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SHORT COMMUNICATIONS

Isolation and Phylogenetic Analysis of the Biologically Active Bacteria Associated with Three South China Sea Sponges[1]

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Sponges have long been recognized as a source of novel compounds with antiviral, antitumor, antimicrobial, or generally cytotoxic properties, which may be of importance in pharmaceuticals and medicine [1]. Sponges are sessile organisms that sequester food particles by filter feeding; they can therefore accumulate abundant microorganisms including heterotrophic bacteria, archaea, cyanobacteria, and unicellular algae, which may constitute 40–60% of the sponge volume or more [2]. Evidence for the involvement of associated microorganisms in the secondary metabolism originally attributed to the sponge host has been accumulated [3]. The isolation of the microorganisms responsible for the origin of the bioactive products of interest will enable large-scale production of marine pharmaceuticals by means of fermentation technology.

Some of the bacteria isolated from sponges have been found to exhibit antimicrobial activity [4]; meanwhile some metabolites of sponge-associated bacteria have been determined [5]. To date, investigations on the sponge *Stelletta* sp. have revealed a limited number of sponge metabolites [6]. We found only one report concerning the chemical compound revealed in the sponge *Stelletta tenui*[7]. *Halichondria* sp. is a source of valuable compounds; for instance, molecular okadaic acid was isolated from the sponge *H. melanodocia* [8]. *Dysidea avara* is known to contain avarol, a potentially active anti-HIV agent [9]. While many studies have been published on *Dysidea avara* metabolites [10], cell culture [11], and mariculture [12], little is known about the bacteria associated with this organism. To our knowledge, there have been no studies on the bacteria associated with sponges *S. tenui* (Lindgren), *H. rugosa*, and *D. avara*, except for our work on the predominant bacterial community using the denaturing gradient gel electrophoresis (DGGE) strategy [13].

In this study, the sponges *S. tenui* (Lindgren), *H. rugosa*, and *D. avara*, studied previously by Li et al.[13], were used for the investigation of associated bacteria with antibacterial or antifungal function and enzymatic potential. The medium used for bacterial isolation contained 10 g/l peptone, 5 g/l beef extract, and artificial sea water (ASW, g/l distilled water: NaCl, 26.518; $MgCl_2$, 2.447; $MgSO_4$, 3.305; $CaCl_2$, 1.141; KCl, 0.725; $NaHCO_3$, 0.202; NaBr, 0.083); pH was adjusted to 7.2–7.4. To prevent fungal growth, the medium was supplemented with 40 mg/ml nystatin. For agar plates, 1.5% agar was added. The inoculated plates were incubated for 2–3 days at 28°C. The designations for the isolated pure cultures were A, B, C (for the sources of isolation *S. tenui*, *H. rugosa*, and *D. avara*, respectively) plus Arabic numerals. Antimicrobial potential was tested using the agar diffusion method with seven indicator strains, including gram-positive and gram-negative bacteria, and fungi: *Escherichia coli* (AS 1.3373), *Staphylococcus aureus* (AS 1.2465), *Pseudomonas fluorescens* (AS 1.55), *Bacillus subtilis* (AS 1.3343), *Candida albicans* (AS 2.2086), *Pacecilomyces variotti* (AS 3.776), and *Aspergillus niger* (AS 3.5487); the test strains were purchased from the Chinese Biodiversity Information Center in the Institute of Microbiology, Chinese Academy of Sciences. Protease, lipase, agarase, and chitinase activity were screened according to the method of Rondon et al. [14]. DNA was extracted according to the modified phenol–chloroform method [13]. PCR amplification was carried out in a Master Cycler Gradient (HYBAID, U.K.) with primer pairs 27f: 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492r: 5'-TAC GGC TAC CTT GTT ACG ACT T-3'. The 50-µl PCR mixture consisted of primer 27f and 1492r, 0.5 µl each; 10× PCR solution (50 mM *Tris*-HCl (pH8.2), 18 mM $MgCl_2$, 500 mM KCl, 0.1% glycerol, 1% TritonX-100), 9 µl; 10 mM dNTP, 2 µl; DNA sample, 1 µl; 2.5U Pfu DNA Polymerase, 0.5 µl; and double distilled H_2O to the final volume. The PCR amplification was performed as follows: 5 min denaturation at 94°C, incubation at 80°C (Pfu DNA Polymerase was added at this stage), and then 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 56°C, 2 min extension at 72°C, followed by a final extension

[1] The text was submitted by the authors in English.

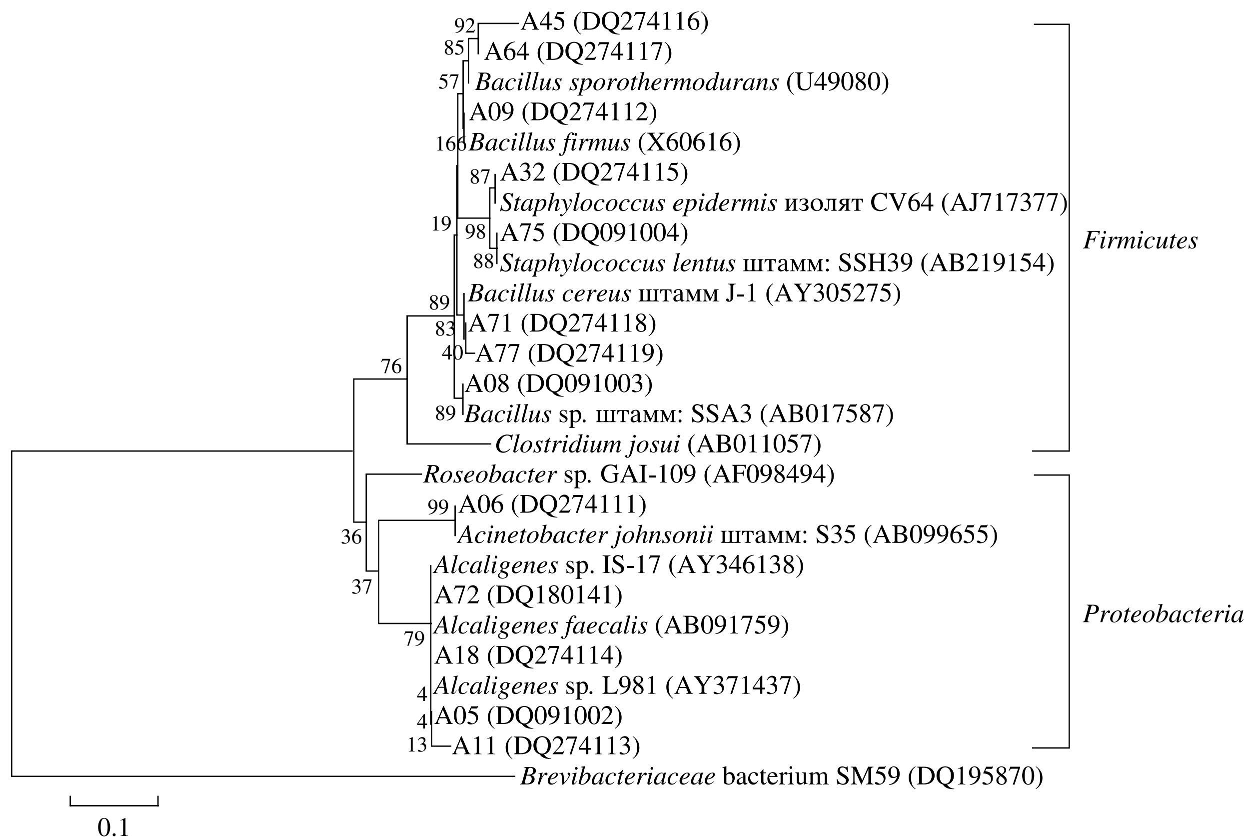

Fig. 1. Phylogenetic tree based on about 600 bp of 16S rRNA gene sequences of bacterial isolates obtained from *Stelletta tenui* (Lindgren). The numbers at the nodes are percentages indicating the levels of bootstrap support based on a neighbor-joining analysis of 100 resampled data sets. Scale bar represents 10% substitutions per nucleotide position. *Brevibacteriaceae bacterium* SM59 (DQ195870) was used as out-group reference.

step at 72°C for 2 min. PCR products were analyzed by electrophoresis in 1% agarose gels and stained with ethidium bromide. The PCR amplification products were sequenced using an ABI3730 DNA Sequencer (USA) with 27f primer. The sequences were compared to those in the GenBank database using the BLAST software package to identify the sequences' similarity. Alignment of 16S rDNA sequences was performed using CLUSTALX software package; the phylogenetic tree was generated using the neighbor-joining algorithms in the Mega III software package. The level of support for the phylogenies derived from neighbor-joining analysis was gauged by 100 bootstrap replicates.

A total of 399 bacterial cultures were isolated. Among these, 13 isolates from *S. tenui*, 42 from *H. rugosa*, and 20 from *D. avara* showed pronounced antimicrobial activities and enzymatic potential. The results for 33 representative bacterial strains are presented in Table 1. Many isolates have a broad spectrum of antimicrobial activity, e.g., A75, A72, B81, B15, C123. The isolates A05, A64, and B81 were active against *E. coli*. Some of the isolates exhibited multiple enzymatic activities, e.g., protease, lipase, agarase, and chitinase. In particular, isolate C117 showed high protease and lipase activities.

The results of BLAST and phylogenetic analysis are shown in Table 2 and Fig. 1–3. The 24 bioactive bacterial strains isolated from the sponges *S. tenu* and *H. rugosa* can be divided in two groups, proteobacteria and firmcutes, whereas isolate A45 (with only 95% similarity to its closest relative) may represent a new taxon. For the sponge *D. avara*, all 9 representative bioactive bacterial isolates belong to *Bacillus* sp. According to our previous study [18], the representatives of the phylum *Proteobacteria* are the main components of the predominant bacterial community associated with sponges *S. tenui, H. rugose*, and *D. avara.* Firmicutes constitute one of the predominant bacterial groups in the sponge *D. avara.* Due to the selectivity of the growth medium used in this work, only seven isolates were found to belong to the phylum *Proteobacteria*; the other 27 isolates belonged to the phylum *Firmicutes*.

Sponges are continuously exposed to a number of potentially harmful factors, which cause them to develop chemical defense systems to protect themselves from toxic compounds, pollution, and invaders. The observed bacteria exhibited multiple antimicrobial

Table 1. Antimicrobial activities and enzymatic potentials of bacterial isolates obtained from sponges

Strain	Antimicrobial potentials							Enzymic potentials			
	E. coli	*S. aureus*	*B. subtilis*	*P. fluorescens*	*C. albicans*	*A. niger*	*P. variotii*	Proatese	Lipase	Chiti-nase	Agarase
A05	++	–	–	–	+++	–	++	+	–	–	–
A06	–	–	+	–	–	–	++	+	–	+	–
A08	–	–	+++	–	+	–	++	+	–	–	–
A09	–	–	++	–	++	–	–	–	+	–	–
A11	–	–	++	–	+	+	+	–	+	–	–
A18	–	–	++	–	–	+	++	–	+	–	–
A32	–	–	–	–	–	++	–	+	–	–	–
A45	–	–	–	–	–	–	++	+	–	–	+
A64	++	–	–	–	++	–	++	+	–	–	+
A71	–	–	–	–	++	++	++	–	+	–	–
A72	–	–	+++	–	+	++	++	–	+	–	–
A75	–	+++	+++	+	+	++	+++	+	+	–	–
A77	–	–	–	–	++	–	–	+	–	–	–
B02	–	+	–	–	–	–	–	+	+	–	+
B04	–	+	–	–	–	++	–	+	+	–	–
B05	–	+++	+	–	–	–	–	–	+	+	+
B12	–	+	–	+	–	+	–	–	–	–	–
B14	–	+	+	–	–	+	–	+	+	–	+
B15	–	+++	–	+	–	+++	–	+	+	–	–
B18	–	–	–	–	–	+++	–	+	+	–	+
B31	–	+	+	–	–	+	–	+	+	–	+
B61	–	–	–	+	–	–	–	+	–	–	–
B81	+++	+++	+++	+++	–	–	–	–	–	–	–
B118	–	++	–	–	–	–	–	–	–	–	–
C77	–	–	–	–	–	++	+	++	+	–	+
C89	–	–	–	–	–	++	+	++	++	–	–
C93								++	++	–	–
C111	–	+	–	+++	–	–	+	+++	–	+	+
C114	–	+	–	+	–	–	++	+++	–	–	–
C117	–	+	–	–	–	+	++	+++	+++	–	–
C123	–	+	+	+++	–	–	+	+++	+	–	–
C130	–	–	–	++	–	–	+	++	–	+	–

Note: (–) not active; (+) with activity; (++) moderate activity; (+++) strong activity.

Table 2. BLAST analysis of 16S rDNA sequences of bacterial isolates obtained from sponges

Strain	Accession no.	Closest relative and its accession number	Similarity, %
A05	DQ091002	*Alcaligenes* sp. L981 (AY371437)	99
A06	DQ274111	*Acinetobacter johnsonii* strain S35 (AB099655)	99
A08	DQ091003	*Bacillus* sp. strain SSA3 (AB017587)	100
A09	DQ274112	*Bacillus firmus* (X60616)	99
A11	DQ274113	*Alcaligenes faecalis* (AB091759)	97
A18	DQ274114	*Alcaligenes faecalis* (AB091759)	99
A32	DQ274115	*Staphylococcus epidermidis* isolate CV64 (AJ717377)	100
A45	DQ274116	*Bacillus sporothermodurans* (U49080)	95
A64	DQ274117	*Bacillus sporothermodurans* (U49080)	98
A71	DQ274118	*Bacillus cereus* strain J-1 (AY305275)	100
A72	DQ180141	*Alcaligenes* sp. IS-17 (AY346138)	98
A75	DQ091004	*Staphylococcus lentus* strain SSH39 (AB219154)	100
A77	DQ274119	*Bacillus cereus* strain J-1 (AY305275)	98
B02	DQ277980	*Bacillus* sp. Con a/4 (AJ784845)	100
B04	DQ277981	*Bacillus licheniformis* strain CICC10181 (AY842871)	99
B05	DQ277982	*Bacillus licheniformis* ATCC 14580 (CP000002)	99
B12	DQ277983	*Providencia* sp. OP1 (AM040495)	100
B14	DQ277984	*Bacillus* sp. XL-2004 (AY788910)	100
B15	DQ277985	*Bacillus* sp. IIPON4 (DQ188943)	100
B18	DQ277986	*Bacillus subtilis* strain MO2 (AY553095)	100
B31	DQ277987	*Bacillus anthracis* strain ATCC 4229 (AY920253)	100
B61	DQ277988	*Bacillus* sp. MI-23a1 (DQ180948)	100
B81	DQ277989	*Alcaligenes* sp. IS-18 (AY346137)	100
B118	DQ277990	*Bacillus* sp. AI-15 (AY437624)	100
C51	DQ091005	*Bacillus vallismortis* (AB021198)	99
C77	DQ091006	*Bacillus vallismortis* (AB021198)	100
C89	DQ091007	*Bacillus vallismortis* (AB021198)	100
C93	DQ091008	*Bacillus cereus* strai J-1 (AY305275)	99
C111	DQ091009	*Bacillus cereus* strain ATCC BAA-1005 (AY631056)	99
C114	DQ091010	*Bacillus cereus* strain ATCC BAA-1005 (AY631056)	99
C117	DQ091011	*Bacillus cereus* strain J-1 (AY305275)	99
C123	DQ091012	*Bacillus cereus* strain 2000031486 (AY138272)	99
C130	DQ091013	*Bacillus cereus* strain S-5 (AF390086)	99

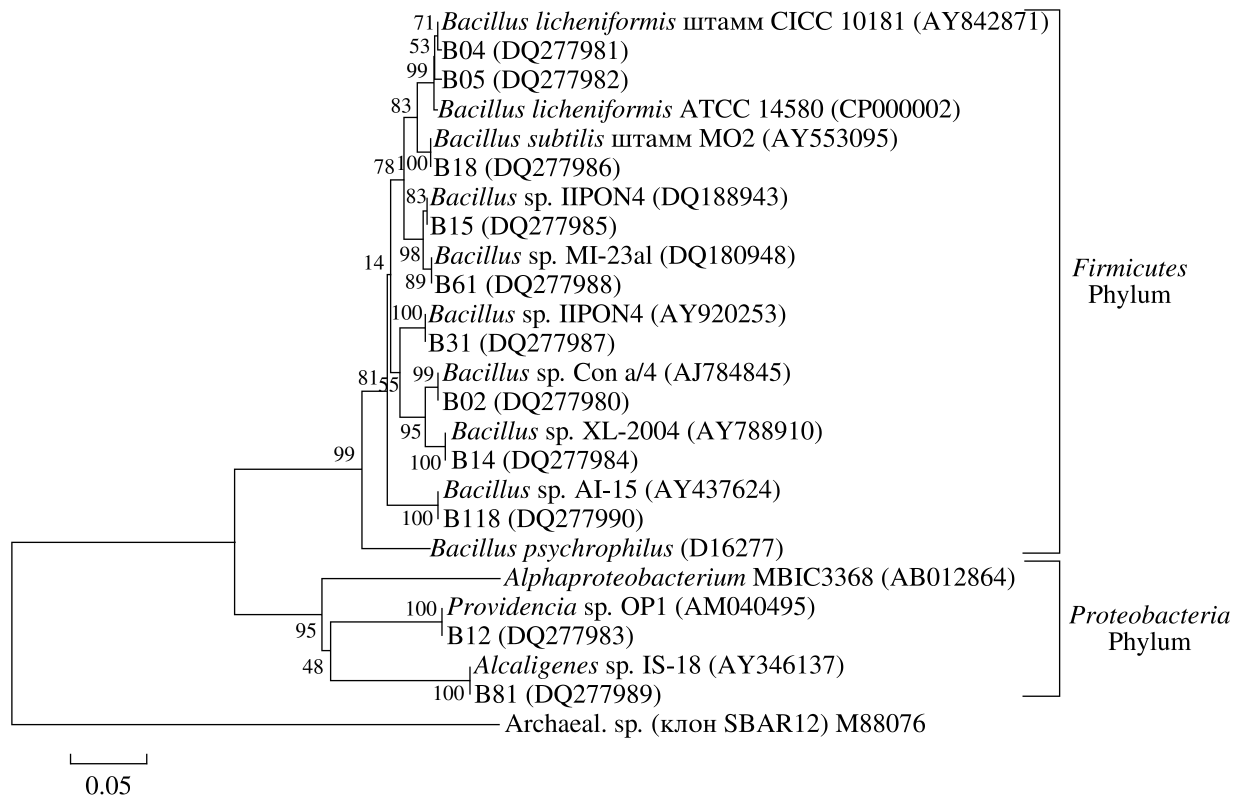

Fig. 2. Phylogenetic tree based on about 600 bp of 16S rRNA gene sequences of bacterial isolates obtained from *Halichondria rugose*. The numbers at the nodes are percentages indicating the levels of bootstrap support based on a neighbor-joining analysis of 100 resampled data sets. Scale bar represents 5% substitutions per nucleotide position. Archaeal clone SBAR12 (M88076) was used as out-group reference.

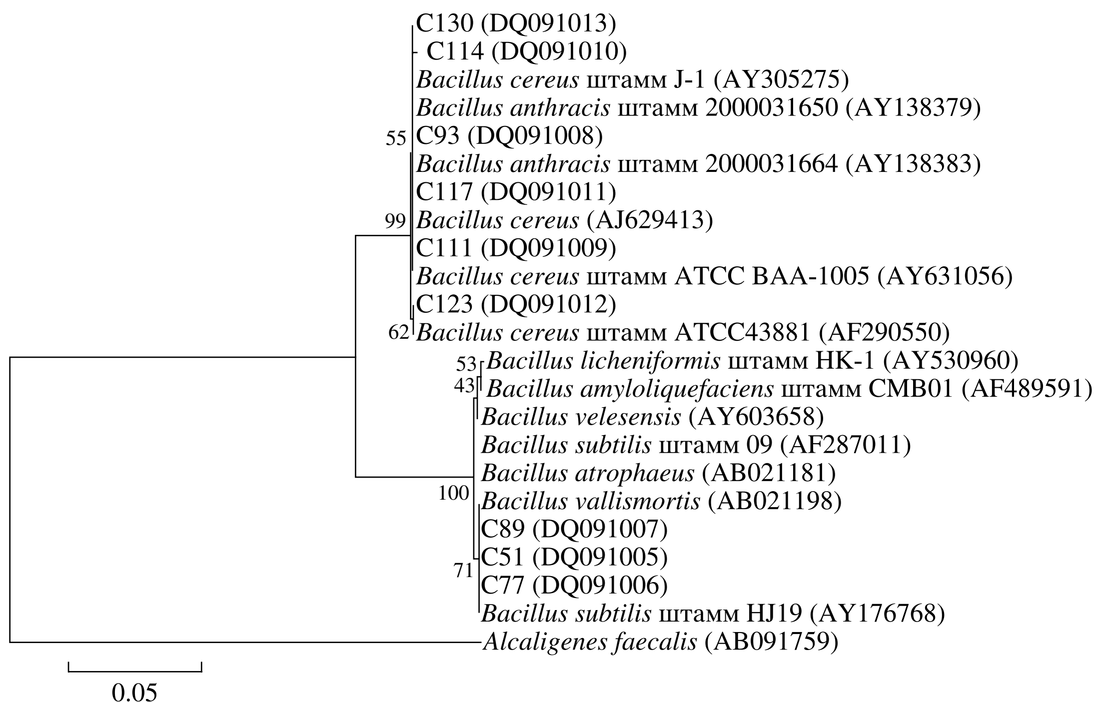

Fig. 3. Phylogenetic tree based on about 600 bp of 16S rRNA gene sequences of bacterial isolates obtained from *Dysidea avara*. The numbers at the nodes are percentages indicating the levels of bootstrap support based on a neighbor-joining analysis of 100 resampled data sets. Scale bar represents 5% substitutions per nucleotide position. *Alcaligenes faecalis* (AB091759) was used as out-group reference.

activities against gram-positive and gram-negative bacteria, and fungi possibly contribute to the chemical defenses of the sponges against harmful microorganisms present in seawater. As the result of the special digestive strategy of sponges, some microbial enzymes should be involved in the nutrition transport of the host. This suggestion is supported by the protease, lipase, chitinase, and agarase activities found in sponge-associated bacteria in this study. For instance, protease, lipase, and chitinase can hydrolyze complex compounds such as proteins, lipids, and carbohydrates of algae or microorganisms swallowed or filtrated by sponges into nutritional low-molecular compounds.

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