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Research article

Beyond traditional screening: Unveiling antibiotic potentials of actinomycetes in extreme environments

Lyudmila P. Trenozhnikova [a,*], Gul B. Baimakhanova [a], Baiken B. Baimakhanova [a], Assya S. Balgimbayeva [a], Saule T. Daugaliyeva [a], Elmira R. Faizulina [a], Larisa G. Tatarkina [a], Gulzhan A. Spankulova [a], Dmitriy A. Berillo [b,c,**], John A. Beutler [d]

[a] LLP Research and Production Center for Microbiology and Virology, Almaty, Republic of Kazakhstan, Kazakhstan
[b] Department of Chemistry and Biochemical Engineering, Satbayev University, Almaty, Republic of Kazakhstan, Kazakhstan
[c] Department of Biochemistry, Asfendiyarov Kazakh National Medical University, Almaty, Republic of Kazakhstan, Kazakhstan
[d] Molecular Targets Program, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Frederick, MD, USA

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ABSTRACT

Extreme ecosystems are a rich source of specialized metabolites that can overcome multidrug resistance. However, the low efficiency of traditional exploratory research in discovering new antibiotics remains a major limitation. We hypothesized that actinomycetes may have the ability to produce antibiotics in the extremes of a changing natural environment. This study introduces a novel approach to screening natural antibiotic producers from extreme habitats based on the relationship between organisms' adaptive traits and their metabolic activities. The antibacterial and antifungal properties of 667 actinomycete isolates, obtained from 160 samples of Kazakhstan's diverse extreme habitats, were studied under neutral, saline, and alkaline conditions against MRSA, *E. coli, C. albicans*, and *A. niger*. Among these isolates, 113 exhibited antibacterial properties, and 109 demonstrated antifungal properties. Notably, one-fifth of the antagonist isolates could produce active substances solely under extreme growth conditions. Fifty-three antagonistic actinomycetes, possessing these characteristics, have been categorized into groups and warrant further investigation as potential producers of new natural antibiotics. Molecular genetic analysis of the selected isolates revealed a high prevalence of *Streptomyces* and *Nocardiopsis* strains. Furthermore, 83.4 % of obtained isolates demonstrated the ability to thrive in all studied habitats—neutral, saline, and alkaline. 96.3 % of actinomycetes isolated from extreme environments exhibited adaptation to neutral conditions, highlighting their inherent versatility. Our findings underscore the nearly complete potential (99.7 %) of isolates to overcome the salinity barrier of 3.5 % NaCl, indicating their capacity to inhabit oceanic environments. We assert that actinomycetes should be perceived as a cohesive, globally adaptive group, capable of migrating between changing conditions or remaining stable within them. These studies lay the groundwork for the development of a new platform for screening natural antibiotics.

* Corresponding author.
** Corresponding author. Department of Chemistry and Biochemical Engineering, Satbayev University, Almaty, Republic of Kazakhstan, Kazakhstan.
E-mail addresses: trenozhnikova@cmvkz.com (L.P. Trenozhnikova), berillo.d@kaznmu.kz (D.A. Berillo).

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1. Introduction

Microorganisms, especially opportunistic pathogens, have developed complex mechanisms to inactivate antibiotics; each new drug compound introduced into medical practice eventually loses its effectiveness [1–4]. This creates an acute need for new drugs that can overcome growing multidrug resistance [5]. Currently, the numbers of existing natural compounds cannot compare with that of synthetic chemical libraries, hence there is a need to search for new natural bioactive products to provide starting points for creating new drugs [6–9]. Microbial resources have contributed enormously to the discovery and development of antibiotics over the past seven decades [10]. In the world of microorganisms, actinomycetes are the most important suppliers of bioactive natural compounds, and are the source of many commercially important drugs [11–14].

Screening procedures for actinomycetes as antibiotic producers have traditionally been conducted under neutral conditions of pH, ionic strength and temperature, and modern antibiotic drugs are the result of this screening. However, it turns out that the arsenal of naturally active compounds produced under neutral conditions is not infinite, and routine approaches to screening new antibiotics too often lead to the rediscovery of known substances [15–17]. Currently, the most frequent objects of research are little-studied unusual sources of isolation of producers of bioactive molecules; screening is gradually changing objects and approaches. Marine environments and their inhabitants, salty and alkaline soils, Arctic regions, and geothermal sources are actively sought in hopes of finding microorganisms producing promising new natural products for medicine [18–21].

In this study, we changed the criteria and approaches of traditional screening to simplify it, make it targeted and universal for searching for new bioactive compounds from the extreme biosphere. We considered the reasons for the low yield of traditional screening, drew conclusions about its problems and new opportunities, and based on the obtained data, propose new hypotheses about the life activities of actinomycetes in nature and their unique ability to produce antibiotics.

The Republic of Kazakhstan accounts for about a fifth of the total area of saline soils worldwide [22]. The numerous and diverse extreme ecosystems of Kazakhstan are a reservoir of many microbial communities that can provide a new generation of medicinal substances. 160 samples of Kazakhstani soils were the basis for identifying new opportunities for traditional screening, studying the behavior of actinomycetes in changing conditions from neutral to salty and alkaline, and their adaptive abilities for the biosynthesis of biologically active molecules.

2. Methods

2.1. Collection of soil samples

Samples of soils were collected from the extreme habitats of Northern, Western, and Southern Kazakhstan during field trips in autumn 2022. Soil and rhizosphere samples of salt-tolerant plants were gathered from solonchaks, solonetz, takyrs, the shores of the Caspian Sea, and salt lakes. Solonchaks (salt marshes) and solonetz are different types of saline soils in structure and distribution [22]. Solonchaks have easily soluble salts, especially in the upper part of their profile. Solonetz differs from solonchaks by the accumulation of exchangeable sodium and sometimes magnesium, with the easily soluble salts located in the lower, deeper horizon. The soil samples were collected at a depth of 10 cm (~4 inches) without removing the surface soil. The samples were packed in sterile plastic containers, transported to the laboratory, and refrigerated at 4 °C (39 °F) until ready for analysis.

2.2. Media for growth

Three variants of modified Bennett's agar were used for the growth of actinomycete isolates in this study: a) glucose (0.2 %), peptone (0.2 %), yeast extract (0.1 %), and agar (2.0 %) at pH 7.0 (neutral), b) the same composition with 3.5 % NaCl, pH 7.0 (saline), or c) with 0.35 % $NaHCO_3$, pH 8.0 (alkaline). Sodium chloride and sodium bicarbonate were used to simulate the salty and alkaline habitats of actinomycetes, with the variant without salts corresponding to a neutral habitat.

Further secondary growth in six further variants of modified Bennett's agar were used for the growth of halophilic and haloalkaliphilic actinomycete isolates: 3 variants of the above composition with 5.0; 7.5; 10.0 % NaCl, pH 7.0, or with 0.5; 0.75; 1.0 % $NaHCO_3$, pH 8.0.

All components, media, and reagents were acquired from HiMedia Laboratories (India).

2.3. Isolation of actinomycetes

Soil samples were plated following a standard microbiological dilution plating method [23]. Actinomycetes were isolated on two variants of modified Bennett's agar: saline and alkaline. One gram of soil sample was serially diluted to 10^{-3} using distilled water as a diluent. The mixture was shaken vigorously using a vortex; 0.1 ml of each dilution was placed on variants of modified Bennett's agar, and the inoculum was spread using a sterile glass spreader. The inoculated plates were allowed to stand at room temperature for 5–10 min to allow the liquid to be absorbed. The plates were incubated at 28 °C and examined for growth after 2 weeks of incubation. The colonies with different cultural-morphological characteristics were inoculated from the variants of modified Bennett's agar into slants with the same variants of the medium. The purity of isolated strains was confirmed by standard microbiological methodologies whereby strains were isolated in axenic cultures from separately growing colonies obtained from plates seeded with soil samples. Purified isolates were maintained at 4 °C for further antagonism tests and morphogenesis investigation.

2.4. Screening of actinomycetes

Screening of actinomycetes with antibacterial and antifungal activity was conducted using the standard agar diffusion method with agar blocks [24]. The isolates were cultured on three variants of modified Bennett's agar (neutral, saline and alkaline) in Petri dishes Y-Plate (3-Section) for 10 days at 28 °C. Agar blocks (7 mm) were cut off by a cork borer and transferred to the surface of agar plates, previously inoculated (10^6 cells/ml) with the test organism (MRSA, *Escherichia coli, Candida albicans*, and *Aspergillus niger*). The petri dishes were kept in a refrigerator for 24 h before incubation to allow the diffusion of antimicrobial substances. The diameters of inhibition zones were measured after incubation for 24 h at 37 °C for bacteria and yeast-like fungi, and for 72 h at 28 °C for filamentous fungi. Sterile nutrient pure media without test microorganism inoculation were used as control. Each test was repeated three times, and the activity was expressed as the mean diameter of the inhibition zones (mm).

2.5. Test microorganisms

The following bacterial strains were used in this study as test organisms for the antimicrobial activity of the isolated actinomycetes strains: hospital strains of *Staphylococcus aureus* (strain code: MV228) a methicillin-resistant strain, resistant to beta-lactams and *Escherichia coli* (strain code: MV603) a strain resistant to beta-lactams and sulfonamides. Antifungal activity was studied against the clinical strain of yeast-like fungi *Candida albicans* (strain code: MV363) and *Aspergillus niger* (wild type isolate). Hospital microbial strains were kindly provided by the staff members of the microbiology laboratory, JSC "Central Clinical Hospital," Almaty, Kazakhstan. Identification of clinical strains of opportunistic pathogens and determination of their drug resistance was performed on the Mini API automatic bacteriological analyzer (Bio Merieux).

2.6. Classification

The classification of actinomycetes into groups was performed based on the visual analysis of their growth character in neutral and extreme conditions [25].

2.7. Molecular characterization

The taxonomic affiliation of actinomycete strains was established based on sequencing the 16S rRNA gene using the Sanger method. Genomic DNA was extracted from the grown culture using the PureLink® Genomic DNA Kits (Invitrogen, USA) according to the manufacturer's protocol. Identification was carried out based on the sequence of the 16S rRNA gene with universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 806R (5′-GGACTACCAGGGTATCTAAT-3′) [26]. The reaction mixture (25 μl) contained: 12.5 μl DreamTaq Hot Start PCR Master Mix (2X) (Thermo Fisher), 1.0 μM each primer, 1 μg DNA and water up to 25 μl. Amplification mode: at 95 °C for 7 min, then 30 cycles consisting of: 95 °C - 30 s, 55 °C - 40 s, 72 °C - 1 min. The final elongation was carried out at 72 °C for 10 min. The amplified product was separated on a 1.2 % agarose gel, the bands were stained with ethidium bromide and visualized on an Infinity VX2 gel documentation system. 1xTAE buffer was used as an electrode buffer. The PCR product was purified using the ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems, USA).

Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's protocol. The sequencing product was purified using the BigDye XTerminator Purification kit (Applied Biosystems, USA). Capillary phoresis of a bacterial 16S rRNA gene fragment was performed on an automatic sequencer 3500 DNA Analyzer (Applied Biosystems, USA).

2.8. Phylogenetic analysis

Sequencing results were processed in the SeqA program (Applied Biosystems). The search for homologous nucleotide sequences of 16S rRNA genes was carried out using BLAST (Basic Local Alignment Search Tool) in the International Gene Bank database, USA [27]. Phylogenetic analysis was performed using MEGA6 software. Nucleotide sequence alignment was performed using the ClustalW algorithm. To construct phylogenetic trees, the Maximum likelihood method was used.

2.9. Statistical analysis

Origin software was used to evaluate the data. The mean values ± standard deviation were used to present quantitative data. ANOVA was employed to compare the data, followed by Tukey's test for pairwise comparisons. Any disparities with a p-value less than 0.05 were regarded as statistically significant.

3. Results

From 160 soil samples, 667 axenic isolates of actinomycetes, capable of growing in extreme conditions on modified Bennett agar saline or alkaline variants and differing in cultural and morphological characteristics were obtained (Fig. 1A and B). Subsequently, to study growth and antagonism, isolates were cultured on modified Bennett's agar neutral, saline and alkaline simultaneously. Various growth types of actinomycetes in neutral, saline, and alkaline conditions were observed, based on which isolates were grouped

(Fig. 2A–C, Table 1).

556 isolates grew in all studied conditions (neutral, saline, and alkaline) and were classified into Group I, 102 isolates showed growth in two media and were classified into Group II, and 9 isolates grew in a single medium, were classified into Group III (Table 1). In Group II, the most numerous was the IIa subgroup (84 isolates), demonstrating growth in neutral and saline conditions. The IIc subgroup of actinomycetes, growing in saline and alkaline conditions, included 16 isolates. Only 2 isolates grew in both neutral and alkaline conditions. Group III was represented solely by isolates growing in saline conditions (IIIa subgroup), with no isolates exclusive to alkaline conditions. Isolates growing only in neutral conditions were not investigated. The presence of neutro-, halo-, and alkali-tolerant actinomycetes in Groups I and II was established.

113 isolates out of 667 exhibited antibacterial properties and 109 isolates demonstrated antifungal properties (Table 1).

111 isolates showed antibacterial properties against gram-positive (*S. aureus* MV228) and 14 isolates against gram-negative (*E. coli* MV603) test microorganisms. 12 isolates were active against gram-positive and gram-negative bacteria. 73 isolates of actinomycetes demonstrated antifungal properties against yeast-like fungi (*C. albicans* MV363) and 60 isolates against filamentous fungi (*A. niger*). 24 isolates were active against yeast-like and filamentous fungi. 30 isolates showed a wide spectrum of action and were active simultaneously against bacteria and fungi.

Of the 113 isolates with antibacterial activity, 92 belonged to group I and could grow in both saline, alkaline and neutral conditions (Table 1). These isolates exhibited neutrophilic or neutrotolerant properties, despite being isolated from extreme sources under extreme conditions, the isolates showed abundant, moderate or poor growth under neutral conditions. 21 isolates were endemic, growing only in two habitats (Group II): 17 in neutral and saline environments (IIa subgroup), 1 in neutral and alkaline environments (IIb subgroup), and 3 in saline and alkaline environments (IIc subgroup). 18 isolates of Group II were capable of growth in neutral environments, demonstrating neutrophilic or neutrotolerant properties, and only 3 isolates grew in absolutely extreme conditions. Out of the 113 isolates with antibacterial activity, 110 showed the ability to grow in a neutral habitat.

Out of the 109 isolates that demonstrated antifungal activity, 96 were categorized into Group I (Table 1). 12 isolates belonged to Group II: 10 grew in neutral and saline environments (subgroup IIa), and 2 in saline and alkaline environments (subgroup IIc). One isolate was a true halophile (subgroup IIIa), grew, and exhibited antifungal properties in saline conditions. In total, out of the 109 isolates with antifungal activity, 106 showed the ability to grow in a neutral habitat.

The adaptability of active isolates to different environmental conditions as well as their distribution across varying numbers of environments is illustrated in Figs. 3 and 4.

In line with our hypothesis, there is a higher likelihood of discovering novel natural bioactive compounds in extreme growth conditions; therefore, groups of isolates with such biological activity could be promising for further investigation.

The analysis of the antagonistic properties of the isolates against bacteria and fungi allowed us to select actinomycetes that were most interesting for further research – obtaining extracts and identifying active natural compounds. 42 isolates had antibacterial or antifungal properties under extreme growth conditions and did not exhibit them when growing in a neutral environment.

Of the 20 isolates with antibacterial activity (Fig. 5), 15 were assigned to Group I. However, despite their ability to grow in neutral conditions, they are considered interesting for further study as these isolates produce antibacterial substances only in extreme growth conditions. 7 isolates form active substances in two environments – saline and alkaline, 2 isolates only in saline, and 6 in alkaline environments. 5 endemic isolates of Group II form antibacterial products only in extreme environments, 2 isolates of subgroup IIa – in saline, 1 isolate of subgroup IIb – in alkaline, and 2 isolates of subgroup IIc – in both extreme environments (3/15/1) and in alkaline (3/34/1). 7 isolates lacked antibacterial activity in saline growth conditions and only 2 in alkaline conditions.

19 isolates with antifungal properties (Fig. 6) out of the 22 selected were part of Group I, of which 3 isolates produce active substances in two extreme environments, and the remaining 16 form antifungal natural products only in alkaline conditions. 2 Group II isolates with antifungal activity form active substances in saline (3/41/1), and in saline and alkaline conditions (3/44/1). Isolate 1/50/2, belonging to subgroup IIIa, was the only halophile among the selected isolates and showed antifungal activity when growing in

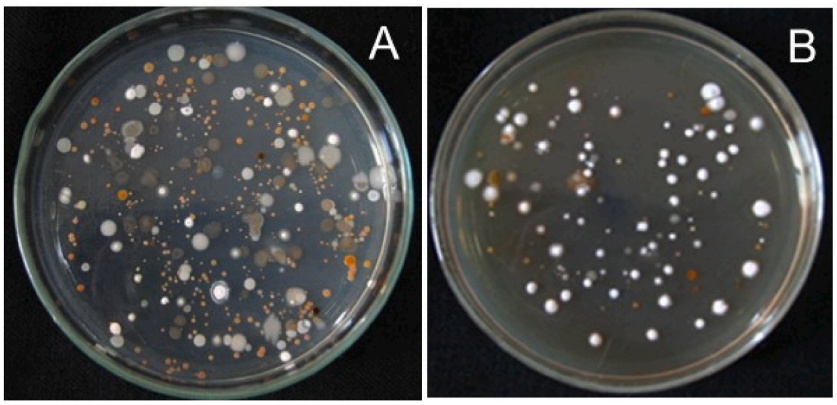

Fig. 1. Isolation stage of actinomycetes from soil samples. A) Growth of actinomycetes on saline modified Bennett's agar with 3.5 % NaCl, pH 7.0. B) Growth of actinomycetes on alkaline modified Bennett's agar with 0.35 % $NaHCO_3$, pH 8.0.

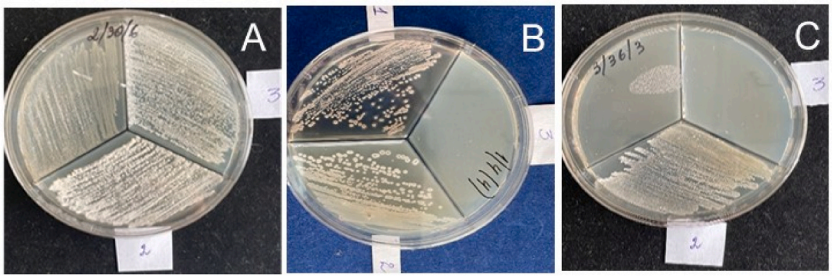

Fig. 2. Classification stage of actinomycetes into groups on the visual analysis of their growth character in neutral and extreme conditions. A) Growth of the isolate on three media, Group I. B) Growth of the isolate on two media, Group II. C) Growth of the isolate on one medium, Group III.

Table 1
Classification of isolated actinomycetes based on their ability to grow under different conditions based on Trenozhnikova and Azizan, 2018 [25] and their antibacterial and antifungal activity.

Actinomycete group	neutral	saline	alkaline	*n*	antibacterial	antifungal
All isolates				667	113	109
I (all conditions)	+	+	+	556	92	96
IIa	+	+	–	84	17	10
IIb	+	–	+	2	1	0
IIc	–	+	+	16	3	2
IIIa	–	+	–	9	0	1
IIIb	–	–	+	0	0	0

+ means growth; −means no growth; n = number of cultures.

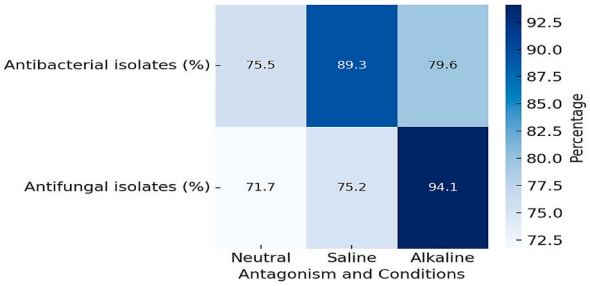

Fig. 3. Distribution of antibacterial and antifungal active isolates under different environmental conditions. The heat map shows the percentage of actinomycetes exhibiting activity in neutral, saline, and alkaline habitats.

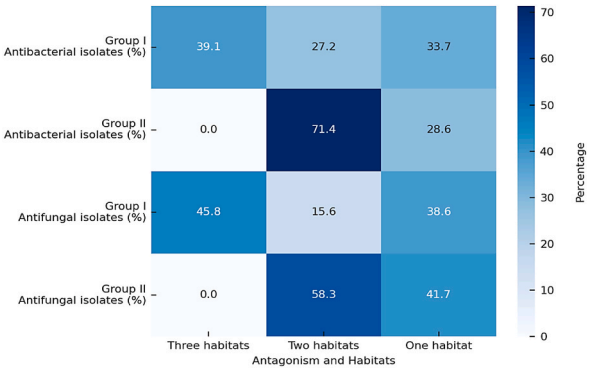

Fig. 4. Distribution of antibacterial and antifungal active isolates across different numbers of environments. The heat map shows the percentage of actinomycetes exhibiting activity in three, two, or one habitat.

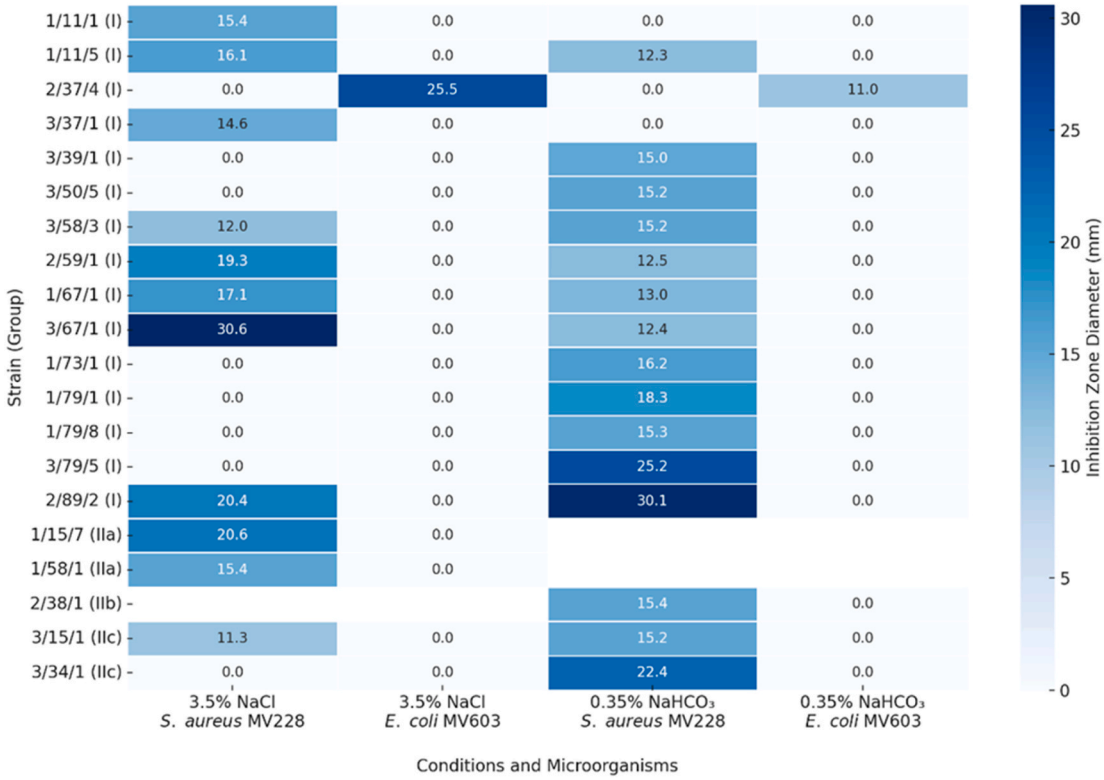

Fig. 5. Selection of promising isolates with antibacterial activity. The heatmap shows antibacterial activity under extreme growth conditions (3.5 % NaCl and 0.35 % $NaHCO_3$) against *Staphylococcus aureus* MV228 and *Escherichia coli* MV603.

saline conditions. 16 isolates lacked antifungal activity in saline growth conditions, while all strains exhibited antifungal activity in alkaline conditions.

Five isolates with a broad spectrum of action, active against both bacteria and fungi, were selected during the screening process (Fig. 7), of which four are Group I isolates. Isolate 2/55/1 exhibits antibacterial and antifungal activity in two extreme environments, isolates 2/15/1 – only in saline environment. Isolate 2/49/2 shows antibacterial activity in saline and antifungal in alkaline environments.

The growth and antagonism of 20 isolates with halophilic and haloalkalophilic properties, which did not exhibit activity within the defined salinity and alkalinity limits, were further investigated at higher salt concentrations in the growth medium. Among them, 12 isolates grew in two extreme environments – saline and alkaline, belonging to IIc subgroup, and 8 isolates were true halophiles, growing only in saline environment and classified into IIIa subgroup. For this group of inactive actinomycetes, the boundaries of salts in the medium were expanded to 5.0 %, 7.5 %, 10.0 % NaCl, pH 7.0, and 0.5 %, 0.75 %, and 1.0 % $NaHCO_3$, pH 8.0, respectively. The isolates did not show activity with increasing $NaHCO_3$ concentration in the medium, but 5 isolates showed activity against *S. aureus* MV228 and 1 isolate against *A. niger* with increasing NaCl concentration (Fig. 8), while they were not active against gram-negative bacteria and yeast-like fungi. Activity of isolate 3/79/8 against *S. aureus* MV228 appeared at 5 % NaCl and reached a maximum at 7.5–10 % NaCl. For isolates 2/19/4 and 3/35/3, the maximum antibacterial activity was also observed at a concentration of 7.5 % NaCl in the medium. Isolate 3/20/3 showed antifungal activity against *A. niger* at a concentration of 10 % NaCl in the medium.

The 31 isolates with antagonistic properties under extreme growth conditions and 6 inactive endemic actinomycetes were identified to the genus level and for 27 isolates their potential closest homologs were determined (Table 2, Fig. 9). All identified active actinomycetes from Groups I and II belonged to the genera *Streptomyces* (20 strains) and *Nocardiopsis* (9 strains), one strain from Group I and one strain from subgroup IIIa attributed to the genus *Isoptericola*. Among the identified actinomycetes of subgroups IIc and IIIa that did not show antagonism, the majority were representatives of the genus *Nocardiopsis* (5 strains), and one strain from subgroup IIc was attributed to the genus *Streptomyces*.

The highest degrees of similarity among active species of *Streptomyces* were observed, the homology levels ranging from 99.3 to 100 %. Four strains were identified as *S. sparsus*. High degrees of similarity were found with two strains of *S. apocyni*, two of *S. thinghirensis,* one of *S. iakyrus*, one of *S. graminofaciens*, and one of *S. ambofaciens*. Eight active strains showed high similarity with species of the genus *Nocardiopsis*: *N. aegyptia*, *N. alba*, *N. halotolerans*, *N. dassonvillei*, and *N. terrae*. Strains of these species of *Nocardiopsis* have previously been described to exhibit various types of activity – antibacterial, antifungal, cytotoxic, among others, and are considered promising objects for biotechnological research [41–44]. Here we report activity for the first time four strains of actinomycetes genetically related to *I. rhizophila* [34] and *S. apocyni* [40].

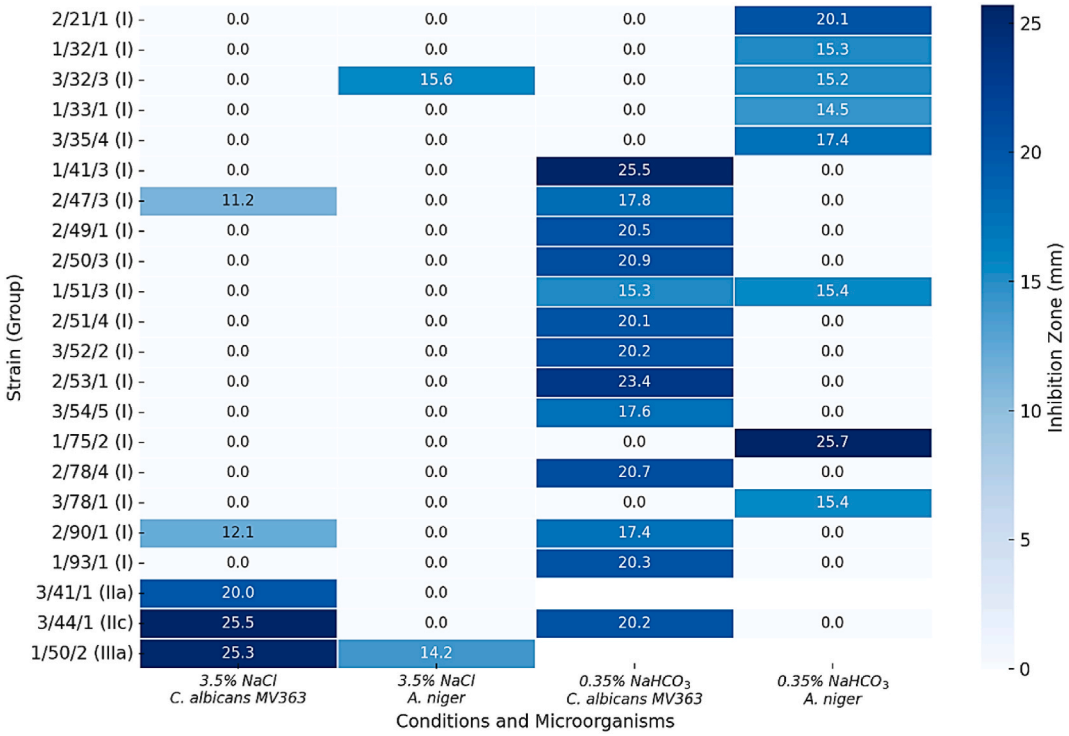

Fig. 6. Selection of promising isolates with antifungal activity. The heatmap shows antifungal activity under extreme growth conditions (3.5 % NaCl and 0.35 % NaHCO3) against *C. albicans* MV363 and *A. niger*.

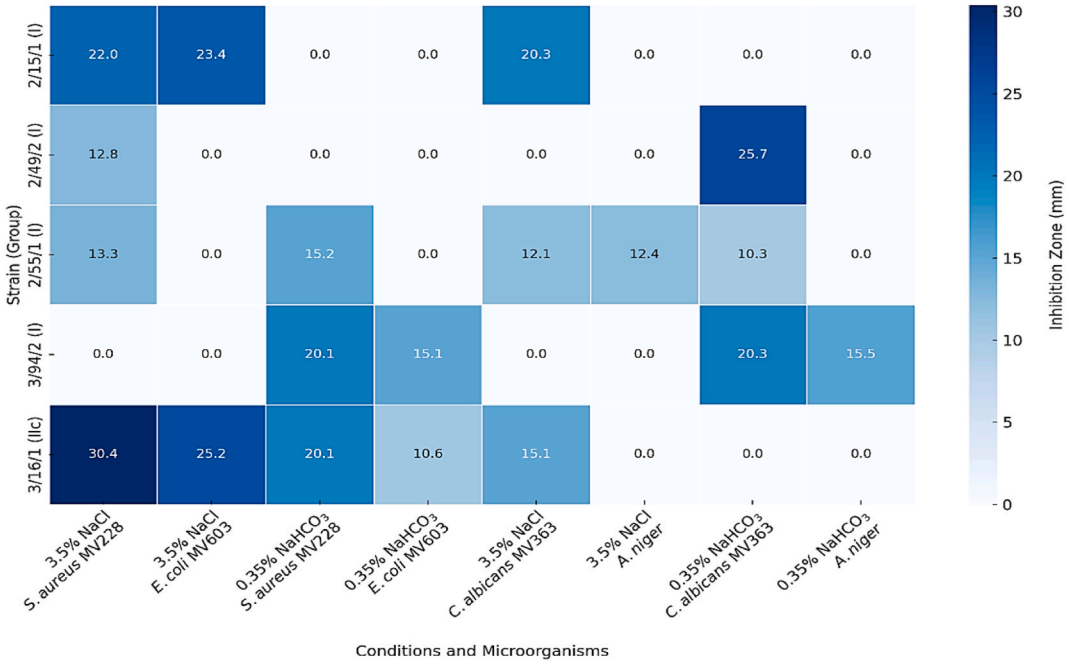

Fig. 7. Selection of promising isolates with a broad spectrum of activity. The heatmap shows activity under extreme growth conditions (3.5 % NaCl and 0.35 % $NaHCO_3$) against *S. aureus* MV228, *E. coli* MV603, *C. albicans* MV363 and *A. niger*.

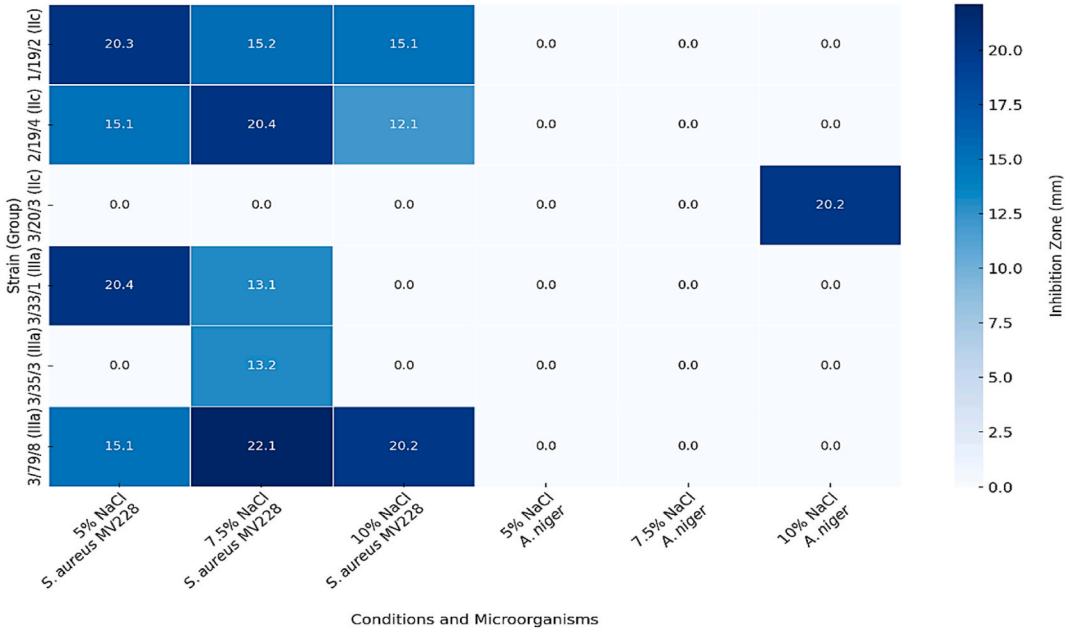

Fig. 8. Selection of promising isolates with activity in hypersaline conditions. The heatmap shows activity under extreme growth conditions (5.0; 7.5; 10.0 % NaCl) against *S. aureus* MV228 and *A. niger*.

Table 2
Phylogenetic diversity of promising *Actinomycetes* isolates.

Strain	Group	Activity	Closest homolog	% homology (bp)	Homolog isolation source
Nocardiopsis sp. 3/54/5	I	af	*N. aegyptia SNG49*	99.0 (616/616)	Marine sediments, Egypt Sabry et al., 2004
Nocardiopsis sp. 1/19/2	IIc	ab	*N. aegyptia* SNG49	99.0 (644/645)	[28]
Nocardiopsis sp. 1/15/7	IIa	ab	*N. aegyptia* SNG49	98.5 (718/722)	
Nocardiopsis sp. 1/22/3, sp. 1/23/4, sp. 3/23/2	IIc	–	*N. aegyptia* SNG49	99.0 (719/720) 99.0 (726/727) 99.0 (734/735)	
Nocardiopsis sp. 3/19/1	IIIa	–	*N. aegyptia* SNG49	98.8 (721/722)	
Nocardiopsis sp. 1/79/1, sp. 3/79/5	I	ab	*N. halotolerans* DSM 44410	100 (663/663) 100 (721/721)	Salt marsh soil, Kuwait Al-Zarban et al., 2002 [29]
Nocardiopsis sp. 3/78/1	I	af	*N. alba* DSM 43377	100 (718/718)	Drainage from hip Grund and Kroppenstedt 1990 [30]
Nocardiopsis sp. 3/44/1	IIc	af	*N. dassonvillei* DSM 43111	100 (612/612)	Soil Gordon and Horan 1968 [31]
Nocardiopsis sp. 3/67/1	I	ab	*N. terrae* YIM 90022	99.7 (619/619)	Saline soil, China Chen et al., 2010 [32]
Nocardiopsis sp. 1/15/5	IIc	–	*N. valliformis* HBUM 20028	99.3 (726/728)	Soil, alkali lake, China Yang et al., 2008 [33]
Isoptericola sp. 3/33/1	IIIa	ab	*I. rhizophila* BKS 3-46	98.9 (709/712)	Mangrove forest, India
Isoptericola sp. 1/79/8	I	ab	*I. rhizophila* BKS 3-46	98.4 (626/627)	Kaur et al., 2014 [34]
Streptomyces sp. 3/58/3	I	ab	*S. sparsus* YIM 90018	99.9 (715/715)	Saline-alkaline soil, China
Streptomyces sp. 2/15/1	I	bs	*S. sparsus* YIM 90018	99.7 (652/652)	Jiang et al., 2011 [35]
Streptomyces sp. 3/16/1	IIc	bs	*S. sparsus* YIM 90018	99.7 (651/651)	
Streptomyces sp. 3/37/1	I	ab	*S. sparsus* YIM 90018	99.7 (606/606)	
Streptomyces sp. 1/15/2	IIc	–	*S. sparsus* YIM 90018	99.1 (644/645)	
Streptomyces sp. 2/59/1	I	ab	*S. thinghirensis* S10	99.5 (601/604)	Rhizosphere soil, Morocco
Streptomyces sp. 1/93/1	I	af	*S. thinghirensis* S10	99.5 (601/604)	Loqman et al., 2009 [36]
Streptomyces sp. 3/32/3	I	af	*S. ambofaciens* NBRC 12836	99.9 (710/710)	Soil, France Pinnert-Sindico 1954 [37]
Streptomyces sp. 3/41/1	IIa	af	*S. iakyrus* NBRC 13401	99.8 (704/704)	Soil, Brazil De Queiroz and Alberto 1962 [38]
Streptomyces sp. 2/50/3	I	af	*S. graminofaciens* NBRC 13455	99.8 (615/616)	Soil, Japan Fukuchi et al., 1995 [39]
Streptomyces sp. 1/73/1	I	ab	*S. apocyni* TRM 66233	99.7 (610/610)	Endogenous, Apocynum venetum
Streptomyces sp. 2/21/1	I	af	*S. apocyni* TRM 66233	99.3 (661/663)	Liu et al., 2020 [40]

(ab), antibacterial activity; (af), antifungal activity; (bs), broad spectrum of activity; (–), not active

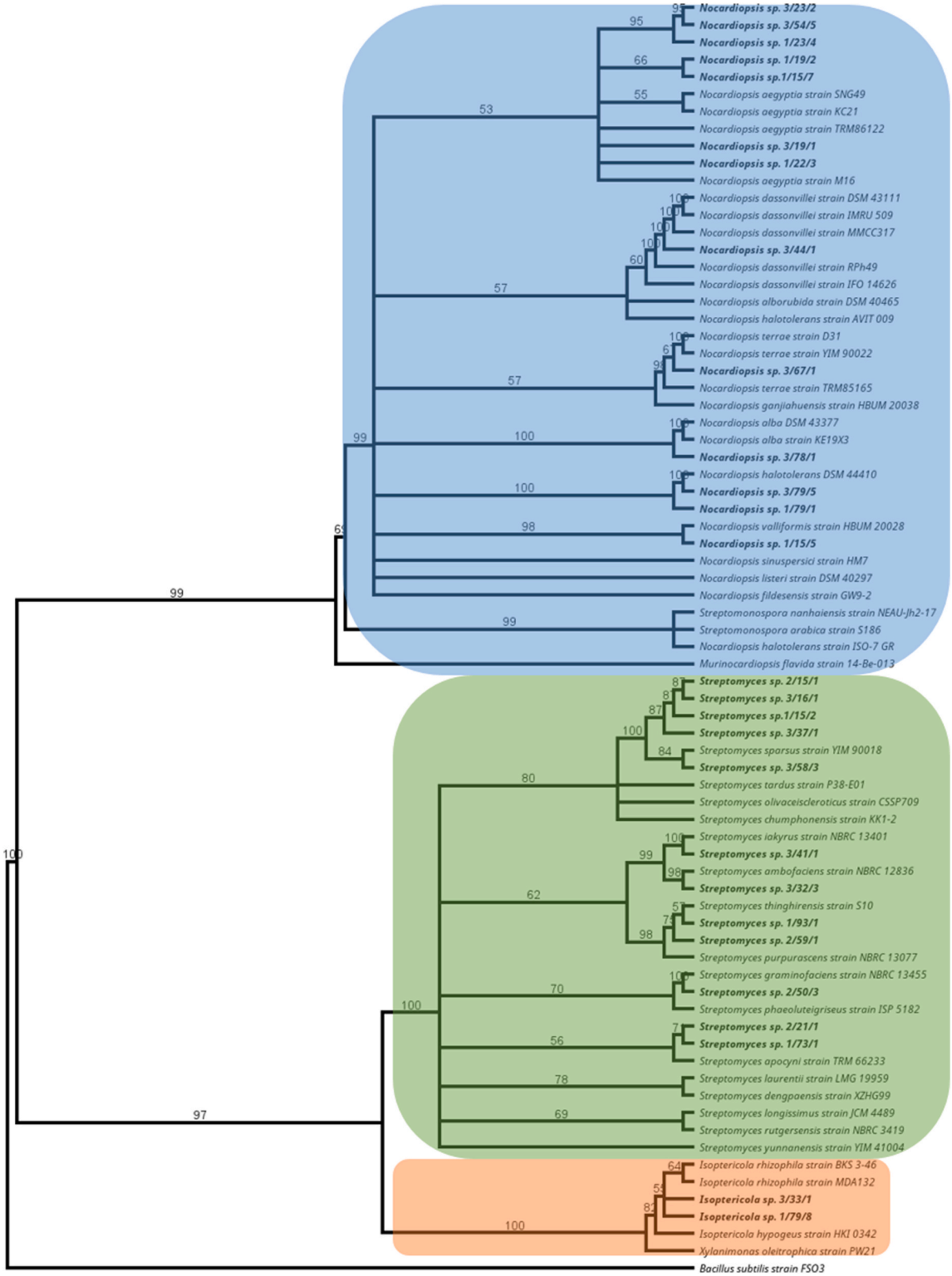

Fig. 9. Neighbor-joining phylogenetic tree generated by distance matrix analysis of 16S rRNA gene sequences from *Nocardiopsis*, *Streptomyces*, *Isoptericola*, and other related *Actinobacteria* strains (highlighted) and their nearest phylogenetic relatives. The numbers on branch nodes indicate bootstrap values (1000 resamplings; only values > 50 % are shown). The bar represents 1 % sequence divergence.

Among the inactive strains identified from subgroups IIc and IIIa (6 strains), the majority were *Nocardiopsis* species with high homology levels ranging from 98.8 to 99.3 %, including four strains of *N. aegyptia* (IIc and IIIa subgroups), and one strain of *N. valliformis* (IIc subgroup). Streptomycetes were a minority in this category of inactive actinomycetes, with the strain 1/15/2 from subgroup IIc showing a high level of homology with *S. sparsus*.

4. Discussion

Under extreme growth conditions, out of 160 soil samples collected in Kazakhstan's extreme biospheres, 667 actinomycete isolates were obtained, 16.9 % of which exhibited antagonism towards bacteria and 16.3 % to fungi. The isolates were classified into groups, 53 promising strains were selected for further research, and the taxonomic position of 31 actinomycete strains was determined. We propose a new approach to screening active natural products based on the relationship between the adaptive properties of actinomycetes and their activity, which involves dereplication at early stages for specific groups of actinomycetes. Based on data analysis, we also propose new hypotheses on the biogeographical distribution of actinomycetes, and ability to produce antibiotics in changing natural environments.

Currently, in the field of screening new natural medicinal compounds, two main problems have arisen – the selection of interesting sources for the isolation of natural product producers and development of an effective methodological approach. The use of classical isolation methods often results in the discovery of already known natural substances [45,46], while the metagenomic approach leads to the creation of large libraries of "silent" genes [47,48]. Therefore, an alternative approach to screening natural substances is increasingly necessary, taking into account the life activity of microorganisms in their natural habitat [12,49–53].

In this study, 556 actinomycete isolates (83.4 %) out of 667 were capable of growth in all studied habitats: neutral, saline, and alkaline. Despite being isolated from extreme environments, 96.3 % of all actinomycetes were able to grow in a neutral habitat. The ability of Group I actinomycetes to grow in any of the studied ecological niches demonstrates their high adaptive capacity and enormous opportunities for distribution and colonization of many zones on Earth, both terrestrial and aquatic. We used a salinity level of 3.5 % in the screening, corresponding to the average salinity level of ocean water, to assess the ability of soil actinomycetes to move and survive in aquatic saline environments. Only 25 % of the earth's land surface is occupied by saline soils, while 70 % of the earth's surface is covered by water, of which 97.5 % is saline. Moreover, saline water is not a fixed environment; it, like the terrestrial environment, is subject to significant variation over time, and microorganisms, including actinomycetes, must be prepared for any "provocations" of the habitat. Actinomycetes successfully overcome this salinity barrier, with 99.7 % of actinomycete isolates in our study potentially capable of colonizing the oceanic environment.

Numerous investigations focus on marine actinomycetes biodiversity, their ability to survive in neutral conditions, and comparative studies of the tolerance of terrestrial and marine actinomycetes to NaCl. It has been shown that marine actinomycetes can grow and synthesize antibiotics both in the presence and absence of seawater, which is fully consistent with our data.

Goodfellow et al. found that actinomycetes isolated from North Atlantic sediments, including *Streptomyces*, *Micromonospora*, and *Rhodococcus* species, have no special seawater requirements and grew equally well on media prepared with both distilled and seawater [54,55]. A seawater requirement for growth was observed in <6 % of 623 strains isolated from California coastal and marine sediments representing six families of the order *Actinomycetales*. The vast majority (88.3 %) of cultivable actinomycetes grew equally well when seawater in the cultivation medium was replaced with deionized water [56]. Out of 100 strains of marine actinomycetes isolated from marine sponges off the coast of Malaysia, 31 strains produced antimicrobial compounds in the presence of seawater, 22 strains - in its absence [57]. The majority of isolates (50 %) from the Trondheim Fjord sediments showed no clear preference for environments with or without seawater. On average, only 9 % of actinomycete isolates in Trondheim study required seawater for growth, and a high biodiversity of actinomycete genera was noted [58,59]. From 275 marine samples collected around the island of Guam, 983 actinomycete strains were isolated, with 58 % of the strains requiring seawater for growth, indicating a high degree of marine adaptation [60]. However, these 58 % of isolates were attributed to a single genus, *Salinispora*, excluding these isolates, only 7 additional actinomycetes were found that needed seawater. Isolation of actinomycetes from marine sediments of the Sea of Japan (241 strains) and terrestrial soil (143 strains) in the presence or absence of 6 % NaCl in the medium demonstrated that the selective pressure of NaCl showed no obvious difference between both groups of isolates [61].

Comparative phylogenetic studies of terrestrial and marine actinomycetes confirm their genetic unity and uniform distribution across both terrestrial and marine environments. The results obtained by authors indicate the presence of terrestrial actinomycetes in marine sediments that occur outside the marine environment, marine isolates show close phylogenetic relationships with terrestrial strains, and the level of phylogenetic novelty and marine specificity does not increase at marine sites [56,58,59,61]. The vast majority of homologs of the identified actinomycetes in our study were isolated not only from various terrestrial geographical environments, but also from diverse marine sources [28,34,62–66]. Moreover, marine actinomycetes can produce antibiotics identical to those produced by soil actinomycetes and vice versa [67]. It was initially believed that the antibiotic abyssomicin was exclusively produced by marine streptomycetes, but it was later shown to be produced by terrestrial streptomycetes as well [68–71]. Salinosporamide A, produced by the marine actinomycete *Salinispora tropica* [72], is highly similar in chemical structure to kinobaramids A-G, which are isolated from the terrestrial strain *Streptomyces* sp. DSM 15324 [73]. To explain the phylogenetic data, it has been hypothesized that marine actinomycetes are of terrestrial origin, the spores or mycelia of terrestrial actinomycetes may be washed into the sea where they remain viable, or terrestrial actinomycetes may adapt to a salt-added environment through stepwise exposure to increasing concentrations of sodium chloride [54,55,74,75].

Understanding the biogeography of actinomycetes is crucial for developing rational strategies for sampling natural substrates to discover new genetic diversity and novel bioactive compounds in natural populations [76]. The hypothesis we propose views

actinomycetes as a unified entity, a singular world capable of thriving in any environment. Actinomycetes are capable of actively moving from one environment to another, or maintaining their vital activity if the natural environment changes. It can be hypothesized that biosynthetic genes clusters, which produce antibiotics, are also universal for both marine and terrestrial environments, a consideration vital for planning modern screening methodologies.

In this study, we demonstrate a framework of a possible actinomycete community in different ecological niches. The primary group in this framework is Group I, consisting of cosmopolitan actinomycetes, freely existing in all the habitats we studied. We also note the presence of Groups II and III, these groups are endemics of specific ecological niches. While Group II actinomycetes can exist in both habitats, Group III actinomycetes are restricted to just one. Our results indicate that Groups II and III are less numerous and occur less frequently in natural niches compared to Group I. 102 isolates showed growth in two media and were classified as Group II, while 9 isolates, growing in a single environment, were attributed to Group III. Most isolates of group II were able to exist simultaneously in neutral and saline environments and belonged to subgroup IIa (84 isolates), which can be explained by the wide distribution of these two ecological niches, both terrestrial and aquatic, on our planet. In this study, identified actinomycetes of Groups I and II were representatives of the genera *Streptomyces* and *Nocardiopsis*, while Group III actinomycetes were associated with the genera *Nocardiopsis* and *Isoptericola*. 90.0 % of the identified active *Streptomyces* strains belonged to Group I and only two strains were assigned to Group II (subgroups IIa and IIc). We cannot conclude that specific adaptive traits are fixed at the species level, as different strains of the same species exhibited varying growth capabilities in experimental models of natural ecological niches.

It should be emphasized that we found seven genetically similar strains of *N. aegyptia* to fall into different adaptive groups and subgroups of actinomycetes – I, IIa, IIc and IIIa, and only three of them showed antifungal or antibacterial activity (3/54/5, Group I; 1/15/7, subgroup IIa and 1/19/2, subgroup IIc). Four genetically similar active strains of *S. sparsus* also differed in affiliation and antimicrobial spectrum, two strains (Group I, 3/37/1, 3/58/3) showed antibacterial activity, and two strains (Group I, 2/15/1; subgroup IIc, 3/16/1) exhibited a broad spectrum of activity. Many researchers have noted similar differences in metabolomics at the level of strains of the same species [77,78]. Analysis of the biosynthetic capabilities of six strains of *S. albus* revealed 16 gene clusters specific to individual strains [79]. Four phylogenetically identical strains of *S. albogriseolus* demonstrated differences in antibiotic and anticancer activity [65]. We also note the high diversity in adaptive and biosynthetic capabilities of strains within the same species, necessitating their primary screening selection based not on species criteria, but rather on classification characteristics – affiliation with a promising group for research and the presence of antibiotic activity in a specific subgroup.

This comprehensive approach underscores the need for a paradigm shift in the screening and classification of actinomycetes, moving beyond traditional taxonomic boundaries to a more dynamic understanding of their ecological roles and genetic potential. By considering actinomycetes as a versatile, adaptive group capable of traversing and thriving in diverse environmental conditions, our study opens new avenues for exploring the vast and largely untapped reservoir of biochemical and genetic diversity within these microorganisms. This approach not only provides a more accurate representation of actinomycete distribution and potential but also facilitates the discovery of novel bioactive compounds with significant implications for medical and environmental applications.

The utilitarian use of antibiotics has led to the emergence of multiple drug resistance in pathogenic microorganisms, posing a potential catastrophic situation for humanity in the future [5]. Screening research is currently underfunded due to its high cost and low yield, while multiple drug resistance continues to rise [80]. Hopes placed on new research subjects—synthetic active biomolecules and new implementation programs—have proven insufficiently successful [53]. Therefore, we are returning to new directions in natural screening using a new level of technical solutions, knowledge, and possibilities [50,52].

A significant problem in recent decades has been the dereplication of already known active natural metabolites, rendering the costs of screening processes unjustifiable. Many authors note the negative impact of commonly isolated actinomycetes, which can be a burden for effective screening and hinder the discovery of new interesting species and individual strains, and are developing new strategies to exclude them [20,52,81–83].

Since the usual neutral terrestrial environment has been well-explored for the production of active substances, it is the marine environment, saline soils, deserts, alkaline and saline lakes, insects, and marine animals are considered promising sources for screening [14,20,84–87]. Recent advances suggest that the diversity of actinomycetes in less-studied, unusual habitats may increase the prospects of discovering new compounds with potential activity that could overcome multiple drug resistance and be valuable for treating chronic diseases, cancer, and viral infections [84,88–91]. Numerous new species of actinomycetes have been identified from extreme sources, with the properties of new natural compounds exhibiting diverse active effects that have been extensively studied [92–97].

However, screening studies of natural antibiotics from extreme habitats have shown that a large number of actinomycetes with neutrophilic or neutrotolerant properties are isolated from these sources. In our research, 97.3 % of isolates with antibacterial activity and 97.2 % of isolates with antifungal activity exhibited neutrophilic or neutrotolerant properties and overwhelming majority belonged to Group I. It can be assumed that it is precisely the numerous adaptive cosmopolitan actinomycetes of Group I that can be isolated with high frequency in laboratory conditions from any ecological niches, and that they may be the carriers of genes for frequently occurring and already known antibiotics. Hence, relying solely on the uniqueness and novelty of extreme habitats is not always justified.

We have determined that Group I, growing in all investigated ecological niches (neutral, saline, and alkaline), is not uniform in its antagonistic properties. Group I actinomycetes exhibited varying adaptive activity types: in all three media, in two media with variations (neutral and saline, neutral and alkaline, saline and alkaline), or in one medium (neutral, saline, or alkaline). Thus, the natural environment and its chemical composition served as the primary and determining factor for the display of actinomycete antagonism. We conclude that groups I and II of actinomycetes with activity under neutral conditions, well studied for antibiotic productivity, may potentially constitute a contaminating background in the contemporary screening process. However, actinomycetes exhibiting activity only in two environments (saline and alkaline) or only in one environment (saline or alkaline) may be promising for further work on

studying the properties of active biomolecules. It is interesting to note that actinomycete endemics of groups II and III mainly did not exhibit activity in extreme ecological niches with set parameters. Only five isolates from Group II showed antibacterial activity, two isolates from Group II and one isolate from Group III showed antifungal activity; one isolate from Group II showed a broad spectrum of activity against bacteria and fungi. However, increasing sodium chloride concentrations in the nutrient medium from 5 % to 10 % led to the appearance of activity in some actinomycetes of groups II and III. Two isolates showed high antibacterial activity against MRSA at a concentration of 5 % NaCl, and three isolates showed activity on a medium with 7.5 % NaCl; one isolate had antifungal activity at 10 % NaCl.

This study demonstrates the need for a more detailed investigation of certain groups of isolates with various adaptive variations of activity. We suggest that this could significantly expedite and simplify the process of discovering new biomolecules. This allows for the main contaminating background to be ignored and creates conditions for the targeted expression of BGC genes, which are silent under neutral conditions, using specific natural factors.

A screening platform based on the magnitude of antimicrobial action and its connection with taxonomy, which has been in use for over 70 years, loses its effectiveness due to the problem of dereplication. The high intraspecific genetic variability of actinomycetes, interconnected with their adaptive properties, indicates the need to develop a new direction of screening research. *In the conducted study, the primary and main criterion for screening was not the magnitude of antibacterial or antifungal activity, but its association with extreme growth conditions.* Interestingly, only about one-fifth of isolates from extreme habitats with antibacterial or antifungal activities were capable of producing active substances only in extreme growth conditions. Moreover, 76.9 % of these isolates with antibacterial activity and 84.6 % of isolates with antifungal activity were representatives of cosmopolitan actinomycetes from Group I. However, the presence of activity only in extreme conditions provides an opportunity to select neutrophilic or neutrotolerant strains that are promising for further study.

In studies of the antagonistic properties of actinomycetes isolated from various sources, including extreme ones, researchers note the presence of both antagonistic actinomycetes and a considerable group of non-antagonistic actinomycetes, with the proportion of active actinomycetes varying from 20 % to 80 % in some studies [23,98–102]. This is also demonstrated in our research, where out of 667 isolates, only 113 exhibited antibacterial properties and 109 antifungal properties, with the rest of the isolated actinomycetes being inactive under the selected parameters.

These results confirm the idea expressed by many authors of cooperation and mutual assistance between microorganisms [103–105], which can provide benefits for both the producer of natural products and the recipient. Considering the relationships within the actinomycete community, an important aspect could be the existence of strongly interacting species (SIS) [3,106] within the regional species pool [107]. According to the Black Queen Hypothesis [108], populations can give rise to various co-evolved interdependent groups, members of which are not interchangeable but form functionally equivalent assemblies [107]. There is considerable evidence of successful mutualistic cross-protection among microorganisms [109], where two strains of *Escherichia coli* protect each other in the presence of two antibiotics (ampicillin and chloramphenicol), allowing the combined culture to survive at antibiotic concentrations that suppress the growth of any strain individually. Ecological interactions are crucial for the growth and survival of bacteria in polymicrobial infection communities and affect their assembly and resilience [110]. Assuming that actinomycete competitors have mutualistic relationships, which result in mutual benefits in survival and development of antibiotic resistance, one can also hypothesize the existence of mutually beneficial relationships within the actinomycete community. It has been found that antibiotic synthesis is associated with quorum sensing in co-cultures [3,111], suggesting that the main outcome of these interactions is related to the construction and stability of the community, and antibiotics can play a more subtle role than just defensive weapons in microbial warfare.

There are active and passive groups within the actinomycete community, with the most active part being the actinomycetes that grow in any investigated habitat - the cosmopolitan actinomycetes of Group I. 66.3 % of actinomycetes with antibacterial activity and 61.4 % of those with antifungal activity from this group participate in antagonism in three or two media. The existence of cosmopolitan actinomycetes, capable of producing antibiotics in many environments, may explain the fact that a large portion of the actinomycetes, which constitutes the passive part of the community under the studied conditions, can utilize the capabilities of the active actinomycetes. The presence of a small but active group of actinomycetes may play a protective role for the entire community.

The high ability of actinomycetes to remain active across various habitats suggests a possible functional differentiation within the actinomycete community, indicating the existence of two interconnected groups: producers of biologically active compounds and genetic reservoirs of BGC genes. It is known that many actinomycetes are carriers of a large number of BGCs [112–114] – on average up to 20, not all of which are expressed in laboratory conditions. Actinomycete producers synthesizing biologically active molecules can allocate only a specific part of their genome to store BGCs, as their genome bears a significant load for biosynthesis of active biomolecules and their own vital processes, or "housekeeping." The passive part of the actinomycete community may serve as carriers of genes, not necessarily possessing the ability to express them. Adam et al. (2018) [95], studying the composition of actinomycetes in "moonmilk," reported that *Nocardia* strains, which had the most BGCs (up to 45), did not exhibit any antibacterial activity. Lewis (2020) [53] notes that an operon silent in one species of streptomycetes may be active in another. For example, actinorhodin is readily expressed in *S. coelicolor* but not in *S. lavendulae* [115]. We hypothesize that inactive strains may be carriers of silent BGC genes, which are not in demand at a certain period. This may be one way of preserving BGCs in nature, since different individuals can perform the important function of a genetic reservoir. Horizontal transfer of BGCs is common among actinomycetes and allows for high variability even among closely related genomes [76,78]. We propose that its use for information exchange between producers and consumers creates favorable conditions for the formation of a community based on mutual benefit and cooperation.

Numerous new directions in screening with advanced technical equipment are being developed, however, research on this vital function of actinomycetes for humanity is often conducted without regard to the processes of their life activities. There is a clear need

to change approaches to screening natural antibiotics from actinomycetes, and to develop new ways to detect the biomolecules we need.

We utilized this screening method to identify antiviral compounds produced by five strains of actinomycetes [116]. Recently, we published a draft genome of *S. anulatus* K-31, a strain with significant antiviral activity that was also discovered using our screening method [117].

In subsequent publications, we plan to provide more comprehensive information about the antibiotic producers, the chemical characteristics of extracts obtained from actinomycetes identified in this study, their identification, antibacterial, antifungal, and antitumor properties, and other targets. We suggest opening the door to the still poorly understood world of actinomycetes, whose architecture may reveal new ways to overcome the resistance of pathogenic bacteria and fungi, treat chronic diseases, viral infections, and cancer.

CRediT authorship contribution statement

Lyudmila P. Trenozhnikova: Writing – review & editing, Writing – original draft, Supervision, Methodology, Visualization, Funding acquisition, Data curation, Conceptualization. **Gul B. Baimakhanova:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation. **Baiken B. Baimakhanova:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Formal analysis. **Assya S. Balgimbayeva:** Writing – review & editing, Writing – original draft, Visualization, Methodology. **Saule T. Daugaliyeva:** Writing – original draft, Methodology, Investigation. **Elmira R. Faizulina:** Writing – original draft, Validation, Resources, Investigation. **Larisa G. Tatarkina:** Writing – review & editing, Validation, Resources, Investigation. **Gulzhan A. Spankulova:** Writing – review & editing, Validation, Resources, Investigation. **Dmitriy A. Berillo:** Writing – review & editing, Visualization, Formal analysis. **John A. Beutler:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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