SUPPLEMENTARY MATERIAL

Ionofore antibiotic polynactin produced by *Streptomyces* sp. 156A isolated from Lake Baikal

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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 Da 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.

Keywords: Secondary metabolites; *Streptomyces*; nactins; Lake Baikal

Experimental

Microorganism isolation and identification

Water was sampled on Lake Baikal in March 2003 at the littoral station near cape Berezovyi (N 51°50'755 E 104°54'152) from the depth of 100 m into sterile 250 mL flasks using a bathometer. The strain *Streptomyces* sp. 156A was isolated on peptone-yeast agar media (PDA, g/L: peptone - 1.0, yeast extract - 1.0, agar - 18.0), the isolation temperature was 20°C. Morphological, biochemical and physiological characteristics of the strain as well as extracellular enzyme activities were studied as described previously (Parfenova et al. 2006). Phosphatase activity was determined using a standard kit Alkaline Phosphatase FL (Vital Diagnostics SPb, Russia). The reagent containing p-nitrophenyl phosphate (0.25 mL) was added to each well of plates together with the cultures studied. Phosphatase activity was determined by the intensity of yellow staining of the resulting suspension after the incubation for 1 h and 24 h at 20°C. Proteinase activity was assayed on milk agar containing defatted milk in 1.8% starvation agar (15 mL per 100 mL). Lecithinase (phospholipase C) activity was determined using thoroughly suspended chicken egg yolk. The medium was poured into sterile Petri dishes and the studied cultures were stab-inoculated. A clearance zone was observed in the presence of proteolytic or lecithinase activity around stabs. The results were recorded after the incubation for 3 days at 20°C. Amylase activity was determined on starch agar (KH₂PO₄, 0.5 g/L; K₂HPO₄, 0.5 g/L; MgSO₄×7H₂O, 0.2 g/L; (NH₄)₂SO₄, 0.2 g/L; starch, 10 g/L, and agar, 15 g/L) (Rodina 1965, pp. 159–161) sterilized and poured into sterile Petri dishes. The cultures tested were stab-inoculated and incubated for 3–5 days. Starch hydrolysis was visualized as a clearance zone around stabs after staining with Lugol's solution. Lipase activity was determined on 20 g/L tributyrin agar (Fluka no. 91015). After the sterilization at 121°C for 15 min, the medium was cooled to 80°C, complemented with tributyrin (Fluka no. 91010), stirred on a magnetic stirrer for 2–3 h until complete homogenization, and poured into Petri dishes. The cultures tested were stab-inoculated and incubated for 5 days at 20°C. Clearance zones were indicative of lipase activity.

The identification of the strain was performed by two independent methods: ribosomal phylogeny (Parfenova et al. 2006) and MALDI Biotyper. Accession number of the 16S rDNA sequence is EU186098 *Streptomyces* sp. Lake Baikal T156.

The standard procedure recommended by Bruker Daltonics (the direct transfer method (Seng et al. 2010) was used for the bacterial MALDI-TOF MS identification. Protein profiles were obtained from a single colony of bacteria directly deposited on the MALDI-TOF target plate and overlaid with matrix solution (i.e., a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid, after air-drying at room temperature for 5

min). The analysis was carried out using a mass spectrometer UltrafleXtreme MALDI-TOF (Bruker Daltonics GmbH, Germany). The resulting protein profile was compared to that of database Bruker Daltonics (MALDI Bio-typer, version 3.1, and flexAnalysis, version 3.4) in the automatic mode.

Strain cultivation and antibiotic extraction

The small agar slice bearing mycelia of *Streptomyces* sp. 156A was placed in 250 mL Erlenmeyer flask containing 100 mL medium (g/L: glycerol – 3.0; fish-peptone broth – 0.5; NaCl – 0.45; CaCO₃ – 0.35, pH 7.0) and incubated at 22–25°C, at 120 rpm for 15 days. An aliquot was placed in 5 L Erlenmeyer flasks containing 2 L of the same medium and incubated for 6 days at the same conditions. Total 10 L culture broth was performed.

The culture broth with cells was extracted three times with EtOAc (75:25; v/v) at ambient temperature. The resulting extracts were combined and concentrated under vacuum at 40°C. Crude extract was dissolved in hexane (at the rate of 1.5 mg/g), shaken (10 min; BioVortex V1 Plus «BIOSAN», Latvia), and centrifuged (15 min, 12.8 kg). The supernatant was evaporated for the next redistribution in methanol. Flow chart is presented in Fig. S1

The strain *Bacillus subtilis* ATCC 6633 was used as the test microorganism during antibiotic isolation.

Compound structural identification

Fractionation of microbial extracts was performed using high performance liquid chromatograph MiLiChrom A-02 (Econova, Russia). Separation conditions were: column (2 × 75 mm, 5 µm) packed by ProntoSIL 120-5C18 AQ; flow rate of 0.15 mL/min; gradient elution by 1.5 mL of 20 to 100% B, 3 mL of 100% B; solvent A - distilled water, solvent B - acetonitrile (HPLC-gradient grade, “Panreac” EU).

An Agilent 1200 chromatographic system controlled by Chemstation Version B.01.03 (Agilent Technologies, USA) and coupled to Agilent MSD-TOF 6200 with an ESI source operated in the positive mode was used for the separation of hexane fraction. HPLC was carried out using a Zorbax 300 SB-C18 column (2.1 × 150mm, 5 µm) at 35°C, with flow rate of 0.2 mL/min and 90% B isocratic elution of 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in MeCN (solvent B). Other conditions were: capillary voltage 3500 V, nebulizer gas (N₂) at 30 psi, dry gas (N₂) at 5 L/min, dry temperature 325°C, scan range m/z 100-2000.

The confirmation of nactin structures was performed with UltrafleXtreme MALDI TOF/TOF spectrometer (Bruker Daltonics GmbH, Bremen, Germany) using flex control software (Bruker Daltonics). The sample was applied in HCCA (α-cyano-hydroxycinnamic

acid) matrix. The MS measurements were taken in the reflective positive ion mode within the mass range of 100-3500 Da. The MS/MS analyses were performed in LIFT mode with voltage of 19 kV.

IR spectra of antibiotic compounds **1-5** were recorded in the range of 550-3500 cm^{-1} with a laser FTIR spectrometer "Infracum FT-801". NMR spectra of the mixture of compounds **1-5** were recorded in CDCl_3 solution at room temperature employing "Bruker DPX400" 400MHz NMR spectrometer (Bruker Daltonics GmbH, Germany).

The IR and NMR data for the mixture of compounds **1-5** were the following: Yield 25.4 mg, colorless oil; IR ν (cm^{-1}): 2955, 2924, 1734, 1462, 1190, 1082; and other unassigned signals. ^1H NMR, δ : 4.93 (m, 1H), 4.01 (pent, 1H), 3.84 (hex, 1H), 2.52 (m, 1H). 1.85-2.05 (m, 2H), 1.65-1.85 (m, 2H), 1.40-1.65 (unresolved, 5H), 1.23 (d, %3H), 1.10 (d, %3H), 1.08 (d, %3H), 0.88 (t, %3H), 0.75-0.85 (m, 1H). ^{13}C NMR, δ : 174.6 & 174.3 ($\text{C}2''$), 80.2 & 80.0 ($\text{C}5$), 76.6 & 76.5 ($\text{C}2$), 73.4 & 69.2 ($\text{C}2'$), 45.4 & 45.2 ($\text{C}1''$), 42.5 & 40.1 ($\text{C}1'$), 31.6 & 31.51 ($\text{C}3/\text{C}4$), 28.3 & 28.3 ($\text{C}3/\text{C}4$), 27.6 ($\text{R}=\text{Et CH}_2$), 20.6 ($\text{R}=\text{Me } 2'-\text{CH}_3$), 13.3 & 13.0 & 12.8 ($1''-\text{CH}_3$), 9.5 ($\text{R}=\text{Et CH}_3$) (see Figures S4-S6).

Antimicrobial activity assays

To study the antimicrobial activity of the polynactin, the mixture was dissolved in methanol:water 10:90 (v/v) at a concentration of 10, 25, 50 and 100 $\mu\text{g/mL}$. The antimicrobial activity was detected by the agar-well diffusion assay: wells with diameter of 8 mm were done in fish-peptone agar freshly planted with test culture, where aliquot of nactin mixture was added. Plates with opportunistic strains were incubated at 37°C , while original ones were at $22-25^\circ\text{C}$; the values of inhibition zone were measured in mm over 24 hours of incubation. (Guidelines... 2004).

Strains from the laboratory culture collection (Laboratory of Microbiology, Limnological Institute SB RAS) were used as test microorganisms. Opportunistic ones were: *B. subtilis* (DSM and PMBC), *Enterococcus faecium* (CIP 104105), *Enterococcus faecalis* (ATCC 29212), *Listeria monocytogenes*, *Yersinia pseudotuberculosis*, *Klebsiella pneumoniae* (ATCC 700603), *Candida albicans* (90028 and ATCC 10231), including antibiotic-resistant *Streptococcus aureus* (ATCC 25923 and MRSA), *Pseudomonas aeruginosa* (27853 and 190158), *Escherichia coli* (25922 and M17-02) (Table S2). Original strains isolated from Lake Baikal are listed in Table S3.

Figure S1. Flow chart for polynactin isolation from *Streptomyces* sp. 156A.

Figure S2. Total ionic current chromatogram of *Streptomyces* sp.156A hexane fraction (ESI-TOF) (a) and full-scan mass spectrum of *Streptomyces* sp.156A hexane fraction (MALDI-TOF) (b).

Figure S3. MALDI-TOF MS/MS spectra of sodium adduct ions of m/z 787.6 and fragmentation schemes.

Figure S4. MALDI-TOF MS/MS spectra of sodium adduct ions of m/z 773.6 (a), m/z 801.6 (b) and m/z 815.6 (c)

Figure S5. ¹H NMR spectrum of compounds **1-5** (mixture).

Figure S6. ¹³C NMR spectrum of compounds **1-5** (mixture).

Figure S7. IR spectrum of compounds **1-5** (mixture)

Table S1

Polynactin composition (%) of various *Streptomyces* species

Species	Nonactin	Monactin	Dinactin	Trinactin	Tetranactin	Reference
<i>Streptomyces</i> sp. 156A	3	18	36	31	12	present study
<i>Streptomyces chrysomallus</i>	69-79	19-29	1-3	traces	traces	Nefelova et al. 1985
<i>Streptomyces globisporus</i>	9	25	46	20	NI	Jizba et al. 1991
<i>Streptomyces canus</i>	4	24	58	10	4	Wang et al. 2014

NI – not identified

Table S2

Resistance of opportunistic bacteria used in the present study to antibiotics (according to

Terkina et al. 2006)

Strain	Antibiotics
<i>Staphylococcus aureus</i> (ATCC 25923 and MRSA)	ampicillin, streptomycin, erythromycin, tetracycline, kanamycin
<i>Pseudomonas aeruginosa</i> (27853 and 190158)	benzylpenicillin, ampicillin, tetracycline, cefazolin, cefuroxime, carbenicillin
<i>Escherichia coli</i> (25922 and M17-02)	streptomycin, kanamycin, furadonin

Table S3

Isolation origins of natural strains isolated from various ecosystems of Lake Baikal and used for antibacterial activity analysis

Name of the strain	Isolation source	Type of cell wall
<i>Rhodococcus</i> sp. 4	Water collected near sponge <i>Baicalospongia intermedia</i> , the station located near cape Berezovii in the south basin of the lake	G+
<i>Rhodococcus</i> sp. 16	Water collected from the depth of 500 m, the station located at 3 km from sett. Listvianka	G+
<i>Rhodococcus</i> sp. 3	Water collected from the depth of 500 m, the station located at 3 km from sett. Listvianka	G+
<i>Rhodococcus</i> sp. 13	Water collected from the depth of 1000 m, the station located at 3 km from sett. Listvianka	G+
<i>Rhodococcus</i> sp. 155-09	Biofilm formed on quartz plate in the littoral zone of the lake	G+
<i>Microbacterium</i> sp. 10	Sediments collected from the depth of 15-18 cm, the station located at Kanyon Kukuiskyi	G+
<i>Bacillus</i> sp. 44	Sediments collected from the depth of 33-36 cm, the station located at Kanyon Kukuiskyi	G+
<i>Bacillus</i> sp. 2A	Biofilm formed on stone in the littoral zone of the lake	G+
<i>Bacillus</i> sp. P29.2.1	Neustone biofilm collected in Bay Bazarnaya, Lake Baikal	G+
<i>Bacillus</i> sp. P32	Neustone biofilm collected in Bay Bazarnaya, Lake Baikal	G+
<i>Paenibacillus</i> sp. 12A	Biofilm formed on the stone in the littoral zone of the lake	G+
<i>Brevundimonas</i> sp. 19	Water collected from the depth of 500 m at the central station of the south basin of the lake	G–
<i>Brevundimonas</i> sp. 37	Water collected from the depth of 700 m at the central station of the south basin of the lake	G–
<i>Brevundimonas</i> sp. 43	Sediments collected from the depth of 15-18 cm, the station located at Kanyon Kukuiskyi	G–
<i>Brevundimonas</i> sp. 47	Sediments collected from the depth of 30-33 cm, the station located at Kanyon Kukuiskyi	G–
<i>Pseudomonas</i> sp. 38	Water collected from the depth of 500 m, the station located near sett. Listvianka	G–
<i>Pseudomonas</i> sp. 4A	Biofilm formed on urtite plate in the littoral zone of the lake	G–
<i>Pseudomonas</i> sp. 6A	Biofilm formed on granite plate in the littoral zone of the lake	G–
<i>Pseudomonas</i> sp. NA173	Neustone biofilm collected in estuary of the River Sukhaya, the tributary of Lake Baikal	G–
<i>Pseudomonas</i> sp. NA176	Neustone biofilm collected in the estuary of the River Sukhaya, the tributary of Lake Baikal	G–

Table S4

Testing polynactin against the original strains isolated from different ecosystems of Lake Baikal

Name of the strain	Inhibition zone, mm
<i>Rhodococcus</i> sp. 4	12–14
<i>Rhodococcus</i> sp. 16	14–18
<i>Rhodococcus</i> sp. 3	12
<i>Rhodococcus</i> sp. 13	18
<i>Rhodococcus</i> sp. 155-09	12–16
<i>Microbacterium</i> sp. 10	16–18
<i>Bacillus</i> sp. 44	16–18
<i>Bacillus</i> sp. 2A	18–22
<i>Bacillus</i> sp. P29.2.1	10–14
<i>Bacillus</i> sp. P32	16–20
<i>Paenibacillus</i> sp. 12A	12

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