

1 Experimentally-validated correlation analysis reveals new anaerobic
2 methane oxidation partnerships with consortium-level heterogeneity in
3 diazotrophy

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14

15 **Abstract**

16 Archaeal anaerobic methanotrophs ('ANME') and sulfate-reducing Deltaproteobacteria ('SRB')

17 form symbiotic multicellular consortia capable of anaerobic methane oxidation (AOM), and in so

18 doing modulate methane flux from marine sediments. The specificity with which ANME

19 associate with particular SRB partners *in situ*, however, is poorly understood. To characterize

20 partnership specificity in ANME-SRB consortia, we applied the correlation inference technique

21 SparCC to 310 16S rRNA Illumina iTag amplicon libraries prepared from Costa Rica sediment

22 samples, uncovering a strong positive correlation between ANME-2b and members of a clade of

23 Deltaproteobacteria we termed SEEP-SRB1g. We confirmed this association by examining 16S

24 rRNA diversity in individual ANME-SRB consortia sorted using flow cytometry and by imaging

25 ANME-SRB consortia with fluorescence *in situ* hybridization (FISH) microscopy using newly-

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26 designed probes targeting the SEEP-SRB1g clade. Analysis of genome bins belonging to
27 SEEP-SRB1g revealed the presence of a complete *nifHDK* operon required for diazotrophy,
28 unusual in published genomes of ANME-associated SRB. Active expression of *nifH* in SEEP-
29 SRB1g and diazotrophic activity within ANME-2b/SEEP-SRB1g consortia was then
30 demonstrated by microscopy using hybridization chain-reaction (HCR-) FISH targeting *nifH*
31 transcripts and by FISH-nanoSIMS experiments. NanoSIMS analysis of ANME-2b/SEEP-
32 SRB1g consortia incubated with a headspace containing CH₄ and ¹⁵N₂ revealed differences in
33 cellular ¹⁵N-enrichment between the two partners that varied between individual consortia, with
34 SEEP-SRB1g cells enriched in ¹⁵N relative to ANME-2b in one consortium and the opposite
35 pattern observed in others, indicating both ANME-2b and SEEP-SRB1g are capable of nitrogen
36 fixation, but with consortium-specific variation in whether the archaea or bacterial partner is the
37 dominant diazotroph.

38

39 Introduction

40 The partnership between anaerobic, methanotrophic Archaea (ANME) and their associated
41 sulfate-reducing bacteria (SRB) is one of the most biogeochemically-important symbioses in the
42 deep-sea methane cycle [1, 2]. As a critical component of methane seep ecosystems,
43 multicellular consortia of ANME and associated SRB consume a significant fraction of the
44 methane produced in marine sediments, using sulfate as a terminal electron acceptor to perform
45 the anaerobic oxidation of methane (AOM) [1–4]. ANME-SRB consortia are thought to perform
46 AOM through the direct extracellular transfer of electrons between ANME and SRB [5–7]. Along
47 with symbiotic extracellular electron transfer, ANME-SRB consortia also exhibit other traits of
48 mutualism such as the sharing of nutrients. For example, members of the ANME-2 clade have
49 been reported to fix and share N with partner bacteria [8–11], but the extent to which

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50 diazotrophic capability might vary across the diverse clades of ANME and associated SRB is
51 the focus of ongoing research.

52
53 Comparative studies of ANME [12] and associated SRB [13, 14] genomes from multiple ANME-
54 SRB consortia have revealed significant diversity across clades, particularly for SRB genomes
55 falling within subclades of the SEEP-SRB1 [14], common SRB partners to ANME [15]. However,
56 the implications of symbiont diversity for metabolic adaptation in ANME-SRB consortia are
57 obscured by the absence of clearly-established ANME-SRB pairings in the environment. A
58 framework defining these pairings would address this gap in knowledge. Establishing this
59 framework for partnership specificity in ANME-SRB consortia—being the preference that certain
60 ANME exhibit for specific SRB partners—would shed light on the extent to which ANME or SRB
61 physiology may differ in consortia constituted of different ANME-SRB pairs.

62
63 As an aspect of ANME or SRB physiology that may differ in different ANME-SRB pairings,
64 nitrogen anabolism has been observed to be involved in the symbiotic relationship between
65 partners [8, 9] and has been shown to influence niche differentiation of different ANME-SRB
66 consortia via nitrate assimilation ability [16]. Previous evidence documenting active diazotrophy
67 in ANME-SRB consortia by nitrogenase expression [8] and $^{15}\text{N}_2$ fixation by nanoSIMS indicated
68 that ANME-2 are the primary diazotrophs in ANME-SRB consortia and supply fixed nitrogen to
69 SRB partners [8–10]. The diazotrophic potential of syntrophic SRB, however, and their role in
70 nitrogen fixation within consortia is poorly understood. Evidence from SRB genomes [14] and
71 the expression of unidentified nitrogenase sequences in methane seep sediments [8] suggested
72 that seep associated SRB may fix nitrogen, opening up the possibility of variation in diazotrophic
73 activity among taxonomically-distinct ANME-SRB consortia.

74 Previous research characterizing the diversity of partnerships in ANME-SRB consortia have
75 employed fluorescence microscopy, magnetic separation by magneto-FISH, and single-cell

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76 sorting techniques (e.g. BONCAT-FACS) that are robust against false positives, but are often
77 limited in statistical power. Fluorescence *in situ* hybridization (FISH) has helped to establish the
78 diversity of ANME-bacterial associations, with ANME constituting four diverse polyphyletic
79 clades within the Methanomicrobia: ANME-1a/b [4, 17–20], ANME-2a,b,c [3, 20–22] , ANME-2d
80 [23, 24], and ANME-3 [20, 25, 26]. ANME-associated SRB have also observed by FISH to be
81 diverse, representing several clades of Deltaproteobacteria including the
82 *Desulfococcus/Desulfosarcina* (DSS) clade [3–6, 15, 19–22, 27–33], two separate subclades
83 within the Desulfobulbaceae [16, 25, 26], a deeply-branching group termed the SEEP-SRB2
84 [34], and a thermophilic clade of Desulfobacteraceae known as HotSeep-1 [34, 35]. These FISH
85 studies documented associations for different ANME-SRB consortia, including partnerships
86 between members of ANME-1 and SEEP-SRB2 [13] or HotSeep-1 [7, 13, 35], ANME-2a and
87 SEEP-SRB1a [15], ANME-2c and SEEP-SRB1a [5], SEEP-SRB2 [13, 34], or Desulfobulbaceae
88 [29], and ANME-3 and SEEP-SRB1a [15] or Desulfobulbaceae [25, 26]. Conspicuously, SRB
89 found in consortia with ANME-2b have only been identified broadly as members of the
90 Deltaproteobacteria targeted by the probe S-C-dProt-0495-a-A-18 (often referred to as Δ 495) [5,
91 31, 36], leaving little known about the specific identity of this SRB partner. Visualizing ANME-
92 SRB partnerships by FISH has been a valuable aspect of AOM research, but FISH requires the
93 design of probes with sufficient specificity to identify partner organisms and thus will only detect
94 partnerships consisting of taxa for which phylogenetic information is known [22]. Magneto-FISH
95 [29, 37, 38] or BONCAT-enabled fluorescence-activated cell sorting (BONCAT-FACS) of single
96 ANME-SRB consortia [39] complement FISH experiments by physical capture (via magnetic
97 beads or flow cytometry, respectively) and sequencing of ANME and associated SRB from
98 sediment samples. These studies corroborated some of the patterns observed from FISH
99 experiments, showing associations between ANME-2 and diverse members of the DSS [39].
100 Magneto-FISH and BONCAT-FACS observations of ANME-SRB pairings are also highly robust

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101 against false positives but can lack the statistical power conferred by more high-throughput
102 approaches that is necessary to establish a general framework for partnership specificity.
103
104 Recently, a number of correlation analysis techniques have been introduced in molecular
105 microbial ecology studies, providing information about patterns of co-occurrence between 16S
106 rRNA OTUs or ASVs recovered from environmental iTag [40] diversity surveys [41–43].
107 Correlation analysis performed on 16S rRNA amplicon surveys provides a complementary
108 method to Magneto-FISH and/or BONCAT-FACS that can be used to develop hypotheses about
109 potential microbial interactions. While predictions of co-occurrence between phylotypes from
110 these correlation analysis techniques have been reported in a number of diverse environments,
111 they are rarely validated through independent approaches, with a few notable exceptions [44].
112
113 Here, we present a framework for ANME-SRB partnership specificity, using correlation analysis
114 of 16S iTag amplicon sequences from a large-scale survey of seafloor methane seep sediments
115 near Costa Rica to predict potential ANME-SRB partnerships. A partnership between ANME-2b
116 and members of an SRB group previously not known to associate with ANME (SEEP-SRB1g)
117 was hypothesized by correlation analysis and independently assessed using FISH and amplicon
118 data from BONCAT-FACS-sorted ANME-SRB consortia. With this new framework, we were
119 able to identify a novel partnership between ANME-2b and SEEP-SRB1g and map predicted
120 physiological traits of SEEP-SRB1g genomes onto partnership specificity with ANME-2b. Our
121 approach led us to formulate new hypotheses regarding how SEEP-SRB1g physiology may
122 complement ANME-2b physiology, focusing on nitrogen fixation in SEEP-SRB1g. We
123 demonstrate in this study that the symbiotic relationship between ANME and associated SRB
124 can vary depending on the nature of the partner taxa and affirm the importance of characterizing
125 individual symbiont pairings in understanding AOM symbiosis.

126

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127 Materials and Methods

128 Here, we present an abridged description of the methods used in this study. A full description
129 can be found in the Supplemental Materials and Methods.

130

131 *Sample origin and processing*

132 Pushcore samples of seafloor sediment were collected by DSV *Alvin* during the May 20-June 11
133 2017 ROC HITS Expedition (AT37-13) aboard R/V *Atlantis* to methane seep sites southwest of
134 Costa Rica [45–47]. After retrieval from the seafloor, sediment pushcores were extruded aboard
135 R/V *Atlantis* and sectioned at 1-3 cm intervals for geochemistry and microbiological sampling
136 using published protocols [21, 48]. Samples for DNA extraction were immediately frozen in
137 liquid N₂ and stored at -80°C. Samples for microscopy were fixed in 2% paraformaldehyde for
138 24 h at 4°C. A full list of samples used in this study can be found in Supplemental Table 1 and
139 additional location and geochemical data can be found at [https://www.bco-](https://www.bco-dmo.org/dataset/715706)
140 [dmo.org/dataset/715706](https://www.bco-dmo.org/dataset/715706).

141

142 *DNA extraction and iTag sequencing*

143 DNA was extracted from 310 samples of Costa Rican methane seep sediments and seep
144 carbonates (Supp. Table 1) using the Qiagen PowerSoil DNA Isolation Kit 12888 following
145 manufacturer directions modified for sediment and carbonate samples [21, 49]. The V4-V5
146 region of the 16S rRNA gene was amplified using archaeal/bacterial primers, 515F (5'-
147 GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3') with Illumina
148 adapters [50]. PCR reaction mix was set up in duplicate for each sample with New England
149 Biolabs Q5 Hot Start High-Fidelity 2x Master Mix in a 15 µL reaction volume with annealing
150 conditions of 54°C for 30 cycles. Duplicate PCR samples were then pooled and 2.5 µL of each
151 product was barcoded with Illumina NexteraXT index 2 Primers that include unique 8-bp

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152 barcodes. Amplification with barcoded primers used annealing conditions of 66°C and 10
153 cycles. Barcoded samples were combined into a single tube and purified with Qiagen PCR
154 Purification Kit 28104 before submission to Laragen (Culver City, CA, USA) for 2 x 250 bp
155 paired end analysis on Illumina's MiSeq platform. Sequence data was submitted to the NCBI
156 Sequence Read Archive as Bioproject PRJNA623020. Sequence data was processed in QIIME
157 version 1.8.0 [51] following Mason, et al. 2015 [52]. Sequences were clustered into *de novo*
158 operational taxonomic units (OTUs) with 99% similarity [53], and taxonomy was assigned using
159 the SILVA 119 database [54]. The produced table of OTUs detected in the 310 methane seep
160 sediment and seep carbonate amplicon libraries was analyzed using the correlation algorithm
161 SparCC [41]. To examine phylogenetic placement of SRB 16S rRNA gene amplicon sequences
162 predicted by network analysis to associate with particular ANME subgroup amplicon sequences,
163 a phylogeny was constructed using RAxML-HPC [55] on XSEDE [56] using the CIPRES
164 Science Gateway [57] from full-length 16S rRNA sequences of Deltaproteobacteria aligned by
165 MUSCLE [58]. Genomes downloaded from the IMG/M database were searched using tblastn.
166 Chlorophyllide reductase BchX (WP011566468) was used as a reference sequence for a tblastn
167 *nifH* search using BLAST+. Genome trees were constructed using the Anvi'o platform [59] using
168 HMM profiles from a subset [60] of ribosomal protein sequences and visualized in iTOL [61].

169

170 *FISH probe design and microscopy*

171 A new FISH probe was designed in ARB [62]. This new probe, hereafter referred to as Seep1g-
172 1443 (Supp. Table 2), was designed to complement and target 16S rRNA sequences in a
173 monophyletic "*Desulfococcus* sp." clade. Based on phylogenetic analysis (see below), this clade
174 was renamed SEEP-SRB1g. Seep1g-1443 was ordered from Integrated DNA Technologies
175 (Coralville, IA, USA). FISH reaction conditions were optimized for Seep1g-1443, with optimal
176 formamide stringency found to be 35% (Supp. Fig. 1). FISH and hybridization chain reaction
177 (HCR-) FISH was performed on fixed ANME-SRB consortia using previously published density

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178 separation and FISH protocols [22]. FISH was performed overnight (18 hr) using modifications
179 (G. Chadwick, pers. comm.) to previously-published protocols [29, 39, 63, 64]. Structured-
180 illumination microscopy (SIM) was performed on FISH and HCR-FISH (see below) experiments
181 to image ANME-SRB consortia using the Elyra PS.1 SIM platform (Zeiss, Germany) and an
182 alpha Plan-APOCHROMAT 100X/1.46 Oil DIC M27 objective. Zen Black software (Zeiss) was
183 used to construct final images from the structured-illumination data.

184

mRNA imaging using HCR-FISH

186 Hybridization chain reaction FISH (HCR-FISH) is a powerful technique to amplify signal from
187 FISH probes [65, 66]. The protocol used here was modified from Yamaguchi and coworkers
188 [67]. *nifH* initiators, purchased from Molecular Technologies (Pasadena, CA, USA; probe
189 identifier “nifH 3793/D933”) or designed in-house (Supp. Table 2) and ordered from Integrated
190 DNA Technologies, were hybridized to fixed ANME-SRB consortia. Hairpins B1H1 and B1H2
191 with attached Alexa647 fluorophores (Molecular Technologies) were added separately to two 45
192 μ L volumes of amplification buffer in PCR tubes and snap cooled by placement in a C1000
193 Touch Thermal Cycler (BioRad, Hercules, CA, USA) for 3 min at 95°C. After 30 min at room
194 temperature, hairpins were mixed and placed in PCR tubes along with hybridized ANME-SRB
195 consortia. Amplification was performed for 15 min at 35°C. Similar results were observed when
196 the HCR-FISH v3.0 protocol established by Choi et al. [68] was used.

197

Stable Isotope Probing and nanoSIMS

199 Methane seep sediments containing abundant ANME-2b and SEEP-SRB1g consortia (Supp.
200 Fig. 4) were used in stable isotope probing (SIP) experiments to test for diazotrophic activity by
201 SEEP-SRB1g. N sources were removed from the sediment slurry by washing with artificial
202 seawater without an N source (see Supplemental Materials and Methods). Two anoxic
203 incubations were pressurized with 2.8 bar CH_4 with 1.2 mL $^{15}\text{N}_2$ at 1 bar, approximately

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204 equivalent to 2% headspace in 20 mL CH₄ at 2.8 bar (Supp. Table 3). Positive control
205 incubations ($n = 2$) were amended with ¹⁵NH₄Cl and were further pressurized with 2.8 bar CH₄
206 and 1.2 mL natural-abundance N₂ at 1 bar. Incubations were periodically checked for AOM
207 activity via sulfide production using the Cline assay [69] and were chemically fixed for FISH-
208 nanoSIMS analysis [70] after 9 months. Fixed ANME-SRB consortia were separated from the
209 sediment matrix and concentrated following published protocols [5]. Samples were then
210 embedded in Technovit H8100 (Kulzer GmbH, Germany) resin according to published protocols
211 [5, 31] and thin sections (2 μm thickness) were prepared using an Ultracut E microtome
212 (Reichert AG, Austria) which were mounted on Teflon/poly-L-lysine slides (Tekdon Inc., USA).
213 FISH reactions were performed using Seep1g-1443 and ANME-2b-729 probes as described
214 above, with the omission of 10% SDS to prevent detachment of section from slide (G.
215 Chadwick, pers. comm.), and slides were imaged and mapped for subsequent nanoSIMS
216 analysis using a Zeiss Elyra PS.1 platform. After removal of DAPI-Citifuor by washing following
217 published protocols [70], slides were cut to fit into nanoSIMS sample holders and sputter-coated
218 with 40 nm Au using a Cressington sputter coater. NanoSIMS was performed using a Cameca
219 NanoSIMS 50L housed in Caltech's Microanalysis Center, and data was analyzed using
220 look@nanoSIMS [71].

221

222 Results

223 *16S rRNA correlation analysis predicts a specific association between ANME-2b and SEEP-*
224 *SRB1g*

225 Correlation analysis applied to 16S rRNA gene amplicon libraries has been frequently used to
226 identify interactions between microorganisms based on the co-occurrence of their 16S rRNA
227 sequences in different environments or conditions [72–75]. Here, we applied correlation analysis
228 to 16S rRNA amplicon libraries prepared from Costa Rican methane seep sites (Supp. Table 1)

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229 to explore partnership specificity between ANME and associated SRB. QIIME processing of
230 amplicon sequences prepared from 310 Costa Rican methane seep sediment and seep
231 carbonate samples yielded 3,052 OTUs after filtering in R. A table of read abundances for these
232 OTUs across the 310 samples was analyzed by SparCC to calculate correlation coefficients and
233 significance for all possible 4,658,878 OTU pairs using 100 bootstraps (Fig. 1). Of these pairs,
234 9.7% (452,377) had pseudo- p -values < 0.01 , indicating the coefficients for each of these
235 correlations exceeded that calculated for that same OTU pair in any of the 100 bootstrapped
236 datasets [41]. The taxonomic assignment of the constituent OTUs of correlations with pseudo- p
237 < 0.01 were then inspected, where 18% (81,459) of correlations with pseudo- $p < 0.01$ describe
238 those involving ANME. Of these, 32% occur between ANME and OTUs assigned to three main
239 taxa: *Desulfococcus* sp., SEEP-SRB1a, and SEEP-SRB2 (Fig. 1). A complete list of significant
240 correlations, their coefficient values, OTU identifiers, and accompanying taxonomy assignments
241 can be found in Supplemental Table 4.

242
243 16S rRNA phylogenetic analysis revealed the SILVA-assigned “*Desulfococcus* sp.” OTUs
244 comprise a sister clade to the SEEP-SRB1a that is distinct from cultured *Desulfococcus* sp.
245 (e.g. *D. oleovorans* and *D. multivorans*, see below). We therefore reassigned the *Desulfococcus*
246 OTUs to a new clade we termed SEEP-SRB1g following the naming scheme outlined for seep-
247 associated SRB in Schreiber, et al. (e.g. SEEP-SRB1a through -SRB1f) [15]. Furthermore,
248 statistically-significant correlations between OTUs of ANME and SRB taxa suggested that
249 ANME-SRB partnerships in the Costa Rica seep samples could be classified into the following
250 types: ANME-1 with SEEP-SRB1a or SEEP-SRB2, ANME-2a with SEEP-SRB1a, ANME-2b
251 with SEEP-SRB1g, ANME-2c with SEEP-SRB1a or SEEP-SRB2, and ANME-3 with SEEP-
252 SRB1a (Fig. 1). While physical association between different ANME lineages and
253 Deltaproteobacterial clades SEEP-SRB1a and SEEP-SRB2 had been well-documented [5, 13,

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254 15, 31, 34], members of the SEEP-SRB1g had not previously been identified as a potential
255 syntrophic partner with methanotrophic ANME.
256
257 These associations were further examined by detailed network analysis in which the table of
258 correlations with pseudo p -values < 0.01 was further filtered to contain only those correlations
259 with coefficients (a measure of correlation strength) in the 99th percentile of all significant
260 correlations. A network diagram in which nodes represent OTUs and edges between nodes
261 represent correlations was constructed with force-directed methods [76], where edge length
262 varied in inverse proportion to correlation strength. A subregion of this network focused on
263 ANME-associated OTUs is presented in Figure 2a. Cohesive blocks, subsets of the graph with
264 greater connectivity to other nodes in the block than to nodes outside [77], were calculated and
265 revealed 3 primary blocks of ANME and SRB OTUs. Visualization of these 3 blocks by a chord
266 diagram [78] further highlighted the taxonomic identity of ANME-SRB OTU pairs in these blocks:
267 ANME-1 or ANME-2c (one OTU with mean read count < 10) and SEEP-SRB2, ANME-2a or
268 ANME-2c and SEEP-SRB1a, and ANME-2b or ANME-2a and SEEP-SRB1g (Fig. 2b). The
269 predicted associations between ANME-2c and SEEP-SRB2 and between ANME-2a and SEEP-
270 SRB1g were relatively more rare than the other associations; only one rare ANME-2c OTU
271 (mean read count < 10) and four uncommon ANME-2a OTUs (mean read count < 100) were
272 predicted between SEEP-SRB2 and SEEP-SRB1g, respectively. Inferred partnership specificity
273 in two of the blocks has been previously corroborated by FISH studies, namely that exhibited by
274 ANME-1 with SEEP-SRB2 [13, 34], ANME-2c with SEEP-SRB1a [5], and ANME-2a with SEEP-
275 SRB1a [15]. The partnership between SEEP-SRB1g and ANME-2b, however, had no
276 precedent, as the only previous FISH descriptions of ANME-2b had placed it with a partner
277 *Deltaproteobacterium* with taxonomy not known beyond the phylum level [5, 31].
278

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279 *Common patterns of association observed in network analysis and in single ANME-SRB*

280 *consortia*

281 To test if ANME-SRB partnership specificity observed in our iTag correlation analysis from seep
282 samples (Figs. 1, 2) was consistent with data collected from individually-sorted ANME-SRB
283 consortia after BONCAT-FACS [39], we constructed a phylogeny with full-length and amplicon
284 16S rRNA sequences from ANME-associated SRB including SEEP-SRB1g (Fig. 3; Supp. Fig
285 5). 16S rRNA iTag amplicon sequences from the network analysis (Fig. 2) and from BONCAT-
286 FACS sorted consortia (Fig. 3; [39]) were then annotated by ANME subtype and identity of
287 associated phylotypes. In the BONCAT-FACS dataset, 8 out of 11 (72%) of the consortia with
288 ANME-2b OTUs had corresponding deltaproteobacterial OTUs that belonged to the SEEP-
289 SRB1g clade (Fig. 3). Similarly, of the Deltaproteobacteria OTU sequences from the BONCAT-
290 FACS sorted consortia affiliated with SEEP-SRB1g 89% (8/9) had ANME-2b as the archaeal
291 partner (Fig. 3). Notably, we found that these SEEP-SRB1g sequences were also highly-similar
292 to published full-length 16S rRNA clone library sequences (e.g. NCBI accession AF354159)
293 from seep sediments where ANME-2b phylotypes were also recovered [21]. A χ^2 -test for
294 independence was performed on 16S rRNA OTUs recovered from (39) to test the null
295 hypothesis that the presence of a given SRB taxon in a FACS sort is independent of the type of
296 ANME present in the sort. This test demonstrated that the SRB taxon found in a given sort was
297 dependent on the ANME also present in the sort, $\chi^2 = 30.6$ (*d.f.* = 6, *n* = 30), *p* < 0.001. The
298 pattern of association between ANME and SRB OTUs in individual BONCAT-FACS-sorted
299 ANME-SRB consortia thus corroborated the inference from network analysis that ANME-2b and
300 SEEP-SRB1g OTUs exhibit significant partnership specificity. On the basis of amplicon
301 sequence associations found from the BONCAT-FACS sorting dataset as well as those
302 displayed by correlation analysis of amplicons from Costa Rica methane seeps, we designed a
303 set of independent experiments to test the hypothesis that ANME-2b form syntrophic
304 partnerships with the previously-undescribed SEEP-SRB1g deltaproteobacteria.

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305

306 *FISH experiments show SEEP-SRB1g in association with ANME-2b*

307 Specific oligonucleotide probes were designed and tested for the SEEP-SRB1g clade (Supp.

308 Fig. 1) and FISH experiments were used to validate the predicted ANME-2b—SEEP-SRB1g

309 partnership. Simultaneous application of FISH probes targeting SEEP-SRB1a, the dominant

310 deltaproteobacterial partner of ANME (Seep1a-1441 [15]), the newly designed SEEP-SRB1g

311 probe (Seep1g-1443, this work), and a probe targeting ANME-2b (ANME-2b-729 [39])

312 demonstrated that ANME-2b predominantly form consortia with SEEP-SRB1g, appearing as

313 large multicellular consortia in seep sediment samples from different localities at Costa Rica

314 methane seep sites (see Supplemental Materials and Methods for site details) that also contain

315 ANME-2a (Fig. 4b) and ANME-2c (Fig. 4f). ANME-2b was not observed in association with

316 SEEP-SRB1a (Figs. 4a, 4e), and SEEP-SRB1g was not observed in association with ANME-2a

317 (Fig. 4d) or ANME-2c (Fig. 4h) when FISH probes ANME-2a-828 or ANME-2c-760 [20] were

318 substituted for ANME-2b-729 ($n \approx 100$ consortia). Instead, SEEP-SRB1a was found in consortia

319 with ANME-2a (Fig. 4c) and ANME-2c (Fig. 4g), consistent with previous reports [15].

320

321 *Genomic potential for N_2 fixation in sulfate-reducing SEEP-SRB1g deltaproteobacteria*

322 Given the importance of diazotrophy in the functioning of ANME-SRB syntrophy, we screened

323 metagenome-assembled genome bins (MAGs) of SEEP-SRB1g for the presence of the

324 nitrogenase operon. A genome tree constructed from previously published MAGs from Hydrate

325 Ridge and Santa Monica Basin [14, 39] revealed that two closely related MAGs

326 (*Desulfobacterales* sp. C00003104, and C00003106) originally classified as belonging to the

327 Seep-SRB1c clade [14] possessed the nitrogenase operon (Fig. 5). These MAGs did not

328 possess 16S rRNA genes, precluding 16S rRNA-based taxonomic identification. A more

329 detailed look at these reconstructed genomes revealed that the nitrogenase along with a suite of

330 other genes were unique to this subclade and missing in other SEEP-SRB1c MAGs [14],

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331 suggesting they may represent a distinct lineage. In effort to connect these nitrogenase
332 containing SRB MAG's with representative 16S rRNA sequences, we examined mini-
333 metagenome data from individual BONCAT-FACS sorted ANME-SRB consortia which each
334 contained 16S rRNA gene sequences for the ANME and bacterial partner [39]. A genome tree
335 containing deltaproteobacterial MAGs from Skennerton, et al. [14] and reconstructed
336 deltaproteobacterial genomes from the BONCAT-FACS sorts [39] revealed one SRB genome
337 from a FACS-sorted consortium (Desulfobacterales sp. CONS3730E01UFb1, IMG Genome ID
338 3300009064) was closely related to the two putative Seep-SRB1c MAGs containing the
339 nitrogenase operon (Fig. 5). The 16S rRNA amplicon sequence (NCBI accession KT945234)
340 associated with the Desulfobacterales sp. CONS3730E01UFb1 genome was used to construct
341 a 16S rRNA phylogeny and confirmed to cluster within the SEEP-SRB1g clade, providing a link
342 between the 16S rRNA and associated nitrogenase sequences in this lineage (Fig. 3). Given
343 that Desulfobacterales sp. CONS3730E01UFb1, C00003104, and C00003106 genomes
344 appeared highly similar on the genome tree (Fig. 5), we reassigned the previously published
345 Desulfobacterales sp. C00003104 and C00003106 MAGs to the SEEP-SRB1g. Notably, the
346 other 16S rRNA amplicon sequence sampled from the sorted consortium CONS3730E01UF
347 (NCBI accession KT945229) was assigned to ANME-2b [39].

348
349 As noted above, these SEEP-SRB1g MAGs were remarkable for the presence of the *nifHDK*
350 operon involved nitrogen fixation, which had previously not been an area of focus in previous
351 analyses of ANME-associated SRB genomes (Fig. 5). A re-analysis of published *nifH* cDNA
352 sequences from methane seep sediments revealed sequences that were nearly identical to the
353 SEEP-SRB1g *nifH* (NCBI accession KR020451-KR020457, [8]) suggesting active transcription
354 of SEEP-SRB1g *nifH* under *in situ* conditions (Fig. 6). An analysis of published methane seep
355 metaproteomic data [14] also indicated active translation of nitrogenase by SEEP-SRB1g,
356 corroborating evidence from cDNA libraries [8]. Additionally, other *nifH* cDNA sequences in this

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357 study were found to be identical to nitrogenase sequences occurring in 18 SEEP-SRB1a
358 unpublished metagenome bins (Supp. Fig. 6) demonstrating that at least some of the syntrophic
359 SEEP-SRB1a SRB partners also possess and actively express *nifH*.

360

361 *Single-cell nifH expression visualized by HCR-FISH and ¹⁵N₂ FISH-nanoSIMS experiments*
362 *confirm involvement of SEEP-SRB1g in N₂-fixation*

363 The dominant role of ANME-2 in nitrogen fixation reported by previous studies [8–10] motivated
364 our examination of whether the sulfate-reducing SEEP-SRB1g partners of ANME-2b were also
365 involved in diazotrophy, either in concert with the ANME-2b partner, or perhaps as the sole
366 diazotroph in this AOM partnership. Using the *nifH* sequences from SEEP-SRB1g, we designed
367 a specific mRNA-targeted probe set to use in whole-cell hybridization chain reaction FISH
368 (HCR-FISH) assays (Supp. Table 2). HCR-FISH allows for signal amplification and improved
369 signal-to-noise ratio compared to FISH, and has been used in single cell mRNA expression
370 studies in select microbial studies [79–81]. Prior to this study, however, HCR-FISH had not been
371 applied to visualize gene expression in ANME-SRB consortia from methane seep sediments. In
372 the context of experiments with sediment-dwelling ANME-SRB consortia, HCR-FISH provided
373 adequate amplification of the signal to detect expressed mRNA above the inherent background
374 autofluorescence in sediments. Using our HCR-FISH probes targeting SEEP-SRB1g *nifH*
375 mRNA together with the standard 16S rRNA targeted oligonucleotide FISH probes Seep1g-
376 1443 (targeting SEEP-SRB1g) and ANME-2b-729 (targeting ANME-2b), we successfully
377 imaged *nifH* mRNA transcripts by SEEP-SRB1g cells in ANME-2b—SEEP-SRB1g consortia in
378 a sediment AOM microcosm experiment (Fig. 7). Positive HCR-FISH *nifH* hybridization in this
379 sample was observed to be exclusively associated with the SEEP-SRB1g bacterial partner in
380 ANME-2b consortia ($n = 5$), and not observed in ANME-2b stained cells nor in ANME-2a or -2c
381 consortia, supporting the specificity of this assay. Negative control experiments for the HCR-
382 FISH reaction were also performed in which SEEP-SRB1g *nifH* initiator probes were added to

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383 the assay, but the fluorescent amplifier hairpins were absent. In this case, there is no
384 fluorescent signal in either the bacteria or archaeal partners in ANME-2b aggregates confirming
385 that there is no native autofluorescence in Seep-SRB1g that could be responsible for the signal
386 observed in the HCR-FISH experiments (Supp. Fig. 7f-j). We performed another control without
387 the initiator probes that bind the mRNA but with the addition of the fluorescent amplifier hairpins.
388 As in the other negative control, we observed limited non-specific binding of the hairpins that
389 were easy to differentiate from the positively-hybridized SEEP-SRB1g (Supp. Fig. 7a-e).
390 Occasionally, highly localized and small spots of hairpins were observed (Supp. Fig 7d) but
391 these dots were mostly localized outside of aggregates and did not align with either bacteria or
392 archaea in consortia (e.g. Figure 7d). Confirmation of *nifH* expression using HCR-FISH
393 corroborated evidence from cDNA libraries (Fig. 6) that SEEP-SRB1g actively express *nifH*,
394 providing support for diazotrophy in the sulfate-reducing partner in ANME-2b—SEEP-SRB1g
395 consortia.
396
397 To confirm active diazotrophy by ANME-2b-associated SEEP-SRB1g, we prepared stable
398 isotope probing incubations of methane seep sediments recovered from a Costa Rica methane
399 seep. These nitrogen-poor sediment incubations were amended with unlabeled methane and
400 $^{15}\text{N}_2$ and maintained in the laboratory at 10°C under conditions supporting active sulfate-coupled
401 AOM (see Supplemental Materials and Methods). Sediments with abundant ANME-SRB
402 consortia were sampled after 9 months of incubation and separated consortia were analyzed by
403 nanoSIMS to measure single cell ^{15}N enrichment associated with diazotrophy within ANME-
404 2b—SEEP-SRB1g consortia. Representative ANME-2b—SEEP-SRB1g consortia ($n = 4$) were
405 analyzed by FISH-nanoSIMS and shown to be significantly enriched in ^{15}N relative to natural
406 abundance values (0.36%; Fig. 8). Among the consortia analyzed, the ^{15}N fractional abundance
407 in ANME-2b cells were often higher than that measured in SEEP-SRB1g, with ANME-2b cells
408 on the exterior of an exceptionally large consortium (Fig. 8b-c) featuring ^{15}N fractional

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409 abundance of $1.73\% \pm 0.14$ (number of ROIs, $n = 72$), significantly enriched relative to that
410 measured in SEEP-SRB1g cells in the exterior, $0.77\% \pm 0.09$ ($n = 58$). This indicated that
411 ANME-2b were often the primary diazotroph in consortia, consistent with previous reports from
412 ANME-2–DSS consortia [8–11]. Notably, however, in one ANME-2b—SEEP-SRB1g
413 consortium, the SEEP-SRB1g cells were more enriched in ^{15}N relative to the associated ANME-
414 2b cells, with ANME-2b cells containing $1.34\% \pm 0.13$ ^{15}N ($n = 82$) and SEEP-SRB1g containing
415 $3.02\% \pm 0.20$ ^{15}N ($n = 22$, Fig. 8i), suggesting that under certain circumstances the sulfate-
416 reducing partner may serve as the primary diazotroph. This pattern suggests diazotrophic
417 flexibility in ANME-2b—SEEP-SRB1g consortia in which one partner—ANME-2b or SEEP-
418 SRB1g—can serve as the primary diazotroph in the consortium. Additionally, a gradient in ^{15}N
419 enrichment in a the large ANME-2b consortium was observed in which clusters of ANME-2b
420 cells associated with the interior of the consortia were significantly more enriched in ^{15}N relative
421 to ANME-2b clusters near the aggregate exterior, with ^{15}N fractional abundances for ANME-2b
422 cells in the exterior of $1.73\% \pm 0.14$ ($n = 72$), significantly higher than those measured for
423 ANME-2b cells in the interior, $2.64\% \pm 0.14$ ($n = 116$). Notably, no equivalent gradient was
424 observed in the SEEP-SRB1g partner, with SEEP-SRB1g cells in the exterior displaying ^{15}N
425 fractional abundances of $0.77\% \pm 0.09$ ($n = 58$) compared with those measured on the interior,
426 $0.78\% \pm 0.09$ ($n = 62$).

427

428 Discussion

429 The symbiotic relationship between ANME and associated SRB, originally described by Hinrichs
430 [17], Boetius [4], and Orphan [21], has been the focus of extensive study using FISH [5, 7, 13,
431 15, 25, 26, 29, 34, 35], magneto-FISH [29, 37, 38], and BONCAT-FACS [39], techniques that
432 have provided insight into the diversity of partnerships between ANME and SRB. While these
433 fluorescence-based approaches offer direct confirmation of physical association between taxa

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434 and are thus useful for characterizing partnership specificity, they are often constrained by
435 sample size and are comparatively lower-throughput than sequencing-based approaches. Next-
436 generation Illumina iTag sequencing of 16S rRNA amplicon sequences offers advantages in
437 terms of throughput and is rapidly becoming a standard approach in molecular microbial
438 ecology studies. Correlation analysis performed on these large iTag datasets can be an
439 effective hypothesis-generating tool for identifying microbial interactions and symbioses in the
440 environment [75], but most studies employing this approach stop short of validating predictions.
441 As correlation analysis of iTag datasets is known to be sensitive to false positives due to the
442 compositional nature of 16S rRNA amplicon libraries [41, 42, 82], specific correlations predicted
443 between taxa should be corroborated when possible by independent approaches.

444
445 In this study, we used correlation analysis of 16S rRNA iTag data from 310 methane seep
446 sediment and carbonate samples on the Costa Rican Margin to identify well-supported (pseudo-
447 p -values < 0.01) positive correlations between specific OTUs commonly observed in seep
448 ecosystems. Our analysis identified strong correlations between syntrophic partners previously
449 described in the literature, such as that between members of the SEEP-SRB1a and ANME-
450 2a/ANME-2c clades and between ANME-1 and SEEP-SRB2 [5, 7, 13, 15, 25, 26, 29, 34, 35],
451 and uncovered previously unrecognized relationships between members of the ANME-2b clade
452 and OTUs affiliated with an uncultured Desulfobacterales lineage, SEEP-SRB1g (Figs. 1-3). We
453 then validated the specificity of the ANME-2b and SEEP-SRB1g association by FISH (Fig. 4).

454
455 The specificity of the association between ANME-2b and SEEP-SRB1g appeared to extend
456 beyond Costa Rica methane seeps and is likely a widespread phenomenon, as this association
457 was also recovered from BONCAT-FACS datasets originating from methane seep sites off of
458 Oregon, USA (Hydrate Ridge) and from the Santa Monica Basin, California, USA. Our
459 observations of ANME-2b—SEEP-SRB1g partnership specificity in numerous samples is

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460 consistent with published observations of other ANME-SRB partnerships, where consortia
461 composed of specific ANME and SRB clades have been observed in seep ecosystems
462 worldwide [15]. Notably, the syntrophic relationship between ANME-2b and SEEP-SRB1g
463 appears to be highly specific (Fig. 2), as FISH observations from sediment samples from
464 multiple Costa Rica methane seep sites (Supp. Table 1) did not show ANME-2b in consortia
465 with other bacteria besides the SEEP-SRB1g (Fig. 4). This is in contrast with SEEP-SRB1a
466 which, in these same experiments, was found to form associations with both ANME-2a and
467 ANME-2c, indicative of this SRB syntroph having a broader capacity for establishing
468 associations with methanotrophic ANME. Members of the diverse ANME-2c lineage also
469 appeared to display partnership promiscuity in our network analysis, with positive correlations
470 observed between ANME-2c OTUs and both SEEP-SRB1a and SEEP-SRB2 OTUs (Fig. 2).
471 This predicted partnership flexibility in the network analysis was again corroborated by FISH
472 observations of ANME-2c—SEEP-SRB1a consortia (Fig. 4) and prior reports of ANME-2c in
473 association with SEEP-SRB2 from Guaymas Basin sediments [13]. These collective data
474 suggest that partnership specificity varies among different clades of ANME and SRB, which may
475 be the result of physiological differences and/or molecular compatibility, signal exchange, and
476 recognition among distinct ANME and SRB that shape the degree of specificity between
477 particular ANME and SRB partners, as has been observed in other symbiotic associations [83–
478 85]. The degree of promiscuity or specificity for a given syntrophic partner may be influenced by
479 the co-evolutionary history of each partnership, with some ANME or SRB physiologies requiring
480 obligate association with specific partners. A more detailed examination of the genomes of
481 ANME-2b and SEEP-SRB1g alongside targeted ecophysiological studies may provide clues to
482 the underlying mechanism(s) driving specificity within this ANME-SRB consortia. Comparative
483 investigations with ANME-2a and -2c subgroups may similarly uncover strategies enabling
484 broader partner association, perhaps with preference for a SRB partner shaped by
485 environmental variables rather than through pre-existing co-evolutionary relationships.

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486
487 An initial genomic screening of SEEP-SRB1g offered some insight into the distinct metabolic
488 capabilities of the SRB partner which may contribute to the association with ANME-2b. The
489 observation of a complete nitrogenase operon in 3 different SEEP-SRB1g genome bins
490 suggested the potential for nitrogen fixation, a phenotype not previously described for ANME-
491 associated SRB (Fig. 5). While previous work on nitrogen utilization by ANME-SRB consortia
492 has focused on diazotrophy performed by ANME-2 [8–10], environmental surveys of seep
493 sediments have noted active expression of nitrogenase typically associated with
494 Deltaproteobacteria [8, 86]. In these studies, the specific microorganisms associated with the
495 expressed nitrogenase in methane seep sediments were not identified. Prior to our findings
496 presented here, diazotrophy by ANME-associated SRB had not been demonstrated. A
497 phylogenetic comparison of the *nifH* sequences associated with SEEP-SRB1g with sequences
498 of the expressed deltaproteobacterial-affiliated (i.e. Group III) *nifH* transcripts reported in Dekas,
499 et al. [8] revealed a high degree of sequence similarity, with SEEP-SRB1g related *nifH* among
500 the most highly expressed (Figs. 5-6). Explicit tests for nitrogenase expression using HCR-FISH
501 and active diazotrophy using stable isotope probing and FISH-nanoSIMS confirmed the
502 involvement of SEEP-SRB1g in nitrogen fixation. Of the 4 ANME-2b—SEEP-SRB1g consortia
503 analyzed by FISH-nanoSIMS, one had significantly more ¹⁵N enrichment in the SEEP-SRB1g
504 partner relative to the ANME-2b, while the other 3 displayed higher cellular ¹⁵N enrichment in
505 the ANME-2b partner (Fig. 8). This pattern supported our inference of diazotrophic flexibility
506 within ANME-2b—SEEP-SRB1g consortia in which either the ANME or the SRB partner can
507 serve as the primary diazotroph in the consortium. Additionally, our detection of nitrogenase
508 operons in the reconstructed genomes of the dominant syntrophic SRB partner, SEEP-SRB1a
509 (Supp. Fig. 6), suggests the potential for nitrogen fixation may extend to other bacterial partners
510 as well and merits further investigation. Re-examination of nitrogen fixation in these
511 partnerships with new FISH probes and nanoSIMS at single-cell resolution will further illuminate

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512 the full diversity of diazotrophic activity among ANME-SRB consortia and the associated
513 environmental/ physiological controls.
514
515 The factors responsible for determining which partner becomes the primary diazotroph in
516 ANME-2b—SEEP-SRB1g consortia requires targeted study, but our preliminary data suggest
517 this may be influenced in part by the relative position of ANME-2b or SEEP-SRB1g cells,
518 particularly within large (>50 μm) ANME-2b—SEEP-SRB1g consortia. Previous studies of
519 nitrogen fixation in ANME-SRB consortia found no correlation between consortia size and
520 diazotrophic activity in consortia with diameters < 10 μm [10], but larger consortia such as those
521 presented here have not been examined at single-cell resolution. Additionally, consortia with the
522 morphology observed here, in which ANME-2b cells form multiple sarcinal clusters surrounded
523 by SEEP-SRB1g (Figs. 4b, 8), have not been the specific focus of nanoSIMS analysis but
524 appear to be the common morphotype among ANME-2b—SEEP-SRB1g consortia [31]. The
525 frequency with which this morphotype is observed in ANME-2b—SEEP-SRB1g consortia may
526 be related to the underlying physiology of this specific partnership. NanoSIMS analysis of a
527 particularly large ANME-2b—SEEP-SRB1g consortium (~200 μm) with this characteristic
528 morphology (Fig. 8a-f) revealed a gradient in diazotrophic activity in which ANME-2b cells
529 located in the interior of the consortium incorporated far more ^{15}N from $^{15}\text{N}_2$ than ANME-2b cells
530 near the exterior. This pattern may be related to variations in nitrogen supply from the external
531 environment, as similar patterns of nutrient depletion with increasing depth into microbial
532 aggregates have been predicted in modeling studies of nitrate uptake in *Trichodesmium* sp. [87]
533 and directly observed by SIMS in stable isotope probing studies of carbon fixation in biofilm-
534 forming filamentous cyanobacteria [88]. In these examples, modeling and experimental results
535 document declining nitrate or bicarbonate ion availability inwards toward the center of the
536 aggregates resulting from nitrate or bicarbonate consumption. An analogous process may occur
537 in large ANME-2b—SEEP-SRB1g consortia, where cells situated closer to the exterior of the

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538 consortium assimilate environmental NH_4^+ , increasing nitrogen limitation for cells within the
539 consortium core. Interestingly, the single consortium in which the SEEP-SRB1g partner was the
540 inferred primary diazotroph featured SEEP-SRB1g cells in the core of this consortium with
541 ANME-2b cells toward the exterior (Fig. 8). The current nanoSIMS dataset is small and
542 determining the biotic and environmental factors that influence which partner serves as the
543 primary diazotroph in ANME-2b—SEEP-SRB1g consortia necessitates further study, but a
544 reasonable hypothesis is that the proximity of cells in a given ANME-2b—SEEP-SRB1g
545 consortium relative to the consortium exterior (and NH_4^+ availability in the surrounding pore
546 fluid) influences the spatial patterns of diazotrophic activity by both ANME and SRB in large
547 consortia. The concentration of ammonium in seep porefluids can be highly variable over
548 relatively small spatial scales (e.g. between 50 - 300 μM [80]), and rates of diazotrophy
549 estimated from laboratory incubations of methane seep sediment samples indicate different
550 threshold concentrations of $\text{NH}_4^+_{(\text{aq})}$ above which diazotrophy ceases, as low as 25 μM [89] to
551 100-1000 μM [90–92]. In the large consortia observed here, this threshold [$\text{NH}_4^+_{(\text{aq})}$] may be
552 crossed within the consortium as NH_4^+ is assimilated by cells at the consortium exterior,
553 inducing nitrogen limitation and diazotrophy by ANME or SRB near the consortium core. Given
554 the importance of diazotrophy in ANME-SRB consortia for nitrogen cycling at methane seep
555 communities [10, 89], future work should test this hypothesis with SIP incubations with $^{15}\text{N}_2$
556 under variable [$\text{NH}_4^+_{(\text{aq})}$].

557
558 The observed variation in diazotrophic activity in ANME-2b or SEEP-SRB1g cells may also be
559 the result of phenotypic heterogeneity [93] within the multicellular ANME-2b—SEEP-SRB1g
560 consortia, in which expression of the nitrogenase operon that ANME-2b and SEEP-SRB1g
561 partners both possess is an emergent behavior resulting from the spatial organization of ANME-
562 2b and SEEP-SRB1g cells within the consortium. On the basis of nanoSIMS observations of
563 heterogeneous diazotrophy in clonal *Klebsiella oxytoca* cultures, phenotypic heterogeneity was

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564 inferred to confer selective advantage on microbial communities by enabling rapid response to
565 environmental fluctuations [94]. Similar heterogeneity in *nif* expression by ANME-2b or SEEP-
566 SRB1g cells may provide partners with resilience against changes in environmental nitrogen
567 supply. Corroborating these observations in diverse ANME-SRB consortia and direct coupling of
568 single-cell mRNA expression with nanoSIMS-acquired ^{15}N enrichment would further inform the
569 degree to which relative arrangement of the partners and spatial structure within a consortium
570 plays a significant role in determining the mode of nutrient or electron transfer between partners.

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571 Conclusions

572 Here, we present an effective approach to detect novel pairings of microbial symbionts by
573 coupling correlation analysis of 16S rRNA amplicon libraries with FISH and BONCAT-FACS
574 experiments, going beyond amplicon sequencing-based hypothesis generation to experimental
575 validation of hypothesized partnerships using microscopy and single-cell sorting techniques.
576 Correlation analysis performed on a 16S amplicon survey of methane seep sediments near
577 Costa Rica uncovered a novel and highly-specific ANME-SRB partnership between ANME-2b
578 and SEEP-SRB1g. This partnership specificity was then validated by FISH, and further
579 corroborated by 16S rRNA amplicon sequences from BONCAT-FACS-sorted single ANME-SRB
580 consortia from methane seep sediments near Costa Rica, Hydrate Ridge, and Santa Monica
581 Basin in California. Preliminary genomic screening of representative genomes from SEEP-
582 SRB1g uncovered potential for nitrogen fixation in these genomes. Examination of published
583 *nifH* cDNA clone libraries [8] and transcriptomic data [14] prepared from methane seep
584 sediments demonstrated that SEEP-SRB1g actively expresses *nifH in vivo*. Co-localization of
585 signal for *nifH* mRNA and SEEP-SRB1g 16S rRNA by HCR-FISH further corroborated active
586 transcription of *nifH* by SEEP-SRB1g. FISH-nanoSIMS analysis of ANME-2b—SEEP-SRB1g
587 consortia grown with $^{15}\text{N}_2$ headspace documented ^{15}N incorporation in SEEP-SRB1g cells,
588 suggesting that SEEP-SRB1g may fix nitrogen as well. Future work should focus on examining
589 unique aspects of each ANME-SRB partnership to improve our understanding of the diversity of
590 anaerobic methane oxidation symbioses endowed by evolution.

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611

612 Competing Interests

613 The authors declare no competing interests.

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866 Figure 1. Analysis of SparCC-calculated correlations between 16S iTag amplicon sequences
867 (OTUs clustered at 99% similarity) from an ecological survey of 310 methane seep sediment
868 samples from seafloor sites off of Costa Rica. A stacked histogram (A) illustrates the proportion
869 of correlations deemed significant on the basis of pseudo-*p*-values < 0.01 calculated by
870 comparison with 100 bootstrapped correlation tables (see Materials and Methods). Of the
871 correlations with pseudo-*p*-values < 0.01, 18% include ANME with a non-ANME taxon (B).
872 Significant correlations between OTUs with taxonomy assignments that are identical at the
873 genus level (e.g. two *Anaerolinea* OTUs) are indicated by identical taxonomy assignment. 32%
874 of correlations between ANME and non-ANME taxa are represented by OTUs assigned to three
875 groups of sulfate-reducing bacteria: SEEP-SRB1g, SEEP-SRB1a, and SEEP-SRB2 (C).
876 Stacked histograms of correlations between OTUs assigned to SEEP-SRB1g, SEEP-SRB1a, or
877 SEEP-SRB2 and ANME OTUs, parsed by ANME subtype (D), highlights specific associations
878 predicted between ANME-1 and either SEEP-SRB1a or SEEP-SRB2, ANME-2a and SEEP-
879 SRB1a, ANME-2c and SEEP-SRB1a, and between ANME-2b and SEEP-SRB1g.

880
881 Figure 2. Network analysis of the subset of correlations between OTUs calculated by SparCC
882 [41] that are both significant (*pseudo-p*-values < 0.01, 100 bootstraps) and strong ($\geq 99^{\text{th}}$
883 percentile). Correlation strength is proportional to edge length and used to visualize the network
884 (top panel) using force-directed methods [76]. Edges are black where they belong to a set of
885 cohesive blocks of nodes [77] and gray otherwise. Chord diagram [78] visualizing ANME-SRB
886 partnership specificity (bottom panel), with band thickness between SRB (left) and ANME (right)
887 proportional to the number of edges between ANME and SRB OTUs within cohesive blocks.
888 Network analysis supports (cf. Fig. 1) previously-undescribed association between ANME-2b
889 and SEEP-SRB1g.

890 Figure 3. 16S rRNA phylogenetic tree of methane seep Deltaproteobacteria and other lineages,
891 including sequences from recovered metagenome-assembled genomes (MAGs) [14], iTag

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892 amplicons from BONCAT-FACS-sorted ANME-SRB consortia [39], iTag amplicon data from this
893 study, and previously published clone library sequences. Maximum likelihood phylogeny was
894 inferred using 100 bootstraps with >70% or 90% bootstrap support of internal nodes indicated
895 with open or closed circles, respectively. Taxa associated with SRB amplicon iTag sequences
896 were determined from data in Hatzenpichler, et al. 2016 [39] (BONCAT-FACS-sorted ANME-
897 SRB consortia), and by network analysis of iTag amplicon data from methane seep samples (cf.
898 Fig. 2). Taxa in bold represent 16S rRNA sequences from MAG bins acquired from methane
899 seep sediments [14] or from BONCAT-FACS-sorted ANME-SRB consortia, including associated
900 iTag amplicons [39]. The SEEP-SRB1a and -1g clades are operationally defined here by the
901 extent of matches to the respective 16S rRNA FISH probes Seep1a-1441 and Seep1g-1443.
902 Given the low bootstrap values for divergent sequences, the true extent of the SEEP-SRB1g
903 clade is unclear, indicated by the dashed line (cf. Supp. Fig. 5).

904
905 Figure 4. FISH data targeting AOM consortia in seep sediment samples using oligonucleotide
906 probes targeting ANME-2b (ANME-2b-726) and ANME-2a (ANME-2b-828); (in red), a SEEP-
907 SRB1a (Seep1a-1443) probe (in green) and a newly-designed probe (Seep1g-1443) targeting
908 the SEEP-SRB1g clade (in yellow) demonstrating physical association between ANME-2b and
909 SEEP-SRB1g. DAPI counterstain is shown in blue. Examples of ANME-2b—SEEP-SRB1g (B,
910 F) and ANME-2a/ANME-2c—SEEP-SRB1a (C, D, G, H) partnership specificity. Seep sediments
911 harboring ANME-2a and ANME-2b (A-D) host AOM consortia that are composed primarily of
912 either ANME-2a—SEEP-SRB1a or ANME-2b—SEEP-SRB1g (B, C, D). The absence of
913 hybridization with the ANME-2b probe in AOM consortia positively hybridized by the SEEP-
914 SRB1a probe (A) and absence of ANME-2a probe hybridization in SEEP-SRB1g-containing
915 consortia (D) further supports distinct ANME-2—SRB pairings for ANME-2a and ANME-2b.
916 Similarly, FISH analysis of AOM consortia from sediments rich in ANME-2c and ANME-2b (E-H)
917 were composed almost entirely of ANME-2b—SEEP-SRB1g or ANME-2c—SEEP-SRB1a

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918 partnerships (F, G, H); AOM consortia positively hybridized with the SEEP-SRB1g or SEEP-
919 SRB1a probes were not observed to hybridize with probes targeting ANME-2c (H) or ANME-2b
920 (E), respectively. In all panels, the scale bar is 10 μ m.

921
922 Figure 5. Genome tree of ANME-associated Deltaproteobacteria and related organisms inferred
923 from maximum likelihood methods. Bootstrap support for internal nodes was determined using
924 100 bootstraps and depicted on the tree as open (>70% bootstrap support) or closed (>90%)
925 circles. Genome bins containing a 16S rRNA gene or an associated 16S iTag amplicon
926 sequence are highlighted in bold and with a color corresponding to 16S taxonomy assignment.
927 Inferred taxonomy of genome bins closely related to bins containing 16S rRNA sequences are
928 highlighted in a lighter shade. Genome bins containing the nitrogenase operon are annotated
929 with a blue bar. *nifH* sequences found to be expressed in methane seep sediments in cDNA
930 clone libraries [8] are annotated by “cDNA”. As noted in the text, a search of unpublished SEEP-
931 SRB1a MAGs revealed the presence of highly-expressed [8] *nifH* sequences in several
932 unpublished bins (Supp. Fig. 6).

933
934 Figure 6. Phylogeny of *nifH* sequences extracted from *nifH* cDNA (red text) and DNA clone
935 libraries [8], from genome bins acquired from methane seep sediments [14], and from other
936 Deltaproteobacteria genomes using a tblastn search with chlorophyllide reductase BchX
937 (WP011566468) as a query. This BchX sequence along with another BchX (WP012180173)
938 were used as an outgroup to root the tree. Phylogeny was inferred by maximum likelihood
939 methods using 100 bootstraps; bootstrap support of internal nodes is illustrated as open or
940 closed circles, indicating >70% or >90% bootstrap support, respectively. *nifH* recovered from
941 the BONCAT-FACS-sorted genome CONS3730E01UFb1, a bin with an accompanying 16S
942 rRNA amplicon sequence placing it within the SEEP-SRB1g, is highlighted in teal. *nifH* groups
943 (sensu Raymond et al. [95]) were assigned by comparison with Dekas, et al. 2016, and are

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944 annotated either by group number or abbreviated as follows: MSL, Methanosarcina-like; MSG,
945 Methane Seep Group.

946

947 Figure 7. HCR-FISH assays show *in situ* expression of *nifH* in SEEP-SRB1g in association with
948 ANME-2b in methane seep sediment incubations. ANME-2b (B, G) and SEEP-SRB1g (C, H)
949 cells labeled with FISH probes ANME-2b-729 (in red, [39]) and newly-designed Seep1g-1443
950 (in green) with DAPI as the DNA counterstain (A,F). HCR-FISH targeting SEEP-SRB1g *nifH*
951 mRNA (in yellow; Supp. Table 2) demonstrated active expression of *nifH* transcripts localized to
952 SEEP-SRB1g cells (D, I), supporting the hypothesis of diazotrophy by partner SRB. Scale bars
953 in all panels are 5 μm .

954

955 Figure 8. Correlated FISH-nanoSIMS imaging of representative ANME-2b–SEEP-SRB1g
956 consortia demonstrating active diazotrophy by ANME-2b (B, E) and SEEP-SRB1g (H) cells
957 through ^{15}N incorporation from $^{15}\text{N}_2$. FISH images of ANME-2b (pink) and SEEP-SRB1g (green)
958 are shown in panels A, D, G and corresponding nanoSIMS ^{15}N atom percent values are shown
959 in panels B, E, and H. Scale bar is 5 μm in panels A, D, G; raster size in panels B, E, and H is
960 20 μm^2 . Violin plots (C, F, I) of ^{15}N fractional abundance for each type of ROI, representing
961 single ANME-2b or SEEP-SRB1g cells. The number of ROIs measured is indicated by *n* in each
962 panel. Diazotrophic activity in ANME-2b cells appears to be correlated with spatial structure,
963 evidenced by increasing ^{15}N enrichment in cells located within consortia interiors (E, F). SEEP-
964 SRB1g cells are also observed to incorporate ^{15}N from $^{15}\text{N}_2$, and appear to be the dominant
965 diazotroph in the consortium shown in panels G, H, and I, with cellular ^{15}N enrichment in SEEP-
966 SRB1g cells greater than that of the paired ANME-2b partner. Abscissa minima set to natural
967 abundance of ^{15}N (0.36%).

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968

969 Supplemental Table 1. Samples of methane seep sediment used in this study to produce 16S
970 rRNA amplicon libraries.

971

972 Supplemental Table 2. Newly-designed FISH probe (Seep1g-1443) and *nifH* mRNA HCR-FISH
973 probe for labeling ANME-associated members of SEEP-SRB1g or SEEP-SRB1g *nifH*
974 transcripts, respectively. Bolded sequence is complementary to HCR-FISH amplifier B1;
975 nonbolded sequence is complementary to SEEP-SRB1g 16S rRNA or *nifH* RNA. Matches
976 determined by comparison with ARB/SILVA SSU release 128 [54].

977

978 Supplemental Table 3. Stable isotope probing incubation conditions, sample sources and sulfide
979 concentration measurements as a proxy for sulfate reduction activity.

980

981 Supplemental Table 4. SparCC-calculated correlations (pseudo- $p < 0.01$) between OTUs,
982 detailing coefficients, OTU identifiers, and taxonomy assignments.

983

984 Supplemental Figure 1. Optimization of the newly designed Seep1g-1443 probe by FISH
985 hybridization of ANME-2b—SEEP-SRB1g consortia at a range of formamide concentrations.

986 Supplemental Figure 2. Krona chart depicting relative abundance of taxa in Costa Rica seep
987 sediment sample #9279 (Fig. 4) as measured by 16S rRNA iTag amplicon sequencing.

988

989 Supplemental Figure 3. Krona chart depicting relative abundance of taxa in Costa Rica seep
990 sediment sample #9112 (Fig. 4) as measured by 16S rRNA iTag amplicon sequencing.

991

992 Supplemental Figure 4. Krona chart depicting relative abundance of taxa in Costa Rica seep
993 sediment sample #10073 (Fig. 7) as measured by 16S rRNA amplicon sequencing.

Correlation analysis reveals new AOM partnerships

994

995 Supplemental Figure 5. 16S rRNA phylogeny inferred from maximum-likelihood methods using
996 only full-length 16S rRNA sequences. Tree topology shown here is congruent with the
997 phylogeny shown in Figure 3 constructed using a mix of shorter iTag amplicon and full-length
998 16S sequences.

999

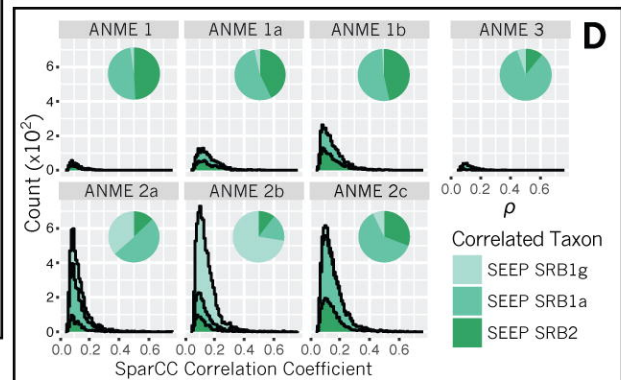
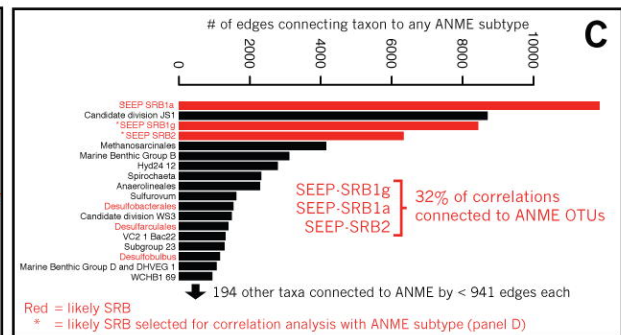
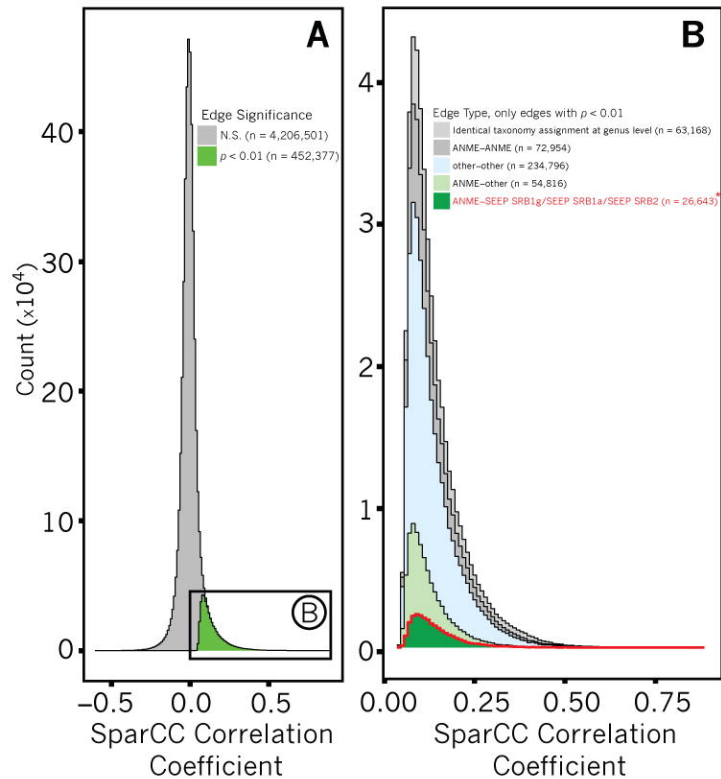
1000 Supplemental Figure 6. Extended *nifH* tree including unpublished SEEP-SRB1a MAGs
1001 possessing *nifH* sequences nearly identical to some recovered in environmental cDNA libraries
1002 (Dekas, et al. 2016).

1003

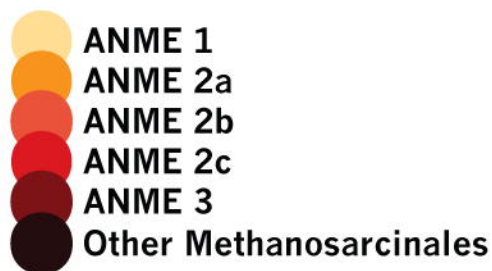
1004 Supplemental Figure 7. Negative controls for HCR-FISH experiments, demonstrating absence
1005 of signal in AOM aggregates with either only initiator or only amplifier added to HCR-FISH
1006 reactions.

1007

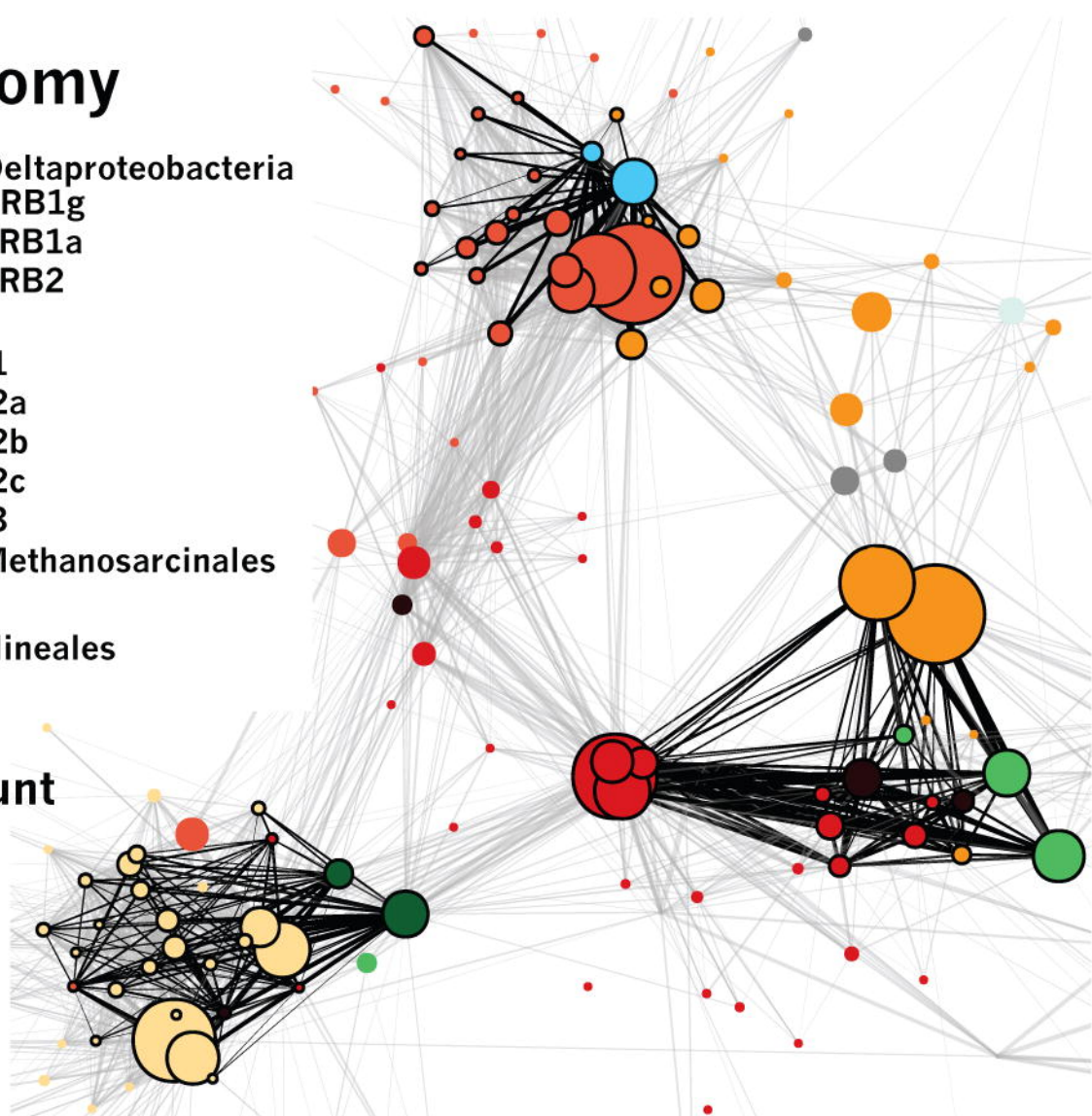
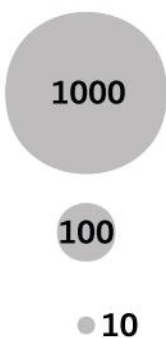
1008 Supplemental File 1. FASTA file containing the translated amino acid sequences for *nifH*
1009 included in Figure 7 in select ANME and SRB genomes (Chadwick, et al., *in prep*) and
1010 transcripts [8].



Taxonomy



Mean Read Count



SRB amplicon
sequence
associated with:

- ANME 1
- ANME 2a
- ANME 2b
- ANME 2c

Desulfobacterales sp. PC51MH44

FACS-sorted ANME-SRB aggregate A03UF iTAG amplicon (KU564230)
 Uncultured bacterium clone LARIS 67-01C09 (FN550063)
FACS-sorted ANME-SRB aggregate C08 iTAG amplicon (KT945204)
FACS-sorted ANME-SRB aggregate A11 iTAG amplicon (KT945219)
 Costa Rica methane seep sediment iTAG amplicon denovo1220845
 Uncultured delta proteobacterium Eel-36e1H1 (AF354164)
 FACS-sorted ANME-SRB aggregate E01 iTAG amplicon (KT945216)
 Uncultured bacterium clone AN 3730incN bac B7 (KM356376)
FACS-sorted ANME-SRB aggregate UD03 iTAG amplicon (KT945207)
FACS-sorted ANME-SRB aggregate A12 iTAG amplicon (KT945203)
FACS-sorted ANME-SRB aggregate H01UF iTAG amplicon (KU564234)
FACS-sorted ANME-SRB aggregate B12 iTAG amplicon (KT945206)
FACS-sorted ANME-SRB aggregate B07 iTAG amplicon (KT945218)
FACS-sorted ANME-SRB aggregate H05UF iTAG amplicon (KU564233)
FACS-sorted ANME-SRB aggregate C08UF iTAG amplicon (KU564231)
FACS-sorted ANME-SRB aggregate C09 iTAG amplicon (KT945202)
FACS-sorted ANME-SRB aggregate C10 iTAG amplicon (KT945210)
 Uncultured bacterium clone AN 3730incN bac A9 (KM356352)
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FACS-sorted ANME-SRB aggregate A03UF iTAG amplicon (KU564229)
 Costa Rica methane seep sediment iTAG amplicon denovo1660670
FACS-sorted ANME-SRB aggregate G01UF iTAG amplicon (KU564235)
FACS-sorted ANME-SRB aggregate B09 iTAG amplicon (KT945205)
 Uncultured Desulfobacteraceae bacterium CH4-cDNA-16S-P1-36 (KR814179)
 Uncultured bacterium clone HR12 (JQ036271)
 Uncultured delta proteobacterium clone SB-24e1E8 (AF354159)
 Uncultured bacterium clone BC-B1 6h (EU622291)
 Costa Rica methane seep sediment iTAG amplicon denovo1857740
 Costa Rica methane seep sediment iTAG amplicon denovo2022355
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FACS-sorted ANME-SRB aggregate C08UF iTAG amplicon (KU564239)
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FACS-sorted ANME-SRB aggregate C10UF iTAG amplicon (KU564238)
FACS-sorted ANME-SRB aggregate A10 iTAG amplicon (KT945217)
FACS-sorted ANME-SRB aggregate C04 iTAG amplicon (KT945213)
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 Uncultured bacterium clone B S2688 12 FL (KM356247)
 Uncultured bacterium MatB0bac98 (GU302433)
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 Desulfatirhabdium butyrativorans DSM 18734
 Desulfonema limicola Jadebusen DSM 2076
 Desulfococcus biacutus KMRActs
 Desulfatibacillum aliphaticivorans DSM 15576
 Desulfatibacillum alkenivorans DSM 16219
 Desulfosarcina variabilis Montpellier
 Desulfococcus oleovorans Hxd3
 Uncultured bacterium clone FeLiveControl B 9 (GQ357013)
 Uncultured bacterium clone 5bav D6 (EU181463)
 Uncultured bacterium clone Fe B 141 (GQ356958)
 Uncultured bacterium clone GB50 Bac A8 (KT152870)
Desulfobacterales sp. S7086C20
Desulfobacterales sp. C00003060
 Desulfobacterium anilini DSM 4660
 Desulfobacca acetoxidans ASRB2 DSM 11109
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 Costa Rica methane seep sediment iTAG amplicon denovo1503198
FACS-sorted ANME-SRB aggregate F03 iTAG amplicon (KT945215)
FACS-sorted ANME-SRB aggregate G01 iTAG amplicon (KT945221)
FACS-sorted ANME-SRB aggregate D05 iTAG amplicon (KT945222)
 Costa Rica methane seep sediment iTAG amplicon denovo3116044
 Uncultured bacterium clone ORI-934-08-P S258-260 086B10 (JX000639)
 Uncultured bacterium clone Elba20 Bac D3 (KT899719)
 Uncultured bacterium clone BAC2 44 KJ569674
 Uncultured bacterium clone AN 5119N bac H2 (KM356386)
 Desulfobulbus elongatus DSM 2908
 Desulfobulbus propionicus 1pr3 DSM 2032
 Desulfobulbus mediterraneus DSM 13871
Desulfobacterales sp. C00003063
 Desulfococcus vexinensis DSM 17965
 Desulfonatronum thioautotrophicum ASO4-1
 Shewanella oneidensis MR-1

SEEP SRB1a

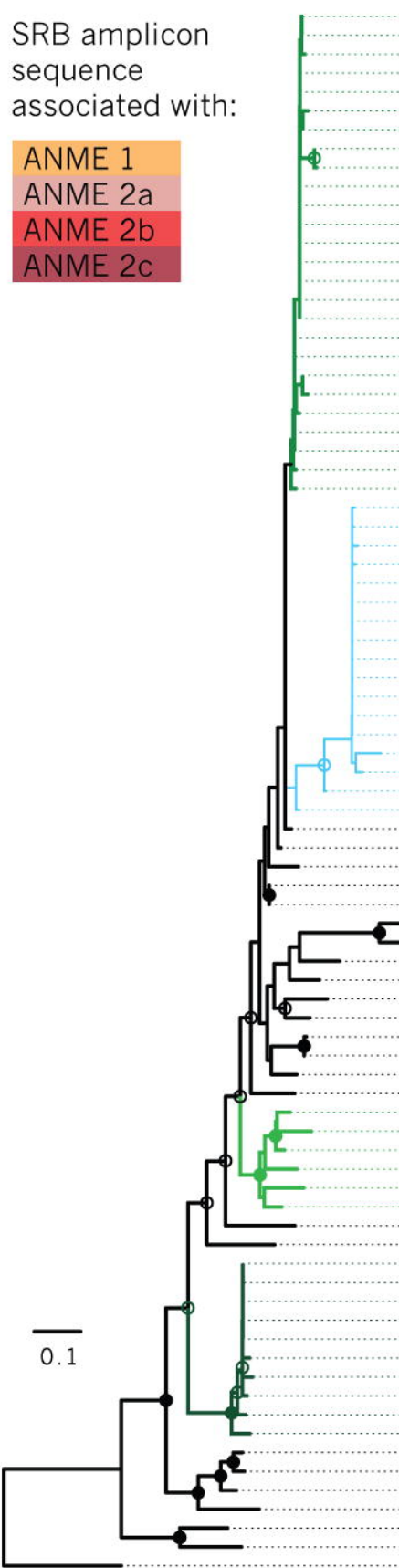
SEEP-SRB1g

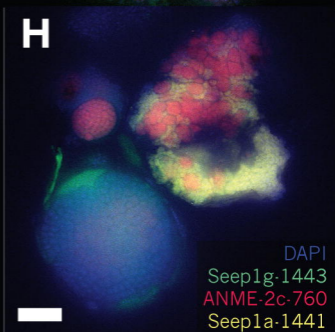
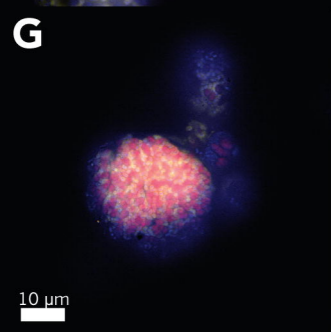
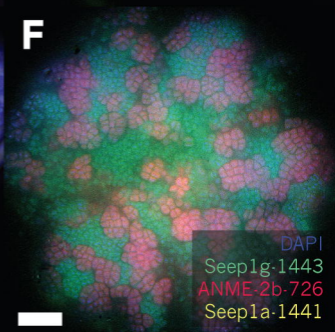
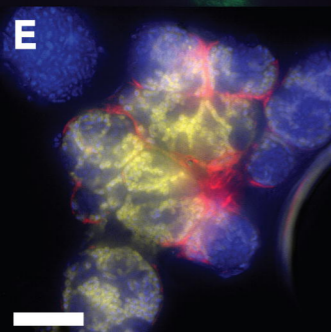
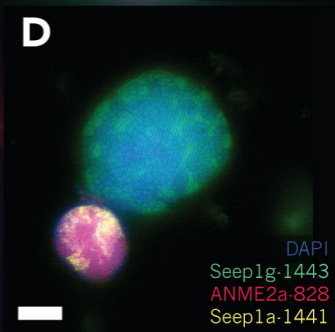
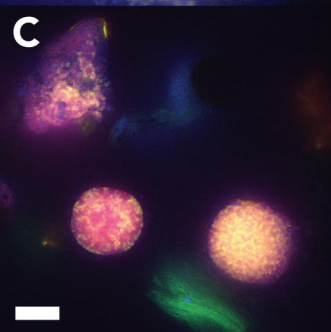
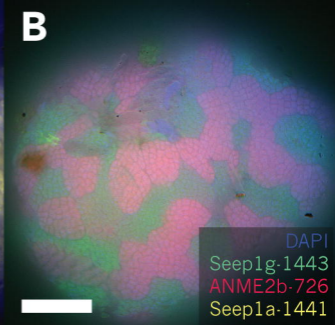
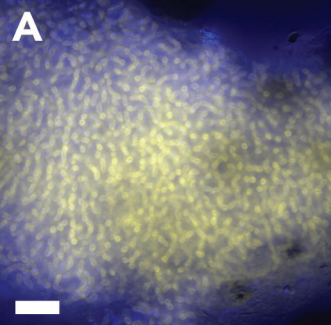
SEEP SRB1c

SEEP SRB2

SEEP SRB4

0.1





Taxonomy by
16S in genome (**G**) or
16S amplicon from FACS (**A**):

SEEP SRB1a
SEEP SRB1c
SEEP SRB1g
SEEP SRB4
SEEP SRB2

taxonomy inferred
16S present

