RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2012) 15:17-32
DOI: 10.2436/20.1501.01.155 ISSN: 1139-6709 **www.im.microbios.org**

INTERNATIONAL MICROBIOLOGY

Bacterial isolates from the bryozoan *Membranipora membranacea*: influence of culture media on isolation and antimicrobial activity

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Received 8 January 2012 · Accepted 13 February 2012

Summary. From specimens of the bryozoan *Membranipora membranacea* collected in the Baltic Sea, bacteria were isolated on four different media, which significantly increased the diversity of the isolated groups. All isolates were classified according to 16S rRNA gene sequence analysis and tested for antimicrobial properties using a panel of five indicator strains and six different media. Each medium featured a unique set of isolated phylotypes, and a phylogenetically diverse collection of isolates was obtained. A total of 96 isolates were assigned to 49 phylotypes and 29 genera. Only one-third of the members of these genera had been isolated previously from comparable sources. The isolates were affiliated with Alpha- and Gammaproteobacteria, Bacilli, and Actinobacteria. A comparable large portion of up to 22 isolates, i.e., 15 phylotypes, probably represent new species. Likewise, 47 isolates (approximately 50%) displayed antibiotic activities, mostly against gram-positive indicator strains. Of the active strains, 63.8 % had antibiotic traits only on one or two of the growth media, whereas only 12.7 % inhibited growth on five or all six media. The application of six different media for antimicrobial testing resulted in twice the number of positive hits as obtained with only a single medium. The use of different media for the isolation of bacteria as well as the variation of media considered suitable for the production of antibiotic substances significantly enhanced both the number of isolates obtained and the proportion of antibiotic active cultures. Thus the approach described herein offers an improved strategy in the search for new antibiotic compounds. [**Int Microbiol** 2012; 15(1):17-32]

Keywords: *Membranipora membranacea* · antimicrobial activity · gene analysis · cultivation media · Baltic Sea

Introduction

Surfaces in the marine environment, whether biotic or abiotic, are exposed to colonization by a multitude of organisms. For example, the encrusting bryozoan *Membranipora membranacea* and related species populate kelps in temperate waters all over the world. The genus *Membranipora* is a potent colonizer and disperser; its global distribution most likely began in the North Pacific several million years ago [42]. In the Baltic Sea, a preferred substrate is provided by phyloids of *Saccharina latissima* (newer synonym of *Laminaria saccharina* [24]). In turn, bryozoan surfaces are themselves subjected to colonizers and grazers. Like other sessile and colony-forming organisms in the marine environment, bryozoans rely on mechanical and chemical defense strategies [13]. As such, bryozoans and their associated microorganisms might be a source of biologically active substances.

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Although the phylum Bryozoa contains several thousands of recent species, studies on natural products have focused only on a few of them [43]. Bryozoan metabolites account only for about 1 % of marine natural products, according to the annually reviews of Blunt et al. [2]. Bryostatins are the most prominent compounds [35] extracted and isolated from bryozoans. However, bryostatins from *Bugula neritina* were show to be produced by the bacterial symbiont "*Candidatus* Endobugula sertula" [9], which is associated with the bryozoan. The resemblance of natural products originally isolated from marine macroorganisms to those identified from microorganisms has led to the assumption that these compounds are of microbial origin [21,22]. This has been a prominent reason to intensify studies on the production of bioactive compounds from bacteria and fungi associated with marine algae, sponges, other invertebrates and, in this study, the bryozoan *Membranipora membranacea*.

Host-associated bacteria, in particular those colonizing surfaces and living in biofilm communities, establish complex interactions with other microorganisms and with their hosts. Communication is mediated chemically, and the sum of all factors shapes the composition of microbial covers, which in many cases have been shown to differ considerably from the surrounding environment [11]. Therefore, regarding the discovery of bioactive compounds, marine surface-associated microorganisms represent excellent sources [10,31]. Bryozoan-associated microorganisms have been studied so far using microscopic [30,48], genetic [20], and cultivation-based [16,36] methods, as well as combinations thereof [14]. So far, no report is available on natural products produced by *M. membranacea* or bacteria associated with this bryozoan [49]. Therefore, the aim of this study was to isolate bacteria from the surface of *M. membranacea* by varying the culture media and growth conditions and then to analyze the ability of these isolates to produce antibacterial compounds.

Materials and methods

Sampling site and sample preparation. Bryozoan samples collected by dredging in the Baltic Sea north of Læsø (Kattegat, coordinates 57° 28.3′ N, 11°10.4′ E, depth 20 m) were identified as *Membranipora membranacea* growing on phylloids of *Saccharina latissima*. Three separate bryozoan colonies were cut out, washed with sterile filtered surrounding sea water, and transferred aseptically into sterile tubes containing 50 % (v/v) glycerol and 3 % (w/v) sodium chloride. The tubes were immediately frozen and stored at –18 °C until further treatment. Excess bryozoan samples growing on algae were placed in a closeable beaker containing local sea water (total volume about one liter) and stored at 4 °C until used for media preparation. In addition, 2 l of seawater from the sampling site were collected before dredging, filtered through a 0.2-µm cellulose acetate filter, and added to selected agar media.

Culture media. For the isolation of microorganisms four media were prepared: "bryozoan extract medium" (BM), "algal extract medium" (AM), diluted "Reasoner's 2A medium" (R2Ad), and diluted "Difco all culture medium" (ACd). For antibiotic activity testing six media were prepared: "Väätänen nine salt solution medium" (VNSS), "*Pseudoalteromonas* specific medium" (PSA), "Reasoner's 2A medium" (R2A), "Difco all culture medium" (AC), "marine broth medium" (MB), and "tryptic soy broth medium" (TSB). Isolation media were prepared as follows: the excess samples from the beaker were used for the media that resembled the natural habitat (BM and AM). Bryozoans were cut out from the algae and minced. An equivalent weight of 3 % (w/v) saline was added. This material was thoroughly blended with an Ultraturrax-homogenizer (IKA Werke, Germany), frozen at –100 °C, and lyophilized to obtain a "bryozoan extract." The remaining algae were recombined with the seawater in a beaker, homogenized, frozen, and lyophilized to yield an "algal extract." Both extracts were dissolved in sea water collected from the sampling site at concentrations of 0.06 % (w/v), yielding BM and AM media. Additionally, R2Ad medium (containing 0.01 % (w/v) Bacto yeast extract, Difco proteose peptone, Difco casamino acids, glucose, soluble starch; 0.006 % (w/v) sodium pyruvate and K_2HPO_4; and 0.00048 % (w/v) $MgSO_4$), and ACd medium [0.06 % (w/v)], both with 3% (w/v) sea salt (Tropic Marin) were prepared.

Six media for the activity tests were prepared (all percentages are w/v): (i) VNSS medium with 0.1 % peptone from soymeal (Merck), 0.05% yeast extract, 0.05 % glucose, 0.5 % soluble starch, 0.001 % $FeSO_4 \cdot 7H_2O$, 0.001 % $Na_2HPO_4 \cdot 2H_2O$, 1.7 6% sodium chloride, 0.147 % Na_2SO_4, 0.008 % $NaHCO_3$, 0.025 % KCl, 0.004 % KBr, 0.187 % $MgCl_2 \cdot 6H_2O$, 0.041 % $CaCl_2 \cdot 2H_2O$, 0.001 % $SrCl_2 \cdot 6H_2O$, and 0.001 % H_3BO_3) (according to Mården et al. [28]); (ii) PSA medium with 0.2 % peptone from soymeal, 0.2 % yeast extract, 0.1 % glucose, 0.02 % KH_2PO_4, 0.005 % $MgSO_4 \cdot 7H_2O$, 0.1 % $CaCl_2 \cdot 2H_2O$, 0.01 % KBr, and 1.8 % sea salt (according to Kalinovskaya et al. [19]); (iii) R2A medium; (iv) AC medium were fivefold concentrated compared to isolation media and 3 % sea salt was added; (v) MB medium with 0.5 % peptone, 0.1 % yeast extract, and 3.14 % sea salt), and (vi) TSB medium with 0.3 % tryptic soy broth (Difco) and 2.5 % sodium chloride). To all media, 1.5 % agar was added for solidification.

Isolation and cultivation of bacteria. For comparison, two different methods of sample preparation were applied. The first two bryozoan samples were crushed with a sterile micropestle, the third was processed with a Precellys 24 lysis & homogenization device with a hard tissue grinding MK28 kit (Bertin Technologies) at 6300 rpm for 20 s. Dilution series with sterile seawater were prepared (10^{-1} to 10^{-5}) [16] and a 100-µl aliquot of each one was spread on agar plates containing four different media. In addition, pieces of the bryozoan samples were placed on plates with all four media. The plates were incubated at 25 °C in the dark until colonies were visible. These were picked and sub-cultured on MB agar plates. For preservation, pure cultures were suspended in liquid MB medium containing 5 % (v/v) DMSO and stored at –100 °C.

Screening for inhibitory activities against indicator organisms. Bacterial isolates were grown on MB agar plates directly from the DMSO stock. Colonies were picked, and suspended in 1 ml sterile 3 % (w/v) saline, and a 15-µl aliquot of each one was pipetted onto agar plates with six different media. After growing for 3–4 days at room temperature (ca. 22 °C), the bacterial colonies were checked for the presence of clearance zones to anticipate false-positive results. The plates were then covered with 5 ml TSB soft agar (with 1 % (w/v) sodium chloride and 0.8 % (w/v) agar) containing one of the following indicator strains: *Escherichia coli* DSM 498, *Bacillus subtilis* subsp. *spizizenii* DSM 347, *Staphylococcus lentus* DSM 6672, *Pseudomonas fluorescens* NCIMB 10586, and the yeast *Candida glabrata* DSM 6425. The presence of inhibition zones was examined the following day as well as on days 3, 7 and 14.

Amplification, sequencing, and classification of the isolates. Amplification, sequencing, and phylogenetic analysis of the 16S rRNA gene sequences from the bacterial isolates were carried out as previously described [16]. Isolates were grouped into phylotypes by sequence similarities ≥99.5 %. Genus affiliation was determined using the RDP classifier [46]. If resulting confidence values were <60 % for the classified genus, the affiliation was specified by constructing phylogenetic trees and comparing BLAST results. This was the case for phylotypes 1 (*Erwinia*), 16 (*Roseobacter*), and 19 (*Ruegeria*).

In the case of strain BB77, a 16S rRNA gene clone library was constructed because direct sequencing of the PCR product was not successful. The PCR product was purified after gel electrophoresis with a MinElute Gel extraction kit (Qiagen, Hilden, Germany) and excision of the band. The purified 16S rRNA gene was cloned into the pCR 2.1-TOPO vector and transformed into One Shot TOP10 chemically competent *E. coli* cells, using the TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Correct insertion was checked by PCR with vector binding primers included in the kit. Fourteen clones were chosen for sequencing and classification of the inserted 16S rRNA gene as described above. The 16S rRNA gene sequences were deposited with the EMBL Nucleotide Sequence Database under the accession numbers FR693269 to FR693364.

Cluster analysis. The distribution patterns of phylotypes and antibiotic activities of isolates were compared by cluster analysis using the Bray-Curtis similarity index. Dendrograms were generated with the program PAST, applying the paired group algorithm [15].

Results

Isolation of *Membranipora membranacea* associated bacteria. Four media were used for the isolation of bacteria, and all colonies grown on the agar plates were picked and purified on MB agar. The results are shown in Table 1. Most isolates (60.4 % of all 96 isolates) derived from media inoculated with a piece of the bryozoan; 30.2 % from dilution step 10^{-1}, 8.3 % from step 10^{-2}, and 1.0 % from step 10^{-4}. Most isolates (43.8 % of all isolates) were obtained from ACd medium, fewer isolates resulted from R2Ad medium (34.4 %), BM medium (15.6 %), and AM medium (6.2 %). Portions of 29, 32 and 39 % of the isolates were obtained from the three bryozoan samples.

Phylogenetic affiliation. All isolates were classified phylogenetically based on 16S rRNA gene sequences and grouped into phylotypes according to sequence similarity values of ≥99.5 %. The resulting 49 phylotypes were affiliated with 28 different genera (Table 2). A cluster analysis regarding the presence and absence of phylotypes within the three bryozoan samples revealed low similarity values at ≤0.3, with samples 1 and 2 as the most related (Fig. 1A). This clearly indicated that distinct phylotypes were obtained from each sample, especially from the third bryozoan specimen, which was prepared differently as described above. The media had a significant influence on the types of bacteria isolated and resulted in dissimilar phylotype patterns. 37 phylotypes (75.5 %) were unique to one of the media, i.e., representatives of these phylotypes were not found elsewhere. Most "unique" phylotypes derived from R2Ad medium (17 of the 23 phylotypes of this medium) followed by ACd medium (11 of 23), BM medium (6 of 12), and AM medium (3 of 6). Accordingly, similarity values for the phylotypes obtained from the different media were low and ranged from 0.1 to 0.3 (Fig. 1B).

The bacterial isolates were affiliated with four classes: Gammaproteobacteria (40 isolates), Alphaproteobacteria (21 isolates), Bacilli (12 isolates), and Actinobacteria (23 isolates). Representatives of these classes were isolated from each bryozoan sample, with the exception of the Alphaproteobacteria, which were not obtained from sample 3 (Fig. 2).

Gammaproteobacteria. The 40 isolates of the Gammaproteobacteria could be grouped into 15 phylotypes and assigned to ten genera: *Erwinia*, *Pseudoalteromonas*, *Vibrio*, *Shewanella*, *Halomonas* (4 phylotypes), *Marinobacter*, *Psychrobacter*, *Microbulbifer* (2 phylotypes), *Alcanivorax*, and *Pseudomonas* (2 phylotypes) (Fig. 3A; Table 2). The majority of these bacteria (85%, covering 14 phylotypes) were picked from media inoculated with a piece of the bryozoan, and most isolates (47.5 %, covering 11 phylotypes) were obtained using ACd medium (Table 1).

Bacteria related to *Halomonas* were isolated from all three bryozoan samples and from all four media. In contrast, all eight isolates assigned to *Pseudoalteromonas* originated from sample 3 but were picked from three different isolation media (BM, R2Ad, Acd). Representatives of *Psychrobacter*, *Microbulbifer*, and *Pseudomonas* each derived from more than one medium and bryozoan sample. Single isolates were obtained of *Vibrio*, *Shewanella*, *Marinobacter*, *Alcanivorax*, and *Erwinia*. The latter (BB49, phylotype 1) could represent a new species, as indicated by 16S similarity values ≤ 97% with validly described species (Table 2). This is below the threshold value of 98.7 % for 16S similarity values proposed by Stackebrandt and Ebers [44], which indicate genomic uniqueness of novel isolates.

Alphaproteobacteria. Members of 16 phylotypes (21 isolates) were affiliated with the *Alphaproteobacteria* and assigned to ten genera: *Roseobacter*, *Roseovarius* (2 phylotypes), *Ruegeria* (4 phylotypes), *Jannaschia*, *Paracoccus*,

Table 1. Origin, affiliation, and antimicrobial activity of *Membranipora membranacea*-associated bacteria

			Isolation		Activity[b]				
Phylotype	Isolates	Affiliation	S[a]	Medium	Bs	Sl	Ec	Pf	Cg
1	BB49	*Erwinia*	2	BM	T				
2	BB66a	*Pseudoalteromonas*	3	BM					
2	BB67	*Pseudoalteromonas*	3	R2Ad					T
2	BB68/ BB69	*Pseudoalteromonas*	3	R2Ad	M				
2	BB71	*Pseudoalteromonas*	3	ACd	M				
2	BB72b/ BB73/ BB74	*Pseudoalteromonas*	3	ACd	TM				
3	BB8	*Vibrio*	1	ACd					
4	BB86	*Shewanella*	1	ACd	TMVA				
5	BB12b	*Halomonas*	1	ACd					
5	BB66b	*Halomonas*	3	BM		A		T	
5	BB85	*Halomonas*	2	ACd		PRA			
5	BB7	*Halomonas*	1	AM				P	
6	BB10/ BB3	*Halomonas*	1	ACd					
6	BB6	*Halomonas*	1	AM					
6	BB65	*Halomonas*	3	BM					
7	BB9	*Halomonas*	1	ACd					
8	BB11b	*Halomonas*	1	ACd					
8	BB81/ BB82b	*Halomonas*	1	R2Ad					
9	BB15	*Marinobacter*	1	ACd	TA				
10	BB13/ BB14	*Psychrobacter*	1	R2Ad					
10	BB20/ BB83	*Psychrobacter*	1	ACd					
10	BB62	*Psychrobacter*	3	BM	TV				
11	BB44	*Microbulbifer*	2	R2Ad	MVPRA	A	A		
12	BB27	*Microbulbifer*	1	AM	R				
12	BB34	*Microbulbifer*	2	ACd	PR				
12	BB48	*Microbulbifer*	2	ACd	R				
13	BB31	*Alcanivorax*	2	R2Ad	*n.t.*	*n.t.*	*n.t.*	*n.t.*	*n.t.*
14	BB5/ BB82a/ BB79	*Pseudomonas*	1	R2Ad					
14	BB80	*Pseudomonas*	1	R2Ad	TVA				
15	BB75a/ BB78	*Pseudomonas*	3	ACd					
16	BB43	*Roseobacter*	2	R2Ad	R				
17	BB22	*Roseovarius*	1	R2Ad	R				
18	BB19	*Roseovarius*	1	AM	RA				
19	BB50b	*Ruegeria*	2	BM	VRA				
20	BB40	*Ruegeria*	2	R2Ad	MVPRA				

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Table 1. (*Continued*) Origin, affiliation, and antimicrobial activity of *Membranipora membranacea*-associated bacteria

			Isolation		Activity[b]				
Phylotype	Isolates	Affiliation	S[a]	Medium	Bs	Sl	Ec	Pf	Cg
21	BB2	*Ruegeria*	1	ACd	MVA				
21	BB29	*Ruegeria*	2	R2Ad	RA				
21	BB45	*Ruegeria*	2	R2Ad	MVR				
22	BB33	*Ruegeria*	2	ACd	MVRA				
23	BB23	*Jannaschia*	1	R2Ad					
24	BB51b	*Paracoccus*	2	BM	TRA				
25	BB54	*Anderseniella*	2	AM	R				
26	BB18	*Amorphus*	1	AM					
27	BB32	*Erythrobacter*	2	R2Ad					
28	BB17	*Erythrobacter*	1	R2Ad	MV				
29	BB1	*Sphingopyxis*	1	ACd	T				
29	BB4/ BB46	*Sphingopyxis*	1/2	ACd					
30	BB24	*Sphingopyxis*	1	R2Ad	VP				
30	BB28	*Sphingopyxis*	2	ACd	M				
31	BB21	*Pelagibius*	1	R2Ad	MPRA				
32	BB58a/ BB58b	*Staphylococcus*	3	ACd	*n.t.*	*n.t.*	*n.t.*	*n.t.*	*n.t.*
32	BB60	*Staphylococcus*	3	R2Ad	P				
32	BB26	*Staphylococcus*	1	ACd					
33	BB41	*Bacillus*	2	R2Ad					
34	BB50c	*Bacillus*	2	BM	P	P			
34	BB51c	*Bacillus*	2	BM					
35	BB42	*Bacillus*	2	R2Ad	TVPRA	TMVPRA		VR	
36	BB52	*Bacillus*	2	R2Ad	T	PA			
37	BB61	*Bacillus*	3	R2Ad	TP				
38	BB75b	*Exiguobacterium*	3	ACd	TMPR				
38	BB76	*Exiguobacterium*	3	ACd	M				
39	BB36	*Mycobacterium*	2	ACd					
39	BB55/ BB56/ BB57	*Mycobacterium*	3	ACd					
39	BB64	*Mycobacterium*	3	BM					
40	BB35	*Mycobacterium*	2	ACd					
41	BB37	*Mycobacterium*	2	ACd		TP			
42	BB38	*Mycobacterium*	2	BM					
43	BB63	*Pseudonocardia*	3	R2Ad					

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Table 1. (*Continued*) Origin, affiliation, and antimicrobial activity of *Membranipora membranacea*-associated bacteria

			Isolation		Activity[b]				
Phylotype	Isolates	Affiliation	S[a]	Medium	Bs	Sl	Ec	Pf	Cg
44	BB16	*Streptomyces*	1	BM	P				
44	BB47a/ BB47b	*Streptomyces*	2	ACd					
44	BB50a/ BB51a	*Streptomyces*	2	BM					
44	BB11a	*Streptomyces*	1	ACd	TMVPRA	TMVPRA			
44	BB84	*Streptomyces*	1	ACd	TMVPRA	TMVPRA		T	
45	BB12a	*Streptomyces*	1	ACd	TMVPRA	TMVPRA			
46	BB72a	*Arthrobacter*	3	ACd					
47	BB59/ BB70	*Arthrobacter*	3	R2Ad					
48	BB77	*Arthrobacter*	3	BM					
49	BB25/ BB30	*Microbacterium*	1/2	R2Ad					

[a]Specimen.
[b]Strains tested: Bs, *B. subtilis*; Sl, *S. lentus*; Ec, *E. coli*; Pf, *P. fluorescens*; Cg, *C. glabrata*. Media used: T, TSB; M, MB; V, VNSS; P, PSA; R, R2A; A, AC, *n.t.*: not tested.

Anderseniella, *Amorphus*, *Erythrobacter* (2 phylotypes), *Sphingopyxis* (2 phylotypes), and *Pelagibius* (Fig. 3B; Table 2). In contrast to the Gammaproteobacteria, these isolates were predominantly picked from R2Ad medium (47.6 %, covering 9 phylotypes) and from the dilution series (71.4 %, covering 12 phylotypes) (Table 1). Except for the isolates affiliated with *Sphingopyxis* (both phylotypes) and *Ruegeria* (phylotype 21), all Alphaproteobacteria were single isolates (Table 2). New species were possibly represented by members of 13 phylotypes: *Roseobacter* (phylotype 16), *Roseovarius* (phylotype 17), *Ruegeria* (phylotypes 19 and 21), *Jannaschia* (phylotype 23), *Paracoccus* (phylotype 24), *Anderseniella* (phylotype 25), *Amorphus* (phylotype 26), *Erythrobacter* (phylotypes 27 and 28), *Sphingopyxis* (phylotypes 29 and 30), and *Pelagibius* (phylotype 31) (Table 2).

Bacilli. This class [27] was represented by seven phylotypes (12 isolates) affiliated with the genera *Staphylococcus*, *Bacillus* (5 phylotypes), and *Exiguobacterium* (Fig. 3C; Table 2). All but one of the isolates were obtained from plates inoculated with bryozoan samples 2 and 3 (41.7 % and 50 %, covering four and three phylotypes, respectively). ACd and R2Ad media yielded five isolates each. *Bacillus* and

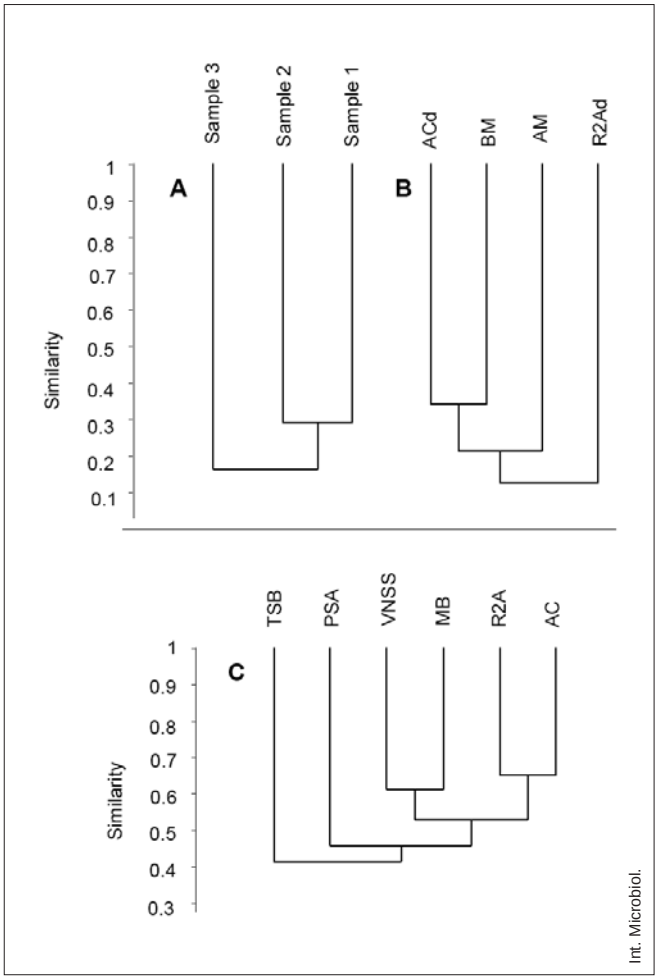

Fig. 1. Similarity dendrograms of shared phylotypes within the three *Membranipora membranacea* specimens (**A**), the four isolation media (**B**), as well as the antimicrobial activities expressed by the isolates on different media (**C**).

Table 2. Phylogenetic affiliation (RDP) and nearest type strains (BLAST)

Phylotype	Representative	No. of isolates	Affiliation	Nearest type strain	Similarity (%)	Accession no.
1	BB49	1	*Erwinia*	*Erwinia tasmaniensis* *Citrobacter gilleni*	97 97	AM055716 AF025367
2	BB66a	8	*Pseudoalteromonas*	*Pseudoalteromonas aliena*	99	AY387858
3	BB8	1	*Vibrio*	*Vibrio tasmaniensis*	98	AJ316192
4	BB86	1	*Shewanella*	*Shewanella kaireitica*	98	AB094598
5	BB66b	4	*Halomonas*	*Halomonas titanicae*	99	FN433898
6	BB65	4	*Halomonas*	*Halomonas boliviensis*	99	AY245449
7	BB9	1	*Halomonas*	*Halomonas boliviensis*	99	AY245449
8	BB82b	3	*Halomonas*	*Halomonas boliviensis*	99	AY245449
9	BB15	1	*Marinobacter*	*Marinobacter algicola*	99	AY258110
10	BB13	5	*Psychrobacter*	*Psychrobacter piscatorii*	99	AB453700
11	BB44	1	*Microbulbifer*	*Microbulbifer thermotolerans*	99	AB124836
12	BB34	3	*Microbulbifer*	*Microbulbifer epialgicus*	98	AB266054
13	BB31	1	*Alcanivorax*	*Alcanivorax venustensis*	99	AF328762
14	BB82a	4	*Pseudomonas*	*Pseudomonas perfectomarina*	100	U65012
15	BB78	2	*Pseudomonas*	*Pseudomonas chloritidismutans*	99	AY017341
16	BB43	1	*Roseobacter*	*Leisingera nanhaiensis* *Seohicola saemankumensis*	97 96	FJ232451 EU221274
17	BB22	1	*Roseovarius*	*Roseovarius aestuarii*	97	EU156066
18	BB19	1	*Roseovarius*	*Roseovarius aestuarii*	99	EU156066
19	BB50b	1	*Ruegeria*	*Ruegeria scottomollicae*	96	AM905330
20	BB40	1	*Ruegeria*	*Ruegeria scottomollicae*	98	AM905330
21	BB2	3	*Ruegeria*	*Ruegeria atlantica*	96	D88526
22	BB33	1	*Ruegeria*	*Ruegeria atlantica*	99	D88526
23	BB23	1	*Jannaschia*	*Jannaschia pohangensis*	97	DQ643999
24	BB51b	1	*Paracoccus*	*Paracoccus homiensis*	97	DQ342239
25	BB54	1	*Anderseniella*	*Anderseniella baltica*	97	AM712634
26	BB18	1	*Amorphus*	*Amorphus coralli*	95	DQ097300
27	BB32	1	*Erythrobacter*	*Erythrobacter longus*	97	AF465835
28	BB17	1	*Erythrobacter*	*Erythrobacter aquimaris*	98	AY461441
29	BB1	3	*Sphingopyxis*	*Sphingopyxis litoris*	98	DQ781321
30	BB24	2	*Sphingopyxis*	*Sphingopyxis litoris*	98	DQ781321
31	BB21	1	*Pelagibius*	*Pelagibius litoralis*	92	DQ401091
32	BB58b	4	*Staphylococcus*	*Staphylococcus epidermidis*	99	D83363
33	BB41	1	*Bacillus*	*Bacillus hwajinpoensis*	98	AF541966

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Table 2. (*Continued*) Phylogenetic affiliation (RDP) and nearest type strains (BLAST)

Phylotype	Representative	No. of isolates	Affiliation	Nearest type strain	Similarity (%)	Accession no.
34	BB50c	2	*Bacillus*	*Bacillus hwajinpoensis*	99	AF541966
35	BB42	1	*Bacillus*	*Bacillus stratosphericus*	99	AJ831841
36	BB52	1	*Bacillus*	*Bacillus licheniformis*	99	CP000002
37	BB61	1	*Bacillus*	*Bacillus cereus*	99	AE016877
38	BB76	2	*Exiguobacterium*	*Exiguobacterium oxidotolerans*	99	AB105164
39	BB64	5	*Mycobacterium*	*Mycobacterium frederiksbergense*	99	AJ276274
40	BB35	1	*Mycobacterium*	*Mycobacterium aurum*	99	X55595
41	BB37	1	*Mycobacterium*	*Mycobacterium aurum*	98	X55595
42	BB38	1	*Mycobacterium*	*Mycobacterium komossense*	97	X55591
43	BB63	1	*Pseudonocardia*	*Pseudonocardia carboxydivorans*	99	EF114314
44	BB51a	7	*Streptomyces*	*Streptomyces griseorubens*	99	AB184139
45	BB12a	1	*Streptomyces*	*Streptomyces praecox*	99	AB184293
46	BB72a	1	*Arthrobacter*	*Arthrobacter parietis*	99	AJ639830
47	BB70	2	*Arthrobacter*	*Arthrobacter tumbae*	97	AJ315069
48	BB77	1	*Arthrobacter*	*Arthrobacter agilis*	98	X80748
49	BB25	2	*Microbacterium*	*Microbacterium schleiferi*	99	Y17237

Exiguobacterium related isolates originated predominantly from agar plates inoculated with a bryozoan piece, while those of *Staphylococcus* derived from the dilution series (Table 1).

Actinobacteria. The 11 phylotypes (23 isolates) affiliated with this class [45] were assigned to the genera *Mycobacterium* (4 phylotypes), *Streptomyces* (2 phylotypes), *Arthrobacter* (3 phylotypes), *Pseudonocardia*, and *Microbacterium* (Fig. 3C; Table 2). The majority of the isolates was obtained from ACd medium (52.2 %, 6 phylotypes). Isolates affiliated with *Streptomyces* and *Arthrobacter* originated from media inoculated with a piece of the bryozoan specimens, while all others were obtained from dilution series (Table 1). Isolates belonging to *Mycobacterium* (phylotype

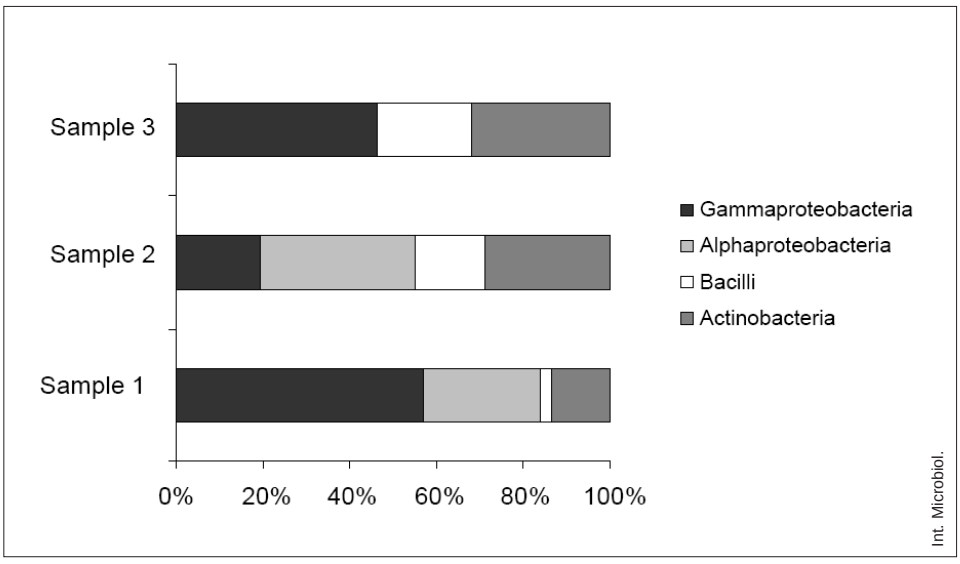

Fig. 2. Relative abundance of isolates from three *Membranipora membranacea* specimens affiliated with the four bacterial classes observed in this study.

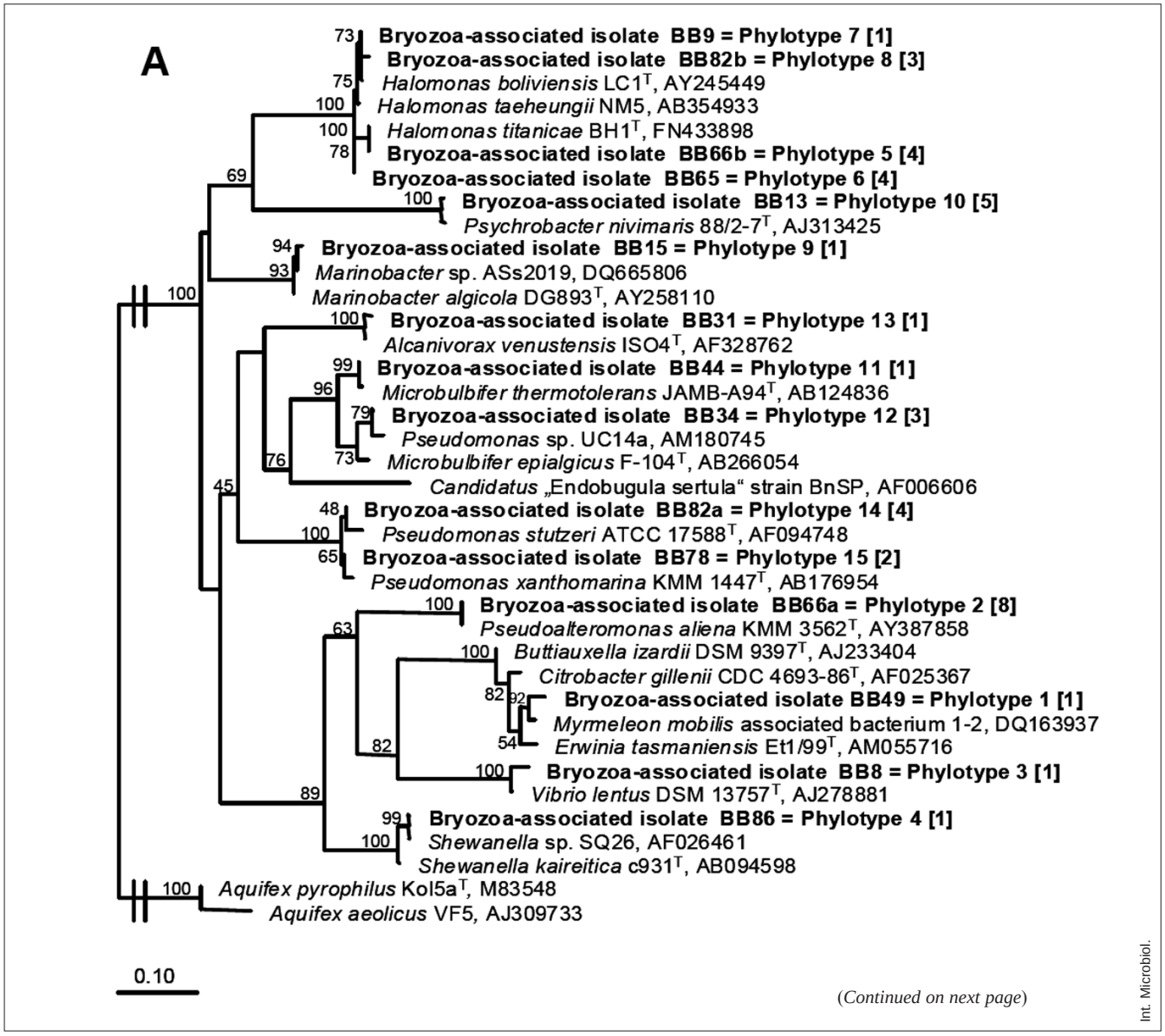

Fig. 3. Maximum-likelihood tree constructed from 16S rRNA gene sequences showing the phylogenetic relationships of isolates from this study with closely related species and some other selected representatives of the Gammaproteobacteria (**A**), the Alphaproteobacteria (**B**) and gram-positive bacteria (**C**). Non-parametric bootstrapping analysis (100 datasets) was conducted. Values ≥ 50 are shown. The scale bar indicates the number of substitutions per nucleotide position. The total number of represented sequences is given in square brackets.

42) and *Arthrobacter* (phylotype 47) might represent new species (Table 2).

Antibiotic activity. Antibiotic activity against at least one indicator strain was shown by 47 out of 93 tested bacteria. The vast majority of the tested isolates inhibited growth of gram-positive test strains (45.2 % *B. subtilis*, 10.8% *S. lentus*, 7.5 % both), while only a minor part (5.4 %) was active against gram-negative indicator bacteria (1.1 % *E. coli*, 4.3% *P. fluorescens*) or against the yeast *C. glabrata* (1.1 %). Three isolates were not analyzed due to insufficient growth on the test media (Table 1).

Activity profiles on different media. The media used for the antibiotic tests had a clear influence on the pattern of antibiotic activities. Antibiotic activities were analyzed on six different media (TSB, MB, VNSS, PSA, R2A, and AC medium). 21 isolates each displayed activity on R2A

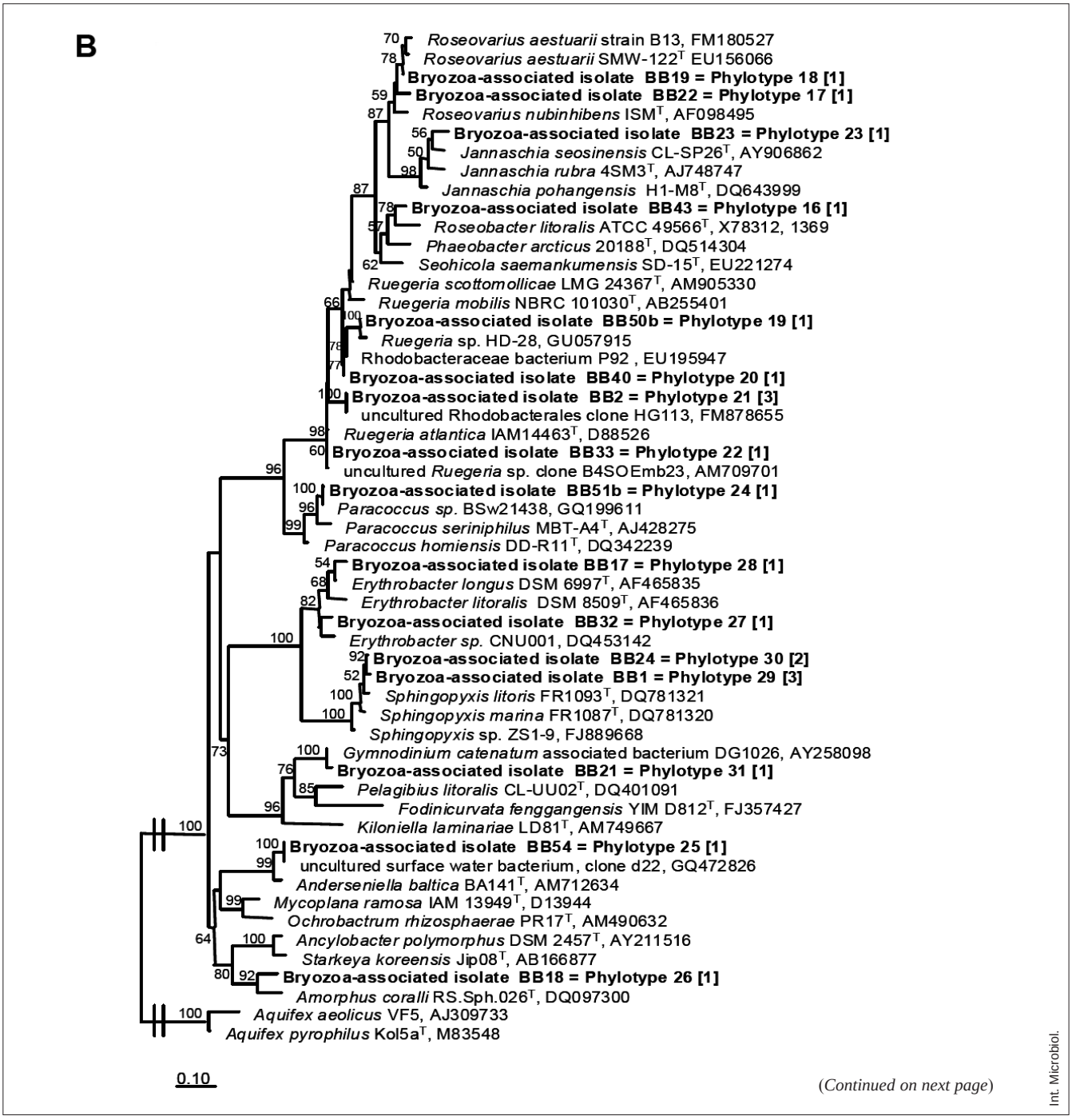

Fig. 3. (*Continued*) Maximum-likelihood tree constructed from 16S rRNA gene sequences showing the phylogenetic relationships of isolates from this study with closely related species and some other selected representatives of the Gammaproteobacteria (**A**), the Alphaproteobacteria (**B**) and gram-positive bacteria (**C**). Non-parametric bootstrapping analysis (100 datasets) was conducted. Values ≥ 50 are shown. The scale bar indicates the number of substitutions per nucleotide position. The total number of represented sequences is given in square brackets.

and MB media, followed by TSB (20 isolates), AC (19 isolates), PSA (18 isolates), and VNSS (15 isolates). The majority (63.8 % of active isolates) inhibited growth of indicator strains on a single medium or on two media. Only six isolates (12.8 % of active isolates) showed antibacterial activities on five or all six media (Table 1). A corresponding cluster analy-

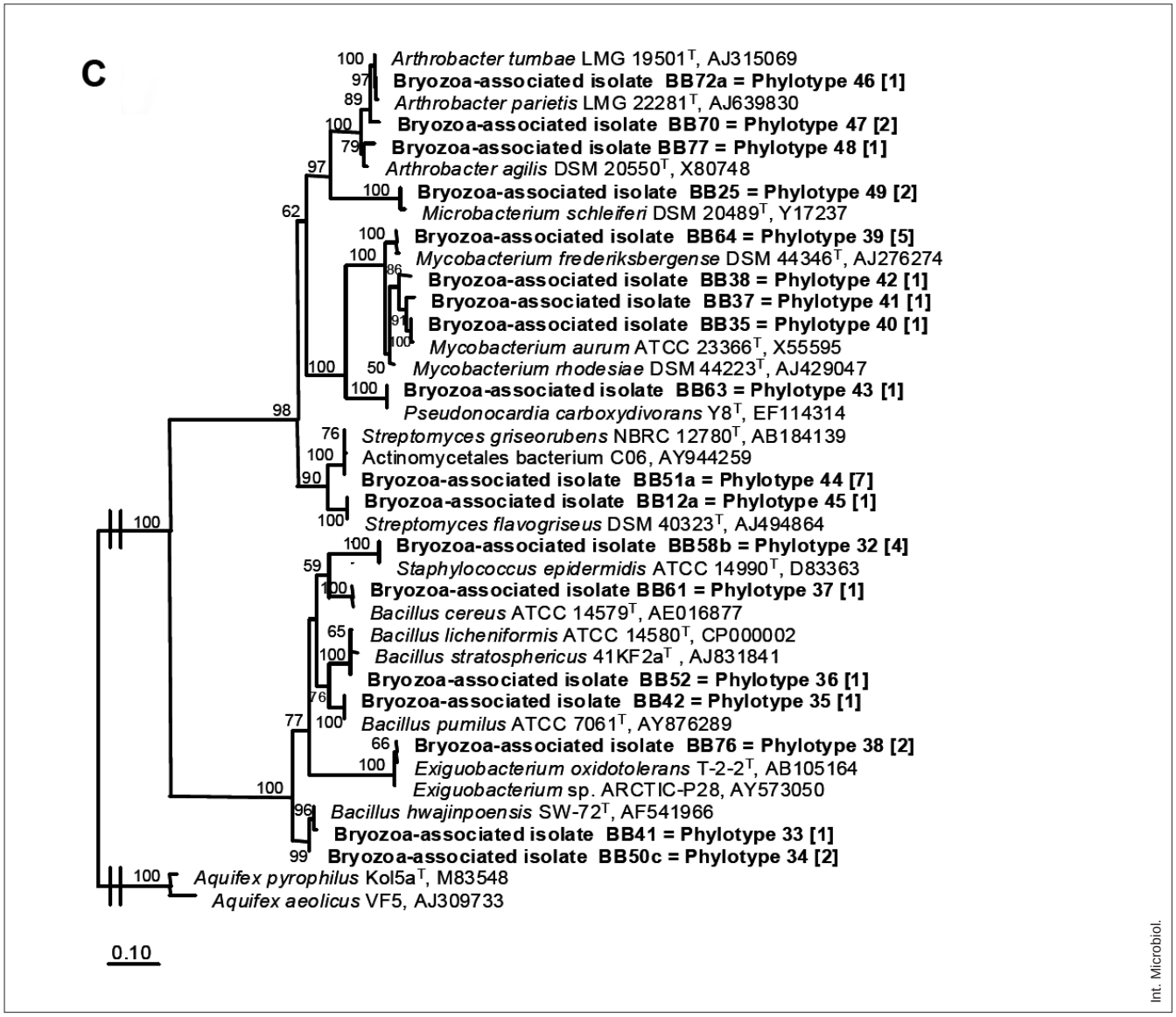

Fig. 3. (*Continued*) Maximum-likelihood tree constructed from 16S rRNA gene sequences showing the phylogenetic relationships of isolates from this study with closely related species and some other selected representatives of the Gammaproteobacteria (**A**), the Alphaproteobacteria (**B**) and gram-positive bacteria (**C**). Non-parametric bootstrapping analysis (100 datasets) was conducted. Values ≥ 50 are shown. The scale bar indicates the number of substitutions per nucleotide position. The total number of represented sequences is given in square brackets.

sis revealed similar activity patterns on R2A and AC as well as on MB and VNSS media, whereas patterns on TSB and PSA media were clearly different from those on the other media (Fig. 1C).

Activity profiles according to phylogenetic affiliation. While the Gammaproteobacteria were mainly active on TSB and MB media, most of the Alphaproteobacteria were active on R2A and AC media, and most of the Bacilli and Actinobacteria were active on PSA and TSB plates. The number of isolates active on the different media and their affiliation with the four bacterial classes are shown in Fig. 4.

Gammaproteobacteria. Almost 50 % of the Gammaproteobacteria (19 of 39 tested strains) displayed antimicrobial properties. Antibiosis was mostly directed against *B. subtilis* (78.9 % of active isolates), followed by *S. lentus* (15.8 %) and *P. fluorescens* (10.5 %). Antibiotically active isolates were affiliated with *Erwinia* (phylotype 1), *Shewanella* (phylotype 4),

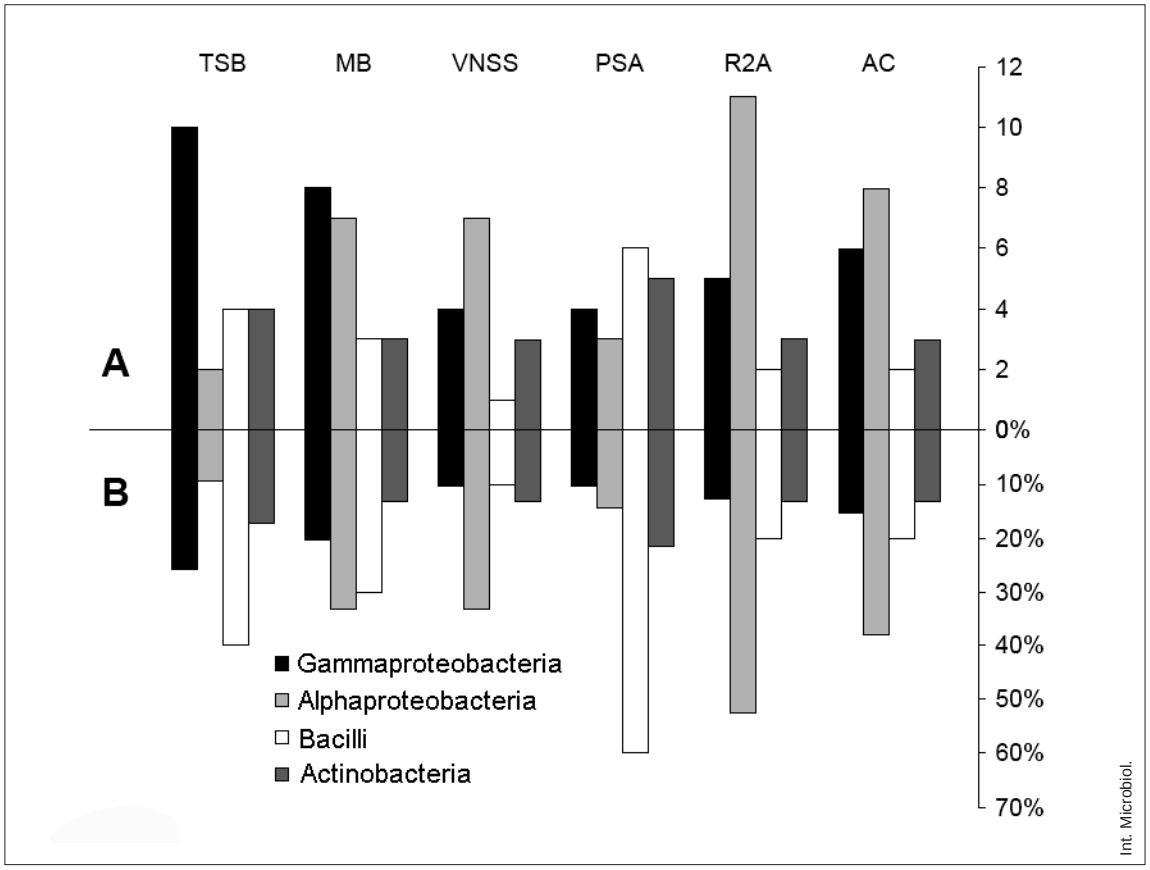

Fig. 4. Number of bioactive isolates on different growth media, (**A**) absolute counts, (**B**) percentage of active isolates within each bacterial class.

Marinobacter (phylotype 9), *Pseudoalteromonas* (phylotype 2; 7 out of 8 isolates), *Halomonas* (phylotype 5; 3 of 4), *Psychrobacter* (phylotype 10; 1 of 5), *Microbulbifer* (phylotypes 11 and 12; all isolates), and *Pseudomonas* (phylotype 14; 1 of 4). All *Microbulbifer* affiliated isolates were active on R2A medium, while most *Pseudoalteromonas* isolates displayed activity on MB agar plates. Whereas activity against *B. subtilis* was shown by almost all active strains, bacteria of phylotype 5 (assigned to *Halomonas*) were active against *S. lentus* and *P. fluorescens* instead. Only single isolates, *Microbulbifer* sp. BB44 (phylotype 11) on AC medium and *Pseudoalteromonas* sp BB67 (phylotype 2) on TSB medium, were active against *E. coli* or *C. glabrata*, respectively. No activity was exhibited by phylotypes 3 (*Vibrio*), 6, 7, 8 (all *Halomonas*), and 15 (*Pseudomonas*).

Alphaproteobacteria. Among the Alphaproteobacteria, > 75 % (16 out of 21 strains) were antimicrobially active. Activity was directed exclusively against *B. subtilis*. Active isolates were members of *Roseobacter* (phylotype 16), *Roseovarius* (phylotypes 17 and 18), *Ruegeria* (phylotypes 19 to 22), *Paracoccus* (phylotype 25), *Erythrobacter* (phylotype 28; 1 of 2 isolates), *Sphingopyxis* (phylotype 29; 1 of 3 isolates; and phylotype 30), and *Pelagibius* (phylotype 31). Most of the active strains (68.8 %) showed antimicrobial activities on R2A medium, followed by AC (50.0 %) medium. Especially isolates of the *Roseobacter*-clade, which was represented by phylotypes 16 to 23, displayed activity (90 % of these isolates) preferentially on R2A medium (88.9 % of the active isolates). The *Jannaschia*-related strain BB23 (phylotype 23) was the only non-active member of the *Roseobacter*-clade. In addition, strains of phylotypes 26 (*Amorphus*) and 27 (*Erythrobacter*) displayed no activity, nor did two strains of phylotype 29 (*Sphingopyxis*).

Bacilli. Antibiotic activity of isolates related to Bacilli was directed against *B. subtilis* (7 strains) or both *B. subtilis* and *S. lentus* (3 strains, all *Bacillus*, phylotypes 34, 35, and 36). One of these isolates (BB42, phylotype 35) additionally showed activity against *P. fluorescens* and also expressed antimicrobial activities on all media. All *Exiguobacterium* affiliated strains (phylotype 38) were active against *B. sub-*

tilis. One representative each of phylotypes 32 (*Staphylococcus*) and 34 (*Bacillus*) as well as phylotype 33 (*Bacillus*) did not impede the growth of any indicator strain.

Actinobacteria. Only five out of 23 Actinobacteria related isolates inhibited the growth of indicator strains. Four strains each were active against *B. subtilis* and *S. lentus*. Three of them (phylotypes 44 and 45, both *Streptomyces*) were active against both gram-positive test strains on all six media. Isolate BB84 (phylotype 44) was additionally active against *P. fluorescens*. The *Mycobacterium*-related strain of phylotype 41 showed anti-*S. lentus* activity. No antibiotic active representatives were found in phylotypes 39, 40, and 42 (all *Mycobacterium*), 43 (*Pseudonocardia*), 46 to 48 (all *Arthrobacter*), and 49 (*Microbacterium*).

Discussion

Phylogenetic affiliation of isolates. A phylogenetically diverse collection of isolates was obtained during this study from three specimens of *Membranipora membranacea* using different isolation media. Each medium featured a rather unique set of isolated phylotypes. This resulted in a highly diverse array of 96 isolates assigned to 49 phylotypes and 29 genera. Only one-third of the members of these genera had been isolated previously from comparable sources. Three of these genera (*Shewanella*, *Pseudoalteromonas*, and *Pseudomonas*) were isolated in all three comparable studies on bryozoans from the North Sea and the Baltic Sea, as well as from Baltic Sea *S. latissima* samples [16,36,47]. Other genera (*Bacillus*, *Arthrobacter*, *Vibrio*, *Psychrobacter*, *Ruegeria*, *Staphylococcus*, *Streptomyces*) were also found, but not consistently, in all three studies.

The use of four different isolation media was undoubtedly an important factor in our success in obtaining such a diverse collection of bacteria. The high number of phylotypes that were exclusively found on one medium (75.5 %) and the resulting large differences in the phylotypes obtained from the different media (Fig. 1B) resulted in a "unique" set of isolates obtained from each medium. This observation correlates with the fact that different bacteria differ in their needs on nutrients, growth factors, salt composition, trace elements, etc., which cannot be covered by a single medium. Nonetheless, clear media preferences could not be narrowed down to a specific group of phylotypes or genera, as all media yielded phylogenetically diverse isolates. However, a certain bias of ACd medium towards the isolation of Gammaproteobacteria and Actinobacteria, as well as of R2Ad medium towards Alphaproteobacteria and Bacilli was noted.

Note that fewer isolates were obtained from media that contained algal or bryozoan extract (BM and AM media), approximately one third of the isolates that grew on the other media (R2Ad and ACd). This may be related to the compounds that originate from bryozoans or algae, which might be involved in the chemical defense mechanisms of these sessile organisms [13,34] and, as such, inhibit bacterial growth. In particular, marine algae are known as producers of a variety of active metabolites that prevent biofouling of their own surfaces [1]. Some of these natural products may be stable enough to express growth inhibiting properties even after autoclaving or long-term storage. Unsaturated fatty acids have been identified as antibacterial active agents from brown algae with activities especially directed against gram-positive bacteria, and they maintain their antibiotic properties even if stored at room temperature for several years [40]. This fact correlates well with our finding that the fewest isolates were obtained from "algal extract medium" (AM) and that no gram-positive bacteria were obtained from this medium. Thus, detrimental effects of inhibitory compounds in this medium might have outbalanced the usually beneficial impact of habitat water demonstrated in previous studies [12,29].

As attempts to isolate bacteria from bryozoans are still very scarce compared to other marine sources, bryozoans provide a good source for the search for new bacteria and new antibiotic compounds. Validly described type strains that were originally isolated from bryozoans include *Tenacibaculum adriaticum* (Flavobacteria), *Marinobacter bryozoorum* (Gammaproteobacteria), and *Paracoccus seriniphilus* (Alphaproteobacteria) [17,37,39]. Single isolates of the latter two genera were also obtained in the present study. Quite significant was the finding that 15 out of 49 phylotypes of this study represented new species (some even new genera), applying the phylogenetic relationship according to Stackebrandt and Ebers [44]. Most significant was the high number of Alphaproteobacteria with 16S rRNA gene sequence similarities of or below 97 % to known species. Moreover, half of the phylotypes (16 to 23) of Alphaproteobacteria isolated in this study were affiliated with members of the *Roseobacter* lineage, which represents typical marine bacteria [6] and is abundant in bacterial communities associated, e.g., with algal blooms, biofilms, and cephalopods.

Note that the three bryozoan specimens yielded a similar amount of exclusive phylotypes: 38 phylotypes (77.6 %) originated from single samples exclusively, which resulted

in clear differences between the samples (Fig. 1A). Similar to the influence of the isolation media, this resulted in "unique" collections of bacteria obtained from each specimen. A previous cultivation-based study on the microbial diversity with samples of the North Sea bryozoan *Flustra foliacea* yielded similar results: although a great array of different isolation media was used and the same procedures were applied to all specimens, such that the distribution of bacterial taxons was highly divergent. Indeed, not a single genus could be found on all three samples [36]. Another culture-independent study on bacterial communities of bryozoans in the North Sea demonstrated species-specific associations for three of the four bryozoan species (*Aspidelectra melolontha*, *Electra monostachys*, and *E. pilosa*). In contrast, a site-dependent influence was observed in *Conopeum reticulum* specimens [20].

Antimicrobial activity. A large proportion, almost 50%, of the bacteria isolated from *Membranipora membranacea* revealed antibiotic activity, predominately against gram-positive test strains. This result is similar to those obtained in our previous study on the antibiotic activities of bryozoan-associated bacteria with the same indicator organisms [16]. However, isolates from the present work, with a few exceptions (*Vibrio, Shewanella, Pseudoalteromonas*, *Pseudomonas*), were affiliated with different genera and included also gram-positive representatives. Activity against the gram-positive bacteria *Bacillus subtilis* and *Staphylococcus lentus* could be advantageous on surfaces in situ, as members of both genera were also isolated from the bryozoan specimens.

The pattern of antibiotic activities was quite variable and strain-specific, phylotype-specific as well as genus-specific activity patterns were observed. All isolates of the genera *Microbulbifer*, *Roseovarius*, *Ruegeria*, and *Exiguobacterium* showed consistent genus-specific activity profiles. Moreover, this antibiosis was expressed on the same media, each with single exceptions of *Ruegeria*-affiliated strains (Table 1). In contrast, only some of the isolates related to *Pseudoalteromonas*, *Psychrobacter*, *Pseudomonas*, *Sphingopyxis*, *Bacillus*, *Staphylococcus*, or *Streptomyces* inhibited target organisms in a strain-specific pattern.

Note that strain-specific activities will more likely be detected if larger subsets of isolates of the considered group are included, such as those related to *Pseudoalteromonas* and *Streptomyces* in this study [25,26]. In addition, growth conditions and media are important factors for the production of bioactive compounds and should be considered in all studies on antibiosis and antibiotic activity of microorganisms. In this study, the influence of test media on antibiotic traits reflected this dependency of the bacterial isolates on a "suitable" environment. Most activities were expressed on one or two media only (63.8 %), whereas a minor fraction of the isolates produced growth inhibitory compounds on all or five media (12.8 %).

The activation of secondary metabolite pathways, which remain silent under standard laboratory conditions, is a feasible way to access new natural products in microorganisms [4,33]. Five isolates related to *Sphingopyxis* expressed activities against *B. subtilis* in different media or did not show activity on any of those used (Table 1). Altogether, the use of six different media resulted in a twofold increase in the discovery of antibiotic active bacteria compared to the results obtained with a single medium.

Microorganisms belong to the prominent producers of natural products in the marine environment. Among the best studied genera in terms of published metabolites are *Streptomyces, Alteromonas, Bacillus, Vibrio, Pseudomonas, Actinomyces,* and *Pseudoalteromonas*, all of which were also isolated from *M. membranacea* in this work. Other genera found in this study, such as *Microbacterium, Marinobacter, Halomonas, Ruegeria*, and *Erythrobacter*, have also contributed to published marine natural products but to a lesser extent [23]. However, only some of these compounds have been reported as antimicrobially active. For example, in the case of *Pseudoalteromonas* and *Pseudomonas* some secondary metabolites display antibiotic properties [3,18]. Sponge- and ascidian-associated *Microbulbifer* strains produce variations of parabens [32,38]. As far as Alphaproteobacteria are concerned, only a few members of this class are known to produce antimicrobial metabolites. Among them are representatives of the *Roseobacter* clade, producing thiotropocin and its precursor tropodithietic acid [5,7,8]. Finally, well-documented producers of antimicrobially active compounds, such as *Streptomyces* strains, can be a source of novel compounds, although only few of the Actinobacteria isolated in this study were antibiotically active.

Recently, the production of the antibacterial compound mayamycin by a marine *Streptomyces* related strain reported to be induced by variation of culture conditions [41], which further supports the requirement for varying the culture conditions to find new antibiotic compounds. Novel species or known taxa of this work with as yet unknown antimicrobial properties, such as members of the genera *Roseobacter*,

Roseovarius, *Ruegeria*, *Paracoccus*, *Anderseniella*, *Erythrobacter*, *Sphingopyxis* and *Pelagibius*, are candidates to be studied more intensively with regard to the production of new antimicrobial compounds.

Acknowledgements. This study was supported by the Ministry of Science, Economic Affairs and Transport of the State of Schleswig-Holstein (Germany) within the framework of the "Future Program of Economy," which is co-financed by EFRE.

Competing interests. None declared.

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