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Diversity and Antagonistic Potential of Bacteria Associated with Bryophytes from Nutrient-Poor Habitats of the Baltic Sea Coast

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Very little is known about the interaction of bryophytes with bacteria. Therefore, we analyzed bacteria associated with three bryophyte species, *Tortula ruralis*, *Aulacomnium palustre*, and *Sphagnum rubellum*, which represent typical moss species of three nutrient-poor plant communities at the southern Baltic Sea coast in Germany. By use of two cultivation-independent techniques, denaturing gradient gel electrophoresis and single-strand conformation polymorphism analysis of the 16S ribosomal DNA, a high degree of moss specificity was found for associated bacterial communities. This specificity could be further evidenced by a cultivation-dependent approach for the following parameters: (i) plate counts of bacteria on R2A medium, (ii) proportion of antagonistic isolates, (iii) antagonistic activity as well as spectrum against pathogens, and (iv) diversity and richness of antagonistic isolates. The proportion of isolates with antagonistic activity against the pathogenic model fungus *Verticillium dahliae* was highest for *S. rubellum* (31%), followed by *A. palustre* (17%) and *T. ruralis* (5%). A high percentage (99%) of moss-associated antagonistic bacteria produced antifungal compounds. The high recovery of antagonistic isolates strongly suggests that bryophytes represent an ecological niche which harbors a diverse and hitherto largely uncharacterized microbial population with yet unknown and untapped potential biotechnological applications, e.g., for biological control of plant pathogens.

The bryophytes, including liverworts, hornworts, and mosses, are a diverse group of land plants that usually colonize habitats with moist or extremely variable conditions. One of their most important features is their life cycle, which involves alternation between a diploid sporophyte and a dominant, free-living haploid gametophyte generation (43). Bryophytes are unique host plants for microorganisms in numerous ways. For example, the small size of mosses results in limited availability of the substratum. Additionally, their indefinite growth in the form of dense overwintering colonies, mats, and cushions without periodic leaf fall enables colonization for long periods. Also, because bryophytes absorb water over much of their surface from atmospheric moisture, most of them display an extraordinarily high tolerance to extreme desiccation and resume normal metabolism very rapidly after rehydration (poikilohydric organization). Hence, successful microbial colonization requires adaptation to these special conditions (11).

Bryophytes represent the simplest extant land plant group and are phylogenetically very old (14). Miller (27) regards them as the oldest extant terrestrial plants, which represent the level of evolution associated with transmigration to the land. Bacterium-host interactions can be symbiotic, commensal, or pathogenic, and while the processes leading to the evolution of symbionts or pathogens are similar, the establishment of a symbiosis requires more time and evolutionary processing (17, 38). Plant-associated bacteria that are able to antagonize other (pathogenic) microorganisms belong to the symbiotic fraction of microorganisms (37, 41).

Traditionally, because of their antimicrobial activity, mosses were used as a natural medicine in Indian culture (15) and as natural diapers (3). Today, mosses represent interesting tools for biotechnological use in medicine, agriculture, and pharmacology (10, 15). However, although mosses are becoming increasingly important in many fields and *Physcomitrella patens* is used as a model organism for genetic studies (30), little is known about moss-associated microorganisms, beneficial as well as pathogenic. The colonization of mosses by ascomycetes is a very frequent though generally neglected phenomenon (for a review, see reference 11). However, no moss-specific pathogenic fungi are known. Therefore, *Verticillium dahliae* KLEB., the causative agent of verticillium wilt, which has an extremely broad spectrum of host plants (39), was selected as the model pathogen for our antagonism studies. The diverse antagonistic bacteria associated with potential *Verticillium* host plants are known to be strongly plant species specific (7, 36). However, much remains to be discovered about the specificity and diversity of moss-associated bacteria (9), their role in influencing the development of bryophytes (19), and their antagonistic and biotechnological potential.

Here, our aim is to analyze and characterize the associated bacterial communities of three different moss species with regard to their diversity and biotechnological potential. The mosses *Tortula ruralis*, *Aulacomnium palustre*, and *Sphagnum rubellum* grow in different natural habitats on the southern Baltic Sea coast (northeast Germany). To examine the impact of the moss species and their ecological background on moss-associated bacteria, two cultivation-independent approaches were used—to our knowledge, for the first time for this purpose. Denaturing gradient gel electrophoresis (DGGE) and single-strand conformation polymorphism (SSCP) analysis of the 16S ribosomal DNA (rDNA) were used to analyze nonculturable bacteria, which generally constitute a high percentage of plant-associated microorganisms (24, 36). Additionally,

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to investigate the abundance and diversity of *Verticillium* antagonists, gametophyte samples of the mosses were analyzed by a cultivation-dependent approach. A comprehensive phenotypic and genotypic characterization of the antagonists allowed us to provide new data on the diversity of moss-dependent *Verticillium* antagonists.

MATERIALS AND METHODS

Sampling and isolation of the bacterial fraction. The mosses were sampled in three different natural habitats on the southern Baltic Sea coast (northeast Germany). *T. ruralis* HEDW. (family *Pottiaceae*) formed lax patches on sand dunes. The plant community in which the moss occurred belongs to *Corynephorus* dune grassland, characterized by grey hairgrass (*Corynephorus canescens*). *A. palustre* HEDW. (family *Aulacomniaceae*) grows on the edge of a noncalcareous mire behind the dunes. In the *Sphagnum*-rich birchwood, the dominant plants are birch trees (*Betula pubescens*), glossy buckthorn (*Frangula alnus*), and purple moorgrass (*Molinia caerulea*). The third moss species, *S. rubellum* HEDW. (family *Sphagnaceae*), was found in a more open part of the mire, in the center, together with common cottongrass (*Eriophorum angustifolium*), small cranberries (*Vaccinium oxycoccus*), and crossleaf heath (*Erica tetralix*) in a *Sphagnum-Eriophorum* mire plant community. These three habitats were characterized by a gradient of abiotic conditions, especially with regard to soil reaction (pH values: moderately acidic, acidic, and strongly acidic, respectively), moisture (dry, medium wet, and wet, respectively), and nutrient content (poor, medium poor, and poor, respectively), described by Precker (28). The gradients were confirmed by species indicator values according to the work of Ellenberg et al. (12). For example, indicator values for soil reaction, expressed on a scale of 1 to 9, are different for *T. ruralis* (6 [moderate]), *A. palustre* (3 [acidic]), and *S. rubellum* (1 [strongly acidic]).

Adult gametophytes of the mosses *T. ruralis*, *A. palustre*, and *S. rubellum* were collected in November 2001 and March 2002 from the natural reserve "Ribnitzer Großes Moor" near Rostock (54°18′N, 12°16′E), Germany. Additionally, *T. ruralis* was collected in March 2002 from the dune in Rostock-Warnemünde (54°05′N, 12°07′E) and in May 2002 from the island of Vilm (54°20′N, 13°30′E); both areas are located in northern Germany directly on the Baltic Sea coast. Gametophytes with adhering soil were placed in sterile petri dishes and transported to the laboratory, and then 5 g was transferred to a sterile stomacher bag. To extract the moss-associated bacteria from the gametophytes, 45 ml of sterile 0.85% NaCl was added, and samples were homogenized in a stomacher laboratory blender for 60 s at high speed (BagMixer; Interscience, St. Nom, France). This suspension was used for cultivation as well as for cultivation-independent investigation procedures. For the latter, after centrifugation at low speed (2 min, 500 × *g*), the supernatant was collected and the resulting pellet was suspended in distilled water, followed by stomacher blending and low-speed centrifugation. This step was repeated once. The supernatants of the three centrifugation steps were combined before centrifugation at high speed (10,000 × *g*) for 30 min to collect the microbial pellet. The resulting microbial pellet was stored at −70°C.

Total-community DNA isolation. DNA of bacterial-cell consortia was extracted by using the FastDNA Spin Kit for Soil (Bio 101, Carlsbad, Calif.) according to the manufacturer's protocol.

Scanning electron microscopy. Gametophytes were fixed in glutaraldehyde-phosphate buffer (2%; 0.1 M; pH 7.2) and postfixed in 2% glutaraldehyde buffer. After removal of the fixative by a wash in phosphate buffer, samples were dehydrated, critical point dried, and coated with gold before undergoing scanning electron microscopy (Carl Zeiss, Oberkochen, Germany).

Molecular analysis by DGGE. Fingerprinting of the bacterial moss communities by DGGE was carried out as described by Heuer and Smalla (18). Briefly, 16S rDNA fragments (positions 968 to 1401 [*Escherichia coli* rDNA sequence]) were amplified by PCR using bacterial DNA isolated from moss tissue as a template, with the primer pair F984GC–R1378 (36). The amplicons were separated in a 40 to 56% denaturing gradient of 7 M urea and 40% (vol/vol) formamide at 60°C. Acid silver staining was used to detect DNA in DGGE gels (31).

Molecular analysis by SSCP. Fingerprinting of the moss communities by SSCP was carried out as described by Schwieger and Tebbe (33). Briefly, 16S rDNA fragments (positions 968 to 1401 [*E. coli* rDNA sequence]) were amplified by PCR using bacterial DNA isolated from moss tissue as the template, with the UniBac 927r primer (18) along with specific primers for *Burkholderia* (32), *Pseudomonas* (42), and *Serratia* (23) spp. The amplicons were separated by using the TGGE Maxi system (Biometra, Göttingen, Germany) at 400 V and 26°C. Silver staining was used to detect DNA in SSCP gels (4).

Identification of SSCP bands. Dominant bands were excised from SSCP gels as described by Schwieger and Tebbe (33). Gel-extracted DNA was reamplified, and products were then ligated into a pGEM-T vector and transformed into *E. coli* DH5α (Promega, Mannheim, Germany). Transformed cells with inserts were selected by blue-white screening. Cloned DNA fragments were amplified from the vector by PCR (under the conditions recommended by the manufacturer) using primers matching the flanking regions of the vector (forward, 5′-CAC GAC GTT GTA AAA CGA C-3′; reverse, 5′-GGA TAA CAA TTT CAC ACA GG-3′). The sizes of the PCR products were determined by agarose gel electrophoresis (0.8% [wt/vol] agarose). Inserts of the expected size were then sequenced by cycle sequencing using the DTCS CEQ Quick Start kit (Beckman Coulter, Fullerton, Calif.). By using primers usp (5′-GTA AAA CGA CGG CCA GT-3′) and rsp (5′-CAG GAA ACA GCT ATG ACC-3′), the fragments were sequenced with the Beckman Coulter system. The sequences were edited and aligned with CEQ 2000 XL analysis systems. For phylogenetic analysis and identification of related sequences, the BLAST algorithm according to Altschul et al. (1) was used. Additionally, sequences were loaded into the ARB program and database (http://www.arb-home.de).

Isolation of moss-associated bacteria. Microbial suspensions obtained by the procedure explained above were serially diluted with sterile 0.85% NaCl and plated onto R2A medium (Difco, Detroit, Mich.). Plates were incubated for 5 days at 20°C, after which CFU were counted to calculate the mean number of colonies ($\log_{10}$ CFU) based on fresh weight. Data were analyzed for significance by using the Mann-Whitney U test ($P \leq 0.05$) by Statistical Product and Service Solutions for Windows, release 9.0.1 (SPSS Inc., Chicago, Ill.). Isolates obtained by plating were purified and stored at −70°C in sterile broth containing 50% glycerol.

Screening of antagonistic bacteria. Bacterial isolates were screened for their activity toward *V. dahliae* KLEB. by a dual-culture in vitro assay on Waksman agar (WA) containing 5 g of proteose-peptone (Merck, Darmstadt, Germany), 10 g of glucose (Merck), 3 g of meat extract (Chemex, Munich, Germany), 5 g of NaCl (Merck), 20 g of agar (Difco), and distilled water (to 1 liter) (pH 6.8). Zones of inhibition were measured after 3 and 7 days of incubation at 20°C according to the method of Berg (5). All strains were tested in three independent replicates with *V. dahliae* V25 (isolated from *Brassica napus* L. by K. Zeise and kept in the culture collection of the University of Rostock, Department of Microbiology). The fungus was routinely grown on Sabouraud medium (Gibco, Paisley, Scotland) and stored at −70°C in sterile broth containing 50% glycerol. In vitro inhibition of *Xanthomonas campestris* DZM 3586 was determined by a dual-culture assay in Luria-Bertani (LB) agar (Difco) in microtiter plates. From an overnight culture of *Xanthomonas*, 10 μl was mixed with LB agar, and bacterial isolates were spotted onto the solidified agar surface. Zones of inhibition were measured after incubation at 20°C for 24 and 48 h.

Identification of bacterial antagonists. Antagonists were identified based on whole-cell fatty acids derivatized to methyl esters (fatty acid methyl esters [FAME]) and analyzed by gas chromatography using the MIDI system (Microbial ID, Inc., Newark, N.J.). In addition, the majority of strains were identified by 16S rDNA sequencing and aligned with the reference 16S rRNA gene sequence by using the BLAST algorithm according to Altschul et al. (1). Species richness, expressed as the number of species (S) as a function (ratio) of the total number of individuals (N), was determined by the index (d) proposed by Menhinick (26). Diversity indices were calculated according to the method of Shannon and Weaver (35). Only isolates which were identified with a similarity index higher than 0.5 by FAME analysis or 97% by sequencing of 16S rDNA genes were used for the calculations of indices.

Screening for strains with macromolecular hydrolytic activity. Chitinase activity (β-1,4-glucosamine polymer degradation) was tested in chitin minimal medium by the method of Chernin et al. (8). Clearing zones were detected 5 days after incubation at 20°C. β-Glucanase activity was tested by using chromogenic (azurine-dyed, cross-linked [AZCL]) substrates (Megazyme, Bray, Ireland). Formation of blue halos was recorded until 5 days after incubation. Protease activity (casein degradation) was determined from clearing zones in skim milk agar (50 ml of sterilized skim milk mixed at 55°C with 50 ml of 1/5 tryptic soy agar and 4% agar) after 5 days of incubation at 20°C.

Production of secondary metabolites with potential antagonistic activity. Antibiosis against *V. dahliae* by the bacterial strains was assayed on WA plates (15 ml) containing 5 ml of sterile culture filtrate (64-h culture, nutrient broth II [Sifin, Berlin, Germany]). The pH was adjusted to between 7 and 8. A 3-mm-diameter plug from a *V. dahliae* agar plate was placed in the center of a WA plate. As a control, WA plates (20 ml) were similarly inoculated with mycelial plugs. Colony diameters were measured daily for 10 days, and the percent reduction in linear growth of the fungi was calculated. Siderophore production was assayed according to the method of Schwyn and Neilands (34).

BOX-PCR genomic fingerprints. Bacterial DNA was prepared by following the protocol of Anderson and McKay (2) modified for genomic DNA. BOX-PCR (fingerprinting based on repetitive BOX elements, of unknown function, in the bacterial genome) was carried out as described by Rademaker and De Bruijn (29). By using the BOXA1R primer (5′-CTA CGG CAA GGC GAC GCT GAC G-3′), PCR amplification was performed with a Peltier Thermal Cycler PTC-200 (Biozym Diagnostic, Hessisch Oldendorf, Germany) with an initial denaturation step at 95°C for 6 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 65°C for 8 min; and a final extension at 65°C for 16 min. A 10-μl aliquot of the amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels in 0.5× Tris-borate-EDTA buffer for 6 h, stained with ethidium bromide, and then photographed under UV transillumination. The reproducibility of the results was verified in three independent experiments.

Computer-assisted cluster analysis. Computer-assisted evaluation of bacterial community profiles obtained by DGGE and SSCP, and of fingerprints generated by BOX-PCR, was performed by using the GelCompar program (version 4.1; Applied Maths, Kortrijk, Belgium). Cluster analysis was performed with the UPGMA (unweighted pair group method with arithmetic averages) algorithm.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the EMBL Data Library under accession numbers AJ575061 to AJ575093, AJ574888 to AJ574896, and AJ5774931 to AJ574938.

RESULTS

Microscopic observation of surfaces of gametophytes. Impressions of the specific microenvironment were obtained by analyzing gametophytes by electron microscopy (Fig. 1). The top panel of Fig. 1 represents the surface of a *Tortula* leaf with characteristic papillae that form a very special microenvironment for bacteria. These unique structures improve the movement of water and its uptake into the cells and are important for mosses living under changing water conditions such as those in the dune. In the central panel of Fig. 1, a typical leaf surface of *Aulacomnium* with conical papillae, colonized by bacteria, is shown. In contrast, *Sphagnum* formed a regular pattern of alternating green and hyaline cells which act as a very special microenvironment for bacteria. The hyaline cells with large pores, normally filled with water, are dead at maturity and now serve to provide water (Fig. 1, bottom panel).

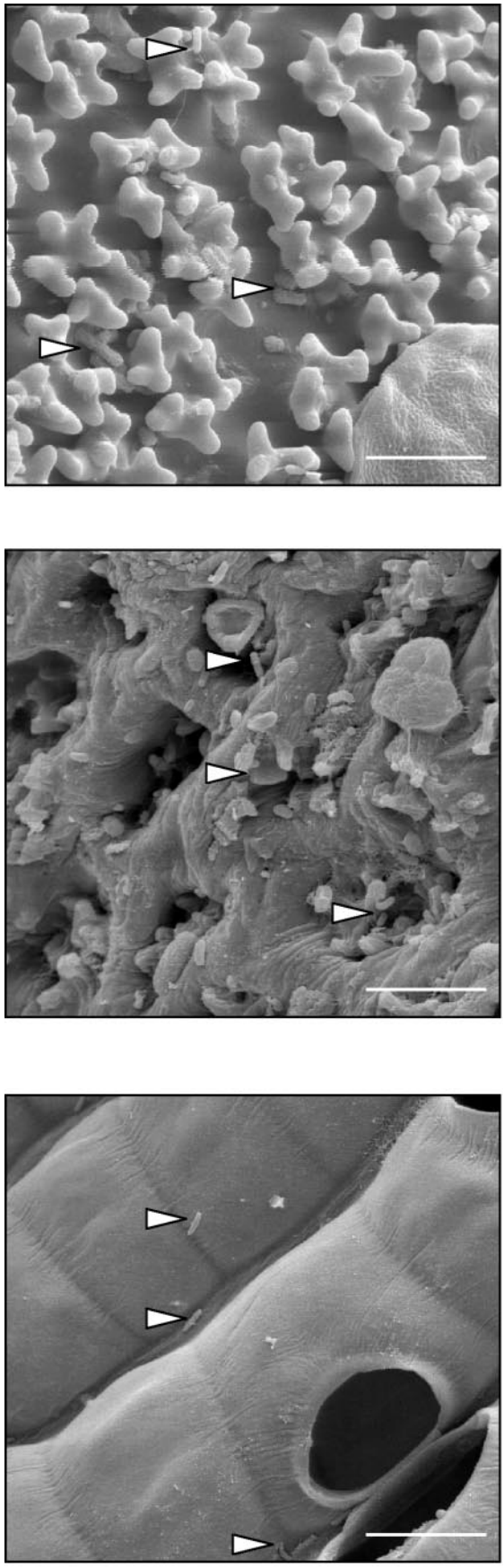
Molecular fingerprinting of moss-associated bacterial communities. For cultivation-independent analysis, total-community DNA was extracted from the microbial pellets recovered from different mosses. 16S rDNA fragments amplified by PCR were analyzed by DGGE and by SSCP. Generally, the patterns of different mosses produced with eubacterial as well as specific primers showed a high degree of diversity. For example, Fig. 2 presents four replicates of DNAs from each of the three bacterial moss-associated communities amplified with universal primers and separated by SSCP. Very similar patterns were obtained for the eubacterial communities by DGGE and SSCP. According to a cluster analysis using the UPGMA algorithm, DGGE patterns exhibited a lower similarity (54%) for all mosses than patterns separated by SSCP (68%). By use of universal primers, the SSCP patterns obtained for *Tortula* from different locations displayed a similarity of 90% and were more similar to each other than to those of the other moss species (Fig. 3). The similarity among all mosses was higher (85%) with *Pseudomonas*-specific primers than with eubacterial primers and confirmed the influence of moss species on the bacterial community. *Tortula*-associated *Pseudomonas* community patterns from different locations exhibited 92% similarity to each other. In contrast, *Serratia*-specific patterns and especially *Burkholderia*-specific patterns showed lower degrees of similarity (48 and 28%, respectively) among the three species and among the three locations (80 and 68%, respectively).

As indicated in Fig. 2, typical bands were excised and sequenced to obtain further information about dominant bacterial populations of the three different moss species. The resulting species list, along with partial sequence analysis data and tentative phylogenetic affiliations, is given in Table 1. DNA sequencing of these bands showed similarities in the range of 90 to 100% to sequences from the database, although five sequences were related to uncultured or unidentified bacteria. For *Aulacomnium*, a high proportion of uncultured or unidentified eubacteria were found. *Photorhabdus luminescens* (SSCP band 1a; 99% similarity), *Collimonas fungivorans* (band 2a; 99% similarity), and diverse *Pseudomonas* spp. (bands 7a and 9a) were identified. The sequences of two dominant bands of *Sphagnum* could be assigned to *Pseudomonas grimontii* with 100% similarity (band 2s) and to *Methylobacterium mesophilicum* (band 4s) with only 93% similarity. Species of *Acetobacter*, *Frateuria*, and *Acidocella*, bacterial genera known for their occurrence in acidic environments, were found for *Sphagnum*. *Tortula*-specific bands were identified as *Pseudomonas aeruginosa* (band 1t; 99% similarity), *Rhodococcus erythropolis* (band 3t; 99% similarity), or *Acidovorax wohlfahrtii* (band 4t; 98% similarity).

Isolation of bacteria from moss gametophytes. CFU determined for moss samples were rather similar for the different species (counts, expressed as $\log_{10}$ CFU per gram [fresh weight] of plant, were 6.7 to 7.1 for *T. ruralis*, 5.9 to 6.4 for *A. palustre*, and 5.4 to 6.1 for *S. rubellum*). However, the highest (and statistically significant) averages were found for *T. ruralis*, with 6.8 $\log_{10}$ CFU g [fresh weight] of plant^{-1}. No statistically significant differences at a P value of ≤ 0.05 could be found between sampling times and sites.

Screening for isolates antagonistic to *V. dahliae*. A total of 710 bacterial isolates were screened for their ability to suppress *V. dahliae* in an in vitro dual-culture assay. Initially, 105 (15%) isolates which were active against *V. dahliae* were found; of these, 23 (22%) were strongly active, with inhibition zones larger than 10 mm. Although similar numbers of isolates from each of the treatments were tested, the proportions of isolates with antagonistic activity were different. The proportion of isolates with antifungal activity was highest for *Sphagnum* (31%), followed by *Aulacomnium* (17%) and *Tortula* (5%). Isolates from *Sphagnum* showed the strongest activity: 44% of the antifungal isolates caused inhibition zones of more than 10 mm. In contrast, no strong antagonist was observed for *Tortula*.

Diversity of *Verticillium* antagonists. The majority of the in vitro antagonists (83) were identified by 16S rDNA sequencing and partly by fatty acid analysis (Table 2). Based on their sequences, 17 different bacterial species were identified; they belonged to nine different genera, among which *Burkholderia*, *Pseudomonas*, and *Serratia* were dominant. The highest numbers of different species with antagonistic activity were isolated from *Sphagnum* (11), whereas only seven and three were found on the gametophytes of *Aulacomnium* and *Tortula*, respectively. The richness and diversity of antagonistic species were moss species dependent. The highest diversity and richness were found for *Sphagnum* (diversity, 6.2; richness, 1.8). Conversely, the lowest indices were calculated for *Tortula* (diver-



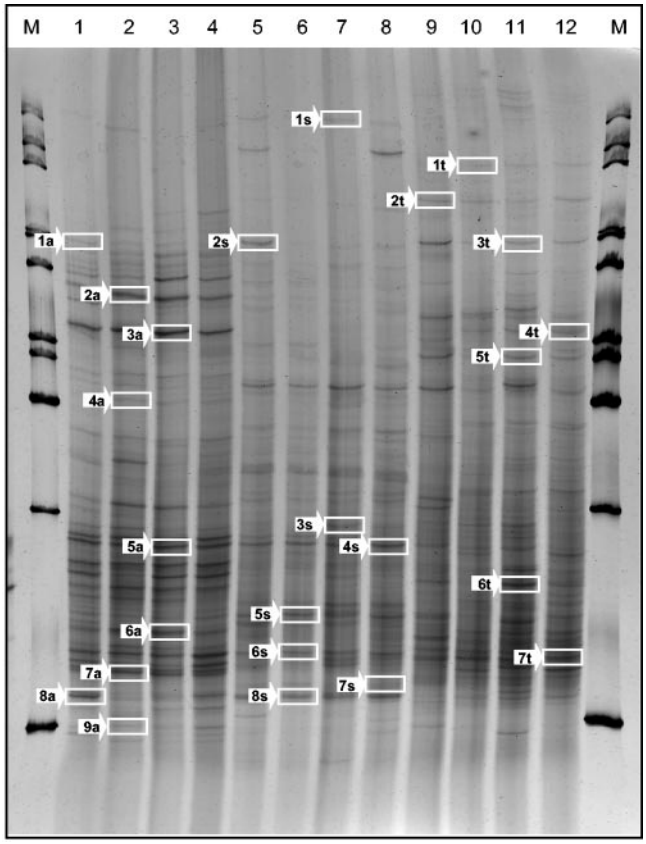


FIG. 2. SSCP profiles showing the bacterial communities of the bryophytes *A. palustre* (lanes 1 to 4), *S. rubellum* (lanes 5 to 8), and *T. ruralis* (lanes 9 to 12). The fingerprints were generated by separation of 16S rDNA fragments amplified with universal primers. As a marker (lanes M), a 1-kb ladder was used. Bands indicated by arrows were purified and sequenced (for results, see Table 1).

sity, 1.2; richness, 0.9). *Aulacomnium*-associated bacteria were characterized by a diversity index of 3.2 and a richness index of 1.5.

No common single species was obtained from the gametophytes of all three mosses. Three species were found on *Sphagnum* and *Aulacomnium*: *Burkholderia fungorum*, *Burkholderia phenazinium*, and *Burkholderia terricola*. For *Sphagnum* and *Tortula*, only *Pseudomonas fluorescens* was detected. No common species were found for *Aulacomnium* and *Tortula.* The most abundant bacterial species were *Burkholderia phenazinium* (31 isolates) and *Serratia liquefaciens* (8 isolates). The dominant species for the single mosses were *B. phenazinium* (25 isolates) for *Sphagnum*, *S. liquefaciens* (8 isolates) for *Aulacomnium*, and *Pseudomonas putida* (7 isolates) for *Tortula*. A high percentage of species (65%) was detected only on one single moss, and a large proportion of species (7) were isolated only once, e.g., *Budvicia aquatica*, *Rahnella aquatilis*, and *Serratia fonticola*.

FIG. 1. Scanning electron micrographs of the surfaces of gametophytes of *T. ruralis* (top), *A. palustre* (center), and *S. rubellum* (bottom). Bars, 8 μm; arrowheads indicate bacterial cells.

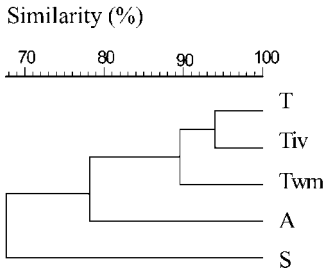


FIG. 3. Dendrogram based on amplified 16S rDNA fragments of the moss-associated communities from *T. ruralis* from Rostock-Warnemünde (Twm), *T. ruralis* from the island of Vilm (Tiv), and *T. ruralis* (T), *S. rubellum* (S), and *A. palustre* (A) from Ribnitzer Großes Moor, obtained by using universal primers and separated by SSCP. The patterns obtained were grouped by UPGMA.

Moss-associated antagonists belong mainly to β-*Proteobacteria* (50 isolates) as well as γ-*Proteobacteria* (18 isolates) and enteric bacteria (13 isolates). Gram-positive antagonistic isolates accounted for only a small proportion of the *Verticillium* antagonists (two isolates). The phylogenetic relationships among moss-associated isolates with antagonistic properties are depicted in Fig. 4. According to the 16S rDNA sequences of the isolates, three cluster groups were found: the *Serratia* cluster, the *Pseudomonas* cluster, and the *Burkholderia* cluster. The *Serratia* cluster contained isolates from *Sphagnum* and *Aulacomnium*, the *Pseudomonas* cluster contained isolates from *Sphagnum* and *Tortula*, and the *Burkholderia* cluster contained isolates from *Sphagnum* and *Aulacomnium*.

Characterization of *Verticillium* antagonists belonging to the *Burkholderia* and *Pseudomonas* groups by BOX-PCR. *Verticillium* antagonists assigned by 16S rDNA sequencing to the genus *Pseudomonas* were isolated from all three of the moss species, whereas, in contrast, *Burkholderia* isolates were isolated only from *Sphagnum* and *Aulacomnium*. A total of 40 *Burkholderia* and 16 *Pseudomonas* isolates were genotypically characterized by their BOX fingerprints to detect moss-specific genotypes (Fig. 5). The intraspecies diversity of BOX patterns analyzed in three independent replicates for each isolate was shown at 89% similarity. Analysis of BOX patterns for *Burkholderia* of more than 85% similarity resulted in 21 different cluster or genotype groups, although 10 of them contained only one isolate. Only five groups contained isolates from *Sphagnum* and *Aulacomnium*. Analysis of BOX patterns for *Pseudomonas* of more than 85% similarity resulted in eight different cluster or genotype groups, although five of them contained only one isolate. The majority of groups contained isolates from only one moss species. Overall analysis of genotypes demonstrated a high degree of moss specificity.

Antifungal activity and production of antifungal metabolites of *Verticillium* antagonists. All 83 antagonists were screened in vitro for their activities against the fungal pathogen *V. dahliae* and the bacterial pathogen *X. campestris* (Table 2). Generally, the fungus grew as well as the bacterial isolates on Waksman agar. Inhibition was clearly discerned by limited growth, or the

TABLE 1. Results of partial sequence analyses and tentative phylogenetic affiliations of bands

Origin and SSCP band	Most closely related sequence(s)	% Identity	GenBank accession no.
A. palustre			
1a	*Photorhabdus luminescens*	99	AY444555
2a	*Collimonas fungivorans*	99	AJ496444
3a	Uncultured eubacterium WD 247	94	AJ292581
4a	*Acinetobacter* sp.	94	AJ633638
5a	Rhizosphere soil bacterium	97	AJ252602
	Flavobacterium heparinum	96	M11657
6a	Unidentified eubacterium	91	AF009975
7a	*Pseudomonas* sp.	99	AY337597
	Pseudomonas grimontii	99	AF268029
8a	Uncultured eubacterium TRB82	97	AF047646
9a	*Pseudomonas migulae*	99	AF501370
S. rubellum			
1s	*Pseudomonas* sp.	99	AY456705
2s	*Pseudomonas grimontii*	100	AF268029
3s	Uncultured eubacterium WD 229	91	AJ292593
4s	*Methylobacterium mesophilicum*	93	AJ400919
5s	*Acidocella* sp.	95	AF376021
6s	*Acetobacter malorum*	96	AJ419844
7s	Gamma proteobacterium	98	AY135638
	Frateuria aurantia	97	AB091201
8s	Uncultured eubacterium WD260	97	AJ292673
T. ruralis			
1t	*Pseudomonas aeruginosa*	99	AY360347
2t	*Bacteroidetes* bacterium	96	AY337604
	Sphingomonas-like sp.	96	X89912
3t	*Rhodococcus erythropolis*	99	AY281114
4t	*Acidovorax wohlfahrtii*	98	AJ400840
5t	*Cytophaga* sp.	96	AB015550
6t	*Bartonella henselae*	90	AY513504
7t	*Flexibacter sancti*	95	AB078068

TABLE 2. Taxonomic classification and characterization of bacterial isolates with antagonistic properties

Strain	Identification by 16S rDNA sequencing or FAME[a]	GenBank accession no.	SI[b]	Taxonomic grouping	Activity[c] against:		Enzyme, siderophore, or antibiotic production[d]				
					V. dahliae	*X. campestris*	Glucanases	Proteases	Chitinases	Siderophores	Antibiotics
1T1	*Pseudomonas putida**		0.429	γ-*Proteobacteria*	++	+++	−	+	−	+	0
1T2	*Pseudomonas putida**		0.919	γ-*Proteobacteria*	++	+++	−	+	−	+	55
1T3	*Pseudomonas putida**		0.919	γ-*Proteobacteria*	+	−	−	+	−	+	59
1T4	*Pseudomonas reactans*	AF320987	97	γ-*Proteobacteria*	++	−	−	+	−	+	55
1T5	*Pseudomonas putida**		0.877	γ-*Proteobacteria*	++	−	−	+	+	+	40
1T6	*Pseudomonas reactans*	AF320987	98	γ-*Proteobacteria*	++	−	−	+	−	+	34
1T7	*Pseudomonas putida**		0.937	γ-*Proteobacteria*	++	−	−	+	−	+	22
1T8	*Pseudomonas putida**		0.888	γ-*Proteobacteria*	++	−	−	+	−	+	10
1T9	*Pseudomonas reactans*	AF320987	98	γ-*Proteobacteria*	+	−	−	+	−	−	ND
T5	*Pseudomonas* sp.	AY089990	98	γ-*Proteobacteria*	+	−	+	+	−	+	20
T6	*Pseudomonas* sp.	AY089990	98	γ-*Proteobacteria*	+	−	−	+	−	+	23
Tiv17	*Pseudomonas* sp.	AY089990	97	γ-*Proteobacteria*	++	+++	−	+	+	+	36
Twm31	*Xanthomonas* sp.	AB016762	97	γ-*Proteobacteria*	++	−	+	+	+	−	26
Twm74	*Pseudomonas fluorescens*	AF368760	97	γ-*Proteobacteria*	+	−	+	+	+	−	20
1A1	*Serratia proteamaculans*	AY040208	98	*Enterobacteria*	+++	−	−	+	+	+	31
1A2	*Serratia liquefaciens**		0.862	*Enterobacteria*	+++	−	−	+	+	+	61
1A3	*Serratia liquefaciens**		0.902	*Enterobacteria*	++	−	−	+	+	+	7
1A4	*Serratia proteamaculans*	AY040208	98	*Enterobacteria*	++	−	−	+	+	+	76
1A5	*Serratia liquefaciens**		0.802	*Enterobacteria*	++	−	−	+	+	+	40
1A6	*Serratia proteamaculans*	AF286867	98	*Enterobacteria*	+++	−	−	+	+	+	10
1A7	*Serratia liquefaciens**		0.680	*Enterobacteria*	+++	−	−	+	+	+	19
1A8	*Serratia liquefaciens**		0.902	*Enterobacteria*	+++	−	−	+	+	+	10
1A9	*Serratia liquefaciens**		0.887	*Enterobacteria*	+++	−	−	+	+	+	16
1A10	*Serratia liquefaciens**		0.762	*Enterobacteria*	+++	−	−	+	+	+	31
1A11	*Burkholderia fungorum*	AAAC01000192	97	β-*Proteobacteria*	+	−	−	−	−	+	3
1A16	*Burkholderia phenazinium*	AY154375	98	β-*Proteobacteria*	+	−	−	+	−	+	3
A1	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	++	−	+	+	−	−	20
A2	*Burkholderia* sp.	AJ300693	98	β-*Proteobacteria*	++	−	−	−	−	−	52
A3	*Burkholderia phenazinium*	U96936	97	β-*Proteobacteria*	++	+	+	+	−	−	23
A5	*Burkholderia* sp.	AJ300693	98	β-*Proteobacteria*	++	−	−	−	−	−	ND
A6	*Burkholderia phenazinium*	AY154375	98	β-*Proteobacteria*	++	−	+	+	−	−	13
A8	*Bacillus pumilus*	AY167882.1	99	β-*Proteobacteria*	++	−	−	−	−	−	ND
A11	*Pseudomonas* sp.	AY089990	98	γ-*Proteobacteria*	+	−	−	−	−	−	ND
A12	*Burkholderia phenazinium*	AB021394	97	β-*Proteobacteria*	++	−	+	+	−	−	30
A14	*Burkholderia terricola*	AY040362	97	β-*Proteobacteria*	+++	−	−	−	−	−	42
A15	*Xanthomonas* sp.	AB016762	97	γ-*Proteobacteria*	++	−	−	−	−	−	ND
A16	*Pseudomonas* sp.	AY089990	97	γ-*Proteobacteria*	+	−	−	−	−	−	39
A17	*Burkholderia phenazinium*	AY154375	98	β-*Proteobacteria*	+	−	−	−	−	−	ND
A20	*Burkholderia* sp.	AJ300693	98	β-*Proteobacteria*	+	−	−	−	−	−	ND
A21	*Burkholderia phenazinium*	U96936	97	β-*Proteobacteria*	+	−	−	−	−	−	ND
A22	*Burkholderia* sp.	AJ300693	97	β-*Proteobacteria*	+	−	−	−	−	+	36
A23	*Collimonas fungivorans*	AJ496444	98	β-*Proteobacteria*	+	−	−	−	−	−	39
A24	*Burkholderia* sp.	AJ300693	98	β-*Proteobacteria*	+	−	−	−	−	+	23
A25	*Burkholderia terricola*	AY040362	98	β-*Proteobacteria*	+	−	−	−	−	−	ND
1S1	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	+++	−	−	−	−	+	10
1S2	*Micrococcus lylae**		0.770		+++	−	−	−	−	+	31
1S3	*Burkholderia gladioli**		0.436	β-*Proteobacteria*	+++	−	−	−	−	+	13
1S4	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	+++	−	−	−	−	+	10
1S5	*Burkholderia terricola*	AY040362	99	β-*Proteobacteria*	+	−	−	+	−	+	31
1S8	*Burkholderia gladioli**		0.437	β-*Proteobacteria*	+++	−	−	−	−	−	64
1S9	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	++	−	−	−	−	+	66
1S10	*Burkholderia gladioli**		0.680	β-*Proteobacteria*	+++	−	−	−	−	+	64
1S11	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	+++	−	−	−	−	+	10
1S12	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	++	−	−	−	−	+	ND
1S13	*Paenibacillus pabuli**		0.827	*Firmicutes*	+++	−	−	−	−	+	16
1S15	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	+++	−	−	−	−	+	13
1S16	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	+++	−	−	−	−	+	10
1S18	*Burkholderia cepacia*		0.399	β-*Proteobacteria*	+++	−	−	−	−	+	10
S1	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	+++	−	−	−	−	+	55
S2	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	+++	−	−	−	−	++	19
S3	*Rahnella aquatilis*	AY253919.1	98	*Enterobacteria*	+++	−	−	−	−	++	7
S4	*Pseudomonas* sp.	AF094725	97	γ-*Proteobacteria*	++	−	−	−	−	+	55
S5	*Burkholderia terricola*	AY040362	97	β-*Proteobacteria*	+++	−	−	−	−	++	10

Continued on facing page

TABLE 2—*Continued*

Strain	Identification by 16S rDNA sequencing or FAME[a]	GenBank accession no.	SI[b]	Taxonomic grouping	Activity[c] against:		Enzyme, siderophore, or antibiotic production[d]				
					V. dahliae	*X. campestris*	Glucanases	Proteases	Chitinases	Siderophores	Antibiotics
S6	*Rahnella aquatilis*	U90758	98	*Enterobacteria*	++	−	−	−	−	−	ND
S7	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	++	−	−	−	−	+	10
S9	*Burkholderia phenazinium*	U96939	98	β-*Proteobacteria*	++	−	−	+	−	+	23
S11	*Burkholderia* sp.	AY134849	97	β-*Proteobacteria*	+++	−	−	−	−	−	7
S12	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	++	+	−	−	−	+	20
S13	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	++	−	−	−	−	+	26
S14	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	+++	−	−	−	−	+	10
S15	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	++	−	−	−	−	++	23
S16	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	++	−	−	−	−	++	20
S17	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	+	−	−	−	−	−	4
S18	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	+	−	−	−	−	++	23
S19	*Serratia fonticola*	AF286869	97	*Enterobacteria*	++	−	−	−	−	+	26
S20	*Burkholderia phenazinium*	U96936	99	β-*Proteobacteria*	++	−	−	−	−	+	26
S22	*Burkholderia phenazinium*	U96936	99	β-*Proteobacteria*	++	+	−	−	−	++	17
S23	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	++	+	−	−	−	++	20
S25	*Burkholderia phenazinium*	U96936	97	β-*Proteobacteria*	++	+	−	−	−	++	ND
S26	*Burkholderia phenazinium*	U96936	97	β-*Proteobacteria*	+++	−	−	−	−	++	26
S28	*Burkholderia fungorum*	AAAC01000192	98	β-*Proteobacteria*	++	−	−	−	−	+	23
S31	*Burkholderia phenazinium*	U96936	97	β-*Proteobacteria*	++	+	−	−	−	++	25
S32	*Burkholderia phenazinium*	U96936	98	β-*Proteobacteria*	++	−	−	−	−	++	23

[a] Asterisks indicate isolates identified by FAME analysis.
[b] SI, similarity index. For isolates identified by FAME analysis, values range from 0 to 1; for isolates identified by 16S rDNA sequencing, values range from 0 to 100%.
[c] Antagonism toward *V. dahliae* and *X. campestris* was determined by dual-culture assay. +, 0 to 5-mm-wide zone of inhibition; ++, 5 to 10-mm-wide zone; +++, 10 to 15-mm-wide zone.
[d] Production of glucanases, proteases, chitinases, and siderophores was determined by plate assays. +, hydrolysis; −, no hydrolysis. Antibiotic production was determined by in vitro bioassays with sterilely filtered culture supernatants; results are expressed as percentages of fungal growth inhibition. ND, not determined.

complete absence of fungal mycelium or bacterial growth in the inhibition zone surrounding a bacterial colony. Only nine isolates possessed antifungal as well as antibacterial activity. Three strong *Xanthomonas* antagonists were selected from *Tortula* and were found to be pseudomonads. Additionally, in vitro production of antagonistic metabolites was analyzed. Only seven isolates with glucanolytic activity were found; none of the *Sphagnum* antagonists was able to produce glucanase. Production of proteases was moss specific: all of the *Tortula*-associated antagonists were proteolytic, but only two proteolytic antagonists were isolated from *Sphagnum*. Twenty-two percent of the isolates possessed chitinolytic properties, and all strongly chitinolytic isolates were associated with *Aulacomnium*. The production of siderophores (77% of the isolates) and antifungal antibiotics (99% of the isolates) was extremely widely distributed. *Verticillium* antagonists assigned to the same species often displayed different patterns of antagonistic activity, as was the case, for example, with *S. liquefaciens*.

Concluding assessment of the in vitro screening. All of the parameters tested were used to evaluate the strains by means of a point system. For antagonistic activity, three points were awarded for high activity against either of the two pathogens (totaling a possible six points). One point each was awarded for production of an enzyme (chitinase, glucanase, or proteinase) or siderophore (for a possible four points), and antibiotic production received zero to three points depending on the level. Based on this system, the number of points for each isolate was established in order to evaluate the strains. Among the *Sphagnum* isolates, *B. phenazinium* S22 was the most efficient (nine points). *Pseudomonas* sp. strain Tiv17 was the most efficient isolate from the *Tortula* gametophyte (11 points). Three *Serratia* isolates, 1A1, 1A2, and 1A10, identified as *Serratia proteamaculans*/*liquefaciens*, were the most effective antagonists from *Aulacomnium* (nine points).

DISCUSSION

In the present study, a high degree of specificity of moss-associated bacteria was found. Here we combined cultivation-dependent techniques with two cultivation-independent methods to analyze the moss-associated bacterial populations. The latter were based on the extraction of bacterial DNA and amplification of the 16S rDNA fragments, which were then separated by DGGE and/or SSCP. Both methods gave the same picture, showing clearly the moss specificity of the associated bacterial communities. To our knowledge, this is the first report which documents an analysis of moss-associated bacteria with these particular molecular tools. For various crop species, the specificity of associated bacterial communities was demonstrated by Smalla et al. (36) and Berg et al. (7); that for wild plants was demonstrated by Kowalchuk et al. (20). Cultivation on R2A medium was also used to isolate bacteria and to investigate their properties. By this approach, moss specificity could be confirmed by using the following parameters: (i) plate counts of bacteria on R2A medium, (ii) percentage of antagonistic isolates, (iii) antagonistic activity as well as spectrum against pathogens, and (iv) diversity and richness of antagonistic isolates. Overall, the combination of methods was employed successfully to define and to illustrate the specificity and diversity of moss-associated bacteria.

For this study, samples were isolated from bryophytes inhabiting natural nutrient-poor habitats on the southern Baltic Sea coast. Interestingly, correlations were found between the natural abiotic gradients in the ecosystem pH, moisture, and

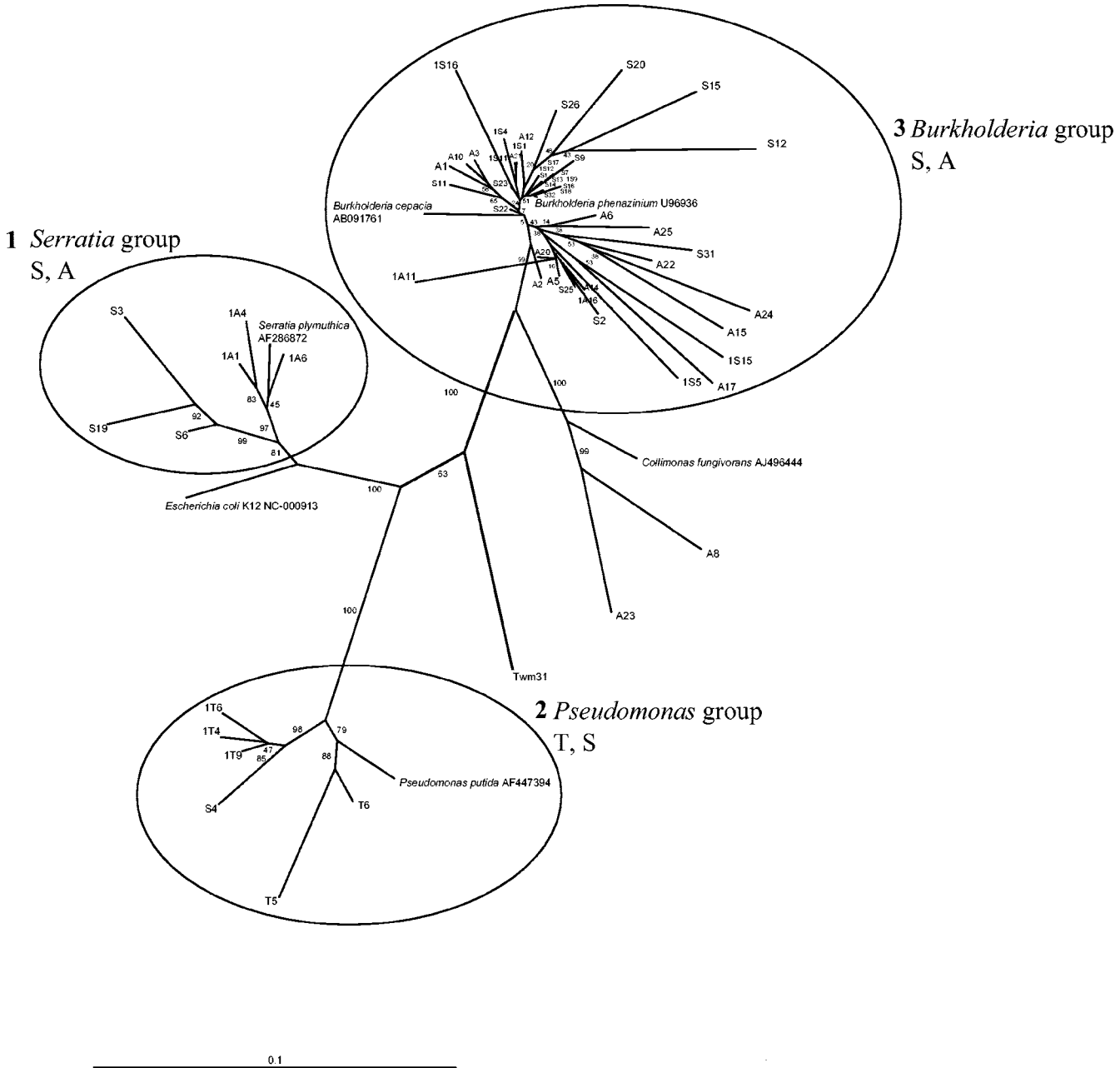


FIG. 4. Phylogenetic dendrogram calculated with all 16S rDNA sequences that were recovered from bacteria associated with the mosses *T. ruralis* (T), *A. palustre* (A), and *S. rubellum* (S), grouped by the neighbor-joining method. Additionally, type strain sequences of *Burkholderia cepacia*, *Collimonas fungivorans*, *E. coli*, *P. putida*, and *Serratia plymuthica* were added. Most of the isolates belong to three different cluster groups. Bar indicates 10% sequence divergence.

nutrient content and the colonization of bryophytes. The abundance of culturable bacteria increased with pH. In contrast, the percentage of antagonistic bacteria, along with the intensity of antagonism, richness, and diversity, increased with decreasing pH and moisture of these microenvironments. Different structures of leaf surfaces could be another reason for the specificity and diversity of moss-associated bacteria. As shown on micrographs, leaf surfaces formed highly specific microenvironments.

The second key aim of our investigation was to ascertain whether moss-associated bacteria displayed any biotechnological potential. Plant-associated bacteria and those with antagonistic properties are important not only for plant health and growth but also for biotechnological applications. For example, they can be used directly, for biological control of plant pathogens (41), or indirectly, for the production of active substances, e.g., antibiotics or enzymes (16). In comparison to plant-associated bacteria, approximately twofold higher active-substance production was detected for moss-associated bacteria, especially those from *Sphagnum*. The proportion of antagonistic

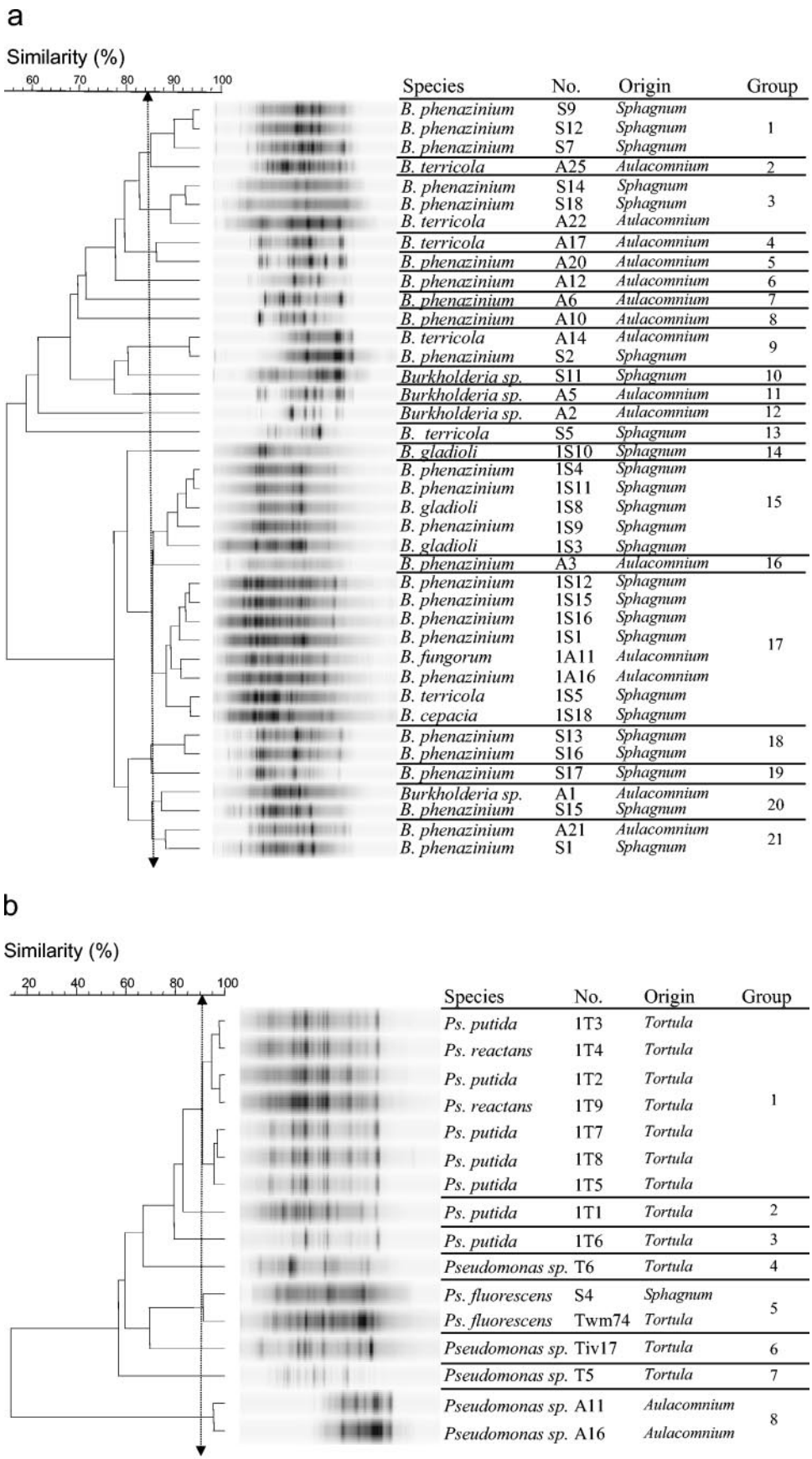


FIG. 5. Dendrogram showing the relationships of 38 *Burkholderia* isolates (a) and 16 *Pseudomonas* isolates (b) from *Tortula*, *Aulacomnium*, and *Sphagnum* based on BOX-PCR fingerprints grouped by UPGMA. Double-headed vertical arrows indicate the similarity for the groupings.

bacteria was 3 to 9% in the rhizosphere of *Verticillium* host plants (7), 16% in the rhizosphere of oilseed rape (5), and as much as 18% for various weeds (22). The high proportion is much more surprising when the rhizosphere effect is taken into consideration. This is the well-known phenomenon that, in comparison to that in other plant-associated microenvironments or in bulk soil in the rhizosphere, the proportion of microorganisms (including those with antagonistic properties) is enhanced because of the rich exudation of roots (21, 36). For bryophytes, which possess only a root-like rhizoid, no exudation of nutrients is known. Most of the mosses are ectohydric, which means that the gametophytes can absorb water and dissolved minerals over their surfaces. In this way, the leaf surface of mosses resembles the rhizosphere. This may be one reason for bacterial colonization. While *Pseudomonas* and *Serratia* are well-known antagonistic genera (6, 25), *Burkholderia* is an interesting and rarely mentioned genus. In 2001, Estrada-De Los Santos et al. (13) demonstrated that nitrogen fixation is a common property in the genus *Burkholderia*. In addition, *Burkholderia* strains have been shown to be plant growth-promoting rhizobacteria (40). Here, we selected five isolates with very high biotechnological potential, namely, *Pseudomonas* sp. strain Tiv17, *B. phenazinium* S22, and the *Serratia* isolates 1A1, 1A2, and 1A10. Generally, a high percentage of antibiotic-producing bacteria was found.

Recently, mosses have been proposed as ideal models for genetic studies and biotechnological applications (10). Therefore, it is essential to establish whether the mosses were colonized with a variety and abundance of microorganisms as well as whether these microorganisms performed important functions in the mosses' life cycle, health, and growth. Additionally, moss-associated bacteria have great potential for biotechnological applications. Our results indicate that bryophyte-associated bacteria are important potential sources of antifungal antibiotics and that some of them are interesting candidates for biological control agents against plant pathogens.

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