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Antibacterial activities of marine epibiotic bacteria isolated from brown algae of Japan

Manmadhan KANAGASABHAPATHY[1*], Hideaki SASAKI[2], Soumya HALDAR[3], Shinji YAMASAKI[3], Shinichi NAGATA[1]

[1]Environmental Biochemistry Division, Research Center for Inland Seas, Kobe University, 5-1-1 Fukaeminamicho, Higashinada-ku, Kobe 658-0022, Japan; [2]Department of Life and Environment Science, College of Science and Engineering, Iwaki Meisei University, 5-5-1, Chuodaiiino, Iwaki, Fukushima 970-8551, Japan; [3]Laboratory of International Prevention of Epidemics, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1, Gakuen-cho, Sakai-shi, Osaka 599-8531, Japan

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Abstract - One hundred and sixteen epibiotic bacteria were isolated from the surface of nine species of brown algae *Sargassum serratifolium, Sargassum fusiforme, Sargassum filicinum, Padina arborescens, Undaria pinnatifida, Petalonia fascia, Colpomenia sinuosa, Scytosiphon lomentaria* and *Ecklonia cava* which were collected at Awaji Island, Japan. Primary screening results using disc-diffusion assay revealed that, among the bacteria isolated 20% of epibiotic bacteria exhibited antibacterial activity. Among them, 10 bacteria which showed high antibacterial activity were further studied for their ability against (i) a set of fouling bacteria isolated from marine natural biofilm, (ii) some luminescent *Vibrio* and *Photobacterium* species and (iii) a panel of pathogenic bacteria. In general, inhibitory activities were high or moderate against fouling bacteria, *Vibrio* and *Photobacterium* species, while they were found to be low against pathogenic bacteria tested. The phylogenetic analysis using 16S rRNA sequencing revealed that all of the bacteria with high antibacterial activity showed a close affiliation with genus *Bacillus*. This result suggested that the genus *Bacillus* is efficient producers of antibacterial compounds and these epibiotic bacteria isolated are highly successful colonizers on macroalgal surfaces.

Key words: macroalgae, epibiotic bacteria, antibacterial activity, *Bacillus*.

INTRODUCTION

Novel secondary metabolites from marine epibiotic bacteria are attracting attention because of the growing demand for new compounds of natural origin, having potential applications in pharmaceutical or industrial fields. Bacteria growing on the surfaces of marine algae live in a highly competitive environment where space and access to nutrients are limited. Such bacteria living on the surfaces of marine organisms have been documented to include a percentage of antibiotic producing bacteria higher than that observed in free-living bacteria isolated from marine environments (Lemos *et al*., 1986; Zheng *et al*., 2005). For example, in a survey conducted in Scottish coastal waters, 35% of surface-associated bacteria isolated from various seaweed and invertebrate species were found to produce antimicrobial compounds. It has been speculated (Burgess *et al*., 1999) that competition for space and nutrients might be a selective force that may have led to the evolution of a variety of effective adaptation in several attached bacteria. These bacteria can produce a variety of secondary metabolites that inhibit the settlement of potential competitors such as fouling bacteria and invertebrate larvae (Holmstrom *et al*., 1996; Burgess *et al*., 1999).

* Corresponding author. Phone: +81 78 431 8399;
Fax: +81 78 431 6342; E-mail: manmadhan_k@yahoo.co.uk

The surfaces of many marine organisms remain relatively clean. Many sessile algae and animals have evolved defence mechanisms against fouling by producing metabolites that can influence the settlement, growth and survival of other microorganisms potentially able to attach on their surface (McCaffrey and Endean, 1985; de Nys *et al*., 1994). However, some algae and animals lacking chemical and non-chemical defences such as surface shedding are thought to rely on secondary metabolites produced by surface-associated bacteria as their defence against fouling. Bacteria isolated from the surface of the seaweeds have been shown to release compounds that repel other fouling bacteria, suggesting they may protect the seaweed from fouling by other organisms (Boyd *et al*., 1999; Burgess *et al*., 1999). Bacteria belonging to the genus *Pseudoalteromonas* isolated from green alga *Ulva lactuca* were capable of preventing the settlement of invertebrate larvae and inhibited the growth of a variety of bacteria (Egan *et al*., 2000, 2001). Marine epibiotic bacteria have shown to produce antimicrobial compounds, such as phenazine produced by a strain of *Pelagiobacter variabilis* isolated from seaweed *Pocockiella variegata* (Imamura *et al*., 1997). Thus, marine epibiotic bacteria can provide novel antimicrobial compounds.

There are limited studies of the bacteria collected from different algal species, especially those living as epiphytic bacteria on brown algae. To our knowledge, no information is still available on the antibacterial or bactericidal activity of marine epibiotic bacteria from Japanese marine brown algae.

On the other hand, while there is abundant evidence (Burgess *et al*., 1999, Hentschel *et al*., 2001, Zheng *et al*., 2005) that supernatants and solvent extracts of marine epibiotic bacteria exhibit antimicrobial activity against bacteria of clinical significance, the susceptibility of ecologically relevant bacteria to such activity has rarely been studied. In the present work, we have screened marine epibiotic bacteria possessing antibacterial activity, isolated from the surfaces of nine species of brown algae, against a range of ecologically relevant bacteria as well as against some pathogenic bacteria. Their phylogenetic identification was performed by 16S rRNA gene sequencing method.

MATERIALS AND METHODS

Isolation of algal epibiotic bacteria, preliminary screening and preparation of bacterial supernatant. Specimens of the marine brown algae *Sargassum serratifolium, Sargassum fusiforme, Sargassum filicinum, Padina arborescens, Undaria pinnatifida, Petalonia fascia, Colpomenia sinuosa, Scytosiphon lomentaria* and *Ecklonia cava* were collected from the intertidal zone of Esaki (34º36′N, 134º59′E), Iwaya port (34º35′N, 135º1′E), and Oiso (34º33′N, 135º0′E) in Awaji Island, Hyogo, Japan. Portions of these specimens were thoroughly washed three times with sterile seawater to remove loosely attached bacteria. The surface of seaweeds was scraped with a sterile cotton swab to obtain epibiotic bacteria. The swab was then used to directly inoculate Marine agar plates 2216 (Difco Laboratories, Detroit, Michigan, USA) and incubated at 30 ºC for 7 days. Colonies with different morphologies on this medium were selected. Altogether one hundred and sixteen isolates were obtained from the surfaces of a range of marine algae (Table 1). Axenic cultures were obtained by restreaking on agar plates and subsequently stored at 4 ºC.

For the preliminary screening, pure cultures of epibiotic bacteria were inoculated into 10 ml aliquots of nutrient broth which contained (per litre) bactopeptone (Difco) 5 g, yeast extract (Difco) 3 g, NaCl 30 g, $CaCl_2$ 2 H_2O 0.1 g, $MgSO_4 \cdot 7$ H_2O 0.7 g, NH_4Cl 1.0 g, K_2HPO_4 35 mg and KH_2PO_4 15 mg, pH 7.5 (Nagata, 1988). At the exponential phase of growth (48 h) at 30 ºC, each culture was centrifuged (15000 × *g*, 4 ºC, 5 min) and the supernatants, collected and 10 fold concentrated using a rotavapor, were stored as extracts containing natural bioactive substances. Antibacterial testing of the extracts was performed by the paper disc diffusion technique in agar coated Petri dishes as previously described (Bauer *et al*., 1966). A volume of 50 µl of concentrated supernatant was then used to saturate sterilized paper disc (Whatman, 6 mm), allowed to dry at room temperature, and placed on the surface of the Marine agar plates which had been freshly swabbed with the liquid cultures of a marine isolate (FB1), a standard strain of *Vibrio fischeri* DSM 7151 and a pathogenic *Staphylococcus aureus* MTCC 1430. The diameter of the inhibition halos was used as an index of antibacterial activity. Among the bacteria isolated, 10 bacteria, which showed high activity against two or more test strains, were selected for further experiments. For these bioassays, each of these strains was inoculated into 100 ml nutrient broth followed by incubation for 24 h at 30 ºC and the supernatants were collected and treated as previously described.

Isolation of fouling bacteria. The fouling bacteria used in this study were isolated from a marine biofilm formed on the surface of test panels coated with antifouling agents. Zinc pyrithione and pyridine triphenylborane were the antifouling agents of this paint coating, which was immersed in seawater for 2 months at Osaka Bay (34º43′N, 135º17′E), Japan. The surface of the plate, which was covered by a thin biofilm, was washed with sterilized seawater before removing the biofilm using a swab. The swab was used immediately to directly inoculate plates of Marine agar medium. Based on differences in colony morphology and pigmentation we isolated 12 strains, which were designated with progressive numbers from FB-1 to FB-12 in this study.

Environmental bacteria (*Vibrio* and *Photobacterium*). As representative of marine bacteria, five bacterial species belonging to the genera *Vibrio* and *Photobacterium* were used in this study. These were *Vibrio fischeri* DSM 7151, *Vibrio harveyi* IFO 15634, *Vibrio alginolyticus* 138-2 (from Dr. H. Tokuda, The University of Tokyo), *Photobacterium phosphoreum* IFO 13896, and *Photobacterium damselae* IFO 15633.

TABLE 1 – Summary of bacterial species and their antibacterial activity profile isolated from 9 species of brown algae

Brown algae species	Isolated strains strains (No.)	Active strains (No.)*	Highly active strains (No.)**	Code given to selected strains
Sargassum serratifolium	17	3	1	SS1
Sargassum fusiforme	15	5	2	SF3, SF6
Sargassum filicinum	8	1	0	-
Padina arborescens	10	3	1	PA6
Undaria pinnatifida	5	0	0	-
Petalonia fascia	12	5	3	PF5, PF6, PF7
Colpomenia sinuosa	19	3	1	CS10
Scytosiphon lomentaria	9	1	1	SL2
Ecklonia cava	21	2	1	EC1
TOTAL	116	23	10	

* Number of strains exhibiting antibacterial activity in the disc diffusion assay against at least one of the test bacteria. ** Number of strains exhibiting antibacterial activity in the disc diffusion assay against two or more test bacteria.

Bacterial pathogenic strains. Pathogenic strains were obtained from Microbial Type Culture Collections (MTCC), Chandigarh, India. The strains used in the present study were *Staphylococcus aureus* MTCC 1430*, Staphylococcus epidermidis* MTCC 155*, Serratia marcescens* MTCC 86*, Salmonella typhi* MTCC 733*, Klebsiella pneumoniae* MTCC 109*,* and *Salmonella enteritidis* MTCC 453.

Antimicrobial assay against fouling bacteria, environmental and pathogenic bacteria. For the screening of antibacterial substances, each of the twelve fouling bacteria (FB-1 to FB-12), *Vibrio* spp., *Photobacterium* spp., and pathogenic bacteria were grown separately in Nutrient broth for 24 h, then 0.1 ml aliquots were spread over the agar medium. After incubation for 24 h, antibacterial activity was evaluated by measuring the inhibition zone (in mm) from the edge of the disc.

Extraction of genomic DNA. Selected colonies were inoculated in LB broth (Difco) supplemented with 3% NaCl and were grown at 37 °C in shaking condition for 6-8 h. Genomic DNA was extracted using phenol-chloroform extraction method. The culture was centrifuged at 4000 × *g* for 3 min. The pellet was resuspended in 400 μl of sucrose Tris-EDTA buffer. Lysozyme was added to a final concentration of 8 mg/ml and the solution was incubated for 1 h at 37 °C. Into the tube, 100 μl of 0.5M EDTA pH 8.0, 60 μl of 10% SDS and 3 μl of proteinase K were added and incubated overnight at 55 °C. Genomic DNA was extracted with equal volume of phenol:chloroform (1:1), then the mixture was centrifuged (10000 × *g*, 10 min) and the supernatant was transferred to a sterile tube. The supernatant was extracted twice with phenol:chloroform and once with chloroform:isomethyl alcohol (24:1) and precipitated with ethanol. The DNA pellet was resuspended in sterile distilled water and stored at 4 °C for immediate use and at −20 °C for long term storage.

PCR amplification, sequencing of partial 16S rDNA and phylogenetic analysis. PCR was performed by MicroSec® 500 16S rDNA Bacterial Identification Kits (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instruction. The method comprises addition of 15 μl of template DNA with 15 μl of PCR module (including dNTP, buffer, Taq DNA polymerase). Positive and negative controls were prepared using *Escherichia coli* genomic DNA and nuclease free water respectively, supplied by the manufacturer. PCR conditions were fixed as initial denaturation for 10 min at 95 °C followed by 30 cycles of melting for 30 s at 95 °C, annealing for 30 s at 60 °C, extension for 45 s at 72 °C. A final extension was carried out for 10 min at 72° C. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, England) according to manufacturer's instruction. Band size and concentration of PCR products were measured by electrophoresis using 2% agarose gel after staining with ethidium bromide (10 mg/ml) for 10 min, purified PCR products were amplified using 7 μl of purified PCR products and 13 μl of sequencing modules according to manufacturer's instruction for each sample. PCR Conditions were fixed for 25 cycles (melting 96 °C for 10 s, annealing 50 °C for 5 s, extension 60 °C for 4 min).

After cycle sequencing, excess dye terminators and primers were removed by purification with Clean Seq Kit (Agencourt Bioscience Corporation, Beverly, MA01915, USA) and sequencing was done in ABI PRISM®3100 and 3100-Avant Genetic Analyzer, USA. The 16S rRNA gene sequences were compared with all other known rRNA gene sequences through a BLAST search (http://www.ddbj.nig.ac.jp/search/blast-j.html). Similar rDNA sequences were downloaded from the database and aligned with our sequences. The Clustal W program (Thompson *et al*., 1994) was used for preliminary DNA sequence alignment, followed by a manual final alignment. The aligned sequences were subjected to maximum parsimony (MP) analyses in a general heuristic search using PAUP v. 4.0b3a (Swofford, 1999). Twenty random taxon addition replicates were performed in each heuristic search with the Goloboff fit criterion (k = 2), using the TBR branch-swapping option. Gaps were considered as missing data in the MP analysis. From the same alignment, two-parameter distances between taxa were estimated, and a phylogenetic tree was constructed with the neighbour-joining (NJ) method, using PAUP. The robustness of the resulting trees was tested by a bootstrap analysis (Felsenstein, 1985) with 1000 resamplings.

Nucleotide sequence accession numbers. The nucleotide sequences of the isolates sequenced in this study have been added to the DNA Data Bank of Japan (DDBJ)/GenBank with the following accession numbers:
SS1(AB244179), SF3(AB244180), SF6(AB244181), PA6(AB244182), PF5(AB244183), PF6(AB244184), PF7(AB244185), CS10(AB244186), SL2(AB244187) and EC1(AB244188).

RESULTS AND DISCUSSION

Primary screening of macro-algae epibiotic bacteria

In the present investigation 116 strains of epibiotic bacteria were collected from 9 species of macroalgae. For the primary screening all of the bacterial supernatants (10 fold concentrated) were collected and tested for the production of antimicrobial metabolites using the disc diffusion assay against a marine isolate FB1, *Vibrio fischeri* DSM 7151 and a pathogenic *Staphylococcus aureus* MTCC 1430. The antimicrobial assay showed that 23 strains corresponding to 20% of the total of the strains isolated inhibited at least one test microorganism. Ten strains, which showed high activity, inhibiting 2 or 3 test bacteria, were selected for more detailed study. The number of bacterial isolates from each algal sample and the bacterial code given to the selected bacteria are shown in Table 1. Boyd *et al*. (1999) and our results confirmed that algae-associated bacteria frequently display antimicrobial activities.

Activity against fouling bacteria

To characterize the antimicrofouling activity of the macroalgal isolates, the selected 10 bacterial supernatants were first tested against a set of fouling bacteria collected from a natural marine biofilm. This collection of fouling bacteria was chosen to represent the natural community of fouling bacteria in the marine environment. To ensure that the bacteria collected were actually fouling bacteria, we isolated these bacteria from a natural biofilm formed on plates coated with an antifouling paint. Gram staining revealed that the strains of fouling bacteria FB-1, FB-4, FB-8 and FB-9 were Gram-positive, while the other fouling bacteria were Gram-negative. Here, we defined as weak, moderate and strong antibacterial activities those producing an inhibition zone of <2 mm, 2-5 mm and ≥ 5 mm, respectively. In general, all

TABLE 2 – Antibacterial activity of brown algae-associated bacterial supernatant against fouling bacteria

Bacterial code*	Antibacterial activity of bacterial supernatant (inhibition zone in mm)**											
	Gram-positive bacteria				Gram-negative bacteria							
	FB1	FB4	FB8	FB9	FB2	FB3	FB5	FB6	FB7	FB10	FB11	FB12
SS1	4.5	1	0	0.5	2.5	4	5.5	6	5.5	3.5	6	2.5
SF3	4	0.5	0	1.5	3.5	2	3	4.5	2.5	5.5	2.5	3
SF6	3	0	0	1	5	4.5	4	5	3.5	3	5	2
PA6	2	0.5	1	1	4	2.5	6	4.5	3	6.5	3.5	3
PF5	0.5	0.5	0	2	2	3.5	4.5	5.5	6	2.5	5	1.5
PF6	4.5	2	1	0	3.5	2	2.5	5	2.5	2.5	4	2.5
PF7	3.5	0	0	1	2	5.5	4	2.5	5.5	3	2.5	4
CS10	4	1.5	0	1.5	3	3	3.5	5	4.5	4.5	3.5	2
SL2	3.5	1	0	0	5	2.5	3	4.5	4	3	4.5	2.5
EC1	3	0	0	0.5	2.5	3	6.5	3.5	5	6	2.5	4

* The bacteria indicated with the codes FB-1 to FB-12 were fouling bacteria isolated from a natural biofilm. ** Antibacterial activities were determined as inhibition zones (in mm) measured from the edge of the disc after 24 h of incubation.

the bacterial supernatants were active mostly against Gram-negative bacteria compared to Gram-positive bacteria (Table 2). Only one Gram-positive bacterium (FB-1) was inhibited by all of the ten bacterial supernatants with weak to moderate activity (0.5 mm to 4.5 mm). A weak activity was found against other 3 Gram-positive bacteria (FB-4, FB-8, FB-9) with an inhibition zone ranging from 0.5 mm to 2 mm. Moderate and high activities were found against Gram-negative bacteria and all the ten epibiotic bacterial supernatants inhibit at least one of the fouling bacteria with high activity (≥ 5 mm) in this study. The differences in antibacterial properties may reflect the ability of individual isolates to interact and compete with other bacteria.

Recent studies have increasingly indicated that some epibiotic bacteria evoke an inhibitory effect on the growth and attachment of co-occurring bacterial species competing for the same ecological niche (Holmstrom *et al*., 1996; Boyd *et al*., 1999; Holmstrom and Kjelleberg, 1999; Thakur and Anil, 2000). In this study algae-associated bacteria showed some specificity in their antimicrobial activity against fouling bacteria, therefore they might contain some metabolites potentially able to inhibit the attachment of fouling microorganisms in natural environments. In addition these natural substances provide the host plants with a tool to control biofouling and could find applications as natural antifoulants (Clare, 1996).

Activity against *Vibrio* and *Photobacterium* species

In the second panel of test, a collection of both *Vibrio* and *Photobacterium* species was used to assess the antibacterial activity of different algal-epibiotic bacteria. Table 3 summarizes the effect of each of the strains. Generally all the bacterial supernatants showed activity against *Vibrio* and *Photobacterium* species. Particularly the supernatants of strains SS1, PA6, EC1 and SF3 were able to inhibit the growth of most of the target strains used in this experiment with high or moderate activity, while strain PF6 supernatant was found to be weakly active or inactive against the tested strains.

An earlier study indicated that a strain of *Vibrio* isolated from a marine biofilm promotes the settlement of spores (Patel *et al*., 2003). These luminous *Photobacterium* species used in this study were reported to contribute to the micro-

TABLE 3 – Antibacterial activity of brown algae-associated bacterial supernatant against *Vibrio* and *Photobacterium* species

Bacterial code	Antibacterial activity of bacterial supernatant (inhibition zone in mm)*				
	V. fischeri	*V. harveyi*	*V. alginolyticus*	*P. phosphoreum*	*P. damselae*
SS1	4	6	3.5	4	2.5
SF3	4.5	2.5	6	3.5	2
SF6	3.5	4	2.5	3	1.5
PA6	5	3	5.5	4	4.5
PF5	3	4.5	2.5	2.5	3
PF6	2	2	0	1.5	0
PF7	4	3.5	2.5	1	1.5
CS10	3.5	3	1.5	2.5	0.5
SL2	2.5	4.5	3	4	3.5
EC1	4	5.5	5	3	2.5

* Antibacterial activities were determined as inhibition zones (in mm) measured from the edge of the disc after 24 h of incubation.

TABLE 4 – Antibacterial activity of brown algae-associated bacterial supernatant against bacterial pathogens

Bacterial code	Antibacterial activity of bacterial supernatant (inhibition zone in mm)*					
	Staphylococcus aureus	*Staphylococcus epidermidis*	*Serratia marcescens*	*Klebsiella pneumoniae*	*Salmonella typhi*	*Salmonella enteritidis*
SS1	3.5	1.5	0	1	1.5	2
SF3	0	4	1.5	2	3	0
SF6	2	1	0	0.5	1.5	0
PA6	5	3.5	4.5	2.5	3.5	3
PF5	2	0	3	2.5	1.5	0.5
PF6	0	2	1.5	0	0	1.5
PF7	2	0.5	1.5	0.5	0	0
CS10	3	0	0.5	2.5	3.5	4
SL2	0	1.5	1	1.5	0	1
EC1	4.5	2	5	3	3.5	2.5

* Antibacterial activities were determined as inhibition zones (in mm) measured from the edge of the disc after 24 h of incubation.

fouling community in the marine environment (Li *et al*., 2003). The broad antibacterial activity expressed by the epibiotic bacteria in our experiments suggests that these algal-associated bacteria have the potential to control the growth of likely competitors in the surface environment and thus exert some control over the composition of the microbial population at the seaweed surface. It was also proposed that epibiotic bacteria on macroalgae can produce antifouling compounds that work in synergy with the seaweed derived compounds to protect the seaweed surface (Armstrong *et al*., 2001). Therefore these isolated epiphytic bacteria may be potentially able to influence the composition of the microbial community present on seaweed surfaces through the production of secondary metabolites with antimicrobial properties (Boyd *et al*., 1999). These findings support the hypothesis that epibiotic bacteria may regulate biofouling events on surfaces of marine organisms.

Activity against pathogens

As there is a growing interest in the usage of microbial secondary metabolites in medical applications, various pathogenic strains were also included in this study as other target organisms. Table 4 summarizes the effect of each isolate upon the growth of 6 different pathogenic strains. The supernatants from isolates PA6 and EC1 inhibited all the pathogenic strains, but with a moderate antibacterial activity ranging from 2 to 5 mm. Less pronounced moderate activities were exhibited by the supernatants of strains SS1, SF3, PF5 and CS10, while those of other isolates (SF6, PF6, PF7 and SL2) displayed weak or no activity. In general the inhibition ability against pathogens was lower than that detected against the environmental strains or against *Vibrio-Photobacterium* species.

Microbial competition for limiting natural resources within a community is thought to be an important selective force that promotes biosynthesis of antimicrobial compounds (Slattery *et al*., 2001). From an ecological point of view, the inhibition of other marine bacterial species competing for the same niche will give a selective advantage during colonization. In this study the weak inhibitory effect against other non-marine bacteria, therefore, can provide evidence of a lack of competition for the same niche.

Phylogeny of the isolates

An attempt was made to perform the 16S rRNA analysis to clarify the phylogenetic status of epibiotic bacteria with high antibacterial activity. For phylogenetic characterization, the 16S rRNA sequences of 23 species were compared with the DDBJ database using the blast program, including 10 newly reported sequences. The aligned sequences comprised a total of 588 sites and contained 89 parsimony-informative nucleotide positions. In MP analysis, 8 maximum parsimonious (316 steps, CI = 0.6912, RI = 0.8161) trees were found. Tree topologies were essentially the same for maximum parsimony and neighbour-joining analysis (Fig. 1). Molecular phylogenetic analysis demonstrated all bacterial isolates with antibacterial activity belonged to the genus *Bacillus* family Bacillaceae. Neighbor-joining analysis of 16S rRNA sequences revealed that strains PF5, PF7, SF6, SS1, and SL2 belonged to *B. cereus*, strain PF6 belonged to *B. clausii*, strains EC1, SF3, and CS10 belonged to *B. pumilus* and strain PA6 belonged to *B. subtilis*. Strains PF5, PF7, SF6, SS1, and SL2 showed 98.7-99.6% similarity (2-6 bp difference) to *B. cereus*. The isolate PF6 showed 96.5% similarity (17 bp difference) to *B. clausii*. Strains EC1, SF3, and CS10 showed 99.0-99.8% sequence similarity (1-5 bp difference) to *B. pumilus*. The isolate PA6 showed 98.4% similarity (8 bp difference) to *B. subtilis*. Based on their 16S rRNA genes, some isolates were closely related to each other or even identical. In our experiments, however, all isolates were treated as distinct entities since they differed in their individual inhibition ability and/or growth patterns.

The high number of Gram-positive bacterial isolates found in the present study may be due to our primary screening procedure, in which we selected the strains based on their broad activity against tested strains. Since we carefully rinsed all our specimens with sterile seawater, we presume that the vast majority of our isolates were indeed attached to macro-algae. However, our Gram-positive isolates were able to grow at the high salt concentrations typical of seawater we used for cultivation and hence we assume that they are able to efficiently compete with other marine bacteria.

Bacillus species are well known to be the producers of metabolites with antimicrobial and antifungal activities (Zuber *et al*., 1993). Bioactive metabolites have also been reported in a variety of other *Bacillus* (Lebbadi *et al*., 1994;

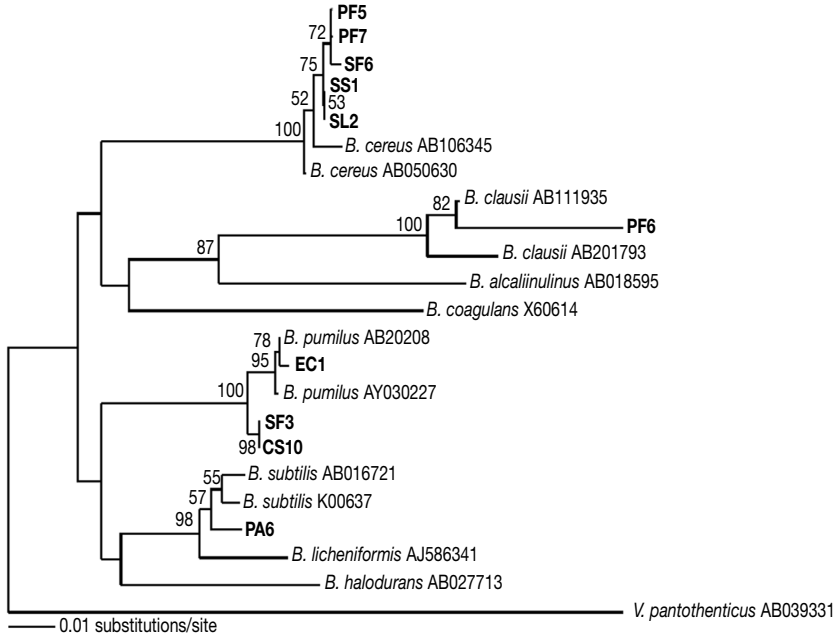

Fig. 1 – Neighbor-joining analysis of 16S rRNA gene sequences from 23 species of Bacillaceae, including 10 strains showing high antibacterial activity, isolated from 9 brown algal species. The scale bar indicates 1% sequence divergence. Numbers at nodes represent bootstrap values in percentage terms based on 1000 replicates.

Hathout *et al*., 1999), rendering this genus one of the most active producers of bioactive metabolites. *Bacillus* species have primarily been isolated from soil but have been frequently found also in several marine habitats (Trischman *et al*., 1994; Ivanova *et al*., 1999; Siefert *et al*., 2000) on multiple occasions. In our earlier study, 4 strains of *Bacillus* were isolated from the surface of a marine sponge *Pseudoceratina purpurea* in Indian waters (Manmadhan *et al*., 2005). *Bacillus* species have also been isolated from marine macroalgae. Burgess *et al*. (2003) isolated several bacteria with high antifouling activity and found that most of these bacteria belonged to the genus *Bacillus*, such as *B. pumilus*, *B. licheniformis* and *B. subtilis*. Their supernatants incorporated in sea paints were able to inhibit microfouling as well as macrofouling. *Bacillus cereus* isolated from soil was reported to produce a novel aminopolyol antibiotic called zwittermicin A (Stabb *et al*., 1994) and large number of peptide antibiotics (Bizani and Brandelli, 2002). In this study five active strains have been identified as belonging to the species *B. cereus*, which is usually found in soil. According to our knowledge this is the first report concerning the antibacterial activity of a strain of *B. clausii* isolated from macro-algal surface.

CONCLUSIONS

Although there were many studies and ecological investigations on marine microbial screening for medically important compounds attributed to macroalgae epibiotic bacteria, this study particularly focused on the isolation and phylogenetic identification of bacteria with antimicrobial activities from several brown algal species collected from Japanese waters. This study has also demonstrated that the *Bacillus* species display specific inhibitory activities in different bioassays. Although a previous study (Burgess *et al*., 1999) proved that the inclusion of culture supernatants in the growth medium stimulated the antimicrobial activity in marine epibiotic bacteria, the variation in the levels of activity of the *Bacillus* species against different target strains suggests that different antibacterial compounds are produced by each species. This suggests that the members of *Bacillus* are successful competitors with other microorganisms for surface, space and nutrient in the marine environment.

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