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Ecological and genomic analyses of candidate phylum WPS-2 bacteria in an unvegetated soil

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Journal

Environmental Microbiology, 22(8)

ISSN

1462-2912

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Publication Date

2020-08-01

DOI

10.1111/1462-2920.15054

Peer reviewed

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2 **Ecological and genomic analyses of candidate**
3 **phylum WPS-2 bacteria in an unvegetated soil**

4

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19

20 Running title: **Organoheterotrophic WPS-2 bacteria**

21

22 Keywords: WPS-2, Eremiobacteraeota, acidophile, uncultured bacteria

23

24 Supplementary File 1: Supplementary Tables and Figures

25 Supplementary File 2: Reference genomes and genome predictions

26 **Originality-Significance Statement**

27 The manuscript describes the characterization of bacteria belonging to an
28 uncultured phylum (WPS-2 or Eremiobacteria), using a combination of methods
29 including community analysis, quantitative PCR of environmental samples, single
30 cell genomics and metagenomics. We qualitatively and quantitatively analysed
31 communities in a large sample set taken across different microhabitats of an area
32 affected by iron-sulfur springs. The data revealed a preference of WPS-2 and its
33 associated community network to bare rather than vegetated soils. We then
34 assembled genomes using both SAG and MAG techniques. Cross-referencing of
35 SAGs and MAGs increased the reliability of genomic inferences. We propose that
36 most WPS-2 bacteria in our site are efficient heterotrophic scavengers. **These**
37 **bacteria are therefore physiologically distinct from previously described members of**
38 **the WPS-2 phylum, which have been proposed to be lithoautotrophs and**
39 **photoautotrophs.** In combination with previous studies, these data suggest that
40 phylum WPS-2 includes bacteria with diverse metabolic capabilities. Our
41 combination of ecogeographic community analysis and genome inference provides
42 a detailed analysis for a member of this uncultured bacterial phylum.

43

44 **Summary**

45 **Members of the bacterial candidate phylum WPS-2 (or Eremiobacteraeota)**
46 **are abundant in several dry, bare soil environments. In a bare soil**
47 **deposited by an extinct iron-sulfur spring, we found that WPS-2 comprised**
48 **up to 24% of the bacterial community and up to 10^8 cells per g of soil**
49 **based on 16S rRNA gene sequencing and quantification. A single genus-**
50 **level cluster (*Ca. Rubrimentiphilum*) predominated in bare soils, but was**
51 **less abundant in adjacent forest soils. Nearly complete genomes of *Ca.***
52 ***Rubrimentiphilum* were recovered as single amplified genomes (SAGs) and**
53 **metagenome-assembled genomes (MAGs). Surprisingly, given the**
54 **abundance of WPS-2 in bare soils, the genomes did not indicate any**
55 **capacity for autotrophy, phototrophy, or trace gas metabolism. Genomic**
56 **analysis instead suggesting a predominantly aerobic organoheterotrophic**
57 **lifestyle, perhaps based on parasitizing or scavenging amino acids,**
58 **nucleotides, and complex oligopeptides, along with lithotrophic capacity**
59 **on thiosulfate. Other notable features included many genes encoding**
60 **resistance to antimicrobial compounds. Network analyses of the entire**
61 **community showed that some species of *Chloroflexi*, *Actinobacteria*, and**
62 **candidate phylum AD3 (or Dormibacterota) co-occurred strongly with *Ca.***
63 ***Rubrimentiphilum*, and may represent ecological or metabolic partners.**
64 **We propose that *Ca. Rubrimentiphilum* act as efficient heterotrophic**
65 **scavengers in the site. In combination with previous studies, these data**
66 **suggest that the phylum WPS-2 includes bacteria with diverse metabolic**
67 **capabilities.**

68

69 **Introduction**

70 There are between 112 and 1500 main lineages or phyla within the Domain
71 *Bacteria*, depending on the criteria used for definition (Yarza *et al.*, 2014; Parks *et*
72 *al.*, 2018). However all estimates agree that most bacterial phyla have no cultured
73 members. Recent advances in high-throughput sequencing, single-cell sorting, and
74 bioinformatics have facilitated genomic reconstructions of individual bacteria from
75 many of these uncultivated phyla (Rinke *et al.*, 2013; Eloe-Fadrosh *et al.*, 2016; Hug
76 *et al.*, 2016; Parks *et al.*, 2017). There are two principal approaches to recovering
77 individual genomes of uncultured bacteria. Individual cells can be sorted out of
78 complex microbial communities and then subjected to whole genome amplification
79 and shotgun sequencing yielding single amplified genomes (SAGs); or individual
80 genomes can be computationally separated from a complex metagenome using
81 binning approaches based on composition, coverage, and other contig properties,
82 yielding metagenome assembled genomes (MAGs). These techniques have already
83 proven useful in describing uncultured phyla. The Genomic Encyclopedia of Bacteria
84 and Archaea Microbial Dark Matter project provided substantial SAG data for over
85 20 candidate phyla, and in doing so identified a novel purine synthesis pathway and
86 variants in the genetic code (Rinke *et al.*, 2013). MAG studies have been utilized to
87 describe candidate phyla from contaminated aquifers (Wrighton *et al.*, 2012; Hug *et*
88 *al.*, 2016), deep terrestrial biospheres (Wu *et al.*, 2016), permafrost soils (Taş *et al.*,
89 2014), and other sites (Parks *et al.*, 2017; Holland-Moritz *et al.*, 2018). Drawbacks of
90 these approaches include incomplete genomes, genome contamination, and the
91 difficulty of predicting phenotypes based on annotation alone. Predicting
92 phenotypes is especially problematic with candidate phyla, which are often
93 evolutionarily deeply rooted groups that often contain many hypothetical genes

94 and/or genes with low sequence homology to characterized reference genes.
95 Nevertheless, in combination with ecological data these are powerful approaches to
96 characterizing new microbial groups.

97

98 The Paint Pots, located in British Columbia, Canada, is a system of naturally
99 occurring acidic iron-sulfur springs with high concentrations of heavy metals
100 (Grasby *et al.*, 2013). It is an important site to the indigenous Ktunaxa people, and a
101 tourist destination in Kootenay National Park. Geological studies have shown that as
102 the spring precipitates iron oxide at its outlet, the hydraulic pressure increases,
103 eventually causing the spring source to migrate and leaving a relic spring feature
104 behind. Although the site has geochemical similarities to acid mine drainage (AMD),
105 the microbial community is not typical of AMD (Grasby *et al.*, 2013). Instead, the
106 community is more similar to communities in natural acidic soils, with one
107 peculiarity being an elevated abundance of the candidate phylum WPS-2 in some
108 areas.

109

110 WPS-2 is recognizable as a phylum-level grouping in most bacterial taxonomy
111 databases and classification systems (Parks *et al.*, 2018). Ji *et al.* (2017) have
112 suggested the alternate name Eremiobacteraeota for WPS-2. Since neither name
113 has validity in official nomenclature, we will primarily use “WPS-2” here due to its
114 precedence. WPS-2 bacteria were originally detected in polychlorinated biphenyl
115 (PCB) polluted soil (Nogales *et al.*, 2001), and later in other soils (Costello *et al.*,
116 2009; Lin *et al.*, 2012; Grasby *et al.*, 2013; Pascual *et al.*, 2016; Hermans *et al.*,
117 2017), various temperate to Arctic peatlands (Bragina *et al.*, 2015; Holland-Moritz *et*
118 *al.*, 2018; Woodcroft *et al.*, 2018), gas-producing shale (Trexler *et al.*, 2014), the

119 canine oral microbiome (Dewhurst *et al.*, 2012; Camanocha and Dewhurst, 2014).
120 They are abundant in several organic-poor soil environments such as bare Antarctic
121 soils (Ji *et al.*, 2016), Arctic cryoconite (Stibal *et al.*, 2015), extremely dry volcanic
122 soils (Costello *et al.*, 2009), and bare metal-contaminated soils (Grasby *et al.*, 2013).
123 WPS-2 MAGs were recently described from bare soils in Antarctica and the potential
124 ecological role of these bacteria in autotrophic CO₂ fixation and scavenging of
125 atmospheric H₂ was proposed (Ji *et al.*, 2016). MAGs of WPS-2 bacteria associated
126 with boreal mosses also contained genes encoding the Calvin Cycle, as well as key
127 genes for bacteriochlorophyll-based anoxygenic photosynthesis (Holland-Moritz *et*
128 *al.*, 2018; Ward *et al.*, 2019).

129

130 The unusually high relative abundance of WPS-2 at the Paint Pots location, higher
131 than reported anywhere else, provides an ideal situation for studying members of
132 this candidate phylum, and simplifies genome recovery. In this study we performed
133 a detailed environmental survey of microbial communities, and WPS-2 specifically,
134 throughout the site. Forested sites adjacent to the bare soils served as a control for
135 assessing the role of vegetation and soil organic matter in shaping the bacterial
136 communities. MAG and SAGs were constructed to predict the physiology of the
137 WPS-2 bacteria.

138

139 **Results**

140 *Environmental distribution of candidate phylum WPS-2*

141 Based on 16S rRNA gene amplicon sequencing, microbial communities in vegetated
142 soils of the experimental area generally had low relative abundances of WPS-2
143 (<2.5 % of total reads), while bare ochre-coloured soils supported higher relative

144 abundances of the phylum (Figure 1; Supplementary Table 1). In transects, WPS-2
145 relative abundance increased from the forest edge outwards into the bare Mound
146 soil, reaching maxima of 15%-24% (Figure 1, Supplementary Figure 1).

147

148 Multiple 16S rRNA gene-based OTUs of WPS-2 were detected, which
149 phylogenetically fell into two distinct clusters (Supplementary Figure 2). The
150 primary cluster we denote as *Ca. Rubrimentiphilales*, and its most abundant OTU as
151 *Ca. Rubrimentiphilum* (from L. neut. n. rubrimentum, red ink; N.L. neut. adj. philum
152 [from Gr. neut. adj. philon], friend, loving). The *Ca. Rubrimentiphilales* bacteria
153 comprised 72% of the WPS-2 16S rRNA gene reads, on average, over all samples
154 collected (Supplementary Table 1). The other WPS-2 cluster we refer to as AS-11
155 based on Silva (Supplementary Figure 2).

156

157 The absolute abundance of bacteria belonging to the *Ca. Rubrimentiphilales* cluster,
158 based on a specific qPCR assay, was significantly higher in bare soils than in
159 vegetated soils (Figure 2A). This higher absolute abundance was measured in bare
160 soils despite the fact that significantly less DNA was recoverable, indicating a lower
161 overall bacterial load (Figure 2A). The relative abundance of *Ca. Rubrimentiphilales*
162 (based on its share of reads in 16S rRNA amplicons) was positively correlated with
163 absolute abundance (based on qPCR), and negatively correlated with the total
164 community DNA (Figure 2B). Its predominance in bare soil was therefore a
165 combined effect of higher *Ca. Rubrimentiphilales* populations and lower populations
166 of other bacteria.

167

168 Compared to forested soils, bare soils had higher relative abundances of the phyla
169 *Chloroflexi* and candidate phylum AD3 along with WPS-2, while *Proteobacteria*,
170 *Acidobacteria*, and *Verrucomicrobia* were lower (Supplementary Figure 1). An OTU
171 co-occurrence analysis (Figure 3) similarly showed a small network of OTUs
172 connected to *Ca. Rubrimentiphilum*, including *Thermogemmatissporaceae*
173 (*Chloroflexi*), candidate phylum AD3, *Conexibacter* (*Actinobacteria*),
174 *Beijerinckiaceae* (*Proteobacteria*) and AS11 (WPS-2).

175

176 WPS-2 SAGs and MAGs

177 Binning of several metagenomes made from DNA extracts of bare Mound soils
178 (Supplementary Table 3) failed to produce high-quality MAGs of WPS-2. We
179 therefore applied a differential centrifugation procedure to preferentially recover
180 small cells from the soil. This procedure depleted communities of *Actinobacteria*,
181 *Acidobacteria*, and *Chloroflexi*, while WPS-2 and TM7 became more predominant
182 (Supplementary Figure 3). The overall diversity was also lowered by the
183 centrifugation procedure, as shown by the Shannon Index (Supplementary Figure
184 3). This simplified community dominated by WPS-2 was metagenome sequenced in
185 an attempt to obtain high-quality MAGs. MetaBAT binning yielded 12 bins
186 (Supplementary Figure 4), two of which (bins 6 and 7) contained 16S rRNA gene
187 fragments (<120 bp) identified as WPS-2. Bin 6 was particularly well assembled,
188 with only 48 contigs and an N_{50} of 185 kb. However, MAG Bin 6 had two copies of
189 nearly every single-copy marker gene based on CheckM. The most likely
190 explanation was the presence of two similarly abundant strains of WPS-2 in bin 6. As
191 this bin was comprised of only 48 contigs, we applied a manual curation procedure
192 to divide it into 2 sub-bins (6A and 6B). Details of the procedure and results are

193 given in Supplementary Table 4. The resulting sub-bins were 2.34-2.58 Mbp and
194 estimated to be 95% complete (Table 1).

195

196 Eight WPS-2 SAGs were also generated. These ranged from 0.58 bp to 1.66 Mbp,
197 with estimated completeness from 12% to 54% (Table 1). The SAGs were used
198 primarily to verify the MAG data. Local BLAST and ANI searches against the
199 combined SAG data demonstrated that all 48 contigs in MAG bin 6 could be
200 recruited to these SAGs at high (>75%) nucleotide identity (Supplementary Figure
201 5), suggesting that the WPS-2 MAGs contained no contaminating DNA sequences
202 from other bacteria.

203

204 Pairwise average nucleotide identity (ANI) and average amino acid identity (AAI)
205 comparisons between the 8 SAGs and 2 MAGs were performed using IMG/M
206 (Supplementary Table 5). ANIs were never <78%, and AAls never <76%, which
207 represent roughly genus level thresholds (Konstantinidis and Tiedje, 2005;
208 Rodriguez-R and Konstantinidis, 2014; Rodriguez-R *et al.*, 2018). We conclude that
209 all SAGs and MAGs belong to a single genus (Rodriguez-R *et al.*, 2018), for which we
210 suggest the name *Candidatus* Rubrimentiphilum. However, 5 separate putative
211 species of this genus were identified based on a ANI threshold of 95% (Richter and
212 Rosselló-Móra, 2009).

213

214 Phylogenetic analyses verified the close relationship of the SAGs, MAGs, and sub-
215 OTUs from the amplicon analyses. One SAG (H17) contained a full-length 16S rRNA
216 gene sequence, which matched perfectly to a 16S rRNA gene fragment in MAG bin 6
217 and to a sub-OTU (rubr5) from the V3-V4 amplicon analyses (Supplementary Figure

218 2). This sequence was closely related to the most predominant sub-OTU (rubr1).
219 Highly resolved phylogenies were also created for the SAGs and the MAGs based on
220 a set of 56 conserved genomic markers (Figure 4), verifying that all SAGs and MAGs
221 from this study were closely related and belonged to phylum WPS-2.

222

223 *Phylogenetic and functional comparisons of WPS-2 genomes*

224 A phylogenetic reconstruction using 56 conserved genomic marker genes, including
225 all available WPS-2 genomes along with selected reference genomes, placed the
226 SAGs and MAGs from our study into a monophyletic cluster denoted as *Ca.*
227 *Rubrimentiphilum* (Figure 4). Several WPS-2 MAGs from a large-scale study of Arctic
228 peatlands (Woodcroft *et al.* 2018) fell into this group as well. Potential hydrogenase-
229 encoding genes and key genes encoding ribulose biphosphate carboxylase are
230 widespread among WPS-2 genomes, but were not found in any of the *Ca.*
231 *Rubrimentiphilum* genomes.

232

233 The abundance profiles of Pfams in the two *Ca.* *Rubrimentiphilum* MAGs 6A and 6B
234 were compared to a taxonomically balanced reference database consisting of 2363
235 genomes of cultured bacteria. This analysis aimed to identify genomes with a
236 similar functional gene complement (i.e. a similar niche) to *Ca.* *Rubrimentiphilum*.
237 The most similar genomes to WPS-2 based on this analysis belonged to various soil
238 organoheterotrophs, mostly within the phyla *Acidobacteria*, *Armatimonadetes*,
239 *Actinobacteria*, and *Chloroflexi* (Figure 5; Supplementary Figure 6). All of the most
240 closely related bacteria were aerobic organoheterotrophs (Zarilla and Perry, 1984;
241 Sako *et al.*, 2003; Urios *et al.*, 2006; Barabote *et al.*, 2009; Johnson *et al.*, 2009;
242 Ward *et al.*, 2009; Wu *et al.*, 2009; Pati *et al.*, 2010; Dunfield *et al.*, 2012; Kielak *et*

243 *al.*, 2016), and none were autotrophic. Most were also thermoacidophiles, which
244 may indicate an abundance of polyextremophilic tolerance mechanisms in common
245 with *Ca. Rubrimentiphilum*.

246

247 Compared to the 2363 reference genomes, the *Ca. Rubrimentiphilum* genomes had
248 a large proportion of genes dedicated to amino acid and protein metabolism (Figure
249 6 and Supplementary Figure 7). Additionally, genes for “Cell wall, membrane,
250 envelope biogenesis” comprised more than 9% of *Ca. Rubrimentiphilum* genomes,
251 comparable only to *Acidobacteria* and significantly higher than the average in the
252 other selected lineages (Figure 6). Another notable property of *Ca.*
253 *Rubrimentiphilum* was the low number of genes responsible for inorganic ion
254 complexing and import, which could reflect the metal rich environment.

255

256 *Metabolic potential of Ca. Rubrimentiphilum*

257 Metabolic predictions for *Ca. Rubrimentiphilum* were made based on the nearly
258 complete MAG bins 6A and 6B, and the different SAGs (Figure 7). The genomic data
259 indicate rod-shape, flagella, P and type IV pili, and an outer membrane with a
260 typical lipid A core. A chromosomal cluster for biosynthesis of peptidoglycan
261 includes the usual enzymes for biosynthesis, translocation and cross-linking of a
262 disaccharide-pentapeptide monomeric unit, but in addition encodes a protein with
263 low similarity to the CofE enzyme, which catalyzes GTP-dependent glutamylation of
264 coenzyme F420 precursor. The presence of this enzyme may indicate unusual
265 peptidoglycan structure with peptide units modified with amino acids or polyamines.
266 Biosynthetic pathways for lysine, arginine, cysteine, branched-chain and aromatic
267 amino acids, folate, riboflavin, thiamin, biotin, pyridoxine, heme, and CoA are

268 complete. While cobalamin-dependent methionine synthase and ribonucleoside
269 diphosphate reductase are encoded, the genes for de novo cobalamin biosynthesis
270 appear to be lacking, suggesting the possibility of cross-feeding with other
271 populations in the community.

272

273 The genomes encode proteins for glycolysis, the oxidative pentose-phosphate
274 shunt, a complete TCA cycle, and an electron transport chain including an NADH
275 dehydrogenase complex, quinol-cytochrome c reductase (cytochrome bc₁
276 complex), aa₃-type cytochrome c oxidase, and an F₀-F₁-type ATPase (Figure 7). The
277 respiratory chain could utilize both ubiquinone and menaquinone, the latter
278 synthesized via a futasine pathway. The respiratory chain is likely to be even more
279 complex due to the presence of periplasmic and membrane-associated multi-copper
280 oxidase enzymes and other proteins of unknown function with cupredoxin domains.
281 The presence of all these genes suggests aerobic metabolism with the capability to
282 adapt to varying oxygen concentrations.

283

284 Some functions previously proposed to be important for WPS-2 bacteria, i.e.
285 autotrophy, phototrophy, and trace gas metabolism, were not evident in *Ca.*
286 *Rubrimentiphilum* (Figure 4; Supplementary Table 6). Homologues of genes
287 encoding the large subunit of ribulose biphosphate carboxylase, although common
288 in other WPS-2 bacteria, were not found in any *Ca. Rubrimentiphilum* SAG or MAG,
289 including those detected in Arctic peats (Figure 4), ruling out the Calvin Benson
290 Bassham Cycle. Key steps for other inorganic carbon fixation pathways were also
291 missing. There was also no evidence of phototrophy in *Ca. Rubrimentiphilum*.
292 Indeed, there was no evidence for chlorophyll or bacteriochlorophyll-based

293 phototrophy in the entire soil metagenome (although there are predicted
294 bacteriorhodopsin-encoding genes), suggesting that phototrophy is not driving the
295 community as a whole. Trace gas metabolism-encoding genes were abundant in the
296 soil metagenome, over 800 genes were annotated as hydrogenase components.
297 However, none were associated with *Ca. Rubrimentiphilum*. Only a weak homologue
298 to a gene for aerobic CO/xanthine dehydrogenase (<40% identity) was detected.

299

300 These bacteria appear to prefer nucleotides and amino acids as growth substrates.

301 We predict that it can degrade glycine via glycine dehydrogenase complex,

302 branched-chain amino acids via branched-chain oxoacid dehydrogenase complex,

303 threonine via threonine-3-dehydrogenase and threonine aldolase; histidine via

304 histidine ammonia-lyase; tyrosine via homogentisate pathway; tryptophan via

305 kynurenine, and lysine via lysine aminomutase pathway. A high proportion of

306 protein processing pfams/COGs was noted in comparison to other bacteria (Figure

307 6). Particularly abundant individual COGs identified via analysis of heatmaps in IMG/

308 M included: peptide/Ni ABC transporter substrate binding proteins; dipeptidyl

309 aminopeptidase/acylaminoacyl peptidase; amino acid transporters; and TonB C-

310 terminal (Supplementary Table 6). TonB_C interacts with outer membrane

311 transporters to facilitate the transport of large molecules like siderophores, vitamin

312 B₁₂, Ni, or large polymers such as oligopeptides (Schauer *et al.*, 2008).

313

314 A few enzymes for metabolism of complex carbohydrates are encoded, including a

315 putative beta-hexosaminidase of the glycosyl hydrolase (GH) 20 family, putative GH

316 family 5 and GH43/DUF377 proteins, a secreted protein with low similarity to 1,3-

317 beta-glucanase, and an unusual protein with fused GH family 1 and a periplasmic

318 substrate-binding protein. Similar fusion proteins are also present in several
319 Cyanobacteria, but their enzymatic activity and physiological function remain
320 unexplored. N-acetyl-D-glucosamine kinase and N-acetylglucosamine-6-phosphate
321 deacetylase for peptidoglycan degradation are encoded. A third gene required for
322 this pathway, encoding glucosamine-6-phosphate deaminase, was not annotated,
323 but predicted copper amine oxidase-encoding genes are located near the N-
324 acetylglucosamine-6-phosphate deacetylase genes in each MAG that may serve this
325 function. Other potential functions for degradation of polymers include a predicted
326 peptidoglycan/xylan/chitin deacetylase, an N-acylglucosamine-6-phosphate 2-
327 epimerase involved in the N-acetylmannosamine (ManNAc) utilisation pathway
328 found in pathogenic bacteria, and β -N-acetylhexosaminidase (Supplementary Table
329 6). β -N-acetylhexosaminidases cleave and transfer diverse substrates including the
330 β -1,4 bond between N-acetylglucosamine and anhydro-N-acetylmuramic acid
331 (Slámová *et al.*, 2010).

332

333 Other energy sources may include formate and thiosulfate, the latter being oxidized
334 to tetrathionate by thiosulfate dehydrogenase. Genes expected in an iron oxidising
335 organism, i.e. those encoding ferric reductase or rusticyanin (Hedrich *et al.*, 2011)
336 were not predicted.

337

338 Many gene products were classified within beta lactamase pfam categories
339 (Supplementary Table 6). Indeed, the peptidoglycan degradation mechanisms
340 described above may be involved in the recycling of peptidoglycan units after the
341 action of beta lactams. In addition, four genes in each *Ca. Rubrimentiphilum* MAG
342 (and up to 5 in each SAG) were annotated as virginiamycin B lyase or streptogramin

343 lyase, enzymes active against actinomycetal antibiotics. Remarkably, this COG
344 (COG4257) was found in only 489 of 6992 finished bacterial genomes on IMG, and
345 never at more than 2 copies per genome. Each of the 4 copies in the MAGs are
346 different, with as little as 38% amino acid identity to each other.

347

348 *Enrichment and cultivation efforts*

349 The *Ca. Rubrimentiphilum*-specific qPCR assay was used for rapid screening of
350 potential enrichments (Supplementary Table 7). Incubations failed to yield
351 conclusive growth under any of the conditions tested (data not shown).

352

353 **Discussion**

354 An extensive 16S rRNA-gene based survey of microbial communities in the Paint
355 Pots area revealed an abundance of bacteria belonging to the candidate phylum
356 WPS-2. The most predominant WPS-2 OTUs belonged to a cluster we designated as
357 *Ca. Rubrimentiphilum*. These were particularly abundant in bare soils rich in iron
358 oxides deposited by former springs. The acidity, high metal content, and extremely
359 compact nature of these soils prevent vegetation from establishing (Grasby *et al.*,
360 2013). Recent studies have reported high relative abundances of WPS-2 in other
361 organic-poor soils, indicating that these may be a preferred habitat (Costello *et al.*,
362 2009; Stibal *et al.*, 2015; Ji *et al.*, 2016). Here we quantified this observation by
363 comparisons of adjacent bare and vegetated areas. Bacterial communities in
364 vegetated soil not only supported lower relative abundances of *Ca.*
365 *Rubrimentiphilum*, but also lower absolute abundances based on a qPCR assay,
366 indicating that these bacteria do indeed prefer bare soils.

367

368 There are similarities between the bacterial communities in the bare Paint Pots soils
369 and communities recently described in bare Antarctic soils by Ji *et al.* (2017). Both
370 show predominant WPS-2 and AD3 candidate phyla, along with
371 *Thermogemmatissporaceae* (*Chloroflexi*) and *Actinobacteria*. These assemblages are
372 likely adapted to stresses such as metal toxicity, low pH, extreme dryness, high
373 radiation, and very limited organic matter. Ji *et al.* (2017) present convincing
374 evidence that the Antarctic soil community is supported largely by trace gas
375 metabolism and autotrophy, and analysis of one WPS-2 MAG (bin 22) verified the
376 presence of genes encoding these functions (Ji *et al.*, 2017). Additionally, these
377 genes are found in multiple WPS-2 genomes reported by Woodcroft *et al.* (2018) as
378 summarized in Figure 4. Photoautotrophy has also been predicted in WPS-2 MAGs
379 recovered from *Sphagnum* wetlands (Holland-Moritz *et al.*, 2018; Ward *et al.*, 2019).
380 These MAGs contain 16S rRNA gene fragments too short to be unambiguously
381 placed on SSU phylogeny. However, genome-based phylogenies place them in
382 distant clusters of WPS-2 compared to the *Ca. Rubrimentiphilum* we analysed in this
383 study.

384

385 Genes for autotrophy and trace gas metabolism (but not phototrophy) were
386 conspicuous in our bare soil metagenome, and these processes likely provide much
387 of the primary productivity. For example, relatives of the *Thermogemmatissporaceae*
388 co-occurred strongly with *Ca. Rubrimentiphilum* in the bare soil. These bacteria
389 have been shown to oxidise atmospheric CO (King and King, 2014), and both
390 publicly available genomes of this group: *Thermogemmatispora* sp. T81 (Stott *et al.*,
391 2008) and *Thermogemmatispora carboxidovorans* PM5 (King and King, 2014) also
392 contain hydrogenases (but not the CBB cycle). However, although these processes

393 are undoubtedly critical to the soil community as a whole, they are not present in
394 the predominant WPS-2 cluster designated as *Ca. Rubrimentiphilum*. These
395 genomes lacked any obvious genes encoding methanotrophy, H₂ oxidation,
396 autotrophy, or phototrophy. We note that a second WPS-2 MAG predicted by Ji *et al.*
397 (2017) (bin 23) from Antarctic soil is closely related to our *Ca. Rubrimentiphilum*
398 group (91% identity, Supplementary Figure 2), and also does not show any evidence
399 of autotrophy or trace gas metabolism (Supplementary Table 6).

400

401 Instead, genome annotation of *Ca. Rubrimentiphilum* indicated an aerobic
402 heterotroph, perhaps growing on amino acids, polypeptides, nucleotides or some
403 complex polymers. Thiosulfate lithotrophy may provide additional energy. This
404 niche is also indicated by comparisons of the genomic pfam content to reference
405 cultured organisms. *Ca. Rubrimentiphilum* genomes were functionally most similar
406 to versatile organoheterotrophic bacteria, not lithoautotrophs. The most functionally
407 similar bacteria (Figure 5) are aerobic heterotrophs with versatile organic
408 substrates: for example *Acidothermus cellulolyticus* grows on diverse cell wall
409 polymers (Barabote *et al.*, 2009), while all known *Armatimonadetes* (Dunfield *et al.*,
410 2012) and most *Acidobacteria* (Ward *et al.*, 2009; Kielak *et al.*, 2016) grow on
411 several complex polymeric substrates. It seems counterintuitive for such an organic-
412 poor environment, but genomic comparisons with other bacteria, and specific
413 inferences based on genome annotations, both indicate a primarily
414 organoheterotrophic lifestyle for *Ca. Rubrimentiphilum*. The soil does experience
415 some input from deadfall, and a close examination did show the incorporation of
416 conifer needles. However, we did not detect elevated amounts of *Ca.*

417 Rubrimentiphilum associated with this organic material (data not shown). They may
418 instead grow in association with other bacteria.

419

420 A key to obtaining high quality MAGs in this study was differential extraction of
421 small cells from soil before metagenome sequencing. A similar filtration approach
422 has proven effective in characterizing members of the Patescibacteria (Rinke *et al.*,
423 2013; Parks *et al.*, 2018) or Candidate Phylum Radiation (Wrighton *et al.*, 2012; Hug
424 *et al.*, 2016). SAGs were also generated to verify the MAGs. All contigs in the MAGs
425 could be recruited to SAG DNA with high identity, indicating that MAGs were not
426 contaminated with DNA from other organisms. Metagenome binning always runs
427 the risk of contamination, but the combination of single cell genomics with
428 metagenomic binning of simplified communities in our study makes contamination
429 very unlikely, and adds confidence to our metabolic interpretations.

430

431 The estimated genome size of *Ca. Rubrimentiphilum* (max 2.6 Mb) is small but not
432 atypical of a free-living extremophile (Podar *et al.*, 2008). The bacterium co-occured
433 with a simple network of OTUs belonging to the *Chloroflexi*, AD3, *Acidobacteria*,
434 *Proteobacteria*, *Actinobacteria* and another group of WPS-2 (AS11). This network
435 may reflect direct biotic relationships such as parasitism, or it may simply reflect
436 common environmental adaptations. A remarkable feature of the *Ca.*

437 *Rubrimentiphilum* genomes was the number of genes encoding resistance against
438 antimicrobial agents, including more genes encoding for streptogramin lyase than
439 found in any other genome. These bacteria may live in close association with
440 antimicrobial producing bacteria, and survive in part by scavenging cell components
441 of these other microbes. The most conspicuous partners of the *Ca.*

442 Rubrimentiphilum in our site are not well studied in terms of antibiotic production
443 capabilities. However, there are indications that some *Chloroflexi* may have this
444 capacity (Nett and König, 2007). *Thermosporothrix hazakensis* SK20-1T, a relative
445 of one *Chloroflexi* OTU closely co-occurring with *Ca. Rubrimentiphilum*, has recently
446 been shown to produce thiazoles as secondary metabolites (Park *et al.*, 2015).

447

448 Attempts to develop an enrichment of *Ca. Rubrimentiphilum* failed. Previous SIP
449 based studies of the soil using cellulose and other EPS also did not demonstrate any
450 enrichment of WPS-2 (Wang *et al.*, 2015). *Ca. Rubrimentiphilum* may not utilize any
451 of the provided substrates, or precise conditions for growth were not met, although
452 by performing the enrichment directly in the soil we attempted to bypass this issue.
453 It is also possible that these are slowly growing bacteria and growth could simply
454 not be seen at the time scales of these experiments. Finally, it is possible the
455 substrate concentrations added (around 0.025-0.3% w/w, Supplementary Table 7)
456 were too high. Oligotrophic bacteria can be inhibited by excessive substrate
457 availability, particularly in the case of amino acids (Kuznetsov *et al.*, 1979). For
458 example the acidobacteria *Edaphobacter* spp. can grow on casamino acids and
459 peptone only when provided at very low concentrations around 0.01% w/v (Koch *et*
460 *al.*, 2008).

461

462 In summary, we noted that there is a predominant genus of the candidate phylum
463 WPS-2 (*Ca. Rubrimentiphilum*) in bare acidic soils of the Paint Pots site, and applied
464 a combination of ecological and genomic studies to understand its niche. Based on
465 our data, the bacteria prefer the bare, extreme soil to more organic forest soil.
466 Despite the obvious hypothesis that they should survive via lithoautotrophy,

467 photoautotrophy, or trace gas metabolism in bare soils, they were instead predicted
468 to be aerobic heterotrophs, possibly scavenging cell components from hardy
469 autotrophic bacteria as energy substrates. As other bacteria from the WPS-2 phylum
470 have been predicted to be primary producers (photoautotrophs or autotrophic
471 hydrogenotrophs), our study suggests that there is considerable metabolic
472 versatility across the WPS-2 phylum, with alternative autotrophic/heterotrophic and
473 phototrophic/lithotrophic/organotrophic phenotypes.

474 We propose the following tentative species description for the bacterium *Ca.*
475 *Rubrimentiphilum* (from L. neut. n. *rubrimentum*, red ink; N.L. neut. adj. *philum*
476 [from Gr. neut. adj. *philon*], friend, loving). Gram negative, aerobic heterotrophic
477 bacterium. Motile via flagella. Forms type P and type IV pili. Amino acids and
478 nucleotides are the presumed preferred substrates. Other substrates include
479 thiosulfate, formate, and possibly some oligopeptides or oligosaccharides.
480 Prototrophic for most amino acids and vitamins, auxotrophic for vitamin B12.

481

482 **Experimental Procedures**

483 *Sampling*

484 Samples from the Paint Pots, Kootenay National Park, B.C (N 51.16991°, W
485 116.14735°) were collected into sterile 50-mL Falcon tubes (VWR, Mississauga,
486 Canada) on Aug. 13, 2012, Oct. 20, 2014, July 19, 2016 and May 25, 2017. The
487 geology and geochemistry of the area have been described elsewhere (Everdingen,
488 1970; Grasby *et al.*, 2013). Forested soils are Podzols (USDA: Spodosols) with a thick
489 O horizon, a distinct E horizon, and a deep organic A horizon. However, areas
490 covered with iron oxide depositions from active or extinct springs are generally
491 unvegetated mineral soils with no O horizon, although various grasses do grow on

492 the fringes of the forest or in flooded marshy areas. Over 70 samples were taken
493 from various locations as shown in Figure 1 and Supplementary Table 1. The Source
494 is the present site of spring water discharge. The Relic Spring is a water-filled pool
495 still connected to the water source but no longer discharging water. The Mound and
496 the Ancient Mound are former spring sources that are now dry. Acidity, metal
497 toxicity, and compactness due to the solidified iron oxides are the most likely
498 reasons for the lack of vegetation on the Mound and Ancient Mound areas (Grasby
499 *et al.*, 2013). Samples were transported back to the laboratory on ice, and 0.5-g
500 subsamples immediately frozen at -80 °C until DNA extractions were performed. Soil
501 samples for enrichments were stored at 4 °C in the dark until use.

502

503 *Microbial community analyses*

504 Community analyses were performed as described previously (Ruhl *et al.*, 2018).
505 Briefly, DNA was extracted from 500 mg of soil with the FastDNA® SPIN Kit for Soil
506 (MP Biomedicals, Santa Ana, CA, USA), with an additional 5.5 M guanidine
507 isothiocyanate wash to remove humic acids (Knief *et al.*, 2003). 16S rRNA gene
508 amplicon libraries were prepared as per the Illumina (San Diego, CA, USA) standard
509 library preparation protocol: "16S Metagenomic Sequencing Library Preparation"
510 (Part # 15044223 Rev. B), except that Taq polymerase was used in the second PCR
511 reaction. Primers targeted the V3-V4 region (341fw and 785r). Amplicon libraries
512 were quantified using the Qubit HS kit (Invitrogen, Carlsbad, CA, USA), diluted to 4
513 nM, pooled, and prepared for sequencing on a MiSeq instrument as per Illumina's
514 standard protocol "Preparing Libraries for Sequencing on the MiSeq" protocol (Part
515 # 15039740 Rev. D). Libraries were sequenced using the MiSeq Reagent Kit v3, 600
516 cycles (Illumina part number MS-102-3003). The Qiime2 software 2019.4 was used

517 to analyse 16S rRNA sequence data (Bolyen *et al.*, 2019). Raw reads were quality
518 controlled and denoised sub-OTUs were formed using the deblur plugin (Amir *et al.*,
519 2017). Taxonomic assignment was performed with the feature-classifier plugin
520 (Bokulich *et al.*, 2018) employing a naïve Bayes classifier approach. The taxonomy
521 classifier for the analysis was trained on thte Silva database, release 132 (Quast *et*
522 *al.*, 2013) for the V3-V4 region, after manual edits to to delineate *Ca.*
523 *Rubrimentiphilales* and AS-11 lineages of phylum WPS-2 (Supplementary Figure 2).

524

525 *Co-occurrence network analysis*

526 The sub-OTUs detected from the total of 67 samples collected in 2014, 2016 and
527 2017 (Supplementary Table 1) were used to construct a co-occurrence network. An
528 inclusion threshold of average abundance of 0.25% was applied to select the major
529 lineages (See Supplementary Table 2 for full taxon strings of the sub-OTUs). The
530 network topology was constructed using igraph from the Jaccard similarity
531 adjacency matrix using the picante package in R (Csardi and Nepusz, 2006; Hardy,
532 2008). The network was visually rendered with Gephi software using the
533 ForceAtlas2 algorithm for its layout (Jacomy *et al.*, 2014). Network modularity was
534 computed using the Louvain algorithm for community detection (Blondel *et al.*,
535 2008).

536

537 *Quantitative PCR-based estimation of Ca. Rubrimentiphilum abundance*

538 The probe design function of the ARB software (Ludwig *et al.*, 2004) and ARB-Silva
539 111 database were used to identify paired oligonucleotide primer sequences
540 targeting *Ca. Rubrimentiphilales* and excluding the AS-11 group. A primer pair
541 specifically targeting a 242 bp fragment was designed: WPS-2_for (5'-

542 GCACTCACTCGAGACCGCCGTT - 3') and WPS-2_rev (5' -
543 GGGAACGTATTCACCGCAGCGT -3'). These were quality controlled against dimer and
544 hairpin formation via OligoCalc (Kibbe, 2007) and searches against the ARB-Silva
545 111 database showed a minimum of 4 mismatches to non-target sequences
546 (including AS11). Primers were obtained from Invitrogen (Waltham, MA, USA).
547 Illumina sequencing of these qPCR amplicons from 4 Paint Pots samples confirmed
548 the primer specificity (data not shown). To create standards, PCR amplification was
549 performed on soil DNA and the resulting amplicon cloned into a pJET 3.0 plasmid
550 (ThermoFisher, Waltham, MA, USA). Sanger sequencing confirmed that the plasmid-
551 borne sequence matched the target sequence. Standard dilutions were constructed
552 with a purified amplicon from the plasmid. The maximum primer annealing
553 temperature was determined using gradient PCR (Veriti 96 Well Thermal Cycler,
554 Applied Biosystems, Waltham, MA, USA) to maximise the stringency of the qPCR
555 assay. qPCR was performed on a Rotor-Gene 6000 (QIAGEN, Velno, Netherlands)
556 using a SYBR Green qPCR master mix (QIAGEN, Velno, Netherlands) in 12.5- μ l total
557 reaction volumes with 1 μ M of each primer included. qPCR runs were performed
558 under the following conditions: Initial denaturation at 95 °C for 10 minutes; 40
559 cycles at 95 °C for 20s, 72°C for 20s and 72 °C for 20s; a pre-melt conditioning step
560 at 72 °C for 90s; and a melt ramp from 72 °C to 95 °C increasing 0.5 °C every 5s.
561 Amplicon melt profiles (0.5°C increments) did not show evidence of non-specific
562 amplification.

563

564 *Single cell genomics*

565 Soil samples taken on Aug. 13, 2012 from the Mound were cryopreserved with 5%
566 glycerol solution in TE buffer and frozen on dry ice. Single cells were isolated with

567 fluorescence-activated cell sorting (FACS), lysed and whole genome amplified using
568 multiple displacement amplification (MDA), and MDA products were screened with
569 16S rRNA gene PCR according to standard JGI protocols (Rinke *et al.*, 2014). From
570 397 wells that amplified with MDA, 58 produced 16S rRNA gene PCR amplicons, of
571 which 8 were positively identified as WPS-2 and genome sequenced (Table 1).
572 Preparation of the libraries, sequencing and assembly procedures are summarized
573 in Supplementary Table 4.

574

575 *Metagenomic sequencing and MAG binning*

576 Metagenomic binning of several Mound soil metagenomes (listed in Supplementary
577 Table 3) did not yield high-quality and low-contamination genomes of *Ca.*
578 *Rubrimentiphilum*. In order to obtain better MAGs, we performed cell size
579 fractionation prior to DNA extraction. Briefly, 20 g of soil was mixed with 25 ml of
580 cell detachment buffer (Eichorst *et al.*, 2007) supplemented with 10 mM
581 $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ and 1 mM dithiothreitol, and stirred for 1 hour. The soil suspension
582 was transferred into a 50-ml conical centrifuge tube left to settle for 5 min, then
583 decanted and used in 2-3 sequential centrifugation steps. A short spin at $1,500 \times g$
584 for 2 min pelleted large debris and soil particles (Designated Fraction 0). The clear
585 supernatant was gently transferred to new centrifuge tubes for a further 1-2 steps
586 of centrifugation. In one (3-step) trial, centrifugation was applied at $3,000 \times g$ for 5
587 min and the resulting pellet (Fraction 1) resuspended in PBS buffer; transferred
588 supernatants were centrifuged at $19,000 \times g$ for 30 min, and pellets (Fraction 2)
589 resuspended in PBS buffer. Alternatively, for a 2-step procedure the middle
590 centrifugation step was skipped, and Fraction 1 was collected after centrifugation at
591 $19,000 \times g$ for 30 min. Microbial communities in each fraction were analyzed as

592 described above. A fraction with a low-diversity community enriched in WPS-2
593 (NM2-5_DC) was selected for metagenome sequencing and assembly as described
594 in Supplementary Table 4. MAG binning and quality control were performed with
595 MetaBAT v2.12.1 (Kang *et al.*, 2015) and CheckM v1.0.9 (Parks *et al.*, 2015),
596 respectively.

597

598 *Functional comparisons of WPS-2 genomes with other genomes*

599 MAGs and SAGs were annotated via the JGI standard pipeline (Huntemann *et al.*,
600 2015, 2016) and further analysed using the IMG/M platform (Chen *et al.*, 2019). The
601 Pfam and COG profiles of WPS-2 MAGs were compared to a reference database that
602 included 2363 bacterial genomes from IMG (available in Supplementary File 2). To
603 minimize taxonomic bias in the database, this reference set included one bacterium
604 (whenever possible with a finished status) from each described genus. The Pfam
605 and COG content profiles of the MAGs were compared against the reference
606 bacteria via the calculation of Jaccard and Bray-Curtis similarity indices (Legendre
607 and Legendre, 2012). Two-tailed t-tests were used to calculate significance.

608

609 *Phylogenetic analyses*

610 A maximum likelihood concatenated marker gene tree was created by taking all
611 bacterial isolate genomes in the Integrated Microbial Genomes (IMG) database,
612 reducing the 61,619 bacterial genomes to a manageable set by clustering the RNA
613 Polymerase beta subunit gene at 65% and using this as the reference database for
614 tree inference. Ten WPS-2 genomes from the current study (8 SAGs and 2 MAGs)
615 and 2 MAGs from Ji *et al.* (2017), 3 MAGs from (Holland-Moritz *et al.*, 2018) and 53
616 MAGs from (Woodcroft *et al.*, 2018) were added to the total set of genomes used for

617 the bacterial tree in Supplementary Figure 8. Briefly, trees were constructed in the
618 following manner. Proteins were called using Prodigal v.2.6.3. Phylogenetic markers
619 were extracted from the resulting faa files using HMMs of each of the 56 markers
620 with HMMER v.3.1b2, then aligned using MAFFT v.7.221 and concatenated using an
621 internal python script. Phylogenetic trees were inferred using IQ tree tree to
622 produce maximum likelihood trees with 1000 bootstraps (Nguyen *et al.*, 2015).
623 Visualization was produced in R using ape and ggtree (Yu *et al.*, 2017) packages.

624

625 For the 16S rRNA gene analysis, full length sequences of WPS-2 from the Nr99 Silva
626 database, release 132 (Quast *et al.*, 2013), were iteratively aligned to WPS-2 sub-
627 OTUs with Muscle v.3.8.425. WPS-2 sub-OTUs of average abundance >0.25% in the
628 entire sequencing dataset were included. Bayesian 16S rRNA gene phylogeny was
629 constructed with MrBayes v.3.2.6 (Huelsenbeck and Ronquist, 2001). Posterior
630 probabilities of the tree were estimated using a Markov Chain Monte Carlo of 1×10^6
631 cycles with first 1×10^5 states discarded and used for re-initialization (burn-in). Data
632 was analyzed with a 4by4 nucleotide substitution model with a GTR structure. Rate
633 variation was set to gamma-distributed with a proportion of invariable sites.

634

635 *Enrichment and cultivation*

636 Enrichments efforts for WPS-2 used 4-5 g (wet weight) amounts of soil in 15-mL
637 Falcon tubes, with addition of various monosaccharides, polysaccharides, protein
638 digests, and organic acids (Supplementary Table 7). Duplicates of each substrate
639 were incubated at room temperature in the dark. 0.5-g soil samples were taken
640 biweekly for a period of up to 2 months for DNA extraction and quantification using
641 the WPS-2 qPCR assay.

642

643 **Acknowledgements**

644 This work was supported by a Natural Sciences and Engineering Research Council of
645 Canada (NSERC) Discovery Grant (2019-06265). We are indebted to Parks Canada
646 and the Ktunaxa Nation for allowing us to sample in Kootenay National Park. The
647 work conducted by the U.S. Department of Energy Joint Genome Institute, a DOE
648 Office of Science User Facility, is supported by the Office of Science under Contract
649 No. DE-AC02-05CH11231.

650

651 **Conflict of Interest**

652 The authors declare no conflict of interest.

653

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904

905 **TABLES**

906

907

908 **Table 1.** Summary of WPS-2 SAGs and MAGs obtained from the Paint Pots Mound site. Genome completeness and
 909 contamination were estimated with CheckM. Genome qualities were assigned according to the standards outlined in
 910 (Bowers *et al.*, 2017).

911

Name	IMG ID	Assembly Size, Mb	Number of Scaffolds	N ₅₀ , kb	GC Content, %	Completeness estimate, %	Contamination estimate, %	Genome quality	Gene Count	Genes with Pfam	Genes with COG
MAGs from differential soil extraction metagenome ¹											
6	2781125698	4.92	48	185	60.9	100	100		5132	3671	2939
6A	2781125701	2.58	19	253	60.7	95	2.78	High-quality draft	2688	1900	1523
6B	2781125702	2.34	29	160	61.0	95	2.78	High-quality draft	2444	1771	1415
SAGs											
H17	2617270725	1.66	134	28	60.6	54	0.0	Medium-quality draft	1798	1015	728
I5	2706794755	1.47	118	23	60.5	43	0.0	Low-quality draft	1621	970	653
G15	2616644938	1.03	105	28	60.0	44	0.0	Low-quality draft	1131	680	427
C21	2706794749	1.20	94	18	60.9	29	0.0	Low-quality draft	1311	843	549
F4	2616644933	1.13	168	46	60.6	44	0.0	Low-quality draft	1306	789	501
C6	2616644939	0.59	75	19	60.5	22	0.0	Low-quality draft	678	461	271
B5	2616644827	0.58	70	16	60.1	12	0.0	Low-quality draft	648	414	269
I16	2616644826	0.74	113	30	60.5	37	0.0	Low-quality draft	863	551	354

912

913 ¹CheckM analysis suggests that bin 6 contained two closely related strains. Bin 6 was separated into bins 6A and 6B,
 914 each representing a single strain.

915

916 **FIGURES**

917

918 **Figure 1: A:** Overview map of the Paint Pots area, with orange areas representing

919 ochre coloured soils, and white areas representing forested podzols. **B** is a blow-up

920 of the area indicated by the dashed square in A and, **C** is blow-up of the bare Mound

921 indicated by the dashed square in B. Percentages are 16S rRNA gene relative

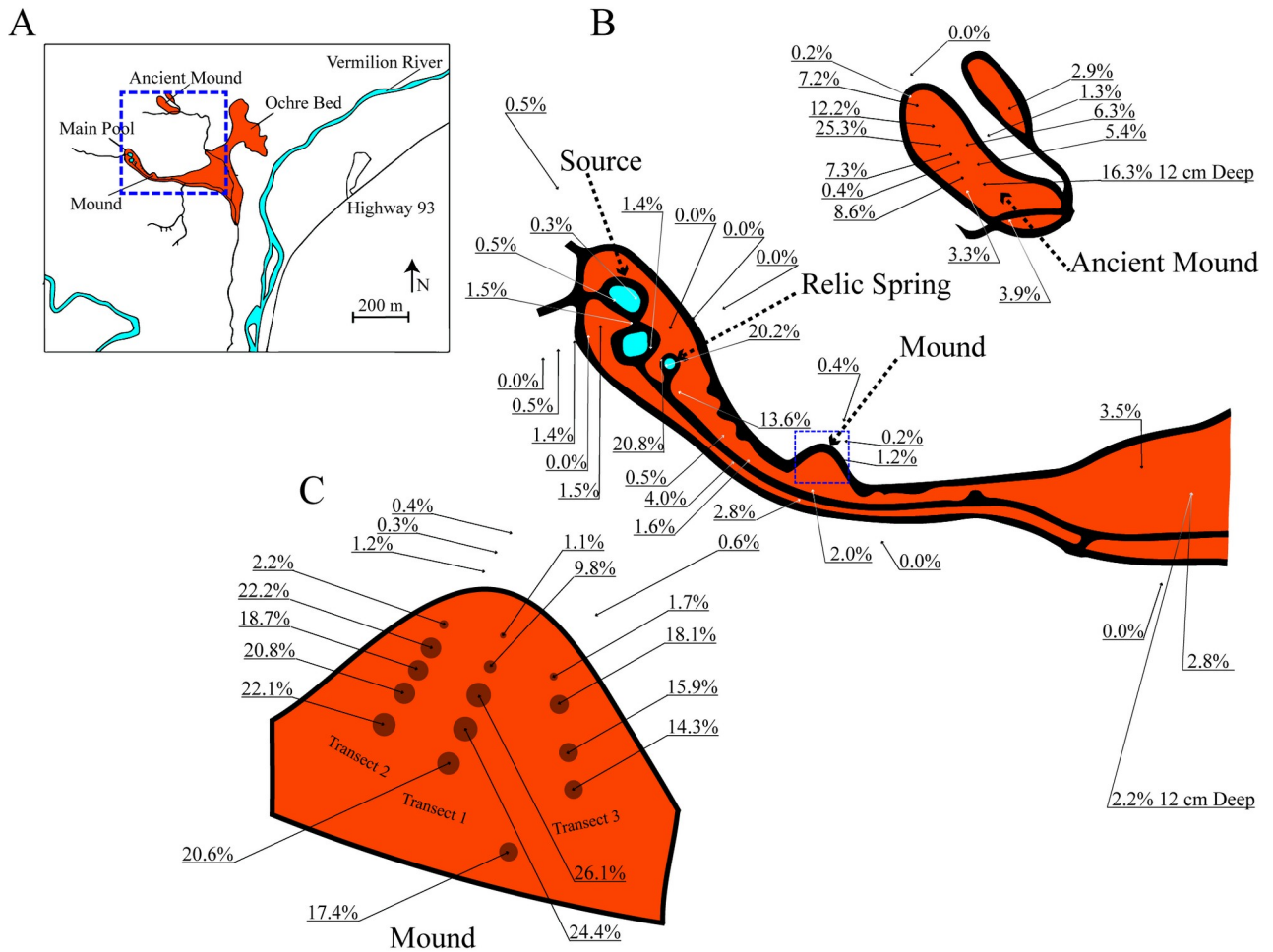
922 abundances of WPS-2 bacteria in different sampling points. Soil samples were taken

923 from the surface 0-5 cm layer unless otherwise specified. The circle diameters in C

924 are proportional to the WPS-2 relative abundances. Additional information on the

925 samples is provided in Supplementary Table 1.

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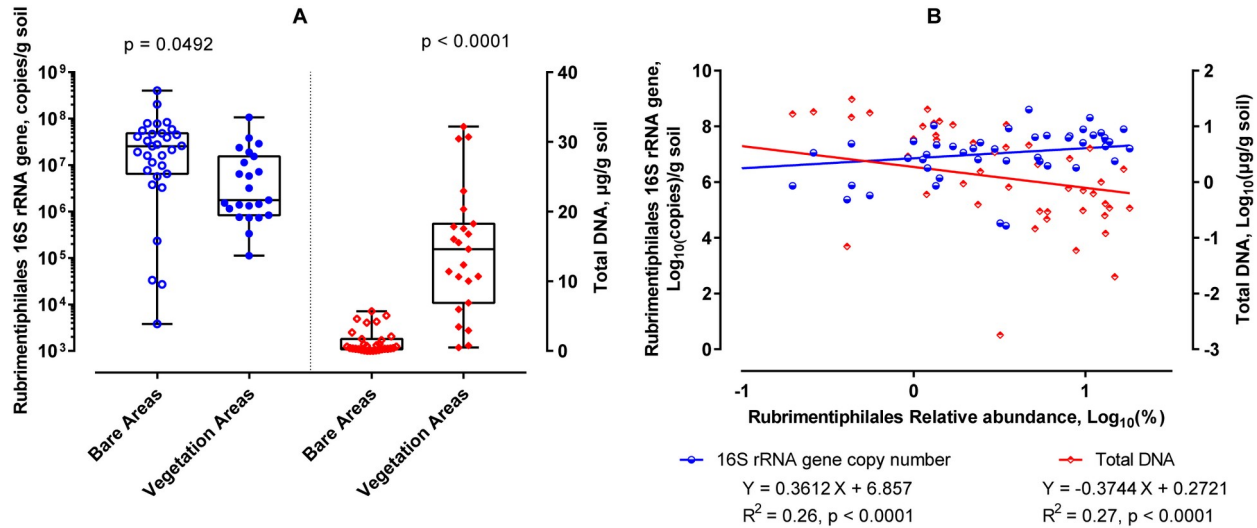


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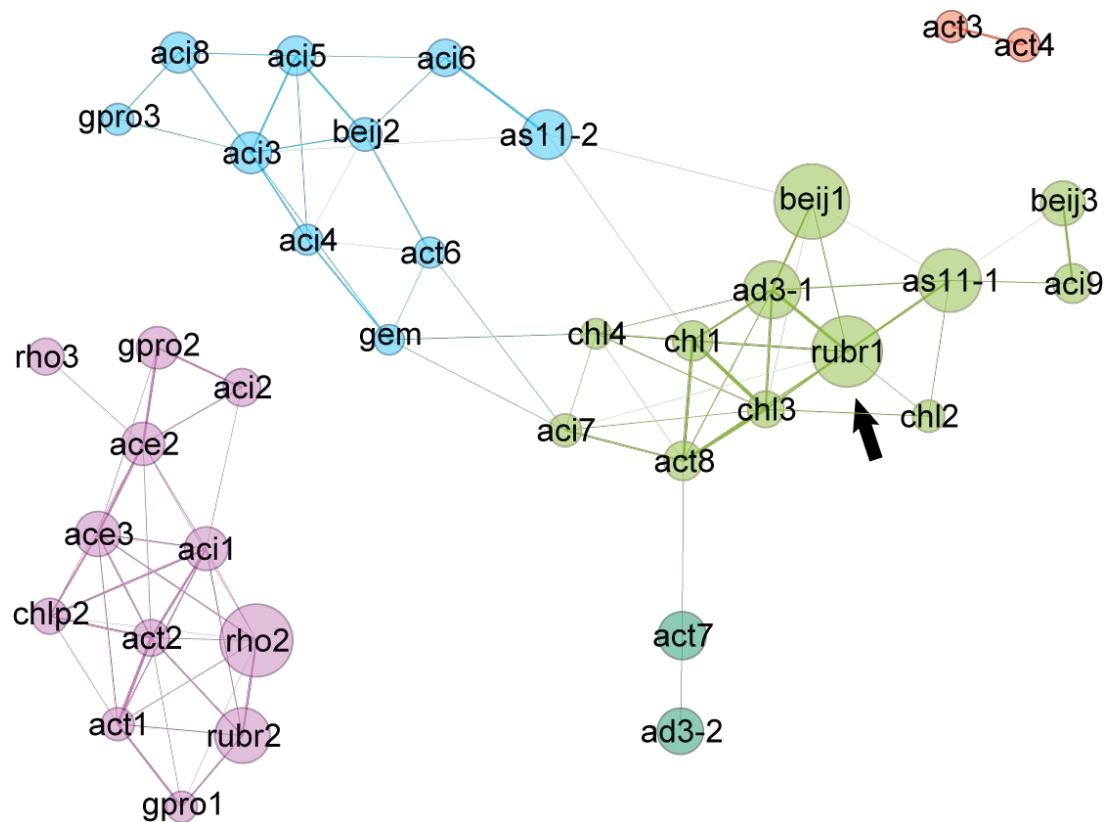
931 **Figure 2:** DNA recovery and abundance of *Ca. Rubrimentiphilales* in bare versus
 932 vegetated soils. **A:** Absolute abundance determined via a specific 16S rRNA gene
 933 targeted qPCR assay (blue) and total DNA yield (red). Unpaired t-tests were applied
 934 to determine statistical significance between bare and vegetated soils. **B:**
 935 Relationship of absolute WPS-2 abundance determined via qPCR (blue) and total
 936 DNA obtained from the samples (red), to the relative WPS-2 abundance determined
 937 via 16S rRNA gene amplicon sequencing. The slopes of both regression lines
 938 differed significantly from zero. Data are plotted as a log-log scale to ensure they
 939 are parametric.
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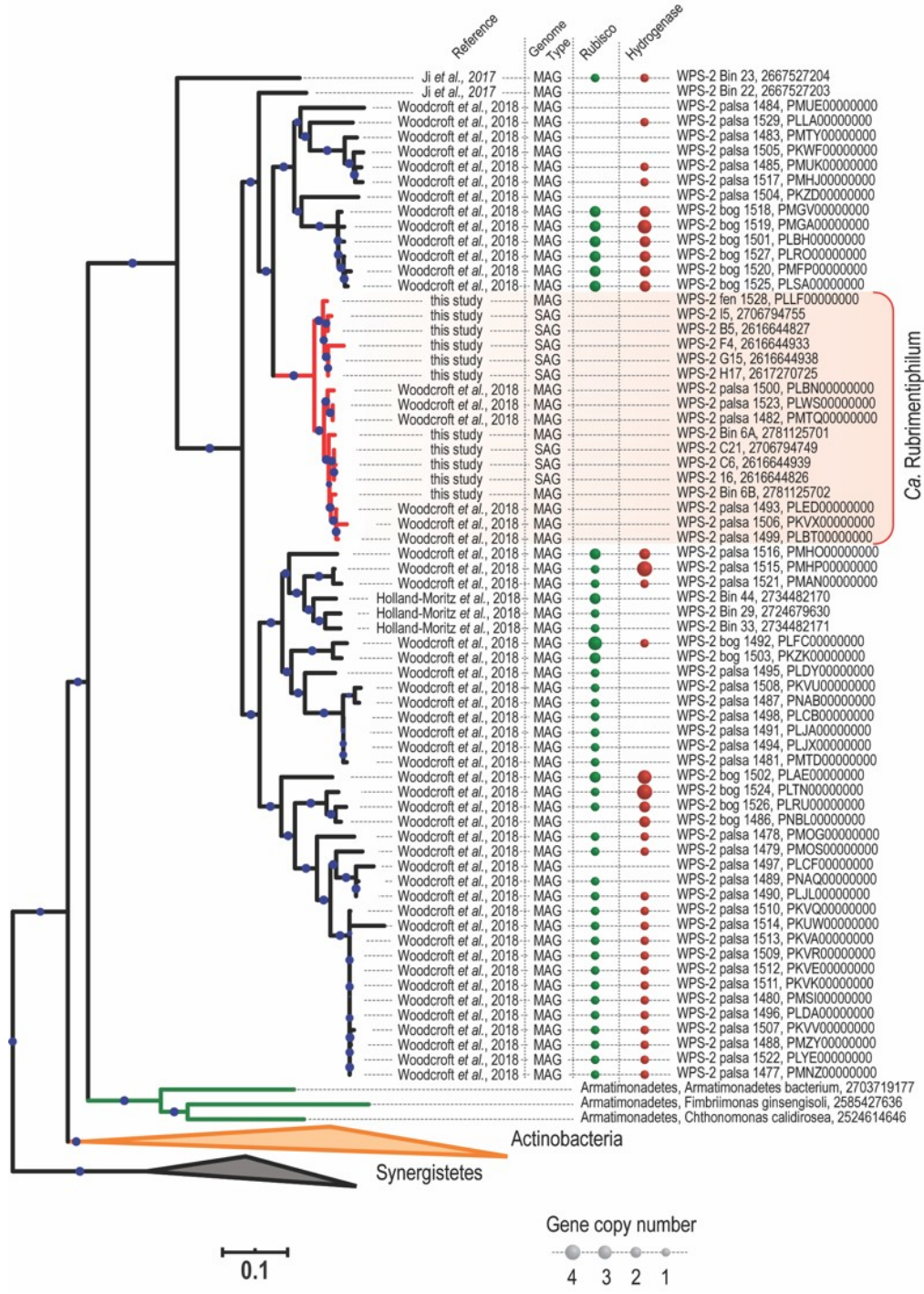
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Figure 3: Co-occurrence network analysis of 16S rRNA gene sub-OTUs, based on 67 soil Paint Pots samples (Supplementary Table 1). Sub-OTUs of average relative abundance >0.25% across all samples were used to construct the network, unconnected nodes are not shown. The edges represent positive association which is proportional to line thickness. Node sizes are proportional to the average abundances of sub-OTUs. All analyzed *Chloroflexi* sub-OTUs were placed inside the same sub-network, which also contains the most abundant WPS-2 sub-OTU rubr1 (i.e. *Ca. Rubrimentiphilum*, indicated by the arrow). The strongest associations of rubr1 were formed with chl1 (*Ktedonobacteria*), chl3 (*Thermosporothrix* sp.), ad3-1 (candidate phylum AD3) and the most abundant WPS-2 sub-OTU from the AS-11 clade. Weaker associations included beij1 (*Beijerinckiaceae*), chl2 (*Ktedonobacteria*) and aci7 (*Bryobacter*). See Supplementary Table 2 for full taxon strings of the OTUs in the graph.



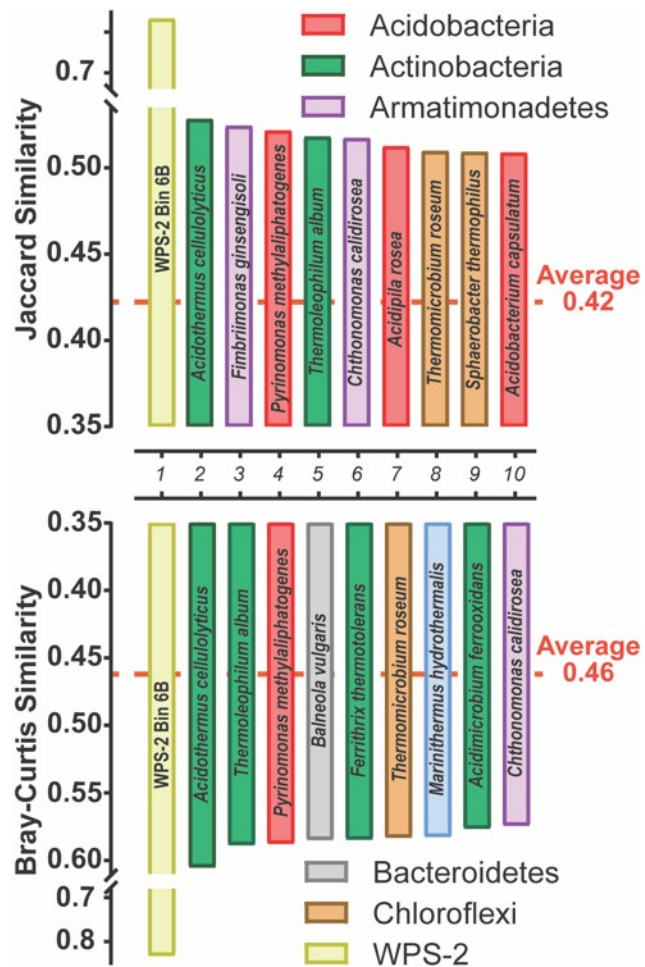
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965 **Figure 4:** Phylogenetic tree based on a set of 56 conserved marker genes showing
966 the relationships of 69 available WPS-2 genomes in relationship to reference
967 bacteria. Phylogenetic trees were inferred using IQ tree to produce maximum
968 likelihood trees with 1000 bootstraps. Bootstrap support is shown as a solid blue
969 circles for nodes with greater than 50% support with sizes proportional to the node
970 supports. The presence of Rubisco or hydrogenases within a genome is indicated
971 with bubbles of the sizes proportional to the copy number of a corresponding gene.
972 Neither gene was found within any *Ca. Rubrimentophilum*, but both are usually
973 present in other WPS-2 genomes.



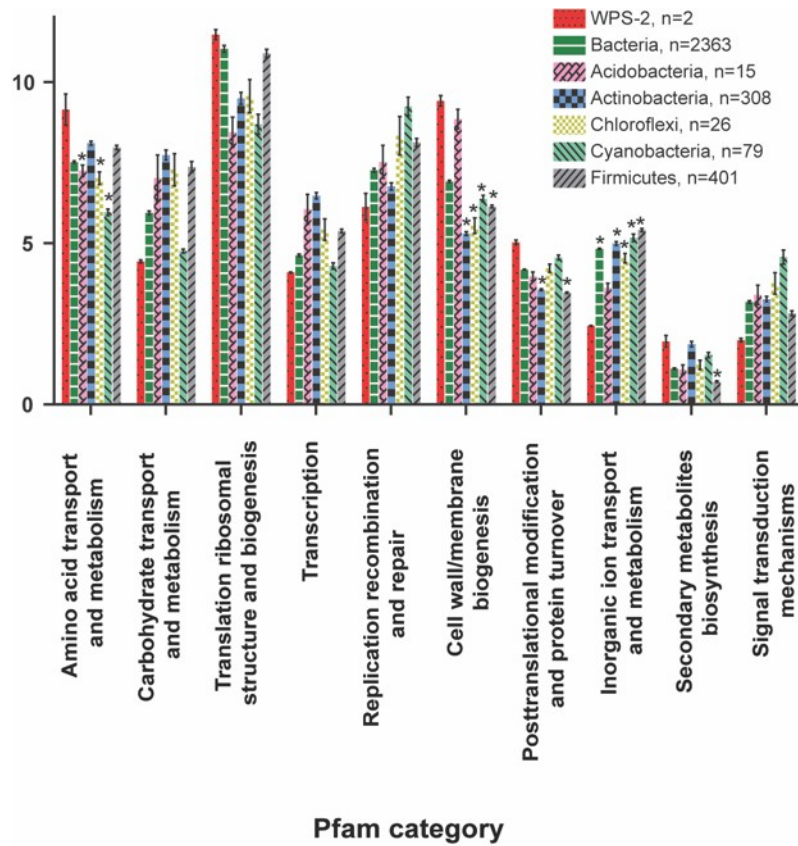
975 **Figure 5.** Bacterial genomes most similar to the WPS-2 genome (Bin 6A) based on
 976 Jaccard and Bray-Curtis similarities of Pfam profiles. Jaccard distances require only
 977 the presence or absence of a given Pfam, while Bray-Curtis distances include
 978 quantitative relationships of the Pfam profiles. Average distances over the entire
 979 dataset are indicated by red dashed lines. Extended data are shown in
 980 Supplementary Figure 6.

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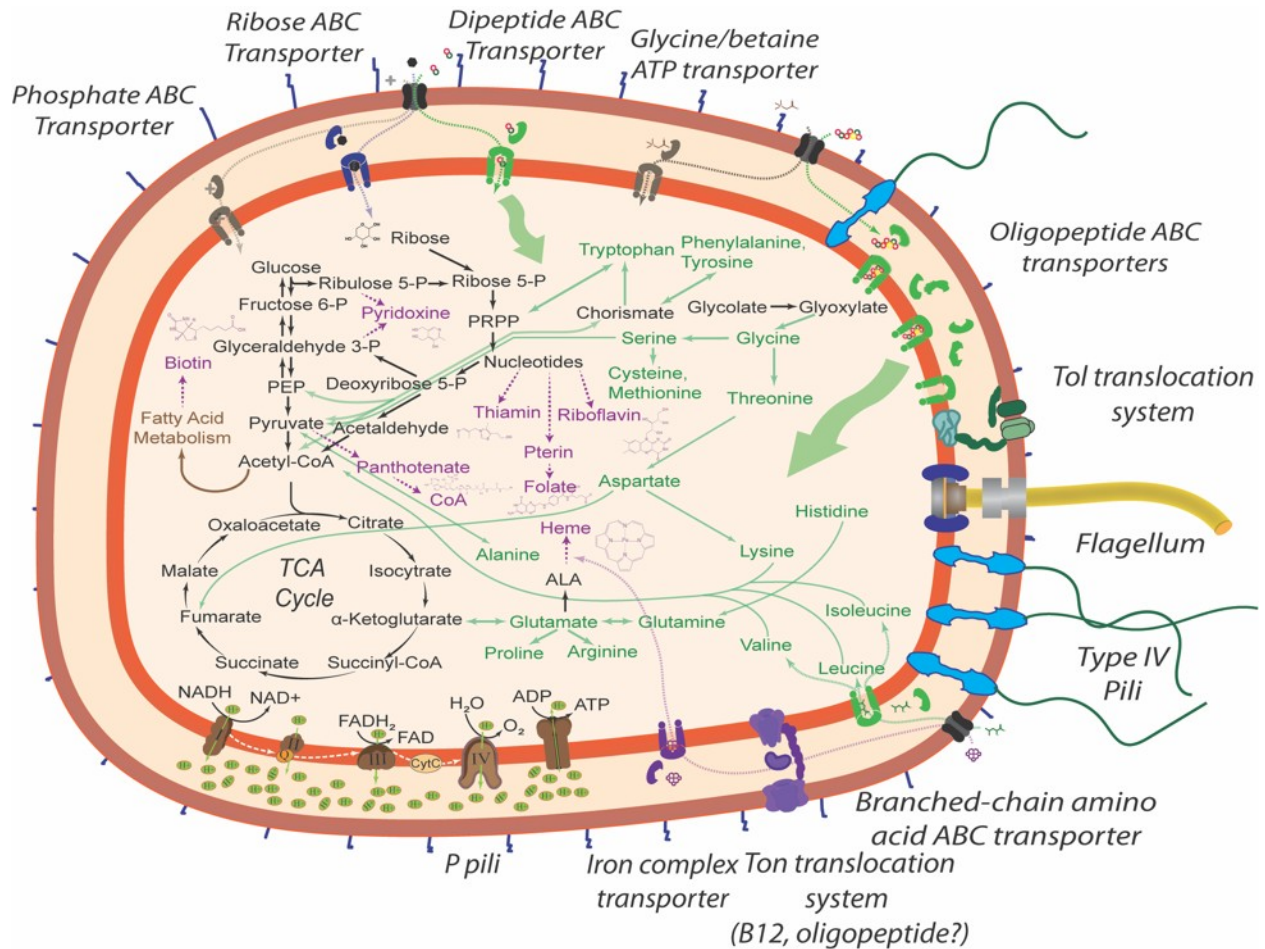
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986 **Figure 6.** Comparison of gene distributions in selected Pfam categories for the *Ca.*
 987 *Rubrimentiphilum* MAGs versus different bacterial lineages based on a reference
 988 dataset of 2363 genomes (Supplementary File 2). Bars represent the mean
 989 percentages (± 1 standard error) of the total genes that fall into a category for a
 990 taxonomic lineage. Group significance between the mean percentages within a
 991 given category were tested with one-way ANOVA. A post-hoc Bonferroni correction
 992 was used to detect significant differences between *Ca. Rubrimentiphilum* and other
 993 lineages (indicated by asterisks). Extended data are shown in Supplementary Figure
 994 7.



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1002 **Figure 7.** Predicted metabolic potential of *Ca. Rubrimentiphilum* based on the SAG
 1003 and MAG data recovered in our study. The microorganisms exhibit Gram-negative
 1004 cell wall structure, flagella, P and type IV pili. Transporters are scarce, and are
 1005 mostly related to amino-acid and peptide transport. Amino acids are a likely growth
 1006 substrate. The genomes show biosynthetic pathways for most vitamins, while
 1007 crucial steps of B12 biosynthesis were missing.
 1008



1009