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Isolation and Characterization of Novel Marine-Derived Actinomycete Taxa Rich in Bioactive Metabolites†

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A unique selective enrichment procedure has resulted in the isolation and identification of two new genera of marine-derived actinobacteria. Approximately 90% of the microorganisms cultured by using the presented method were from the prospective new genera, a result indicative of its high selectivity. In this study, 102 actinomycetes were isolated from subtidal marine sediments collected from the Bismarck Sea and the Solomon Sea off the coast of Papua New Guinea. A combination of physiological parameters, chemotaxonomic characteristics, distinguishing 16S rRNA gene sequences, and phylogenetic analysis based on 16S rRNA genes provided strong evidence for the two new genera (represented by strains of the PNG1 clade and strain UMM518) within the family *Micromonosporaceae*. Biological activity testing of fermentation products from the new marine-derived actinomycetes revealed that several had activities against multidrug-resistant gram-positive pathogens, malignant cells, and vaccinia virus replication.

The order *Actinomycetales* is composed of approximately 80 genera, nearly all from terrestrial soils, where they live primarily as saprophytes (30, 36). All members of the order are characterized in part by their high G+C DNA content, and most exhibit a highly differentiated developmental life cycle (30). Several recent systematic reevaluations of the order reflected more closely the integration of molecular data (16S rRNA gene sequences). This reorganization resulted in the placement of the two most prolific producers of secondary metabolites, *Streptomyces* and *Micromonospora*, in the families *Streptomycetaceae* and *Micromonosporaceae*, respectively (30).

The diversity of actinomycete secondary metabolites is unrivaled and unmatched in medical significance. Structurally and functionally diverse bioactive compounds have also been isolated from other prokaryotes, including members of the myxobacteria (e.g., *Sorangium*) and cyanobacteria (e.g., *Nostoc*), as antibiotics with antimicrobial, antiviral, and antitumor activities (16, 21, 24, 25). Recently, the rate of discovery of new compounds from existing genera obtained from terrestrial sources has decreased, while the rate of reisolation of known compounds has increased. Moreover, the rise in the number of drug-resistant pathogens and the limited success of strategies such as combinatorial chemistry in providing new agents indicate an uncertain forecast for future antimicrobial therapy (17, 18). Thus, it is critical that new groups of microbes from unexplored habitats be pursued as sources of novel antibiotics and other small-molecule therapeutic agents (3).

The oceans cover more than 70% of the earth's surface, and little is known about the microbial diversity of marine sediments. As with terrestrial soils, marine sediments contain limited amounts of readily available organic matter, with most sources of carbon being present in complex forms (i.e., cellulose and chitin). However, culture-independent methods have demonstrated that marine sediments contain a wide range of unique microorganisms not present in the terrestrial environment (20, 29). These studies and the recent isolation of the MAR1 clade show that new actinobacteria are present widely in marine subtidal habitats (14). Isolation strategies directed toward new marine-derived actinomycetes have been lacking, but it is clear that such procedures will increase the understanding of marine bacterial diversity.

Here we describe a new selective enrichment procedure that has resulted in the discovery of two novel actinomycete taxa from marine sediments collected from the Bismarck Sea and the Solomon Sea off the coast of Papua New Guinea. A polyphasic approach including physiological and chemotaxonomic analyses as well as phylogenetic analysis based on 16S rRNA gene sequences has led to the identification of two proposed new genera within the family *Micromonosporaceae*. The enrichment method that we describe is highly selective for such strains; of the 102 strains isolated in this study, over 90% represented the two new groups of microorganisms. Evidence is also provided that these new marine-derived actinobacteria produce a variety of bioactive metabolites.

MATERIALS AND METHODS

Environmental sampling. A total of 192 sediment samples were collected on two separate expeditions (2001 and 2002) to the Bismarck Sea, in Madang Province, off the northern coast of Papua New Guinea, and to the Solomon Sea, in Milne Bay Province. Samples were obtained from the first 1 to 5 cm of sediment by scuba collection at depths of 10 to 40 m and placed in sterile 50-ml conical tubes. Samples were kept at room temperature during the expedition and at 4°C upon return to the laboratory.

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Enrichment and isolation of slowly growing marine microorganisms. Approximately 50 mg of each wet sediment sample was used to inoculate an NaST21Cx agar plate. NaST21Cx is derived from ST21Cx (25) by elimination of yeast extract and replacement of distilled water with artificial seawater. The complete composition of NaST21Cx agar is solution A (750 ml of artificial seawater containing 1 g of K_2HPO_4 and 10 g of Bacto Agar) and solution B (250 ml of artificial seawater containing 1 g of KNO_3, 1 g of M_gSO_4, 1 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of $FeCl_3$, and 0.1 g of $MnSO_4 \cdot 7H_2O$). Solutions A and B are autoclaved separately for 30 min, mixed together following autoclaving, and supplemented with 1 ml of trace element solution (5) and 25 μg of cycloheximide/ml. Whatman no.1 sterile filter paper disks (precut to fit the agar surface) were placed on the agar, and the sediment material was dispersed evenly on the surfaces of the cellulose disks. The plate was incubated in a humidified chamber at 30°C for 30 to 90 days. Following incubation, selected colonies were streaked on ISP-2 medium (BD Diagnostics, Sparks, Md.) prepared with artificial seawater (ISP-2/ASW) and containing cycloheximide (25 μg/ml) and nalidixic acid (25 μg/ml).

Propagation and fermentation media consisted of the following: M1 medium prepared with artificial seawater (M1/ASW), which contained 10 g of starch, 4 g of yeast extract, 2 g of peptone, 18 g of agar, and 1 liter of artificial seawater; ISP-2/ASW; and tryptic soy broth (TSB) agar (BD Diagnostics) prepared with artificial seawater (TSB/ASW). Pure cultures were stored temporarily on agar slants (TSB/ASW); for long-term storage, cultures were frozen in 20% glycerol at −80°C.

Testing for cellulolytic ability of marine-derived actinomycetes. Strains were tested for the ability to degrade cellulose by two different plate methods. In each, a loopful (two or three well-developed colonies on ISP-2 medium plus 3.0% NaCl) of the marine-derived actinomycete of interest was transferred to test petri plates. The first procedure involved the use of a two-layer agar plate; the bottom layer was composed of TSB agar plus 3% NaCl, and the top layer was 1.5% water-agar supplemented with 0.6% carboxymethyl cellulose (a semisynthetic, soluble ether derivative of cellulose). After 14 days of incubation, the colonies were examined with a dissecting microscope for zones of clearing (22). The second procedure involved the use of nutrient salts agar supplemented with 3% (wt/vol) powdered cellulose and incubation of cultures for 14 to 21 days (27).

Electron microscopy. Cultures grown in ISP-2 medium plus 3% NaCl were centrifuged, and the pellet was fixed with 2.5% (wt/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer and postfixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer. Samples were dehydrated with a gradient series of ethanol concentrations (50, 70, 80, 95, 95, 100, 100, and fresh 100%). The resulting preparations were dried at the critical point of liquid CO_2, sputter coated with platinum (1 to 3 nm), and examined with a Hitachi model 4700 scanning electron microscope. For analysis of submerged sporulation in a simulated marine environment, strains of the marine-derived actinomycetes were grown for 14 days at 30°C with shaking in 20 ml of low-nutrient medium prepared with artificial seawater (MN1/ASW); this medium contained 0.25 g of glucose, 0.25 g of yeast extract, 0.64 g of malt extract, and 1 liter of artificial seawater and was adjusted to pH 7. Five milliliters of cell suspension was transferred to a 50-ml Oak Ridge tube and centrifuged at 4,000 rpm for 5 min in a Centra 4 model centrifuge (International Equipment Company). The cell pellet was resuspended in 1 ml of MN1/ASW and prepared for electron microscopy as described above.

Isolation of marine-derived actinomycete DNA. A protocol was devised for efficient actinomycete genomic DNA extraction. Cultures were grown in 20 ml of TSB in 50-ml conical tubes for 7 days and then centrifuged at 4,000 rpm (Centra 4 model centrifuge; International Equipment Company) for 5 min. The mycelial pellet was resuspended in 500 μl of 5 M NaCl and transferred to a 2-ml Eppendorf tube. The cells were centrifuged (Eppendorf model 5415 centrifuge) at 10,000 rpm for 30 s, and the pellet was resuspended in 1 ml of 10 mM Tris-Cl–1 mM EDTA (pH 7.5) (TE) containing 20 mg of lysozyme/ml and 20 mg of RNase A/ml and incubated at 37°C for 1 h. Following incubation, 250 μl of 0.5 M EDTA, 250 μl of TE containing 5 mg of proteinase K/ml, and 100 μl of 10% sodium dodecyl sulfate were added to each tube and incubated at 37°C for 1 h. The tubes were mixed by inversion after the addition of 250 μl of 5 M NaCl. Immediately thereafter, 200 μl of cetyltrimethylammonium bromide (CTAB) solution (10% CTAB plus 0.7 M NaCl) was added, and the tubes were heated in a 65°C water bath for 10 min. Cellular debris was removed by centrifugation (8,000 rpm for 5 min in an Eppendorf model 5415 centrifuge), and the supernatant solution was transferred to a new 2-ml microcentrifuge tube. Proteins and lipids were removed by the addition of 0.3 volume of phenol-chloroform, and the phases were mixed by inversion and centrifuged at 12,500 rpm for 5 min. The aqueous phase was transferred to a new tube, and the DNA was precipitated with an equal volume of isopropanol. After the genomic DNA was centrifuged, the pellet was rinsed with 70% ethanol to remove traces of salt, dried, and redissolved in 200 μl of TE for immediate use or storage at −20°C.

16S rRNA gene amplification and DNA sequencing. The 16S rRNA gene was amplified from genomic DNA obtained from bacterial cultures by PCR with previously described primers FC27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RC1492 (5′-TACGGCTACCTTGTTACGACTT-3′) (19). The reaction mixture contained 25 to 50 ng of DNA, *Ex*Taq PCR buffer, 1.5 mM $MgCl_2$, 10 mM deoxynucleoside triphosphate mixture, 50 pmol of each primer, and 0.5 U of Ex*Taq* polymerase. PCR conditions consisted of an initial denaturation at 80°C for 5 min; 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and a final 7-min extension at 72°C. The amplification products were examined by agarose gel electrophoresis and purified by using a QIAquick PCR cleanup kit with the protocol suggested by Qiagen Inc., Chatsworth, Calif. The nearly complete 16S rRNA gene was sequenced by using the PCR products directly as sequencing templates with the following primers: FC27 and RC1492 (used in template amplification), F357 (5′-TACGGGAGGCAGCAG-3′), F803 (5′-ATTAGATA CCCTGGTAG-3′), F1114 (5′-GCAACGAGCGCAACCC-3′), RM519 (5′-GT CTTACCGCGGCTGCTG-3′), RM907 (5′-CCGTCAATTCCTTTGAGTTT-3′), and RM1378 (5′-CGGTGTGTACAAGGCCCGGGAACG-3′). The design of the sequencing primers and the methodology used were described previously (14, 19). All sequencing reactions were carried out with an ABI 3700 automated DNA sequencer at the Advanced Genetic Sequencing Center, University of Minnesota.

Molecular taxonomy determined by sequences and phylogenetic analysis. Nucleotide sequences were analyzed and edited by using BioEdit software (8). The nearly complete 16S rRNA gene sequences (averaging 1,445 nucleotides) were used to search the GenBank database with the BlastN algorithm to determine relative phylogenetic positions. Sequences then were aligned by using ClustalX software (version 1.8.1) with representative actinomycete 16S rRNA gene sequences and the complete 16S rRNA gene sequence of *Escherichia coli* K-12 (34). The genus-level signature nucleotides of the emended family *Micromonosporaceae* were obtained from alignments as described by Koch et al. (10). Phylogenetic trees were constructed by first generating a complete alignment of 16S rRNA gene sequences of selected members of each genus within the family *Micromonosporaceae* by using ClustalX (34). The aligned sequences then were incorporated into Phylo_Win, and neighbor-joining and maximum-parsimony methods were used to generate bootstrap values (from 1,000 resamplings) and consensus trees (7). Manipulation and tree editing were done by using TreeView (15).

To assess the genetic diversity within the PNG 1 clade, ribotype analysis was done by using a RiboPrinter microbial characterization system (DuPont Qualicon, Wilmington, Del.). For each ribotype analysis, approximately two 10-μl loopfuls containing two to five actinomycete colonies were treated by existing protocols (23). Ribotypes were derived from PvuII digests (ribotype VCC) of the resulting genomic DNAs and the entire *E. coli* ribosomal operon used as a DNA probe according to the manufacturer's instructions (Dupont Qualicon). The exact ribotype of each strain then was downloaded into a database and analyzed within the ribotype software (Dupont Qualicon) to determine the level of divergence between the strains of interest.

Analysis of chemotaxonomic characteristics. Cultures were grown on TSB agar, and cells were harvested before the appearance of spores. The major fatty acids present were analyzed by using a microbial identification system according to the manufacturer's instructions (MIDI, Newark, Del.) The resulting profiles were analyzed and grouped on the basis of their chemical structures (9). The major membrane-associated menaquinones were determined by established protocols (33). Cell wall amino acids and sugars were isolated and analyzed by previously described protocols (31).

Production and testing of marine microbial secondary metabolites. Fermentations of purified strains first involved the generation of a seed culture in TSB prepared with 3% NaCl. The seed culture (20 ml) was grown with shaking in a 40-ml glass tube for 14 days. Following this initial growth stage, 2.5 ml of the seed culture was transferred to a 250-ml Erlenmeyer flask containing either 70 ml of M1/ASW (rich medium) or MN1/ASW. M1/ASW is an adaptation of M1 medium described by Sieburth (26) and used previously (14). Approximately 24 to 48 h after inoculation, 2.5 g of wet XAD-16 absorber resin (Aldrich, St. Louis, Mo.) contained within a nylon mesh bag measuring 4 by 4 cm was added to the culture, which was incubated with shaking (250 rpm) at 30°C for 10 to 21 days (depending on average cell density). The resin bag was removed, and the samples were extracted with 10 ml each of a graded series of methanol (30, 60, and 100%), acetone, and ethyl acetate. Each fraction was concentrated in vacuo, reconstituted in 100% dimethyl sulfoxide, and transferred to a 96-well plate for storage at −80°C until analysis for the production of biologically relevant activities. Disk diffusion assays were used to determine the antimicrobial activities of solvent extracts.

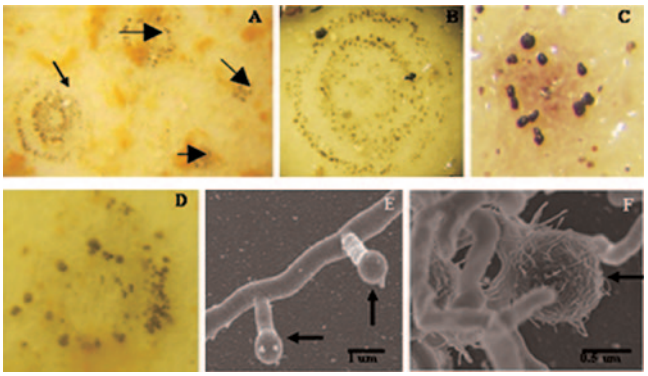

FIG. 1. Colony and spore morphologies of marine-derived actinomycetes of group I, group II, and group III. (A) NaST21Cx plate containing different marine actinomycete colonies (arrows) radiating outward from marine sediment particulate matter. (B) Isolation of UMM486 (group I) showing the growth pattern, phenotype characteristics, and concentric ring formation of group I members on NaST21Cx. (C) Isolation of UMM500 (group II) showing the growth pattern and phenotype characteristics of group II members on NaST21Cx. (D) Isolation of UMM518 (group III) showing the growth pattern and phenotype characteristics of group III members on NaST21Cx. (E) Scanning electron micrograph of UMM486 submerged spores (arrows). (F) Scanning electron micrograph of UMM518 submerged spores (arrows).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences determined for 22 strains in this study have been deposited in the GenBank database with the accession numbers indicated in parentheses: UMM478 (AY552771), UMM479 (AY552773), UMM480 (AY552770), UMM481 (AY552766), UMM483 (AY552761), UMM485 (AY552762), UMM486 (AY552774), UMM500 (AY552775), UMM502 (AY552757), UMM509 (AY552772), UMM514 (AY552758), UMM518 (AY552777), UMM526 (AY552759), UMM527 (AY552760), UMM537 (AY552763), UMM539 (AY552776), UMM543 (AY552769), UMM553 (AY552767), UMM559 (AY552756), UMM566 (AY552764), UMM573 (AY552765), and UMM576 (AY552768).

RESULTS

Enrichment and selective isolation of novel marine actinomycetes. During our examination of marine sediment collections from the 2001 Papua New Guinea expedition, with an emphasis on the isolation of marine myxobacteria (in particular, marine *Sorangium*), it became apparent that organisms not related to any members of the myxobacteria frequently appeared. The colonies were visually similar to the fruiting bodies characteristic of *Sorangium cellulosum* but instead were found to be gram-positive, filamentous organisms resembling several types of previously described terrestrial actinomycetes (i.e., *Micromonospora*) and actinomycetes of the marine MAR1 clade (Fig. 1A) (10, 14). In the subsequent expedition (2002 Papua New Guinea), we sought to determine whether this isolation procedure was indeed selective for this group of marine-derived actinomycetes. This turned out to be the case and presented an opportunity to proceed with an expanded effort to isolate and characterize new marine-derived actinomycetes.

Among 192 samples of marine sediments collected in the 2001 and 2002 expeditions to Papua New Guinea, approximately 100 yielded colony types characteristic of the two new genera that we have described. Such colonies were observed with the aid of a dissecting microscope after 15 to 30 days but became visible to the naked eye after 45 to 75 days. The colonies not only had a characteristic morphology but also were always arranged in concentric rings (Fig. 1A). Colonies from the outer rings were transferred to a Difco marine agar 2216 plate containing cycloheximide (25 μg/ml) and nalidixic acid (25 μg/ml). The colonies were similar in pigmentation (orange turning brown or black with age) and texture (waxy and leathery tough) and were typical of many actinobacteria of the family *Micromonosporaceae*.

After 45 to 60 days of incubation, colonies could be grouped into three distinct phenotypes, the most predominant being a group referred to as group I (Fig. 1; also see below). A predominant feature of all types was the concentric ring pattern with a central "parent colony " (Fig. 1A). Colonies from the growth rings were determined to be derived from the central colony by sequencing and comparing the 16S rRNA genes from each (data not shown). The ability to form concentric rings on this medium appears to be a chemotropic response to a growth-induced nutrient gradient and not the result of gliding motility. The formation of such concentric rings of colonies was not observed on other commonly used actinomycete isolation agars, such as International Streptomyces Project medium 2 (ISP-2) agar, chitin agar, oatmeal agar-ISP3, and marine agar 2216.

General characteristics of marine-derived actinomycetes. The first and most common group (Fig. 1B) was comprised of glistening colonies ranging from pale to bright orange and usually turning increasingly dark (deep brown to black) with age on all NaCl-containing media tested (ISP-2 medium, marine agar 2216, and TSB). The cells of these strains exhibited a finely branched filamentous pattern (filaments with diameters of <0.5 μm) when grown in both liquid and on agar plates (data not shown). Strains of this group were gram positive, did not make aerial mycelium, did not produce sporangia, and produced nonmotile spores that were borne singly off branched hyphae. Strains of this group were tolerant of salt concentrations up to 4% but did not require the presence of salt for growth. The strains were unable to decompose cellulose, as determined by two methods (see below). The singly borne spores contained, on average, three to six small spikes but had an otherwise smooth appearance (Fig. 1F). These characteristics are consistent with those of the genus *Micromonospora*; however, the ability of the strains to grow well at pHs 4 to 9 is not typical for the genus. Sensitivity to pHs below 6 is a defining characteristic of all previously described members of the genus *Micromonospora* (28).

The second group (Fig. 1C) formed glistening colonies that were purple on the isolation medium but turned orange to dark brown with age on other NaCl-containing media tested (ISP-2 medium, marine agar 2216, and TSB). When examined by light microscopy, all strains exhibited a finely branched filamentous pattern (filaments with diameters of <0.5 μm) when grown in both liquid and on agar plates (data not shown). Strains of this group were gram positive, did not produce aerial mycelium, did not produce sporangia, and produced nonmotile spores that were borne singly off branched hyphae. Strains of this group were tolerant of salt concentrations up to 4% but did not require salt for growth. The strains grew well at pHs 6 to 9, were not cellulolytic, and had growth rates faster than those of group I strains but not as fast as those of group III

strains. These characteristics are consistent with those of the genus *Micromonospora*; strain sensitivity to pHs below 6, unlike the first phenotype, is consistent with the previously described properties of *Micromonospora* (28).

Strains of the third group (Fig. 1D) had a dull orange pigmentation on all media tested; however, as sporulation occurred, the tops of the colonies turned from light tan to dark brown on other NaCl-containing media tested (ISP-2 medium, marine agar 2216, and TSB). These strains exhibited a finely branched filamentous pattern (filaments with diameters of <0.5 μm) when grown in both liquid and on agar plates. Strains of this group were gram positive, did not make aerial mycelium, did not produce sporangia, and formed nonmotile spores that were borne singly off branched hyphae. Strains of this group were tolerant of salt concentrations up to 5% but did not require salt for growth. The spore surface differed from those of groups I and II, having a "hairy" spore coat (Fig. 1F). The overall spore morphology was most similar to that of spores from the genus *Verrucosispora* (22). The strains grew well at pHs 5 to 9, were not cellulolytic, and had growth rates faster than those of group I and II strains.

Overall, the physiological characteristics of each group were comparable in most respects to those of existing members of the family *Micromonosporaceae*, such as the genera *Catellatospora*, *Verrucosispora*, and *Micromonospora* and the marine MAR1 clade (14, 30).

Molecular taxonomy of marine-derived actinomycetes. (i) Group I. A comparison of the nearly complete 16S rRNA gene sequences (averaging 1,445 nucleotides) of the strains tested here against sequences in the GenBank database revealed homologies of greater than 95% to members of the family *Micromonosporaceae*. The closest matches were to species belonging to the genus *Micromonospora*, in particular, *Micromonospora nigra* DSM 43818 (35). This match was consistently the top match, ranging from 97.9 to 98.5%; other, less close matches were to members of the MAR1 clade (14). The signature nucleotides of members of the *Micromonosporaceae* revealed differences from previously described members. A comparison of the signature nucleotides from one strain (UMM486) belonging to group I with all signature nucleotides of genera of the *Micromonosporaceae* revealed that the UMM486 sequences did not match the consensus signature nucleotide sequences in existing genera (which contained 11 of 12 of the *Micromonospora* signature nucleotides). All other sequenced strains of group I were examined and had the same 12 signature nucleotides, a requirement for generic membership in this family (10). In addition, the group I strains had another conserved nucleotide (outside the recognized 12 signature nucleotides) that was thus an additional unifying molecular signature of this clade (U at position 1491, according to *E. coli* numbering). This finding provides another level of distinction from other members of the *Micromonosporaceae*.

Both neighbor-joining and parsimony phylogenetic methods yielded consensus tree topologies that grouped all 16S rRNA gene sequences obtained from strains of group I into a single coherent clade (Fig. 2). The grouping was supported by high bootstrap values (98% neighbor joining and 89% parsimony from 1,000 resamplings), but relationships within the clade could not be determined accurately. The high intraclade homology (99%) typical of other genera within the family was also evident in the phylogenetic tree with UMM486 as the anchoring strain and candidate strains UMM559, UMM573, and UMM537 as the most divergent members of the clade (12). Differences within the PNG1 clade, on the basis of more than 16S rRNA gene sequences alone, were determined by riboprint analysis.

Riboprinting, a validated bacterial taxonomic technique, determines the restriction fragment length polymorphisms of the entire ribosomal operon by heterologous hybridization with the entire *E. coli* ribosomal operon as a DNA probe (1, 4). The riboprints of the PNG1 clade strains are shown in Fig. 3. Intraclade divergence was suggested by visual comparison of the hybridization banding pattern. An algorithm was also used to calculate the relative divergence from UMM486 (the anchoring PNG1 clade strain) (Fig. 3). The ribotype of UMM559 was the most different from that of UMM486 and, as a result, emerged as the most deeply branching member of the clade. Strains UMM480, UMM485, and UMM526 displayed highly similar ribotypes and thus were considered the same species and possibly subspecies. Another notable intraclade ribotype subgroup included strains UMM481, UMM483, and UMM573, which shared similar patterns; this finding, in addition to close 16S rRNA gene sequence relationships, suggested that they represented one species or subspecies. Strains UMM478, UMM537, and UMM559 had vastly different ribotypes, and it is plausible that these strains are unique species of the PNG1 clade. To represent these relationships graphically, a jackknifing method (6) was used to create a dendrogram from the gel banding patterns obtained from the numerous PNG 1 clade ribotypes with BioNumerics software (version 1.5; Applied Maths, St. Martens-Latem, Belgium) (Fig. 3).

(ii) Group II. Two strains were selected from group II for molecular taxonomic evaluation. Using the nearly complete 16S rRNA gene sequences obtained from these two strains as query sequences against the GenBank database, we found that there was significant similarity to members of the family *Micromonosporceae*. UMM500 had 99.5% nucleotide sequence identity with terrestrial *Micromonospora rhodorangea*, whereas UMM539 was 98.7% identical to another saltwater marsh strain, *Micromonospora halophytica* DSM 43171, and showed similar levels of identity to other species of *Micromonospora*. Examination of the dendrograms revealed that strains with this phenotype did not cluster with group I (PNG1 clade) strains but instead clustered with existing *Micromonospora* strains possibly representing new species or subspecies within this well-characterized genus (Fig. 2). Examination of their sequences for signature nucleotides revealed that both strains had perfect matches to the proposed *Micromonospora* signature nucleotides, further validating the phylogenetic identity of the strains.

(iii) Group III. One strain (UMM518) was selected from group III for molecular taxonomic evaluation. Using 16S rRNA gene sequences obtained from UMM518 in a search of the GenBank database with BlastN, we found homologies to peat bog *Verrucosispora gifhornensis* (98.3%) and to marine sediment MAR1 isolate CHN440 (97.5%). The 16S rRNA gene sequence of UMM518 was compared with those of members of the family *Micromonosporaceae* by neighbor-joining and parsimony methods. The resulting consensus tree (Fig. 2) did not provide a clear position for UMM518 within monophyletic groups of the family. The position of UMM518 does

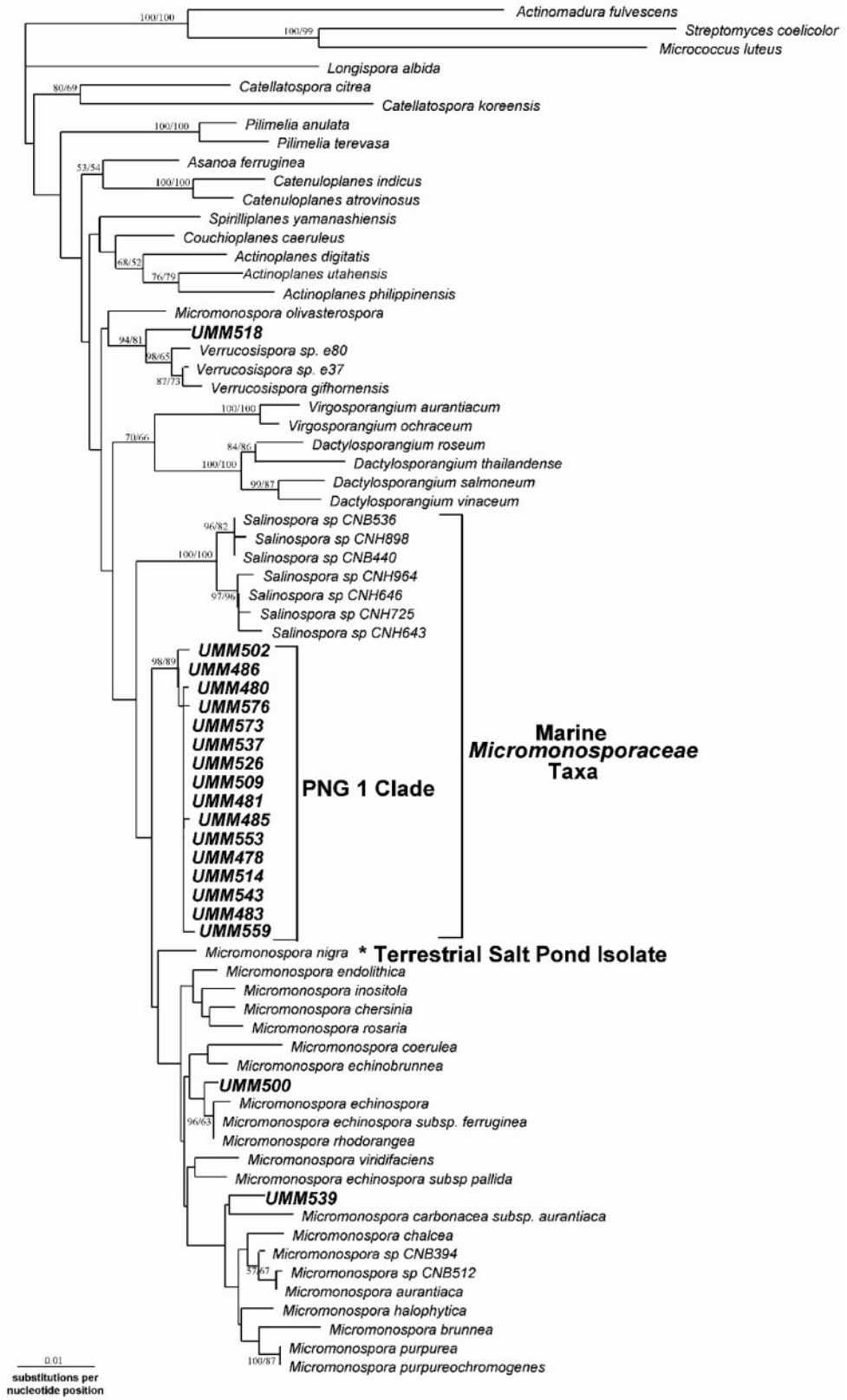

FIG. 2. Phylogram depicting the phylogenetic relationships based on the 16S rRNA gene sequences of all described members of the family *Micromonosporaceae* and selected strains belonging to groups I, II, and III (10, 12–14, 22, 32). Bootstrap values (those calculated to be greater than 50% by both methods) from 1,000 resamplings are shown at their respective nodes with neighbor-joining values on the left and maximum-parsimony values on the right. *Actinomadura fulvescens*, *Streptomyces coelicolor*, and *Micrococcus luteus* were used as outgroups in tree constructions. Marine clades of the family *Micromonosporaceae* are enclosed in brackets. The closest terrestrial relative of the marine *Micromonosporaceae* clades, *M. nigra*, a salt pond isolate (Syracuse, N.Y.) (35), is indicated by an asterisk. The relative distances of branch lengths are a reflection of the substitutions per nucleotide position within the 16S rRNA gene sequences (scale bar).

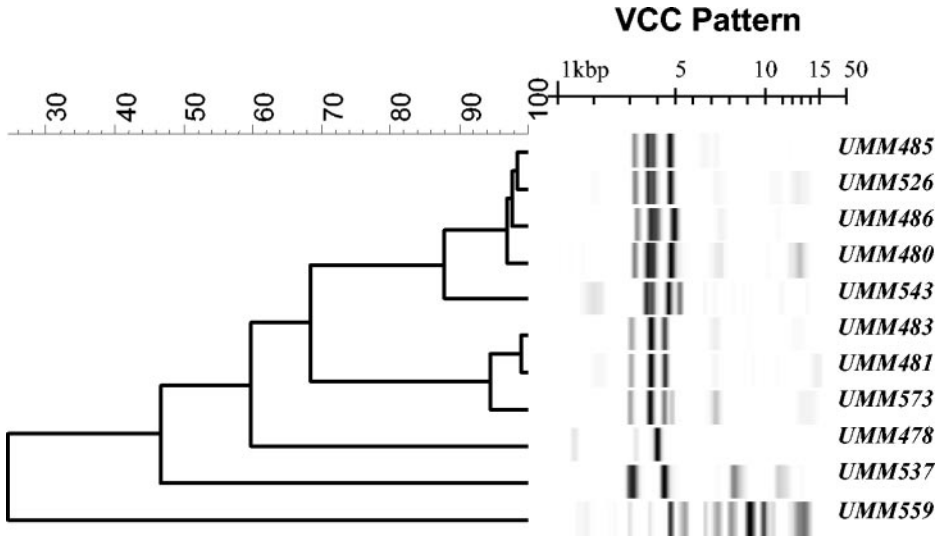

FIG. 3. Ribotype analysis of PNG1 clade strains (based on PvuII digestion). (Left) Riboprint dendrogram generated by comparing the VCC riboprint patterns of various PNG1 clade strains by using BioNumerics software. (Right) VCC riboprint for PNG1 clade strains.

share a branch with the most deeply branching member of the genus *Micromonospora*, *Micromonospora olivasterospora*, or *Verrucosispora* strains (Fig. 2) (22). The position of UMM518 prompted us to compare the molecular signatures of *Verrucosispora* and *Micromonospora* strains with sequences within UMM518. The UMM518 16S rRNA gene sequence was examined for the presence of a loop found within the predicted RNA secondary structure of *V. gifhornensis* and for the presence of *Verrucosispora* signature nucleotides (22). UMM518 did contain the loop, but the nucleotides did not match the characteristic sequences, sharing only 10 of 12 of the *Verrucosispora* signature nucleotides present in the three previously sequenced strains of this genus. Further, UMM518 contained 11 of the 12 signature nucleotides common to the genus *Micromonospora* (10).

Chemotaxonomic studies. (i) PNG1 clade (group I). To establish further the biochemical characteristics of the PNG1 clade, we analyzed cell wall, menaquinone, and fatty acid components of the microorganisms. The amino acids in the cell wall were *meso*-diaminopimelic acid, alanine, glycine, and glutamic acid. Strains of this clade contained arabinose, xylose, glucose, galactose, and one unknown as predominant whole-cell sugars. Analysis of menaquinones and fatty acids for the PNG1 clade included three strains, UMM486, UMM543, and UMM566. The predominant menaquinones of PNG1 clade strains were MK-9(H_4) and MK-10(H_4) (11). Each member contained iso-branched, anteiso-branched, and saturated fatty acids at ratios corresponding to type 2c (Table 1) (11). The fatty acid profile of the PNG1 clade differed from that of the closest monophyletic group of the family *Micromonosporaceae*, *Micromonospora* (type 2c versus type 3b) (11). The clustering of PNG1 clade 16S rRNA gene sequences as a monophyletic grouping, 16S rRNA gene signature nucleotides, chemotaxonomic distinctions (i.e., fatty acid content), and certain physiological characteristics (i.e., growth below pH 6) provide the basis for considering the PNG1 clade to be a distinct genus within the family *Micromonosporaceae*.

(ii) UMM539 (group II). The cell wall amino acid composition of the representative of group II, UMM539, was determined to include *meso*-diaminopimelic acid, alanine, glycine, and glutamic acid. The major menaquinones were MK-9(H_4) and MK-10(H_4) (11). The fatty acid profile of UMM539 was determined to be type 3b (Table 1). These chemotaxonomic characteristics, along with the phylogenetic placement of group II strains based on 16S rRNA gene sequence homology, led to the classification of this group as a member of the genus *Micromonospora*.

(iii) UMM518 (group III). The cell wall amino acids in UMM518 were determined to be *meso*-diaminopimelic acid, alanine, glycine, and glutamic acid. UMM518 contained galactose, xylose, arabinose, and mannose as the predominant whole-cell sugars. The major respiratory menaquinones were MK-9(H_4) and MK-10(H_4). UMM518 had an overall fatty acid profile of type 3b; it contained saturated, unsaturated, and terminally branched fatty acids (iso-branched and anteiso-branched fatty acids), along with 10-methyl-heptadecanoic acid, a product generated by a combination of two fatty acid pathways (Table 1) (11). The iso-branched fatty acid i-$C_{16:0}$ was the predominant fatty acid, accounting for greater than 60% of the total cellular fatty acid content. In comparison, *Verrucosispora*, the genus most similar to UMM518 based on 16S rRNA gene sequence homology, lacks 10-methyl-branched fatty acids, has i-$C_{15:0}$ as the predominant fatty acid, and has a type 2c fatty acid profile. *V. gifhornensis*, the type strain of *Verrucosispora*, lacks arabinose in the cell wall and the membrane respiratory menaquinone MK-10(H_4), both of which are present in UMM518 (11, 22). These differences in chemotaxonomic characteristics are consistent with the results of the 16S rRNA gene sequence analysis and provide strong support for considering UMM518 to be a distinct phylotype within the family *Micromonosporaceae*.

Spore formation in simulated marine environments. Strains of group I and group III were examined for the ability to complete their developmental life cycle in liquid medium (ISP-

TABLE 1. Comparison of cell-associated fatty acids from selected members of the family *Micromonosporaceae*

Organism	Cellular fatty acid content:														10-Methyl ($C_{17:0}$)	Fatty acid profile (type)
	Iso-branched					Anteiso-branched				Saturated or unsaturated						
	i-$C_{15:0}$	i-$C_{16:0}$	i-$C_{17:0}$	i-$C_{17:1}$	i-$C_{18:0}$	ai-$C_{15:0}$	ai-$C_{17:0}$	ai-$C_{17:1}$	ai-$C_{19:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$		
PNG1 clade (group I)																
UMM 486		43	3				21	4				7	9	1.8		2c
UMM543		50			5		21	5	4			14				
UMM566		48			4		22					10.5	7.6	3		
Group II (UMM539)	10	21				4	4		14						8	3b
Group III (UMM518)	14	40	3				5		12						2.5	3b
V. gifhornensis HR1-2	31.1	18.6	8.3	4		2.2	16			1	1	1		1.8		2b

2/ASW). Representatives of each group were capable of producing spores under these submerged culture conditions. The spores produced in submerged cultures were identical in structure to those produced on the agar surface; scanning electron micrographs of submerged spores of group I and group III are shown in Fig. 1E and F, respectively.

Testing the selectivity basis of NaST21Cx agar. Strains of group I and group III were examined for growth on NaST21Cx agar with or without a cellulose disk. Strains of each group were streaked on respective agar plates, incubated at 30°C, and examined for growth after 21 days. Strains grew much faster when inoculated on NaCT21Cx agar with cellulose disks (Fig. 4). However, it was not apparent that the faster growth was the result of cellulose decomposition. It is thus unlikely that the difference in growth was a nutritional effect but rather was due to the nature of the growth surface itself (i.e., agar versus a cellulose matrix).

DISCUSSION

The diversity of terrestrial actinomycetes has been of extraordinary significance in several areas of science and medicine. The decreasing rate of discovery of novel drugs from established terrestrial sources has motivated the evaluation of new sources of chemically diverse bioactive compounds (2). The oceans represent an underexplored environment for microbial discovery, and although new methods are under development (9), relatively few have been applied to reveal the microbial diversity of the ocean environment. Marine sediments, in particular, have been largely overlooked.

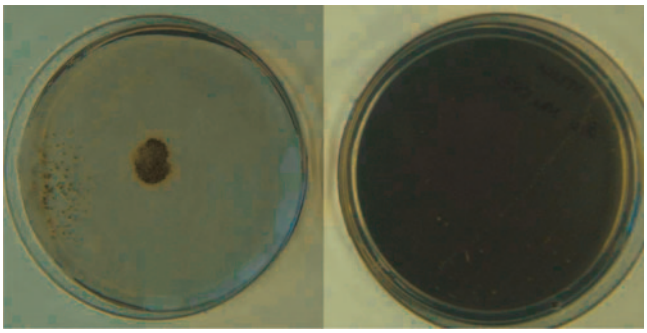

FIG. 4. Selectivity of the NaST21Cx isolation method. Growth differences of PNG1 clade member UMM553 on NaST21Cx with cellulose (left panel) and NaST21Cx without cellulose (right panel) following 14 days of incubation at 30°C.

We have described a novel selective enrichment procedure that has resulted in the isolation of large numbers of new actinobacteria (i.e., >90% of the isolated strains did not match known actinomycete genera). This cellulose- and/or agar-based procedure was initially selected in order to isolate cellulose-decomposing marine myxobacteria (e.g., *S. cellulosum*). The procedure is simple, does not rely on extinction culturing, requires no pretreatments, and still allows a high level of selectivity of slowly growing actinobacteria. Unlike *S. cellulosum*, the isolated actinomycete strains did not degrade the cellulose matrix but did develop concentric rings extending outward from the origin of the sediment material. Representatives of each group were tested for their ability to degrade cellulose, but none was evidently able to do so. When the marine sediments were inoculated on conventional media for the isolation of actinomycetes (e.g., ISP-2 agar or chitin agar), we were not able to isolate members of groups I and III (data not shown). However, culturing of the actinobacterial isolates on NaST21Cx medium with or without cellulose clearly demonstrated that growth was enhanced on plates containing cellulose filter paper (Fig. 4). We suggest that this procedure selects for strains that may be oligotrophs requiring only trace amounts of a nutrient present as impurities in the agar, with the cellulose paper serving as a physical matrix promoting the chemotrophic spread of filamentous bacteria over the surface. The formation of the concentric rings of colonies on this medium, a characteristic not observed on other media, may reflect the gradual outward growth of the cells as nutrients are depleted, facilitated in some fashion by the cellulose matrix.

Initially, strains fell into three phenotypic groups based on pigmentation and colony morphology (Fig. 1). Analysis of 16S rRNA gene sequences supported this grouping and further suggested that two of the groups did not fit into established genera. All three groups, however, could be placed into the family *Micromonosporaceae* based on all of the parameters that we examined (Fig. 4 and Table 1). One phenotypic class (group II), when examined on the molecular and chemotaxonomic levels (fatty acids, cell wall amino acids, and respiratory menaquinones), conformed to the description of the genus *Micromonospora*. This classification was supported by phyloge-

netic analysis based on 16S rRNA gene sequences (Fig. 2). When strains of groups I and III were compared on the basis of their 16S rRNA gene sequences, clear differences at the genus level were identified (i.e., genus-level signature nucleotides). These distinctions were supported by the generation of a phylogenetic tree including representatives of existing genera of the *Micromonosporaceae* and isolates belonging to the MAR1 clade ("*Salinospora*") (10, 14, 30). The branching points of the sequenced members of these two groups clearly demonstrated that they cannot be classified as genera of the family *Micromonosporaceae* or of the recently described MAR1 clade. In addition, comparisons of the fatty acid profiles, respiratory menaquinones, and cell wall sugars of strains from the two groups (PNG1 clade and UMM518) revealed differences from strains of the most closely related genera (based on 16S rRNA gene sequence data). We thus conclude that these strains represent new genera of the family *Micromonosporaceae* (Table 1). The polyphasic taxonomic analysis of the PNG1 clade and the group III member (UMM518) warrants the conclusion that each comprises a new genus. For the PNG1 clade, we suggest "*Solwaraspora*" as an adequate generic epithet, as the prefix *solwara* is Motu (a language native to Papua New Guinea) for salt water. Further analyses of additional 16S rRNA gene sequences from group III strains are planned to help establish whether these strains form a distinct clade and how such additional sequences compare to 16S rRNA gene sequences of other members of the family *Micromonosporaceae*. Based on the classification of UMM518 presented here, it is clear that this group III member is a distinct phylotype within the family *Micromonosporaceae*.

To determine intraclade differences for the PNG1 clade, strains were analyzed by ribotyping, a procedure that allows for species and subspecies level differences to be detected through restriction fragment length polymorphisms of the entire ribosomal DNA operon. The results of this analysis revealed genetic differences within the clade that may be indicative of species and subspecies within the clade.

The absence of existing strains with similar characteristics in the terrestrial environment suggests that these newly described actinobacteria (PNG1 clade and UMM518) are unique to the marine environment. The MAR1 clade has been shown to have an obligate growth requirement for salt, which confirms the designation of organisms in this clade as bona fide marine organisms (14). The PNG1 clade and UMM518 do not have an obligate requirement for salt but do complete a full developmental cycle in submerged cultures (liquid saltwater growth medium containing 3% NaCl) (Fig. 1E and F). Both of these slowly growing actinomycetes were capable of sporulating under submerged conditions in the presence of a salt concentration of 3%, typical of seawater, consistent with their ability to grow and divide in the marine environment.

It is interesting to consider the evolutionary history of these strains in comparison to that of terrestrial actinomycetes and the new marine clade, MAR1 (14). Because of the confidence (high bootstrap values) in the relative position of the PNG1 clade, it is the most suitable for this comparison. The branching of this clade based on a rooted bootstrap tree suggests that the PNG1 clade and the ubiquitous MAR1 clade share a common ancestor (Fig. 2). Interestingly, based on phylogenetic analysis, the genus *Micromonospora* branches at a position suggesting that it is the closest accepted genus relative to these two new marine clades (MAR1 clade and PNG1 clade). Of the *Micromonospora* species whose 16S rRNA genes have been sequenced (over 150), *M. nigra* is the closest relative. Interestingly, this halotolerant strain was isolated from the bottom sediment of a salt marsh in Syracuse, N.Y. (35). Conceptually, this evolutionary linkage is not unexpected and, depending on which diverged later, the terrestrial or the marine actinobacteria, a common linkage with saltwater would invariably be expected.

A major goal of examining this previously uncultured group of marine actinomycetes was to establish new sources of antibiotics and other bioactive compounds. The long-held thesis in terrestrial microbial natural product drug discovery has been and continues to be that microbial diversity translates to chemical diversity. We suggest that this will hold true for marine microbial natural product drug discovery, and we anticipate that several of the secondary metabolites from this new group of actinomycetes will be novel in terms of structure and function.

To determine the potential of prospective new genera (PNG1 clade and group III) to produce secondary metabolites, organic solvent extracts from liquid cultures were tested with a variety of antimicrobial, antiproliferative, and enzyme-based assays. Strains from each group were grown in rich (M1/ASW) and minimal (MN1/ASW) liquid media, extracted, fractionated, and dispensed into 96-well microtiter plates for activity testing. Multidrug-resistant gram-positive pathogens, vancomycin-resistant enterococci (VRE), and methicillin-resistant *Staphylococcus aureus* (MRSA) were tested. *Cryptococcus neoformans* and *Candida albicans* were also tested to determine the antifungal activities of the extracts. Antiproliferative activity against a murine leukemia cancer cell line (P388) was determined. A vaccinia virus topoisomerase assay (37) was used as a model to identify molecules capable of inhibiting the replication of a related orthopoxvirus that causes smallpox.

The majority of fermentation extracts were derived from strains belonging to the PNG1 clade due to the predominance of this group during isolation. Strikingly, over 80% of the extracts generated from 75 members of the PNG1 clade were active in such assays (Table 2). Of particular interest were antibiotics that were active against MRSA and VRE (e.g., extracts obtained from UMM481, UMM491, UMM521, and UMM543) and metabolites that were active against vaccinia virus topoisomerase I (e.g., extract obtained from UMM481). The single extract derived from a group III member (UMM518) exhibited good activity against MRSA and *C. neoformans*. The active metabolites from UMM518 are being isolated for complete structural analysis. Additional strains from group III are being cultured to generate a wide-ranging representation of the biological activities of bioactive metabolites from this group.

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TABLE 2. Biological activities of marine actinobacterial extracts derived from members of groups I, II, and III

Group	Strain	Result[a] for:					
		MRSA (mm)	VRE (mm)	vtop (% inhibition)	*C. neoformans* (mm)	P388 (% inhibition)	*C. albicans* (mm)
PNG1 clade (I)	UMM483	28	0	0	20	37	ND
	UMM483	9	0	100	ND	0	ND
	UMM484	20	0	0	ND	0	ND
	UMM491	20	12	0	18	ND	ND
	UMM492	13	0	0	14	12	ND
	UMM499	0	0	0	0	50	ND
	UMM520	0	0	0	0	55	ND
	UMM521	12	10	0	14	ND	ND
	UMM529	12	0	0	4	18	ND
	UMM534	0	0	0	0	47	14
	UMM535	0	0	0	0	33	25
	UMM543	12	13	0	14	ND	ND
	UMM553	9	0	0	8	0	ND
	UMM558	0	0	0	0	24	12
II	UMM500	0	0	0	0	7	ND
	UMM539	13	0	0	2	0	ND
III	UMM518	12	0	0	9	ND	ND

[a] For antimicrobial assays (MRSA, VRE, *C. neoformans*, and *C. albicans*), activity is given as the zone produced (in millimeters) with fermentation extracts (250 μg) in disk diffusion assays. For murine leukemia cell line P388 and vaccinia virus topoisomerase (vtop) assays, 5 μg of extract/ml was used, and the percent inhibition resulting from each extract is given. ND, extract activity was not determined.

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