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ISOLATION OF *PHOTOBACTERIUM KISHITANII* TAIGALEON FROM A LOCAL FISH MEHIKARI (GREENEYE) FOUND NEAR IWAKI CITY JAPAN, AND POSSIBLE APPLICATION FOR WATER QUALITY ASSESSMENT

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Abstract – *Chlorophthalmus* is a greeneye genus of deep-sea fish harboring symbiotic luminous bacteria near their anus. *Chlorophthalmus borealis* called "*Mehikari*" in Iwaki City (Fukushima Prefecture, Japan) is a symbol of the City but its morphological and ecological features are poorly understood, including the generality of this symbiosis with luminous bacteria. We performed molecular phylogenetic analysis of a symbiotic luminescent bacterial strain isolated from *C. borealis* collected near the coast of Fukushima. The results showed that the luminous bacterium was *Photobacterium kishitanii*, and a novel culture strain was established as *P. kishitanii* Taigaleon. The emission intensity of *P. kishitanii* Taigaleon, an emission maximum at 475 nm, differed depending on the age and state of the culture as determined by confocal laser microscopy and fluorescence spectrophotometer. In this study we tried to detect and evaluate the harm of ammonia by biological assays using living-whole cell *P. kishitanii* Taigaleon, because they respond to external factors and their emission intensity changes. In this paper, we discuss the possibility of using *P. kishitanii* Taigaleon to assess water samples for harmful ammonia.

INTRODUCTION

Iwaki City is located on the southeast coast of Fukushima Prefecture in the Tohoku region of Japan (Fig. 1). The greeneye species *Chlorophthalmus borealis* is distributed in the deep-sea near Iwaki and is locally known as "Mehikari," after their apparently shining eyes (Fig. 2). However, the fish in fact emit pale blue light due to the luminous symbiotic bacteria residing near the anus (Somiya, 1977). *Chlorophthalmus borealis* is an important fish for the citizens of Iwaki because it is part of their food culture and because it is used as a tourism mascot and appears in souvenirs. However, the ecological and morphological features of *C. borealis* remain poorly understood throughout its habitat range. Therefore, one of aims of this report is to provide information about *C. borealis* and the symbiotic bacteria.

Chlorophthalmus borealis is most similar to *C. albatrossis* morphologically, and they have been reported to be genetically identical (Saruwatari *et al*., 2005). However, in some reports, for example Nakabo (2002) and Kobylyanskii (2013), both species are regarded as distinct species. Around Japanese water, it is thought that *C. albatrossis* is distributed southward from Sagami Bay, and *C. borealis* is distributed northward from Choshi City, Chiba Prefecture (Nakabo, 2002). Hence, in this study, we considered the greeneye collected near off Iwaki as *C. borealis*.

Numerous luminous organisms, including invertebrates, have been identified in deep-sea environments (Widder, 2010; Haddock *et al*., 2010; Hellinger *et al*., 2017). There are two types of luminous fish: those that emit light themselves, and those whose luminescence is dependent on symbionts. *Chlorophthalmus* species were reported to have luminous bacteria as symbionts in a bag-like structure near the anus and their luminescence is

dependent on these symbionts (Somiya, 1977). The bioluminescent light is based on oxidation of a light emitting luciferin in the presence of oxygen and the enzyme luciferin (Widder *et al*., 2010; Hellinger *et al*., 2017). Symbiosis between luminous bacteria and deep-sea fish is well known and has been researched for a century. *Photobacterium kishitanii* used in this study were firstly described by Ast*et al*., 2007. They found in association with marine fishes and the morphological features were described as follows: gram-negative, catalase-positive, and weakly oxidative-positive or oxidative-negative (Ast *et al*., 2007). The cells were coccoid or coccoid-rods, occurring singly or in pairs, and motile by flagella (Ast *et al*., 2007).

Biosensors using luminous bacteria were first developed in 2000 in Europe (Girotti *et al*., 2008). For example, the toxicity of many organic compounds is evaluated by *in vivo* experiments using luminescent bacteria directly (Ren and Frymier, 2002); examples include evaluation of the toxicity of tritium (Selivanova *et al*., 2013) and phenol (Kudryasheva *et al*., 2002). Luminous bacteria are also used in applied experiments by incorporating them into chips used as a detection system (Yoo *et al*., 2007) as well as by freeze-drying them (Choi and Gu, 2002). One example of applied research is the detection of metal ions by transfecting a plasmid containing genes encoding the bacterial protein responsible for luminescence into *Escherichia coli* (Ivask *et al*., 2009). Studies utilizing luminous bacteria as applied tools were reviewed by Girotti *et al*. (2008). Despite many applications of luminous bacteria for assessing toxicity, this approach has not been applied to detecting or evaluating the toxicity of ammonia, which has been reported to impair the ability of aquatic animals to survive (Camargo and Alonso, 2006), in seawater. Therefore, the another scope of this study is to develop the method to evaluate the harm of ammonia for water assessment. Today, the analytical methods such as high-performance liquid chromatography (HPLC) and gas chromatography coupled with mass spectrometry (GC/MS) for water assessment including detection and quantify toxic chemicals, however, these methods have proved to be time-consuming, expensive, and difficult to perform (Xu *et al*., 2014). In addition, they cannot measure bioavailability and biological impact (Xu *et al*., 2014). On the other hand, the biological assay by using luminous bacteria can provide more biologically relevant information (Xu *et al*., 2014) and it is easy and fast to perform (Ren and Frymier, 2002; Kratasyuk and Esimbekova, 2015). Therefore, in this study, we demonstrate the features of luminous bacteria isolated from *C. borealis* collected near off Iwaki as well as their application to assessing water quality by detecting toxic ammonia.

MATERIALS AND METHODS

Collection and morphological identification of fish samples

Chlorophthalmus borealis is a host for luminous bacteria and specimens were collected by bottom trawling net by the *R/V Iwakimaru*, a marine research ship operated by Fukushima Prefecture. The fish were caught at off Fukushima Prefecture [37.5174N, 141.3956E (200 m depth)] (Fig. 1) in a day. The morphological identification was followed by Nakabo (2002).

Isolation and purification of bioluminescent bacteria Sample collection and culture

A single species of luminous bacteria was isolated by swabbing method from a tissue slice of a fish. It was dissected from near the anus of the fish within 24 h of capture of the fish and transferred to high salinity LB medium (LB-HS) (0.5% yeast extract, 0.5% tryptone, 30% NaCl, 0.4 mM NaOH) agar plates (1.5 % agar added to LB-HS) and incubated for 1 day at 25 °C. Emitting colonies were transferred to new agar LB-HS plates and this process was repeated to screen the luminous bacteria. Pure cultures of the isolated luminous bacterial species were maintained in LB-HS or on LB-HS agar plates.

Evaluation of emission intensity and growth curve preparation

The emission spectra and emission intensities of the isolated luminous bacteria were measured using a fluorescence spectrophotometer (FP-6500, Jusco, Japan). The excitation light was blocked and the fluorescence emission spectrum of *P. kishitanii* was immediately measured after the samples put into the fluorescence spectrophotometer using a spectral bandwidth of 1 nm. The fluorescence intensity was maximum at 475 nm. The 12–24 hours cultured bacteria culture were used in the experiments.

The bacterial concentration of *P. kishitanii* was obtained by inoculating LB-HS with an isolated colony and shaking overnight, and then the culture was re-suspended at a density of OD_{600} = 0.03 as

determined using a spectrophotometer (BioSpec-1600, Shimadzu, Japan). The bacterial density was calculated from OD_{600} as 1×10^8 cells/mL (MacFarland, 1907). Both the OD_{600} and the emission intensity were measured every 3 to 6 h. Data points and attached bars correspond to the means of 4 identical samples and their standard errors.

DNA extraction and phylogenetic analysis

DNA of luminous bacteria was extracted from 12 hours cultured bacteria suspension (OD_{600}= 1.0) originated a single emitting colony of plate culture. DNA extraction was carried out using PrepMan Ultra Reagent Kit (Thermo Fisher Scientific, Japan) following manufacture's instruction. The partial sequences of 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the extracted DNA as a template. The primer set was used for PCR: 5'-AGAGTTTGATCMTGGCTCAG-3' as forward primer and 5'-AAGGAGGTGATCC ARCCGCA-3' as reverse primer. The reaction time was 10 seconds at 98 °C, 30 seconds at 55 °C, and 60 seconds at 72 °C were repeated 30 times. The DNA electrophoresis was carried out using 3.5 % agarose gel in TBP buffer. For determine of the sequence, the PCR product was purified using Fast Gene Gel/PCR Extraction Kit (Thermo Fisher Scientific, Japan). The cycle sequence PCR was performed by using Big Dye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Japan). The primer set for cycle sequence PCR was F1 (5'-AGAGTTTGATCM TGGCTCAG-3'), F2 (5'-TGCCAGCAGCCGCGGTA-3'), F3 (5'-GGTTAAGTCCCGCAACG-3'), R1 (5'-TACCGCGGCTGCTGGC-3'), R2 (5'-TCGTTGCGG GACTTAACC-3'), R3 (5'-AAGGAGGTGATCCA RCCGCA-3').

Continuously the product of cycle sequence PCR was purified using BigDyeXterminator Purification Kit (Thermo Fisher Scientific, Japan), then the base sequences were determined by using Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific, Japan). The sequence was assembled in the software CLC Mainworkbench (Filgen, Japan) and carried out alignment using CLUSTAL W program (Thompson *et al*., 1994), Bio Edit (Hall, 1999) used for repairing the gap. Molecular phylogenetic tree was constructed by Neighbor-Joining method (Saitou and Nei, 1987) using MEGA 7.0 (Kumar *et al*., 2016), and based on Kimura 2-parameter base substitution model (Kimura, 1980). The tree reliability was assessed using bootstrap of 1000 replicates. In order to construct the phylogenetic tree, the sequences of some species were cited by Okuzumi *et al*. (1994); Ruimy *et al*. (1994); Kato *et al*. (1995); Nogi *et al*. (1998); Ast and Dunlap (2004); Ast and Dunlap (2005), Seo *et al*. (2005a); Seo *et al*. (2005b); Dunlap and Ast (2005); Ast *et al*., (2007); Yoshizawa *et al*. (2009); Gomez-Gil *et al*. (2011); Lo *et al.* (2014); Moreira *et al*. (2014); Machado *et al.* (2015); Hilgarth *et al*. (2018). *Vibrio cholera* CECT 514^T (accession no. X76337) (Aznar *et al*., 1994) was used for the outer group.

Evaluation of water quality

Water quality was evaluated using the *P. kishitanii* Taigaleon. The bacteria 12–24h cultured were used the experiments. The 1 mL of each ammonia samples (3×10^{-4} mol/L, 1×10^{-3} mol/L and 1×10^{-2} mol/L) containing double the amount of ammonia compared to the target final concentration were prepared in LB-HS and mixed with an equal volume (1 mL) of bacterial culture in LB-HS (OD_{600} = 1 to 1.5) in a sample tubes. In case of no incubation samples, the mixed samples transferred into a cuvette and the emission intensity of the mixture was measured immediately. On the other hand, 1h-incubation samples, the mixed samples transferred into a cuvette and the emission intensity of the mixture was measured immediately subsequent to 1 h incubation at 25 °C. The four identical samples were prepared and the statics analysis was performed by Mann-Whitney U test.

In regards to experiments presuming fresh water samples, the 1 mL of culture medium or pure water mixed with an equal volume (1 mL) of bacterial culture in LB-HS (OD_{600} = 1 to 1.5) in a sample tubes. In this case, the salinity concentration was reduced to half. In addition, the luminescent bacteria were re-suspended in pure water following centrifugation. The culture medium was added instead of pure water as control. For no incubation samples, the mixed samples transferred into a cuvette and the emission intensity of the mixture was measured immediately. On the other hand, 1h-incubation samples, the mixed samples transferred into a cuvette and the emission intensity of the mixture was measured immediately subsequent to 1 h incubation at 25 °C. The four identical samples were prepared and the statics analysis was performed by Mann-Whitney U test.

RESULTS AND DISCUSSION

Establishment of *Photbacterium kishitanii* Taigaleon

Fig. 1. Map of the oceans near Japan. The arrowhead shows where the *C. borealis* sample was collected. The net point was (37.5174N, 141.3956E) and the bottom depth was 200 m.

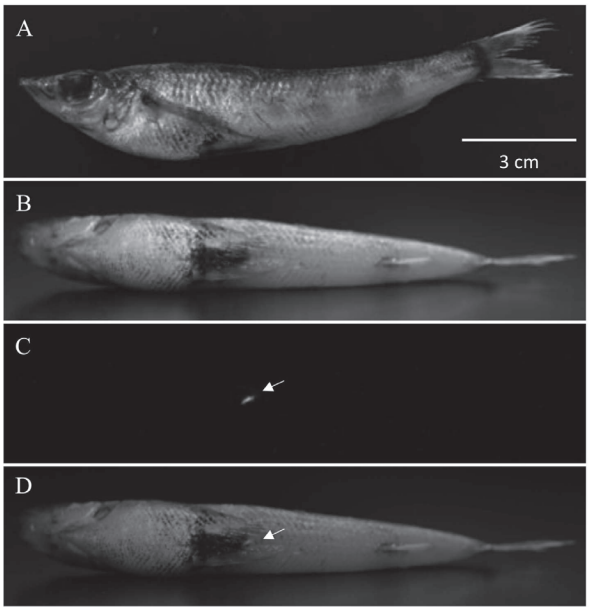

Fig. 2. Photo of *C. borealis* collected in this study. The lateral view (A) and ventral view (B) are shown under bright-field illumination. The ventral side is shown under dark-field illumination (C) and an overlay of B and C is shown in (D). The arrows indicate emitting spots. The pictures reproduced from Shimizu *et al.* (2018).

The site of fish (*C. borealis*) sample collection is shown in Figure 1. A sample fish is shown in Figure 2. *Chlorophthalmus borealis* and *C. albatrossis* are very similar to each other morphologically but the sample fishes were collected from off Fukushima Prefecture where is northward from Choshi City and thus were identified as *C. borealis* (Nakabo, 2002). The fishes were preserved on ice, and its photos were taken in the dark (Fig. 2). The luminescence from *C. borealis* was difficult to visualize directly but long exposure times confirmed spot-like luminescence (Fig. 2C) emitted from the vicinity of the anus (Fig. 2D). This result showed that *C. borealis* collected off Fukushima harbors a light emitting symbiont near the anus, as reported for *Chlorophthalmus* species. (Somiya, 1977).

Pure cultures of the isolated bacterium were obtained using LB-HS plates and liquid LB-HS medium. Bright-field images are shown in Fig. 3A a-1, b-1 and the colonies were luminous under dark-field conditions (Fig. 3A a-2, b-2). The bacteria were also luminous when observed by confocal laser microscopy (Fig. 3B). The morphological features were in agreement with those of *P. kishitanii* described by Ast *et al*. (2007). In addition, the emission spectrum (Fig. 3C) showed a maximum around 475 nm as reported in previously (Widder, 2010).

We identified the species of the luminous bacteria by amplifying a partial region of the 16S rRNA gene, followed by electrophoresis. A clear single band of about 1600 bp was detected (Fig. 4A). A partial sequence of the 16S rRNA gene (1497 bp) was determined, and it is deposited in DDBJ with acceciation no. LC421662. Molecular phylogenetic trees were created using the neighbor- joining method (Fig. 4B). The phylogenetic tree shows that the partial sequence of the 16S rRNA gene of the luminous bacterium isolated in this study from *C. borealis* was included in a clade consisted of previously reported sequences of *P. kishitanii* indicating that it was *P. kishitanii*. Hence, we established a new culture strain, *P. kishitanii* Taigaleon. This finding was in agreement with previous studies that have reported *P. kishitanii* as the symbiotic luminous bacterium in *C. albatrossis*, *C. acutifrons*, and *C. nigromarginatus* (Dunlap and Ast, 2005; Ast *et al*., 2007), suggesting that all members of the genus *Chlorophthalmus* harbor the same bacterial species regardless of the distribution of the host.

Analysis of relationship between bacterial situation and luminescent intensity

We simultaneously measured the bacterial concentration and emission intensity to understand the relationship between the growth state and luminescence in *P. kishitanii* Taigaleon (Fig. 5A).

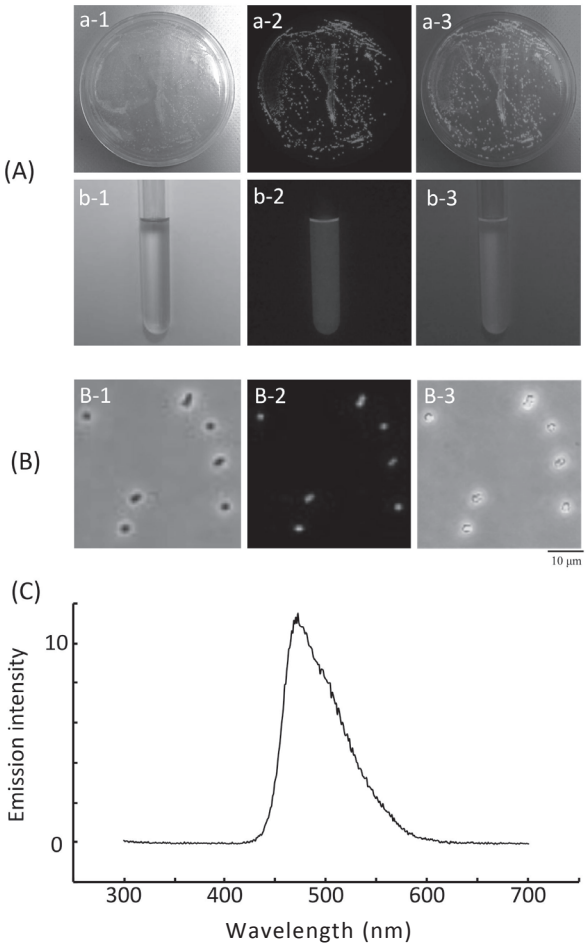

Fig. 3. Photos of pure cultures of the luminous bacterial strain (A), individual bacteria (B), and emission spectrum of the luminous bacteria (C). Plate cultures of the luminous bacteria (a-1 to a-3): a-1: bright-field image; a-2: dark-field image; a-3: overlay of a-1 and a-2. Liquid culture of the luminous bacteria (b-1-3): b-1: bright-field image; b-2: dark-field image; b-3: overlay of b-1 and b-2. Individual bacteria observed by phase contrast under bright-field (B-1), dark-field (B-2), and overlay of B-1 and B-2 (B-3). Scale bar is 20 μm.

Emission intensity increased with increasing cell number in stationary phase, logarithmic growth phase, and the early stage of the stable phase (Fig. 5A). In contrast, there was no relationship between growth and emission intensity after the logarithmic growth phase ended as the rates of cell proliferation and cell death were similar. In the late stable phase and death phase, the emission intensity apparently decreased and the cells gradually died, perhaps due to external environmental factors such as reduced nutrients and an increase in toxic compounds, resulting in *P. kishitanii* Taigaleon cultures losing luminescence. To confirm this, *P. kishitanii* Taigaleon in the phase after the stable period was stained with propidium iodide (PI) and observed by confocal laser microscopy (Fig. 5B). We confirmed that bacterial particles with suitable condition were emit their luminous (Fig. 5B-1) without staining PI (Fig. 5B-2), and dead bacterial particles were stained PI (Fig. 5B-2) without their luminous (Fig. 5B-1). The others did not emit their luminous and also were not stained with PI (Fig. 5B-3, 5B-4). These bacteria were alive but stressed. These results suggest that *P. kishitanii* Taigaleon responds to external factors and its emission intensity changes. Thus, a harmful external environment is reflected in the intensity of their luminescence.

Assessment of water samples for harmful ammonia

We used this property of *P. kishitanii* Taigaleon to assess the utility of luminous bacteria for detecting toxic ammonia, a biohazardous compound found in seawater. Previous studies reported the detection of various organic compounds such as alcohols, phenols, acids and amines in pure water (Ren and Frymier, 2002), but not of ammonia. In addition, there have been few reports of the detection of toxic compounds in seawater using luminous bacteria. We first evaluated how the state of the bacteria would affect the results, as the intensity of luminescence would likely fluctuate during measurement due to cell to cell physical contact. Therefore, we first monitored emission intensity at 5-s intervals (Fig. 6A) and observed that the luminescence intensity apparently decreased within 100 s from the start of measurement without toxic ammonia. Hence, measurements should be performed immediately, before emission intensity decreases (within 1 min of mixing the samples). Luminescence intensity decreases as the concentration of ammonia increases (Fig. 6B). The addition of 3×10^{-4} mol/L ammonia to the bacteria resulted in a significant decrease in the emission intensity (compare 0 with 3×10^{-4} mol/L; $p < 0.05$), indicating that *P. kishitanii* Taigaleon can detected ammonia at 3×10^{-4} mol/L. Other concentrations of ammonia also resulted in the emission intensity significantly decreasing, suggesting that *P. kishitanii* Taigaleon can be used to assess water for ammonia based on the decrease in emission intensity depending on the ammonia concentration (Fig. 6B). We demonstrated that ammonia is harmful to

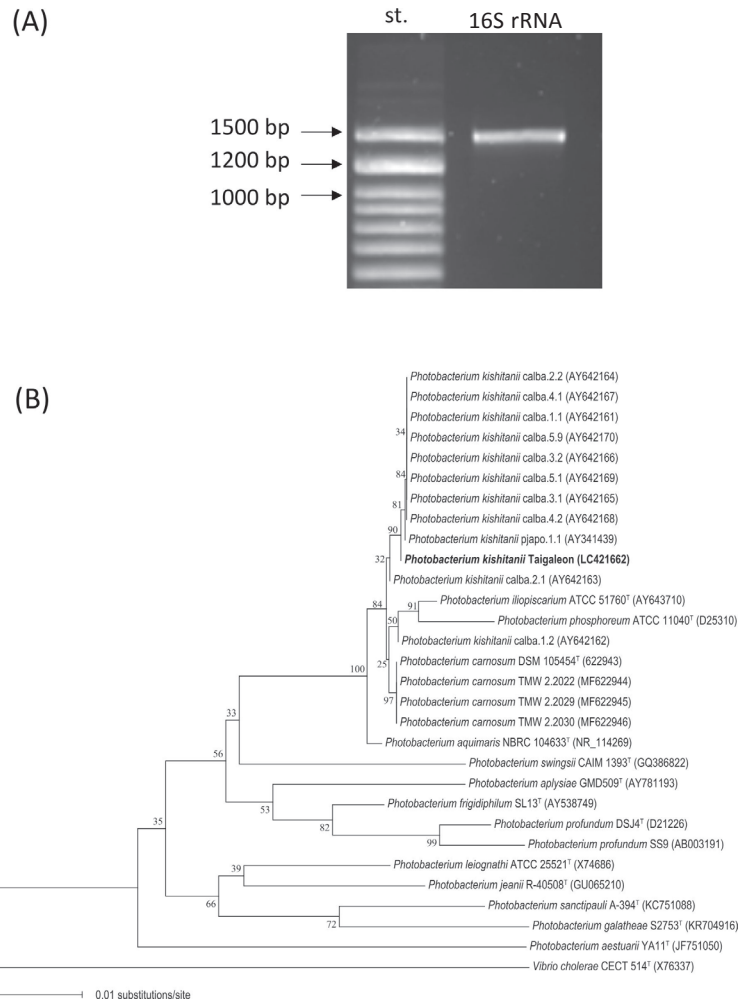

Fig. 4. Photograph of an electrophoresis gel of *P. kishitanii* Taigaleon partial 16S rRNA gene amplified by PCR (A). "St" is molecular weight markers and "16S rRNA" was amplified by PCR. Neighbor-joining (NJ) tree of the partial sequences of the 16S rRNA gene of the luminous bacteria (B). The scale indicates genetic distance of Kimura 2-parameter model. Numbers near the internal branches are percentage bootstrap value. The bold letters show *C. borealis* culture strain established in this study. The accession number of each sequence is indicated in parentheses.

organisms by mixing culture medium containing ammonia with an equal volume of luminescent bacteria culture. We also considered the possibility of assessing freshwater samples using *P. kishitanii* Taigaleon (Fig. 7). When the emission intensity was measured by mixing equal volumes of pure water and luminescent culture, the salinity concentration was reduced to half and there was no immediate change in luminescence intensity. A comparison of culture medium and pure water is presented in Fig. 7A. This result suggests that *P. kishitanii* Taigaleon can be used to assess freshwater samples despite being marine bacteria. However, luminescence decreased substantially 1 h after mixing [Fig. 7A: compare pure water with pure water (after 1 h)], although the sample mixed with culture medium showed no significant difference in luminescence intensity after 1 h [Fig. 7A: compare culture medium with culture medium (1 h later)]. This result suggested that *P. kishitanii* Taigaleon can be used to assess fresh water if the measurement is performed quickly by mixing equal volumes of *P. kishitanii* Taigaleon culture and the freshwater sample. Further experiments were conducted using pure freshwater samples. A culture of *P. kishitanii* was centrifuged, the cell pellet was re-suspended with the same volume of pure water, and the emission intensity was then measured (Fig. 7B). The emission

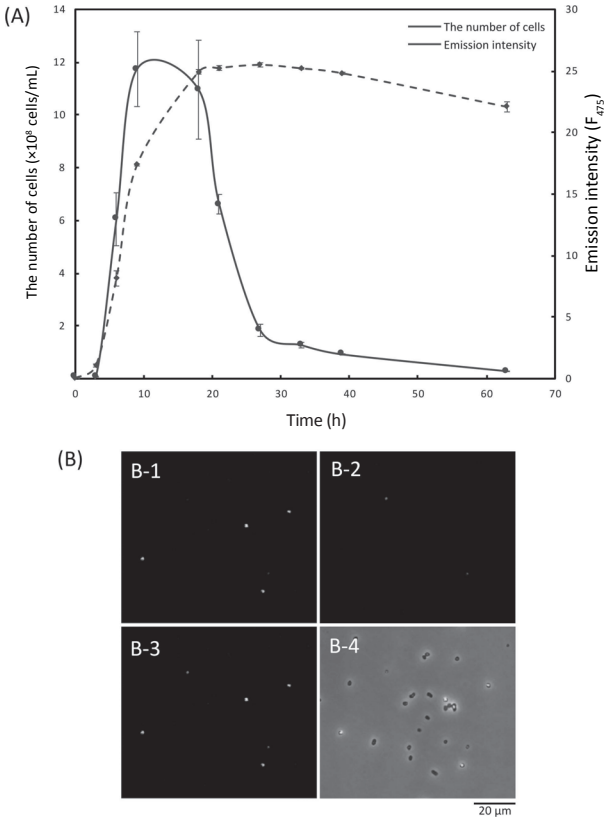

Fig. 5. The relationship between bacterial concentration and luminescence intensity (A) and microscopic observation of PI-stained luminous bacteria (B). (A) Graph of the bacterial concentration (solid line) and emission intensity (dotted line) of the luminous bacteria. Horizontal axis is cultivation time, left vertical axis is cell number, and right vertical axis is emission intensity at the emission maximum (475 nm). Data points and attached bars correspond to the means of 4 identical samples and their standard errors. (B) Photograph of *P. kishitanii* detected at 475 nm (B-1), detected by PI fluorescence (620 nm), overlay of B-1 and B-2, overlay of B-1, B-2, and bright-field illumination. Scale bar is 20 μm.

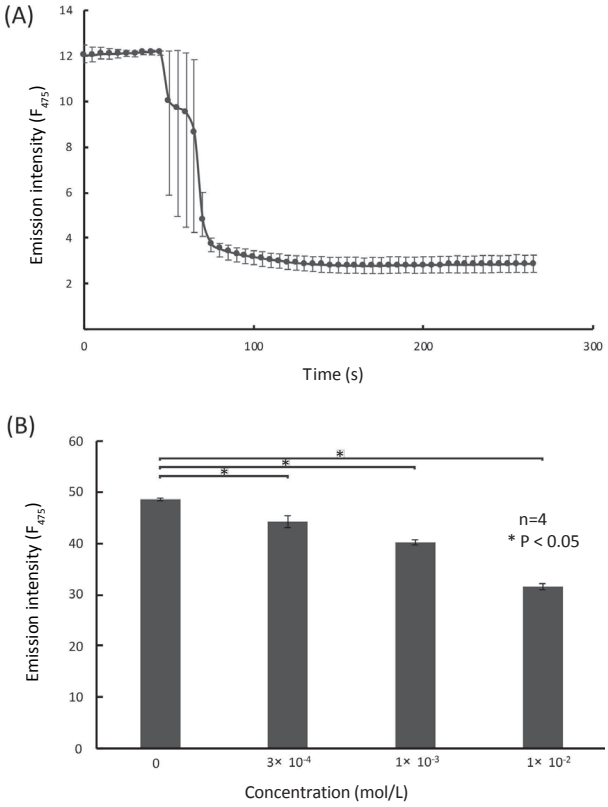

Fig. 6. Time-dependent change in emission intensity (A) and ammonia concentration-dependent emission intensity of the luminous bacteria (B). (A) The emission intensity of the luminescence was measured every 5 s from the start of the test. Data points and attached bars correspond to the means of 4 identical samples and their standard errors. (B) Emission intensities of a bacterial suspension in culture medium containing ammonia (3×10^{-4} M, 1×10^{-3} M, and 1×10^{-2} M final concentration) immediately after mixing. Columns and attached bars correspond to the means of 4 identical samples and their standard errors. Asterisks correspond to the means of significant differences ($p< 0.05$) by statistical analysis (Mann-Whitney U test).

intensity of the sample was almost zero even immediately after preparation and the bacteria died immediately (Fig. 7B: pure water). The results were similar 1 h after mixing [Fig. 7B: compare the culture medium (after 1 h) and pure water (after 1 h)]. These results suggest that *P. kishitanii* Taigaleon was difficult to be used to assess freshwater samples if the freshwater samples were direct mixed with bacterial pellets, however, they can be used if the volume of fresh water added was equal to that of the bacterial culture. In addition, since ammonia is not the only contaminant present in a given water sample collected from natural environment, it can be that other contaminants can affect the luminescence intensity. Hence, we also might have to establish a method to remove the influence by substance except for ammonia. The biological assays using living-whole cell bacteria can provide biologically relevant information (Xu *et al*., 2014), however, the failure to maintain the suitable condition of bacterial culture results in low accuracy of measurement (Kratasyuk and Esimbekova, 2015) Therefore, further more work

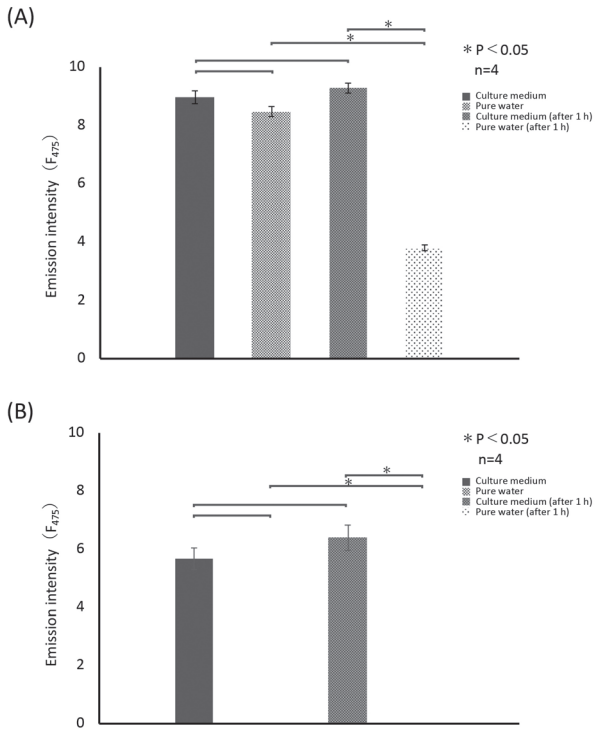

Fig. 7. Detection of luminescence intensity using freshwater samples. Emission intensity of a suspension of luminescent bacteria following the addition of an equal volume of pure water (A) and luminescent bacteria following centrifugation, followed by re-suspension in freshwater (B). "Culture medium" is control (culture medium was added instead of pure water). Each sample was measured immediately or 1 h after mixing ("after 1 h"). Columns and attached bars correspond to the means of 4 identical samples and their standard errors. Asterisks correspond to the means of significant differences ($p< 0.05$) by statistical analysis (Mann-Whitney U test).

will be required to improve accuracy of measurement and method for maintenance of stable state of bacterial culture.

CONCLUSION

In this study we identified a luminous bacterial symbiont of a deep sea fish [greeneye (*C. borealis*)] commonly found in the waters near Iwaki, Fukushima Prefecture. The bacterium was identified as *P. kishitanii* by phylogenetic analysis using the partial sequence of the 16S rRNA gene. The new culture line *P. kishitanii* Taigaleon was isolated from *C. borealis*. We demonstrated that *P. kishitanii* Taigaleon responds to external stress and is useful for assessing water quality by detecting dissolved ammonia. Nevertheless, ammonia is not the only contaminant present in a given water sample collected from natural environment, and other contaminants may affect the luminescence intensity. Hence, further more work will be required to establish a method to remove the influence by substance except for ammonia and to improve accuracy of measurement and method for maintenance of stable state of bacterial culture.

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