熊本大学学術リポジトリ

生理学的特性化の微生物学系Neptunomonas spp. から分離した赤藻から...
Physiological characterization of epiphytic bacteria Neptunomonas spp. isolated from the red alga Pyropia yezoensis and the seagrass Zostera marina
(紅藻スサビノリおよび海草アマモから単離したネプチュノモナス属細菌における生理特性の解析)

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主論文要旨
In marine environment, epiphytic bacteria are widely distributed and abundant commonly on the leaf-surface of macroalgae. Various beneficial effects of epiphytic bacteria on macroalgae were proposed, but direct evidence for these effects using *in vitro* culture is limited. The red alga *Pyropia yezoensis*, which is cultivated for laver farming in Japan, 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. Two culture lines of *P. yezoensis* (TU-1 and U-51) were predominantly used in Japan. The EST database was constructed using TU-1 line and draft genome sequences were identified using U-51. Unlike seed plants, the gametophytes of *P. yezoensis* are maintained with epiphytic bacteria in culture because aseptically grown gametophytes do not development the normal leafy form. Several bacteria capable of inducing normal morphogenesis were isolated from two lines. Although various taxonomic bacteria were identified from gametophytes, physiological role was not reported except induction of normal development of host plants.

Recently, in our laboratory, an epiphytic bacterium *Neptunomonas* sp. BPy-1 was isolated from TU-1 strain. The 16S rRNA gene sequences of BPy-1 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. It is expected that BPy-1 has a beneficial effect on host plant. In our laboratory, the BPy-1 closest bacterium *Neptunomonas* sp. BZm-1 was isolated from a seagrass *Zostera marina* grown in Yatsushiro Sea (Japan). *Neptunomonas* is a relatively new genus and its physiological property remains unclear. In this study, to examine the physiological role of two
Neptunomonas clones on P. yezoensis, growth property of BPy-1 (chapter 2) and BZm-1 (chapter 3) were analyzed, and effect of epiphytic bacteria on growth of P. yezoensis was examined using co-culture experiment. In chapter 4, physiological properties of isolated clones were compared.

In chapter 2, biochemical and physiological property of Neptunomonas sp. BPy-1 was analyzed. Physiological tests revealed that 22 characters were identical between BPy-1 and 0536, but that 4 characters differed. 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. Isolated 60 clones 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. PCR analysis suggested that BPy-1 was detected as minor epiphyte. However, only BPy-1 could grow on marine broth (MB) agar, suggesting that the growth of BPy-1 is restricted on normal gametophytes. 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 assess the effect of Neptunomonas sp. BPy-1 on gametophytes, bacteria-less gametophytes were prepared using a multi-enzyme cleaner with 0.05%, 0.075% and 0.1% in concentration. The results suggested that treatment with 0.05% and 0.075% multi-enzyme solution caused relatively mild and severe damage to the gametophytes, respectively. The effect of enzyme treatment on the amount of epiphytic bacteria was determined by CFUs. Three treatments using varying concentrations of the enzyme solution reduced the amount of Neptunomonas sp. BPy-1 by as much as 60%. Incubating n-LBG (0.05% enzyme concentration) and c-LBG (0.075% enzyme concentration) with the varying concentration of Neptunomonas sp. BPy-1 was resulted in positive effect on n-LBG while c-LBG was less affected by Neptunomonas sp. BPy-1. It is suggesting that attachment of BPy-1 on the gametophyte may be required for growth promotion.

In chapter 3, BPy-1 closest bacterium, Neptunomonas sp. BZm-1 was isolated from a seagrass Zostera marina in Yatsushiro Sea. BZm-1 showed 99% 16S rRNA sequences identity to that of BPy-1. Twenty-seven physiological characters used for bacterial classification were analyzed in Neptunomonas sp. BZm-1. BZm-1 is Gram-negative bacterium that displays motility, moderate tolerance to salinity, and catalase/oxidase reactions, similar to BPy-1 and 0536. On the other hand, BZm-1 differed from 0536 in three characteristics: gelatin liquification, maltose assimilation and glucose acidification. BZm-1 showed the same alcohol utilization preference observed in BPy-1. BZm-1 showed high tolerance to multiple drugs. Co-culture experiment was done to determine the role of BZm-1 on the gametophytes. From the
two types of damaged gametophytes, only the n-LBG type was promoted. However, since inconsistence result was observed, additional experiment is needed to confirm whether this assay method by using *P. yezoensis* gametophytes is available for *Neptunomonas* sp. BZm-1.

In chapter 4, physiological properties of isolated two *Neptunomonas* clones were compared and discussed. Although BZm-1 and BPy-1 differ in their origin, BZm-1 showed 99% 16S rRNA sequences identity to that of BPy-1, with 27 identical characteristics in physiological properties between two strains. Like BPy-1, BZm-1 possessed two prominent features: ethanol or butanol utilization as the sole carbon source and the promotion of growth in damaged gametophyte of *P. yezoensis*. While the physiological properties of *Neptunomonas* species have been reported, no isolates have been shown to utilize ethanol. BPy-1 and BZm-1 were the first example of ethanol-utilization among *Neptunomonas* bacteria. Another unique feature of BPy-1 and BZm-1 is their promotion of damaged *P. yezoensis* gametophyte growth. Epiphytic bacteria capable of inducing normal development of gametophytes of *P. yezoensis* were isolated. In that study, protoplasts were prepared from gametophytes. In contrast, in this study, effect of epiphytic bacteria on the gametophytes was examined using mature gametophytes. Although BZm-1 did not habituate in natural environment, it promoted the growth of damaged *P. yezoensis* gametophytes. Therefore, this method will be useful for identification of beneficial bacteria. In Japan, *P. yezoensis* is used for laver farming. As the seagrass used in this study inhabits regions flanking laver farming, seagrass-inhabiting BZm-1 may contribute to the success of this industry. Considering the probiotic role of *Neptunomonas* sp. 0536 for green shell mussels, further characterization of the associations between BZm-1 and other marine animals will provide important information on related bacteria derived from macroalgae and marine animals including shellfish.