



Contents lists available at ScienceDirect

Current Research in Microbial Sciences

journal homepage: www.elsevier.com/locate/crmicr

Draft whole genome sequence for four highly copper resistant soil isolates *Pseudomonas lactis* strain UKR1, *Pseudomonas panacis* strain UKR2, and *Pseudomonas veronii* strains UKR3 and UKR4

Olesia Havryliuk^{a,*}, Vira Hovorukha^a, Marianna Patrauchan^b, Noha H. Youssef^b, Oleksandr Tashyrev^a

^a Department of Extremophilic Microorganisms Biology, D. K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, 154 Zabolotny St., Kyiv 03143, Ukraine

^b Department of Microbiology and Molecular Genetics, Oklahoma State University, 307 LSE, Stillwater, Oklahoma 74075, United States of America

ARTICLE INFO

Keywords:

Copper-resistant microorganisms
Environmental biotechnologies
Soil isolates
Arctic
Antarctic, and Ukrainian soils

ABSTRACT

Environmental copper pollution causes major destruction to ecological systems, which require the development of environmentally friendly biotechnological, in particular, microbial methods for copper removal. These methods rely on the availability of microorganisms resistant to high levels of copper. Here we isolated four bacterial strains with record resistance to up to 1.0 M Cu(II). The strains were isolated from ecologically diverse soil samples, and their genomes were sequenced. A 16S rRNA sequence-based phylogenetic analysis identified that all four isolates belong to the genus *Pseudomonas*. Particularly, strains UKR1 and UKR2 isolated from Kyiv region in Ukraine were identified as *P. lactis* and *P. panacis*, respectively, and strains UKR3 and UKR4 isolated from Svalbard Island in the Arctic Ocean and Galindez Island in Antarctica, respectively, were identified as *P. veronii*. Initial *in-silico* screening for genes encoding copper resistance mechanisms showed that all four strains encode copper resistance proteins CopA, CopB, CopD, CopA3, CopZ, as well as two-component regulatory system CusRS, all known to be associated with metal resistance in *Pseudomonas* genus. Further detailed studies will aim to characterize the full genomic potential of the isolates to enable their application for copper bioremediation in contaminated soils and industrial wastewaters.

1. Introduction

Environmental copper pollution causes major destruction to ecological systems (Abraham and Susan, 2017; Brennecke et al., 2016; Fashola et al., 2016). Copper-polluted sites are commonly associated with the urbanized areas in many countries, including Ukraine and the USA (Dovgalyuk, 2013; Schiff et al., 2007), and result from the heavy applications of copper-containing pesticides to control bacterial and fungal plant diseases (Husak, 2015). Other sources of copper contamination include industrial wastewaters (Al-Saydeh et al., 2017), metal mines, and tailing sites (Abraham and Susan, 2017; Fashola et al., 2016). Chemical and physical copper removal methods, such as adsorption, cementation, electro dialysis, electro-winning, photocatalysis, and membrane filtration have been developed (Al-Saydeh et al., 2017). However, these methods are expensive and environmentally hazardous. An environmentally friendly alternative is the development of biotechnological, in particular, microbial methods for copper removal (Cydzik-Kwiatkowska

and Zielińska, 2016; Parungao et al., 2007; Rajbanshi, 2008; Yang et al., 2017). These methods require microorganisms that are resistant to high levels of copper. For bioremediation efforts, identifying such microorganisms and understanding their molecular mechanisms of resistance and detoxification of hazardous copper compounds are of utmost importance (Volentini et al., 2011; Andreatza et al., 2010; Ölmezoğlu et al., 2012). In aqueous solutions, copper levels are affected by its solubility that can be altered during biochemical conversions, for example, reduction to insoluble and non-toxic copper(I) compounds (Volentini et al., 2011). Bioavailability of copper compounds is impacted by their complexation, precipitation, or adsorption (C.A.Flemming, 1989). The ability of microorganisms to withstand high levels of copper relies on adopting one or more of these mechanisms in natural ecosystems and is often exploited for bioremediation of heavy metal contaminated ecosystems (Andreatza et al., 2010; Ölmezoğlu et al., 2012).

Recent studies of copper-resistant microorganisms using biochemical, bioinformatics and metalloproteomics approaches revealed several

* Corresponding author.

E-mail addresses: gav_olesya@ukr.net (O. Havryliuk), vira-govorukha@ukr.net (V. Hovorukha), m.patrauchan@okstate.edu (M. Patrauchan), noha@okstate.edu (N.H. Youssef), tach2007@ukr.net (O. Tashyrev).

<https://doi.org/10.1016/j.crmicr.2020.06.002>

Received 19 May 2020; Received in revised form 20 June 2020; Accepted 22 June 2020

2666-5174/© 2020 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license.

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

genetic determinants of copper resistance. They include *cop* genes encoding tightly regulated Cu⁺-sensing transcriptional regulators, chaperones, transporters, sequestering molecules that together constitute copper resistance mechanisms in various bacteria. Cop proteins can reside in the membrane, periplasm, or be secreted into the extracellular space, where they control the copper levels preventing its toxicity to bacterial cells (Argüello et al., 2013). For example, CopA and CopB belong to the family of P-type ATPases and couple the unidirectional Cu⁺ efflux to the hydrolysis of ATP (Argüello et al., 2013; Palmgren and Nissen, 2011; Samanovic et al., 2012). The Cu⁺-sensing transcriptional regulator CueR together with the two homologous metal chaperones CopZ1 and CopZ2 form a copper resistance system, whereby CopZ1 delivers Cu⁺ to the CueR sensor, while CopZ2 functions as a fast-response Cu⁺-sequestering storage protein (Novoa-Aponte et al., 2019). In eukaryotes, the common soil fungus *Rhizoglyphus irregularis* was shown to express two genes encoding copper transporters of the CTR family, RiCTR1 and RiCTR2 (Gómez-Gallego et al., 2019). Some of the genes are encoded on plasmids such as *copA* in *Sphingomonas* sp. and *Stenotrophomonas* sp. isolated from copper-polluted agricultural soils (Altimira et al., 2012), others are chromosomally encoded, such as *cue* and *cus*, identified in *Escherichia coli* (Outten et al., 2001).

Despite the considerable scientific advances in the understanding of the mechanisms of microbial copper resistance, the number of copper-resistant organisms and their resistance to copper are limited. To our knowledge, the maximum reported levels of Cu(II) enabling bacterial growth is 472 mM determined for *Thiobacillus ferrooxidans* ATCC 19859 (Brahmaharakash et al., 1998).

This study aimed to isolate bacterial strains with a greater resistance to copper and sequence their genomes to potentiate discoveries of new genetic determinants of copper detoxification. We report the isolation and genomic sequencing of four highly copper resistant *Pseudomonas* strains, and the preliminary analysis of their genomic determinants of copper resistance and detoxification.

2. Theory

Microbial growth and interaction with metals are theoretically permissible if the redox potential of the metal transformation reaction is within the zone of thermodynamic stability of water (the standard redox potential E_0' is from -414 to +814 mV) even at high metal concentration (Hovorukha et al., 2018). In this study, we used Pourbaix diagrams (Pourbaix, 1974) and Nernst equations (Lehninger et al., 1993) to determine the relationships between pH and solubility of Cu(II) compounds as well as the range of redox potential (Eh) values and the concentrations of Cu(II) compounds during their reduction to Cu(I). These calculations enabled the thermodynamic prognosis of theoretical possibility of microbial interactions with Cu(II) at the concentrations of up to 1.0 M. Guided by this novel approach, we isolated four strains with record level of resistance to Cu(II).

3. Materials and methods

3.1. Soil samples

Soil samples were collected from three geographic location: Kyiv region of Ukraine in April, 2018, Galindez Island in Antarctica in January, 2008 and Svalbard Island in the Arctic Ocean in August, 2010. Immediately upon collection, the samples were frozen and stored at -20 °C until further use. The concentration of copper in soil samples was determined by atomic absorption spectroscopy method (da Silva Medeiros et al., 2020).

¹ A standard redox potential (E_0' , mV) – the redox potential of the reaction at 1M concentration of both the oxidized and reduced forms of the reacting compounds at the pH=7.0

3.2. Preparation of stock solution of Cu(II) and copper containing nutrient medium

To avoid precipitation of Cu(II), we optimized the preparation of growth media as follows. Stock solution of Cu(II) (1.333 M) in citrate was prepared by dissolving CuSO₄·5H₂O in an aqueous solution of 1.94 M Na₃C₆H₅O₇ (pH 3.5). The pH of the solution was adjusted to 6.5 by adding NaHCO₃. The obtained solution was transferred to a borosilicate glass bottle, hermetically sealed, and sterilized by boiling for 20 min in a water bath. This solution was then added to the previously autoclaved nutrient broth or nutrient agar (kept liquid by incubating at 45 °C) to adjust the final concentration of Cu(II) to 1.0 M. The sterility of the media was tested by incubating a control nutrient agar plate and 5 mL of nutrient broth in a tube at 30 °C for 7 days, and no growth was observed. The level of Cu(II) in the solutions was tested by colorimetric determination (Prekrasna and Tashyrev, 2015). The maximum levels of Cu(II) in nutrient agar (tested prior solidifying) and nutrient broth were 236 mM and 1.1 M, respectively. The concentration of Cu(II) in liquid media remained stable over time.

3.3. Isolation of highly copper resistant microorganisms

For strains isolation, 1.0 g of soil was inoculated into 10 mL of nutrient broth containing up to 1.0 M Cu(II) followed by incubation at 30 °C for 10 days. Adding soil to nutrient broth was used only as an initial step. To avoid any effect that soil may have on complexing copper and hence decreasing its effective concentration in solution, no soil was added during all the subsequent sub-culturing steps performed by transferring 0.5 ml collected from the top of the enriched culture into a fresh nutrient broth containing up to 1.0 M Cu(II). After at least three passages, individual isolates were obtained by streaking on nutrient agar containing increasing levels of Cu(II) up to 157 mM and incubation at 30 °C. The isolates were passaged on Cu(II)-containing nutrient agar until pure cultures were obtained. The isolated strains were tested for their ability to grow in nutrient broth containing 1.0 M Cu²⁺. Microbial cell growth was monitored by light microscopy and counting colony forming units (CFU) on nutrient agar.

3.4. Studying the ability of isolates to interact with Cu(II)

To test the ability of isolates to accumulate Cu(II) or reduce Cu(II) to Cu(I) in colonies, they were grown on nutrient agar plates amended with 80 mM Cu²⁺. Accumulation of Cu(II) in colonies was determined by hydrogen sulfide test (Prekrasna and Tashyrev, 2015) and observation of blue coloration of the colonies (Irawati et al., 2018). The ability of microorganisms to reduce Cu(II) was determined by the formation of insoluble brown Cu₂O↓ that was visible within the colonies (Hovorukha et al., 2018).

3.5. DNA extraction and whole genomic sequencing

Genomic DNA was extracted from each of the four isolates using standard phenol/chlorophorm method (Neumann et al., 1992) and sequenced on the Illumina MiSeq platform using 2 × 300 paired-end chemistry (Novogene). Generated reads were quality filtered using standard Illumina settings resulting in 10,025,497-13,114,132 reads. All quality-filtered reads were assembled using the short read de Bruijn graph assembly (Compeau et al., 2011) program Velvet (Zerbino and Birney, 2008). Velvet assembly run-time settings were a k-mer value of 31 bp and a minimum contig coverage value of 10 ×. In an attempt to identify plasmid sequences, the quality-filtered reads were assembled using the pipeline plasmidSpades (Antipov et al., 2016) using the default parameters. The phylogenetic position of the isolates was determined by 16S rRNA gene sequence analysis. The initial screening of the genomes for genes encoding copper resistance mechanisms was conducted through the IMG platform (Chen et al., 2019).

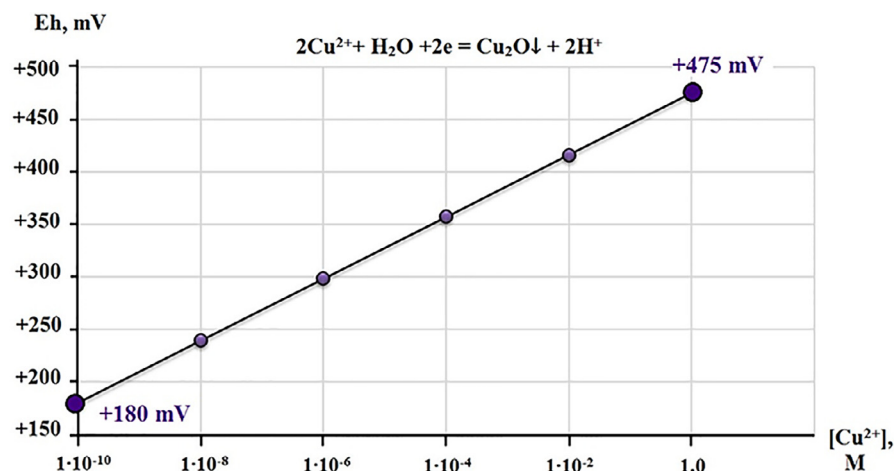


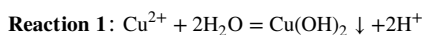
Fig. 1. Redox potential of Cu(II) solutions as concentration increases from 0.0001 μM to 1.0 M at pH = 4.6 (the level of Eh was calculated at pH 4.6 since the Cu(II) compounds precipitate at higher pH values).

3.6. Data accession

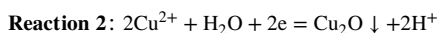
This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the Bioproject accession number PR-JNA565195, Biosample accession numbers SAMN12736602, SAMN12736603, SAMN12736604, and SAMN12736605, and genome accession numbers VWXW00000000, VWXV00000000, VWXU00000000, VWXT00000000 for *Pseudomonas lactis* UKR1, *Pseudomonas panacis* UKR2, *Pseudomonas veronii* UKR3 and *Pseudomonas veronii* UKR4, respectively.

4. Results and discussion

4.1. Thermodynamic calculations of the redox potential of the reaction of Cu^{2+} reduction to Cu_2O



We calculated the pH value below which Cu(II) compounds are highly soluble and stable in aqueous solutions using the Pourbaix diagrams (Pourbaix, 1974) that characterize the thermodynamically stable forms of chemical compounds in aqueous solutions at pH and Eh coordinates. The pH of the transformation of soluble Cu(II) compounds into insoluble $\text{Cu}(\text{OH})_2 \downarrow$ (according to Reaction 1) was calculated as follows: $\log[\text{Cu}^{2+}] = 9.21 - 2\text{pH}$; from which, $\text{pH} = 4.6$, indicating that Cu^{2+} precipitates at $\text{pH} > 4.6$.



The Eh of Cu^{2+} reduction (according to Reaction 2) was calculated at pH 4.6 and 1.0 M Cu^{2+} concentration applying the equation:

$$E_0 = 0.203 + 0.0591 \text{pH} + 0.0591 \log [\text{Cu}^{2+}] = +0.475 \text{ V.} \quad (1)$$

The Nernst equation was used to calculate the effect of the increased Cu^{2+} concentration on the value of Eh of Reaction 2 by substituting in the equation:

$$\text{Eh} = E_0 + 0.059/n \times \log [\text{Cu}^{2+}], \quad (2)$$

where E_0 – the redox potential of the reaction, V; n – the number of electrons involved in the reaction.

The values of Eh for different copper concentrations was calculated and the results are shown in Fig. 1.

The calculated redox potential of Cu(II) reduction according to Reaction 2 increased from +180 mV to +475 mV as the concentration of Cu(II) increased from 0.0001 μM to 1.0 M (Fig. 1). These redox potential values are within the zone of water thermodynamic stability, and hence we expected the feasibility of microbial life at concentrations up to 1.0 M Cu(II), with the reduction of Cu(II) to Cu(I) (Hovorukha et al., 2018, Pourbaix, 1974) being the most probable outcome.

4.2. Isolated strains are resistant to and interact with Cu(II)

Guided by the calculations above, we attempted and successfully isolated four strains resistant to the presence of up to 1.0 M Cu(II). These highly copper resistant bacterial strains named UKR1, UKR2, UKR3, and UKR4 were isolated from diverse ecological niches including soils from central Ukraine, Galindez Island in Antarctica, and Svalbard Island in the Arctic Ocean (Table 1). The soil samples were tested for Cu(II) levels and showed concentrations ranging from 0.11 to 0.37 mM/dm^3 of dry sample (Table 1). All four strains were able to grow in nutrient broth containing up to 1.0 M Cu(II) (Table 1), which, to our knowledge, is a record resistance to Cu(II) in bacteria that significantly exceeds the previously reported resistance levels to Cu(II) (up to 472 mM) (Rajbanshi, 2008, Brahmaharakash et al., 1998, Andrade et al., 2019). When grown on nutrient agar in the presence of high Cu^{2+} concentrations, the four strains accumulated Cu(II) within the colonies as indicated by the blue coloration of the colonies (Fig. 2A). While in the presence of lower Cu^{2+} concentrations (2.0 mM to 20 mM), the isolates were able to reduce Cu(II) to Cu(I) compounds that are insoluble and appear brown (Fig. 2B). Thus, the type interaction with Cu(II) depended on its concentration in the growth media.

4.3. Phylogenetic analysis and general genomic features of the isolated strains

Small subunit rRNA sequence-based phylogenetic analysis identified that all four strains belonged to the genus *Pseudomonas*. BlastN against the GenBank 16S ribosomal DNA database (limited to species) identified *Pseudomonas* sp. strain UKR1 from Kyiv region as *P. lactis* (100% identity to *P. lactis* strain DSM 29167; NR_156986), *Pseudomonas* sp. strain UKR2 from Kyiv region as *P. panacis* (99.21% identity to *P. panacis* strain CG201106; NR_043195), and the two isolates from Svalbard and Galindez Islands (*Pseudomonas* sp. strain UKR3 and UKR4) as *P. veronii* (99.15%, and 99.61% identity with *P. veronii* strain CIP 104663; NR_028706.1, respectively). These phylogenies were confirmed by alignment and Maximum Likelihood tree construction (Fig. 3).

The genomes of the four strains were sequenced, and the assembly statistics and general genomic features are summarized in Table 2.

4.4. Initial screening of the genomes revealed genes encoding copper resistance mechanisms

Initial screening of the four genomes for genes encoding copper resistance proteins was done through the IMG platform (Chen et al., 2019). All four strains encode for the copper resistance proteins A, B, C, and D (Chan et al., 2016; Chong et al., 2016; Cooksey, 1993), copper exporting ATPase *copA3* (Chong et al., 2016), copper chaperone *copZ*

Table 1
Features of resistance and interaction of isolated *Pseudomonas* strains with Cu(II).

Isolated strains	Colony accumulation of Cu(II) ^a	Reduction of Cu(II) to Cu(I) ^b	Growth in Nutrient Broth at 1000 mM Cu(II) ^c	Growth on Nutrient Agar with Cu(II), mM ^d	Cu(II) concentration, mM/dm ³ of dry sample	Isolation source and location
<i>P. lactis</i> UKR1	+	+	+	157	0.13	Chernozem soil, Kyiv region, Ukraine
<i>P. panacis</i> UKR2	+	+	+	110	0.11	Chernozem soil, Kyiv region, Ukraine
<i>P. veronii</i> UKR3	+	+	+	157	0.36	Arctic soil, Svalbard Isl., Norway, Arctic
<i>P. veronii</i> UKR4	+	+	+	157	0.37	Antarctic soil, Galindez Isl., Antarctica

^a Observed as blue colonies.

^b Observed as brown colonies.

^c Observed by light microscopy and sowing on nutrient agar followed by colony counting.

^d The maximum Cu(II) concentration supporting growth on solid media. The maximum solubility of Cu(II) in nutrient agar was 236 mM. Note that all four isolates were able to grow in liquid media in the presence of 1.0 M Cu(II).

Table 2
Genome assembly statistics and general genomic features of the four *Pseudomonas* isolates sequenced in this study.

	<i>P. lactis</i> strain UKR1	<i>P. panacis</i> strain UKR2	<i>P. veronii</i> strain UKR3 ^a	<i>P. veronii</i> strain UKR4 ^a
Assembly statistics				
N50 (bp)	7122	10124	11442	12356
Largest assembled contig (bp)	53476	68185	125256	148322
Total number of contigs assembled	1139	839	927	884
General genomic features				
Genome Size (bp)	5939518	6257067	7204846	7102407
Gene Count	5648	5936	6961	6765
% GC	60	60	60	61
% Coding Bases	89.6	89.51	89.54	89.72
Number of 16S rRNA genes	1	1	1	1
Number of 23S rRNA genes	1	1	1	1
Number of tRNA genes	25	29	30	28
Number of genes				
with function prediction	4696	4760	5673	5559
without function prediction	872	1089	1187	1107
with COGs	3922	3949	4628	4657
GenBank WGS accession number	VWXW01	VWXV01	VWXU01	VWXT01

^a The plasmid assembly algorithm plasmidSPAdes identified and assembled a few contigs in the two strains UKR3 and UKR4 as belonging to plasmids. These contigs ranged in length between 1.1-1.5 Kb and encoded 1-2 genes each. The IMG scaffold IDs for these potential plasmids are: 2806336179 and 2806336314 in strain UKR3, and 2806337104 and 2806337222 in strain UKR4.

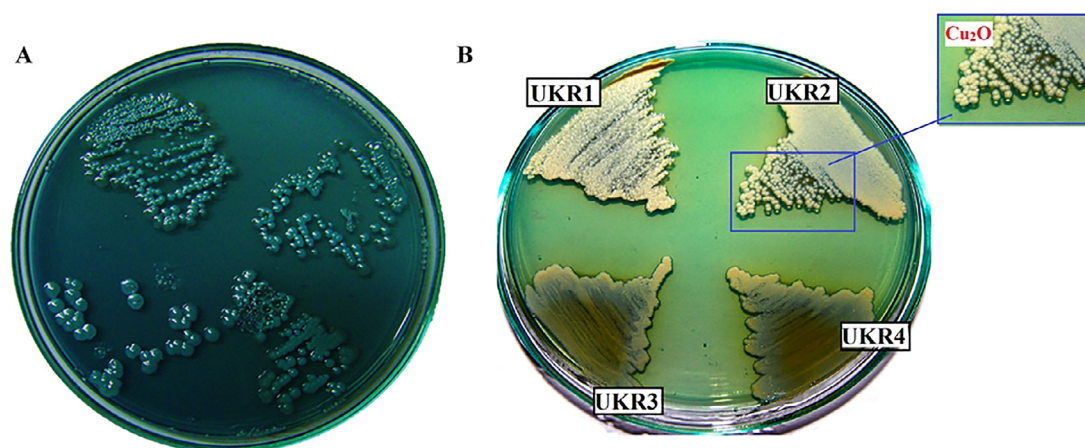


Fig. 2. (A) Growth of UKR1 strain on nutrient agar containing 80 mM of Cu(II). The blue coloration of the colonies is indicative of Cu(II) accumulation (Irawati et al., 2018). UKR2, UKR3, and UKR4 behaved similarly. (B) Reduction of Cu(II) to Cu(I) by the four isolates during growth in presence of 16 mM of Cu(II). The brown colour is indicative of the formation of insoluble brown Cu₂O within the colonies (Hovorukha et al., 2018).

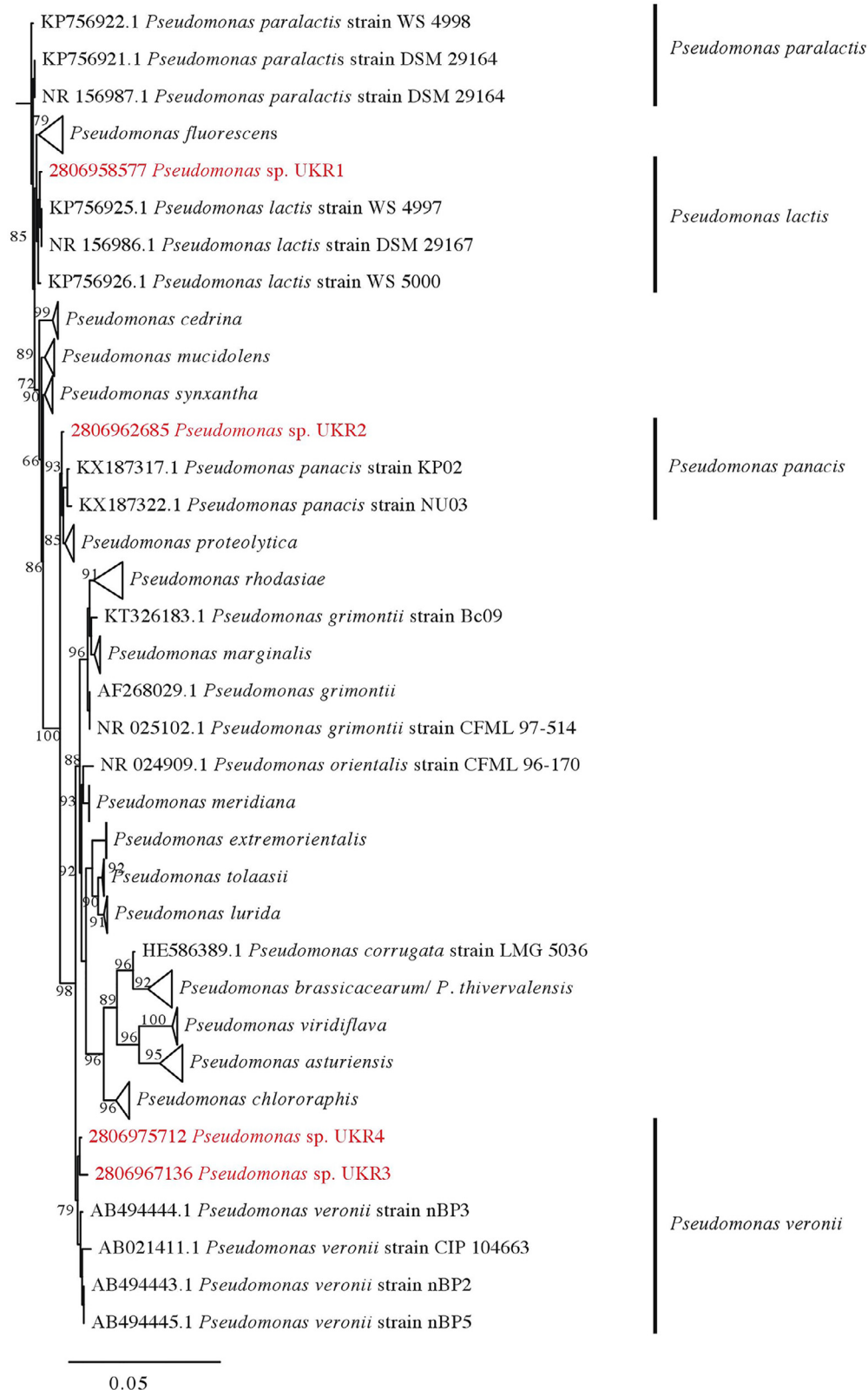


Fig. 3. A maximum likelihood tree based on 16S rRNA genes alignment was constructed in Mega (Kumar et al., 2016) and showed the phylogenetic position of the isolated *Pseudomonas* strains (shown in red). GenBank or IMG gene accession numbers are shown for unwedged species. Bootstrap values (from 100 replicates) are shown for nodes with more than 50 bootstrap support. The tree was rooted (but the outgroup is not shown for better visualization) using the 16S rRNA sequence of *Sphingomonas paucimobilis* (GenBank accession number AM237364.1).

(Quintana et al., 2017), as well as the two-component regulatory system *cusRS* (Adams, 2016), all known to determine copper resistance in *Pseudomonas* genus (Fig. 4). Compared to previously studied copper-resistant or copper-tolerant microorganisms (reference sequences shown in Fig. 5), percentage similarities of CopA, CopB, CopC, and CopD encoded by the four *Pseudomonas* strains studied here ranged within 49.1–

100%, 34.7–100%, 47.5–98.4%, and 34–99.6%, respectively. Maximum likelihood phylogenetic trees comparing proteins encoded by the four *Pseudomonas* strains studied here to other copper resistant species are shown in Fig. 5.

Interestingly, the copper resistance genes of *P. lactis* strain UKR1 (encoded on scaffold ID 2806333723), *P. veronii* strain UKR3 (encoded on

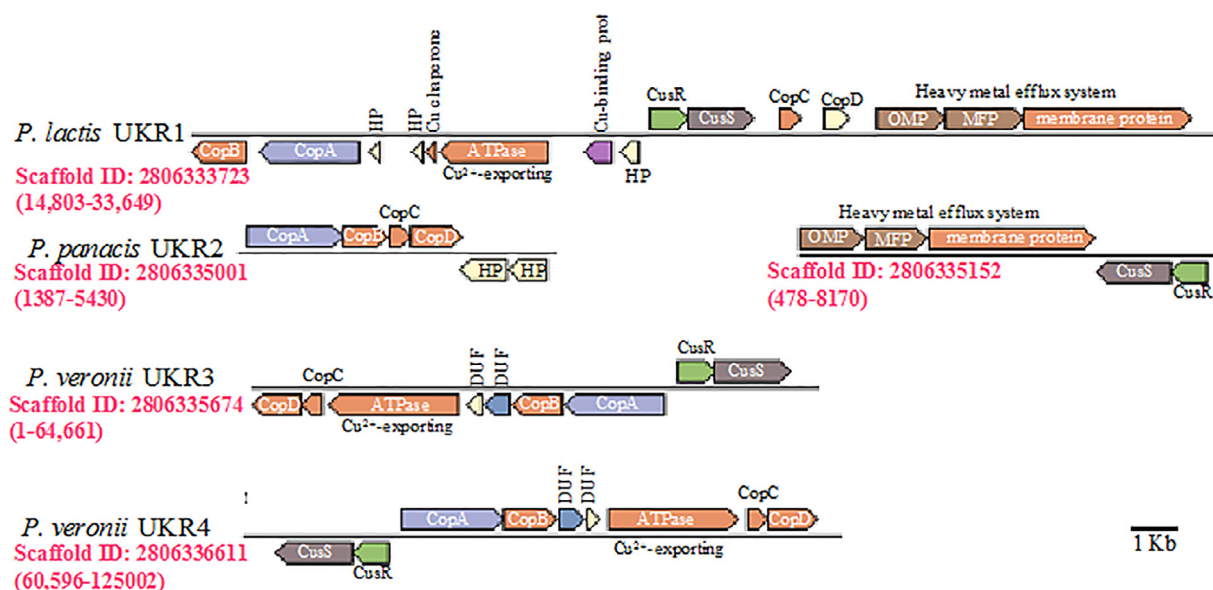


Fig. 4. Genes predicted to encode copper resistance in the genomes of the four *Pseudomonas* species studied. Shown are regions of the chromosome encoding the copper resistance *Cop* system (*copA*, *copB*, *copC*, and *copD*), the Cu^{2+} -exporting ATPase, the two-component signal transduction system *Cus* (*cusRS*), and the type I secretion system heavy metal efflux system (OMP, outer membrane protein; MFP, membrane fusion protein; and efflux system membrane protein). HP, hypothetical protein; DUF, domain of unknown function. IMG scaffold IDs are shown below each scaffold in red. Copper resistance genes in strain UKR2 were encoded on two scaffolds and are shown as separate regions. The scaffolds shown are most probably chromosomal as they were not identified by the plasmidSPades as belonging to plasmids. A scale bar is shown in the lower right corner.

Table 3

Number of genomic copies and intra- and inter-strain percentage similarities of CopA, CopB, CopC, and CopD copper-resistance proteins encoded by the four *Pseudomonas* genomes in the current study.

Copper resistance Protein	Genome	# of copies per genome	Percentage similarity			
			<i>Pseudomonas</i> sp. UKR1	<i>Pseudomonas</i> sp. UKR2	<i>Pseudomonas</i> sp. UKR3	<i>Pseudomonas</i> sp. UKR4
CopA	<i>Pseudomonas</i> sp. UKR1	2	71.5			
	<i>Pseudomonas</i> sp. UKR2	1	74–84	100		
	<i>Pseudomonas</i> sp. UKR3	4	52.7–89.8	55.3–82.6	51.9–75.6	
	<i>Pseudomonas</i> sp. UKR4	4	52.7–90	55.3–82.6	51.9–99.8	51.9–75.6
CopB	<i>Pseudomonas</i> sp. UKR1	2	69.6			
	<i>Pseudomonas</i> sp. UKR2	1	67.4–73.1	100		
	<i>Pseudomonas</i> sp. UKR3	2	42.4–83.4	51–70.7	50.5	
	<i>Pseudomonas</i> sp. UKR4	2	42.4–83.4	51–70.7	50.5–99.8	50.52
CopC	<i>Pseudomonas</i> sp. UKR1	2	68.5			
	<i>Pseudomonas</i> sp. UKR2	1	67.7–77.3	100		
	<i>Pseudomonas</i> sp. UKR3	3	61.3–100	57.6–73.4	58.8–68.6	
	<i>Pseudomonas</i> sp. UKR4	3	61.3–100	57.6–73.4	58.8–100	58.7–68.5
CopD	<i>Pseudomonas</i> sp. UKR1	2	56.8			
	<i>Pseudomonas</i> sp. UKR2	1	61.9–62.7	100		
	<i>Pseudomonas</i> sp. UKR3	2	39.79–74	43.3–63.6	41.96	
	<i>Pseudomonas</i> sp. UKR4	2	39.79–74	43.3–63.6	41.96–100	41.96

scaffold ID 2806335674), and *P. veronii* strain UKR4 (encoded on scaffold ID 2806336611) are all flanked by integrative conjugative mobile elements (including the genes encoding for excisionase, integrase, and conjugative transfer ATPase necessary for the excision and subsequent integration into the chromosome) suggestive of their transfer by conjugation.

Notably, several copies of the copper resistance genes *copA*, *copB*, *copC*, and *copD* were encoded in three *Pseudomonas* genomes belonging to strains UKR1, UKR3, and UKR4 (only one such copy is depicted in Fig. 4 for each of these genomes). Intra-strain copies of the same gene were not identical, and ranged in similarity between 42 and 75.6% at the protein level (Table 3). The different copies of the copper resistance genes *copA*, *copB*, *copC*, and *copD* encoded by *Pseudomonas lactis* strain UKR1 were phylogenetically similar (Fig. 5) and belonged to

a clade that included other Gamma- and Alpha-Proteobacteria species (Clade A in Fig. 5). This clade also included the genes from *Pseudomonas panacis* strain UKR2. Interestingly, *Pseudomonas veronii* strains UKR3, and UKR4 encoded phylogenetically distinct copies of each of the copper resistance genes (Fig. 5), one of which belonged to the Gamma/Alpha-Proteobacteria clade (Clade A in Fig. 5) that also included the *Pseudomonas lactis* strain UKR1, and *Pseudomonas panacis* strain UKR2 genes, while the other was more closely related to Beta-Proteobacteria species (Clade B in Figure 5). Whether these multiple copies were all acquired by horizontal gene transfer from other copper resistant microorganisms, or originated by gene birth via gene duplication remains to be investigated. Regardless of their origin, the presence of multiple copies of copper-resistance genes in these strains strongly suggest their potential importance in the presence of high copper concentration.

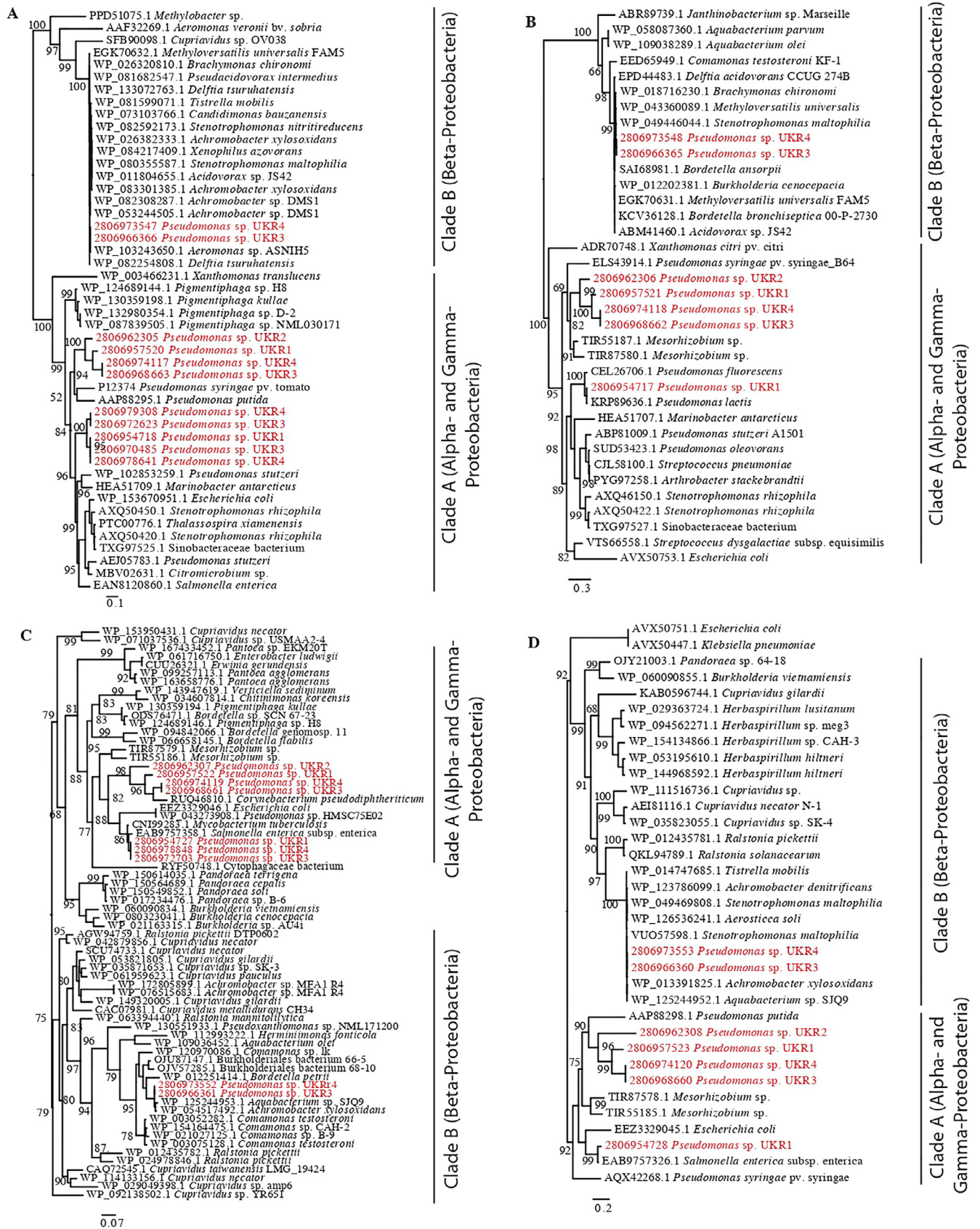


Fig. 5. Maximum likelihood phylogenetic trees based on the alignment of (A) CopA, (B) CopB, (C) CopC, and (D) CopD proteins encoded in the four studied *Pseudomonas* genomes (shown in red text) and other copper-resistant species. GenBank accession numbers are shown for reference sequences, while IMG gene IDs are shown for the *Pseudomonas* genes from this study. Alignment and tree construction were conducted in Mega (Kumar et al., 2016), and bootstrap values (from 100 replicates) are shown for nodes with more than 50 bootstrap support. Trees are mid-point rooted. Clades A and B refer to the phylogenetically distinct copies of Cop proteins encoded by strains UKR3 and UKR4 as explained in text.

Conclusions

Based on thermodynamic calculations, we hypothesized the theoretical possibility of microbial growth in the presence of 1.0 M Cu(II). This was experimentally verified, and here we report the isolation of four strains resistant to 1.0 M Cu(II) from diverse non-polluted soils. The genomes of the strains were sequenced, and the initial screening for genes encoding copper resistance mechanisms in *Pseudomonas* spp. revealed several genomic determinants of Cu(II) resistance. The isolates may serve as potential candidates for copper-containing wastewaters purification and bioremediation of copper-contaminated ecosystems.

Declaration of Competing Interest

The authors have no competing interests.

CRediT authorship contribution statement

Olesia Havryliuk: Investigation, Writing - original draft. **Vira Hovorukha:** Investigation, Supervision. **Marianna Patrauchan:** Resources, Conceptualization, Writing - review & editing. **Noha H. Youssef:** Resources, Data curation, Investigation, Writing - review & editing. **Oleksandr Tashyrev:** Conceptualization, Methodology, Resources, Supervision.

Acknowledgments

The authors are thankful to Anatoliy Shakhovskiy (Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine, Kyiv) for help in genomic DNA extraction and to Anastasiya Sachko (Yuriy Fedkovych Chernivtsi National University, Chernivtsi, Ukraine) for help in atomic absorption spectroscopy analysis of soil samples.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2020.06.002.

References

- Abraham, M.R., Susan, T.B., 2017. Water contamination with heavy metals and trace elements from Kilembe copper mine and tailing sites in Western Uganda; implications for domestic water quality. *Chemosphere* 169, 281–287.
- Adams, M.W.W., 2016. Novel metal cation resistance systems from mutant fitness analysis of denitrifying *Pseudomonas stutzeri*. *Appl. Environ. Microbiol.* 82, 6046–6056. <https://doi.org/10.1128/AEM.01845-16>.
- Al-Saydeh, S.A., El-Naas, M.H., Zaidi, S.J., 2017. Copper removal from industrial wastewater: a comprehensive review. *J. Ind. Eng. Chem.* 56, 35–44. <https://doi.org/10.1016/j.jiec.2017.07.026>.
- Altimira, F., Yáñez, C., Bravo, G., González, M., Rojas, L.A., Seeger, M., 2012. Characterization of copper-resistant bacteria and bacterial communities from copper-polluted agricultural soils of central Chile. *BMC Microbiol.* 12, 193. <https://doi.org/10.1186/1471-2180-12-193>.
- Andrade, C.J., Andrade, L.M., Dias, M., Mendes, M.A., Nascimento, C.A.O., 2019. Isolated *Pseudomonas aeruginosa* from mining site: proteome changes due to presence of copper. *Current Trends Anal. Bioanal. Chem.* 1 (1), 42–49. <https://doi.org/10.36959/525/438>.
- Andrezza, R., Pieniz, S., Wolf, L., Lee, M.K., Camargo, F.A.O., Okeke, B.C., 2010. Characterization of copper bioreduction and biosorption by a highly copper resistant bacterium isolated from copper-contaminated vineyard soil. *Sci. Total Environ.* 408, 1501–1507. <https://doi.org/10.1016/j.scitotenv.2009.12.017>.
- Antipov, D., Hartwick, N., Shen, M., Raiko, M., Lapidus, A., Pevzner, P.A., 2016. plasmidSPAdes: assembling plasmids from whole genome sequencing data. *Bioinformatics* 32 (22), 3380–3387. <https://doi.org/10.1093/bioinformatics/btw493>.
- Argüello, J.M., Raimunda, D., Padilla-Benavides, T., 2013. Mechanisms of copper homeostasis in bacteria. *Front. Cell. Infect. Microbiol.* 4, 1–14. <https://doi.org/10.3389/fcimb.2013.00073>.
- Brahmhrakash, G.P., Devasia, P., Jagadish, K.S., Natarajan, K.A., Ramananda Rao, G., 1998. Development of *Thiobacillus ferrooxidans* ATCC 19859 strain tolerant to copper and zinc. *Bull. Mater. Sci.* 10 (5), 461–465.
- Brennecke, D., Duarte, B., Paiva, F., Caçador, I., Canning-Clode, J., 2016. Microplastics as vector for heavy metal contamination from the marine environment. *Estuar. Coast. Shelf. Sci.* 178, 189–195. <https://doi.org/10.1016/j.ecss.2015.12.003>.
- C.A.Flemming, J.T.Trevors, 1989. Copper toxicity and chemistry in the environment: a review. *Water Air Soil Pollut.* 44, 143–158. <https://doi.org/10.1007/BF00228784>.
- Chan, K.-G., Chong, T.-M., Adrian, T.-G.-S., Kher, H.L., Grandclément, C., Faure, D., Yin, W.-F., Dessaux, Y., Hong, K.-W., 2016. *Pseudomonas lini* strain ZBG1 revealed carboxylic acid utilization and copper resistance features required for adaptation to vineyard soil environment, a draft genome analysis. *J. Genom.* 4, 26–28. <https://doi.org/10.7150/jgen.16146>.
- Chen, I.A., Chu, K., Palaniappan, K., Pillay, M., Ratner, A., Huang, J., Hunte-mann, M., Varghese, N., White, J.R., Seshadri, R., Smirnova, T., Kirtan, E., Jung-bluth, S.P., Woyke, T., Eloë-Fadrosh, E.A., Ivanova, N.N., Kyrpides, N.C., 2019. IMG/M v.5.0: an integrated data management and comparative analysis system for microbial genomes and microbiomes. *Nucl. Acids Res.* 47 (D1), D666–D677. <https://doi.org/10.1093/nar/gky901>.
- Chong, T.M., Yin, W.-F., Chen, J.-W., Mondy, S., Grandclément, C., Faure, D., Dessaux, Y., Chan, K.-G., 2016. Comprehensive genomic and phenotypic metal resistance profile of *Pseudomonas putida* strain S13.1.2 isolated from a vineyard soil. *AMB Express* 6 (1), 1–7. <https://doi.org/10.1186/s13568-016-0269-x>.
- Compeau, P.E.C., Pevzner, P.A., Tesler, G., 2011. How to apply de Bruijn graphs to genome assembly. *Nat. Biotechnol.* 29, 987–991. <https://doi.org/10.1038/nbt.2023>.
- Cooksey, D.A., 1993. Copper uptake and resistance in bacteria. *Mol. Microbiol.* 7 (1), 1–5. <https://doi.org/10.1111/j.1365-2958.1993.tb01091.x>.
- Cydzik-Kwiatkowska, A., Zielińska, M., 2016. Bacterial communities in full-scale wastewater treatment systems. *World J. Microbiol. Biotechnol.* 32, 66. <https://doi.org/10.1007/s11274-016-2012-9>.
- da Silva Medeiros, D.C.C., Piechontcoski, F., da Rocha Watanabe, E.R.L., Chaves, E.S., Inglez, S.D., 2020. Fast and effective simultaneous determination of metals in soil samples by ultrasound-assisted extraction and flame atomic absorption spectrometry: assessment of trace elements contamination in agricultural and native forest soils from Paraná – Brazil. *Environ. Monit. Assess.* 192 (111), 1–15. <https://doi.org/10.1007/s10661-020-8065-0>.
- Dovgalyuk, A., 2013. Environmental contamination by toxic metals and its indication by plant test systems. *Biol. Stud.* 7 (1), 197–204. <https://doi.org/10.30970/sbi.0701.269>.
- Fashola, M., Ngole-Jeme, V., Babalola, O., 2016. Heavy metal pollution from gold mines: environmental effects and bacterial strategies for resistance. *Int. J. Environ. Res. Public Health* 13 (11), 1047. <http://doi.org/10.3390/ijerph13111047>.
- Gómez-Gallego, T., Benabdellah, K., Merlos, M.A., Jiménez-Jiménez, A.M., Alcon, C., Berthomieu, P., Ferrol, N., 2019. The *Rhizophagus irregularis* genome encodes two ctr copper transporters that mediate cu import into the cytosol and a ctr-like protein likely involved in copper tolerance. *Front. Plant Sci.* 10, 1–16. <https://doi.org/10.3389/fpls.2019.00604>.
- Hovorukha, V., Havryliuk, O., Tashyeva, H., Tashyrev, O., Sioma, I., 2018. Thermodynamic substantiation of integral mechanisms of microbial interaction with metals. *Ecol. Eng. Environ. Protect* 2, 55–63. <https://doi.org/10.32006/eeep.2018.2.5563>.
- Husak, V., 2015. Copper and copper-containing pesticides: metabolism, toxicity and oxidative stress. *J. Vasyly Stefanyk Precarpathian Natl. Univ.* 2 (1), 38–50. <https://doi.org/10.15330/jpnu.2.1.38-50>.
- Irawati, W., Ompusunggu, N.P., Yuwono, T., 2018. Influence of bacterial consortium for copper biosorption and accumulation. *AIP Conf. Proc.* 2002. 020072-1–020072-8. <https://doi.org/10.1063/1.5050168>.
- Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33 (7), 1870–1874. <https://doi.org/10.1093/molbev/msw054>.
- Lehninger, A.L., Nelson, D.L., Cox, M.M., 1993. *Principles of Biochemistry*, second ed. Worth Publishers, New York.
- Neumann, B., Pospiech, A., Schairrer, H.U., 1992. Rapid isolation of genomic DNA from Gram-negative bacteria. *Trends Genet.* 8, 332–333. [https://doi.org/10.1016/0168-9525\(92\)90269-a](https://doi.org/10.1016/0168-9525(92)90269-a).
- Novoa-Aponte, L., Ramírez, D., Argüello, J.M., 2019. The interplay of the metalloprotein CueR with two distinct CopZ chaperones defines copper homeostasis in *Pseudomonas aeruginosa*. *J. Biol. Chem.* 294, 4934–4945. <https://doi.org/10.1074/jbc.RA118.006316>.
- Ölmezoğlu, E., Kiratlı Herand, B., Öncel, M.S., Tunç, K., Özkan, M., 2012. Copper bioremoval by novel bacterial isolates and their identification by 16S rRNA gene sequence analysis. *Turkish J. Biol.* 36, 469–476. <https://doi.org/10.3906/biy-1104-15>.
- Outten, F.W., Huffman, D.L., Hale, J.A., O'Halloran, T.V., 2001. The independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. *J. Biol. Chem.* 276, 30670–30677. <https://doi.org/10.1074/jbc.M104122200>.
- Palmgren, M., Nissen, P., 2011. P-type ATPases. *Annu. Rev. Biophys.* 40, 243–266. <https://doi.org/10.1146/annurev.biophys.093008.131331>.
- Parungao, M.M., Tacata, P.S., Tanayan, C.R.G., Trinidad, L.C., 2007. Biosorption of copper, cadmium and lead by copper-resistant bacteria isolated from Mogpog river, Marinduque. *Philippine J. Sci.* 136 (2), 155–165.
- Pourbaix, M., 1974. Atlas of electrochemical equilibria in aqueous solutions. Houston:NACE International. Mater. Sci. Forum 43–54. <http://dx.doi.org/10.4028/www.scientific.net/msf.251-254.143>.
- Prekrasna, I.P., Tashyrev, O.B., 2015. Copper resistant strain *Candida tropicalis* RomCu5 interaction with soluble and insoluble copper compounds. *Biotechnol. Acta* 8, 93–102. <https://doi.org/10.15407/biotech8.05.093>.

- Quintana, J., Novoa-Aponte, L., Argüello, J.M., 2017. Copper homeostasis networks in the bacterium *Pseudomonas aeruginosa*. *J. Biol. Chem.* 292 (38), 15691–15704. <https://doi.org/10.1074/jbc.m117.804492>.
- A. Rajbanshi, Study on heavy metal resistant bacteria in guheswori sewage treatment plant, *Our nature* 6 (2008) 52–57. <https://doi.org/10.3126/on.v6i1.1655>.
- Samanovic, M.I., Ding, C., Thiele, D.J., Darwin, K.H., 2012. Copper in microbial pathogenesis: meddling with the metal. *Cell Host Microbe* 11, 106–115. <https://doi.org/10.1016/j.chom.2012.01.009>.
- Schiff, K., Brown, J., Diehl, D., Greenstein, D., 2007. Extent and magnitude of copper contamination in marinas of the San Diego region, California, USA. *Mar. Pollut. Bull.* 54 (3), 322–328. <https://doi.org/10.1016/j.marpolbul.2006.10.013>.
- Volentini, S.I., Farias, R.N., Rodríguez-Montelongo, L., Rapisarda, V.A., 2011. Cu(II)-reduction by *Escherichia coli* cells is dependent on respiratory chain components. *BioMetals* 24, 827–835. <https://doi.org/10.1007/s10534-011-9436-3>.
- Yang, Y., Hu, M., Zhou, D., Fan, W., Wang, X., Huo, M., 2017. Bioremoval of Cu²⁺ from CMP wastewater by a novel copper-resistant bacterium *Cupriavidus gilardii* CR3: characteristics and mechanisms. *RSC Adv.* 7, 18793–18802. <https://doi.org/10.1039/c7ra01163f>.
- Zerbino, D.R., Birney, E., 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res.* 18, 821–829. <https://doi.org/10.1101/gr.074492.107>.