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Bacteria with antimicrobial properties isolated from the Mediterranean sponges *Chondrilla nucula* and *Petrosia ficiformis*

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ABSTRACT: Bacteria were isolated seasonally from the Mediterranean sponges *Chondrilla nucula* and *Petrosia ficiformis* and screened for antibacterial activities. Selected isolates were taxonomically identified by 16S rRNA gene sequencing. A total of 416 different bacterial strains were isolated, 60 (14.4%) of which displayed variable degrees of antimicrobial activity. Of the bioactive strains, 58.3% were able to inhibit *Staphylococcus aureus*, 6.7% were active against *Bacillus subtilis*, 11.7% against both *Enterococcus faecalis* and *Escherichia coli*, 38.3% against *Pseudoalteromonas atlantica* and 33.3% against *Pseudomonas elongata*. 16S rRNA gene sequence analysis showed that 2 isolates, 1 from seawater samples and 1 from *P. ficiformis,* were most closely related to *Bacillus subtilis* (99% similarity) and that another isolate from *P. ficiformis* was most closely related to a previously described sponge-associated *Alphaproteobacterium* NW001 (98% similarity). Two isolates from *C. nucula* were most closely related to *Brachybacterium paraconglomeratum* (99% similarity) and *Shewanella algae* (89% similarity). The high percentage of bioactive isolates derived from the 2 sponges suggests that marine microorganisms, whether animal-associated or planktonic, are promising sources for drug discovery.

KEY WORDS: Antimicrobial activity · Associated bacteria · *Chondrilla nucula* · *Petrosia ficiformis* · Porifera · 16S rRNA

INTRODUCTION

Members of the Phylum Porifera (sponges) are often associated with large microbial communities comprising autotrophic and heterotrophic bacteria. Sponge-associated microorganisms are dispersed in the intercellular mesohyl matrix or contained within specialised cells called bacteriocytes; bacterial concentrations reach 2 to 4 orders of magnitude higher than in surrounding seawater (Friedrich et al. 2001, Hentschel et al. 2003, 2006, Scheuermayer et al. 2006). Functions of sponge-associated bacteria include nutritional support, skeletal support, prevention of photo-oxidation and defense against potential predators and biofouling (for a summary, see Hentschel et al. 2003 and references therein).

An increasing number of bioactive compounds originally isolated from sponges are actually produced by sponge-associated bacteria (Piel et al. 2004). As sponge-derived bioactive compounds are attracting applied scientific interest, the potential of obtaining these secondary molecules through cultivation of microorganisms offers promising solutions to the marine invertebrate supply problem. Typically, the

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compounds of interest are found only in traces and harvesting in the field poses ethical and operational constraints.

We focus here on the isolation of bacteria from 2 Mediterranean sponges. *Chondrilla nucula* Schmidt 1862 (*Demospongiae*, *Chondrosida*) is a thick encrusting sponge that forms large patches of interconnected clones on well-lit substrata. It occurs from the sea surface to ~30 m depth. In addition to populations of *Synechococcus* (*Cyanobacteria*) in the light-exposed sponge surface layer, there are extracellular heterotrophic bacteria in the mesohyl matrix. The heterotrophs are sometimes enclosed within bacteriocytes (Usher et al. 2004). The bacterial community associated with *C. nucula* was recently characterized by 16S rDNA-based methods (Thiel et al. 2007). Interestingly, 79% of all *C. nucula*-derived phylotypes were most closely related to other sponge-specific microbial lineages, which reflects the highly specific nature of the microbial community associated with marine 'bacteriosponges' (Hentschel et al. 2006). Cytotoxic and deterrent activities have been demonstrated in Mediterranean and Caribbean *C. nucula* specimens (see references in Milanese et al. 2003).

Petrosia ficiformis (Poiret 1789) (*Demospongiae*, *Haplosclerida*) is a massive sponge living on hard substrata to ~50 m depth. The color of the sponge can be either dark-violet or white depending on the presence or absence of pigmented, symbiotic *Synechococcus* (Usher et al. 2004). Cyanobacteria symbiotic in *P. ficiformis* play a metabolic role and are also involved in screening excessive solar radiation (Regoli et al. 2000). Heterotrophic bacteria are present in the mesohyl and within specialised sponge-cells. The genus *Petrosia* is rich in bioactive compounds with cytotoxic, antibacterial and antifungal properties (e.g. Lim et al. 2001).

MATERIALS AND METHODS

Sponge samples. We collected samples at Paraggi in the Portofino Marine Protected Area (Ligurian Sea, NW Mediterranean Sea) by SCUBA diving. All *Chondrilla nucula* samples were obtained from a single patch at 8 m depth, while the *Petrosia ficiformis* samples were taken from a large individual at 9 m depth. Sponge samples of approximately 5 cm^3 were cut underwater with scalpels, transferred into previously sterilised vials and transported to the laboratory in cooled bags (12 to 15°C). Replicates (1 l) of surrounding seawater (SW) were also collected on each sampling occasion.

Bacterial isolation. Bacteria were isolated from sponge and seawater samples collected in the period December 2002 to August 2003. Each sponge specimen was rinsed in sterile seawater prior to further analysis. Viable heterotrophic bacteria from the surface and from the mesohyl were isolated as previously described (Chelossi et al. 2004). Epibiotic bacteria were obtained by swabbing a small area (ca. 1 cm^2) of each sponge specimen with a sterile cotton swab, diluted in 2 ml of filtered natural seawater (FNSW, 0.2 µm, Millipore) and vortexed. Mesohyl sub-samples (1 cm^3) were cut from sponges and homogenized under sterile conditions in 4 ml of FNSW. Homogenates were briefly centrifuged (30 × *g*, 5 min) and the resulting supernatant fluid was used for analyses. Three 1 ml sub-samples were taken from each 1 l seawater sample (SW) and prepared as below. Serial 10-fold dilutions of each solution (sponge-supernatant and SW sub-samples) were prepared and aliquots (0.1 ml) were plated onto marine agar (MA, Microbiol). Plates were incubated at room temperature (20 to 25°C) for 7 d. Viable heterotrophic bacteria were counted as colony-forming units (CFU) on agar plates. For each sample, isolates were chosen from the widest variety of colony morphologies to increase bacterial diversity. All isolates were reisolated and subcultured. Strains were preserved in agar stabs or as lyophilised samples.

Antimicrobial screening. Sponge isolates were tested for antimicrobial activity against 6 indicator strains: *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Pseudoalteromonas atlantica* ATCC19262, *Pseudomonas elongata* LMG2182, *Bacillus subtilis* ATCC10774 and *Enterococcus faecalis* ATCC10741. Antimicrobial activities were screened using the spot-on-lawn method. Briefly, 100 µl overnight cultures of each indicator strain were seeded as a lawn on MA plates. We spotted 10 µl of overnight culture (10^8 cells ml^{-1}) from each strain onto agar plates seeded with actively growing cells of the indicator organism. Following overnight incubation (ON) at 25°C, plates were checked for the formation of inhibition zones around spots. The zone of inhibition around the spot was measured and the assay was scored positive (+) if the inhibition zone was ≤10 mm, double positive (++) if between 10 and 19 mm, triple positive (+++) if ≥20 mm, and negative (–) if there was no inhibition of reference strains. The most active strains were chosen for further analysis.

16S rRNA gene sequencing and phylogenetic analysis. Genomic DNA was extracted after an overnight incubation (20°C, with shaking) of each strain in 3 ml of Marine Broth 2216 (Difco) using the modified protocol of Grimberg et al. (1989). In total, 2 ml of ON culture were centrifuged for 4 min at 7741 × *g*, the pellet was washed with 1 ml TNE buffer and spun down again. The pellet was resuspended in 270 µl TNEX buffer and 25 µl lysozime. After a 30 min incubation at 37°C, 50 µl Proteinase K were added and samples were

incubated at 60°C for 2 h. Samples were then added to 15 µl NaCl (5 M), and 500 µl ice-cold EtOH 100% were added to samples that were then spun down for 15 min at 20 442 × *g*, washed with 500 µl of 70% EtOH and air dried. DNA was resuspended in 200 µl of sterile water. The universal primers 27f and 1492r were used for 16S rRNA gene amplification. PCR amplification was performed using a Mastercycler Gradient (Eppendorf) as follows: one initial denaturation step for 10 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 54°C, 1.5 min at 72°C; one final elongation step for 10 min at 72°C. The PCR mix consisted of 5 µl of 10× reaction buffer (Qiagen), 1 µl (2 mM) of each primer, 1 µl (0.2 mM) of dNTPs, 0.25 µl (1.25 U) of *Taq* DNA polymerase (Qiagen), 1 µl of DNA template and distilled water to reach a final volume of 50 µl. A total of 10 µl of each PCR product were checked by electrophoresis (run for 30 min at 300 V on a 1% agarose gel). PCR products were purified with the Qiagen PCR purification kit (Qiagen) according to the manufacturer's protocol. Purified amplification products were subjected to sequencing PCR reactions using the universal primers 27f or 1492r and the reaction kit BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems). The products were further purified using ABI Prism sequencing reagents and protocol. Sequencing was performed on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer). The sequences were aligned to nearly full length 16S rRNA gene contigs using the ABI Prism software (Perkin-Elmer) and compared to 16S rRNA genes in the GenBank database using the BLASTN search algorithm (www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

Viable counts of the heterotrophic bacterial communities associated with the surface and mesohyl of *Chondrilla nucula* and *Petrosia ficiformis* and from ambient seawater are given in Table 1. Viable counts were largely consistent over sampling times. A weak positive trend was noted for *C. nucula* from winter to summer. Viable counts from mesohyl were about one order of magnitude higher than those for the surface which were in turn one to 2 orders of magnitude higher than those from ambient seawater. A total of 416 bacterial strains was isolated from colonies with different phenotypic characteristics. Sixty strains (14.4% of isolates) displayed antibacterial activity against one or more reference strains. Of these, 58.3% were active against *Staphylococcus aureus*, 6.7% against *Bacillus subtilis*, 11.7% against *Enterococcus faecalis*, 11.7% against *Escherichia coli*, 38.3% against *Pseudoalteromonas atlantica* and 33.3% against *Pseudomonas elongata* (Table 2). Forty percent of the bioactive strains were isolated from seawater, 31.7% from *C. nucula* and 28.3% from *P. ficiformis*. In *C. nucula*, 63.2% and 36.8% of bioactive isolates were from the surface and mesohyl, respectively (36.8%); in *P. ficiformis*, 41.2% and 58.8% were from the surface and mesohyl, respectively (Fig. 1). No bioactive strains were isolated in December 2002; 13.3% of the total number of bioactive isolates across all months (n = 60) were isolated in January and in March 2003, 28.3% in June 2003 and 45.5% in August 2003 (Fig. 2). Of the total number of January isolates (bioactive + non-bioactive), 12.3% were bioactive; the respective frequency in March was 8.9%, 11.3% in June and 34.2% in August 2003 (Fig. 2). Fig. 2 also shows the relative contributions in each month of sponges and seawater as a proportion (%) of total number of bioactive isolates across all dates (n = 60) and as a percentage of total isolates in each month. There was a seasonal trend from

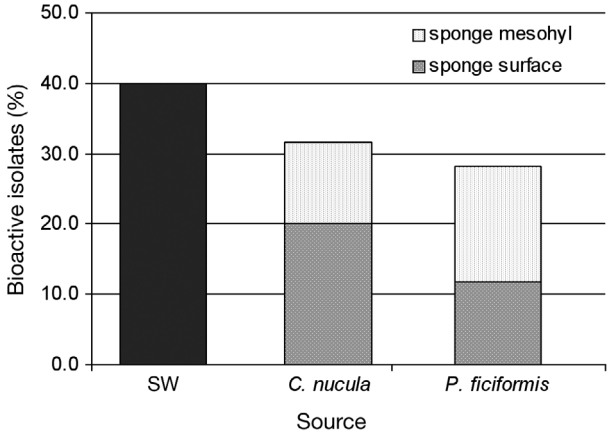

Fig. 1. Percentage of bioactive isolate recovered from sponge tissues (mesohyl, surface) and seawater (SW). *C.* = *Chondrilla*, *P* = *Petrosia*

Table 1. Viable counts of heterotrophic bacteria (as CFU × 10^3 cm^{-2} on marine agar medium; CFU = colony-forming units) associated with the surface and the mesohyl of *Chondrilla nucula* and *Petrosia ficiformis* and derived from seawater. Units are expressed as means (SE); n = 6; nd = not determined

Sampling period	Seawater	*Chondrilla nucula*		*Petrosia ficiformis*	
		Surface	Mesohyl	Surface	Mesohyl
Dec 2002	0.3 (0.1)	0.2 (0.1)	40 (2.7)	nd	35 (3.9)
Jan 2003	0.48 (0.12)	2.6 (0.3)	49 (3.2)	102 (18)	6300 (250)
Ma. 2003	5.3 (1.7)	50 (8.2)	240 (3.7)	15 (2.3)	370 (38)
Jun 2003	0.74 (0.12)	17 (2.0)	270 (21)	150 (21)	3800 (340)
Aug 2003	0.39 (0.06)	54 (13)	790 (65)	62 (3.7)	830 (98)

Table 2. Antimicrobial activities of sponge and seawater isolates. *S. aur*: *Staphylococcus aureus*; *B. sub*: *Bacillus subtilis*; *E. fae*: *Enterococcus faecalis*; *E. coli*: *Escherichia coli*; *P. alt*: *Pseudoalteromonas atlantica*; *P. elong*: *Pseudomonas elongata*. Bold: isolates whose 16S rRNA gene was sequenced. Percentage inhibition refers to the total of 60 bioactive isolates. +, ++ and +++ = inhibition zones ≤10, 10 to 19 and ≥20 mm, respectively; – = no inhibition

Sample date	Isolate	*S. aur*	*B. sub*	*E. fae*	*E. coli*	*P. atl*	*P. elong*
Jan 2003	CH37	+++	–	–	–	–	+
	PA7	**++**	**–**	**–**	**–**	**+++**	**+++**
	PB7	–	–	–	–	+	–
	AD3	–	–	–	–	–	+
	AD4	–	–	–	–	–	++
	AD9	–	–	–	–	–	++
	AD17	–	–	–	–	+	–
	AE14	–	–	–	–	–	+
Mar 2003	PTB9	++	–	–	+	+	–
	PTB16	–	–	–	–	–	+
	TCA1	–	–	–	–	+	–
	TCA8	+	–	+++	+	+	+++
	SWA3	–	–	–	–	++	–
	SWA12	**+**	**–**	**+++**	**+**	**–**	**+++**
	SWC11	–	–	+	–	+	+++
	TCC2	–	–	–	–	+	–
Jun 2003	SWB1	+	+	++	–	+	+
	SWB2	+++	+	–	–	–	–
	SWB3	+	–	–	–	–	–
	SWB6	–	–	–	–	–	+
	SWB7	–	–	–	–	–	++
	SWB11	+	–	–	–	–	++
	SWB12	++	–	–	–	–	–
	SWB14	+	–	–	–	–	–
	SWB15	–	–	–	–	+	–
	SWB16	++	–	–	–	–	+
	SWA4	++	–	–	–	–	–
	SWA7	–	–	–	–	++	–
	PA3	+++	–	–	–	+	–
	PC10	–	–	–	–	–	+
	TCC4	–	–	–	–	++	–
	TCC5	–	–	–	–	++	–
	TCC6	–	–	–	–	++	–
Aug 2003	SWA3	+++	–	–	–	–	–
	SWA4	+++	–	–	–	+	–
	SWA7	+	–	–	–	–	–
	SWA11	+++	–	–	–	–	–
	TPA2	–	–	+	–	–	–
	TPA4	+++	–	–	+	–	–
	TPA5	+++	–	–	–	–	–
	TPA6	+++	–	–	–	–	–
	TPA7	+++	–	–	+	+	–
	PA1	+	–	–	–	–	+
	PA3	–	–	–	–	+	–
	PC2	++	–	–	–	–	–
	PC3	++	+	–	–	–	–
	PC4	**+++**	**–**	**–**	**++**	**+**	**–**
	PC6	–	+	–	–	–	–
	TCA1	+++	–	+	–	++	–
	TCA3	++	–	–	–	–	–
	TCA5	–	–	+	–	–	–
	TCC1	++	–	–	–	–	–
	TCC2	–	–	–	–	–	+
	TCC3	–	–	–	–	–	+
	CHB1	++	–	–	+	+	–
	CHB2	+++	–	–	–	–	–
	CHC1	+++	–	–	–	–	–
	CHC2	**+++**	**–**	**–**	**–**	**+**	**+**
	CHC3	++	–	–	–	–	–
	CHC8	**+++**	**–**	**–**	**–**	**++**	**+**
	% inhibition	**58.3**	**6.7**	**11.7**	**11.7**	**38.3**	**33.3**

winter to summer of increasing relative sponge contribution to the number of bioactive isolates.

Of the bioactive strains from sponge and seawater samples, 23.3% and 11.7%, respectively, inhibited ≥3 test strains of bacteria. Partial 16S rDNA sequences of 5 selected bioactive isolates were obtained and compared to known database entries in GenBank. The sequences of 2 Gram-positive, spore-forming rods (strains PA7 and SWA12) were most closely related to that of *Bacillus subtilis* (99%) (Table 3). The 16S rRNA gene sequence of strain CHC2 was 89% similar to both the marine Gram-negative bacterium *Shewanella algae* and to an uncultured bacterial clone. Strain PC4 was most closely related (98% similarity) to the culturable *Alphaproteobacterium* strain NW001 that seems to be specifically associated with at least 8 different marine sponges (Scheuermayer et al. 2006) and with sponge larvae (Enticknap et al. 2006). The 16S rDNA sequence of strain CHC8 was most similar (99%) to *Brachybacterium paraconglomeratum* (*Actinobacteria*) (Table 3).

DISCUSSION

We focused on recovery and seasonal variation of bioactive bacterial isolates associated with the Mediterranean sponges *Chondrilla nucula* and *Petrosia ficiformis*. In culture, there was a 2 order of magnitude difference between the heterotrophic microbial communities from the mesohyl of 2 sponges and those from ambient seawater (Table 1), in accordance with previous work in temperate areas (Friedrich et al. 2001). Furthermore, surface bacterial densities were approximately one order of magnitude lower than those in the sponge mesohyl (Table 1), as previously shown for *P. ficiformis* (Chelossi et al. 2004). Because a single large *P. ficiformis* specimen and the same large *C. nucula* patch were repeatedly sampled over time, no information can be

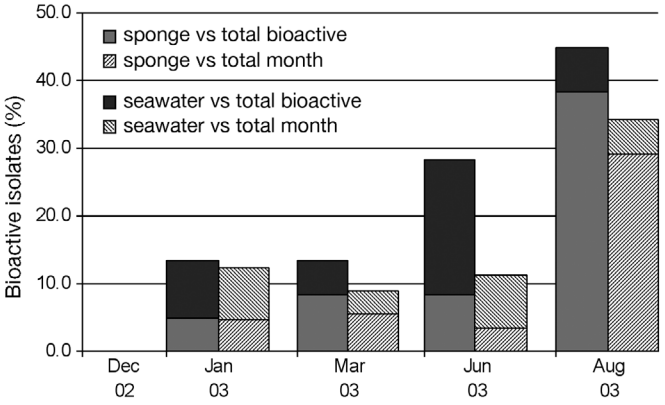

Fig. 2. Recovery of bioactive bacteria over time. Total number of bioactive samples (n) across all dates was 60. Solid bars indicate the percentage of total bioactive isolates (60) found in each month, with relative contributions from sponges (grey) and seawater (black). Cross-hatched bars show the percentage at each collection time of bioactive isolates as a proportion of total monthly isolates (bioactive + non-bioactive: December 2002, N = 32; January 2003, N = 65; March 2003, N = 90; June 2003, N = 150; August 2003, N = 79, grand total = 416). Cross-hatched bars are divided to show relative contributions of bioactive bacteria isolated from sponges and seawater as percentage of total isolates in each month

gained about intra-individual variation. CFU of total isolates are slightly lower than CFU titres from 10 different Mediterranean sponges including *C. nucula* and *P. ficiformis* (Muscholl-Silberhorn et al. 2007).

Of 416 heterotrophic strains isolated, 14.4% were able to inhibit at least one reference strain. This number is strikingly congruent with that of Muscholl-Silberhorn et al. (2007) who found that 15.2% of all subcultured colonies were active against at least 1 test strain. Almost 60% of bioactive isolates we retrieved were active against *Staphylococcus aureus*, which opens interesting avenues in the search for novel compounds against multidrug-resistant pathogenic staphylococci. The high frequency of activity against Gram-negative bacteria is intriguing. In a comparable study, the Gram-negative test bacterium *Escherichia coli* was inhibited by >75% of bioactive isolates from sponges (Muscholl-Silberhorn et al. 2007). In our study however, the Gram-negative bacteria *Pseudoalteromonas atlantica* (38.3% inhibition) and *Pseudomonas elongata* (33.3% inhibition) were more susceptible than *E. coli* (11.7% inhibition) (Table 2). Gram-negative bacteria are generally less susceptible to antimicrobials than Gram-positive bacteria because of the presence of an outer membrane and LPS (lipopolysaccharide) which together act as an efficient barrier against hydrophobic and lipophilic molecules. Among Gram-negative bacteria, *Pseudomonas* species are generally more resistant to antibiotic drugs than other genera. Although *P. elongata* strain LMG2182 used in our screening is more closely related to the genus *Microbulbifer* than to authentic pseudomonads (Yoon et al. 2003), it is intrinsically resistant to beta-lactam antibiotics and yet inhibited by a large percentage of our isolates.

Surprisingly, a large fraction of bioactive isolates was retrieved from seawater (Fig. 1), indicating that the water column microbial community can be considered a suitable source for novel antimicrobial compounds. Bioactive bacteria have been identified previously in seawater samples at numbers that are well within the range of those reported for sponges (e.g. Muscholl-Silberhorn et al. 2007). The benefits of anti-infective substances for the producing microbe in the seawater environment are not clear. It appears that competition among microbes may also occur in seawater, for example during settlement of the bacteria on particles such as detritus or marine snow.

About one-third of the bioactive isolates were recovered from each of *Chondrilla nucula* and *Petrosia ficiformis* (Fig. 1), consistent with reports by Chelossi et al. (2004) yet contrasting with results of Muscholl-Silberhorn et al. (2007), who did not recover bioactive

Table 3. 16S rRNA gene sequence analysis of 5 selected bioactive isolates. *C.* = *Chondrilla*, *P.* = *Petrosia*

Isolate	Source	GenBank accession no.	Closest match in GenBank	Accession no.	Identity (%)	Phylogenetic affiliation
PA7	*P. ficiformis* mesohyl	EF192100	*Bacillus subtilis* strain ZJUT	EF694950	99	*Firmicutes, Bacillaceae*
PC4	*P. ficiformis* mesohyl	EF192102	Great Barrier Reef sponge-associated *Alphaproteobacterium* strain NW001	AF295099	98	*Alphaproteobacteria*
CHC2	*C. nucula* mesohyl	EF202836	Uncultured bacteria clone ctg NISAA41, *Shewanella algae* strain YJ06114	DQ396218, EF542799	89	*Gammaproteobacteria*, *Alteromonadaceae*
CHC8	*C. nucula* mesohyl	EF192098	*Brachybacterium paraconglomeratum* strain LMG 19861T from deteriorated parts of a medieval wall painting	AJ415377	99	*Actinobacteria, Dermabacteraceae*
SWA12	Seawater	EF081005	*Bacillus subtilis* strain JKC-13 from Korean fermented food	EF517121	99	*Firmicutes*, *Bacillaceae*

bacteria from these 2 sponge species. We found that about half of the sponge bioactive strains were derived from the sponge surfaces. The role of surface bacteria in the control of fouling has been already elucidated for marine algae and invertebrates including sponges (Lee & Quian 2004), but only few studies have investigated the microbial composition of sponge surfaces and their role in chemical defense (e.g. Chelossi et al. 2004, Thakur et al. 2004).

The number of bioactive isolates increased from winter to summer (Fig. 2) apparently due to an increased general diversity rather than to one of few dominant bioactive strains. The contribution of bioactive strains by sponges (relative to seawater) tended to increase concomitantly. These results suggest that the warmer seasons might increase competitive interactions between bacteria, which in turn may lead to a predominance of bacteria able to produce bioactive compounds. In fact, invertebrate diseases in the Mediterranean Sea resulting from opportunistic/pathogenic bacteria usually break out at the end of the summer season (Gaino & Pronzato 1989, Cerrano et al. 2000). Mass mortality events related to the presence of invasive bacteria have been recorded in the recent decades for Mediterranean sponge populations (Gaino & Pronzato 1989).

Sequencing of isolates that inhibited ≥3 reference strains revealed their affiliation with the genera *Bacillus*, *Brachybacterium*, *Shewanella* and *Alphaprotebacteria* (Table 3). Marine *Bacillus* species are usually isolated from sediments including mud and some strains have been found in association with marine sponges (Thakur et al. 2004, Muscholl-Silberhorn et al. 2007). Most *Bacillus* strains are able to produce peptidic compounds with antibacterial activity (e.g. Jaruchoktaweechai et al. 2000). The genus *Brachybacterium* comprises Gram-positive coryneform bacteria with high G + C contents. This genus and related species are common in soil, but have also been cultivated from marine sediments. Recently, *Actinobacteria* (belonging to the genus *Brachybacterium*) strains have also been cultured from marine sponges (Montalvo et al. 2005). Antimicrobially active strains of this genus have not been found before. The genus *Shewanella* comprises Gram-negative, facultative anaerobic bacteria belonging to the *Gammaproteobacteria*. Marine *Shewanella* strains are known to produce bioactive compounds, and a new species has been recently isolated from Mediterranean sponges (Lee et al. 2006).

The *Alphaproteobacterium* strain PC4 belongs to the previously described, apparently sponge-specific clade of *Alphaproteobacterium* MBIC 3368 and related strains (Thiel & Imhoff 2003, Enticknap et al. 2006, Scheuermayer et al. 2006 and references cited therein, Muscholl-Silberhorn et al. 2007). This clade is closely related to *Pseudovibrio denitrificans*, which is a novel taxon of heterotrophic and facultative anaerobes (Shieh et al. 2004). Antimicrobial activity has been demonstrated for at least 2 *Alphaproteobacterium* MBIC3368-related isolates from marine sponges (Thiel & Imhoff 2003, Hentschel et al. 2006). Retrieving the closely related, bioactive isolate PC4 from *Petrosia ficiformis* further supports the hypothesis that sponges provide a microhabitat for specific microorganisms. To our knowledge, neither of the strains isolated and sequenced from *Chondrilla nucula* mesohyl (CHC2 and CHC8) has a close match with any of the sponge-derived uncultured bacterial clones sequenced by other authors and reported in GenBank. Whether the bioactivity of these isolates plays a functional role in the chemical defence of the sponge host will be subject to further investigations.

CONCLUSIONS

In the present work, 60 of 416 bacterial strains isolated from 2 Mediterranean sponges and ambient seawater had antimicrobial activities against bacterial strains that are potential pathogens for humans and animals. These antimicrobial characteristics offer relevant opportunities for applied sciences. The observed intra-individual variability, the high fraction of seawater bioactive isolates and the seasonal trend of antimicrobial-producing bacteria might indicate that the bioactive strains are rather transiently associated with the sponge from which they were isolated. Whether *Petrosia ficiformis* and *Chondrilla nucula* may rely additionally on stably-associated bioactive bacterial symbionts for defense is still an open question. Further long-time observations of marine sponge-associated microbial communities should be undertaken to better define their role in sponge ecology, symbiosis and with respect to seasonal trends.

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