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BIOLOGICALLY ACTIVE COMPOUNDS FROM LAKE BAIKAL STREPTOMYCETES

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Data on the biological activity of ethylacetate extracts of Lake Baikal streptomycetes were obtained for the first time. The extracts of isolated actinobacteria of the genus Streptomyces *were tested for the presence of compounds that inhibit the growth of pathogenic and conditionally pathogenic microorganisms and that exhibit a cytotoxic effect on Erlich carcinoma tumor cells. The ability of streptomycetes to produce inhibitors of enzymes isolated from marine organisms was also investigated. The production of bioactive compounds as a function of medium composition was given.*

Key words: actinomycetes, *Streptomyces*, biological activity, Lake Baikal.

Gram-positive bacteria of the actinomycete line are among the leading producers of antibiotics and other biologically active compounds. The most significant and practically important compounds are obtained from actinobacteria of the genus *Streptomyces* [1-4]. Much has been published on the chemical structure determination of antitumor and antibacterial compounds produced by streptomycetes isolated from soil samples and marine sources [5, 6]. Until now, metabolites of actinobacteria isolated from freshwater aquifers have not been studied.

Herein we report data on the biological activity of extracts of actinomycetes inhabiting Lake Baikal. Cultures of the pathogens *Listeria ivanovii*, *L. monocytogenes* 4-B, *Staphylococcus aureus* 3515, *Yersinia pseudotuberculosis* H-2781, *Y. pseudotuberculosis* 282, and the conditionally pathogenic strains *L. seeligeri*, *S. epidermidis* 2482, *Proteus mirabilis* 5216, *P. vulgaris* 4848, *Salmonella enteritidis* 124-6, *Klebsiella pneumoniae* 181.0.15, *Pseudomonas fluorescens* 282, *P. aeruginosa* H-1515, *Escherichia coli* 384, and *E. coli* DSM 3901 from the museum collection of the Institute of Epidemiology and Microbiology (Vladivostok) and museum strains *Staphylococcus aureus* ATCC 21027, *Bacillus subtilis* ATCC 6633, *Enterococcus faecium* CIP 104105, and *Candida albicans* KMM 455 from the collection of marine microorganisms (Vladivostok, PIBOC) were used as test cultures to determine the antimicrobial activity of the ethylacetate extracts of streptomycetes.

Ethylacetate extracts of seven actinomycetes (76S, 128A, 150A, 251A, 156A, 178A, and 243S) exhibited specific activity only toward gram-positive test cultures. Extracts of cultures 5S, 12S, 125A, 229S, and 172A were active toward both gram-positive and gram-negative test cultures. One streptomycete (229S) synthesized compounds that were active only toward gram-negative bacteria (Table 1). Of the tested strains, 11 (5S, 76S, 125A, 128A, 12S, 172A, 150A, 251A, 156A, 178A, and 243S) synthesized compounds that were strong inhibitors of the pathogenic bacteria *L. monocytogenes* 4B, *L. ivanovii*, and *S. aureus* 3535. Extracts of actinomycetes (5S, 12S, 156A, and 178A) suppressed growth of all three *Listeria* species. The synthesis of antibiotics depended on the cultivation medium for 86% of the investigated streptomycetes.

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TABLE 1. Antimicrobial Activity of Ethylacetate Extracts of Culture Liquid from Lake Baikal Actinobacteria

Strain No. (isolation source)	Medium (MD)	*Listeria monocytogenes* 4-B	*Listeria ivanovii*	*Listeria seeligery*	*S. aureus* 3535	*S. epidermidis* 4282	*B. subtilis* ATCC 6633	*S. aureus* ATCC 21027	*C. albicans* KMM 455	*Ent. faectum* CIP 104105
5S	I	10/100	-	-	-	-	16/100	23/100	-	10/100
(water)	II	16/100	24/200	25/200	12/100	15/200	10/100	30/100	-	16/100
	III	-	-	-	-	-	18/100	25/100	-	18/100
	IV	-	-	-	-	-	-	-	-	-
76S	I	-	-	-	-	-	-	-	-	-
(water)	II	-	-	-	12/100	-	14/100	23/100	-	20/100
	III	7/100	-	-	7/100	-	+/100	-	-	20/100
	IV	-	-	-	-	-	-	-	-	-
125A	I	-	-	-	-	-	-	-	-	17/100
(water)	II	-	-	-	9/100	-	-	12/100	17/100	24/100
	III	-	-	-	-	-	-	13/100	17/100	25/100
	IV	-	-	-	11/30	-	-	18/100	16/100	30/100
128A	I	-	-	-	-	-	-	-	-	-
(water)	II	-	-	-	15/100	8/200	-	17/100	-	20/100
	III	7/100	-	-	8/100	-	-	-	-	18/100
	IV	8/100	-	-	7/100	-	+/100	15/100	-	20/100
12S	I	15/100	18/200	25/200	16/100	20/200	-	-	-	+/100
(water)	II	6/100	-	-	12/30	-	16/100	18/100	-	20/100
	III	8/300	-	-	16/10	6/10	-	25/100	-	16/100
	IV	-	-	-	-	-	-	11/100	-	-
172A	I	-	-	-	-	-	-	-	-	34/100
(water)	II	10/100	-	-	-	-	-	-	-	30/100
	III	-	-	-	-	-	-	-	-	20/100
	IV	-	-	-	-	-	-	-	-	-
150A	I	-	-	-	-	-	-	-	-	-
(water)	II	-	-	-	15/200	10/200	18/100	8/100	-	20/100
251A	I	-	-	-	-	-	-	-	-	-
(water)	II	11/100	-	18/200	12/100	-	13/100	18/100	-	21/100
	III	9/100	-	-	-	-	-	-	-	18/100
	IV	10/100	-	-	-	-	-	-	-	14/100
156A	I	-	-	-	-	-	-	-	-	-
(water)	II	12/100	8/200	24/200	14/100	10/200	-	-	-	-
	III	11/100	-	-	13/100	-	20/100	13/100	14/100	20/100
	IV	-	-	-	-	-	17/100	-	-	25/100
178A	I	-	-	-	10/100	-	20/100	-	-	20/100
(water)	II	18/100	10/200	25/200	12/100	12/200	20/100	10/100	-	-
243S	I	-	-	-	-	-	-	-	-	16/100
(sediment)	II	-	-	-	14/100	8/200	-	-	-	-
	III	-	-	-	-	-	-	-	-	-
	IV	7/100	-	-	-	-	-	-	-	15/100

(-), Lack of antimicrobial activity; (+), slight antimicrobial activity.

[1]For strains 125A, medium IV; 12S, IV; and 229S, II, the band of growth inhibition of *Y. pseudotuberculosis* H-2781 was 15 mm (300 μg), 9 mm (300 μg), and 15 mm (300 μg), respectively.

Strain 229S (sponge) in media (I, III, IV) did not synthesize compounds that stimulated bacteria growth in all test cultures.

Strain 172A (medium III) synthesized compounds that stimulated growth of *Y. pseudotuberculosis* H-2781 over a 9-mm band of growth stimulation (100 μg).

[2]Strain 5S (medium IV) gave a 12-mm band of growth inhibition for *E. coli* 384 (30 μg).

The anti-listeria activity was found mainly for cultures grown on semisynthetic (II) and synthetic (III) media. Most actinomycetes on these same media synthesized compounds that were active toward *S. aureus* 3535. Strains *S.* sp. 125A and *S.* sp. 12S cultivated on synthetic (IV) medium synthesized compounds that inhibited growth of *Y. pseudotuberculosis* H-2781. Strain *S.* sp. 5S produced compounds that suppressed growth of *E. coli* 384. Fermentation of these bacteria on organic (I) and semisynthetic (II) media led to the synthesis of compounds that were active toward most gram-positive bacteria.

Strain *S.* sp. 172A grown on medium (III) synthesized compounds that stimulated growth of *Y. pseudotuberculosis* H-2781; on medium (I), slightly active compounds toward gram-positive bacteria. Strain *S.* sp. 229S produced biologically active compounds that suppressed growth of *Y. pseudotuberculosis* H-2781 only on semisynthetic medium (II). Extracts of strains (125A, 156A) suppressed growth of the yeast-like fungus *C. albicans* KMM 455. Strain *S.* sp. 125A synthesized anticandidal compounds if grown on semisynthetic and synthetic media whereas *S.* sp. 156A produced such compounds only if cultivated on medium (III). Streptomycetes (strains 131A and 138A) did not synthesize active compounds on any of the media used. It was found that medium (II) was most favorable for cultivation of actinomycetes and production of bioactive compounds by them.

We used the following enzymes to search for producers of enzyme inhibitors: amylase, 1→3-β-D-glucanase from *Trichoderma aureviride*, 1→3-β-D-glucanase from *Strongylocentrotus intermedius*, arylsulfatase from *Littorina kurila*, and 1→3-β-D-glucanase (LIV) from crystalline style *Spisula sachalinensis*. Incubation of these enzymes with the studied extracts led to the synthesis by strains *S.* sp. 156A and *S.* sp. 229S of extracellular inhibitors of 1→3-β-D-glucanase (LIV) for fermentation in all media (I), (II), (III), and (IV). Bacteria *S.* sp. 150A and *S.* sp. 172A produced inhibitors of this enzyme only if cultivated on semisynthetic medium. Strain *S.* sp. 5S fermented on synthetic medium (IV) synthesized an enzyme inhibitor; on natural medium (I), compounds that activated enzyme. Strain *S.* sp. 12S synthesized inhibitors if grown on semisynthetic medium (II) and enzyme activators on synthetic medium (IV). Strain *S.* sp. 243S was a source of enzyme activators if cultivated on synthetic medium (IV). Actinomycete *S.* sp. 178A produced inhibitors only if grown on organic medium (I). Thus, eight actinomycete strains produced 1→3-β-D-glucanase (LIV) effectors.

Endo 1→3-β-D-glucanase is widely distributed in nature [7]. In the last decade it has been continuously studied because of the role of these compounds in plant and animal immunity [8]. Interest in finding new enzyme effectors in marine organisms and plants is growing [9, 10]. Marine plants [9] and sponges [11-13] are rich sources of glucanase inhibitors. Data on effectors of endo 1→3-β-D-glucanase that are synthesized by aqueous streptomycetes have not been reported. Thus, we investigated for the first time the effect of compounds produced by Lake Baikal bacteria on the process catalyzed by endo 1→3-β-D-glucanase. During the experiment we did not observe effectors of enzymes such as amylase, 1→3-β-D-glucanase from the marine fungus *Trichoderma aureviride*, 1→3-β-D-glucanase from the sea urchin *Strongylocentrotus intermedius*, and arylsulfatase from the marine mollusk *Littorina kurila*.

The study of the cytotoxic action of ethylacetate extracts from streptomycetes found that practically all extracts at a concentration of 100 µg/mL exhibited cytotoxic activity in vitro toward ascitic Erlich carcinoma tumor cells, inhibiting cell proliferation up to 80% compared with controls (Table 2).

It is known that the level of synthesis of high- and low-molecular-weight compounds depends substantially on the microorganism cultivation conditions because of the high adaptability of bacteria to the environment [14, 15]. In the present work, we investigated the dependence of the production of cytotoxic compounds on the nutrient medium composition. All extracts from streptomycetes grown on organic (I) and semisynthetic (II) media produced a high level of cytotoxins. Inhibition of growth and tumor-cell proliferation by the studied extracts varied from 67 to 83%. Production of cytotoxic compounds decreased only for strain 251A. The extract of this streptomycete inhibited DNA synthesis in tumor cells by only 49%. For streptomycetes 131A and 172A, cytotoxin production decreased sharply, down to its complete absence, if cultivated on medium (III). On medium (IV), cytotoxin production decreased for strains 178A, 229S, and 251A (Table 2). Despite the noted variations in cytotoxin production, cytotoxin production was relatively stable for streptomycetes grown on various media.

The results established that 86% of the studied actinobacteria are potential sources of biologically active compounds. For streptomycetes, antibiotic synthesis depends on the cultivation medium composition. Most studied strains produced compounds with a broad spectrum of antibacterial activity if cultivated on semisynthetic medium (II). Of all streptomycetes, 57% synthesized antimicrobial compounds and glucanase effectors. Of these, 36% of streptomycetes produced inhibitors, 7% activators, and 14% inhibitors and activators of enzymes.

TABLE 2. Cytotoxic Activity of Streptomycetes Extracts Toward Erlich Carcinoma Tumor Cells *in vitro*, %

Strain No.	Inhibition of tumor growth			
	I	II	III	IV
150A	80	83	-	-
131A	74	70	-11	76
178A	79	72	75	11
243S	72	70	76	72
76S	72	67	67	74
138A	72	73	72	71
172A	75	71	-11	79
229S	69	71	72	-13
251A	49	81	71	22
5S	65	75	77	62
125A	71	73	73	61
156A	71	-	82	81
12S	-	73	74	82
128A	-	71	67	75

The ability of actinobacteria *S.* sp. 5S and 12S to synthesize antimicrobial compounds with a broad spectrum of action and activators and inhibitors of glucanase in all media used in the experiments should especially be noted. The number of streptomycetes that synthesize only compounds with antimicrobial activity was 21%. The remaining bacteria did not synthesize compounds with the desired activity on any of the media used. Not one of the studied strains synthesized compounds that caused lysis of murine erythrocytes. Keeping in mind the cytotoxic action of low-molecular-weight compounds of streptomycetes and the complete lack of lytic activity toward blood erythrocytes, further study of the antitumor activity and chemical composition of low-molecular-weight compounds isolated from streptomycetes is interesting for the selection of promising antitumor agents.

EXPERIMENTAL

Isolation and Identification of Actinomycetes. The 14 studied strains of actinomycetes (5S, 76S, 125A, 128A, 12S, 229S, 172A, 150A, 251A, 156A, 178A, 243S, 131A, 138A) are representatives of the Lake Baikal microbial community. They all belong to the genus *Streptomyces* according to morphological and cultural signatures [16, 17]. Twelve cultures were isolated from water samples taken near the shore (near Berezovyi) and in the depths (central point of Listvyanka and Tankhoi) of the lake. Strain *S.* sp. 243S was isolated from sediments taken near Posol′sk Bank from a depth of 108 cm; strain *S.* sp. 229S, from the Baikal sponge *L. baicalensis*. The following selective media were used to isolate microorganisms: starch—casein agar diluted 10 times [18], fish-peptone agar diluted 10 times (FPA:10) [19], and peptone-yeast agar (0.01%).

Actinomycete Cultivation. Actinomycetes were cultivated in Ehrlenmeyer flasks (250 cc) with medium (100 mL) of the given composition on a UVMT-12-250 apparatus for growing microorgamisms (rocking rate 110 rpm). The cultivation time was 5 d; cultivation temperature, 22°C. Cultivation was carried out in modified media (I-IV). Medium (I): fish-peptone bullion (2 g/L); medium (II): glycerine (3 g), fish-peptone bullion (0.5 g), peptone (0.5 g), NaCl (0.45 g), $CaCO_3$ (0.35 g), H_2O (1 L), pH 7.2; medium (III): glucose (10 g), K_2HPO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KNO_3 (1 g), $CaCO_3$ (2 g), $FeSO_4 \cdot 7H_2O$ (trace), H_2O (1 L), pH 7.2; medium (IV): saccharose (10 g), KNO_3 (5.5 g), $MgSO_4$ (0.5 g), NaCl (1 g), K_2HPO_4 (0.4 g), $ZnSO_4$ (0.002 g), $FeSO_4$ (0.002 g), H_2O (1 L), pH 7.2.

Preparation of Extracts. Culture liquid was extracted with ethylacetate, which was concentrated in vacuo to produce a dry solid. The activity of extracts was determined by preparing ethylacetate and aqueous-alcoholic (less than 10% alcohol in water) solutions at concentrations of 4 mg/mL. Activity of extracts toward enzymes was tested using dry solid (1 mg) of the ethylacetate fractions dissolved in dry DMSO (1 mL).

Antimicrobial activity of extracts was estimated by diffusion in agar and disk loading [20].

Determination of Enzyme Activity. Activity of enzymes that catalyze hydrolysis of polysaccharides (amylopectin, laminaran) was found from the increase in the amount of reduced sugars using the Nelson method [21]. The standard reaction mixture for determination of glucanase activity contained enzyme (50 μL) and the corresponding substrate (450 μL, 1 mg/mL) in succinate buffer (0.05 M) at pH 5.2 and 37°C. Activity of arylsulfatase was determined under the same conditions from the amount of released *p*-nitrophenol [22].

Determination of Inhibitory Activity of Extracts. The inhibitory activity was estimated from the ability of the extracts to stop the reaction of enzyme with substrate. A mixture of a solution (50 μL) of the appropriate enzyme and a solution (40 μL) of the inhibitor in DMSO (1 mg/mL) was held for 10 min at 20°C, treated with a solution (410 μL) of substrate (1 mg/mL) in succinate buffer (0.05 M) at pH 5.2, and incubated for 20 min at 37°C. The residual activity in the mixture was determined as described above. The control was treated with DMSO (40 μL) instead of the inhibitor solution.

Determination of Hemolytic Activity. Hemolytic activity was determined using a suspension (2%) of mouse erythrocytes that were rinsed beforehand by centrifuging twice in isotonic NaCl solution. The studied compounds in aqueous alcohol (5%) were placed successively by a double serial dilution method into the wells of a 96-well planchet (10 μL each) with subsequent addition of a suspension of erythrocytes (200 μL) to the well. The system was incubated for 1 and 2 h in a thermostat at 37°C. The hemolytic activity of the compounds was estimated visually from the appearance of the blood "lake" effect in doses of 1, 10, 25, 50, 100, and 200 μg/mL.

Determination of Cytotoxic Activity of Extracts. Cytotoxic activity of extracts from streptomycetes toward ascitic Erlich carcinoma tumor cells was estimated *in vitro* by a radiometric method using inhibition of incorporation of a labeled DNA-biosynthesis precursor in tumor cells. Ascitic tumor cells taken from allogenic mice seven days after tumor xenografting were rinsed by centrifugation. The precipitate was resuspended in medium 199. Methyl-[^{3}H]-thymidine (specific activity 1.85×10^6 Bq/mg) was added to the suspension containing 2×10^6 cells/mL calculating 1×10^5 Bq per mL of incubation medium and diluted to 90 μL in the microplate wells into which the studied compuonds (10 μL) had been placed beforehand into the well. The cytotoxic activity of the extracts dissolved in DMSO was determined for a single dose of 100 μg/mL. The DMSO content in the incubation medium was less than 10%. Samples were incubated in a thermostat at 37°C for 4 h. They were transfered after incubation to filter paper (Whatman, 3 mm), treated three times with TCA solution (5%) (calculated 5 mL on the filter), washed with ethanol, dried, and placed into vials with scintillation cocktail. The radioactivity was measured in a Mark-III β-counter (USA). The results were evaluted relative to control samples into which only solvent (10% DMSO solution) was placed instead of the studied compounds [23].

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