

Tropical Soil Metagenome Library Reveals Complex Microbial Assemblage

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2 **Abstract**

3 In this work, we characterized the metagenome of a Malaysian mangrove soil sample via next
4 generation sequencing (NGS). Shotgun NGS data analysis revealed high diversity of microbes
5 from Bacteria and Archaea domains. The metabolic potential of the metagenome was
6 reconstructed using the NGS data and the SEED classification in MEGAN shows abundance of
7 virulence factor genes, implying that mangrove soil is potential reservoirs of pathogens.

8

9 Keywords: Metagenomics, Mangrove, Soil, Proteobacteria

10

11 **Introduction**

12 Mangrove forests are usually located at the tropical and subtropical latitudes. They are present at
13 the transition of land and sea which makes them susceptible to tidal change and salinity.
14 Mangrove soils generally comprise soft, muddy and anaerobic sediment with thin top layer of
15 aerobic sediment. They also function as heavy metal sink¹ which acts as natural sink and
16 filtration system. Mangrove swamps are the habitat for a diverse variety of fauna especially
17 juvenile fishes, and they also act as breeding and nursery grounds for these aquatic animals.

18 Microorganisms in the mangrove habitat play an important role in maintaining the productivity,
19 conservation and nutrients of this ecosystem. Microorganisms are involved in biogeochemical
20 cycles that supply nutrients to plants and animals^{2,3}. Mangroves are rich in organic matters but
21 usually lack phosphorus and nitrogen⁴⁻⁷. Their microorganisms' activities are high because they
22 are very efficient in recycling the nutrients contained therein. Microorganisms are directly
23 involved in nitrogen fixation, phosphate solubility, photosynthesis, sulfate reduction and
24 production of other substances.

25 The mangrove environment is highly susceptible to anthropogenic effects such as pollution,
26 deforestation and human activity. These could change the dynamic mangrove ecosystem which in
27 turn affects the mangrove community and disturbs the microorganism community that maintains
28 the productivity and conservation of mangrove.

29 This study aimed to investigate the metagenome of a mangrove soil sample and their ecological
30 role via metabolic reconstruction. We used the Illumina HiSeq 2000 platform to carry out
31 shotgun metagenome next generation sequencing (NGS). This method avoided bias of PCR
32 amplification as in the case of amplicon sequencing and it enabled parallel study on both the

33 taxonomic and functional diversities. We hypothesized that the abundance and diversity of
34 microbes and their functional attributes to be similar to those of previous studies^{8,9}.

35 **Materials and Methods**

36 Sampling was done on a soil sample obtained in the east coast of Peninsular Malaysia, namely
37 Rantau Abang (RA) (N04° 54.189' E103° 22.208'). No specific permissions were required for the
38 chosen locations and such research activities. Our work also did not involve endangered or
39 protected species. The top 5 to 20 cm of soil was collected and stored at -20°C until processing. A
40 portion of the soil sample was sent for biochemical analyses of its pH, carbon nitrogen ratio, and
41 contents of phosphorus, sulfur and heavy metals, like arsenic, cadmium, lead and mercury, as
42 described previously¹⁰.

43 DNA extraction was carried out according to the protocol as described previously¹¹ with
44 modifications. Traces of plant materials were removed from the soil prior to extraction. Briefly,
45 5g of soil was added with 13.5ml of DNA extraction buffer (Tris-HCl, pH8 100mM; EDTA, pH8
46 100mM; Na₂HPO₄, pH7.8 100mM; NaCl, 1.5M; CTAB, 1% w/v), 100µl of proteinase K
47 (10mg/µl), and 200µl of lysozyme (10mg/µl). The mixture was incubated horizontally at 37°C
48 with orbital shaking (225rpm). After 30min, 0.5ml of SDS (20% w/v) was added and the mixture
49 further incubated in a 65°C water bath for 2h with gentle mixing by inverting the tube at 15min
50 intervals. The supernatant was collected by centrifugation at 6000 × g for 10min. The pellet was
51 suspended in 4.5ml of DNA extraction buffer and 0.5ml of SDS (20% w/v) and vortexed for 10s
52 followed by incubation at 65°C for 10min. The supernatant was then collected by centrifugation
53 and pooled with the supernatant collected previously. Equal volume of chloroform:isoamyl
54 alcohol (24:1, vol/vol) was added to the pooled supernatant and the mixture was gently mixed by

55 inversion. The aqueous phase was transferred to a clean, sterile tube after centrifugation at $6000 \times$
56 g for 10min. The chloroform:isoamyl alcohol step was repeated once. For DNA precipitation, 0.6
57 volume of cold isopropanol was added and the resultant mixture incubated at -20°C for 30min.
58 DNA was collected by centrifugation at $16000 \times g$ for 20min, followed by washing with 70%
59 (v/v) ethanol and kept in -20°C for 15min. Ethanol was removed by centrifugation at top speed in
60 a table top centrifuge for 10min and the pellet was air dried aseptically. The DNA pellet was then
61 dissolved in elution buffer (Roche High Pure PCR Product Purification Kit).

62 The soil metagenomic DNA was further purified by gel elution in a 3% (w/v) low melting
63 temperature agarose electrophoresis. The metagenomic DNA was mixed with 80% (v/v) glycerol
64 and $6\times$ loading dye and the mixture was then loaded into a well. Electrophoresis was carried out
65 at 15V for 16 to 20h. DNA was excised from the gel with a sterile blade and recovered using the
66 Qiagen Gel Extraction Kit (Venlo, Netherlands). DNA concentration and purity were determined
67 using Qubit and Nanodrop 2000c by Life Technologies, respectively. The purified DNA was then
68 subject to NGS using Illumina HiSeq 2000.

69 For taxonomic analysis, the metagenomic nucleotide sequences obtained were trimmed using
70 CLC Bio Genomic Workbench 5.5.2 (Aarhus, Denmark) at 50-nucleotide length to remove short
71 low quality reads. The trimmed data were then blasted against the NCBI Microbial database
72 (dated 22 Jan 2013) using Blastall 2.2.25 (NCBI) at the expected value of 1×10^{-20} .

73 For functional gene study, the trimmed nucleotide sequences were assembled using *de novo*
74 assembly in CLC Bio Genomic Workbench at the minimum contig length of 400 nucleotides.
75 The assembled data were extracted at coverage of $\geq 10\%$. Gene prediction was performed on the
76 extracted sequences using Prodigal 2.60¹², and each predicted gene was annotated using
77 RAPsearch 2.09^{13,14} (against the NCBI NR database (dated 22 January 2013)). Data obtained for

78 both taxonomic and functional distributions were analyzed in MEGAN 4.70.4¹⁵. Taxonomic
79 analysis was done according to the percentage identity filter to get the best sequence match. In
80 MEGAN, functional analysis was accomplished with the SEED¹⁶ classification.

81 The data of this study are available as NCBI database accession number SRR748204.

82 **Results**

83 Biochemical Analyses

84 Table 1 shows the biochemical properties of the Rantau Abang (RA) soil sample. The pH for RA
85 sample was recorded as pH of 5.1.

86 Metagenomic Library Analysis

87 The RA sample metagenome library shows very high nucleotides sequenced after editing and
88 >500,000 contigs generated at the coverage at approximately 41 times (Table 2).

89 Microbial Taxonomic Distribution

90 A total of 98% of the reads from RA sample was assigned to the domains level by MEGAN and
91 they excluded the “No hits” reads category. The majority of the assigned reads from RA samples
92 was of the domain Bacteria while the remaining were of the domain Archaea with 78.52% reads
93 assigned to Bacteria and 21.48% to Archaea (Figure 1).

94 There were 27 phyla hits from the domain Bacteria for RA metagenome library. The phylum
95 Proteobacteria dominated other phyla in (43.72%) in the RA sample. There were 10 phyla with
96 abundance percentages of more than 1% in the RA sample. In this sample, the phyla detected
97 were Proteobacteria, Acidobacteria (17.68%), Firmicutes (13.45), Actinobacteria (4.55%),

98 Nitrospirae (4.22%), Planctomycetes (3.06%), Chloroflexi (2.88%), Verrucomicrobia (2.69%),
99 Spirochaetes (1.70%), Chlamydiae (1.32%) and Bacteroidetes (1.31%) (Figure 2a). In RA sample,
100 unclassified bacteria were clustered as Caldithrix, Haloplasmatales and some phototrophic
101 bacteria.

102 Forty-three classes of bacteria were detected in RA sample (Figure 2b) where the first two most
103 abundant classes were Deltaproteobacteria (19.29%) and Alphaproteobacteria (16.89%), followed
104 by Acidobacteria (16.61%) (Figure 3b). The other minor classes included Clostridia (9.32%),
105 Gammaproteobacteria (5.50%), Actinobacteria (4.58%), Nitrospira (4.24%), Bacilli (3.24%),
106 Planctomycetia (3.08%), Spirochaetia (1.71%), Betaproteobacteria (1.69%), Ophitidae (1.52%),
107 Chlamydia (1.33%), Verrucomicrobiae (1.19%), Holophagae (1.17%), Anaerolineae (1.06%),
108 and Ktedonobacteria (1.02%).

109 At the genus level, *Acidobacterium* of the Acidobacteria phylum was the dominant genus in RA
110 sample. The abundance frequencies of this genus is 10.01% (Figures 2c).

111 Five classes of Proteobacteria namely Alphaproteobacteria, Betaproteobacteria,
112 Deltaproteobacteria, Gammaproteobacteria and Epsilonproteobacteria, were detected in RA
113 sample). Among these, Deltaproteobacteria (43.88%) was the major class, followed by
114 Alphaproteobacteria (38.43%), Gammaproteobacteria (12.51%) Betaproteobacteria (RA 3.85%)
115 and Epsilonproteobacteria (RA 1.33%) (Figure 3). The segregation of orders within
116 Deltaproteobacteria (Figure 4) showed Syntrophobacterales was the most abundant order in this
117 soil sample. At the genus level of this order *Syntrophobacter* was the most abundant genus
118 (Figures 5).

119 The RA sample showed present of archaea but only phyla Crenarchaeota and Euyarchaeota were
120 detected. In the RA sample, Crenarchaeota (63.78%) was present at a higher percentage as
121 compared to Euyarchaeota (36.22%) (Figure 6). A total of eight classes of archaea were detected
122 in both soil samples and among them, Thermoprotei (RA 63.78%) and Methanomicrobia (RA
123 17.85%) were the two dominant classes. Other minor archaea classes present in the RA sample
124 were Thermococci (5.48%), Methanococci (5.35%), Thermoplasmata (3.52%), Methanobacteria
125 (2.17%), Archaeoglobi (1.36%) and Halobacteria (0.47%).

126 The taxonomic diversity for the domains Bacteria and Archaea was estimated at the genus level
127 using the Shannon-Weaver diversity index, H' , in MEGAN and the H' value RA samples was
128 7.765.

129 Metabolic Functional Analysis via Reconstruction of Metagenome Library

130 The gene anthology was derived from the SEED classifications. Using this approach, the most
131 abundant gene detected in the RA sample was associated with carbohydrate metabolism (12.97%).
132 The second most abundant genes in the RA sample were associated with protein metabolism
133 (9.89%), virulence (9.53%), respiration (8.39%) and amino acids and their derivatives (8.19%)
134 (Figure 7).

135 **Discussion**

136 The application of the NGS enables the study of microbial diversity and function in metagenomes
137 without the need of culturing bacteria thus bypassing the growing of fastidious bacteria, which
138 are often unculturable, on laboratory media. However, this method depends on the reliability of
139 NGS data generated.

140 Most bacteria detected in the RA metagenomes were either anaerobic or facultative anaerobic.
141 They were predominantly from the domain Bacteria. However, in the RA metagenome, a
142 significant number of bacteria belonging to the domain Archaea were detected and the percentage
143 of archaeal abundance (21.48%) is significantly higher than the percentage values reported for
144 other soil metagenomes¹⁷. Crenarchaeota is known to be present at high sulfur content
145 environment and for its ability to utilize sulfur¹⁸. The high percentage of this phylum detected in
146 the RA sample is consistent with the high concentration of sulfur in the RA sample.

147 The bacterial diversity detected in the RA sample conforms to the common soil bacteria present
148 in other types of soil in other geographical locations¹⁹. In our RA sample, Proteobacteria remains
149 the most abundant bacteria and it comprised five different classes. However, the distribution of
150 Proteobacteria classes in this study differs from the distributions of Proteobacteria classes
151 reported for other mangrove habitats^{8,20}. In contrast to other mangrove metagenomes reported to
152 date, both the Malaysian mangrove metagenome possessed Deltaproteobacteria as the dominant
153 class of Proteobacteria. Even though Proteobacteria was the dominant phylum in RA
154 metagenome, *Acidobacterium* of the Acidobacteria phylum was the most abundant genus in our
155 soil sample.

156 The presence of the high frequency of genes associated with carbohydrate metabolism in our soil
157 metagenome analysis is not surprising because these genes are commonly detected in abundance
158 in most studies of soil metagenomes^{8,21}. However, the presence of the high frequency of
159 virulence factor genes in our soil metagenomes is unusual because they are not commonly
160 reported^{8,22}. This leads to the speculation that mangrove soil is a potential reservoir of pathogenic
161 bacteria but further work is required to verify this finding.

162 In our RA soil metagenome, antibiotic and toxic compound resistance genes were also detected
163 frequently. Their abundance may be related to the high percentages of Actinobacteria, which are
164 known to produce a myriad of antibacterial compounds, and Deltaproteobacteria, members of
165 which are known to be resistant to heavy metals and to oxidize heavy metals to their benign
166 forms²³, in our two tropical mangrove soil samples. We also obtained a high hit rate of the stress
167 response gene in our soil metagenomic libraries, suggesting that the tropical mangrove
168 environment is harsh for microorganisms. This is most probably due to polluted marine waters
169 and high salinity and low aeration available in the muddy mangrove soil.

170 The biochemical tests showed considerably high amount of phosphorus and sulfur in our soil
171 sample. However, the gene ontology analysis revealed that the genes associated with the
172 metabolism of compounds containing phosphorus and sulfur were of relatively low abundance,
173 suggesting that these compounds may mainly be involved in redox reactions in electron transport
174 but not in microbial metabolism. This also implies that sulfur and phosphorus compounds exist in
175 stable forms as mangroves are sink for inorganic compounds.

176 Although Actinobacteria and Firmicutes were two of the detected sub-dominant phyla, our
177 analysis showed low frequency of spore producing bacteria such as *Bacillus* and *Streptomyces*.
178 This may explain why the genes for sporulation and dormancy were not detected in our soil
179 metagenomic DNA.

180 **Conclusion**

181 This study demonstrated the high level of microbial diversity in mangrove swamps compared to
182 the limited vegetation that is able to survive in this environment. The high abundance of members
183 of Deltaproteobacteria and heavy metal and toxic compound resistance genes indicates that

184 microorganisms have potential for bioremediation of heavy metals. The differences in the
185 distribution of microorganisms compared with other previous studies on mangrove soils are most
186 likely due to the different geographical locations. To the best of our knowledge, this is the first
187 study of microbial diversity of mangrove soil in Malaysia using the NGS metagenomic approach.
188 More mangrove soil samples collected from different locations in Malaysia are required to be
189 analysed by this approach before a more defined conclusion on the microbiome of Malaysian
190 mangrove soils and its functional genes can be reached.

191

192 **Acknowledgements**

193 Kok-Gan Chan thanks the University of Malaya for the High Impact Research Grants
194 (UM.C/625/1/HIR/MOHE/CHAN/01, Grant No. A-000001-50001 and UM-MOHE HIR Grant
195 UM.C/625/1/HIR/MOHE/CHAN/14/1, no. H-50001-A000027). There are no conflicts among
196 authors regarding this research.

197

198 **References**

- 199 [1] Tama NFY, Wong YS., Retention and distribution of heavy metals in mangrove soils
200 receiving wastewater. *Environ Pollut*, 1996, **94**(3): 283-291.
- 201 [2] Holguin G, Vazquez P, Bashan Y., The role of sediment microorganisms in the productivity,
202 conservation, and rehabilitation of mangrove ecosystems: an overview. *Biol Fert Soils*, 2001,
203 **33**:265–278.
- 204 [3] Sahoo K, Dhal NK., Potential microbial diversity in mangrove ecosystems: a review. *Indian J*
205 *Mar Sci*, 2009, **38**(2): 249-256.
- 206 [4] Sengupta A, Chaudhuri S., Ecology of heterotrophic denitrogen fixation in the rhizosphere of
207 mangrove plant community at the Ganges river estuary in India. *Oecologia*, 1991, **87**: 560-
208 564.
- 209 [5] Holguin G, Guzman MA, Bashan Y., Two new nitrogen-fixing bacteria from the rhizosphere
210 of mangrove trees: their isolation, identification and in vitro interaction with rhizosphere
211 *Staphylococcus* sp. *FEMS Microbiol Ecol*, 1992, **101**: 207-216.
- 212 [6] Alongi DM, Christoffersen P, Tirendi F., The influence of forest type on microbial nutrient
213 relationships in tropical mangrove sediments. *J Exp Mar Biol Ecol*, 1993, **171**(2): 201-223.
- 214 [7] Vazquez P, Holguin G, Puente ME, Lopez-Cortes A, Bashan Y., Phosphate-solubilizing
215 microorganisms associated with the rhizosphere of mangroves in semiarid coastal lagoon.
216 *Bio Fertil Soils*, 2000, **30**: 460-468.
- 217 [8] Andreote FD, Jiménez DJ, Chaves D, Dias ACF, Luvizotto DM, Dini-Andreote F, Fasanella
218 CC, Lopez MV, Baena S, Taketani RG, Melo IS., The microbiome of Brazilian mangrove
219 sediments as revealed by metagenomics. *PLoS ONE*, 2012, doi:
220 10.1371/journal.pone.0038600.

- 221 [9] Xu Z, Hansen MA, Hansen LH, Jacquiod S, Sørensen SJ., Bioinformatic Approaches Reveal
222 Metagenomic Characterization of Soil Microbial Community. *PLoS ONE*, 2014, 9(4):
223 e93445. doi: 10.1371/journal.pone.0093445
- 224 [10] Paul EA., Soil Microbiology, Ecology and Biochemistry (Third Edition). *Elsevier Inc.*, 2007,
225 pp 389-430.
- 226 [11] Zhou J, Bruns MA, Tiedje JM., DNA recovery from soils of diverse composition. *Appl*
227 *Environ Microbiol*, 1996, **62**(2): 316-322.
- 228 [12] Hyatt D, LoCascio PF, Hauser LJ, Uberbacher EC., Gene and translation initiation site
229 prediction in metagenomic sequences. *Bioinformatics*, 2012, **28**(17): 2223-2230.
- 230 [13] Ye Y, Choi JH, Tang H., RAPSearch: a fast protein similarity search tool for short reads.
231 *BMC Bioinformatics*, 2011, doi:10.1186/1471-2105-12-159.
- 232 [14] Zhao Y, Tang H, Ye Y., RAPSearch2: a fast and memory-efficient protein similarity search
233 tool for next generation sequencing data. *Bioinformatics*, 2012, **28**(1): 125-126.
- 234 [15] Huson DH, Mitra S, Weber N, Ruscheweyh HJ, Weber N, Schuster SC., Integrative analysis
235 of environmental sequences using MEGAN4. *Genome Res*, 2011, **21**: 1552-1560.
- 236 [16] Disz T, Akhter S, Cuevas D, Olson R, Overbeek R, Vonstein V Stevens R, Edwards RA.,
237 Accessing the SEED genome databases via web services API: tools for programmers. *BMC*
238 *Bioinformatics*, 2010, doi:10.1186/1471-2105-11-319.
- 239 [17] Bates ST, Berg-Lyons D, Caporaso JG, Walters WA, Knight R, Fierer N., Examining the
240 global distribution of dominant archaeal populations in soil. *ISME J*, 2011, **5**(5): 908-917.
- 241 [18] Hou W, Wang S, Dong H, Jiang H, Briggs BR, Peacock JP, Huang Q, Huang L, Wu G, Zhi
242 X, Li W, Dodsworth JA, Hedlund BP, Zhang C, Hartnett HE, Dijkstra P, Hungate BA., A
243 comprehensive census of microbial diversity in hot springs of Tengchong, Yunnan Province

244 China using 16S RNA gene pyrosequencing. *PLoS ONE*, 2013, doi:
245 10.1371/journal.pone.0053350.

246 [19] Janssen PH., Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S
247 rRNA genes. *Appl Environ Microbiol*, 2006, **72**(3): 1719-1728.

248 [20] dos Santos HF, Cury JC, do Carmo FL, dos Santos AL, Tiedje J, van Elsas JD, Rosado AS,
249 Peixoto RS., Mangrove bacterial diversity and the impact of oil contamination revealed by
250 pyrosequencing: bacterial proxies for oil pollution. *PLoS ONE*, 2011, doi:
251 10.1371/journal.pone.0016943

252 [21] Jeffries TC, Seymour JR, Gilbert JA, Dinsdale EA, Newton K, et al., Substrate Type
253 Determines Metagenomic Profiles from Diverse Chemical Habitats. *PLoS ONE*, 2011, *6*(9):
254 e25173. doi: 10.1371/journal.pone.0025173

255 [22] Ghosh A, Dey N, Bera A, Tiwari A, Sathyaniranjan KB, Chakrabarti K, Chattopadhyay D.,
256 Culture independent molecular analysis of bacterial communities in the mangrove sediment
257 of Sundarban, India. *Saline Syst*, 2010, **6**: 1 doi: 10.1186/1746-1448-6-1.

258 [23] Blöthe M, Akob DM, Kostka JE, Göschel K, Drake HL, Küsel K., pH gradient-induced
259 heterogeneity of Fe(III)-reducing microorganisms in coal mining-associated lake sediments.
260 *Appl Environ Microbiol*, 2008, **74**(4): 1019-1029.

261

262

263

Parameter	Unit	RA
Arsenic (As)	mg/kg	ND(<0.5)
Cadmium (Cd)	mg/kg	0.10
Lead (Pb)	mg/kg	25.34
Mercury (Hg)	mg/kg	ND(<0.05)
pH (10% w/w)	-	5.1
Phosphorus	mg/kg	449.70
Sulfur	mg/kg	1374.52
Carbon:Nitrogen	%	5.19:0.31

264 **Table 1. Results of biochemical analyses of the soil sample.** ND: not detected.

265

266

Parameters	RA
Total no. of sequences (bp)	24 227 393 584
Total no. of quality sequences (bp)	20 907 568 942
Average quality read length (bp)	96.81
Total no. of contigs (>400bp)	552 541
Total no. of contigs length (bp)	502 888 532
Coverage	41.57
Percentage of hits against NCBI 16S Microbial database (%)	0.03
Total CDS assigned to SEED categories (%)	34.71

267 **Table 2. Reads statistics.** The numbers of reads were generated by Illumina HiSeq 2000. The
268 RA sample showed good quality of reads in term of number, length and number of contigs
269 generated.

270

271 Titles and legends to figures

272 **Figure 1 Bacteria and Archaea.** The majority of the reads were assigned to the domain Bacteria
273 by MEGAN. The number of reads detected for archaea was significantly higher than previously
274 reported.

275 **Figure 2a The segregation of phyla in the domain Bacteria.** Proteobacteria was the dominant
276 phylum in the samples with almost half of the reads assigned to this particular phylum.

277 **Figure 2b Classes of bacteria.** Classes of bacteria that were present in the RA sample at more
278 than 1%.

279 **Figure 2c Bacterial genera.** The genera shown are those that had more than 1% reads. Bacterial
280 genera detected in RA sample in which *Acidobacterium* was the most abundant genus.

281 **Figure 3 Classes of Proteobacteria.** Deltaproteobacteria was the most abundant class in RA
282 samples.

283 **Figure 4 Order level of Deltaproteobacteria.** Syntrophobacterales were the most abundant
284 order.

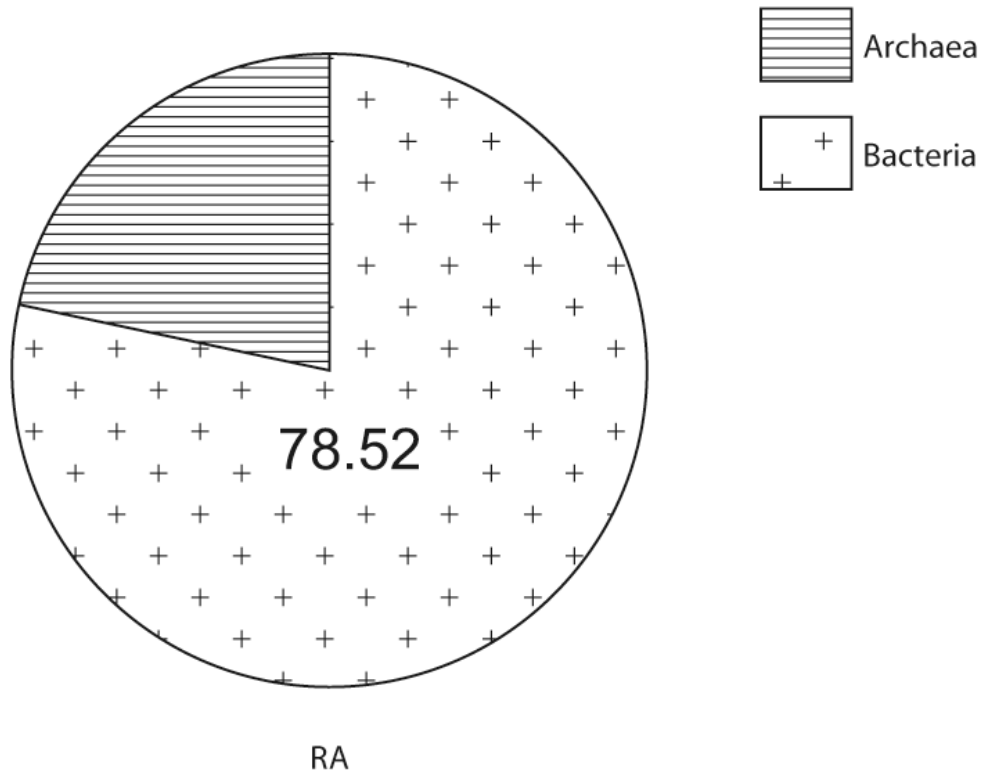
285 **Figure 5 The most abundant genera in Syntrophobacterales.** *Syntrophobacter* was the most
286 abundant genus.

287 **Figure 6 Distribution of archaeal phyla in the sample.** RA sample possessed high percentage
288 of Crenarchaeota.

289 **Figure 7 Gene ontology.** The SEED classification by MEGAN.

290

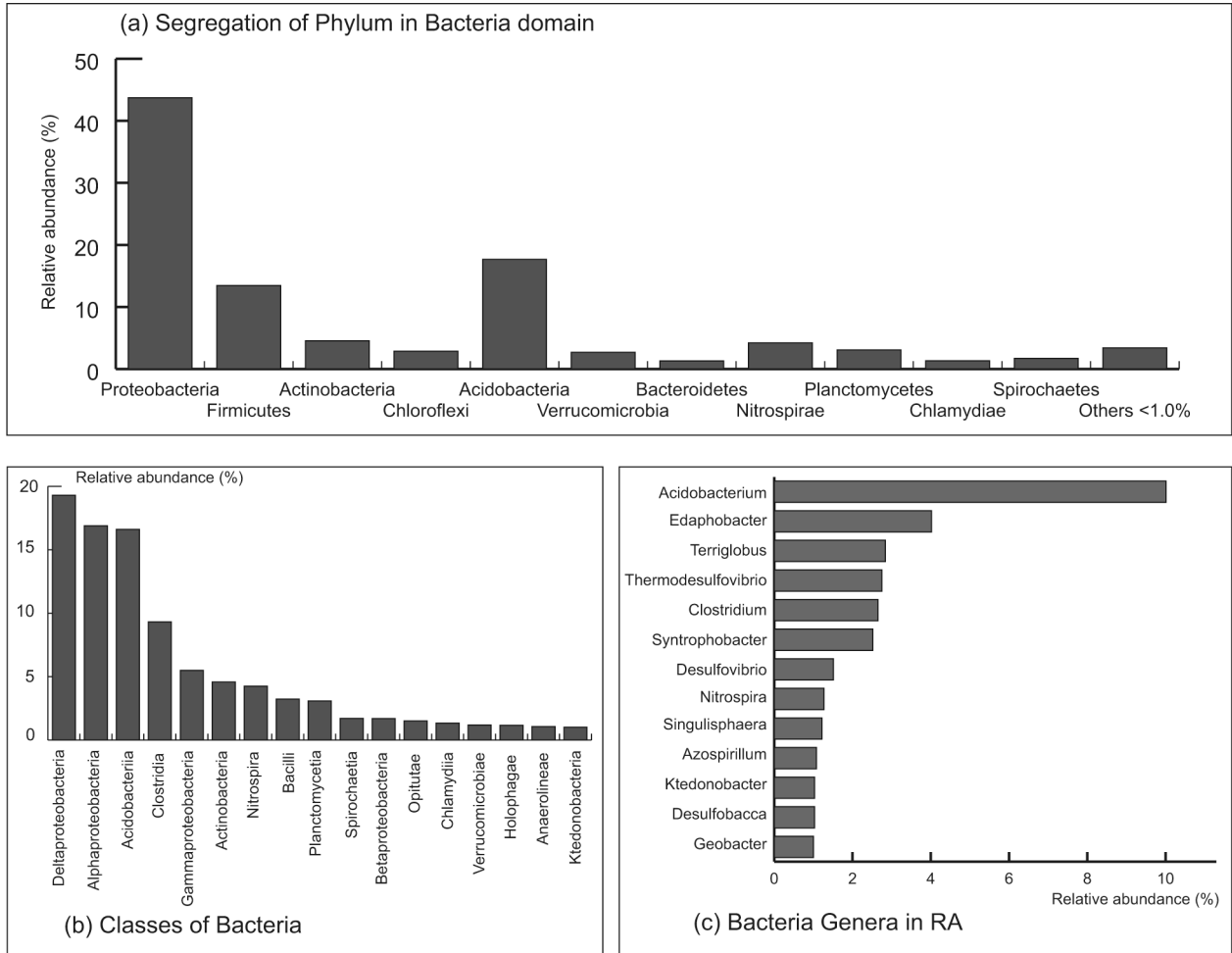
Percentage of assigned reads to domain



291

292 [Figure 1](#)

293

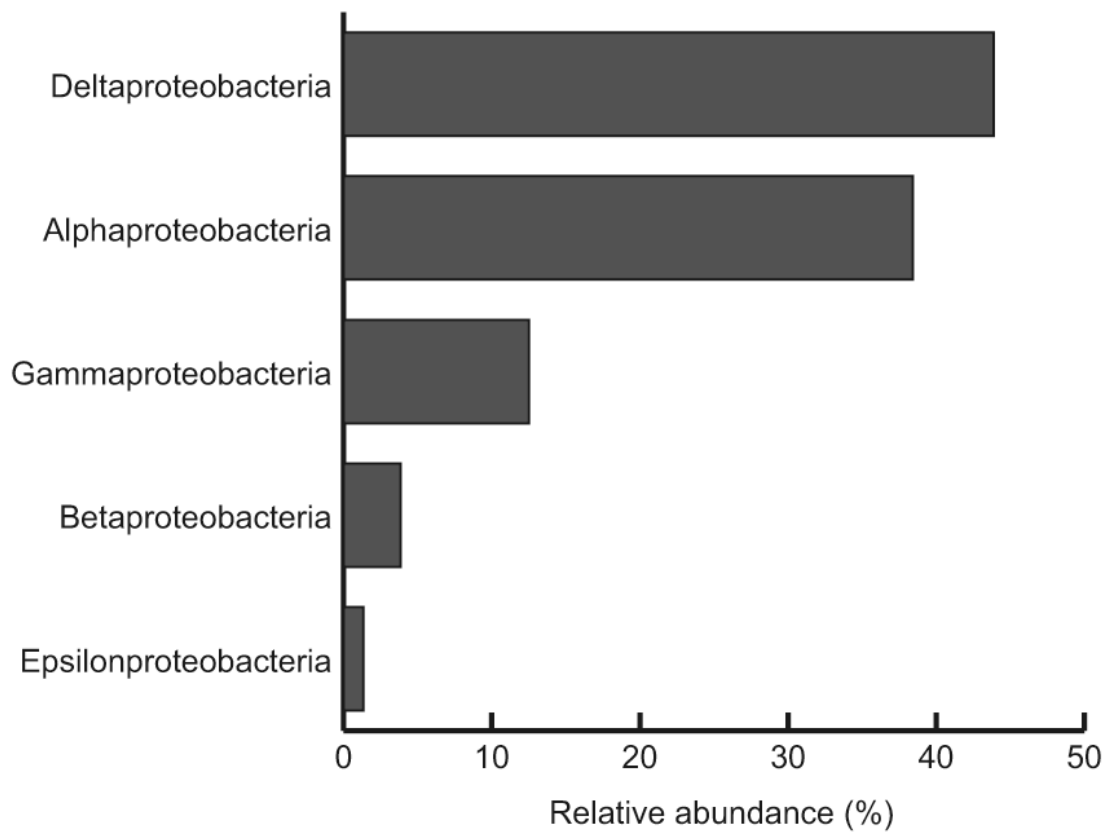


294
295

296 Figure 2

297

Classes of Photobacteria

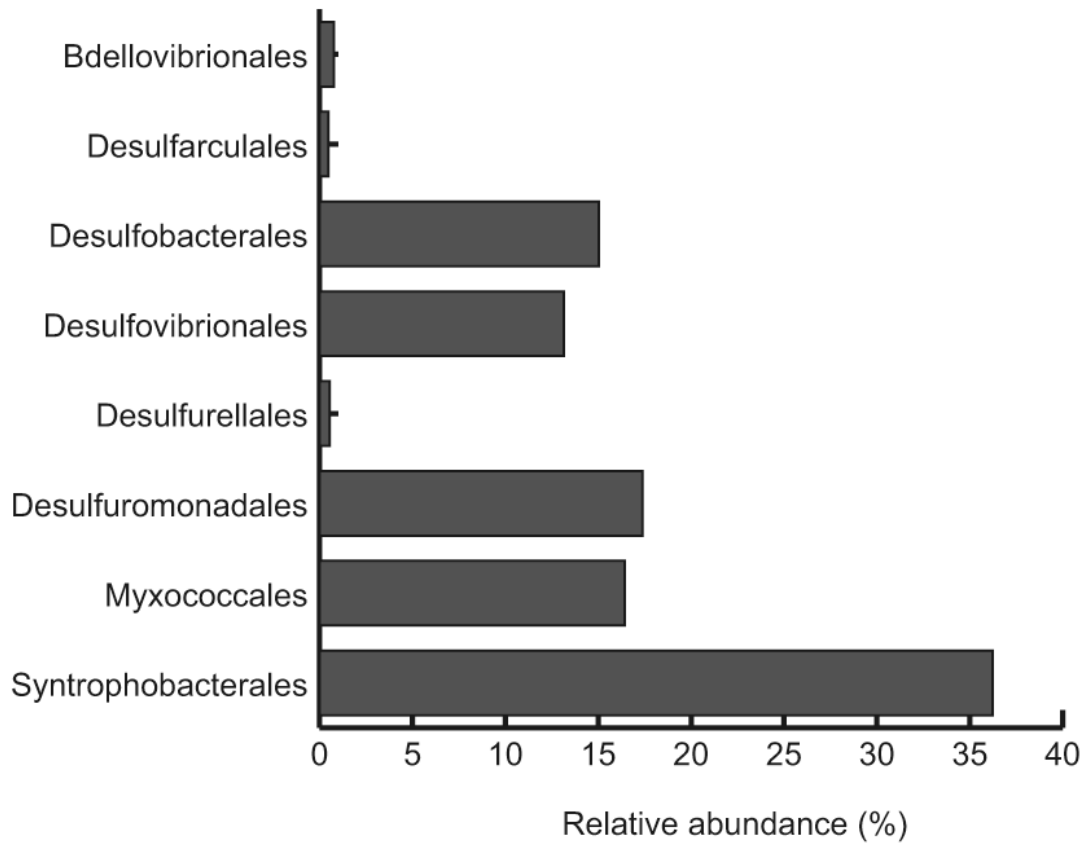


298

299 Figure 3

300

Order level of Deltaprotobacteria

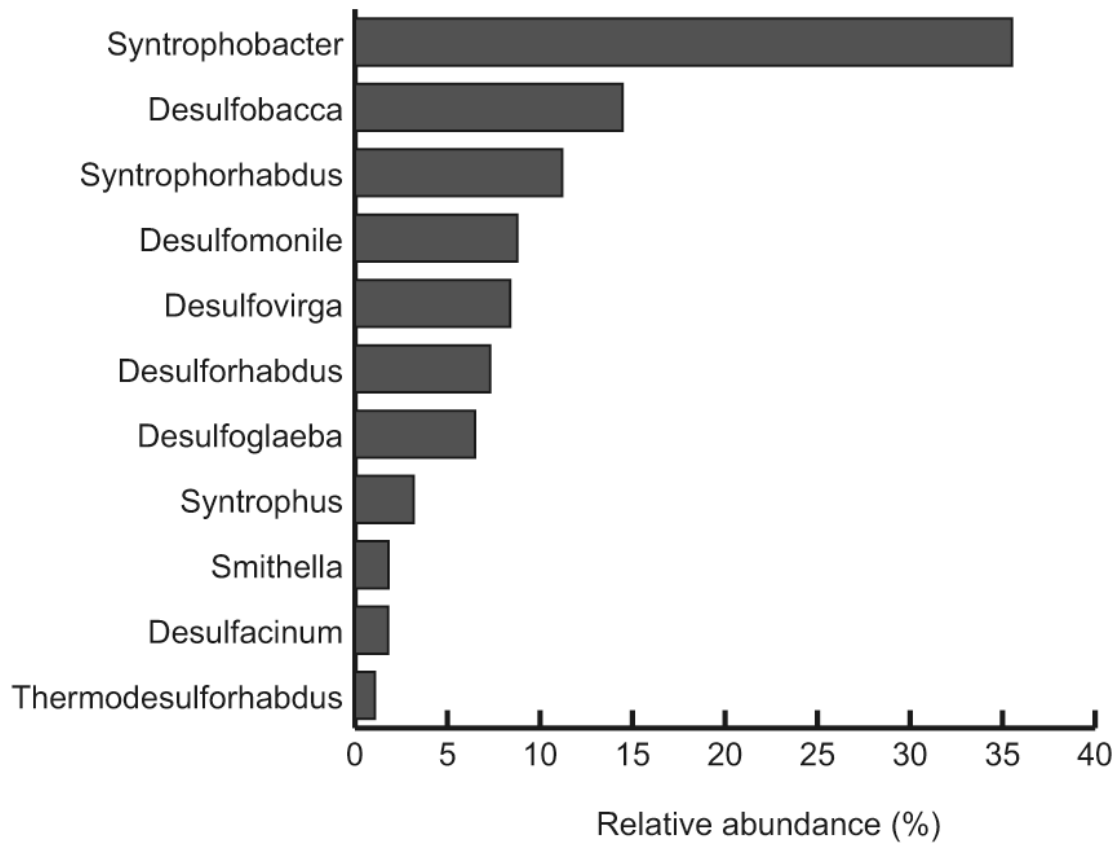


301

302 Figure 4

303

Genus level of Syntrophobacterales

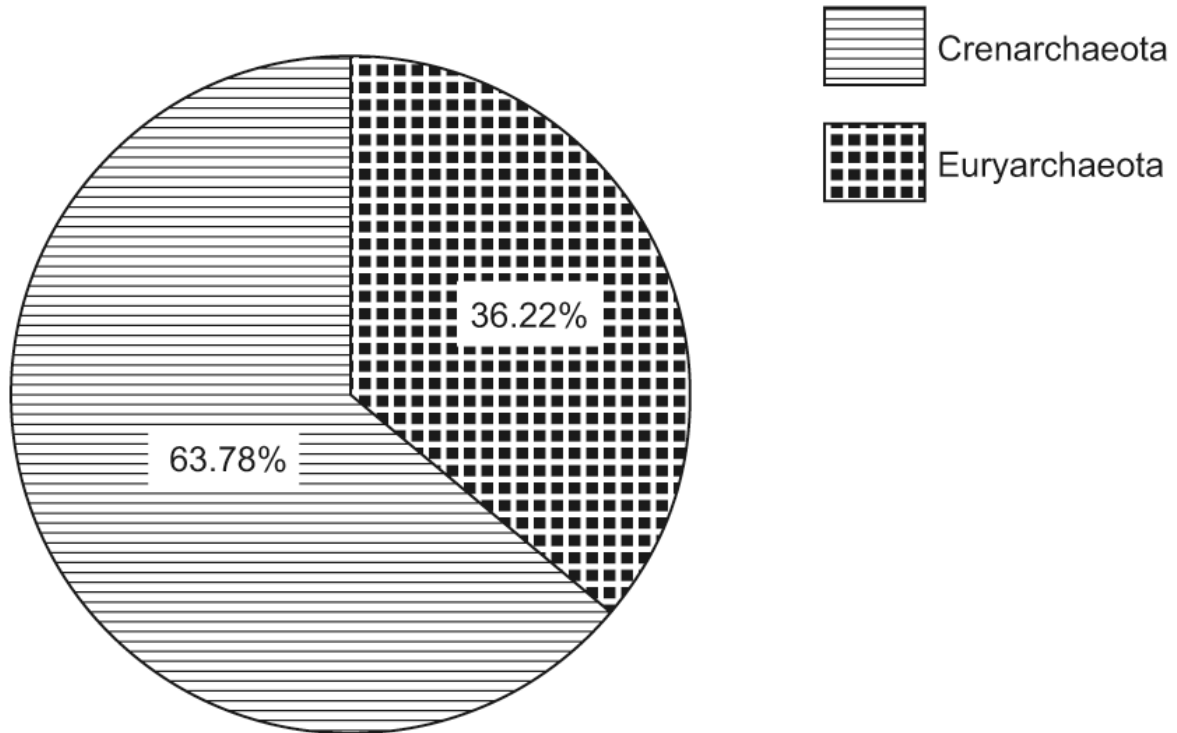


304

305 Figure 5

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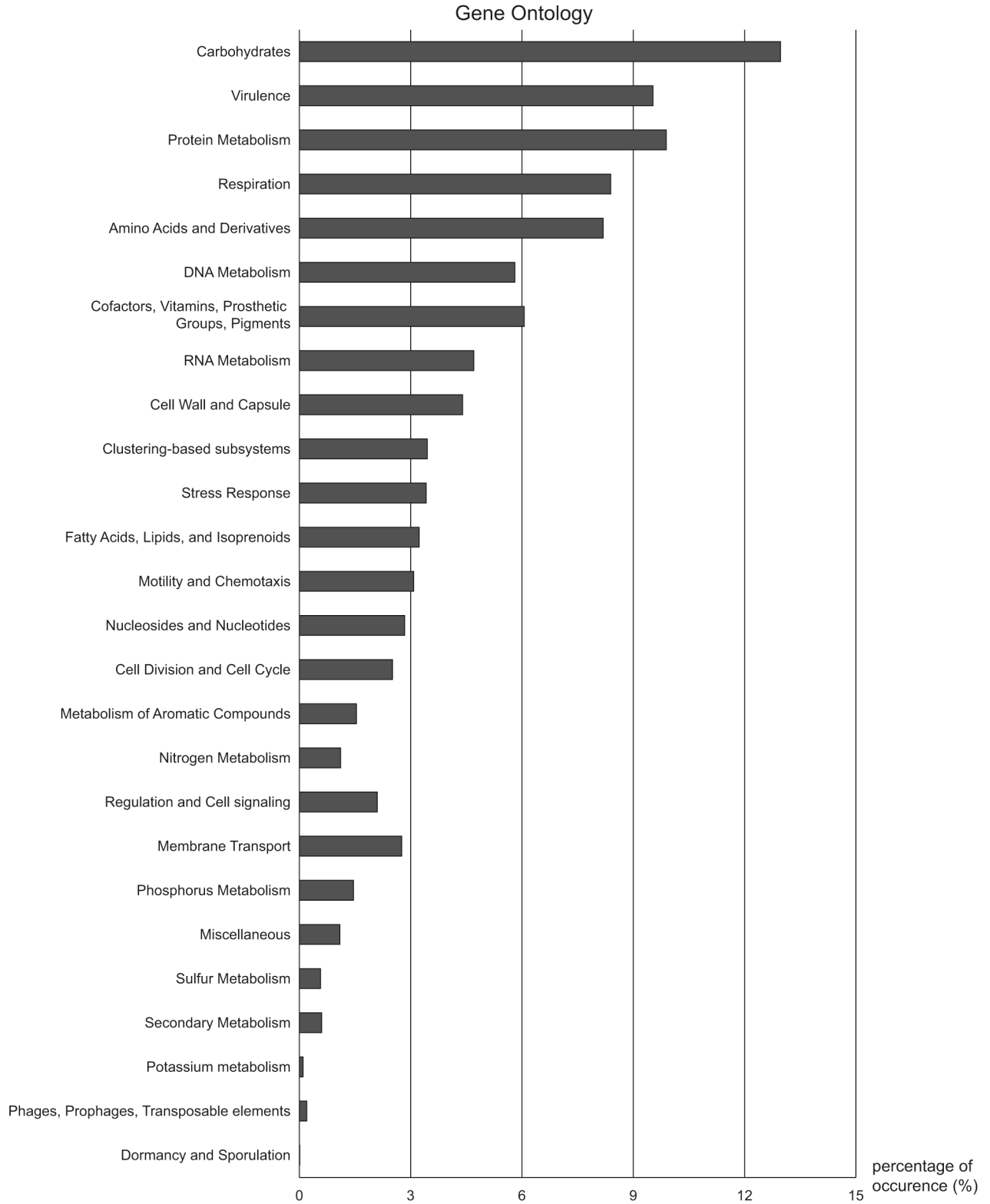
Percentage Distribution of Archaea Phylum



307

308 Figure 6

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311 Figure 7