Development and application of aerobic, chemically defined media for *Dysgonomonas*

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Dysgonomonas defined media

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Impact Statement

Members of the genus *Dysgonomonas* are increasingly prevalent in ecological, medical and biotechnological contexts. To the best of our knowledge, there are currently no formulations for chemically defined or minimal media for *Dysgonomonas*, and limited complex formulations that allow aerobic growth, particularly on solid media. We have created three parallel media formulations (complex, defined & minimal) that permit robust aerobic and anaerobic growth in liquid and agar-solidified media. These formulations remove the necessity for animal blood and expensive equipment such as anaerobic chambers, which can inhibit basic research by groups with biosafety and resource limitations.

Abstract

Members of *Dysgonomonas* are Gram-negative, non-motile, facultatively anaerobic coccobacilli originally described in relation to their isolation stool and wounds of human patients (CDC group DF-3). More recently *Dysgonomonas* have been found to be widely distributed in terrestrial environments and are particularly enriched in insect systems. Their prevalence in xylophagous insects such as termites and wood-feeding cockroaches, as well as in soil-fed microbial fuel cells, elicit interest in lignocellulose degradation and biofuel production, respectively. Their prevalence in mosquito and fruit fly have implications relating to symbiosis, host immunology and developmental biology. Additionally, their prevalence in termite, mosquito and nematode present novel opportunities for pest and vector control. Currently, the absolute growth requirements of *Dysgonomonas* are unknown, and they are cultured solely under anaerobic conditions on complex media containing blood, peptones, tryptones, and yeast, plant or meat extracts. Restrictive & undefined culturing conditions preclude physiological and genetic studies, and thus further understanding of metabolic potential. Here we describe the requirements for growth of termite-derived *Dysgonomonas* isolates and create parallel defined, minimal and complex media that permit vigorous and reliable aerobic growth. Furthermore, we show that these media can be used to easily enrich for *Dysgonomonas* isolates from complex and microbially-diverse environmental samples.

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Keywords

Dysgonomonas, termite, defined media, minimal media, L-cysteine, hemin

Introduction

Members of the genus *Dysgonomonas* (family *Dysgonomonadaceae* [1]) once represented an emerging class of opportunistic pathogens isolated from human sources including stool, abscesses and wounds [2-6]. However, the majority of newly cultivated isolates originate from non-human environmental sources, including a soil-seeded microbial fuel cell [7], higher [8] and lower termites [9,10], marine beach sand [11], paper mill sludge [12] and omnivorous cockroaches [13]. Sequences of the 16S rRNA gene of *Dysgonomonas* are prevalent in insect associations, such as honeybee [14], dipteran flies [15-18], including several *Drosophila* species [19-21], beetles [22-26], and several life stages of three major mosquito genera, *Culex* [27,28], *Aedes* [29,30] and *Anopheles* [31,32]. *Dysgonomonas*-derived 16S rRNA gene sequences are routinely observed associated with xylophagous cockroaches [33-38] and as ectosymbionts of nematodes living in the cockroach digestive system [39], as well as in the hindguts of phylogenetically related termites [40-44]. *Dysgonomonas* have been identified as core microbiota of both higher [45] and lower [46] termites, including *Reticulitermes flavipes* [47]. There is growing interest in *Dysgonomonas* due to their prevalence in biotechnological processes such as lignocellulose degradation and bioconversion of polysaccharides for biofuel development [48,49], microbial fuel and electrolysis cells [50-57], wastewater bioreactors [58,59], biodegradation of food waste [60,61] and pharmaceutical compounds [58,62-64].

Members of genus *Dysgonomonas* are described as fastidious [3,4,65] owing to growth requirements satisfied only by rich, complex media containing whole or digested animal-derived components. For example, both the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ) recommend culture of *Dysgonomona*s on complex media containing enzymatic digests of animal-derived proteins, yeast- or animal-tissue extracts, defibrinated animal blood and other rarely required growth factors used by other fastidious organisms. Pre-mixed media formulations for these recommended media are widely available and can offer convenience, but are relatively expensive and cannot be easily tailored for experimental purposes. In-house preparations of these media can be made, but since many

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79   common recipes were created for the purpose of growing bacteria with diverse nutritional

80   requirements, nearly all available media contain components that can be expensive or difficult to

81   prepare and may be superfluous for the growth of *Dysgonomonas*. The Known Media Database

82   (KOMODO) [66] is a collection of known organism-media pairings, and its online tool

83   GROWREC (http://komodo.modelseed.org/growrec.htm) uses phylogenetic and ecological

84   similarity to predict growth-permitting media for an organism. While this database performs well

85   to determine complex anaerobic media formulations specific to *Dysgonomonas*, it is unable to

86   offer either chemically defined or aerobic media formulations. Defined media created for the

87   closely related genus *Bacteroides* [67,68] have been shown to support the growth *Dysgonomonas*

88   *gadei* and *Dysgonomonas mossii* [69], but we observed poor growth from our isolates in these

89   liquid media when serially cultured (unpublished results). *Dysgonomonas alginatilytica* has been

90   reported to grow on a defined medium consisting only of basal salts and kraft-lignin [12], and

91   while this medium may contain adequate reduced carbon and concomitant micronutrients, it lacks

92   major components of the media known to support the growth of *Dysgonomonas*. Defined media

93   have been described for closely-related *Porphyromonas gingivalis* [70,71], but contain

94   components such as casitone that are not truly defined, or lack known required nutritional

95   components such as B-vitamins. Described species of *Dysgonomonas* are facultatively anaerobic

96   on complex media [3-5,7-9,11,72], but our isolates exhibited slow growth and altered colony

97   morphology when grown aerobically on available complex media formulations. ATCC

98   recommends that members of *Bacteroidaceae* be grown anaerobically using pre-reduced medium

99   containing sodium sulfide, cysteine, or coenzyme M as reducing agents (ATCC Bacterial Culture

100   Guide, 2015). These reductants, and expensive equipment such as anaerobic chambers, may be

101   unnecessary given the facultative nature of *Dysgonomonas*.

102   The lack of chemically defined media for members of *Dysgonomona*s precludes a deeper

103   understanding of physiological and metabolic potential and dampens the ability to perform

104   phenotypic analyses. Furthermore, defined media complement the development of genetic tools,

105   such as the addition or omission of specific components for the purpose of genetic selections. Here,

106   we developed chemically defined media formulations based on the minimal growth requirements

107   of four *Dysgonomonas* isolates belonging to differing phylogenetic clades within the genus. The

108   parallel media formulations *Dysgonomonas* <u>C</u>omplex Medium (DCM), *Dysgonomonas* <u>Defined</u>

109   Medium (D<u>D</u>M) and *Dysgonomonas* <u>Minimal</u> Medium (D<u>M</u>M) make use of widely available

110   components, offer straightforward preparation, allow growth in liquid or solid culture and permit

111   growth under aerobic or anaerobic conditions. Finally, we demonstrate the ease with which our

112   minimal media (DMM) allows enrichment of *Dysgonomonas* from microbially-complex

113   environmental samples such as the lower termite hindgut.

Materials and methods

Isolation & maintenance of bacteria from termite hindguts

116   *Dysgonomonas* spp. BGC7 and HGC4 were generously provided by Dr. Michael C. Nelson and

117   Dr. Joerg Graf at the University of Connecticut. *R. flavipes* termites were collected from decaying

118   oak tree stumps in Granby, CT, USA (Lat: 41.999316, Long: -72.789053) during September 2017.

119   All isolations were performed in a laminar flow hood using sterile instruments and reagents.

120   *Dysgonomonas* spp. GY75 and GY617 were isolated by washing ten worker termites in 95%

121   ethanol and aseptically extirpating hindguts using the technique described by Matson et al. [73].

122   Hindgut contents were pooled into 1 ml sterile 1X M9 salts (5.8 g/L Na_2HPO_4 (Fisher, Pittsburgh,

123   PA, USA), 3.0 g/L KH_2PO_4 (Fisher), 0.50 g/L NaCl (Fisher), 1.0 g/L NH_4Cl (Fisher), 0.011 g/L

124    $CaCl_2$ (Acros Organics, Geel, Belgium), 0.25 g/L $MgSO_4$ (Acros)) and the entire volume added to

125   a 2-ml screw-capped microcentrifuge tube (USA Scientific, Ocala, FL, USA) containing 200 mg

126   of 1 mm glass beads (Biospec, Bartlesville, OK, USA) and processed for 30 seconds on a Mini-

127   Beadbeater-16 (Biospec). Homogenized samples were serially diluted in sterile 1X M9 salts and

128   plated onto peptone-yeast extract-blood-glucose medium (PYBG; 6 g/L $Na_2HPO_4 \cdot 7H_2O$, 10 g/L

129   proteose peptone #3 (US Biological, Salem, MA, USA), 10 g/L yeast extract (Bacto, Mt Pritchard,

130   NSW, Australia), 10% whole sheep blood (Lampire, Pipersville, PA, USA), 0.5% D-glucose

131   (Acros), 50 μg/ml kanamycin sulfate (Gibco, Dublin, Ireland) and 1.5% w/v agar (Bacto)). Plates

132   were incubated anaerobically for 5 days at 22°C in a gloveless anaerobic chamber (Coy, Grass

133   Lake, MI, USA) under an atmosphere of 5.5% CO_2, 5.5% H_2 and 89% N_2. Isolates with colony

134   morphology similar to *Dysgonomonas* to were serially sub-streaked twice onto the same medium

135   and incubated under the same conditions. Purified isolates of two strains, GY75 and GY617, were

136   grown under anaerobic conditions at 22°C in rich peptone-hemin-glucose medium (rPHG; 6 g/L

137    $Na_2HPO_4 \cdot 7H_2O$, 30 g/L proteose peptone #3, 10 g/L yeast extract, 50 mg/L porcine ferric hemin

138   (MP Biomedicals, Santa Ana, CA, USA; prepared as an aqueous solution of 0.5 g/L in 10 mM

139   NaOH (Fisher)), 0.5% w/v D-glucose, 50 μg/ml kanamycin sulfate, 1% v/v Wolfe's Mineral

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140   Solution [74] (WMS; 3.0 g/L $MgSO_4·7H_2O$ (Acros), 1.5 g/L nitrilotriacetic acid (Acros), 1.0 g/L

141   NaCl, 0.5 g/L $MnSO_4·2H_2O$ (Acros), 0.1 g/L $CoCl_2·6H_2O$ (Sigma, St. Louis, MO, USA), 0.1 g/L

142    $ZnSO_4·7H_2O$ (Research Organics, Cleveland, OH, USA), 0.1 g/L $CaCl_2·2H_2O$ (Acros), 0.1 g/L

143    $FeSO_4·7H_2O$ (Research Organics), 0.025 g/L $NiCl_2·6H_2O$ (Acros), 0.02 g/L $KAl(SO_4)_2·12H_2O$

144   (Fisher), 0.01 g/L $CuSO_4·5H_2O$ (Sigma), 0.01 g/L H_3BO_3 (Fisher), 0.01 g/L $Na_2MoO_4·2H_2O$

145   (Sigma), 0.3 g/L $Na_2SeO_3·5H_2O$ (Sigma)) and 5% v/v Wolfe's Vitamin Solution [74] (WVS; 2.0

146   mg/L biotin (Fisher), 2.0 mg/L folic acid (Sigma), 10 mg/L pyridoxine-HCl (Sigma), 5.0 mg/L

147   thiamine-HCl (Sigma), 5.0 mg/L riboflavin (Sigma), 5.0 mg/L nicotinamide (Sigma), 5.0 mg/L

148   calcium-D-pantothenate (Sigma), 100 μg/L cyanocobalamin (Fisher), 5.0 mg/L p-aminobenzoic

149   acid (PABA; Sigma), 5 mg/L α-lipoic acid (Sigma)), pH 7.5) before being frozen at -80°C in 20%

150   (v/v) sterile glycerol (Acros). Isolates were cultured form glycerol stocks and maintained on solid

151   PYBG medium incubated anaerobically for 72 hours at 22°C. Unless otherwise stated, cultures

152   used as growth curve inocula were grown anaerobically at 22°C to stationary phase in Falcon 15

153   ml polypropylene conical tubes (Corning, Corning, NY, USA) in 5 ml volumes.

154   **Identification of bacterial isolates**

155   The identity of four strains of *Dysgonomonas* (isolates BGC7, HGC4, GY75 & GY617) were

156   confirmed by sequencing nearly full-length 16S rRNA gene sequences (~1455 of 1535 bp,

157   hereafter called 'full-length'). Single colonies from pure isolates were inoculated into 5 ml rPHG

158   medium in 18 x 150 mm glass culture tubes (Fisher) containing 50 μg/ml kanamycin sulfate and

159   incubated ~18 hours at 30°C aerobically without shaking. Overnight cultures were used for

160   genomic DNA (gDNA) preparation using Promega Wizard Genomic DNA Purification Kit

161   (Promega, Madison, WI, USA) or Epicenter MasterPure Complete DNA and RNA Purification

162   Kit (Lucigen, Madison, WI, USA) according to manufacturer instructions. Isolated gDNA was

163   checked for quality and concentration by gel electrophoresis and by spectrophotometry using a

164   Take3 Micro-Volume Plate (BioTek, Winooski, VT, USA). Approximately 100 ng of gDNA was

165   used as template for PCR using Q5 Hot Start DNA Polymerase (New England Biolabs (NEB),

166   Ipswich, MA, USA) and primers Eubact_27F (5'-AGAGTTTGATCMTGGCTCAG-3') and

167   Eubact_1492R (5'-TACGGYTACCTTGTTAC-3') (modified from [75]). A no-template control

168   contained water in place of gDNA. PCR was performed using the manufacturer's recommended

169   procedure with a primer annealing temperature of 50°C and a 45 second extension time, for a total

170 of 35 cycles. An aliquot of each PCR reactions was checked by gel electrophoresis for single

171 amplicons before purifying the remaining reaction using Monarch DNA Gel Extraction Kit (NEB),

172 per manufacturer instructions. PCR amplicons were A-tailed by incubating 750 ng of DNA with 2

173 Units of Taq DNA Polymerase (NEB) and 0.2 mM dATP (Thermo Fisher Scientific, Inc.,

174 Waltham, MA, USA) at 72°C for 30 minutes. A-tailed PCR amplicons were cloned into p-GEM-

175 T Easy Vector System (Promega) per manufacturer instructions and ligation reactions introduced

176 directly into electrocompetent XL1-Blue *E. coli* (Stratagene, La Jolla, California, USA) by

177 electroporation. After a 1 hour recovery at 37°C in liquid SOC (10 g/L tryptone (Bacto), 2.5 g/L

178 yeast extract, 0.6 g/L NaCl, 0.2 g/L KCl (Fisher), 3.6 g/L D-glucose, 2 g/L $MgCl_2 \cdot 6H_2O$ (Research

179 Organics), cells were diluted 10-fold and plated on agarose-solidified LB (10 g/L tryptone, 5 g/L

180 yeast extract, 10 g/L NaCl, 15 g/L agar) containing 100 μg/ml ampicillin sodium salt (Fisher), 47.6

181 μg/ml (0.2 mM) isopropyl β-D-thiogalactopyranoside (IPTG; Fisher), 40 μg/ml (97.8 μM) 5-

182 bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Fisher). Plates were incubated ~18

183 hours at 37°C and individual white colonies screened for insert by colony PCR using primers

184 M13_F (5'-ACGACGTTGTAAAACGACGGCCAGT-3') and M13_R (5'-

185 ATTTCACACAGGAAACAGCTATGACCA-3') GoTaq DNA Polymerase (Promega).

186 Reactions were separated by gel electrophoresis, and insert-positive clones were cultured ~18

187 hours in 5 ml LB broth containing 100 μg/ml ampicillin. Plasmid DNA was isolated using Promega

188 PureYield Plasmid Miniprep System (Promega) and sent to Genewiz (South Plainfield, NJ, USA)

189 for bidirectional dideoxy sequencing using primers M13_F and M13_R. Overlapping sequencing

190 reads were assembled and 2-4X sequence coverage was obtained for each clone.

Growth experiments

192 Experiments were all performed in a basal media containing 1X M9 salts, and when required,

193 amended by adding components to the following final concentrations (unless otherwise stated):

194 1% w/v proteose peptone #3, 1% w/v casamino acids (Fisher), 0.1 mg/ml L-tryptophan (Sigma),

195 0.1 mg/ml L-cysteine-HCl·H$_2$O (Fisher), 0.1 mg/ml L-methionine (Sigma), 0.1 mg/ml each of

196 nucleobases adenine, guanine, cytosine, thymine, uracil, hypoxanthine (Acros), 0.2 mg/ml L-

197 ornithine-HCl (Acros), 0.2 mg/ml beta-nicotinamide adenine dinucleotide reduced disodium salt

198 (NAD; Alfa Aesar, Lancashire, United Kingdom), 0.2 mg/ml diaminopimelic acid (DAP; Sigma),

199 1% v/v WMS, 5% v/v WVS, 0.5% w/v D-glucose, 50 μg/ml kanamycin sulfate, 10% v/v hemin

200 solution. Unless otherwise stated, cell cultures used for growth experiments were pre-grown

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201 anaerobically without shaking for ~18 hours in 5 ml volumes of rPHG medium. Cultures were

202 centrifuged and the cell pellet washed three times with 1X M9 salts. Optical density of the cultures

203 at 595 nm was determined by spectrophotometry using BioTek Synergy HT or H1 automated plate

204 readers (BioTek) then diluted to an OD_{595} of 0.1 (200 μl of 1X M9 salts in 96-well microtiter plate

205 (Corning #35-1172)) before being added to media at a final OD_{595} of approximately 0.01 in 1X

206 M9 salts. When stated, cells were starved of particular media components prior to performing

207 growth curves as follows. Cultures pre-grown in rPHG medium were washed and diluted to OD_{595}

208 of 0.1 as described above, and subcultured 1:100 into 50 ml volume of defined medium or minimal

209 medium lacking the specific component(s). Cultures were grown to component-limiting stationary

210 phase before being washed, diluted and used as inoculum as stated above. When the presence of

211 sulfate or ammonium was of concern, cells were washed and resuspended in sterile 1X phosphate-

212 buffered saline (PBS; 8.0 g/L NaCl, 0.20 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L mM KH_2PO_4, pH

213 7.4) in place of 1X M9 salts. Water used to prepare all components and media was Fisher Optima

214 HPLC-grade water (Fisher). Aerobic growth curves were performed using BioTek HT and H1

215 Synergy microplate readers, using 220 μl total volume in 96-well micro-titer plates at 30°C.

216 Automated optical density readings at 595 nm (OD_{595}) were taken every 10 minutes following a

217 30-second orbital shake. Where described, duplicate plates were inoculated and each plate grown

218 in parallel under aerobic or anaerobic conditions as described above. Endpoint OD_{595} readings

219 were taken at the start and finish of each experiment, preceded by a 2-minute orbital shake. Unless

220 otherwise indicated, all experiments included 2 biological replicates per plate with the exception

221 of (i) auxanography for amino acids (**Figures S1 & S2**) & vitamins (**Figure S7**) which were

222 performed as preliminary screens and subsequently followed by experiments to validate observed

223 phenotypes; (ii) experiments examining a broad range of component concentrations such as L-

224 cysteine or ferric hemin (**Figures S3, S5 & S6**). All experiments regardless of biological replicates

225 were performed multiple times. Generation of graphs, as well as regression and statistical analyses

226 were performed using GraphPad Prism v8 (GraphPad Software, La Jolla, CA, USA).

227 **Amino acid auxanography**

228 To reveal possible amino acid auxotrophies in wild-type *Dysgonomonas* isolates, a modified

229 version of auxanography [76] was performed (**Figures S1 & S2**). To reduce experimental

230 complexity, B-vitamins were supplied. Nucleobases, NAD and PABA were omitted, as they were

231 not required for growth (**Figure 1**). Twenty-two individual amino acids (obtained from Sigma

232   except for L-cysteine-HCl·H$_2$O), were prepared as 10 mg/ml aqueous solutions using concentrated

233   NaOH or HCl to allow dissolution as necessary. All were free acids with the exception of the

234   following chloride salts: L-ornithine-HCl, L-arginine-HCl, L-histidine-HCl, L-lysine-HCl and L-

235   cysteine-HCl·H$_2$O. Equal volumes of each amino acid in a pool were combined to 6 mL total

236   volume, with each amino acid at a final concentration of 1.67 mg/ml. Pools can be found in **Table**

237   **S1** and as follows: Pool 1: L-phenylalanine, L-serine, L-tryptophan, L-tyrosine, L-glutamine; Pool

238   2: L-alanine, L-cysteine, L-threonine, L-asparagine, L-methionine, DAP; Pool 3: L-arginine, L-

239   ornithine, L-aspartic acid, L-proline, L-glutamic acid; Pool 4: L-leucine, L-glycine, L-isoleucine,

240   L-histidine, L-lysine, L-valine; Pool 5: L-phenylalanine, L-alanine, L-arginine, L-leucine; Pool 6:

241   L-serine, L-cysteine, L-ornithine, L-glycine; Pool 7: L-tryptophan, L-threonine, L-aspartic acid,

242   L-isoleucine; Pool 8: L-tyrosine, L-asparagine, L-proline, L-histidine; Pool 9: L-methionine, L-

243   glutamic acid, L-lysine; Pool 10: L-glutamine, DAP, L-valine. Media were supplemented with

244   amino acid pools at a ratio of 1:5 such that each amino acid was present at 0.33 mg/ml each. The

245   final media consisted of 1X M9 supplemented with 5% v/v hemin solution, 5% WVS, 0.5% v/v

246   WMS, 0.5% w/v D-glucose, 50 μg/ml kanamycin sulfate and a single amino acid pool. Cells were

247   pre-grown in rPHG, washed and used as inoculum as described above, and growth curve analysis

248   performed.

Determination of sulfur, nitrogen and iron requirements

250   To determine L-cysteine and sulfate requirements (**Figure 2**), cells were pre-grown in DDM

251   containing 0.569 mM L-cysteine, 1 mM MgSO$_4$, 5% v/v WVS and 10% v/v hemin solution before

252   being washed and diluted in 1X PBS and used for growth curves. Modified WMS (mWMS) was

253   created by substituting magnesium chloride or manganese chloride (MnCl$_2$·4H$_2$O, Acros) salts for

254   the magnesium and manganese sulfate salts in the standard WMS recipe. Growth curves were

255   performed in either sulfate-replete (0.01% v/v WMS, 1 mM total SO$_4^{2-}$) or sulfate-limited (0.01%

256   v/v mWMS, 83 nM total SO$_4^{2-}$) conditions. To determine if L-cysteine was able to be utilized as a

257   sole source of nitrogen (**Figure S4**), cultures were pre-grown in rPHG, washed and resuspended

258   in PBS, and used as inoculum for growth curves in the presence or absence of excess ammonium

259   (18.7 mM from 1X M9) and/or 1.7 mM L-cysteine. For determination of growth response to hemin

260   concentration (**Figures S5 & S6**) and for determination of alternate sources of iron beside ferric

261   hemin (**Figure 3**), cells were pre-grown in rPHG, then washed and subcultured 1:100 in 50 ml

262   DDM without hemin and grown to hemin-limited stationary phase. Cells were again washed,

263   diluted and then used for growth curves.

Determination of vitamin and mineral requirements

265   To determine vitamin auxotrophy (**Figure S7**), twelve variations of DDM media were prepared.

266   All variations lacked WVS and contained 9 out of 10 individual vitamins, which were prepared as

267   0.1 mg/ml aqueous stock solutions and added to final concentration equal to that in 5% v/v WVS:

268   (0.1 mg/L (0.41 μM) biotin, 0.1 mg/L (0.23 μM) folic acid, 0.5 mg/L (2.4 μM) pyridoxine-HCl,

269   0.25 mg/L (0.74 μM) thiamine-HCl, 0.25 mg/L (0.66 μM) riboflavin, 0.25 mg/L (2.0 μM)

270   nicotinamide, 0.25 mg/L (1.0 μM) calcium-D-pantothenate, 5.0 μg/L (3.7 nM) cyanocobalamin,

271   0.25 mg/L (1.8 μM) p-aminobenzoic acid, 0.25 mg/L (1.2 μM) α-lipoic acid). Cells were pre-

272   grown in rPHG, washed and subcultured 1:100 in 50 ml DDM without WVS and grown to

273   stationary phase. Starved cells were washed, diluted and used for growth curves as previously

274   described. To validate requirements for specific vitamins observed during auxanography (**Figure**

275   **4**), cells were first starved of vitamins as described and subsequently inoculated into DDM without

276   WVS but containing combinations of biotin, thiamine, cyanocobalamin and L-methionine. We

277   included trace metals (provided by WMS) in our defined media formulations due to the use of

278   highly-purified molecular grade water in our experiments, and also to provide metal-replete

279   conditions inclusive to the potential requirements of yet unknown enzymatic functions,

280   particularly in the context of redox reactions, oxygen tolerance and lignocellulose degradation.

281   WMS can be diluted 100-fold (0.01% v/v WMS) relative to the standard amount in DMM (1% v/v

282   WMS) with no change in growth phenotype in defined media (**Figure 2**). If desired, trace metals

283   may be omitted from media, particularly in the presence of complex components such as yeast

284   extract or if using distilled or impure water sources.

Serial culture in DMM

286   To confirm that DMM contained all nutritional requirements, cultures were serially transferred in

287   DMM (**Figure 5**) as follows. Isolates were pre-grown anaerobically in 5 ml volume of liquid

288   rPHG, washed, diluted as previously described and inoculated 1:20 into 200 μl freshly prepared

289   DMM in 96-well microtiter plates. Cultures were grown aerobically to stationary phase (48 hours)

290   and endpoint OD_{595} readings taken after a two-minute orbital shake. Each transfer event reflected

291   a direct, unwashed 1:10 subculture into fresh DMM media. The transfer from rPHG to DMM

292   represented transfer #0 and serial transfers were repeated 10 additional times. Datapoints represent

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293   (OD final) − (OD initial) at 595 nm for each transfer. Linear regression analysis was performed

294   using individual datapoints from all replicates and the best fit curves along with 95% confidence

295   intervals were plotted. The slope of the linear fit is provided, along with the p-values indicating

296   the probability of the slope being significantly non-zero.

Growth kinetics in complex and minimal liquid media

298   To create a set of interchangeable, parallel liquid media, we used DDM as a basal media to which

299   10% w/v aqueous solutions of proteose peptone #3 and yeast extract were each added to a final

300   concentration of 1% v/v. To demonstrate expected growth behaviors within and between media,

301   DMM- or DCM-adapted cells were subcultured into either DMM or DCM, as follows. Several

302   DCM-grown colonies of each isolate were used to inoculate 5 ml volumes of liquid DMM and

303   grown to stationary phase as described. DMM cultures were pelleted, washed and subcultured

304   1:100 into either DCM or DMM and again allowed to reach stationary phase. Subcultures were

305   centrifuged, washed and resuspended as previously described and inoculated separately into DMM

306   and DCM in triplicate. Growth curves were generated as previously described (**Figure 6**). Blank-

307   corrected OD_{595} values for the entire 96 hour duration were used as input data to measure growth

308   kinetics in R [77] package Growthcurver [78] with default settings. Growth rates, generation times

309   and carrying capacities along with standard error values are listed for each isolate in each condition

310   in **Table 1**. Recipes for DMM, DDM and DCM as well as all required components can be found

311   in **Tables S2 & S3.**

Growth on agar-solidified media

313   To create parallel, agar-solidified media, single antioxidants were added to agar-solidified DDM

314   according to Dione et al. [79]. Aqueous solutions of reduced glutathione (Fisher, 10 mg/ml) L-

315   ascorbic acid sodium salt (Acros; 10 mg/ml) or uric acid potassium salt (Sigma; 10 mg/ml in 1M

316   NaOH) were prepared and added to 1.5% w/v agar-containing DDM at final concentrations of 0.1

317   mg/ml, 1 mg/ml or 0.4 mg/ml, respectively. Media was pH-adjusted to 7.5 as necessary using

318   concentrated HCl or NaOH. Isolates were cultured from rPHG-grown glycerol stocks onto agar-

319   solidified complex medium and several colonies used to inoculate liquid DDM cultures.

320   Stationary-phase cells were washed and resuspended to OD 0.1 and spotted onto agar-solidified

321   DDM plates with and without antioxidant amendment. Plates were loosely bagged in plastic petri

322   dish bags (Fisher) and incubated for 12 days aerobically at 22°C and photographed at 7 and 12

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323   days post inoculation (dpi) (**Figure 7**). To test growth on solid DCM, single colonies of each isolate

324   were taken from rPHG plates and streaked to isolation on DCM amended with 1.5% w/v agar and

325   0.1 mg/ml reduced glutathione. Plates were incubated loosely bagged aerobically at 22°C or 30°C,

326   or anaerobically at 22°C for 4 days (**Figure S8**). To test solid DMM, several colonies of each

327   isolate were taken from solid DDM plates and individually resuspended into 200 µl 1X M9 salts,

328   spotted onto agar-solidified DMM containing 0.1 mg/ml reduced glutathione and streaked to

329   isolation. Plates were incubated with either no bag (unrestricted atmosphere) or sealed in a plastic

330   bag (restricted atmosphere) and incubated aerobically at 30°C for 5 days (**Figure S9**).

Enrichment of *Dysgonomonas isolates* from termite hindgut

332   Termites were collected from the same site and colony as isolates GY75 and GY617 in June 2019

333   during an alate (winged reproductive) swarming event. Hindguts from freshly collected worker

334   (n=25) and alate (n=50) castes were extirpated and the contents separately prepared and diluted as

335   previously described. Dilutions were plated on agar-solidified DMM containing 0.1 mg/ml

336   reduced glutathione, 50 µg/ml kanamycin sulfate and 100 µg/ml cycloheximide (Acros), loosely

337   bagged and incubated aerobically or anaerobically at 22°C for 4 days. Well-isolated colonies that

338   exhibited morphology characteristic of *Dysgonomonas* (circular, convex, opaque, glossy, white-

339   cream or light-brown in color) were purified by serially streaking onto the same medium, and 30

340   isolates were selected for identification. We used a high-throughput screen to quickly identify the

341   presence of *Dysgonomonas* spp. within the collection of isolates. Crude gDNA was prepared

342   individually for each isolate, in which several colonies were resuspended in 100 µl sterile

343   molecular-grade water, boiled at 95°C for 15 minutes and briefly centrifuged. The supernatant was

344   used as template for PCR targeting the V4 region of the bacterial 16S rRNA gene. Each isolate

345   was individually PCR amplified using Q5 Polymerase and primers 16S_F (5'-

346   GTGCCAGCMGCCGCGGTAA-3') and 16S_R (5'-GGACTACHVGGGTWTCTAAT-3'), each

347   flanked by 5' extensions containing unique combinations of forward or reverse indexing sequence

348   and high-throughput sequencing adapters [80]. The negative control replaced lysate supernatant

349   with water. Positive controls included lysate supernatant from *Dysgonomonas* spp. BGC7 &

350   HGC4 and isolates GY75 & GY617. Amplicons were pooled, column-purified and sent to the

351   UConn MARS Facility (University of Connecticut, Storrs, CT, USA) where libraries were

352   prepared and sequenced on the Illumina MiSeq platform. Raw reads were processed in R using the

353   package DADA2 [81] to quality filter and trim, merge, chimera-check, determine read error rates,

354 generate Amplicon Sequence Variants (ASVs) [82] and to assign taxonomy to ASVs using the

355 SILVA rRNA database v132 [83]. Taxonomic assignments were manually inspected and

356 preliminary identification was determined by the taxonomic group with which >95% of reads were

357 placed, with the remaining reads attributed to PCR and sequencing errors that passed the filtering

358 process. Isolates that were identified as belonging to genus *Dysgonomonas* (24 of 30), along with

359 the controls, clustered into 4 discrete 'ASV-groups' (ASV_1, ASV_2, ASV_4, and ASV_5), each

360 of which contain 253 bp sequences with 100% identity with each other. ASVs from this study can

361 be found in **Table S4**. To aid in resolving subtle variations in 16S rRNA gene heterogeneity within

362 ASV-groups, a subset of the isolates (13 of 24) containing representatives from each ASV-group

363 were selected for bidirectional dideoxy sequencing to obtain full length 16S rRNA gene sequences.

364 gDNA template was prepared and quantified for each isolate within the subset. PCR, cloning and

365 sequencing were carried out as previously described. Sequences were trimmed of primer sequence

366 and base-calls manually inspected and curated using the generated ASV sequences as a reference.

367 Full-length 16S rRNA gene sequences for *Dysgonomonas* isolates obtained or used in this work

368 (AAn1, AAn3, AAn4, AAn6, AAn7, AAn9, AAn11, BGC7, GY75, GY617, HGC4, WAe4,

369 WAe5, WAe6, WAe3, WAn2, WAn3) were submitted to GenBank under accession numbers

370 MT340871-MT340887, respectively. Isolates were named by isolation source (<u>W</u>orker or <u>A</u>late),

371 oxygen condition (<u>Ae</u>robic or <u>An</u>aerobic), and isolate number. For example, isolate AAn1 was

372 isolated from alate hindguts under anaerobic conditions, while WAe3 was isolated from a worker

373 hindgut under aerobic conditions. Colonies with morphology consistent with that of

374 *Dysgonomonas* were obtained aerobically from alate hindguts (A<u>Ae</u>) but were discarded due to

375 fungal overgrowth. Reference sequences from cultured members of *Dysgonomonas* and from

376 representative taxa from within the *Bacteroidetes* greater than 1.2 kb in size were obtained from

377 RefSeq or GenBank, in order of preference, on April 8, 2020. Reference sequences were trimmed

378 of primers and aligned with those from *Dysgonomonas* spp. BGC7 and HGC4 and isolates from

379 this study using the MUSCLE algorithm in Geneious R9 with default settings (see **File S1** for

380 multiple sequence alignment of 16S genes and **File S2** for pairwise nucleotide identifies). IQ-

381 TREE v2.0 [84] was used for substitution model testing and maximum likelihood phylogenetic

382 reconstruction using model TIM3e+I+G4. Branch support values were calculated using 1000

383 Ultrafast Bootstraps and SH-aLRT testing. Isolation sources for *Dysgonomonas* isolates not from

384 this study were obtained from the published reference when available or from data associated with

13

385   the RefSeq/GenBank entry. Formatting and metadata were applied in R using packages dplyr [85],

386   ggplot2 [86], treeio [87], ggtree [88], and ggrepel [89]. Explanations of formatting and metadata

387   can be found in the legend for **Figure 8**.

Results and discussion

Growth in complex vs. defined media

390   We tested four phylogenetically diverse termite-derived *Dysgonomonas* isolates for their ability to

391   grow in aerobic, liquid media consisting of M9 salts supplemented with trace minerals, ferric

392   hemin, B-vitamins, D-glucose and proteose peptone (**Figure 1**). Previous experiments determined

393   that removal of peptone, and thus amino acids, from the medium resulted in no growth from all

394   isolates (data not shown), suggesting a growth requirement for one or more components found in

395   peptone. To determine which components of peptone were necessary to sustain growth, cultures

396   were supplied with B-vitamins (WVS) and trace metals (WMS), and proteose peptone was

397   replaced with either casamino acids or a mixture of twenty individual free amino acids, each at 0.1

398   mg/ml. Casamino acids were supplemented with 0.1 mg/ml L-tryptophan and L-cysteine to

399   replenish amino acids lost during acid hydrolysis. In comparison to growth in a medium with

400   peptone, isolates BGC7, HGC4 and GY617 exhibited slight decreases in growth rate and yield

401   when grown with casamino acids. Isolate GY75 displayed growth kinetics most comparable to that

402   on peptone, demonstrating a very slight decrease in growth rate, and no discernible reduction in

403   yield. Free amino acids were able to be substituted for peptone or casamino acids for all isolates,

404   though growth rates and yields were decreased. Additionally, isolates HGC4 and GY617 exhibited

405   a slight diauxie-like phenotype when grown with casamino acids which was exacerbated by growth

406   on free amino acids. This phenotype may be attributed to differences the relative abundance of

407   particular amino acids or oligopeptides between the supplements. Nucleobases, NAD and non-

408   proteinogenic amino acids L-ornithine and DAP were not required for growth, nor did they

409   contribute to additional yield increases beyond that of free proteinogenic amino acids alone.

410   Nucleobases had a negative effect on all measures of growth from isolate BGC7, perhaps due to

411   interference between biosynthesis and salvage pathways. Together these results suggested that

412   growth could occur on oligopeptides or free amino acids in the presence of trace metals and B-

413   vitamins, and suggested a possible amino acid auxotrophy.

14

Amino acid auxanography

Amino acid auxanography was performed to determine the growth requirement for one or more amino acids. Isolates HGC4, GY75 and GY617 exhibited growth only on amino acid pool 2 (L-alanine, L-cysteine, L-threonine, L-asparagine, L-methionine, DAP) & pool 6 (L-serine, L-cysteine, L-ornithine, glycine), which suggested L-cysteine as the single limiting amino acid (**Figures S1 & S2**). Additionally, isolates HGC4 & GY617 again exhibited a diauxie-like growth phenotype, likely indicating limitation of one of more preferred nutrients in the auxanography growth medium. Isolate BGC7 exhibited growth on pools 2, 6 & 9 (L-methionine, L-glutamic acid, L-lysine), with greatest yield on the L-cysteine- and L-methionine-containing pool 2. Pool 6, which contained L-cysteine but not L-methionine, was growth-permitting for isolate BGC7, but both growth rate and yield suffered. Isolate BGC7 also exhibited weak, linear growth on pool 9, suggesting that L-methionine alone, while not optimal, is growth-permitting under the conditions tested (**Figure S2**). These results suggest that L-cysteine alone or in combination with L-methionine can replace complex sources of amino acids and allow exponential aerobic growth of *Dysgonomonas*.

L-cysteine is required during growth under aerobic or anaerobic conditions

To examine relationship between growth kinetics and L-cysteine, isolates were grown aerobically and anaerobically across a range of L-cysteine concentrations. Freshly prepared trace metals, B-vitamins and ferric hemin were provided and L-cysteine supplemented between 0-5.69 mM. All cultures exhibited concentration-dependent phenotypes in relation to L-cysteine (**Figure S3**) under both aerobic and anaerobic conditions. With respect to growth rate under aerobic conditions, isolates displayed either a positive correlation with L-cysteine concentration (isolate BGC7) or a binary response (isolates HGC4, GY75 & GY617) in which nearly maximal growth rate was achieved at the lowest concentration tested (0.285 mM). When L-cysteine was absent from aerobic media, isolates exhibited extended lag phase, periods of linear growth and early entry to stationary phase suggestive of L-cysteine limitation. Similar to the results from amino acid auxanography, isolate BGC7 grew modestly under aerobic conditions in the absence of L-cysteine and exhibited extended lag phase, low growth rate and low yield. Isolate BGC7 exhibited reduced lag time and an increase in growth yield, as well as an increase in growth rate that was positively correlated with L-cysteine concentration up to 3.42 mM, beyond which no gains in growth rate, or yield, were obtained. Anaerobic growth of isolate BGC7 showed positive correlation between L-cysteine

15

445   concentration and final yield up to 4.56 mM. Aerobically, isolate HGC4 and isolates GY75 &

446   GY617 did not exhibit the same relationship between L-cysteine and growth rate as isolate BGC7,

447   and instead displayed a binary response which required only 0.285 mM to achieve near-maximal

448   growth rates and yields. For isolates HGC4 and GY617, 0.285 mM L-cysteine was yield-limiting,

449   but 0.596 mM was considered L-cysteine replete under the conditions tested. Anaerobic growth

450   was similarly binary for these isolates, with no growth occurring in the absence of L-cysteine. The

451   replacement of peptones or casamino acids with L-cysteine as a component of the basal salt-

452   vitamin-hemin-glucose medium led to the development of a chemically defined medium, which

453   was later refined to become DDM.

454   As previous experiments demonstrated L-cysteine-dependent growth, we sought to determine

455   whether pre-growth in rich medium provided the means for cells to intracellularly store sulfur and

456   permit moderate growth in media without L-cysteine. Forthcoming draft genome assemblies for

457   *Dysgonomonas* isolates BGC7, HGC4, GY75 & GY617 all contain genes encoding sulfate

458   permease, but pathways for assimilatory sulfate reduction are absent. As such, we were further

459   interested to determine whether sulfate was necessary in the presence of a reduced, assimilable

460   source of organic sulfur such as L-cysteine. Cultures were pre-grown to stationary phase in DDM

461   with 0.57 mM L-cysteine, washed several times and diluted into PBS. Prepared cells were diluted

462   1:100 into fresh media with and without sulfate or L-cysteine and grown under aerobic and

463   anaerobic conditions. Sulfate-limited DDM (0.01% v/v mWMS) contained 83 nM total sulfate,

464   which is ~300-fold less than the required 26.5 μM for *Salmonella typhimurium* to achieve an OD_{420}

465   of 1 in MOPS medium [90], while sulfate-replete DDM (0.01% v/v WMS) contained 1 mM total

466   sulfate. L-cysteine concentrations were either 0 or 1.71 mM, and kanamycin sulfate was omitted

467   from all media. It should be noted that both sulfate-replete and sulfate-limited DDM contain the

468   vitamins biotin, thiamine and lipoic acid (found in WVS). These cofactors contain organic reduced

469   sulfur, and together contribute ~1.15 μM to the pool of putatively available reduced sulfur. All

470   isolates failed to grow either aerobically or anaerobically in the in the absence of L-cysteine,

471   regardless of the presence of sulfate in the medium, which suggested a requirement for a reduced

472   form of assimilable sulfur (**Figure 2**). These results further suggested that the growth of isolate

473   BGC7 in the absence of L-cysteine observed in **Figures S2 & S3** may be attributed to intracellular

474   sulfur stores present as a consequence of pre-growth in complex medium. Aerobic growth

475   phenotypes were nearly identical for all isolates in the presence of L-cysteine, regardless of the

476 presence of sulfate in the medium, which suggested that sulfate is not required aerobically in the

477 presence of L-cysteine. Although sulfate was unable to replace L-cysteine as a sulfur source,

478 anaerobic growth was stimulated by the presence of sulfate, particularly for isolate HGC4 and

479 isolate GY75. The role of sulfate during anaerobic growth is unclear, but it is perhaps used as an

480 alternative electron acceptor during anaerobic respiration. All isolates were able to utilize the

481 tripeptide glutathione (glutamate-cysteine-glycine) to some degree in place of L-cysteine, and

482 isolate BGC7 was further able to utilize L-methionine as a sulfur source. No growth occurred when

483 L-cysteine was replaced with thiosulfate, thioglycolate, 2-mercaptoethanol or dithiothreitol (not

484 shown). Sodium sulfide was not tested, but was found to be a suitable sulfur source for *Bacteroides*

485 *fragilis* [67].

486 The precise roles of L-cysteine are difficult to discern over the range of concentrations tested here

487 (0.1-10 mM), as it can be imported and used directly as a substrate for peptide synthesis, as a

488 source of assimilable reduced sulfur [67], or as a reducing agent [79,91] to lower the redox

489 potential of the medium. Draft genome assemblies contain complete L-cysteine and other

490 proteinogenic amino acid biosynthesis pathways suggesting that the isolates are not amino acid

491 auxotrophs, and the ability of L-cysteine concentration to modulate final growth yield also

492 suggests that it is an exhaustible nutrient, assimilated into biomass, or both. Moreover, the

493 requirement for L-cysteine anaerobically suggests that its function as a reductant provides a minor

494 contribution to its role in growth. The inability of other sulfur-containing reducing agents to permit

495 growth may be due simply to the inability of *Dysgonomonas* to effectively assimilate reduced

496 sulfur under the provided conditions. Taken together, these results suggest that L-cysteine is

497 utilized as an easily assimilable source of reduced sulfur, and that other roles that it may provide

498 are secondary to this function.

499 **Growth using L-cysteine as the sole source of nitrogen**

500 To determine if L-cysteine could also be utilized as a sole source of nitrogen, cultures were grown

501 in the presence or absence of excess ammonium and/or 1.7 mM L-cysteine, which should not be

502 significantly yield-limiting as the sole source of nitrogen [90] under these conditions. All isolates

503 were able to grow in the absence of ammonium, using L-cysteine as a sole source of nitrogen under

504 aerobic and anaerobic conditions (**Figure S4**). Using L-cysteine as a nitrogen source, isolate GY75

505 exhibited a 24-hour lag before exponential growth using L-cysteine under aerobic conditions.

506 Isolates BGC7, HGC4 & GY617 exhibited similar growth rates to their counterparts grown with

17

507   ammonium and L-cysteine, but entered stationary phase early, perhaps due to nitrogen limitation

508   or buildup of toxic end products of L-cysteine catabolism such as hydrogen sulfide [92]. Under

509   aerobic conditions in the absence of both ammonium and L-cysteine, some weak growth occurred

510   aerobically, but not anaerobically, which could be indicative of scavenged and assimilated trace

511   nitrogen from the medium (hemin, 0.3 mM; kanamycin, 0.34 mM; WMS, 0.078 mM; biotin, 0.82

512   μM; thiamine, 2.96 μM; cyanocobalamin, 51.6 nM; totaling ~0.7 mM nitrogen) or that the growth

513   was enabled by utilizing intracellular nitrogen stores. Further analysis of nitrogen sources was

514   precluded by the requirement for L-cysteine for growth and its ability to be used as a nitrogen

515   source. Under anaerobic conditions, isolates exhibited approximately half the yield as compared

516   to those grown under ammonium-replete conditions. We speculate that assimilation of organic

517   nitrogen could be unfavorable during anaerobic growth under the provided conditions.

Requirement for ferric hemin

519   Our *Dysgonomonas* isolates had previously been observed to require ferric hemin when grown

520   under aerobic or anaerobic conditions, even when grown in complex media such as rPHG (author

521   observation). Hemin, a ferric-iron carrying porphyrin, is biosynthesized through a costly and

522   complex pathway, making it a valuable commodity. Respiration requires both sufficient quantities

523   of iron for redox reactions, and iron-containing porphyrins for synthesis b-type cytochromes,

524   [67,90,93] which hemin could provide. We sought to determine growth-limiting concentrations of

525   hemin for our *Dysgonomonas* isolates when D-glucose was used as a carbon source (**Figure S5**).

526   Cultures were hemin-starved and prepared as described in Materials & Methods before being used

527   for growth curves. Hemin was present in media at final concentrations between 0 and 153.4 μM

528   (0-20% v/v hemin solution). Similar to that observed in *Bacteroides fragilis* [94], growth of

529   *Dysgonomonas* under aerobic conditions exhibited a positive correlation between hemin

530   concentration and growth yield, up to the growth-saturating concentration for that organism where

531   hemin became excessive and yield remained constant. Isolates did not grow when hemin

532   concentration was below 7.67 μM (1% v/v hemin solution), demonstrating a hemin requirement,

533   though growth-permissive concentrations differed among the isolates tested. All isolates also

534   exhibited poor growth at their respective lowest growth-permissive concentration. Poor growth

535   included extended lag time, slower growth rate and lower yield. Isolates BGC7 and HGC4 required

536   38.4 μM hemin for growth to occur, with 61.3 μM sufficient for near-maximal growth rate and

537   yield. Isolate GY75 required the least ferric hemin for growth (7.67 μM) and again achieved near-

18

maximum growth rate and yield in the presence of 38.4 μM. Isolate GY617 required the greatest concentration of ferric hemin (46.0 μM) for growth to occur, and near-maximal growth rate required at least 76.7 μM hemin. Ferric hemin had no deleterious effects on growth of any isolates at the higher concentrations tested under these conditions. Hemin requirements may change depending on carbon source, particularly if growth requires extensive oxidation of substrates performed by iron-cofactor dependent electron carriers.

Under anaerobic conditions, the hemin concentration-yield relationship was consistent with a third-order polynomial in which concentration was positively correlated with yield at lower concentrations, but yields either plateaued or decreased over a range of concentrations before reaching maximal yield in the presence of 153 μM hemin (**Figure S6**). Though all isolates exhibited the greatest yield in the presence of 153 μM hemin (20% v/v hemin solution), isolates BGC7, HGC4, GY75 and GY617 each also exhibited a secondary, local maximal yields at 46.0 μM, 30.67 μM, 46.0 μM and 76.7 μM hemin, respectively. Lower concentrations of hemin were required under anaerobic than under aerobic conditions for all isolates, consistent with a switch from aerobic respiration to fermentation, which could require fewer iron-containing electron carriers and respiratory cytochromes. Isolates HGC4 and GY617 required 15.4 μM to exhibit growth, roughly half of that required aerobically. Isolate BGC7 required only 3.83 μM hemin but growth was limited to ~2.5 generations. Isolate GY75 was able to grow in the absence of hemin, consistent with having the least requirement for hemin during aerobic growth, but was growth was limited to under two generations. It is noteworthy that all isolates can exhibit characteristics consistent with hemin accumulation during growth in hemin-replete media similar to that of close relative *Porphyromonas gingivalis* ([95] and references therein), such as the formation of brown cell pellets from liquid culture or darkening of colonies on agar-solidified media. Isolate GY75 routinely exhibits the greatest cell darkening (author observation) and its ability to grow using the least amount of hemin of the four isolates under aerobic and anaerobic conditions is consistent with the ability to sequester ferric hemin within the periplasm or at the outer cell membrane.

We further sought to determine whether ferric hemin was able to be replaced with alternate sources of iron (**Figure 3**). In addition to ferric hemin, we tested soluble ferric chloride and two ferric chelates; ferric-EDTA and ferric-citrate, as well as soluble ferrous sulfate alone. All conditions were considered replete for ferrous iron by the addition of 1% v/v WMS which provided 3.6 μM ferrous sulfate, the standard for DDM. For all isolates, ferrous iron alone was insufficient to permit

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569 growth, and similar to *Prevotella intermedia* [96], none of the soluble or ferric chelates were able

570 to replace ferric hemin under aerobic or anaerobic conditions. Hemin was ultimately used at 76.7

571 μM (10% v/v hemin solution) in our final media recipes as it was not significantly rate- or yield-

572 limiting and reduced the risk for heme-toxicity which can occur at high concentrations [93].

573 Although beyond the scope of this study, it would be interesting to determine the ability for

574 *Dysgonomonas* utilize non-heme iron in the presence of alternate porphyrins such as

575 protoporphyrin IX such as related bacteria such as *P. intermedia* [96] *P. ruminicola* [97], *P.*

576 *gingivalis* [98] and are able.

Vitamin requirements

578 Previous experiments determined that WVS was required for growth by all isolates when grown

579 without peptone (data not shown), indicative of auxotrophy for one or more B-vitamins. To screen

580 for vitamin auxotrophies, cultures were vitamin-starved and prepared as previously described

581 before being inoculated into 12 different B-vitamin-replete media, each with an omission of a

582 single cofactor (**Figure S7**). Removal of biotin, thiamine or cyanocobalamin limited growth from

583 all isolates with the exception of isolate BGC7, which exhibited significantly slower growth rate

584 but similar yield in the absence of cyanocobalamin. All isolates were able to grow to some degree

585 in the absence of biotin, which suggested intracellular reserves in the inocula were not yet biotin-

586 limited during the vitamin pre-starvation procedure. All isolates exhausted their intracellular biotin

587 stores through cell growth and dilution within a few generations and growth ceased. We

588 subsequently tested the requirements for these three cofactors by omitting WVS from the medium,

589 amending only with biotin, thiamine and cyanocobalamin and then removing each cofactor

590 individually (**Figure 4**). Consistent with previous findings, all isolates displayed the expected

591 growth phenotypes when provided biotin, thiamine and cyanocobalamin together. Omission of all

592 vitamins resulted in absence of growth from all isolates. Thiamine and biotin were required for the

593 growth of all isolates, while cyanocobalamin was required for all but isolate BGC7. Some growth

594 was observed from all isolates in the absence of biotin, again suggesting that cells in the vitamin-

595 starved inocula contained an exhaustible intracellular pool of biotin. Isolates also exhibited some

596 degree of oscillatory growth in the absence of biotin alone. For example, isolates HGC4 and

597 GY617 ultimately entered a death phase after oscillation, while isolate BGC7 continued a pattern

598 of linear growth, indicative of cofactor limitation. Isolate GY75 exhibited oscillatory growth in the

599 absence of biotin for the duration of the 96-hour assay. Cyanocobalamin was required for growth

20

600   for all strains except BGC7, which exhibited a lower growth rate but nearly equivalent yield

601   compared to cells grown with cobalamin. Cobalamin-independent growth of isolate BGC7 was

602   confirmed by diluting washed cells 1:100 into fresh medium without cyanocobalamin and allowed

603   to reach stationary phase. Cells were able to be serially passaged three times in this manner (not

604   shown), which confirmed that isolate BGC7 can indeed grow in the absence of cyanocobalamin.

605   Genomic data show the absence of de novo cobalamin biosynthetic pathways, but we cannot rule

606   out the ability of isolate BGC7 to biosynthesize cobalamin through non-canonical pathways. It

607   also cannot be ignored that hemin and cobalamin share the intermediate compound

608   uroporphyrinogen-III, which could be, although energetically costly, created by dissimilation of

609   hemin. This conundrum has been previously explored in *Porphyromonas gingivalis* [98], although

610   clear answers were also not derived. Alternatively, it is possible that isolate BGC7 could grow in

611   the absence of cobalamin due to the presence of genomic features such as cobalamin-independent

612   methionine synthase, for example. In the presence of thiamine and biotin, the substitution of

613   cyanocobalamin with L-methionine allowed high growth rates for all isolates, although the

614   provided 0.67 mM L-methionine appeared yield-limiting for isolate GY75. This result suggests

615   that although cyanocobalamin may be required to perform multiple functions, a major role for

616   cellular cobalamin during aerobic and anaerobic growth of some *Dysgonomonas* species is likely

617   biosynthesis of L-methionine [99].

618   **Serial culture in DMM**

619   To be certain that DMM did not lack growth factors required only in trace amounts, we performed

620   ten serial aerobic transfers of four *Dysgonomonas* isolates in DMM. Cells from previous DMM

621   cultures were diluted 1:10 into fresh DMM without washing and allowed to reach stationary phase

622   before being transferred again. Each transfer allowed contaminating nutrients that were either

623   intracellular, bound to the cell surface or carried along with culture supernatant to be diluted into

624   fresh medium, and then diluted by redistribution amongst actively growing cells within the

625   population. All four isolates were able to be serially transferred ten times in DMM without a loss

626   in final yield (**Figure 5**). Cultures were not shaken during these experiments and thus endpoint

627   OD_{595} readings did exhibit some fluctuation due to cell clumping and biofilm formation. Linear

628   regression analysis demonstrated that the slope of each fit to final OD_{595} values was positive, which

629   indicated that final yield did not decrease during successive transfers. The linear fit was tested for

630   significant deviation from zero, and all isolates exhibited p-values >0.22, which indicated that the

631 slopes of the linear fits were not significantly different from zero. Taken together these data

632 confirmed that liquid DMM was indeed sufficient to meet the growth needs for the *Dysgonomonas*

633 isolates used here. Although we tested *Dysgonomonas* isolates with diverse phylogenetic

634 placement, it is possible that other strains may exhibit auxotrophy for specific amino acids or

635 vitamins. Using DMM as a basal minimal medium to perform further amino acid or cofactor

636 auxanography will allow simple and quick elucidation of any alternate growth requirements.

637

Growth kinetics in DCM & DMM

639 To create a parallel complex media, we amended DDM (B-vitamin replete) with 1% v/v each of

640 peptone and yeast extract to create DCM and established a baseline for the expected growth

641 kinetics in these media. Each *Dysgonomonas* isolate was grown in DMM broth, washed, diluted

642 and subcultured into either DMM or DCM broth and grown to stationary phase. These pre-growth

643 cultures were again washed and diluted into the same or opposite media and incubated aerobically

644 and anaerobically. Aerobically, as expected, all isolates exhibited faster growth rates and higher

645 yield when grown in DCM than in DMM, regardless of the pre-growth medium used (**Figure 6**).

646 Under anaerobic conditions, isolates also achieved greater final yield when grown in DCM rather

647 than DMM, though there was variability between isolates regarding which pre-growth medium

648 allowed the greater yield. These results are unsurprising given reduction in biosynthetic costs of

649 amino acids, vitamins and cofactors provided by the amendments in DCM. Isolates pre-grown in

650 DMM and switched to DCM performed near identically to DCM pre-grown cultures in all cases

651 under aerobic conditions, displaying identical yields and only slight differences in growth rates

652 (**Table 1**). During anaerobic growth there was again variability between isolates with respect to

653 which pre-growth medium resulted in greater final yield. Aerobic cultures pre-grown in DCM and

654 switched to DMM exhibited slightly greater growth rates for all isolates except for GY75, for

655 which DMM-conditioned cells displayed the greater rate. Final yields in DMM during anaerobic

656 growth differed based on pre-growth medium between the isolates, with isolate BGC7 and isolate

657 GY75 displaying greater yield when pre-growth occurred in DCM, while isolate HGC4 and

658 GY617 displayed greater yield when pre-growth occurred in DMM. Perhaps unexpectedly, the

659 switch from DCM to DMM did not elicit an observable lag phase during aerobic growth, which

660 suggested that gene expression related to growth and cell division can shift rapidly following

661 transfer between the two media. Within media types, carrying capacities of the media were highly

22

662   consistent across isolates and allowed stable population kinetics during stationary phase to at least

663   96 hours.

664   **Growth on agar-solidified media**

665   Growth of *Dysgonomonas* isolates on DDM or DMM solidified with 1.5% w/v agar resulted in

666   delayed and overall weak growth when incubated aerobically, while plates incubated anaerobically

667   exhibited good colony growth (author observation). Due to differences in exposure to ambient

668   atmosphere between cultures grown on solid media versus liquid culture, oxidative stress was

669   suspected as a growth-limiting factor on our solid, defined medium. As described by Dione et al.

670   [79], we amended agar-solidified DDM with a single antioxidant (ascorbic acid, uric acid or

671   glutathione) and adjusted pH to 7.5. Isolates were streaked onto each medium, loosely bagged and

672   incubated aerobically. Each antioxidant alone was sufficient to permit aerobic growth on agar-

673   solidified DDM (**Figure 7**). Due to the characteristics of aqueous solutions of ascorbic and uric

674   acid, media required additional pH amendments using concentrated NaOH or HCl. Both ascorbic

675   acid and uric acid changed the hue of the medium to an orange or green-grey color, respectively,

676   which may be inhibitory for some biochemical or genetic analyses. Additionally, the presence of

677   uric acid caused colonies from some isolates to exhibit umbonate morphology (**Figure 7, insets**).

678   Glutathione did not contribute to visible changes in the media or to alterations in colony

679   morphology, and was selected for final media formulations. Addition of ascorbic acid, glutathione

680   or uric acid to liquid media did not significantly alter growth phenotypes in liquid media, with the

681   exception that ascorbic acid lowered growth rate and yield for isolate BGC7 (not shown).

682   Additionally, the *Dysgonomonas* isolates tested did not utilize L-cysteine, ascorbic acid, uric acid

683   or glutathione as a sole source of carbon in DMM (not shown).

684   To create a parallel complex solid medium, agar-solidified DCM was also amended with

685   glutathione and incubated loosely bagged under common laboratory conditions. Expected growth

686   phenotypes were observed from all isolates on solid DCM after 3 days of incubation under all

687   conditions with the exception that isolates BGC7 and GY75 grew somewhat slower aerobically at

688   22°C than did the other isolates and required an additional day of incubation (**Figure S8**).

689   Importantly, the creation of DCM, particularly when solidified in the presence of an antioxidant,

690   abolishes the requirement for animal blood when growing *Dysgonomonas*, which may be a

691   limiting factor for some research groups.

692   When incubated under aerobic conditions, cultures on solid DMM containing glutathione still

693   exhibited unimpressive growth compared to that on DDM, and we speculated that the presence of

694   excess B-vitamins could have contributed to maintaining a reduced intracellular environment

695   during growth on solid DDM. To determine if additional protection from oxidative stress was

696   required, we grew aerobic cultures on solid DMM containing glutathione and incubated plates

697   aerobically, with either unrestricted access to ambient atmosphere (un-bagged plates) or with

698   restricted access to ambient atmosphere (sealed tightly in a plastic bag) (**Figure S9**). Colonies from

699   plates that were un-bagged displayed sparse growth with small colonies which never reached the

700   size of those grown on DDM under similar conditions. In contrast, colonies from plates that were

701   incubated in sealed plastic bags exhibited the typical colony phenotype observed on DDM. We

702   suspect that restricting access to ambient atmosphere could provide several advantages for growth

703   of *Dysgonomonas*; (i) moisture from the media is retained during extended incubation; (ii) oxygen

704   is likely consumed during aerobic respiration faster than it can be replaced in a sealed plastic bag,

705   creating microaerophilic conditions with reduced oxidative pressure; (iii) the buildup of volatile

706   metabolic end products such as carbon dioxide may provide beneficial conditions for growth, as

707   has been demonstrated for several *Dysgonomonas* spp. [9].

Enrichment of *Dysgonomonas* isolates from termite hindgut

709   In addition to providing access to 'cleaner' physiological and genetics studies, we were interested

710   to determine if DMM could be used to selectively enrich for *Dysgonomonas* from environmental

711   samples. We collected worker and alate termites from the same colony during a swarming event

712   and performed bacterial strain isolation, purification, high-throughput V4 16S rRNA gene

713   screening and ASV generation as described in Materials and Methods. Thirteen isolates

714   representative of four ASV-groups (**Table S4**) were selected for full-length 16S rRNA genes

715   sequencing alongside isolates BGC7, HGC4, GY75 and GY617. We reconstructed a 16S rRNA

716   gene maximum-likelihood phylogeny containing genes from our own isolates and those from other

717   cultured members of *Dysgonomonas* and overlaid ASV-group and isolation source metadata

718   (**Figure 8**). Our small collection of *Dysgonomonas* isolates displayed congruency between ASV

719   groups and full length 16S rRNA gene sequences; that is, all full-length 16S gene sequences from

720   our isolates were also clustered by ASV group. Representative genes obtained from sequence

721   databases that shared 100% identity to, and over the entire length of, our ASVs were considered

722   part of an ASV-group, even if there was disparity between full-length sequences. Sequences in

723   ASV-group 3* represent a subset of 16S rRNA genes from species which were not isolated or used

724   in this study but are identical over the ~250 bp of the V4 region of 16S rRNA gene that is used to

725   generate ASVs. ASV-group 5 contained only the sequence derived from isolate BGC7, we did not

726   obtain any novel isolates with this ASV during this work.

727   *Dysgonomonas* isolates that belonged to ASV-group 1 also shared 100% identity between full-

728   length 16S rRNA gene, which included the type strain *D. gadei* (NR_113134.1). Interestingly, we

729   obtained ASV-group 1 isolates from worker and alate hindguts that shared 100% identity with

730   GY75, which was isolated 21 months previous from the same colony. ASVs from isolates within

731   ASV-group 4 shared 98% identity to that of ASV-group 1 (5 substitutions) and clustered separately

732   with *Dysgonomonas termitidis* (AB971823.1) to the exclusion of ASV-group 1 members, based

733   on full-length 16S gene sequences. Full-length sequences belonging to ASV-group 4 shared 98.3%

734   identity with *D. termitidis* and 98.0% identity to those sequences comprising ASV-group 1,

735   representing what could be a novel species (**Files S1 & S2**). Isolates that belonged to ASV-group

736   2 contained isolate HGC4, isolate GY617, five additional isolates from worker and alate hindguts

737   (WAn2, WAn3, AAn4, AAn7 & AAn9), sp. zg-930 (MN933917.1) and *D. alginatilytica*

738   (NR_137388.1), although *D. alginatilytica* clustered separately based on full-length 16S gene

739   sequence. All termite-derived sequences within ASV-group 2 from this work share >99.9%

740   identity, exhibiting only 1-2 bp substitutions along the full-length 16S gene and together shared

741   >99.9% identity to isolate HGC4, which was isolated from *R. flavipes* termites by a separate

742   laboratory several years prior to our study. Moreover, isolates WAn2 and WAn3 showed 100%

743   identity along the full-length 16S rRNA gene sequence with isolate GY617, which was isolated

744   twenty-one months prior at the same timepoint and from the same termite colony as GY75.

745   That isolates belonging to ASV-groups 1 and 2 were obtained from worker hindguts from the same

746   colony over multiple seasons, and that isolates belonging to both of these ASV-groups are also

747   able to be obtained from actively-swarming alate hindguts (also from the same termite colony),

748   suggested that these particular strains of *Dysgonomonas* may be stable members of the termite

749   hindgut community within this colony. This is supported by ASV analysis of data from [47]

750   (PRJEB5527) and [100] (PRJEB20463) (conducted using the same methods as described in

751   Materials & Methods) which contained ASVs from termite hindguts within geologically distinct

752   locations across New England which were identical to those found in ASV-groups 1, 2 and 4 from

753   our study (data not shown). Additionally, recent work from our lab [101] which used hindgut DNA

754   from worker termites trapped from the same colony as used in this study, identified

25

755 Bacteroidetes_ASV008, which was associated with several species of eukaryotic protists, to be

756 identical to the independently generated ASV_4 from this study. Although the diversity of

757 *Dysgonomonas* ASVs in sequencing data from termites suggests many strains are likely acquired

758 horizontally from the environment, our finding that isolates from ASV-groups 1, 2 and 4 contain

759 members isolated from both workers and alates may point to the possibility that some strains of

760 *Dysgonomonas* could gain a competitive advantage in the termite hindgut by being vertically

761 transmitted to new founder populations via alates.

762 Although studies have shown that high-throughput sequencing of full-length 16S rRNA genes can

763 provide within-species resolution [102], here we have only provided representative sequences

764 pertaining to dominant clones from 16S rRNA gene libraries for each organism. As such, we also

765 do not intend our phylogenetic reconstruction to delimit species or strains. Additionally, we found

766 that isolates which shared ASVs (and in the case of *D. gadei*, 100% similarity over >1.4kb

767 fragments of the 16S rRNA gene) were found in vastly different environments. For example, ASV-

768 group 1 contains sequences from isolates obtained from termite and human sources; ASV-group

769 3* contains isolates from sludge, microbial fuel cells, aquatic and human sources. It would simple,

770 but incorrect, to imply that ASV groups were correlated with natural reservoir, genomic content

771 or metabolic function, and we do not intend to convey this point.

772 The use of solid DMM to culture *Dysgonomonas* isolates distributed widely across a 16S rRNA

773 gene phylogeny demonstrates that our chemically defined media are broadly applicable and are

774 likely suitable and otherwise easily amenable for the growth of most strains of *Dysgonomonas*.

Conclusion

776 Although *Dysgonomonas* are considered fastidious organisms, their minimal requirements for

777 growth are quite simple. The termite-hindgut derived and phylogenetically diverse *Dysgonomonas*

778 isolates tested in this work exhibit requirements for L-cysteine, ferric hemin, biotin, thiamine and

779 nearly all required cyanocobalamin. Our isolates exhibit preference for media containing

780 ammonium and sulfate and required additional antioxidants when growing aerobically on solid

781 media.

782 Robust growth on minimal medium is the cornerstone for many physiological and genetic studies

783 using bacteria, and during preparation of this manuscript we were able to further utilize DMM to

784 screen for naturally occurring nucleotide, amino acid and vitamin auxotrophies, examine resistance

26

785 phenotypes to antibiotics of interest and observe growth phenotypes using specific animal- and

786 plant-derived carbon sources, including polysaccharides associated with lignocellulose

787 (manuscripts in preparation). The media formulations described here provide robust and reliable

788 growth in complex, defined or minimal variations that limit animal-derived components and

789 laboratory equipment such as anerobic chambers. These media can be used in liquid culture, or

790 agar-solidified under both aerobic or anaerobic conditions. The results from this work provide

791 three different, but parallel, media for the growth of members of genus *Dysgonomonas* and will

792 aid in facilitating further physiological and genetic characterizations to determine their functional

793 roles within particular ecological systems.

Acknowledgements

Competing interest statement

798 The authors declare no competing interests.

Funding source declaration

Figure & table legends:

Figure 1. Growth of *Dysgonomonas* isolates in defined medium containing amino acids and B-vitamins. *Dysgonomonas* isolates BGC7 (A), HGC4 (B), GY75 (C) or GY617 (D) grown in 1X M9 salts supplemented with 5% v/v hemin solution, 5% v/v WVS, 0.5% v/v WMS 0.5% w/v D-glucose, 50 μg/ml kanamycin sulfate, pH 7.5. Amendments are shown in the legend in panel A. Error bars represent standard error of the mean (n=2).

Figure 2. L-cysteine is required for aerobic and anaerobic growth. *Dysgonomonas* isolates BGC7 (A), HGC4 (B), GY75 (C) or GY617 (D) were pre-grown in DDM and diluted into sulfate-replete medium (1X M9 salts, 0.01% mWMS, 10% v/v hemin solution, 5% v/v WVS, 0.5% w/v D-glucose, pH 7.5; 1mM total sulfate) or sulfate-limited medium (1X M9 salts with 1mM $MgCl_2$ replacing $MgSO_4$, 0.01% WMS, 10% v/v hemin solution, 5% v/v WVS, 0.5% w/v D-glucose, pH 7.5; 83 nM total sulfate). Media contained either 0 or 1.7 mM L-cysteine as stated. Kanamycin sulfate was omitted for all conditions. Insets show final corrected OD_{595} under anaerobic conditions; the y-axis is in linear units. L-cysteine and sulfate combinations can be found in the legend in panel A. Error bars represent standard error of the mean (n=2)

Figure 3. Ferric hemin is a preferred source of iron for *Dysgonomonas*. Isolates BGC7 (A), HGC4 (B), GY75 (C) or GY617 (D) were pre-grown in DDM washed and diluted into 1X M9 salts supplemented with 1% v/v WMS, 0.5% w/v D-glucose, 1.7 mM L-cysteine, 50 μg/ml kanamycin sulfate, pH 7.5, and contained iron sources according to the legend in panel A. Insets show final corrected OD_{595} under anaerobic conditions; the y-axis is in linear units. Error bars represent standard error of the mean (n=2).

Figure 4. *Dysgonomonas* exhibit growth requirements for thiamine, biotin and cyanocobalamin. *Dysgonomonas* isolates BGC7 (A), HGC4 (B), GY75 (C) or GY617 (D) were pre-grown in DDM lacking vitamins, washed and diluted into 1X M9 salts supplemented with 1% v/v WMS, 0.5% w/v D-glucose, 1.7 mM L-cysteine, 10% v/v hemin solution, 50 μg/ml kanamycin sulfate, pH 7.5, containing 0.41 μM biotin, 0.74 μM thiamine, 3.7 nM cyanocobalamin and 0.67 mM L-methionine and amended as described in the legend in panel A. Insets show final corrected

28

833   OD$_{595}$ under anaerobic conditions; the y-axis is in linear units. Error bars represent standard error

834   of the mean (n=2).

835

836   **Figure 5. Serial transfer of *Dysgonomonas* in DMM liquid cultures.** Cultures were grown to

837   stationary phase in DMM and diluted 1:10 into fresh DMM media and again allowed to reach

838   stationary phase. Cultures underwent a total of 10 serial transfers, where transfer #0 represents

839   inoculation from complex medium. Error bars represent standard error of the mean (n=2). Slopes

840   and their associated p-values (representing significant deviation from zero) for the endpoint

841   readings were generated using linear regression analysis in Prism8.

842

843   **Figure 6. Comparison of growth phenotypes in complex and minimal media.** Isolates were

844   pre-grown to stationary phase in either complex (DCM) or minimal (DMM) media and transferred

845   separately into the same or opposite media. Cultures were grown in triplicate to stationary phase

846   aerobically or anaerobically. Pre-growth and growth combinations are located in the legend in

847   panel A. Insets show final corrected OD$_{595}$ under anaerobic conditions; the y-axis is in linear units.

848   Error bars represent standard error of the mean (n=3). Growth rates and carrying capacity were

849   measured using the R package Growthcurver, and results are reported in **Table 1**.

850

851   **Table 1. Aerobic growth metrics in DCM and DMM.** Isolates were inoculated from solid

852   complex media into DMM broth and allowed to reach stationary phase. Cultures were washed,

853   diluted and subcultured into either DMM or DCM (DMM- or DCM-adjusted cultures) before being

854   washed, diluted and used as inoculum for growth curves (Figure 10). Growth data were fit to the

855   logistic growth model and metrics calculated using the R package Growthcurver. Reported values

856   represent the mean ± standard error (n=3).

857

858   **Figure 7. Antioxidants are required for aerobic growth on defined medium.** *Dysgonomonas*

859   isolates BGC7, HGC4, GY75 and GY617 were grown on agar-solidified DDM containing: No

860   supplemental antioxidants (A), 0.1 mg/ml glutathione (B), 1 mg/ml L-ascorbic acid (C), 0.32

861   mg/ml uric acid (D). Overnight cultures were grown in DCM to stationary phase and washed and

862   diluted to OD$_{595}$ of 0.1 and 20 µl spots were allowed to dry onto media before streaking to single

863   colonies. Plates were loosely bagged and incubated aerobically at 22°C for 12 days. Photographs

29

864   were taken at 7 dpi (main panels) or 12 dpi (insets). Scale bars represent 10 mm (main panels) or

865   5 mm (insets).

866

867   **Figure 8. Maximum-likelihood phylogenetic reconstruction of near-full length 16S rRNA**

868   **gene sequences from cultured members of genus *Dysgonomonas*.** Sequences of length 1.2-1.5

869   kb were acquired from RefSeq and GenBank and combined with quality-controlled sequences

870   from *Dysgonomonas* isolates from this study. Nodes containing filled circles represent branch

871   support values; small, medium or large node shapes represent 70%/85%, 80%/90% or 90%/95%

872   SH-aLRT/UFBoot support, respectively. The scale bar represents 0.05 substitutions per site. Type

873   strains are designated by a superscript 'T' following the strain designation. Isolates used or

874   obtained in this study are bolded. Bolded *Dysgonomonas* isolates beginning with 'A' are derived

875   from alates while those beginning with 'W' are worker-derived. Highlighted clades represent ASV-

876   groups, which share common ASVs (contain 100% identity over the 250 bp sequence containing

877   the V4 region of the 16S rRNA gene). *Dysgonomonas* spp. not obtained or used in this study were

878   included as part of an ASV-group if they also exhibited 100% identity to ASVs generated in this

879   study. ASV-group 3* represents taxa which share 100% identity over the same 250 bp sequence of

880   16S rRNA gene, but were not true ASVs generated in this study. Square tip points are colored by

881   isolation source according to the legend. Source category 'Other' contains *Dysgonomonas* isolates

882   from marmot (MT065889.1), bird (MT065888.1), crayfish (MN933917.1) and beetle

883   (MN099400.1 & MN099399.1).

884

885   **Figure S1. Amino acid auxanography using defined medium.** Heat map of corrected growth

886   measurements of *Dysgonomonas* isolates BGC7 (A), HGC4 (B), GY75 (C) or GY617 (D) at

887   twelve-hour intervals. Isolates were grown in 1X M9 salts supplemented with 5% v/v hemin

888   solution, 5% v/v WVS, 0.5% v/v WMS, 0.5% w/v D-glucose, 50 µg/ml kanamycin sulfate, pH 7.5,

889   amended with a single combinatorial amino acid pool, with each amino acid at 0.33 mg/ml. Amino

890   acid pools are as follows: Pool 1: L-phenylalanine, L-serine, L-tryptophan, L-tyrosine, L-

891   glutamine; Pool 2: L-alanine, L-cysteine, L-threonine, L-asparagine, L-methionine, DAP; Pool 3:

892   L-arginine, L-ornithine, L-aspartic acid, L-proline, L-glutamic acid; Pool 4: L-leucine, glycine, L-

893   isoleucine, L-histidine, L-lysine, L-valine; Pool 5: L-phenylalanine, L-alanine, L-arginine, L-

894   leucine; Pool 6: L-serine, L-cysteine, L-ornithine, glycine; Pool 7: L-tryptophan, L-threonine, L-

895 aspartic acid, L-isoleucine; Pool 8: L-tyrosine, L-asparagine, L-proline, L-histidine; Pool 9: L-

896 methionine, L-glutamic acid, L-lysine; Pool 10: L-glutamine, DAP, L-valine, and can also be found

897 in **Table S1**.

898

899 **Figure S2. Amino acid auxanography.** Growth curves of *Dysgonomonas* isolates BGC7 (A),

900 HGC4 (B), GY75 (C) or GY617 (D) in 1X M9 salts supplemented with 5% v/v hemin solution,

901 5% v/v WVS, 0.5% v/v WMS, 0.5% w/v D-glucose, 50 µg/ml kanamycin sulfate, pH 7.5, amended

902 with a single combinatorial amino acid pool, with each amino acid at 0.33 mg/ml. Amino acid

903 pools can be found in **Table S1**.

904

905 **Figure S3. Aerobic growth in defined medium is L-cysteine-dependent.** Growth of

906 *Dysgonomonas* isolates BGC7 (A), HGC4 (B), GY75 (C) or GY617 (D) in 1X M9 salts

907 supplemented with 5% v/v hemin solution, 5% v/v WVS, 1% v/v WMS, 0.5% w/v D-glucose, 50

908 µg/ml kanamycin sulfate, pH 7.5. L-cysteine concentrations are shown in the legend in panel A.

909 Insets show final corrected OD_{595} under anaerobic conditions; the y-axis is in linear units.

910

911 **Figure S4. *Dysgonomonas* can utilize L-cysteine as the sole source of nitrogen.** *Dysgonomonas*

912 isolates BGC7 (A), HGC4 (B), GY75 (C) or GY617 (D) washed and resuspended in 1X PBS and

913 diluted into 1X M9 salts with or without ammonium chloride, supplemented with 1% v/v WMS,

914 0.5% w/v D-glucose, 50 µg/ml kanamycin sulfate, pH 7.5, with and without 1.7 mM L-cysteine or

915 L-methionine, as outlined in the legend in panel A. Insets show final corrected OD_{595} under

916 anaerobic conditions; the y-axis is in linear units. Error bars represent standard error of the mean

917 (n=2).

918

919 **Figure S5. Aerobic growth is dependent on ferric hemin concentration.** *Dysgonomonas*

920 isolates BGC7 (A), HGC4 (B), GY75 (C) or GY617 (D) were pre-grown in DDM without hemin,

921 washed and diluted into 1X M9 salts supplemented with 5% v/v WVS, 1% v/v WMS, 0.5% w/v

922 D-glucose, 1.7 mM L-cysteine, 50 µg/ml kanamycin sulfate, pH 7.5. ferric hemin amendments can

923 be found in the legend in panel A.

924

925 **Figure S6. Anaerobic growth yield is dependent on ferric hemin concentration.**

31

926 *Dysgonomonas* isolates BGC7 (A), HGC4 (B), GY75 (C) or GY617 (D) were pre-grown on DDM

927 lacking hemin, washed and diluted into 1X M9 salts supplemented with 5% v/v WVS, 1% v/v

928 WMS, 0.5% w/v D-glucose, 1.7 mM L-cysteine, 50 μg/ml kanamycin sulfate, pH 7.5, and

929 incubated anaerobically. Shown are the final growth yields (difference in OD_{595} at t=0h and t=85h)

930 across a range of hemin concentrations during anaerobic growth. Identical concentrations as in

931 Fig. 5 were used to determine final yield. The data were fit using third order polynomial regression

932 (solid line) and coefficients of determination for the fit are given as R^2 values as indicated.

933 Confidence intervals of 95% are represented by shading bounded by dashed lines.

934

935 **Figure S7. Isolates exhibit multiple B-vitamin auxotrophies.** *Dysgonomonas* isolates BGC7

936 (A), HGC4 (B), GY75 (C) or GY617 (D) were pre-grown in DDM lacking B-vitamins, washed

937 and diluted into 1X M9 salts supplemented with 1% v/v WMS, 0.5% w/v D-glucose, 1.7 mM L-

938 cysteine, 10% v/v hemin solution, 50 μg/ml kanamycin sulfate, pH 7.5, containing vitamin pools

939 with all, none or a single cofactor omission, as outlined in the legend in panel A. Insets show final

940 corrected OD_{595} under anaerobic conditions; the y-axis is in linear units.

941

942 **Figure S8. Agar-solidified complex medium (DCM) supports growth under common**

943 **incubation conditions.** *Dysgonomonas* isolates were streaked from solid DDM onto agar-

944 solidified DCM containing 0.1 mg/ml reduced glutathione and streaked to isolation. Plates were

945 loosely bagged, incubated aerobically at 22°C or 30°C or anaerobically at 22°C for 4 days and

946 photographed. Scale bar represents 10 mm.

947

948 **Figure S9. Incubation in atmosphere-restricted conditions promotes growth on agar-**

949 **solidified DMM containing antioxidants.** Several colonies of each *Dysgonomonas* isolate were

950 taken from solid DDM and separately pooled in M9 salts, spotted and streaked onto agar-solidified

951 DMM containing 0.1 mg/ml glutathione. Plates were incubated at 30°C with either unrestricted

952 access (un-bagged) or restricted access (sealed in plastic bag) to ambient atmosphere for 5 days

953 and photographed. Scale bar represents 5 mm.

954

955 **Table S1. Amino acid auxanography pools.** Twenty-two amino acids were prepared as aqueous

956 10 mg/ml solutions. Ten pools were constructed such that concentrations of each component was

957 1.67 mg/ml. Pools were added to media such that final concentration of each amino acid was 0.33

958 mg/ml.

959

960 **Table S2. Recipes for stock salt, metal and vitamin solutions.** Recipe for concentrated stock

961 solutions are listed in mass per liter and molarity. Stock solutions can be prepared and safely stored

962 at 4°C for weeks to months with minimal effect on growth. See references for WMS and WVS in

963 Materials and Methods for preparation instructions.

964

965 **Table S3. Media recipes for DCM, DDM & DMM.** Media recipes for DMM, DDM and DCM

966 are provided along with final concentrations in mass per liter and molarities. Suggestions for

967 preparation are also provided, but we assume some knowledge and experience with media

968 preparation.

969

970 **Table S4. ASVs generated from *Dysgonomonas* isolates used in this study.** Thirty isolates were

971 obtained from worker and alate termites and purified on DMM. The V4 region of the 16S rRNA

972 gene was amplified and sequenced using dual-barcoded primers on an Illumina MiSeq platform.

973 The R package DADA2 was used to generate ASVs. Molecular-grade water was used as a no-

974 template control, and *Dysgonomonas* spp. BGC7 and HGC4 as well as isolates GY75 and GY617

975 were used as biological controls. Read counts less than 5 percent of the total attributed to a

976 particular isolate were considered amplification or sequencing errors that passed filtering and

977 disregarded. ASV_3* was not generated from high-throughput sequencing data, but was observed

978 to represent a subset of dideoxy-sequenced 16S rRNA genes which share 100% similarity over the

979 same V4 region as ASVs generated with DADA2 (see **Figure 13**).

980

981 **File S1. Multiple sequence alignment of 16S rRNA genes used in this study (FASTA format).**

982

983 **File S2. Pairwise nucleotide identities for 16S rRNA genes used in this study (CSV format).**

984 # Figures and tables:
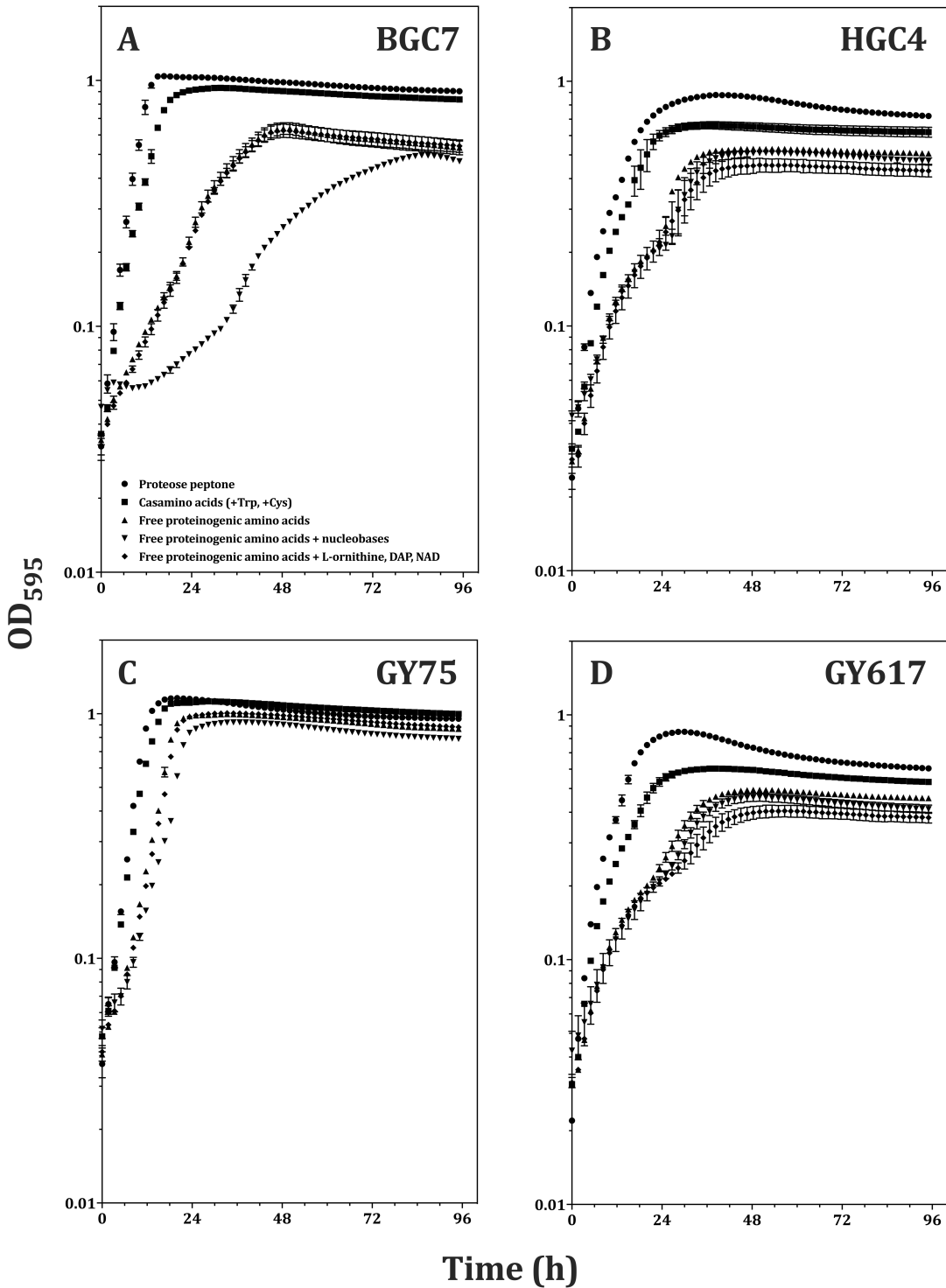
Figure 1. Growth of *Dysgonomonas* isolates in defined medium containing amino acids and B-vitamins.

Figure 2. L-cysteine is required for aerobic and anaerobic growth.
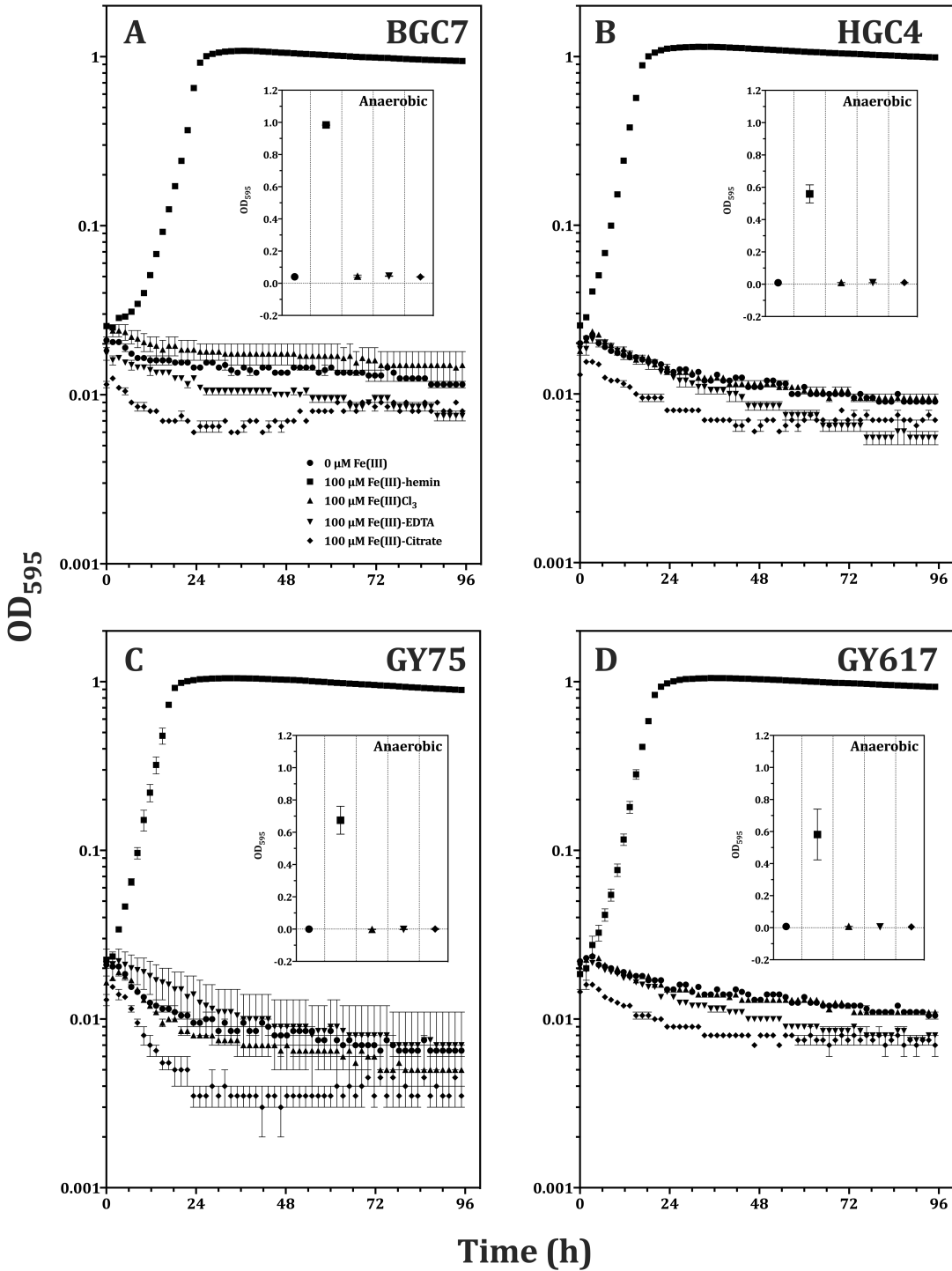
Figure 3. Ferric hemin is a preferred source of iron for *Dysgonomonas*
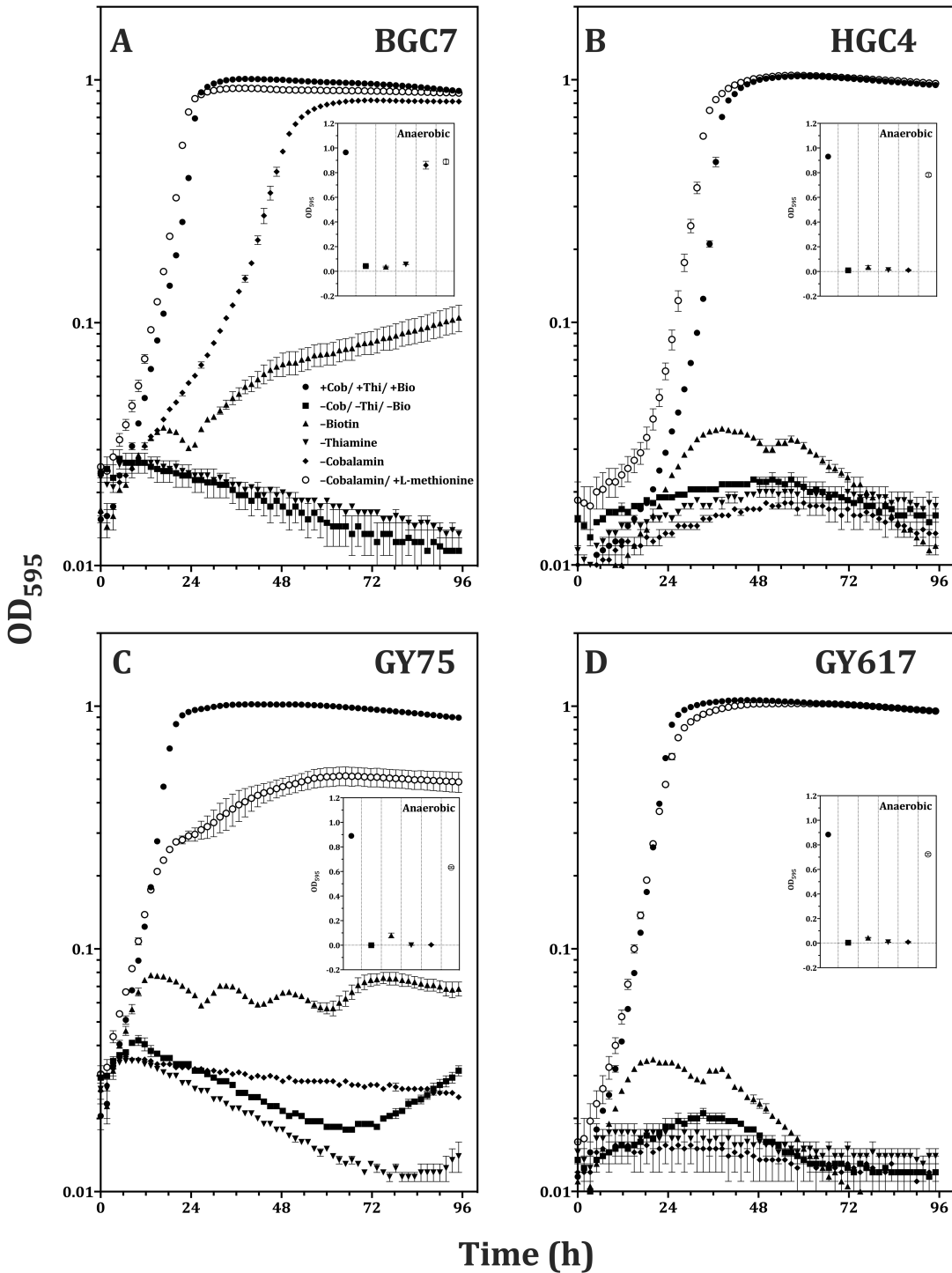
Figure 4. *Dysgonomonas* **exhibit growth requirements for thiamine, biotin and cyanocobalamin.**

**Figure 5. Serial transfer of *Dysgonomonas* in DMM liquid cultures.**
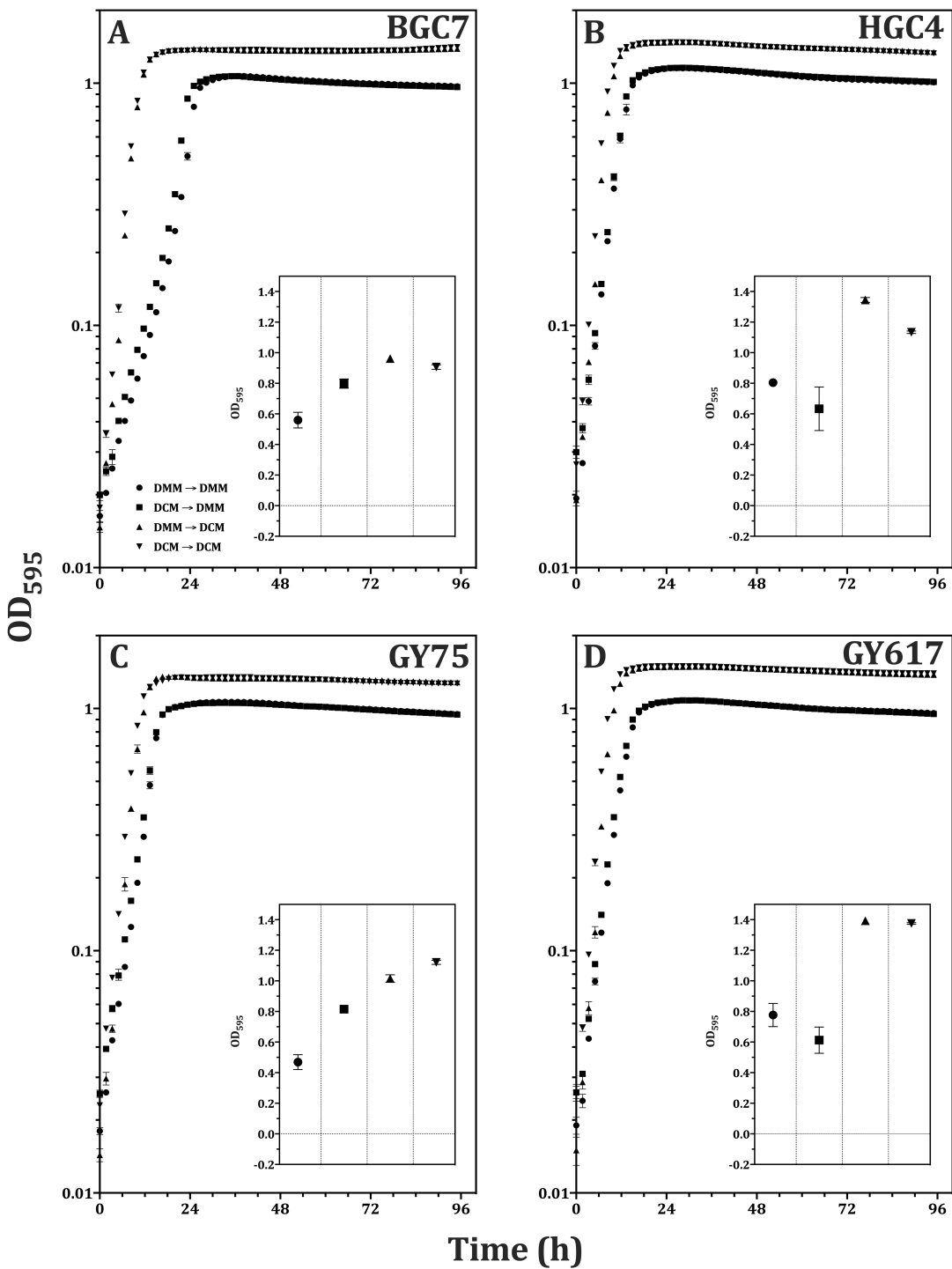
Figure 6. Comparison of growth phenotypes in complex and minimal media.

Isolate	Pregrowth Medium	Growth Medium	Maximum growth rate (h^{-1})	Generation time (h)	Carrying capacity (OD_{595})
BGC7	DMM	DMM	0.454 ± 0.005	1.53 ± 0.016	1.02 ± 0.005
		DCM	0.582 ± 0.003	1.19 ± 0.005	1.37 ± 0.003
	DCM	DMM	0.483 ± 0.001	1.44 ± 0.004	1.02 ± 0.004
		DCM	0.547 ± 0.016	1.27 ± 0.038	1.37 ± 0.015
HGC4	DMM	DMM	0.513 ± 0.017	1.35 ± 0.043	1.08 ± 0.006
		DCM	0.652 ± 0.005	1.06 ± 0.008	1.42 ± 0.011
	DCM	DMM	0.530 ± 0.011	1.31 ± 0.027	1.09 ± 0.003
		DCM	0.664 ± 0.013	1.05 ± 0.020	1.41 ± 0.013
GY75	DMM	DMM	0.540 ± 0.007	1.29 ± 0.016	1.02 ± 0.006
		DCM	0.617 ± 0.004	1.12 ± 0.007	1.32 ± 0.015
	DCM	DMM	0.484 ± 0.002	1.43 ± 0.007	1.01 ± 0.006
		DCM	0.572 ± 0.025	1.22 ± 0.053	1.31 ± 0.019
GY617	DMM	DMM	0.460 ± 0.006	1.51 ± 0.019	1.02 ± 0.010
		DCM	0.637 ± 0.001	1.09 ± 0.001	1.44 ± 0.005
	DCM	DMM	0.462 ± 0.004	1.50 ± 0.012	1.02 ± 0.006
		DCM	0.652 ± 0.002	1.06 ± 0.003	1.45 ± 0.009

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1000 **Table 1. Aerobic growth metrics in DCM and DMM.**

40
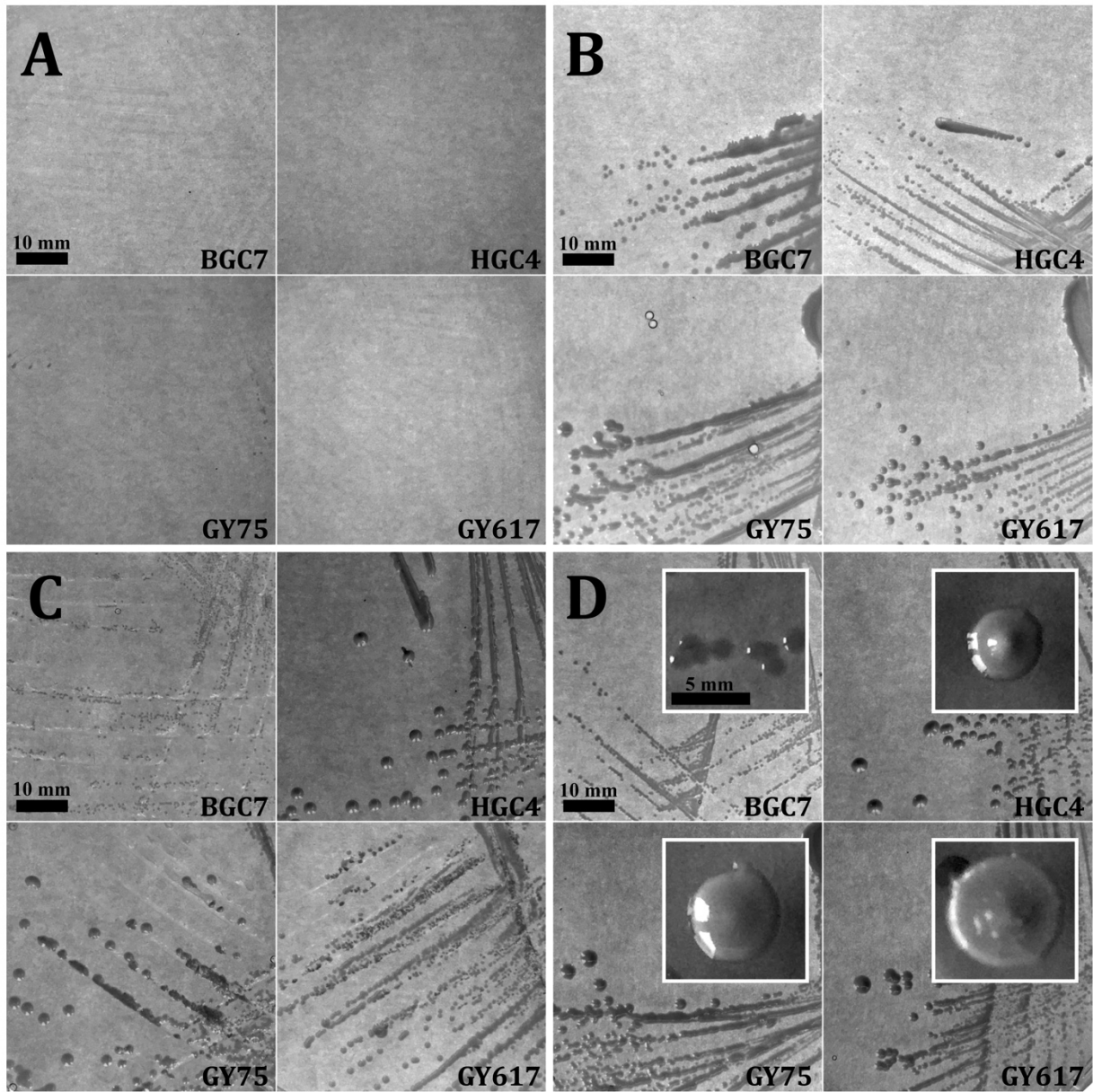
Figure 7. Antioxidants are required for aerobic growth on defined medium.

Figure 8. Maximum-likelihood phylogenetic reconstruction of near-full length 16S rRNA gene sequences from cultured members of genus *Dysgonomonas*.

Figure S1. Amino acid auxanography using defined medium.

Figure S2. Amino acid auxanography.

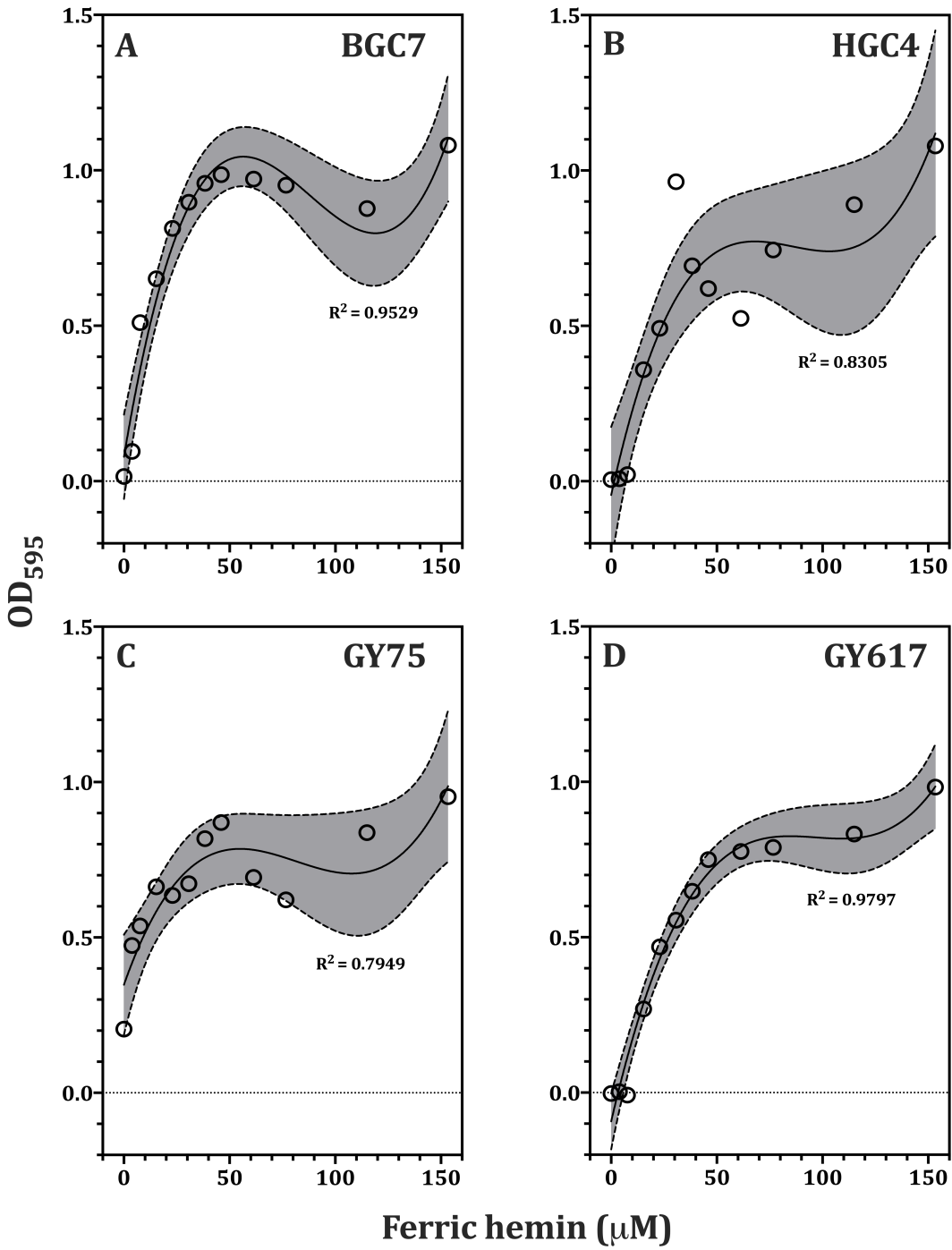**Figure S3. Aerobic growth in defined medium is L-cysteine-dependent.**

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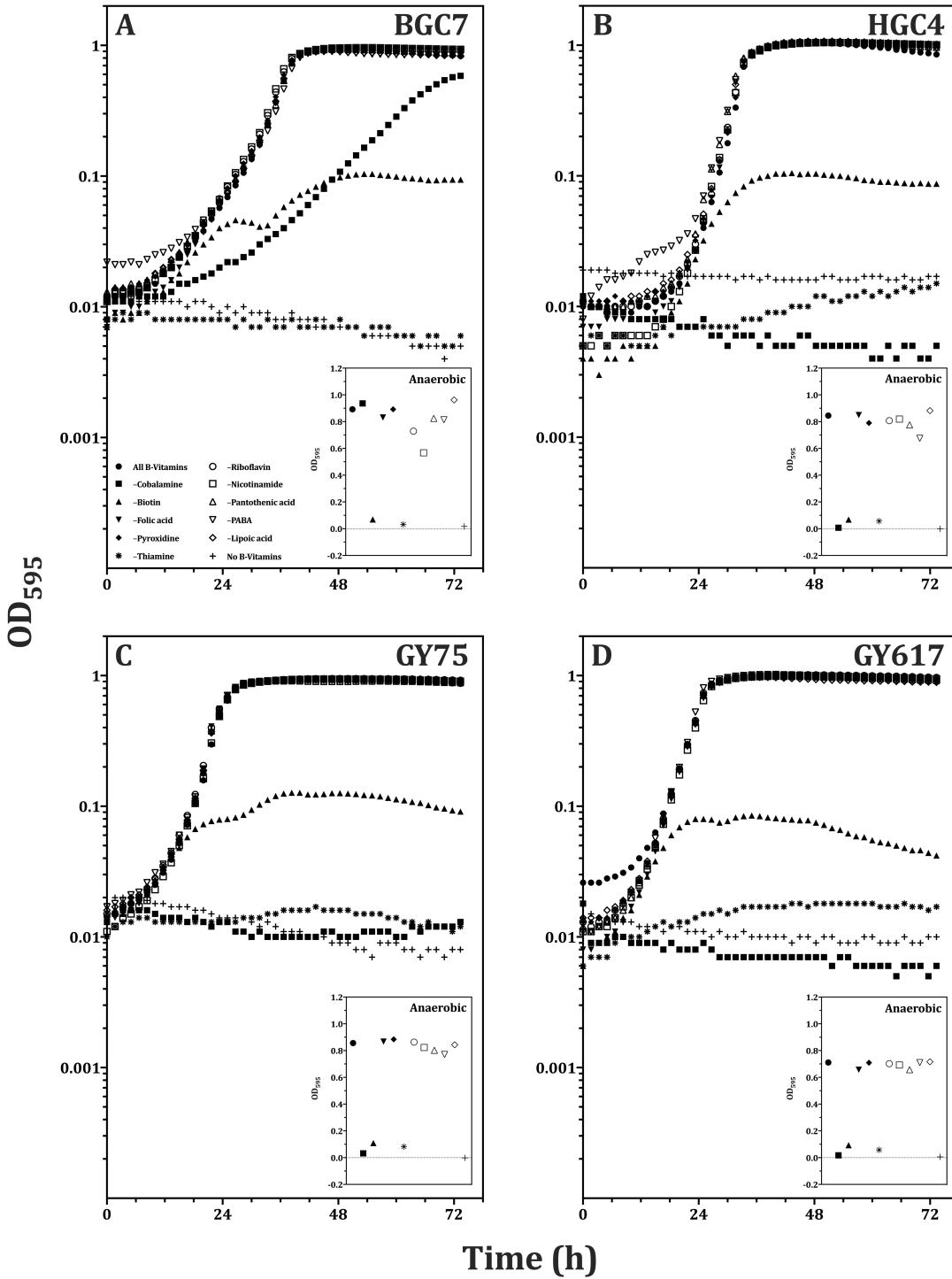**Figure S4. *Dysgonomonas* can utilize L-cysteine as the sole source of nitrogen.**

Figure S5. Aerobic growth is dependent on ferric hemin concentration.

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Figure S6. Anaerobic growth yield is dependent on ferric hemin concentration.

Figure S7. Isolates exhibit multiple B-vitamin auxotrophies.

Figure S8. Agar-solidified complex medium (DCM) supports growth under common incubation conditions.

Figure S9. Incubation in atmosphere-restricted conditions promotes growth on agar-solidified DMM containing antioxidants.

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