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Phylogenetic analysis of epiphytic marine bacteria on Hole-Rotten diseased sporophytes of *Laminaria japonica*

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Abstract During an occurrence of Hole-Rotten Disease of *Laminaria japonica* in a cultivating farm in Ma Shan Shandong province, China, 42 Gram-negative epiphytic marine bacteria were isolated and purified on Zobell 2216E marine agar medium. Morphological and biochemical characteristics of each isolated bacterium were studied, and molecular identification of bacterial strains was conducted with polymerase chain reaction amplification to 16S rRNA gene sequence analysis. Based on nearly full length of 16S rRNA gene sequence analysis, the isolated strains were bacteria that belong to genus *Pseudoalteromonas*, *Vibrio*, *Halomonas* and *Bacillus*. The percentage of each group was 61.9%, 28.6%, 7.1% and 2.4% respectively. The results of pathogenicity assay showed that 12 strains could cause the disease symptoms in sporophytes of *L. japonica*. They belonged to the genera *Pseudoalteromonas*, *Vibrio* and *Halomonas* with 58.3%, 33.3%, 8.3% respectively. The results suggest that these bacteria are the dominant marine bacteria on diseased sporophytes of *L. japonica* and may be the potential pathogenic bacteria associated with Hole-Rotten Disease of *L. japonica*.

Keywords 16S rRNA gene · *Laminaria japonica* · Marine bacteria · Phylogenetic analysis

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Introduction

Laminaria is one of the commercial sources of industrial alginic acid and about 13,000 tons of alginic acid are produced annually in China (Tseng 2001). Even though cultivation has been successfully implemented, disease usually influences seedling production and even makes cultivation yields drop. Therefore, it is essential to study the distribution of bacterial communities on diseased *Laminaria japonica*, and eventually to decipher the physiological and pathological process for the disease occurrences.

Information on bacterial pathogens of algae (Andrews 1976), especially algae of the genus *Laminaria* is limited. Ezura et al. (1988) reported that a marine bacterium isolated from the red-spot disease culture beds of *L. japonica*, was assigned to the genus *Alteromonas*. Yumoto et al. (1989) studied the annual distributional variations of *Alteromonas* sp., and discussed the pathogen's invasion route in *L. japonica* cultivating areas. Recently, by using phenotypic features and molecular identification, *Pseudoalteromonas bacteriolytica* was verified to be correlated with red-spot disease of *L. japonica* (Sawabe et al. 1998). In addition, another marine bacterium *P. elyakovii* was considered to be associated with the spot-wounded fronds of *L. japonica* (Sawabe et al. 2000).

Over the past 25 years in China, intensive studies on the relationship between bacteria and diseased juveniles of

L. japonica have been conducted (Chen et al. 1979, 1981, 1984, 1986; Ding 1990). *Pseudomonas* sp. with alginic acid decomposing ability is regarded as a main pathogen of diseased juveniles during *L. japonica* sporeling production (Chen et al. 1986; Ding 1990). Chen et al. (1986) believe that *Pseudomonas* sp. can secrete alginase to decompose the kelp cell walls and cause rotten disease. Sun et al. (1984) reported that the alginic acid decomposing bacteria became the dominant bacterial community under a bad growing seawater environment, and finally resulted in detrimental effects on normal growth and development in the *Laminaria* nursery. Similarly, Lin et al. (2004) investigated the distribution and reinfection effects of alginic acid-decomposing bacteria on juveniles of *L. japonica*.

Epidemic disease occurred in Ma Shan cultivating farm, Shandong province, China, in June of 2004. The diseased sporophytes of *L. japonica* fell from the cultivating ropes, with symptoms of green-rotting on the sporophytic edges and rotten holes spread on the blade. This happened near harvesting time and had a serious effect on *L. japonica* production.

To reduce severe damage for *L. japonica* in production, it is necessary first to investigate the distribution of epiphytic marine bacteria on the diseased sporophytes. The objectives of our study were to isolate and verify the epiphytic marine bacteria on *L. japonica* sporophytes with Hole-Rotten Disease, and had 16S rRNA gene sequence for phylogenetic analysis. It will be of significance for analyzing marine bacterial communities during the disease occurrence in *L. japonica* cultivation.

Materials and methods

In June 2004, freshly diseased sporophytes of *L. japonica* were collected from Ma Shan cultivating farm, located at the eastern part of Shandong province. The diseased sporophytes were processed within 3 h after collection.

Isolation

The diseased sporophytes of *L. japonica* were prewashed softly in sterilized seawater to eliminate the pollutions on algae, and then cut into small sections with a sterile surgical blade and tweezers. Microbial isolations were made by grinding 2–3 g prewashed diseased sporophytes into a homogenized slurry, followed by suspended in 4–5 mL sterilized seawater. Different appropriate dilutions were plated on Zobell 2216E agar medium (Oppenheimer and ZoBell 1952), and incubated at 25°C for 7–10 days. Colonies were selected and purified on the same medium. All the subcultures were carried out at least three times at 25°C for 18–20 h.

Morphological and biochemical characterization

Cell size and morphology were determined microscopically. The conventional biochemical tests included: Gram reaction, flagella and motility, production of pigment, luminescence, oxidase reactions and glucose catabolism. To observe the bacterial flagella, a diluted suspension of freshly cultured bacteria (25°C for 18–20 h) were rinsed with PBS for three times, and re-suspended in PBS. A drop of bacteria suspension was placed onto a carbon-coated grid for 1 min, then negatively stained with 20 $g.L^{-1}$ uranyl acetate for 20 s. Samples were observed with a JEM-1200EX (JEOL Corp. Japan) transmission electron microscope. All biochemical tests were performed according to Bain and Shewan (1968).

Pathogenicity assay

Pathogenicity assays were conducted to confirm whether the isolated strains were bacterial pathogens on *L. japonica* according to the methods described by Wang et al. (2004). Fresh juvenile sporophytes (ca. 15x20 cm in length) of *L. japonica* were collected from the Qingdao coast, Shandong Province, China, on 8 Jan 2007. The algae were cleaned thoroughly with autoclaved seawater. Marginal parts were cut into small sections (1.0×1.0 cm) with a sterile surgical blade and tweezers, 0.4x0.5 cm incised wound were made on each tissue, and were put into 24-well Costar cell culture cluster (Corning Inc. USA). The cut tissues were infected with individually with an isolated strain. The solution for inoculation was 1×10^7 $cfu.mL^{-1}$, and the controls were treated with autoclaved seawater only. The plates were kept at 20°C and 20 μmol photons. $m^{-2}.s^{-1}$ irradiance. The treated tissues were collected to detect the disease symptoms at 4 h after the infection. Three parallel experiments were conducted for each strain of bacterium at each time.

PCR amplification and sequencing

The molecular identification of the isolated strains was by use of 16S rRNA gene sequences according to Fisher (1998). Bacterial template DNAs were extracted by using UNIQ-10 kit (Sangon, Shanghai), Usually about 100 ng DNA template was used in a PCR reaction. The nearly full-length 16S rRNA gene was amplified with universal primers 27F (5′-AGAGTTT GATCCTGGCTCAG-3′) and 1492R 5′-TACGGTACC TTGTTACGACTT-3′) (Uchida et al. 2002). PCR mixture consisted of 2 μL of 10×PCR mix (final concentrations: 50 mM KCl, 0.01% gelatin, 10 mM Tris-HCl pH 9.0); 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP dGTP, and dTTP; 1 μL each of two primers (20 mM); 1 μL of DNA sample (100 $ng.\mu L^{-1}$); 2.0 units

of *Taq* polymerase (Promega, USA) in a final volume of 20 μL. Reactions were performed in Mastercycler Gradient (Eppendorf, Germany) programmed as follows: 1 cycle of 5 min pre-denaturation at 94°C; 30 cycles with denaturation at 94°C for 1 min; annealing at 58.6°C for 1 min; and extension at 72 °C for 2 min. A final extension was performed at 72°C for 10 min. Sequencing analysis of the amplified DNA fragments was conducted by Sangon Ltd. Co., Shanghai, with an Applied Biosystems 377 DNA Analyzer.

Phylogenetic analysis

The sequences were aligned and the bootstrap neighbor-joining analysis was constructed using a MEGA 3.1 program. In all phylogenetic analyses, we used the sequences determined in this study obtained from the EMBL/GenBank databank. Sequences are shown in Fig. 1.

Nearly full sequences of 16S rRNA genes cloned from the epiphytic marine bacteria from the diseased *L. japonica* were deposited to GenBank with the accession numbers: DQ517529, DQ642805, DQ642806, DQ642807, DQ642808, DQ642809, DQ642810, DQ642811, DQ642812, DQ642813, DQ642814, DQ642815, DQ642816, DQ642817, DQ642818, DQ642819, DQ642820, DQ642821, DQ642822, DQ642823, DQ642824, DQ642825, DQ642826, DQ642827, DQ642828, DQ642829, DQ642830, DQ642831, DQ642832, DQ642833, DQ642834, DQ642835, DQ642836, DQ642837, DQ642838, DQ642839, DQ642840, DQ642841, DQ642842, DQ642843, DQ642844, DQ647665.

Results

A total of 42 strains of bacteria were isolated from Hole-Rotten diseased sporophytes of *L. japonica*. They had polar or peritrichous flagella, and were Gram-negative and rod shaped. Other phenotypic properties, such as, cell size, motility, luminescence, oxidase and glucose catabolism are listed in Table 1.

Pathogenicity assay

The pathogenicity assay indicated that 12 bacterial strains (indicated with asterisk in Fig. 1) caused consistent disease symptoms within 4 h after the incubation in the three parallel tests. Meanwhile, bacteria-free control tests remained dark and healthy over 4 h incubation. The pathogenicity assay proved that these 12 isolated bacterial strains were indeed the potential pathogens of *L. japonica*. Phylogenetic analysis of these 12 pathogenic bacteria indicated that they belonged to the genera *Pseudoalteromonas*, *Vibrio Halomonas* with percentage of 58.3%, 33.3%, 8.3% respectively.

A phylogenetic tree was constructed based on 16S rRNA sequences (Fig. 1), and all the isolated 42 bacterial strains were identified as belonging to *Pseudoalteromonas*, *Vibrio*, *Halomonas* and *Bacillus*, with the percentage of each group 61.9%, 28.6%, 7.1% and 2.4% respectively. Most of the isolates are members of the gamma subclass of the Proteobacteria (data not shown), except for strain 17. These data clearly indicate *Pseudoalteromonas* was the dominant bacterial community isolated from the diseased sporophytes of *L. japonica*.

Discussion

In our study, the distribution of bacterial communities on diseased *Laminaria japonica* was phylogenetically analyzed, and indicated that 41 of the isolated bacterial strains belonged to Gammaproteobacteria (Table 1). DeLong et al. (1993) have reported that the bacteria that belong to *Cytophaga*, *Planctomyces* and Gammaproteobacteria were the main epiphytic bacteria on algal surfaces. Our data are consistent with his work.

Pseudoalteromonas is a newly established genus and many species of *Pseudoalteromonas* exist on marine algae (Holmstrom and Kjelleberg 1999). Recently, new *Pseudoalteromonas* species were isolated from diseased *L. japonica*. Sawabe et al. (1998) suggested that the new species *P. bacteriolytica* could be the pathogen causing red-spot disease of *L. japonica*. By DNA hybridization, phenotypic characterization and phylogenetic analyses, Sawabe et al. (2000) assigned *Alteromonas elyakovii* KMM 162^T and five strains isolated from spot-wounded fronds of *L. japonica* to *Pseudoalteromonas elyakovii*. In our investigation, among the 42 isolated strains, 25 were determined as belonging to the genus *Pseudoalteromonas*, however, only 12 strains could be assigned to *Pseudoalteromonas* according to the alignment similarities (99.0%). Separated branches of 13 of the isolated strains (Fig. 1) suggest that they might be new *Pseudoalteromonas* species. The separate branch of strain No. 11 indicates that it might belong to another genus (Fig. 1).

It has been reported that many species of *Pseudoalteromonas* can produce bacteriolytic enzymes and hemolysins which are repellent to other bacteria (Grant et al. 1986). This could be the reason that *Pseduoalteromonas* becomes dominant microbial community on sporophytes of *L. japonica*. Other reports (Takamoto et al. 1994; Ivanova et al. 2002) address proteinases and lipases secreted by *Pseudoalteromonas* which could decompose the cell walls of *L. japonica* and cause the disease. This has been verified

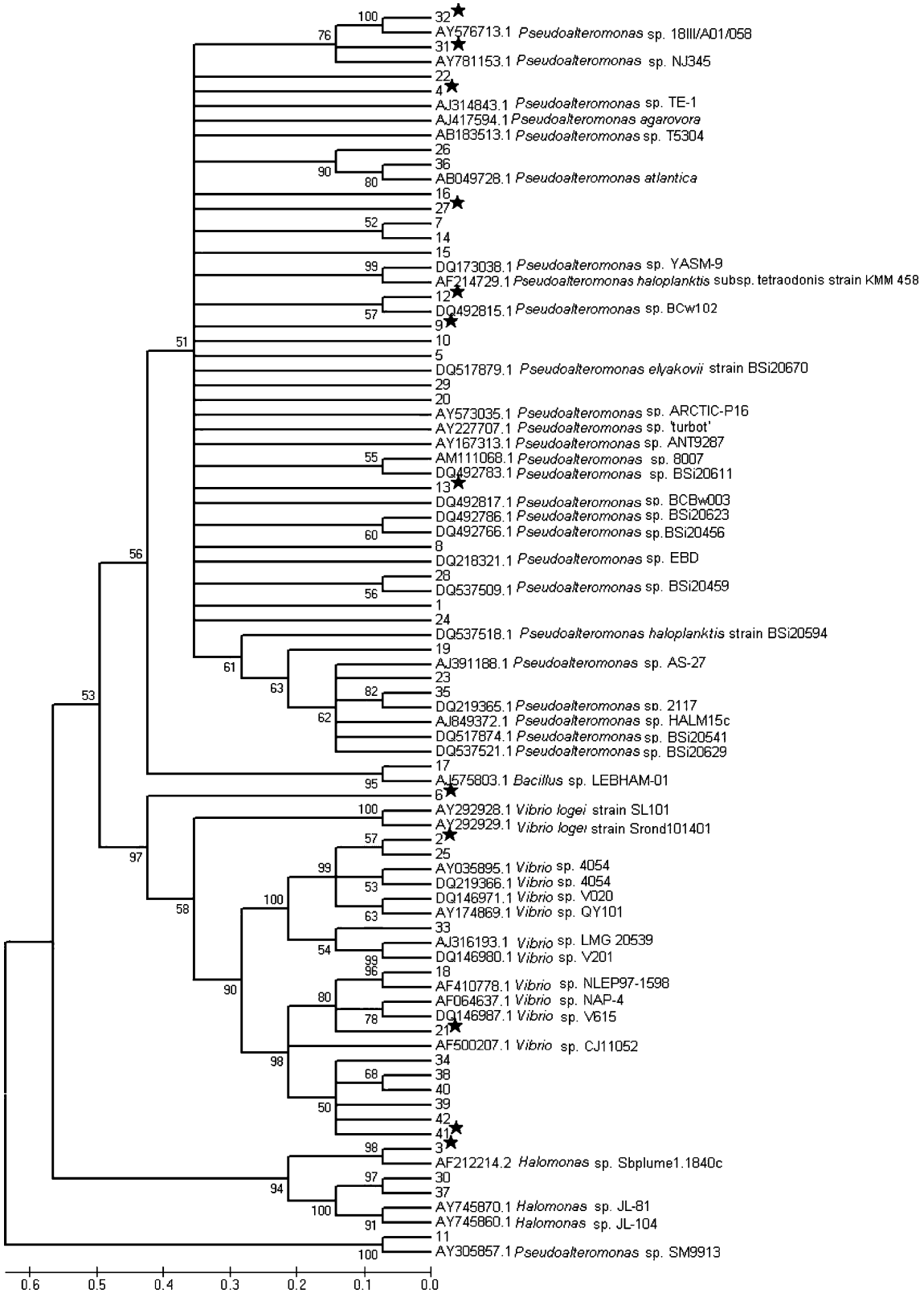

Fig. 1 Phylogenetic tree showing the position of the 42 strains and potential bacterial pathogens based 16S rRNA gene sequence similarity data

Table 1 Morphological and biochemical characterization of bacteria isolated from the Hole-Rotten Disease sporophytes of *Laminaria japonica*

Bacterial strains	Colour and morphology	Size (μm)	Gram reaction	Flagella	Motility	Lumi-nescence	Oxidase	Glucose catabolism
1	Round, non-pigmented, lucent	2.5–3.0×0.9–1.0	–	Peritrichous	+	–	+	–
2	Round, non-pigmented, lucent	2.0×0.9–1.0	–	Polar	+	–	+	+
3	Round, yellow, lucent	1.0–2.0×0.9–1.0	–	Peritrichous	+	–	+	–
4	Round, light yellow, lucent	1.0–2.5×0.9–1.0	–	Polar	–	–	+	+
5	Round, non-pigmented, lucent	2.0–3.0×0.9–1.0	–	Polar	–	–	+	–
6	Round, light yellow, lucent	3.0–3.5×0.9–1.0	–	Polar	+	–	+	+
7	Round, light yellow, lucent	2.0–3.0×0.9–1.0	–	Polar	+	–	+	–
8	Round, non-pigmented, lucent	1.2–3.5×0.9–1.0	–	Polar	–	–	+	–
9	Round, non-pigmented, translucent	2.0×0.9–1.0	–	Peritrichous	+	–	+	–
10	Round, non-pigmented, translucent	3.5–4.0×0.7–0.8	–	Polar	+	–	+	–
11	Round, non-pigmented, cloudy zone	2.0–3.0×0.9–1.0	–	Polar	+	–	+	–
12	Round, light yellow, translucent	2.0–3.0×0.9–1.0	–	Polar	+	–	+	–
13	Round, non-pigmented, translucent	2.0–3.0×0.4–0.5	–	Polar	+	–	+	+
14	Round, light yellow	2.0–3.0×0.8–1.0	–	Polar	+	–	+	+
15	Round, non-pigmented	1.0–2.0×0.9–1.6	–	Polar	+	–	+	–
16	Round, light yellow, translucent	1.5–2.0×0.5–1.0	–	Polar	+	–	+	–
17	Round, light yellow, cloudy zone	1.2–2.0×0.9–1.0	–	Peritrichous	+	–	+	+
18	Round, light yellow, cloudy zone	1.0–1.5×0.9–1.0	–	Peritrichous	+	–	+	+
19	Round, non-pigmented	1.1–2.5×1.1–1.5	–	Polar	+	–	+	–
20	Round, non-pigmented	Diameter: 0.5	–	Polar	+	–	–	+
21	Round, light yellow	1.0–2.2×0.8–1.0	–	Polar	+	–	+	+
22	Round, light yellow, cloudy zone	3.5–4.0×0.8–1.0	–	Polar	+	–	+	+
23	Round, light yellow, cloudy zone	2.0–3.0×1.0–1.2	–	Polar	+	–	+	–
24	Round, light yellow	2.0–3.0×0.4–0.5	–	–	–	–	–	–
25	Round, light yellow, lucent	2.0–3.0×0.8–1.0	–	Polar	+	–	+	+
26	Round, light yellow, translucent	1.0–2.0×0.9–1.0	–	Polar	+	–	–	–
27	Round, light yellow	2.0–3.0×0.5–0.6	–	Polar	+	–	+	–
28	Round, light yellow	1.5–2.0×0.5–1.0	–	Polar	+	–	+	–
29	Round, light yellow	1.2–2.0×0.9–1.0	–	Polar	+	–	+	–
30	Round, light yellow,	1.0–2.2×0.8–1.0	–	Polar(two)	+	–	+	–
31	Round, light yellow	Diameter: 0.5	–	Polar	+	–	+	–
32	Round, light yellow, cloudy zone	3.5–4.0×0.8–1.0	–	Polar	+	–	+	–
33	Round, light yellow, cloudy zone	2.0–3.0×1.0–1.2	–	Polar	+	–	+	+
34	Round, light yellow, cloudy zone	1.0–2.5×0.7–0.8	–	Polar	+	–	+	+
35	Round, non-pigmented	1.1–1.8×0.5–0.6	–	Polar	+	–	+	–
36	Round, non-pigmented	1.0–3.0×0.8–0.9	–	Polar	+	–	+	–
37	Round, yellow, smooth	1.2–2.0×0.9–1.0	–	Polar	–	–	+	–
38	Round, light yellow, cloudy zone	1.3–2.5×1.2–1.5	–	Polar	+	–	+	+
39	Round, light yellow, cloudy zone	Diameter: 0.5	–	Polar	+	–	+	+
40	Round, light yellow, cloudy zone	1.0–2.0×0.8–0.9	–	polar	+	–	+	+
41	Round, light yellow, cloudy zone	1.0–2.0×0.5–0.6	–	Polar	+	–	+	+
42	Round, light yellow, cloudy zone	0.7–1.5×0.7–0.8	–	Polar	+	–	+	+

+: positive test reaction; –: negative test reaction

by showing that the crude enzyme from alginic acid-decomposing bacteria could decompose blades of *L. japonica* completely within 3–5 days (Chen et al. 1979). Although the pathogenic mechanisms for the Hole-Rotten Disease is not understood, we believe that *Pseudoalteromonas* is closely involved with disease of *L. japonica*.

So far, many possible pathogenic bacteria isolated from diseased seaweeds have been identified (Uyenco et al. 1981; Lobban et al. 1985; Kusuda et al. 1992; Lavilla-Pitogo 1992; Largo et al. 1995; Jaffray and Coyne 1996), however reinfection of healthy seaweeds usually failed to cause the similar disease. We believe that one main reason for this is that the marine pathogenic bacteria from diseased seaweeds are opportunistic. Another possibility is the existence of unculturable bacteria. Usually some microorganisms, especially some bacteria, can be isolated under the specific

growth conditions (Margulis et al. 1986; Pace 1996). These microorganisms are visible under the microscope, but can not be cultured on plates (Xu et al. 1982; Eilers et al. 2000). Recently, novel isolation techniques were applied to obtain the ubiquitous marine bacterioplankton SAR11 (Rappe et al. 2002; Morris et al. 2002) and thermophilic Nanoarchaeota (Huber et al. 2002). This may provide better ways for the isolation of unculturable microorganisms. It is therefore necessary to conduct further study to investigate the unculturable pathogenic bacteria based on our present work.

Besides the *Peduoalteromonas*, four *Vibrio* strains and one *Halomonas* strain also caused disease symptoms. *Vibrio* has been isolated and identified as one of the opportunistic pathogens from diseased *Porphyra* (Tsukidate 1977; Lobban et al. 1985), *Eucheuma* (Uyenco et al. 1981) and *Gracilaria*. *Halomonas marina* has also been shown to have the ability to degrade the thallus of the brown alga, *Fucus evanescens* (Ivanova et al. 2002). Our results were consistent with these studies. It suggested that *Vibrio* and *Halomonas* were the potential pathogens causing Hole-Rotten Disease of *L. japonica*. We speculate that it is possible that *Pseudoalteromonas, Vibrio* and *Halomonas* cause the disease in *L. japonica* simultaneously.

Usually, pure cultures of the potential pathogens involved in diseased seaweeds are obtained through conventional incubation and molecular identification. Here in our study, the 42 isolated bacterial strains were identified to genus by combined methods. Conventional identification of marine bacteria has a low degree of reproducibility and stability, and characterization systems for marine bacteria are time consuming. Compared with conventional methods, molecular identification has proven to be more feasible and reliable (Paillard et al. 2006). Sawabe et al. (1998, 2000) and Uchida et al. (2002) used 16S rRNA gene sequences for pathogenic bacteria analysis to diseased *L. japonica*. Even though 16S rRNA gene sequence analysis can also be applied to analyze the bacterial communities on the diseased seaweed, we believe that it should be combined with conventional identification data to improve the results.

In conclusion, *Pseudoalteromonas*, *Vibrio* and *Halomonas* were the dominant bacterial communities on Hole-Rotten diseased sporophytes of *L. japonica*. However, it is necessary to investigate their degradation mechanisms during the pathogenic infection to algal tissue, and to abate the occurrence of kelp disease during cultivation.

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