Title: Development of a free radical scavenging probiotic to mitigate coral bleaching

Running title: Making a probiotic to mitigate coral bleaching

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ABSTRACT

Corals are colonized by symbiotic microorganisms that exert a profound influence on the animal's health. One noted symbiont is a single-celled alga (from the family *Symbiodiniaceae*), which provides the coral with most of its fixed carbon. During thermal stress, hyperactivity of photosynthesis results in a toxic accumulation of reactive oxygen species (ROS). If not scavenged by the antioxidant network, ROS may trigger a signaling cascade ending with the coral host and algal symbiont disassociating; this process is known as bleaching. Our goal was to construct a probiotic comprised of host-associated bacteria able to neutralize free radicals such as ROS. Using the coral model, the anemone *Exaiptasia diaphana*, and pure bacterial cultures isolated from the model animal, we identified six strains with high free radical scavenging ability belonging to the families *Alteromonadaceae*, *Rhodobacteraceae*, *Flavobacteriaceae*, and *Micrococcaceae*. In parallel, we established a "negative" probiotic consisting of genetically related strains with poor free radical scavenging capacities. From their whole genome sequences, we explored genes of interest that may contribute to their potential beneficial roles, which may help facilitate the therapeutic application of a bacterial probiotic. In particular, the occurrence of key pathways that are known to influence ROS in each of the strains has been inferred from the genomes sequences and are reported here.

IMPORTANCE

Coral bleaching is tightly linked to the production of reactive oxygen species (ROS), which accumulates to a toxic level in algae-harboring host cells leading to coral-algal dissociation. Interventions targeting ROS accumulation, such as the application of exogenous antioxidants, have shown promise for maintaining the coral-algal partnership. With the feasibility of administering antioxidants directly to corals being low, we aim to develop a probiotic to neutralize toxic ROS during a thermal stress event. This probiotic can be tested with corals or a coral model to assess its efficacy in improving coral resistance to environmental stress.

44 **INTRODUCTION**

45 Coral reefs are among the most biologically and economically valuable ecosystems on Earth (1-
46 3). While they cover less than 0.1% of the ocean floor (4), coral reefs support fisheries, tourism,
47 pharmaceuticals and coastal development with a global value of $8.9 trillion "international
48 $"/year (5). Corals and other reef organisms have been dying, largely due to anthropogenic
49 influences such as climate change (6, 7), which has led to an increased frequency, intensity and
50 duration of summer heat waves that cause coral bleaching (8, 9).

51 The coral holobiont, which is the sum of the coral animal and its associated microbiota,
52 including algae, fungi, protozoans, bacteria, archaea and viruses (10), is an ecosystem engineer.
53 By secreting a calcium carbonate skeleton, the reef structure rises from the ocean floor,
54 forming the literal foundation of the coral reef ecosystem. The success of corals to survive and
55 build up reefs over thousands of years (11) is tightly linked to their obligate yet fragile symbiosis
56 with endosymbiotic dinoflagellates of the family *Symbiodiniaceae* (12).

57 Intracellular *Symbiodiniaceae* translocate photosynthetically fixed carbon to the coral host (13,
58 14) in exchange for inorganic nutrients and location in a high light environment with protection
59 from herbivory (15, 16). During periods of thermal stress, the relationship between the coral
60 host and their *Symbiodiniaceae* can break down, resulting in a separation of the partners and
61 significantly, fixed carbon shortage for the host. This phenomenon, 'coral bleaching', is
62 devastating to the host and detrimental to the reef system. The ecosystem-wide effects of
63 bleaching on the coral include reduced skeletal growth and reproductive activity, a lowered
64 capacity to shed sediments, and an inability to resist invasion of competing species and
65 diseases. Severe and prolonged bleaching can cause partial to total colony death, resulting in
66 diminished reef growth, the transformation of reef-building communities to alternate, non-reef
67 building community types, bioerosion and ultimately the disappearance of reef structures (12).

68 There are several hypotheses detailing the mechanisms driving bleaching (see 17, 18-20), with a
69 common theme being the overproduction and toxic accumulation of reactive oxygen species
70 (ROS) from the algal symbiont. Excess ROS are generated by a number of pathways including

71 heat damage to both chloroplast and mitochondrial membranes (21, 22), and are shown to play
72 a central role in inter-partner communication of a stress response (17). Once generated, ROS
73 causes damage to many cell components including photosystem II reaction centers in the
74 *Symbiodiniaceae*, specifically at the D1 and D2 proteins (23, 24), thus interfering with the
75 supply of fixed carbon to the holobiont. Once damaged, *Symbiodiniaceae* are no longer able to
76 maintain their role in the symbiotic relationship with corals and separate from the host tissue
77 via *in situ* degradation, exocytosis, host cell detachment, host cell apoptosis or host cell necrosis
78 (17).

79 Probiotics are preparations of viable microorganisms that are introduced to a host to alter their
80 microbial community in a way that is beneficial to the system. Microbiome engineering through
81 the addition of probiotics has been postulated as a key strategy to facilitate adaptation to
82 changing environmental conditions by enhancing corals with the metabolic capabilities of the
83 introduced probiotic bacterial strains (25-30). The differences in the bacterial species
84 composition of healthy and thermally stressed corals (31-36) and the coral model *Exaiptasia*
85 *diaphana* (37-39) suggest a role for microbiome engineering in cnidarian health. A disruption to
86 the bacterial community of *Pocillopora damicornis* with antibiotic treatment diminished the
87 resilience of the holobiont during thermal stress, whereas intact microbial communities
88 conferred resilience to thermal stress and increased the rate of holobiont recovery after
89 bleaching events (40). The relative stability of coral-associated bacterial communities has also
90 been linked to coral heat tolerance; for instance, the bacterial community of heat sensitive
91 *Acropora hyacinthus* corals shifted when transplanted to thermal stress conditions, whereas
92 heat-tolerant *A. hyacinthus* corals harbored a stable bacterial community (41).

93 In recent years, researchers have begun to explore the use of probiotics in corals and the model
94 organism for corals, *E. diaphana*. To inhibit the progression of white pox disease, caused by
95 pathogenic *Serratia marcescens*, an *Alphaproteobacteria* cocktail containing several
96 *Marinobacter* spp. isolates was applied to *E. diaphana* (42). These introduced strains were able
97 to inhibit both biofilm formation and swarming of *S. marcescens,* which halted disease
98 progression. The *Marinobacter*-based probiotic was deemed effective as anemones exposed to

99 both the cocktail and pathogen survived after seven days, while anemones in the *S. marcescens*
100 control treatment died. A bacterial consortium native to the coral *Mussismilia harttii* was
101 selected to degrade water-soluble oil fractions(43). This bioremediation strategy reduced the
102 negative impacts of oil on *M. harttii* health and accelerated the degradation of petroleum
103 hydrocarbons (43). Coral microbiomes have also been manipulated through addition of a
104 consortium of native or seawater derived bacteria to the surface of *P. damicornis* to mitigate
105 the effects of thermal stress (44). The results from this study suggest the consortium was able
106 to partially mitigate coral bleaching.

107 Our goal was to identify bacterial strains suitable for use in a probiotic to mitigate the effects of
108 thermal stress in *E. diaphana*. Given the potential role of ROS in the bleaching process, our
109 focus was to select diverse *E. diaphana*-sourced bacterial strains with antioxidant properties
110 while avoiding potential pathogens. Antioxidant properties were measured using the stable
111 free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), which is reduced in the presence of an
112 antioxidant molecule, undergoing a color change from a violet to a colorless solution.

113 **RESULTS**

114 **Diversity of culturable bacteria associated with *E. diaphana*.** A total of 842 isolates were
115 obtained from four genotypes of Great Barrier Reef (GBR)-sourced *E. diaphana*. There were no
116 significant differences in bacterial colony forming units (CFUs) between the four genotypes,
117 regardless of growth medium, with 5.9-10.3 x 10^3 cells per anemone on Reasoner's 2A agar
118 (R2A) and 6.3-10.4 x10^3 cells per anemone on marine agar (MA) ($p>0.05$). Partial 16S rRNA gene
119 sequences (~1000 bp) were used to identify the closest matches from the NCBI database using
120 the Basic Local Alignment Search Tool (BLASTn). In total there were 109 species in 64 genera, 27
121 families and six phyla (Fig. 1). The most abundant genera were *Alteromonas, Labrenzia*, and
122 *Ruegeria* (Table 1). Gram-positive bacteria comprised 23 species, including *Microbacterium* (31
123 isolates) and *Micrococcus* (28 isolates). Eight genera were found to be associated with all four
124 genotypes (Table 1); these eight genera made up 59.4% of all *E. diaphana*-associated bacterial
125 isolates.

126 **Table 1:** Bacterial genera associated with all four genotypes of GBR-sourced *E. diaphana*
127 (AIMS1-4).

Genus	Class	No. of species	AIMS1	AIMS2	AIMS3	AIMS4	*Total Isolates
Alteromonas	*Gamma-proteobacteria*	6	10	52	24	30	116
Labrenzia	*Alpha-proteobacteria*	4	11	10	26	38	85
Marinobacter	*Gamma-proteobacteria*	4	7	25	10	13	55
Muricauda	*Flavobacteriia*	1	16	11	10	5	42
Roseovarius	*Alpha-proteobacteria*	3	9	12	5	6	32
Ruegeria	*Alpha-proteobacteria*	3	29	8	5	39	81
Shimia	*Alpha-proteobacteria*	2	2	3	9	40	54
Vibrio	*Gamma-proteobacteria*	3	3	7	31	15	56

128 *521 out of the 842 isolates obtained from the four *E. diaphana* genotypes sourced from the
129 Great Barrier Reef.

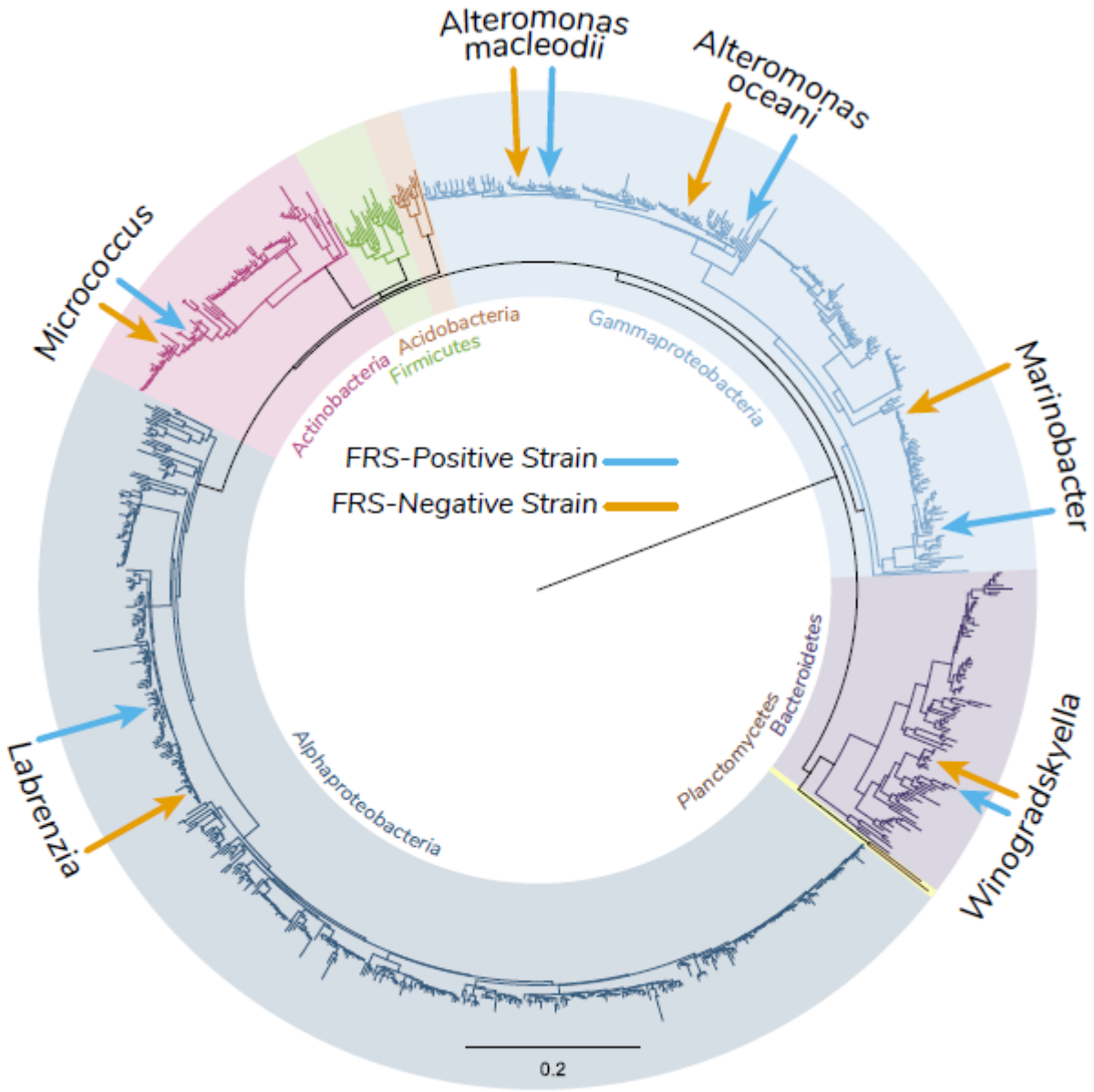

FIG 1: Neighbor-Joining tree showing an overview of the phylogenetic relationship between the 842 *E. diaphana*-associated bacterial isolates inferred using partial 16S rRNA sequences. These isolates covered six phyla indicated by shading over the tree with *Proteobacteria* split into the classes *Gammaproteobacteria* and *Alphaproteobacteria*. The positions of selected probiotic strains are highlighted by arrows with blue arrows indicating the high FRS strains and orange arrows indicating low FRS strains.

137 **Bacterial probiotic selection.** Of the original 842 isolates, 709 were screened for their ability to
138 scavenge free radicals. Those isolates were divided into three categories, positive (144), weakly
139 positive (121), and negative (444). Ninety-eight strains representing eight families and 18
140 genera were then quantitatively assessed for FRS. There was no clear pattern of free radical
141 scavenging (FRS) capacity at the family level (Fig. 2) with strain-specific responses evident. From
142 these isolates, probiotic members were selected by choosing *E. diaphana*-associated bacteria,
143 where conspecific or congeneric pairs of strains displayed a high ("positive") and low
144 ("negative") FRS ability (Fig. 2; Table 2). Of the 12 selected probiotic members, seven were
145 catalase positive and five were catalase negative (Table 2). In each probiotic set (i.e., high or
146 low FRS strains), none of the selected isolates showed antagonistic activity against one other as
147 evidenced by the absence of any zone of inhibition and growth from each combination of
148 isolates on a plate.

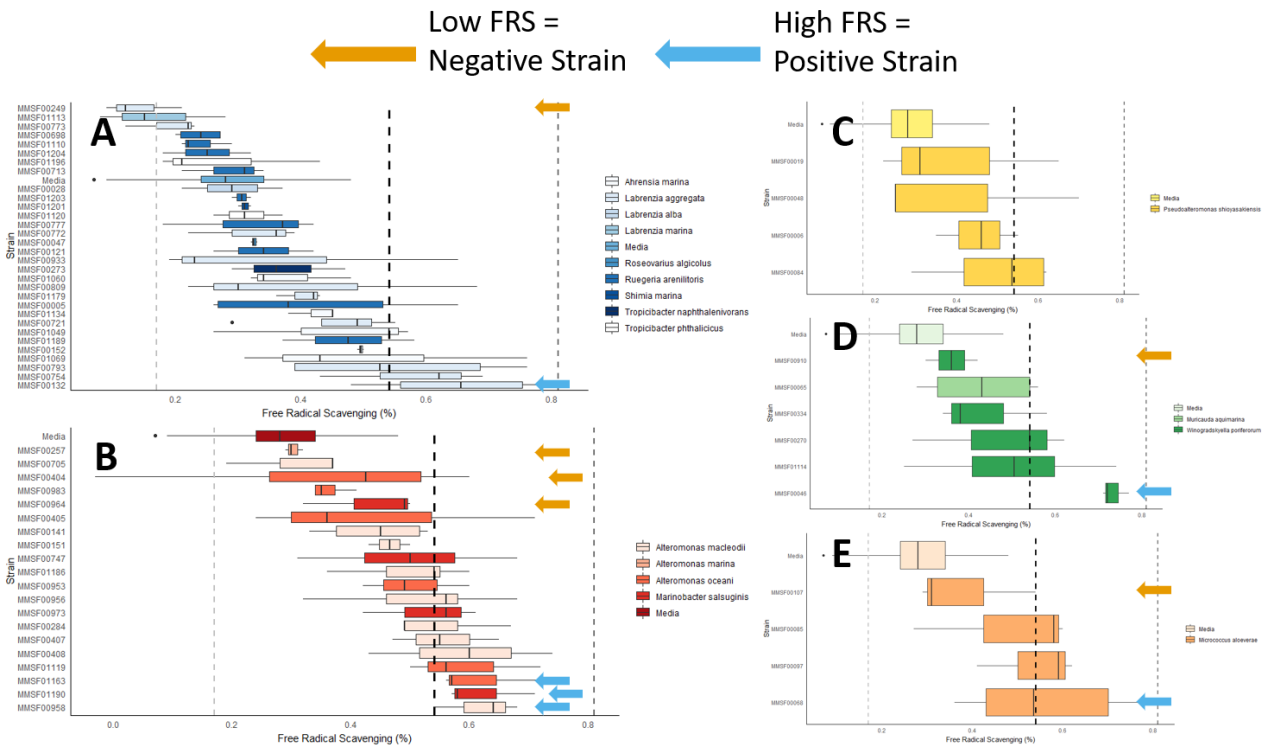

FIG 2: Quantitative FRS ability of *E. diaphana*-associated bacteria isolates, separated by Family. Families with high relative abundance among all cultured bacteria (*Rhodobacteraceae* – A, *Alteromondaceae* – B, *Pseudoalteromonadaceae* – C, *Flavobacteraceae* – D, and *Micrococcaceae* – E) were separately analyzed to identify strains with a high FRS ability (blue arrows) and a corresponding conspecific or congeneric strain with a low FRS ability (orange arrows). In each panel, the light dashed vertical line on the left represents the mean FRS of a 0.025% (w/v) ascorbic acid standard, the middle dark dashed vertical line is the mean FRS for 0.05% (w/v) ascorbic acid standard, and the far right dashed line is the mean FRS of the 0.075% (w/v) ascorbic acid standard.

156 **Table 2:** Overview of probiotic high FRS bacterial strains and conspecific/congeneric low FRS control strains. All sequence data can
157 be found under BioProject# PRJNA574193. References to each probiotic candidate at the Genus level are identified in the last three
158 columns.

Strain	Bacteria species (Phylum or Class)	#FRS (% ± SE)	*Positive vs Negative	*Strain vs Growth Medium	Catalase	*E. diaphana* Literature	Coral Literature	Probiotic Literature
MMSF01163 (n=3)	*Alteromonas oceani* (*Gamma-proteobacteria*)	61.7 ± 5.2	0.065	**0.004**	Negative	(45-47)	(27, 48-50)	(51-54)
MMSF00404 (n=4)		35.5 ± 13.7		0.571	Negative			
MMSF00958 (n=3)	*Alteromonas macleodii* (*Gamma-proteobacteria*)	62.0 ± 4.2	0.175	**0.017**	Negative			
MMSF00257 (n=3)		30.3 ± 0.9		0.431	Positive			
MMSF00132 (n=8)	*Labrenzia aggregata (55)* (*Alpha-proteobacteria*)	53.6 ± 8.7	**0.016**	0.101	Negative	(46)	(35, 50)	(56)
MMSF00249 (n=3)		14.0 ± 3.6		0.103	Positive			
MMSF01190 (n=3)	*Marinobacter salsuginis* (*Gamma-proteobacteria*)	62.0 ± 4.5	**0.041**	**<0.0001**	Negative	(45, 46, 57)	(49, 58, 59)	(42)
MMSF00964 (n=3)		43.7 ± 5.8		**0.020**	Positive			
MMSF00068 (n=6)	*Micrococcus luteus* (*Actinobacteria*)	56.3 ± 7.3	0.210	**0.002**	Positive	(46)	(60)	(51, 61, 62)
MMSF00107 (n=3)	*Micrococcus yunnanensis* (*Actinobacteria*)	38.0 ± 8.0		0.306	Positive			

MMSF00046 (n=3)	*Winogradskyella poriferorum (63)* (*Bacteroidetes*)	73.3 ± 1.9	0.284	**0.010**	Negative	^	(43, 64)	NA
MMSF00910 (n=3)		36.0 ± 3.5		0.174	Negative			

[#] Mean R2A FRS (% ± SE) was 27.2 ± 2.3% (n=12) and it was catalase negative.

^While there are no instances of *Winogradskyella* specifically identified in current *E. diaphana* literature, there are several *Flavobacteriaceae* that are not resolved to the genus level from metabarcoding data *(38, 39, 45, 47)*.

*Indicates p values for pairwise comparisons from respective one-way analysis of variance (Tukey HSD) or Kruskal-Wallis rank sum test (Dunn test) with **bold** values representing significant differences.

164 **Comparative genomics.** As part of the characterization of the 12 isolates (six positive and six
165 conspecific or congeneric negative FRS isolates), a draft genome sequence was determined for
166 each isolate. A summary of the data and metrics for the draft genome sequences is presented
167 in Table S1. The diversity of the six pairs of isolates is indicated by the %G+C range (35% to 72%)
168 and genome size (2.4 Mb to 6.8 Mb). Each isolate pair was classified as the same species except
169 the *Micrococcus* stains. Isolates MMSF00068 (high FRS strain) and MMSF00107 (low FRS strain)
170 are classified as *Micrococcus luteus* by both 16S rRNA gene and genome-based methods, but
171 MMSF00107 is classified as *Micrococcus yunnanensis* by NCBI. These isolates have the smallest
172 genomes among the isolates (2.43 Mb and 2.48 Mb, respectively) and the highest G+C content
173 of 72.8% and 72.4%, respectively. The classification of isolate MMSF00107 is uncertain,
174 particularly given that the genome sequence for the type strain for *M. yunnanensis* is not yet
175 available.

176 Core genome comparison of the draft genome sequences provides an overview of the genetic
177 relationship between the conspecific or congeneric isolate pairs. A wide range of genome
178 variation between pairs was observed, this ranged from ~190,000 core genome single
179 nucleotide polymorphisms (SNP) differences between the *Alteromonas oceani* strains, to fewer
180 than five core genome SNPs between the *Labrenzia aggregata* isolates (Table S2). Importantly,
181 this is not a comprehensive estimate of isolate identity, e.g. the genome sequences of
182 *Winogradskyella poriferorum* isolates MMSF00910 and MMSF00046 are nearly identical with
183 fewer than ten pairwise SNP differences in the core genome; but there are accessory genome
184 differences.

185 **Genes of interest.** The annotated genome sequences of each selected probiotic member were
186 searched for key genes of interest (Table S3-S4). Dimethylsulfoniopropionate (DMSP) cleavage
187 to dimethylsulfide (DMS) was identified by presence of one or more of the DMSP lyase genes;
188 *dddP*, *dddD*, *dddL*, *dddW*, and *dddQ*. Only *L. aggregata* strains contained DMSP lyase genes
189 (*dddP* and *dddL*) in their whole genome sequences. DMSP biosynthesis was identified by the
190 presence of *dsyB*, which is the only described gene for an enzyme in the DMSP biosynthesis
191 pathway. The *cobP* gene was used as an indicator for the presence of the dynamic vitamin B_{12}

192 pathway, which contains 27 genes (Table S4). Again, only the *L. aggregata* isolates contained
193 *cobP*. Catalase positive strains were identified by the presence of *katG*; all positive and negative
194 FRS strains contained *katG* except the *Micrococcus* spp. strains, in which *katA* and *katE* were
195 detected.

196 **16S rRNA gene copy number.**

197 The 16S rRNA gene copy numbers of the 12 draft genomes were estimated using a read depth
198 approach (Table S1). The copy numbers were similar within pairs of isolates, in which the pair of
199 isolates MMSF00257 and MMSF00958 contained the most copies (5.15 and 4.79, respectively),
200 corresponding with copy numbers in a known closed genome of *A. macleodii* (65).
201 *Marinobacter* isolates MMSF00046 and MMSF00910 contained the fewest copies (1.03 and
202 0.77, respectively).

203 **DISCUSSION**

204 The 842 *E. diaphana* bacterial isolates reported here comprise 109 species from 64 genera and
205 six phyla. Using metabarcoding, studies of microbiomes associated with *E. diaphana* have
206 revealed a similar diversity at the phylum level for *E. diaphana* sourced from the GBR (37),
207 Hawaii (strain H2; 45), Pacific and Caribbean (57), Atlantic (strain CC7; 47) and Red Sea (38), as
208 well as stony corals (66). Thus, our culture collection of *E. diaphana* bacterial isolates captures
209 the diversity of the *E. diaphana*-associated microbiome. The *E. diaphana* probiotics generated
210 in this study were assembled from 12 isolated bacterial strains selected based on their FRS
211 ability since free radical production, specifically ROS, is relevant in coral bleaching. The broader
212 culture collection contains bacteria with a wide range of FRS capacity, and our probiotic
213 comprises greater bacterial diversity compared to others (42, 44).

214 The consistent and frequent reporting of our probiotic bacteria genera in *E. diaphana* and coral
215 studies (Table 2) suggests these bacteria likely have key functions in cnidarian holobionts.
216 Among these potential functions are the production of the antioxidant DMSP and the
217 breakdown of DMSP to other antioxidants (67). *L. aggregata* has been reported to produce

218 DMSP in the absence of any methylated sulfur compounds with *dsyB* identified as the first
219 DMSP biosynthesis gene in any organism (68). The *dsyB* gene was found in the genome
220 sequences of both high and low FRS *L. aggregata* strains (Table S3). Many *E. diaphana*-sourced
221 bacterial species, specifically relatives of our selected probiotic members, are implicated in the
222 degradation of DMSP to DMS (*Alteromonas* spp., (69); *Labrenzia* spp., (70)). *dddP* codes for the
223 enzyme responsible for cleaving DMSP to DMS and acrylate and was used (from the Prokka
224 annotation) as an indicator of a DMSP degradation genotype. Only the *L. aggregata* isolates
225 contained genes responsible for DMSP degradation (Table S3).

226 Carotenoids are among the strongest antioxidants and are highly reactive against both ROS and
227 free radicals (71-75). Carotenoids are lipid-soluble pigments, and in bacteria they give an
228 orange-yellow hue to colonies. Two of the five selected probiotic genera produce
229 orange/yellow colonies (*Winogradskyella*, *Micrococcus*), and there is evidence of carotenoid
230 production by marine *Flavobacteriaceae* (75, 76) and *Micrococcus* (77). A marine
231 *Flavobacteriaceae* (strain GF1) was found to produce the potent antioxidant carotenoid
232 zeaxanthin that protected *Symbiodiniaceae* from thermal and light stress (78).

233 Vitamin B_{12} is a cofactor involved in the production of the amino acid methionine, which is
234 needed to synthesize every protein and in diverse metabolic pathways including generation of
235 the antioxidants glutathione and DMSP (79). Vitamin B_{12} is synthesized by many heterotrophic
236 bacteria (80). Genomic evidence suggests that *Symbiodiniaceae* have lost the capacity to
237 synthesize vitamin B_{12} (81), which is in agreement with other work showing that free-living
238 *Symbiodiniaceae* rely on bacterial symbionts for this important cofactor (82). The genes
239 involved in the biosynthesis of vitamin B_{12} have been found in coral-associated bacteria,
240 specifically *L. aggregata* cultured from the Caribbean coral, *Orbicella faveolata* (83). Eighteen
241 genes, including the *cobP* gene (80), are annotated in each of *L. aggregata* isolates
242 (MMSF00132 & MMSF00249), suggesting both are capable of vitamin B_{12} biosynthesis. None of
243 the other sequenced isolated had genes related to vitamin B_{12} biosynthesis, except in the
244 *Marinobacter salsuginis* isolates, where a *cobO* gene was detected, strongly indicating that
245 none of the remaining ten isolates were capable of vitamin B_{12} biosynthesis (Table S4).

246 Bacteria have developed highly specific mechanisms to protect themselves against oxidative
247 stress, in particular using enzymatic antioxidants such as catalase/peroxidase and superoxide
248 dismutase (84). It has been suggested that increasing the *in hospite* concentration of catalase in
249 the coral holobiont by the application of a probiotic with catalase-positive organisms, could
250 possibly minimize the impact of thermal stress by neutralizing hydrogen peroxide (H_2O_2) (29).
251 Here we tested all probiotic candidates for catalase production using a standard H_2O_2 assay
252 (85). Catalase participates in cellular antioxidant defense by enzymatically breaking down H_2O_2
253 to H_2O and O_2. Hydroperoxidase I (HPI, *katG*) is present during aerobic growth and is
254 transcriptionally controlled at different levels, and hydroperoxidase II (HPII, *katE*) is induced
255 during stationary phase (86, 87). The phenotypically determined catalase positive strains
256 (MMSF00249, *L. aggregata*; MMSF00257, *A. macleodii*; MMSF00964, *M. salsuginis*) had a
257 catalase-peroxidase gene (HPI, *katG*); but all strains had at least one *katG*, *katA* (catalase), or
258 *katE* (catalase HPII) gene (Table S3).The lack of correlation between genotype and phenotype in
259 relation to catalase activity may be associated with the culture conditions used or the bacterial
260 growth phase during the catalase assay. The inconsistency between the catalase and DPPH
261 phenotype and FRS ability (in many cases high FRS isolates were catalase negative while the low
262 FRS strain was catalase positive), justifies our approach of using FRS analysis, instead of using
263 the presence of catalase genes as a proxy for FRS.

264 A critical characteristic in the selection of probiotic members is the maintenance and
265 proliferation of the inoculated bacteria in the host over time (51) and their potential
266 transmission to the next generation. There is evidence that corals release bacteria with their
267 offspring such as *Alteromonas* (48), *Flavobacteriaceae* (48), *Rhodobacteraceae* (88), and
268 *Marinobacter* (58). While broadcast spawning corals do not appear to transfer their bacterial
269 symbionts with their gametes (vertical transfer) (89), the brooding coral *Porites astreoides*
270 transmits some bacteria vertically to planulae with two bacterial taxa (*Roseobacter* clade-
271 associated bacteria and *Marinobacter* spp.) consistently and stably associating with juvenile
272 *P. astreoides* (58). *Marinobacter* potentially has antioxidant properties (90), while *Roseobacter*
273 spp. might be beneficial in facilitating larval settlement. If adult corals stably associate with

274 inoculated probiotic candidates like *Marinobacter, Alteromonas,* and *Winogradskyella*, they
275 may be passed on to offspring and thus have a long-term positive impact on these individuals.

276 The probiotic members were chosen from a highly diverse pool of *E. diaphana*-sourced
277 bacterial isolates. While the selected probiotic members are phylogenetically diverse,
278 potentially promising probiotic bacteria in the culture collection were omitted based on our
279 selection criteria. For example, *Ruegeria* spp. were reported to breakdown DMSP and
280 participate in denitrification (83). *Muricauda* isolates had high FRS abilities, but they did not
281 grow consistently in the selected medium and therefore were excluded from the consortium.
282 *Muricauda* isolates have genes for denitrification (83), can breakdown DMSP (70), and produce
283 potent carotenoids (91) that can mitigate thermal and light stresses in *Symbiodiniaceae*
284 cultures (78). *Muricauda* will be included in future probiotic evaluations.

285 Interactions among members of the microbiota associated with marine animals are
286 undoubtedly complex. Results presented in this manuscript show that pure cultured bacteria
287 from *E. diaphana* can scavenge free radicals, albeit at a strain-specific rate. Inoculation with
288 high FRS strains could be beneficial to the host under high oxidative stress conditions, such as
289 those that contribute to coral bleaching. Outlined here is the start of a complex process to
290 identify, evaluate, and select durable and useful probiotics that can buffer the coral host
291 against climactic variation. Conspecific or congeneric pairs of bacteria provide an opportunity to
292 determine the genetic basis for measured phenotypic differences between the pairs. An
293 essential element of future work will be to investigate the stability of the phenotypic
294 differences observed and this stability may be reflected in the nature of the genetic differences
295 between the pairs of strains.

296 **MATERIALS AND METHODS**

297 **Isolation of bacterial isolates.** GBR origin *E. diaphana* were maintained in the laboratory at
298 26 ^{o}C (92) and used to isolate probiotic candidates. Sixteen individuals from each of four
299 *E. diaphana* genotypes (AIMS1-4) were collected using sterile disposable pipets and gently
300 transferred to filter-sterilized (0.2 µm) reverse osmosis (RO) water reconstituted Red Sea Salt™

301 (Red Sea; RSS) at ~34 parts per thousand (ppt) salinity (fRSS). This was done to remove some of
302 the influence of the external seawater on the bacterial community. After 30 min, each
303 anemone was transferred to a sterile glass homogenizer with 1 mL of fRSS. Each homogenate
304 was used to prepare serial dilutions from 10^{-1} to 10^{-4}. From each dilution, 50 µL was spread
305 plated onto three replicate plates each of MA (Difco™ Marine Agar 2216) and R2A (CM0906,
306 Oxoid) supplemented with 40 g L^{-1} RSS and incubated at 26 ˚C. After one week, CFU counts
307 were completed. Individual bacterial isolates were sub-cultured to purification from plates with
308 <100 CFUs onto the initial isolation medium. All purified bacterial isolates were resuspended in
309 40% glycerol, aliquoted into 1.2 mL cryotubes and stored at -80 °C.

310 **Identification of *E. diaphana*-sourced isolates.** Colony PCR with the universal bacterial primers
311 27f (5' – AGA GTT TGA TCM TGG CTC AG – 3') and 1492r (5' – TAC GGY TAC CTT GTT ACG ACT T
312 – 3') (93) was used to generate 16S rRNA gene amplicons from each isolate. Briefly, cells from
313 each pure culture were suspended in 20 µL Milli-Q water and denatured at 95 °C for 10 min.
314 The suspension was then centrifuged at 2,000 x *g* at 4 °C for two minutes and the supernatant
315 was used as the DNA template for PCR amplification. The PCR was performed with 20 µL Mango
316 Mix™ (Bioline), 0.25 µM of each primer and 2 µL of DNA template in a final volume of 40 µL.
317 The thermal cycling protocol was as follows: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 50 °C
318 for 1 min, and 72 °C for 1 min; and a final extension of 10 min at 72 °C. Amplicons were purified
319 and Sanger sequenced on an ABI sequencing instrument by Macrogen Inc. (Seoul, South Korea)
320 or by the Australian Genome Research Facility (AGRF) using the 1492r primer. Trimmed high
321 quality read data from each isolate was used for presumptive identification by querying the 16S
322 rRNA gene sequences via BLASTn. For some isolates the near-complete 16S rRNA gene
323 sequence was determined by sequencing with additional primers (27f, 357f (5'-CCT ACG GGA
324 GGC AGC AG-3', (94)), 926f (5'-CCG TCA ATT CMT TTR AGT TT-3', (95)), 519r (5'-GWA TTA CCG
325 CGG CKG CTG-3', (94)), 926r (5'-AAA CTR AAA MGA ATT GAC GG-3', (95)), and 1492r). The six
326 reads for each isolate were aligned using Geneious Prime 2019.1.2
327 (https://www.geneious.com) via the Geneious global alignment default settings with automatic
328 determination of read direction. From this alignment, a consensus sequence for the 16S rRNA
329 gene was constructed based on the frequency of a base and its quality (from chromatogram

330 data) in each alignment column. The consensus sequence length for each of the six isolate pairs
331 varied from 1352 to 1495 nucleotides. GenBank accession numbers for sequences are shown in
332 Table 1.

333 **Qualitative free radical scavenging assay.** DPPH is a stable free radical that is purple in its
334 oxidized state but becomes white-yellow when reduced by antioxidants, and has been used to
335 identify FRS marine bacteria (96, 97). To qualitatively assess *E. diaphana*-associated bacteria
336 isolates for FRS ability, a sterile Whatman #1 filter paper was gently pressed against fresh (2-4
337 days old) colonies from a streak plate. Plates (with filter paper) were then incubated overnight
338 at 26 °C. The following day, filter papers were removed with forceps, allowed to dry in a fume
339 hood for 30 min, and 500 µL of a 0.2 mM DPPH (Cat# D9132, Sigma-Aldrich) solution in
340 methanol was applied with a pipette over individual colonies. As a positive control, a few drops
341 of 0.1% (w/v) L-ascorbic acid (Cat# A7631, Sigma-Aldrich) were placed on a separate filter. The
342 response of each isolate to DPPH was recorded within 3 min of DPPH application; a positive
343 response was recorded if a white-yellow halo appeared around individual colonies within 1 min,
344 a weak positive response was assigned to strains that had a halo form between 1 and 3 min
345 after DPPH application, and a negative response was listed for strains that failed to form a halo
346 (Fig. 3). Approximately 700 isolates were screened using the qualitative DPPH assay.

347 A.

B.

348 **FIG 3:** DPPH is a stable free radical that is purple in its oxidized state. When reduced by an
349 antioxidant, a white-yellow halo will appear around individual bacteria colonies (A), this was
350 qualitatively deemed a positive response. Isolates that did not have a halo around colonies
351 within 3 min of DPPH application were deemed negative (B).

352 **Quantitative free radical scavenging assay.** To quantitatively assess the FRS ability, select
353 isolates were grown in R2A broth (Table S5). A volume of 50 mL of autoclaved medium was
354 added to sterile 250 mL Erlenmeyer flasks and each flask was inoculated with an isolate colony
355 grown from a R2A or MA plate culture. Cultures were grown with shaking (150 rpm; Ratek
356 orbital incubator) at 37˚C for 48 h. Sterile uninoculated R2A was used as a control. A minimum
357 of three replicate cultures were grown per isolate. After 48 h, the optical density of each
358 culture was measured at 600 nm (OD_{600}, CLARIOstar PLUS, BMG Labtech), and the cultures
359 (including negative medium controls) were centrifuged at 3000 x *g* at 4˚C for 30 min (Allegra X-
360 12R) to pellet the bacterial cells. The cell-free supernatants (CFSs) were collected, frozen at -
361 80 ˚C, freeze dried (Alpha 1-4 LDplus, Martin Christ), and stored under inert gas in a dark, dry
362 environment until analysis. Antioxidants were extracted from the CFSs by resuspending at 50
363 mg mL^{-1} in 100% methanol, sonicating (Branson 2510) for 5 min, then centrifuging at 3000 x *g*
364 at 4 ˚C for 5 min. Quantitative DPPH assays were run by creating a 1:1 solution of 0.2 mM DPPH
365 in methanol and CFS extract to a final volume of 1 mL, vortexing, and incubation in the dark at
366 room temperature for 30 min. Samples were then vortexed briefly, and three 300 µL replicates
367 of each sample were transferred into a well of a 96 well plate. Decolourization of DPPH was
368 determined by measuring the decrease in absorbance at 517 nm (Enspire 2300 plate reader,
369 Perkin Elmer), and the FRS activity was calculated according to the formula, % DPPH scavenging
370 activity = (Control – Sample) / Control ×100, where, Control is the absorbance of the DPPH
371 control (1:1 0.2 mM DPPH:methanol), and Sample is the absorbance of CFS extract in DPPH. All
372 samples were measured against a 100% methanol blank. Positive controls consisting of 0.01 -
373 0.001% (w/v) L-ascorbic acid were run on each 96-well plate. FRS activity ranged from 0-90%.

374 **Catalase assay.** The pelleted cells from above were resuspended in 2 mL fRSS and 500 µL H_2O_2
375 giving a final concentration of 16 mM. If bubbles appeared, the organism was considered
376 catalase positive. If there were no bubbles, the organism was classified as catalase negative.

377 **Inhibition testing.** Each paired set of high and low FRS strains were inoculated crosswise along
378 the middle of MA plates to test for antagonism. Plates were kept at 26 °C and monitored daily

379 for up to 7 days for antagonistic activity by documenting the presence or absence of both
380 inoculated isolates and if there was a zone of inhibition between them.

381 **Phylogenetic analysis.** All partial 16S rRNA gene sequences (842) were aligned with reference
382 sequences (72) of closely related organisms using Geneious Prime 2019.1.2
383 (https://www.geneious.com). This alignment was used to construct a neighbor-joining
384 phylogenetic tree using the Jukes-Cantor method. Maximum-likelihood dendrograms were
385 generated with bootstrap values of 1000.

386 **Whole genome sequence analysis.** Positive FRS strains along with conspecific or congeneric
387 negative FRS strains were selected for genome sequencing; in total, six pairs of isolates were
388 sequenced. Genomic DNA was isolated from a single colony using a JANUS Chemagic
389 Workstation and Chemagic Viral DNA/RNA kit (PerkinElmer). Libraries were prepared with the
390 Nextera XT DNA sample preparation kit (Illumina). Readsets were produced using the Illumina
391 sequencing platform (Instrument: Illumina NextSeq 500, 150 base, paired-end) and the whole
392 genome shotgun (WGS) method. Read depth coverage was approximately 100 times assuming
393 a genome size of 4 M bases.

394 Illumina readsets for each isolate were assembled using Skesa (98) and the draft genome
395 sequence annotated using Prokka (99). A genome sequence based taxonomic classification for
396 each isolate was determined using Kraken2 (100) with the Genome Taxonomy Database (GTDB;
397 101) as the curated genomic data source. Classification was primarily based on the genome
398 sequence of related isolates (within the relevant species where possible), which were obtained
399 from GenBank. In situations where genomes of taxonomically relevant individuals were
400 available, a species level classification was possible. Where available, closed genome sequences
401 from GenBank were used for comparative genomics analysis. For each of the six pairs of
402 isolates, core genome (i.e. genes shared between the isolate pair) comparisons were
403 performed, as implemented in Nullarbor (https://github.com/tseemann/nullarbor), , with
404 phylogenies inferred using SNP differences. Genes of interest for DMSP synthesis and
405 degradation, vitamin B_{12} synthesis, and catalase were identified from the annotated genome

406 sequence (GFF format) produced by Prokka; specific genes were identified by both name and
407 Refseq accession number.

408 **16S rRNA gene copy number estimation.**

409 The 16S rRNA gene copy number of the 12 draft genomes was predicted by the 16Stimator
410 pipeline (102). Briefly, all the 12 genomic assemblies were submitted to the rapid annotations
411 using subsystems technology (RAST) server (103), and the positions of 16S rRNA and a set of
412 single-copied housekeeping genes (Table S6) were extracted from the RAST annotations. The
413 clean read sets were mapped back to the corresponding genomic assemblies by Bowtie 2 (104)
414 to determine the read depth of each position. Finally, the 16S copy number of each isolate was
415 calculated by dividing the median depth of 16S gene by the median depth of the single-copied
416 housekeeping genes after the read depths were calibrated by the model parameters provided
417 by 16Stimator.

418 **Statistical analysis.** CFU counts were analyzed in R (v3.6.2, 105) by first checking the
419 assumptions of equal variance and homogeneity. An analysis of variance test was used to
420 detect differences in the mean number of bacterial colonies from each anemone genotype by
421 solid growth media (R2A or MA). A one-way analysis of variance (one-way ANOVA; 106) was
422 used to determine if there were significant differences between FRS abilities of selected
423 positive (high FRS), negative (low FRS), and media controls, and pairwise comparisons were
424 performed using Tukey's HSD (107, 108). Each probiotic pair and media control was tested to
425 determine if data met the assumptions of normality and homoscedasticity. If either assumption
426 was violated, the non-parametic Kruskal-Wallis rank sum test (109) was used with a Dunn test
427 (110) for multiple comparisons (p-values adjusted with the Benjamini-Hochberg method (111))
428 with the R package "FSA" (112).

429 **Data availability.** WGS raw reads are freely available in the Sequence Read Archive under
430 BioProject PRJNA574193; the complete data set is listed in Table S1.

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440 AMD, MvO and LLB conceived and designed the study. AMD performed the sampling and
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442 draft. All authors edited and approved the final manuscript.

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731 **Table S1**: Isolate Genome Sequence Data Summary. Strains are presented as high FRS (grey) followed by low FRS (white). 16S rRNA
732 gene presumptive identity is derived from the NCBI classification of near-complete 16S rRNA gene sequences. *We were unable to
733 determine the 16S rRNA copy number of isolate MMSF00068 due to the presence of contaminant sequences in the WGS.

Isolate	Genus Level Classification	Genus % Confidence	Family	Family % Confidence	SRA Accession	Sample Accession
MMSF00257	*Alteromonas*	96.65	*Alteromonadaceae*	96.74	SRR10186803	SAMN12851724
MMSF00958	*Alteromonas*	92.93	*Alteromonadaceae*	92.98	SRR10186806	SAMN12851731
MMSF01163	*Alteromonas*	9.28	*Alteromonadaceae*	12.01	SRR10186808	SAMN12851721
MMSF00404	*Alteromonas*	8.15	*Alteromonadaceae*	10.49	SRR10186805	SAMN12851732
MMSF00132	*Labrenzia*	90.9	*Rhodobacteraceae*	90.91	SRR10186800	SAMN12851727
MMSF00249	*Labrenzia*	90.83	*Rhodobacteraceae*	90.83	SRR10186799	SAMN12851728
MMSF00964	*Marinobacter*	59.99	*Alteromonadaceae*	60.1	SRR10186802	SAMN12851725
MMSF01190	*Marinobacter*	65.77	*Alteromonadaceae*	65.85	SRR10186797	SAMN12851730
MMSF00068	*Micrococcus*	71.81	*Micrococcaceae*	72.88	SRR10186807	SAMN12851722
MMSF00107	*Micrococcus*	83.18	*Micrococcaceae*	84.87	SRR10186798	SAMN12851729
MMSF00046	*Winogradskyella*	49.63	*Flavobacteriaceae*	49.72	SRR10186801	SAMN12851726
MMSF00910	*Winogradskyella*	54.44	*Flavobacteriaceae*	54.53	SRR10186804	SAMN12851723

734

Isolate	16S rRNA gene presumptive identity	Confidence	Reads	Total Bases	G+C%	Avg. Read Length	Max Read Length	Avg Quality
MMSF00257	*Alteromonas macleodii*	1.00	2233080	334079592	44.7	149	151	30.9
MMSF00958	*Alteromonas macleodii*	1.00	2971718	443870758	44.6	149	151	31.9
MMSF01163	*Alteromonas oceani*	0.83	2757340	412741366	48.7	149	151	33.7
MMSF00404	*Alteromonas oceani*	0.83	2067800	306005114	48.7	147	151	33.3
MMSF00132	*Labrenzia aggregata*	0.98	3758944	557529936	59.2	148	151	31.1
MMSF00249	*Labrenzia aggregata*	0.98	3333098	497032699	59.3	149	151	30.7
MMSF00964	*Marinobacter salsuginis*	1.00	1713198	256635737	57.1	149	151	31
MMSF01190	*Marinobacter salsuginis*	1.00	3239568	483874048	57.1	149	151	33.1
MMSF00068	*Micrococcus luteus*	0.99	2658898	398496031	72.4	149	151	30.3

MMSF00107	*Micrococcus yunnanensis*	0.99	2323654	348617190	72.8	150	151	31.9
MMSF00046	*Winogradskyella poriferorum*	0.59	1930584	285978916	35	148	151	31.8
MMSF00910	*Winogradskyella poriferorum*	0.59	3939880	579510587	35.6	147	151	33.9

735

Isolate	Est. Read Coverage	Contigs in Draft Genome	Bases in Draft Genome	Min Contig	Avg Contig	Max Contig	N50	16S rRNA gene copy number
MMSF00257	69	136	4831263	515	35523	210048	72973	5.15
MMSF00958	94	42	4732026	620	112667	580903	303003	4.79
MMSF01163	75	100	5507488	526	55074	394030	155957	4.18
MMSF00404	51	111	6014142	502	54181	256548	93725	3.97
MMSF00132	82	34	6792087	855	199767	1019681	302139	3.57
MMSF00249	73	44	6791310	851	154347	1286212	294458	3.02
MMSF00964	56	41	4588310	512	111910	932206	291012	3.67
MMSF01190	110	40	4404819	529	110120	769441	404351	3.21
MMSF00068	160	316	2484978	509	7863	78799	12670	*
MMSF00107	143	501	2435904	515	4862	42365	7717	1.75
MMSF00046	83	104	3446386	553	33138	299826	78549	1.03
MMSF00910	168	50	3456468	553	69129	355406	162623	0.77

736

737 **Table S2**: Pairwise comparison of the genome sequences between the pairs of isolates

NCBI Classification	Type strain	Core Genome	SNP difference
Alteromonas macleodii	ATCC 27126	85%	~60000
Alteromonas oceani	S35	60%	~190000
Labrenzia aggregata	IAM12614	55%	5
Marinobacter salsuginis	SD14B	15%	~120000
Micrococcus spp.	NCTC2665	70%	~35000
Winogradskyella poriferorum	NA	NA	10

738

739 **Table S3:** Search outcomes for genes of interest.

Isolate	NCBI Classification	Gene	Gene Classification	Contig	Start	End	Strand
MMSF00132	*Labrenzia aggregata*	*dddL*	positive	Contig_16_56.34	104067	104756	-
MMSF00249	*Labrenzia aggregata*	*dddL*	positive	Contig_18_45.1167	104045	104734	-
MMSF00132	*Labrenzia aggregata*	*dddP*	positive x2 (Copy 1)	Contig_6_57.2063	50787	52046	+
MMSF00132	*Labrenzia aggregata*	*dddP*	positive x2 (Copy 2)	Contig_6_57.2063	650583	651881	-
MMSF00249	*Labrenzia aggregata*	*dddP*	positive x2 (Copy 1)	Contig_17_46.7174	317316	318575	+
MMSF00249	*Labrenzia aggregata*	*dddP*	positive x2 (Copy 2)	Contig_17_46.7174	917112	918410	-
MMSF00132	*Labrenzia aggregata*	*dsyB*	positive	Contig_32_56.2994	210397	211419	+
MMSF00249	*Labrenzia aggregata*	*dsyB*	positive	Contig_4_46.0396	460215	461237	+
MMSF00046	*Winogradskyella poriferorum*	*katG*	positive	Contig_15_55.0851	16430	18661	+
MMSF00068	*Micrococcus luteus*	*katG*	ND; katA & katE detected	NA	NA	NA	NA
MMSF00107	*Micrococcus yunnanensis*	*katG*	ND; katA & katE detected	NA	NA	NA	NA
MMSF00132	*Labrenzia aggregata*	*katG*	positive	Contig_6_57.2063	425873	428044	-
MMSF00249	*Labrenzia aggregata*	*katG*	positive	Contig_17_46.7174	692402	694573	-
MMSF00257	*Alteromonas macleodii*	*katG*	positive	Contig_101_46.6864	22130	24502	+
MMSF00404	*Alteromonas oceani*	*katG*	positive x2 (Copy 1)	Contig_26_39.8992	70873	73035	-
MMSF00404	*Alteromonas oceani*	*katG*	positive x2 (Copy 2)	Contig_52_41.1626	55994	58225	-
MMSF00910	*Winogradskyella poriferorum*	*katG*	positive	Contig_39_101.483	25621	27852	+
MMSF00958	*Alteromonas macleodii*	*katG*	positive	Contig_21_66.072	22086	24458	+
MMSF00964	*Marinobacter salsuginis*	*katG*	positive	Contig_25_36.8412	50391	52565	-
MMSF01163	*Alteromonas oceani*	*katG*	positive x2 (Copy 1)	Contig_46_63.2119	54248	56479	-
MMSF01163	*Alteromonas oceani*	*katG*	positive x2 (Copy 2)	Contig_86_59.9301	369720	371882	-
MMSF01190	*Marinobacter salsuginis*	*katG*	positive	Contig_22_84.8909	7780	9954	+
MMSF00132	*Labrenzia aggregata*	*cobA*	positive	Contig_6_57.2063	552920	553741	-

MMSF00249	*Labrenzia aggregata*	*cobA*	positive	Contig_17_46.7174	819449	820270	-
MMSF00132	*Labrenzia aggregata*	*cobB*	positive	Contig_6_57.2063	551611	552930	-
MMSF00249	*Labrenzia aggregata*	*cobB*	positive	Contig_17_46.7174	818140	819459	-
MMSF00132	*Labrenzia aggregata*	*cobC*	positive	Contig_6_57.2063	842748	843392	-
MMSF00249	*Labrenzia aggregata*	*cobC*	positive	Contig_17_46.7174	1109277	1E+06	-
MMSF00132	*Labrenzia aggregata*	*cobD*	positive x2	Contig_26_56.6932	208207	209184	-
MMSF00132	*Labrenzia aggregata*	*cobD*	positive x2	Contig_26_56.6932	209194	210195	-
MMSF00249	*Labrenzia aggregata*	*cobD*	positive x2	Contig_15_47.504	84229	85230	+
MMSF00249	*Labrenzia aggregata*	*cobD*	positive x2	Contig_15_47.504	85240	86217	+
MMSF00132	*Labrenzia aggregata*	*cobH*	positive	Contig_6_57.2063	545294	545950	+
MMSF00249	*Labrenzia aggregata*	*cobH*	positive	Contig_17_46.7174	811823	812479	+
MMSF00132	*Labrenzia aggregata*	*cobI*	positive	Contig_6_57.2063	547369	548139	+
MMSF00249	*Labrenzia aggregata*	*cobI*	positive	Contig_17_46.7174	813898	814668	+
MMSF00132	*Labrenzia aggregata*	*cobK*	positive	Contig_6_57.2063	555025	555786	+
MMSF00249	*Labrenzia aggregata*	*cobK*	positive	Contig_17_46.7174	821554	822315	+
MMSF00132	*Labrenzia aggregata*	*cobL*	positive	Contig_6_57.2063	546203	547417	+
MMSF00249	*Labrenzia aggregata*	*cobL*	positive	Contig_17_46.7174	812732	813946	+
MMSF00132	*Labrenzia aggregata*	*cobM*	positive	Contig_6_57.2063	550028	550801	+
MMSF00249	*Labrenzia aggregata*	*cobM*	positive	Contig_17_46.7174	816557	817330	+
MMSF00132	*Labrenzia aggregata*	*cobN*	positive	Contig_26_56.6932	201965	205723	+
MMSF00249	*Labrenzia aggregata*	*cobN*	positive	Contig_15_47.504	88701	92459	-
MMSF00132	*Labrenzia aggregata*	*cobO*	positive	Contig_26_56.6932	205720	206358	+
MMSF00249	*Labrenzia aggregata*	*cobO*	positive	Contig_15_47.504	88066	88704	-
MMSF00964	*Marinobacter salsuginis*	*cobO*	positive	Contig_25_36.8412	168373	168930	+
MMSF01190	*Marinobacter salsuginis*	*cobO*	positive	Contig_21_84.3861	85977	86534	+
MMSF00132	*Labrenzia aggregata*	*cobP*	positive	Contig_26_56.6932	200255	200806	+
MMSF00249	*Labrenzia aggregata*	*cobP*	positive	Contig_15_47.504	93618	94169	-
MMSF00132	*Labrenzia aggregata*	*cobQ*	positive	Contig_26_56.6932	206715	208205	+
MMSF00249	*Labrenzia aggregata*	*cobQ*	positive	Contig_15_47.504	86219	87709	-

MMSF00132	*Labrenzia aggregata*	*cobU*	positive	Contig_24_56.1446	78969	79994	+
MMSF00249	*Labrenzia aggregata*	*cobU*	positive	Contig_13_45.8339	40345	41370	+
MMSF00132	*Labrenzia aggregata*	*cobV*	positive	Contig_24_56.1446	77990	78862	-
MMSF00249	*Labrenzia aggregata*	*cobV*	positive	Contig_13_45.8339	39366	40238	-
MMSF00132	*Labrenzia aggregata*	*cbiD*	positive	Contig_6_57.2063	553941	555035	+
MMSF00249	*Labrenzia aggregata*	*cbiD*	positive	Contig_17_46.7174	820470	821564	+
MMSF00964	*Marinobacter salsuginis*	*cbiO*	positive	Contig_36_38.8121	420124	420720	+

742 **Table S4:** Summary of vitamin B_{12} biosynthesis pathway genes. A "+" indicates the presence of the gene in the respective isolate,
743 whereas a "-" represents the absence of that gene. Genes in red were not found in any isolate.

Isolate	Genus Level Classification	CobP as indicator	Gene Count	*CobA*	*CobB*	*CobC*	*CobD*	*CobE*	*CobF*	*CobG*
MMSF01163	*Alteromonas*	-	0	-	-	-	-	-	-	-
MMSF00257	*Alteromonas*	-	0	-	-	-	-	-	-	-
MMSF00958	*Alteromonas*	-	0	-	-	-	-	-	-	-
MMSF00404	*Alteromonas*	-	0	-	-	-	-	-	-	-
MMSF00132	*Labrenzia*	+	18	**+**	**+**	**+**	**+ x2**	-	-	-
MMSF00249	*Labrenzia*	+	18	**+**	**+**	**+**	**+ x2**	-	-	-
MMSF00964	*Marinobacter*	-	2	-	-	-	-	-	-	-
MMSF01190	*Marinobacter*	-	1	-	-	-	-	-	-	-
MMSF00068	*Micrococcus*	-	0	-	-	-	-	-	-	-
MMSF00107	*Micrococcus*	-	0	-	-	-	-	-	-	-
MMSF00910	*Winogradskyella*	-	0	-	-	-	-	-	-	-
MMSF00046	*Winogradskyella*	-	0	-	-	-	-	-	-	-

744

Isolate	Genus Level Classification	*CobH*	*CobI*	*CobJ*	*CobK*	*CobL*	*CobM*	*CobN*	*CobO*	*CobP*	*CobQ*
MMSF01163	*Alteromonas*	-	-	-	-	-	-	-	-	-	-
MMSF00257	*Alteromonas*	-	-	-	-	-	-	-	-	-	-
MMSF00958	*Alteromonas*	-	-	-	-	-	-	-	-	-	-
MMSF00404	*Alteromonas*	-	-	-	-	-	-	-	-	-	-
MMSF00132	*Labrenzia*	**+**	**+**	-	**+**	**+**	**+**	**+**	**+**	**+**	**+**
MMSF00249	*Labrenzia*	**+**	**+**	-	**+**	**+**	**+**	**+**	**+**	**+**	**+**
MMSF00964	*Marinobacter*	-	-	-	-	-	-	-	**+**	-	-
MMSF01190	*Marinobacter*	-	-	-	-	-	-	-	**+**	-	-
MMSF00068	*Micrococcus*	-	-	-	-	-	-	-	-	-	-

MMSF00107	*Micrococcus*	-	-	-	-	-	-	-	-	-	-
MMSF00910	*Winogradskyella*	-	-	-	-	-	-	-	-	-	-
MMSF00046	*Winogradskyella*	-	-	-	-	-	-	-	-	-	-

745

Isolate	**Genus Level Classification**	***CobP***	***CobQ***	***CobR***	***CobS***	***CobT***	***CobU***	***CobV***	***CobW***	***CobX***	***CobY***	***CobZ***	***Cbi***
MMSF01163	*Alteromonas*	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00257	*Alteromonas*	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00958	*Alteromonas*	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00404	*Alteromonas*	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00132	*Labrenzia*	**+**	**+**	-	-	-	**+**	**+**	-	-	-	-	**cbiD**
MMSF00249	*Labrenzia*	**+**	**+**	-	-	-	**+**	**+**	-	-	-	-	**cbiD**
MMSF00964	*Marinobacter*	-	-	-	-	-	-	-	-	-	-	-	**cbiO**
MMSF01190	*Marinobacter*	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00068	*Micrococcus*	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00107	*Micrococcus*	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00910	*Winogradskyella*	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00046	*Winogradskyella*	-	-	-	-	-	-	-	-	-	-	-	-

746

747 **Table S5:** Composition of R2A broth adjusted to suit marine bacteria. Final pH = 7.2 +/- 0.2 at
748 26 °C. R2A broth was made by suspending 43.12 g of combined reagents in 1 L of MilliQ water,
749 dissolving the medium completely, and sterilization by autoclaving at 121˚C for 15 min.

Component	**grams L^{-1}**	**Supplier**
Casein acid hydrolysate	0.500	Cat#C0501, Sigma-Aldrich
Yeast extract	0.500	Cat#LP0021, Oxoid
Proteose peptone	0.500	Cat#211684, ThermoFisher
Dextrose	0.500	Cat#G360, PhytoTech Laboratories
Starch, soluble	0.500	Cat#AJA526, Univar
Dipotassium phosphate	0.300	Cat#P3786, Sigma-Aldrich
Magnesium sulfate	0.024	Cat#M2643, Sigma-Aldrich
Sodium pyruvate	0.300	Cat#P2256, Sigma-Aldrich
Red Sea Salt™	40.00	Cat#R11065, Red Sea

750

751 **Table S6:** Single-copy housekeeping genes extracted from the RAST annotations

Gene	Function	NCBI
16S	SSU rRNA ## 16S rRNA, small subunit ribosomal RNA	16S ribosomal RNA
23S	LSU rRNA ## 23S rRNA, large subunit ribosomal RNA	23S ribosomal RNA
fusA	Translation elongation factor G	elongation factor G
gyrB	DNA gyrase subunit B (EC 5.99.1.3)	DNA gyrase subunit B
gyrA	DNA gyrase subunit A (EC 5.99.1.3)	DNA gyrase subunit A
pyrG	CTP synthase (EC 6.3.4.2)	CTP synthetase
lepA	Translation elongation factor LepA	GTP-binding protein LepA
recA	RecA protein	recA protein
recG	ATP-dependent DNA helicase RecG (EC 3.6.4.12)	ATP-dependent DNA helicase RecG
rpoB	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6)	DNA-directed RNA polymerase subunit beta
rpoD	RNA polymerase sigma factor RpoD	RNA polymerase sigma factor RpoD
atpD	ATP synthase beta chain (EC 3.6.3.14)	F0F1 ATP synthase subunit beta
ppK	Polyphosphate kinase (EC 2.7.4.1)	polyphosphate kinase
polA	DNA polymerase I (EC 2.7.7.7)	DNA polymerase I
pheSa	Phenylalanyl-tRNA synthetase alpha chain (EC 6.1.1.20)	phenylalanyl-tRNA synthetase subunit alpha
pheSb	Phenylalanyl-tRNA synthetase beta chain (EC 6.1.1.20)	phenylalanyl-tRNA synthetase subunit beta
ileS	Isoleucyl-tRNA synthetase (EC 6.1.1.5)	isoleucyl-tRNA synthetase
leuS	Leucyl-tRNA synthetase (EC 6.1.1.4)	leucyl-tRNA synthetase
aspS	Aspartyl-tRNA synthetase (EC 6.1.1.12)	aspartyl-tRNA synthetase
alaS	Alanyl-tRNA synthetase (EC 6.1.1.7)	alanyl-tRNA synthetase
argS	Arginyl-tRNA synthetase (EC 6.1.1.19)	arginyl-tRNA synthetase
hisS	Histidyl-tRNA synthetase (EC 6.1.1.21)	histidyl-tRNA synthetase
valS	Valyl-tRNA synthetase (EC 6.1.1.9)	valyl-tRNA synthetase
srp1	SSU ribosomal protein S1p	30S ribosomal protein S1
srp2	SSU ribosomal protein S2p (SAe)	30S ribosomal protein S2
srp3	SSU ribosomal protein S3p (S3e)	30S ribosomal protein S3
srp4	SSU ribosomal protein S4p (S9e)	30S ribosomal protein S4
srp5	SSU ribosomal protein S5p (S2e)	30S ribosomal protein S5
srp6	SSU ribosomal protein S6p	30S ribosomal protein S6
srp7	SSU ribosomal protein S7p (S5e)	30S ribosomal protein S7
srp8	SSU ribosomal protein S8p (S15Ae)	30S ribosomal protein S8
srp9	SSU ribosomal protein S9p (S16e)	30S ribosomal protein S9
srp10	SSU ribosomal protein S10p (S20e)	30S ribosomal protein S10

srp11	SSU ribosomal protein S11p (S14e)	30S ribosomal protein S11
srp12	SSU ribosomal protein S12p (S23e)	30S ribosomal protein S12
srp13	SSU ribosomal protein S13p (S18e)	30S ribosomal protein S13
srp14	SSU ribosomal protein S14p (S29e) @ SSU ribosomal protein S14p (S29e), zinc-independent	30S ribosomal protein S14
srp15	SSU ribosomal protein S15p (S13e)	30S ribosomal protein S15
srp16	SSU ribosomal protein S16p	30S ribosomal protein S16
srp17	SSU ribosomal protein S17p (S11e)	30S ribosomal protein S17
srp18	SSU ribosomal protein S18p @ SSU ribosomal protein S18p, zinc-independent	30S ribosomal protein S18
srp19	SSU ribosomal protein S19p (S15e)	30S ribosomal protein S19
srp20	SSU ribosomal protein S20p	30S ribosomal protein S20
srp21	SSU ribosomal protein S21p	30S ribosomal protein S21
lrp1	LSU ribosomal protein L1p (L10Ae)	50S ribosomal protein L1
lrp2	LSU ribosomal protein L2p (L8e)	50S ribosomal protein L2
lrp3	LSU ribosomal protein L3p (L3e)	50S ribosomal protein L3
lrp4	LSU ribosomal protein L4p (L1e)	50S ribosomal protein L4
lrp5	LSU ribosomal protein L5p (L11e)	50S ribosomal protein L5
lrp6	LSU ribosomal protein L6p (L9e)	50S ribosomal protein L6
lrp7	LSU ribosomal protein L7/L12 (P1/P2)	50S ribosomal protein L7
lrp8	LSU ribosomal protein L8p	50S ribosomal protein L8
lrp9	LSU ribosomal protein L9p	50S ribosomal protein L9
lrp10	LSU ribosomal protein L10p (P0)	50S ribosomal protein L10
lrp11	LSU ribosomal protein L11p (L12e)	50S ribosomal protein L11
lrp12	LSU ribosomal protein L12p	50S ribosomal protein L12
lrp13	LSU ribosomal protein L13p (L13Ae)	50S ribosomal protein L13
lrp14	LSU ribosomal protein L14p (L23e)	50S ribosomal protein L14
lrp15	LSU ribosomal protein L15p (L27Ae)	50S ribosomal protein L15
lrp16	LSU ribosomal protein L16p (L10e)	50S ribosomal protein L16
lrp17	LSU ribosomal protein L17p	50S ribosomal protein L17
lrp18	LSU ribosomal protein L18p (L5e)	50S ribosomal protein L18
lrp19	LSU ribosomal protein L19p	50S ribosomal protein L19
lrp20	LSU ribosomal protein L20p	50S ribosomal protein L20
lrp21	LSU ribosomal protein L21p	50S ribosomal protein L21
lrp22	LSU ribosomal protein L22p (L17e)	50S ribosomal protein L22
lrp23	LSU ribosomal protein L23p (L23Ae)	50S ribosomal protein L23
lrp24	LSU ribosomal protein L24p (L26e)	50S ribosomal protein L24
lrp25	LSU ribosomal protein L25p	50S ribosomal protein L25
lrp26	LSU ribosomal protein L26p	50S ribosomal protein L26
lrp27	LSU ribosomal protein L27p	50S ribosomal protein L27
lrp28	LSU ribosomal protein L28p	50S ribosomal protein L28

lrp29	LSU ribosomal protein L29p (L35e)	50S ribosomal protein L29
lrp30	LSU ribosomal protein L30p (L7e)	50S ribosomal protein L30
lrp31	LSU ribosomal protein L31p @ LSU ribosomal protein L31p, zinc-independent	50S ribosomal protein L31

752