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2 **Alternative hydrogen uptake pathways**

3 **suppress methane production in ruminants**

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27

28 Abstract

29 Farmed ruminants are the largest source of anthropogenic methane emissions
30 globally. The methanogenic archaea responsible for these emissions use molecular
31 hydrogen (H₂), produced during bacterial and eukaryotic carbohydrate fermentation,
32 as their primary energy source. In this work, we used comparative genomic,
33 metatranscriptomic, and co-culture-based approaches to gain a system-wide
34 understanding of the organisms and pathways responsible for ruminal H₂ metabolism.
35 Two thirds of sequenced rumen bacterial and archaeal genomes encode enzymes
36 that catalyze H₂ production or consumption, including 26 distinct hydrogenase
37 subgroups. Metatranscriptomic analysis confirmed that these hydrogenases are
38 differentially expressed in sheep rumen. Electron-bifurcating [FeFe]-hydrogenases
39 from carbohydrate-fermenting Clostridia (e.g. *Ruminococcus*) accounted for half of all
40 hydrogenase transcripts. Various H₂ uptake pathways were also expressed, including
41 methanogenesis (*Methanobrevibacter*), fumarate reduction and nitrate ammonification
42 (*Selenomonas*), and acetogenesis (*Blautia*). Whereas methanogenesis predominated
43 in high methane yield sheep, alternative uptake pathways were significantly
44 upregulated in low methane yield sheep. Complementing these findings, we observed
45 significant differential expression and activity of the hydrogenases of the
46 hydrogenogenic cellulose fermenter *Ruminococcus albus* and the hydrogenotrophic
47 fumarate reducer *Wolinella succinogenes* in co-culture compared to pure culture. We
48 conclude that H₂ metabolism is a more complex and widespread trait among rumen
49 microorganisms than previously recognized. There is evidence that alternative
50 hydrogenotrophs, including acetogens and selenomonads, can prosper in the rumen
51 and effectively compete with methanogens for H₂ in low methane yield ruminants.
52 Strategies to increase flux through alternative H₂ uptake pathways, including animal
53 selection, dietary supplementation, and methanogenesis inhibitors, may lead to
54 sustained methane mitigation.

55 Introduction

56

57 Methane production by livestock accounts for over 5% of global greenhouse gas
58 emissions annually ¹. These emissions mostly originate from the activity of
59 methanogens within ruminants, which generate methane as an obligate end-product
60 of their energy metabolism ². Several lineages of methanogenic archaea are core
61 members of the microbiome of the ruminant foregut ^{3–5}. Of these, hydrogenotrophic
62 methanogens are dominant in terms of both methane emissions and community
63 composition ^{6,7}, with global surveys indicating that *Methanobrevibacter gottschalkii*
64 and *Methanobrevibacter ruminantium* comprise 74% of the rumen methanogen
65 community ⁵. These organisms use molecular hydrogen (H₂) to reduce carbon dioxide
66 (CO₂) to methane through the Wolfe cycle of methanogenesis ^{8,9}. Rumen
67 methanogens have also been identified that use formate, acetate, methyl compounds,
68 and ethanol as substrates, but usually do so in conjunction with H₂ ^{5,10–12}. Given their
69 major contribution to greenhouse gas emissions, multiple programs are underway to
70 mitigate ruminant methane production ^{13,14}. To date, most strategies have focused on
71 direct inhibition of methanogens using chemical compounds or vaccines ^{15–18}. A
72 promising alternative strategy is to modulate the supply of substrates to methanogens,
73 such as H₂, for example through dietary or probiotic interventions ^{14,19,20}. To achieve
74 this, while maintaining productivity of the host animal, requires an understanding of
75 the processes that mediate substrate supply to methanogens within the rumen.

76

77 H₂, the main substrate supporting ruminal methanogenesis, is primarily produced
78 through fermentation processes ⁶. Various carbohydrate fermentation pathways lead
79 to the production of H₂ as an end-product, together with volatile fatty acids (VFAs) and
80 CO₂ ^{21–23}. This process is supported by hydrogenases, which reoxidize cofactors
81 reduced during carbohydrate fermentation and dispose of the derived electrons by
82 producing H₂. While it is unclear which rumen microorganisms mediate H₂ production
83 *in situ*, a range of isolates have been shown to produce H₂ *in vitro* ^{24–28}. For example,
84 the model rumen bacterium *Ruminococcus albus* 7 reoxidizes the reduced ferredoxin
85 and NADH formed during glucose fermentation using two different [FeFe]-
86 hydrogenases depending on environmental conditions ²⁹. In addition, it is well-
87 established that some rumen fungi and ciliates produce H₂ *via* hydrogenosomes ^{30,31}.

88 A further potential source is the nitrogenase reaction, which produces one H₂ for every
89 N₂ fixed; however, while numerous rumen microorganisms encode putative
90 nitrogenases²¹, there is no convincing *in situ* evidence that N₂ fixation occurs in the
91 rumen³². A large proportion of the H₂ produced by hydrogenogenic fermenters is
92 directly transferred to hydrogenotrophic methanogens, in an ecological process known
93 as interspecies hydrogen transfer^{25,33}. Particularly remarkable are the endosymbiotic
94 and ectosymbiotic associations of methanogens, such as *M. ruminantium*, with rumen
95 ciliates^{34–36}. In addition to providing a continual substrate supply for methanogens,
96 such symbioses benefit fermenters by maintaining H₂ at sufficiently low concentrations
97 for fermentation to remain thermodynamically favorable³⁷.

98
99 Various hydrogenotrophic bacteria are thought to compete with methanogens for the
100 rumen H₂ supply. Most attention has focused on homoacetogens, which mediate
101 conversion of H₂/CO₂ to acetate using [FeFe]-hydrogenases³⁸. Several genera of
102 homoacetogens have been isolated from the rumen, including *Eubacterium*³⁹, *Blautia*
103⁴⁰, and *Acetitomaculum*⁴¹. However, molecular surveys indicate their abundance is
104 generally lower than hydrogenotrophic methanogens^{42–44}. This is thought to reflect
105 that methanogens outcompete acetogens due to the higher free energy yield of their
106 metabolic processes, as well as their higher affinity for H₂. The dissolved H₂
107 concentration fluctuates in the rumen depending on diet, time of feeding, and rumen
108 turnover rates, but is generally at concentrations between 400 to 3400 nM⁴⁵; these
109 concentrations are typically always above the threshold concentrations required for
110 methanogens (< 75 nM) but often below those of homoacetogens (< 700 nM)⁴⁶.
111 Despite this, it has been proposed that stimulation of homoacetogens may be an
112 effective strategy for methane mitigation in methanogen-inhibited scenarios^{14,20,47,48}.
113 Various microorganisms have also been isolated from cows and sheep that support
114 anaerobic hydrogenotrophic respiration, including dissimilatory sulfate reduction (e.g.
115 *Desulfovibrio desulfuricans*)^{49,50}, fumarate reduction and nitrate ammonification (e.g.
116 *Selenomonas ruminantium*, *Wolinella succinogenes*)^{51–58}, and trimethylamine *N*-oxide
117 reduction (e.g. *Denitrobacterium detoxificans*)⁵⁹. The first described and most
118 comprehensively studied of these hydrogen oxidizers is *W. succinogenes*, which
119 mediates interspecies hydrogen transfer with *R. albus*²⁵. In all cases, respiratory
120 electron transfer *via* membrane-bound [NiFe]-hydrogenases and terminal reductases
121 generates a proton-motive force that supports oxidative phosphorylation⁶⁰. It is

122 generally assumed that these pathways are minor ones and are limited by the
123 availability of oxidants. Promisingly, it has been observed that dietary supplementation
124 with fumarate, sulfate, or nitrate can significantly reduce methane production in cattle,
125 likely by stimulating alternative pathways of H₂ consumption^{61,62}.

126

127 We postulate that mitigating methane emissions, while maintaining animal
128 productivity, depends on understanding and controlling H₂ utilization by methanogens.
129 This requires a system-wide perspective of the schemes for production and
130 concomitant utilization of H₂ in the rumen. To facilitate this, we determined which
131 organisms and enzymes are primarily responsible for H₂ production and consumption
132 in rumen. Firstly, we screened genome, metagenome, and metatranscriptome
133 datasets^{21,63,64} to resolve the microbial genera, metabolic pathways, and hydrogenase
134 classes^{65,66} that mediate H₂ metabolism. We demonstrate that ruminants harbor a
135 diverse community of hydrogenogenic fermenters and hydrogenotrophic
136 methanogens, acetogens, sulfate reducers, fumarate reducers, and denitrifiers.
137 Secondly, we used the model system of the H₂-producing carbohydrate fermenter
138 *Ruminococcus albus* 7 and the H₂-utilizing fumarate-reducing syntrophic partner
139 *Wolinella succinogenes* DSM 1740^{25,53,54,67} to gain a deeper mechanistic
140 understanding of how and why ruminant bacteria regulate H₂ metabolism. We
141 observed significant differences in the growth, transcriptome, and metabolite profiles
142 of these bacteria in co-culture compared to pure culture. Finally, we compared gene
143 expression profiles associated with H₂ metabolism between low- versus high-methane
144 yield sheep⁶³. It was recently proposed, on the basis of community structure analysis,
145 that fewer H₂-producing bacteria inhabit low methane yield sheep⁶⁸. In this work, we
146 present an alternative explanation: H₂ uptake through non-methanogenic pathways
147 accounts for these differences. Whereas the enzymes mediating fermentative H₂
148 production are expressed at similar levels, those supporting H₂ uptake through
149 acetogenesis, fumarate reduction, and denitrification pathways are highly upregulated
150 in low methane yield sheep. In turn, these findings support that strategies to promote
151 alternative H₂ uptake pathways, including through dietary modulation, may
152 significantly reduce methane emissions.

153

154 Results

155

156 **H₂ metabolism is a common and diverse trait among rumen bacteria, archaea,**
157 **and eukaryotes**

158

159 We searched the 501 reference genome sequences of rumen bacteria and archaea ²¹
160 for genes encoding the catalytic subunits of H₂-consuming and H₂-producing enzymes
161 **(Table S1 & S2)**. Of these, 65% encoded the capacity to metabolise H₂ via [FeFe]-
162 hydrogenases (42%), [NiFe]-hydrogenases (31%), [Fe]-hydrogenases (2.4%), and/or
163 nitrogenases (23%). This suggests that H₂ metabolism is a widespread trait among
164 rumen microorganisms. We also identified multiple partial sequences of group A1
165 [FeFe]-hydrogenases in the incomplete genomes of six rumen fungi and ciliates. This
166 is consistent with the known ability of these microorganisms to produce H₂ during
167 cellulose fermentation ³¹. The 329 hydrogenase- and nitrogenase-positive genomes
168 spanned 108 genera, 26 orders, 18 classes, and 11 phyla **(Figure 1; Figure S1; Table**
169 **S1 & S2)**.

170

171 We then classified the hydrogenases identified into subgroups. To do so, we used the
172 phylogeny-based, functionally-predictive classification scheme of HydDB ⁶⁶, which has
173 been used to understand H₂ metabolism in a range of organisms and ecosystems ^{69–}
174 ⁷². In total, 273 strains encoded hydrogenases from classes that primarily evolve H₂
175 under physiological conditions **(Table S2)**. These include group A1 and B [FeFe]-
176 hydrogenases and group 4e [NiFe]-hydrogenases that couple ferredoxin oxidation to
177 H₂ production in anaerobic bacteria ^{73–75}. However, the most widespread
178 hydrogenases are the group A3 [FeFe]-hydrogenases, which were encoded in 43
179 genera, among them well-characterized carbohydrate fermenters such as
180 *Ruminococcus*, *Lachnoclostridium*, and *Bacteroides*. These hydrogenases form
181 heterotrimeric complexes, together with diaphorase subunits, that mediate the
182 recently-discovered process of electron-confurcation: coupling co-oxidation of NADH
183 and ferredoxin produced during fermentative carbon degradation to production of H₂
184 ^{29,76}. This reversible complex can also support hydrogenotrophic acetogenesis ⁷⁷. By
185 retrieving the genes immediately upstream and downstream, we verified that the
186 diaphorase subunits (HydB) of this complex were co-encoded with the retrieved
187 hydrogenase subunits **(Figure 1; Table S2)**.

188

189 In addition, multiple organisms encoded hydrogenases and terminal reductases
190 known to support hydrogenotrophic growth (**Figure 1**). All 21 methanogen genomes
191 surveyed harbored [NiFe]-hydrogenases together with the signature gene of
192 methanogenesis (*mcrA*) (**Figure 1; Table S2**). These include 14 *Methanobrevibacter*
193 strains, which encoded a complete set of enzymes for mediating hydrogenotrophic
194 methanogenesis through the Wolfe cycle ⁸, including the [Fe]-hydrogenase and the
195 groups 3a, 3c, 4h, and 4i [NiFe]-hydrogenases. Seven genomes encoded both [FeFe]-
196 hydrogenases (A2, A3) and the marker gene for acetogenesis (*acsB*) (**Table S2**),
197 including known hydrogenotrophic acetogens *Blautia schinkii* ⁴⁰ and *Acetitomaculum*
198 *ruminis* ⁴¹. Several subgroups of the group 1 [NiFe]-hydrogenases, all membrane-
199 bound enzymes known to support hydrogenotrophic respiration ^{65,78}, were also
200 detected. Most notably, various *Selenomonas*, *Mitsuokella*, and *Wolinella* strains
201 encoded such hydrogenases together with the signature genes for fumarate reduction
202 (*frdA*) and nitrate ammonification (*narG*, *napA*, *nrfA*). As anticipated, the group 1b
203 [NiFe]-hydrogenase and *dsrA* gene characteristic of hydrogenotrophic sulfate
204 reduction were also encoded in the three genomes of ruminal *Desulfovibrio* isolates
205 (**Figure 1; Table S2**).

206

207 **H₂ is mainly produced by clostridial electron-bifurcating [FeFe]-hydrogenases** 208 **and consumed by [NiFe]-hydrogenases of methanogens and selenomonads**

209

210 We then investigated the relative abundance and expression levels of the retrieved
211 hydrogenases in rumen communities. To do so, we used 20 pairs of metagenomes
212 and metatranscriptomes that were previously sequenced from the rumen contents of
213 age- and diet-matched sheep ⁶³ (**Table S3**). Screening these datasets with
214 hydrogenases retrieved from the rumen microbial reference genomes yielded 15,464
215 metagenome hits (0.015% of all reads) and 40,485 metatranscriptome hits (0.040%)
216 (**Table S4**). Across the metagenomes, the dominant hydrogenase reads originated
217 from eleven subgroups (A1, A2, A3, B, 3a, 3c, 4e, 4g, 4h, 4i, Fe) (**Figure 2a & S2a**)
218 and three taxonomic orders (Clostridiales, Methanobacteriales, Selenomonadales)
219 (**Figure 2c & S3a**); this is concordant with the hydrogenase content in the genomes
220 of the dominant community members ^{63,64} (**Table S2**). Metatranscriptome analysis
221 indicated these genes were differentially expressed: whereas A3, 1d, 3a, 3c, and 4g
222 genes were highly expressed (RNA / DNA expression ratio > 4), others were

223 expressed at moderate (A1, A2, Fe; ratio 1.5 – 2.5) or low levels (B, 4e, 4h, 4i; ratio <
224 1.5) (**Figure S2 & S3**). Though putative nitrogenase genes (*nifH*) were detected,
225 expression ratios were low (av. 0.45), suggesting nitrogen fixation is not a significant
226 H₂ source in sheep (**Figure S4**).

227

228 Accounting for 54% of hydrogenase transcripts detected (**Figure 2b, 3a, S2**), group
229 A3 [FeFe]-hydrogenases appear to be the primary catalysts of H₂ production in
230 ruminants. We assigned the retrieved transcripts to taxa based on their closest hits to
231 the rumen genome hydrogenase dataset (**Table S4**). Clostridia accounted for the
232 majority of the hits (**Figure 2d**), including *Ruminococcus* (22%), *Saccharofermentans*
233 (9.2%), and *Lachnoclostridium* (7.4%) species known to fermentatively produce H₂
234 ^{29,33,79} (**Figure S5 & S6**). Transcripts from the characterized fermentative genera
235 *Bacteroides*, *Butyrivibrio*, *Clostridium*, and *Sarcina* were also moderately abundant. A
236 further 21% of group A3 [FeFe]-hydrogenase hits were assigned to three
237 uncharacterized cultured lineages within the Clostridia: Clostridiales R-7,
238 Ruminococcaceae P7, and Lachnospiraceae YSB2008 (**Figure S5 & S6**). This is
239 compatible with our previous studies showing unclassified microorganisms, especially
240 from R-7 group, are abundant in rumen ²¹. H₂-evolving hydrogenases from the A1 and
241 B subgroups were also detected, but their RNA/DNA expression ratios were threefold
242 lower than the A3 hydrogenases. Rumen ciliates such as *Epidinium* dominated A1
243 reads (**Figure 2d & S5**), but it is likely that their abundance in the datasets is
244 underestimated due to the minimal genome coverage of these organisms to date.

245

246 The metatranscriptome datasets indicate that multiple H₂ uptake pathways operate in
247 ruminants (**Figure 2 & 3**). In agreement with historical paradigms ⁶, hydrogenotrophic
248 methanogenesis appears to be the largest sink of H₂; methanogens accounted for
249 5.3% of normalized hydrogenase reads (**Figure 2d**) and methyl-CoM reductase
250 (*mcrA*) is the most expressed of the reductases surveyed (**Figure 3c**). Consistent with
251 their central roles in the CO₂-reducing pathway of methanogenesis ⁹, the F₄₂₀-reducing
252 [NiFe]-hydrogenase (3a) ⁸⁰ and the heterodisulfide reductase-associated [NiFe]-
253 hydrogenase (3c) ⁸¹ of *Methanobrevibacter* species were among the most transcribed
254 of all H₂ uptake enzymes (**Figure 3a & S5**). In contrast, the Eha-type (4h), Ehb-type
255 (4i), and [Fe]-hydrogenases were expressed at lower levels (**Figure 3a & S5**),
256 reflecting their secondary roles in the physiology of methanogens ^{82–84}. There was also

257 strong evidence that hydrogenotrophic acetogenesis may be a more significant
258 ruminal H₂ sink than previously recognized. Across the dataset, acetyl-CoA synthases
259 (*acsB*; 1135 normalized reads) were expressed at a quarter of the level of methyl-CoM
260 reductases (*mcrA*; 5246 normalized reads) (**Figure 3c**). For 74% of the reads, the
261 closest matches were to predicted hydrogenotrophic acetogens isolated from rumen,
262 including *Blautia*, *Acetitomaculum*, and *Oxobacter* (**Figure S8 & Table S5**).
263 Consistently, group A2 and group A3 [FeFe]-hydrogenases from the same genera
264 were moderately expressed in the metatranscriptomes (3.7%) (**Figure S5**). The other
265 *acsB* reads likely originate from acetogens that use other electron donors, such as
266 formate.

267
268 Surprisingly, however, the most highly expressed H₂ uptake hydrogenase overall is
269 the group 1d [NiFe]-hydrogenase of Selenomonadales (4.1%) (**Figure 3a, 3b & S5**).
270 This enzyme is likely to mediate the long-known capacity of *Selenomonas* species to
271 grow by hydrogenotrophic fumarate reduction and denitrification^{51,52,57}. Consistently,
272 fumarate reductases (*frdA*), nitrate reductases (*narG*), and ammonia-forming nitrite
273 reductases (*nrfA*) homologous to those in *S. ruminantium* were expressed in the
274 metatranscriptomes (**Figure 3c**). Normalized *nrfA* expression was fivefold higher than
275 *narG*, suggesting selenomonads may preferentially use external nitrite; while further
276 studies are required to determine the source of nitrite, this compound is known to
277 accumulate in the rumen depending on nitrate content of feed⁸⁵. Reads corresponding
278 to the group 1b [NiFe]-hydrogenase, periplasmic nitrate reductase (*napA*), *nrfA*, and
279 *frdA* from *Wolinella* was also detected, but at low levels (**Table S4 & S5; Figure S5**).
280 Several other pathways in low abundance in the metagenome were also highly
281 expressed, notably group 1b [NiFe]-hydrogenases and *dsrA* genes from *Desulfovibrio*
282 species, as well as group 1i [NiFe]-hydrogenases from metabolically flexible
283 Coriobacteriia (e.g. *Slackia*, *Denitrobacterium*) (**Figure S4 & S5**). The expression
284 levels of the 1b and 1d hydrogenases, together with the functionally-unresolved 4g
285 hydrogenases, were the highest of all hydrogenases in datasets (RNA / DNA ratio >
286 10) (**Figure S3**). Though these findings need to be validated by activity-based studies,
287 they suggest that respiratory hydrogenotrophs are highly active and quantitatively
288 significant H₂ sinks in the rumen despite often being detected in low abundance⁵.

289

290 **Culture-based studies demonstrate that hydrogenases mediating H₂ production**
291 **and uptake are differentially regulated in response to hydrogen levels**

292

293 In order to better understand how rumen bacteria regulate H₂ metabolism, we
294 performed a culture-based study using *Ruminococcus albus* 7 and *Wolinella*
295 *succinogenes* DSM 1740, a model system for interspecies hydrogen transfer²⁵. We
296 compared the growth, transcriptome, and extracellular metabolite profiles of these
297 strains in either pure culture or co-culture when grown on modified fumarate-
298 supplemented Balch medium (**Table S6**). The concentrations of the metabolites
299 consumed and produced by the strains varied between the conditions (**Table S1**;
300 **Figure S8**) in a manner consistent with the transcriptomic results (**Figure 4**) and
301 historical paradigms^{24,25,29,53}. Pathway reconstruction indicated that *R. albus*
302 fermentatively degraded cellobiose to H₂, acetate, and ethanol in pure culture (glucose
303 + 3.3 ADP + 3.3 P_i → 2.6 H₂ + 1.3 acetate + 0.7 ethanol + 2 CO₂ + 3.3 ATP²⁹) and H₂
304 and acetate in co-culture (glucose + 4 ADP + 4 P_i → 4 H₂ + 2 acetate + 2 CO₂ + 4 ATP
305²⁹) (**Figure 4a, 4b, 4c**). *W. succinogenes* grew by hydrogenotrophic fumarate
306 respiration under both conditions by using exogenously supplied H₂ in pure culture
307 and syntrophically-produced H₂ in co-culture (**Figure 4d, 4e, 4f**). Hence, *R. albus*
308 channels fermentation through the pathway that yields stoichiometrically more ATP,
309 H₂, and acetate, provided that H₂ concentrations are kept sufficiently low through
310 interspecies hydrogen transfer for this to be thermodynamically favorable.

311

312 Transcriptome profiling revealed that *R. albus* tightly regulates the expression of its
313 three hydrogenases (**Figure 4a, 4b**). Overall, 133 genes were differentially expressed
314 (fold change > 2, *q*-value < 0.05) in co-culture compared to pure culture (**Table S7**).
315 Of these, the greatest fold-change was the 111-fold downregulation of a putative eight-
316 gene cluster encoding the ferredoxin-only hydrogenase (group A1 [FeFe]-
317 hydrogenase), a bifunctional alcohol and aldehyde dehydrogenase, and regulatory
318 elements including a putative sensory hydrogenase (group C [FeFe]-hydrogenase)
319 (**Figure 4a & 4b**). By suppressing expression of these enzymes, *R. albus* can divert
320 carbon flux from ethanol production to the more energetically efficient pathway of
321 acetate production; acetate fermentation produces equimolar levels of NADH and
322 reduced ferredoxin, which can be simultaneously reoxidized by the electron-
323 bifurcating hydrogenase (group A3 [FeFe]-hydrogenase) (**Figure 4c**). Glycolysis

324 enzymes and the phosphate acetyltransferase, acetate kinase, and electron-
325 bifurcating hydrogenase of the acetate production pathway were expressed at similarly
326 high levels under both conditions (**Figure 4a & 4b**). However, there was a significant
327 increase in the biosynthesis of thiamine pyrophosphate, a cofactor for pyruvate
328 dehydrogenase complex, in co-culture (**Figure 4a**).

329

330 The fermentation stoichiometries of *R. albus 7* measured in pure culture compared to
331 co-culture (**Table 1**) were the same as we previously reported for the bacterium at high
332 vs low concentrations of H₂²⁹. This suggests that the differences in regulation are
333 primarily determined by H₂ levels, rather than by direct interactions with syntrophic
334 partners. This regulation may be achieved through direct sensing of H₂ by the putative
335 sensory group C [FeFe]-hydrogenase co-transcribed with the ferredoxin-only
336 hydrogenase and alcohol dehydrogenase (**Figure 4e**). In common with other enzymes
337 of this class^{65,86,87}, this enzyme contains a H-cluster for H₂ binding, a PAS domain for
338 signal transfer, and a putative serine or threonine phosphatase that may modify
339 downstream regulators. Thus, analogous to the well-studied regulatory hydrogenases
340 of aerobic bacteria^{88,89}, this enzyme may directly sense H₂ levels and induce
341 expression of the alcohol / aldehyde dehydrogenase and ferredoxin-only hydrogenase
342 when H₂ concentrations are high through a feedback loop. H₂ sensing may be a
343 general mechanism regulating hydrogenase expression in ruminants, given group C
344 [FeFe]-hydrogenases are abundant in ruminant genome (**Figure 1**), metagenome
345 (**Figure 2a**), and metatranscriptome datasets (**Figure 2b & 3a**).

346

347 The transcriptome results also clarified understanding of hydrogenotrophic fumarate
348 respiration by *W. succinogenes* (**Figure 4d**). In both pure culture and co-culture, the
349 group 1b [NiFe]-hydrogenase, fumarate reductase, and F₁F_o-ATPase that mediate this
350 process were expressed at high levels (**Table S7; Figure 4f**). A periplasmic
351 asparaginase, aspartate ammonia-lyase, and dicarboxylate-binding proteins were
352 also highly expressed; this suggests that the organism can efficiently produce and
353 import additional fumarate from amino acid sources (**Table S7**). In total, 352 genes
354 were significantly differentially regulated in co-culture (fold-change > 2, q-value <
355 0.05). The respiratory hydrogenase was among the upregulated genes (**Figure 4e**),
356 which may reflect the strain's faster growth rate in co-culture (**Table 1**). The
357 periplasmic nitrate reductase and ammonia-forming nitrite reductase (**Figure 4f**) were

358 also induced, indicating some plasticity in oxidant usage, in line with the
359 metatranscriptomic findings. Two formate dehydrogenases and a formate
360 hydrogenlyase (group 4a [NiFe]-hydrogenase) were highly expressed in co-culture
361 **(Figure 4d & 4e)**. This suggests the bacterium can potentially use formate, known to
362 be produced through formate pyruvate lyase by *R. albus*, as an additional electron
363 donor **(Figure 4a)**. However, the significance of these findings is unclear given no
364 formate was detected under any condition **(Table 1)** and the expression of formate-
365 dependent hydrogenases was extremely low in metatranscriptome datasets **(Figure**
366 **3c)**.

367

368 **Hydrogenotrophic acetogenesis, fumarate reduction, and denitrification** 369 **pathways are significantly upregulated in low methane yield ruminants**

370

371 Finally, we tested whether the abundance and expression of hydrogenases and H₂
372 uptake pathways differed between high and low methane yield sheep. The current
373 leading hypothesis, proposed on the basis of community composition⁶⁸, asserts that
374 H₂ production levels account for differences in methane yield between sheep. To the
375 contrary, the expression levels of the dominant H₂-evolving hydrogenases (e.g. group
376 A3 [FeFe]-hydrogenases) and taxonomic orders (e.g. Clostridiales) were in fact
377 extremely similar between the groups **(Figure 3a & 3b; Table S8 & S9)**.

378

379 We therefore formulated an alternative hypothesis: H₂ utilization through non-
380 methanogenic pathways can reduce methane yield. In line with this hypothesis, the
381 expression levels of the five methanogen hydrogenases and methyl-CoM reductase
382 are significantly reduced in low methane yield sheep **(Figure 3a & 3c; Table S8 & 10)**,
383 confirming a strong correlation with measured phenotypes **(Table S3)**. Concurrent
384 increases in the gene expression for two major alternative H₂ sinks were detected,
385 namely acetogenesis (*acsB*; $p < 0.0001$) and fumarate reduction (*frdA*; $p = 0.002$)
386 **(Figure 3c; Table S10)**, concomitant with significant increases in the expression levels
387 of *Blautia* and *Selenomonas* hydrogenases **(Figure S5)**. Expression levels of *nrfA*
388 were also on average 1.8-fold higher in low methane yield sheep, though there was
389 much inter-sample variation in the read count for this gene. Whereas there are more
390 transcripts of *mcrA* than other terminal reductases combined in high methane yield
391 sheep, the transcript levels of *acsB* and *nrfA* together exceed those of *mcrA* in low

392 methane yield sheep. Depending to what extent expression levels predict activity,
393 hydrogenotrophic acetogens and selenomonads may therefore be more active than
394 methanogens in low methane yield sheep and may significantly limit substrate supply
395 for methanogenesis. Two other potential H₂ sinks are also upregulated in the low
396 methane yield sheep: the putative group 1i [NiFe]-hydrogenase of *Coriobacteriia* and,
397 consistent with previous observations⁶⁴, the functionally-unresolved group A2 [FeFe]-
398 hydrogenase of *Sharpea*, *Olsenella*, and *Oribacterium* (**Figure 3a, 3b, S5**).

399

400 Discussion

401

402 To summarize, H₂ metabolism is a more widespread and complex process in
403 ruminants than previously realized. Together, the genomic, metagenomic, and
404 metatranscriptomic surveys suggest that multiple orders of bacteria, archaea, and
405 eukaryotes encode and express enzymes mediating H₂ production and consumption
406 in the rumen. We infer that fermentative Clostridia are the main source of H₂ in the
407 rumen, which largely agrees with findings from activity-based and culture-based
408 studies^{6,25,26,29}. However, a surprising finding is that uncharacterized lineages within
409 the Clostridia account for a large proportion of hydrogenase reads, emphasizing the
410 need for physiological and bacteriological characterization of these organisms. Further
411 studies are also needed to better account for the role of rumen ciliates and fungi, which
412 to date are underrepresented in genomic datasets.

413

414 One of the most important findings of this work is that the recently-characterized
415 electron-bifurcating hydrogenases appear to primarily mediate ruminal H₂ production.
416 These enzymes are highly upregulated compared to ferredoxin-only hydrogenases *in*
417 *situ* and constitute over half of hydrogenase reads in metatranscriptomes. We provide
418 a rationale for this finding by showing that *Ruminococcus albus*, a dominant H₂
419 producer within the rumen, expresses its electron-bifurcating hydrogenase and
420 suppresses its ferredoxin-only hydrogenase when grown syntrophically with *Wolinella*
421 *succinogenes*. In this condition, H₂ concentrations remain sufficiently low that the
422 fermentation pathway producing higher levels of ATP, H₂, and acetate remains
423 thermodynamically favorable. In the rumen, where tight coupling of hydrogenogenic
424 and hydrogenotrophic processes usually keeps H₂ at sub-micromolar concentrations

425 ⁴⁵, Clostridia will also preferentially oxidize carbohydrates through higher ATP-yielding
426 pathways and reoxidize the NAD and ferredoxin reduced using the electron-bifurcating
427 hydrogenase. It is likely that the ferredoxin-only hydrogenases are preferentially
428 upregulated during the transient periods where H₂ levels are high, for example
429 immediately after feeding ⁴⁵. Based on these findings and previously published results
430 ^{29,65,86,87}, we propose that the hydrogenases and fermentation pathways are
431 differentially regulated as a result of direct H₂ sensing by putative sensory [FeFe]-
432 hydrogenases.

433

434 The other major finding of this work is that there are multiple highly active H₂ sinks in
435 the rumen. We provide evidence, based on transcript levels of their hydrogenases and
436 terminal reductases, that acetogens (*Blautia*, *Acetitomaculum*), fumarate reducers and
437 denitrifiers (*Selenomonas*, *Wolinella*), and sulfate reducers (*Desulfovibrio*) are
438 quantitatively significant H₂ sinks in sheep. In support of these findings, our culture-
439 based study confirmed that the enzymes mediating hydrogenotrophic fumarate
440 reduction and potentially nitrate ammonification are highly expressed by *W.*
441 *succinogenes* in co-culture with *R. albus*. While alternative H₂ uptake pathways have
442 been previously detected *in vitro* ^{40,41,49–52,56,57}, it has generally been assumed that
443 they are quantitatively insignificant compared to hydrogenotrophic methanogenesis
444 ^{5,6,45}. To the contrary, hydrogenase and terminal reductase transcripts from alternative
445 H₂ uptake pathways are more numerous than those of methanogens in low methane
446 yield sheep, and hence these pathways may collectively serve as a larger H₂ sink than
447 methanogenesis under some circumstances. These findings justify activity-based
448 studies to quantify H₂ flux within ruminants between the pathways. There is also
449 evidence of other novel pathways operating in the rumen, mediated by the functionally
450 unresolved group 1i [NiFe]-hydrogenases (*Slackia*, *Denitrobacterium*), group 4g
451 [NiFe]-hydrogenase (*Clostridium*), and group A2 [FeFe]-hydrogenases (*Sharpea*,
452 *Oribacterium*, *Olsenella*).

453

454 The strong correlation between H₂ uptake pathways and methane yield phenotypes
455 suggests that modulating H₂ metabolism may be an effective methane mitigation
456 strategy. One strategy is to develop inhibitors that redirect electron flux from H₂
457 production towards volatile fatty acid production. However, given the central role of H₂
458 metabolism in the physiology and ecology of most rumen microorganisms, this would

459 be challenging to achieve without compromising rumen function and consequently
460 ruminant nutrition. Furthermore, such strategies may have a converse effect on
461 methane production, given lower H₂ concentrations restrict acetogens more than
462 methanogens⁴⁵. Instead, our metatranscriptome analyses suggest a more promising
463 approach may be to stimulate alternative H₂ pathways such as fumarate, nitrate, and
464 sulfate respiration. Selective breeding of low methane yield sheep is an option, given
465 methane yield and in turn metatranscriptome profiles have been shown to be a
466 quantitative heritable trait to some extent^{63,90}. However, the similar metagenome
467 profiles between the sheep, combined with the metatranscriptome profiles of the
468 phenotype-switching sheep, indicate alternative H₂ uptake pathways are also
469 inducible. Another solution may be to supplement animal feeds with electron
470 acceptors, such as fumarate, nitrate, or sulfate, that stimulate the dominant respiratory
471 hydrogenotrophs. Such approaches have shown some promise in mitigating methane
472 production both *in vitro*⁹¹⁻⁹³ and in field trials^{61,62,94,95}. These strategies may
473 complement methanogenesis inhibitors^{16,17} by facilitating the redirection of H₂ flux
474 from methanogens to other pathways.

475

476 **Materials and Methods**

477

478 **Comparative genomic analysis**

479 The protein sequences of the 501 genomes of cultured rumen bacteria (410 from
480 Hungate Collection²¹, 91 from other sources) were retrieved from the Joint Genome
481 Institute (JGI) genome portal. These sequences were then screened against local
482 protein databases for the catalytic subunits of the three classes of hydrogenases
483 (NiFe-hydrogenases, FeFe-hydrogenases, Fe-hydrogenases), nitrogenases (NifH),
484 methyl-CoM reductases (McrA), acetyl-CoA synthases (AcsB), adenylylsulfate
485 reductases (AprA), dissimilatory sulfite reductases (DsrA), alternative sulfite
486 reductases (AsrA), fumarate reductases (FrdA), dissimilatory nitrate reductases
487 (NarG), periplasmic nitrate reductases (NapA), ammonia-forming nitrite reductases
488 (NrfA), DMSO / TMAO reductases (DmsA), and cytochrome *bd* oxidases (CydA).
489 Hydrogenases were screened using the HydDB dataset^{66,96}, targeted searches were
490 used to screen six protein families (AprA, AsrA, NarG, NapA, NrfA, DmsA, CydA), and
491 comprehensive custom databases were constructed to screen five other protein

492 families (NifH, McrA, AcsB, DsrA, FrdA) based on their total reported genetic diversity
493 ^{97–101}. A custom Python script incorporating the Biopython package ¹⁰² for producing
494 and parsing BLAST results was used to batch-submit the protein sequences of the
495 501 downloaded genomes as queries for BLAST searches against the local
496 databases. Specifically, hits were initially called for alignments with an e-value
497 threshold of 1e-50 and the resultant XML files were parsed. Alignments producing hits
498 were further filtered for those with coverage values exceeding 90% and percent
499 identity values of 30% to 70%, depending on the target, and hits were subsequently
500 manually curated. **Table S1** provides the FASTA protein sequences and alignment
501 details of the filtered hits. For hydrogenases, the protein sequences flanking the
502 hydrogenase large subunits were also retrieved; these sequences were used to
503 classify group A [FeFe]-hydrogenases into subtypes (A1 to A4), as previously
504 described ⁹⁶, and retrieve diaphorase sequences (HydB) associated with the A3
505 subtype. Partial [FeFe]-hydrogenase protein sequences from six incompletely
506 sequenced rumen ciliates and fungi genomes were retrieved through targeted blastP
507 searches ¹⁰³ in NCBI.

508

509 **Metagenomic and metatranscriptomic analysis**

510 We analyzed previously published datasets of twenty paired metagenomes and
511 metatranscriptomes of sheep rumen contents ⁶³. All profiles were derived from the
512 rumen contents of age-matched, pelleted lucerne-fed rams that were collected four
513 hours after morning feeding and subject to paired-end sequencing on the HiSeq 2000
514 platform ⁶³. The samples were taken from ten rams at two different sampling dates
515 based on their measured methane yields ^{63,90}; four rams were consistently low yield,
516 four were consistently high yield, and two others switched in methane yield between
517 the sampling dates (**Table S3**). The metagenome and metatranscriptome datasets
518 analyzed are accessible at the NCBI Sequence Read Archive (SRA;
519 <http://www.ncbi.nlm.nih.gov/sra>) accession numbers SRA075938, and SRX1079958 -
520 SRX1079985 under bioproject number PRJNA202380. Each metagenome and
521 metatranscriptome was subsampled to an equal depth of 5 million reads using seqtk
522 (<https://github.com/lh3/seqtk>) seeded with parameter -s100. Subsampled datasets
523 were then screened in DIAMOND (default settings, one maximum target sequence per
524 query) ¹⁰⁴ using the protein sequences retrieved from the 507 rumen microbial
525 genomes (NiFe-hydrogenases, FeFe-hydrogenases, Fe-hydrogenases, HydB, NifH,

526 McrA, AcsB, AprA, DsrA, AsrA, FrdA, NarG, NapA, NrfA, DmsA, CydA). Results were
527 then filtered (length of amino acid > 40 residues, sequence identity > 65%). Subgroup
528 classification and taxonomic assignment of the hydrogenase reads was based on their
529 closest match to the hydrogenase dataset derived from the 507 genomes at either
530 65% or 85% identity. The number of reads with the rumen-specific hydrogenase
531 dataset (15464 metagenome hits, 40485 metatranscriptome hits) exceeded those
532 obtained by screening with the generic dataset from HydDB ⁶⁶ (12599 metagenome
533 reads, 31155 metatranscriptome reads), verifying the rumen dataset comprehensively
534 captures hydrogenase diversity. For each dataset, read count was normalized to
535 account for the average length of each gene using the following formula: Normalized
536 Read Count = Actual Read Count × (1000 / Average Gene Length). Independent two-
537 group Wilcoxon rank-sum tests were used to determine whether there were significant
538 differences in the targets analyzed between low and high methane yield sheep.
539 Separate analyses were performed based on gene abundance, transcript abundance,
540 and RNA/DNA ratio.

541

542 **Bacterial growth conditions and quantification**

543 The bovine rumen isolates *Ruminococcus albus* 7 ⁶⁷ and *Wolinella succinogenes* DSM
544 1740 ⁵³ were cultured anaerobically at 37°C in modified Balch medium ¹⁰⁵ (**Table S6**).
545 Pre-cultures were grown in Balch tubes (18 × 150 mm; Chemglass Life Sciences,
546 Vineland, NJ) containing 20% v/v culture medium and sealed with butyl rubber
547 stoppers crimped with aluminium caps. Cultures were grown in Pyrex side-arm flasks
548 (Corning Inc., Corning, NY) containing 118 mL modified Balch medium. Two pre-
549 cultures were grown before final inoculation, and all inoculum transfers were 5% (v/v).
550 The headspace consisted of 20% CO₂ and 80% N₂ for *R. albus* pure cultures and the
551 co-cultures, and 20% CO₂ and 80% H₂ for *W. succinogenes* pure cultures. Cultures
552 were periodically sampled at 0, 3, 5, 7, 9, and 11 h for metabolite analysis and bacterial
553 quantification. Culture samples were immediately centrifuged (16,000 × *g*, 10 min) in
554 a bench-top centrifuge (Eppendorf, Hamburg, Germany). For metabolite analysis, the
555 supernatant was collected and further centrifuged (16,000 × *g*, 10 min) before HPLC
556 analysis. For bacterial quantification, DNA was extracted from each pellet using the
557 Fungal/Bacterial DNA MiniPrep kit according to the manufacturer's instructions (Zymo
558 Research, Irvine, CA). Quantitative PCR (qPCR) was used to quantify the number of
559 copies of the Rumal_2867 (*R. albus* glucokinase gene; FW:

560 CTGGGATTCCTGAACTTTCC; RV: ATGCATACTGCGTTAG) and WS0498 (*W.*
561 *succinogenes* *flgL* gene; FW: CAGACTATACCGATGCAACTAC; RV:
562 GAGCGGAGGAGATCTTTAATC) against pGEM-T-Easy standards of each gene of
563 known concentration. DNA was quantified using the iTaq Universal SYBR Green Mix
564 (Bio-Rad) using a LightCycler 480 (Roche Holding AG, Basel, Switzerland).

565

566 **Liquid and gas metabolite analysis**

567 The concentrations of acetate, ethanol, fumarate, succinate, and formate in the culture
568 supernatants were analyzed using an Ultra-Fast Liquid Chromatograph (UFLC;
569 Shimadzu, Kyoto, Japan). The UFLC consisted of a DGU-20A5 degasser, a SIL-
570 20A-CHT autosampler, an LC-20AT solvent delivery unit, an RID-10A refractive index
571 detector, a CBM-20A system controller, and a CTO-20AC column oven. The mobile
572 phase was 5 mM H₂SO₄ passed through an Aminex HPX-87H ion exclusion column
573 (Bio-Rad, Hercules, CA) at a flow rate of 0.4 mL min⁻¹, 25°C. Each culture was also
574 sampled at 0 h and 24 h to analyze H₂ percentage mixing ratios using a gas
575 chromatograph (GC; Gow-Mac Series 580 Thermal Conductivity Gas Chromatograph,
576 Gow-Mac Instrument Co., Bethlehem, PA). Samples were withdrawn directly from the
577 culture tube in a gas-tight syringe and 0.5 mL was injected into GC for analysis using
578 N₂ as the carrier gas. The flow rate was 60 mL min⁻¹, the detector was set to 80°C, the
579 injector was set to 80°C, and the oven was set to 75°C. For both liquid and gas
580 analyses, peak retention times and peak area were compared to standards of known
581 concentration.

582

583 **RNA extraction and sequencing**

584 Each pure culture and co-culture used for transcriptome analysis was grown in
585 duplicate in Balch tubes. Growth was monitored until the cultures were in mid-
586 exponential phase; the change in OD₆₀₀ at this phase was 0.14 for *W. succinogenes*,
587 0.20 for *R. albus*, and 0.35 for the co-culture. At mid-exponential phase, 5 mL cultures
588 were harvested by centrifugation (13,000 x *g*, 4°C). Cell pellets were resuspended in
589 400 µL fresh lysis buffer (5 mM EDTA, 0.5% SDS, 25 mM lysozyme, 250 U mL⁻¹
590 mutanolysin, and 150 µg mL⁻¹ proteinase K in 25 mM sodium phosphate buffer, pH
591 7.0) and incubated 30 minutes at 55°C with periodic vortexing. RNA was subsequently
592 extracted using an RNeasy Mini Kit following the manufacturer's protocol, including all
593 optional steps (Qiagen, Hilden, Germany) and eluted with 50 µL ultra-pure DEPC-

594 treated water (Invitrogen, Carlsbad, CA). RNA quantity, quality, and integrity were
595 confirmed by Qubit Fluorometry (Invitrogen, Carlsbad, CA), Nanodrop UV-Vis
596 Spectrophotometry (Thermo Fisher Scientific, model 2300c), and agarose gel
597 electrophoresis respectively. Bacterial rRNA was removed from 1 µg of total RNA with
598 the MicroExpress Kit (Life Technologies, Carlsbad, CA). Libraries were prepared on
599 the enriched mRNA fraction using the Tru-Seq Stranded RNA Sample Prep Kit
600 (Illumina, San Diego, CA). The barcoded libraries were pooled in equimolar
601 concentration the pool and sequenced on one lane for 101 cycles on a HiSeq2000
602 using a TruSeq SBS Sequencing Kit (Version 3). Fastq files were generated and
603 demultiplexed with the bc12fastq Conversion Software (Illumina, version 1.8.4). The
604 RNA-seq data were analyzed using CLC Genomics Workbench version 5.5.1 (CLC
605 Bio, Cambridge, MA). RNA-seq reads were mapped onto the reference genome
606 sequences of *Ruminococcus albus* 7¹⁰⁶ and *Wolinella succinogenes* DSM 1740¹⁰⁷
607 **(Table S7 & S11)**. The RNA-seq output files were analyzed for statistical significance
608 as described¹⁰⁸ and q-values were generated using the qvalue package in R¹⁰⁹.
609 Predicted subsystems and functions were downloaded and aligned to the RNA-seq
610 transcriptional data using the RAST Server¹¹⁰.

611

612 **Footnotes**

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624

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626 conceived this study. C.G., R.I.M., G.M.C., I.C., S.E.M., M.J.M., and X.C.M. designed

627 research, supervised students, and analyzed data. C.G., L.C.W., M.J.M., and S.C.L.
628 performed the comparative genomic analysis. C.G., C.W., S.E.M., G.M.C., R.R.G.,
629 G.T.A., W.J.K., S.C.L., and X.C.M. performed the metagenomic and
630 metatranscriptomic analysis. R.G. performed the co-culture experiments and R.G.,
631 R.I.M., I.C., and C.G. analyzed the results. C.G. wrote and illustrated the paper with
632 input from all authors.

633

634 The authors declare no conflict-of-interest.

635 **Figures**

636 **Figure 1. Heatmap showing distribution of enzymes mediating H₂ production**
637 **and H₂ consumption in orders of rumen microorganisms.** Results are shown
638 based on screens of the 501 genomes of cultured rumen bacteria and archaea (410
639 from the Hungate collection plus 91 other genomes). Partial hydrogenase sequences
640 were also retrieved and classified from four rumen ciliates and two rumen fungi. The
641 left-hand side of the heatmap shows the distribution of the catalytic subunits of
642 enzymes that catalyze H₂ oxidation and production. These are divided into
643 fermentative hydrogenases (H₂-producing; group A1, A2, B FeFe-hydrogenases),
644 bifurcating hydrogenases (bidirectional; group A3, A4 FeFe-hydrogenases),
645 respiratory hydrogenases (H₂-uptake; group 1b, 1c, 1d, 1f, 1i, 2d NiFe-hydrogenases),
646 methanogenic hydrogenases (H₂-uptake; group 1k, 3a, 3c, 4h, 4i NiFe-hydrogenases,
647 Fe-hydrogenases), energy-converting hydrogenases (bidirectional; group 4a, 4c, 4e,
648 4f, 4g NiFe-hydrogenases), sensory hydrogenases (group C FeFe-hydrogenases),
649 and nitrogenases (H₂-producing; NifH). The right-hand side shows the distribution of
650 the catalytic subunits of key reductases in H₂ consumption pathways. They are genes
651 for methanogenesis (McrA, methyl-CoM reductase), acetogenesis (AcsB, acetyl-CoA
652 synthase), sulfate reduction (DsrA, dissimilatory sulfite reductase; AprA,
653 adenylylsulfate reductase; AsrA, alternative sulfite reductase), fumarate reduction
654 (FrdA, fumarate reductase), nitrate ammonification (NarG, dissimilatory nitrate
655 reductase; NapA, periplasmic nitrate reductase; NrfA, ammonia-forming nitrite
656 reductase), dimethyl sulfoxide and trimethylamine *N*-oxide reduction (DmsA, DMSO
657 and TMAO reductase), and aerobic respiration (CydA, cytochrome *bd* oxidase). Only
658 hydrogenase-encoding orders are shown. **Table S2** shows the distribution of these
659 enzymes by genome, **Figure S1** depicts hydrogenase subgroup distribution by class,
660 and **Table S1** lists the FASTA sequences of the retrieved reads.

661

662 **Figure 2. Hydrogenase content in the metagenomes and metatranscriptomes of**
663 **the microbial communities within rumen contents of high and low methane yield**
664 **sheep.** Hydrogenase content is shown based on hydrogenase subgroup (a, b) and
665 predicted taxonomic affiliation (c, d) for metagenome datasets (a, c) and
666 metatranscriptome datasets (b, d). Hydrogenase-encoding sequences were retrieved
667 from 20 paired shotgun metagenomes and metatranscriptomes randomly subsampled
668 at five million reads. Reads were classified into hydrogenase subgroups and
669 taxonomically assigned at the order level based on their closest match to the
670 hydrogenases within the genomes screened (**Figure 1**). L01 to L10 are datasets for
671 sheep that were low methane yield at time of sampling, H01 to H20 are datasets from
672 sheep that were high methane yield at time of sampling (see **Table S3** for full details).
673

674 **Figure 3. Comparison of expression levels of H₂ production and H₂ uptake**
675 **pathways in low and high methane yield sheep.** Results are shown for ten
676 metatranscriptome datasets each from low methane yield sheep (orange) and high
677 methane yield sheep (blue) that were randomly subsampled at five million reads. (a)
678 Normalized count of hydrogenase transcript reads based on hydrogenase subgroup.
679 (b) Normalized count of hydrogenase transcript reads based on predicted taxonomic
680 affiliation. (c) Normalized count of transcript reads of key enzymes involved in H₂
681 production and H₂ consumption, namely the catalytic subunits of [NiFe]-hydrogenases
682 (NiFe), [FeFe]-hydrogenases (FeFe), [Fe]-hydrogenases (Fe), hydrogenase-
683 associated diaphorases (HydB), nitrogenases (NifH), methyl-CoM reductases (McrA),
684 acetyl-CoA synthases (AcsB), adenylylsulfate reductases (AprA), dissimilatory sulfite
685 reductases (DsrA), alternative sulfite reductases (AsrA), fumarate reductases (FrdA),
686 dissimilatory nitrate reductases (NarG), periplasmic nitrate reductases (NapA),
687 ammonia-forming nitrite reductases (NrfA), DMSO / TMAO reductases (DmsA), and
688 cytochrome *bd* oxidases (CydA) are provided. For FrdA, NrfA, and CydA, the
689 numerous reads from non-hydrogenotrophic organisms (e.g. Bacteroidetes) were
690 excluded. Each boxplot shows the ten datapoints and their range, mean, and quartiles.
691 Significance was tested using independent two-group Wilcoxon rank-sum tests (* $p <$
692 0.05; ** $p <$ 0.01; *** $p <$ 0.001; **** $p <$ 0.0001; full p values in **Table S8**, **Table S9**
693 and **Table S10**). Note the metagenome abundance and RNA / DNA ratio of these
694 genes is shown in **Figure S2** (hydrogenase subgroup), **Figure S3** (hydrogenase
695 taxonomic affiliation), and **Figure S4** (H₂ uptake pathways). A full list of metagenome
696 and metatranscriptome hits is provided for hydrogenases in **Table S4** and H₂ uptake
697 pathways in **Table S5**.

698

699 **Figure 4. Comparison of whole genome expression levels of *Ruminococcus***
700 ***albus* and *Wolinella succinogenes* in pure culture and co-culture.** Pure cultures
701 and co-cultures of *Ruminococcus albus* 7 (a, b, c) and *Wolinella succinogenes* DSM
702 1740 (d, e, f) were harvested in duplicate during mid-exponential phase and subject
703 to RNA sequencing. (a & d) Volcano plots of the ratio of normalized average transcript
704 abundance for co-cultures over pure cultures. Each gene is represented by a grey dot
705 and key metabolic genes, including hydrogenases, are highlighted as per the legend.
706 (b & d) Predicted operon structure of the three hydrogenases of *R. albus* and two
707 hydrogenases of *W. succinogenes*. (e) Comparison of dominant fermentation
708 pathways of *R. albus* in pure culture (left) and co-culture (right) based on transcriptome
709 reads and metabolite profiling. The three enzymes downregulated in co-culture are in
710 red font. (f) Respiratory chain composition of *W. succinogenes* in pure culture and co-
711 culture based on transcriptome reads. Metabolite profiling indicated that the
712 respiratory hydrogenase and fumarate reductases were active in both conditions. A
713 full list of read counts and expression ratios for each gene is provided in **Table S**

714 **Table 1. Comparison of growth parameters and metabolite profiles of**
 715 ***Ruminococcus albus* and *Wolinella succinogenes* in pure culture and co-**
 716 **culture.** Growth of pure cultures and co-cultures of *Ruminococcus albus* 7 and
 717 *Wolinella succinogenes* DSM 1740 was monitored by qPCR. Values show means \pm
 718 standard deviations of three biological replicates. Also shown is the change in
 719 extracellular pH, percentage hydrogen gas (measured by gas chromatography), and
 720 concentrations of fumarate, succinate, acetate, ethanol, and formate (measured by
 721 ultra-fast liquid chromatography) between 0 hours and 12 hours. Growth media was
 722 the same between the three conditions, except 80% H₂ was added for *W.*
 723 *succinogenes* growth, whereas no H₂ was added for the other conditions. Full liquid
 724 metabolite measurements are shown in **Figure S8**. BDL = below detection limit.

725

	<i>Ruminococcus albus</i>	<i>Wolinella succinogenes</i>	Co-culture
Growth parameters			
Growth yield (OD ₆₀₀)	0.79 \pm 0.01	0.36 \pm 0.01	0.93 \pm 0.01
Specific growth rate (h ⁻¹)	0.58 \pm 0.19	0.33 \pm 0.06	0.57 \pm 0.34 (<i>Ra</i>) 0.54 \pm 0.11 (<i>Ws</i>)
Concentration changes of extracellular metabolites			
Hydrogen (%)	+5.3	-78.4	BDL
Fumarate (mM)	-5.5	-46.3	-43.1
Succinate (mM)	+2.2	+54.6	+55.4
Acetate (mM)	+21.8	0	+32.4
Ethanol (mM)	+8.7	0	+0.3
Formate (mM)	BDL	BDL	BDL
pH	-0.4	-0.4	-0.6

726

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Bacteria

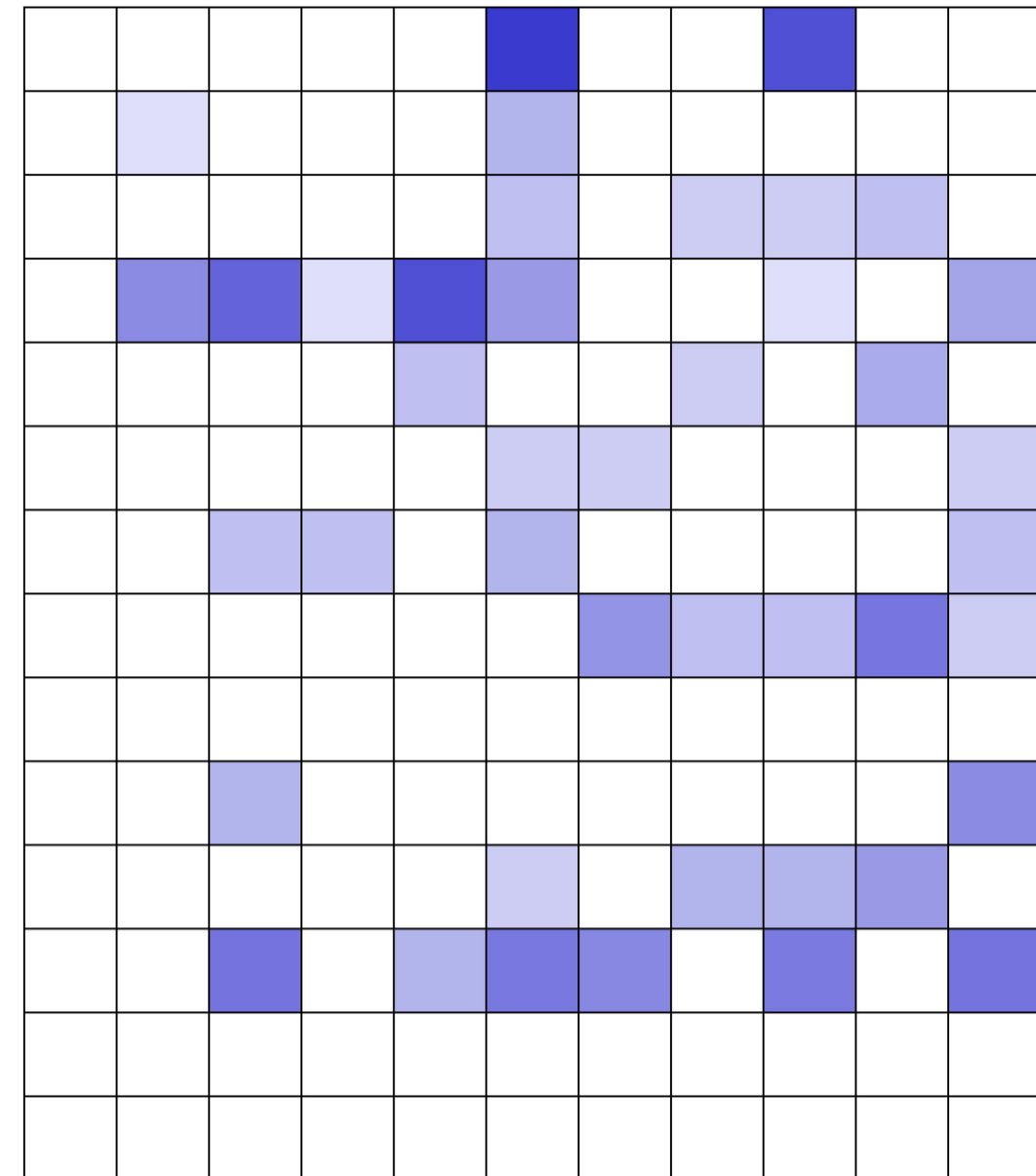
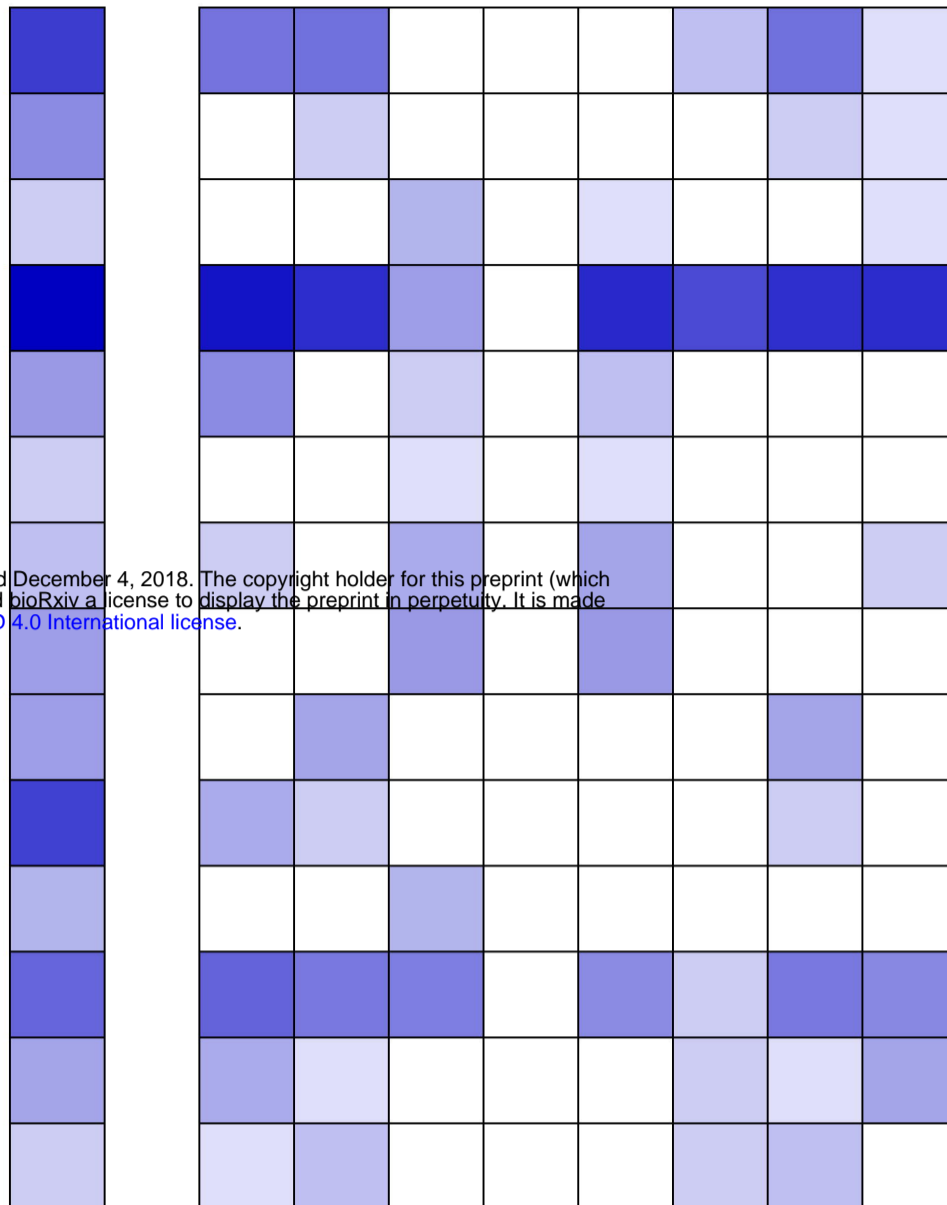
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Archaea

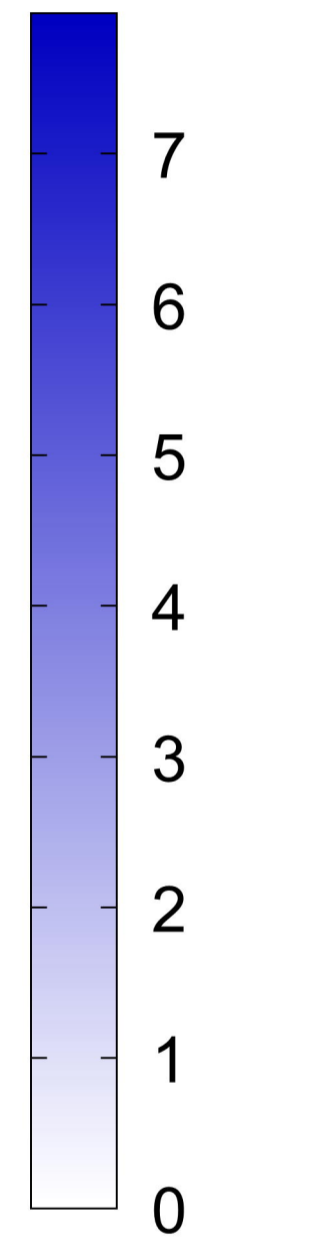
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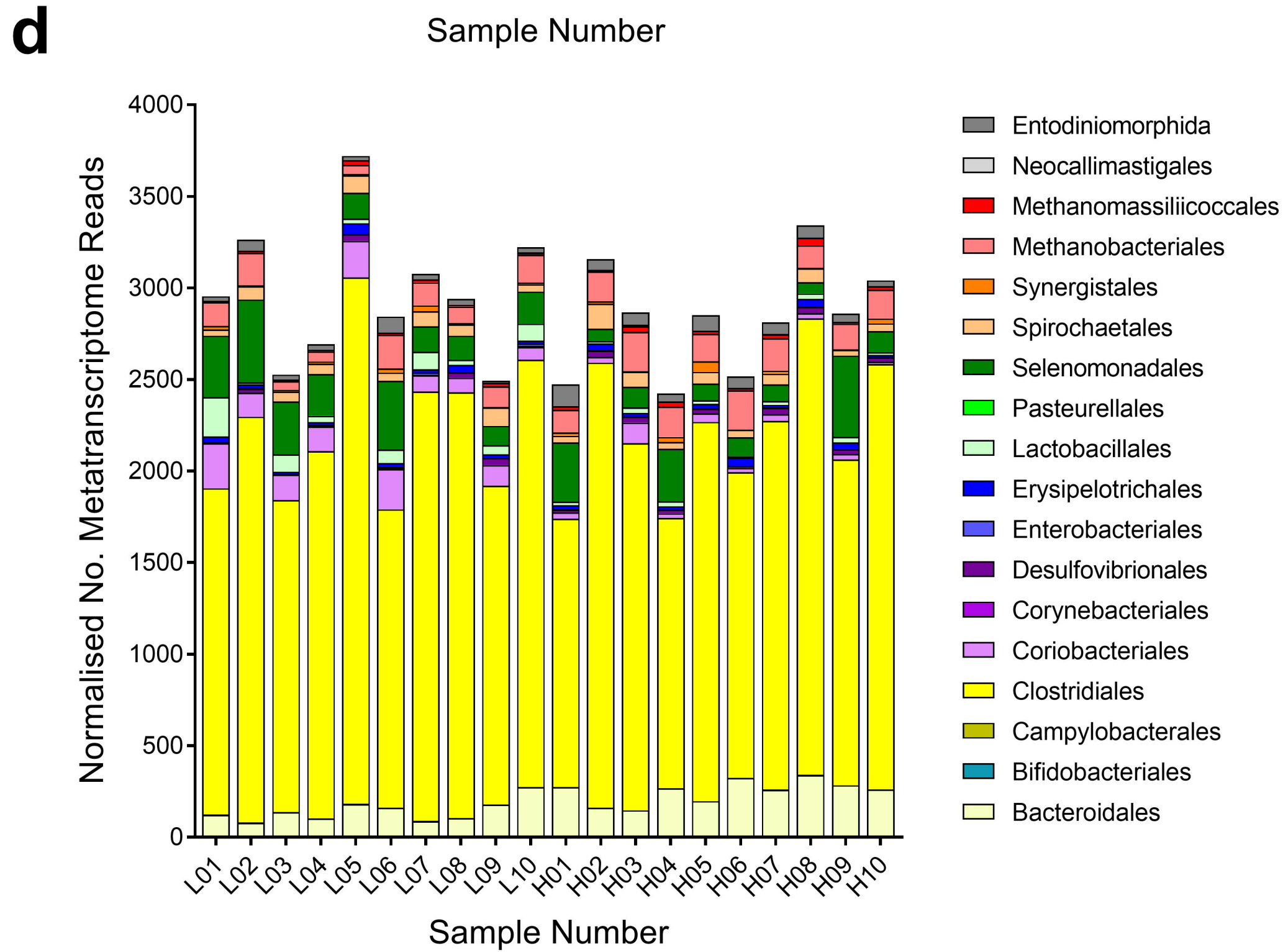
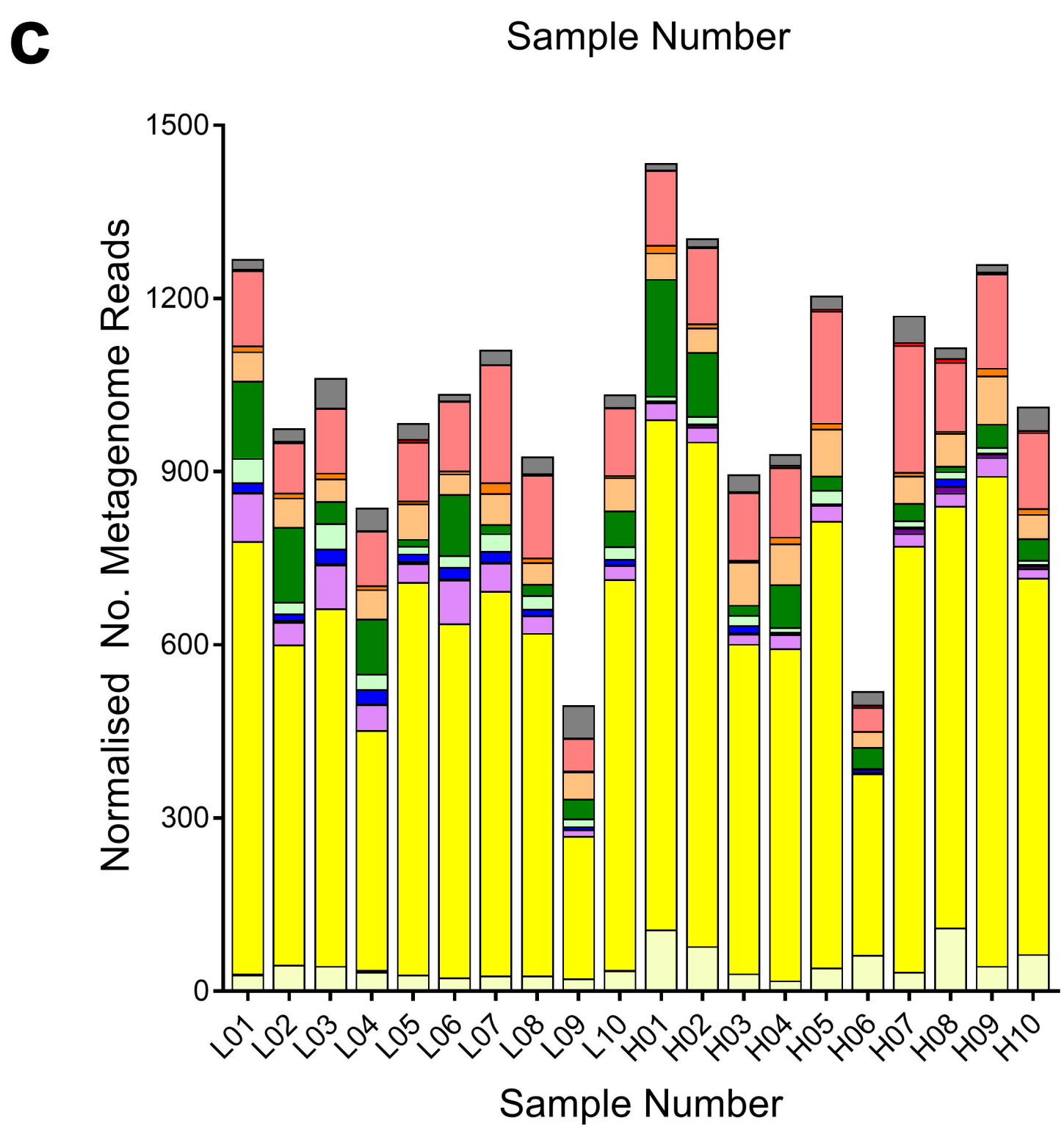
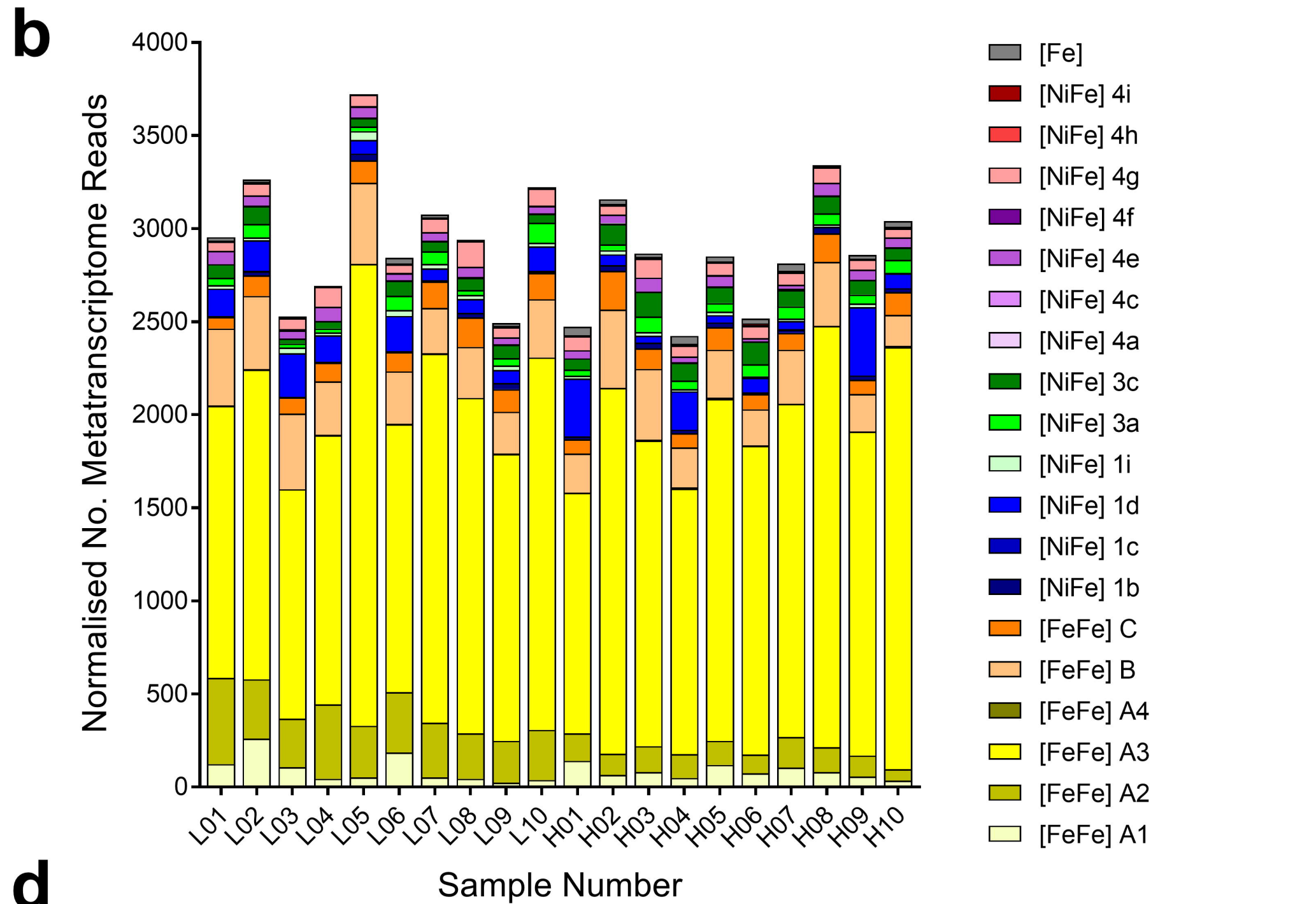
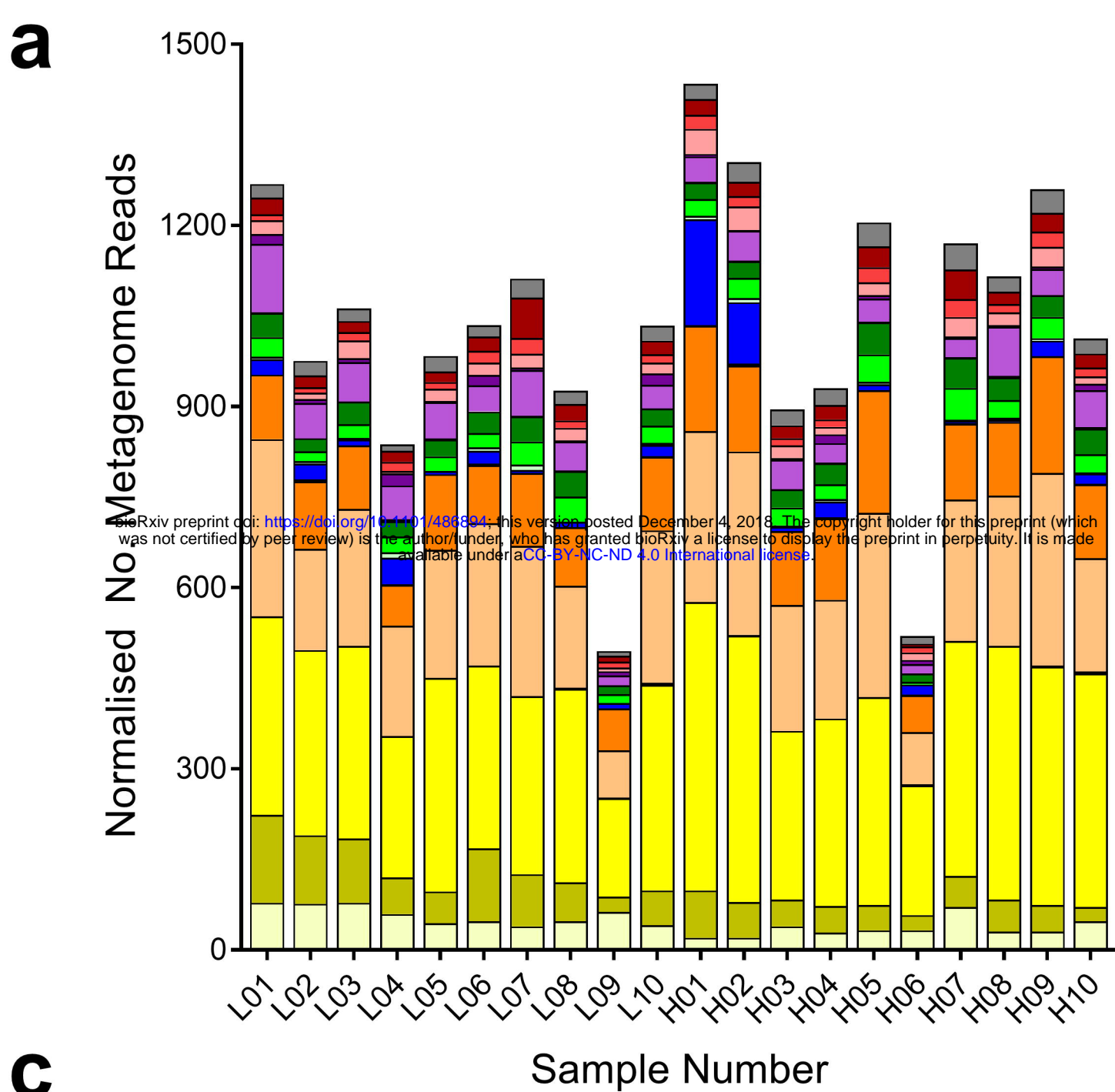


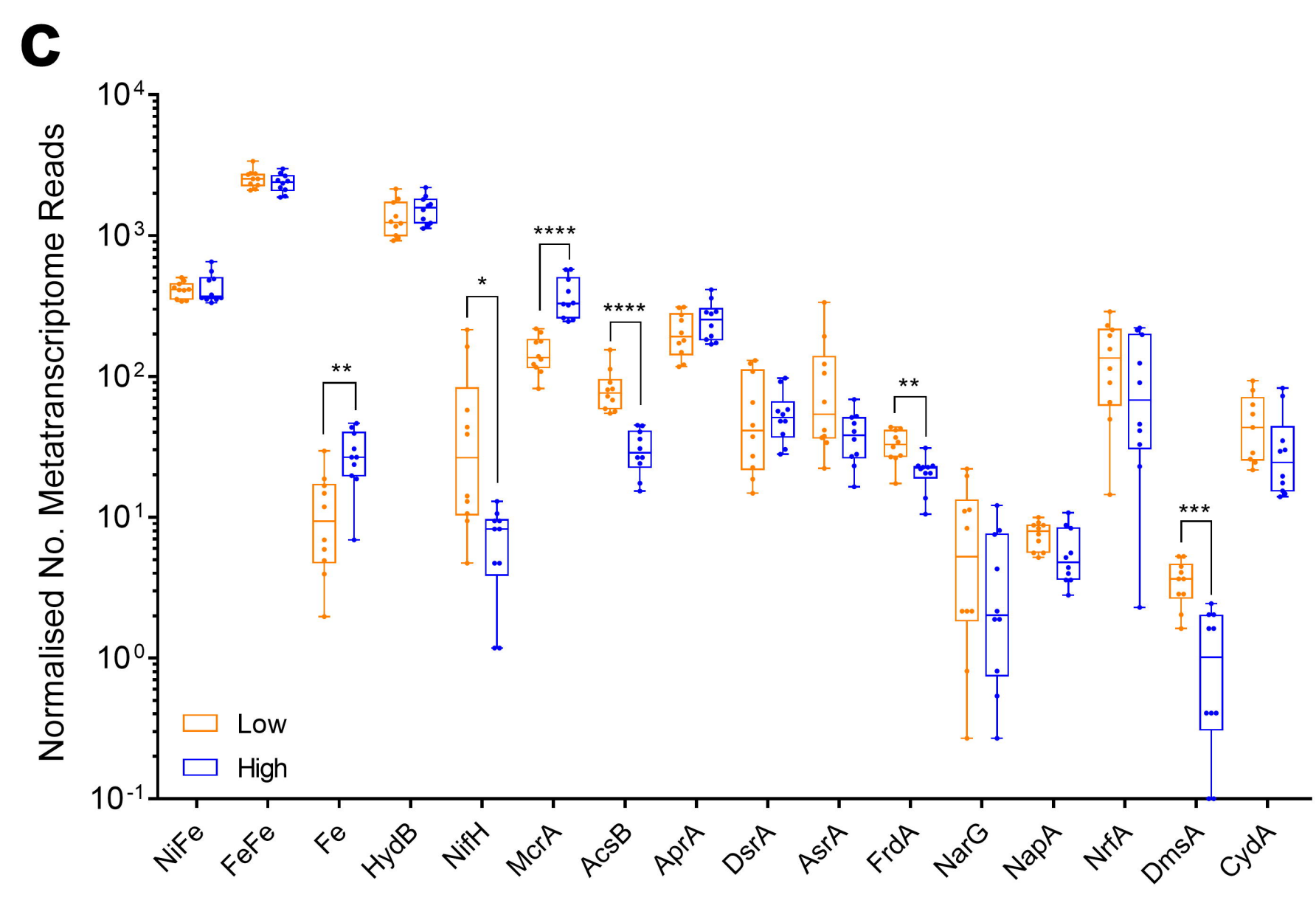
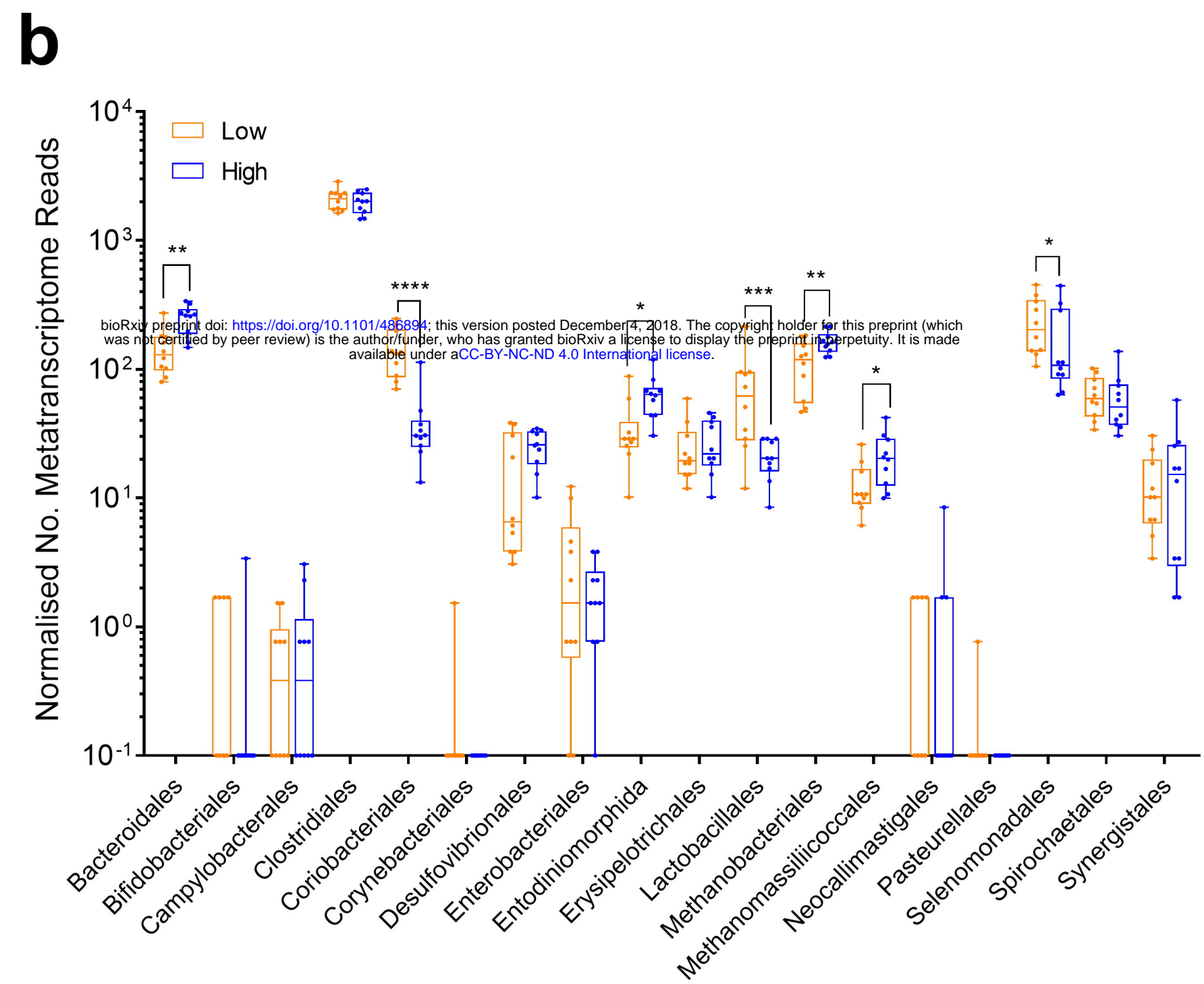
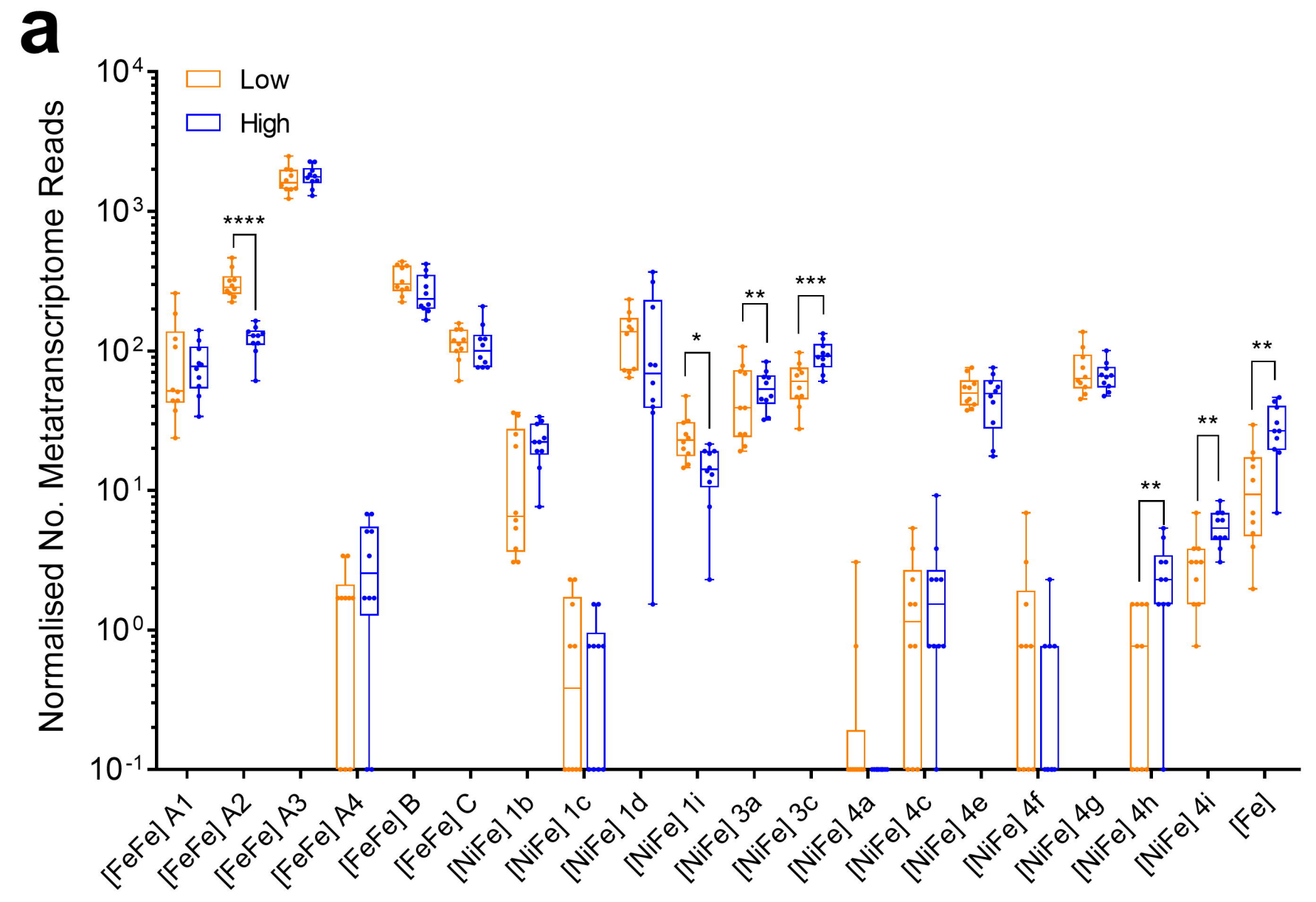
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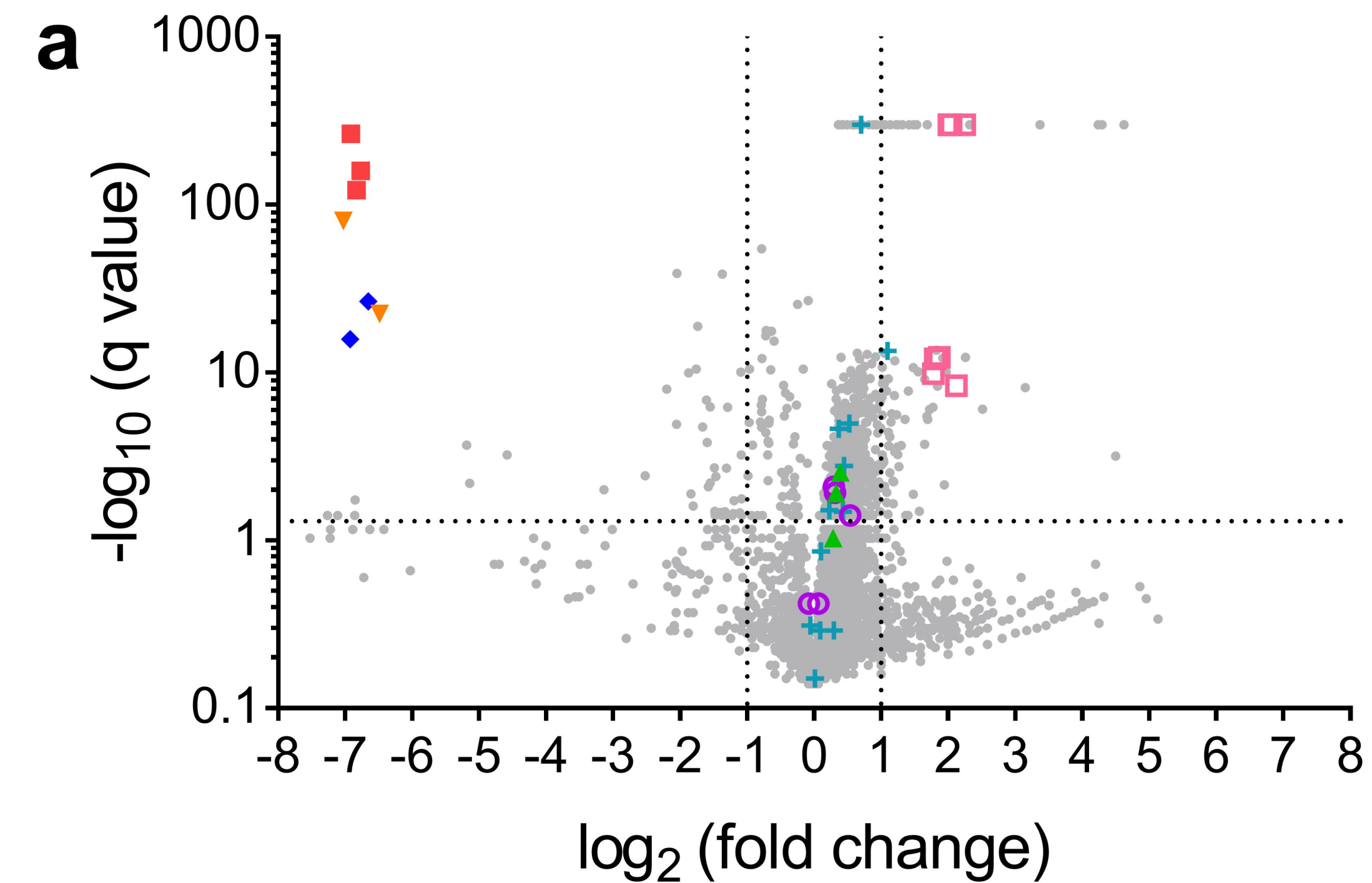


Number of genomes
Fermentative hydrogenase
Bifurcating hydrogenase
Respiratory hydrogenase
Methanogenic hydrogenase
Energy-converting hydrogenase
Sensory hydrogenase
Hydrogenase-associated diaphorase
Nitrogenase
Methyl-CoM reductase
Acetyl-CoA synthase
Adenylylsulfate reductase
Dissimilatory sulfite reductase
Alternative sulfite reductase
Fumarate reductase
Dissimilatory nitrate reductase
Periplasmic nitrate reductase
Ammonia-forming nitrite reductase
DMSO / TMAO reductase
Cytochrome *bd* oxidase

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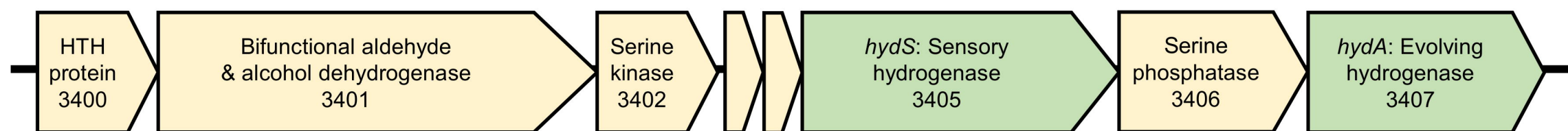




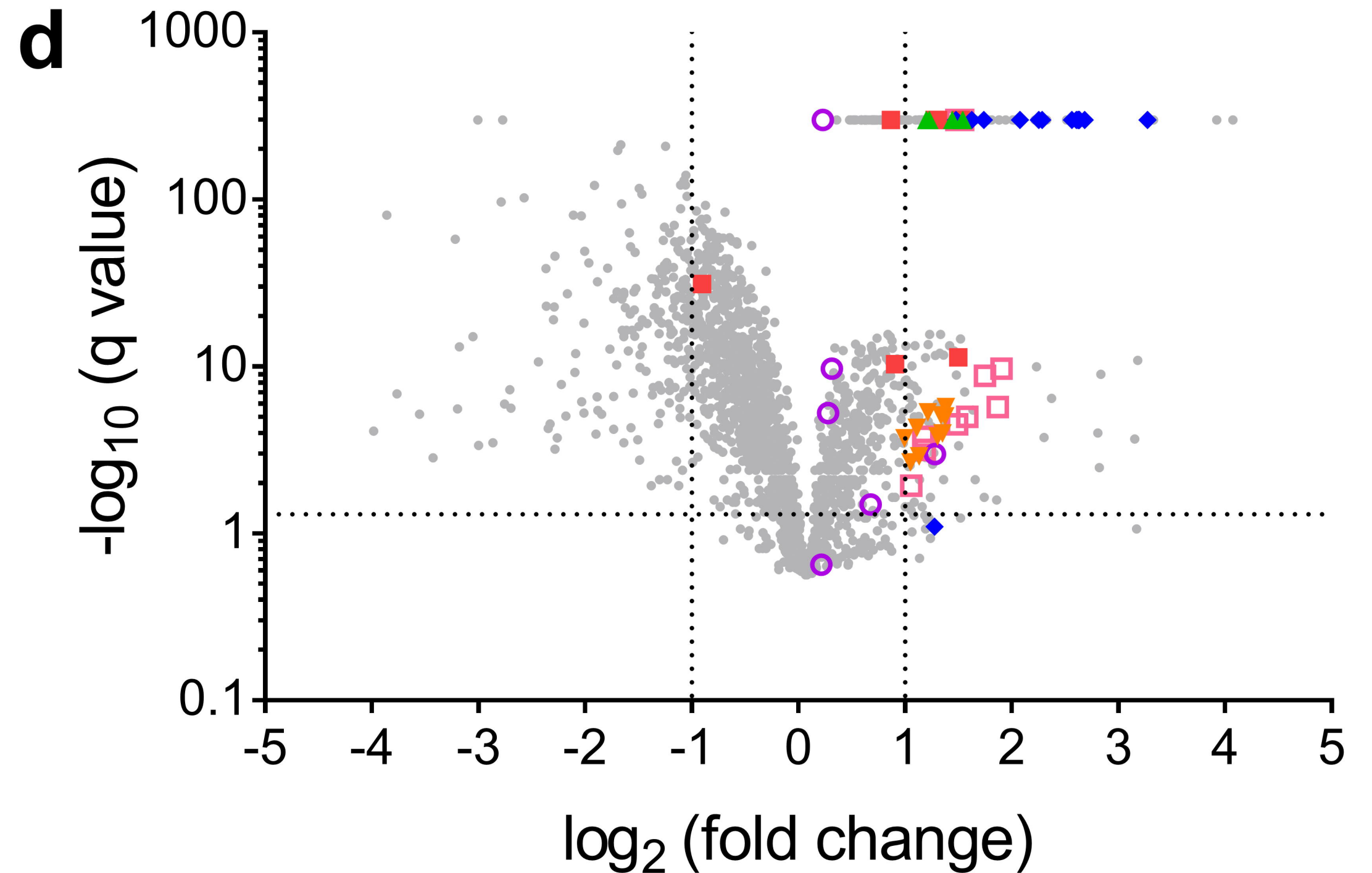
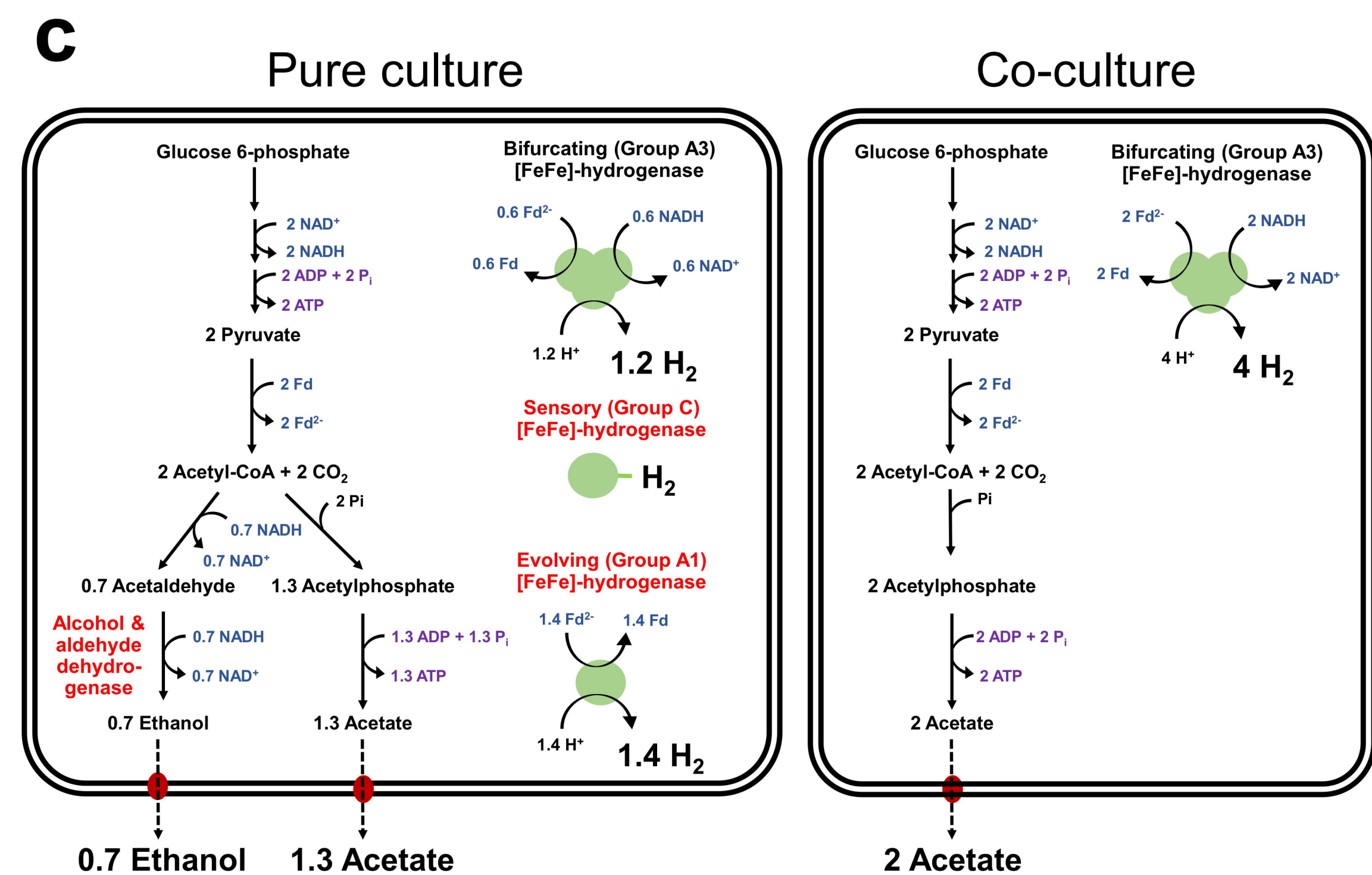
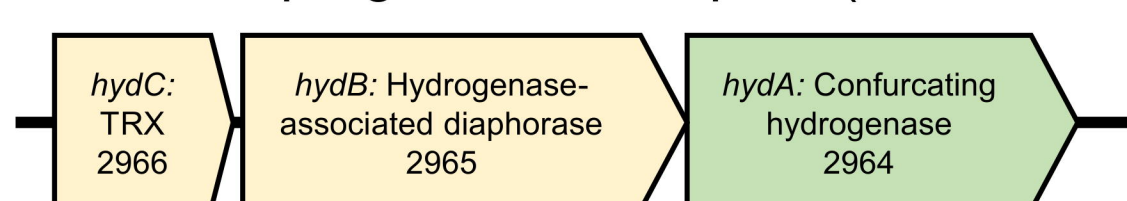
- ▲ Bifurcating hydrogenase
- ▼ Fermentative hydrogenase
- ◆ Sensory hydrogenase
- Alcohol dehydrogenase
- Pyruvate formate lyase
- TPP biosynthesis
- + Glycolysis enzymes

b

111 × downregulated: Group A1 (fermentative) and Group C (sensory) [FeFe]-hydrogenases



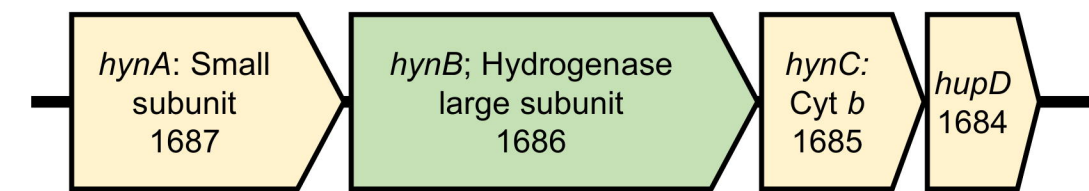
1.26 × upregulated: Group A3 (electron-bifurcating) [FeFe]-hydrogenase



- ▲ Respiratory hydrogenase
- ▼ Hydrogen formatelyase
- ◆ Formate dehydrogenases
- Fumarate acquisition
- Fumarate reductases
- Nitrate ammonification

e

2.7 × upregulated: Group 1b (respiratory uptake) [NiFe]-hydrogenase



2.4 × upregulated: Group 4a (formate hydrogenlyase) [NiFe]-hydrogenases

