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 carbon. During thermal stress, the algae's photosystems are impaired, resulting in a toxic accumulation of reactive oxygen species (ROS) that cause cellular damage to both the host and symbiont. As a protective mechanism the coral host and algal symbiont disassociate; 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 explore genes of interest that may contribute to the potential beneficial roles of these putative probiotic members, which may help facilitate the therapeutic application of a bacterial probiotic. Probiotics is one of several interventions currently being developed with the aim of augmenting climate resilience in corals and increasing the likelihood of coral reef persistence into the future.

IMPORTANCE

Coral bleaching is tightly linked to the production of reactive oxygen species (ROS), whereby ROS accumulates to a toxic level in host-harboring algae cells leading to coral-algal dysbiosis. Interventions targeting toxic 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 low, we have applied bioengineering strategies to develop a probiotic to neutralize toxic ROS during a thermal stress event. This probiotic can then be tested with corals or a coral model to assess its efficacy in improving coral resistance to environmental stress.

46 **INTRODUCTION**

47 Coral reefs are among the most biologically and economically productive ecosystems on Earth
48 [1, 2]. Though they make up less than 0.1% of the ocean floor [3], coral reefs support fisheries,
49 tourism, pharmaceuticals and coastal development with a global value of $8.9 trillion
50 "international $"/year [4]. Corals and other reef organisms have been dying, largely due to
51 anthropogenic influences such as climate change[5, 6], which has led to an increased
52 frequency, intensity and duration of summer heat waves that cause coral bleaching [7, 8].

53 The coral holobiont [the sum of the coral animal and its symbiotic partners, including
54 intracellular algae, endolithic algae, fungi, protozoans, bacteria, archaea and viruses; 9] is an
55 ecosystem engineer. By secreting a calcium carbonate skeleton, the reef structure rises from
56 the ocean floor, forming the literal foundation of the coral reef ecosystem. The success of
57 corals to survive and build up reefs over thousands of years [10] is tightly linked to their
58 obligate yet fragile symbioses with endosymbiotic dinoflagellates of the family *Symbiodiniaceae*
59 [11].

60 Intracellular *Symbiodiniaceae* translocate photosynthetically fixed carbon to the coral host [12]
61 in exchange for inorganic nitrogen, phosphorus and carbon and location in a high light
62 environment with protection from herbivory [13, 14]. During periods of intense thermal stress,
63 the relationship between the coral host and their *Symbiodiniaceae* breaks down, resulting in a
64 separation of the partners and a state of dysbiosis. This phenomenon, 'coral bleaching', is
65 devastating to the host and detrimental to the reef system. Debilitating effects of bleaching on
66 the coral include reduced skeletal growth and reproductive activity, a lowered capacity to shed
67 sediments, and an inability to resist invasion of competing species and diseases. Severe and
68 prolonged bleaching can cause partial to total colony death, resulting in diminished reef
69 growth, the transformation of reef-building communities to alternate, non-reef building
70 community types, bioerosion and ultimately the disappearance of reef structures [11].

71 Although there are several hypotheses detailing the mechanisms driving bleaching [see 15, 16-
72 18], a common theme is the overproduction and toxic accumulation of reactive oxygen species

73 (ROS) from the algal symbiont. Excess ROS are generated by a number pathways including heat
74 damage to both photosynthetic and mitochondrial membranes [19, 20], and are shown to play
75 a central role in injury to both symbiotic partners and to inter-partner communication of a
76 stress response [15]. Once generated, ROS causes damage to many cell components including
77 photosystem II (PSII) reaction centers in the *Symbiodiniaceae*, specifically at the D1 and D2
78 proteins , [see review in 21]. Exposure to elevated temperatures [22] can result in
79 photoinhibition of photosynthesis in *Symbiodiniaceae*. Once damaged, *Symbiodiniaceae* are no
80 longer able to maintain their role in the symbiotic relationship with corals and separate from
81 the host tissue via *in situ* degradation, exocytosis, host cell detachment, host cell apoptosis or
82 host cell necrosis [15].

83 Probiotics are preparations of viable microorganisms that are introduced to alter a microbial
84 community in a way that is beneficial to the host. Microbiome engineering through the addition
85 of probiotics has been postulated as a key strategy to manipulate host phenotypes and
86 ecosystem functioning for coral reefs [23-28]. The differences in the bacterial species
87 composition of healthy and thermally stressed corals [29-34] and the coral model *Exaiptasia*
88 *diaphana* [35-37] suggest a role for microbiome engineering in cnidarian health. A disruption to
89 the bacterial community of *Pocillopora damicornis* with antibiotic treatment diminished the
90 resilience of the holobiont during thermal stress, whereas intact microbial communities
91 conferred resilience to thermal stress and increased the rate of recovery after bleaching events
92 to the coral holobiont [38]. The relative stability of coral-associated bacterial communities have
93 also been linked to coral heat tolerance; the bacterial community of heat sensitive *Acropora*
94 *hyacinthus* corals shifted when transplanted to thermal stress conditions, whereas heat-
95 tolerant *A. hyacinthus* corals harbored a stable bacterial community [39].

96 In recent years, researchers have begun to explore the use of probiotics in corals and the model
97 organism for corals, *E. diaphana*. To inhibit the progression of white pox disease in *E. diaphana*,
98 caused by the pathogen *Serratia marcescens*, an *Alphaproteobacteria* cocktail containing
99 several *Marinobacter* spp. isolates was applied [40]. These strains were able to inhibit both
100 biofilm formation and swarming in *S. marcescens,* which halted disease progression in

101 *E. diaphana*. The probiotic was deemed effective as anemones exposed to both the cocktail and
102 pathogen survived after seven days, while anemones in the *S. marcescens* control experiment
103 died. A bacterial consortium native to the coral *Mussismilia harttii* was selected to degrade
104 water-soluble oil fractions[41]. This bioremediation strategy reduced the negative impacts of oil
105 on *M. harttii* health and accelerated the degradation of petroleum hydrocarbons [41]. Coral
106 microbiomes have also been manipulated to mitigate the effects of thermal stress. This
107 manipulation of the coral-associated microbiome was facilitated through addition of a
108 consortium of native or seawater derived bacteria to the surface of *P. damicornis* [42]. The
109 results from this study suggest the consortium was able to partially mitigate coral bleaching and
110 provides promising initial results in the field of coral probiotics.

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

118 **RESULTS**

119 **Diversity of culturable bacteria associated with *E. diaphana*.** A total of 842 isolates were
120 obtained from four genotypes of GBR-sourced *E. diaphana*, with no significant differences in
121 bacterial colony forming units (CFUs) between the four genotypes, regardless of growth
122 medium, with (mean±SE) 5.9-10.3 x 10^3 cells per anemone on Reasoner's 2A agar (R2A) and 6.3-
123 10.4 x10^3 cells per anemone on marine agar (MA) ($p>0.05$). Partial 16S rRNA gene sequences
124 (~1000 bp) were used to identify the closest matches from the NCBI database using BLASTn. In
125 total there were 109 species in 64 genera, 27 families and six phyla (Fig. 1). The most abundant
126 genera were *Alteromonas, Labrenzia*, and *Ruegeria* (Table 1). Gram-positive bacteria comprised
127 23 species, including *Microbacterium* (31 isolates) and *Micrococcus* (28 isolates). Eight genera
128 were found to be associated with all four genotypes (Table 1); these eight genera made up
129 59.4% of all *E. diaphana*-associated bacterial isolates.

130

131 **Table 1:** Details of cultured bacteria associated with all four genotypes of GBR-sourced
132 *E. diaphana* (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

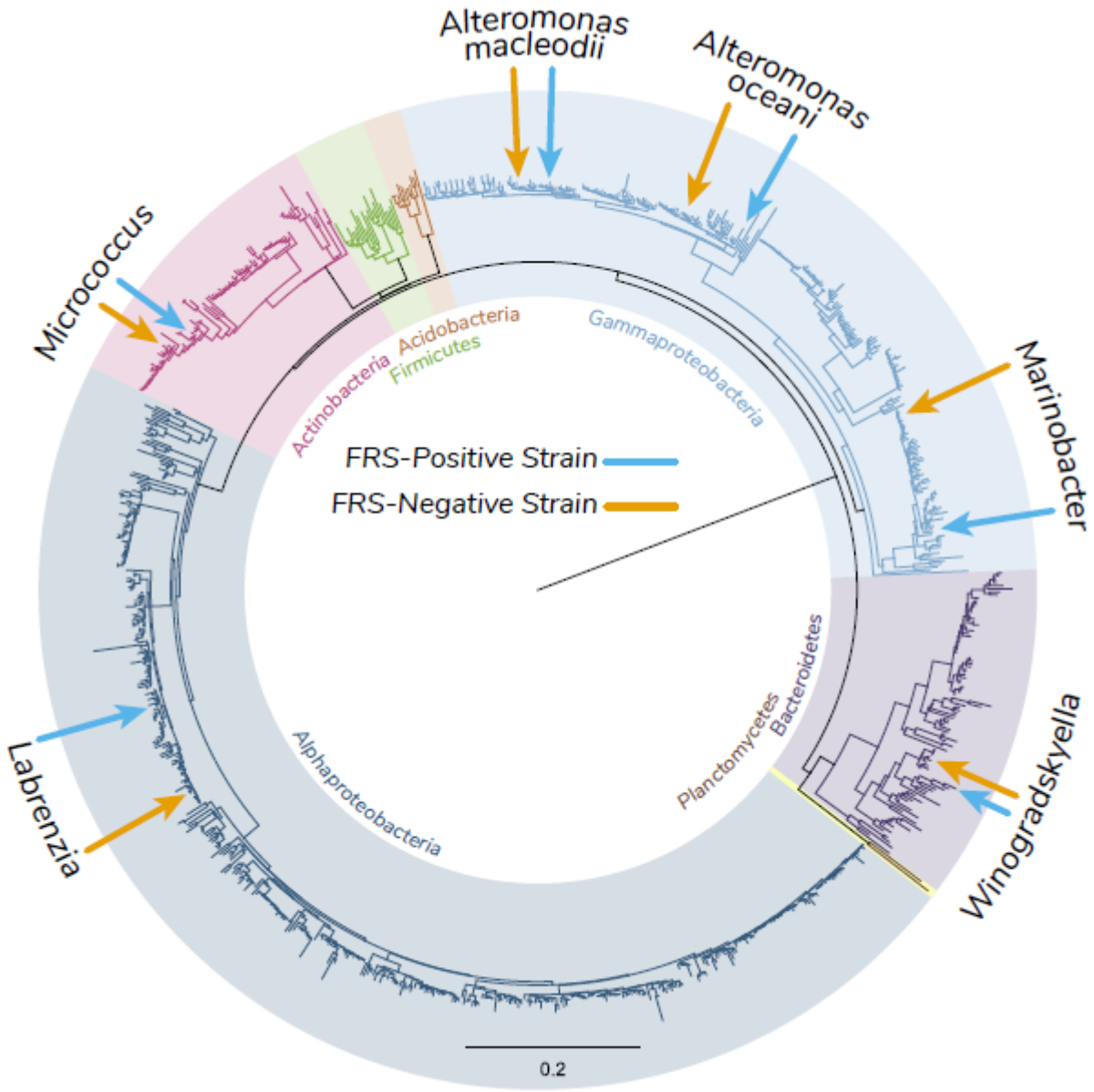
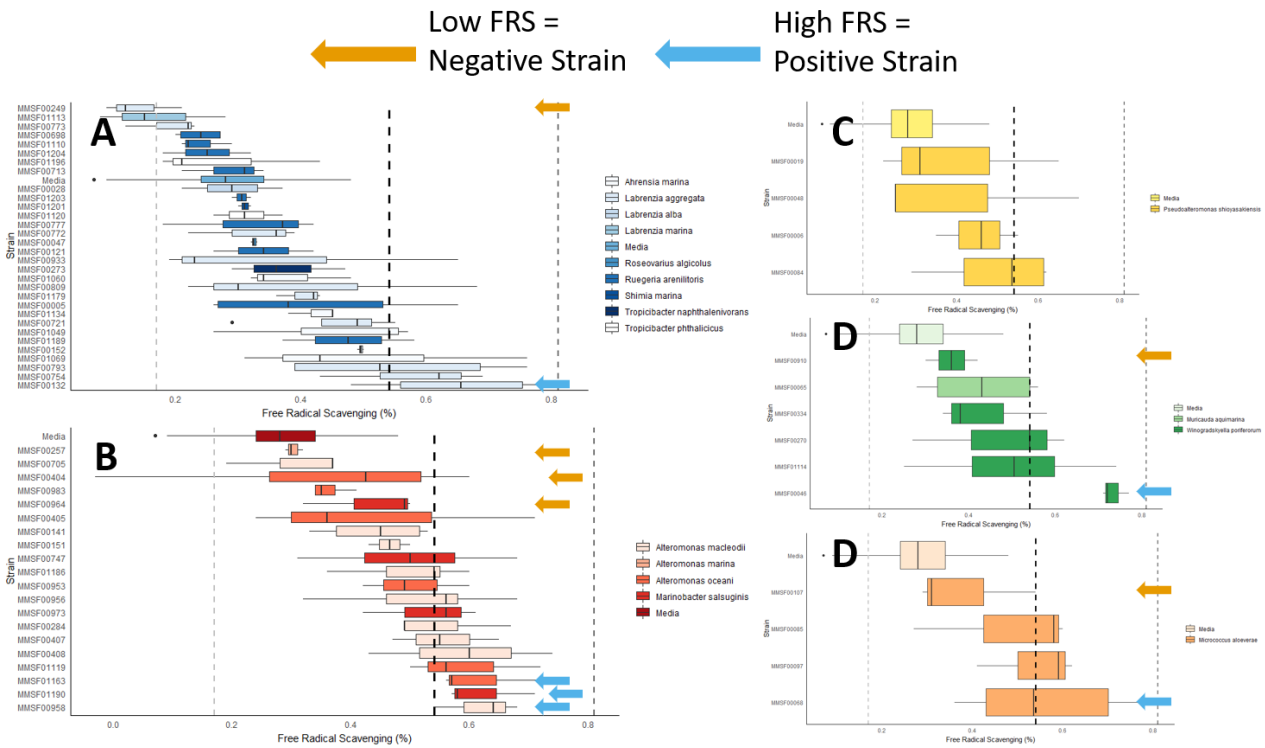


FIG 1: Neighbor-Joining phylogenetic tree showing the diversity and relatedness of 842 *E. diaphana*-associated bacterial isolates inferred using partial 16S rRNA sequences. These isolates covered six phyla indicated by shading over the tree with *Proteobacteria* split into the classes *Gamma* and *Alphaproteobacteria*. 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.

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



151

152 **FIG 2:** Quantitative FRS ability of *E. diaphana*-associated bacteria isolates, separated by Family. Families with high relative
153 abundance among all cultured bacteria (*Rhodobacteraceae* – A, *Alteromondaceae* – B, *Pseudoalteromonadaceae* – C,
154 *Flavobacteraceae* – D, and *Micrococcaceae* – E) were separately analyzed to identify strains with a high FRS ability (blue arrows) and
155 a corresponding strain of the same species* with a low FRS ability (red arrows). *The two strains selected from *Micrococcaceae*
156 belonged to the Genus *Micrococcus* but were not the same species. In each panel, the light dashed vertical line on the left
157 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%
158 (w/v) ascorbic acid standard, and the far right dashed line is the mean FRS of the 0.075% (w/v) ascorbic acid standard.

159 **Table 2:** Details on probiotic strains. Mean media FRS (% ± SE) was 27.2 ± 2.3% (n=12) and was catalase negative. All sequence data
160 can be found under BioProject# PRJNA574193. References to each probiotic candidate at the Genus level are identified in the last
161 three columns.

Strain	Bacteria species	FRS (% ± SE)	*Positive VS Negative	*Strain VS Growth Medium	Catalase	*Exaiptasia* Literature	Coral Literature	Probiotic Literature
MMSF01163 (n=3)	*Alteromonas oceani* (Gamma-proteobacteria)	61.7 ± 5.2	0.065	**0.004**	Negative	[43-45]	[25, 46-48]	[49-52]
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 [53]* (Alpha-proteobacteria)	53.6 ± 8.7	**0.016**	0.101	Negative	[44]	[33, 48]	[54]
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	[43, 44, 55]	[47, 56, 57]	[40]
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	[44]	[58]	[49, 59, 60]
MMSF00107 (n=3)	*Micrococcus yunnanensis* (Actinobacteria)	38.0 ± 8.0		0.306	Positive			

MMSF00046 (n=3)	*Winogradskyella poriferorum [61]* (Bacteroidetes)	73.3 ± 1.9	0.284	**0.010**	Negative	^	[41, 62]	NA
MMSF00910 (n=3)		36.0 ± 3.5		0.174	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 *[36, 37, 43, 45]*.

*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.

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

178 Direct pairwise comparison of the genome sequences between the pairs of isolates revealed a
179 wide range of genome variation, ranging from ~190,000 single nucleotide polymorphisms (SNP)
180 differences between the *Alteromonas oceani* strains, to fewer than five SNPs between the
181 genomes of *Labrenzia aggregata* (Table S2). The genome sequences of *Winogradskyella*
182 *poriferorum* isolates MMSF00910 and MMSF00046 are nearly identical with fewer than ten
183 pairwise core SNP differences; but there were some accessory genome differences.

184 **Genes of interest.** The annotated genome sequences of each selected probiotic member were
185 searched for key genes of interest (Table S3-S4). Dimethylsulfoniopropionate (DMSP) cleavage
186 to dimethylsulfide (DMS) was identified by presence of one or more of the DMSP lyase genes;
187 *dddP*, *dddD*, *dddL*, *dddW*, and *dddQ*. Only *L. aggregata* strains contained DMSP lyase genes
188 (*dddP* and *dddL*) in their whole genome sequences (WGS). DMSP biosynthesis was identified by
189 the presence of *dsyB*, which is the only described gene for an enzyme in the DMSP biosynthesis
190 pathway. The presence of *dsyB* in both *L. aggregata* isolates suggests that they have the
191 capacity to produce DMSP. *cobP* was used as an indicator gene for the presence of the dynamic
192 vitamin B_{12} pathway, which contains 27 genes (Table S4). Again, only the *L. aggregata* isolates
193 contained *cobP*. Catalase positive strains were identified by the presence of *katG*; all positive

194 and negative FRS strains contained *katG* except the *Micrococcus* spp. strains, in which *katA* and
195 *katE* were 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 close between each other within pairs of isolates,
199 in which the pair of isolates MMSF00257 and MMSF00958 contained the most copies (5.15 and
200 4.79, respectively) and the isolates MMSF00046 and MMSF00910 contained the fewest copies
201 (1.03 and 0.77, respectively).

202 **DISCUSSION**

203 The 842 *E. diaphana* bacterial isolates reported here and based on 16S rRNA gene-based
204 taxonomic classification comprise 109 species from 64 genera and six phyla. Using
205 metabarcoding, studies of microbiomes associated with *E. diaphana* have revealed a similar
206 diversity at the phylum level for *E. diaphana* sourced from the GBR [35], Hawaii [strain H2; 43],
207 Pacific and Caribbean [55], Atlantic [strain CC7; 45] and Red Sea [36], as well as stony corals
208 (see a review by Blackall, Wilson [63]). Thus, our culture collection of *E. diaphana* bacterial
209 isolates suitably represents the diversity of the *E. diaphana*-associated microbiome. Though
210 previous studies have used only a narrow bacterial species diversity to develop a probiotic [40,
211 42], our culture collection is sufficiently diverse to better select potential probiotics.

212 Inoculated bacteria can be acquired by developing coral larvae [25] and probiotic mixes applied
213 to cnidarians have been shown to be effective in inhibiting disease progression [40] and
214 increasing resistance to the negative effects of oil [41] and heat exposure [42]. The *E. diaphana*
215 probiotics generated in this study were assembled from 12 isolated bacterial strains selected
216 based on their FRS ability since free radical production, specifically ROS, is relevant in coral
217 bleaching. The broader culture collection contains bacteria with a wide range of FRS capacity.

218 The consistent and frequent reporting of our probiotic bacteria genera in *E. diaphana* and coral
219 studies (see Table 2) suggests these bacteria may have key functions in cnidarian holobionts.

220 Among these potential functions are the production of antioxidants such as DMSP, the
221 breakdown of DMSP to other antioxidants (dimethyl sulfide (DMS), acrylate, dimethyl sulfoxide
222 (DMSO), and methane sulfinic acid (MSA) [64]. *L. aggregata* has been reported to produce
223 DMSP in the absence of any methylated sulfur compounds with *dsyB* identified as the first
224 DMSP biosynthesis gene in any organism [65]. *dsyB* was found in the whole genome sequences
225 of both high and low FRS *L. aggregata* strains (Table S3). Many *E. diaphana*-sourced bacterial
226 species, specifically relatives of our selected probiotic members, are implicated in the
227 degradation of DMSP to DMS (*Alteromonas* spp., [66]; *Labrenzia* spp., [67]). *dddP* codes for the
228 enzyme responsible for cleaving DMSP to DMS and acrylate and was used (from the Prokka
229 annotation) as an indicator of a DMSP degradation genotype. Only the *L. aggregata* isolates
230 were found to be able to degrade DMSP (Table S3).

231 Carotenoids are among the strongest antioxidants and are highly reactive against both reactive
232 oxygen species and free radicals [68-72]. Carotenoids are lipid-soluble pigments, and in bacteria
233 they give an orange-yellow hue to colonies. Two of the five selected probiotic genera produce
234 orange/yellow colonies (*Winogradskyella*, *Micrococcus*), and there is evidence of carotenoid
235 production by marine *Flavobacteriaceae* [72, 73] and *Micrococcus* strains [74]. A marine
236 *Flavobacteriaceae* (strain GF1) was found to produce the potent antioxidant carotenoid
237 zeaxanthin that protected *Symbiodiniaceae* from thermal and light stress [75].

238 Vitamin B_{12} is a cofactor involved in the production of the amino acid methionine, which is
239 needed to synthesize every protein and in diverse metabolic pathways including generation of
240 the antioxidants glutathione and DMSP [76]. Vitamin B_{12} is synthesized by many heterotrophic
241 bacteria [77]. Genomic evidence suggests that *Symbiodiniaceae* may have lost the capacity to
242 synthesize vitamin B_{12} due to changes in their metabolic enzymes during their evolution [78],
243 agreeing with other work that free-living *Symbiodiniaceae* depend on bacterial symbionts to
244 gain access to this important cofactor [79]. The genes involved in the biosynthesis of vitamin B_{12}
245 have been found in coral-associated bacteria, specifically *L. aggregata* cultured from the
246 Caribbean coral, *Orbicella faveolata* [80].From the annotated draft genome sequence, using the
247 *cobP* gene [77] as the genotypic indicator, both *L. aggregata* isolates are capable of Vitamin B_{12}

248 biosynthesis. The *Marinobacter salsuginis* isolates lack this gene but have other detectable
249 vitamin B_{12} synthesis genes (Table S4).

250 Bacteria have developed highly specific mechanisms to protect themselves against oxidative
251 stress with enzymes such as catalase/peroxidase and superoxide dismutase (SOD), small
252 proteins like thioredoxin and glutaredoxin, and molecules such as glutathione (GSH) in
253 combination with glutathione peroxidase and glutathione reductase [see review 81]. It has
254 been suggested that increasing the *in hospite* concentration of catalase in the coral holobiont
255 by the application of a probiotic with catalase-positive organisms, could possibly minimize the
256 impact of thermal stress by neutralizing hydrogen peroxide [27]. While this is a justifiable
257 hypothesis worth investigating further, to our knowledge, no studies have shown that catalase
258 producing bacteria can reduce the concentration of ROS *in hospite*. Here we tested all probiotic
259 candidates for catalase production using a standard hydrogen peroxide assay [82]. Catalase
260 participates in cellular antioxidant defense by decomposing hydrogen peroxide; this
261 decomposition is enzymatically driven by two catalases (yielding H_2O and O_2): hydroperoxidase
262 I (HPI),which is present during aerobic growth and transcriptionally controlled at different
263 levels, and hydroperoxidase II (HPII), which is induced during stationary phase [83]. Most
264 phenotypically determined catalase positive strains (MMSF00249, *L. aggregata*; MMSF00257,
265 *A. macleodii*; MMSF00964, *M. salsuginis*) had homologs for the catalase-peroxidase gene, *katG*;
266 the *Micrococcus* spp. isolates did not (Table S3). However, *katG* homologs were found in all
267 strains that were phenotypically catalase negative, indicating that these genes may not be
268 active during culture. Given the inconsistency between the catalase and DPPH results (in many
269 cases high FRS isolates were catalase negative while the low FRS strain was catalase positive)
270 the catalase results were not used as a primary factor in selecting members of the positive and
271 negative probiotic.

272 Members of the selected probiotic consortium (e.g., *Marinobacter* spp. and *Winogradskyella*
273 spp.) have some described roles in coral/*E. diaphana* health. For example, coral-bleaching
274 events are often followed by disease outbreaks [84-86] such as white pox [87]. When
275 inoculated in the presence of the white pox pathogen, *S. marcescens*, *Marinobacter* strains

276 were able to inhibit the progression of the infection in *E. diaphana* [40]. In a bioremediation
277 study on coral and oil, *Winogradskyella* sp. showed a significant decrease in abundance during
278 oil treatment, which was correlated with a decrease in coral holobiont health measured via
279 maximum quantum yield [41]. The presence of this microbiome member could play a role in
280 protecting the *Symbiodiniaceae* photochemical ability during periods of stress.

281 A critical characteristic in the selection of probiotic members is the maintenance and
282 proliferation of the inoculated bacteria in the host over time [49] and their potential
283 transmission to the next generation. There is evidence that corals release bacteria with their
284 offspring such as *Alteromonas* [46], *Flavobacteriaceae* [46], *Rhodobacteraceae* [88], and
285 *Marinobacter* [56]. While the majority of broadcast spawning corals do not transfer their
286 bacterial symbionts with their gametes (vertical transfer) [89], the brooding coral *Porites*
287 *astreoides* transmits bacteria vertically to planulae with two bacterial taxa (*Roseobacter* clade-
288 associated bacteria and *Marinobacter* spp.) consistently and stably associating with juvenile *P.*
289 *astreoides* [56]. In addition to the potential antioxidant properties of *Marinobacter* [90], others
290 like *Roseobacter* spp. might be beneficial in facilitating larval settlement. If adult corals stably
291 associate with inoculated probiotic candidates like *Marinobacter, Alteromonas,* and
292 *Winogradskyella*, they may be passed on to offspring and thus have a long-term positive impact
293 on these individuals.

294 Interactions within the microbiota associated with marine holobionts are undoubtedly complex.
295 Results presented in this manuscript show that pure cultured bacteria from *E. diaphana* can
296 scavenge free radicals, albeit at a strain-specific rate. This suggests that the selection and
297 inoculation of these high FRS strains could be beneficial to the host under high oxidative stress
298 conditions, such as those that contribute to coral bleaching. Conspecific pairs of six bacteria
299 provide an opportunity to determine the genetic basis for measured phenotypic differences
300 between the pairs. An essential element of this future work will be to investigate the stability of
301 the phenotypic differences observed and this stability may be reflected in the nature of the
302 genetic differences between the pairs of strains. Where isolate pairs are distantly related, it is

303 unlikely that the genetic basis for the phenotypic difference will be identified using comparative
304 genomics.

305 The probiotic members were chosen from a highly diverse pool of *E. diaphana*-sourced
306 bacterial isolates. While the selected probiotic members are phylogenetically diverse,
307 potentially promising probiotic bacteria in the culture collection were omitted based on our
308 selection criteria. For example, *Ruegeria* spp., which were excluded based on the absence of a
309 high FRS strain, have the ability to breakdown DMSP and can participate in denitrification [80].
310 *Muricauda* isolates had high FRS abilities, but they did not grow consistently in the selected
311 medium and therefore were excluded from the consortium. Like *Ruegeria*, *Muricauda* also has
312 genes for denitrification [80], can oxidize DMS to produce DMSO [67], and produces potent
313 carotenoids [91] that can mitigate thermal and light stresses in *Symbiodiniaceae* cultures [75].
314 *Muricauda* will be involved in future probiotic evaluations.

315 At present there is no biological treatment that can minimize coral bleaching in the field.
316 Management priorities for coral reefs must move beyond documenting their declines and
317 toward investigating potential approaches for mitigating coral bleaching, such as the
318 application of coral probiotics. We believe that the application of a coral probiotic specifically
319 tailored to address coral bleaching by neutralizing ROS could provide hope for the future of
320 coral reefs. We also understand that this form of intervention may not work alone, but could
321 benefit by pairing with other strategies such as enhancing coral resistance and resilience using
322 other assisted evolution approaches such as assisted gene flow, hybridization and experimental
323 evolution of the algal symbionts [24, 26, 92-98].Climate warming will continue even with the
324 most drastic reductions in greenhouse gas emissions, thus, additional interventions such as
325 coral probiotics present an alternative that could lead to relief from coral bleaching in real time.

326 **MATERIALS AND METHODS**

327 **Isolation of bacterial isolates.** Great Barrier Reef (GBR) origin *E. diaphana* were maintained in
328 the laboratory at 26°C [99] and used to isolate probiotic candidates. Sixteen individuals from
329 each of four *E. diaphana* genotypes (AIMS1-4) were collected using sterile disposable pipets

330 and gently transferred to filter-sterilized (0.2 µm) reverse osmosis (RO) water reconstituted Red
331 Sea Salt™ (Red Sea; RSS) at ~34 parts per thousand (ppt) salinity (fRSS) and placed in the dark
332 for 30 min. This was done to remove some of the influence of the external seawater on the
333 bacterial community. After 30 min, each anemone was transferred to a sterile glass
334 homogenizer with 1 mL of fRSS. Each homogenate was used to prepare serial dilutions from 10^{-}
335 ^{1} to 10^{-4}. From each dilution, 50 µL was spread plated onto three replicate plates each of MA
336 (Difco™ Marine Agar 2216) and R2A (CM0906, Oxoid) supplemented with 40 g L^{-1} RSS and
337 incubated at 26˚C. After one week, CFU counts were completed. Individual bacterial isolates
338 were sub-cultured to purification from plates with <100 CFUs onto the initial isolation medium.
339 All purified bacterial isolates were resuspended in 40% glycerol, aliquoted into 1.2 mL
340 cryotubes and stored at -80°C.

341 **Identification of *Exaiptasia*-sourced isolates.** Colony PCR with the universal bacterial primers
342 27f (5' – AGA GTT TGA TCM TGG CTC AG – 3') and 1492r (5' – TAC GGY TAC CTT GTT ACG ACT T
343 – 3') [100] was used to generate 16S rRNA gene amplicons from each isolate. Briefly, cells from
344 each pure culture were suspended in 20 µL Milli-Q water and denatured at 95°C for 10 min. The
345 suspension was then centrifuged at 2,000 x *g* at 4°C for two minutes and the supernatant was
346 used as the DNA template for PCR amplification. The PCR was performed with 20 µL Mango
347 Mix™ (Bioline), 0.25 µM of each primer and 2 µL of DNA template in a final volume of 40 µL.
348 The thermal cycling protocol was as follows: 95°C for 5 min; 35 cycles of 95°C for 1 min, 50 °C
349 for 1 min, and 72°C for 1 min; and a final extension of 10 min at 72°C. Amplicons were purified
350 and Sanger sequenced on an ABI sequencing instrument by Macrogen Inc. (Seoul, South Korea)
351 or by the Australian Genome Research Facility (AGRF) using the 1492r primer. Trimmed high
352 quality read data from each isolate was used for presumptive identification by querying the 16S
353 rRNA gene sequences via the Basic Local Alignment Search Tool (BLASTn). For some isolates the
354 near-complete 16S rRNA gene sequence was determined by sequencing with additional primers
355 (27f, 357f (5'-CCT ACG GGA GGC AGC AG-3', [101]), 926f (CCG TCA ATT CMT TTR AGT TT, [102]),
356 519r (5'-GWA TTA CCG CGG CKG CTG-3', [101]), 926r (5'–AAA CTR AAA MGA ATT GAC GG–3',
357 [102]), and 1492r). The six reads for each isolate were aligned using Geneious Prime 2019.1.2
358 (https://www.geneious.com) via the Geneious global alignment default settings with automatic

359 determination of read direction. From this alignment, a consensus sequence for the 16S rRNA
360 gene was constructed based on the frequency of a base and its quality (from chromatogram
361 data) in each alignment column. The consensus sequence length for each of the six isolate pairs
362 varied from 1352 to 1495 nucleotides. GenBank accession numbers for sequences are shown in
363 Table 1.

364 **Qualitative free radical scavenging assay.** DPPH is a stable free radical that is purple in its
365 oxidized state but becomes white-yellow when reduced by antioxidants, and has been used to
366 identify FRS marine bacteria [103, 104]. To qualitatively assess *E. diaphana*-associated bacteria
367 isolates for FRS ability, a sterile Whatman #1 filter paper was gently pressed against fresh (2-4
368 days old) colonies from a streak plate. Plates (with filter paper) were then incubated overnight
369 at 26°C. The following day, filter papers were removed with forceps, allowed to dry in a fume
370 hood for 30 min, and 500 µL of a 0.2 mM DPPH (Cat# D9132, Sigma-Aldrich) solution in
371 methanol was applied with a pipette over individual colonies. As a positive control, a few drops
372 of 0.1% (w/v) L-ascorbic acid (Cat# A7631, Sigma-Aldrich) were placed on a separate filter. The
373 response of each isolate to DPPH was recorded within 3 min of DPPH application; a positive
374 response was recorded when a white-yellow halo appeared around individual colonies within 1
375 min, a weak positive response was assigned to strains that had a halo form between 1 and 3
376 min after DPPH application, and a negative response was listed for strains that failed to form a
377 halo (Fig. 3). Approximately 700 isolates were screened using the qualitative DPPH assay.

378 A.

B.

379 **FIG 3:** DPPH is a stable free radical that is purple in its oxidized state. When reduced by an
380 antioxidant, a white-yellow halo will appear around individual bacteria colonies (A), this was

381 qualitatively deemed a positive response. Isolates that did not have a halo around colonies
382 within 3 min of DPPH application were deemed negative (B).

383 **Quantitative free radical scavenging assay.** To quantitatively assess the FRS ability, select
384 isolates were grown in R2A broth (see Table S5 for composition) made by suspending 43.12 g in
385 1 L of MilliQ water, dissolving the medium completely, and sterilization by autoclaving at 121˚C
386 for 15 min. Fifty mL aliquots of autoclaved medium were distributed into sterile 250 mL
387 Erlenmeyer flasks and each flask was inoculated with an isolate colony grown on agar (R2A or
388 MA). Cultures were grown with shaking (150 rpm; Ratek orbital incubator) at 37˚C for 48 h. A
389 minimum of three replicate cultures were grown per isolate. After 48 h, the optical density of
390 each culture was measured at 600 nm (OD_{600}, CLARIOstar PLUS, BMG Labtech), and the cultures
391 (including negative medium controls) were centrifuged at 3000 x *g* at 4˚C for 30 min (Allegra X-
392 12R) to pellet the bacterial cells. The cell-free supernatants (CFSs) were collected, frozen at -
393 80˚C, freeze dried (Alpha 1-4 LDplus, Martin Christ), and stored under inert gas in a dark, dry
394 environment until analysis. Antioxidants were extracted from the CFSs by resuspending at 50
395 mg mL^{-1} in 100% methanol, sonicating (Branson 2510) for 5 min, then centrifuging at 3000 x *g*
396 for 5 min at 4˚C. Quantitative DPPH assays were run by creating a 1:1 solution of 0.2 mM DPPH
397 in methanol and CFS extract to a final volume of 1 mL, vortexing, and reaction in the dark for 30
398 min at room temperature. Samples were then vortexed briefly, and three 300 µL replicates of
399 each sample were transferred into a well of a 96 well plate. FRS was measured by determining
400 absorbance at 517 nm (Enspire 2300 plate reader, Perkin Elmer). Decolourization of DPPH was
401 determined by measuring the decrease in absorbance at 517 nm, and the FRS activity was
402 calculated according to the formula, % DPPH scavenging activity = (Control – Sample) / Control
403 ×100, where, Control is the absorbance of the DPPH control (1:1 0.2 mM DPPH:methanol), and
404 Sample is the absorbance of CFS extract in DPPH. All samples were measured against a 100%
405 methanol blank. Positive controls consisting of 0.01 - 0.001% (w/v) L-ascorbic acid were run on
406 each 96-well plate. FRS activity ranged from 0-90%.

407 **Catalase assay.** The pelleted cells from above were resuspended in 2 mL fRSS and 500 µL
408 hydrogen peroxide giving a final concentration of 16 mM. If bubbles appeared, the organism

409 was considered catalase positive. If there were no bubbles, the organism was classified as
410 catalase negative.

411 **Inhibition testing.** Each paired set of high and low FRS strains were inoculated crosswise along
412 the middle of MA plates to test for antagonism. Plates were kept at 26 °C and monitored daily
413 for up to 7 days for antagonistic activity by documenting the presence or absence of both
414 inoculated isolates and if there was a zone of inhibition between them.

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

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

428 Illumina readsets for each isolate were assembled using Skesa [105] and the draft genome
429 sequence annotated using Prokka [106]. No evidence of mixed colonies or sequence
430 contamination was detected. A genome sequence based taxonomic classification for each
431 isolate was determined using Kraken2 [107] with the Genome Taxonomy Database [GTDB; 108]
432 as the curated genomic data source. Classification was primarily based on the genome
433 sequence of related isolates (within the relevant species where possible), which were obtained
434 from GenBank. In situations where genomes of taxonomically relevant individuals were
435 available, a species level classification was possible. Where available, closed genome sequences

436 from GenBank were used for comparative genomics analysis. Core genome comparisons were
437 performed, as implemented in Nullarbor (https://github.com/tseemann/nullarbor), for each of
438 the six pairs of isolates, with phylogenies inferred using core SNP differences. Genes of interest
439 for DMSP synthesis and degradation, vitamin B_{12} synthesis, and catalase were identified from
440 the annotated genome sequence (GFF format) produced by Prokka; specific genes were
441 identified by both name and Refseq accession number.

442 **16S rRNA gene copy number estimation.**

443 The 16S rRNA gene copy number of the 12 draft genomes was predicted by the 16Stimator
444 pipeline [109]. Briefly, all the 12 genomic assemblies were submitted to the RAST server [110],
445 and the positions of 16S rRNA and a set of single-copied housekeeping genes (Table S6) were
446 extracted from the RAST annotations. The clean readsets were mapped back to the
447 corresponding genomic assemblies by Bowtie 2 [111] to determine the read depth of each
448 position. Finally, the 16S copy number of each isolate was calculated by dividing the median
449 depth of 16S gene by the median depth of the single-copied housekeeping genes after the read
450 depths were calibrated by the model parameters provided by 16Stimator.

451 **Statistical analysis.** CFU counts were analyzed in R [v3.6.2, 112] by first checking the
452 assumptions of equal variance and homogeneity. An analysis of variance test was used to
453 detect differences in the mean number of bacterial colonies from each anemone genotype by
454 solid growth media (R2A or MA). A one-way analysis of variance [one-way ANOVA; 113] was
455 used to determine if there were significant differences between FRS abilities of selected
456 positive (high FRS), negative (low FRS), and media controls, and pairwise comparisons were
457 performed using Tukey's HSD [114, 115]. Each probiotic pair and media control was tested to
458 determine if data met the assumptions of normality and homoscedasticity. If either assumption
459 was violated, the non-parametic Kruskal-Wallis rank sum test [116] was used with a Dunn test
460 [117] for multiple comparisons (p-values adjusted with the Benjamini-Hochberg method [118])
461 with the R package "FSA" [119].

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

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473 AMD, MvO and LLB conceived and designed the study. AMD performed the sampling and
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742 **Table S1**: Isolate Genome Sequence Data Summary. Strains are presented as high FRS (grey) followed by low FRS (white). 16S rRNA
743 gene presumptive identity is derived from the NCBI classification of near-complete 16S rRNA gene sequences. *We were unable to
744 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

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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

746

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

747

748 **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

749

750 **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	+

753 **Table S4:** Summary of vitamin B12 biosynthesis pathway genes. 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*	+	16	+	+	+	+ x2	-	-	-
MMSF00249	*Labrenzia*	+	16	+	+	+	+ x2	-	-	-
MMSF00964	*Marinobacter*	-	2	-	-	-	-	-	-	-
MMSF01190	*Marinobacter*	-	1	-	-	-	-	-	-	-
MMSF00068	*Micrococcus*	-	0	-	-	-	-	-	-	-
MMSF00107	*Micrococcus*	-	0	-	-	-	-	-	-	-
MMSF00910	*Winogradskyella*	-	0	-	-	-	-	-	-	-
MMSF00046	*Winogradskyella*	-	0	-	-	-	-	-	-	-

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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*	-	-	-	-	-	-	-	-	-	-

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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*	-	-	-	-	-	-	-	-	-	-	-	-

756

757 **Table S5:** Composition of R2A broth adjusted to suit marine bacteria. Final pH = 7.2 +/- 0.2 at
758 26 °C.

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

759

760 **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

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