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Antiangiogenic, Antimicrobial, and Cytotoxic Potential of Sponge-Associated Bacteria

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Abstract

The bacteria associated with marine invertebrates are a rich source of bioactive metabolites. In the present study bacteria associated with the sponge *Suberites domuncula* and its primmorphs (3-dimensional aggregates containing proliferating cells) were isolated and cultured. These bacteria were extracted, and the extracts were assayed for antiangiogenic, hemolytic, antimicrobial, and cytotoxic activities. Our studies revealed that extract obtained from the bacterium (PB2) isolated from sponge primmorphs is a potent angiogenesis inhibitor. In the chick chorio-allantoic membrane (CAM) assay, it showed 50% activity at 5 μg ml^{-1} and 100% activity at 10 and 20 μg ml^{-1} concentrations. Extracts obtained from 5 bacterial strains isolated from sponge and its primmorphs showed hemolytic activity. The sponge-associated bacteria belonging to the α subdivision of *Proteobacteria* and the primmorph-associated bacterium identified as a possible novel *Pseudomonas* sp. displayed remarkable antimicrobial activity. It is important to note that these bacterial extracts were strongly active against multidrug-resistant clinical strains such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, isolated from hospital patients. The bacterial extracts having antimicrobial activity also showed cytotoxicity against HeLa and PC12 cells. In summary, this investigation explores the importance of sponge-associated bacteria as a valuable resource for the discovery of novel bioactive molecules.

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Introduction

In recent years the ocean has been considered as a rich source of compounds possessing novel structures and biological activities. Biologically active molecules isolated from marine flora and fauna have applications in pharmaceuticals, nutritional supplements, cosmetics, agrochemicals, molecular probes, enzymes, and fine chemicals (Faulkner, 2002). Microorganisms and in particular bacteria have had a profound effect on the development of medical science. Microorganisms are not only the cause of infections; they can also produce organic substances that can cure infections (Jensen and Fenical, 2000). Marine bacteria constitute approximately 10% of the living biomass of carbon in the biosphere (Parkes et al., 1994). However, only a small percentage of marine bacteria have been studied for their bioactive potential. These bacteria originate mainly in sediments but also occur in the open ocean and in association with marine organisms.

Microorganisms associated with marine invertebrates are reported to be involved in the production of bioactive molecules (Proksch et al., 2002, and references cited therein). Bioactive compound production in these bacteria could be attributed to the competition among them for space and nutrition (Burgess et al., 1999). Though these bioactive compounds may be important for epibiotic defense of marine invertebrate hosts (Holmström et al., 2002; Thakur et al., 2003), they also have significant medical and industrial applications.

Sponges (*Porifera*), being evolutionarily ancient, multicellular, sessile organisms, inhabit every type

of marine benthic environment. In particular, demosponges contain large amounts of bacteria that can amount to 40% of the biomass of the animal (Vacelet, 1975), which may exceed the bacterial concentration of the seawater by 2 orders of magnitude (Friedrich et al., 2001). As marine sponges are a rich source of diverse bacterial populations, the present investigation was planned to explore the antiangiogenic, antimicrobial, and cytotoxic potential of sponge-associated bacteria.

Angiogenesis is a multistep process, leading to the formation of new blood vessels from the existing ones. It occurs during embryonic development, endometrial regulation, the reproductive cycle, and wound healing. Angiogenesis also plays a critical role in many disease conditions like solid tumor progression, metastasis, diabetic retinopathy, arthritis, psoriasis, hemangiomas, and atherosclerosis (Folkman, 1995). Folkman and co-workers pioneered the concept of antiangiogenesis as a strategy for preventing the growth of tumors (Folkman, 1971). Solid tumors cannot grow without inducing the formation of new blood vessels to satisfy the nutritional needs of new tumor tissue (Folkman, 1971). In view of this, it was thought that the growth of the tumor could be stopped by blocking the development of new blood vessels with the help of antiangiogenic drugs. Antiangiogenic drugs have also been found useful for the treatment of other angiogenesis-dependent diseases (Harris, 1998).

More than 300 angiogenesis inhibitors have been discovered so far, and more than 80 drugs derived from them are in clinical trials (Madhusudan and Harris, 2002). Though these angiogenesis inhibitors are effective against a variety of tumors, high dosages of these substances are necessary to suppress tumor growth. Other disadvantages of antiangiogenic protein therapy include the need for repeated injections and prolonged treatment, the transmission of toxins and infectious particles, and the high manufacturing cost (Gastl et al., 1997). In this context natural resources for the exploration of antiangiogenic substances hold great promise. A variety of antiangiogenic substances have also been isolated from natural sources, including shark cartilage, curcumin, the ω-3 and ω-6 fatty acids, green tea, licorice, quercetin, squalamine, and vitamin D3 (Marwick, 2001). Apart from shark cartilage (Cho and Kim, 2002; Gingras et al., 2003), some marine natural compounds from sponges (Zhou et al., 2000; Fujita et al. 2001; Shin et al., 2001; Rodriguez-Nieto et al., 2002; Williams et al., 2002) and sponge-associated bacteria (Müller et al., 2004) have also been reported to posses antiangiogenic potential. In view of the importance of antiangiogenic substances, the extracts of sponge-associated bacteria were screened for antiangiogenic assay.

In the present investigation the marine sponge *Suberites domuncula* (Demospongiae, Hadromerida, Suberitidae) was chosen as a source for the recovery of bacteria having bioactive potential. This sponge typically grows on snail shells and has a compact, smooth, waxy, colorful surface. Bacteria were isolated from the sponge surface, as well as from its laboratory-developed primmorphs (3-dimensional aggregates containing proliferating cells), and subjected to different bioassays.

Materials and Methods

Isolation and Culture of Bacteria. *Suberites domuncula* sponges were collected from depths of between 15 and 25 m, from the Northern Adriatic, near Rovinj, Croatia (45°07′ N; 13°39′ E), and kept in recirculating seawater aquaria in Mainz (Germany) under continuous aeration. Sponge primmorphs were obtained from dissociated cells of *S. domuncula* as described previously (Müller et al., 1999). The primmorphs were cultivated in seawater (Sigma), supplemented with 0.2% RPMI 1640-medium and 60 μM silicate in the presence of antibiotics (Krasko et al., 2000). Seven days after transfer of the cells into seawater, primmorphs were used for the isolation of bacteria.

The bacteria from sponge surface and sponge primmorphs were isolated as described previously (Thakur et al., 2003). These bacterial strains were purified and cultured on B1 agar medium (0.25% peptone, 0.15% yeast extract, 0.15% glycerol, 1.6% agar, 100% seawater) (Newbold et al., 1999).

Preparation of Organic Extracts. Sponge and primmorphs–associated bacteria were extracted in n-butanol following the method of Elyakov et al. (1996). Bacterial isolates were inoculated into conical flasks (1-L capacity) with 500 ml of culture broth. The culture broth contained peptone (0.25%), yeast extract (0.15%), and glycerol (0.15%) in seawater. The pH was adjusted to between 7.2 and 7.5. The flasks were incubated at 30°C for 3 days with shaking (100 rpm). After incubation bacterial cultures (500 ml each) were mixed with 150 ml of n-butanol. The mixtures were kept at 40°C for 24 hours stirred for 20 minutes, and centrifuged, and the butanol layer was separated and then evaporated using a rotary evaporator. Dry residues (100–150 mg) were stored below 5°C until further use.

Antiangiogenic Assay. The original chick chorio-allantoic membrane (CAM) assay (Crum et al., 1985) was modified and developed in our laboratory (Pathare, 2001). The bacterial extracts were tested at 3 different concentrations: 5, 10, and 20 $\mu g\ ml^{-1}$. Bacterial extract of the desired concentration (volume, 10 μl) was dissolved in (2.5%) agar. This solution was air dried on a Teflon-coated tray, and dried agar disks of 4 mm were prepared. Fertilized, 5-day-incubated eggs were obtained from the central poultry breeding farm, Aarey colony, Mumbai. In the laboratory these eggs were surface sterilized by wiping them with 70% alcohol and kept in an incubator at 38°C. The fertilized eggs were candled to locate the developing embryo, and a window of cm^2 was marked. The marked window was cut in order to see the embryo and the surrounding blood vessels on CAM.

An agar disk impregnated with a known concentration of the bacterial extract was placed in the outer third portion of the CAM, approximately 2 to 3 mm away from the central blood vessel. After placing the disk on the CAM, we resealed the window with Parafilm and incubated the eggs at 38°C for 48 hours. Subsequently the eggs were opened and the antiangiogenic response was assessed by measuring the avascular zone on the CAM beneath the disk. An avascular zone larger than 4 mm in diameter was considered as positive. Results are presented as the percentage of embryos that showed antiangiogenesis in response to the treatment with bacterial extract (number of Embryos with Avascular Zone/Total Number of Embryos Tested in the Assay × 100). A positive control (10 $\mu g\ ml^{-1}$) was kept using a mixture of 60 μg of hydrocortisone (Hi-media Laboratory, Cat. No. RM556) and 50 μg of heparin (Hi-media Laboratory, Cat. No. RM 639). An agar disk with saline was used as negative control, which did not show activity. Ten eggs were used for each dose, and the experiment was performed in triplicate to ensure reproducibility.

Antimicrobial and Hemolytic Assays. Antibacterial and antifungal activities were tested in triplicate using the standard paper disk diffusion method against gram-positive bacteria, gram-negative bacteria, and yeast. Hemolytic activity was tested by using sheep blood agar (Oxoid). Stock solutions of extracts were prepared by dissolving 50 $mg\ ml^{-1}$ of methanol. The extracts (1 mg per disk) were applied to sterile paper disks (6 mm in diameter). The solvent was evaporated before they were placed onto agar plates that had been seeded with reference bacterial strains. The diameters of the inhibition zones (diameter of inhibition zone minus diameter of disk) were measured in millimeters after incubation at 30°C for 24 hours. Solvent control disks without extracts prepared in the same manner were never observed to inhibit bacterial growth.

Cytotoxicity Assay. Bacterial extracts were tested for cytotoxicity against PC12 cells (ATCC CRL 1721) and human cervix HeLa S3 cells (ATCC CCL 2.2) as described previously (Bringmann et al., 2003). The PC12 cells were grown in medium DMEM (supplemented with 10% horse serum and 5% fetal calf serum) and HeLa cells in RPMI (with 10% fetal calf serum). The cells were seeded into 96-well plates at a concentration of 1.4×10^4 cells cm^{-2} and incubated at 37°C for 24 hours. The bacterial extracts were reconstituted in distilled water at a concentration of 1 $mg\ ml^{-1}$ of solvent and assayed at different concentrations: 3, 10, 30, and 100 $\mu g\ ml^{-1}$ ($n = 4$ for each concentration). The desired concentrations were achieved by introducing different quantities of reconstituted extracts in multi-wells containing incubated cells. The final volume in each well was 200 μl. The plates were then incubated at 37°C for 72 hours. Cell viability was determined using the methylthiazolyldiphenyl-tetrazolium (MTT) bromide colorimetric assay (Scudiero et al., 1988). The plates were read at 595 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio Rad 3550, equipped with the program NCIMR IIIB), after overnight incubation at 37°C. Percentage inhibition of the cells was calculated with respect to the control.

Antibiotic Resistance Assay. All 5 bioactive bacterial strains were studied for their resistance to the standard antibiotics penicillin and streptomycin. The assay was carried out using the well-diffusion method in triplicate. Wells of 1-cm diameter were made on agar plates, and these plates were swabbed with test bacterial isolates. Three different stock solutions were prepared in sterile distilled water in order to obtain antibiotic concentrations of 50, 100, and 150 $\mu g\ ml^{-1}$. The wells in the agar plates were subsequently filled with 100 μl of antibiotic stock solution. Controls were run by putting sterile distilled water into the wells. The diameters of the inhibition zones (diameter of inhibition zone minus diameter of well) were measured in millimeters after incubation at 30°C for 24 hours.

Results and Discussion

Sponge and Primmorph–Associated Bacteria. Previous studies have focused on bacteria isolated from whole sponges or from the sponge mesohyl (Kobay-

Table 1. Inhibitory Effects (percentage of embryos) of Bacterial Extracts and Positive Control (mixture of hydrocortisone and heparin) on Angiogenesis in the CAM Assay[a]

	Concentration ($\mu g\ ml^{-1}$)		
Test material	*5*	*10*	*20*
SB1	—	—	—
SB2	—	50%	50%
SB6	Toxic	Toxic	Toxic
PB1	Toxic	Toxic	Toxic
PB2	50%	100%	100%
Positive control	NT	100%	NT

[a]NT = not tested.

ashi and Ishibashi, 1993, and references cited therein; Hentschel et al., 2001) for the production of bioactive metabolites. The present study was an attempt to investigate the bioactive potential of sponge surface-associated bacteria and sponge primmorph-associated bacteria, which are thought to be involved in the epibacterial chemical defense of the sponge *S. domuncula* (Thakur et al., 2003). Sponge primmorphs were developed in the laboratory, in order to isolate the bacteria. It is assumed that the primmorph bacteria, which can be retained after dissociation of sponge cells in the presence of antibiotics, have an obligatory association with the sponge cells. Altogether 6 bacterial isolates (SB1–SB6) were obtained from the sponge surface, whereas 2 bacterial isolates (PB1 and PB2) were obtained from sponge primmorphs. Of the 6 sponge-associated bacteria, SB3, SB4, and SB5 did not show any bioactivity.

Antiangiogenic Assay. A number of endogenous inhibitors targeting the tumor vasculature have recently been identified using in vitro and in vivo antiangiogenesis models. The chick CAM assay has been extensively used in the study of angiogenesis. In the present investigation this assay was used to determine the ability of bacterial extracts to inhibit angiogenesis in vivo. Preliminary results showed that 2 of 5 bacterial extracts tested for angiogenesis inhibition were active (Table 1). These active extracts were obtained from SB2 (sponge-associated bacterium) and PB2 (primmorph bacterium). The SB1 extract was inactive. The SB2 extract showed 50% angiogenesis inhibition at 10 and 20 $\mu g\ ml^{-1}$ concentrations. SB6 and PB1 extracts were highly toxic to the eggs at concentrations above 5 $\mu g\ ml^{-1}$. These extracts completely stopped the development of the embryo by reducing its blood supply. In some cases half of the embryo had reduced blood supply, whereas the other half was normal. The extract obtained from PB2 was a potent angiogenesis inhibitor: it showed 50% activity at 5 $\mu g\ ml^{-1}$ and 100% activity at 10 and 20 $\mu g\ ml^{-1}$.

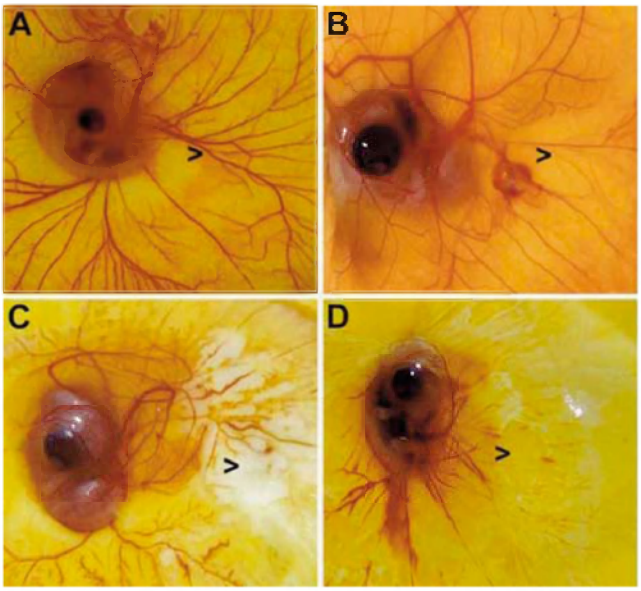

Fig. 1. Effect of bacterial extract on vascularization in chick embryo (CAM assay). **A**: Chick CAM after incubation with an agar disk without bacterial extract for 48 hours (negative control). **B**: CAM incubated for the same period with 10 $\mu g\ ml^{-1}$ concentration of a mixture of hydrocortisone and heparin as positive control. **C:** CAM incubated for 48 hours with 10 $\mu g\ ml^{-1}$ concentration of PB2 extract. Distinct disorganization of the vessel formation (avascular zone) is seen at the location where the extra-treated disk was placed. **D:** Toxic effect on CAM incubated for the same period with 5 $\mu g\ ml^{-1}$ concentration of PB1 extract. This extract stopped the development of embryo by reducing blood supply. Arrowhead indicates the place of agar disk on CAM.

The mixture of hydrocortison and heparin, which was used as a positive control, and our PB2 crude extract showed 100% antiangiogenic activity at 10 $\mu g\ ml^{-1}$. On close examination, the effect of this extract showed the presence of discontinuous and disrupted blood vessels, in a larger area surrounding the disk. Figure 1 shows chick CAM after incubation with an agar disk without bacterial extract for 48 hours (negative control, A), the CAM with positive control (B), the avascular zone caused by PB2 extract (C), and the toxic effects of PB1 extract on the CAM (D). Interestingly, PB2 extract disrupted only newly forming blood vessels without affecting the preexisting vasculature. These results are exciting, and further studies are warranted to investigate the mechanism by which these extracts inhibit angiogenesis.

Antimicrobial and Hemolytic Assays. There is ample evidence documenting the existence of bacteria associated with sponges that produce antibacterial metabolites (Kobayashi and Ishibashi, 1993; Thakur and Anil, 2000). In the present study the extracts obtained from sponge-associated bacteria

Table 2. Antimicrobial Activity of Bacterial Extracts

Test bacterial strain	*SB1*	*SB2*	*SB6*	*PB1*	*PB2*
S. aureus WT	+	+	–	–	+++
S. aureus 118	+	+	–	–	++
S. aureus A134	+	+	–	–	++
S. epidermidis 40	+	++	–	–	++
S. epidermidis RP62A	+	+	–	–	+++
S. lentus 84	–	–	–	–	+
Escherichia coli DH5α	–	–	–	–	+++
Marine *Vibrio* (SB1)	–	+	–	–	–
Candida albicans	–	–	–	–	+++

[a]SB indicates sponge-associated bacteria; PB, primmorph-associated bacteria.
Symbols: + indicates inhibition zone ≤ 4 mm; ++, 5–9 mm; +++, >9 mm.

SB1 and SB2 and primmorph-associated bacterium PB2 displayed antimicrobial activity (Table 2). An important observation of this study is that these bacterial extracts were active against multidrug-resistant clinical isolates such as *Staphylococcus aureus* and *Staphylococcus epidermidis* strains isolated from hospital patients. These 2 bacteria are the cause of most common hospital-acquired infections and account for numerous complications following surgical interventions (vascular grafts, catheters, and other implants). Among those, the methicillin-resistant *S. aureus* (MRSA) strains pose a particular threat as they are resistant to all known antibiotics except vancomycin (Raad et al., 1998; De Lalla, 1999; Smith and Jarvis, 1999). In this regard, it is noteworthy that the chosen strains of *S. aureus* and *S. epidermidis* were effectively inhibited by the extracts obtained from the sponge-associated bacteria. Further chemical isolation and characterization of active compounds from these bacterial extracts will be the aim of future experiments.

Out of 6 bacteria isolated from sponge surface, SB1, SB2, and SB6 showed hemolytic activity, whereas the extracts of both the primmorph bacteria were hemolytic.

Cytotoxicity Assay. In vitro cytotoxicity assays are a potentially useful tool in the study of toxic compounds of complex mixtures. PC12 and HeLa cells are widely used in toxicity assays. We found that the extract obtained from sponge surface bacterium SB2 was toxic against PC12 cells (Figure 2). However, the extracts from both the primmorph-associated bacteria PB1 and PB2 exhibited potent cytotoxicity against HeLa cells (Figure 2). These results suggest that the bacterial isolates SB2, PB1, and PB2 are good candidates for further activity-monitored fractionation to identify active principles.

Antibiotic Resistance Assay. The results of the antibiotic resistance assay are given in Table 3. All 5 bacterial isolates having bioactive potential were resistant to penicillin and streptomycin at 50 μg ml^{-1} concentration. However, primmorph-associated bacterium PB2 was resistant to both of these standard antibiotics up to 150 μg ml^{-1}. This may be the reason why PB2 was retained in the sponge primmorphs, which were developed in sterile conditions by growing in a medium containing antibiotics. It is noteworthy that the bacterium PB2, which was resistant to standard antibiotics, was sensitive to PB1 as well as PB2 (autoinhibition) extracts (Thakur et al., 2003). The antibiotic resistance of these bacteria was not surprising, as they grow in an environment where they have to compete with other microbes for space and nutrients.

Identification of Potential Bacteria. All the bacteria isolated were previously identified using 16S ribosomal DNA sequences (Thakur et al., 2003). The isolates SB1 and SB2 belong to the *α-Proteobacterium* MBIC3368. Interestingly, the *α-Proteobacterium* MBIC3368 strain has been reported from diverse marine sponges irrespective of their taxonomic identity, geographic location, or natural products profile (Hentschel et al., 2001; Webster and Hill, 2001; GenBank accession number AB012864). The isolate SB6 (having hemolytic activity) revealed species-level similarity to *Idiomarina ioihiensis* (Alteromonadaceae). However, the primmorph-associated bacteria belong to unidentified *Pseudomonas* sp. Though the primmorph-associated bacteria PB1 and PB2 showed similar sequences, only PB2 displayed antiangiogenic and antibacterial activities. It is possible that PB1 and PB2 are different strains belonging to the same species.

Conclusion

The current study shows that bacterial isolates from the sponge (*S. domuncula*) surface and from the primmorphs possess bioactive properties. Among all the isolates, PB2 displayed potential antiangiogenic, antimicrobial, hemolytic, and cyto-

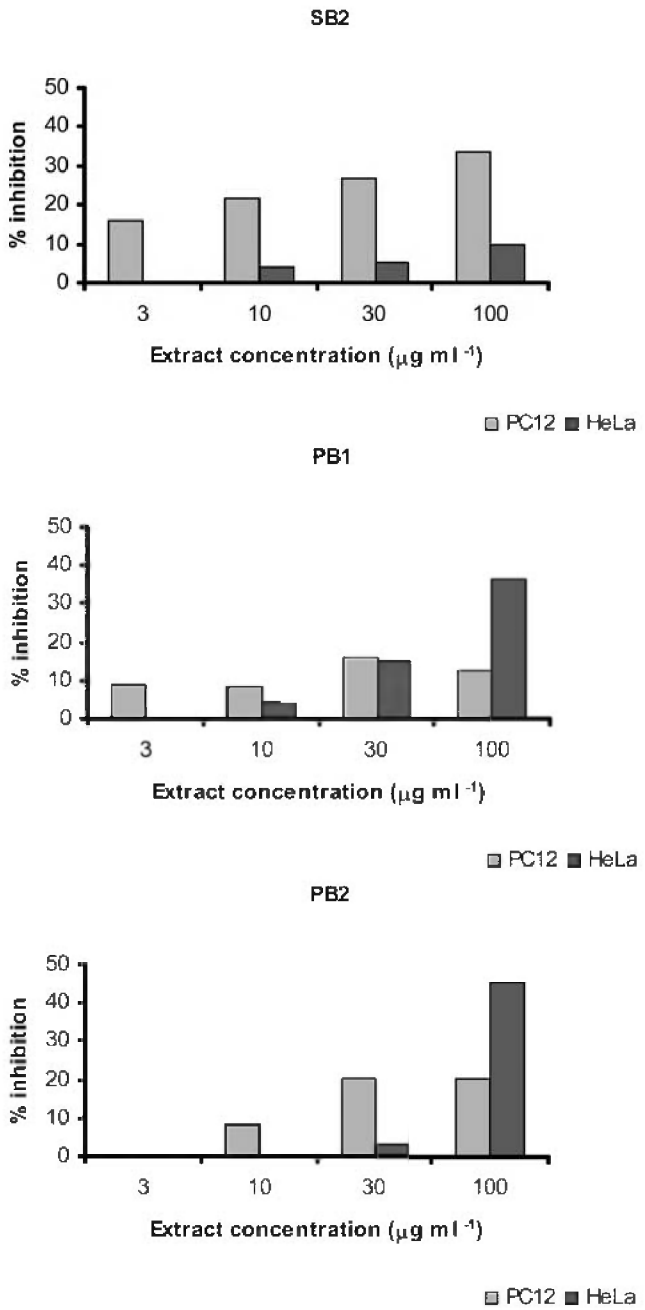

Fig. 2. Cytotoxicity of SB2, PB1, and PB2 bacterial extracts against PC12 cells (ATCC CRL 1721) and human cervix HeLa S3 cells (ATCC CCL 2.2) at 4 different concentrations. Cell viability was determined using the MTT bromide colorimetric assay. The plates were read at 595 nm using an ELISA plate reader, after overnight incubation at 37°C. Percentage inhibition of the cells was calculated with respect to the control.

toxic activities. It was also found to be resistant to the standard antibiotics and was identified as a possible novel *Pseudomonas* sp. Thus this investigation highlights the importance of bacteria associated with the sponge *S. domuncula* as a valuable resource for the discovery of novel bioactive molecules. Further chemical isolation and characterization of active compounds from these bacterial extracts is under investigation, and findings will be reported in due course.

Table 3. Antibiotic Resistance Assay of Sponge and Primmorphs–Associated Bacteria[a]

Bacterial strain	*Penicillin* ($\mu g\ ml^{-1}$)			*Streptomycin* ($\mu g\ ml^{-1}$)		
	50	*100*	*150*	*50*	*100*	*150*
SB1	R	+	+	R	+	+
SB2	R	R	+	R	+	+
SB6	R	R	+	R	+	++
PB1	R	+	+	R	++	++
PB2	R	R	R	R	R	R

[a]SB indicates sponge-associated bacteria; PB, primmorph-associated bacteria. R, resistant.
Symbols: + indicates inhibition zone = 4 mm; ++, >4 mm.

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