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Scientific Research

Characterization of an Epiphytic Bacterium *Neptunomonas* sp. BPy-1 on the Gametophytes of a Red Alga *Pyropia yezoensis*

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Abstract

The established culture of gametophytes of the red alga *Pyropia yezoensis* (TU-1) is superficially colonized by epiphytic bacteria. By 16S rRNA sequencing, 6 bacterial species were identified, and a culturable bacterium, *Neptunomonas* sp. BPy-1, was isolated. The 16S rRNA sequences of BPy-1 showed 100% identity with that of *Neptunomonas* sp. 0536, a probiotic bacterium found in greenshell mussels in New Zealand. Physiological tests revealed that 22 characters were identical between BPy-1 and 0536, but that 4 characters differed. BPy-1 cannot grow in the artificial seawater used for the culture of gametophytes. BPy-1 can grow in the artificial seawater with ethanol or butanol but not in methanol or propanol. To determine the effect of BPy-1 on gametophyte growth, BPy-1 colonization was reduced by 80% using a multi-enzyme cleaner. Changing the cleaner concentration yielded two types of gametophytes, a compressed or callus-like form and a nearly normal form. BPy-1 promoted the growth of the treated gametophytes with relatively normal form, while it showed less effect on compressed gametophytes. These findings suggested that BPy-1 promotes the growth of damaged gametophytes but does not affect the development of normal gametophytes.

Keywords

***Neptunomonas*, *Pyropia yezoensis*, Ethanol-Eating Bacteria, Epiphytic Bacteria**

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1. Introduction

The red alga *Pyropia yezoensis* has been extensively cultivated in many Asian countries, especially Japan. In addition to its industrial importance, *P. yezoensis* is currently regarded as a model plant in marine sciences due to its short life cycle, small genome size, and availability for *in vitro* culture [1] [2]. For molecular biological studies, 20,779 EST clones were isolated from gametophytes and sporophytes of *P. yezoensis* strain TU-1 (http://est.kazusa.or.jp/en/plant/porphyra/EST/index.html). More recently, draft genome sequences were identified using gametophytes of *P. yezoensis* U-51 strain [3]. Unlike seed plants, which are cultured in isolation, the gametophytes of *P. yezoensis* are maintained with epiphytic bacteria in culture because aseptically grown gametophytes do not development the normal leafy form required to generate a callus-like mass [4]. From the gametophytes of strain U-51, 32 bacterial 16S rRNA gene sequences were identified [5] and several *Hyphomonas* strains capable of inducing normal morphogenesis in protoplast of *P. yezoensis* were isolated [6]. Various beneficial effects of epiphytic bacteria on macroalgae were proposed [7], including growth promotion, induction of normal development, and support of zoospore settlement. However, direct evidence for these effects using *in vitro* culture is limited.

Although epiphytic bacteria are colonized on gametophytes of *P. yezoensis*, they do not overgrow in the usual culture media, such as artificial seawater. In our laboratory, gametophytes of strain TU-1 have been maintained in aseptic medium without overgrowth of epiphytic bacteria for more than 10 years. However, seven years ago upon building renovation, the gametophyte cultures turned into a milky suspension when incubated in rooms in which other plants were maintained. Although the gametophytes of *P. yezoensis* were cultured with continuous air bubbling, contamination by environmental bacteria was impossible, because the air was filtrated with sterilized filter. However, contamination by volatile organic compounds from indoor air was possible. A preliminary analysis of the indoor air by GC-MS and isolation of bacteria from troubled cultures suggested that the gametophyte cultures were contaminated by bubbles of ethanol-polluted air from the culture room and that the trouble-causing bacterium was an epiphytic bacterium of gametophytes. In this study, we show that a new epiphytic bacterial strain isolated from gametophytes of *P. yezoensis* promotes the growth of damaged gametophytes.

2. Materials and Methods

2.1. Gametophyte Culture

Gametophytic blades of *P. yezoensis* (strain TU-1) were grown in artificial seawater (ASW) (Sealife, Marinetech Co., Ltd.) supplemented with 1% ESS_2 stock solution [8], with the concentration of nitrate changed to 2.8 mM. ESS2 stock solution consisted of 420 µM HEPES, 37 µM disodium glycerophosphate, 7.8 µM Fe-EDTA, 120 nM KI, 10.7 µM Na_2EDTA, 0.7 µM $FeCl_3$, 74 µM H_3BO_3, 2.9 µM $MnCl_2$, 0.31 µM $ZnCl_2$, 68 nM $CoCl_2$ and vitamins (0.74 µM vitamin B12, 409 µM biotin, 0.96 µM thiamine-HCl, 81 µM nicotinic acid, 21 µM Ca-pantotheate, 7.29 µM *p*-aminobenzoic acid, 555 µM myo-inositol, 79 µM thymine). Cultures were maintained with constant bubbling of air (sterilized using a filter within the plant growth camber) at 15˚C on a photocycle of 10 h of light (intensity of 30 µmol $m^{-2}\cdot s^{-1}$):14 h of dark. The culture medium was refreshed weekly.

2.2. Isolation of Bacterial DNA from the Gametophytes

Epiphytic bacterial DNA was isolated by the method of Burke *et al.* [9] with some modifications. Gametophytes (100 mg fresh weight) were briefly washed with artificial seawater, blotted with filter paper, cut into ~5 mm pieces, and incubated with 900 µl of calcium- and magnesium-free ASW (CMFSW) containing 0.45 M NaCl, 7 mM Na_2SO_4, and 10 mM $NaHCO_3$ supplemented with 10 mM EDTA and 1% filter-sterilized rapid multi-enzyme cleaner (70508A, 3M Health Care Ltd.) in a 1.5 ml microtube. The tube was shaken at 80 rpm at room temperature for 2 h, vortexed for 2 min, and gametophyte fragments were discarded. To remove any residual cell debris, the remaining suspension was centrifuged at 300 × *g* for 15 min. The supernatant was treated with RNase A and DNA was extracted using phenol/chloroform/isoamylalcohol (25:24:1). The DNA was purified using the same phenol/chloroform/isoamylalcohol mixture and stored in TE solution. For the extraction of genomic DNA from isolated bacterial clones, each clone was cultured in liquid Marine Broth medium with reciprocal shaking 130 rpm at 32˚C. After incubation for overnight, the suspension was centrifuged at 10,000 × *g* for 10 min at room temperature. The collected bacteria were suspended in 900 µl of CMFSW supplemented with the ions described above; and bacterial DNA was isolated as described above.

2.3. PCR Amplification of 16S rRNA Genes

16S rRNA genes were amplified by PCR using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [10] or the primers 75F (5'-ATGCAAGTCGAACGGTAACA-3') and 1492R [5]. After the electrophoresis, bands of the expected size (1.5 kb) were purified (Wizard SV Gel and PCR Clean-Up System, Promega) and ligated into the T-vector pMD20 (Takara, Japan). Plasmid DNA harboring the amplified sequence was prepared using the Wizard Plus SV Minipreps DNA Purification System (Promega) and used as the templates for sequencing by an external contractor (SolGent Co., Ltd., Korea). 16S rRNA gene sequences obtained in this study were deposited into GenBank (http://www.ncbi.nlm.nih.gov/) with accession number BPy-1 (AB751619), BPyGA1 (AB751620), BPyGA2 (AB751623), BPyGB5 (AB751622), BPyA3 (LC002976) and BPyG-1 (LC002975). 16S rRNA gene sequences were aligned using ClustalW. A phylogenetic tree was constructed using MEGA 5.

2.4. Analysis of the Indoor Air by GC-MS

Air samples (100 l) from rooms containing contaminated and non-contaminated cultures were collected on charcoal filters using a GSP-2LFT sampler (Gas Tech, Japan) over a 2 h period. The volatile organic compounds collected were subjected to GS-MS using a PerkinElmer Elite 624 column (PerkinElmer, USA). The flow was initiated at 40˚C (5 min) and increased by 5˚C /min until reaching 200˚C using helium gas.

2.5. Phenotypic Profiling of BPy-1

Tests for oxidase, motility, acid or gas production from glucose, oxidation-fermentation, and catalase, as well as Gram staining, were carried out according to Barrow and Feltham [11]. The remaining 21 tests employed API 20NE strips (BioMérieux, Hazelwood, MO, USA). BPy-1 was further profiled for phenotypic characteristics, including temperature growth range (4˚C, 37˚C and 45˚C), salt tolerance (7% and 8%), pH (5, 7, and 9), anaerobic growth, and lipase activity (TechnoSuruga Laboratory Co., Ltd.).

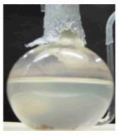
2.6. Preparation of Low-Bacteria Gametophytes and Co-Culture Experiments

The gametophytes were gently washed with ASW medium and aliquots (200 mg fresh weight) were incubated in 1 ml of varying concentration of multi-enzyme solution for 20 min with shaking at 80 rpm at room temperature. After vortexing for 30 s, the gametophytes were transferred to a new 50-ml tube and washed twice with ASW. The treated gametophytes were maintained in normal ASW. For the co-culture experiments, BPy-1 was added to cultures of the low-bacteria gametophytes and incubated for 2 - 3 weeks. The growth of epiphytic bacteria on gametophytes was measured by suspending normal and low-bacteria gametophytes in ASW with quartz sand, vortexing for 10 min at 180 rpm at room temperature, spreading on agar (MB or ASW with ethanol), and counting the colonies. The bacterial composition of the low-bacteria gametophytes was determined by analyzing the 16SrRNA sequences of each colony (grown on MB agar after vortexing and incubating in MB liquid medium overnight at 23˚C). The effect of BPy-1 on gametophyte growth was examined by incubating BPy-1 (10 µl, freshly prepared by incubation in liquid MB medium, concentration adjusted to 0.2 OD_{600}) with normal and low-bacteria gametophyte fragments (5 mm; 5 per well in 6 ml of ASW) on a rotary shaker at 85 rpm under the condition used for gametophyte culture.

3. Results

3.1. Isolation of Overgrowth Bacteria from Pyropia Gametophyte Culture

Upon noticing that *Pyropia yezoensis* (TU-1) gametophyte cultures became cloudy if the incubators were moved to certain rooms (**Figure 1(a)** and **Figure 1(b)**), we analyzed the air in the culture rooms by GC-MS, which revealed that ethanol (1.6 ppm) was present in the room where clouded culture was observed but not in those where the culture remained clear. Therefore, it was expected that overgrowth bacteria might be ethanol-eating epiphytic bacteria. The addition of 0.1% ethanol to gametophyte cultures in the clean room led to bacterial overgrowth after incubation for several days, as observed in the room where clouded culture was observed (**Figure 1(c)**). To determine the composition of epiphytic bacteria, bacterial DNA was isolated from the gametophytes grown under normal conditions and 16S rRNA gene sequences were amplified using the primers

Gametophyte cultures under different conditions			
Culture type	(a)	(b)	(c)
Indoor air quality	Normal	Polluted	Normal
Culture medium	ASW	ASW	ASW +Ethanol
Culture image			

Identification of epiphytic bacteria			
	Number of colony		
Clone name (taxon)	Gametophytes of type (a)	Medium of type (b)	Medium of type (c)
BPyGA2 (Sphingobacteria)	-	-	-
BPyG-1 (Flavobacteria)	-	-	-
BPyA3 (Y-Proteobacteria)	-	-	-
BPy-1 (Y-Proteobacteria)	25	25	18
BPyGA1 (δ-proteobacteria)	-	-	-
BPyGB5 (β-Proteobacteria)	-	-	-

Figure 1. Gametophyte cultures under different conditions (upper) and identification of epiphytic bacteria (lower panel). Gametophytes were cultured in rooms with unpolluted (a) (c) and ethanol-polluted (b) air. In the culture (c), 0.1% ethanol was supplemented with ASW medium. A bacterial analysis employed gametophytes (a) and culture media (b) (c). Epiphytic bacteria released from gametrophytes (a) and overgrowth bacteria in the media (b) (c) were cultured on MB agar at 37˚C, and 16S rRNA sequences of each colony was analyzed.

27F/1492R [10] and 75F/1492R [5]. Of 72 clones, 12 were identified as chloroplast 16SrRNA gene of *P. yezoensis*, and the remaining 60 consisted of 6 bacteria species. Although the isolation frequencies of six species differed with varying experimental conditions, such as the PCR primers used and gametophyte age, multiple species were detected in each analysis. However, only BPy-1 could grow on MB agar at 37˚C. When culture medium from the room (b) or (c) was spread on MB agar and incubated at 37˚C, all colonies were identified as BPy-1, suggesting that the growth of BPy-1 is restricted on normal gametophytes, and that it grows as a free-living bacterium under ethanol-enriched conditions.

The 16S rRNA gene sequences of BPy-1 (1463 bp) were identical to that of a γ-proteobacterium *Neptunomonas* sp. 0536, which was newly isolated as the probiotics from the green mussel aquaculture in New Zealand [12]. *Neptunomonas* is a relatively new genus, proposed by Hedlund *et al.* [13] in a study that demonstrated the ability of *N. naphthavorans* to degrade many polycyclic aromatic hydrocarbons. A phylogenetic tree of *Neptunomonas* species was constructed based on 16SrRNA sequences (**Figure 2**). BPy-1 was found to belong a clade that included recently identified species, which were classified based on their location and environment [*Neptunomonas* sp. 0536 (green mussels in New Zealand), sp. VSG922 (hydrothermal vent of Espalamaca, Azores), and sp. S1 (seawater in Korea)]. Although other *Neptunomonas* species have been isolated in various places, this group is most related to *N. naphthavorans*.

The physiological characteristics of BPy-1 were compared with those of *Neptunomonas* sp. 0536 (**Table 1**). Although 20 of BPy-1's characteristics resembled those of *Neptunomonas* sp. 0536, 4 were different. A sample of BPy-1 was deposited at the NITE Biological Resource Center (*Neptunomonas* sp. BPy-1, ID: NBRC 108560).

3.2. Carbon Sources of BPy-1

As shown in **Figure 3(a)**, BPy-1 grew well in both shaken and standing cultures in MB medium, consistent with the mobility of BPy-1 (**Table 1**). The gametophyte culture medium consisted of commercial ASW supple-

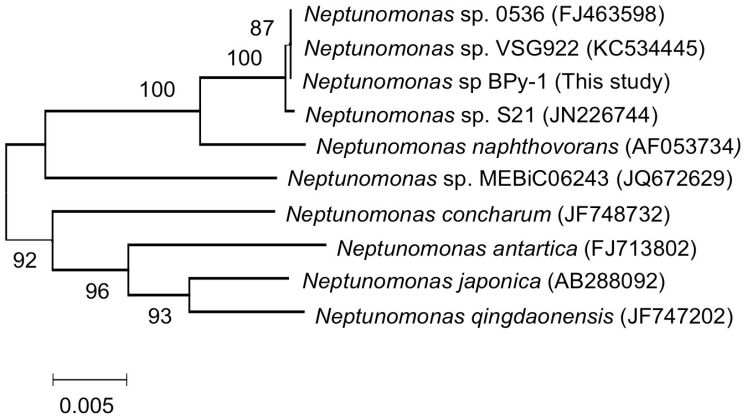


Figure 2. Phylogenetic tree of 16S rRNA gene sequences of *Neptunomonas* species. A neighbor-joining phylogram analysis was performed using ClustalW with MEGA5 software. Bootstrap values based on 1000 replications are provided at the nodes. The values less than 50 were removed. Accession numbers are shown in parentheses.

Table 1. Comparison of physiological traits of two *Neptunomonas* bacteria.

Test	*Neptunomonas* sp. BPy-1	*Neptunomonas* sp. 0536
Gram	−	−
Motility	+	+
Oxidase reaction	+	+
Cytochrome oxidase	+	+
Catalase reaction	+	+
NaCl tolerance	7% (+), 8% (−)	0.5% - 7% (+)
Temperature growth range	4˚C (−), 37˚C (+), 45˚C (−)	17˚C - 40˚C (+)
Gelatin liquefaction	−	+
Maltose assimilation	−	+
Glucose acidification	−	+
Citrate assimilation	+	−
Indole production, nitrate reduction, glucose assimilation, N-acetyl-glucosamine assimilation, arabinose assimilation, mannose assimilation, mannitol assimilation, capric acid assimilation, arginine dihydrolase, urea hydrolysis, esculin hydrolysis, ONPG, potassium gluconate assimilation, adipic assimilation, malate assimilation, phenylacetic acid assimilation	−	−

mented with ESS_2 nutrient solution, which includes nitrate, some metals, phosphate and vitamins. BPy-1 did not grow in liquid ASW medium (**Figure 3(a)**), but did grow on ASW agar (data not shown), suggesting that BPy-1 grows under carbon-limiting conditions similar to oligotrophic bacteria.

To examine alcohol utilization by BPy-1, BPy-1 was cultured in ASW supplemented with different alcohol species and concentrations (**Figure 3(b)** and **Figure 3(c)**). BPy-1 grew in medium with ethanol and butanol, but not in the medium with methanol and propanol. Maximum growth was observed in 1% ethanol and 0.3% butanol. BPy-1 continued to produce the same culture yield upon repeated (three times) subculture in medium containing ethanol and butanol, suggesting that the strain can use ethanol or butanol as its sole carbon source.

3.3. Promotion of Damaged Gametophyte Growth by BPy-1

To assess the effect of BPy-1 on gametophytes, bacteria-less gametophytes were prepared using a multi-enzyme

cleaner normally used to isolate bacterial DNA (**Figure 4**). To determine the effect of enzyme treatment on the amount of epiphytic bacteria, CFUs were counted upon culture on agar with ASW plus 0.1% ethanol. Since only BPy-1 was culturable under these conditions, this value likely reflects the BPy-1 concentration. Three treatments using varying concentrations of the enzyme solution reduced the amount of BPy-1 by as much as 60%.

To determine the damage done to the gametophytes by enzyme treatment, microscopic images of cells of treated and untreated gametophytes were compared (**Figure 5**). In contrast to the amount of residual epiphytic bacteria, damage done to the gametophyte cells differed by varying the concentration of the enzyme solution.

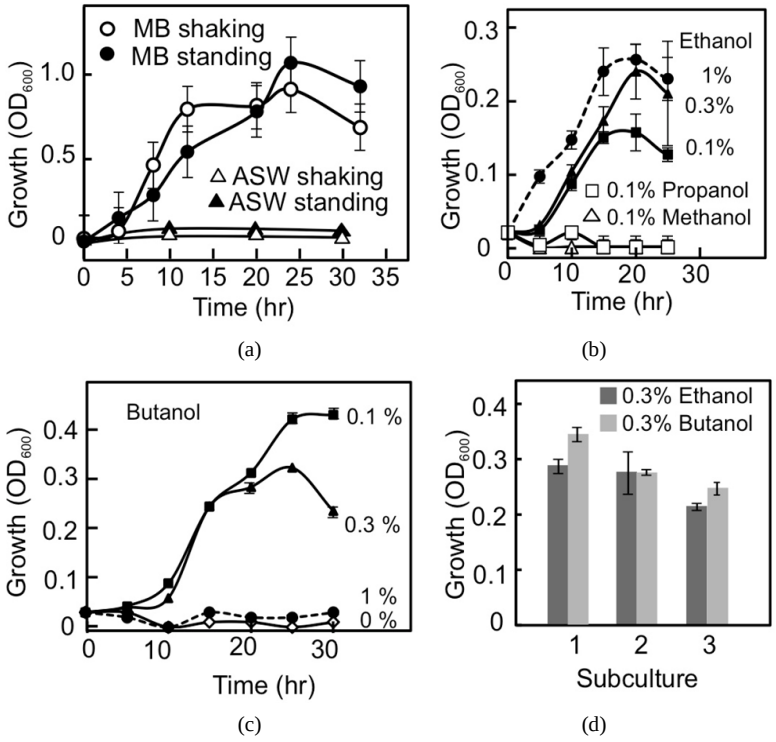


Figure 3. Growth properties of BPy-1. (a) BPy-1 cultured in liquid MB medium was inoculated into fresh MB and ASW at 0.02 OD_{600} and incubated at 37˚C (for MB) or 23˚C (ASW) with shaking (125 rpm) or standing. (b) (c) Growth of BPy-1 in liquid ASW media including varying concentrations of ethanol, 0.1% propanol, or 0.1% methanol at 23˚C and 125 rpm. (d) BPy-1 was subcultured three times (inoculated at 0.02 OD_{600} each time) in fresh ASW medium with 0.3% ethanol or butanol by at 23˚C and 125 rpm for 1 day. All values are the mean (n = 3) ± standard error.

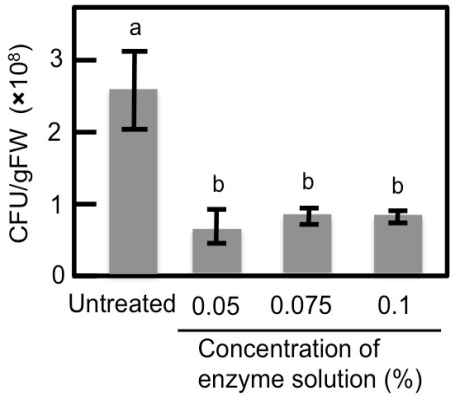


Figure 4. CFUs on ASW agar with 0.1% ethanol from enzyme-treated gametophytes (incubated at 37˚C). Each data point is the mean (n = 3) ± standard error. Data points labeled with different letters are significantly different based on Tukey's HSD test ($P < 0.05$).

Star-shaped chloroplasts were observed in untreated cells (**Figure 5(a)** and **Figure 5(e)**). Just after treatment with 0.05% multi-enzyme solution, the vacuoles were swollen and the cell volumes increased (**Figure 5(b)**). Cells in the partial region had shrunk, and the major cellular space was occupied by chloroplasts. After treatment with 0.075% multi-enzyme solution, the shrunken cells with cellular spaces occupied by chloroplasts expanded (**Figure 5(c)**). Treatment with 0.1% solution seemed to cause cell death (**Figure 5(d)**). After incubation of gametophytes for six days, cells of gametophytes treated with 0.05% solution recovered (**Figure 5(f)**). On the other hand, cells of gametophytes treated with 0.075% solution became either dead cells or irregularly dividing cells (**Figure 5(g)**). After incubation for 3 weeks, gametophytes consisted of multi-layered or callus-like cells (data not shown). After incubation of gametophytes treated with 0.1% solution for several days, gametophyte cells changed to white (**Figure 5(h)**). These results suggest that treatment with 0.05% and 0.075% multi-enzyme solution caused relatively mild and severe damage to the gametophytes, respectively.

The surviving gametophytes grew slower and had an abnormal morphology, although the concentration of enzyme solution causing these effects varied among gametophytes (**Figure 6**). Treatment with 0.075% enzyme solution yielded compressed (or callus-like) gametophytes, named c-LBG (compressed lower bacteria gametophyte), while 0.05% led to gametophytes with a relatively normal morphology, named n-LBG (normal LBG). Incubating these gametophytes with varying concentrations of BPy-1 partially promoted their growth. In the case of n-LBG, the positive effect of BPy-1 was observed after one week of incubation at all concentrations of BPy-1 (**Figure 6(a)**) and continued (**Figure 6(b)**). On the other hand, c-LBG was less affected by BPy-1. BPy-1 did not promote the growth of untreated gametophytes (data not shown). Neither boiled BPy-1 nor medium from BPy-1 cultures promoted the growth of n-LBG (data not shown), suggesting that attachment of BPy-1 on the gametophyte may be required for growth promotion.

4. Discussion and Conclusions

Neptunomonas sp. BPy-1 is distinguished by two prominent features: ethanol utilization as the sole carbon source (**Figure 3**) and the promotion of host plant growth (**Figure 6**). While the physiological properties of *Neptunomonas* species have been reported, none have been shown to utilize ethanol, generally produced as the final products by fermentation under anaerobic environment. Although ethanol utilization in bacteria is rare, ethanol-eating bacteria have unique metabolic properties. For example, *Pseudomonas fluorescens* strain S227 produces antifungal antibiotics when grown on ethanol as a sole carbon source [14]. As shown in **Figure 3(c)**, **Figure 3(d)**, BPy-1 can also use butanol as a carbon source. Up to now, reports on n-butanol degradation have been limited. In anaerobic bacteria, butanol is used for energy production during fermentation [15]. *Enterobacter* sp. VKGH12 grows in mineral medium with up to 0.4% 1-butanol under aerobic conditions, but its growth is inhibited at higher concentrations [16]. Considering that BPy-1 can use 0.3% butanol as its sole carbon source, BPy-1 has a higher tolerance to butanol toxicity.

As shown in **Figure 6**, high concentration of BPy-1 did not inhibit gametophyte growth, suggesting that BPy-1 uses a limited amount of host nutrients, although it seems to use the host cell wall as a carbon source. However, under ethanol-enriched conditions, BPy-1 proliferates as a free-living bacterium. The average concentration of

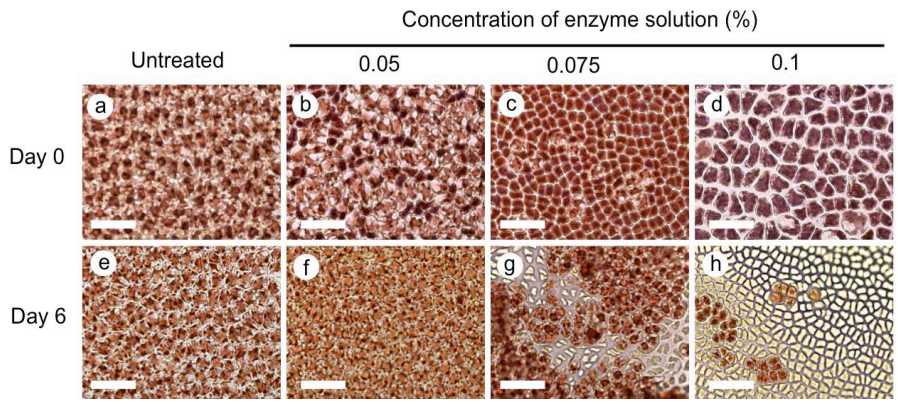


Figure 5. Microscopic image of gametophyte cells. Photos were captured just after treatment with varying concentrations of enzyme solution and after incubation of gametophytes for 6 days. Scale bars 50 μm.

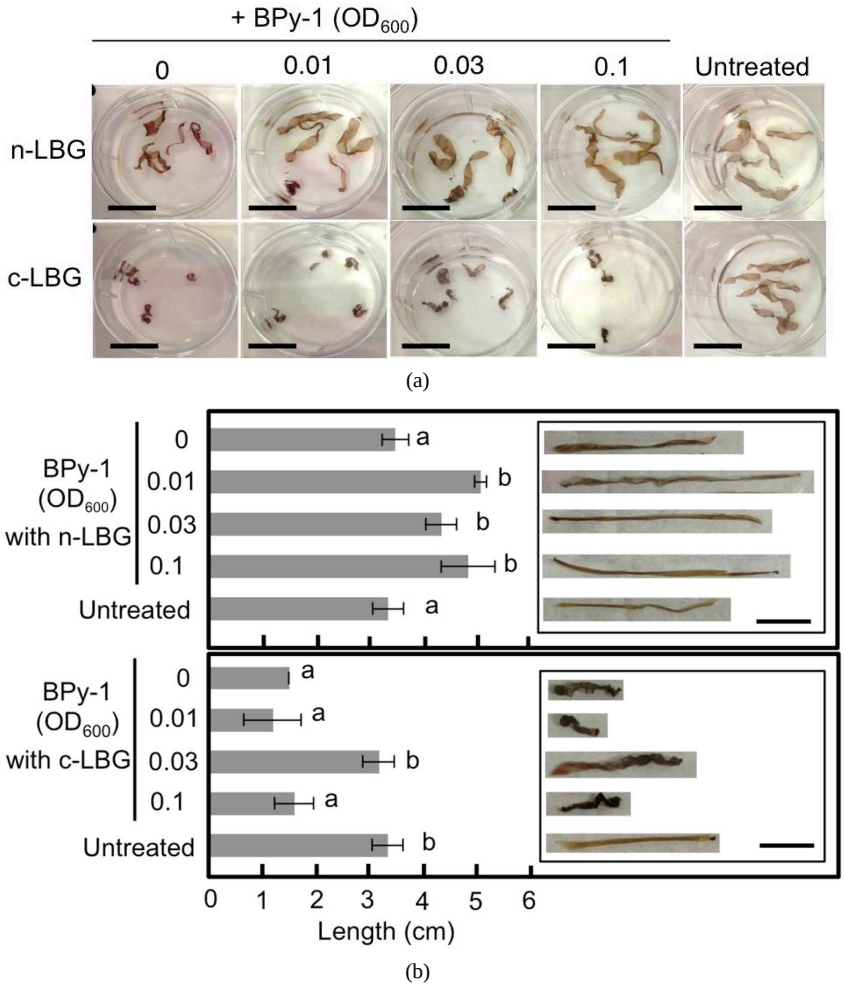


Figure 6. Co-culture of low-bacteria gametophytes and BPy-l. Morphology and size of damaged gametophytes (n-LBG and c-LBG; prepared by enzyme treatment) following co-culture with varying concentrations of BPy-1 in fresh ASW medium for 8 (a) or 21 days (b). Five gametophyte fragments (5 mm) were incubated with BPy-1, shaken at 85 rpm under the same conditions used for gametophyte culture. Each data point is the mean (n = 5) ± standard error. Data points labeled with different letters are significantly different based on Tukey's HSD test ($P < 0.05$). Scale bars 1 cm.

ethanol in indoor air ranges 0.03 - 0.1 ppm [17], making it the most prevalent indoor air pollutant. We detected 1.6 ppm ethanol in the BPy-1 overgrowth-causing room but not in the room where cloudy cultures were not observed. Considering the usual concentration of ethanol in indoor air, it is probable that trace amount of ethanol might sometimes contaminate cultures even when overgrowth is not noticeable, which could contribute to the long-term survival of BPy-1 in gametophyte culture. These epiphytes may also colonize *Pyropia* in their natural oceanic habitat, where they are sometimes subjected to anaerobic conditions by phytoplankton blooms [18].

The second unique feature of BPy-1 is the promotion of host plant growth (**Figure 6**). Recently, several *Hyphomonas* strains isolated from gametophytes of *P. yezoensis* were reported to induce normal morphogenesis in protoplasts [6]. Since the protoplasts were nearly free from bacteria in that experiment, the effect of bacteria was clear. On the other hand, more complex effects of epiphytic bacteria on settlement of zoospore of the host algae were reported [19]; specifically, settlement was dependent on the bacterial density and the threshold of the bacterial density was indicated. In addition, the effect was also dependent on particular species or clones. Similarly, our results should be interpreted with caution. First, the enzyme cleaner affected the viability of the gametophytes and epiphytic bacteria; the host plants were partially damaged and bacteria were partially eliminated (**Figure 4** and **Figure 5**). Second, treatment with 0.05% and 0.075% enzyme solution induced different types of damage in gametophytes (**Figure 5**). Third, the residual amount of BPy-1 on gametophytes was the same fol-

lowing the two treatments (**Figure 4**), but the response of other epiphytes remains unknown. Considering this, the recovery of weakly damaged gametophytes (n-LBG) by BPy-1 may involve the direct promotion of gametophyte repair or indirect effects caused by a change in the composition of other epiphytic bacteria. In contrast to its effect on n-LBG, BPy-1 only slightly improved the morphology of the more severely damaged gametophytes (c-LBG). This small effect suggests that other epiphytic bacteria inducing normal gametophyte were also eliminated by the higher enzyme concentration. If so, recovery of the severely damaged gametophyte would include two steps, the induction of normal development followed by growth promotion, and BPy-1 may contribute to the latter step. For further characterization of physiological role of BPy-1 will require the isolation of other epiphytic bacteria.

Neptunomonas is a relatively new genus, proposed by Hedlund *et al.* [13] in a study of *N. naphthavorans* upon isolation from creosote-contaminated estuary sediment in US, which uses many polycyclic aromatic hydrocarbons as the carbon source. On the other hand, *Neptunomonas* sp. 0536 was isolated as a novel probiotics for green mussel aquaculture in New Zealand; this strain protects green mussel larvae against pathogenic bacteria, the first evidence of a probiotic role for this genus. Recently, *Neptunomonas* species closely related to BPy-1 were isolated from seawater in Korea (*Neptunomonas* sp. S21) and shallow water hydrothermal vent near Espalamaca, Azores (*Neptunomonas* sp. VSG922) (**Figure 2**). Although *Neptunomonas* four clones, BPy-1, 0356, N21, and VSG922, were grouped into the same clade in the phylogenetic tree, they are distributed worldwide under very different conditions, suggesting that this group has broad physiological flexibility. The recent deterioration in coastal environment has threatened various marine organisms, increasing the importance of isolating and characterizing beneficial bacteria from aquacultures [7] [20]. Considering the probiotic role of *Neptunomonas* sp. 0536, further characterization of the association of BPy-1 and its relatives to macroalgae and marine animals will provide important information regarding related bacteria from the macroalgae and marine animals will provide important information.

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