Candidatus Nealsonbacteria (OD1) in a methanogenic benzene-degrading enrichment culture is likely an ectosymbiotic biomass recycler

Xu Chen[1], Olivia Molenda[1], Christopher T. Brown[2,3†], Courtney R. A. Toth[1], Shen Guo[1], Fei Luo[1††], Jane Howe[4], Camilla L. Nesbø[1], Christine He[5], Elizabeth A. Montabana[2,6], Jamie H. D. Cate[2,6,7], Jillian F. Banfield[5,6,8,9], and Elizabeth A. Edwards[1,*]

[1]Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, M5S 3E5, Canada.
[2]Department of Molecular and Cell Biology, University of California, Berkeley, CA, USA
[3]California Institute for Quantitative Biosciences, University of California, Berkeley, CA, USA
[4]Department of Materials Science and Engineering, University of Toronto, 184 College Street, Toronto, Ontario M5S 3E4, Canada
[5]Innovative Genomics Institute, University of California, Berkeley, CA, USA
[6]Molecular Biophysics and Integrated Bioimaging, Lawrence Berkeley National Laboratory, Berkeley, CA, USA
[7]Department of Chemistry, University of California, Berkeley, CA, USA
[8]Department of Earth and Planetary Science, University of California, Berkeley, CA, USA;
[9]Environmental Science, Policy and Management, University of California, Berkeley, CA, USA;
†Current Address: Metagenomi, Inc., 1545 Park Avenue, Emeryville, CA, USA
††Current Address: Liven Proteins Corporation, 200 College Street, Toronto, ON, Canada.

Keywords: Candidate Phyla Radiation, Nealsonbacteria, genomics, genome-resolved metagenomics, fluorescent in situ hybridization, CryoEM, methanogenic benzene biodegradation, biomass recycling

*Corresponding author mailing address: University of Toronto, 200 College St., Toronto, Ontario, M5S 3E5, Canada. Phone: (416) 946-0996. Fax: (416) 978-8605.
E-mail: elizabeth.edwards@utoronto.ca;

Abstract

The Candidate Phyla Radiation (CPR, or superphylum Patescibacteria) is a very large group of bacteria with few cultivated representatives first discovered by culture-independent metagenomic analyses. Within the CPR, the candidate phylum Parcubacteria (previously OD1) is prevalent in anoxic lake sediments and groundwater. We identified a bacterium belonging to the Parcubacteria in a methanogenic benzene-degrading enrichment culture originally derived from oil-contaminated sediments. *Candidatus* Nealsonbacteria DGGOD1a is the only bacterium other than a previously identified benzene-degrading fermenter (*Deltaproteobacteria* Candidate Sva0485 clade ORM2) consistently and abundantly detected in all active benzene-degrading transfers of this culture. Therefore, we hypothesized that DGGOD1a must serve an important role in sustaining anaerobic benzene metabolism in the consortium. Growth experiments using a variety of possible substrates suggested that it is involved in biomass recycling. Microscopic observations supported by molecular analyses and a closed genome revealed an epibiont lifestyle with very small *Ca.* Nealsonbacteria DGGOD1a closely associated with much larger *Methanosaeta*. The images reveal a first example of cross-domain episymbiosis that may apply to other *Ca.* Nealsonbacteria found in diverse environments.

23 **Introduction**

24 A methanogenic benzene degrading enrichment culture referred to as consortium OR (for
25 Oil Refinery) was derived from contaminated sediments in 1995 and has since been maintained
26 with only benzene as added electron donor and carbon source. Multiple bottles and lineages of
27 the culture have been maintained with periodic transfers in anaerobic pre-reduced minimal
28 medium (1). This culture harbors benzene-degrading fermenting organisms
29 (Deltaproteobacterium ORM2), Parcubacteria (OD1, further classified as *Candidatus*
30 Nealsonbacteria in this study), acetogens, methanogens and other less numerous microbes which
31 together enable anaerobic benzene degradation at rates from 0.2 to 10 mg benzene/L/day (2-4).
32 The mechanism for anaerobic benzene biodegradation in the OR consortium is still poorly
33 understood. A recent study describes the current state of knowledge (3), revealing coordinated
34 metabolic interaction between fermenters, acetoclastic methanogens, and hydrogenotrophic
35 methanogens. The chief benzene-degrading fermenter was identified as a member of the
36 Candidate clade Sva0485 in the Deltaproteobacteria, present in at least two clonal variants
37 denoted ORM2a and ORM2b. Interestingly, besides the chief benzene degrader, a member of
38 *Ca.* Nealsonbacteria is the only other microbe consistently found in all different consortium OR
39 lineages (2). However, no direct correlation was found between *Ca.* Nealsonbacteria and
40 benzene degradation rate. Given *Ca.* Nealsonbacteria's persistence and the high biomass
41 turnover rates in anoxic hydrocarbon- and organohalide-contaminated habitats (5), perhaps as a
42 result of exposure to toxic concentrations of hydrocarbons, we hypothesized that this
43 microorganism might play a role in biomass recycling. The OR consortium provided us an
44 opportunity to study *Ca.* Nealsonbacteria's metabolic potential *in vivo*.

45 The Parcubacteria belong to the superphylum referred to as the Candidate Phyla
46 Radiation (CPR) which was first discovered by culture-independent metagenomic analysis (6).
47 The CPR, believed to comprise over 15% of the Bacteria domain, is prevalent in lakes, sediment,
48 and groundwater (6, 7). The discovery of CPR recast the tree of life and further emphasizes the
49 need for better functional characterization. To date, Parcubacteria remain uncultured and are only
50 available as metagenome assembled genomes (MAGs), with 14 closed MAGs or cMAGs
51 publicly available as of Dec. 9th, 2021 (8-12). Parcubacteria genomes are extremely small (~1
52 Mb) and include a large portion of hypothetical proteins which lack homologs from well-studied
53 microbes. The reduced genomes of Parcubacteria betray limited metabolic ability, lacking
54 complete pathways for the biosynthesis of vitamins, lipids amino acids, and nucleotides (11, 13).
55 The lack of complete biosynthetic pathways or electron transport system suggests a fermentative
56 symbiotic lifestyle where Parcubacteria obtain nutrients, metabolites, and energy directly from
57 their host or partner cells, a process known as ectosymbiosis. The recent example of an enriched
58 aerobic co-culture of a member of the CPR (Saccharibacteria) together with *Actinomyces*
59 *odontolyticus* XH001 revealed a symbiont or parasite lifestyle (14). Saccharibacteria genomes
60 shares similarities with the Parcubacteria in that they are small and lack complete biosynthetic
61 pathways. Metagenomic analyses suggests that Parcubacteria may also be associated with a host
62 microbe (11, 12, 15, 16) but experimental evidence has yet to be gleaned because of the
63 difficulty in cultivating and enriching these tiny microorganisms.
64 Using both Illumina short reads and PacBio long reads, we successfully reconstructed the
65 fully closed genome sequence of the uncultured Parcubacteria DGGOD1a in our methanogenic
66 benzene-degrading culture. A maximum likelihood concatenated ribosomal protein tree (Figure
67 S1) places the genome within the candidate group Nealsonbacteria, which was first defined by

68 Anantharaman et al. (17). This study aimed to identify substrates for the *Ca.* Nealsonbacteria
69 DGGOD1a in benzene-degrading OR consortium, and to visualize and enumerate these cells in
70 the mixed culture using various microscopic techniques. We tested various potential substrates
71 for *Ca.* Nealsonbacteria and found that most significant enrichment occurred when the culture
72 was amended with crude cell lysate, increasing relative abundance from 5% to ~30% and
73 absolute abundance up to 10 times. Although specific components present in lysed cells used to
74 enrich *Ca.* Nealsonbacteria were not identified, the results suggest a potential role as a biomass
75 recycler. Fluorescence *in situ* hybridization (FISH) and electron microscopy (EM) revealed *Ca.*
76 Nealsonbacteria as an epibiont on the surface *Methanosaeta*, a methanogenic archaeon essential
77 for completing the biotransformation of benzene to methane. These discoveries begin to shed
78 light on the uncharacterized majority of the CPR.

Materials and Methods

Chemicals and microbial cultures. All chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada) at the highest purity available unless specified otherwise. The OR consortium is maintained in 100 mL to 2 L batch culture bottles at the University of Toronto and in larger vessels (>100 L) at SiREM laboratories (Guelph, Ontario, Canada); the scaled-up culture is referred to as DGG-B (3). Two OR consortium subculture bottles (or lineages) were used in the experiments described herein: a bottle called OR-1bBig was the inoculum for *Ca.* Nealsonbacteria enrichment Donor Trials #1 & #2 and a bottle called OR-p5 was the inoculum for the augmentation experiment. All cultures were grown in an iron sulfide (FeS)-reduced, bicarbonate-based mineral medium (MM medium) amended regularly (approx. 1 per month) with 10 mg/L benzene (2, 3).

Preparation of electron donor solutions for *Ca.* Nealsonbacteria enrichment trials. Two crude cell lysate solutions from the OR consortium and a culture of *Escherichia coli* were prepared using tangential flow filtration and French press as described in Text S1. Next, we prepared two solutions of specific biomass components, nucleic acids (salmon sperm DNA; Sigma-Aldrich, Deoxyribonucleic acid sodium salt) and a membrane lipid (L-α-phosphatidylethanolamine; Sigma-Aldrich, Type II, ≥97%, lyophilized powder from egg yolk). Acetate, pyruvate and hydrogen were also tested as donors as they are intermediates of benzene degradation. Stock solution concentrations are listed in Table S1a.

***Ca.* Nealsonbacteria Enrichment – Donor Trial #1.** To test if *Ca.* Nealsonbacteria DGGOD1a could grow on crude lysed biomass or specific sub-cellular components, 25 mL culture tubes with 9 mL MM medium were inoculated with 1 mL OR-1bBig culture and amended with 7 different

102 electron donor solutions (Table S1a) at approximately the same electron equivalents, targeting ~30
103 mg COD/L. Each condition was tested in quadruplicate and incubated in a Coy anaerobic glove
104 box for 7 months (supplied with 10% H_2, 10% CO_2, and 80% N_2). DNA samples were collected
105 at 2, 4, and 7 months after inoculation by sacrificing an entire replicate from each treatment.
106
107 ***Ca.* Nealsonbacteria enrichment – Donor Trial #2.** After observing successful growth and
108 enrichment of *Ca.* Nealsonbacteria with crude cell lysate from the benzene culture in Donor Trial
109 #1, a second experiment was set up to test the impact of various biomass fractions of cell lysate.
110 Culture (500 mL) was concentrated by tangential flow and cells were lysed using the same French
111 press method described earlier (Text S1). The cell lysate was then divided into four fractions, as
112 seen in Figure S2. One fraction was used "as is" (BL for Benzene culture Lysate). The remaining
113 lysate was centrifuged (at 13,000 × g for 20 mins) to create a pellet fraction and a supernatant
114 fraction. The supernatant fraction was further separated by tangential flow filtration with 50kDa
115 cut-off membrane (Pellicon® XL cassette with Durapore® membrane) to create a permeate fraction
116 (< 50 kDa) and a retentate fraction (>50 kDa). Each of these fractions was tested separately (Table
117 S1b). Two additional treatments were included in Donor Trial #2. One consisted of pyruvate plus
118 the antibiotics kanamycin and vancomycin (1 mM) to inhibit bacteria without inhibiting
119 methanogens. A second additional treatment was amended with *E. coli* extract instead of benzene
120 culture extract. We ensured that the *E. coli* pellet was well washed with MM medium to remove
121 any residual *E. coli* rich growth medium that could confound results. Controls without substrate
122 (starved control; SC) and crude benzene culture lysate without inoculum (no inoculum; NI) were
123 included. All treatment were conducted in biological duplicates except the starved control which
124 was a singleton.

125

126 ***Ca.* Nealsonbacteria augmentation trial.** This experiment made use of an inoculum from bottle
127 OR-p5 with a very slow benzene degradation rate (~0.1 mg/L/day). Eight 25-mL culture vials were
128 each filled with a 10 mL aliquot of this slow culture (see Text S2 for details). Subsequently, 5 mL
129 of OR-p5 culture was removed from vials #1-2 and replaced with 5 mL of *Ca.* Nealsonbacteria
130 enrichment culture BL (replicate #1) from Donor Trial #2. In vials #3-5, 5 mL of culture was
131 removed and replaced with 5 mL of culture supernatant from a very active benzene culture
132 (degradation rate of ~10 mg/L/day), prepared by centrifugation at 13,000 × g for 20 min. The
133 remaining vials (#6-8) of slow culture were left as is. All vials were amended with neat benzene
134 targeting 15 mg/L (liquid concentration) and reamended whenever concentrations decreased below
135 5 mg/L. Benzene and methane concentrations were monitored by GC/FID (see below). DNA
136 samples were collected on day 217 for qPCR analysis.

137

138 **DNA extraction, quantitative PCR, and Illumina amplicon sequencing.** DNA from Donor
139 Trial #1 was extracted from 10 mL culture samples; otherwise, DNA was extracted from 1 mL
140 culture samples. Cells were harvested by centrifugation at 13,000 × g for 20 min at 4°C and pellets
141 resuspended in residual (~0.05 mL) supernatant. DNA was extracted using the DNeasy PowerSoil
142 Pro Kit (QIAGEN) following the manufacturer's protocol. Real-time quantitative polymerase
143 chain reaction (qPCR) assays were performed to track the gene copy numbers of Bacteria and
144 Archaea using universal 16S rRNA gene primers; abundances of *Ca.* Nealsonbacteria and Sva0485
145 clade Deltaproteobacteria (ORM2) were also tracked using specific 16S rRNA gene primers
146 (Table S2). Standard curves for all the qPCR studies are reported in Table S3. 16S rRNA gene
147 amplicon sequencing with primers targeting the V6-V8 region was conducted at the Genome

148 Quebec Innovation Centre (McGill University) as previously described (3). Raw reads were
149 processed into amplicon sequence variants (ASVs) and analyzed as described in Text S3.
150
151 **Metagenome analysis.** The OR consortium/DGG culture was sequenced using a combination of
152 Illumina paired-end (~150 bp reads, ~300 bp insert) and PacBio long-read library (~7000 bp
153 reads). Metagenomes were submitted to IMG under sequencing project Gp0324998. Detailed
154 description of assembly, binning and functional and taxonomic annotation can be found in Text
155 S4.
156
157 **Epifluorescence microscopy and fluorescence in situ hybridization (FISH).** The FISH probe
158 ARCH915 was used for visualizing all Archaea (18). Eight FISH probes (Table S4) targeting the
159 16S rRNA gene of *Ca.* Nealsonbacteria were newly designed but none yielded a signal
160 distinguishable from background noise. The protocol for epifluorescence microscopy is provided
161 in Text S5 and details into the design and testing of FISH probes in Text S6. A LIVE/DEAD™
162 BacLight™ Bacterial Viability kit (ThermoFisher) was used to determine the ratio of viable cells
163 in consortium OR (Text S5).
164
165 **Scanning Electron Microscopy with Ionic Liquid and Cryo-EM.** To maintain the wet native
166 state of microbial cells and to eliminate artifacts that may be introduced by sample drying and
167 coating, culture samples were prepared with an ionic liquid and examined by SEM, as detailed in
168 Text S7. Cryo-EM was also used to explore intracellular structure and spatial associations. Sample
169 preparation and Cryo-EM conditions are described in Text S8.
170

171 **Analytical procedures.** Benzene and methane were measured in culture bottles and vials by
172 injecting 0.3 mL headspace samples into a Hewlett-Packard 5890 Series II GC fitted with a GSQ
173 column (30 m x 0.53 mm I.D. PLOT column; J&W Scientific, Folsom, CA) as previously
174 described (2). External standards were used for calibration.
175
176 **Accession numbers.** The closed genome sequence of *Candidatus* Nealsonbacteria DGGOD1a is
177 available at the Joint Genome Institute (IMG) under Taxon ID 2791354853 and at NCBI under
178 accession no. CP092821. A Genbank accession number is being generated for amplicon sequences
179 and will be added when available.
180
181 ## Results and Discussion
182
183 **qPCR reveals abundance of *Ca.* Nealsonbacteria and Live/Dead staining confirms**
184 **abundance of dead cells**
185 A qPCR survey of 15 different methanogenic benzene-degrading subculture bottles was
186 conducted to measure the abundance of the two known strains of *Deltaproteobacteria* ORM2
187 (ORM2a and ORM2b) and *Ca.* Nealsonbacteria (formerly referred to as OD1), as well as total
188 bacteria and total archaea. The culture bottle named "OR-1bBig" was selected as the parent
189 culture for this study because it already had a high relative abundance of *Ca.* Nealsonbacteria
190 (~5% based on 16S rRNA amplicon sequencing results) and sufficient culture volume (1.5 L).
191 Microscopy using live/dead staining (Figure S3) revealed large proportions of dead cells in all
192 subculture bottles. Detailed cell counts were conducted for two OR subcultures (~20 microscopic
193 images per sample) yielding ~46-62% dead cells (Table S5). This is not all surprising given that
194 these cultures are maintained as fed-batch, with infrequent transfers into fresh medium and
195 correspondingly long residence times. The products of lysed cells are clearly a source of electron

196 donor in addition to benzene in these bottles. This led us to hypothesize that components from
197 lysed cells might serve as substrates for *Ca.* Nealsonbacteria.
198
199 ***Ca.* Nealsonbacteria found most enriched in vials amended with lysed cells.**
200 In Donor Trial #1, we found that *Ca.* Nealsonbacteria became most enriched in vials amended with
201 lysed benzene culture (Figure 1a). In these vials, relative and absolute abundances of *Ca.*
202 Nealsonbacteria increased ~6-10 times over 4 months, reaching a maximum 30% relative
203 abundance and 5.1×10^6 copies/mL (Figure 1b; Table S6a). Thus *Ca.* Nealsonbacteria may grow
204 on components in lysed biomass. None of the other tested substrates, including salmon sperm
205 DNA, acetate, and a long-chain fatty acid resulted in noticeable enrichment of *Ca.* Nealsonbacteria
206 relative to benzene-amended controls. Some increases (5x) were measured in bottles amended with
207 pyruvate (up to 9.8×10^5 copies/mL), but the abundances of other microbes increased even more
208 such that the average relative abundance of *Ca.* Nealsonbacteria dropped from ~5% to 1% (Figure
209 1b; Table S7). It is not clear whether the growth of *Ca.* Nealsonbacteria resulted from the direct
210 utilization of pyruvate or as an indirect consequence of the increases in biomass and corresponding
211 lysed cells. Either way, sodium pyruvate was not selective for *Ca.* Nealsonbacteria.
212
213 Other observations were gleaned from this first trial from looking at the entire microbial. First, we
214 clearly see that relative abundances of the chief benzene degrader (ORM2) dropped significantly
215 when benzene was absent (Figure 1b). Considering the vials amended with cell lysate, the only
216 two bacterial ASVs that co-enriched with *Ca.* Nealsonbacteria belong to the *Syntrophaceae* and
217 *Spirochaetaceae*. The *Syntrophaceae* ASV was found to share 100% 16S RNA gene sequence
218 identity to an uncultured *Syntrophaceae* (MH665869) from a methanogenic pyrite forming culture

219 fed with FeS, H_2S, and CO_2 (19). It is also distantly related (96.4%) to *Smithella propionica*
220 (AF126282), a syntrophic propionate degrader (20). The *Spirochaetaceae* ASV we identified is
221 distantly related (95.1%) to a spirochaetes (KJ535432) from a methanogenic bioreactor (21).
222 Spirochaetes are also thought to be involved in biomass recycling and may use protein and
223 polysaccharides to produce ethanol and acetate (22).
224
225 Nonmetric dimensional scaling (NMDS) was applied to further analyze Donor Trial #1 ASV data
226 (Figure S4a), displaying the impact of adding different electron donors on microbial community
227 composition. Methane production in each treatment indicated that the added COD was largely
228 consumed within 4 months (Table S8). Therefore, data collected after 7 months are long-starved
229 and marked as "+" in NMDS analysis. The "starved control" (unfed culture) community is very
230 close to the communities from vials amended with cell lysate indicating that cell decay in the OR
231 culture leads to a similar community as the vials amended with benzene culture cell lysate – which
232 makes intuitive sense. Interestingly, we found that communities after 7 months of incubation did
233 not converge, even though they should all be enriching on dead biomass after such a long
234 incubation time, prompting consideration that different sources of cell biomass might enrich for
235 different recyclers. In fact, we expected to see all vials shift to a scavenger-dominated microbial
236 community after 7 months of incubation, and we were hoping to see the enrichment of *Ca.*
237 Nealsonbacteria in every vial after prolonged starvation. However, such enrichment was only
238 clearly observed in acetate and pyruvate-amended vials (Figure 2). It is possible that there was
239 insufficient biomass produced in the other vials to detect enrichment. Indeed, total cell
240 concentrations were highest in acetate and sodium pyruvate amended vials. Interestingly, we
241 observed two different *Ca.* Nealsonbacteria ASVs (labeled a and b) in the acetate- and pyruvate-

242 amended cultures, where *Ca.* Nealsonbacteria ASVb became more abundant than *Ca.*
243 Nealsonbacteria ASVa – the strain originally detected in benzene cultures (Figure 2) (2). These
244 two *Ca.* Nealsonbacteria ASVs show only 91.2% 16S rRNA sequence identity. This result
245 intriguingly suggests that different strains may exhibit a specific preference for the type of lysed
246 cell material they can metabolize, or that the a and b variants have different hosts, because the
247 acetate- and pyruvate-amended cultures selected for very different communities.
248
249 To further investigate the identity of specific substrates for *Ca.* Nealsonbacteria, a second trial
250 (Donor Trial #2) was conducted where benzene culture lysate and also *E. coli* cell lysate were
251 compared. Moreover, the lysed benzene culture was further separated into three sub-fractions by
252 centrifugation and tangential flow filtration with a 50kDa cut off (Figure S2) and each fraction was
253 amended to the benzene culture separately. The pyruvate treatment was repeated with added
254 antibiotics (1 mM each kanamycin and vancomycin) to try to increase the selectivity of pyruvate.
255 The results of Donor Trial #2 (raw data in Table S6b) were similar to those of Donor Trial #1, with
256 cell lysate resulting in significant (>10×) growth (Figure S5) regardless of source. The sub-
257 fractions of benzene culture lysate also resulted in enrichment although we observed considerable
258 variability between duplicates (Figure S5). The *E. coli* crude lysate appeared most stimulatory, but
259 it was also provided at 10× the concentration of the lysate from the benzene culture based on
260 measured COD (Table S1c). The fact that of the three fractions, the >50 kDa fraction (supernatant
261 retentate) was most effective after 4 months suggests that larger molecules are important; perhaps
262 we should have tested proteins. An NMDS analysis was also conducted on amplicon data from
263 Donor Trial #2 (Figure S4b). These results show that the microbial communities enriched on each
264 fraction of dead biomass are a bit different after the first two months (triangles), but that they

265 converged after four months (squares). Perhaps after four months, only larger biomolecules
266 remain.
267
268 To look more closely at the microbial composition in each treatment, a heatmap of absolute
269 abundance (Figure 3) was generated showing the top 18 ASVs in Donor Trial #2, with raw data
270 reported in Table S9. *Ca.* Nealsonbacteria ASVa was the abundant sequence variant detected, and
271 *Methanosaeta* and *Spirochaetaceae* were the only other two microbes consistently enriched with
272 *Ca.* Nealsonbacteria in all cell lysate amended vials. Vials amended with lysed *E. coli* enriched
273 some ASVs belonging to *Clostridium* and *Bacteroides* that were not enriched in other treatments
274 (Figure 3). The vials amended with antibiotics and pyruvate successfully inhibited the growth of
275 most bacteria including *Ca.* Nealsonbacteria. However, it is interesting to note that the culture's
276 benzene fermenter (ORM2) seems to have persisted in those treatments. The growth of
277 *Methanomethylovorans* and *Methanobacterium* in the presence of antibiotics confirms their
278 resistance and points to a possible means of enriching for ORM2. Taken together, the two donor
279 trials have confirmed that compound(s) present in cell lysate can be used to enrich *Ca.*
280 Nealsonbacteria. The identity of the compounds remains elusive, but the most parsimonious
281 answer would be certain higher MW proteins and amino acid products, as they would be found in
282 all of the fractions tested. Further studies will focus on these substrates.
283
284 **Microscopy reveals *Ca.* Nealsonbacteria as an epibiont of *Methanosaeta*.**
285 Seeing is believing, and to date, *Ca.* Nealsonbacteria has never been successfully visualized. Eight
286 specific FISH probes were designed targeting the 16S rRNA gene of *Ca.* Nealsonbacteria
287 DGGOD1a. However, none of the probes yielded a distinguishable signal from background noise.

288 This problem was also reported in a previous study with a different set of FISH probes to
289 Parcubacteria (23). Although FISH was unsuccessful for *Ca.* Nealsonbacteria, we found that using
290 a combination of a FISH probe targeting all Archaea and DAPI staining could distinguish the
291 archaea (methanogens) from other microbes, i.e., bacteria. Given that *Ca.* Nealsonbacteria was the
292 most abundant bacterial phylotype in several of our enrichment cultures (Figure 3), we aimed to
293 positively identify these cells based on their known abundance. We selected cell lysate enrichment
294 culture (Lysate replicate #2 at 2 months) because *Ca.* Nealsonbacteria abundance was above 10^6
295 cells/mL in this culture and all other bacterial phylotypes detected were more than an order of
296 magnitude less abundant (Figure 3). A representative image of this culture is shown in Figure 4(a).
297 This method was used to examine both the parent culture and the *Ca.* Nealsonbacteria enrichments.
298 In the *Ca.* Nealsonbacteria enrichments, we observed many DAPI-stained ultra-small (~0.2 μm)
299 cells associated with a single filamentous structure that stained with the archaeal FISH probe, and
300 therefore must be a filamentous methanogen. The ultra-small cell size matches the typical size of
301 CPR members (11, 15) and is most likely *Ca.* Nealsonbacteria. Using the approach, we counted
302 the number of small cells attached to long archaeal filaments. This cell count was conducted
303 thoroughly on two independent samples from *Ca.* Nealsonbacteria enrichment used for
304 microscopy. Twenty fields of view were imaged and counted for each sample. These small cells
305 were by far the most numerous bacteria in all views, and thus must correspond to *Ca.*
306 Nealsonbacteria, as this bacterial ASV was most numerous as well. The cell counts obtained were
307 2.3×10^6 and 4.7×10^6 cells/mL in the two samples (Table S10). Enumeration of gene copies by
308 qPCR in samples of DNA extracted from the same vials using primers specific for *Ca.*
309 Nealsonbacteria gave a result of 8.3×10^6 copies/mL which is of the same order of magnitude as
310 cell counts. Thus, we are confident that the tiny cells attached to Archaea are *Ca.* Nealsonbacteria.

311 Interestingly, the archaeal FISH probe was observed to bind more strongly to methanogens that
312 had many *Ca.* Nealsonbacteria cells attached, compared to naked methanogens (Figure 4a; bottom
313 right). Naked methanogens stained brightly with DAPI but only faintly with the archaeal FISH
314 probe. Since the FISH probe intensity indicates metabolic activity, perhaps methanogens with *Ca.*
315 Nealsonbacteria are more active and have a higher number of ribosomes per cell (24). *Ca.*
316 Nealsonbacteria's role as either a parasite or commensal organism is still unknown. Perhaps
317 methanogens without *Ca.* Nealsonbacteria are dying or non-viable, or that *Ca.* Nealsonbacteria
318 specifically target the most active cells.
319
320 SEM examination of the parent methanogenic benzene-degrading culture also showed a similar
321 association of very small *Ca.* Nealsonbacteria-like cells attached to a large filamentous cell
322 structure consisting of a chain of rod-shaped, blunt-ended cells. This filament was enclosed in a
323 tubular sheath and the flat ends distinctively match the characteristics of *Methanosaeta* (25),
324 shown in Figure 4b. The *Ca.* Nealsonbacteria-like cells are spherical with a diameter of 0.2 µm to
325 0.8 µm and they are always found in budding pairs, forming a peanut shape. Cryo-EM was also
326 used to exam the *Ca.* Nealsonbacteria enrichments. The Cryo-EM images (Figure 4c) show the
327 same association of peanut-shaped cells with *Methanosaeta*. The large black rod-shaped blurry
328 cells at the center of the image are *Methanosaeta* forming a sheathed chain. Multiple peanut-
329 shaped ultra-small cells were observed attached to the chain of *Methanosaeta* (Figure 4c). The *Ca.*
330 Nealsonbacteria-like cells are always found in pairs and the one attached to the methanogen is
331 usually bigger than the second one. A zoomed-in image (Figure 4c; right side) reveals the detailed
332 cell structure of this *Ca.* Nealsonbacteria-like cell. It has a very interesting membrane structure
333 with a thick sheath and an apparent S-layer. This thick sheath might explain why FISH probes

334 failed, as they may not be able to enter the cells. Additional FISH, SEM, Cryo-EM images are
335 provided in Figures S6 and S7.
336
337 We had attempted to enrich *Ca.* Nealsonbacteria by filtration through a 0.22-µm filter. However,
338 qPCR results of the filtrate showed no such enrichment: both the total bacterial and the *Ca.*
339 Nealsonbacteria concentrations decreased 100 fold (data not shown). This is in contrast to other
340 studies where 0.22-µm filters were successful at enriching ultra-small microbes (6, 23, 26).
341 Perhaps the strong attachment of *Ca.* Nealsonbacteria to its host caused this approach to fail. The
342 majority of *Ca.* Nealsonbacteria were seen associated with *Methanosaeta* while very few were
343 observed free-living, perhaps indicating that it is an obligate epibiont.
344
345 **Impact of *Ca.* Nealsonbacteria on benzene degradation**
346 To further study its role in benzene degradation, we decided to mix a *Ca.* Nealsonbacteria
347 enrichment (containing about 10^7 *Ca.* Nealsonbacteria cells/mL) with a "slow" benzene-degrading
348 culture bottle (containing about 10^7 ORM2 cells/mL). Raw qPCR and monitoring data for this
349 experiment are reported in Table S11. We observed a significant enhancement of benzene
350 degradation rate in the two replicates that were a 50:50 mix of the two cultures (Figure 5a), even
351 though mixing diluted the benzene degrader (ORM2) by 50%. Benzene culture amended with
352 supernatant from a healthy culture (degrading benzene at 10 mg/L/day benzene) as well as the
353 (slow) culture-only controls all showed variable, but overall slower benzene degradation (Figure
354 5a). We tried to see if the rate of benzene degradation would relate to the abundance of *Ca.*
355 Nealsonbacteria and ORM2 (Figure 5b). In general, benzene biodegradation rates were highest in
356 cultures with the highest measured ORM2 concentrations at Day 217, except for one culture

357 replicate (slow culture #1) with abundant ORM2 but no benzene degradation activity. In hindsight
358 we should have monitored these cultures by qPCR more often. The two vials amended with the
359 *Ca.* Nealsonbacteria enrichment do show highest cell concentrations for both ORM2 and *Ca.*
360 Nealsonbacteria. Therefore, *Ca.* Nealsonbacteria did enhance benzene degradation, maybe by
361 supplying ORM2 with nutrients or co-factors recycled from lysed biomass or some specific
362 metabolites. Further studies using isotope-labeled cell lysate and additional replicates may help to
363 investigate these complex interactions.
364

365 ***Ca.* Nealsonbacteria DGGOD1a's genome matches with symbiotic epibiotic lifestyle**

366 We successfully assembled and closed the genome of *Ca.* Nealsonbacteria DGGOD1a (Genbank:
367 CP092821 and IMG ID: 2791354853). The complete genome is tiny: a mere 1.16 Mbp with 1145
368 predicted coding regions, that includes 18 pairs of perfect repeats over 400 bp. Interestingly, the
369 majority of these repeats are predicted transposases, and in fact, this genome has an
370 overrepresentation of predicted transposases, a total of 34, which is the most found in any
371 Parcubacteria genome and possibly of the highest number of transposases per base pair of genomes
372 sequenced to date. Most of metabolic pathways annotated by automatic pipelines are incomplete
373 (Table S12). Key findings are summarized in Figure 6. As inferred from genomes of other
374 members of the CPR group, it can use the pentose phosphate pathway to bypass glycolysis. Also,
375 basic transport pathways, and pathways for the biosynthesis of most amino acids, vitamins, and
376 lipids are incomplete. The large ribosomal RNA subunit L1 is missing; a common feature of
377 members of the OD1-L1 group (6, 11). It is also worth mentioning that two toxin and five antitoxin
378 genes were found in the genome which could participate in its symbiotic or parasitic lifestyle (27,
379 28). Many pili, cell surface, and secretion genes were found in this genome which could also

380 support an epibiont lifestyle. A CRISPR system with the complex of cas4, cas1, and cas2 was also
381 found with 49 spacers. Unfortunately, a spacer target search against all available OR consortium
382 metagenomic DNA did not return a single hit other than the CRISPR matrix itself.
383
384 **Implications**
385 This study revealed that *Ca.* Nealsonbacteria likely contributes to biomass recycling in a
386 methanogenic benzene degrading enrichment culture, and in so doing enhances the growth of
387 benzene fermenters. Although we were unable to target *Ca.* Nealsonbacteria cells using specific
388 FISH probes, the substantial enrichment of this phylotype on lysed biomass provided a culture
389 background in which to be certain that tiny DAPI-stained cells observed by epifluorescence were
390 in fact *Ca.* Nealsonbacteria. Once identified, we could observe their association with
391 *Methanosaeta*, which was also consistent with relative abundance from amplicon sequencing data.
392 SEM and CryoEM further confirmed and refined these observations.
393
394 *Ca.* Nealsonbacteria grew and became enriched when provided with cell lysate in the
395 absence of benzene in this methanogenic benzene-degrading culture. This result strongly supports
396 a biomass recycling role for *Ca.* Nealsonbacteria in this and likely in other anaerobic environments
397 where *Ca.* Nealsonbacteria are found. Diverse microscopic observations combined with a highly
398 reduced genome corroborate a reliance on a host which in this culture was *Methanosaeta* in a clear
399 example of an unusual cross-kingdom episymbiosis. The observed direct attachment or connection
400 between *Ca.* Nealsonbacteria to the surface of *Methanosaeta* implies metabolite exchange or direct
401 electron transfer between the two cell types. Perhaps the pili encoded in the genome of *Ca.*
402 Nealsonbacteria are used for attachment to the host and possibly as a conduit electron transfer. *Ca.*

403 Nealsonbacteria were also found to be beneficial for the growth of benzene-metabolizing
404 *Deltaproteobacteria* ORM2 from Candidate clade Sva0485. Perhaps *Ca.* Nealsonbacteria help
405 recycle nutrients or cofactors required to initiate and sustain higher benzene biodegradation rates.
406 These results guide future experiments into specific relationships between *Ca.* Nealsonbacteria
407 and benzene degradation in these enrichment cultures, as well as to suggest possible analogous
408 roles in other anaerobic communities, particularly where energy input is limited, such as deep
409 subsurface environments.
410
411
412 **References**

413 1. Ulrich AC, Edwards EA. Physiological and molecular characterization of anaerobic
414 benzene-degrading mixed cultures. Environ Microbiol. 2003;5:92-102.
415 2. Luo F, Devine CE, Edwards EA. Cultivating microbial dark matter in benzene-degrading
416 methanogenic consortia. Environ Microbiol. 2016;18:2923-36.
417 3. Toth CRA, Luo F, Bawa N, Webb J, Guo S, Dworatzek S, et al. Anaerobic benzene
418 biodegradation linked to the growth of highly specific bacterial clades. Environ Sci
419 Technol. 2021;55(12):7970-80.
420 4. Guo S, Toth CRA, Luo F, Chen X, Xiao J, Edwards EA. Transient oxygen exposure causes
421 profound and lasting changes to a benzene-degrading methanogenic community. BioRxiv.
422 2022.
423 5. Griebler C, Lueders T. Microbial biodiversity in groundwater ecosystems. Freshw Biol.
424 2009;54(4):649-77.

425 6. Brown CT, Hug LA, Thomas BC, Sharon I, Castelle CJ, Singh A, et al. Unusual biology
426 across a group comprising more than 15% of domain Bacteria. Nature.
427 2015;523(7559):208-11.
428 7. Hug LA, Baker BJ, Anantharaman K, Brown CT, Probst AJ, Castelle CJ, et al. A new view
429 of the tree of life. Nat Microbiol. 2016;1:16048.
430 8. Kantor RS, Wrighton KC, Handley KM, Sharon I, Hug LA, Castelle CJ, et al. Small
431 genomes and sparse metabolisms of sediment-associated bacteria from four candidate
432 phyla. mBio. 2013;4(5):e00708-13.
433 9. Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng JF, et al. Insights
434 into the phylogeny and coding potential of microbial dark matter. Nature.
435 2013;499(7459):431-7.
436 10. Nelson WC, Stegen JC. The reduced genomes of Parcubacteria (OD1) contain signatures
437 of a symbiotic lifestyle. Front Microbiol. 2015;6:713.
438 11. Castelle CJ, Brown CT, Thomas BC, Williams KH, Banfield JF. Unusual respiratory
439 capacity and nitrogen metabolism in a Parcubacterium (OD1) of the Candidate Phyla
440 Radiation. Sci Rep. 2017;7:40101.
441 12. Tian R, Ning D, He Z, Zhang P, Spencer SJ, Gao S, et al. Small and mighty: adaptation of
442 superphylum *Patescibacteria* to groundwater environment drives their genome simplicity.
443 Microbiome. 2020;8:51.
444 13. Wrighton KC, Thomas BC, Sharon I, Miller CS, Castelle CJ, VerBerkmoes NC, et al.
445 Fermentation, hydrogen, and sulfur metabolism in multiple uncultivated bacterial phyla.
446 Science. 2012;337(6102):1661-5.

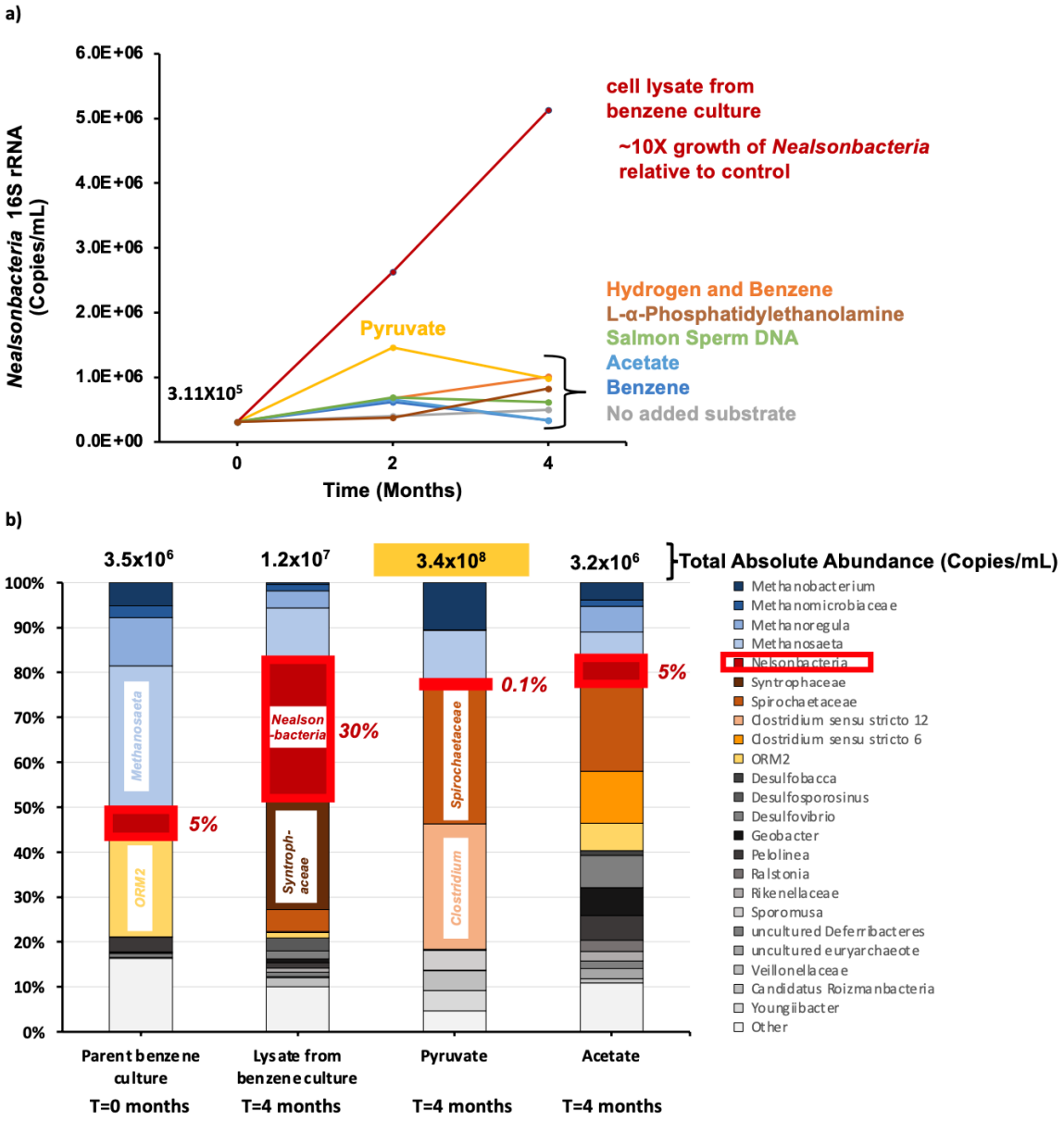
447 14. He X, McLean JS, Edlund A, Yooseph S, Hall AP, Liu SY, et al. Cultivation of a human-
448 associated TM7 phylotype reveals a reduced genome and epibiotic parasitic lifestyle. Proc
449 Natl Acad Sci U S A. 2015;112(1):244-9.
450 15. He C, Keren R, Whittaker ML, Farag IF, Doudna JA, Cate JHD, et al. Genome-resolved
451 metagenomics reveals site-specific diversity of episymbiotic CPR bacteria and DPANN
452 archaea in groundwater ecosystems. Nat Microbiol. 2021;6(3):354-65.
453 16. Jaffe AL, Castelle CJ, Matheus Carnevali PB, Gribaldo S, Banfield JF. The rise of diversity
454 in metabolic platforms across the Candidate Phyla Radiation. BMC Biol. 2020;18:69.
455 17. Anantharaman K, Brown CT, Hug LA, Sharon I, Castelle CJ, Probst AJ, et al. Thousands
456 of microbial genomes shed light on interconnected biogeochemical processes in an aquifer
457 system. Nat Commun. 2016;7:13219.
458 18. Raskin L, Poulsen LK, Noguera DR, Rittmann BE, Stahl DA. Quantification of
459 methanogenic groups in anaerobic biological reactors by oligonucleotide probe
460 hybridization. Appl Environ Microbiol. 1994;60(4):1241-8.
461 19. Thiel J, Byrne JM, Kappler A, Schink B, Pester M. Pyrite formation from FeS and H_2S is
462 mediated through microbial redox activity. Proc Natl Acad Sci U S A. 2019;116(14):6897-
463 902.
464 20. Liu Y, Balkwill DL, Aldrich HC, Drake GR, Boone DR. Characterization of the anaerobic
465 propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and
466 *Syntrophobacter wolinii*. International Journal of Systematic Bacteriology. 1999;49:545-
467 56.

468 21. Nobu MK, Narihiro T, Rinke C, Kamagata Y, Tringe SG, Woyke T, et al. Microbial dark
469 matter ecogenomics reveals complex synergistic networks in a methanogenic bioreactor.
470 ISME J. 2015;9(8):1710-22.
471 22. Dong X, Greening C, Brüls T, Conrad R, Guo K, Blaskowski S, et al. Fermentative
472 Spirochaetes mediate necromass recycling in anoxic hydrocarbon-contaminated habitats.
473 ISME J. 2018;12:2039-50.
474 23. Luef B, Frischkorn KR, Wrighton KC, Holman HY, Birarda G, Thomas BC, et al. Diverse
475 uncultivated ultra-small bacterial cells in groundwater. Nat Commun. 2015;6:6372.
476 24. Poulsen LK, Ballard G, Stahl DA. Use of rRNA fluorescence in situ hybridization for
477 measuring the activity of single cells in young and established biofilms. Appl Environ
478 Microbiol. 1993;59(5):1354-60.
479 25. Beveridge TJ, Patel GB, Harris BJ, Sprott GD. The ultrastructure of *Methanothrix concilii*,
480 a mesophilic aceticlastic methanogen. Can J Microbiol. 1986;32(9):703-10.
481 26. Miyoshi T, Iwatsuki T, Naganuma T. Phylogenetic characterization of 16S rRNA gene
482 clones from deep-groundwater microorganisms that pass through 0.2-micrometer-pore-
483 size filters. Appl Environ Microbiol. 2005;71(2):1084-8.
484 27. Magnuson RD. Hypothetical functions of toxin-antitoxin systems. J Bacteriol.
485 2007;189(17):6089-92.
486 28. Van Melderen L, Saavedra De Bast M. Bacterial toxin-antitoxin systems: more than selfish
487 entities? PLoS Genet. 2009;5(3):e1000437.

488

489 **Figures and Figure Legends**

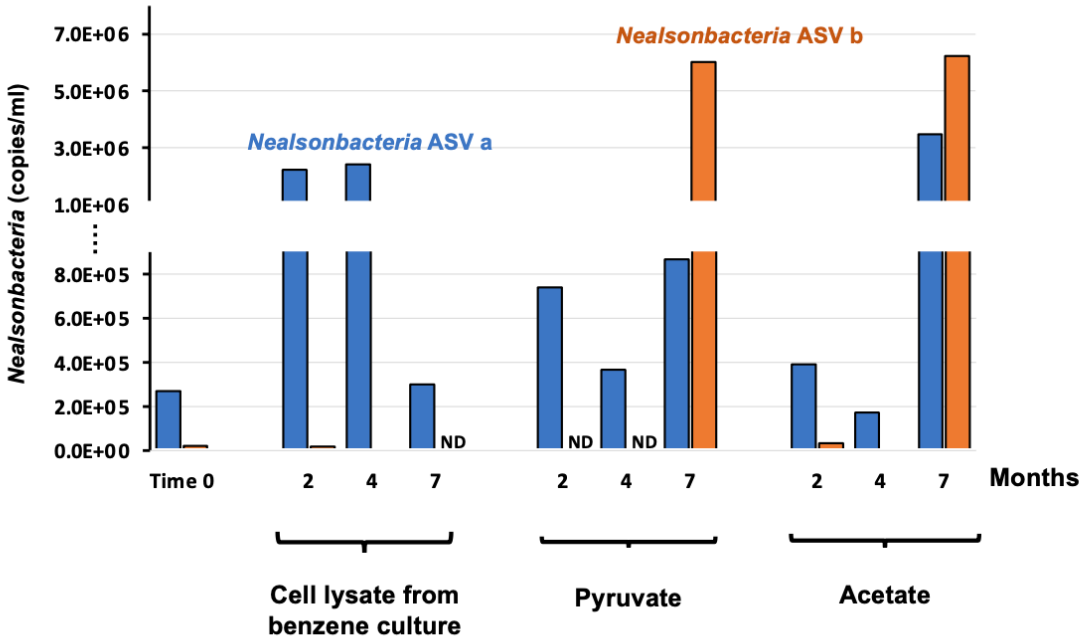
490



491

492 **Figure 1. Results of Growth Trial #1.** a) Absolute *Ca.* Nealsonbacteria abundance measured by
493 qPCR in copies/mL, and b) microbial community profiles (relative abundance) as a function of
494 substrate after 4 months of incubation, with total abundance shown above bars. In panel a) one
495 DNA sample was collected on day 1 to represent initial conditions. Subsequent DNA samples were
496 collected by sacrificing one entire bottle at each time point for each treatment. In panel b) ASVs
497 with relative abundance below 1% were combined as "other". ASVs with the same taxonomy at
498 the genus level were combined. Total abundance was obtained by adding the qPCR results for
499 general bacteria, general archaea and *Ca.* Nealsonbacteria together. "Parent Benzene culture"
500 column is a 10% transfer of the parent culture at time zero.

501



502

503 **Figure 2. *Ca.* Nealsonbacteria strain abundance over 7 months of incubation.** Figure shows
504 treatments amended with benzene culture lysate, acetate, or pyruvate from Donor Trial #1.
505 Absolute abundance of *Ca.* Nealsonbacteria strains ASVa and ASVb were calculated by
506 multiplying the relative abundance of these ASVs (obtained from amplicon sequencing) by the
507 estimate of total bacterial abundance.

508

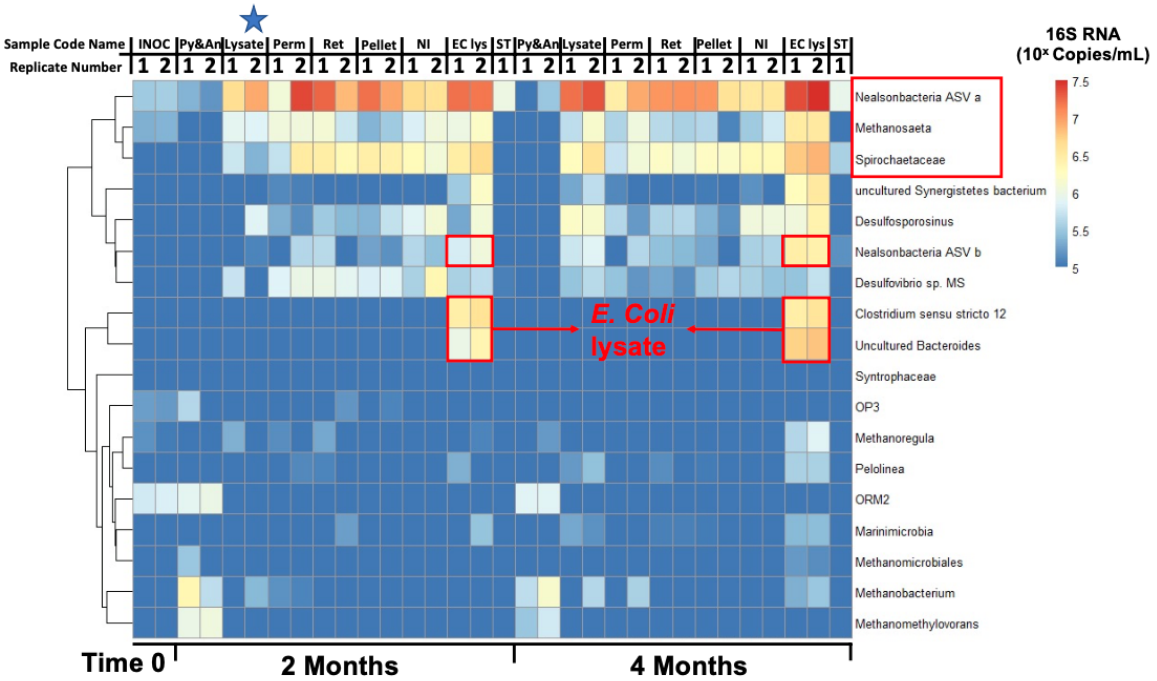


Figure 3. Heatmap of absolute abundance of top 18 ASVs in growth Trial #2. Rows were clustered using a hierarchical clustering algorithm "hclust" in R based on similarity of absolute abundance. The columns represent different treatment bottles: INOC: Bottle with only 10% inoculum at time 0; NI: no inoculum (lysate only); Py&An: pyruvate & antibiotics; Lysate: French pressed benzene culture; Perm: lysate supernatant permeate through 50 kDa membrane; Ret: lysate supernatant retentate; Pellet: lysate pellet after centrifugation at 13,000 x g for 20 min; EC lys: French pressed lysate of *E. coli* culture; ST: Starved culture.

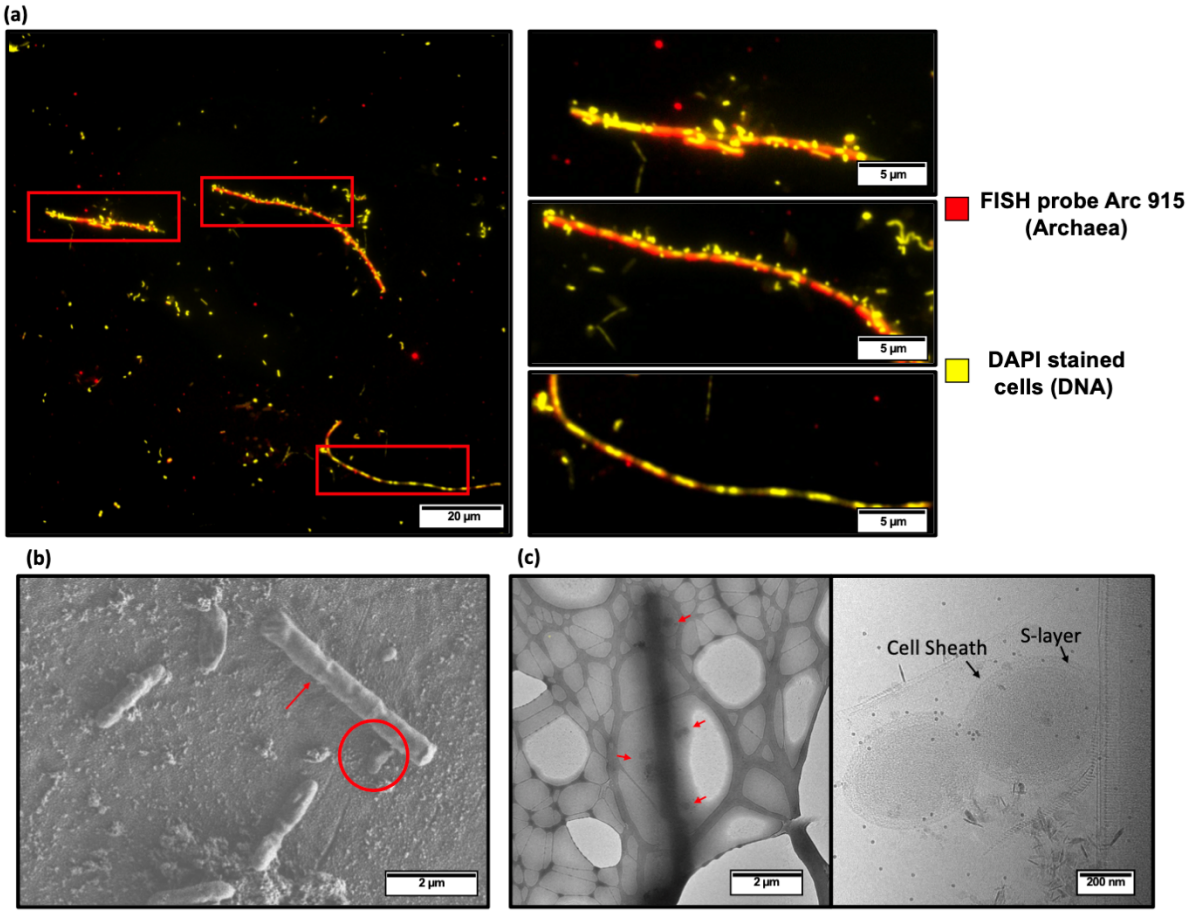


Figure 4. Various microscopic images of *Ca.* Nealsonbacteria in enrichment cultures. (a) Epifluorescence microscopy showing archaea (red) and DAPI (yellow). Images are false colored and overlayed using ImageJ. Images on the right are zoomed in views of red rectangular area in left image highlighting archaeal filaments with and without attached cells. (b) SEM images of methanogenic benzene-degrading culture prepared by ionic liquid exchange. *Methanosaeta* filament shown with red arrow. *Ca.* Nealsonbacteria-like cell is marked with a red circle. (c) Cryto-TEM images of *Ca.* Nealsonbacteria enrichments. *Ca.* Nealsonbacteria-like cells are marked by red arrows and the right-side shows a zoomed in view of two cells that appear to be budding. Additional microscopic images are shown in Figures S6 and S7.

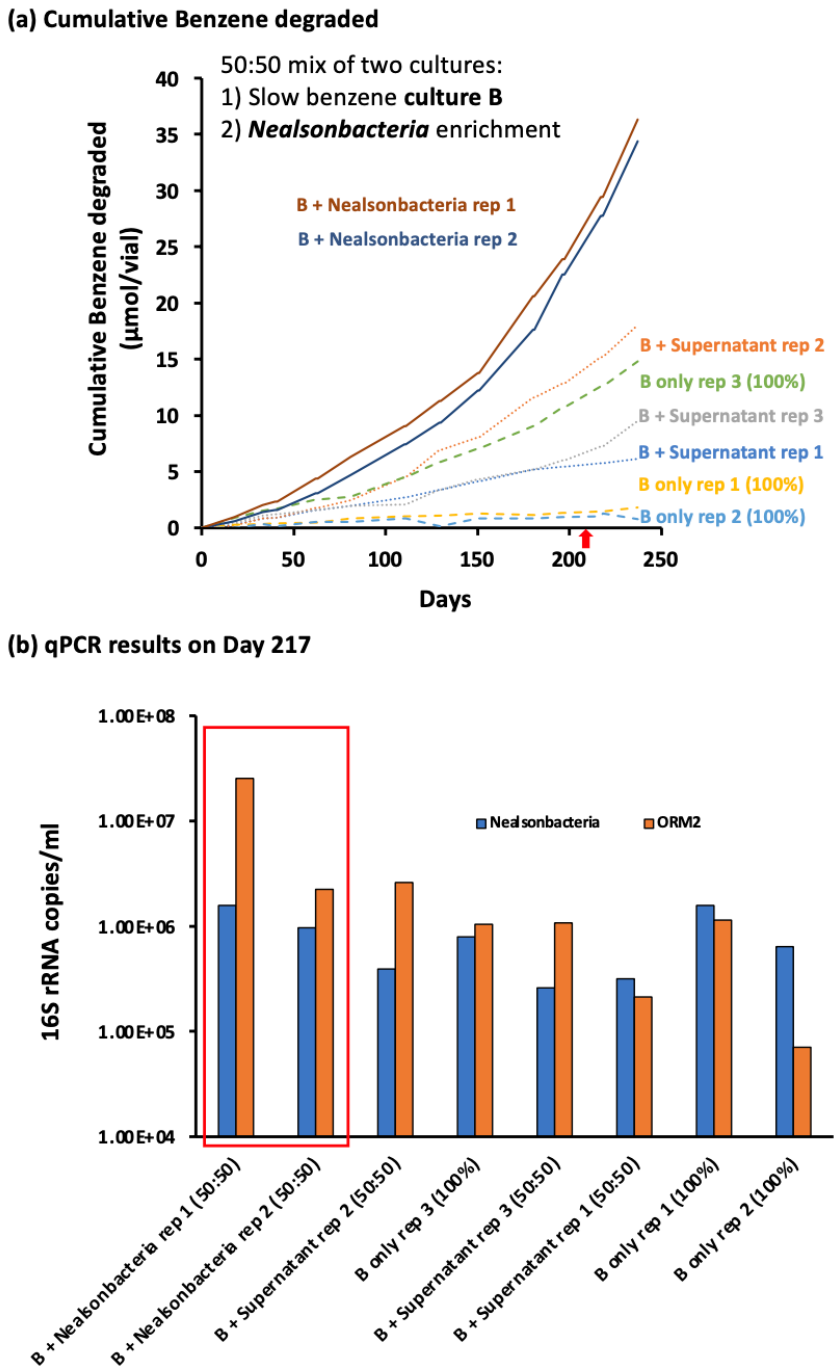
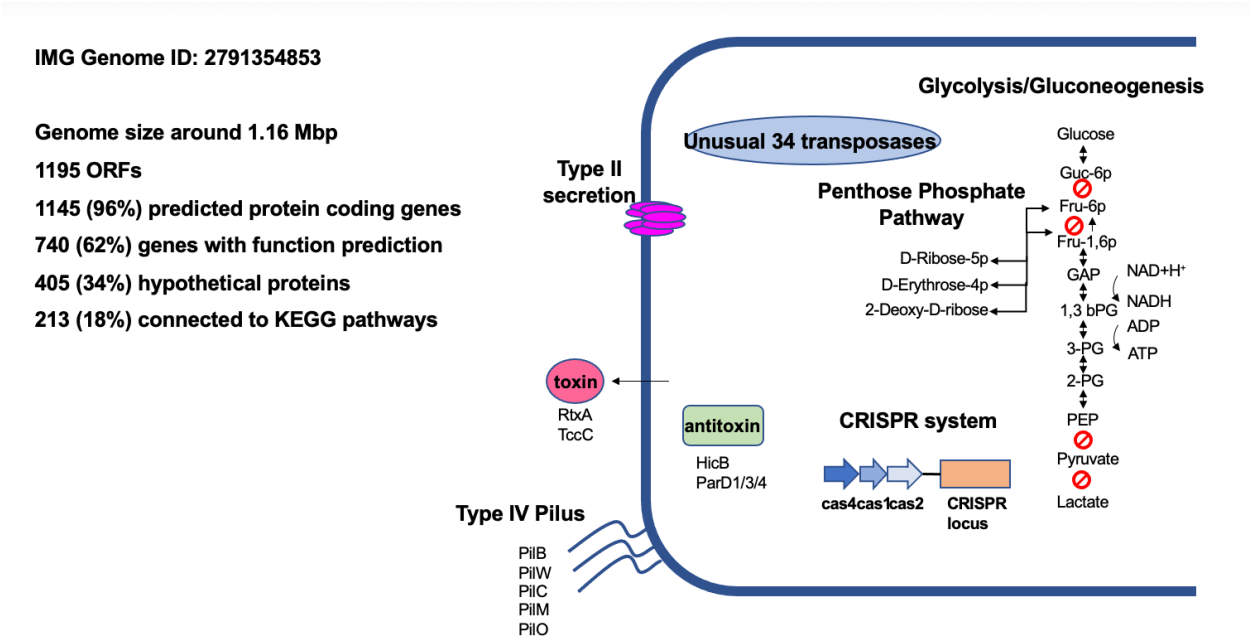


Figure 5. Impact of adding *Ca.* Nealsonbacteria enrichment to a slow benzene degrading culture. a) Cumulative benzene consumption and b) qPCR results tracking *Ca.* Nealsonbacteria and ORM2 on Day 217. The qPCR results are sorted by descending order of benzene degradation rate.



535
536 **Figure 6. A summary of key findings from the genome of *Ca.* Nealsonbacteria DGGOD1a**.
537 Detailed annotations for each ORF are in Table S12.