

Diversity and antimicrobial potential of bacterial isolates associated with the soft coral *Alcyonium digitatum* from the Baltic Sea

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Abstract It is well recognized that microorganisms associated with marine invertebrates, in particular sponges and hard corals, are an excellent source of new natural products. Therefore, the diversity of bacteria associated with marine invertebrates and their potential to produce bioactive compounds have received much attention in recent years. We report here for the first time on the biodiversity of bacteria associated with the soft coral *Alcyonium digitatum*, which is abundant in the Baltic Sea. In order to increase the cultured diversity, bacteria were isolated using four different media, identified with support of 16S rRNA gene sequences and screened for antimicrobial activity using two different media. Activity of crude extracts was tested against *Bacillus subtilis*, *Staphylococcus epidermidis*, *Escherichia coli*, and the yeast *Candida albicans*. A total of 251 coral-associated bacterial isolates were classified and found to belong to 41 species in 14 genera of the *Firmicutes*,

Actinobacteria, *Gammaproteobacteria*, and *Alphaproteobacteria*. The genus *Bacillus* was most abundant and diverse with 17 recognized species. Forty-eight percent of all 251 isolates exhibited antimicrobial activity. All isolates of *Bacillus methylophilicus* and *Bacillus amyloliquefaciens* displayed inhibition of at least three out of the four tested microorganisms. It became obvious during this study that the production of antibiotic substances not only is strain-specific, but in many cases also depends on the media composition and growth conditions. In addition, the antimicrobial potential of bacteria associated with *A. digitatum* may represent a promising source for antimicrobial substances.

Keywords Soft coral *Alcyonium digitatum* · Associated bacteria · Bacterial diversity · Antimicrobial activity

Introduction

Microorganisms associated with marine invertebrates such as sponges and corals, as well as with algae, are known as potent producers of biologically active substances and represent a rich source of novel compounds (Imhoff et al. 2011). Research on marine microbial communities and their antimicrobial compounds has focussed on bacteria associated with sponges (Muscholl-Silberhorn et al. 2008; Thiel et al. 2007; Thiel and Imhoff 2003), algae

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(Staufenberger et al. 2008; Wiese et al. 2009), ascidians (Tait et al. 2007), holothurians (Ward-Rainey et al. 1996), bryozoa (Heindl et al. 2010) and molluscs (Romanenko et al. 2008). A few studies on bacteria associated with hard corals demonstrated high species richness based on genetic diversity. Culture-based methods, 16S rRNA gene sequence analysis, biochemical testing and antimicrobial susceptibilities of the hard coral *Fungia scutaria* in the Red Sea (Lampert et al. 2006) and in the cold water coral *Lophelia pertusa* in the deep sea (Galkiewicz et al. 2011) revealed *Gammaproteobacteria*, *Alphaproteobacteria*, and *Actinobacteria* as the major groups, but also *Betaproteobacteria*, *Bacteroidetes*, and *Firmicutes*. Similar findings were reported from other hard corals (Gray et al. 2011; Lampert et al. 2008; Rohwer et al. 2001). A high proportion (51.6 %) of these microbial isolates displayed distinct antibacterial and antifungal activities (Zhang et al. 2012).

Among the few studies of bacterial communities of soft corals are those on *Dendronephthya* sp. in Port Shelter, Hong Kong. A distinctive epibiotic community was found, of which 55 % belonged to the *Gammaproteobacteria*, 27 % to the *Alphaproteobacteria*, and 18 % to the *Bacteroidetes* (Harder et al. 2003). These bacteria inhibited the larval settlement of the tubeworm *Hydroides elegans* and were considered to contribute to antifouling mechanisms (Dobretsov and Qian 2004). The diversity of bacteria associated with the soft coral *Alcyonium antarcticum* from the Ross Sea (Southern Ocean in Antarctica) was investigated across three different sites by culture-based and molecular techniques i.e. denaturing gradient gel electrophoresis, 16S rRNA gene sequence analysis, and fluorescence in situ hybridization (Webster and Bourne 2007). Phylogenetic analysis of these bacteria showed a close affiliation with psychrophiles from the Antarctic region and high abundance of *Gammaproteobacteria* clades known to be associated with sponges (Webster and Bourne 2007). The soft coral *Alcyonium digitatum*, or dead man's finger, is a common boreal species of the *Alcyoniidae* family. Its habitats are found around the Atlantic coast of northwest Europe from Portugal to Norway, with further reports from Britain, Ireland and North America. This soft coral species was well-studied in terms of taxonomy, distribution and reproduction (Hartnoll 1975).

This is the first study analysing the diversity of bacteria associated with the soft coral *A. digitatum* and their antimicrobial activities using culture-based

approaches and 16S rRNA gene analysis to identify the isolates.

Methods

Sampling processes

Nine living soft coral colonies of *A. digitatum* with white or orange colour were randomly collected at three different sampling sites on 4th May 2011 in 23–30 m depth by dredging (140 cm × 30 cm). All the sampling sites were located in the Kattegat, Baltic Sea (Germany), with the following coordinates: site Baltic 1 (57°00.773N–11°34.835E), site Baltic 2 (56°58.805N–11°35.166E), and site Baltic 3 (56°42.041N–11°44.185E). Three soft coral colonies were collected at each station. To remove loosely attached microbes from their surfaces, the soft corals were washed three times with 0.2- μ m-filtered sterile seawater for 5 min. Each colony was cut into four branches with a sterile scalpel. These branches were kept in 16 ml of filtered sterile Baltic Sea water in a 50 ml tube and stored at 4 °C prior to isolation experiments. The identification of the coral was performed according to Gosner (1978).

Isolation of bacteria from coral samples

The soft coral samples in 16 ml of filter-sterilized Baltic Sea water were homogenized with an ultraturax (IKA T25, Staufen, Germany) for 30 s at 17,500 U/min. Before use the steel tip was sterilized with 70 % ethanol and washed with sterile seawater. Ten-fold serial dilutions of the homogenized samples were prepared to a dilution of 10^{-5} , and 100 μ l of each dilution was spread in triplicate on 4 different culture media. Tropic Marine Medium (TM): 5.0 g peptone from soya bean (Merck, Darmstadt, Germany), 1.0 g Bacto yeast extract, 15.0 g agar (Difco, Becton–Dickinson, Heidelberg, Germany), 30.0 g Tropic Marine Salt (Tropic Marin, Wartenberg/Angersbach, Germany), 1000 ml distilled water. Baltic Sea water medium (BSW): 15.0 g Tropic Marine Salt, 15.0 g agar, 1000 ml Baltic Sea water (filtrated, 0.2 μ m; 15 %). R2A: 0.5 g Bacto yeast extract, 0.5 g Bacto proteose peptone, 0.5 g Difco casamino acids, 0.5 g glucose × H₂O, 0.5 g soluble starch, 0.3 g sodium pyruvate, 0.3 g K₂HPO₄, 0.05 g MgSO₄ × 7H₂O,

30.0 g Tropic Marine Salt, 15.0 g agar, 1000 ml distilled water. SWM: 5.0 g Bacto proteose peptone, 2.5 g yeast extract, 1.0 g glucose, 0.2 g K₂HPO₄, 0.05 g MgSO₄, 15.0 g agar, 15.0 g Tropic Marine Salt, 1000 ml filtered Baltic Sea water. Plates were incubated at 28 °C and at 10 °C for several months. Visible colonies were selected and representatives of each colony morphotype were serially streaked onto fresh media to obtain pure isolates. The pure isolates were cryopreserved using the Microbank System at –80 °C (MAST DIAGNOSTIKA, Reinfeld, Germany) following procedures recommended by the manufacturer.

DNA extraction and phylogenetic classification

Single colonies from pure cultures were suspended in 100 µl of DNA-free water for molecular biology-grade water (Sigma-Aldrich, Taufkirchen at Munich, Germany). The bacterial suspension was centrifuged at 10,000 rpm/min for 15 min at 4 °C. After removing the supernatant, the cells were resuspended into 200 µl of DNA-free water (water for molecular biology, DEPC-treated and sterile filtered; Sigma-Aldrich). Silica beads (0.1 mm diameter; BioSpec Products, Roth, Germany) were added and the suspension was shaken for 6 min at 30/s frequency (Retsch MM200, Retsch, Haan, Germany). The lysed bacterial suspensions were centrifuged at 10,000 rpm for 5 min and the supernatant was stored at 4 °C for short term usage or at –20 °C for subsequent analyses. Amplification of 16S rRNA gene sequences and sequencing procedures were performed according to Heindl et al. (2010). Amplification of the 16S rRNA gene fragment was performed using puReTaqTMReady-To-GoTMPCR Beads (Heathcare) with the universal primers 27f (5'-GAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTACCTTGTACGACTT-3') or 1525r (5'-CGGGCGGTGTGTACAAGG-3') (Lane 1991). The polymerase chain reaction (PCR) conditions were: initial denaturation (2 min at 94 °C) followed by 30 cycles of primer annealing (40 s at 50 °C), primer extension (90 s at 72 °C), and denaturation (1 min at 42 °C), a final primer annealing (1 min at 42 °C), and a final extension (5 min at 72 °C). Sequencing was performed with two forward primers 342f (5'-TACGGGAGGCAGCAG-3'), 790f (5'-GATACCTGGTAGTCC-3'), and one reverse primer 543r (5'-ATTA CCGCGGCTGCTGG-3'). All sequences were done at

the Institute for Clinical Molecular Biology (University Medical Center Schleswig–Holstein, Kiel, Germany). Sequence data were edited with SeqManTMII (DNASTar) and closest relatives were determined by comparison to 16S rRNA gene sequences from the National Center for Biotechnology Information (NCBI) GenBank database using BLAST searches (Altschul et al. 1990). Type strain relatives of all isolates were identified by comparison of 16S rRNA gene sequences of the Ribosomal Database Project II (RDP-II) Sequence Match Program (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) with the tool “Align two or more sequences”/Nucleotide blast/NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Isolates related to the same type strain were grouped into arbitrary taxonomic units (ATUs) (Table 2). In the following the term ATU is used as equivalent for species, if the 16S rRNA gene sequence similarity with recognized type strains is >99 %. All 16S rRNA gene sequences were deposited in GenBank under accession numbers KT583311–KT583561.

Preparation of cell extracts from the isolates

The bacterial strains were grown in Tropic Marine Medium (TM) for 3–5 days and then transferred to 300 ml Erlenmeyer flasks containing of 100 ml medium GYM 4 medium (glucose 4 g/l, yeast extract 4 g/l, malt extract 4 g/l, Tropic Marine Salt 15 g/l) and Bannett medium (yeast extract 1 g/l, beef extract 1 g/l, tryptone Bacto 2 g/l, Tropic Marine Salt 20 g/l). After 3 days of incubation at 28 °C with shaking at 120 rpm cultures were extracted. The culture broth was extracted by ethyl acetate 1/1 (v/v). After homogenizing and breaking the cells at 17,500 U/min for 30 s using an ultraturax metabolites from both culture supernatant and from bacterial cells were extracted. Extracts were dried and re-suspended in 1 ml of methanol. The methanolic extracts were used for antimicrobial tests with four microorganisms as indicated below.

Antimicrobial activity of the extracts

The determination of the antimicrobial activity was carried out on extracts from all isolates. The following test organisms were used: *Bacillus subtilis* DSM 347 and *Staphylococcus epidermidis* DSM 20044 as representatives of Gram-positive bacteria, *Escherichia*

coli DSM 498 as a Gram-negative bacterium, and the yeast *Candida albicans* DSM 1386, all obtained from the German Culture Collection (DSMZ, Braunschweig, Germany). Cultivation was done in TSB medium (1.2 % tryptic soy broth, 0.5 % NaCl) for the bacteria and in YM medium (0.1 % yeast extract, 0.1 % malt extract, 0.17 % peptone from soybeans, 0.33 % glucose \times H₂O) for the yeast. Overnight cultures of the test organisms were prepared and diluted to an optical density (600 nm) of 0.01–0.05. 10 μ l of the crude extracts were transferred into a 96-well microplate and 200 μ l of the cell suspension cultures were added to each well. The microplates were incubated for five h at 37 °C then 10 μ l of a resazurin solution (0.2 mg ml⁻¹ phosphate-buffered saline) was added to each well and the plates were incubated again at 37 °C for 5–30 min. To evaluate cell viability, the reduction of resazurin to resorufin was assessed by measuring the fluorescence at 560 nm after excitation at 590 nm. The resulting values were compared with a positive (10 μ M chloramphenicol for bacteria; 10 μ M nystatin for the yeast) and a negative control (no compound) on the same plate. All tests were carried out in duplicates and the mean was determined. The threshold for a positive activity was defined as 25 % in comparison to the positive controls exhibiting 100 % inhibition.

Results

Phylogenetic analysis and identification of the isolates

The bacterial community of the soft coral *A. digitatum*, based on 251 pure cultures, is represented by *Firmicutes* (4 genera, 23 species), *Actinobacteria* (2 genera, 4 species) *Gammaproteobacteria* (5 genera, 11 species) and *Alphaproteobacteria* (3 genera, 3 species). Information on sampling sites as well as numbers of total and of antimicrobial-active isolates and arbitrary taxonomic units (ATUs) is given in Tables 1 and 2. The most abundant isolates (226 strains) were *Firmicutes*, including representatives of the genera *Bacillus* (17 ATUs: BA1 to BA17, 192 isolates), *Exiguobacterium* (1 ATU: EX, 1 isolate), *Paenibacillus* (1 ATU: PA, 4 isolates), and *Lysinibacillus* (4 ATUs: LY1 to LY4, 29 isolates). Among 17 distinct *Bacillus* species isolated, the most

abundant species were *Bacillus methylotrophicus* (BA8, 52 isolates), *Bacillus thuringiensis* (BA16, 35 isolates), and *Bacillus amyloliquefaciens* (BA2, 33 isolates) (see Table 2). Out of the 41 identified species only five were found in all soft coral specimens or in all but one. These are *B. amyloliquefaciens*, *B. methylotrophicus*, *Bacillus pumilus*, *B. thuringiensis*, and *Lysinibacillus fusiformis*. All other species were less frequent or occurred only occasionally.

The *Actinobacteria* (7 strains) isolated in the present study belong to *Blastococcus* and *Micrococcus*. The genus *Blastococcus* is represented by a single strain (ATU: BL), which is closely related to the type strain of *Blastococcus saxosidens*. Two isolates each of three *Micrococcus* species were found, *Micrococcus flavus* (ATU: MIF), *Micrococcus luteus* (ATU: MIL), and *Micrococcus yunnanensis* (ATU: MIY).

In total 18 *Proteobacteria* isolates were affiliated with 11 ATUs of *Gammaproteobacteria* and three ATUs of *Alphaproteobacteria*. The *Gammaproteobacteria* were identified as *Pseudoalteromonas aliena* (PSA1, 3 isolates), *Pseudomonas peli* (PSM2, 2 isolates), *Psychrobacter nivimaris* (PSY2, 2 isolates) and one isolate each of *Pseudoalteromonas prydzensis* (PSA2), *Pseudomonas guineae* (PSM1), *Psychrobacter alimentarius* (PSY1), *Psychrobacter piscatorii* (PSY3), *Shewanella baltica* (SH1), *Shewanella colwelliana* (SH2), *Shewanella putrefaciens* (SH3), and *Vibrio lentus* (VI). The *Alphaproteobacteria* were identified as *Rhizobium vignae* (RH, 1 isolate), *Labrenzia alba* (LA, 1 isolate) and *Sulfitobacter marinus* (SU, 1 isolate).

Almost all isolates of this study (96 %) had a sequence similarity of >99 % with the closest related type strain. Five isolates affiliating to the genera *Lysinibacillus*, *Bacillus*, *Pseudoalteromonas*, and *Shewanella* displayed a sequence similarity in the range of 98–99 % (Table 3). Isolates with 16S rRNA gene sequence similarities less than 98 % to the validly described species may be considered to represent new species (Stackebrandt and Ebers 2006), which applies to four isolates of this study. Isolate A-1-28 had a sequence similarity of 97.5 % to the type strain of *Psychrobacter nivimaris* 88/2-7^T (GenBank accession number AJ313425). Isolate C-1-32 was closely related to *Rhizobium vignae* CCBAU 05176^T (GenBank accession number GU128881) with 97.6 % sequence similarity. In addition, isolate A-1-2B affiliated with *Lysinibacillus fusiformis* DSM 2898^T with 97.8 %

Table 1 The numbers of total isolates, active isolates, total and active ATU

Sampling sites	Sample ID	Number of isolates		Number of ATU**	
		Total	Bioactive*	Total	Bioactive*
Baltic 1	A-1	30	8	13	5
	B-1	35	13	14	6
	C-1	27	16	12	5
Baltic 2	A-2	29	12	12	5
	B-2	17	9	10	5
	C-2	31	13	14	7
Baltic 3	A-3	19	13	7	2
	B-3	32	20	13	6
	C-3	31	18	9	3
Total		251	122	41	21

The samples were collected in 23–30 m depth by dredging in the Kattegatt, Baltic Sea

* Bioactive is defined as number of isolates displaying an antibiotic activity against at least one of the test organisms

** ATU (arbitrary taxonomic unit) is defined as equivalent for species, if the 16S rRNA gene sequence similarity with recognized type strains is >99 %

sequence similarity and isolate B-2-18 to *Paenibacillus lautus* JCM 9073^T with 97.5 % sequence similarity.

Antimicrobial activities

Antimicrobial activities were found in 21 out of 37 species (122 isolates or 48.6 % from a total of 251 isolates) against at least one of four indicator microorganisms. The bioactive species included members of the *Firmicutes*, comprising 105 isolates of *Bacillus* (8 ATUs: BA1, BA2, BA8, BA10, BA11, BA14, BA15, BA17), two isolates of *Lysinibacillus* (2 ATUs: LY1, LY4), one isolate of Actinobacteria identified as *Blastococcus* (1 ATU: BL), six isolates of *Micrococcus* (3 ATUs: MIF, MIL and MIY), and eight isolates of the *Proteobacteria* (7 ATUs: PSA1, PSY2, SH1, SH2, SH3, SU, VI) (see Table 3).

Eight out of 17 distinct species of the genus *Bacillus* showed antimicrobial activities. In particular all isolates of *B. amyloliquefaciens* (BA2) and *B. methylotrophicus* (BA8) inhibited at least three of the four indicator microorganisms. All of them inhibited *B. subtilis*, *S. epidermidis* and *E. coli*, and some of them, five isolates of BA2 and three isolates of BA8, also were inhibitory against *C. albicans*.

All *Actinobacteria* isolated in the present study exhibited antimicrobial activities. *M. yunnanensis*

inhibited only *S. epidermidis* whereas *M. flavus* and *M. luteus* were active against *B. subtilis* and *S. epidermidis* after growth in both GYM and BM media. *B. saxosidens* showed antimicrobial activity against *B. subtilis*, *S. epidermidis* and *E. coli*, but only if grown in BM medium.

Seven out of 15 *Gammaproteobacteria* isolates displayed antimicrobial activities. They were assigned to *P. aliena*, *P. nivimaris*, *Shewanella* spp., and *V. lentus*. *P. aliena* and *V. lentus* possessed antimicrobial activities against the three bacterial test organisms but not against the yeast *C. albicans* (Table 3). Isolates of the three *Shewanella* species showed different activity profiles, but all were active against *B. subtilis* and *S. epidermidis*. Notably *S. baltica* (SH1) inhibited *C. albicans* in addition to the Gram-positive bacteria only after growth in GYM medium, while *S. colwelliana* (SH2) inhibited *E. coli* after cultivation in both BM and GYM media. Inhibition of *B. subtilis* and *S. epidermidis* by *S. putrefaciens* (SH3) was found only when it was grown in BM medium. One out of two isolates of *P. nivimaris* showed weak inhibition of *B. subtilis* (31 % in BM medium). Among the *Alphaproteobacteria*, only *S. marinus* (SU) inhibited just *S. epidermidis* and was active (88 %) only after growth in GYM medium.

Most of the bioactive isolates (119 out of 122 isolates) including all 14 isolates of *B. pumilus* (BA10)

Table 2 Number of isolates affiliating with the same species (type strain)

ATUs	Next related type strains	Baltic coral sample 1			Baltic coral sample 2			Baltic coral sample 3			Total number
		Number of isolates			Number of isolates			Number of isolates			
		A-1	B-1	C-1	A-2	B-2	C-2	A-3	B-3	C-3	
BA1	<i>Bacillus aerophilus</i> 28K ^T	0	0	0	0	0	0	0	1	0	1
BA2	<i>Bacillus amyloliquefaciens</i> NBRC 15535 ^T	4	3	3	4	2	1	3	7	6	33
BA3	<i>Bacillus cereus</i> ATCC 14579 ^T	1	1	0	1	3	2	0	2	0	10
BA4	<i>Bacillus fusiformis</i> NRS-350 ^T	0	0	1	0	0	2	0	2	0	5
BA5	<i>Bacillus hwajinpoensis</i> SW-72 ^T	0	0	0	0	0	0	0	1	0	1
BA6	<i>Bacillus licheniformis</i> DSM 13 ^T	0	5	3	4	0	0	2	4	3	21
BA7	<i>Bacillus megaterium</i> IAM 13418 ^T	0	0	0	0	0	0	1	0	0	1
BA8	<i>Bacillus methylotrophicus</i> CBMB205 ^T	1	6	9	3	0	5	10	9	9	52
BA9	<i>Bacillus niabensis</i> 4T19 ^T	0	1	1	4	0	1	1	0	1	9
BA10	<i>Bacillus pumilus</i> ATCC 7061 ^T	1	1	2	1	4	1	0	1	3	14
BA11	<i>Bacillus safensis</i> FO-036b ^T	0	0	1	0	0	1	0	1	0	3
BA12	<i>Bacillus simplex</i> DSM 1321 ^T	0	0	0	0	0	1	0	0	0	1
BA13	<i>Bacillus stratosphericus</i> 41KF2a ^T	0	1	1	0	0	0	0	1	0	3
BA14	<i>Bacillus subtilis</i> DSM10 ^T	0	0	0	0	1	0	0	0	0	1
BA15	<i>Bacillus subtilis</i> NRRL B-23049 ^T	0	0	0	0	0	0	0	1	0	1
BA16	<i>Bacillus thuringiensis</i> IAM 12077 ^T	8	9	1	4	1	5	0	1	6	35
BA17	<i>Bacillus vallismortis</i> DSM11031 ^T	0	0	0	0	1	0	0	0	0	1
EX	<i>Exiguobacterium oxidotolerans</i> T-2-2 ^T	1	0	0	0	0	0	0	0	0	1
PA	<i>Paenibacillus lautus</i> JCM 9073 ^T	0	1	0	0	2	0	0	0	1	4
LY1	<i>Lysinibacillus boronitolerans</i> 10a ^T	4	0	0	2	1	0	0	0	0	7
LY2	<i>Lysinibacillus fusiformis</i> DSM 2898 ^T	4	0	3	1	1	2	1	1	1	14
LY3	<i>Lysinibacillus sphaericus</i> DSM 28 ^T	0	0	0	1	0	0	0	0	0	1
LY4	<i>Lysinibacillus xylanilyticus</i> XDB9 ^T	0	2	0	0	0	4	0	0	1	7
BL	<i>Blastococcus saxobsidens</i> DSM 44509 ^T	0	0	1	0	0	0	0	0	0	1
MIF	<i>Micrococcus flavus</i> LW4 ^T	0	0	0	2	0	0	0	0	0	2
MIL	<i>Micrococcus luteus</i> DSM 20030 ^T	0	0	0	2	0	0	0	0	0	2
MIY	<i>Micrococcus yunnanensis</i> YIM 65004 ^T	0	0	0	0	0	2	0	0	0	2
PSA1	<i>Pseudoalteromonas aliena</i> KMM 3562 ^T	0	0	0	0	0	3	0	0	0	3
PSA2	<i>Pseudoalteromonas prydzensis</i> MB8-11 ^T	1	0	0	0	0	0	0	0	0	1
PSM1	<i>Pseudomonas guineae</i> LMG 24016 ^T	1	0	0	0	0	0	0	0	0	1
PSM2	<i>Pseudomonas peli</i> R-20805 ^T	0	2	0	0	0	0	0	0	0	2
PSY1	<i>Psychrobacter alimentarius</i> JG-100 ^T	1	0	0	0	0	0	0	0	0	1
PSY2	<i>Psychrobacter nivimaris</i> 88/2-7 ^T	2	0	0	0	0	0	0	0	0	2
PSY3	<i>Psychrobacter piscatorii</i> T-3-2 ^T	1	0	0	0	0	0	0	0	0	1
SH1	<i>Shewanella baltica</i> NCTC 10735 ^T	0	1	0	0	0	0	0	0	0	1
SH2	<i>Shewanella colwelliana</i> ATCC 39565 ^T	0	0	0	0	0	1	0	0	0	1
SH3	<i>Shewanella putrefaciens</i> LMG 26268 ^T	0	1	0	0	0	0	0	0	0	1
VI	<i>Vibrio lentus</i> DSM 13757 ^T	0	0	0	0	1	0	0	0	0	1
LA	<i>Labrenzia alba</i> CECT 5094 ^T	0	0	0	0	0	0	1	0	0	1
SU	<i>Sulfitobacter marinus</i> SW-265 ^T	0	1	0	0	0	0	0	0	0	1
RH	<i>Rhizobium vignae</i> CCBAU 05176 ^T	0	0	1	0	0	0	0	0	0	1
Total		30	35	27	29	17	31	19	32	31	251

exhibited antimicrobial activities against *S. epidermidis* (Table 3). Under the conditions tested, a number of strains did not display any antimicrobial activity. These included a large number of Firmicutes: *Bacillus* species (including the ATUs BA3, BA4, BA5, BA6, BA7, BA9, BA12, BA13, and BA16), *L. fusiformis* (LY2, 14 isolates), *Lysinibacillus sphaericus* (LY3, 1 isolate), *P. lautus* (PA, 4 isolates), and *Exiguobacterium oxidotolerans* (EX, 1 isolate). No antimicrobial activity was found as well in extracts of *P. prydzensis* PSA2, *P. guineae* PSM1, *Pseudomonas peli* PSM2, *P. alimentarius* PSY1, and *P. piscatorii* PSY3. The antimicrobial activity patterns of all bioactive isolates are shown in Table 3.

Discussion

Phylogenetic classification of bacteria associated with corals

The aim of this study was to isolate and identify bacteria associated with the soft coral *A. digitatum* living in the Baltic Sea, as well as the assessment of their production of antimicrobial compounds. In order to obtain a maximum diversity of distinct characteristic isolates of the soft coral, colonies were picked randomly from different media. As a result, this diversity is reflected by representatives of four major groups, the Firmicutes, Gammaproteobacteria, Alphaproteobacteria, and Actinobacteria. A related Alcyonium species, *Alcyonium antarcticum*, revealed a high diversity of associated bacteria including of Alpha-, Beta- and Gammaproteobacteria, Bacteroidetes, Firmicutes, Actinomycetales, Planctomycetes, as well as sulfate-reducing bacteria and *Chlorobi* as shown by the analysis of 16S rRNA gene sequences from the metagenome associated with this coral (Webster and Bourne 2007). In a culture-dependent analysis of the same coral, Gammaproteobacteria represented the dominant group (Webster and Bourne 2007).

In the present study, representatives of *Bacillus* together with *Lysinibacillus*, *Paenibacillus* and *Exiguobacterium* isolates were found as the dominant Firmicutes in *A. digitatum*. *Paenibacillus* and *Exiguobacterium* have rarely been found in association with invertebrates. *Paenibacillus* was associated with the black coral *A. dichotoma* from the South China Sea (Zhang et al. 2012) and with *Mussismilia hispida* from

eastern Brazil (de Castro et al. 2010). *Exiguobacterium* was considered to undergo a mutualistic relationship with the coral *Acropora palmata* from the Florida Keys (Ritchie 2006). The genus *Bacillus* was most abundant among the isolates from the black coral *Antipathes dichotoma* from South China Sea (Zhang et al. 2012) and from the four sympatric gorgonian corals *Dichotella gemmacea*, *Melitodes squamata*, *Muricella flexuosa*, and *Subergorgia suberos*, obtained from the South China Sea (Peng et al. 2013). In contrast, representatives of the genus *Bacillus* were not reported in a study on bacteria associated with the coral *F. scutaria* from the Red Sea (Lampert et al. 2006).

Gammaproteobacteria such as *Pseudoalteromonas* spp., *Vibrio* spp., and *Shewanella* spp. have been commonly isolated from corals (Gray et al. 2011; Lampert et al. 2006). *Pseudoalteromonas*, *Pseudomonas*, *Shewanella*, *Psychrobacter*, and *Vibrio* species were associated with cold water coral *L. pertusa* from the northeastern Gulf of Mexico (Galkiewicz et al. 2011). Representatives of *Spongiobacter*, *Vibrio*, and *Alteromonas* were among the dominant groups of bacteria isolated from the corals *M. aequituberculata* and *A. millepora* from Davies Reef, Great Barrier Reef by using media with either dimethylsulfoniopropionate (DMSP) or dimethyl sulfide (DMS) as sole carbon source (Raina et al. 2009). It was inferred that the coral-associated bacteria might be involved in cycling of sulfur in the coral reef as well as in coral health (Raina et al. 2009, 2010). *Pseudoalteromonas* and *Vibrio* were present in reef-building corals and *Psychrobacter* spp. were reported as being dominant in *Acropora palmata* (Mexican Caribbean), in *Acropora formosa* and *Porites lutea* (Indonesia) (McKew et al. 2012). Representatives of the genus *Vibrio* were described as pathogens causing the bleaching of the corals *Pocillopora damicornis* from the Red Sea and *Oculina patagonica* from the Mediterranean Sea (Ben-Haim et al. 2003; Kushmaro et al. 1996, 1997). They were found in the Red Sea coral *F. scutaria* and in the Caribbean coral *Montastraea franksi* by cultivation-independent approaches (Lampert et al. 2008; Rohwer et al. 2001). *Vibrio* spp. also were dominant in cultures obtained from the coral *A. digitifera* from the Gulf of Mannar (Nithyanand and Pandian 2009) and were recovered from healthy corals, i.e. *Pocillopora damicornis* from the Great Barrier Reef (Bourne and Munn 2005), *M. hispida*

Table 3 ATUs, media and antimicrobial activities of the bioactive isolates

ATU	Isolates	Media for isolation	Next related type strain (RDPII)	<i>B. subtilis</i> *	<i>S. epidermidis</i> *	<i>E. coli</i> *	<i>C. albicans</i> *
BA1	B-3-8	TM 28 °C	<i>B. aerophilus</i> 28K ^T (AJ831844; 99.2 %)	0/0	86/80	0/0	0/0
BA10	A-2-32	SW 28 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.4 %)	0/0	57/0	0/0	0/0
BA10	A-1-32	TM 10 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.7 %)	88/0	85/79	0/0	0/0
BA10	B-2-15	SW 10 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.7 %)	0/0	67/0	0/0	0/0
BA10	B-2-16	TM 10 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.7 %)	0/0	81/84	0/0	0/0
BA10	B-2-17	TM 10 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.7 %)	0/0	76/84	0/0	0/0
BA10	C-2-21	TM 10 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.7 %)	0/0	100/0	0/0	0/0
BA10	C-1-27	SW 28 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.8 %)	0/25	100/100	0/0	0/0
BA10	C-3-34	R2A 10 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.8 %)	58/0	90/91	0/0	0/0
BA10	B-1-33	SW 28 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.9 %)	91/0	86/84	0/0	0/0
BA10	C-1-16	SW 28 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.9 %)	93/0	96/77	0/0	0/0
BA10	B-2-12	R2A 10 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.9 %)	0/0	91/55	0/0	0/0
BA10	C-3-36	R2A 10 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.9 %)	0/0	45/0	0/0	0/0
BA10	C-3-33	R2A 10 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.9 %)	66/0	85/89	0/0	0/0
BA10	B-3-17	TM 28 °C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289; 99.7 %)	0/0	97/97	32/42	0/0
BA11	C-1-15	R2A 28 °C	<i>B. safensis</i> FO-036b ^T (AF234854; 99.9 %)	0/0	82/82	0/0	0/0
BA11	B-3-34A	SW 10 °C	<i>B. safensis</i> FO-036b ^T (AF234854; 99.9 %)	0/0	92/0	0/0	0/0
BA14	B-2-7	TM 28 °C	<i>B. subtilis</i> DSM10 ^T (AJ276351; 99.8 %)	0/0	25/86	0/0	0/0
BA15	B-3-37	TM 10 °C	<i>B. subtilis</i> NRRL B-23049 ^T (AF074970; 99.9 %)	0/30	100/100	0/0	0/0
BA17	B-2-4	SW 28 °C	<i>B. vallismortis</i> DSM11031 ^T (AB021198; 99.6 %)	71/43	76/74	86/90	26/31
BA2	A-2-4	SW 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669; 100 %)	73/84	79/78	0/78	0/0
BA2	A-2-23A	TM 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669; 100 %)	44/42	85/97	66/98	0/0
BA2	C-3-39	R2A 10 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669; 100 %)	79/87	83/79	87/92	0/0
BA2	A-1-13	R2A 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669; 99.4 %)	41/63	66/68	34/73	0/0
BA2	B-2-5	TM 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669; 99.4 %)	73/82	72/74	87/0	0/20
BA2	A-3-18	TM 10 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669; 99.4 %)	36/84	70/80	89/100	0/0
BA2	C-2-14	R2A 10 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669; 99.5 %)	86/94	85/90	80/90	0/0
BA2	C-3-21	R2A 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669; 99.5 %)	78/90	77/85	67/85	0/0
BA2	C-3-20	R2A 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669; 99.5 %)	92/96	95/87	88/100	0/0
BA2	A-1-36	SW 10 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669; 99.6 %)	44/72	58/71	42/80	0/0
BA2	A-1-6A	TM 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669; 99.6 %)	0/79	0/76	0/86	0/0

Table 3 continued

ATU	Isolates	Media for isolation	Next related type strain (RDPII)	<i>B. subtilis</i> *	<i>S. epidermidis</i> *	<i>E. coli</i> *	<i>C. albicans</i> *
BA2	B-1-8	R2A 10 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.6 %)	90/87	87/85	87/91	0/0
BA2	B-1-11	TM 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.6 %)	70/82	70/78	91/100	0/0
BA2	B-3-1	TM 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.6 %)	91/82	81/76	0/51	0/0
BA2	C-1-11	TM 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.7 %)	90/95	78/80	87/92	0/0
BA2	A-3-17	SW 10 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.7 %)	0/84	79/76	0/77	0/0
BA2	B-3-6	SW 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.7 %)	90/100	87/89	30/89	0/24
BA2	B-3-33	SW 10 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.7 %)	46/89	97/99	99/100	0/45
BA2	C-3-23	TM 10 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.7 %)	91/63	89/87	0/93	0/0
BA2	C-3-30	R2A 10 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.7 %)	74/0	82/89	86/0	0/0
BA2	A-1-18	SW 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8 %)	84/89	80/81	73/93	0/0
BA2	B-1-28 B	SW 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8 %)	84/87	68/76	98/100	0/0
BA2	C-1-10	TM 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8 %)	88/80	94/99	96/100	0/33
BA2	A-2-5	SW 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8 %)	59/88	85/89	95/100	0/0
BA2	A-2-14	R2A 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8 %)	39/68	89/99	88/99	0/0
BA2	B-2-2	R2A 10 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8 %)	84/0	88/83	89/91	0/0
BA2	A-3-13	SW 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8 %)	87/88	81/83	0/85	0/0
BA2	B-3-12	R2A 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8 %)	91/92	84/87	94/93	0/0
BA2	B-3-21	R2A 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8 %)	53/90	96/99	97/100	0/0
BA2	B-3-16	SW 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8 %)	89/91	83/85	83/93	0/0
BA2	B-3-32	R2A 10 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8 %)	84/91	94/100	36/100	0/0
BA2	C-3-35	R2A 10 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8 %)	69/86	82/88	77/81	0/0
BA2	C-1-9	R2A 28 °C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.9 %)	43/28	66/69	80/76	0/0
BA8	B-3-26	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897: 100 %)	89/88	83/77	91/91	0/0
BA8	C-1-17	SW 10 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897: 99.5 %)	87/93	79/83	89/89	0/0
BA8	C-2-10	R2A 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897: 99.5 %)	70/82	71/80	91/100	0/0
BA8	B-3-36	TM 10 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897: 99.5 %)	92/97	88/100	84/100	0/0
BA8	C-2-5A	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897: 99.6 %)	87/86	81/83	82/80	0/0
BA8	C-2-9	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897: 99.6 %)	87/85	80/80	93/91	0/0
BA8	B-3-18	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897: 99.6 %)	67/93	57/88	97/100	0/0
BA8	C-2-32	R2A 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897: 99.6 %)	80/84	89/95	97/99	0/0
BA8	A-1-34	SW 10 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897: 99.7 %)	64/85	78/84	87/92	0/0

Table 3 continued

ATU	Isolates	Media for isolation	Next related type strain (RDPII)	<i>B. subtilis</i> *	<i>S. epidermidis</i> *	<i>E. coli</i> *	<i>C. albicans</i> *
BA8	B-1-13	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	59/51	85/80	0/87	0/0
BA8	B-1-27	R2A 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	79/80	66/77	35/100	0/0
BA8	B-1-31	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	86/88	77/82	0/46	0/0
BA8	C-1-7	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	32/91	90/93	95/100	0/0
BA8	C-1-21	R2A 10 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	36/33	74/71	93/82	0/0
BA8	C-1-22	R2A 10 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	34/89	76/80	88/90	0/0
BA8	C-1-25	TM 10 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	73/85	62/71	92/91	0/0
BA8	C-1-5	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	90/0	81/81	0/61	0/0
BA8	A-2-2	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	77/87	82/90	99/100	0/0
BA8	C-2-5B	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	88/95	89/91	83/78	0/0
BA8	A-3-3	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	0/91	75/82	80/95	0/0
BA8	A-3-6C	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	72/90	95/96	99/99	0/0
BA8	A-3-22	TM 10 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	62/78	68/77	70/85	0/0
BA8	A-3-23A	R2A 10 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	82/86	83/85	87/90	0/0
BA8	A-3-23B	R2A 10 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	86/77	74/75	89/80	36/0
BA8	A-3-6A	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	45/72	82/85	98/99	0/*
BA8	A-3-6B	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	66/84	68/73	92/100	0/0
BA8	A-3-8	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	51/96	89/99	70/100	0/0
BA8	B-3-24	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	48/80	71/74	96/100	0/49
BA8	B-3-13	R2A 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	37/50	81/83	99/99	0/0
BA8	B-3-23	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	66/90	81/81	0/87	0/0
BA8	C-3-14	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.7 %)	79/74	83/82	0/91	0/0
BA8	B-1-14	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	60/88	78/85	51/100	0/0
BA8	C-1-12	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	72/0	75/73	45/64	0/0
BA8	C-1-13	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	0/94	76/85	0/93	0/0
BA8	C-1-24	R2A 10 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	88/83	100/82	97/92	0/0
BA8	A-2-23B	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	0/47	71/80	58/99	0/28
BA8	A-3-2	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	57/87	81/82	0/83	0/0
BA8	A-3-7B	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	22/0	88/88	100/100	0/0
BA8	B-3-2	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	76/90	91/99	95/100	0/0
BA8	B-3-28	R2A 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	73/79	73/86	75/99	0/0

Table 3 continued

ATU	Isolates	Media for isolation	Next related type strain (RDPII)	<i>B. subtilis</i> *	<i>S. epidermidis</i> *	<i>E. coli</i> *	<i>C. albicans</i> *
BA8	B-3-20	R2A 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	37/86	63/89	95/99	0/*
BA8	C-3-2	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	24/42	84/81	0/92	0/0
BA8	C-3-4	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	88/82	82/85	89/93	0/0
BA8	C-3-6	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	42/79	83/91	81/93	0/0
BA8	C-3-10	R2A 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	86/88	80/82	89/91	0/0
BA8	C-3-19	R2A 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	72/61	80/77	0/91	0/0
BA8	C-3-7	TM 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	74/68	81/87	64/94	0/0
BA8	C-3-9	R2A 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	66/0	80/87	91/82	0/0
BA8	C-3-17	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.8 %)	98/91	93/86	91/91	0/0
BA8	B-1-28A	R2A 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.9 %)	80/79	85/83	0/88	0/0
BA8	B-1-30	SW 28 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.9 %)	82/89	79/84	0/82	0/0
BA8	A-2-17	R2A 10 °C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897; 99.9 %)	75/71	86/88	94/100	0/0
LY1	A-1-40B	TM 28 °C	<i>L. boronitolerans</i> 10a ^T (AB199591; 98.6 %)	0/0	0/0	0/0	34/42
LY4	C-2-29	TM 10 °C	<i>L. xylanilyticus</i> XDB9 ^T (FJ477040; 99.0 %)	0/0	0/0	0/0	57/68
BL	C-1-31	BSW 28 °C	<i>B. saxobsidens</i> DSM 44509 ^T (FN600641; 98.8 %)	50/0	94/0	86/0	0/0
MIF	A-2-19A	SW 28 °C	<i>M. flavus</i> LW4 ^T (DQ491453; 99.2 %)	0/0	35/60	0/0	0/0
MIF	A-2-28	SW 10 °C	<i>M. flavus</i> LW4 ^T (DQ491453; 99.3 %)	0/0	30/68	0/0	0/0
MIL	A-2-7	TM 28 °C	<i>M. luteus</i> DSM 20030 ^T (AJ536198; 99.7 %)	0/0	60/74	0/0	0/0
MIL	A-2-19B	SW 28 °C	<i>M. luteus</i> DSM 20030 ^T (AJ536198; 99.8 %)	0/0	60/62	0/0	0/0
Miy	C-2-28	TM 10 °C	<i>M. yunnanensis</i> YIM 65004 ^T (FJ214355; 99.4 %)	0/0	0/97	0/35	0/0
Miy	C-2-27	TM 10 °C	<i>M. yunnanensis</i> YIM 65004 ^T (FJ214355; 99.9 %)	0/0	0/83	0/0	0/0
PSA1	C-2-1	R2A 10 °C	<i>P. aliena</i> KMM 3562 ^T (AY387858; 99.2 %)	72/69	78/61	0/22	0/0
PSA1	C-2-19	SW 10 °C	<i>P. aliena</i> KMM 3562 ^T (AY387858; 99.3 %)	76/76	56/29	0/0	0/0
PSY2	A-1-30	SW 10 °C	<i>P. nivimaris</i> 88/2-7 ^T (AJ313425; 99.0 %)	31/0	0/0	0/0	0/0
SH1	B-1-21	R2A 10 °C	<i>S. baltica</i> NCTC 10735 ^T (AJ000214; 98.5 %)	0/67	0/41	0/0	0/32
SH2	C-2-3	R2A 10 °C	<i>S. colwelliana</i> ATCC 39565 ^T (AY653177; 99.9 %)	74/62	84/88	89/89	0/0
SH3	B-1-22	R2A 10 °C	<i>S. putrefaciens</i> LMG 26268 ^T (X81623; 99.0 %)	91/0	92/0	0/0	0/0
SU	B-1-18	BSW 10 °C	<i>S. marinus</i> SW-265 ^T (DQ683726; 99.7 %)	0/0	0/88	0/0	0/0
VI	B-2-1	R2A 10 °C	<i>V. lentus</i> DSM 13757 ^T (AJ278881; 98.8 %)	79/00	85/87	90/84	0/0

* The value indicate percent (%) inhibition of cell growth by extracts obtained after cultivation of the coral associated bacteria in BM/GYM media

from eastern Brazil (de Castro et al. 2010), *Acropora hyacinthus*, and *Stylophora pistillata* from Great Barrier Reef (Kvennefors et al. 2010). In contrast, only a single isolate of *Vibrio* was obtained from *A. digitatum* in the present study.

The alphaproteobacterium *Silicibacter lacuscaerulensis* (Rhodobacteraceae) was associated with the healthy coral *M. franksis* from the Caribbean at five different reefs within a distance of up to 10 km in Bocas del Toro (Panama), which suggests that it may play a specific role for growth and health of the host coral (Rohwer et al. 2001). *Silicibacter* spp. were also dominant in *Acropora* sp. and *Porites* sp. living in the Mexican Caribbean (McKew et al. 2012). Representatives of the genus *Sulfitobacter* (Rhodobacteraceae) were reported from corals from Sampela, Indonesia (McKew et al. 2012). Members of the genus *Roseobacter* (Rhodobacteraceae) were one of the major groups of bacteria retrieved from clone libraries of the corals *M. aequituberculata* and *A. millepora* from Davies Reef, Great Barrier Reef (Raina et al. 2009). In the present study, representatives of the *Rhodobacteraceae* family closely related to *S. marinus* SW-265^T, *L. alba* CECT 5094^T, and *Rhizobium vignae* CCBAU 05176^T were isolated from *A. digitatum*.

Actinobacteria were found to be associated with several corals such as the stony coral *F. scutaria* from the Red Sea (Lampert et al. 2006), the reef building coral *A. digitifera* from the Gulf of Mannar (Nithyanand and Pandian 2009), the reef coral *M. hispida* from eastern Brazil (de Castro et al. 2010), the cold water coral *L. pertusa* from the northeastern Gulf of Mexico (Galkiewicz et al. 2011), the black coral *Anthipathes dichotoma* from the South China Sea (Zhang et al. 2012), and a deep sea Gorgonian coral from the South China Sea (Gray et al. 2011) in culture-dependent studies. Cultivation-independent studies reported the association of *Actinobacteria* with the reef-building coral *P. damicornis* from the Great Barrier Reef (Bourne and Munn 2005), the hard coral *F. scutaria* from Red Sea (Lampert et al. 2008), with the cold water coral *L. pertusa* from the Trondheimsfjord in Norway (Neulinger et al. 2008), the reef-building coral *A. palmata*, and with *Porites asteroides* from the Mexican Caribbean (McKew et al. 2012). Representatives of *Blastococcus* and *Micrococcus* which were observed in our study were not found in the soft coral *A. antarcticum* (Webster and Bourne 2007).

Antimicrobial activity of isolates

At present, our knowledge on the antimicrobial potential of coral-associated bacteria is poor and the role of bioactive compounds from soft coral-associated bacteria is not well understood. A large proportion of bacteria have antimicrobial activity and the broad range of activity over several phyla suggest that the bacteria and their bioactive compounds indeed play a significant role in the life of corals.

Studies of four sympatric Gorgonian corals in China Sea and the black coral *A. dichotoma* from the South China Sea identified representatives of the genus *Bacillus* as producers of antimicrobial compounds (Peng et al. 2013; Zhang et al. 2012). Interestingly, one of our isolates (strain B-2-4) is related to *Bacillus vallismortis* DSM 11031^T and inhibited all four indicator microorganisms. As the metabolite profile of strain B-2-4 (data not shown) differed from those of other *Bacillus* isolates of this study, this strain may be a promising candidate for further studies on the antibiotic-active compounds.

All isolates of *Bacillus megaterium* as well as *Paenibacillus* sp. and *Exiguobacterium* sp. did not produce antimicrobial substances whether grown in GYM or BM media, despite the fact that the production of antibiotic substances has been demonstrated in other studies, in which *B. megaterium* exhibited antimicrobial activity against *B. subtilis*, whilst *Exiguobacterium* sp. showed activity against *Serratia marcescens* PDL100, causing the coral white pox disease (Ritchie 2006). A mutualistic relationship was postulated of *B. megaterium* and *Exiguobacterium* sp. with the host *A. palmata* on the basis of their production of antibiotic compounds (Ritchie, 2006).

The study of Palomo et al. (2013) demonstrated that *Micrococcus yunnanensis* YIM 65004^T (FJ214355), isolated from an unidentified sponge from Florida Keys (USA), was the producer of antimicrobial compounds inhibiting the growth of methicillin-resistant *Staphylococcus aureus* (MRSA). The *Micrococcus* isolate produced kocurin, a new member of the thiazolyl peptide family of antibiotics, and possessed the genes of type II polyketide synthase (PKS-II) (Palomo et al. 2013). One of the isolates of the present study affiliated with this species and showed antibacterial activity against both Gram-negative (*E. coli*) and Gram-positive (*S. epidermidis*) indicator bacteria after cultivation on GYM medium. Another actinobacterium in our study,

identified as *B. saxobsidens* (C-1-31, ATU BL), showed antimicrobial activity only if grown in BM medium. Further investigations are required to identify and characterize the bioactive compounds produced by both of these strains.

Coral-associated *Gammaproteobacteria* such as species of *Vibrio* and *Pseudoalteromonas* exhibited antimicrobial activity against both Gram-positive and Gram-negative strains (Shnit-Orland et al. 2012; Shnit-Orland and Kushmaro 2009). A *Pseudoalteromonas* isolate from the Mediterranean coral *O. patagonica* inhibited the coral pathogen *Vibrio shiloi* and may have a probiotic function for the coral (Nissimov et al. 2009). All bacterial *Pseudoalteromonas* isolates from bryozoans from the Baltic Sea inhibited at least one out of two Gram-positive indicator bacteria, whereas only two out of 37 tested *Pseudoalteromonas* strains showed antimicrobial activity against the Gram-negative *E. coli* (Heindl et al. 2010). A *Pseudoalteromonas* strain isolated from brown algae in the Baltic Sea had a similar antimicrobial profile and only a few isolates inhibited *E. coli* as well as the yeast *C. glabrata* (Wiese et al. 2009). Two out of three isolates of *P. aliena* in the present study had the same pattern of antimicrobial activities whereas the third did not reveal any inhibition of the four test organisms. The isolate B-2-1 (ATU VI—*Vibrio lentus*) from the present study exhibited strong antimicrobial activity against *B. subtilis*, *S. epidermidis*, and *E. coli*. Other *Vibrio* species such as *Vibrio parahaemolyticus* and *Vibrio natrigens*, isolated from *A. digitifera* from the Gulf of Mannar, showed strong antimicrobial activity as well (Nithyanand and Pandian 2009). A number of *Shewanella* isolates from bryozoans in Baltic Sea inhibited Gram-positive bacteria (Heindl et al. 2010). In contrast, representatives of this genus that were associated with a brown alga from the Baltic Sea did not show activity against Gram-positive test strains, but inhibited *E. coli* and *C. glabrata* (Wiese et al. 2009). The activity spectra of all *Shewanella* isolates in the present study were different from the profiles of the *Shewanella* isolates from bryozoans and from brown alga in Baltic Sea habitat (Heindl et al. 2010; Wiese et al. 2009) by showing inhibition of both Gram-positive and Gram-negative bacteria as well as of *C. albicans*. These results suggest that different bioactive compounds are produced by various *Shewanella* strains and further studies on the identification of these compounds and their production need to be carried out.

Three representatives of genera belonging to the family *Rhodobacteraceae* in our study were identified as *S. alba*, *S. marinus*, and *R. vignae*. The isolates of *S. alba* and *R. vignae* showed no antimicrobial activity to any indicator microorganism, whereas *S. marinus* inhibited only *S. epidermidis* after growth in GYM medium. Studies on the antibiotic activities of *Sulfitobacter* species are scarce and the few available obtained different results. A *Sulfitobacter* isolate derived from the brown alga *L. saccharina* from the Baltic Sea inhibited *C. glabrata* (Wiese et al. 2009), while a representative of *Sulfitobacter pontiacus*, isolated from the sponge *Haliclona simulans* from Irish water, did not reveal any antimicrobial activity (Kennedy et al. 2009).

It is obvious that the production of antibiotic substances by bacteria often is dependent on growth conditions and media. Most of the strains isolated from the soft coral *A. digitatum* in this study showed identical antibiotic activities after growth in both media, whereas in some of the strains these activities were strictly dependent on the growth medium.

In addition, strain-specific antimicrobial activity may play a significant role. Whereas most of the isolates identified as *B. amyloliquefaciens* and *B. methylotrophicus* revealed antibacterial activities against all three test strains, including the Gram-negative *E. coli*, those identified as *B. pumilus* showed strain-specific inhibition. All of them inhibited *S. epidermidis*, but only some of them were also active against *B. subtilis*, mostly after cultivation on BM medium (Table 3). A strict dependence on the production of bioactive compounds from the growth medium was also seen with isolates of *M. yunnanensis*, *P. nivimaris*, *S. baltica*, *S. putrefaciens*, *S. marinus*, and *B. saxobsidens* (Table 3). These findings clearly indicate that many bacteria produce antimicrobial agents only under specific conditions and that the production may be triggered by ecological factors and biological interactions with other microorganisms and/or the host organism.

Conclusions

By using four different media for isolation of bacteria from *A. digitatum*, a diverse number of isolates of Firmicutes, Actinobacteria, Gammaproteobacteria, and Alphaproteobacteria was obtained including

potential new species of the genera *Lysinibacillus*, *Paenibacillus*, *Rhizobium*, and *Psychrobacter*.

Without doubt the bacteria isolated from *A. digitatum* represent a valuable resource for antibiotic-active substances. A large portion of the isolates (49 %), which covers a wide range of phylogenetic groups, is able to produce antimicrobial compounds. Extracts from most of the isolates were inhibitory against the bacterial test strains, though only a few showed weak inhibitory effects to the yeast and these were dependent on the growth medium. A remarkable exception is given by the two isolates of *Lysinibacillus*, *L. boronitolerans* and *L. xylanilyticus*, which revealed good inhibition of the yeast in both media but no inhibition of the bacterial test organisms (Table 3). Antibiotic profiles and the production of antibiotic substances apparently are strain-specific properties and in many of the isolates depend on the media applied for the production of metabolites.

Though soft coral associated bacteria may play a role in coral health and can contribute to the chemical defense of the corals by producing antimicrobial substances, further studies are needed to clarify the role of microbes in the production of antibiotic compounds in the living coral and the interrelationship between the bacteria and the coral. Thus, corals and their associated communities remain attractive objects for future research.

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