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ORIGINAL ARTICLE

Diversity of Antibiotic-Active Bacteria Associated with the Brown Alga *Laminaria saccharina* from the Baltic Sea

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Abstract Bacteria associated with the marine macroalga *Laminaria saccharina*, collected from the Kiel Fjord (Baltic Sea, Germany), were isolated and tested for antimicrobial activity. From a total of 210 isolates, 103 strains inhibited the growth of at least one microorganism from the test panel including Gram-negative and Gram-positive bacteria as well as a yeast. Most common profiles were the inhibition of *Bacillus subtilis* only (30%), *B. subtilis* and *Staphylococcus lentus* (25%), and *B. subtilis*, *S. lentus*, and *Candida albicans* (11%). In summary, the antibiotic-active isolates covered 15 different activity patterns suggesting various modes of action. On the basis of 16S rRNA gene sequence similarities >99%, 45 phylotypes were defined, which were classified into 21 genera belonging to *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. Phylogenetic analysis of 16S rRNA gene sequences revealed that four isolates possibly represent novel species or even genera. In conclusion, *L. saccharina* represents a promising source for the isolation of new bacterial taxa and antimicrobially active bacteria.

Keywords *Laminaria saccharina* · Alga-associated bacteria · Isolation · Phylogeny · Novel taxa · Antimicrobial activity

Introduction

Laminaria species provide a rich habitat for different epiphytic, endophytic, and epizoobenthic organisms (Bartsch et al. 2008). Epiphytic bacteria have been studied by microscopic methods (Corre and Prieur 1990) and by genetic and cultivation approaches. Bacterial cell numbers of up to 10^7 colony-forming units (CFU) per centimeter squared were reported for *Laminaria digitata* from the coast of Brittany (France) and *Laminaria pallida* on the Bengal upwelling region of southern Africa (Corre and Prieur 1990; Mazure and Field 1980). However, the interactions between members of the epiphytic and endophytic communities and the relationships between these communities and *Laminaria* spp. as well as the type of association (specific or unspecific) are only poorly understood. It is assumed that the bacterial communities in part are specifically associated but also include opportunistic commensal as well as algae-degrading microorganisms (Staufenberger et al. 2008) and in consequence *Laminaria*-associated bacteria might affect the alga positively or negatively in different ways. Some bacteria affect the alga in a deleterious manner by decomposing cell material, like alginate, laminaran, or mannitol (Dimitrieva and Dimitriev 1996; Ivanova et al. 2003; Laycock 1974; Sawabe et al. 1997, 1998b, 2000) and/or by causing diseases such as those triggered by species of *Alteromonas* and *Pseudoalteromonas* species and others (Wang et al. 2006; Sawabe et al. 1998a; Vairappan et al. 2001). A favorable, growth-promoting effect of bacteria on *Laminaria* was shown for *Pseudoalteromonas porphyrae* isolated from *Laminaria japonica* in the Sea of Japan (Dimitrieva et al. 2006). This bacterium induced improved spore germination of the alga and extended the thallus length. An additional beneficial effect of *Laminaria*-associated bacteria could be the protection of the alga against microbial pathogens by the production of antimicrobial substances.

Various novel compounds with antibiotic activity have been identified from alga-associated bacteria. These chemically diverse substances include new lipopeptides such as

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massetolide A, novel antibacterial lactones (macrolactines G–M), phenazines (i.e., pelagiomycin A), and korormicin, which exhibit a variety of activities against bacteria and fungi pathogenic to man and plants as well as leukemic cells (Gerard et al. 1997; Imamura et al. 1997; Yoshikawa et al. 1997; Tran et al. 2007).

No detailed study concerning the characterization of alga-associated bacteria exploring antibiotic effects is available to date. In this study, we focussed on the isolation, identification, and phylogenetic analysis of antimicrobially active bacteria associated with the marine macroalga *Laminaria saccharina*.

Materials and Methods

Sampling Site and Sampling Procedures Samples of *L. saccharina* were taken from the Kiel Fjord (Baltic Sea, Germany). The algae were collected from October 2002 to June 2004 at approximately 6 m depth by scuba diving. Complete algae were removed carefully from the substrate using a knife and transferred into sterile plastic bags. All samples were kept in the dark and at 4°C until subsequent processing in the laboratory within 4 h of sampling.

Isolation, Cultivation, and Storage of Bacterial Strains Fresh *L. saccharina* samples were cut into pieces of approximately 10 cm^2, suspended in sterile sea water, and homogenized using an Ultraturrax T25 (IKA Werke, Germany). The suspension was diluted in sterile sea water and plated on tryptic soy broth (TSB) medium A (3.0 g/l Difco tryptic soy broth, 15 g/l Difco agar, 7, 10, 15, or 25 g/l NaCl, respectively), TSB medium B (0.3 g/l Difco tryptic soy broth, 15 g/l Difco agar, 7, 15, or 25 g/l NaCl, respectively), or MW medium (15 g/l Difco agar in sea water), respectively. Further media used were AIA-S15 (22 g/l Difco *Actinomyces* isolation agar, 15 g/l NaCl), CPS-S15 modified after Collins and Willoughby (1962; 0.5 g/l Bacto peptone, 0.5 g/l casitone, 0.5 g/l starch, 1.0 ml/L glycerine, 2.0 ml/L 10% K_2HPO_4 solution, 0.5 ml/L 10% $MgSO_4 \times 7\ H_2O$ solution, four drops 0.01% $FeCl_3$ solution, 15.0 g/l agar, 15 g/l NaCl; pH 7.0), MHA-S15 (10.0 g/l malt extract, 5.0 g/l Bacto yeast extract, 15 g/l NaCl, 15.0 g/l agar; pH 6.5), MA (18.0 g/l Bacto marine broth, 15.0 g/l agar; pH 7.6), WM-S5 modified after Wickerham (1951; 10.0 g/l glucose$\times H_2O$, 5.0 g/l Bacto peptone, 3.0 g/l Bacto yeast extract, 3.0 g/l Bacto malt extract, 5.0 g/l NaCl, pH 6.3), and GPY (1.0 g/l glucose$\times H_2O$, 0.5 g/l Bacto peptone, 0.1 g/l Bacto yeast extract, 15.0 g/l NaCl, 15 g/l agar; pH 7.2). In addition, a semisynthetic polycarbon (HSPC) medium was used (Muscholl-Silberhorn et al. 2008). The incubation was performed in the dark at 22°C for 14 days. Pure cultures were obtained by several subsequent isolation steps on TSB medium A (with 10 g/l NaCl). The isolates were stored at −80°C using the Cryobank System (Mast Diagnostica GmbH, Reinfeld, Germany) according to the manufacturer.

Determination of the Antimicrobial Activity of the Isolates The following test organisms were used: *Escherichia coli* DSM 498 as a Gram-negative strain, *Staphylococcus lentus* DSM 6672 and *Bacillus subtilis* DSM 347 as representatives of Gram-positive bacteria, and the yeast *Candida glabrata* DSM 6425, all obtained from the German Culture Collection (DSMZ, Braunschweig, Germany).

The activity of *L. saccharina*-associated isolates against the test strains was tested by using an overlay method. The isolates were inoculated onto TSB medium A agar plates (with 10 g/l NaCl) by streaking out cell material on a circular area with a diameter of 1 cm. The cultures were incubated at 22°C for 5 days before they were covered with an overlay containing the test strains in TSB agar C (3.0 g/l Difco tryptic soy broth, 8 g/l Difco agar, 10 g/l NaCl, pH 7.2). Overnight cultures of each test strain with approximately 10^9 cells per milliliter were mixed (all bacteria 1% *v*/*v*, *C. glabrata* 10% *v*/*v*) with TSB agar C, which was then poured onto the agar surface previously inoculated with alga-associated isolates. The plates were incubated at 22°C for 5 days. Antibacterial activity was defined by the formation of inhibition zones determined as a distance of ≥1 mm between the circular area (=lawn of the isolate) and the end of the clear zone bounded by the lawn of the test strain.

DNA Extraction To obtain genomic DNA, cell material was transferred from the agar plate into 500-µl DNA-free water (Sigma-Aldrich) and homogenized (2×6,300 rpm/min for 20 s) using the Precellys24 homogenizer (PEQLAB Biotechnologie GmbH, Erlangen, Germany). After centrifugation for 10 min at 8,000×*g*, the supernatant was collected and the DNA extract was stored at −20°C.

16S rRNA Gene Amplification The amplification of the 16S rRNA gene sequence was performed using puReTaq Ready-To-Go polymerase chain reaction (PCR) Beads (Amersham Biosciences) with the eubacterial primers 27f and 1492r (Lane 1991). The PCR profile included the following steps: 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 (40 s at 94°C) as well as a final primer annealing (40 s at 50°C) and extension step (5 min at 72°C).

Purification of PCR products was carried out with Exonuclease I (Exo I, GE Healthcare) and Shrimp Alkaline Phosphatase (SAP, Roche). For each reaction, 1.5 U of Exo I and 0.3 U of SAP were added to the PCR product and

incubated for 15 min at 37°C, followed by heat inactivation of the enzymes for 15 min at 72°C. Sequencing was performed using the BigDye Terminator v1.1 Sequencing Kit (Applied Biosystems) in a 3730-DNA-Analyzer (Applied Biosystems) as specified by the manufacturer. Sequencing was performed with the primers 342f (Lane 1991), 790f 5'-GATACCCTGGTAGTCC-3', and 543r (Muyzer et al. 1993). The 16S rRNA gene sequences were submitted to the European Molecular Biology Laboratory (EMBL) database with the accession nos. AM913880–AM913982.

Phylogenetic Analysis Next relatives of the bacterial isolates were determined by comparison to 16S rRNA gene sequences in the NCBI GenBank and the EMBL databases using Basic Local Alignment Search Tool (BLAST) and the "Seqmatch" program of the Ribosomal Database Project II (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) restricted to type strains. Sequences were aligned using the FastAlign function of the alignment editor implemented in the ARB software package (http://www.arb-home.de; Ludwig et al. 2004) and refined manually employing secondary structure information. For phylogenetic calculations, the PhyML software (Guindon and Gascuel 2003) as well as the online version of PhyML (Guindon et al. 2005) were used. Trees were calculated by the maximum likelihood method (Felsenstein 1981) using the general time reversal model with the estimated proportion of invariable sites and the Gamma distribution parameter. Isolates with 16S rRNA gene sequences sharing ≥99% sequence similarity were grouped into arbitrary taxonomic units (ATUs; Fig. 1). For phylogenetic analysis, only one representative sequence of each ATU was used. Sequence similarity values were determined using the "BLAST 2 SEQUENCES" tool of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi; Tatusova and Madden 1999). Isolates with sequence similarities <97.2% to the next validly described type strain are assumed to be representatives of potentially novel species.

Results

Phylogenetic Analysis of Antibiotic-Producing Isolates In total, 210 isolates were obtained and tested for antibiotic activity. For all 103 biologically active bacteria isolated, 16S rDNA sequences were obtained. Phylogenetic analysis according to the sequence data demonstrated that the bacteria isolated from *L. saccharina* were affiliated to six major groups of the bacterial domain, the Gram-positive *Actinobacteria* (high G + C) and *Firmicutes* (low G + C), the Gram-negative *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*, and the *Bacteroidetes*. The isolates were assigned to 45 ATUs of >99% sequence similarity belonging to 21 different genera. For the calculation of phylogenetic trees, only one representative of each ATU was used (Fig. 1, Table 1).

Representatives of the *Proteobacteria* were most abundant (45 isolates), the majority of which were affiliated with the γ-subgroup (40 isolates). Two ATUs belong to the genus *Pseudoalteromonas*, one of which was related to *Pseudoalteromonas tunicata* (ATU PA2 with three isolates and L28 as representative). This isolate L28 shared 97.5% sequence similarity to the most closely related type strain, *P. tunicata* D2^T (GenBank EMBL DNA Databank of Japan (DDBJ) accession no. Z25522). Isolate LD86 is proposed to represent a novel species of the family *Alteromonadaceae* because the 16S rRNA gene sequence similarity to the most closely related validly described type strains, *Glaciecola mesophila* KMM 241^T and *Glaciecola polaris* LMG 21857^T (GenBank EMBL DDBJ accession nos. AJ488501 and AJ293820), was 96.3%.

Among four isolates affiliated to the *Alphaproteobacteria*, two may represent new species. The comparison of the 16S rRNA gene sequence of strain L96 with validly described type strains revealed an identity of 96.8% to *Mesorhizobium chacoense* PR5^T (GenBank EMBL DDBJ accession no. AJ278249), which suggests that it may represent a new *Mesorhizobium* species. The affiliation of strain LD81 to a novel genus or even family of the *Alphaproteobacteria* related to *Rhodospirillales* is supported by a low 16S rRNA gene sequence similarity values to various validly described type strain of *Alphaproteobacteria* (90.7% to the most closely related type, which is *Pseudovibrio denitrificans* DN34^T, GenBank EMBL DDBJ accession no. AY486423). The highest sequence similarity (96%) was found, however, to *Kopriimonas byunsanensis*, a proposed new species so far not validly described.

Betaproteobacteria are rarely found in association with *Laminaria*. Just a single isolate (LD114) was obtained with *Alcaligenes faecalis* as the next relative.

Two representatives of the Bacteroidetes were isolated. One (LD83) was identified as *Olleya marilimosa*; the second (LD84) was related to the genus *Cellulophaga* and shared 97.1% sequence similarity with the closest type strain, *Cellulophaga baltica* NN015840^T (GenBank EMBL DDBJ accession no. AJ005972) and possibly represents a new species.

Among the Actinobacteria, most of the isolates were identified as *Streptomyces* species (Table 1) and all of the *Firmicutes* belong to the genus *Bacillus*, of which (one L91) shares 97.5% sequence similarity with its closest described

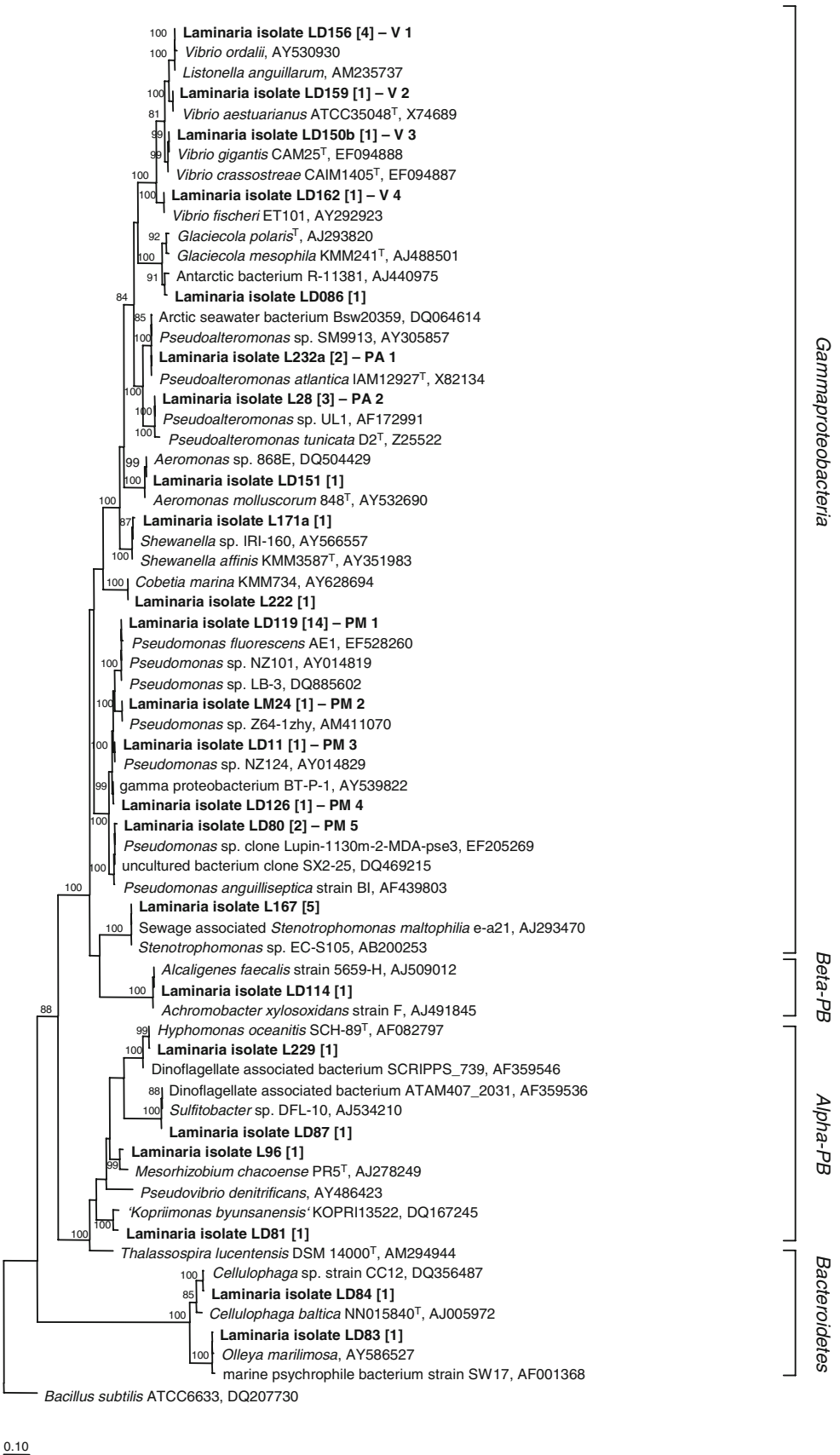

Fig. 1 Phylogenetic trees of *Laminaria saccharina*-associated antimicrobially active bacterial strains calculated with the maximum likelihood method. The trees include the closest relative determined by BLAST search and next type strain relatives to the isolates as well as representatives of closely related marine-derived strains or 16S rDNA clone sequences. Bootstrap values are given in percent (only numbers above 50 are shown). *Numbers* in *square brackets* give the number of represented sequences

Fig. 1 (continued)

relative, *Bacillus patagoniensis* PAT 05^T (GenBank–EMBL–DDBJ accession no. AY258614).

Antimicrobial Profiles of the Isolates From a total of 210 bacterial isolates, 103 displayed antimicrobial activity against at least one of the test strains used in this study (Tables 2 and 3). The majority of the *L. saccharina*-associated isolates were active against the Gram-positive *B. subtilis* and *S. lentus*. Of the isolates, 83.5% showed an inhibitory effect against *B. subtilis* and 47.7% against *S.*

Table 1 Taxonomic identification and phylogenetic position of the isolate

Genus	ATU	Number of isolates	Representatives	Next related strain in tree (similarity)	Bacterial group
Pseudomonas	PM1	14	LD119	*Pseudomonas* sp. LB-3, DQ885602.1 (99.86%)	Proteobacteria
	PM2	1	LM24	*Pseudomonas* sp. Z64-1zhy, AM411070 (100%)	
	PM3	1	LD11	*Pseudomonas* sp. NZ124, AY014829 (100%)	
	PM4	1	LD126	Gamma proteobacterium BT-P-1, AY539822 (100%)	
	PM5	2	LD80	*Pseudomonas* sp. clone Lupin-1130m-2-MDA-pse3, EF205269 (99.4%)/ *Pseudomonas anguilliseptica* strain BI, AF439803 (99.4%)	
Pseudoalteromonas	PA1	2	L232a	Arctic seawater bacterium Bsw20359, DQ064614 (99.86%)/ *Pseudoalteromonas atlantica* IAM12927T, X82134 (99.72%)	
	PA2	3	L28	*Pseudoalteromonas* sp. UL1, AF172991 (99.92%)	
Stenotrophomonas	ST	5	L167	*Stenotrophomonas* sp. EC-S105, AB200253 (99.86%)	
Vibrio	V1	4	LD156	*Vibrio anguillarum*, AM235737 (99.86%)	
	V2	1	LD159	*Vibrio aestuarianus* ATCC35048, X74689 (99.57%)	
	V3	1	LD150b	*Vibrio gigantis* CAM25, EF094888 (99.78%)	
	V4	1	LD162	*Vibrio fischeri* ET101, AY292923 (99.86%)	
Aeromonas	AE	1	LD151	*Aeromonas molluscorum* 848, AY532690 (99.44%)	
Shewanella	SH	1	L171a	*Shewanella* sp. IRI-160, AY566557 (99.15%)	
Cobetia	CO	1	L222	*Cobetia marina* KMM734, AY628694 (100%)	
Glaciecola	GL	1	LD86	Antarctic bacterium R-11381, AJ440975 (97.77%)/ *Glaciecola mesophila* KMM241, AJ488501 (96.57%)	
Alcaligenes	AL	1	LD114	*Alcaligenes faecalis* strain 5659-H, AJ509012 (99.93%)	
Sulfitobacter	SU	1	LD87	*Sulfitobacter* sp. DFL-10, AJ534210 (99.92%)	
Hyphomonas	HY	1	L229	*Hyphomonas oceanitis* SCH-89, AF082797 (99.84%)	
Mesorhizobium	ME	1	L96	*Mesorhizobium chacoense* PR5, AJ278249 (96.76%)	
Proposed new genus *Kiloniella*	KI	1	LD81	*Kopriimonas byunsanensis* KOPRI13522, DQ167245 (96.57%)	
Cellulophaga	CE	1	LD84	*Cellulophaga* sp. strain CC12, DQ356487 (99.33%)	Bacteroidetes
Olleya	OL	1	LD83	*Olleya marilimosa*, AY586527 (99.12%)	
Streptomyces	S1	5	L105	*Streptomyces flavofungini*, EF571003 (100%)	Actinobacteria
	S2	2	L94	Marine *Streptomyces* sp. GY-2006, AM421779 (100%)/ *Streptomyces fungicidicus* YH04, AY636155 (100%)	
	S3	4	LD101	Sponge-associated *Actinomycetales* bacterium HPA72, DQ144231 (99.92%)/*Streptomyces gougerotii* NBRC 13043, AB249982 (99.92%)	
	S4	1	L92	Sponge-associated *Actinomycetales* bacterium C06, AY944259 (99.93%)/ *Streptomyces variabilis* NRRL B-3984^T, DQ442551 (99.59%)	
	S5	1	L98	'*Streptomyces maritimus*' BD26, AF233338 (99.04%)/ *Streptomyces aurantiogriseus* NRRL B-5416, AY999773 (99.04%)	
	S6	15	L93	*Streptomyces* sp. YIM8, AF389344 (99.92%)/ *Streptomyces griseus* 52-1, EF571001 (99.85%)	
	S7	1	L87	*Streptomyces globosus* 12620-1, EF371433 (99.73%)	
	S8	1	L155	*Streptomyces* sp. KN-1220, AY029699 (99.71%)/ *Streptomyces tauricus*, AB045879 (98.74%)	
	S9	1	L142	*Streptomyces* sp. 98-62, DQ450946 (99.56%)/ *Streptomyces flavogriseus* DSM 40323, AJ494864 (99.12%)	
	S10	1	L103	*Streptomyces* sp. 3490, EF063500 (99.79%)/ *Streptomyces albogriseolus* NRRL B-1305, AJ494865 (98.71%)	
Leifsonia	LE	1	L228	Marine bacterium P_wp0234, AY188942 (100%)/ *Leifsonia rubeus* CMS 76r, AJ438585 (97.59%)	
Amycolatopsis	AM	1	L140	*Amycolatopsis palatopharyngis* 1Bdz, AF479268 (100%)	
Arthrobacter	AR	1	L134	*Arthrobacter parietis*, AJ639830 (99.85%)	

Table 1 (continued)

Genus	ATU	Number of isolates	Representatives	Next related strain in tree (similarity)	Bacterial group
Micrococcus	MI	1	LD4	*Micrococcaceae* bacterium KVD-unk-39, DQ490457 (99.85%)/ *Micrococcus luteus* DSM 20030, AJ536198 (99.70%)	
Bacillus	B1	4	LD153	*Bacillus subtilis* ATCC 6633, DQ207730 (100%)	Firmicutes
	B2	6	LD125	*Bacillus pumilus* CICCHLJ Q74, EF528287 (99.93%)	
	B3	4	L157	*Bacillus licheniformis* BCRC 12826, EF423608 (99.93%)	
	B4	1	L244	*Bacillus* sp. BM-11_1, AY635875 (99.52%)/*Bacillus humi* LMG22167, AJ627210 (98.71%)	
	B5	1	L135	Marine *Bacillus* sp. JL1082, DQ985062 (99.59%)/*Bacillus aquaemaris* TF-12 (), AF483625 (99.51%)	
	B6	2	LD160	*Bacillus cereus* strain Delaporte, AF155958 (99.92%)/*Bacillus thuringiensis* 4Q281, AF155954 (100%)	
	B7	1	L89	*Bacillus odysseyi* 34hs, AF526913 (98.30%)	
	B8	1	L91	*Bacillus* sp. 17-1, AB043843 (97.54%)/*Bacillus patagoniensis* PAT05, AY258614 (97.45%)	

lentus, respectively; 30.1% of the isolates were effective against *B. subtilis* only; 4.8% were effective against *S. lentus* only and 25.2% inhibited the growth of both *B. subtilis* and *S. lentus*. Inhibition of the Gram-negative *E. coli* was observed for 19.4% of the isolates with 4.8% exclusively inhibiting the growth of *E. coli*. In total, 25.4% of the isolates were active against the yeast *C. glabrata*, 5.8% exclusively. Most strains displayed antimicrobial activity against either one (45.5%) or two (34%) of the test strains. Of the isolates, 19.5% were active against three, and one of the bacteria showed activity against all four test strains (Table 2).

Table 2 Antimicrobial activity profiles of *Laminaria saccharina*-associated bacterial isolates (103 strains)

Inhibition of test strains	Activity pattern	Number of isolates	Percentage
Only *B. subtilis*	a	31	30.1
Only *S. lentus*	b	5	4.8
Only *E. coli*	c	5	4.8
Only *C. glabrata*	d	6	5.8
B. subtilis and *S. lentus*	e	26	25.2
B. subtilis and *E. coli*	f	3	2.9
B. subtilis and *C. glabrata*	g	3	2.9
S. lentus and *E. coli*	h	1	1.0
S. lentus and *C. glabrata*	i	1	1.0
E. coli and *C. glabrata*	j	1	1.0
B. subtilis, *S. lentus*, and *E. coli*	k	5	4.8
B. subtilis, *S. lentus*, and *C. glabrata*	l	11	10.8
B. subtilis, *E. coli*, and *C. glabrata*	m	1	1.0
S. lentus, *E. coli*, *and C. glabrata*	n	3	2.9
B. subtilis, *S. lentus*, *E. coli*, and *C. glabrata*	o	1	1.0

The proportion of isolates, which inhibited the growth of a single test strain or a combination of test strains is given as percentages of total number tested

There was no correlation between distinct ATUs and the 15 activity patterns (Table 3). Inhibition of *B. subtilis* exclusively (pattern "a") as the most common one was observed for 31 out of 57 isolates belonging to 18 different ATUs. Out of 64 members of 13 ATUs, 26 represented the activity pattern "e", inhibiting the growth of *B. subtilis* and *S. lentus*. Eleven isolates, which inhibited the growth of *B. subtilis*, *S. lentus*, and *C. glabrata* (pattern "l"), affiliated to eight ATUs consisting of total 36 strains. Further antibiotic profiles were observed infrequently (Table 3). The inhibition of *C. glabrata* only (pattern "d") was exhibited by six strains each representing a single ATU, which were affiliated to the genera *Cellulophaga*, *Glaciecola*, *Pseudomonas*, *Streptomyces*, and *Sulfitobacter*. The activity pattern "b" (active against *S. lentus* only) and "k" (active against *B. subtilis*, *S. lentus*, and *E. coli*) were shown for five ATUs each. Inhibitory effects on the growth of *E. coli* only (pattern "c") were observed for five isolates belonging to four ATUs. Three members of three ATUs each exhibited the activity pattern "f" (active against *B. subtilis* and *E. coli*), "g" (active against *B. subtilis* and *C. glabrata*), and "n" (active against *S. lentus*, *E. coli*, and *C. glabrata*), respectively. The pattern "h," "i," "j," "m," and "o" were represented by single strains belonging to different ATUs (Table 3).

Table 3 Affiliation of *Laminaria saccharina*-associated isolates to ATUs, bacterial groups, and activity pattern (a–o), respectively

Isolate	ATU	Bacterial group	Activity pattern	Activity against test strains			
				B. subtilis	*E. coli*	*S. lentus*	*C. glabrata*
L134	*Arthrobacter* AR	Gram-positive, high GC	a	+	−	−	−
LD82	*Bacillus* B2	Gram-positive, low GC	a	+	−	−	−
L164	*Bacillus* B3	Gram-positive, low GC	a	+	−	−	−
L166	*Bacillus* B3	Gram-positive, low GC	a	+	−	−	−
L244	*Bacillus* B4	Gram-positive, low GC	a	+	−	−	−
L135	*Bacillus* B5	Gram-positive, low GC	a	+	−	−	−
L91	*Bacillus* B8	Gram-positive, low GC	a	+	−	−	−
L222	*Cobetia* CO	Gammaproteobacteria	a	+	−	−	−
L96	*Mesorhizobium* ME	Alphaproteobacteria	a	+	−	−	−
L28	*Pseudoalteromonas* PA2	Gammaproteobacteria	a	+	−	−	−
L35	*Pseudoalteromonas* PA2	Gammaproteobacteria	a	+	−	−	−
LM24	*Pseudomonas* PM2	Gammaproteobacteria	a	+	−	−	−
LD11	*Pseudomonas* PM3	Gammaproteobacteria	a	+	−	−	−
LD80	*Pseudomonas* PM5	Gammaproteobacteria	a	+	−	−	−
L104	*Streptomyces* S1	Gram-positive, high GC	a	+	−	−	−
L108	*Streptomyces* S1	Gram-positive, high GC	a	+	−	−	−
L97	*Streptomyces* S3	Gram-positive, high GC	a	+	−	−	−
L101	*Streptomyces* S3	Gram-positive, high GC	a	+	−	−	−
L107	*Streptomyces* S3	Gram-positive, high GC	a	+	−	−	−
L98	*Streptomyces* S5	Gram-positive, high GC	a	+	−	−	−
L99	*Streptomyces* S6	Gram-positive, high GC	a	+	−	−	−
L100	*Streptomyces* S6	Gram-positive, high GC	a	+	−	−	−
L102	*Streptomyces* S6	Gram-positive, high GC	a	+	−	−	−
L131	*Streptomyces* S6	Gram-positive, high GC	a	+	−	−	−
L132	*Streptomyces* S6	Gram-positive, high GC	a	+	−	−	−
L136	*Streptomyces* S6	Gram-positive, high GC	a	+	−	−	−
L137	*Streptomyces* S6	Gram-positive, high GC	a	+	−	−	−
L138	*Streptomyces* S6	Gram-positive, high GC	a	+	−	−	−
L139	*Streptomyces* S6	Gram-positive, high GC	a	+	−	−	−
L165	*Stenotrophomonas* ST	Gammaproteobacteria	a	+	−	−	−
LD7a	*Vibrio* V1	Gammaproteobacteria	a	+	−	−	−
LD152	*Bacillus* B1	Gram-positive, low GC	b	−	−	+	−
L240	*Bacillus* B3	Gram-positive, low GC	b	−	−	+	−
L110	*Bacillus* B6	Gram-positive, low GC	b	−	−	+	−
LD83	*Olleya* OL	Bacteroidetes	b	−	−	+	−
L87	*Streptomyces* S7	Gram-positive, high GC	b	−	−	+	−
L232	*Pseudoalteromonas* PA1	Gammaproteobacteria	c	−	+	−	−
LD126	*Pseudomonas* PM4	Gammaproteobacteria	c	−	+	−	−
LD17	*Vibrio* V1	Gammaproteobacteria	c	−	+	−	−
LD18	*Vibrio* V1	Gammaproteobacteria	c	−	+	−	−
LD162	*Vibrio* V4	Gammaproteobacteria	c	−	+	−	−
LD84	*Cellulophaga* CE	Bacteroidetes	d	−	−	−	+
LD86	*Glaciecola* GL	Gammaproteobacteria	d	−	−	−	+
LD119	*Pseudomonas* PM1	Gammaproteobacteria	d	−	−	−	+
L76	*Streptomyces* S2	Gram-positive, high GC	d	−	−	−	+
L77	*Streptomyces* S6	Gram-positive, high GC	d	−	−	−	+
LD87	*Sulfitobacter* SU	Alphaproteobacteria	d	−	−	−	+
LD151	*Aeromonas* AE	Gammaproteobacteria	e	+	−	+	−
LD153	*Bacillus* B1	Gram-positive, low GC	e	+	−	+	−
LD125	*Bacillus* B2	Gram-positive, low GC	e	+	−	+	−
LD4	*Micrococcus* MI	Gram-positive, high GC	e	+	−	+	−
L145	*Pseudoalteromonas* PA2	Gammaproteobacteria	e	+	−	+	−
LD45	*Pseudomonas* PM1	Gammaproteobacteria	e	+	−	+	−
LD46	*Pseudomonas* PM1	Gammaproteobacteria	e	+	−	+	−

Table 3 (continued)

Isolate	ATU	Bacterial group	Activity pattern	Activity against test strains			
				B. subtilis	*E. coli*	*S. lentus*	*C. glabrata*
LD47	*Pseudomonas* PM1	Gammaproteobacteria	e	+	−	+	−
LD49	*Pseudomonas* PM1	Gammaproteobacteria	e	+	−	+	−
LD51	*Pseudomonas* PM1	Gammaproteobacteria	e	+	−	+	−
LD52	*Pseudomonas* PM1	Gammaproteobacteria	e	+	−	+	−
LD53	*Pseudomonas* PM1	Gammaproteobacteria	e	+	−	+	−
LM59	*Pseudomonas* PM1	Gammaproteobacteria	e	+	−	+	−
LM62	*Pseudomonas* PM1	Gammaproteobacteria	e	+	−	+	−
LD118	*Pseudomonas* PM1	Gammaproteobacteria	e	+	−	+	−
L112	*Streptomyces* S1	Gram-positive, high GC	e	+	−	+	−
L148	*Streptomyces* S1	Gram-positive, high GC	e	+	−	+	−
L95	*Streptomyces* S3	Gram-positive, high GC	e	+	−	+	−
LM78a	*Streptomyces* S6	Gram-positive, high GC	e	+	−	+	−
L111	*Streptomyces* S6	Gram-positive, high GC	e	+	−	+	−
L130	*Streptomyces* S6	Gram-positive, high GC	e	+	−	+	−
L133	*Streptomyces* S6	Gram-positive, high GC	e	+	−	+	−
L155	*Streptomyces* S8	Gram-positive, high GC	e	+	−	+	−
L142	*Streptomyces* S9	Gram-positive, high GC	e	+	−	+	−
L167	*Stenotrophomonas* ST	Gammaproteobacteria	e	+	−	+	−
LD156	*Vibrio* V1	Gammaproteobacteria	e	+	−	+	−
LD12	*Pseudomonas* PM5	Gammaproteobacteria	f	+	+	−	−
L94	*Streptomyces* S2	Gram-positive, high GC	f	+	+	−	−
L93	*Streptomyces* S6	Gram-positive, high GC	f	+	+	−	−
LD81	*'Kiloniella'* KI	Alphaproteobacteria	g	+	−	−	+
LD114	*Alcaligenes* AL	Betaproteobacteria	g	+	−	−	+
L105	*Streptomyces* S1	Gram-positive, high GC	g	+	−	−	+
L158	*Stenotrophomonas* ST	Gammaproteobacteria	h	−	+	+	−
L74	*Bacillus* B2	Gram-positive, low GC	i	−	−	+	+
L171	*Shewanella* SH	Gammaproteobacteria	j	−	+	−	+
L149	*Bacillus* B2	Gram-positive, low GC	k	+	+	+	−
LD160	*Bacillus* B6	Gram-positive, low GC	k	+	+	+	−
L92	*Streptomyces* S4	Gram-positive, high GC	k	+	+	+	−
LD159	*Vibrio* V2	Gammaproteobacteria	k	+	+	+	−
LD150b	*Vibrio* V3	Gammaproteobacteria	k	+	+	+	−
L140	*Amycolatopsis* AM	Gram-positive, high GC	l	+	−	+	+
LD116	*Bacillus* B1	Gram-positive, low GC	l	+	−	+	+
LD154	*Bacillus* B1	Gram-positive, low GC	l	+	−	+	+
LD121	*Bacillus* B2	Gram-positive, low GC	l	+	−	+	+
LD117	*Bacillus* B2	Gram-positive, low GC	l	+	−	+	+
L157	*Bacillus* B3	Gram-positive, low GC	l	+	−	+	+
L89	*Bacillus* B7	Gram-positive, low GC	l	+	−	+	+
LM63	*Pseudomonas* PM1	Gammaproteobacteria	l	+	−	+	+
LD120	*Pseudomonas* PM1	Gammaproteobacteria	l	+	−	+	+
L103	*Streptomyces* S10	Gram-positive, high GC	l	+	−	+	+
L127	*Stenotrophomonas* ST	Gammaproteobacteria	l	+	−	+	+
L169	*Stenotrophomonas* ST	Gammaproteobacteria	m	+	+	−	+
L228	*Leifsonia* LE	Gram-positive, high GC	n	−	+	+	+
L229	*Hyphomonas* HY	Alphaproteobacteria	n	−	+	+	+
L230	*Pseudoalteromonas* PA1	Gammaproteobacteria	n	−	+	+	+
LD115	*Pseudomonas* PM1	Gammaproteobacteria	o	+	+	+	+

Different ATUs within the genera *Bacillus*, *Pseudoalteromonas*, *Pseudomonas*, *Streptomyces*, and *Vibrio* were numbered
+ antibiotic activity, − no antibiotic activity

Discussion

L. saccharina occurs mainly in cold to temperate waters of the Baltic Sea, North Sea, and the North Pacific as well as the North and South Atlantic (Kain 1979; Lüning 1990). Studies concerning the bacterial communities associated with *L. saccharina*, their ecological role, their interactions with the algae or other organisms, and their biotechnological potential are still limited. It is known from microscopic and cultivation experiments, which were carried out with other *Laminaria* species, that the algae are colonized by bacteria (Corre and Prieur 1990; Mazure and Field 1980). Recently, molecular data led to the assumption that there is a specific association of the bacterial community with various parts of the algal thallus, i.e., rhizoid, cauloid, meristem, and phylloid (Staufenberger et al. 2008).

The main topic of this study was the characterization of the cultured bacterial community of *L. saccharina* exhibiting antimicrobial activity. In order to determine the correlation between phylogenetic affiliation of the isolates and their antibiotic activity, all 103 antibiotically active strains were grouped into 45 ATUs on the one hand and to 15 different antibiotic patterns on the other hand. Out of 45 ATUs, 31 contained only a single strain and represented therefore only one antibiotic pattern. The antibiotic profiles within the remaining 14 of the ATUs were not uniform but showed up to five different antibiotic patterns each. This clearly indicates a strain-specific production of biologically active secondary metabolites. As a consequence, it was not possible to infer the antibiotic activity from the phylogenetic identification of the isolates.

The inhibition of Gram-positive bacteria by the *L. saccharina*-associated isolates was more common than the inhibition of Gram-negative bacteria and yeast. Especially, the activity against *S. lentus* led to the assumption that *L. saccharina*-associated isolates produce compounds, which might also inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) strains. These strains are known to cause severe diseases and belong to the most common infectious agents in hospitals (Klevens et al. 2006). Furthermore, a proportion of isolates might be able to produce secondary metabolites against human pathogenic *E. coli* strains and/or against members of clinically relevant *Candida* species, like *Candida albicans*. Organic extracts of 16 antibiotic-producing isolates were tested against human pathogens and revealed that 11 isolates inhibited the growth of MRSA, *E. coli*, or *C. albicans* (data not shown). Potentially new antibiotic substances active against these pathogenic strains thus display high clinical importance and biotechnological potential.

The phylogenetic analysis revealed the affiliation of antimicrobially active isolates to a variety of bacterial taxa including different potentially novel species or even genera. Representatives of the Gram-positive divisions Firmicutes and the Actinobacteria as well as members of the Gram-negative *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* were found associated with the alga. More than half (54%) of the *L. saccharina*-associated isolates belong to the Gram-positive bacterial divisions, to *Actinobacteria* and to *Firmicutes*, which include well-known producers of antibiotic substances. In particular, the *Actinomycetes* are known as important sources for pharmaceutical drugs. Of the presently known antibiotic-active compounds, over 8,700 substances, 53% were isolated from members of the *Actinomycetales*. Moreover, approximately 70% of all antibiotics used worldwide as therapeutic drugs are produced by *Actinomycetales* (Berdy 2005). Within the *Firmicutes*, especially strains of the genus *Bacillus*, are common producers of antimicrobial compounds. Approximately 800 metabolites with antibiotic activity, including the important group of peptide antibiotics like bacitracin, gramicidin, and polymyxin B are produced by *Bacillus licheniformis*, *Bacillus brevis*, and *Bacillus polymyxin*, respectively (Berdy 2005; Ishihara et al. 2002; Vandamme and Demain 1976; Crisley 1964).

Within the *Actinomycetales*, the genus *Streptomyces* represents the most frequent producers of antibiotic agents. Examples are tetracycline (*Streptomyces viridifaciens*), vancomycin (*Streptomyces orientalis*), fosfomycin (*Streptomyces fradiae*), streptomycin (*Streptomyces griseus*), and the macrolide erythromycin (*Streptomyces erythreus*; Cheng et al. 1999; Zheng et al. 2000; Watve et al. 2001). Also, representatives of other groups of *Actinobacteria*, including the genera *Amycolatopsis*, *Arthrobacter*, and *Micrococcus* identified in this study, are known as producers of pharmaceutically important antibiotics. Two antibiotics in clinical use are produced by *Amycolatopsis* species. Vancomycin, a glycopeptide antibiotic which is used as a drug of last resort in the treatment of life-threatening infections by Gram-positive bacteria, is produced by *Amycolatopsis orientalis* (Hubbard and Walsh 2003) and a vancomycin-like antibiotic, balhimycin, is produced by *Amycolatopsis mediterranei* (Recktenwald et al. 2002). The biological activity of the natural products of these bacteria is not limited to the inhibition of other bacteria but also can affect eukaryotic organisms, such as the human parasite *Plasmodium falciparum* which is inhibited by micrococcin produced by *Micrococcus varians* (Rogers et al. 1998).

Despite the great variety of known antibiotics, novel chemical classes, produced by these bacteria (*Actinobacteria* and *Firmicutes*), continue to be discovered. Among the newly discovered natural products of *Streptomyces* species, the lipopeptide daptomycin produced by *Streptomyces roseopurpureus* was brought into market in 2003 (Baltz et al. 2005) and platensimycin produced by *Streptomyces*

platensis represents a promising candidate of a new antibiotic drug (Wang 2006). Other antimicrobial active substances belonging to new structural classes of antibiotics include azicemicin A and B as well as epoxyquinomicin A, B, C, and D, isolated from strains closely related to *Amycolatopsis sulphurea* (Tsuchida et al. 1995; Matsumoto et al. 1997) and a novel quinolone antibiotic, YM-30059, active against multiple drug-resistant *S. aureus* and *Staphylococcus epidermidis* strains, isolated from an *Arthrobacter* species (Kamigiri et al. 1996).

A number of secondary metabolites with antimicrobial activity also have been identified in members of the *Proteobacteria* and the *Bacteroidetes*. Within the *Alphaproteobacteria*, a major clade of marine bacteria (Giovannoni and Rappé 2000; Gonzalez and Moran 1997), production of antibiotic substances has been identified in members of the *Roseobacter* lineage, *Sulfitobacter pontiacus*, *Roseovarius* sp., and *Oceanibulbus indoliflex*. *Roseobacter* species produce tropodithietic acid, a novel antibiotic, effective against marine bacteria and algae (Brinkhoff et al. 2004). Tryptanthrin, first discovered in 1987 as an antimicrobial plant metabolite and later patented as an antimalaria pharmacophore (Bhattacharjee et al. 2004), is also produced by the marine alphaproteobacterium *O. indoliflex* (Wagner-Döbler et al. 2004). Antibiotic activity against *E. coli*, *S. aureus*, and *C. glabrata* was found in *S. pontiacus*, related to the alga-derived isolate LD87 (Toledo et al. 2006) and also in *Alphaproteobacteria* closely related to *P. denitrificans* and *Ruegeria atlantica*, which were found to dominate the cultured bacteria isolated from Mediterranean sponges (Muscholl-Silberhorn et al. 2008).

Members of the *Betaproteobacteria* have been detected mainly in freshwater habitats but rarely in oceanic environments (Nold and Zwart 1998). We isolated a marine alga-associated strain of the betaproteobacterial genus *Alcaligenes* (LD114). Other members of this genus, such as *Alcaligenes xylosoxidan*, display biotechnological potential in antifungal biocontrol by inhibition of two fungal plant pathogens *Rhizoctonia bataticola* and *Fusarium* sp. (Vaidya et al. 2001).

Also, representatives of the *Gammaproteobacteria*, which have been isolated in this study, species of *Pseudomonas*, *Pseudoalteromonas*, *Stenotrophomonas*, *Vibrio*, *Aeromonas*, and *Shewanella*, yielded antimicrobial substances. From a total of 22,500 biologically active substances derived from bacteria and fungi, 610 (2.7%) are produced from *Pseudomonas* species (Berdy 2005), among these massetolide A (Gerard et al. 1997). *Pseudoalteromonas* strains are less frequently reported as producers of antibiotic substances. Longeon et al. (2004) highlighted a *Pseudoalteromonas* isolate as producer of a novel antimicrobial protein, which inhibited human pathogenic strains causing dermatologic diseases. Minkwitz and Berg (2001) demonstrated antifungal activities of *Stenotrophomonas maltophilia* strains against the yeast *C. albicans* and phytopathogenic fungi. *Vibrio* strains are known to produce antibiotic-active peptides like andrimid (Oclarit et al. 1994), which represent a new class of antibiotics targeting bacterial fatty acid biosynthesis (Pohlmann et al. 2005). An *Aeromonas* isolate was found to produce a glucosidic cyclic lactone showing an antifungal activity (Afonso et al. 1999).

Representatives of the *Bacteroidetes* group from aquatic habitats are known as surface-associated bacteria, as they were found predominantly in floating aggregates (Nold and Zwart 1998). As reviewed by Michel et al. (2006), *Flavobacteria* were found to produce carrageenases and agarase and are hence able to degrade algal compounds. Thus, the algal isolates affiliated with the *Bacteroidetes* possibly represent opportunistic alga-degrading bacteria. Nevertheless, CFB group members have also been shown to produce secondary metabolites with biotechnological potential. Fucoidan hydrolases (Urvantseva et al. 2006) and other substances with algaecidal properties can be of great use to prevent shellfish farms from closing due to toxic dinoflagellate blooms (Skerratt et al. 2002).

In addition to members of known antibiotic-producing taxa, especially representatives of novel species or genera isolated from marine habitats may be valuable sources of novel biologically active metabolites, which have not been derived from terrestrial environments (Bernan et al. 2004; Fiedler et al. 2005; Jensen et al. 2005; Lam 2006). As reviewed recently by Bull and Stach (2007), marine *Actinobacteria* harbor an unrivaled capacity to produce exploitable natural products. Especially members of novel marine genera, such as the recently described genus *Salinispora* (Fenical and Jensen 2006), exhibit a high potential to produce new antibiotics. *Salinispora tropica* produces the antitumor agent salinosporamide A, which went into preclinical trials against cancer (Newman and Cragg 2006).

Not only chemical analysis of culture broth and cell mass but also genome sequences of marine bacteria provided valuable information on the potential to produce promising secondary metabolites (Hopwood 2007; Udwary et al. 2007). In addition, variation of the cultivation conditions (e.g., cultivation on substrate surfaces or in liquid broth, cocultivation with other organisms) can influence the production of secondary metabolites (Yan et al. 2003; Diggle et al. 2007). Studies on the impact of cultivation conditions for the production of antimicrobial compounds by the alga-derived isolates are expected to be a valuable tool in the search for new antibiotically active substances.

In summary, we have demonstrated that *L. saccharina*-associated bacteria have a great potential to produce antimicrobial compounds. The large variation of antimicrobial activity patterns among our isolates (even within single ATUs), the large number of phylogenetically distinct ATUs,

and the presence of new species and a new genus among the isolates are promising results for future work on antibiotically active compounds produced by these bacteria. Following studies with these isolates will focus on functional genetic studies concerning biosynthetic pathways of their secondary metabolites and the identification of chemical structures of the produced substances in order to unravel their biotechnological potential.

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