culture was diluted 100-fold in a 2-L baffled shake

413flask containing 1L LB broth supplemented with 50 µg/mL kanamycin, and grown aerobically at

41437°C with constant shaking (190 rpm). At OD_{600} ~0.7, the culture was induced with isopropyl β-

17

415D-1-thiogalactopyranoside (IPTG; IBI Scientific, Peosta, IA) to a final concentration of 0.5 mM

416and supplemented with an aqueous solution of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (Sigma, St. Louis, MO;

417prepared anaerobically) to a final concentration of 200 µM. Following induction, the temperature

418was decreased to 18°C and the culture was propagated overnight at this temperature for ~18

419hours. Cells were then harvested by centrifugation and cell pellets were stored at -80°C until

420lysis.

421   For overexpression of PhdB, strain AS019 (Extended Data Table 1) was cultivated in

422autoinduction medium[41]. A frozen glycerol stock was used to inoculate 50 mL ZYP-0.8G

423medium containing 100 µg/mL kanamycin in a 250-mL shake flask incubated overnight at 30°C

424with constant shaking (200 rpm). The starter culture was diluted 100-fold into a 2-L baffled

425shake flask containing 1-L ZYP-5052 medium with 100 µg/mL kanamycin and grown

426aerobically at 37°C with constant shaking at 190 rpm. At OD_{600} ~1.5, the temperature was

427decreased to 18°C and the culture was propagated overnight at this temperature for ~18 hours.

428Cells were then harvested by centrifugation and cell pellets were stored at -80°C until lysis.

429   All purification steps were carried out under strictly anaerobic conditions. For lysis, cells

430were passed three times through a French pressure cell (138 MPa) under anaerobic conditions.

431Sealed lysates were centrifuged under anaerobic conditions at 19,000 rpm at 4°C for 40 min.

432Clarified lysates were purified within an anaerobic glove box as described below using an

433Econo-Gradient pump coupled with a model 2110 fraction collector (Bio-Rad).

434   For PhdA purification, strain AS013 cell pellets were resuspended in buffer A [50 mM

435TRIS (pH 7.5; EMD Millipore, Billerica, MA), 300 mM NaCl (EMD Millipore), 10 mM

436imidazole (Sigma), 0.1 mM DL-dithiothreitol (DTT; VWR, Visalia, CA)] and mixed with

437powdered protease inhibitors (Pierce EDTA-free tablets, Thermo Scientific), chicken egg

18

438lysozyme (300 µg/mL, Sigma) and DNaseI (10 µg/mL, Sigma). This mixture was incubated for

43920 min followed by cell lysis and clarification of the lysate as described above. The clarified

440lysate was filtered through a 0.45-µm filter (EMD Millipore) and loaded onto a 5-mL HisTrap

441HP column (GE Healthcare, Chicago, IL) that was pre-equilibrated with buffer A. The column

442was then washed with 3 column volumes (CV) of buffer A to remove unbound proteins and

443eluted using a stepwise imidazole gradient made by mixing buffer A with buffer B [50 mM TRIS

444(pH 7.5), 300 mM NaCl, 500 mM imidazole, 0.1 mM DTT] using stepwise concentrations of 20

445mM, 50 mM, 250 mM, and 400 mM imidazole. Each step was set to 1.6 CV and 2-mL fractions

446were collected. Fractions containing PhdA were dark red-brown and eluted at a concentration of

447250 mM imidazole. The purity of PhdA fractions was confirmed by SDS-PAGE. Elution

448fractions were pooled and DTT was added to a final concentration of 2 mM. To keep the protein

449anoxic during concentration outside the glove box, a 10-kDa molecular weight cutoff (MWCO)

450concentrator (EMD Millipore) was sealed inside a 250-mL centrifuge bottle (Nalgene, Rochester,

451NY) with an O-ring-sealed cap. Concentrated protein was exchanged into buffer C [50 mM TRIS

452(pH 7.5), 300 mM NaCl, 5 mM DTT] using a pre-equilibrated PD-10 desalting column (GE

453Healthcare). Protein concentration was determined using the Bradford assay (Bio-Rad).

454Collected UV-visible spectra (UV-2450; Shimadzu Scientific, Pleasanton, CA) indicated the

455presence of [2Fe-2S] clusters bound to the protein (Extended Data Figure 118)[42].

456   For reconstitution of [4Fe-4S] clusters in PhdA, which are required for activity, the

457protein was diluted to 0.2 mM in buffer C in a stoppered serum bottle and cooled to 4°C. DTT

458was then added to a final concentration of 10 mM and the solution was incubated at 4°C for ~1

459hour. Aqueous $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was added to a final concentration of 1 mM and incubated

460at 4°C for ~3-4 hours. Aqueous $Na_2S \cdot 9H_2O$ was then added to a final concentration of 0.9 mM

19

461and the mixture was incubated at 4°C overnight (~18 hr). The protein mixture was then filtered

462through a 0.45-µm filter, concentrated, and diluted 15-fold in buffer D [50 mM TRIS (pH 7.5),

46320 mM NaCl, 2 mM DTT]. The diluted protein was then loaded onto a 5-mL Bioscale High Q

464column (Bio-Rad) that was pre-equilibrated with buffer D and eluted using buffer E [50 mM

465TRIS (pH 7.5), 1 M NaCl, 2 mM DTT] with a stepwise NaCl gradient of concentrations 40 mM,

466100 mM, 500 mM, and 800 mM NaCl. Each step was set to 1.6 CV and 2-mL fractions were

467collected. PhdA eluted at a concentration of ~500 mM NaCl and fractions were yellow-brown.

468Purity of eluted fractions was confirmed by SDS-PAGE. Pooled fractions were concentrated and

469exchanged into assay buffer [50 mM TRIS (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$ (Sigma), 5

470mM $(NH_4)_2SO_4$ (Sigma), 5 mM DTT] using a pre-equilibrated PD-10 column and stored at 4°C in

471a stoppered serum bottle. Protein concentration was determined using the Bradford assay. UV-

472visible spectra confirmed the presence of [4Fe-4S] clusters bound to the protein (Extended Data

473Figure 118)[42].

474   For PhdB purification, strain AS019 (Extended Data Table 1) cell pellets were washed in

475buffer containing 50 mM TRIS (pH 7.5), 150 mM NaCl, and 0.5 mM dithionite. For purification,

476cell pellets were resuspended in buffer A [20 mM TRIS (pH 7.5), 200 mM NaCl, 1 mM EDTA

477(EMD Millipore), 1 mM DTT] and mixed with powdered protease inhibitors, chicken egg

478lysozyme (300 µg/mL) and DNaseI (10 µg/mL). This mixture was incubated for 20 minutes,

479followed by cell lysis with a French pressure cell under anaerobic conditions and clarification of

480the lysate as described for PhdA. The clarified lysate was filtered through a 0.45-µm filter

481(Millipore) and loaded on to a 5 mL-MBPTrap HP column (GE Healthcare) that was pre-

482equilibrated with buffer A. The column was then washed with 3 CV of buffer A to remove

483unbound proteins and eluted using a program consisting of a stepwise maltose gradient made by

484mixing buffer A with buffer B [20 mM TRIS (pH 7.5), 200 mM NaCl, 1 mM EDTA, 10 mM

485maltose (Sigma), 1 mM DTT] using concentrations of 0.4 mM, 1 mM, 5 mM, and 8 mM

486maltose. Each step was set to 1.6 CV and 1-mL fractions were collected. PhdB eluted at a

487concentration of ~1 mM maltose and purity of fractions was confirmed by SDS-PAGE. Elution

488fractions were pooled and DTT was added to a final concentration of 2 mM and the protein was

489concentrated anaerobically as described for PhdA (except with a 50-kDa MWCO rather than 10-

490kDa MWCO). Concentrated protein was exchanged into assay buffer [50 mM TRIS (pH 7.5),

491150 mM NaCl, 1 mM MgCl$_2$, 5 mM (NH$_4$)$_2$SO$_4$, 5 mM DTT)] using a pre-equilibrated PD-10

492desalting column (GE Healthcare). Protein concentration was determined using the Bradford

493assay (Bio-Rad). During initial purifications, the identity of the protein was confirmed by

494Western blotting using HRP-conjugated anti-MBP antibody (New England Biolabs).

495**Anaerobic *in vitro* assays for PhdA activity with recombinant protein**

496   In an anaerobic chamber at ambient temperature, 0.7 mM reconstituted PhdA was

497incubated in assay buffer [50 mM TRIS (pH 7.5), 150 mM NaCl, 1 mM MgCl$_2$, 5 mM

498(NH$_4$)$_2$SO$_4$, 5 mM DTT)] with 2 mM dithionite (Sigma) for 1 hour in 4-mL screw-capped glass

499vials (Supelco). This was followed by the addition of 2 mM SAM [*S*-(5'-adenosyl)-L-methionine

500chloride dihydrochloride; Sigma]. The reaction mixture (1.2 mL) was shaken at low speed on a

501tabletop orbital shaker. After initiation of the PhdA reaction by SAM addition, sampling was

502conducted from 0 to 180 min at 30-min intervals. Immediately upon sampling, 75 µL of reaction

503mixture was quenched by addition of 75 µL LC/MS grade methanol (Honeywell Research

504Chemicals, Muskegon, MI) and gentle bubbling of 0.5 mL of air (from a sealed serum bottle).

505Control reaction mixtures excluding PhdA were assayed in an identical manner. Post quenching,

506samples were centrifuged at 13,000 rpm for 15 min, then diluted in 50% (v/v) methanol in

507LC/MS grade water (J.T. Baker, Phillipsburg, NJ) in preparation for LC/MS measurement.

508Replicates involved separate assays rather than multiple analyses of a given assay sample.

509   For analysis of methionine produced by PhdA activity with SAM, external standard

510quantification was performed with five-point calibration standards ranging from 0.25-10 µM

511methionine (Sigma) in 50/50 (v/v) methanol/water. Samples were run on an LC/MSD SL

512(Agilent, Santa Clara, CA) equipped with a model 1260 Infinity Binary Pump and operated in

513the electrospray ionization, positive-ion mode. The mobile phase initially flowed at 0.6 mL/min

514(0 - 13 min), and later at 1 mL/min (13-15 min), through a Kinetex HILIC column (2.6-µm

515particle size, 4.6-mm inner diameter x 50-mm length; Phenomenex, Torrance, CA). The initial

516mobile phase composition was 10 vol% A (20 mM ammonium acetate in water) and 90 vol% B

517(10 mM ammonium acetate in 90% acetonitrile, 10% water), which was decreased linearly to

51870% B at 4 minutes, then decreased linearly to 40% B from 6 - 11.5 minutes, and then increased

519linearly to 90% B from 12 - 15 minutes to re-equilibrate the column to initial conditions. Sample

520injection volume was 2 µL. Source conditions included 3.5 kV capillary voltage, 250°C drying

521gas temperature, 12 L/min drying gas flow, and 25 psig nebulizer pressure. Data acquisition for

522methionine was in the selected ion monitoring (SIM) mode at *m/z* 150.2. Peak areas were

523integrated using Mass Hunter software (Agilent, version B.05.00).

524**Anaerobic *in vitro* assays for phenylacetate decarboxylase activity with recombinant PhdA**

525**and PhdB**

526   Assays for phenylacetate decarboxylase activity were performed under strictly anaerobic

527conditions within a glove box. Assays, which were performed in 4-mL glass vials sealed with 13-

528mm diameter PTFE Mininert screw-cap valves (Sigma-Aldrich), contained 250 µM PhdA in

529assay buffer [50 mM TRIS (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 5 mM

530DTT)], to which 2 mM dithionite was added and incubated for ~1 hour, followed by the addition

531of 2 mM SAM, 2.5 µM PhdB in assay buffer, and 2.5 mM phenylacetic acid-2-^{13}C in a final

532volume of 1 or 1.5 mL (depending on the specific experiment). Quantitative standards contained

533the same headspace/liquid ratios as assays and a dimensionless Henry's constant of 0.27[43] was

534used to calculate aqueous concentration. Negative controls were run concurrently and were

535identical except for the absence of SAM (Figure 3b) or other conditions specified in Extended

536Figure 4. The vials were shaken on a tabletop orbital shaker at low speed. Gaseous headspace

537samples (100 µL) were taken within the glove box using a 500-µL gastight syringe (Sample-Lok

538series A-2; Sigma-Aldrich) and analyzed immediately by GC/MS, as described previously[8].

539Briefly, toluene was analyzed by static headspace, electron ionization (EI) GC/MS using a model

5407890A GC (Agilent, Santa Clara, CA) with a DB-5 fused silica capillary column (30-m length,

5410.25-mm inner diameter, 0.25-µm film thickness; Agilent) coupled to an HP 5975C series

542quadrupole mass spectrometer. As described elsewhere[8], the identity of [*methyl*-^{13}C]toluene was

543confirmed with the expected *m/z* 93/92 ratio of 0.6. Replicates involved separate assays rather

544than multiple analyses of a given assay sample. In assays testing whether PhdB could

545decarboxylate *p*-hydroxyphenylacetate to *p*-cresol, conditions were as described above except

546that equimolar amounts (2.5 mM) of *p*-hydroxyphenylacetic acid (Sigma) and phenylacetic acid-

5472-^{13}C were added, and GC/MS analysis of *p*-cresol in 1-µL liquid injections of concentrated

548hexane extracts were conducted as described previously[8]. The identity of *p*-cresol was assessed

549using retention time and the expected *m/z* 108/107 ratio of 0.83 based on authentic standards.

550**PCR amplification of *phd* gene cluster from genomic DNA from lake sediment culture**

551   *phdA, phdB,* and an adjacent putative transcription factor were PCR-amplified from

552genomic DNA extracted from the lake sediment community using primers shown in Extended

23

553Data Table 2. Primer design was guided in part by partial gene sequences available from 554metagenomes (IMG Taxon ID 2100351000 and 3300001865). Amplified and gel-purified DNA 555was sequenced by Genewiz.

556**Construction of maximum likelihood tree of glycyl radical enzymes in sewage-derived** 557**culture**

558   The maximum-likelihood tree in Fig. 1 encompasses protein sequences of putative glycyl 559radical enzymes (GREs) detected in the sewage culture metagenome (IMG Taxon ID 5603300001865) based on BLASTP[31] searches against known GREs (> 30% sequence identity), 561searches for the glycyl radical motif (FIMO[44]), and a minimum length of 171 amino acids (not all 562were full length). The following model sequences were also included in the tree to provide 563context (accession numbers in parenthesis): PflB (GenBank: NP_415423), HpdB (GenBank: 564AJ543425.1), CsdB (GenBank: ABB05046.1), CutC (PDB: 5A0Z), NrdD (GenBank: 565NP_418659), and Gdh (PDB: 1R8W). The collected set of model and putative GRE sequences 566(n=81, mean = 675±194 aa) were aligned using MUSCLE v. 3.8.31[45]. The resulting MSA was 567screened for ambiguous C and N termini as well as columns with >97% gaps. The final 568alignment spanned 1138 columns. A maximum likelihood phylogenetic tree was inferred with 569RAxML v. 7.6.3[46], under the LG plus Gamma model of evolution as follows:

570raxmlHPC-PTHREADS-SSE3 -# 50 -m PROTGAMMAGTR -p 777 -x 2000 -f a

571The tree was constructed with iTOL[47].

572**Binning of sewage culture metagenomes and recovery of *Acidobacteria* strain Tolsyn** 573**genome**

574   For binning, two groups of sewage metagenomes (Group 1 from SRA accession numbers 575SRP077640, SRP072654, and SRP099295 and Group 2 from SRA accession numbers

576SRP105442 and SRP105443) were separately co-assembled using metaSPAdes v3.6[48] with the

577"--careful" option. The two co-assemblies were separately binned using MaxBin 2.0[49] with

578default parameters (-min_contig_len 1000). The *Acidobacteria* strain Tolsyn bins were separately

579identified within the two co-assemblies, and scaffolds that were shared (with >98% identity)

580were selected to constitute the draft *Acidobacteria* genome. The scaffolds were further refined by

581mapping against the hybrid assemblies of the sewage sludge samples (IMG Taxon ID

5823300017643, 3300017642, and 3300017814) and extracting scaffolds that unambiguously

583connected two or more sequences in the draft *Acidobacteria* genome. Genes were predicted from

584the genome using Prodigal (parameter: -p meta)[50]. Amino acid sequence identity between the

585draft Tolsyn genome and the *Ca.* Koribacter versatilis genome was carried out by comparing

586predicted proteins from the two genomes using BLASTP[31] with an e-value cutoff of 1e-10 and

587coverage cutoff 0.4. Annotation was performed by matching identical genes identified by the

588IMG pipeline (IMG Taxon ID 3300001865) using BLASTP with minimum amino acid identity

589set to 95% and minimum coverage set to 40%; the best matching IMG annotations were then

590assigned for those genes. CheckM software[51] reported that the genome was 96.35% complete

591with a contamination ratio of 1.69%. The circular genome plot (Fig. 6a) was made using

592Circos[52]. The 16S rRNA gene was identified as follows. A partial 16S rRNA gene (756 bp) was

593identified in a 1.7-kb scaffold and was 100% identical to a 16S rRNA gene identified from 16S

594rRNA iTag analysis: *Acidobacteria* OTU (Operational Taxonomic Unit) #9 (Supplementary Data

595File 3). When OTU9 was used as query sequence for BLASTN searches of the sewage culture

596metagenome (IMG Taxon ID 3300001865), it had a 100% match with scaffold

597JGI2065J20421_1000212, which contained a 1382-bp 16S rRNA gene

25

598(JGI2065J20421_10002126). As a result, the partial 16S rRNA gene in the *Acidobacteria* strain

599Tolsyn genome was replaced by the 1382-bp 16S rRNA gene.

600**Construction of phylogenetic trees for *Acidobacteria* strain Tolsyn**

601   The 16S rRNA tree (Extended Data Fig. 96) was constructed by aligning selected 16S

602rRNA gene sequences using MUSCLE[45] and then applying FastTree[53] to the alignment file. The

603concatenated   protein   tree   (Fig.   6b)   was   constructed   with   ezTree

604(https://github.com/yuwwu/ezTree; manuscript under review), a pipeline for identifying single-

605copy marker genes from a collection of complete or draft genomes and using the marker genes to

606generate a concatenated protein tree.

607**Molecular modeling of PhdB in complex with its phenylacetate substrate**

608   A molecular model of PhdB (Extended Figure 8) was created based on homology

609modeling of three-dimensional protein structures implemented in the program SWISS-

610MODEL[54]. The GRE 1,2-propanediol dehydratase from *Roseburia inulinivorans* (PDB ID:

6115I2A), which shares 32% sequence identity with PhdB, was used as a template to generate the

612molecular model of PhdB. Superposition of the CsdB in complex with *p*-hydroxyphenylacetate

613(PDB ID: 2YAJ)[28] against the molecular model of PhdB with the program COOT[55] was used to

614extract the binding position of phenylacetate. A structure idealization of PhdB-phenylacetate

615using REFMAC[56] enabled the final molecular model of the complex. The overall stereochemical

616quality of the final models was assessed using the program MolProbity[57].

617**Data Availability**

618 Data that support the findings of this study are available within the paper, its supplementary

619information   files,   and   data   repositories   cited   therein   [including   JGI's   IMG-M   site

620(https://img.jgi.doe.gov/cgi-bin/mer/main.cgi),   NCBI's   Sequence   Read   Archive

621(https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi), and the public version of the JBEI Registry

622(https://public-registry.jbei.org), which contains strains, plasmids, and their associated

623information].

624 **References**

625

626 1 Galperin, M. Y. & Koonin, E. V. From complete genome sequence to 'complete'

627   understanding? *Trends Biotechnol.* **28**, 398-406, doi:10.1016/j.tibtech.2010.05.006

628   (2010).

629 2 Gerlt, J. A. *et al.* The Enzyme Function Initiative. *Biochemistry* **50**, 9950-9962,

630   doi:10.1021/bi201312u (2011).

631 3 Anton, B. P. *et al.* The COMBREX project: design, methodology, and initial results.

632   *PLoS Biol.* **11**, e1001638, doi:10.1371/journal.pbio.1001638 (2013).

633 4 Lespinet, O. & Labedan, B. Orphan enzymes? *Science* **307**, 42,

634   doi:10.1126/science.307.5706.42a (2005).

635 5 Sorokina, M., Stam, M., Medigue, C., Lespinet, O. & Vallenet, D. Profiling the orphan

636   enzymes. *Biol. Direct* **9**, 10, doi:10.1186/1745-6150-9-10 (2014).

637 6 McKenna, R. & Nielsen, D. R. Styrene biosynthesis from glucose by engineered *E. coli*.

638   *Metab. Eng.* **13**, 544-554, doi:10.1016/j.ymben.2011.06.005 (2011).

639 7 Jüttner, F. & Henatsch, J. J. Anoxic hypolimnion is a significant source of biogenic

640   toluene. *Nature* **323**, 797-798 (1986).

641 8 Zargar, K. *et al. In vitro* characterization of phenylacetate decarboxylase, a novel enzyme

642   catalyzing toluene biosynthesis in an anaerobic microbial community. *Scientific Reports*

643   **6**, 31362, doi:10.1038/srep31362 (2016).

644 9 Fischer-Romero, C., Tindall, B. J. & Juttner, F. *Tolumonas auensis* gen. nov., sp. nov., a

645   toluene-producing bacterium from anoxic sediments of a freshwater lake. *Int. J. Syst.*

646   *Bacteriol.* **46**, 183-188, doi:10.1099/00207713-46-1-183 (1996).

28

647	10	Pons, J. L., Rimbault, A., Darbord, J. C. & Leluan, G. [Biosynthesis of toluene in *Clostridium aerofoetidum* strain WS]. *Ann. Microbiol. (Paris)* **135B**, 219-222 (1984).

649	11	Akhtar, M. K., Turner, N. J. & Jones, P. R. Carboxylic acid reductase is a versatile enzyme for the conversion of fatty acids into fuels and chemical commodities. *Proc. Natl. Acad. Sci. U S A* **110**, 87-92, doi:10.1073/pnas.1216516110 (2013).

652	12	Schirmer, A., Rude, M. A., Li, X., Popova, E. & del Cardayre, S. B. Microbial biosynthesis of alkanes. *Science* **329**, 559-562, doi:10.1126/science.1187936 (2010).

654	13	Selmer, T. & Andrei, P. I. *p*-Hydroxyphenylacetate decarboxylase from *Clostridium difficile*. A novel glycyl radical enzyme catalysing the formation of *p*-cresol. *Eur. J. Biochem.* **268**, 1363-1372 (2001).

657	14	Yu, L., Blaser, M., Andrei, P. I., Pierik, A. J. & Selmer, T. 4-Hydroxyphenylacetate decarboxylases: properties of a novel subclass of glycyl radical enzyme systems. *Biochemistry* **45**, 9584-9592, doi:10.1021/bi060840b (2006).

660	15	Selmer, T., Pierik, A. J. & Heider, J. New glycyl radical enzymes catalysing key metabolic steps in anaerobic bacteria. *Biol. Chem.* **386**, 981-988, doi:10.1515/BC.2005.114 (2005).

663	16	Shisler, K. A. & Broderick, J. B. Glycyl radical activating enzymes: structure, mechanism, and substrate interactions. *Arch. Biochem. Biophys.* **546**, 64-71, doi:10.1016/j.abb.2014.01.020 (2014).

666	17	Leuthner, B. *et al.* Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. *Mol. Microbiol.* **28**, 615-628 (1998).

29

669   O'Brien, J. R. *et al.* Insight into the mechanism of the B12-independent glycerol
670   dehydratase from *Clostridium butyricum*: preliminary biochemical and structural
671   characterization. *Biochemistry* **43**, 4635-4645, doi:10.1021/bi035930k (2004).
672   Beller, H. R. & Spormann, A. M. Substrate range of benzylsuccinate synthase from
673   *Azoarcus* sp. strain T. *FEMS Microbiol. Lett.* **178**, 147-153 (1999).
674   Becker, A. *et al.* Structure and mechanism of the glycyl radical enzyme pyruvate formate-
675   lyase. *Nat. Struct. Biol.* **6**, 969-975, doi:10.1038/13341 (1999).
676   Larsson, K. M., Andersson, J., Sjoberg, B. M., Nordlund, P. & Logan, D. T. Structural
677   basis for allosteric substrate specificity regulation in anaerobic ribonucleotide reductases.
678   *Structure* **9**, 739-750 (2001).
679   Heider, J., Spormann, A. M., Beller, H. R. & Widdel, F. Anaerobic bacterial metabolism
680   of hydrocarbons. *FEMS Microbiology Reviews* **22**, 459-473 (1998).
681   Feliks, M., Martins, B. M. & Ullmann, G. M. Catalytic mechanism of the glycyl radical
682   enzyme 4-hydroxyphenylacetate decarboxylase from continuum electrostatic and
683   QC/MM calculations. *J. Am. Chem. Soc.* **135**, 14574-14585, doi:10.1021/ja402379q
684   (2013).
685   Kalnins, G. *et al.* Structure and function of CutC choline lyase from human microbiota
686   bacterium *Klebsiella pneumoniae*. *J Biol Chem* **290**, 21732-21740,
687   doi:10.1074/jbc.M115.670471 (2015).
688   Craciun, S. & Balskus, E. P. Microbial conversion of choline to trimethylamine requires a
689   glycyl radical enzyme. *Proc. Natl. Acad. Sci. U S A* **109**, 21307-21312,
690   doi:10.1073/pnas.1215689109 (2012).

30

691 26   Levin, B. J. *et al.* A prominent glycyl radical enzyme in human gut microbiomes

692   metabolizes *trans*-4-hydroxy-l-proline. *Science* **355**, doi:10.1126/science.aai8386 (2017).

693 27   Funk, M. A., Marsh, E. N. & Drennan, C. L. Substrate-bound structures of

694   benzylsuccinate synthase reveal how toluene is activated in anaerobic hydrocarbon

695   degradation. *J. Biol. Chem.* **290**, 22398-22408, doi:10.1074/jbc.M115.670737 (2015).

696 28   Martins, B. M. *et al.* Structural basis for a Kolbe-type decarboxylation catalyzed by a

697   glycyl radical enzyme. *J. Am. Chem. Soc.* **133**, 14666-14674, doi:10.1021/ja203344x

698   (2011).

699 29   Kielak, A. M., Barreto, C. C., Kowalchuk, G. A., van Veen, J. A. & Kuramae, E. E. The

700   Ecology of *Acidobacteria*: Moving beyond Genes and Genomes. *Front. Microbiol.* **7**,

701   744, doi:10.3389/fmicb.2016.00744 (2016).

702 30   Ward, N. L. *et al.* Three genomes from the phylum *Acidobacteria* provide insight into the

703   lifestyles of these microorganisms in soils. *Appl. Environ. Microbiol.* **75**, 2046-2056,

704   doi:10.1128/AEM.02294-08 (2009).

705 31   Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment

706   search tool. *J. Mol. Biol.* **215**, 403-410, doi:10.1016/S0022-2836(05)80360-2 (1990).

707 32   Dawson, L. F., Stabler, R. A. & Wren, B. W. Assessing the role of *p*-cresol tolerance in

708   *Clostridium difficile*. *J. Med. Microbiol.* **57**, 745-749, doi:10.1099/jmm.0.47744-0

709   (2008).

710 33   Schneider, S., Mohamed, M. E. S. & Fuchs, G. Anaerobic metabolism of L-phenylalanine

711   via benzoyl-CoA in the denitrifying bacterium *Thauera aromatica*. . *Arch. Microbiol.*

712   **168**, 310-320 (1997).

31

713 34 Carmona, M. *et al.* Anaerobic catabolism of aromatic compounds: a genetic and genomic

714 view. *Microbiol. Mol. Biol. Rev.* **73**, 71-133, doi:10.1128/MMBR.00021-08 (2009).

715 35 Molenaar, D., Bosscher, J. S., ten Brink, B., Driessen, A. J. & Konings, W. N. Generation

716 of a proton motive force by histidine decarboxylation and electrogenic

717 histidine/histamine antiport in *Lactobacillus buchneri*. *J Bacteriol* **175**, 2864-2870

718 (1993).

719 36 Pereira, C. I., Matos, D., San Romao, M. V. & Crespo, M. T. Dual role for the tyrosine

720 decarboxylation pathway in *Enterococcus faecium* E17: response to an acid challenge and

721 generation of a proton motive force. *Appl Environ Microbiol* **75**, 345-352,

722 doi:10.1128/AEM.01958-08 (2009).

723 37 Beller, H. R., Legler, T. C. & Kane, S. R. Genetic manipulation of the obligate

724 chemolithoautotrophic bacterium *Thiobacillus denitrificans*. *Methods Mol. Biol.* **881**, 99-

725 136, doi:10.1007/978-1-61779-827-6_5 (2012).

726 38 Huntemann, M. *et al.* The standard operating procedure of the DOE-JGI Microbial

727 Genome Annotation Pipeline (MGAP v.4). *Stand. Genomic Sci.* **10**, 86,

728 doi:10.1186/s40793-015-0077-y (2015).

729 39 Edgar, R. C. UPARSE: highly accurate OTU sequences from microbial amplicon reads.

730 *Nat. Methods* **10**, 996-998, doi:10.1038/nmeth.2604 (2013).

731 40 Quast, C. *et al.* The SILVA ribosomal RNA gene database project: improved data

732 processing and web-based tools. *Nucleic Acids Res.* **41**, D590-596,

733 doi:10.1093/nar/gks1219 (2013).

734 41 Studier, F. W. Protein production by auto-induction in high density shaking cultures.

735 *Protein Expr. Purif.* **41**, 207-234 (2005).

736 42 Gao, H. *et al. Arabidopsis thaliana* Nfu2 accommodates [2Fe-2S] or [4Fe-4S] clusters

737 and is competent for *in vitro* maturation of chloroplast [2Fe-2S] and [4Fe-4S] cluster-

738 containing proteins. *Biochemistry* **52**, 6633-6645, doi:10.1021/bi4007622 (2013).

739 43 Mackay, D. & Shiu, W. Y. A critical review of Henry's Law constants for chemicals of

740 environmental interest. *Journal of Physical and Chemical Reference Data* **10**, 1175-1199

741 (1981).

742 44 Grant, C. E., Bailey, T. L. & Noble, W. S. FIMO: scanning for occurrences of a given

743 motif. *Bioinformatics* **27**, 1017-1018, doi:10.1093/bioinformatics/btr064 (2011).

744 45 Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high

745 throughput. *Nucleic Acids Res.* **32**, 1792-1797, doi:10.1093/nar/gkh340 (2004).

746 46 Stamatakis, A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of

747 large phylogenies. *Bioinformatics* **30**, 1312-1313, doi:10.1093/bioinformatics/btu033

748 (2014).

749 47 Letunic, I. & Bork, P. Interactive tree of life (iTOL) v3: an online tool for the display and

750 annotation of phylogenetic and other trees. *Nucleic Acids Res.* **44**, W242-245,

751 doi:10.1093/nar/gkw290 (2016).

752 48 Bankevich, A. *et al.* SPAdes: a new genome assembly algorithm and its applications to

753 single-cell sequencing. *J. Comput. Biol.* **19**, 455-477, doi:10.1089/cmb.2012.0021 (2012).

754 49 Wu, Y. W., Simmons, B. A. & Singer, S. W. MaxBin 2.0: an automated binning algorithm

755 to recover genomes from multiple metagenomic datasets. *Bioinformatics* **32**, 605-607,

756 doi:10.1093/bioinformatics/btv638 (2016).

757 50 Hyatt, D. *et al.* Prodigal: prokaryotic gene recognition and translation initiation site

758 identification. *BMC bioinformatics* **11**, 119, doi:10.1186/1471-2105-11-119 (2010).

33

759   Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P. & Tyson, G. W. CheckM:

760   assessing the quality of microbial genomes recovered from isolates, single cells, and

761   metagenomes. *Genome Res.* **25**, 1043-1055, doi:10.1101/gr.186072.114 (2015).

762   Krzywinski, M. *et al.* Circos: an information aesthetic for comparative genomics.

763   *Genome Res.* **19**, 1639-1645, doi:10.1101/gr.092759.109 (2009).

764   Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree: computing large minimum evolution

765   trees with profiles instead of a distance matrix. *Mol. Biol. Evol.* **26**, 1641-1650,

766   doi:10.1093/molbev/msp077 (2009).

767   Biasini, M. *et al.* SWISS-MODEL: modelling protein tertiary and quaternary structure

768   using evolutionary information. *Nucleic acids research* **42**, W252-258,

769   doi:10.1093/nar/gku340 (2014).

770   Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta*

771   *Crystallogr D Biol Crystallogr* **60**, 2126-2132, doi:10.1107/S0907444904019158 (2004).

772   Vagin, A. A. *et al.* REFMAC5 dictionary: organization of prior chemical knowledge and

773   guidelines for its use. *Acta Crystallogr D Biol Crystallogr* **60**, 2184-2195,

774   doi:10.1107/S0907444904023510 (2004).

775   Davis, I. W. *et al.* MolProbity: all-atom contacts and structure validation for proteins and

776   nucleic acids. *Nucleic acids research* **35**, W375-383, doi:10.1093/nar/gkm216 (2007).

777   Sievers, F. *et al.* Fast, scalable generation of high-quality protein multiple sequence

778   alignments using Clustal Omega. *Mol. Syst. Biol.* **7**, 539, doi:10.1038/msb.2011.75

779   (2011).

780

781**Supplementary Information** is linked to the online version of the paper at

782.

783**Acknowledgments**

796**Author Contributions**

797H.R.B., A.V.R., K.Z. and R.M.S. conceived of and designed the experiments. A.V.R., K.Z.,

798H.R.B., A.K.S., and R.S. performed the experiments. H.R.B., Y.W.W., and A.V.R. analyzed the

799data. S.G.T. oversaw metagenomic data production and C.J.P. oversaw metaproteomic data

800production. The manuscript was written by H.R.B. (primarily) and all authors, including J.D.K.,

801contributed to refining the text.

802

803

804

805 Author Information

809

810
811
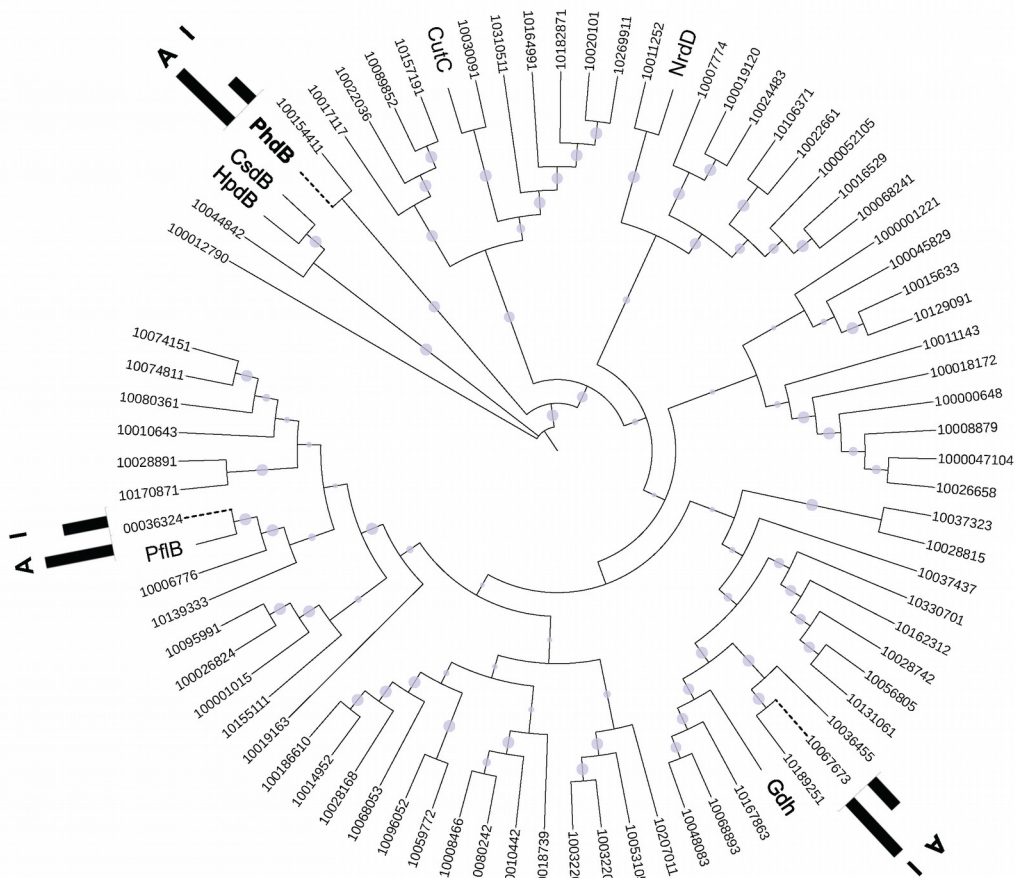
Figure 1 | **Glycyl radical enzymes encoded in the toluene-producing sewage culture metagenome and their association with *in vitro* toluene synthase activity.** This maximum-likelihood tree is based on protein sequences of putative glycyl radical enzymes (GREs) detected in the sewage-derived metagenome [IMG Taxon ID 3300001865 on JGI's IMG-M site (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi)]. Numerical values on the leaves represent locus tags in the metagenome from which the prefix "JGI2065J20421_" has been truncated for brevity. Leaves with protein names rather than locus tags are known GREs provided for context (see Methods for details). The leaf marked PhdB represents the GRE characterized in this study. Leaves with dashed lines represent proteins detected by LC/MS/MS in active FPLC fractions, and the histograms on these leaves represent the maximum abundance of this protein in (A) the two most active fractions and (I) the two flanking inactive or less active fractions (Supplementary Data File 1); histograms are normalized to the greatest of the A and I values. Purple circles on leaves represent bootstrap support values for each node (largest symbols are 100).

826
827
828
829

830
831
832
833
834
835
836
837
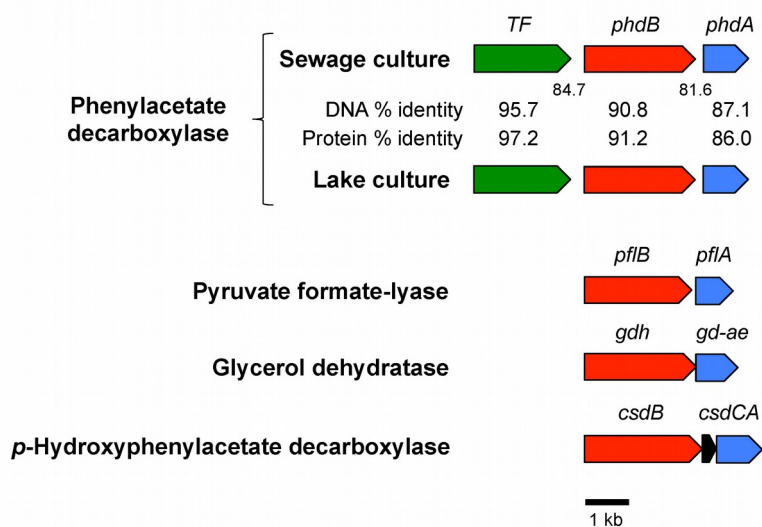838
839
840
841
842
843
844
845
846

847**Figure 2** | **Homologous** ~~toluene synthas~~phenylacetate decarboxylasee **gene clusters from**
848**sewage and lake sediment cultures.** *phdB*, phenylacetate decarboxylase (a glycyl radical
849enzyme); *phdA*, a cognate activating enzyme for *phdB*; *TF*, putative transcription factor.
850Sequence identity is shown for the coding sequences as well as the two intergenic regions. Gene
851clusters for selected GREs (in red) and their cognate activating enzymes (in blue) are shown for
852comparison, including pyruvate formate-lyase (*pflB*, *pflA*), glycerol dehydratase (*gdh*, *gd-ae*),
853and *p*-hydroxyphenylacetate decarboxylase (*csdB*, *csdC*, *csdA*). A 1-kb scale bar is included.
854
855
856

38

857
858
859
860
861
862
863
864
865
866
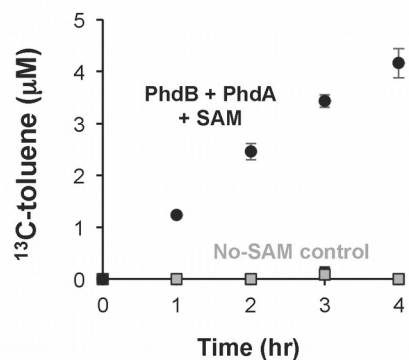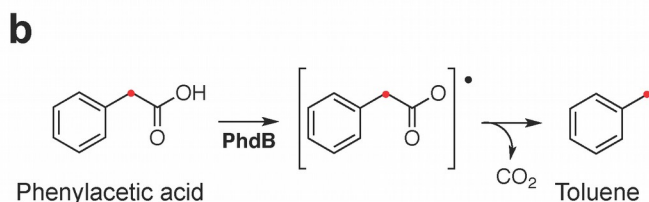867
868
869
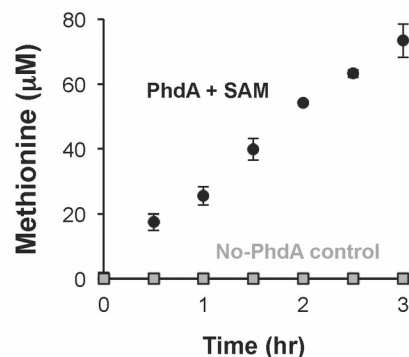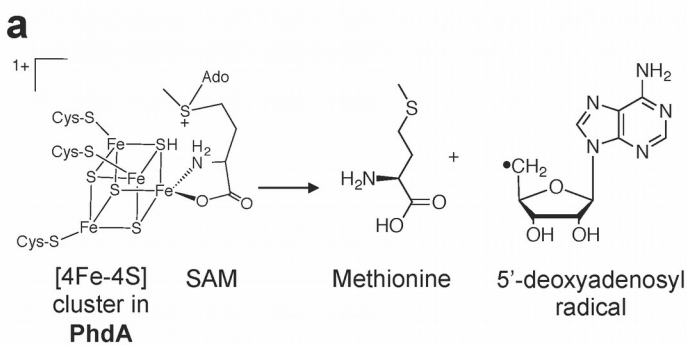870
871
872
873
874
875
876
877
878
879
880 **Figure 3** | **Reactions catalyzed by PhdA and PhdB. a,** proposed reaction of PhdA with SAM,
881 as supported *in vitro* by methionine production by re-constituted and purified recombinant PhdA
882 (black circles). Controls without PhdA are also shown (gray squares). **b**, proposed reaction of
883 PhdB with phenylacetic acid-2-^{13}C, as supported *in vitro* by [*methyl*-^{13}C]toluene production by
884 partially purified PhdB in combination with PhdA and SAM (black circles). Controls without
885 SAM are also shown (gray squares). ^{13}C-labeled C atoms in the proposed reaction are
886 highlighted with a red circle. Data points represent means and error bars represent one standard
887 deviation (*n*=3). Experiments demonstrating PhdA-catalyzed production of methionine from
888 SAM were replicated ~~twice~~ three times and experiments demonstrating labeled toluene
889 production from labeled phenylacetate in the presence of PhdB and PhdA were performed 6
890 times (four times with no-SAM negative controls).
891
892
893
894
895
896
897

39

898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
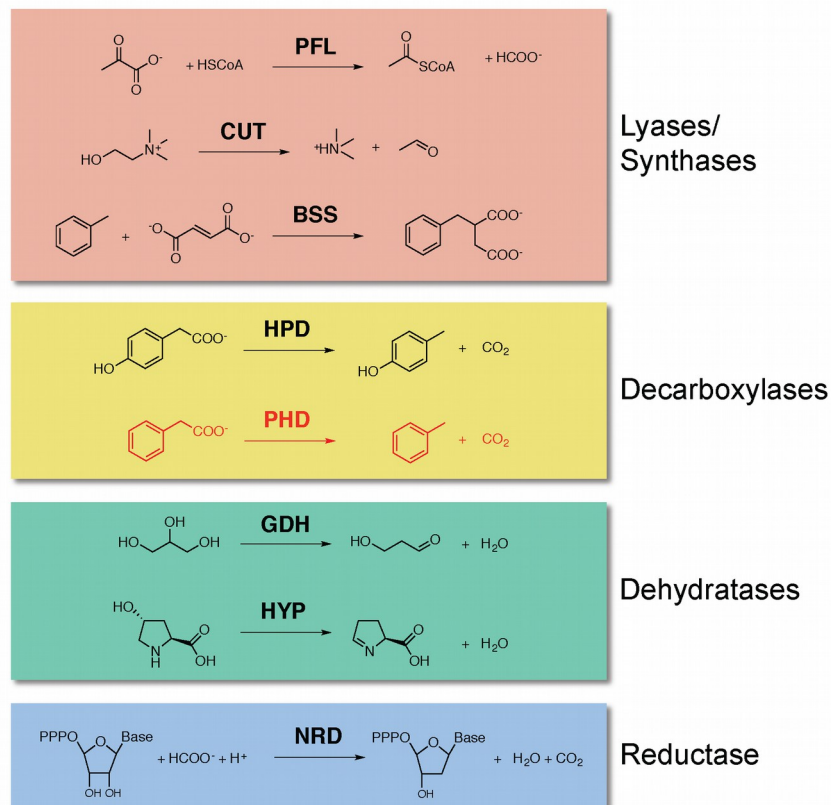
922**Figure 4** | **Reactions catalyzed by characterized GREs.** PFL, pyruvate formate-lyase; CUT, 923choline trimethylamine-lyase; BSS, benzylsuccinate synthase; HPD, *p*-hydroxyphenylacetate 924decarboxylase; PHD, phenylacetate decarboxylase (this study); GDH, glycerol dehydratase; 925HYP, *trans*-4-hydroxy-L-proline dehydratase; and NRD, anaerobic ribonucleotide reductase.
926
927
928

40

a

```
         PflB     MLLDAMENPEKYPQLTIRVSGYAVRFNSLTKEQQQDVITRTFTQSM     760
         CsdB     TLRDAQLTPEKYRELMVRVAGFTQYWCEIGKPIQDEVIYRTEYDK     897
         BssA     EMRAAQREPEKHHDLIVRVSGYSARFVDIPTYGQNTIIARQEQDFSASDL 857
         Gdh      ILLAAQKNPEKYQDLIVRVAGYSAQFISLDKSIQNDIIARTEHVM     787
         CutC     VLKKAQQEPEKYRDLIVRVAGYSAYFVELCKEVQDEIISRTVIEKF     1128
         HypD     VLLEAQKNPQDYKDLIVRVAGYSDHFNNLSRTLQDEIIGRTEQTF     789
         PhdB-s   TLRAAQKDPDSYRDLIVRVAGFSAYFITLCPEVQDEIVSRTCQTW     839
         PhdB-l   TLRAAQKDPDSFRDLIVRVAGFSAYFITLCPEVQNEIVSRTSQQW     839
```

b

```
         PflB     DDYAIACCVSPMIVG--------KQM-----QF-----FGARANLAKTML 444
         CsdB     RAWCLGGCLESAPGCFLPLEYNGKVTMIPGGASPTCGTGVHFIGMPKVLE 545
         BssA     HNWVNVLCMSPGIHG--------RRK-----TQKTRSEGGGSIFPAKLLE 521
         Gdh      RDYGIIGCVEPQKPG--------KTE-----GWH----DSAFFNLARIVE 458
         CutC     RDYCLMGCVEPQKSG--------RIY-----QWT----STGYTQWPIAIE 796
         HypD     RLGGTSGCVETGCFG--------K-E-----AYV----LTGYMNIPKILE 458
         PhdB-s   RDQAVAGCVQSIIGG--------KTD-----GT-----WEARFNMTKMME 506
         PhdB-l   RDQAVAGCVQSIIGG--------KTD-----GT-----WEARFNMCKMIE 506
```

c

```
         NrdG      MNYHQYYPVDIVNGPGTRCTLFVSGCVHECPGCYNKS 37
         BssD      MKIPLITEIQRFSLQDGPGIRTTIFLKGCPLRCPWCHNPE 40
         PflA      MSVIGRIHSFESCGTVDGPGIRFITFFQGCLMRCLYCHNRD 36
         CutD      MIAKQELTGRIFNIQKYSIYDGDGIRTLVFFKGCNIRCPWCANPE 45
         CsdA      MKEKGLIFDIQSFSVHDGPGCRTSVFFIGCPLQCKWCANPE 41
         GD-AE     MSKEIKGVLFNIQKFSLHDGPGIRTIVFFKGCSMSCLWCSNPE 43
         HypD-AE   MNPLVINLQKCSIHDGPGIRSTVFFKGCPLECVWCHNPE 39
         PhdA-s    MGTNELTGMVFNIQGYSVQDGPGIRTTVFLKGCPLRCLWCSNPE 44
         PhdA-l    MGTSELTGTNELTGMVFNIQGYSIQDGPGIRTTIFLKGCPLRCLWCSNPE 50
```

Figure 5 | **Multiple sequence alignments comparing PhdB and PhdA with other glycyl radical enzymes and glycyl radical activating enzymes, respectively. a,** C-terminal region of GREs containing the conserved glycyl radical motif, with the glycyl radical site highlighted in red with an asterisk and other conserved residues in bold. **b**, mid-sequence region of GREs containing conserved, active-site cysteine residue (which bears the thiyl radical that interacts with the substrate), highlighted in red with an asterisk, along with other conserved residues shown in blue. **c**, N-Terminal region of activating enzymes highlighting the CxxxCxxC motif (highlighted with asterisks) coordinating with the [4Fe-4S] cluster. Sequences used in these alignment comparisons include the following GREs and AEs [PDB (Protein Data Bank) or GenBank accession number]: PflB (GenBank: NP_415423), PflA (GenBank: NP_415422), CsdB (GenBank: ABB05046.1), CsdA (GanBank: 2580384209), BssA (PDB: 4PKC:A), BssD (GenBank: CAA05050.2), Gdh (PDB: 1R8W), GD-AE (GenBank: AAM54729), CutC (PDB: 5A0Z), CutD (GenBank: EPO20361.1), HypD (UniProt: A0A031WDE4), HypD-AE (UniProt: A0A069AMK2), NrdG (GenBank: NP_418658). The "s" and "l" suffixes for PhdB and PhdA stand for sewage and lake, respectively. Alignment was performed with Clustal Omega[58].

973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
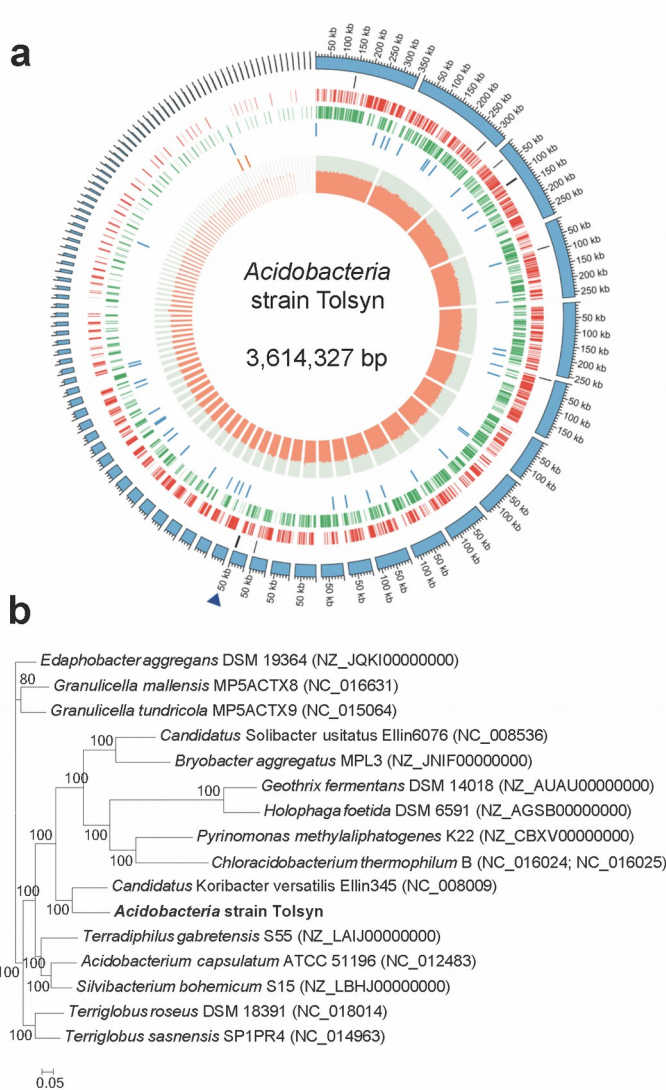
Figure 6 | **Characterization of the putatively toluene-producing *Acidobacterium* strain Tolsyn based on its recovered genome. a**, schematic circular diagram of the genome, with contigs in size order, displaying contigs and their corresponding lengths (outer ring), genes encoding radical-related enzymes (second ring; the contig containing *phdA* and *phdB* is indicated with a filled triangle), genes on the forward strand (third ring), genes on the reverse strand (fourth ring), tRNA genes (fifth ring), rRNA genes (sixth ring), and GC content (seventh ring; GC is averaged every 1000 bp and is represented as orange, whereas AT is light green). **b**, Phylogenetic relationships among *Acidobacterium* strain Tolsyn and the most closely related *Acidobacteria* sequenced isolates based upon 129 concatenated marker proteins (GenBank accession numbers for species are shown in the tree). Numbers at nodes represent bootstrap support values. The scale bar represents substitution rate per site.