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Article

Mechanism-based Approach to New Antibiotic Producers Screening among Actinomycetes in the Course of Civil Science

Inna A. Volynkina [1,2,*], Yuliya V. Zakalyukina [3,4], Vera A. Alferova [5,6], Albina R. Belik [3], Daria K. Yagoda [7], Arina A. Nikandrova [1], Yuliya A. Buyuklyan [3], Andrei V. Udalov [3], Evgenii V. Golovin [3], Maxim A. Kryakvin [7], Dmitrii A. Lukianov [1,2], Mikhail V. Biryukov [1,3,8], Petr V. Sergiev [1,2], Olga A. Dontsova [1,2,5] and Ilya A. Osterman [1,2,3,*]

1 Center of Life Sciences, Skolkovo Institute of Science and Technology, Bolshoy Boulevard 30, bld. 1, 121205 Moscow, Russia; Inna.Volynkina@skoltech.ru (I.A.V.); arinanikandrova@mail.ru (A.A.N.); Dmitrii.Lukianov@skoltech.ru (D.A.L.); metrim@gmail.com (M.V.B.); petya@genebee.msu.ru (P.V.S); olga.a.dontsova@gmail.com (O.A.D); i.osterman@skoltech.ru (I.A.O.)

2 Department of Chemistry, Lomonosov Moscow State University, Leninskie Gory 1, 119991 Moscow, Russia; Inna.Volynkina@skoltech.ru (I.A.V.); Dmitrii.Lukianov@skoltech.ru (D.A.L.); petya@genebee.msu.ru (P.V.S); olga.a.dontsova@gmail.com (O.A.D); i.osterman@skoltech.ru (I.A.O.)

3 Center for Translational Medicine, Sirius University of Science and Technology, Olympic Avenue 1, 354340 Sochi, Russia; juline@soil.msu.ru (Y.V.Z.); albinabelik32@gmail.com (A.R.B.); buyuklyan.ya@talantiuspeh.ru (Y.A.B.); udalov.av@talantiuspeh.ru (A.V.U.); golovin.ev@talantiuspeh.ru (E.V.G.); metrim@gmail.com (M.V.B.); i.osterman@skoltech.ru (I.A.O.)

4 Department of Soil Science, Lomonosov Moscow State University, Leninskie Gory 1, 119991 Moscow, Russia; juline@soil.msu.ru (Y.V.Z.)

5 Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Miklukho-Maklaya 16/10, 117997 Moscow, Russia; alferovava@gmail.com (V.A.A.); olga.a.dontsova@gmail.com (O.A.D)

6 Gause Institute of New Antibiotics, B. Pirogovskaya 11, 119021 Moscow, Russia; alferovava@gmail.com (V.A.A.)

7 School of Bioengineering and Bioinformatics, Lomonosov Moscow State University, Leninskie Gory 1, 119991 Moscow, Russia; yagodadasha@mail.ru (D.K.Y.); maxim.kryakvin@gmail.com (M.A.K.)

8 Department of Biology, Lomonosov Moscow State University, Leninskie Gory 1, 119991 Moscow, Russia; metrim@gmail.com (M.V.B.)

* Correspondence: Inna.Volynkina@skoltech.ru (I.A.V.); i.osterman@skoltech.ru (I.A.O.)

Abstract: Since streptomycin discovery, actinomycetes were the main source for new antibiotics, but after the Golden age (1950-1960th) the discovery rate significantly decreased. The high probability to rediscover well-known antibiotics led to a reduction in interest in soil bacteria as a source for new antibiotics. At the same time, actinomycetes remain a very promising reservoir for searching for new active molecules. In this work, we present several reporters containing eye-visible fluorescent protein genes, which can be used to increase the efficiency of determining the mechanism of antibiotics at the very initial stage of screening. Presented reporters and the following pipeline were optimized given the involvement of citizen scientists without specialized skills and equipment in order to utilize the reservoir of soil bacteria in the search for new antibiotic producers. The combination of mechanism-based approaches and civil science has proved its effectiveness in practice revealing a significant increase in the screening rate. Two new strains *Streptomyces sp.* KB-1 and BV113 were found to produce antibiotics pikromycin and chartreusin, respectively, demonstrating the efficiency of the pipeline.

Keywords: civil science; antibiotic producers screening; actinomycetes; reporter systems; chartreusin; pikromycin

1. Introduction

The spread of antibiotic resistance is one of the major problems for modern antibacterial therapy. The most serious threats are methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), multidrug-resistant (MDR) *Acinetobacter baumannii* and β-lactam-resistant *Enterobacteriaceae* [1]. The discovery of new molecules with antibacterial activity may help to solve this problem, and soil

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bacteria still represent one of the most promising sources [2]. Involving citizens without specialized skills and equipment in the search for new antibiotics could greatly increase the efficiency of this process.

A mechanism-oriented approach for new antibiotics screening can significantly speed up the identification of natural products, which is critical because of the high probability of rediscovering known antibiotics. In this study, we developed a set of constructs containing eye-visible fluorescent proteins and β-galactosidase. With these tools, civilian researchers could conduct a mechanism-oriented screening on their own, in educational laboratories or even at home.

We have proposed and tested a pipeline for new antibiotic producers screening, starting from a soil sample and ending with the antibiotic structure. The whole process could be divided into two stages: (a) isolation and screening for new producing strains; and (b) identification of the active compound. Involvement of civilian researchers at the first stage of high-throughput screening will significantly increase the speed of research and increase the chance of detecting new antibiotics. The identification of the active compound (the second stage) should be done by a specialist in the research laboratory because it requires the usage of high-pressure liquid chromatography (HPLC), mass spectrometry etc. Two examples of the pipeline application are presented in this article: new pikromycin (translation inhibitor) and chartreusin (SOS-response inducer) producers. The combination of civil science with a mechanism-based approach and modern analytical methods allows quick and efficient discovery of new antibiotic producers.

2. Results

2.1. New reporter systems for antibiotic mechanism determination in application to civil science

Recently we developed a double reporter system pDualrep2 [3] consisting of two fluorescent protein genes: *turbo-rfp* which expression is controlled by the SOS-inducible *sulA* promoter, and *katushka2S* which expression is controlled by the modified *trpL2A* attenuator sequence [4] in a way that it is activated in the presence of protein synthesis inhibitors (Figure 1A). Previously, this system was successfully used to discover antibiotic producers [5,6]. However, for the efficient reporter application, specific equipment is required to distinguish the fluorescent signals of TurboRFP (553/574 nm) and Katushka2S (588/633 nm) (Figure 1B).

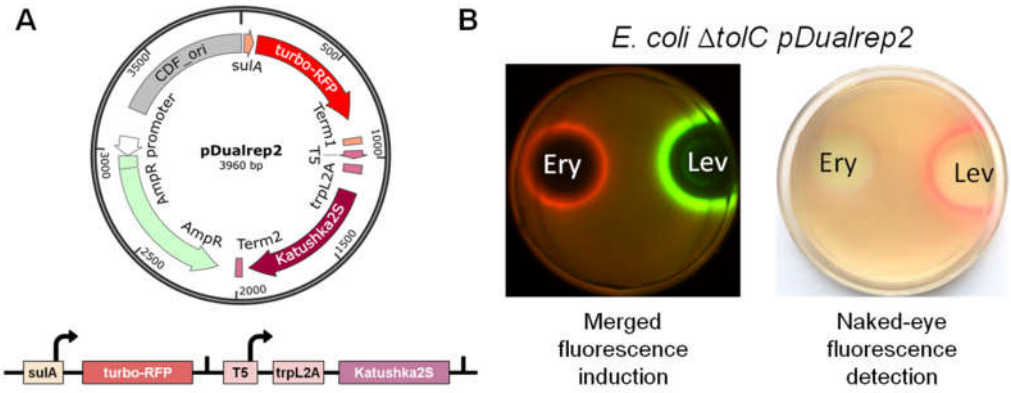

Figure 1. Double reporter system pDualrep2. (**A**) pDualrep2 plasmid map and the reporter scheme. CDF_ori, CloDF13-derived CDF replicon; sulA, promoter of the *sulA* gene; T5, bacteriophage T5 promoter; trpL2A, modified *trpL* leader open reading frame carrying W10A and W11A substitutions. Transcription start sites are shown by arrows. Transcription terminators are shown by vertical dashes. (**B**) Comparison of fluorescence induction detection using an imaging system (left) and with the naked eye (right). Agar plate coated with the *E.coli ΔtolC* strain transformed with the pDualrep2 plasmid and spotted with erythromycin (Ery) and levofloxacin (Lev). The plate was scanned in TurboRFP (Cy3) and Katushka2S (Cy5) channels, shown as green and red pseudocolors, respectively.

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In order to keep the advantage of the dual reporter system pDualrep2, but to avoid the necessity of specific equipment, the *katushka2S* gene was replaced by the β-galactosidase gene (*lacZ*), which expression could be visually detected by the blue-colored product in the presence of X-gal substrate (Figure S1). The *turbo-rfp* gene was left unmodified since its expression is clearly visible in orange-red color to the naked eye. The new plasmid was named pDualrep3. In practice, it demonstrated a high background signal due to the nonspecific hydrolysis of X-gal and partial leakage of the *trpL2A* regulatory sequence so that only potent protein synthesis inhibitors (such as chloramphenicol and fusidic acid) could be revealed.

This problem and the inability to resolve the situation when one sample induces expression of both reporter genes prompted us to design single reporter constructs. Based on fluorescent protein genes *turbo-rfp* and *katushka2S* we created three new plasmids, named pTrpL2A-RFP, pTrpL2A-Katushka2S and pSulA-RFP, and validated them with a set of antibiotics (Figure 2). Both pTrpL2A-RFP and pTrpL2A-Katushka2S demonstrated strong reporter induction upon treatment with antibiotics that inhibit protein biosynthesis during the elongation step, such as chloramphenicol, puromycin, tetracycline, erythromycin, fusidic acid and lincomycin (Figure 2A,B). Consistent with previous data, spectinomycin and clindamycin demonstrated barely visible reporter induction while streptomycin and kanamycin showed no induction at all [3]. It is worth considering that only antibiotics that cause ribosome stalling on the *trpL2A* attenuator sequence could be detected by means of these reporters. For this reason, many aminoglycoside antibiotics, which predominantly cause mRNA misreading, such as streptomycin and kanamycin would stay undercover [7]. Besides, we observed that cytotoxic antibiotic doxorubicin, which is known to intercalate DNA and inhibit topoisomerases in bacteria and eukaryotic cells, induced *trpL2A*-containing reporters [8-10]. We suggest that doxorubicin may bind to ribosomal RNA thus impeding the ribosome to synthesize polypeptides.

The construct pSulA-RFP demonstrated clear reporter induction upon treatment with doxorubicin and levofloxacin (Figure 2C). Both of them are known to function by inhibiting bacterial DNA gyrase, contributing to DNA damage and subsequent SOS-response [8,11]. Moreover, we observed a barely visible reporter induction in case of rifampicin which is known to inhibit bacterial RNA polymerase [12].

Plasmids pTrpL2A-Katushka2S and pSulA-RFP were selected as the best reporter constructs for use in the civil science pipeline.

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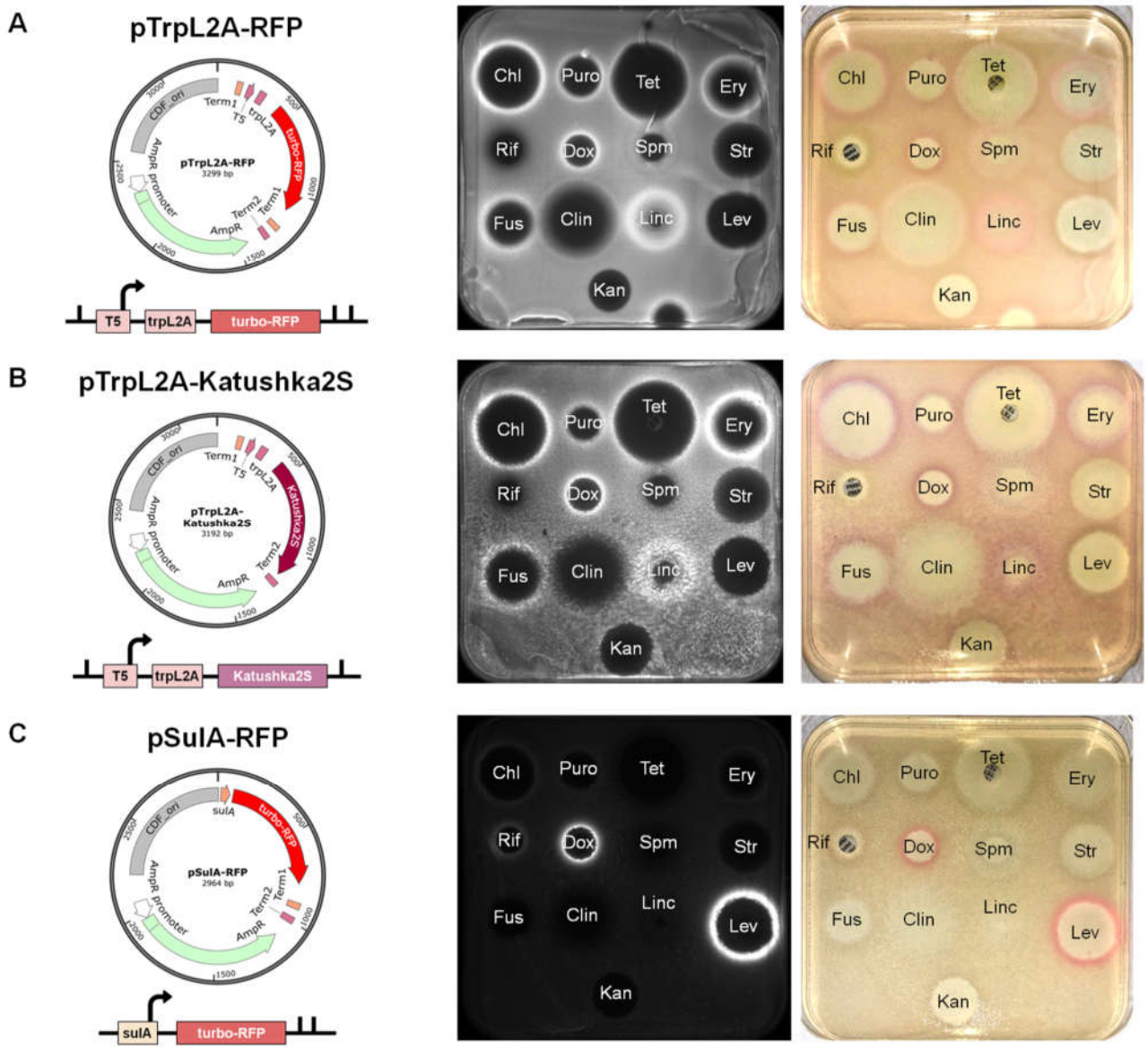

Figure 2. New reporter constructs pTrpL2A-RFP (**A**), pTrpL2A-Katushka2S (**B**) and pSulA-RFP (**C**) were created, transformed into the *E.coli ΔtolC* strain and validated with a panel of antibiotics. The plates (**A**) and (**C**) were scanned in the TurboRFP (Cy3) channel, the plate (**B**) was scanned in the Katushka2S (Cy5) channel (middle). TurboRFP and Katushka2S are visible to the naked eye in orange-red and lilac colors, respectively, in the zone of antibiotic sublethal concentrations (right). The panel of antibiotics: chloramphenicol (Chl), puromycin (Puro), tetracycline (Tet), erythromycin (Ery), rifampicin (Rif), doxorubicin (Dox), spectinomycin (Spm), streptomycin (Str), fusidic acid (Fus), clindamycin (Clin), lincomycin (Linc), levofloxacin (Lev) and kanamycin (Kan).

2.2. Stocks of freeze-dried E.coli reporter cells are suitable for application in civil science

In order to be able to work with reporter cells (*E.coli* strains transformed with reporter plasmids) far away from a laboratory in non-sterile conditions, the freeze-drying method was worked out. This method was previously described for *E.coli* cells in ATCC and some publications [13]. In the current research we examined the freeze-drying procedure on the *E.coli ΔtolC* strain transformed with the pDualrep2 plasmid. As a result, we observed that freeze-dried cells maintain reporter activity for at least eight and a half months (Figure 3). Besides, they can be stored both at room temperature and at 4°C, which makes the stocks of freeze-dried reporter cells suitable for application in the course of civil science.

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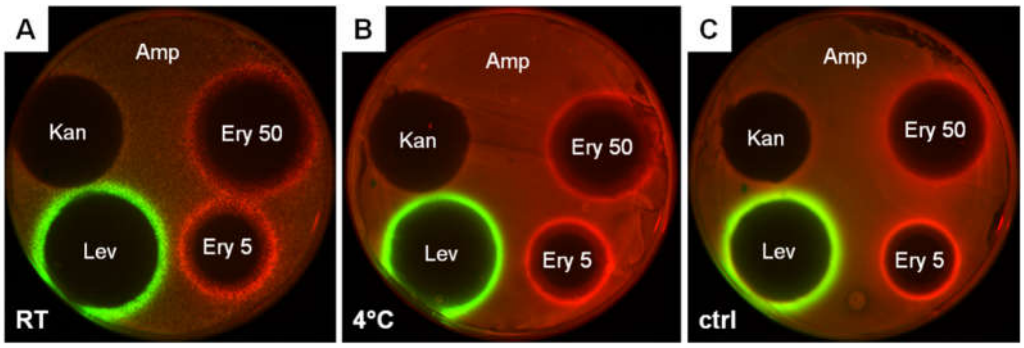

Figure 3. Comparison of fluorescence induction after the freeze-dried cells were kept for 8.5 months (257 days) at room temperature (**A**), at 4°C (**B**) and overnight culture as a control (**C**). Agar plates coated with the *E.coli ΔtolC* strain transformed with the pDualrep2 plasmid and spotted with antibiotics at the following concentrations: ampicillin (Amp, 100 mg/ml), erythromycin at two concentrations (Ery 50, 50 mg/ml) and (Ery 5, 5 mg/ml), levofloxacin (Lev, 25 μg/ml) and kanamycin (Kan, 50 mg/ml). Plates were scanned in TurboRFP (Cy3) and Katushka2S (Cy5) channels, shown as green and red pseudocolors, respectively.

2.3. The pipeline of Civil Science project

The Civil Science project inspired by the Ministry of Science and Higher Education of the Russian Federation (075-15-2021-1085) provides schoolchildren, students, teachers and just the interested public throughout Russian regions a unique opportunity to work with scientists as part of the global initiative to discover new antibiotics from soil actinobacteria.

The first and most valuable stage of the project is to choose the environmental locus from which sampling will be carried out: it can be either a poorly studied natural niche or a farmland, an urban landscape or a hard-to-reach area. Volunteers collect samples of soil or sediments in sterile containers (Figure 3(1)) and transport them to a school laboratory, educational center or home. All information about the selected location, description of the territory, relief, vegetation, etc. are recorded on a special website.

By means of the Isolation Kit containing sterile plates and reagents, the volunteers prepare a serial 10-fold dilutions of samples and spread 50 μL of each suspension onto different agar plates supplemented with antibiotics to limit the growth of Gram-negative bacteria and fungi. The inoculated plates should be wrapped with Parafilm® M and incubated either in a thermostat at 28°C or at room temperature for 2-3 weeks until noticeable colonies of actinobacteria appear, which can be recognized by a fluffy, velvety or leathery surface (Figure 3(2)).

Individual colonies are picked according to their cultural properties and transferred by sterile toothpicks onto the surface of different agarized media known to promote the synthesis of natural products by actinomycete strains (Table S3). No pharmaceutical antibiotics are added to cultivation media. Acting carefully near the alcohol lamp or gas burner, the experimenter can streak 4-5 isolates on one plate, arranging them by sectors (Figure 3(3)). The same cultures are inoculated into separate plates for transfer to the Research Center.

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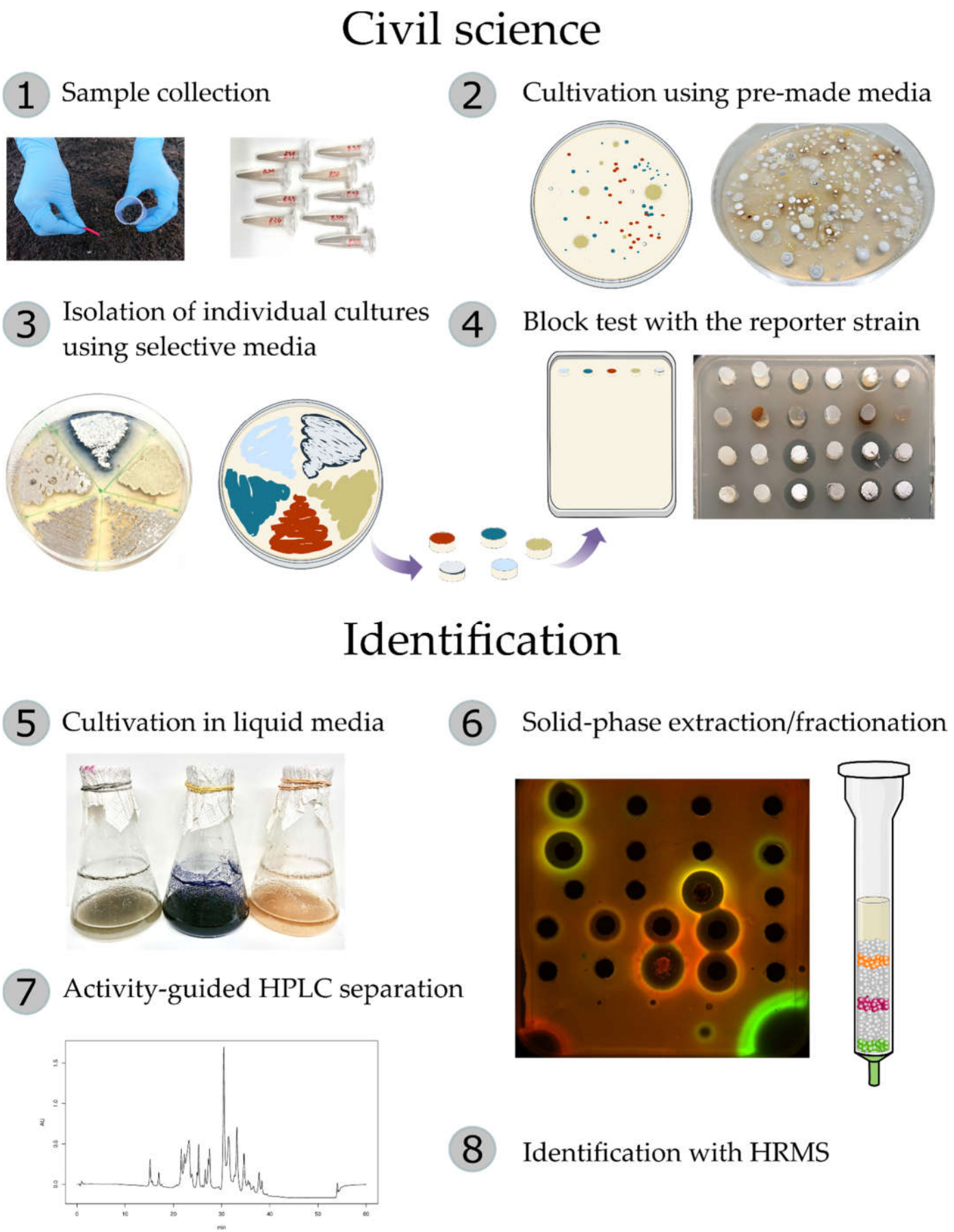

Figure 4. Schematic research pipeline of Civil Science project.

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After 10-14 days of cultivation, agar blocks with mycelium are cut out of the grown lawns of actinobacteria and placed on the surface of media coated with a reporter strain. A day later, one can observe not only a possible delay in the growth of the test organism, but also a characteristic response (reporter induction) providing information about the mechanism of action of metabolites with antibiotic properties (Figure 3(4)). At all stages, the volunteers record the results: they mark and describe isolates, photograph plates with colonies, streaks and reporter tests, and enter information on the project website. Sending out Petri dishes with active antagonists to the Research Center completes the block of civil science.

In the Research Center laboratory the isolates are transferred to fresh media, their purity is confirmed and activity is verified on the reporter strains. Then the cultures proceed to the production stage of target substances using deep cultivation in liquid media that have resulted in the greatest antibiotic effect (Figure 3(5)). Fermentation broths retaining activity in scaled-up cultivation are then subjected to solid-phase extraction on LPS-500-H sorbent. After initial sorption on the column, it is eluted stepwise with an ascending concentration of acetonitrile in water (Figure 3(6)). This approach makes it possible to combine the extraction and rough fractionation steps, providing active concentrates suitable for further HPLC analysis. In order to assess the stability of active components in acidic conditions, samples are treated with 0.1% trifluoroacetic acid (TFA) and tested for retention of antibiotic activity. The active fractions are then subjected to semi-preparative HPLC procedure, with acetonitrile and water being used as eluents under neutral or acidic conditions. Newly collected fractions are concentrated *in vacuo* and tested on the reporter strains (Figure 3(7)). For each analyte, HPLC conditions are refined to identify the exact localization of the active substance. Then the active pure peak is collected and analyzed with HRMS in positive and negative ionization modes with fragmentation of the three most abundant ions (Figure 3(8)).

Mass spectra are processed manually with deconvolution and adduct analysis. The resulting dataset on the active compounds includes: (a) UV-Vis spectrum; (b) HPLC retention time; (c) plausible mechanism of action, deduced from the reporter strain testing results; (d) exact mass of the substance, supported by the analysis of the adduct pattern in mass spectra; (e) fragmentation of the abundant ions in both positive and negative modes. The resulting dataset is used to identify the compound with various databases on natural products [14]. If the purified active substance has not been previously described in the literature data, then it is further subjected to NMR spectroscopy to reveal the molecular structure.

Optionally, the pipeline can be supplemented with an additional analysis of fractions on a panel of bacterial strains specifically resistant to known antibiotics. Such an option may help not to waste time on rediscovering already known compounds.

2.3. Phenotypic, phylogenetic and physiological characteristics of two new producing actinobacteria strains

In the course of the Civil Science project, two strains KB-1 and BV113 were isolated from the urban soil of Moscow and moss from Sochi, respectively. Both strains demonstrated prominent antibiotic activity in tests on the reporter cells (Figure S2). KB-1 exhibited strong pTrpL2A-Katushka2S reporter induction, indicating that the active compound produced by the isolate functions as an inhibitor of protein biosynthesis. Whereas BV113 exhibited the induction of pSulA-RFP, which points at its ability to produce a substance that elicits the SOS-response in bacteria nearby. According to the phenotypic features, KB-1 and BV113 strains were assumed as mycelial actinobacteria (Figures 5, S3, S4).

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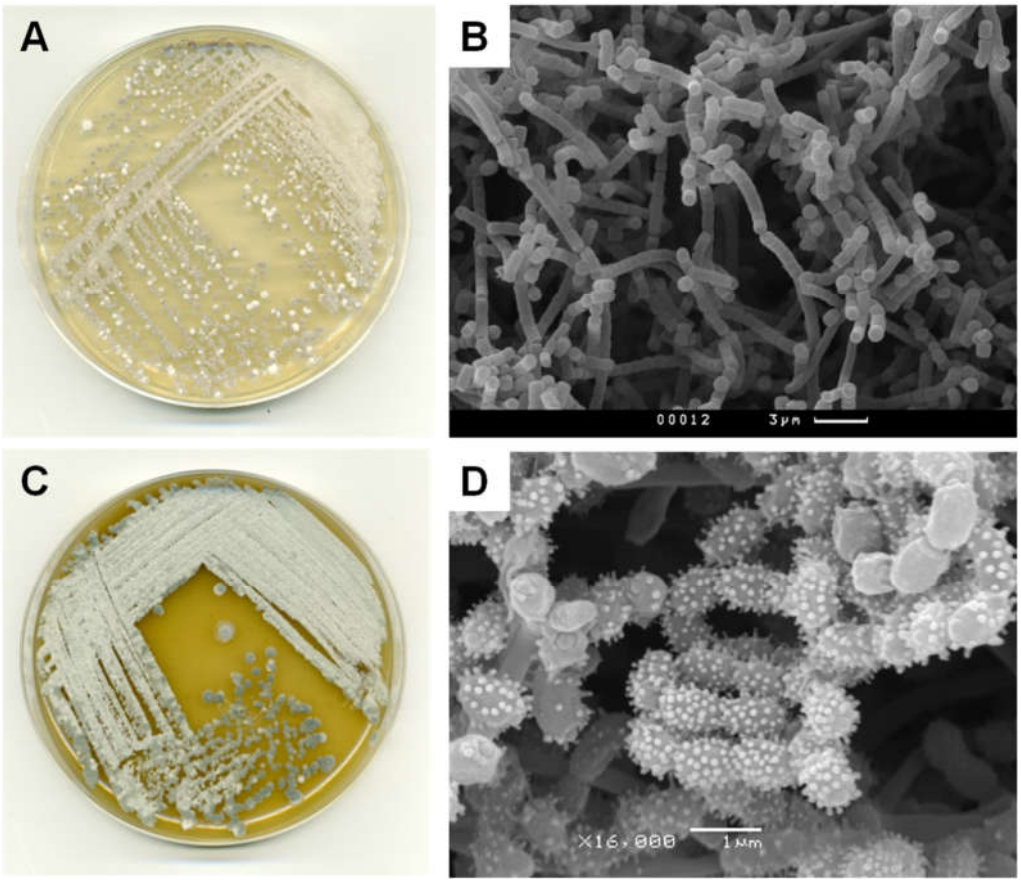

Figure 5. Morphological properties of *Streptomyces sp.* KB-1 grown on ISP4 medium at 28°C for 14 days (**A**, **B**) and *Streptomyces sp.* BV113 grown on ISP3 medium at 28°C for 14 days (**C**, **D**). (**A**, **C**) Photographs of plates with strain streaks. (**B**, **D**) Mycelium micrographs taken on scanning electron microscope (SEM).

Comparative analysis of 16S rRNA sequences of KB-1 and BV113 strains with representatives of the family *Streptomycetaceae* confirmed that the isolates are closely related to species of the genus *Streptomyces*. The Maximum Likelihood tree (Figure 6) based on 16S rRNA gene sequences indicated that KB-1 formes a tight cluster with strains *S. zaomyceticus* NRRL B-2038^T, *S. exfoliatus* NBRC 13191^T, *S. venezuelae* ATCC 10712^T and *S. viridobrunneus* NBRC 15902^T (100% sequence similarity). The ability to synthesize pikromycin was noted earlier in *S. zaomyceticus* [15] and *S. venezuelae* ATCC 15439 [16]. High levels of 16S rRNA gene sequence similarity were also found between strain BV113 and *S. osmaniensis* OU-63^T, *S. longwoodensis* NBRC 14251^T and *S. galbus* JCM 4570^T (99.7%), which constitute a well supported cluster on phylogenetic tree with 91% bootstrap value (Figure 6). The isolate BV113 was also found to share relatively high 16S rRNA gene similarity with *S. chartreusis* JCM 4570^T (99.2%), known as a producer of chartreusin [17].

The position of strains KB-1 and BV113 in the phylogenetic tree was unaffected by the choice of tree-making algorithm or outgroup strains used.

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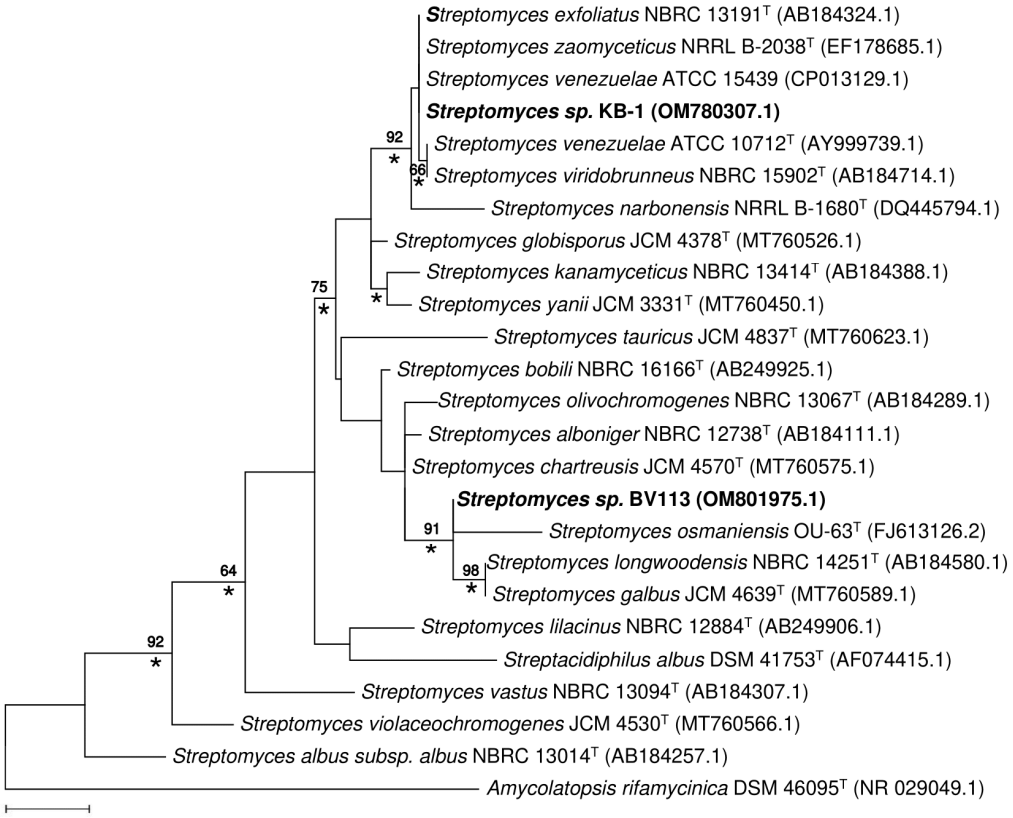

Figure 6. Maximum Likelihood phylogenetic tree based on Tamura-Nei model, showing the relationship between strains KB-1 and BV113 and representative members of the genus *Streptomyces* (based on 1147 unambiguously aligned nucleotides of 16S rRNA gene sequence). Numbers at nodes are bootstrap support percentages based on 1000 sampled datasets; only values above 60% are shown. Asterisks indicate the branches that were also found in the Neighbor-Joining tree. Bar, 0.01 substitutions per nucleotide position.

The physiological and biochemical characteristics of KB-1 and BV113 strains compared with closely related strains are given in Tables S1, S2.

2.4. Identification of active components produced by Streptomyces sp. KB-1 and BV113 strains

The first object of the study was *Streptomyces sp.* KB-1, which demonstrated inhibition of protein biosynthesis on the reporter strains (Figure S2). The culture liquid of this isolate was used for solid-phase extraction, with an active fraction being eluted from the LPS-500-H sorbent with a 20% aqueous acetonitrile (MeCN). HPLC analysis of the fraction followed by activity validation revealed a specific active metabolite with a UV maximum at 274 nm (Figure 7). The mass spectrum of this compound contained the main adduct $[M+H]^+$ with m/z value 526.3370, exhibiting a sole fragment ion observed at m/z 158.1184 in the MS/MS spectrum. The exact mass of the detected metabolite corresponds to the composition $C_{28}H_{47}NO_8$ (the calculated m/z value for $[M+H]^+$, 526.3374).

The search for candidates was carried out based on the accumulated data using the NPAtlas, Dictionary of Natural Products and PubChem databases. The characteristic fragmentation allowed us to conclude that the active compound is a known inhibitor of protein biosynthesis, **pikromycin** (Figure 7) [18,19].

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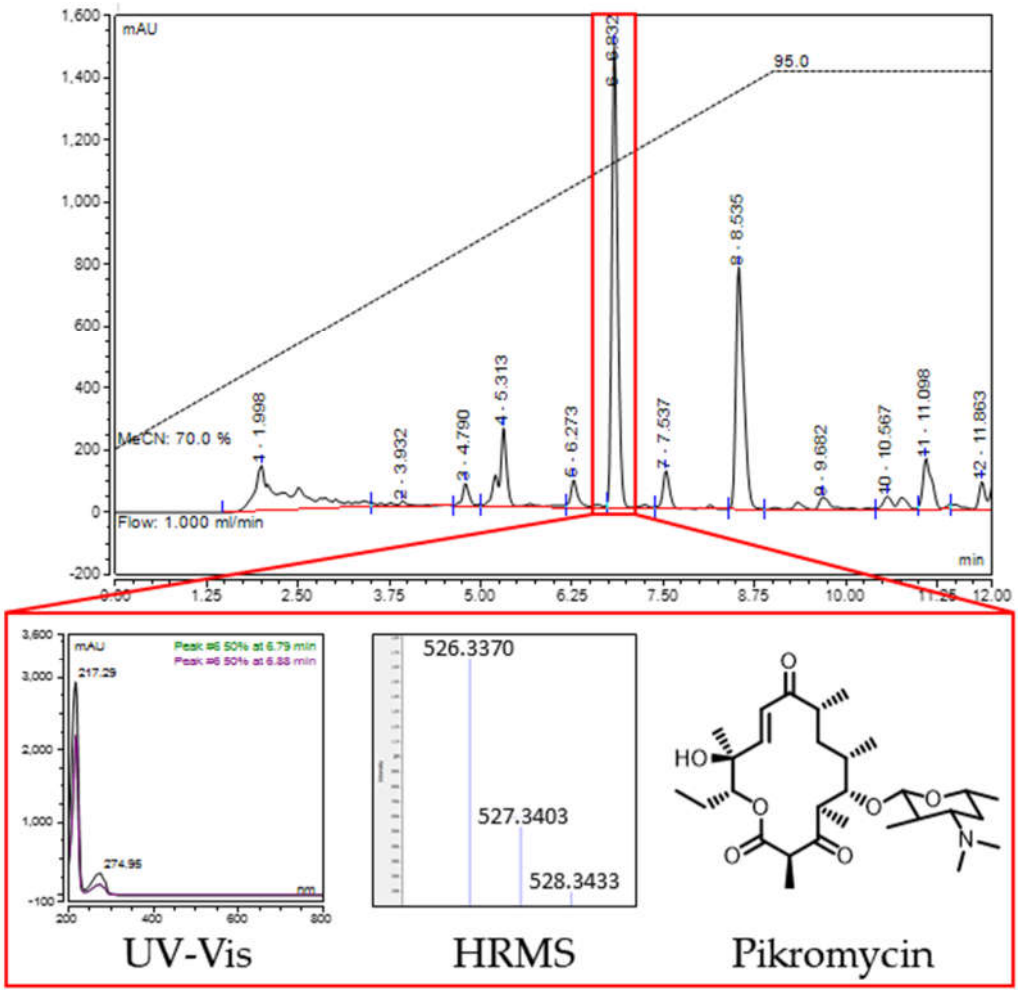

Figure 7. HPLC analysis of the active fraction of *Streptomyces sp.* KB-1 culture liquid. Elution with 70→95% MeCN in water for 9 min followed by 3 min of 95% MeCN. Red box indicates the active component on the HPLC profile.

The second object of study was *Streptomyces sp.* BV113, which demonstrated the induction of SOS-response in tests on the reporter strains (Figure S2). Solid-phase extraction of the *Streptomyces sp.* BV113 strain fermentation broth using the LPS-500-H sorbent yielded an active fraction eluted with 30% acetonitrile in water. HPLC analysis of the fraction (Figure 8) showed that the activity was associated with a hydrophobic compound with characteristic long wavelength maxima in the UV-Vis spectrum (400, 422 nm). Mass spectrometric analysis of this compound revealed a prominent $[M+NH_4]^+$ ion peak at m/z 658.213 in positive ion mode, corresponding to the molecular formula $C_{32}H_{32}O_{14}$ (the calculated m/z value for $[M+NH_4]^+$, 658.2130).

Glycosylated benzochromenone **chartreusin** (Figure 8) was selected as the most appropriate candidate. The structural hypothesis was corroborated by comparing the fragmentation of this compound with the literature data. Fragmentation of the $[M+Na]^+$ ion with m/z value 663.1677 contains key fragment ions with m/z values 329.1207 and 503.0943, which correspond to the loss of glycosidic fragments of the molecule [20]. Consistent with the results of the reporter strains test, chartreusin is known to bind to GC-rich tracts in DNA and cause single-strand breaks [21].

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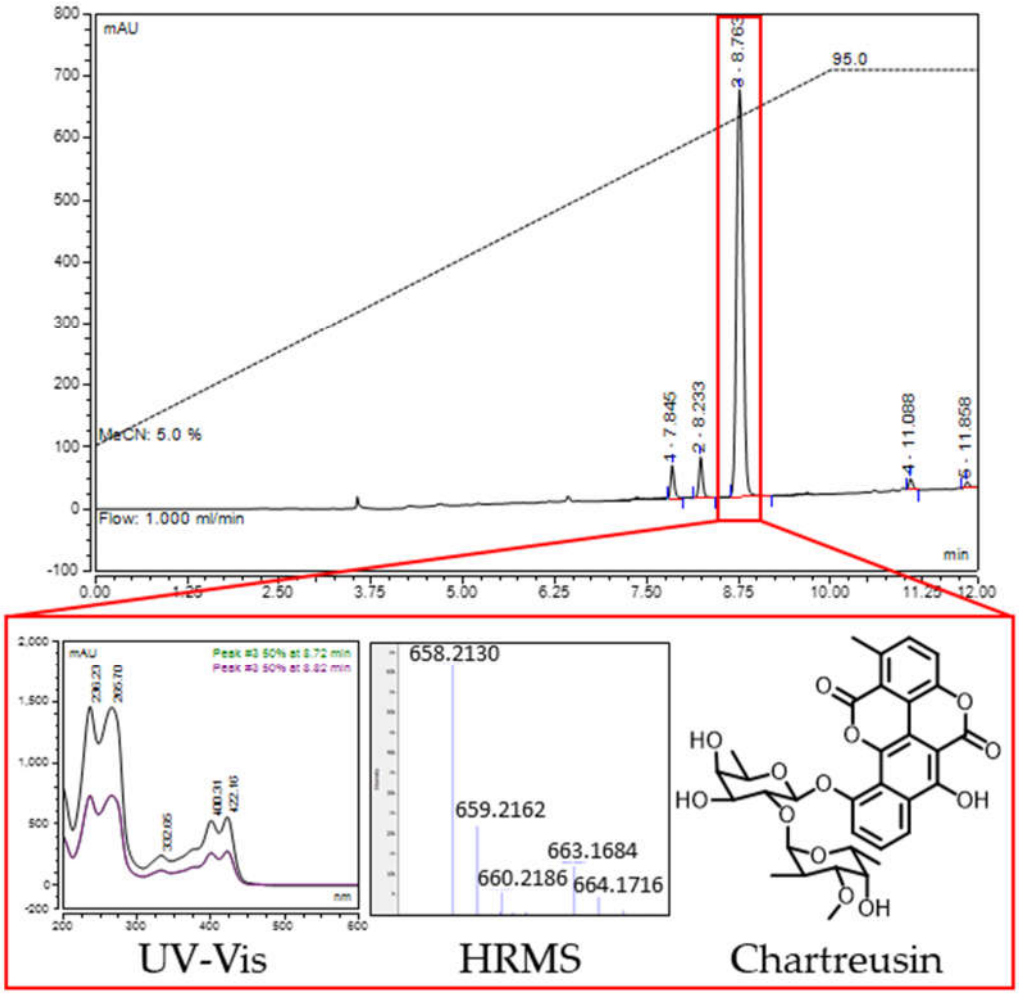

Figure 8. HPLC analysis of the active fraction of *Streptomyces sp.* BV113 culture liquid. Elution with 5→95% MeCN in water for 10 min followed by 2 min of 95% MeCN. Red box indicates the active component on the HPLC profile.

3. Materials and Methods

3.1. Reporter strains and medium

E.coli JW5503 strain with deletion of the *ΔtolC* gene (referred to here as *E.coli ΔtolC*) was kindly provided by Hironori Niki, National Institute of Genetics, Japan [22]. *E.coli* BW25113 strain with partial deletion of the *lptD* gene, codons 330 to 352, (referred to here as *E.coli lptD*) was kindly provided by Alexander S. Mankin, University of Illinois at Chicago, USA [23]. Both strains were transformed with either new reporter plasmids or a double reporter system pDualrep2 [3].

E.coli strains were grown at 37°C in LB medium supplied with 100 μg/ml ampicillin if required.

3.2. Plasmids and cloning

To create the construct pDualrep3, the vector backbone was amplified by high-fidelity PCR from the pDualrep2 plasmid [3] using primers 5′-CCAGCACAGTGGTCGAAG-3′ and 5′-CATATGTTGTGTTTGCATTGTTATTCTC-3′. The *lacZ* gene was amplified by PCR from the pJC27 plasmid [24] with 5′-CAATGCAAACACAACATATGACCATGATTACGCCAAGC-3′ forward and 5′-TTCTTCGACCACTGTGCTGGAATACGGGCAGACATGGC-3′ reverse primers. The joining of two DNA fragments was performed with the NEBuilder® HiFi DNA Assembly technique (NEB).

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The plasmid pTrpL2A-Katushka2S was obtained by PCR amplification with primers 5′-GGGCCCGCGACTCTAGATCATAATCA-3′ and 5′-GGTTCAGTAGAAAAGATCAAAGGATC-3′ using pDualrep2 as a template followed by blunt-end DNA ligation.

The plasmid pTrpL2A-RFP was obtained from pTrpL2A-Katushka2S by replacing the *katushka2S* gene with *turbo-rfp*. The vector was amplified by PCR with primers 5′-CCAGCACAGTGGTCGAAG-3′ and 5′-TTCTCCTTGATCAGCTCGCCCATATGTTGTGTTTGCATTGTTATTCTC-3′. The *turbo-rfp* gene was amplified from the pDualrep2 plasmid with primers 5′-GGCGAGCTGATCAAGGAG-3′ and 5′-TTCTTCGACCACTGTGCTGGAAGCTTGTCGACCTGCAG-3′. The joining of two DNA fragments was performed using the NEBuilder® HiFi DNA Assembly technique (NEB).

The reporter construct pSulA-RFP was obtained from the pDualrep2 plasmid by PCR amplification with primers 5′-CCAGCACAGTGGTCGAAG-3′ and 5′-TTCTTCGACCACTGTGCTGGAAGCTTGTCGACCTGCAG-3′ and subsequent NEBuilder® HiFi DNA Assembly technique (NEB).

The *E.coli* JM109 strain was used for DNA cloning. Sequences of intermediate products and final constructs were confirmed by sequencing with appropriate primers. Plasmid maps were visualized using the program SnapGene® Viewer (version 5.2.4).

3.3. Reporter cells freeze-drying and storage

For freeze-drying, an overnight culture (OD_{600} 0.9-1.0) of *E.coli ΔtolC* transformed with the pDualrep2 plasmid was used. 500 µl of the overnight culture was transferred into sterile 2.0 mL centrifuge tubes, cells were harvested by centrifugation at 7000 rpm for 2 minutes (~8400 × g, Centrifuge 5418 R, Eppendorf), washed and resuspended in lyophilization medium: 1% gelatin (w/v), 1% monosodium glutamate (w/v), 10% sucrose (w/v) and distilled H_2O. Prepared lyophilization medium was sterilized by filtration through a 0.45 µm filter prior to using.

Opened centrifuge tubes with the suspension of reporter cells were covered with Parafilm® M. Thereafter, tubes were subjected to gradual freezing: 20 min at 4°C, then freezing in liquid nitrogen. Drying of samples was carried out at a pressure below 0.370 mBar for 24 hours (FreeZone Plus 2.5 Liter Cascade Benchtop Freeze Dry System, Labconco). The parafilm was removed from dried samples, half of them were stored at room temperature and the other half - at 4°C. On the day of testing, freeze-dried stocks were restored by adding 2 ml of fresh sterile LB medium.

3.4. Reporter assays on agar plates

The overnight culture of reporter cells diluted 5-10 times with fresh sterile LB medium or the restored freeze-dried stocks were plated on LB agar medium supplied with 100 µg/ml ampicillin. The test samples were placed on the surface of dried agar plates covered with a reporter strain. For reporter activity validation tests, 1.5 µl of the following antibiotics were used: chloramphenicol (1 mg/ml), puromycin (2 mg/ml), erythromycin (5 mg/ml), doxorubicin (2 mg/ml), spectinomycin (5 mg/ml), streptomycin (5 mg/ml), fusidic acid (5 mg/ml), clindamycin (10 mM), lincomycin (10 mM), levofloxacin (30 µg/ml), kanamycin (5 mg/ml) and ampicillin (100 mg/ml); as well as disks soaked in antibiotic solution were used for tetracycline (30 µg) and rifampicin (5 µg).

In order to perform reporter activity tests with actinobacteria isolates grown on different nutrient media, agar blocks with mycelium were cut out of the grown lawns by means of the wide end of sterile 1000 µl pipette tips and placed on the surface of dried plates coated with a reporter strain.

To test the reporter and antibacterial activity of liquid samples, such as culture liquids or fractions obtained after solid-phase extraction or HPLC analysis, dried plates

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covered with a reporter strain were subjected to cutting wells out of the agar medium by means of the wide end of sterile 1000 µl pipette tips. The free volume of the resulting wells was 100 µl. For culture liquids and HPLC fractions, 100 µl of solution per well was used; for solid-phase extraction eluates, 10 µl of solution mixed with 90 µl of distilled H_2O per well was used in the agar diffusion assay.

Following overnight incubation at 37°C, the Petri dishes were photographed or scanned by a ChemiDoc™ Imaging System (Bio-Rad) if possible.

3.5. Sampling and isolation of actinobacteria

Soils, sea sediments and plant samples for the study were collected from different regions of Russia during the summer of 2021. The samples were placed into sterile containers to prevent contamination, delivered to the educational laboratory as soon as possible and stored at 4°C until investigation. Sample solutions were prepared by dissolving 1 g of materials in 99 ml distilled water. To facilitate dissolution, sample flasks were shaken at 200 rpm for 10 min (Innova® 44 Shaker, New Brunswick Scientific). Aliquots of serial dilutions were spread on isolation media: Mineral agar Gauze 1 [25], Organic medium 79 [26], M490 [27] and HV agar [28] (Table S3) supplemented with nystatin (250 µg/ml) and nalidixic acid (10 µg/ml) to prevent the growth of fungi and Gram-negative bacteria, respectively. After 14-21 days of incubation at 28°C or at room temperature the powdery-surfaced and leathery colonies were recognized as actinobacteria strains, picked and restreaked on the fresh ISP3 agar medium (Table S3) [29]. The isolated strains were kept on Oatmeal agar (ISP3) slants at 4°C and stored as suspensions of spores in 20% glycerol (v/v) at -20°C.

Individual actinobacteria isolates were restreaked and grown on a set of nutrient media: Mineral agar Gauze 1 (G1), Organic medium 79 (Org79), Glucose-asparagine agar (GA), Soy flour mannitol agar (SFM) and Oatmeal agar (ISP3) for 10 days at 28°C (Table S3) to test agar blocks for antibiotic activity against reporter strains.

3.6. Phenotypic, morphological and physiological characterization of new producing actinobacteria strains

Aerial spore-mass color, substrate mycelial pigmentation, the production of diffusible pigments and melanin were recorded after incubation at 28°C for 14 days on media recommended by the International Streptomyces Project (ISP) [29]. Morphological characteristics of aerial hyphae of KB-1 and BV113 strains were analyzed after 2 weeks of incubation at 28°C on ISP4 with CamScan S2 scanning electron microscope (Cambridge Instruments) and on ISP3 medium with JSM-6380LA scanning electron microscope (JEOL Ltd.), respectively.

Carbon source utilization was assessed after incubation at 28°C for 14 days on Basal agar medium (ISP9) (Table S3) [29] supplemented with bromocresol purple indicator solution, 0.04% (w/v). Cellulose decomposition, starch hydrolysis, nitrate reduction, milk peptonisation, gelatin liquefaction and H_2S production were examined as described previously [30].

3.7. Phylogenetic analysis of new producing actinobacteria strains

Genomic DNA extraction from actinobacteria isolates was carried out according to the procedures described previously [31]. Amplification of the 16S rRNA gene was performed by high-fidelity PCR using primers 5′-GGATGAGCCCGCGGCCTA-3′ (243F) and 5′-CCAGCCCCACCTTCGAC-3′ (A3R) [6]. The sequencing was conducted with ABI Prism® BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and detected with Applied Biosystems® 3730 DNA Analyzer (Life Technologies) in the Center for Collective Use "Genome" (Moscow, Russia). The contigs were processed and assembled with the GeneStudio™ Pro software (Version 2.2.0.0). GenBank accession numbers for the 16S rRNA gene sequences of *Streptomyces sp.* KB-1 and BV113 strains are OM780307.1 and OM801975.1, respectively.

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Taxonomic affiliation of the isolates was assessed using the 16S rRNA gene sequences as a query in the BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzTaxon (http://www.ezbiocloud.net) web services. The 16S rRNA gene sequences of the most closely related species of the genus *Streptomyces* (more than 99% of identity) were used to build the alignment. In total, it comprised 25 nucleotide sequences, with *Amycolatopsis rifamycinica* DSM 46095^T as an outgroup. The alignment was manually trimmed to 1147 bp and the phylogenetic tree was reconstructed by the Maximum Likelihood (ML) method based on the Tamura-Nei model [32] as well as by the Neighbor-Joining (NJ) method [33] based on the Kimura two-parameter model [34]. The phylogenetic tree was visualized using MEGA 7.0 software [35].

3.8. Cultivation and extraction of secondary metabolites

Actinobacteria isolates were first inoculated in 20 ml of liquid Organic medium 79 (Org79, no agar) and cultivated at 28°C with constant shaking (180 rpm, Innova® 44 Shaker, New Brunswick Scientific) for 3 days. The resulting starters were used to inoculate 200 ml of different liquid media (Table S3) and then suspensions were incubated at 28°C with constant shaking (220 rpm, Innova® 44 Shaker, New Brunswick Scientific) in 750 ml Erlenmeyer flasks to determine the optimal cultivation conditions.

For *Streptomyces sp.* KB-1, the greatest production of an antibacterial metabolite was observed upon cultivation in a modified ISP3 liquid medium for 6 days. Composition of the modified ISP3 medium (g/L): oatmeal - 20, powder chalk - 5, $FeSO_4 \cdot 7H_2O$ - 0.001, $MnCl_2 \cdot 4H_2O$ - 0.001, $ZnSO_4 \cdot 7H_2O$ - 0.001, pH 7.2.

For *Streptomyces sp.* BV113, the greatest production of an antibacterial metabolite was observed upon cultivation in a modified Org79 liquid medium for 4 days. Composition of the modified Org79 medium (g/L): maltose - 10, peptone - 10, casein hydrolysate - 2, yeast extract - 2, NaCl - 6, pH 7.2.

Culture liquids were separated from biomass by centrifugation at 20000 × g for 5 minutes (Centrifuge 5810 R, Rotor FA-45-6-30, Eppendorf). The supernatants were subjected to solid-phase extraction and primary fractionation on LPS-500-H sorbent (LLC "Technosorbent") using water-acetonitrile mixtures as eluents.

3.9. Antibiotic identification

HPLC analysis and fractionation was performed with Vanquish Flex UHPLC System using Diode Array Detector (Thermo Fisher Scientific), equipped with Luna® 5 µm C18(2) 100 Å, 250 x 4.6 mm column (Phenomenex).

Mass spectra were collected using Bruker maXis II 4G ETD mass spectrometer, UltiMate 3000 chromatograph, equipped with Acclaim RSLC 120 C18 2.2 µm 2.1 x 100 mm column. Spectrum registration mode: ESI ionization mode, full scan from 100-1500 m/z, MS/MS with selection of the three most intense ions, dissociation type: CID 10-40 eV, nitrogen collision gas. Mass spectra were processed using OpenChrom Lablicate Edition (1.4.0.202201211106), TOPPView v. 2.6.0 [36]. The structure was established using the GNPS [37], NPAtlas [38,39] and Dictionary of Natural Products 31.1 (https://dnp.chemnetbase.com/) [14] databases.

4. Conclusions

In order to speed up high-throughput screening for new antibiotic producing strains, we created reporter plasmids that can be utilized in the course of the Civil Science project. The ability to detect the reporter induction with the naked eye makes it possible to involve civilian researchers at the early stages of the process, which would subsequently increase the screening productivity. Consistent with the idea to give citizen scientists an opportunity to collect and analyze natural samples near their homes, we developed a special optimized pipeline that was successfully applied in practice. Two examples of antibiotic-producing strains discovered *de novo* are presented in the article: *Streptomyces sp.* KB-1 (pikromycin) and BV113 (chartreusin).

Supplementary Materials: Figure S1: double reporter system pDualrep3; Figure S2: agar blocks with isolated actinomycetes (BV113, KB-1, Nm4) were tested on the reporter strains; Figure S3: utilization of different sugars (1.0%, w/v) as sole carbon source by strain KB-1; Figure S4: utilization of different sugars (1.0%, w/v) as sole carbon source by strain BV113; Table S1: physiological and biochemical characteristics of KB-1 isolate and closely related *Streptomyces sp.* strains; Table S2: physiological and biochemical characteristics of BV113 isolate and closely related *Streptomyces sp.* strains; Table S3: composition of some nutrient media used.

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