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NOTE

Marine Bacteria Associated with the Korean Brown Alga, *Undaria pinnatifida*

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Several marine bacterial strains were isolated from *Undaria pinnatifida* (Miyok in Korean). Sixty-six strains were isolated on R2A agar media at 10°C and identified by a phylogenetic analysis of the 16S rRNA gene sequences. They were grouped into 10 different sequence types based on the initial sequence analysis of the 5' domain of the gene (approximately 500 bp). Full sequences of 16S rRNA gene were obtained from one strain in each sequence type and the species-affiliation was determined using phylogenetic and sequence similarity analyses. The results of the analyses indicated that they were closely related to *Psychrobacter aquimaris, P. celer, P. nivimaris, P. pulmonis, Psychromonas arctica* or *Bacillus psychrodurans*. These bacteria are marine or psychrotrophic bacteria. Because the sporophytes of *U. pinnatifida* are cultured on the costal area during winter, the *U. pinnatifida*-associated bacteria appeared to grow at low temperatures. *U. pinnatifida* sporophytes can be a good source for the isolation of psychrotrophic bacteria.

Keywords: *Bacillus*, marine bacteria, *Psychrobacter, Psychromonas,* psychrotrophic bacteria, *Undaria pinnatifida*

Marine bacteria are closely associated with brown algae. Several bacterial species have been isolated from the spot-wounded fronds of *Laminaria japonica* (Sawabe *et al.,* 2000), the surface of *Fucus serratus* (Johansen *et al.,* 1999) and the rotten thallus of *Fucus evanescens* (Ivanova *et al.,* 2004a, 2004b, 2004c). Some brown algae-associated bacteria may degrade algal polysaccharides, such as fucoidan (Nedashkovskaia *et al.,* 2002), or alginates (Preston *et al.,* 1985, 1986; Brown and Preston 1991). On the other hand, brown algae can produce biologically active compounds that are capable of killing bacteria or inhibiting bacterial growth (Bennamara *et al.,* 1999; Nagayama *et al.,* 2002).

Undaria pinnatifida (Korean, Miyok) is an annual brown alga that exhibits heteromorphic alternation of generation. The sporophyte generally grows 1.5 ~ 2 m long, occasionally up to 3 m, within a year. The microscopic gametophytes are composed of several cells. The dioecious gametophytes produce male or female reproductive cells. *U. pinnatifida* is widely cultivated in Korea, China and Japan where the sporophyte is a commercially important food (Guiry and Blunden, 1991). Two different forms of *U. pinnatifida* are commercially cultivated in Korea: *U. pinnatifida* f. *typica* and *U. pinnatifida* f. *distans* (Okamura, 1936). The two forms show differences in stipe and sporophyll morphology; however, the fucoidan composition is quite similar between the two forms (Lee *et al.,* 2006).

U. pinnatifida growth is temperature dependent. Natural samples of *U. pinnatifida* inhabit cold temperate coastal areas. The outgrowing of *U. pinnatifida* young sporophytes begins in autumn when the water temperature is about 20°C. The optimum range of temperature for sporophyte growth is approximately 5 ~ 10°C (Zhang, 1984). Sporophyte degradation has been reported to occur at temperatures above 20°C and the sporophytes die at temperatures higher than 23°C. Therefore, *U. pinnatifida* is cultured during winter.

In this study, we isolated and identified marine bacteria from the brown alga *Undaria pinnatifida*,

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which had been cultured on the costal area during winter.

Undaria pinnatifida were maricultured around Wando Island (*U. pinnatifida* f. *typica*) and Kijang (*U. pinnatifida* f. *distans*). Mature sporophytes of *U. pinnatifida* were collected on February 15, 2006 and transported to the Korea Polar Research Institute (KOPRI) in an icebox under low temperature conditions. The thalli of *U. pinnatifida* were washed in autoclaved seawater to remove microorganisms that are often loosely attached to the surface of *U. pinnatifida*. The thalli were then cut into 1 × 1 cm^2 square pieces. Each piece was chopped with a sterile razor and transferred into 1.5 ml micro-centrifuge tubes to which 1 ml of sterilized seawater was added. After vigorous vortexing and centrifugation, 50 µl of aqueous phase and 1/10, 1/100, 1/1,000 diluted solutions were spread onto R2A agar media supplemented with distilled water. The plates were cultured at 10°C for 7 days, and the visible colonies were selected and subcultured until a pure culture was obtained. The pure colonies were cultured on R2A agar media for 6 days at 4°C, 10°C, 20°C 25°C, and 30°C, and growth was scored by measuring the colony diameter. The strains were maintained in a glycerol suspension (20%, w/v) at −80°C.

Total genomic DNA was extracted from 1 ml of isolates cultured in R2A broth using an AccuPrep genomic DNA Extraction kit (Bioneer, Korea). Nearly full-length 16S rRNA gene sequences were amplified by PCR using primers 27F; 5'-AGA GTT TGA TCN TGG CTC AG-3' and 1518R; 5'-AAG GAG GTG ATC CAN CCR CA-3'. The PCR mixture consisted of 5 µl of 10× PCR mixture (final concentrations: 50 mM KCl, 0.01% gelatin, 10 mM Tris-HCl pH 9.0), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µl of each primer, 1 µl of template DNA, and 2.5 units of *Taq* polymerase (TaKaRa, Japan) in a final volume of 50 µl. The PCR reaction was performed in a thermal cycler

Table 1. Marine bacteria associated with *Undaria pinnatifida* that had been cultivated around Korea

Strain	Origin	The closest species (Acc. No.)	Similarity (%)	Sister strains*	Temperature (°C)†
KOPRI 24924	*U. pinnatifida* f. *distans*	*Psychrobacter nivimaris* (EF101543)	99.93	N2, N3, N4, N5, N6, N7, N8, N9, N10, N11, N12, N13, N14, N15, N16, N17, N18, N19, N20, N21, N22, N23	4, 10, 20, 25, 30
KOPRI 24925	*U. pinnatifida* f. *typica*	*Psychrobacter nivimaris* (EF101544)	99.86	S2, S3, S4, S5, S6, S7	10
KOPRI 24927	*U. pinnatifida* f. *distans*	*Psychrobacter aquimaris* (EF101545)	99.60		4, 10, 20, 25, 30
KOPRI 24928	*U. pinnatifida* f. *typica*	*Psychrobacter aquimaris* (EF101546)	99.67		10
KOPRI 24929	*U. pinnatifida* f. *distans*	*Psychrobacter aquimaris* (EF101547)	99.67	N25, N26, N27, N29	4, 10, 20, 25, 30
KOPRI 24930	*U. pinnatifida* f. *distans*	*Psychromonas arctica* (EF101548)	98.93	N31, N32, N33, N34, N35, N36, N37, N38, N39, N40, N41, N42, N43, N44	10, 20, 25
KOPRI 24931	*U. pinnatifida* f. *distans*	*Psychromonas arctica* (EF101549)	98.93	N45, N47, N48, N49, N50, N51, N52	10
KOPRI 24932	*U. pinnatifida* f. *typica*	*Psychrobacter celer* (EF101550)	99.46		10
KOPRI 24933	*U. pinnatifida* f. *typica*	*Psychrobacter pulmonis* (EF101551)	99.78		10
KOPRI 24934	*U. pinnatifida* f. *typica*	*Bacillus psychrodurans* (EF101552)	99.93	S12, S13, S14	10

* Strains showing identical 16S rRNA gene sequences with the KOPRI strains. "N" means that the strain was isolated from *U. pinnatifida* f. *distans*, which were maricultured around Kijang, and "S" indicates that this strain was isolated from *U. pinnatifida* f. *typica*, which were maricultured around Wando Island.

† Temperature at which *Undaria pinnatifida*-associated bacteria were able to form colonies on R2A agar media after incubation for 6 days

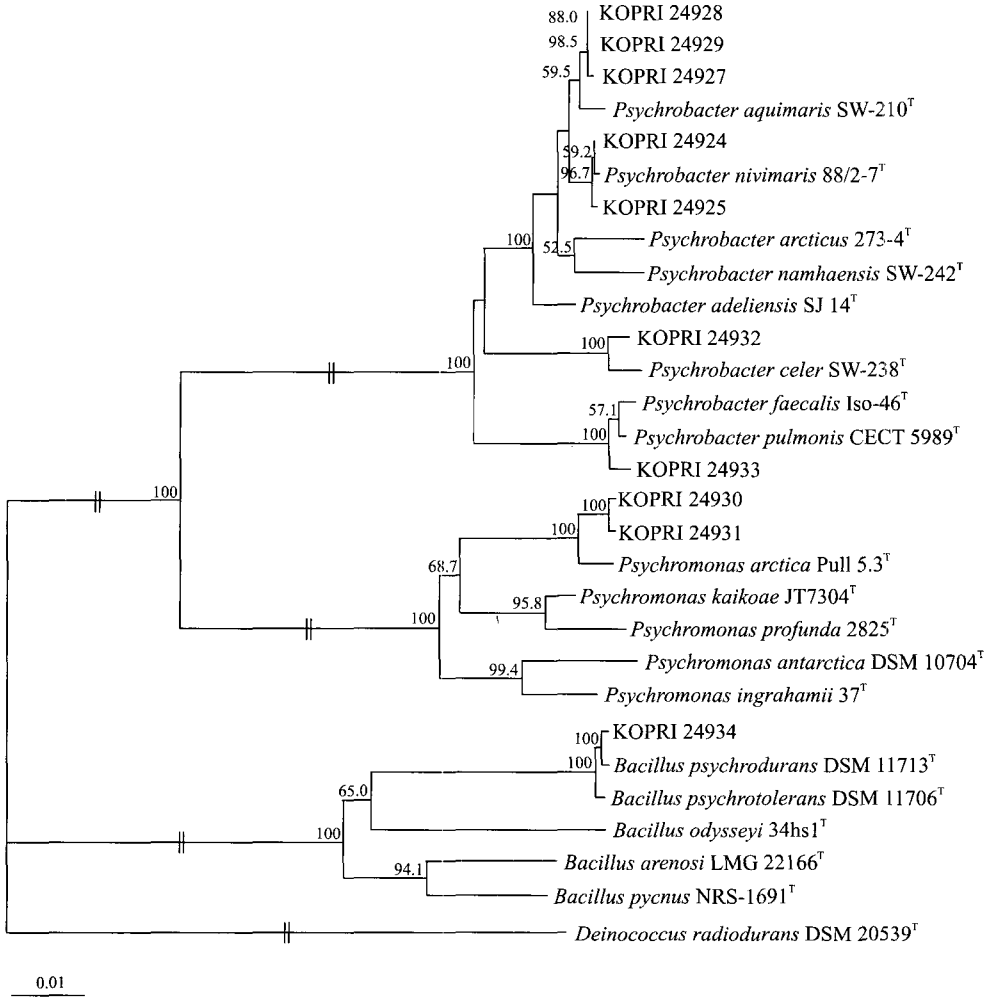

Fig. 1. Neighbor-joining tree showing *Undaria pinnatifida*-associated with marine bacteria. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at the branch points.

(Biometra, Germany) using cycling conditions consisting of an initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis and purified with an AccuPrep PCR purification kit (Bioneer, Korea).

The full-length sequences of amplified 16S rRNA genes were deposited in the GenBank database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). The sequences were submitted to the Advanced BLAST search program to identify the sequences of any closely related organisms. The related sequences were preliminarily aligned with CLUSTALW (Thompson *et al.*, 1994) and manually adjusted with the aid of 16S rRNA secondary structure information using PHYDIT (Chun, 1995). The phylogenetic analyses were performed using PHYLIP (Felsenstein, 1993), and the phylogenetic trees were determined using the neighbor-joining method with Kimura's two-parameter (Saitou and Nei, 1987). The resultant tree topology was evaluated by bootstrap analyses of the neighbor-joining method based on 1,000 resamplings.

A total of 66 strains were isolated from Korean *Undaria pinnatifida*. Among them, 16S rRNA gene sequences of 56 strains were identical to those of representative strains isolated in this study; therefore, 10 representative strains were finally identified. The phylogenetic analysis based on the 16S rRNA gene sequences showed that these 10 stains shared high sequence similarities with 6 species: *Psychrobacter aquimaris, P. celer, P. nivimaris, P. pulmonis, Psychromonas arctica,* and *Bacillus psychrodurans* (Table 1).

Seven of the ten selected strains are members of the genus *Psychrobacter,* which belongs to the *Gammaproteobacteria* (Fig. 1). Two strains, KOPRI 24924 and KOPRI

24925, showed high similarity (more than 99.8%) with *Psychrobacter nivimaris*. *P. nivimaris* 88/2-7 ^{T} was isolated from organic particles in a sea water sample from the southern Atlantic (Antarctic) Ocean (Heuchert *et al.* 2004). The 16S rRNA gene sequences of KOPRI 24924 and KOPRI 24925 differed at only a single site out of 1,477 total sites. Three strains, KOPRI 24927, KOPRI 24928 and KOPRI 24929, showed high similarity (more than 99%) with *Psychrobacter aquimaris*. The 16S rRNA gene sequences of KOPRI 24927 and KOPRI 24928 differed at just a single site out of 1,506 total sites. KOPRI 24928 and KOPRI 24929 have identical sequences, but the growth temperature differed between them (Table 1). KOPRI 24932 showed high similarity with *Psychrobacter celer* (99.46%). *Psychrobacter aquimaris* SW-210^{T} and *P. celer* SW-238^{T} were isolated from seawater collected from the South Sea in Korea (Yoon *et al.* 2005a, 2005b). KOPRI 24933 showed high similarity with *Psychrobacter pulmonis* (99.78%) and *P. faecalis* (99.26%).

Two strains, KOPRI 24930 and KOPRI 24931, showed high similarity (98.93%) with *Psychromonas arctica,* which belongs to the *Gammaproteobacteria* (Fig. 1). *Psychromonas arctica* Pull 5.3^{T} was isolated from a seawater sample taken near Svalbard, Spitzbergen in the Arctic region. *Psychromonas arctica* Pull 5.3^{T} grew in dense aggregates enclosed by fibrous exopoly saccharides within a few days at any cultivation temperature between 4 and 20°C (Groudieva *et al.,* 2003). KOPRI 24930 and KOPRI 24931, however, did not produce any exopolysaccharides. KOPRI 24934 showed high similarity with *Bacillus psychrodurans* (99.93%) and *B. psychrotolerans* (99.73%), which belong to the *Firmicutes* (Fig. 1). *Bacillus psychrodurans* 68E3^{T} was isolated from garden soil in Egypt, and *B. psychrotolerans* 3H1^{T} was isolated from field soil in Germany (Abd El-Rahman *et al.,* 2002).

The *Undaria pinnatifida*-associated bacteria shared high sequence similarities with marine or psychrotrophic bacteria. *Psychrobacter aquimaris, P. celer, P. nivimaris* and *Psychromonas arctica* are marine bacteria (Groudieva *et al.,* 2003; Heuchert *et al.,* 2004; Yoon *et al.,* 2005a; 2005b). While *Psychrobacter nivimaris, Psychromonas arctica* and *Bacillus psychrodurans* are psychrotrophic bacteria (Abd El-Rahman *et al.,* 2002; Groudieva *et al.,* 2003; Heuchert *et al.,* 2004).

All KOPRI strains isolated from *U. pinnatifida* f. *typica* exhibited narrow temperature ranges for growth, growing only at 10°C after incubation for 6 days. On the other hand, the majority of KOPRI strains isolated from *U. pinnatifida* f. *distans* have broad temperature ranges and were able to grow between 4 and 30°C. KOPRI 24930 and KOPRI 24931 were both isolated from the same *U. pinnatifida* thallus and their 16S rRNA gene sequences were the same; however, they exhibited different growth temperature ranges.

All KOPRI strains isolated from *U. pinnatifida* f. *typica* grew at lower temperatures than the other previously reported type strains. The colonies of *P. aquimaris* SW-210^{T} and *P. celer* SW-238^{T} were 1.5 ~ 4.0 mm in diameter after incubation on marine agar 2216 medium for 3 days at 30°C (Yoon *et al.,* 2005a; 2005b). *P. nivimaris* 88/2-7^{T} and *P. pulmonis* S-606^{T} can grow at temperatures up to 35°C and 37°C, respectively (Vela *et al.,* 2003; Heuchert *et al.,* 2004). *Bacillus psychrodurans* 68E3^{T} can grow at temperatures up to 30°C (Abd El-Rahman *et al.,* 2002). On the other hand, KOPRI 24925, KOPRI 24928, KOPRI 24932, KOPRI 24933, KOPRI 24934 and their sister strains could not form a single colony at a temperature over 20°C after incubation for 6 days.

It is interesting that *Undaria pinnatifida*-associated bacteria shared high sequence similarities with psychrotrophic bacteria. Since the sporophytes of *U. pinnatifida* are cultured during winter, many of *U. pinnatifida*-associated bacteria seemed to share high sequence similarities with psychrotrophic bacteria and grew at low temperatures. *U. pinnatifida* sporophytes can be a good source for the isolation of psychrotrophic bacteria.

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