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Metal Resistance of Microorganisms as a Crucial Factor for Their Homeostasis and Sustainable Environment

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Abstract: Heavy metals are prevalent environmental components, but when their concentrations exceed critical thresholds, they pose environmental hazards, disrupting the sustainability of ecosystems. Microorganisms are among the first to encounter the toxic effects of metals. Therefore, it is crucial to understand both the levels and mechanisms of their resistance to maintain their homeostasis under the pressure of extreme factors as well as contribute to increasing the sustainability of ecosystems. The aim of the study was to examine two soil bacterial strains, *Brevundimonas vesicularis* USM1 and *Pseudomonas putida* USM4, to assess their resistance levels to toxic metals and to identify the mechanisms behind this resistance. For this purpose, microbiological, statistical, and bioinformatics methods were used. The comparative analysis of the two strains revealed that *P. putida* USM4 exhibited greater resistance to Cr(VI), Co(II), Cu(II), Ni(II), and Fe(III) compared to *B. vesicularis* USM1. This was confirmed by the metal concentrations at which the strains could survive, their growth dynamics, and the genetically based resistance mechanisms. These findings enhance our understanding of microbial metal resistance and contribute to the advancement of microbial-based environmental biotechnologies.

Keywords: metal resistance; environmental protection; sustainability of ecosystems; homeostasis; environmental biotechnology; heavy metals; genome analysis

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1. Introduction

Heavy metals are ubiquitous environmental components of the environment as an integral part of rocks, soil, and water reservoirs [1]. At low concentrations, they are essential for living organisms [2,3]. They are involved in biochemical reactions and metabolic pathways as co-factors of enzymes, electron carriers, etc. [2,4]. Metals such as cobalt, copper, iron, and manganese in trace concentrations are essential for proper cellular function and promote biomass growth [4]. However, due to natural phenomena (e.g., dissolution of minerals containing heavy metals, volcanic eruptions) or anthropogenic activity (e.g., mining enterprises, inefficient agriculture, waste disposal, military activities), the concentration of heavy metals in the environment can be significantly increased [1,5]. This leads to the accumulation of toxic metals in ecosystems and can be dangerous for living organisms. At high concentrations, metals can form toxic complex compounds, induce oxidative stress and DNA damage, disrupt natural metabolic reactions, and provoke the death of both macro- and microorganisms [2,6,7]. Microorganisms are essential for the maintenance of natural biogeochemical cycles, soil health, and, correspondently, crop yields [3,8]. Therefore, it is of great importance to study the mechanisms that provide the resistance of microorganisms

to the harmful effects of heavy metals and maintain the homeostasis that is the stability of their function in the presence of toxic metals.

Microorganisms are the oldest living organisms on the planet. Their ability to maintain their homeostasis and adapt to changing environmental conditions has allowed them to survive and play a crucial role in the sustainability of all ecosystems [9]. Resistance of microorganisms to heavy metals is a complex phenomenon. Developing mechanisms to protect against the harmful effects of these metals is a critical aspect of their survival in various environments. Microorganisms have evolved several strategies to maintain homeostasis and mitigate the toxic effects of heavy metals. These include extracellular or intracellular sequestration to prevent interaction with essential life-supporting structures of the cell, active export outside the cell to minimize the concentration, and enzymatic detoxification via reduction or oxidation to reduce toxicity via the formation of insoluble compounds [2,6]. Extracellular sequestration of heavy metal ions consists of preventing their entry into the cell by binding them to external components of the cell wall, such as proteins, polysaccharides, and lipids. Additionally, it includes transforming metals into inactive forms through interactions with molecules like glutathione or by precipitating them as sulfides. Intracellular sequestration of metal ions also involves their detoxification through the formation of insoluble sulfides, binding to cysteine-rich metalloproteins, etc. Efflux systems of microorganisms, consisting of transporter proteins, such as ABC transporters, provide active transport of metals from the cell, reducing their concentration and toxic effect. Enzymatic detoxification occurs during oxidation or reduction of a metal to reduce its toxicity. For instance, the toxicity of soluble Cr(VI) in the form CrO_4^{2-} is reduced via its reduction to insoluble Cr(III) hydroxide. Some of the mechanisms of metal resistance are non-specific, such as metal detoxification through the formation of insoluble complexes with sulfides, which are formed during the metabolism of sulfate-reducing microorganisms [6]. Other mechanisms, such as transport systems, may be specifically activated in the presence of certain metals. For example, chromium resistance operon in *Ochrobactrum tritici* consists of four genes chrBACF activated by the presence of Cr(VI) [9].

For humans, the metal resistance of microorganisms can be highly beneficial for environmental biotechnologies, particularly in purifying soil and water from toxic metals and facilitating their bioremediation. Depending on the purpose, the strategies for the treatment of heavy metals via microorganisms include bioaccumulation, bioleaching, oxidation, reduction, biomineralization, etc. [10]. Since bacteria offer advantages such as rapid growth rates, high activity, and ease of handling, they are highly promising for use in environmental biotechnologies. A series of approaches based on the metabolic properties of *Pseudomonas*, *Escherichia*, and *Bacillus* have already been implemented [6]. On the other hand, metal resistance in bacteria is associated with linked resistance to other antimicrobial compounds, such as antibiotics, which can pose a threat to human health. Such a phenomenon can take place via cross- or co-resistance mechanisms. Cross-resistance happens when one mechanism (such as an efflux pump) grants resistance to multiple compounds at the same time, and co-resistance occurs when two or more distinct resistance genes are situated on the same genetic element, like a plasmid or a transposon, or are found within the same bacterial strain, with each gene conferring resistance to different compounds. Another mechanism involves different resistance genes controlled by a single regulatory gene: protein CzcR regulates the expression of the CzcCBA efflux pump providing resistance to cations of zinc (Zn), cadmium (Cd), and cobalt (Co), and represses the synthesis of OprD porin preventing the entry of carbapenems to microbial cell [11].

Therefore, the investigation of the phenomenon of metal resistance and the pathways of interaction of microorganisms with metals and their detoxification is important for understanding the metabolic pathways of microorganisms and developing approaches for their effective and safe application. The aim of this study was to examine two soil bacterial strains, *B. vesicularis* USM1 and *P. putida* USM4, to assess their resistance levels to toxic metals and to identify the mechanisms behind this resistance.

2. Materials and Methods

2.1. Preparation of Metal Solutions

Initial metal solutions were prepared via the dissolution of metal salts in distilled water. The volume of each metal solution was 100 mL. To prepare solutions of Cr(VI), Co(II), Cu(II), and Ni(II) with the concentration 10,000 ppm, 3.7 g of K_2CrO_4, 4.0 g of $CoCl_2 \times 6H_2O$, 3.9 g of $CuSO_4 \times 5H_2O$, and 4.5 g of $NiSO_4 \times 6H_2O$ were used. The concentration of Fe(III) in the initial solution was 50,000 ppm. For its preparation, the salt of $FeSO_4 \times 7H_2O$ (25.0 g) was dissolved in distilled water obtaining the solution of Fe(II). It was chelated by trisubstituted sodium citrate in the weight ratio of 1:1 and then oxidized with the oxygen of air while boiling. The Fe(II) traces were completely oxidized to Fe(III) by adding 3% H_2O_2. To conduct sterilization, flasks with metal solutions were boiled in a water bath for 30 min. To obtain the required concentrations of metals in nutrient media, an aliquot of the initial metal solutions was added to the appropriate volume of the medium.

2.2. Bacterial Strains

Two bacterial strains isolated from the roots of wheat grown in soil contaminated with cadmium were used to study the effect of heavy metals: *Brevundimonas vesicularis* USM1 (GenBank accession no. JABTYI000000000) and *Pseudomonas putida* USM4 (GenBank accession no. JABTYF000000000) [12]. They showed high resistance to Cd^{2+} compounds up to 200 ppm. In this connection, it was assumed that the strains would also be highly resistant to other metals.

2.3. Determination of the Maximum Tolerable Concentrations of Heavy Metals for the Strains

The maximum tolerable concentration (MTC) refers to the highest concentration of a metal or toxic substance at which microorganisms can still grow and exhibit normal metabolic activity [13]. It helps to define the upper limit of exposure that the microorganisms can withstand without experiencing substantial growth inhibition or toxicity. The MTCs were determined on peptone agar (PA) (BioMaxima S.A., Lublin, Poland) in Petri plates. PA (20 mL) with the different concentrations of metals was added to Petri plates. The resistance of microorganisms was studied by seeding them on PA with metals. The initial concentration of each metal was 25 ppm. Since the strains grew in the presence of metals at this concentration, the following step was its increase to 50 ppm. Further increase in concentration with a step of 50 ppm was dictated by the growth of strains at the studied concentration. If the strain did not grow at a certain concentration of metal, the MTC was considered the previous one, at which the growth was observed. The highest concentrations were observed for Fe(III) as the less toxic metal for microorganisms—2600 ppm.

2.4. Growth of the Strains in the Presence of Heavy Metals

The impact of heavy metals on microbial growth was examined by measuring the optical density of peptone broth (PB) (BioMaxima S.A., Lublin, Poland) with varying metal concentrations as well as without metals. The measurement of optical density was carried out by a SPECTROstar Nano microplate reader (BMG LABTECH, Ortenberg, Germany) at 600 nm (OD_{600}) for 36 h at 25 °C. Sterile 96-well microtiter plates (TPP—Techno Plastic Products AG, Trasadingen, Switzerland) were used for cultivation. Pre-cultivated (24 h) cultures were adjusted to an $OD_{600} \approx 0.9–1.0$ in sterile PB and used as inoculum (20 µL). The final volume of nutrient medium for cultivation in each well was 300 µL. The concentration of metals in PB was in the range of 50–250 ppm of Cr(VI), 500–2500 ppm of Fe(III), 25–150 ppm of Co(II), 50–500 ppm of Cu(II), and 50–500 ppm of Ni(II). Growth curves were analyzed via MARS data analysis software 4.01 R2 (BMG LABTECH, Ortenberg, Germany).

2.5. Analysis of Genes Responsible for Metal Resistance

The sequencing of the genomes of the original strains was performed earlier [12]. The initial analysis of the genomes of *B. vesicularis* USM1 and *P. putida* USM4 for genes encoding metal resistance mechanisms was carried out through the Bacterial and Viral Bioinformatics

Resource Center (BV-BRC) platform (https://www.bv-brc.org/, accessed on 1 September 2024), resulting in identifications given as BRC IDs for particular genes and products.

2.6. Data Analysis

The experiments were performed in triplicate. Statistical analysis of experimental data was carried out via Microsoft Excel professional plus 2010 (Microsoft Corporation, Redmond, WA, USA). Mean values and standard deviations (SDs) were determined with a 95% confidence level. The values were presented as the mean ± SD. The level of significance of differences between the data sets grouped in accordance with each studied metal was determined via the one-way ANOVA test with the post hoc test (Bonferroni correction). Groups also included the yield of biomass in PB without metals as the control of growth.

3. Results

3.1. Level of Microbial Resistance to Metals

An analysis of the resistance of two bacterial strains, *B. vesicularis* USM1 and *P. putida* USM4, to soluble compounds of representative toxic metals Cr(VI), Co(II), Cu(II), Ni(II), and Fe(III) was carried out. For this, maximum tolerable concentrations (MTCs) of metals in the PA were determined (Table 1).

Table 1. Maximum tolerable concentration of metals for *B. vesicularis* USM1 and *P. putida* USM4.

Strain	MTC, ppm				
	Cr(VI)	**Fe(III)**	**Co(II)**	**Cu(II)**	**Ni(II)**
B. vesicularis USM1	50	1500	25	150	50
P. putida USM4	250	2500	100	600	500

P. putida USM4 was revealed to be more resistant to metals. A range of metals in order of increasing toxicity for this strain was Fe(III) < Cu(II) < Ni(II) < Cr(VI) < Co(II). For *B. vesicularis* USM1, the metal range was as follows: Fe(III) < Cu(II) < Ni(II) = Cr(VI) < Co(II). Co(II) was the most toxic among the studied metals. The MTCs of Co(II) were 25 and 100 ppm for *B. vesicularis* USM1 and *P. putida* USM4, respectively. Fe(III) was the least toxic, since microorganisms survived at 1500 and 2500 ppm.

The MTC values are an important indicator of the level of microbial stability and the ability of microorganisms to maintain homeostasis.

3.2. Dynamics of Microbial Growth in the Presence of Heavy Metals

The extent of microbial resistance is important for assessing ecosystem stability and for the application of microorganisms in environmental biotechnologies. Furthermore, the analysis of the dynamics of microbial growth in the presence of heavy metals is important to assess microbial metabolic activity, investigate interaction patterns between microorganisms and metals, and elucidate the pathways involved in metal detoxification.

Growth curves of the strains showed the general patterns of microbial resistance where Co(II) was shown to be among the most toxic and Fe(III) the least (Figures 1 and 2). The dynamics of *B. vesicularis* USM1 growth revealed the inhibition of bacteria in the presence of 250 ppm of Cr(VI), whereas at 50 and 100 ppm no changes were observed (Figure 1a). The strain *P. putida* USM4 was shown to be resistant to 50 ppm of Cr(VI) and sensitive to 250 ppm. The concentration of 150 ppm of Cr(VI) also showed growth inhibition after 19 h of cultivation (Figure 1b). Fe(III) was the least toxic for both strains. Even at a concentration of 2500 ppm, the growth of microorganisms was not inhibited (Figures 1b and 2b). Co(II) suppressed growth of *B. vesicularis* USM1 even at the minimum studied concentration of 25 ppm (Figure 1c), while 50 ppm of Co(II) did not inhibit *P. putida* USM4 (Figure 2c). At 100 and 150 ppm, Co(II) prevented the growth of *B. vesicularis* USM1 and *P. putida* USM4, respectively (Figures 1c and 2c). *P. putida* USM4 was also more resistant to Cu(II), withstanding 100 ppm (Figure 2d), whereas *B. vesicularis* USM1 was resistant only to 50 ppm (Figure 1d). Complete inhibition of *B. vesicularis* USM1 was caused by 150 ppm of

Cu(II) (Figure 1d) and *P. putida* USM4—400 ppm (Figure 2d). Ni(II) was observed to cause inhibitory action on *B. vesicularis* USM1 even at 50 ppm (Figure 1e). *P. putida* USM4 was able to grow at 100 ppm of Ni(II); however, the higher concentrations inhibited the growth (Figure 2e).

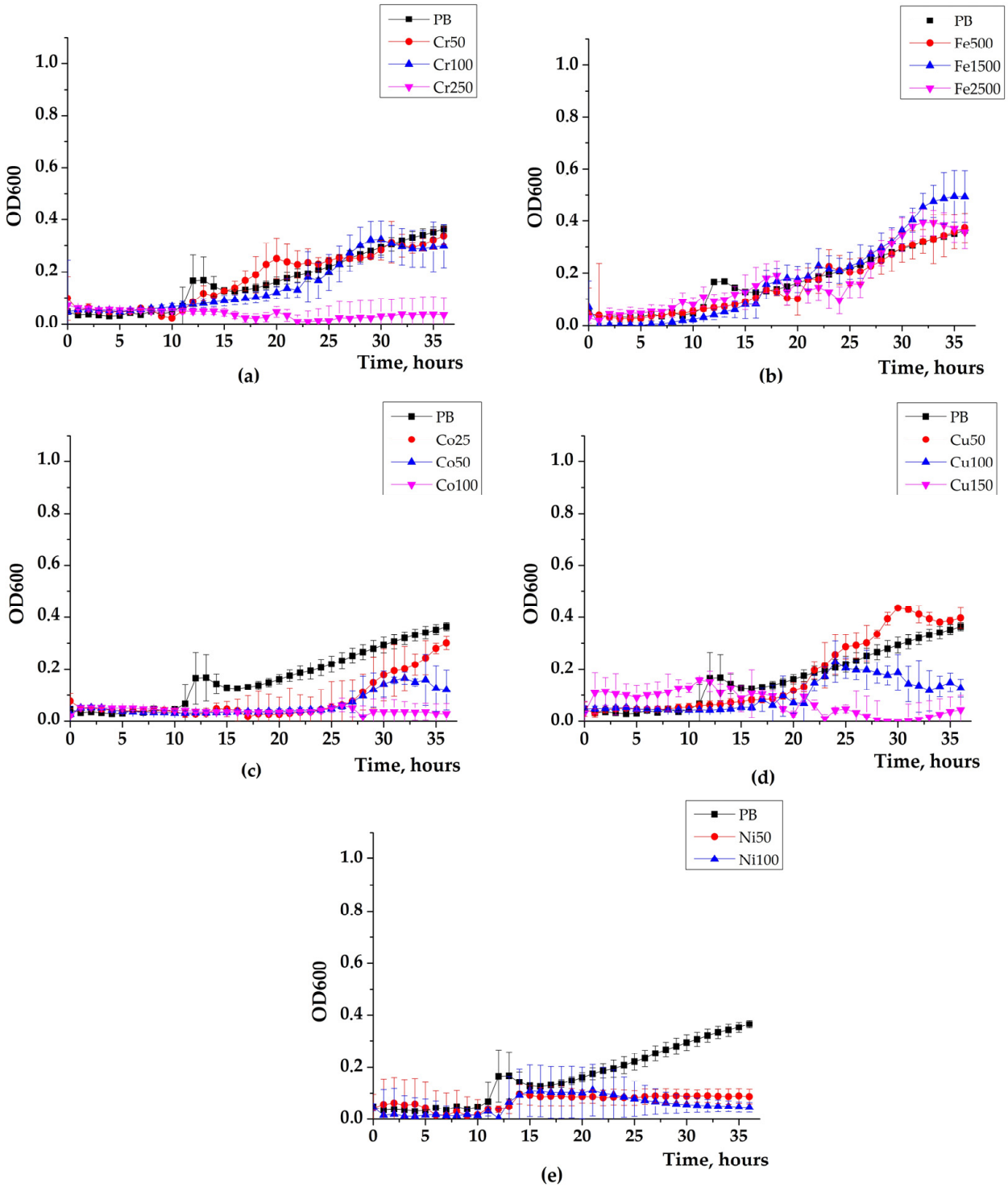

Figure 1. Dynamics of growth of *B. vesicularis* USM1 in the presence of Cr(VI) (**a**), Fe(III) (**b**), Co(II) (**c**), Cu(II) (**d**), and Ni(II) (**e**).

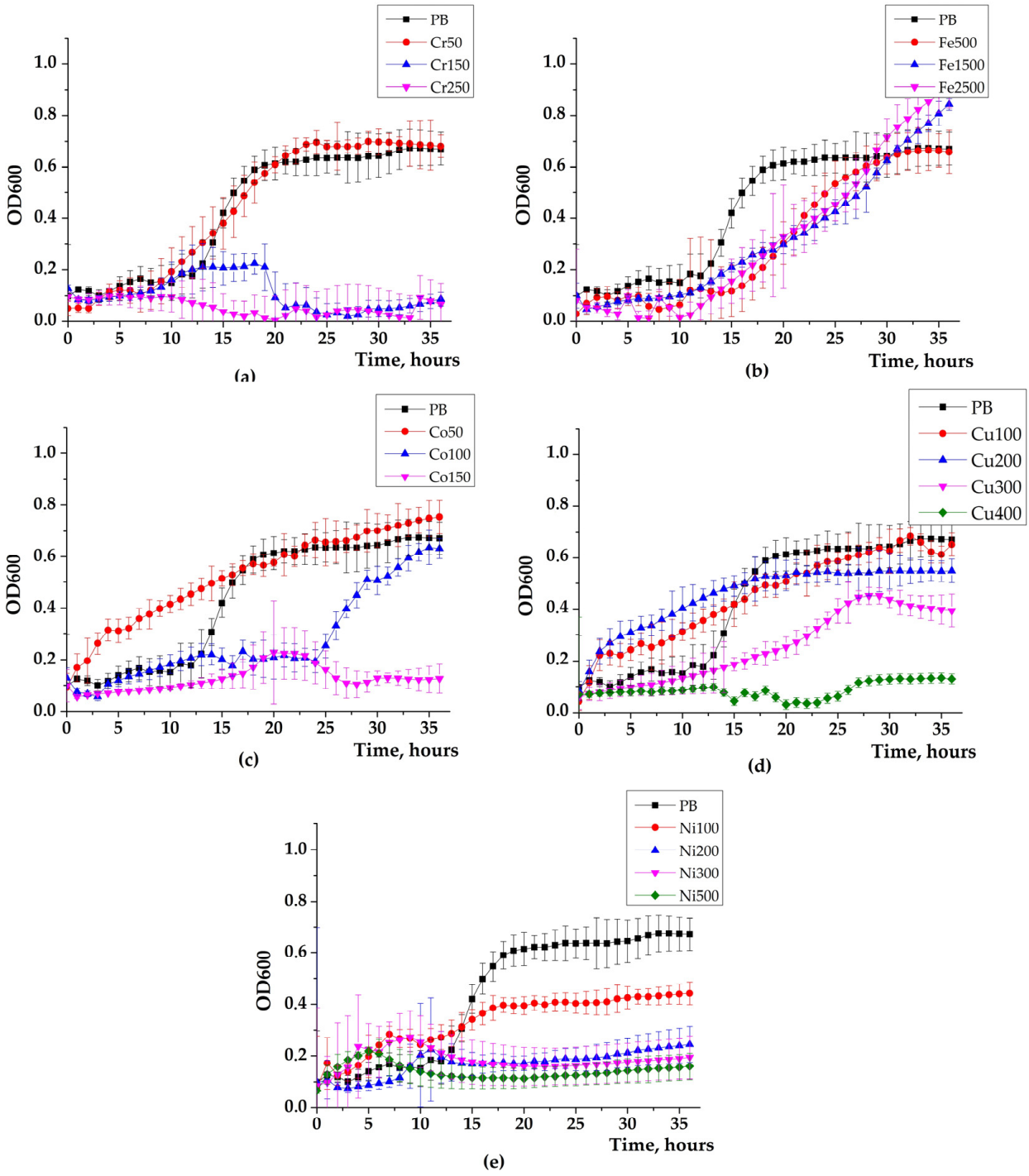

Figure 2. Dynamics of growth of *P. putida* USM4 in the presence of Cr(VI) (**a**), Fe(III) (**b**), Co(II) (**c**), Cu(II) (**d**), and Ni(II) (**e**).

The toxicity of metals was also evaluated based on the duration of the lag phase that indicated the time required for microorganisms to adapt to the presence of metal in the nutrient medium: a higher metal concentration results in a longer lag phase, reflecting increased time needed for adaptation. Generally, the duration of the lag phase showed the common patterns where the higher metal concentration caused its extension (Table 2). For example, 50 ppm of Cr(VI) did not provoke the inhibition of the growth of *B. vesicularis* USM1, while 100 ppm caused a 1.9-fold extinction of the lag phase (up to 19 h). For Fe(III), the lag phase was close to that without metals (10–11 h), showing that iron did not cause

significant inhibitory action on both strains. However, in the case of Cu(II), the duration of the lag phase of *P. putida* USM4 was much shorter in the presence of metal at 100 and 200 ppm, which may be related to the possible stimulating effect of copper in a certain concentration range.

Table 2. Duration of the lag phase of *B. vesicularis* USM1 and *P. putida* USM4 in the presence of different concentrations of metals.

Metal	Concentration, ppm	Lag Phase Duration, Hours	
		***B. vesicularis* USM1**	***P. putida* USM4**
PB without metal	0	10	10
Cr(VI)	50	10	8
	100	19	NA
	150	NA [1]	9
	250	GA [2]	GA
Fe(III)	500	10	10
	1500	10	11
	2500	10	11
Co(II)	25	25	NA
	50	26	1
	100	GA	24
	150	NA	15
Cu(II)	50	16	NA
	100	16	1
	150	GA	NA
	200	NA	1
	300	NA	15
	400	NA	GA
Ni(II)	50	11	NA
	100	12	3
	200	NA	8
	300	NA	3
	500	NA	2

[1] NA—not applicable—the concentration of metal was not studied. [2] GA—growth absence—the growth of microorganisms was not detected.

Another important indicator was the changes in the yield of microbial biomass, measured as the ratio of the optical density of the medium without metal and with it. The inhibitory effect of metals was studied based on the biomass yield. With the biomass yield in the medium without metals set as 100%, the biomass amount in the presence of metals, calculated after 36 h of cultivation, was compared to assess the extent of metal-induced inhibition (Figure 3).

The patterns of biomass yield for both strains showed the inhibition of microbial growth with the increase in the concentration of Cr(VI), Co(II), Cu(II), and Ni(II). Fe(III) was confirmed to not inhibit microbial growth. Moreover, it was supposed to stimulate the growth of strains, since the biomass yield was higher compared to the growth in PB.

Thus, *B. vesicularis* USM1 and *P. putida* USM4 were shown to be highly resistant to Cr(VI), Co(II), Cu(II), Ni(II), and Fe(III). Comparing the MTCs of metals, growth dynamics, and duration of the lag phase, *P. putida* USM4 was observed to be more resistant. This may be due to the metabolic characteristics and genetic determinants of the strain.

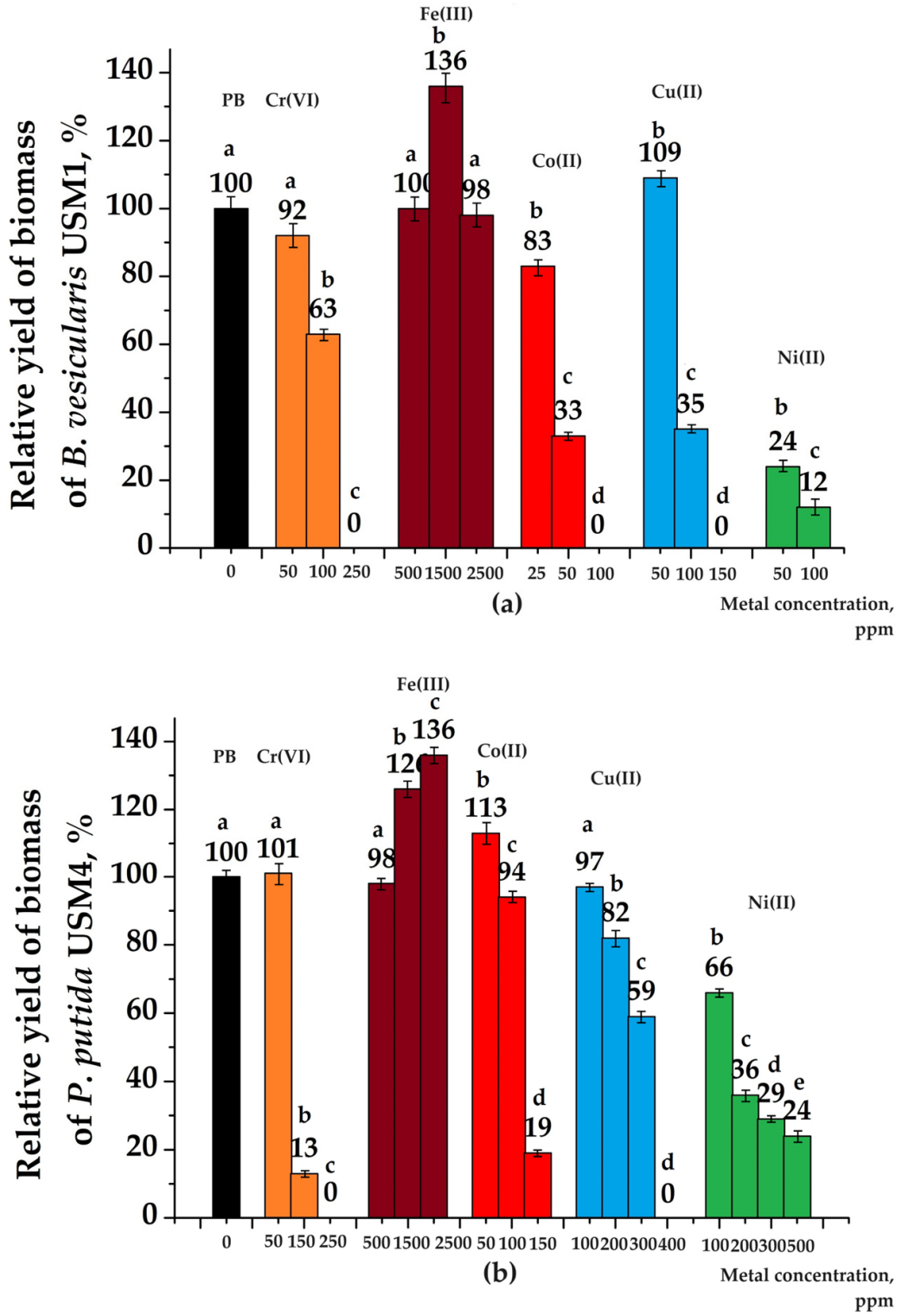

Figure 3. Relative yield of the biomass of *B. vesicularis* USM1 (**a**) and *P. putida* USM4 (**b**) in the presence of metals. Letters a–e show the statistical difference between the data sets grouped in accordance to each studied metal, where each group also included the yield of biomass in PB without metals as the control of growth; $p < 0.05$ with Bonferroni correction.

3.3. Genetic Background of Microbial Resistance to Metals

The pathways of microbial interaction with metals as well as the possible mechanisms of resistance to Cr(VI), Co(II), Cu(II), Ni(II), and Fe(III) were studied based on the analysis of genes found in the genomes of *B. vesicularis* USM1 and *P. putida* USM4. The analysis

revealed the presence of at least 18 genes in the genome of *B. vesicularis* USM1 (Table 3) and 51 genes in *P. putida* USM4 (Table 4).

Table 3. The list of genes encoding metals resistance mechanisms of *B. vesicularis* USM1.

No.	BRC ID	Product
	Copper resistance genes	
1	fig|41276.14.peg.852	Lead-, cadmium-, zinc-, and mercury-transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); copper-translocating P-type ATPase (EC 3.6.3.4)
2	fig|41276.14.peg.2064	Apolipoprotein N-acyltransferase/Copper homeostasis protein CutE
3	fig|41276.14.peg.3039	Copper resistance protein B
4	fig|41276.14.peg.3043	Lead-, cadmium-, zinc-, and mercury-transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); Copper-translocating P-type ATPase (EC 3.6.3.4)
5	fig|41276.14.peg.3048	Copper resistance protein CopD
6	fig|41276.14.peg.3049	Copper resistance protein CopC
	Cobalt and Nickel resistance genes	
1	fig|41276.14.peg.574	Cobalt/zinc/cadmium resistance protein CzcD
2	fig|41276.14.peg.854	Nickel–cobalt–cadmium resistance protein NCCN
3	fig|41276.14.peg.860	Cobalt/zinc/cadmium resistance protein CzcD
4	fig|41276.14.peg.2683	Nickel–cobalt–cadmium resistance protein NCCN
5	fig|41276.14.peg.3024	Cobalt/zinc/cadmium resistance protein CzcD
6	fig|41276.14.peg.3025	RcnR-like protein clustered with cobalt–zinc–cadmium resistance protein CzcD
7	fig|41276.14.peg.3052	Nickel–cobalt–cadmium resistance protein NCCN
	Chromate resistance genes	
1	fig|41276.14.peg.1538	Chromate reductase (EC 1.6.5.2)
2	fig|41276.14.peg.1882	Chromate transport protein ChrA
3	fig|41276.14.peg.2841	Chromate transport protein ChrA
	Iron resistance genes	
1	fig|41276.14.peg.718	Outer membrane receptor proteins, mostly Fe transport
2	fig|41276.14.peg.1238	Ferrous iron efflux pump FieF

Table 4. The list of genes encoding metals resistance mechanisms of *P. putida* USM4.

No.	BRC ID	Product
	Copper resistance genes	
1	fig|303.690.peg.47	Apolipoprotein N-acyltransferase/copper homeostasis protein CutE
2	fig|303.690.peg.358	Lead-, cadmium-, zinc-, and mercury-transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); copper-translocating P-type ATPase (EC 3.6.3.4)
3	fig|303.690.peg.1569	Copper resistance protein B
4	fig|303.690.peg.1570	Blue copper oxidase CueO precursor
5	fig|303.690.peg.1574	Copper-sensing two-component system response regulator CusR

Table 4. *Cont.*

No.	BRC ID	Product
6	fig|303.690.peg.1575	Copper sensory histidine kinase CusS
7	fig|303.690.peg.1576	Copper/silver efflux RND transporter, outer membrane protein CusC
8	fig|303.690.peg.1577	Copper/silver efflux RND transporter, membrane fusion protein CusB
9	fig|303.690.peg.1578	Copper/silver efflux RND transporter, transmembrane protein CusA
10	fig|303.690.peg.1581	Copper-sensing two-component system response regulator CusR
11	fig|303.690.peg.1587	Lead-, cadmium-, zinc-, and mercury-transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); copper-translocating P-type ATPase (EC 3.6.3.4)
12	fig|303.690.peg.1593	Lead-, cadmium-, zinc-, and mercury-transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); copper-translocating P-type ATPase (EC 3.6.3.4)
13	fig|303.690.peg.2116	Copper-sensing two-component system response regulator CpxR
14	fig|303.690.peg.3556	Heavy-metal-associated domain (N-terminus) and membrane-bounded cytochrome biogenesis cycZ-like domain, possible membrane copper tolerance protein
15	fig|303.690.peg.4168	Copper sensory histidine kinase CusS
16	fig|303.690.peg.4335	Copper resistance protein B
17	fig|303.690.peg.4357	Copper tolerance protein
18	fig|303.690.peg.4358	Copper-sensing two-component system response regulator CusR
19	fig|303.690.peg.5088	Copper(I) chaperone CopZ
20	fig|303.690.peg.5090	Lead-, cadmium-, zinc-, and mercury-transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); copper-translocating P-type ATPase (EC 3.6.3.4)
Cobalt and Nickel resistance genes		
1	fig|303.690.peg.46	Magnesium and cobalt efflux protein CorC
2	fig|303.690.peg.347	Mg/Co/Ni transporter MgtE, CBS domain-containing
3	fig|303.690.peg.1024	Mg/Co/Ni transporter MgtE, CBS domain-containing
4	fig|303.690.peg.1583	Cobalt/zinc/cadmium efflux RND transporter, outer membrane protein CzcC
5	fig|303.690.peg.1584	Cobalt/zinc/cadmium efflux RND transporter, membrane fusion protein CzcB
6	fig|303.690.peg.1585	Cobalt/zinc/cadmium efflux RND transporter, transmembrane protein CzcA
7	fig|303.690.peg.1607	Cobalt/zinc/cadmium resistance protein CzcD
8	fig|303.690.peg.1746	Cobalt–zinc–cadmium resistance protein
9	fig|303.690.peg.2184	Predicted cobalt transporter CbtA
10	fig|303.690.peg.2257	ABC transporter, permease protein 2 (cluster 5, nickel/peptides/opines)
11	fig|303.690.peg.2258	ABC transporter, permease protein 1 (cluster 5, nickel/peptides/opines)

Table 4. *Cont.*

No.	BRC ID	Product
12	fig|303.690.peg.2259	ABC transporter, substrate-binding protein (cluster 5, nickel/peptides/opines)
13	fig|303.690.peg.2879	ABC transporter, ATP-binding protein (cluster 5, nickel/peptides/opines)/ABC transporter, ATP-binding protein (cluster 5, nickel/peptides/opines)
14	fig|303.690.peg.3397	Nickel-binding accessory protein UreJ-HupE
15	fig|303.690.peg.3529	Magnesium and cobalt transport protein CorA
16	fig|303.690.peg.4187	Cobalt ABC transporter, ATP-binding protein CbtL
17	fig|303.690.peg.4188	Cobalt ABC transporter, permease protein CbtK
18	fig|303.690.peg.4189	Cobalt ABC transporter, substrate-binding protein CbtJ
19	fig|303.690.peg.4434	Magnesium and cobalt transport protein CorA
20	fig|303.690.peg.4684	Nickel ABC transporter, ATP-binding protein NikE (TC 3.A.1.5.3)
21	fig|303.690.peg.4685	Nickel ABC transporter, ATP-binding protein NikD (TC 3.A.1.5.3)
22	fig|303.690.peg.4686	Nickel ABC transporter, permease protein NikC (TC 3.A.1.5.3)
23	fig|303.690.peg.4687	Nickel ABC transporter, permease protein NikB (TC 3.A.1.5.3)
24	fig|303.690.peg.4688	Nickel ABC transporter, substrate-binding protein NikA (TC 3.A.1.5.3)
25	fig|303.690.peg.4689	Nickel-responsive regulator NikR
	Chromate resistance genes	
1	fig|303.690.peg.2867	Chromate reductase (EC 1.6.5.2)
2	fig|303.690.peg.4676	Chromate transport protein ChrA
	Iron resistance genes	
1	fig|303.690.peg.31	Ferrous iron efflux pump FieF
2	fig|303.690.peg.354	Ferric iron ABC transporter, iron-binding protein
3	fig|303.690.peg.355	Ferric iron ABC transporter, permease protein
4	fig|303.690.peg.356	Ferric iron ABC transporter, ATP-binding protein

The strain *B. vesicularis* USM1 (Table 3) was found to have genes encoding proteins of the Cut (*CutE*) and Cop (*BCD*) families, which are responsible for the uptake and export of copper [14] as well as copper-translocating ATPases. Resistance to nickel and cobalt may involve genes encoding the proteins CzcD [15] and protein NCCN [16], which function as a cation efflux pump. Resistance to chromium could be determined by genes encoding a reductase that reduces toxic Cr(VI) to non-toxic Cr(III), as well as *ChrA*, encoding a chromium efflux protein [17]. Iron resistance was determined by transport proteins and efflux systems.

P. putida USM4 (Table 4) was found to have more genes that promote resistance to the studied metals. This explains the strain's higher resistance to them. Copper resistance genes included copper-translocating ATPases, copper uptake and export protein families (Cut and Cop) [14], as well as RND-type efflux pump (Cus proteins) [18]. Nickel and cobalt resistance could involve cation efflux pump (Czc system) [19], efflux protein CorC [20], transporter MgtE [21], as well as ABC transporters [22]. Chromium resistance was also determined by reductases and transporters. Iron resistance genes involve the ABC transporters family and ferrous iron efflux *FieF* belonging to the cation diffusion facilitator family (CDF) [23].

The expression of metal resistance genes requires further research. However, the presence of genes encoding certain proteins helps to determine the pathways of interaction of microorganisms with metals. The general arrangement of genes in the bacterial genomes is shown in Figure 4.

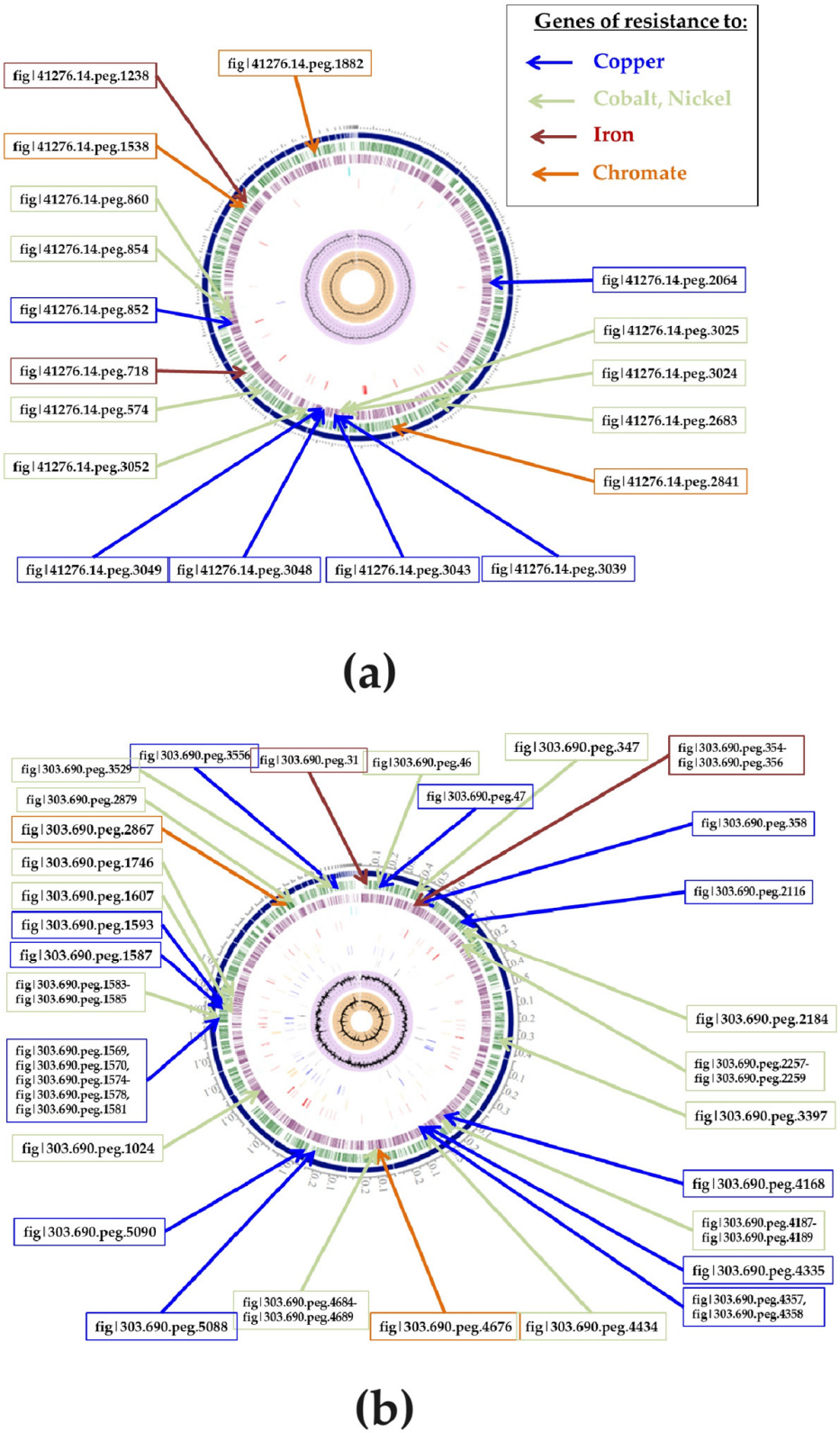

Figure 4. The arrangement of genes in the genomes of *B. vesicularis* USM1 (**a**) and *P. putida* USM4 (**b**) encoding resistance to Cr(VI), Co(II), Cu(II), Ni(II), and Fe(III).

It illustrates that metal resistance genes are dispersed across the genome rather than clustered in a distinct region. Additionally, certain genes conferring resistance to different metals are found in close proximity, which could lead to linked resistance against multiple metals simultaneously. Therefore, further investigation into the genetic basis of microbial metal resistance is crucial for enabling the regulation of microbial metabolism and facilitating their application in environmental biotechnology.

4. Discussion

The wide variety of microorganisms and their metabolic activities play a crucial role in every ecosystem. They drive essential biochemical cycles of elements and adapt to various environmental challenges, providing the stability and sustainability of ecosystems [24,25]. In this regard, the ability of microorganisms to survive at high concentrations of toxic metals is indispensable for maintaining the homeostasis of ecosystems. Understanding how microorganisms interact with metals enables us to explore processes involved in the formation and dissolution of minerals, the contamination or purification of soils and water bodies, and the development of environmental biotechnologies [26].

Two strains (*B. vesicularis* USM1 and *P. putida* USM4) were studied to reveal the level of their resistance to the representative toxic metals (Cr(VI), Co(II), Cu(II), Ni(II), and Fe(III) as well as the pathways of interaction with metals. The obtained results showed different levels of metal resistance in tested microorganisms. *P. putida* USM4 was shown to be more resistant to metals. This was evident in the higher maximum permissible metal concentrations on PA at which the strain remained viable, as well as in the more vigorous growth of microbial biomass in PB with metals with shorter lag phases. Analysis of the genomes of the strains confirmed the experimental data obtained. Significantly more genes encoding different resistance pathways were found in the *P. putida* USM4 genome.

A literature search confirmed our findings. *B. vesicularis* was reported on its low metal resistance [27]. *Brevundimonas* sp. U22 was shown to have the lowest efficacy of mercury-removing and incapability of nickel removal [28]. The strain Brevundimonas sp. B10 revealed moderate resistance to Cu (150 mg L^{-1}) and Cr (150 mg L^{-1}), which was probably provided by the expression of the corresponding genes (including the resistance proteins CopC, CzcC, CzcB, and CzcA) [29]. We obtained similar results in terms of the level of *B. vesicularis* USM1 resistance to metals, as well as the presence of genes that provide it. Among the genes, we found mainly those encoding proteins responsible for the transport of toxic metals, apparently limiting the entry of toxicants into microbial cells. In the literature, there are data on the interaction of *Brevundimonas* sp. with compounds of mercury and arsenic [28,29], although there is little information regarding other metals. Our research expands knowledge of the metal resistance of these microorganisms by showing levels of resistance to chromium, nickel, cobalt, iron, and copper as well as by analyzing genes that can provide pathways of interaction with metals.

In contrast to *B. vesicularis* USM1, *P. putida* USM4 showed high metabolic activity and levels of resistance. *Pseudomonas putida* is characterized by branched metabolic pathways, metabolic flexibility, and the ability to withstand physicochemical stress [30]. Our research has confirmed this. *P. putida* USM4 showed a high level of resistance to metals both in liquid and agarized media as well as possessed a large number of genes that could provide it. The analysis of the genome of *P. putida* USM4 revealed that the resistance to metals was connected to the genes encoding different types of efflux pumps and transporters. It is consistent with literature data that have shown the presence of genes that encode P-type ATPases, Czc system [31], transport protein ChrA, Cop protein families, etc. [32,33]. These mechanisms allowed the studied strain *P. putida* USM4 to exhibit resistance to metals in the range of 100 ppm of Co(II) to 2500 ppm of Fe(III). Literature data also showed the high level of resistance of the strains of the species: 1000 mg/L for chromium (*Pseudomonas putida* S4) [34], 300 mg/L for Cu^{2+} [35], and 165 mg/L for nickel [36]. Although there is variation in the concentrations of metals to which different strains are resistant, the general pattern of high levels of resistance

remains. Such heterogeneity may be associated both with specific cultivation conditions and with the metabolic characteristics of individual strains.

The comparative analysis of the two strains, *B. vesicularis* USM1 and *P. putida* USM4, revealed that metal resistance can vary significantly depending on microbial metabolism. Genetically encoded mechanisms of microbial interaction with metals enable microorganisms to maintain homeostasis in metal-contaminated environments. This capability, along with specific pathways of metal interaction, holds potential for advancing environmental biotechnologies. Microorganisms can either immobilize metals to purify metal-contaminated waters or mobilize them to expedite their removal from soils, depending on the requirements. Effective microbial biotechnologies rely on the appropriate selection of microorganisms and the regulation of their metabolic activity. The research conducted enhances our understanding of microbial resistance levels to metals and the mechanisms of their interactions, contributing to the development of environmental biotechnologies.

Literary data from the last three years confirm the high resistance of microorganisms of the genus *Brevundimonas* sp. to toxic metals. In addition to the metals we studied, the resistance of *Brevundimonas* species to other metals has been demonstrated: tellurium, selenium, vanadium [37], arsenic [29], mercury, and lead [38]. Moreover, they have been shown to have a growth-promoting effect on plants and can also protect plants from the toxic effects of metals [29]. In this regard, *Brevundimonas* sp. is being studied as a promising microorganism for soil bioremediation and increasing their fertility [39]. The strain we studied, although it showed lower resistance to metals compared to *P. putida* USM4, also showed a high level of activity in the presence of a wide range of metals with different mechanisms of damaging effects on microbial cells. Based on the literature data and our findings, *B. vesicularis* USM1 is a promising strain for further research and use in environmental biotechnology.

Pseudomonas strains have always been characterized by active metabolism and high resistance to stress factors. The strain *P. putida* USM4 we studied is not an exception, but confirms this pattern and is promising for biotechnology. Recent studies are similar to our findings that high resistance is associated with the presence of various types of efflux systems that protect cells [40]. In addition, it has been shown that biofilm formation by *P. putida* strains increases resistance to metals, which is a valuable property of microorganisms for the development of biotechnologies for metal removal via accumulation [41]. These bacteria have been shown to enhance plant growth and protect them from the toxic effects of metals [42]. Based on the properties of closely related strains shown in the literature, it can be assumed that the studied strain *P. putida* USM4 is capable of participating with high efficiency in metal detoxification and increasing soil productivity.

Although the species has been known for a long time, the study of isolated strains allows us to discover new properties of known bacteria. The knowledge gained accumulates, creating a complete picture and showing the patterns of high metabolic activity and resistance of these microorganisms to toxic metals, making them indispensable in the development of effective environmental technologies.

5. Conclusions

Based on the experimental data and genome analysis, the comparison of *B. vesicularis* USM1 and *P. putida* USM4 strains revealed the difference in the levels of resistance to soluble compounds of Cr(VI), Co(II), Cu(II), Ni(II), and Fe(III). The range of metals in order of increasing toxicity in accordance with the maximum tolerable concentrations for *B. vesicularis* USM1 was as follows: Fe(III) (1500 ppm) < Cu(II) (150 ppm) < Ni(II) (50 ppm) = Cr(VI) (50 ppm) < Co(II) (25 ppm). For *P. putida* USM4, the following range was obtained: Fe(III) (2500 ppm) < Cu(II) (600 ppm) < Ni(II) (500 ppm) < Cr(VI) (250 ppm) < Co(II) (100 ppm). The growth patterns of the strains also confirmed the high level of resistance to metals. Genomic analysis revealed that the strains possess genes primarily responsible for transporting metals out of the cells, which could explain the observed resistance. *P. putida* USM4 exhibited greater resistance on both solid and liquid nutrient media. It can be attributed

to the higher metabolic activity of the strain and the presence of a more diverse array of genes encoding metal resistance mechanisms. This strain is promising for further research aimed at developing microbial-based approaches for environmental protection and the remediation of metal-contaminated sites.

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