Inhibitory Activities of Surface Associated Bacteria Isolated from the Marine Sponge *Pseudoceratina purpurea*

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The effects of four strains of Gram positive bacteria (PS2, PS9, PS11, and PS79) isolated from the sponge *Pseudoceratina purpurea* on the growth of a set of fouling bacteria and standard strains of genera *Vibrio*-*Photobacterium* were examined. Using a paper disc diffusion method, 4 sponge isolates were found to have inhibitory activity against 4 of 12 isolates of the fouling bacteria. All of the four bacterial supernatants also showed inhibitory activity against *Vibrio alginolyticus* and *V. fischeri*, while sponge extracts inhibited every strain tested. Phylogenetic analysis using 16S rRNA gene sequences revealed that three of the sponge isolates (PS2, PS9 and PS79) belonged to the genus *Bacillus* and one (PS11) belonged to the genus *Virgibacillus*. The isolation of strains possessing inhibitory activity against fouling bacteria suggests that sponge-associated bacteria could be potential sources of novel natural products.

Key words: marine sponge, *Pseudoceratina purpurea*, sponge associated bacteria, antibacterial activity, *Bacillus*, *Virgibacillus*

Marine sponges are known to produce a wide array of bioactive secondary metabolites. Sponges house archaea, cyanobacteria and unicellular algae⁸, many of which play supporting roles in nutrition²⁹, defense against predators and biofouling^{2,26}. Sponges are also known to maintain numerous bacteria which can amount to up to 40% of the biomass of a sponge²⁸. It has been reported that the ratio of bacteria with antimicrobial activity isolated from marine invertebrates was higher than that from other sources³. Bacteria associated with marine invertebrates might be a chemical defense for their host. Considering the cooperative relationships between bacterium and sponge in chemical defense, there is further potential to seek for natural products in bacteria associated with marine invertebrates.

The marine sponge *Pseudoceratina purpurea* (Order Verongida, Family Aplysinellidae) found in shallow waters in the Gulf of Mannar, India, is almost free of fouling or-

ganisms and at the same time has proven to be a rich source of interesting metabolites. A number of sponge species, such as *Psammopemma* sp., *Psammoplysilla purpurea*, *Aplysina aerophoba*, and *Dysidea herbacea*, produce brominated aromatic metabolites, bromoindoles, bromophenols, polybrominated diphenyl ethers and di-bromodibenzo-*p*-dioxins²⁷. Many sponge or sponge symbiont-derived metabolites are potent antibacterial, antifungal, antifeeding and antifouling compounds¹. Some marine bioproducts originally isolated from macroorganisms, such as sponges, have been subsequently discovered to be present in microbial associates². The role of sponge-associated microorganisms in the synthesis of compounds of biological interest is a subject of scientific debate, as in some cases these bacteria are reported to produce bioactive metabolites¹². The marine surface-associated bacterium *Pseudoalteromonas tunicata* strain D2 produces a number of extracellular inhibitors during the stationary phase of growth that affect the normal settlement and growth of a variety of common marine fouling organisms¹⁰. The *Vibrio* spp. associated with the sponge

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Dysidea species were shown to synthesize cytotoxic and antibacterial tetrabromodiphenyl ethers⁵). In addition, dike-topiperazines produced by a *Micrococcus* species associated with the sponge *Tedania ignis* were found to have potent antimicrobial activity²²).

Many previous studies on antimicrobial activity have tested sponge metabolites against terrestrial or medically important strains in order to search for novel antimicrobial agents. But metabolites may affect ecologically relevant bacteria in a different manner. From an ecological point of view, it is also important to test sponge extracts and surface associated bacterial extracts against bacteria that sponges would naturally encounter in the environment. While there is abundant evidence that crude organic extracts of marine invertebrates exhibit antimicrobial activity against medically important bacteria, the susceptibility of ecologically relevant bacteria to such activity has rarely been studied⁸). Bacteria of the genus *Vibrio* are typically found in marine habitats where they are often attached to surfaces. Luminescent bacteria such as *Photobacterium* species are commonly used to test the effect of antifouling compounds³⁰). To test the inhibitory activities of sponge-associated bacteria we have selected a series of fouling bacteria isolated from a natural biofilm formed on antifouling paint-coated plates and a set of *Vibrio* and *Photobacterium* species. The aim of this study was to isolate bacteria with antimicrobial activities from the *P. purpurea* sponge and to identify potent bacterial strains using a 16S rRNA phylogenetic analysis.

Materials and methods

Sponge materials

The marine sponge *P. purpurea* was collected from a depth of 5 m along the coastal waters of Mandapam, Gulf of Mannar (09°16'N, 79°08'E), India and washed with sterile seawater to remove adhering debris and associated biota. Then the sponge was squeezed softly to eliminate most of the extraneous water and soaked in methanol before being transported to the laboratory. Final processing of the sponge was carried out by crushing it into smaller pieces and filtering through a fine mesh using 90% methanol by percolation 3 times at ambient temperature. These extracts were evaporated dry under reduced pressure. The concentrated methanol extracts were stored and used to check for antibacterial properties.

Isolation of sponge-associated bacteria, preliminary screening, and preparation of bacterial supernatant

Additional *P. purpurea* individuals were collected and

rinsed thoroughly with sterile seawater to remove loosely-attached bacteria before swabbing with a sterilized cotton swab from different portions of the sponge surface. The swab was then used to directly inoculate standard Marine agar medium (Himedia, Bombay, India) which contained (per liter) peptone 5 g, yeast extract 1 g, ferric citrate 0.1 g, K₂HPO₄ 0.5 g, and agar 15 g (pH 7.5). Colonies with different morphologies on agar medium after incubation at 30°C were selected. Altogether, 102 strains were recovered from the sponge surface and stored for further screening.

For the preliminary screening, pure cultures of surface-associated bacteria were inoculated into 10-ml aliquots of nutrient broth which contained (per liter) Bactopeptone (Difco Laboratories, Detroit, MI, USA) 5 g, yeast extract (Difco) 3 g, NaCl 30 g, CaCl₂·2H₂O 0.1 g, MgSO₄·7H₂O 0.7 g, NH₄Cl 1.0 g, K₂HPO₄ 35 mg and KH₂PO₄ 15 mg (pH 7.5). At an exponential phase of growth at 30°C, each culture was centrifuged (15,000×g, 4°C, 5 min). Antibacterial testing of the extracts which were concentrated from supernatants 10 fold was performed by the paper disc diffusion technique in agar plated Petri dishes as previously described⁷). A volume of 60 µl of concentrated supernatant was then used to saturate a sterilized paper disc (Whatman, 6 mm), allowed to dry at room temperature, and placed on the surface of Marine agar plates which had been freshly swabbed with the liquid cultures of *Escherichia coli* K-12 IFO 3301. Among 102 bacteria, 4 which were highly active against *E. coli* IFO 3301 were selected for further experiments.

To check their resistance to sponge metabolites, these four bacteria were spread over the Marine agar plates and the sponge crude extracts (60 µl) were used to test their activity with the same paper disc diffusion assay. All four bacteria showed resistance to the sponge crude extracts (no inhibition zone) and these strains were designated PS2, PS9, PS11 and PS79. For further bioassay experiments, each of these strains was inoculated into 100 ml of nutrient broth and incubated for 24 h at 30°C. The supernatants were then collected and concentrated 10 fold.

Isolation of fouling bacteria

The fouling bacteria were isolated from an artificially developed biofilm on the surface of steel plates coated with zinc pyrithione and pyridine triphenylborane, two antifouling agents found in paint. The plates coated with the antifouling agents were left for 2 months in Osaka Bay (34°43'N, 135°17'E), Japan. The thin biofilm that developed on the steel plate surface was washed with sterilized seawater, and a swab was used immediately to directly inoc-

ulate plates of Marine agar medium. Based on differences in colony morphology and pigmentation we isolated 12 strains, designated FB-1, FB-2, FB-3, FB-4, FB-5, FB-6, FB-7, FB-8, FB-9, FB-10, FB-11 and FB-12 in this study.

Vibrio and Photobacterium species

As representative of marine bacteria, five species belonging to the genera *Vibrio* and *Photobacterium* were used in this study. These were *V. harveyi* IFO 15634, *Photobacterium phosphoreum* IFO 13896, *P. damsela* IFO 15633, *V. fischeri* DSM 7151, and *V. alginolyticus* 138-2 (from H. Tokuda, The University of Tokyo)²⁵.

Bioassay against growth of fouling bacteria and Vibrio species

Sponge extracts and bacterial supernatant (60 µl) loaded on each of the paper discs were allowed to dry at room temperature. *Vibrio* spp., *Photobacterium* spp. and the twelve fouling bacteria, FB-1 to FB-12, were grown in nutrient broth for 24 h, at which point 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. Control tests with methanol alone were performed for every assay, but showed no inhibition.

DNA extraction and PCR amplification

Bacteria were grown in a modified LB medium (pH 7.0) consisting of 5 g/l dry yeast extract D-3 (Nihon Pharmaceuticals, Tokyo, Japan), 10 g/l polypepton (Nihon Pharmaceuticals) and 2 g/l NaCl. Bacterial pellets were collected from 1 ml of culture by centrifugation (18,000×g, 4°C) for 5 min. These pellets were used for extracting genomic DNA which was achieved using a Blood and Tissue Genomic DNA Extraction Miniprep System (Viogene, Illkirch Cedex, France), following the manufacturer's instructions.

PCR amplification of the 16S rRNA gene was carried out using GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The total reaction volume of 30 µl included 3.0 µl of 10×*Taq* Buffer with MgCl₂ (Promega, Madison, Wisconsin, USA), 3.0 µl of dNTP mixture (Takara-Bio, Shiga, Japan), 2.1 pmol of each primer, 0.8 units of *Taq* polymerase (Promega) and 1.0 µl of DNA solution. The oligonucleotide primers used (27f; 5'-GAGTTTGATCCTGGCTCA-3', 1385r; 5'-CGGTGTGTRCAAGGCC-3') were based on a previous report⁸). The conditions for PCR were as follows; initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 30 s; and a final extension at 72°C for

7 min. The PCR products were ligated into the pT7Blue T-vector (EMD Biosciences, San Diego, CA, USA). Plasmids were introduced into *Escherichia coli* strain JM109 by electroporation using a Micro Pulser (Bio-Rad Laboratories, Hercules, CA, USA). Plasmids were sequenced using the BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems) and ABI PRISM 377 Genetic Analyzer (Applied Biosystems).

DNA sequencing and phylogenetic analysis

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 program²⁴ 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. 4.0b3a²³. 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 analysis⁶ with 1000 resamplings.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences have been deposited into the DNA Data Bank of Japan (DDBJ)/GenBank with the following accession numbers: PS2 (AB196350), PS9 (AB196351), PS11 (AB196352) and PS79 (AB196353).

Results and Discussion

Primary screening of sponge isolates

In our earlier study¹⁷ we demonstrated that some surface-associated bacteria isolated from the marine sponge *Psammaphysilla* (= *Pseudoceratina*) *purpurea* have the ability to inhibit pathogenic bacteria. In the present investigation, four bacteria (PS2, PS9, PS11 and PS79) were selected after a screening based on their resistance to host metabolites and inhibition of strain IFO 3301. The sponge crude extracts were tested against these four bacterial cultures using the paper disc diffusion method, which indicated that none of the bacteria was inhibited by sponge extracts. The results of the primary screening based on their potential tolerance of

host metabolites suggested that these four bacteria were not common free-living bacteria (planktonic) but instead associated with the surface of the host. Earlier reports stated that the production of secondary metabolites by marine organisms may serve to protect against colonization, and hence bacteria attaching to such surfaces are likely to resist host metabolites as well as to express phenotypes which will enable the cells to adhere to the surface¹¹). By analogy, bacteria that are resistant to the bioactive metabolites of their host might be capable of producing antimicrobial compounds themselves¹⁹).

Activity against fouling bacteria

To characterize the antimicrofouling activity of the *P. purpurea* sponge isolates, bacterial supernatants were first tested against a set of fouling bacteria collected from a natural marine biofilm. To compare bacterial inhibitory activity, sponge crude extracts were also included in the bioassay. To ensure that the bacteria collected from the marine environment are actually fouling bacteria we have isolated 12 fouling bacteria from a natural biofilm formed on plates coated with antifouling paint. Their characteristics and partial identification are summarized in Table 1. Gram staining experiments revealed that the fouling bacteria FB-1, FB-4, FB-8 and FB-9 were Gram-positive, while the rest were Gram-negative. In the first test against fouling bacteria, the sponge extracts were active against all 12 fouling bacteria and the supernatants of sponge-associated bacteria inhibited only some of the fouling bacteria as shown in Table 2. The supernatant obtained from four bacteria had no activity against fouling bacteria such as FB-4, FB-5, FB-8, FB-9, and FB-11, including three Gram positive and two Gram negative

strains. Here, we define mild, moderate and strong activities as an inhibition zone of <2 mm, 2–5 mm and ≥5 mm, respectively. Mild activity was found against FB-3, FB-10 and FB-12 (all Gram negative) and even this inhibitory effect differed from strain to strain, *i.e.* supernatant from strain PS11 had no effect against strain FB-3, but inhibited strains FB-10 and FB-12. Moderate activity was found against strains FB-1, FB-2 and FB-7, which include one Gram positive and two Gram negative strains. The greatest inhibition was found against strain FB-6 (Gram negative) where the zone produced by the supernatant of PS9 was larger than that of the sponge extract. The inhibitory effects of 4 isolates against strain FB-7 were also strong or almost equivalent to that of sponge extracts. At least one of the supernatants from sponge-associated bacteria was active against strains FB-1, FB-2, FB-3, FB-6, FB-7, FB-10 and FB-12 (Fig. 1). In general, all the bacterial supernatants were active mostly against Gram negative bacteria and only one Gram-positive bacterium (FB-1) was inhibited by any of the four bacterial compounds.

In screening for substances with antifouling activity, the paper disc diffusion assay employing fouling bacteria as the target organism has been widely used as an indicator of antifouling potential⁴). These four associated bacteria showing some specificity in their antimicrobial activity therefore may contain some metabolites which have the potential to inhibit the attachment of fouling microorganisms in natural environments.

Activity against *Vibrio* and *Photobacterium* species

In the second panel of the test, the sponge crude extracts were highly active against all the *Vibrio* species used in this

Table 1. Fundamental features of bacterial species isolated from marine natural biofilms formed on the surface of plates coated with antifouling paint

Fouling bacteria	Gram	Color	Shape	Elevation
FB-1	+	yellow	cocci	convex
FB-2	–	pale orange	rods	convex
FB-3	–	fluorescent	short rods	flat
FB-4	+	white	cocci	convex
FB-5	–	white	rods	convex
FB-6	–	yellowish cream	rods	raised
FB-7	–	pale yellow	short rods	flat
FB-8	+	ivory	rods	convex
FB-9	+	white	cocci	convex
FB-10	–	pale white	rods	convex
FB-11	–	colorless, transparent	rods	flat
FB-12	–	pale white	filamentous	raised

Table 2. Antimicrofouling activities* of supernatants from sponge-associated bacteria and sponge extract from *Pseudoceratina purpurea*

Fouling Bacteria	Supernatants of sponge-associated bacteria				Sponge extract
	PS2	PS9	PS11	PS79	
FB-1	3	6	1.5	4	6.5
FB-2	4.5	4	5	6	7
FB-3	0.5	0.5	0	0.5	8
FB-4	0	0	0	0	9
FB-5	0	0	0	0	2
FB-6	0	10	0	0	9
FB-7	5	9	5	6	7
FB-8	0	0	0	0	8
FB-9	0	0	0	0	7
FB-10	0	0	0.5	0	5
FB-11	0	0	0	0	4
FB-12	0	0.5	0.5	0	12

* Antimicrofouling activity was determined as a zone of inhibition (in mm) measured from the edge of the disc after 24 h of incubation; 0 = no activity.

Fig. 1. Supernatant activity of sponge surface-associated bacteria against fouling bacteria FB-1, FB-2, FB-6 and FB-7. Fouling bacteria were collected from the biofilm formed on plates coated with antifouling paint. 2C, 9C, 11C and 79C indicate 60 µl of 10-fold concentrated bacterial supernatant from strains PS2, PS9, PS11 and PS79 that were isolated from the marine sponge *P. purpurea*, respectively.

study (Table 3). The supernatants of four sponge-associated bacteria were not active against the strain *V. harveyi*. Moderate and high levels of activity were recognized against *V. fischeri* and *V. alginolyticus*, respectively. The supernatant of the isolate PS79 was almost as active against *V. algi-*

nolyticus as the sponge extracts. An earlier study indicated that a strain of *Vibrio* isolated from a marine biofilm promotes the settlement of spores²⁰. In addition, marine *Vibrio* species were found to be a major cause of bleaching in corals through effects on the coral's symbiotic zooxanthellae¹⁵.

Sponge extracts inhibited the growth of both *Photobacterium* species but none of the bacterial supernatants was active against them (Table 3). Both of these luminous bacteria were reported to contribute to the microfouling community in the marine environment. In addition, *Photobacterium* species have been widely used in assessing the toxicity of antifouling compounds. The antibacterial effect of the supernatant was species specific, *i.e.*, it inhibited the growth of only some fouling bacteria as well as two *Vibrio* species but did not inhibit *Photobacterium* species.

In this study, the production of compounds by bacteria on the surfaces of marine sponge suggests that there are some interactions taking place between the sponge and bacteria and these associated bacteria may play a role in the defensive mechanisms of the host. Such interactions are not uncommon in the marine environment with epibiotic bacterial species implicated in the production of toxic metabolites originally attributed to their host²⁶.

Thus, it seems plausible that some surface-associated bacteria have adapted to defend their position on the sponge surface through the production of bioactive metabolites which are active against other bacteria. In this study it has been demonstrated that bacteria which are associated with the sponge *P. purpurea* are capable of affecting the surface ecosystem through the production of (chemical) metabo-

Table 3. Activities of supernatants from sponge-associated bacteria and sponge extract from *Pseudoceratina purpurea* against *Vibrio* and *Photobacterium* species*

Fouling strains	Supernatants of sponge-associated bacteria				Sponge extract
	PS2	PS9	PS11	PS79	
<i>Vibrio alginolyticus</i> 138-2	8	5	4	12	13
<i>Vibrio harveyi</i> IFO 15634	0	0	0	0	6.5
<i>Vibrio fischeri</i> DSM 7151	3	4	0	2.5	11
<i>Photobacterium damsela</i> IFO 15633	0	0	0	0	4.5
<i>Photobacterium phosphoreum</i> IFO 13896	0	0	0	0	9

* Antibacterial activity was determined as a zone of inhibition (in mm) measured from the edge of the disc after 24 h of incubation; 0 = no activity.

Fig. 2. Neighbor-joining analysis of 16S rRNA gene sequences from several species of *Bacillaceae* and strains PS2, PS9, PS11 and PS79 isolated from the marine sponge *P. purpurea*. The scale bar indicates 1% sequence divergence. Numbers at nodes represent bootstrap values in percentage terms based on 1000 replicates.

lites. The sponge seems to have selective control of the bacterial epibiosis on its surface. This relation may help the sponge in governing epibacterial populations on its surface by deterring settlement of other microbes.

Phylogenetic characterization of isolates

For phylogenetic characterization, the 16S rRNA gene sequences of 24 species were compared with the GenBank database using the BLAST programme, including 4 newly reported sequences. The aligned sequences comprised a to-

tal of 1399 sites and contained 214 parsimony-informative nucleotide positions. In the MP analysis, one maximum parsimonious (770 steps, CI=0.6078, RI=0.7127) tree was found. Tree topologies were essentially the same for maximum parsimony and neighbour-joining analyses (Fig. 2). Molecular phylogenetic analysis showed that all bacterial isolates belong to the family *Bacillaceae*. Neighbor-joining analysis of 16S rRNA gene sequences revealed that the isolates PS2 and PS79 belong to *Bacillus licheniformis*. In addition, the isolates PS9 and PS11 belong to *Bacillus pumilus*

and *Virgibacillus pantothenicus*, respectively. There is 99.7% similarity (4 bp difference) between PS2 and PS79, and both isolates showed greater than 99.7% similarity to *Bacillus licheniformis*. The isolate PS9 showed greater than 99.7% similarity to *Bacillus pumilus*. The isolate PS11 showed 98.9% sequence similarity to *Virgibacillus pantothenicus*.

Bioactive metabolites have been reported in a variety of *Bacillus* species¹⁶, rendering this genus one of the most active producers of bioactive metabolites. Even though Bacilli are reported to be terrestrial bacteria, they have been isolated from marine coastal and open ocean environments on multiple occasions¹³. Bacilli have also been isolated from marine sponges, such as *Ircinia felix*²¹, *Aplysina aerophoba*⁸, and soft corals. Furthermore, *Bacillus* species are well known producers of metabolites with antimicrobial, antifungal, or generally cytotoxic properties³¹. Surfactin possessing a dipeptide moiety was initially isolated from a marine sponge, and thereafter it was shown that the bacterium, *Bacillus pumilus*, which is associated with the sponge, is the real producer of these compounds¹⁴. Also *B. pumilus* has been reported to produce lipopeptides, such as pumilacidin A and B, which contain antiviral activity¹⁸. As with most other *Bacillus* species, marine *Bacillus* species are able to produce peptide compounds with antimicrobial activity⁸.

Among the numerous *Bacillus* species, only *B. badius*, *B. subtilis*, *B. cereus*, *B. licheniformis*, *B. firmus*, *B. pumilus*, *B. mycoides*, and *B. lentus* were reported to be detected in marine environments¹³. The genus *Virgibacillus* was recently created with *V. pantothenicus*, formerly known as *B. pantothenicus* as the type species⁹. This is the first time that *Virgibacillus pantothenicus* has been isolated from a marine sponge, as far as we know.

In conclusion, this study demonstrated that four surface-associated bacteria from *P. purpurea* suppressed fouling bacteria isolated from natural biofilms and inhibited the growth of two *Vibrio* species. Based on the present findings, it is envisaged that the bioassay-guided purification and fractionation of these bacterial metabolites will provide leads for the development of environmentally-friendly antifouling compounds.

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