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ABSTRACT

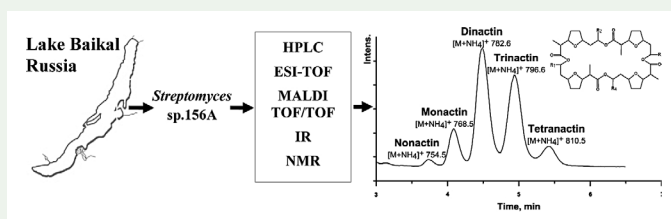
The potential antibacterial activity of secondary metabolites produced by *Streptomyces* sp. 156A isolated from Lake Baikal was investigated. The selective liquid–liquid extraction method was applied to obtain a mixture of nactins (polynactin) produced by the strain. The polynactin consisted of nonactin (3%), monactin (18%), dinactin (36%), trinactin (31%) and tetranactin (12%). The compounds were identified by MS/MS, ^1H and ^{13}C NMR methods. The loss of neutral 184 and 198 Da fragments from a sodiated molecular ion, $[\text{M} + \text{Na}]^+$, of nactins was observed in the MS/MS spectrum. The polynactin was shown to possess the antibiotic activity against Gram-positive strains including opportunistic strains and strains isolated from various ecosystems of Lake Baikal.

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Streptomyces; nactins; Lake Baikal



1. Introduction

Bacterioplankton plays an important role in the breakdown of organic compounds in aquatic ecosystems and thus is an important part of food web (Teeling et al. 2012). Despite the intensive study of freshwater bacterial biodiversity (Tang et al. 2014), the concept of the mechanisms that lead to the change in bacterioplankton community composition is still far from being understood. Nevertheless, there are two factors supposed to be the main causes of the changes in the microbial community. They are (1) the variation in the phytoplankton structure (Teeling et al. 2012) and (2) presence of microorganisms producing biologically active compounds (Bhatnagar & Kim 2010) in the aquatic ecosystem. The synthesis of antibiotic compounds assists microorganisms, such as actinomycetes, to participate in the antagonistic microbial interactions (Terkina et al. 2006; Mokni-Tlili et al. 2011).

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Lake Baikal is well known for its oligotrophic environment (Grachev 2002). The specific ecological conditions in Lake Baikal, first and foremost, the low content of dissolved organic matter and biogenic elements, are likely to affect the vital functions of microorganisms and cause competition between bacteria for nutrient sources.

Actinomycetes are widely distributed in all the ecological niches of Lake Baikal: in the water column, in sediments and in various aquatic organisms (as associates) (Terkina et al. 2002; Bel'kova et al. 2003; Parfenova et al. 2008). Actinomycetes of *Streptomyces* and *Micromonospora* genera were reported to possess the antagonistic activity against bacteria isolated from water and sediments of Lake Baikal, as well as against selected antibiotic-resistant microorganisms (Terkina et al. 2006). These biologically active compounds produced by the Lake Baikal actinomycetes have not been previously isolated and characterised.

Streptomyces is a large genus of bacteria, which is well known for its production of secondary metabolites. Indeed, about 34% of all known microbial natural products are produced by *Streptomyces* (Bérdy 2005). Among them are cyclic dipeptides (Wattana-Amorn et al. 2015), anthracyclines (Hu et al. 2016), pyrrol compounds (Chen et al. 2015; Yang et al. 2015) and lipopeptides (Yang et al. 2014).

This study aimed at (1) isolation of secondary metabolites responsible for *in vitro* antagonistic activity of the strain *Streptomyces* sp. 156A using bioassay-guided fractionation and selective liquid–liquid extraction; (2) identification of the isolated compounds by MALDI-MS/MS and NMR spectroscopy; (3) probing of these compounds against micro-organisms isolated from multiple ecosystems of Lake Baikal; and (4) probing of these compounds against laboratory test cultures, including antibiotic-resistant strains.

2. Results and discussion

2.1. Isolation and identification of bacteria

Bacterioplankton was collected from the coastal zone of Lake Baikal and strain 156A was isolated as described (see Sobolevskaya et al. 2006 and supplementary material). According to our studies, it showed a wide range of extracellular enzymatic activities, namely phosphatase, protease, lipase and phospholipase.

Molecular genetic identification based on ribosomal phylogeny allowed the strain to be assigned to *Streptomyces* genus. Automatic comparison of the protein profile of the strain 156A with Bruker Daltonics database showed score at 1.9 and the best match with *Streptomyces griseus*. Such score value allows the identification of strain at the genus level (1.7–2.0), though identification at the species level is not achieved. Previous studies demonstrated MALDI-TOF-MS profiling to be useful for rapid identification of environmental bacteria with high accuracy (Cayrou et al. 2010). Our MALDI-TOF-MS results confirm the accuracy of the assignment of the strain 156A to genus *Streptomyces*.

2.2. Isolation and identification of bioactive compounds

It has been previously shown that the ethyl acetate extract obtained from the liquid culture of the strain 156A possesses antibacterial activity against Gram-positive bacteria, such as *Listeria seeligeri*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Enterococcus faecium*, *Enterococcus faecalis* and pathogenic *Staphylococcus aureus*, *Listeria monocytogenes* and

Listeria ivanovii (Sobolevskaya et al. 2006). Antifungal activity of the extract against diploid fungi *Candida albicans* has also been reported (Sobolevskaya et al. 2006).

Therefore, the ethyl acetate extract of *Streptomyces* sp. 156A was fractionated using C18 column. Based on the activity of obtained fractions against *B. subtilis*, the compounds responsible for antibiotic activity were eluted in the range of high retention times ($T_R = 10\text{--}20$ min, 100% eluent B). Subsequently, liquid–liquid hexane extraction was used to isolate hydrophobic antibiotic compounds from the ethyl acetate extract. The hexane fraction obtained (Figure S1) and the residue after the extraction were further tested against *B. subtilis* and showed 15–18 and 0 mm zones of bacterial growth inhibition, respectively. The latter value indicates a high extraction degree of antibiotic compounds by hexane. Further LC-ESI-TOF analysis of the hexane fraction showed the presence of five major compounds with m/z 754.5, 768.5, 782.6, 796.6 and 810.5 (Figure S2(a)), while m/z 759.6, 773.6, 787.6, 801.6 and 815.6 were detected by MALDI-TOF (Figure S2(b)). The 14 Da difference between the m/z values was the same for both types of ionization and indicated the presence of homologous compounds. The 5 Da difference between ESI and MALDI, e.g. m/z 754.5 (ESI) and m/z 759.6 (MALDI), is assigned to the mass difference between ions $[M + NH_4]^+$ and $[M + Na]^+$.

MALDI-MS/MS spectra allowed the identification of cyclic copolymerisation of nonactin and homononactin acids on the basis of fragments lost (Figures S3 and S4). The main fragmentation pathway is C–O bond cleavage in an ester moiety and the formation of fragment ions corresponding to the loss of the acid units of 184 or 198 Da.

According to the comparison of the obtained data to those published previously, the compounds **1–5** were referred to nonactin, monactin, dinactin, trinactin and tetranactin, respectively. The methylation vs. ethylation patterns were suggested analogously to those published for nactins. (Řezanka et al. 2010; Crevelin et al. 2014) (Figures 1 and S5–S7). The

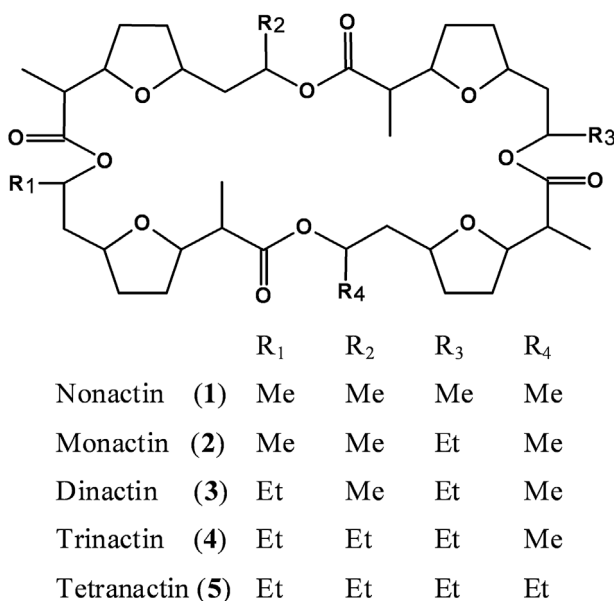


Figure 1. Structures of the naturally occurring macrotetrolides (Řezanka et al. 2010).

polynactin of *Streptomyces* sp. 156A was found to contain nonactin (3%), monactin (18%), dinactin (36%), trinactin (31%) and tetranactin (12%) according to MALDI-TOF (measured with $\pm 5\%$ accuracy), while its content in the hexane fraction was estimated to be 96.3% using total ionic current chromatogram of ESI-MS. The polynactin production by the *Streptomyces* sp. 156A under periodic cultivation was about 2 mg/L.

Previously, nactins were isolated from various *Streptomyces* species as a polynactin or individual compounds of different composition and in various proportions (Table S1). It has to be noted that among nactins, antibacterial activity was reported for polynactin and tetranactin (compound **5**) only (Žižka 1998).

2.3. Antimicrobial activity

According to the published data, polynactin exhibited a wide spectrum of biological activities: antibacterial, insecticidal, antifungal, immunosuppressive and antitumor (Borrel et al. 1994; Žižka 1998). Biological activity of nactins was usually associated with their ionophoric properties (Kusche et al. 2009), and the potencies of these activities appeared in parallel to the size of the alkyl substituents (Jizba et al. 1991). Moreover, tetranactin was shown to be the most potent member of the nactin's homologues (Žižka 1998).

We tested polynactin against *B. subtilis* at concentrations of 10, 25, 50 and 100 $\mu\text{g/mL}$, and the diameter of bacterial growth inhibition zone from 10 to 25 mm was observed. The highest inhibition concentration (100 $\mu\text{g/mL}$) was chosen for further analysis to achieve the stable inhibition effect. This concentration was based on the published observations, particularly suppression of phytopathogenic fungi by nactin-containing crude extract (100 $\mu\text{g/mL}$) (Silva et al. 2014), and inhibition of *Escherichia coli* growth by polynactin (25 $\mu\text{g/mL}$) (Nefelova et al. 1985). Further, we tested polynactin against opportunistic strains (see supplementary material for details), including antibiotic-resistant ones (Table S2). The inhibition zone of Gram-positive *B. subtilis*, *E. faecium* and *E. faecalis* ranged from 20 to 24 mm. The smaller inhibition zone was observed for *L. monocytogenes* (14 mm) and *S. aureus* (10–12 mm). Among the Gram-negative opportunistic bacteria, *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, *Klebsiella pneumoniae* and *E. coli* were tested. All Gram-negative strains tested as well as fungi *C. albicans* showed resistance to polynactin. Thus, the inhibition of growth was revealed only for Gram-positive test cultures.

Naturally occurring strains of *Proteobacteria*, *Firmicutes* and *Actinobacteria* phyla were selected for further study of the polynactin biological activity. Several strains were isolated from various ecological habitats of Lake Baikal: water, sediments and biofilms (Table S3). As a result of further probing, the specific inhibition of Gram-positive bacteria belonging to the two most populated phyla (*Actinobacteria* (*Rhodococcus* spp., *Microbacterium* sp.) and *Firmicutes* (*Bacillus* spp., *Paenibacillus* sp.)) was confirmed. The diameter of the bacterial growth inhibition zone was defined by individual features of cell interactions. It was found to vary from 10 to 20 mm and to be independent on the strain origin (Table S4). Among Gram-negative bacteria, representatives of two widespread genera (*Brevundimonas* and *Pseudomonas*) were tested. They belong to *Alphaproteobacteria* and *Gammaproteobacteria* classes of *Proteobacteria*, respectively. All of the nine strains tested showed no sensitivity to polynactin.

3. Conclusions

Summarising the results of our study, a mixture of the antibiotic compounds produced by Baikalian strain *Streptomyces* sp. 156A was isolated and identified by MALDI-MS/MS, LC/ESI-TOF-MS, IR and NMR spectroscopy as polynactin. It contained 3% nonactin, 18% monactin, 36% dinactin, 31% trinactin and 12% tetranactin. Polynactin showed a suppressive effect on all tested Gram-positive bacteria, including those isolated from different ecosystems of Lake Baikal (*Rhodococcus* spp., *Microbacterium* sp., *Bacillus* spp. and *Paenibacillus* sp.) and those from opportunistic laboratory cultures. None of the Gram-negative strains tested showed the sensitivity to polynactin.

Lake Baikal is well known for its oligotrophic environments where high competition for nutrients exists. Such a competition is most extensive between bacteria belonging to the same taxon. Thus, the production of secondary metabolites, such as polynactin, might give an advantage to *Streptomyces* sp. 156A to compete for substrate and thereby to survive in microbial community of Lake Baikal.

Supplementary material

Experimental details related to this article are available online, alongside Figures S1–S7 and Tables S1–S4.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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