Research Article

Cultivating *in situ* triggers growth of previously uncultivated microorganisms via a growth initiation factor in nature

Dawoon Jung[1], Koshi Machida[2], Yoichi Nakao[2,3], Tomonori Kindaichi[4], Akiyoshi Ohashi[4], Yoshiteru Aoi[1*]

[1]Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Japan

[2]Research Institute for Science and Engineering, Waseda University, Japan

[3]Department of Chemistry and Biochemistry, Graduate School of Advanced Science and Engineering, Waseda University, Japan

[4]Department of Civil and Environmental Engineering, Graduate School of Engineering, Hiroshima University, Japan

Running title: Growth initiation by *in situ* cultivation

[*]Corresponding author: Yoshiteru Aoi (yoshiteruaoi@hiroshima-u.ac.jp)

Keywords: uncultured microbes, cultivation, resuscitation, nongrowing, dormancy, diffusion chamber

Abstract

Most microorganisms resist cultivation under standard laboratory conditions. On the other hand, to cultivate microbes in a membrane-bound device incubated in nature (*in situ* cultivation) is an effective approach. In the present study, we applied *in situ* cultivation to isolate diverse previously uncultivated marine sponge-associated microbes and comparatively analyzed this method's efficiencies with those of the conventional method. Then, we attempted to clarify the key and unknown mechanism of *in situ* cultivation by focusing on growth triggering via growth initiation factor. We hypothesized that majority of environmental microorganisms are in nongrowing state and requiring "growth initiation factor" for the recovery and that can be provided from environments. Consequently, significantly more novel and diverse microbial types were isolated via *in situ* cultivation than by standard direct plating (SDP). Next, the effect of the sponge extract on starvation recovery was compared between strains derived from *in situ* and SDP cultivation. Adding small amounts of the sponge extracts to the medium elevated the colony-formation efficiencies of the *in situ* strains at the starvation recovery step, while it showed no positive effect on that of SDP strains. Conversely, specific growth rates or carrying capacities of all tested strains were not positively affected. These results indicate that, 1) the sponge extract contains chemical compounds that facilitate starvation recovery, these substances selectively worked on the *in situ* strains, and 2) growth initiation factor in the sponge extract did not continuously promote growth activity but worked as triggers for regrowth (resuscitation from dormancy).

Importance

Most microbial species resist cultivation under laboratory condition. This is critical

impediment for both academic and applied microbiology, and thus clarification of the mechanism of microbial uncultivability is highly demanded. Several evidences have been reported that to cultivate microbes in a membrane-bound device incubated in nature (*in situ* cultivation) is an effective approach. However, the mechanism behind this approach has not been clarified. The present study shows the evidence that 1) initiating growth is a key for cultivating previously uncultivated microbes rather than simple growth promotion, and 2) growth initiation factor (signaling-like compounds) in natural environments stimulate microbial resuscitation from a nongrowing state. Since no study has focused on growth initiation for cultivation of previously uncultivated microorganisms, the discovery shown in the present study provides a new insight into microorganisms previously considered uncultivable and a microbial growth controlling system in nature.

Introduction

Most microbes remain uncultivated and have been referred to as ‘mysterious dark matter’ of the microbial world (1, 2). Cultivation independent survey have demonstrated great diversity among these uncultivated species (3-5). Accessing this “missing” microbial diversity is of great interest to both basic and applied sciences and has been regarded as a major challenge for microbiology.

Standard agar plating as a conventional method of cultivating microorganisms is limited because a significantly low proportion (usually less than 1%) of the plated microbes readily form visible colonies on the agar plates, thus leading to plate count anomalies (6, 7). To overcome the limitations, much effort has been devoted to developing alternative approaches, including physically separating cells to decrease competition or inhibitors (8-10), using

69 modification to prepare agar media, using alternative gelling agents or antioxidants to
70 minimize unfavorable compounds (11-13), and adding signal molecules or cocultivating with
71 recruiter organisms to better reflect the natural environment (14-16). Nevertheless, most
72 postulated extant microbes in nature remain uncultivated; thus, other essential factors for
73 microbial cultivation that exist in nature are likely absent from those artificial conditions.

74 One simple solution for approaching the unknown growth factors is to incubate the
75 microbes in their natural environment. Applying this idea to microbial cultivation led to
76 developing *in situ* cultivation methods aiming to better simulate the natural environment (for
77 a review, see Epstein et al., 2010 [17]). Several *in situ* cultivation methods with similar basic
78 concepts have been applied to various environments, including sediment, activated sludge,
79 alkaline soda lakes, sponges, soil, and a hot spring environment, and have been demonstrated
80 to be highly capable of microbial cultivation (18-24). However, why these *in situ* cultivation
81 techniques enable cultivating previously uncultivated microbes that are otherwise difficult or
82 impossible to grow using conventional methods remains unclear. Answering this question
83 would provide a key factor for microbial cultivation and contribute to developing additional
84 advanced cultivation techniques.

85 In the present work, we applied *in situ* cultivation to the marine sponge to isolate
86 previously uncultivated microbial species. Marine sponges are rich sources of bioactive
87 secondary metabolites of biotechnological interest for their antiviral, antitumoral,
88 antimicrobial and cytotoxic properties (25-27). It has been suggested that symbiotic microbes
89 in marine sponges produces some of these bioactive metabolites (28-32). Additionally,
90 recently developed culture-independent approaches have provided molecular evidence of
91 these microorganisms' functional roles (33-35). However, most of these producers remain
92 unexplored and unavailable because the microbes associated with the sponges cannot be

93 easily cultivated (32).

94 One focus of the present study was to determine an effective cultivation method to
95 broaden the accessible marine sponge-associated microbes. To do this, we employed one of *in*
96 *situ* cultivation method, diffusion chamber to isolate previously uncultivated marine sponge-
97 associated microbes and comparatively analyzed this method's efficiencies with those of the
98 conventional method (direct agar plating). Although the applicability of *in situ* cultivation to
99 marine (22) and freshwater sponges (21) has been demonstrated in previous studies, this
100 approach's potential for cultivating novel, previously uncultivated microbial species from
101 sponge samples remains unknown.

102 Another aim of the study is clarifying the reason why *in situ* cultivation enables growing
103 previously uncultivated microbes that cannot be isolated by conventional methods. We
104 hypothesized that inactive or nongrowing microbial cells are stimulated and aroused to begin
105 regrowing during *in situ* incubation by a growth initiation factor from the outside
106 environment and enriched inside an *in situ* cultivation chamber with nutrients. This brings
107 different microbial groups, which mostly resist cultivation by conventional methods, to the
108 culture collection. To verify our hypothesis, recovery from the nongrowing state in response
109 to the marine sponge extract was comparatively analyzed between microbes isolated via *in*
110 *situ* cultivation and those isolated via the conventional method.

111

112 ## Results

113 **Identifying isolates based on the 16S rRNA gene.**

114 Figures 1 presents the overall experimental design for the cultivation experiments (Fig. 1a),

115 structure and application of the device (DC) (Fig. 1b and c), and principle of *in situ*
116 cultivation method (Fig. 1d). One hundred twenty bacterial isolates (60 per cultivation
117 method) were randomly selected and identified. *In situ* cultivation (DC) enabled isolating 37
118 species (defined as operational taxonomic units [OTUs] composed of 16S rRNA gene
119 sequences sharing over 97% identity) from six taxonomic groups (*Actinobacteria*,
120 *Bacteroidetes*, *Firmicutes*, *Alphaproteobacteria*, *Epsilonproteobacteria* and
121 *Gammaproteobacteria*) (Fig. 2, Table S1). Standard direct plating (SDP) cultivation enabled
122 isolating 13 species from three taxonomic groups (*Bacteroidetes*, *Alphaproteobacteria* and
123 *Gammaproteobacteria*) (Table S2). These results suggested that *in situ* cultivation (DC)
124 yielded significantly higher diversity among the isolates at the species level than among those
125 obtained via SDP cultivation (Fig. 2). In addition, a few species overlapped among the
126 isolates from each method (Fig. 2). One species belonging to *Ruegeria atlantica* (99%
127 similarity to the closest known species) was shared between the DC and SDP isolates.

128 Suggested location for Fig. 1 and Fig. 2

129 The ratios of novel species, defined as a strain with ≤97% 16S rRNA similarity to the
130 closest known relative among the isolates, differed between the SDP and *in situ* cultivation
131 methods. Only one SDP isolate (1.7%) was a novel species, while 40% (24 isolates belonging
132 to 12 species) of the *in situ* isolates was novel species (Fig. 2, 3).

133 Suggested location for Fig. 3

134 **Microbial community compositions among the samples: comparison of the culture-**
135 **dependent and -independent methods.**

136 The microbial community compositions of the marine sponge (*Theonella swinhoei*) samples
137 were analyzed by Illumine-MiSeq sequencing based on the 16S rRNA gene. A total of

114,624 reads with a median length of 252 base pairs (bp) (V4~533–786 bp) assigned to 1625 OTUs were obtained from the sample. Ten major taxonomic groups were detected (mostly at the phylum level but at the class level for Proteobacteria): *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes*, *Poribacteria*, *Alphaproteobacteria*, *Epsilonproteobacteria*, *Gammaproteobacteria* and SBR1093 (with a cut-off of <1.0%; Fig. 4). The *in situ* isolates belonged to six groups, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Alphaproteobacteria*, *Epsilonproteobacteria* and *Gammaproteobacteria*, while the SDP isolates belonged to three groups: *Bacteroidetes*, *Alphaproteobacteria* and *Gammaproteobacteria*.

Suggested location for Fig. 4

Effect of the sponge extract on starvation recovery.

For further investigation, we picked 28 and 11 strains obtained from *in situ* and SDP cultivation, respectively, which are representing identical species of all identified isolates except a few lost species (we hereafter define those selected stains as *in situ* and SDP strains, respectively). Figure 5a shows the effect of adding the sponge extract (0.1% of the total volume) to the medium on starvation recovery (colony formation efficiency). Each strain's colony efficiency ratio between the two culture conditions (with and without the sponge extract) was calculated. Adding the sponge extract to the medium exerted different effects on the recoveries between the *in situ* and SDP strains. Adding the sponge extract positively affected the recoveries of all tested *in situ* strains. Among them, 11 strains (11/28, 39%) more than doubled in colony numbers on the agar media with the sponge extract compared with that on the media with no sponge extract. In contrast, none of SDP strains was not positively affected or rather negatively affected on the recovery by the sponge extract addition, except

two strains were slightly positively affected (Fig. 5a). Note that closest species of one SDP strain (SDP8) showed positive is *Ruegeria atlantica*, which is commonly found in *in situ* isolates (Table S1 and S2).

Effect of the sponge extract on specific growth rates and carrying capacities.

Figure 5b and c shows the effect of adding the sponge extract (0.1% of the total volume) to the medium on the specific growth rate and carrying capacity, respectively. Each strain's specific growth rate and carrying capacity were measured and compared under two medium conditions (with and without the sponge extract) to examine the sponge extract's effect on growth activity (Fig. S1). In contrast to its effect on starvation recovery (colony forming efficiencies), adding the sponge extract to the medium did not significantly elevate or decrease both the specific growth rate and carrying capacity of any tested strain. The addition slightly negatively affected the specific growth rates for more than half the tested *in situ* strains (15/28, 54%) and all SDP strains except SDP7 and SDP8 (SDP8 is commonly isolated species from *in situ* cultivation).

Suggested location for Fig. 5

Discussion

Effectiveness of *in situ* cultivation techniques for isolating novel species from marine sponges.

Marine sponges are sources of many bioactive natural products (36-38), which are often produced by host-specific microbes that are mostly unknown because of their uncultivability (32, 39). Molecular surveys have shown that sponges host rich microbial communities (40,

41); however, only a minor component of this richness has been cultivated via the conventional cultivation method (32). Extensive cultivation efforts have made use of several alternative techniques to isolate previously uncultured sponge-associated bacteria, including adding the sponge extracts or its skeleton to the media (42, 43), using oligotrophic media (36), adding antibiotics to inhibit fast-growing bacteria (44), using alternative gelling agent (45), and using *in situ* diffusion devices (21, 22) and floating filters (42). These alternative approaches yielded an increased novelty of sponge isolates and improved cultivability rates up to 14% in some cases (42). However, most postulated extant microbes in sponges remain uncultivated; thus, further efforts to discover such novel microbes are needed. Here, we isolated previously uncultivated microorganisms from marine sponges more efficiently than in previous studies (40% of *in situ* isolates were novel species in this study). Newly discovered microbes are candidates as sources for valuable secondary metabolites, and the newly established approach would be a strong tool for further accessing untapped microbial resources.

Hypothesis: a key mechanism of *in situ* cultivation for growing previously uncultivated microbial types.

In situ cultivation approach via diffusion chamber (DC) enabled obtaining a different culture collection that was larger and more novel than that obtained by standard direct plating (SDP) (Fig. 2, 3). Only one species was common in culture collections between the *in situ* and SDP cultivation methods. These results indicated that most isolated species were unique to their isolation approach, and *in situ* cultivation enabled cultivating specific microbial types that have rarely been isolated by conventional cultivation approaches.

206 These results raised the question, “what mechanism from the alternative approach yielded
207 these previously uncultivated microbial groups that differed entirely from the standard
208 cultivation approach?”. A simple explanation is that during *in situ* cultivation, inoculated
209 microbes receive unknown growth components from the natural environment necessary for
210 their growth but absent from the artificial medium (46). However, the present study found
211 that isolates unique to *in situ* cultivation grew stably under the same conditions as those of the
212 SDP cultivation at the subcultivation step, which lacks such growth components. This
213 phenomenon also occurred in previous studies that used *in situ* cultivation (18, 20, 21, 24).
214 The only the difference between the *in situ* and SDP cultivation methods in the experimental
215 procedure was whether a one-week incubation was performed in the natural environment
216 (inside the marine sponge in this study) prior to agar plate cultivation (Fig. 1, a). We then
217 explored what mechanism could explain this observation.

218 We hypothesized that 1) most environmental microbes (in this case, microbes associated
219 with marine sponges) are in nongrowing states (viable but nonculturable [VBNC], dormancy,
220 near-zero growth, inactive state) to survive under nutrient-limited conditions (47), and 2) they
221 require a specific signaling-like compounds, so called growth initiation factor to recover from
222 the nongrowing state and begin regrowth together with nutrient (here, it is supplied by
223 medium in diffusion chamber). Such growth initiation factor exists in natural environments
224 but is usually absent from artificial media. We expected that this factor stimulated and
225 aroused inactive microbial cells (in a nongrowing state), resulting in the growth of specific
226 microbial groups that could not be isolated via standard cultivation (Fig. 6). However,
227 although considered essential for microbial cultivation, the microbial awakening mechanism
228 remains unclear (48).

229 Suggested location for Fig. 6

Growth triggering by growth initiation factor in nature.

To verify the hypothesis and clarify the mechanism behind the *in situ* cultivation results, the effect of adding small amounts of the sponge extract on the starvation recoveries and specific growth rates of representative isolates (from every species) were compared between the strains derived from *in situ* and SDP cultivation. Most species (represented isolates) from the *in situ* and SDP cultivations were used for the test, except a few strains which had been lost.

The results supported the hypothesis as follows. Adding the sponge extract to the medium (only 0.1% of the total volume) elevated the colony formation efficiencies of all tested *in situ* strains at the starvation recovery step, while it only negatively affected that of the SDP strains (Fig. 5a). In contrast, adding the sponge extract did not elevate the specific growth rates in either the *in situ* or SDP strains but rather slightly suppressed them, especially the SDP strains (Fig. 5b). In addition, adding the sponge extract did not positively affect the carrying capacity of every tested strain (Fig. 5c). These results show that 1) the sponge extract contains a growth initiation factor that facilitate starvation recovery, and these substances selectively worked on the *in situ* strains, and 2) the growth initiation factor in the sponge extract did not continuously promote growth activity but worked as triggers for regrowth, which was likely resuscitation from dormancy (nongrowing state).

Therefore, during the *in situ* cultivation, such signaling-like compounds, defined as "growth initiation factor", was provided from the natural environment (i.e., the marine sponge), while nutrients were provided inside the chamber from the beginning. These factors enabled microbes begin to growing again and finally become enriched inside the chamber (Fig. 6b). In contrast, standard agar plate medium lacks such growth initiation factor; thus, significant numbers of inoculated microbial cells did not resuscitate from dormancy and thus

did not form colonies (Fig. 6c, upper part). Because sponge-associated microbes produce various chemical compounds, such growth initiation factor are likely also produced by specific microbes associated with the sponge (32).

In contrast, because SDP strains require no growth initiation factor to begin regrowing, these strains are frequently isolated via conventional approaches (the SDP method in the present study) and have been discovered in previous studies. Therefore, microbial types in isolates differ completely between methods, and the novelty and diversity of isolates derived from *in situ* cultivation are higher than are those derived from the conventional approach.

Additionally, the sponge extract was highly toxic to the microbes. Adding the sponge extract to the media at higher concentrations than that of the original test condition (e.g., 1%, which was 10 times higher than the original test condition) strongly inhibited isolate growth (data not shown). This was likely due to antimicrobial substances in the sponge, such as secondary antimetabolites or high concentrations of toxic trace elements, which can accumulate in the sponge body (43, 49, 50). Furthermore, SDP strains are more sensitive to that toxicity than are *in situ* strains as described above and shown in Fig. 5. In other words, under the tested condition (0.1%), the negative effect of adding the sponge extract appeared only for SDP strains. This suggests that microbial types that are susceptible to being enriched by *in situ* cultivation are less sensitive to the toxic substances that are likely to be excreted from the sponge in nature; thus, microbial types derived from *in situ* cultivation would be more competitive in the original environment (inside the sponge). We reported a similar observation in our previous study, suggesting that *in situ* isolates are more competitive under their original conditions (natural environment) than are SDP isolates (23). Therefore, *in situ* isolates might outcompete the microbes that are frequently isolated via SDP cultivation. This might be another reason why *in situ* isolates are novel and differ phylogenetically from SDP

277 isolates.

278

279 **Significance of the present study relative to solving microbial uncultivability.**

280 Various *in situ* cultivation methods with similar basic rationales have been applied to various
281 environmental samples and have been shown to be highly capable of microbial cultivation
282 compared with conventional approaches because the method led to isolating some
283 uncultivated and phylogenetically new and industrially important isolates (18-21, 51-53).
284 Furthermore, mining “microbial dark matter” using this new approach remains highly
285 expected (2). However, no study has clarified the growth facilitation mechanism of *in situ*
286 cultivation or the reason why *in situ* cultivation yields more phylogenetically novel and
287 diverse microbial types than does the conventional method. This has limited further
288 advancing the method regardless of its potential. The present study resolved this issue for the
289 first time.

290 Furthermore, the discovery in the present study, especially regarding microbial
291 resuscitation from a nongrowing state in response to the growth initiation factor gives us an
292 important clue toward solving this challenging issue of why most environmental microbes
293 cannot grow on standard media. This has been described as "the great plate count anomaly"
294 (6, 7). Although most microbial types that resist cultivation are assumed to be fastidious and
295 require culture conditions within a narrow range, the results of the present study suggest that
296 microbial uncultivability cannot simply be explained by the unfitness of the specific strain to
297 certain culture conditions such as medium composition, gas composition, temperature, and
298 pH (54).

299 Several studies have been conducted to determine the reasons for microbial

uncultivability, such as inoculum size (55) and phosphate-catalyzed hydrogen peroxide formation from agar (12, 13, 56). A few attempts have been made to discover microbial interactions via signaling molecules that promote the growth of specific microbial types. Short peptides (57), siderophores (58) and quinones (59) have been identified as growth-promoting factors for microbial types that cannot grow independently.

However, the causes of the uncultivability and the mechanism facilitating growth of previously uncultivated microbes shown in the present study differ from those reported in previous studies in several aspects as follows. First, no study has focused on growth triggering or distinguished this from simple growth promoting, yet that must be the key to solving the microbial uncultivability as shown herein. Second, no study has provided experimental evidence showing that the growth of diverse environmental microbes is controlled by a growth initiation factor.

Previous studies have reported various types of microbial nongrowing states such as viable but nonculturable (VBNC), near-zero growth (NZG) and dormancy (60-62), which are suspected to cause microbial uncultivability. Wide species distributions in the VBNC state have been detected in broad environments such as sediment (63), estuarine water (64), seawater (65) and soil (66). The broad distribution of inactive microbial cells in environments reinforce that this state is a common mechanism for coping with unfavorable environmental conditions (60, 62). Although microbial resuscitation from dormancy remains unclear, a few studies on resuscitation or germination of gram-positive bacteria via signaling compounds have been reported (67-69). Therefore, many environmental microorganisms may possess similar systems for microbial awakening from nongrowing states via signaling compounds. Furthermore, the “scout hypothesis” theory has been proposed, which states that 1) dormant microbes stochastically and spontaneously awaken, and 2) awakened cells trigger the

awakening of other dormant cells (70, 71). However, no study has experimentally confirmed that growth triggering of environmental microbes from a nongrowing state is a key phenomenon of microbial cultivation.

Concluding remarks and perspectives.

We demonstrated that *in situ* cultivation effectively isolates previously uncultivated microbial types from the marine sponge. We further clarified that *in situ* cultivation facilitates isolating novel and different microorganisms compared with the conventional approach because of the discovery of the growth-triggering phenomenon from a nongrowing state by growth initiation factor in the natural environment. To our knowledge, the present study shows the first evidence that 1) triggering growth is a key for cultivating previously uncultivated microbes, and 2) the growth initiation factor (signaling-like compounds) in natural environments (likely produced by microbes) stimulate microbial resuscitation from a nongrowing state.

Next challenge beyond the present study include 1) identifying the growth initiation factor via natural chemical approaches and 2) clarifying the donors and accepters of signaling compounds (i.e., who produces what and to whom). This will provide a new insight into microorganisms previously considered uncultivable and the complex networking system for controlling growth among microbes in nature.

Materials and Methods

Sample collection.

Marine sponge (*Theonella swinhoei*) specimens were collected while scuba diving at 15-20 m

346 depth by hand in August 2015, near Okino island, Kochi prefecture, Japan. Sponges were
347 kept in a cooling box with seawater and transported to the aquarium and laboratory for
348 further experiments.

349

350 **Media.**

351 For the microbial cultivation experiments, we used the following four media: 1) 1:10 diluted
352 Reasoner's 2A (R2A) media (10% of the manufacturer's suggested concentration, Nihon
353 Seiyaku, Japan); 2) marine media (Difco, Franklin Lakes, NJ, USA); 3) fish extract media
354 (0.2 g fish extract and 0.1 g yeast extract per liter); and 4) sponge extract media (0.01 g
355 peptone and 40 ml aqueous sponge extract per liter). The sponge extract was prepared by
356 mixing homogenized sponge and sterile distilled water including 3.5% artificial sea salt at a
357 1:1 (vol/vol) ratio, then vortexing for 1 min, spinning down (10 min, 8,000 rpm), and filter
358 sterilization using a 0.2-μm pore-size filter. All media contained 1.5% agar to produce solid
359 medium, and all media except the marine media were supplemented with 3.5% artificial sea
360 salt, SEALIFE (Marine Tech, Tokyo, Japan). Colonies grown on these media were
361 subcultured for purification on 1.5% agar plates supplemented with 1:10 R2A broth. All
362 isolates grew well on 1:10 diluted R2A agar media. Some isolates were derived from
363 different media at the subcultivation or direct plating steps.

364

365 **Diffusion chamber *in situ* cultivation.**

366 Diffusion chamber (DC) i*n situ* cultivation method was performed to isolate marine-sponge
367 associated microbes. The DC was prepared as described previously (18). The DC is an *in situ*

368 cultivation device that allows exchanging chemical compounds, thereby simulating a natural
369 environment (Fig. 1. d). To produce a chamber, a 0.1-μm pore-size filter polycarbonate
370 membrane (Millipore, Darmstadt, Germany) was glued to a rectangular-shaped silicone flame
371 (w = 3 cm × 1.2 cm, d = 0.3 cm) with a waterproof adhesive (Cemedain, Tokyo, Japan) (Fig.
372 1. b). To prepare the marine sponge sample inoculum, 20 g of the subsample was rinsed
373 three times with sterile artificial sea water and pulverized using a sterile mortar and pestle
374 with the same volume of sterile artificial sea water. The aliquots were diluted 10^{-4} to 10^{-5}
375 with sterilized water including 3.5% artificial sea salt and mixed with warm (45°C) agar with
376 1:10 diluted R2A. The mix was placed on the chamber membrane, and the second membrane
377 was glued to the other side of the silicone flame, sealing the agar inside to form a chamber.

378 For *in situ* cultivation, the sponges were kept in aquariums in the Takehara station of
379 Hiroshima University in Takehara-city, Japan. The aquarium was a cylindrical container with
380 a holding capacity of ca. 43.3 L (Ø = 0.35 m, d = 0.45 m), and seawater was supplied
381 continuously. To install the DC into the sponge, similar sized grooves as those of the device
382 were made on the specimen's surface by cutting with a scalpel blade, and four DCs were
383 inserted (Fig. 1. c). After one week of *in situ* incubation, DCs were retrieved from the
384 sponges and then transported to the laboratory. The agar material from each device was added
385 to 1 ml of sterilized water including 3.5% artificial sea salt, homogenized with a sterile stick,
386 vortexed, diluted with sterile water including 3.5% artificial sea salt, subcultured on media as
387 described above with 1.5% agar, and incubated at 20°C. After one week, 60 colonies were
388 randomly selected, pure cultured using 1:10 R2A medium and used for further analysis.

389

390 **Standard direct plating cultivation.**

Conventional standard direct plating (SDP) was performed to compare the SDP results with those of the *in situ* cultivation approaches (Fig. 1). Aliquots from sponge samples prepared in the same manner as the DC cultivation were diluted serially and plated directly on the agar media described above. After incubating for one-week at 20°C, 60 colonies were randomly selected and pure-cultured using 1:10 R2A medium for microbial identification.

Identification of isolates based on 16S rRNA gene sequencing.

Taxonomies were identified via sequencing 610- to 712-bp fragments of the 16S rRNA gene. The colony material was used directly as a PCR template. The 16S rRNA gene was amplified using the universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), with a KOD FX Neo system (Toyobo, Osaka, Japan), then purified using a fast gene purification kit (Nippon Genetics, Tokyo, Japan). The purified PCR products were sequenced with a commercial sequencer (Takara Bio, Shiga, Japan) by fluorescent dye terminator sequencing. The sequences were compared with those available in GenBank (www.ncbi.nlm.nih.gov) using Molecular Evolutionary Genetics Analysis (MEGA software, Tempe, AZ, USA) to determine their closest relatives. Distance matrices and phylogenetic trees based on 16S rRNA sequences were calculated among the isolated OTUs (defined at 97% 16S rRNA gene sequence identity) according to the Kimura two-parameter model and neighbor-joining algorithms using the MEGA program (MEGA software, Tempe, AZ, USA). One thousand fast bootstraps were performed to assign confidence levels to the nodes in the trees.

DNA extraction and amplicon sequencing targeting the 16S rRNA gene.

To compare the cultivated bacterial diversity with the microbial molecular signatures in the host sponge, 16S amplicon sequencing was performed based on the 16S rRNA genes. After transferring the marine sponges to the laboratory, samples were washed three times with DNA-free water and homogenized. Genomic DNA from the homogenized tissue was extracted using a FastDNA spin kit for soil (MP Biomedicals, Irvine, CA, USA) per the manufacturer's guidelines. The extracted genomic DNA was amplified using the amplicon forward primer and amplicon reverse primer with a Hifi hot start ready mix PCR (Kapa Biosystems, Wilmington, MA, USA), then purified using a fast gene purification kit (Nippon Genetics, Tokyo, Japan). DNA was sequenced by Hokkaido System Science (Sapporo, Japan) using an Illumina MiSeq System (Illumina, San Diego, CA, USA). Each read was assigned taxonomically using Quantitative Insights Into Microbial Ecology (QIIME) software (72).

Effect of the sponge extract on starvation recovery.

The colony formation efficiency ratios between the two culture conditions (media with and without the sponge extract) were calculated for each tested strain to examine the effect of the sponge extract on starvation recovery. First, one strain per OTU (defined at 97% 16S rRNA gene sequence identity) from the DC and SDP cultivations were selected and cultured in 5 ml of 1:10 diluted R2A broth with 3.5% artificial sea salt at 20°C (liquid cultures). Each culture was diluted 100 times and placed at 5°C to inactivate them. After 3 days, the liquid culture serial dilutions were inoculated in triplicate on two types of agar media: 1:10-diluted R2A agar medium with 0.1% (vol/vol) of the sponge extract and the same medium without the sponge extract. The sponge extract added to the medium before autoclaving. After 5 days of incubation at 20°C, the colony number ratios between the two culture conditions (with and

437 without the sponge extract) were measured for each tested strain.

438

439 **Effect of the sponge extract on the specific growth rate and carrying capacity.**

440 Specific growth rates and carrying capacities under the two culture conditions (medium with
441 and without the sponge extract) were measured and compared for each tested strain to
442 examine the sponge extract's effect on the growth activity. First, one strain per each OTU
443 from the DC and SDP cultivations were selected and cultured (liquid cultures) in the same
444 manner as described above. Next, 5–20 μl of the microbial cell suspension from each liquid
445 culture was inoculated in triplicate into the two media (1:10-diluted R2A medium with 0.1%
446 [vol/vol] of the sponge extract and the same medium without the sponge extract). The sponge
447 extract added to the medium before autoclaving. The cultures were then incubated at 20°C
448 with shaking at 150 rpm. The optical density (OD) was measured at 600 nm using a
449 spectrophotometer (DR 3900, HACH, Metropolitan, MD, USA). The growth curve for the
450 OD 600 value was fitted using the nonlinear regression function with a logistic model in
451 Sigma plot software (San Jose, CA, USA, Systat Software, Inc.). Specific growth rates, as
452 maximum rates of change, were determined to μ by the change in OD 600 on a logarithmic
453 scale as time. The ratio of the specific growth rate (μ) between the values measured under the
454 two culture conditions (with and without the sponge extract) was calculated for each tested
455 strain. The carrying capacity (maximum growth) was determined by its maximum value on
456 the fitted growth curve.

457

458 **Data availability.**

Newly determined sequence data have been deposited in GenBank (www.ncbi.nlm.nih.gov) under accession numbers MK672856 to MK672868 for isolates from diffusion chamber, and MK674856 to MK674892 for isolates from standard direct plate cultivation.

Acknowledgment

This work was supported by JSPS KAKENHI Grant Numbers JP17F17098, JP25630383, JP18K19181. D. J is an International Research Fellow of the Japan Society for the Promotion of Science. We thank Traci Raley, MS, ELS, from Edanz Group for editing a draft of this manuscript. We thank Slava S. Epstein for valuable comments and discussions on our data.

References

1. Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng J, Darling A, Malfatti S, Swan BK, Gies EA, Dodsworth JA, Hedlund BP, Tsiamis G, Sievert SM, Liu W, Eisen JA, Hallam SJ, Kyrpides NC, Stepanauskas R, Rubin EM, Hugenholtz P, Woyke T. 2013. Insights into the phylogeny and coding potential of microbial dark matter. Nature 499: 431-437.

2. Lok C. 2015. Mining the microbial dark matter. Nature 522: 270-273.

3. Rappé MS, Giovannoni SJ. 2003. The uncultured microbial majority. Annu Rev Microbiol 57: 369-394.

4. Handelsman J. 2004. Metagenomics: application of genomics to uncultured microorganisms. Microbiol Mol Biol Rev 68: 669-685.

480 5. Keller M, Zengler K. 2004. Tapping into microbial diversity. Nat Rev Microbiol 2: 141-
481 150.

482 6. Staley JT, Konopka A. 1985. Measurement of in situ activities of nonphotosynthetic
483 microorganisms in aquatic and terrestrial habitats. Annu Rev Microbiol 39: 321-346.

484 7. Amann RI, Ludwig W, Schleifer KH. 1995. Phylogenetic identification and in situ
485 detection of individual microbial cells without cultivation. Microbiol Rev 59:143-169.

486 8. Connon SA, Giovannoni SJ. 2002. High-throughput methods for culturing
487 microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl
488 Environ Microbiol 68: 3878-3885.

489 9. Zengler K, Toledo G, Rappé M, Elkins J, Mathur EJ, Short JM, Keller M. 2002.
490 Cultivating the uncultured. Proc Natl Acad Sci U S A 99: 15681-15686.

491 10. Tandogan N, Abadian PN, Epstein S, Aoi Y, Goluch ED. 2014. Isolation of
492 microorganisms using sub-micrometer constrictions. PLoS One 9: e101429.

493 11. Tamaki H, Sekiguchi Y, Hanada S, Nakamura K, Nomura N, Matsumura M, Kamagata Y.
494 2005. Comparative analysis of bacterial diversity in freshwater sediment of a shallow
495 eutrophic lake by molecular and improved cultivation-based techniques. Appl Environ
496 Microbiol 71: 2162-2169.

497 12. Tanaka T, Kawasaki K, Daimon S, Kitagawa W, Yamamoto K, Tamaki H, Tanaka M,
498 Nakatsu CH, Kamagata Y. 2014. A hidden pitfall in the preparation of agar media
499 undermines microorganism cultivability. Appl Environ Microbiol 80: 7659-7666.

500 13. Kato S, Yamagishi A, Daimon S, Kawasaki K, Tamaki H, Kitagawa W, Abe A, Tanaka M,
501 Sone T, Asano K, Kamagata Y. 2018. Isolation of previously uncultured slow-growing

bacteria by using a simple modification in the preparation of agar media. Appl Environ Microbiol 84: e00807-18.

14. Bruns A, Cypionka H, Overmann J. 2002. Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central Baltic sea. Appl Environ Microbiol 68: 3978-3987.

15. Vartoukian SR, Adamowska A, Lawlor M, Moazzez R, Dewhirst FE, Wade WG. 2016. In vitro cultivation of “Unculturable” oral bacteria, facilitated by community culture and media supplementation with siderophores. PLoS One 11: e0146926.

16. Tanaka Y, Tamaki H, Tanaka K, Tozawa E, Matsuzawa H, Toyama T, Kamagata Y, Mori K. 2018. "Duckweed-microbe co-cultivation method" for isolating a wide variety of microbes including taxonomically novel microbes. Microbes Environ 33: 402-406.

17. Epstein SS, Lewis K, Nichols D, Gavrish E. 2010. New approaches to microbial isolation, p 3-12. In Baltz RH, Davies JE, Demain A (ed), Manual of industrial microbiology and biotechnology, vol 4. ASM Press, Washington, DC.

18. Bollmann A, Lewis K, Epstein SS. 2007. Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. Appl Environ Microbiol 73: 6386-6390.

19. Aoi Y, Kinoshita T, Hata T, Ohta H, Obokata H, Tsuneda S. 2009. Hollow-fiber membrane chamber as a device for in situ environmental cultivation. Appl Environ Microbiol 75: 3826-3833.

20. Jung D, Seo E, Epstein SS, Joung Y, Yim JH, Lee H, Ahn TS. 2013. A new method for microbial cultivation and its application to bacterial community analysis in Buus Nuur,

524 Mongolia. Fundam Appl Limnol 182: 171-181.

525 21. Jung D, Seo E, Epstein SS, Joung Y, Han J, Parfenova VV, Belykh OI, Gladkikh AS, Ahn
526 TS. 2014. Application of a new cultivation technology, I-tip, for studying microbial
527 diversity in freshwater sponges of Lake Baikal, Russia. FEMS Microbiol Ecol 90: 417-
528 423.

529 22. Steinert G, Whitfield S, Taylor MW, Thoms C, Schupp PJ. 2014. Application of diffusion
530 growth chambers for the cultivation of marine sponge-associated bacteria. Mar
531 Biotechnol 16: 594-603.

532 23. Jung D, Aoi Y, Epstein SS. 2016. In Situ Cultivation Allows for Recovery of Bacterial
533 Types Competitive in Their Natural Environment. Microbes Environ 31: 456-459.

534 24. Jung D, Seo E, Owen JS, Aoi Y, Yong S, Lavrentyeva EV, Ahn, TS. 2018. Application of
535 the filter plate microbial trap (FPMT), for cultivating thermophilic bacteria from thermal
536 springs in Barguzin area, eastern Baikal, Biosci Biotechnol Biochem 82: 1624-1632.

537 25. Koopmans M, Martens D, Wijffels HR. 2009. Towards commercial production of sponge
538 medicines. Marine Drugs 7:787-802.

539 26. Mehbub FM, Lei J, Franco C, Zhang W. 2014. Marine sponge derived natural products
540 between 2001 and 2010: Trends and opportunities for discovery of bioactives. Mar Drugs
541 12: 4539-4577.

542 27. Anjum K, Abbas SQ, Shah SAA, Akhter N, Batool S, Hassan SSU. 2016. Marine
543 Sponges as a Drug Treasure. Biomol Ther 24: 347-362.

544 28. Brantley SE, Molinski TF, Preston CM, DeLong EF. 1995. Brominated acetylenic fatty
545 acids from Xestospongia sp., a marine spongebacteria association. Tetrahedron 51: 7667-

546 7672.

547 29. Bewley CA, Holland ND, Faulkner DJ. 1996. Two classes of metabolites from Theonella
548 swinhoei are localized in distinct populations of bacterial symbionts. Experientia 52:
549 716-722.

550 30. Flatt PM, Gautschi JT, Thacker RW, Musafija-Girt M, Crews P, Gerwick WH. 2005.
551 Identification of the cellular site of polychlorinated peptide biosynthesis in the marine
552 sponge Dysidea (Lamellodysidea) herbacea and symbiotic cyanobacterium Oscillatoria
553 spongeliae by CARD-FISH analysis. Mar Biol 147: 761-774.

554 31. König GM, Kehraus S, Seibert SF, Abdel-Lateff A, Müller D. 2006. Natural products
555 from marine organisms and their associated microbes. Chembiochem 7: 229-238.

556 32. Wang G. 2006. Diversity and biotechnological potential of the sponge-associated
557 microbial consortia. J Ind Microbiol Biotechnol 33: 545-551.

558 33. Fan L, Reynolds D, Liu M, Stark M, Kjelleberg S, Webster NS, Thomas T. 2012.
559 Functional equivalence and evolutionary convergence in complex communities of
560 microbial sponge symbionts. Proc Natl Acad Sci U S A 109: 1878-1887.

561 34. Hentschel U, Piel J, Degnan SM, Taylor MW. 2012. Genomic insights into the marine
562 sponge microbiome. Nat Rev Microbiol 10: 641-654.

563 35. Wilson MC, Mori T, Rükert, C., Uria AR, Helf MJ, Takada K, Gernert C, Steffens UAE,
564 Heycke N, Schmitt S, Rinke C, Helfrich EJN, Brachmann AO, Gurgui C, Wakimoto T,
565 Kracht M, Crüsemann M, Hentschel U, Abe I, Matsunaga S, Kalinowski J, Takeyama H,
566 Piel J. 2014. An environmental bacterial taxon with a large and distinct metabolic
567 repertoire. Nature 506: 58-62.

568 36. Schmidt EW, Obraztsova AY, Davidson SK, Faulkner DJ, Haygood MG. 2000.
569 Identification of the antifungal peptide-containing symbiont of the marine sponge
570 Theonella swinhoei as a novel δ-proteobacterium, "Candidatus Entotheonella palauensis".
571 Mar Biol 136: 969-977.

572 37. Piel J, Hui D, Wen G, Butzke D, Platzer M, Fusetani N, Matsunaga S. 2004. Antitumor
573 polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge
574 Theonella swinhoei. Proc Natl Acad Sci U S A 101: 16222-16227.

575 38. Hentschel U, Usher KM, Taylor MW. 2006. Marine sponges as microbial fermenters.
576 FEMS Microbiol Ecol 55: 167-177.

577 39. Taylor MW, Radax R, Steger D, Wagner M. 2007. Sponge-associated microorganisms:
578 evolution, ecology, and biotechnological potential. Microbiol Mol Biol Rev 71: 295-347.

579 40. Schirmer A, Gadkari R, Reeves CD, Ibrahim F, DeLong EF, Hutchinson CR. 2005.
580 Metagenomic analysis reveals diverse polyketide synthase gene clusters in
581 microorganisms associated with the marine sponge Discodermia dissoluta. Appl Environ
582 Microbiol 71: 4840-4849.

583 41. Thomas T, Moitinho-Silva L, Lurgi M, Björk JR, Easson C, Astudillo-García C, Olson
584 JB, Erwin PM, López-Legentil S, Luter H, Chaves-Fonnegra A, Costa R, Schupp PJ,
585 Steindler L, Erpenbeck D, Gilbert J, Knight R, Ackermann G, Victor Lopez J, Taylor
586 MW, Thacker RW, Montoya JM, Hentschel U, Webster NS. 2016. Diversity, structure
587 and convergent evolution of the global sponge microbiome. Nat Commun 7: 11870.

588 42. Sipkema D, Schippers K, Maalcke WJ, Yang, Y, Salim S, Blanch HW. 2011. Multiple
589 approaches to enhance the cultivability of bacteria associated with the marine sponge

590 Haliclona (gellius) sp. Appl Environ Microbiol 77: 2130-2140.

591 43. Keren R, Lavy A, Ilan M. 2016. Increasing the richness of culturable arsenic-tolerant
592 bacteria from Theonella swinhoei by addition of sponge skeleton to the growth medium.
593 Microb Ecol 71: 873-886.

594 44. Selvin J, Gandhimathi R, Kiran GS, Priya SS, Ravji TR, Hema TA. 2009. Culturable
595 heterotrophic bacteria from the marine sponge Dendrilla nigra: isolation and
596 phylogenetic diversity of actinobacteria. Helgol Mar Res 63: 239-247.

597 45. Rygaard AM, Thøgersen MS, Nielsen KF, Gram L, Bentzon-Tilia M. 2017. Effects of
598 gelling agent and extracellular signaling molecules on the culturability of marine bacteria.
599 Appl Environ Microbiol 83: e00243-17.

600 46. Kaeberlein T, Lewis K, Epstein SS. 2002. Isolating "uncultivable" microorganisms in
601 pure culture in a simulated natural environment. Science 296: 1127-1129.

602 47. Jones SE, Lennon JT. 2010. Dormancy contributes to the maintenance of microbial
603 diversity. Proc Natl Acad Sci U S A 107: 5881-5886.

604 48. van Vliet S. 2015. Bacterial dormancy: How to decide when to wake up. Curr Biol 25:
605 R753-R755.

606 49. Hentschel U, Schmid M, Wagner M, Fieseler L, Gernert C, Hacker J. 2001. Isolation and
607 phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean
608 sponges Aplysina aerophoba and Aplysina cavernicola. FEMS Microbiol Ecol 35: 305-
609 312.

610 50. Melander RJ, Liu H, Stephens MD, Bewley CA, Melander C. 2016. Marine sponge
611 alkaloids as a source of anti-bacterial adjuvants. Bioorg Med Chem Lett 26: 5863-5866.

612 51. Sizova MV, Hohmann T, Hazen A, Paster BJ, Halem SR, Murphy CM, Panikov NS,
613 Epstein SS. 2012. New approaches for isolation of previously uncultivated oral bacteria.
614 Appl Environ Microbiol 78: 194-203.

615 52. Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, Mueller A,
616 Schäberle TF, Hughes DE, Epstein S, Jones M, Lazarides L, Steadman VA, Cohen DR,
617 Felix CR, Fetterman KA, Millett WP, Nitti AG, Zullo AM, Chen C, Lewis K. 2015. A
618 new antibiotic kills pathogens without detectable resistance. Nature 517: 455.

619 53. Berdy B, Spoering AL, Ling LL, Epstein SS. 2017. In situ cultivation of previously
620 uncultivable microorganisms using the ichip. Nat Protoc 12: 2232-2242.

621 54. Stewart EJ. 2012. Growing unculturable bacteria. J Bacteriol 194: 4151-4160.

622 55. Davis KER, Joseph SJ, Janssen PH. 2005. Effects of growth medium, inoculum size, and
623 incubation time on culturability and isolation of soil bacteria. Appl Environ Microbiol 71:
624 826-834.

625 56. Kawasaki K, Kamagata Y. 2017. Phosphate-catalyzed hydrogen peroxide formation from
626 agar, gellan, and κ-carrageenan and recovery of microbial cultivability via catalase and
627 pyruvate. Appl Environ Microbiol 83: e01366-17.

628 57. Nichols D, Lewis K, Orjala J, Mo S, Ortenberg R, O'Connor P, Zhao C, Vouros P,
629 Kaeberlein T, Epstein SS. 2008. Short peptide induces an "uncultivable" microorganism
630 to grow in vitro. Appl Environ Microbiol 74: 4889-4897.

631 58. D'Onofrio A, Crawford JM, Stewart EJ, Witt K, Gavrish E, Epstein S. Clardy J, Lewis K.
632 2010. Siderophores from neighboring organisms promote the growth of uncultured
633 bacteria. Chem Biol 17: 254-264.

634 59. Fenn K, Strandwitz P, Stewart EJ, Dimise E, Rubin S, Gurubacharya S, Clardy J, Lewis
635 K. 2017. Quinones are growth factors for the human gut microbiota. Microbiome 5: 161.

636 60. Lennon JT, Jones SE. 2011. Microbial seed banks: the ecological and evolutionary
637 implications of dormancy. Nat Rev Microbiol 9: 119-130.

638 61. Panikov NS, Mandalakis M, Dai S, Mulcahy LR, Fowle W, Garrett WS, Karger BL.
639 2015. Near-zero growth kinetics of Pseudomonas putida deduced from proteomic
640 analysis. Environ Microbiol 17: 215-228.

641 62. Pinto D, Santos M, Chambel L. 2015. Thirty years of viable but nonculturable state
642 research: Unsolved molecular mechanisms. Crit Rev Microbiol 41: 61-76.

643 63. Magarinos B, Romalde JL, Barja JL, Toranzo AE. 1994. Evidence of a dormant but
644 infective state of the fish pathogen Pasteurella piscicida in seawater and sediment. Appl
645 Environ Microbiol 60: 180-186.

646 64. Oliver JD, Hite F, McDougald D, Andon NL, Simpson LM. 1995. Entry into, and
647 resuscitation from, the viable but nonculturable state by Vibrio vulnificus in an estuarine
648 environment. Appl Environ Microbiol 61: 2624-2630.

649 65. Maalej S, Gdoura R, Dukan S, Hammami A, Bouain A. 2004. Maintenance of
650 pathogenicity during entry into and resuscitation from viable but nonculturable state in
651 Aeromonas hydrophila exposed to natural seawater at low temperature. J Appl Microbiol
652 97: 557-565.

653 66. Ben Abdallah F, Lagha R, Bakhrouf A. 2008. Resuscitation and morphological
654 alterations of Salmonella bovismorbificans cells under starvation in soil. World J of
655 Microbiol Biotechnol 24: 1507-1512.

67. Mukamolova GV, Murzin AG, Salina EG, Demina GR, Kell DB, Kaprelyants AS, Young M. 2006. Muralytic activity of Micrococcus luteus Rpf and its relationship to physiological activity in promoting bacterial growth and resuscitation. Mol Microbiol 59: 84-98.

68. Dworkin J, Shah IM. 2010. Exit from dormancy in microbial organisms. Nat Rev Microbiol 8: 890-896.

69. Potgieter M, Bester J, Kell DB, Pretorius E. 2015. The dormant blood microbiome in chronic, inflammatory diseases. FEMS Microbiol Rev 39: 567-591.

70. Epstein SS. 2009. Microbial awakenings. Nature 457: 1083.

71. Buerger S, Spoering A, Gavrish E, Leslin C, Ling L, Epstein SS. 2012. Microbial scout hypothesis and microbial discovery. Appl Environ Microbiol 78: 3229-3233.

72. Kuczynski J, Stombaugh J, Walters WA, González A, Caporaso JG, Knight R. 2011. Using QIIME to analyze 16S rRNA gene sequences from microbial communities. Curr Protoc Bioinformatics. Chapter 10: Unit 10.7.

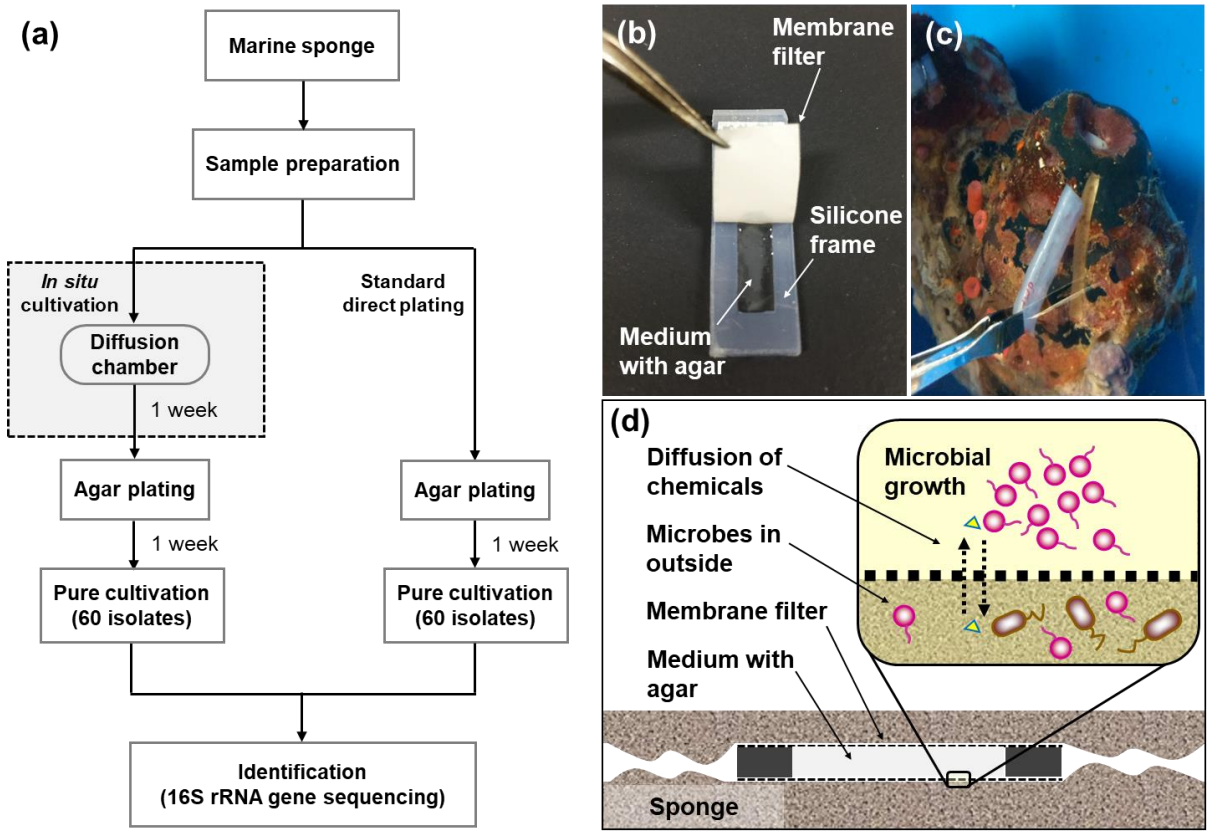
676

677

678

679

680



681

682 Figure 1. Flowchart of the experimental steps for microbial cultivation from marine sponges
683 (a) and diffusion chamber (DC) cultivation method applied in the present study: a photo
684 showing structure of the chamber (b), a photo showing installation of DC device into the
685 marine sponge (c), a schematic image showing the principle of DC (d).

686

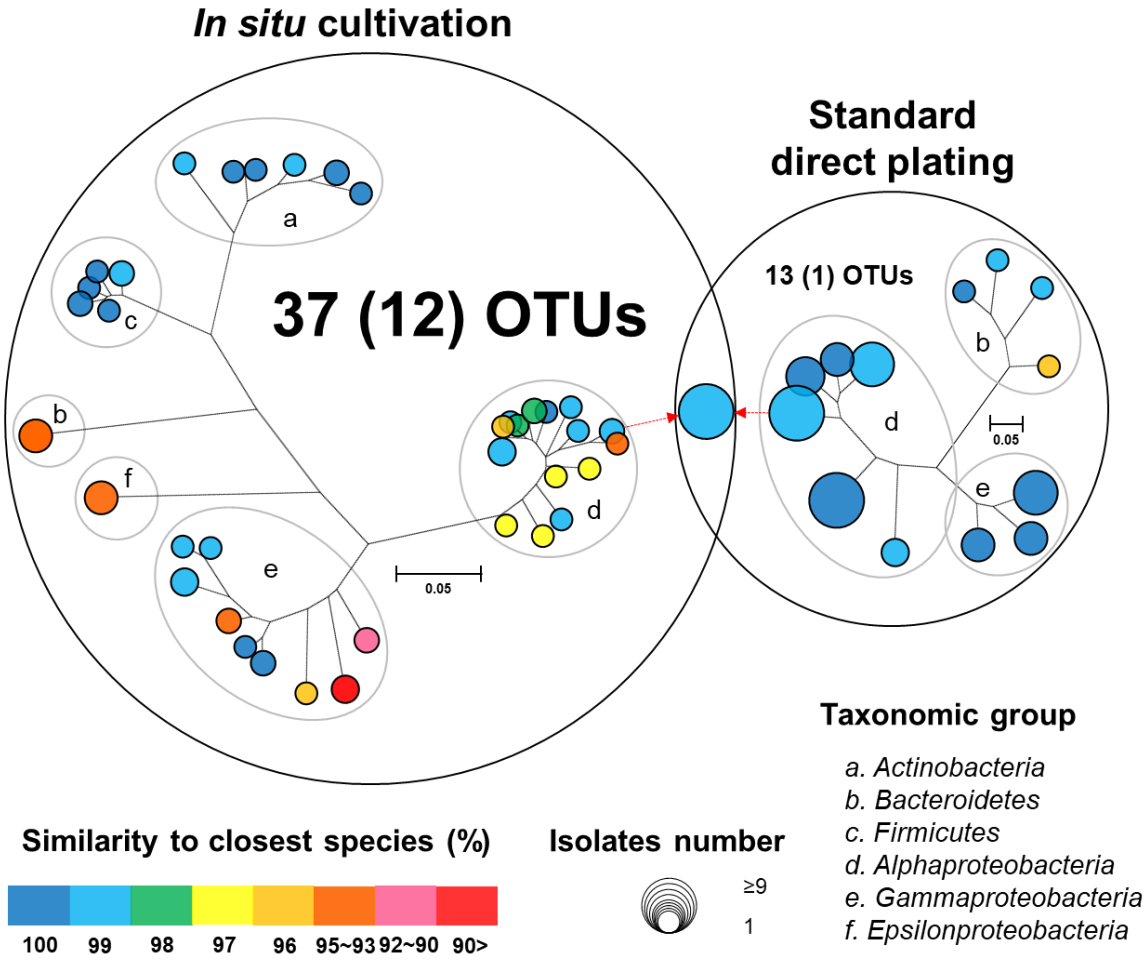
687

688

689

690

691



692

693 Figure 2. Venn diagram consisting of phylogenetic trees based on the 16S rRNA gene of

694 isolates from each cultivation method. The trees are maximum-likelihood trees (fast bootstrap,

1,000 replicates). Circle size and color represent the number of OTUs (defined at 97% 16S rRNA gene sequence identity) and 16S rRNA similarity to the closest known relative in GenBank, respectively. Represented isolates are also grouped into taxonomic groups (a to f) at the phylum level (class level for Alpha, Gamma and Epsilon Proteobacteria) by gray elliptical circles. Circles with pointed arrows indicate species that are overlapped among the isolates in the different cultivation methods. The numbers in parentheses show the numbers of novel species. The outer circle area corresponds to the number of isolated OTUs from each cultivation method.

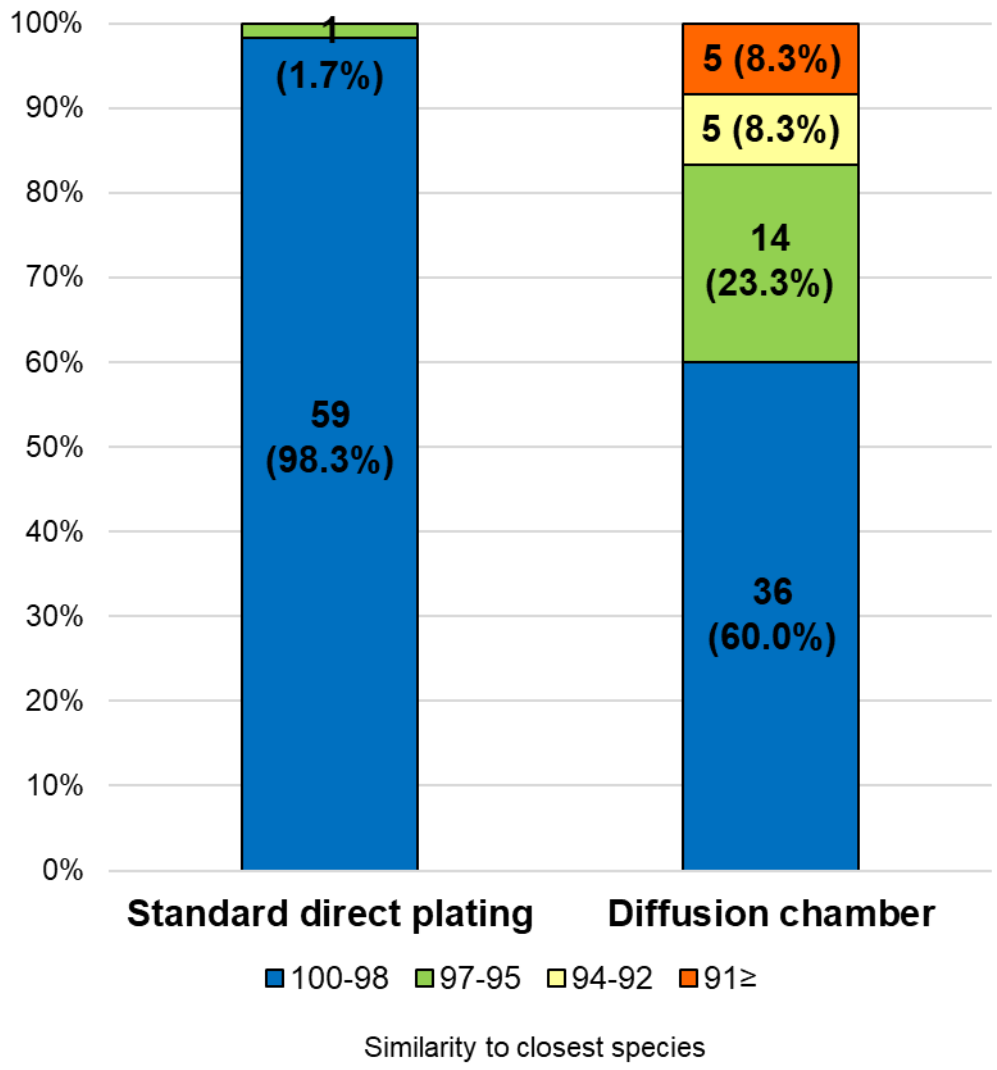


Figure 3. Similarity among the isolates from each cultivation method based on the 16S rRNA gene to the closest known relative in GenBank. Numbers in the bar graphs represent OTUs for each similarity level and its ratio (%).

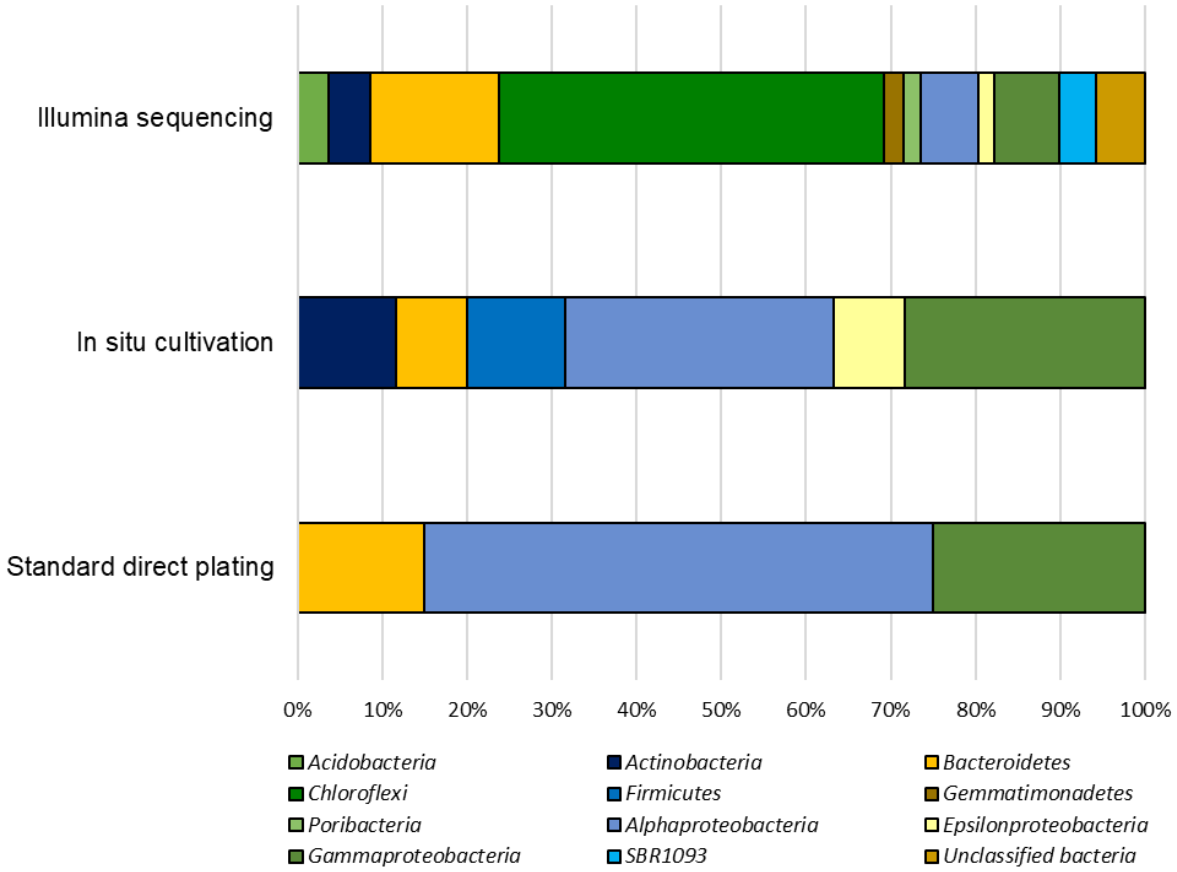
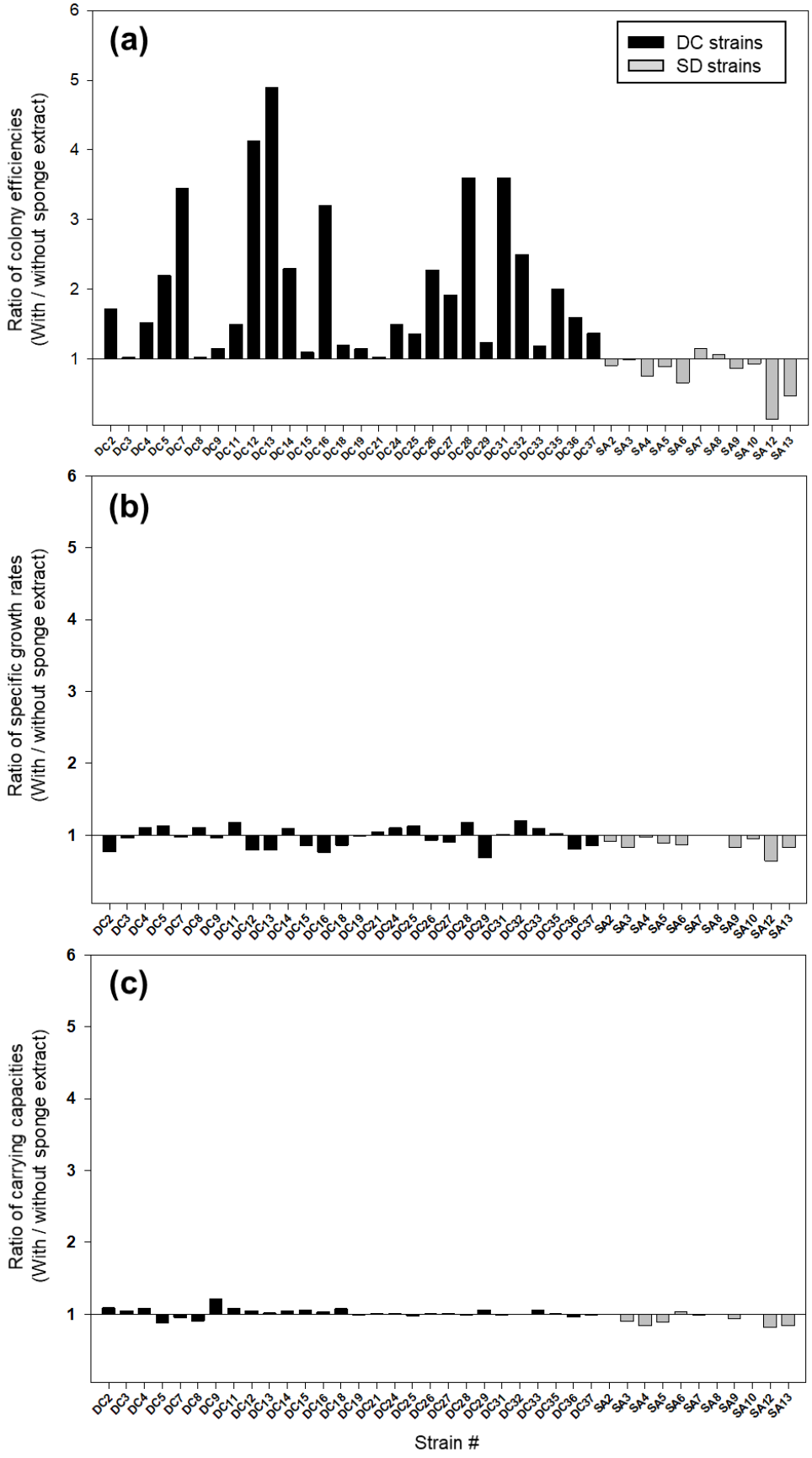


Figure 4. Phylogenetic distribution of 16S rRNA gene sequences of sponge-associated bacteria from the original sample (*Theonella swinhoei*) and isolates obtained by *in situ* cultivation and standard direct plating methods.



722

723 Figure 5. Effect of the sponge extract on starvation recovery (a), specific growth rate (b) and
724 carrying capacity (c) of the *in situ* and SDP strains. The ratio of colony efficiencies between
725 the value measured under two culture conditions (colony numbers on the agar medium with
726 the sponge extract/colony numbers on the agar medium without the sponge extract) was
727 calculated for each selected strain (a). The ratio of the specific growth rate (μ) between the
728 value measured under the two culture conditions (μ with the sponge extract/μ without the
729 sponge extract) was calculated for each tested strain (b). The ratio of the carrying capacity
730 (maximum value of OD600) between the value measured under the two culture conditions
731 (carrying capacity with the sponge extract/ carrying capacity without the sponge extract) was
732 calculated for each tested strain (c).

733

734

735

736

737

738

739

740

741

742

743

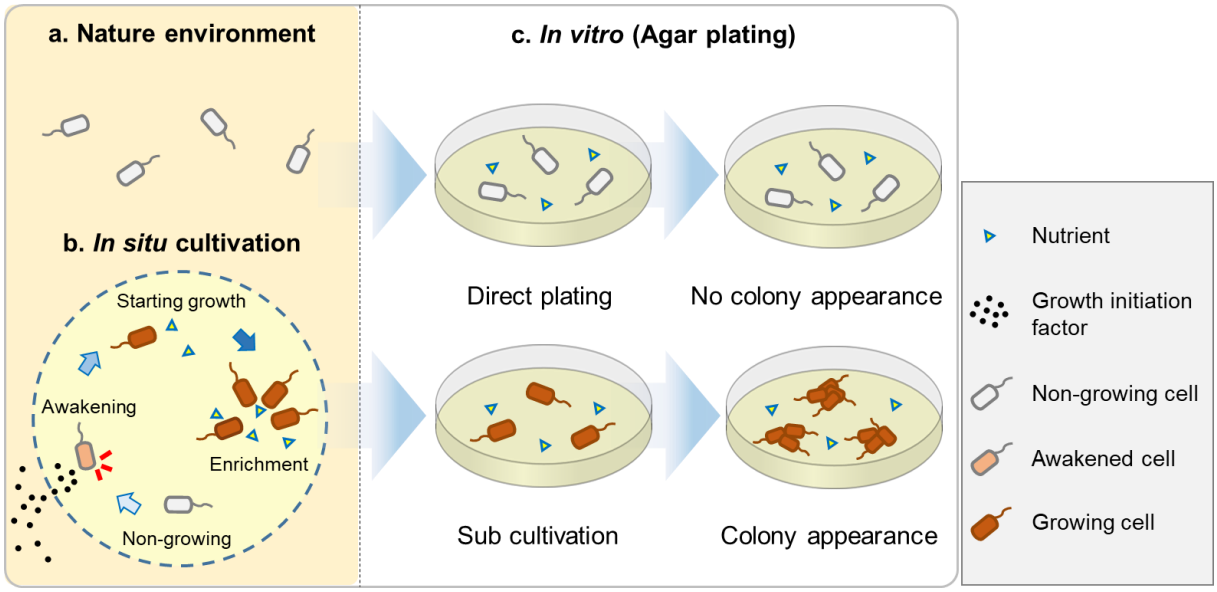


Figure 6. Hypothesis explaining how *in situ* cultivation yields different culture collections from microbial recovery in a natural environment; certain microbial types are non-growing (dormant) to survive unfavorable conditions such as nutrient limited condition (a); during *in situ* cultivation dormant microbes resuscitate from non-growing state stimulated by growth initiation factor from outside environments, then they started to grow and enriched inside supported by sufficient nutrients in the chamber (b); non-growing microbes requiring growth initiation factor for their awakening, do not start to grow when they were directly inoculated on an agar plate, and resulting no visible colony appearance; on the other hand, when they were sub-cultured after the *in situ* cultivation, they continuously grow on an agar plate, and resulting visible colony appearance (c).

Figure Legends

Figure 1. Flowchart of the experimental steps for microbial cultivation from marine sponges (a) and diffusion chamber (DC) cultivation method applied in the present study: a photo showing structure of the chamber (b), a photo showing installation of DC device into the marine sponge (c), a schematic image showing the principle of DC (d).

Figure 2. Venn diagram consisting of phylogenetic trees based on the 16S rRNA gene of isolates from each cultivation method. The trees are maximum-likelihood trees (fast bootstrap, 1,000 replicates). Circle size and color represent the number of OTUs (defined at 97% 16S rRNA gene sequence identity) and 16S rRNA similarity to the closest known relative in GenBank, respectively. Represented isolates are also grouped into taxonomic groups (a to f) at the phylum level (class level for Alpha, Gamma and Epsilon Proteobacteria) by gray elliptical circles. Circles with pointed arrows indicate species that are overlapped among the isolates in the different cultivation methods. The numbers in parentheses show the numbers of novel species. The outer circle area corresponds to the number of isolated OTUs from each cultivation method.

Figure 3. Similarity among the isolates from each cultivation method based on the 16S rRNA gene to the closest known relative in GenBank. Numbers in the bar graphs represent OTUs for each similarity level and its ratio (%).

Figure 4. Phylogenetic distribution of 16S rRNA gene sequences of sponge-associated bacteria from the original sample (*Theonella swinhoei*) and isolates obtained by *in situ* cultivation and standard direct plating methods.

Figure 5. Effect of the sponge extract on starvation recovery (a), specific growth rate (b) and carrying capacity (c) of the *in situ* and SDP strains. The ratio of colony efficiencies between

782 the value measured under two culture conditions (colony numbers on the agar medium with
783 the sponge extract/colony numbers on the agar medium without the sponge extract) was
784 calculated for each selected strain (a). The ratio of the specific growth rate (μ) between the
785 value measured under the two culture conditions (μ) with the sponge extract/μ without the
786 sponge extract) was calculated for each tested strain (b). The ratio of the carrying capacity
787 (maximum value of OD600) between the value measured under the two culture conditions
788 (carrying capacity with the sponge extract/ carrying capacity without the sponge extract) was
789 calculated for each tested strain (c).

790 Figure 6. Hypothesis explaining how *in situ* cultivation yields different culture collections
791 from microbial recovery in a natural environment; certain microbial types are non-growing
792 (dormant) to survive unfavorable conditions such as nutrient limited condition (a); during *in*
793 *situ* cultivation dormant microbes resuscitate from non-growing state stimulated by growth
794 initiation factor provided from outside environments, then they started to grow and enriched
795 inside supported by sufficient nutrients in the chamber (b); non-growing microbes requiring
796 growth initiation factor for their awakening, do not start to grow when they were directly
797 inoculated on an agar plate, and resulting no visible colony appearance; on the other hand,
798 when they were sub-cultured after the *in situ* cultivation, they continuously grow on an agar
799 plate, and resulting visible colony appearance (c).

800

801

802

803

804

805 **Supplemental Materials**

806 Table S1. Phylogenetic affiliations of isolates with the DC method on the basis of 16S rRNA
807 gene sequences.

808 Table S2. Phylogenetic affiliations of isolates with the SDP method on the basis of 16S rRNA
809 gene sequences.

810 Figure S1. The specific growth rate for each tested strain measured under the two culture
811 conditions (the medium with and without the sponge extract).