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Freshwater *Actinobacteria* from sediments of the deep and ancient Lake Baikal (Russia) and their genetic potential as producers of secondary metabolites

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ABSTRACT: *Actinobacteria* from terrestrial and marine environments produce a variety of natural products that mediate inter- and intraspecies interactions. In contrast, the potential of freshwater *Actinobacteria* for secondary metabolite production remains underexplored. Large lakes with a long evolutionary history might contain microflora subjected to unique environmental conditions that favor the evolution of unique metabolic capabilities. One such lake is Lake Baikal (Russia), the deepest lake on earth as well as one of the oldest. In this study, we investigated the genetically encoded secondary metabolic potential of 24 *Actinobacteria* strains isolated from Lake Baikal sediments. PCR-based screening for genes encoding type I and type II polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs), and halogenases confirmed that all strains possessed at least 1 of these biosynthetic genes. Both PKSs and NRPSs were widely distributed, while halogenase-encoding genes were not detected. Phylogenetic comparison of type I PKS ketosynthase (KS) domain sequences between freshwater isolates and marine and terrestrial strains supported overlap between KSs from these 3 groups. Evaluation of antibiotic activity for chemical extracts from all isolates revealed that 75% produced metabolites inhibitory toward model bacteria and/or fungi. To our knowledge, this study is among the first evaluations of the genetically encoded secondary metabolic capabilities of freshwater sediment *Actinobacteria*. Our findings highlight the similarities and differences between freshwater and marine *Actinobacteria* secondary metabolism, suggesting the potential of freshwater *Actinobacteria* for the production of natural products that may play roles as mediators of interactions between organisms in freshwater habitats.

KEY WORDS: Freshwater · *Actinobacteria* · Secondary metabolite · Natural product · Chemical ecology · Biosynthesis · Halogenase · Nonribosomal peptide synthetase · Polyketide synthase

1. INTRODUCTION

Actinobacteria are Gram-positive filamentous bacteria with high G+C content. Members of this phylum are widely distributed in both terrestrial and aquatic ecosystems (Barka et al. 2015). *Actinobacteria*, especially those of the *Streptomyces* genus, are a renowned source of bioactive natural products including myriad antibiotics (Bérdy 2012).

Actinobacterial natural products are assembled via specialized biosynthetic pathways. The assembly of natural product scaffolds is commonly catalyzed by polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs), and/or other specialized en-

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zymes (Wang et al. 2015). Prototypical type I PKSs are multimodular enzymes, where each module minimally contains ketosynthase (KS), acyltransferase (AT), and acyl carrier protein domains responsible for catalyzing 1 condensation reaction from a thioester building block to ultimately yield metabolite skeletons (Helfrich & Piel 2016). In contrast, type II PKSs are iterative enzymes, where each domain catalyzes multiple condensation reaction cycles. Like type I PKSs, NRPSs are multimodular enzymes that typically act noniteratively as molecular assembly lines to catalyze the formation of peptide bonds between amino acids (Helfrich & Piel 2016). Following the assembly of natural product scaffolds via PKSs, NRPSs, or other enzyme classes, tailoring enzymes such as halogenases and glycosyltransferases further enhance molecular complexity.

These biosynthetic pathways typically divert chemical building blocks from primary metabolic pathways. Hence, natural selection is postulated to favor evolution of biosynthetic pathways for natural products that confer competitive advantages (Karlovsky 2008). In support of this, actinobacterial metabolites have been reported to govern biotic antagonism and cooperation (Wietz et al. 2013, Tyc et al. 2017, van der Meij et al. 2017). For example, terrestrial and marine *Actinobacteria* produce a variety of polyketides and nonribosomal peptides that sequester iron, and some of these siderophores have been demonstrated to impede the morphological development of bacterial competitors (Traxler et al. 2012). Antibiotic natural products from a variety of biosynthetic classes have also been proposed as lethal interspecies chemical weapons (Abrudan et al. 2015, Patin et al. 2016). In contrast, some natural products are recognized as cooperative intraspecies signals, mediating quorum sensing within *Actinobacteria* populations (Polkade et al. 2016). Other metabolites mediate symbiotic relationships with hosts (Wietz et al. 2013, Barka et al. 2015). For example, tripartite mutualisms have been reported for leafcutter ants with specific fungi and *Actinobacteria*, with *Actinobacteria* producing antifungal polyketides and nonribosomal peptides that defend the system against attack by fungal invaders (Haeder et al. 2009, Oh et al. 2009). Actinobacterial natural products have also been revealed to influence the structure and function of microbial communities. For example, mesocosm experiments revealed that metabolites from chemically prolific members of the marine *Salinispora* genus had complex effects on marine sediment community composition, with the abundance of some members diminished and others enriched (Patin et al. 2017).

While these previous studies highlight the intriguing ecology and biogeography of *Actinobacteria* from terrestrial and marine ecosystems, *Actinobacteria* from freshwater sediments remain remarkably underexplored from ecological, phylogenetic, biogeographic, and metabolic perspectives (Terkina et al. 2002, Ningthoujam et al. 2009, Gebreyohannes et al. 2013, Mullowney et al. 2015, Shaikh et al. 2015). One potential reason for this disparity is the assumption that most bodies of freshwater are inhabited by soil microorganisms that washed off of land into rivers and lakes. However, large and deep lakes might contain microflora subjected to unique environmental conditions favoring the evolution of unique adaptations, including unusual metabolic capabilities.

One such place is Lake Baikal in the southern part of eastern Siberia, Russia. It is the deepest lake on Earth as well as the largest lake by volume, containing roughly 20% of the world's unfrozen surface freshwater. Lake Baikal features high oxygen content (~10–12 mg l^{-1}) throughout the water column, providing life from the littoral zone to the deepest part of the lake at 1637 m. Water temperatures in the pelagic zone are consistently about 4°C (Rusinek et al. 2012). As a typical oligotrophic lake, Baikal has low concentrations of organic components and inorganic salts with water salinity of about 100 mg l^{-1}. With an approximate age of 25 to 30 million years, it is one of the oldest lakes in the world, offering long potential evolution of organisms under unique selective pressures. In support of this, Lake Baikal is inhabited by more than 2500 species of animals, and about 80% of them are endemics (Timoshkin et al. 2001). The high proportion of endemic animal species suggests that microorganisms highly adapted to the lake's conditions may exist as well. Together, these unique features of Lake Baikal and other large ancient lakes suggest that *Actinobacteria* from these environments may represent unique models for addressing questions associated with *Actinobacteria* natural products, ecology, phylogeny, and biogeography. In support of this possibility, a recent study of *Streptomyces* sp. IB2014/011-12 isolated from Lake Baikal showed the presence of an unusual hybrid NRPS/trans-AT PKS involved in the assembly of new alpiniamides (Paulus et al. 2018). A handful of other strains that produce bioactive metabolites were also recently isolated from the lake's water and animals (Sobolevskay et al. 2006, Axenov-Gribanov et al. 2017, Shishlyannikova et al. 2017).

In the present study, we assessed the prevalence of PKS-, NRPS-, and halogenase-encoding genes, anti-

biotic activity, and salinity tolerance for a collection of newly isolated *Actinobacteria* from Lake Baikal freshwater sediments. To our knowledge, this study is among the first to survey the genetically encoded biosynthetic potential of *Actinobacteria* from freshwater sediments. Our study implicates bacteria from this unique environment as producers of secondary metabolites and suggests some of these bacteria are uniquely adapted to their low-salinity habitat.

2. MATERIALS AND METHODS

2.1. Sediment sampling

Nine sediment samples from Lake Baikal were collected offshore during March 2016 near Bolshie Koty village on the lake's southwestern shore (51.9053° N, 105.0753° E). Samples (about 20 ml) were obtained by Petersen dredge at depths of 50 to 200 m and placed into sterile 50 ml conical tubes.

2.2. Isolation of *Actinobacteria*

Aliquots of 200 µl of freshwater sediments as well as 10^{-1} and 10^{-2} dilutions were plated directly onto MS, ISP2, and Czapek agar media. MS agar was composed of 20 g mannitol, 20 g soy flour, and 20 g agar per 1 l distilled water. ISP2 was composed of 4 g glucose, 4 g yeast extract, 10 g malt extract, and 15 g agar per 1 l deionized (DI) water. Czapek medium was prepared from 3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7\ H_2O$, 30 g sucrose, and 15 g agar per 1 l distilled water. Cyclohexamide (50 µg ml^{-1} final concentration) and phosphomycin (100 µg ml^{-1} final concentration) were added to all media to inhibit the growth of fungi and Gram-negative bacteria, respectively. The plates were incubated for 4 wk at 28°C. *Actinobacteria* were recognized based on colony morphology, including growth into agar and filaments (Kieser et al. 2000). Candidate *Actinobacteria* colonies were repetitively streaked on MS or ISP2 agar to yield pure cultures. Isolates were frozen in 20% glycerol at –80°C for long-term storage.

2.3. 16S rRNA gene sequencing and taxonomic analysis

Strains were cultivated in 5 ml ISP2 broth at 28°C with shaking at ~220 rpm for 3 d. Genomic DNA was extracted from all isolates as described in Kieser et al. (2000). A ~600 bp fragment of the 16S rRNA gene of each isolate was PCR amplified using established *Actinobacteria*-specific primers ACT235 (5′-CGC GGC CTA TCA GCT TGT TG-3′) and ACT848 (5′-CCG TAC TCC CCA GGC GGG G-3′) that amplify the V3 to V5 regions of the 16S rRNA gene (Stach et al. 2003). The reaction mixture (25 µl) contained 1 µl genomic DNA, 2.5 µl 10× New England Biolabs Thermopol buffer, 0.5 µl deoxynucleotide triphosphate mixture (2.5 mM stock), 1 µl of each primer (10 µM stock), 2 µl of DMSO, 0.2 µl of *Taq* Thermopol DNA polymerase (New England Biolabs), and 16.8 µl of molecular biology grade water. PCR conditions consisted of an initial denaturation at 95°C for 2 min; 30 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min; and a final 7 min extension at 72°C. Resulting amplicons were purified using a QIAquick PCR Purification Kit (QIAGEN Sciences) and sequenced by Eurofins.

The forward and reverse sequences were assembled by BioEdit version 7.2.5 (Hall 1999). All sequences were checked for chimeras by DECIPHER (Wright et al. 2012). Assembled partial 16S rRNA gene sequences were queried against the GenBank database using BLAST to determine phylogenetic relatives (Altschul et al. 1990, Johnson et al. 2008). Sequences were aligned with the complete 16S rRNA gene sequence of *Escherichia coli* K-12 using ClustalX (Larkin et al. 2007). Kimura's 2-parameter model was used to calculate evolutionary distance matrices of the phylogenetic tree (Kimura 1980). Bootstrap analysis was performed with 1000 replications (Felsenstein 1985). Phylogenetic analysis based on maximum likelihood and Bayesian methods was performed using the MEGA version 7.0 software package (Saitou & Nei 1987, Tamura et al. 2013). The partial 16S rRNA gene sequences (~600 bp) were deposited in GenBank with accession numbers MG199054 to MG199077 (Table S1 in the Supplement at www.int-res.com/articles/suppl/a084p001_supp.pdf).

2.4. Amplification and cloning of selected biosynthetic gene fragments

PCR screening with 6 previously reported sets of degenerate primers was used to evaluate the genetically encoded biosynthetic potential of investigated strains. Fragments of type I PKS genes were amplified using KSMA-F/KSMB-R, targeting the β-ketoacyl synthase domain (Izumikawa et al. 2003), and

K1/M6, targeting the KS/methylmalonyl-CoA transferase domain (Ayuso-Sacido & Genilloud 2005). Type II PKS KSα domains were amplified using 540F/1100R (Wawrik et al. 2005) and PKS II-FW/PKS II-RV (Hornung et al. 2007). Adenylation domains of NRPSs were amplified with A3/A7 (Ayuso-Sacido & Genilloud 2005), and fragments of genes encoding $FADH_2$-dependent halogenases were amplified with Halo-B4-FW/Halo-B7-RV (Hornung et al. 2007). PCR parameters followed those of the original investigations (Izumikawa et al. 2003, Ayuso-Sacido & Genilloud 2005, Wawrik et al. 2005, Hornung et al. 2007). *Amycolatopsis azurea* NRRL 11412 (PKS I, NRPS), *Nocardiopsis* sp. CMB-M0232 (PKS I + II, NRPS), *Streptomyces venezuelae* ISP-5230 (PKS I + II, NRPS, halogenase), and *Salinispora* sp. DRTO102 (halogenase) were used as positive controls for biosynthetic gene types listed in parentheses.

PCR amplicons for genes encoding putative KS domains of type I PKSs were cloned using the pGEM-T vector (Promega) with *E. coli* JM109 host following manufacturer directions. DNA sequencing of cloned genes was conducted by Eurofins. Resulting DNA sequences were used to deduce amino acid sequences, which were analyzed against the NCBI protein database using the BLASTP algorithm with default parameters. A maximum likelihood tree for the amino acid sequences of KS domains was constructed using the MEGA version 7.0 software package applying 1000 bootstrap resamplings. KS domains were also evaluated using NaPDoS (Ziemert et al. 2012). The nucleotide sequences for all sequenced PCR amplicons were deposited in GenBank with accession numbers MG249993 to MG250007 (Table S2).

2.5. Salinity requirement for growth and chemical extraction

All freshwater *Actinobacteria* strains were evaluated for growth in both saline and nonsaline conditions using M1 media prepared with or without Instant Ocean (28 g l^{-1}). M1 media consisted of 10 g starch, 4 g yeast extract, 2 g peptone, 1 g $CaCO_3$, 35 mg $Fe_2(SO_4)_3$, and 100 mg KBr per 1 l DI water. All strains were grown in both M1 saline and nonsaline media (50 ml) for 5 d at 30°C with shaking at ~225 rpm and visually assessed for growth. For cultures not exhibiting growth after 5 d, incubation time was expanded to 14 d. For strains exhibiting growth, cells were pelleted by centrifugation at 3000 rpm (3500 × *g*) for 5 min and supernatants extracted with an equal volume of ethyl acetate. Resulting chemical extracts were dried *in vacuo*.

2.6. Antimicrobial assays

Antimicrobial assays were conducted in 96-well plates with methicillin-sensitive *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC MYA-2876), and *Aspergillus nidulans* (ATCC 38163). Overnight cultures of *S. aureus* or *P. aeruginosa* in tryptic soy broth (TSB) medium were diluted by a factor of 3 × 10^3 in TSB and 100 µl seeded into each well of a 96-well plate. To each well, chemical extracts (solubilized in a DMSO vehicle) were added at concentrations of 25 µg ml^{-1}. All assays were conducted in duplicate. Gentamycin (50 µg ml^{-1}) was used as a positive control, while wells containing the DMSO vehicle acted as a negative control. Plates were incubated for 24 h at 37°C. Absorbance at 630 nm was measured for each well and absorbance values compared between treatments and vehicle-only controls to determine growth inhibition in response to each chemical extract. Antimicrobial assays with *C. albicans* were conducted analogously, except that RPMI 1640 medium was used instead of TSB, and nystatin (4 µg ml^{-1}) was used as the positive control.

A. nidulans was grown on a Sabouraud dextrose agar plate (100 mm diameter) at 30°C for 2 d to yield a lawn. Then, 5 ml of potato dextrose medium was flooded onto the plate and spores scraped from the plate surface into the liquid. The resulting suspended spore mixture was diluted to a ratio of 1:45 with RPMI 1640 medium. This mixture was used for 96-well plate assays analogously to *C. albicans* assays, except that *A. nidulans* was incubated at 30°C for 48 h before growth was evaluated.

3. RESULTS

3.1. Isolation and phylogenetic diversity of Lake Baikal *Actinobacteria*

A total of 24 strains possessing morphological features suggestive of *Actinobacteria* were isolated from Lake Baikal freshwater sediment samples. *Actinobacteria* comprised a small fraction of overall cultured microbial diversity isolated from the sediments with relative abundance never exceeding 2% (Table S3). The abundance of *Actinobacteria* relative to other bacteria was comparable across all 3 evalu-

ated depths (50, 150, 200 m). Phylogenetic analysis by evaluation of partial 16S rRNA gene sequences supported the fact that the 24 freshwater isolates included representatives of 3 different genera (*Micromonospora*, *Pseudonocardia*, and *Streptomyces*) (Fig. 1, Fig. S1).

The genus *Streptomyces* was predominant among *Actinobacteria* from Lake Baikal sediment samples, comprising 83% of isolated strains. *Streptomyces* strains from this freshwater habitat possessed high 16S rRNA gene sequence similarities with soil-derived strains, including many isolated from rhizospheres as well as isolates from insects, sponges, and wheat shoots (Fig. 1, Table 1). The 2 *Pseudonocardia* strains (IB2016P332-1 and IB2016P332-2) from Lake Baikal shared strong sequence identities with strains from aquatic and terrestrial ecosystems, including cave sediments and termite nests. To our knowledge, our study marks the first report of representatives of the *Pseudonocardia* genus from Lake Baikal sediments. Isolates from the *Micromonosporaceae* family, *Micromonospora* sp. IB2016P328-8 and IB2016P334-1 from Lake Baikal, showed high 16S rRNA sequence similarity with terrestrial and marine sediment strains. Thus, freshwater strains exhibited high similarities with *Actinobacteria* from a variety of ecosystems different from those they originated from in our study. Based on evaluation of 16S rRNA sequences with both maximum likelihood and Bayesian methods (Fig. 1, Fig. S1), Lake Baikal representatives were not found within clades distinct from *Actinobacteria* isolated from other ecosystems.

3.2. Analysis of selected biosynthetic genes from freshwater *Actinobacteria*

To assess the genetically encoded biosynthetic capabilities of freshwater *Actinobacteria*, PCR-based screens were employed to evaluate each strain for genes encoding type I and type II PKSs, NRPSs, and $FADH_2$-dependent halogenases (Table 1). Using 2 sets of previously reported degenerate primers targeting different portions of type I PKS genes (Izumikawa et al. 2003, Ayuso-Sacido & Genilloud 2005), PCR-based screens and sequencing of resulting amplicons supported the fact that type I PKSs are common among evaluated freshwater *Actinobacteria* (Table 1). Using primer set K1/M6, targeting type I PKS KS and methylmalonyl-CoA transferase domains (Ayuso-Sacido & Genilloud 2005), PCR amplicons were found for 19 of 24 evaluated freshwater strains (79%). Using the KSMA-F/KSMB-R primer set, targeting β-ketoacyl synthase (KS) domains (Izumikawa et al. 2003), 16 strains (66%) were supported to possess type I PKSs.

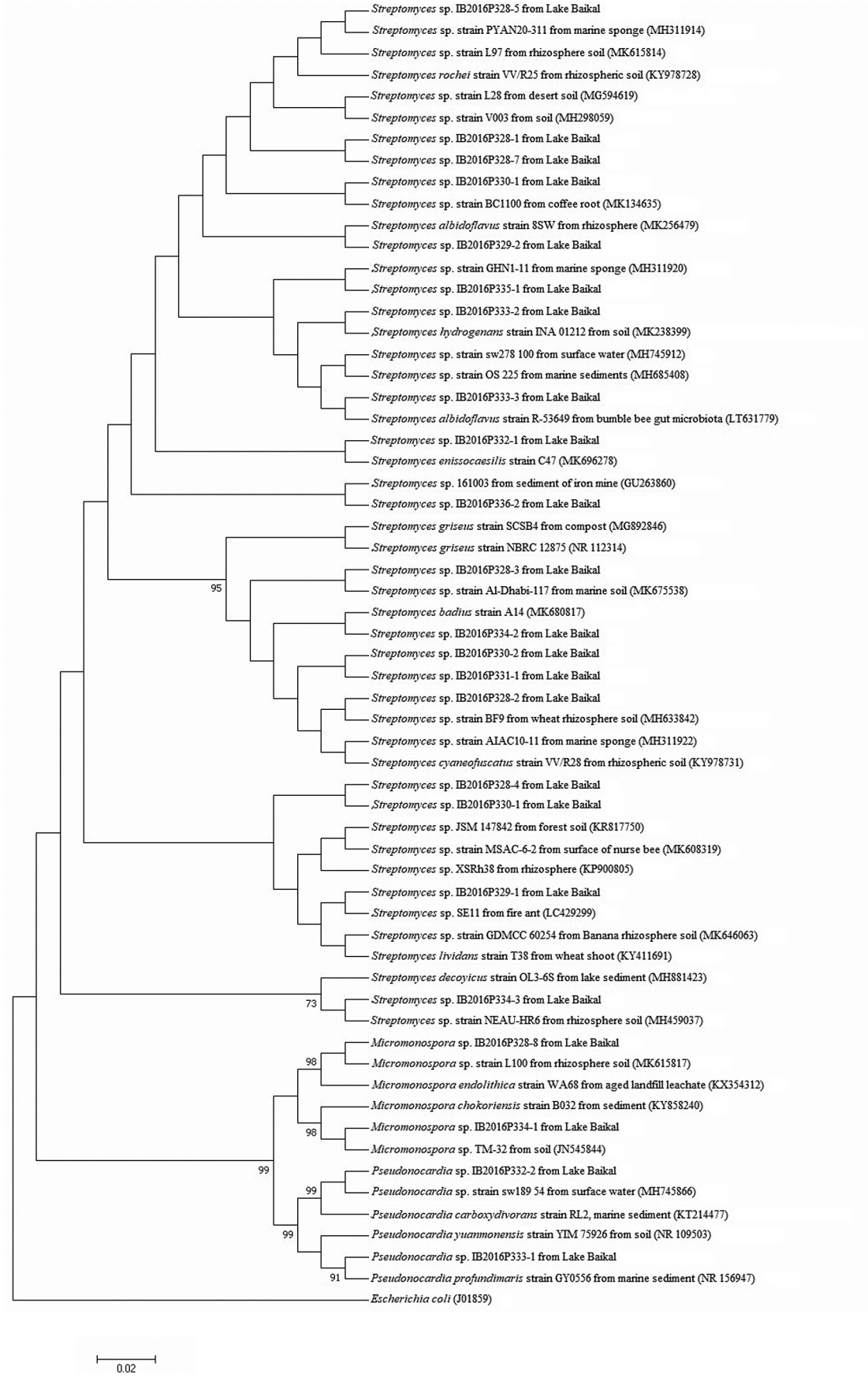
Type I PKS gene fragment amplicons were cloned, 1 to 2 representative cloned genes from each strain sequenced, and these DNA sequences translated into amino acid sequences. Resulting putative KS domains were compared with sequences in the NCBI protein database, revealing that most share moderate or high (69–99%) similarity with previously reported KS domains from *Actinobacteria* isolated from a variety of ecosystems (Fig. 2, Table S2). Phylogenetic analysis of type I PKS KS domains suggested the absence of distinct freshwater clades (Fig. 2). No direct correspondence was found between the positions of representatives within the 16S rRNA gene phylogenetic tree and the KS domain tree (Figs. 1 & 2).

KS domains were additionally evaluated using NaPDoS (Ziemert et al. 2012), which compares previously reported KS sequences with new sequences to predict similarity between known metabolites and molecules encoded by new strains (Table S4). Based on these analyses, KS domains from 5 different Lake Baikal strains were predicted to assemble metabolites most similar to avermectin, an anthelmintic macrolide from a soil-dwelling actinomycete (Ikeda & Ōmura 1997). KS domains from other strains were predicted to be involved in pathways yielding metabolites similar to known antimicrobials, including the antifungals eco-02301 (McAlpine et al. 2005) and candicidin (Gil & Campelo-Diez 2003) as well as the antibacterials tylosin (Fouces et al. 1999) and meridamycin (He et al. 2006) (Table S4).

In addition to type I PKSs, strains were evaluated for type II PKSs by PCR using 2 degenerate primer sets targeting KSα domains (Wawrik et al. 2005, Hornung et al. 2007). Three freshwater (6%) strains yielded PCR amplicons for the PKS II-FW/PKS II-RV primer set (Table 1). No freshwater strains yielded PCR amplicons using type II PKS primers 540F/1100R.

Strains were screened for NRPS genes by PCR using the A3/A7 pair of degenerative primers, which were previously reported to amplify adenylation domains from *Actinobacteria* NRPSs (Ayuso-Sacido & Genilloud 2005). Based on detection of PCR amplicons of the expected 700 bp size, it was revealed that NRPS genes were widely distributed among freshwater strains (96%) (Table 1).

PCR amplification of genes encoding flavin-dependent halogenases from Lake Baikal *Actinobacteria* was conducted using degenerate primer set


Streptomyces sp. IB2016P328-5 from Lake Baikal
Streptomyces sp. strain PYAN20-311 from marine sponge (MH311914)
Streptomyces sp. strain L97 from rhizosphere soil (MK615814)
Streptomyces rochei strain VV/R25 from rhizospheric soil (KY978728)
Streptomyces sp. strain L28 from desert soil (MG594619)
Streptomyces sp. strain V003 from soil (MH298059)
Streptomyces sp. IB2016P328-1 from Lake Baikal
Streptomyces sp. IB2016P328-7 from Lake Baikal
Streptomyces sp. IB2016P330-1 from Lake Baikal
Streptomyces sp. strain BC1100 from coffee root (MK134635)
Streptomyces albidoflavus strain 8SW from rhizosphere (MK256479)
Streptomyces sp. IB2016P329-2 from Lake Baikal
Streptomyces sp. strain GHN1-11 from marine sponge (MH311920)
Streptomyces sp. IB2016P335-1 from Lake Baikal
Streptomyces sp. IB2016P333-2 from Lake Baikal
Streptomyces hydrogenans strain INA 01212 from soil (MK238399)
Streptomyces sp. strain sw278 100 from surface water (MH745912)
Streptomyces sp. strain OS 225 from marine sediments (MH685408)
Streptomyces sp. IB2016P333-3 from Lake Baikal
Streptomyces albidoflavus strain R-53649 from bumble bee gut microbiota (LT631779)
Streptomyces sp. IB2016P332-1 from Lake Baikal
Streptomyces enissocaesilis strain C47 (MK696278)
Streptomyces sp. 161003 from sediment of iron mine (GU263860)
Streptomyces sp. IB2016P336-2 from Lake Baikal
Streptomyces griseus strain SCSB4 from compost (MG892846)
Streptomyces griseus strain NBRC 12875 (NR 112314)
Streptomyces sp. IB2016P328-3 from Lake Baikal
Streptomyces sp. strain Al-Dhabi-117 from marine soil (MK675538)
Streptomyces badius strain A14 (MK680817)
Streptomyces sp. IB2016P334-2 from Lake Baikal
Streptomyces sp. IB2016P330-2 from Lake Baikal
Streptomyces sp. IB2016P331-1 from Lake Baikal
Streptomyces sp. IB2016P328-2 from Lake Baikal
Streptomyces sp. strain BF9 from wheat rhizosphere soil (MH633842)
Streptomyces sp. strain AIAC10-11 from marine sponge (MH311922)
Streptomyces cyaneofuscatus strain VV/R28 from rhizospheric soil (KY978731)
Streptomyces sp. IB2016P328-4 from Lake Baikal
Streptomyces sp. IB2016P330-1 from Lake Baikal
Streptomyces sp. JSM 147842 from forest soil (KR817750)
Streptomyces sp. strain MSAC-6-2 from surface of nurse bee (MK608319)
Streptomyces sp. XSRh38 from rhizosphere (KP900805)
Streptomyces sp. IB2016P329-1 from Lake Baikal
Streptomyces sp. SE11 from fire ant (LC429299)
Streptomyces sp. strain GDMCC 60254 from Banana rhizosphere soil (MK646063)
Streptomyces lividans strain T38 from wheat shoot (KY411691)
Streptomyces decoyicus strain OL3-6S from lake sediment (MH881423)
Streptomyces sp. IB2016P334-3 from Lake Baikal
Streptomyces sp. strain NEAU-HR6 from rhizosphere soil (MH459037)
Micromonospora sp. IB2016P328-8 from Lake Baikal
Micromonospora sp. strain L100 from rhizosphere soil (MK615817)
Micromonospora endolithica strain WA68 from aged landfill leachate (KX354312)
Micromonospora chokoriensis strain B032 from sediment (KY858240)
Micromonospora sp. IB2016P334-1 from Lake Baikal
Micromonospora sp. TM-32 from soil (JN545844)
Pseudonocardia sp. IB2016P332-2 from Lake Baikal
Pseudonocardia sp. strain sw189 54 from surface water (MH745866)
Pseudonocardia carboxydivorans strain RL2, marine sediment (KT214477)
Pseudonocardia yuanmonensis strain YIM 75926 from soil (NR 109503)
Pseudonocardia sp. IB2016P333-1 from Lake Baikal
Pseudonocardia profundimaris strain GY0556 from marine sediment (NR 156947)
Escherichia coli (J01859)
95
73
98
98
99
99
99
91
0.02

Halo-B4-FW/Halo-B7-RV (Hornung et al. 2007). This PCR-based screen revealed no evidence for halogenase genes from evaluated freshwater strains (Table 1), while these genes were readily amplified from positive control strains. To determine whether the absence of halogenases is unique to Lake Baikal strains or whether these are absent across related strains regardless of ecosystem, we evaluated publicly available genome-sequenced strains most closely related to Lake Baikal strains for halogenase homologues. The SynTax bioinformatics tool (Oberto 2013) was used to evaluate 8 complete genomes from relatives of Lake Baikal strains for homologues of $FADH_2$-dependent halogenases from *Amycolatopsis mediterranei* DSM5908 producing balhimycin (Puk et al. 2002), *Streptomyces venezuelae* DSM5908 producing chloramphenicol (Podzelinska et al. 2010), and *Salinispora* sp. DRTO102. Two of 8 terrestrial *Actinobacteria* genomes encoded halogenase homologues, based on percent normalized BLAST scores of 55 to 90% to 1 or more model halogenases (Table S5).

3.3. Freshwater strain viability in saline and nonsaline environments and antimicrobial bioactivities

The growth of each freshwater strain was compared between nonsaline and saline M1 media to evaluate adaptation of these strains to the salinity of the Lake Baikal environment, which is lower than typical terrestrial soils and marine environments. Growth was not observed in saline media for 6 of 24 (25%) freshwater strains (Table 1) over a 2 wk period during which abundant growth in nonsaline media was observed, suggesting these as obligate freshwater *Actinobacteria*. These included 2 *Micromonospora* isolates (IB2016P328-8 and IB2016P334-1), 2 *Streptomyces* strains (IB2016P328-2 and IB2016-P334-3), and 2 *Pseudonocardia* isolates (IB2016P332-2 and IB2016P333-1).

Since most metabolites acting as interspecies chemical signals are expected to be excreted from cells, chemical extracts of culture supernatants were evaluated in antimicrobial assays. These extracts were generated from *Actinobacteria* grown in both nonsaline and saline media to assess the impacts of salinity on the production of antimicrobial metabolites. Chemical extracts were evaluated at a concentration of 25 µg ml^{-1} for inhibition of the Gram-positive bacterium *Staphylococcus aureus*, Gram-negative bacterium *Pseudomonas aeruginosa*, yeast *Candida albicans*, and filamentous fungus *Aspergillus nidulans* (Tables 1 & 2). At least 1 evaluated microorganism was strongly (>75% inhibition relative to vehicle-only control) inhibited by chemical extracts from 18 of 24 strains grown in saline media, nonsaline media, or both (Table 1). Extracts from approximately 62% of strains strongly inhibited at least 1 fungus and 25% inhibited at least 1 bacterium (Tables 1 & 2). All Lake Baikal strains found to produce antimicrobial compound(s) belong to the *Streptomyces* genus.

When grown in nonsaline media, a higher proportion of evaluated *Actinobacteria* (25%) yielded chemical extracts strongly inhibitory toward the model Gram-positive bacterium *Staphylococcus aureus* than when grown in saline media (11%, Table 1). No chemical extracts were strongly inhibitory toward the Gram-negative model bacterium *Pseudomonas aeruginosa*. In some cases, growth of some strains in saline media resulted in enhanced production of antifungal metabolites, especially against the filamentous fungus *A. nidulans* (Tables 1 & 2). Further, 4 of 5 extracts inhibitory toward *A. nidulans* were also inhibitory toward *C. albicans*, potentially suggesting production of broad-spectrum antifungal metabolites.

4. DISCUSSION

As one of the first surveys of freshwater sediment *Actinobacteria* biodiversity and biosynthetic capabilities (Sobolevskay et al. 2006, Ningthoujam et al. 2009, Gebreyohannes et al. 2013), this work adds to the understanding of freshwater *Actinobacteria* from

Fig. 1. Phylogenetic tree of *Actinobacteria* strains isolated from Lake Baikal sediments in the present study. The maximum likelihood tree of ~600 bp fragments of 16S rRNA genes supports representatives from 3 orders within the phylum *Actinobacteria*. Previously reported *Actinobacteria* with 16S rRNA gene sequences most homologous to those from this study are also included, with their 16S rRNA gene sequence GenBank accession numbers given in parentheses. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7. The sequence of *Escherichia coli* K12 was used as an outgroup. Scale bar corresponds to 0.02 substitution per nucleotide position. Accession numbers for isolates reported in the present study are provided in Table S1 in the Supplement

Table 1. Summary of phylogenetic assignment, evaluation of growth in seawater- and freshwater-based media, PCR-based detection of selected biosynthetic genes, and antimicrobial assay results for all evaluated freshwater sediment *Actinobacteria*. For evaluation of the ability of strains to grow in saline or nonsaline media, '+' indicates growth was evident after up to 14 d of cultivation in the indicated media type, and '−' indicates no growth. For biosynthetic genes, '+' indicates the PCR amplicon of the indicated gene fragment was detected using the primer set listed (i.e. KS/MA, K1/M6, FW/RV, 540/1100, A3/A7, or B4-FW/B7-RV), and '−' indicates no amplicon was observed or that DNA sequencing of the amplicon and translation revealed no homology to known secondary metabolism enzymes. For antimicrobial activity assays, '+' indicates that growth of the targeted microorganism was inhibited by at least 75% relative to controls without chemical extract. Percent inhibition values are presented in Table 2. Chemical extracts were evaluated at 25 µg ml^{-1}. No extracts inhibited *Pseudomonas aeruginosa* by at least 75%. S: chemical extract obtained from saline M1 medium; NS: extract obtained from nonsaline M1 medium

Strain	Closest type strain (GenBank accession no.)	Growth in saline medium	Growth in nonsaline medium	Biosynthetic genes						Antimicrobial activity		
				PKS I, KS/MA	PKS I, K1/M6	PKS II, FW/RV	PKS II, 540/1100	NRPS, A3/A7	Halogenase, B4-FW/B7-RV	*Staphylococcus aureus*	*Candida albicans*	*Aspergillus nidulans*
IB2016P328-1	*Streptomyces albidoflavus* strain R-53649, isolate H52-3 (LT631779)	+	+	−	+	−	−	+	−	−	+ (S, NS)	+ (S)
IB2016P328-2	*S. cyaneofuscatus* strain VV/R28 (KY978731)	−	+	+	−	−	−	+	−	+ (NS)	−	−
IB2016P328-3	*S. cyaneofuscatus* strain VV/R28 (KY978731)	+	+	+	−	−	−	+	−	+ (S, NS)	−	−
IB2016P328-4	*S. lividans* strain T38 (KY411691)	+	+	+	+	+	−	+	−	+ (NS)	+ (S, NS)	+ (S)
IB2016P328-5	*S. albidoflavus* strain R-53649, isolate H52-3 (LT631779)	+	+	−	+	−	−	+	−	−	+ (S, NS)	−
IB2016P328-6	*S. cyaneofuscatus* strain VV/R28 (KY978731)	+	+	−	+	−	−	+	−	−	−	−
IB2016P328-7	*S. rochei* strain VV/R25 (KY978728)	+	+	+	+	−	−	+	−	−	+ (S, NS)	+ (S)
IB2016P328-8	*Micromonospora endolithica* strain WA68 (KX354312)	−	+	−	+	+	−	+	−	−	−	−
IB2016P329-1	*S. albidoflavus* strain R-53649, isolate H52-3 (LT631779)	+	+	+	+	−	−	+	−	−	+ (S, NS)	−
IB2016P329-2	*S. albidoflavus* strain R-53649, isolate H52-3 (LT631779)	+	+	+	+	−	−	+	−	−	+ (S)	−
IB2016P330-1	*S. lividans* strain T38 (KY411691)	+	+	+	+	−	−	+	−	−	+ (S, NS)	−
IB2016P330-2	*S. cyaneofuscatus* strain VV/R28 (KY978731)	+	+	+	−	−	−	+	−	−	+ (S)	−
IB2016P330-3	*S. albidoflavus*, strain R-53649, isolate H52-3 (LT631779)	+	+	+	+	−	−	+	−	−	+ (S, NS)	−
IB2016P331-1	*S. cyaneofuscatus* strain VV/R28 (KY978731)	+	+	+	+	−	−	−	−	−	+ (NS)	−
IB2016P332-1	*S. albidoflavus*, strain R-53649, isolate H52-3 (LT631779)	+	+	−	+	−	−	+	−	−	−	+ (S)

Table 1 (continued)

Strain	Closest type strain (GenBank accession no.)	Growth in saline medium	Growth in nonsaline medium	Biosynthetic genes						Antimicrobial activity		
				PKS I, KS/MA	PKS I, K1/M6	PKS II, FW/RV	PKS II, 540/1100	NRPS, A3/A7	Halogenase, B4-FW/ B7-RV	*Staphylococcus aureus*	*Candida albicans*	*Aspergillus nidulans*
IB2016P332-2	*Pseudonocardia carboxydivorans* strain RL2 (KT214477)	–	+	–	+	–	–	+	–	–	–	–
IB2016P333-1	*P. yuanmonensis* strain YIM 75926 (NR_109503)	–	+	+	+	–	–	+	–	–	–	–
IB2016P333-2	*S. albidoflavus*, strain R-53649, isolate H52-3 (LT631779)	+	+	+	+	–	–	+	–	+ (NS)	+ (NS)	–
IB2016P333-3	*S. albidoflavus* strain R-53649, isolate H52-3 (LT631779)	+	+	+	+	–	–	+	–	+ (NS)	+ (NS)	–
IB2016P334-1	*M. chokoriensis* strain B032 (KY858240)	–	+	+	+	–	–	+	–	–	–	–
IB2016P334-2	*S. cyaneofuscatus* strain VV/R28 (KY978731)	+	+	+	–	+	–	+	–	+ (S, NS)	–	–
IB2016P334-3	*S. ramulosus* strain RB113 (KY558692)	–	+	+	–	–	–	+	–	–	–	–
IB2016P335-1	*S. albidoflavus* strain R-53649, isolate H52-3 (LT631779)	+	+	–	+	–	–	+	–	–	+ (S, NS)	–
IB2016P336-2	*S. rochei* strain VV/R25 (KY978728)	+	+	–	+	–	–	+	–	–	+ (S)	+ (S)

large deep lakes with a long evolutionary history (Terkina et al. 2002, Mullowney et al. 2015, Shaikh et al. 2015, Braesel et al. 2018). Further, the high prevalence of natural product biosynthetic genes and antimicrobial activities observed from Lake Baikal actinobacterial isolates (Tables 1 & 2) suggests that natural products may play a role as mediators of biotic interactions in this ancient oligotrophic ecosystem.

In this study, 16S rRNA gene sequencing was used to assess biodiversity of 24 isolates from Lake Baikal freshwater sediments, revealing representatives from *Streptomyces*, *Micromonospora*, and *Pseudonocardia* genera (Fig. 1, Fig. S1). The 16S rRNA gene sequences from 20 freshwater *Streptomyces*, 2 *Micromonospora*, and 2 *Pseudonocardia* isolates exhibited high identity to strains isolated from both terrestrial (e.g. soil and leachate) and aquatic (e.g. marine sediments) environments. Based on both phylogenetic tree construction methods (Fig. 1, Fig. S1), none of the 3 genera isolated from Lake Baikal formed clades distinct from terrestrial and/or marine strains. This suggests the ubiquitous distribution of related members of these genera across diverse terrestrial and aquatic ecosystems. Future application of multi-locus phylogenetic methods (e.g. Alanjary et al. 2019) may offer higher resolution to further distinguish between bacteria from these divergent systems.

The concentration of culturable bacteria from Lake Baikal sediments in our study (Table S3) was 2 orders of magnitude lower than culturable bacteria reported from soil (Vieira & Nahas 2005) and from 1 to 3 orders of magnitude lower than that reported from marine sediments (Bredholt et al. 2008). This lower abundance of culturable bacteria is potentially explained by the oligotrophic conditions of Lake Baikal disfavoring high microbial abundance. Alternatively, it may result from the use of media in our study with antibiotics inhibitory toward Gram-negative bacteria and/or the use of media that were not optimal for the growth of bacteria from oligotrophic freshwater sediments. Our findings set the stage for cul-

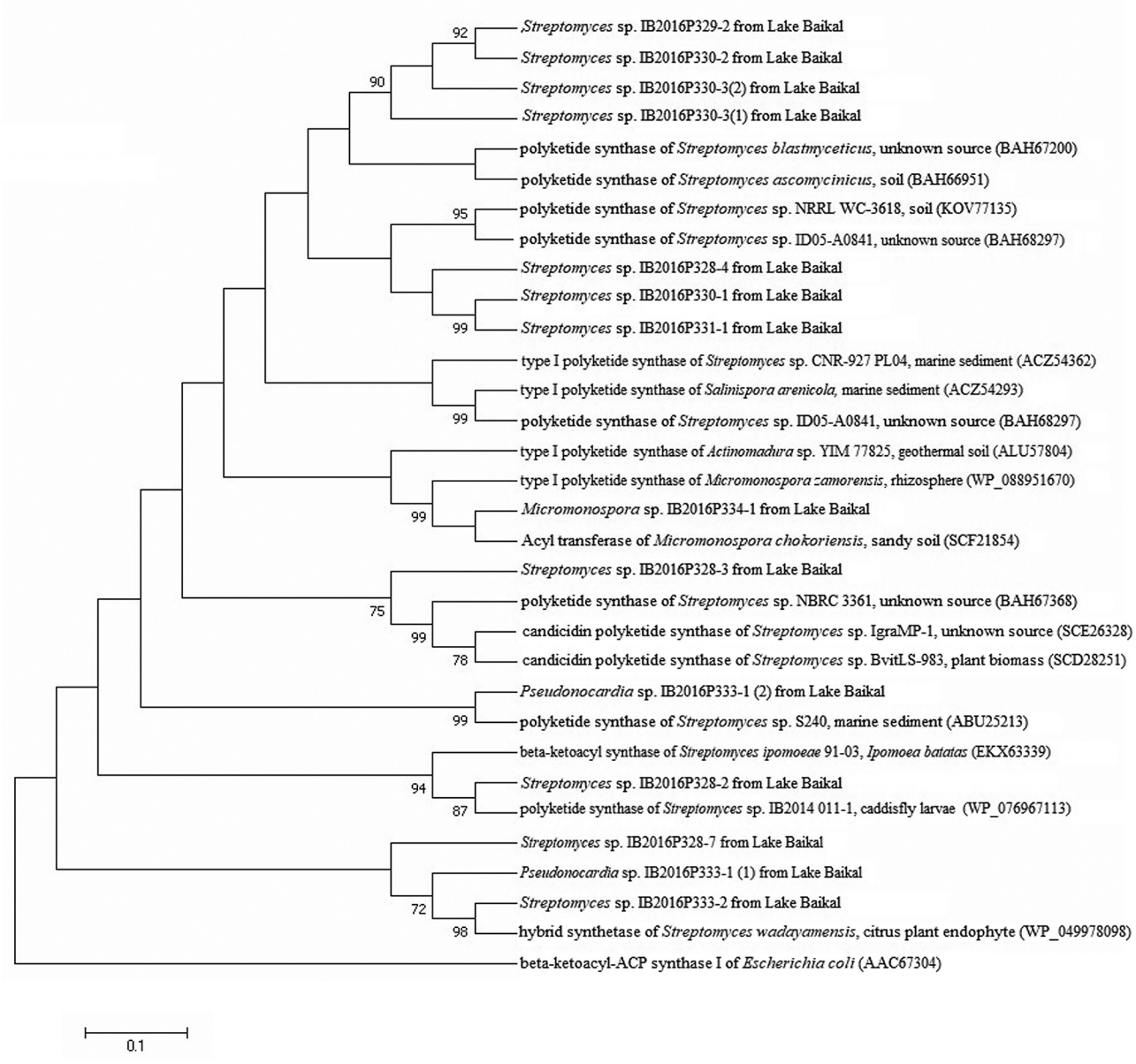


Fig. 2. Maximum likelihood phylogenetic tree for the amino acid sequences of putative ketosynthase (KS) domains from polyketide synthases of freshwater *Actinobacteria* isolates with closest neighbors from the GenBank database. Percentage bootstrap values from 1000 resamplings are indicated at nodes. The scale bar represents 0.1 substitution per site. The sequence of β-ketoacyl-acyl carrier protein (ACP) synthase I of *Escherichia coli* was used as an outgroup. The environment from which each organism was originally isolated is listed in parentheses. When multiple KSs were evaluated from a single isolate, each KS is numbered in parentheses, i.e. (1) or (2) following strain ID. Accession numbers for freshwater KSs from the present study are provided in Table S2 in the Supplement

tivation-independent studies to further highlight microbial abundance and biodiversity from Lake Baikal sediments.

Although *Actinobacteria* strains comprised a small fraction (<2%) of the culturable bacterial realm in freshwater sediments at all sampled depths (Table S3), their rich secondary metabolic potential (Tables 1 & 2, Table S4) suggests that they may play a significant role in chemically mediated biotic interactions. In oligotrophic environments, organisms with slow growth rates and well-developed defense strategies were previously reported to hold a competitive advantage over fast growers (Coley et al. 1985). Hence, in Lake Baikal sediments where resources are scarce, it is plausible that the well-developed chemical repertoire of slow-growing *Actinobacteria* may provide a selective advantage against faster-growing bacteria or fungi.

Table 2. Comparison of the antibiotic activity of chemical extracts from freshwater *Actinobacteria* strains grown in saline and nonsaline media. Darker shades of grey indicate chemical extracts more strongly inhibitory toward evaluated microorganism. Reported values indicate the average percentage (n = 2) inhibition of indicated microorganism growth relative to DMSO vehicle-only controls. Inhibition values of greater than 75% are emphasized in **bold**. All chemical extracts were evaluated at a concentration of 25 µg ml^{-1}. (–) not applicable (antibacterial activities for strains that did not grow in a specified medium were not evaluated)

Strain	*Staphylococcus aureus*		*Pseudomonas aeruginosa*		*Candida albicans*		*Aspergillus nidulans*	
	Saline	Nonsaline	Saline	Nonsaline	Saline	Nonsaline	Saline	Nonsaline
Streptomyces sp. IB2016P328-1	62	0	3	0	**89**	**94**	**78**	44
Streptomyces sp. IB2016P328-2	–	**75**	–	0	–	0	–	55
Streptomyces sp. IB2016P328-3	**79**	**96**	2	19	49	64	32	48
Streptomyces sp. IB2016P328-4	44	**87**	0	0	**95**	**87**	**76**	56
Streptomyces sp. IB2016P328-5	11	8	0	0	**75**	**87**	60	41
Streptomyces sp. IB2016P328-6	14	0	0	12	48	43	32	12
Streptomyces sp. IB2016P328-7	27	22	0	0	**93**	**93**	**78**	58
Micromonospora sp. IB2016P328-8	–	0	–		–	26	–	36
Streptomyces sp. IB2016P329-1	34	10	0	8	**93**	**96**	51	19
Streptomyces sp. IB2016P329-2	18	0	0	0	**94**	54	63	2
Streptomyces sp. IB2016P330-1	20	0	0	11	**94**	**91**	45	17
Streptomyces sp. IB2016P330-2	61	7	12	0	**77**	25	44	22
Streptomyces sp. IB2016P330-3	0	71	0	0	**92**	**85**	49	60
Streptomyces sp. IB2016P331-1	50	13	0	19	61	**81**	55	1
Streptomyces sp. IB2016P332-1	0	0	0	0	48	54	**85**	20
Pseudonocardia sp. IB2016P332-2	–	0	–	11	–	0	–	0
Pseudonocardia sp. IB2016P333-1	–	0	–	0	–	0	–	11
Streptomyces sp. IB2016P333-2	30	**94**	0	0	**76**	**90**	13	48
Streptomyces sp. IB2016P333-3	6	**83**	0	0	61	**82**	21	39
Micromonospora sp. IB2016P334-1	–	5	–	5	–	1	–	0
Streptomyces sp. IB2016P334-2	**100**	**98**	0	0	73	39	63	56
Streptomyces sp. IB2016P334-3	–	0	–	0	–	0	–	0
Streptomyces sp. IB2016P335-1	0	57	0	0	**99**	**86**	43	0
Streptomyces sp. IB2016P336-2	24	4	18	0	**95**	46	**80**	0

Genetically encoded secondary metabolic capabilities of all 24 isolates were surveyed by PCR screening for selected natural product biosynthetic genes (Table 1). Some related strains, based on 16S rRNA gene sequencing (Fig. 1, Fig. S1), exhibited different secondary metabolite gene patterns (Table 1). This finding parallels previous studies of *Streptomyces* isolates from diverse ecosystems (Yuan et al. 2014, Jami et al. 2015, Antony-Babu et al. 2017).

Our PCR-based evaluation of freshwater *Actinobacteria* biosynthetic capabilities supported the fact that type I PKS and NRPS genes were more common than type II PKS and halogenase genes among evaluated strains. Our finding that type I PKSs were more common than type II PKSs corresponds with earlier PCR-based surveys of *Actinobacteria* biosynthetic genes from marine and freshwater environments (Yuan et al. 2014, Jami et al. 2015). Comparison of translated amino acid sequences for KS domains of amplified type I PKS genes with sequences in the NCBI protein database revealed that most share moderate or high (69–99%) similarity with KS domains from various ecosystems (Fig. 2, Table S2). Polyketides from terrestrial and marine *Actinobacteria* have previously been demonstrated as mediators of both cooperative and antagonistic interspecies interactions (Haeder et al. 2009, Traxler et al. 2012, Patin et al. 2016). Hence, the high prevalence of PKSs from Lake Baikal *Actinobacteria* (Table 1) suggests that polyketides are also mediators of ecological interactions in freshwater sediment ecosystems.

Both marine and terrestrial *Actinobacteria* are known as producers of halogenated natural products (Neumann et al. 2008, Gribble 2015). However, no halogenase-encoding genes were detected by PCR from the 24 Lake Baikal freshwater strains (Table 1). To determine whether halogenase-encoding genes were also absent from terrestrial or marine strains related to Lake Baikal isolates, publicly available genome sequences for close relatives of our strains (based on 16S rRNA sequences) were bioinformatically evaluated for halogenase homologues. Of the 8 evaluated genome sequences from terrestrial relatives, 2 (25%) provided strong evidence for haloge-

nase-encoding genes (Table S5). This suggests that the absence of halogenases is a unique feature of Lake Baikal strains rather than a general feature of these groups of *Actinobacteria*. One possible explanation is that the exceptionally low salinity of Lake Baikal (0.43 mg l^{-1} of Cl^-) (Rusinek et al. 2012) disfavors acquisition and/or maintenance of halogenase-encoding genes. Analogous conclusions were previously made for *Rhodobacteraceae* family representatives, in which nonmarine specimens lost genes encoding a Cl^- channel protein and dehalogenase in response to reduced sodium chloride concentration (Simon et al. 2017). Another plausible explanation is that halogenase-encoding genes from Lake Baikal strains diverge from those common in terrestrial and marine *Actinobacteria*, thus evading detection by PCR.

To further evaluate the adaptation of strains to the low-salinity environment of Lake Baikal, growth was assessed in both high- and low-salinity media (Table 1). Six of 24 freshwater isolates thrived in low-salinity media but failed to grow in high-salinity media during a 2 wk period. These included representatives from all 3 genera (*Micromonospora*, *Pseudonocardia*, *Streptomyces*) isolated from Lake Baikal. Although some saline-intolerant *Micromonospora* strains have been previously reported (Carro et al. 2018), most *Actinobacteria* from soils and marine sediments are regarded for tolerance of a broad range of salinities (Ludwig et al. 2012). Further, close relatives of all 6 saline-intolerant isolates in our study (Fig. 1, Fig. S1) were previously reported from marine environments. Hence, our observation may suggest long evolution of saline-intolerant strains in the low-salinity Lake Baikal environment, with selective pressures disfavoring the acquisition and/or maintenance of saline tolerance mechanisms. Our results also imply that observed saline-intolerant strains are biogeographically restricted to Lake Baikal, since surrounding terrestrial habitats possess relatively high salinities. Taken together, our observations of saline intolerance and absence of evidence for halogenases suggest both physiological and metabolic adaptation of some *Actinobacteria* to the low salinity of Lake Baikal.

In total, 75% of freshwater-derived strains grown in at least 1 media type (i.e. saline or nonsaline) yielded chemical extracts that were strongly inhibitory toward 1 or more evaluated microorganisms (Tables 1 & 2). Comparison of bioactivity profiles for *Actinobacteria* grown in saline vs. nonsaline media revealed a higher prevalence of strong antibacterial activity for isolates grown in nonsaline media. In contrast, observation of antifungal activity was more frequent in saline media (Table 1). Differences in metabolic and bioactivity profiles in response to salinity have been previously reported for a variety of marine and terrestrial *Actinobacteria*. For example, previous work demonstrated that some marine-derived actinomycetes exhibited more robust growth and increased production of bioactive metabolites when grown in nonsaline media (Goodfellow & Fiedler 2010). Additionally, the widely studied terrestrial model *Streptomyces coelicolor* A3(2) demonstrated changes in secondary metabolism in response to increased salinity (Sevcikova & Kormanec 2004, Fernández Martínez et al. 2009).

The functions of antimicrobial metabolites in natural environments remain debated. One prevalent hypothesis is that *Actinobacteria* deploy antibiotics, such as those supported by our study, as weapons to inhibit competing bacteria (Karlovsky 2008). Evidence for this classic hypothesis includes numerous studies demonstrating marine and terrestrial actinobacterial natural products as inhibitors of microbial growth in laboratory or mesocosm settings (Wietz et al. 2013, Abrudan et al. 2015, Patin et al. 2016, Tyc et al. 2017, van der Meij et al. 2017). An alternative hypothesis is that antimicrobials at sublethal concentrations, such as those likely common in sediments and other natural environments, cue cooperative behaviors among bacteria (Ratcliff & Denison 2011). This hypothesis has been supported by studies revealing that antibiotics at low concentrations induce responses such as natural product biosynthesis and biofilm formation (Hoffman et al. 2005, Linares et al. 2006, Seyedsayamdost 2014). Hence, while the present study provides evidence for the intriguing phylogeny, biogeography, and biosynthetic potential of *Actinobacteria* from an understudied ecosystem, further studies of antibiotic natural products within lake sediment environments are needed to establish their natural functions.

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