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Metal-Tolerant Bacteria of Wastewater Treatment Plant in a Large City

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Abstract: Biological treatment methods are the most important part of the treatment process for domestic wastewater, the amounts of which are increasing every year due to rapid, unregulated urbanization and the rising number of people living in such areas. At the same time, microorganisms existing in treatment facilities should not only effectively utilize organic pollutants, but also be resistant to a variety of organic and inorganic contaminants. This study's objective is to isolate and identify—using molecular genetic techniques—strains of bacteria that tolerate concentrations of heavy metals (Ni, Cd, Pb, Zn and Cu) in the 3–5 mM range. They were sourced from water and sludge samples obtained from sewage treatment facilities in a large city. Outcomes of phylogenetic analysis of 16S rRNA gene sequences revealed that tolerant strains of bacteria belonged to the genera *Pseudomonas*, *Serratia* and *Klebsiella*; strains belonging to the genus *Pseudomonas* dominated. Of ten resistant strains, nine were isolated from sludge and water samples of the secondary sedimentation tank, and the other one from a treatment plant's digester. Changes in the color of microorganisms' colonies became evident when cultivated on media enriched with heavy metals. Cultivating nonpathogenic strains of these bacteria and their introduction into communities of other activated sludge microorganisms could have practical application to biological decontamination of wastewater.

Keywords: trace elements; sewage sludge; urban wastewater; microorganisms; *Pseudomonas*

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1. Introduction

One of the major global problems linked to industrial development and population growth is the increasing amount of environmental pollution and wastewater produced from domestic activities, industry, and stormwater outflows [1].

Around 330 billion m^3 of municipal wastewater is generated universally every year [2]. Over half of the population living in clusters in the European Union produces on a daily basis wastewater that amounts to 41.5 million m^3. The percentage proportion of people residing in urban areas is anticipated to increase substantially, from 55.3% in 2018 to 60.4% in 2030 [3], which will simply aggravate the already huge volumes of wastewater. Moreover, approximately 1 billion m^3 of treated residential wastewater is discharged, yet this material contains reusable and recyclable nutrients, organic carbon, lipids, and biosolids [4].

Furthermore, urban wastewater contains a great variety of pollutants, such as soluble and particulate organic matter, nutrients, heavy metals (HM), and pathogens [5]. Pollutants in urban wastewater and sludge can be extensively categorized into two groups: organic pollutants and potentially toxic elements [6]. Organic pollutants are categorized into several subgroups [7], while all heavy metal(loid)s fall into the next category. Sources of heavy metals and metalloids found in urban wastewater are industrial discharge, urban rain runoff and some household products, such as detergents.

Currently, there is insufficient decontamination of wastewater, meaning that heavy metals are continuously discharged into the natural environment, along with waters and sewage sludge from treatment plants. This problem has aroused great concern over their potentially dangerous effects by entering the diet, posing a severe threat to ecosystems and people's health [8]. A large amount of wastewater enriched with organic matter and nutrients entering the environment can result in eutrophication and oxygen depletion in receiving waters. This is due to stimulations triggered by microbial activity. This oxygen depletion greatly damages ecosystems and will lead to diminished biodiversity [9]. This also contributes greenhouse gas emissions, including nitrous oxide and methane as well as eutrophication and human health risks due to the discharge of untreated effluent into water bodies. Sewage sludge at acceptable concentrations of pollutants can be effectively used as soil fertilizers. However, utilizing wastewater and its sludge, insufficiently purified from inorganic pollutants, including heavy metals, in agriculture can lead to contamination of food or farm produce and pose considerable risks to human health. It has been reported that wastewater treatment plants constitute the major source for the dissemination of heavy metal-resistant genes into the environment [10].

The conventional treatment of wastewater focuses on the abatement of contaminants through a combination of mechanical, physical, chemical and biological operations [11]. Sewage sludge is produced as solid or semi-solid materials because of urban wastewater treatment processes [7]. Biological wastewater treatment consists of the cultivation of mixed microbial communities of activated sludge that consume wastewater components as nutrient substrates, thereby removing them from wastewater. The main product of the bioconversion of wastewater impurities is biologically purified water, while the by-product is activated sludge biomass [12]. Utilization of inorganic pollutants is also effectively carried out by the activated sludge community, made possible by biosorption and biotransformation processes.

The microbiological community existing in wastewater generally is a combination of human fecal and non-fecal microorganisms that may be residents of the sewer system, as well as microorganisms that enter sewage with domestic, rain and industrial runoff. Some types of industrial wastewater contain specific microorganisms used in the technological processes for producing medicines, meat, dairy products, alcohol, etc. Rain runoff tends to be contaminated with soil microorganisms. Municipal sewage can contain various types of bacteria, among which there are pathogenic forms: opportunistic pathogens (e.g., *Enterobacter cloacae*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* or *Pseudomonas aeruginosa*); obligate pathogens from *Salmonella* and *Shigella* genera; and enteropathogenic strains of *Escherichia coli*. They may also contain viruses, protozoa, fungi, flatworms, roundworms, etc. [13].

Any structure for the biological destruction of pollutants is an artificial ecosystem with certain but often extreme conditions. Ecosystems of wastewater treatment plants differ from natural analogues by cultivating microorganisms at their high density, specific oxygen and temperature conditions, and high concentrations of pollutants contained there. Microbial heavy metal resistance is an important phenomenon because it is relevant to plasmid genetics and the ecology and physiology of microorganisms. Given that heavy metal ions are not biodegradable, it was reported by Nies [14] that there were only three possible mechanisms for a bacteria resistance to them: (1) eflux, an active extrusion of heavy metal ions from the cell; (2) thiol-containing molecules forming nontoxic complex; and (3) transformation into a less toxic oxidation state. Finally, it should be noted that resistance

and homoeostasis involve a combination of two or three of the basic mechanisms mentioned above. Research into genetics has revealed there are several basic mechanisms by which plasmids encode metal resistance: (1) inactivation of the metal (s); (2) alteration of the site of inhibition; (3) impermeability of the metal (s); and (4) metal bypass mechanisms. There are many other physical and chemical factors that influence metal resistance as well [15].

In this regard, strains of microorganisms that are a part of activated sludge, which are capable of destroying organic matter and can resist large concentrations of heavy metals, are of great interest. Of particular interest are the mechanisms by which bacteria are able to adapt to high concentrations of the metals. The objective of this investigation was to isolate and analyze by 16S rRNA gene sequencing strains of bacteria that can tolerate high concentrations of heavy metals, namely Ni, Cd, Pb, Zn and Cu, from the water and sludge being processed in sewage treatment facilities of a large city situated in a temperate climate.

2. Materials and Methods

2.1. Sample Collection

According to information offered by its owner, the sewage treatment plant responsible for the city of Tula, central Russia (population—479,000 people as per the 2019 Census) was designed in 1975 with a capacity of 250,000 m^3/day, which expanded in 1985 to 450,000 m^3/day. The construction of treatment facilities began in 1968, and stage I was put into operation in 1976, while stage II commenced in 1977. In 2012, work was carried out to create a promising scheme for improving the water supply and sanitation up to 2025. To date, sewage treatment facilities in Tula can deal with 180,000 m^3/day. They include: a receiving chamber with gratings, sand traps, primary and secondary sedimentation tanks, digesters (methane tanks), aeration tanks, a mechanical sludge dewatering station, and silt ponds.

In October 2020, wastewater and sludge samples were taken from the secondary sedimentation tank and methane tank (digester) at the sewage treatment plant in Tula to study their microbiological and heavy metal composition. Special scoops served to retrieve the samples from the thickness of the analyzed medium. Of the three samples after homogenization, a mixed sample with a volume of 1 L was created. Determination of the trace element concentration in the collected samples was carried out at the Institute of Physicochemical and Biological Problems in Soil Science, Russian Academy of Sciences, utilizing optical emission spectrometry with inductively coupled plasma (Optima-5300DV, Perkin Elmer, Waltham, MA, USA).

2.2. Bacteria Cultivation

Bacteria from wastewater and sediment samples were inoculated after 7 ten-fold dilutions in physiological solution on the LB medium of the composition (g/L): NaCl—10, yeast extract—5, tryptone—10, agar—15.

To determine the number of colony-forming units (CFU), 100 µL of the microbe suspension from prepared test tubes was inoculated into Petri dishes on agar medium. The cell suspension was rubbed over the surface of the agar medium with a sterile glass spatula. Petri dishes were kept in a thermostat at 28 °C for 24–48 h. Further, the number of microorganisms was determined by direct counting of colonies and expressed as the number of bacteria per 1 mL of the sample.

Bacteria were tested for resistance to six heavy metals, namely Ni, Co, Cd, Zn, Pb, and Cu. Stock standards of heavy metals solutions were prepared from salts: $CoSO_4 \cdot 7H_2O$, $NiCl_2 \cdot 6H_2O$, $Cd(CH_3COO)_2 \cdot 2H_2O$, $Pb(NO_3)_2$, $CuCl_2 \cdot 5H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$ as documented by Cai et al. [8]. Using a sterilized coating stick, 100 µL of the microbe suspension was spread carefully on Petri dishes with twice diluted (to prevent precipitation) LB medium containing 2, 3, 5 mmol of the heavy metal compounds. Having been cultured for 24 h at 37 °C in a constant-temperature incubator, the bacterial colonies on the dishes were examined, and colonies with different morphologies, including size, edge appearance, and color, were marked for purification and isolation. To streak the marked colonies on a fresh LB Petri dish containing 2, 3, and 5 mmol of the heavy metal compounds, a sterilized

coating stick was employed. Then, the dishes were incubated in a thermostat as described above, and the same steps were replicated until a single colony was formed. Bacteria cultures generated from the liquid LB medium were assembled and blended with sterilized glycerol and then stored at −20 °C.

From the isolated bacteria, 10 strains were selected based on their being the most resistant to various HMs and differing in phenotype properties. Genomic DNA was isolated from the selected strains using the Quick-DNA Miniprep Kit (Zymo Research, Irvine, CA, USA).

2.3. PCR Analysis of Target Genes

The isolated DNA was used to amplify the 16SrRNA gene. PCR was performed with the following primers: forward 27f 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse 1492r 5′-GGTTACCTTGTTACGACTT-3′. Amplification of the 16S rRNA gene was carried out on a GeneAmp PCR System 9700 device (Applied Biosystems, Foster City, CA, USA) under the following conditions: primary denaturation: 95 °C, 5 min; further 30 cycles: 95 °C—30 s, 55 °C—30 s (primer annealing temperature), 72 °C—40 s; final elongation—72 °C, 5 min. The PCR product was 1465 bp in size. The reaction products were separated by agarose gel electrophoresis (1.0%) at a voltage of 10 V/cm. The gel was stained with a solution of ethidium bromide (5 μg/mL) and photographed under UV light using a Gel DocTM XR gel-documentation system (Bio-Rad, Hercules, CA, USA). Taq DNA polymerase, PCR components, concentrated electrophoresis buffer, and GeneRuler 1kb DNA Ladder marker (SM0311) were manufactured by Fermentas (Lithuania). PCR products were purified using DNA Clean & Concentrator (Zymo Research, USA) according to the manufacturer's instructions.

Sanger sequencing of the 16S rRNA gene of the strains was performed with PCR products using the primers stated above. Sequencing was completed on an ABI Prism 373 3130XL automatic sequencer (Applied Biosystems, USA). Preliminary phylogenetic screening for the similarity of the 16S rRNA gene nucleotide sequences was carried out in the GenBank database, National Center for Biotechnology Information (NCBI), using the Basic Local Alignment Search Tool (BLAST) software package. To more accurately determine the phylogenetic position of the studied strain, the subsequent 16S rRNA gene sequence was aligned with the corresponding sequences of the closest bacterial species using the CLUSTAL W program [16].

2.4. Anaylsis of the Evolutionary Distances of the Studied Bacteria

Multiple alignments of the nucleotide sequences of the 16S rRNA genes were performed using the MUSCLE algorithm integrated into the Unipro UGENE software package [17]. Evolutionary distances were studied by the maximum likelihood method based on the Tamura–Nei model [18]. The initial dendrograms for heuristic search were performed automatically by employing the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated utilizing the maximum likelihood method (MCL). Evolutionary analysis and construction of a phylogenetic tree were executed using the MEGA v. 7.0 [19].

3. Results

The elemental composition of the samples (water, suspension of sludge in the water of secondary sedimentation tank and media of digester) is shown in Table 1. The water of the secondary sedimentation tank did not meet the standards for cadmium concentration accepted in the Russian Federation [20] (Table 1). For other elements, no excess concentrations in the water of the secondary sedimentation tank are observed. Thus, at the time of sampling, wastewater from the studied parts of the treatment facilities was not contaminated with most heavy metals.

CFU levels in sludge suspension from the secondary sedimentation tank were higher (2.8×10^6 cells/mL) in samples compared to water from the secondary sedimentation tank (2.4×10^5 cells/mL) and suspension from the digester (1.6×10^5 cells/mL). Based on the results of inoculation on a nutrient medium with HM from the water and sludge

of the secondary sedimentation tank, bacteria were isolated that were resistant to the concentrations of Ni, Cd, Zn, Pb, and Cu at 3 mM. No growth of colonies was noted on the medium with Co. Among cultivated bacteria, some indicated resistance to the largest concentrations (5 mM) of Pb and Zn. Only one HM-resistant strain, namely Ni (5 mM), was isolated from the digester. Some strains, developing on a medium enriched with heavy metals, changed the color of their colonies. From the isolated strains, 10 were selected for further identification. Phylogenetic trees for the isolated strains of microorganisms resistant to HM are shown in Figures 1 and 2.

The results of phylogenetic analysis of 16S rRNA gene sequences confirmed that most of the isolated HM-tolerant strains belonged to the genus *Pseudomonas* (Table 2, Figure 1). The exceptions were, firstly, strains resistant to Ni from the digester; and secondly, strains resistant to Pb and Cu from sludge of the secondary sedimentation tank. It is important to note that the Zn-resistant *Pseudomonas gessardii* 9 strains also grew on a medium with a Cd concentration of 2 mM/L. Lead-resistant strains (*Pseudomonas brenneri* 7 and *Pseudomonas fragi* 6) grew on a medium containing 3 mM/L of Zn.

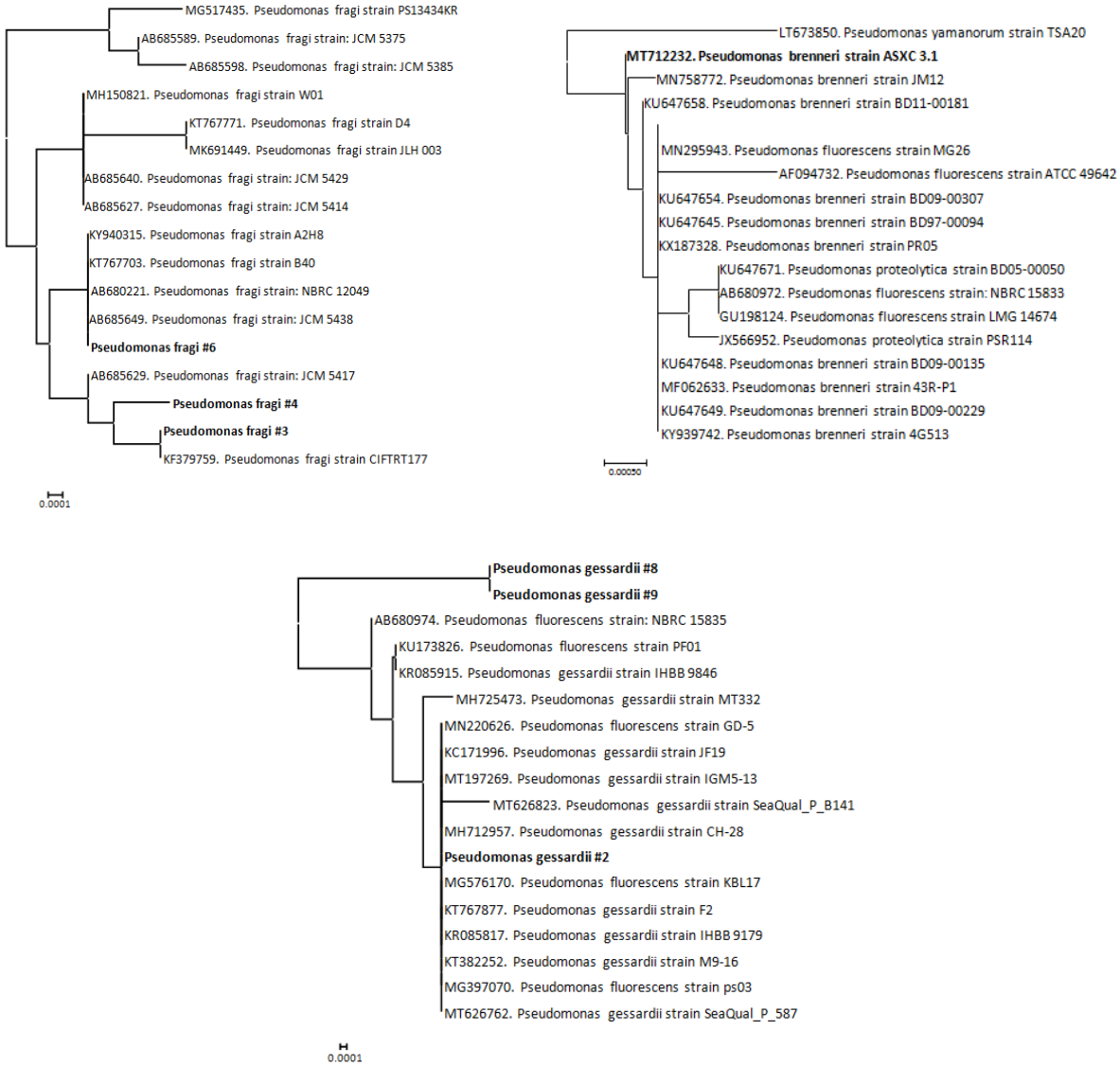

Figure 1. Phylogenetic tree of metal-tolerant bacteria of the genus *Pseudomonas*.

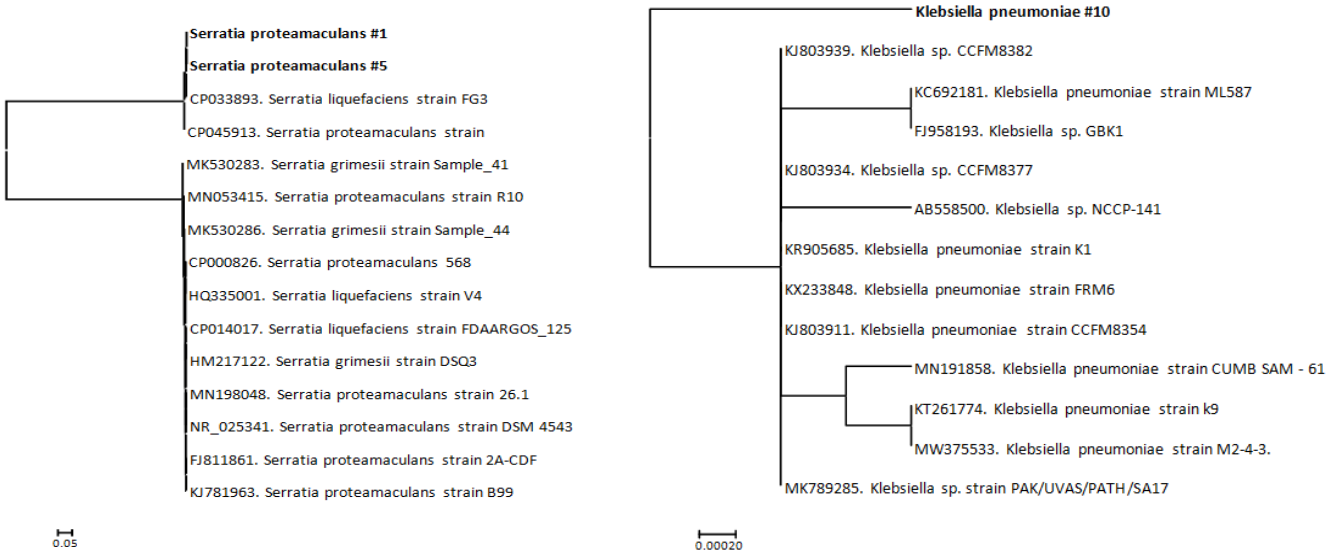

Figure 2. Phylogenetic tree of metal-tolerant bacteria of the genus *Serratia* and genus *Klebsiella*.

Table 1. Concentrations of heavy metals in the media of the secondary sedimentation tank and digester of the sewage treatment plant.

Heavy Metal	Concentration, mg/L			
	Maximum Permissible Concentrations for Waters of Household and Cultural Water Use [20]	Secondary Sedimentation Tank (Water)	Secondary Sedimentation Tank (Suspension)	Digester (Suspension)
Cd	0.001	0.0053	0.0037	0.0186
Cu	1.0	0.0056	0.0037	0.0248
Ni	0.02	0.0089	0.0041	0.1204
Pb	0.01	<0.0001	0.0038	0.0050
Zn	1.0	0.0002	0.00001	0.0002

Table 2. Isolated strains of metal-tolerant bacteria from a wastewater treatment plant.

N	Species (According to Sequencing Results)	Source	Element	Maximal Tolerance Concentration
1	*Serratia proteamaculans*	Digester	Ni	5 mM
2	*Pseudomonas gessardii*	Secondary sedimentation tank (sludge)	Ni	3 mM
3	*Pseudomonas fragi*	Secondary sedimentation tank (sludge)	Cd	3 mM
4	*Pseudomonas fragi*	Secondary sedimentation tank (water)	Cd	3 mM
5	*Serratia proteamaculans*	Secondary sedimentation tank (sludge)	Pb	5 mM
6	*Pseudomonas fragi*	Secondary sedimentation tank (water)	Pb	3 mM
7	*Pseudomonas brenneri*	Secondary sedimentation tank (water)	Pb	3 mM
8	*Pseudomonas gessardii*	Secondary sedimentation tank (sludge)	Zn	5 mM
9	*Pseudomonas gessardii*	Secondary sedimentation tank (water)	Zn	5 mM
10	*Klebsiella pneumonia*	Secondary sedimentation tank (sludge)	Cu	3 mM

4. Discussion

Chemical composition of wastewater is generally determined by the sources of their entry to the treatment plant. Data on the number of heavy metals in them vary greatly. For example, heavy metal concentrations in wastewater of the Eastern Industrial Zone, central Ethiopia, ranged from 0.04–0.08 mg/L for Cd, 0.07–0.21 mg/L for Zn, 3.11–45 mg/L for Pb

and 0.30–0.99 mg/L for Cu [21]. For wastewaters being treated in plants in China, the respective ranges for these elements were 0.001–0.009 mg/L, 0.05–0.37 mg/L, 0.01–0.11 mg/L, 0.01–0.36 mg/L, and 0.06–0.4 mg/L for Ni [22]. The major wastewater sources that the treatment plant in Tula deals with originate from domestic wastewater (70%) and non-industrial wastewater from manufacturing plants (30%). The microelement composition of wastewater and sludge reflects what was really happening at the sampling time. Nevertheless, regular sporadic discharges containing large concentrations of heavy metals are possible and subsequently recorded. In effect, the habitat conditions of the bacteria isolated by us altered from non-toxic concentrations of heavy metals to toxic ones.

Interest has grown in recent times on the issue of microorganisms living in the conditions of wastewater treatment plants [8,10,13,23,24] etc. For the molecular genetic study of bacteria living in wastewater, two main strategies are used—metagenomic whole genome sequencing [10] and the isolation of functionally culturable bacteria followed by sequencing of 16S rRNA genes of the isolated strains [8,23,24]. Moreover, there are flaws with metagenomic analysis due to the bias in the DNA extraction, which can affect incomplete or incorrect explanations of how microbial performances are related to metal pollutants [8]. Consequently, from an application perspective, isolating functionally culturable bacteria and rRNA gene sequencing of the isolated strains are greatly valued [8].

The bacterial strains we have isolated belong to the genera *Pseudomonas*, *Serratia* and *Klebsiella*. Representatives of these genera have specific ecological and physiological features. The genus *Pseudomonas* contains 294 species as of May 2022 [25] (https://bacterio.net/; accessed on 22 May 2022). The Pseudomonadaceae family incorporates gram-negative rods that grow under aerobic conditions or obtain energy from anaerobic respiration or from bound oxygen (denitrification), but not from fermentation. Pseudomonades use a wide range of organic substances, including cyclic compounds (heterocyclic and aromatic). Due to their "omnivorous nature", these bacteria are ubiquitous: in water, soil, silt, and are transported with air flows. Many of them form water-soluble and fluorescent pigments. Phytopathogenic species of *Pseudomonas* cause a wide variety of diseases [26]. They can generate necrotic lesions or spots on stems, leaves and fruits. They can also cause gills, rot and vascular infections. A group of *P. syringae* attacks mainly leaves. For example, in onions they can produce leaf spots and bulb rot. Pathogenicity for humans in *Pseudomonas* is weakly expressed, but even such a saprophyte, usually well known to microbiologists, as *P. aeruginosa*, is often the cause of secondary infection of long-term non-healing wounds and ulcers in humans and animals.

Heavy metal pollution-tolerant strains of species *Pseudomonas putida* and *Pseudomonas guariconensis* were isolated from wastewater irrigated agricultural soils near Pakistan's major industrial cities [27]. The strain *Pseudomonas aeruginosa* KUCd1 was discovered to be remarkably resistant to heavy metals. The order of toxicity of the metals to the bacterium on both solidified and liquid medium was Cr > Co > Cu > Cd > Ni > Zn > Mn [28]. A total of 80% of the 45 strains of genera *Pseudomonas* isolated from soil harbored resistance to Cu, while 73% of the isolates demonstrated their ability to resist Cd; 71% were able to resist Cr and Zn [29]. Thus, representatives of this genus, isolated from polluted and unpolluted habitats, have a lot of practical potential with reference to high concentrations of heavy metals. In our research, strains resistant to heavy metals belonged to the species *P. brenneri* (tolerant to Pb), *P. fragi* (tolerant to Cd and Pb) and *P. gessardii* (tolerant to Ni and Zn) (Table 2).

P. brenneri is a gram-negative, rod-shaped, fluorescent, motile bacterium with one polar flagellum first isolated from natural mineral waters in France [30]. Based on 16S rRNA analysis, *P. brenneri* belongs to the *P. fluorescens* group. The strain *P. brenneri* 7 isolated by us on the pseudomonad phylogenetic tree is included in the *P. brenneri* cluster; the closest related species are *P. proteolytica* and *P. fluorescens* (Figure 1). Authors highlighted that the microbial isolate *Pseudomonas brenneri* MF957286 isolated from one mining area survived in Cr(VI)-tainted solution at a concentration up to 140 mg/L. The isolated bacterium showed much promise for the bio-treatment of Cr(VI)-laden wastewater [31].

P. fragi is a gram-negative psychrophilic bacterium responsible for spoilage of dairy products [32]. Unlike many other representatives of the genus *Pseudomonas*, *P. fragi* does not produce siderophores [33]. The ideal temperature for its growth is 30 °C, but it can grow at temperatures from 0 to 35 °C [34]. *P. fragi* was assigned to the *P. chlororaphis* group based on the analysis of 16SrRNA [35]. The three strains of *P. fragi* 3, *P. fragi* 4, and *P. fragi* 6 that we have identified are part of the *P. fragi* cluster (Figure 1). The strain *P. fragi* 6 grown on 3 mmol of Pb changed the color of colonies to red-brown. There are several opinions for why pigments are produced in response to heavy metals' presence. It is possible that bacterial pigments can protect against photooxidative damage in microorganisms [36]. Often, however, it is not possible to establish a linear relationship between the color intensity and metal concentration in the medium, as it only appears in a certain concentration range [23]. It has been suggested that small concentrations of toxic metals may discourage bacterial pigmentation [37]. In some scenarios, the ability to produce pigment may be directly linked to metal tolerance. In their research, Fugimore et al. [38] observed that a red pigment-deficient white mutant of *Pseudomonas* K-62 strain betrayed greater sensitivity to Hg^{2+} than the parent wild-type reddish strain.

P. gessardii is a gram-negative, fluorescent, rod-shaped bacterium first isolated from natural mineral waters in France [39]. Based on the analysis of 16SrRNA, *P. gessardii* was assigned to the *P. fluorescens* group [35]. The three strains of *P. gessardii* 2, *P. gessardii* 8, and *P. gessardii* 9 that we have isolated are part of the *P. gessardii* cluster; the closest related species is *P. fluorescens* (Figure 1). Previously, it was shown that *Pseudomonas gessardii* strain LZ-E isolated from the wastewater discharge site of a petrochemical company simultaneously degraded naphthalene and reduced Cr(VI) [40]. In a study conducted in Tunisia, *Pseudomonas* sp. strain AHD-1 (closely related to *P. azotoformans*, *P. gessardii*, and *P. libanensis*) was isolated from soil contaminated with wastewater [41].

Our findings strongly suggest that a strain of the genus *Klebsiella* can resist copper at a concentration of 3 mM. The genus *Klebsiella* contains 13 species as of May 2022 [25]. *Klebsiella pneumoniae* is a gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. This is similar to a mucoid lactose fermenter on MacConkey agar. Although it was noticed in the normal flora of the mouth, skin, and intestines [26], it can cause devastating changes in the lungs of humans and animals when aspirated, especially in the alveoli, resulting in bloody, brownish, or yellow jelly-like sputum. Clinically, it is the most critical member of the *Klebsiella* genus from the Enterobacteriaceae family. *K. oxytoca and K. rhinoscleromatis* have also been demonstrated in human clinical specimens. In recent years, *Klebsiella* species have become important causative agents of nosocomial infections. In nature, *Klebsiella* occurs in the soil, and about 30% of strains can fix nitrogen under anaerobic conditions [42].

As a free-living diazotroph, its nitrogen fixation system is well understood and of agricultural interest, since *K. pneumoniae* has been shown to increase agricultural yields [43]. The *K. pneumoniae* #10 strain we isolated is located on the phylogenetic tree in the *K. pneumoniae* cluster, although at some distance. This may be due to the small number of *Klebsiella* species identified to date; a more accurate identification requires a genome-wide sequencing of the strain (Figure 2). *Klebsiella pneumoniae* Kpn555, isolated from coffee pulp waste, displayed tolerance to metals, for example cadmium (Cd), lithium (Li) and mercury (Hg). The bacterium exposed a lowest inhibitory concentration of 150 mg/L, 250 mg/L and 10 mg/L of Cd, Li and Hg, correspondingly. Researchers have argued that *K. pneumoniae* Kpn555 could be employed for bioremediation of soil and water polluted by various heavy metals [44]. Elsewhere, authors reported toxic metals tolerance and bioremediation potentials of novel bacteria of a genus *Klebsiella* isolated from Nigeria's Uburu Salt Lake. The bacteria isolate confirmed their capacity to tolerate 50.0 mM Hg^{+2} and Pb^{+2}, 17.0, 12.5, 4.0 mM and 4.0 mM Ni^{+2}, Cd^{+2}, Cu^{2+} and Zn^{+2}, respectively, in solid media [45].

The *S. proteamaculans* strain indicated its resistance to maximum concentrations of Ni and Pb used by us—5 mM. The presence of heavy metal tolerance genes *czcA* (cadmium, zinc and cobalt efflux pump), *merA* (mercuric reductase), *silA* (silver efflux pump), and

pcoD (copper efflux pump) was noted in species of this genus [46]. The genus *Serratia* has 23 species [25] (May 2022). *Serratia* is a genus of gram-negative, facultative anaerobic rod-shaped bacteria of the Yersiniaceae family [47]. As per the List of Prokaryotic Names with Constant Nomenclature (LPSN), at present, 19 *Serratia* species were reliably reported with exact names, as of 2020. They are generally 1–5 µm in length, do not form spores and can be detected in water, soil, plants and animals [48]. Several members of this genus formed a featured red pigment, prodigiosin, and they can be distinguished from other members of the Enterobacterales order by the unique production of three enzymes: DNase (nucA), lipase, and gelatinase (serralisin) [47]. *Serratia* was considered to be a harmless ecological bacterium until the most common species of the genus, *S. marcescens*, surfaced as an opportunistic pathogen that could affect living organisms [47].

In humans, *S. marcescens* is mainly associated with nosocomial infections, but can also cause urinary tract infections, pneumonia, and endocarditis [49]. *S. marcescens* is usually observed in showers, toilets, and around wet tiles as a pinkish to red biofilm, which causes illness in immunocompromised people. In addition to *S. marcescens*, some rare strains of *Serratia* species (*S. plymuthica*, *S. liquefaciens*, *S. rubidaea*, and *S. odoriferae*) have been known to cause infections, including osteomyelitis and endocarditis [50]. *Serratia proteamaculans* is a gram-negative, facultative anaerobic, rod-shaped bacterium. *S. proteamaculans* HY-3, isolated from the digestive tract of a spider, produces an extracellular protease named arazim, with a projected molecular weight of 51.5 kDa [51]. The closest neighboring species in the *S. proteamaculans* 1 and *S. proteamaculans* 5 strains we isolated on the Serratia phylogenetic tree are *S. liquefaciens* and *S. grimesii* (Figure 2). The strain *S. proteamaculans* 5, when grown on a medium with a Pb, also changed the colonies' colors to red-brown.

5. Conclusions

In this study, strains of microorganisms resistant to concentrations of Ni, Cd, Pb, Zn and Cu in the range 3–5 mM were isolated from various parts (water and sludge of secondary sedimentation tank, digester) of a wastewater treatment plant in the eastern European city of Tula. The total number of colony-forming units of bacteria in the water of the secondary sedimentation tank, sludge of secondary sedimentation tank, and the contents of the digester was 2.4×10^5 cells/mL, 2.8×10^6 cells/mL and 1.6×10^5 cells/mL, respectively. Molecular genetic methods have shown that the isolated strains belong to the genera *Pseudomonas*, *Serratia* and *Klebsiella*, with the largest number belonging to the genus *Pseudomonas.* Strains *Serratia proteamaculans* 1, *Serratia proteamaculans* 5, *Pseudomonas gessardii* 8 and *Pseudomonas gessardii* 9, when cultivated in laboratory conditions, proved their resistance to the maximum used heavy metal concentrations (5 mM)—nickel, lead, zinc and zinc, respectively. Some bacteria could alter the color of their colonies when cultivated on media enriched with heavy metals. It is possible that cultivating nonpathogenic strains of these bacteria and their introduction into communities of other activated sludge microorganisms could improve the efficiency of biological treatment of wastewater containing organic and inorganic pollutants. Some from these species, or rather their individual strains, are opportunistic pathogens. This issue requires further study in detail. Obviously, strains with pathogenic properties cannot be used for water purification.

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