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EFFECTS OF HEAVY METAL IONS ON SOME GRAM-NEGATIVE MULTIRESISTANT STRAINS

O.D. Ianieva, H.F. Smyrnova

Вплив іонів важких металів на ріст деяких грам-негативних мультирезистентних бактерій. – О.Д. Янєва, Г.Ф. Смирнова. – *Була досліджена стійкість декількох мультирезистентних штамів грам-негативних бактерій до важких металів. Мультирезистентні штами проявили різний характер росту в рідкому середовищі з іонами міді, кадмію, ванадію та срібла. Присутність іонів міді, кадмію і ванадію в середовищі приводила до збільшення тривалості генерації для більшості штамів. Вірогідно, що штами А17, А03 і С25а осаджують кадмій у вигляді сульфіду.*

Ключові слова*: стійкість до важких металів, мультирезистентні штами, грам-негативні бактерії*

Адреса: *Інститут мікробіології і вірусології НАН України, вул. Заболотного, 154, Київ, D03680, Україна, e-mail: yaneva@serv.imv.kiev.ua*

Effects of heavy metal ions on some gram-negative multiresistant strains. – O.D. Ianieva, H.F. Smyrnova. – *The tolerance to heavy metals in several gram-negative multi-resistant bacterial strains have been studied. The multiresistant strains showed different patterns of growth in a liquid medium supplemented with copper, cadmium, vanadium and silver ions. The presence of cadmium, copper and vanadium in the medium resulted in a longer generation time for most strains. The possible mechanism for cadmium detoxification was sulfide precipitation by strains A17, A03 and C25a.*

Key words: *heavy metal resistance, multiresistant strains, gram-negative bacteria*

Address: *Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Zabolotnogo Str, 154, Kyiv, D03680, Ukraine, e-mail: yaneva@serv.imv.kiev.ua*

Rapid industrialization has led to an increasing release of the various chemicals into the environment by agriculture and industry. Heavy metals belong to the class of the most hazardous and abundant environmental pollutants. As microorganisms are known to adapt to the presence of toxic metal ions in their habitat the interest in metal-microbe interactions in recent years has increased. Most heavy metals are highly toxic to living organisms even at very low levels. However, such metals as copper, zinc, cobalt and some others may participate in catalytic functions but at high concentrations they become toxic to microorganisms as well [7].

Metal-resistant microorganisms have evolved several distinct mechanisms to tolerate toxic metal ions. Some gram-negative and gram-positive bacteria possess specific efflux pumps, which eject divalent cations through the cell membrane [14]. Other ways to detoxify heavy metal ions by microorganisms include binding of ions to various sites on the cell surface, sequestration through intracellular “trapping” by proteins or polymers, precipitation of heavy metals as insoluble phosphates, sulfides and carbonates. Finally, some bacteria have enzymes that specifically detoxify heavy metals by changing their valence [15].

Bacterial tolerance to heavy metals can be plasmid-determined as well as chromosome-determined [4, 10, 12]. In many cases plasmid-encoded heavy metal resistance is correlated with resistance to antibiotics [21].

Though mostly metal-resistant microorganisms are isolated from metal-polluted areas some reports show the presence of bacteria tolerant to heavy metals in metal-free environments. Several authors suggest that microorganisms have acquired toxic metal resistance systems at early evolutionary stages [3, 11].

Here we describe seven strains that show multiple resistance to heavy metals. The growth in liquid medium supplemented with metals in these strains is studied.

Materials and methods

Bacterial strains and growth conditions. Previously, we isolated 110 bacterial strains resistant to high concentrations of copper, cadmium, silver and vanadium from samples of soil, water, air and other environmental sources [1]. Among them 7 multiresistant strains have been chosen for further experiments; their characteristics are listed in Table 1.

All strains were mantained on a simple mineral medium (MM) (pH 7.0-7.2) containing (g/L): NaH_2PO_4 – 0.5, NH_4NO_3 – 0.5, $CaCl_2$ – 0.1, yeast extract – 0.5, sodium citrate - 10, agar - 15.

Effect of metal ions on bacterial growth. All experiments on bacterial growth were performed in liquid mineral medium of the same content as described above, supplemented with $CuSO_4x7H_2O$ (1 g/L of Cu^{2+}), $Cd(NO_3)_2x4H_2O$ (0.5 g/L of Cd^{2+}), $AgNO_3$ (0.1 g/L of Ag^+) or NH_4VO_3 (1 g/L of V^{5+}). In experiments on the effect of silver ions on microorganisms $Ca(NO_3)_2$ instead of $CaCl_2$ has been used to minimize the precipitation of silver ions by chloride ions. 1 ml of overnight cultures was transferred to tubes containing 9 ml of LMM supplemented with the appropriate metal. Cultures were cultivated on a rotary shaker at 240 rpm at 28-30^{0}C for 8 days. The growth was monitored turbidimetricallycally at 540 nm every 4 hours during first 24 hours and every 24 hours after that. All tests were performed in triplicates.

Sulfide production. Ability of the studied strains to produce sulfide was monitored on Kligler's agar; the blackening of the medium indicated sulfide production.

Vanadium reduction. To determine whether multiresistant strains could reduce ions of vanadium (V), overnight cultures of every strain were inoculated into mineral medium containing 1g/L V^{5+} as NH_4VO_3 under semi-anaerobic conditions (tubes were tightly sealed to avoid excessive oxigen penetration). Tubes were incubated at 30^{0}C for 14 days. Vanadium reduction was indicated by the change of the colour of the medium to blue, attributable to the presence of vanadyl ion (IV).

Results and discussion

Previously, we reported the isolation of 110 metal-resistant strains from different environmental niches [10]. To further research the effect of heavy metal ions on bacteria and the possible mechanisms of resistance we selected 7 strains, which showed multiple resistance to heavy metals. These strains were able to grow on the solid MM in the presence of 1 g/L of Cu^{2+}, 0.5 g/L of Cd^{2+}, 0.1 g/L of Ag^+, 1 g/L of V^{5+} and the mixture of 0.5 g/L of Cu^{2+}, 0.25 g/L of Cd^{2+} and 0.05 g/L of Ag^+. Phenotypically, all these strains were gram-negative motile aerobic or facultative anaerobic rods. The characteristics of metal-resistant cultures used in the study are represented in Table 1.

Table 1. Multiresistant bacterial strains used in this study

Strain	Source of isolation	Systematical position
A17	Faeces of the cattle	Pseudomonas sp.
35	Faeces of the cattle	Enterobacteriaceae
A03	Field soil	Pseudomonas sp.
C25a	Soil from machine-building plant	Pseudomonas sp.
C25b	Soil from machine-building plant	Not determined
OC11a	Faeces of the cattle	Enterobacteriaceae
044	Laboratory air	Enterobacteriaceae

It is of interest that these strains were isolated from metal-stressed as well as from non-contaminated sources. Though it is generally believed that metal-resistant microorganisms are mostly isolated from metal-exposed environments [18, 20], some authors reported that heavy metal resistance is wide-spread [16, 19] while others found no difference in metal susceptibility patterns between strains isolated from metal-stressed environments and strains found in non-contaminated sources [13].

The growth responses of the selected multiresistant strains to high concentrations of copper, cadmium, vanadium and silver in liquid medium were studied (Fig. 1). Growth curves of 7 multiresistant isolates suggest that generation times were far lower in the presence of heavy metal ions (Table 2). For example, in the case of the strain A17 the Cu^{2+}-amended culture was characterized by a 2.5-fold increase in generation time while Cd^{2+}-exposed cells showed almost a 6-fold increase. Vanadium was the least toxic for all the selected strains having a profound effect on the growth rate only in the case of the strains A03 and OC11a. No significant increase in optical density in a liquid medium with silver was detected in any strains. Although the microscopic examination of the selected strains after 48 h incubation in the medium supplemented with 0.1 g/L of silver showed that cells did not lose their motility, nevertheless the seeding of these cultures onto plates with MM did not result in any visible growth (data not shown). The strains 35, OC11a and 04 were not capable of the growth in the presence of cadmium ions while copper ions inhibited the growth of OC11a and 044 isolates.

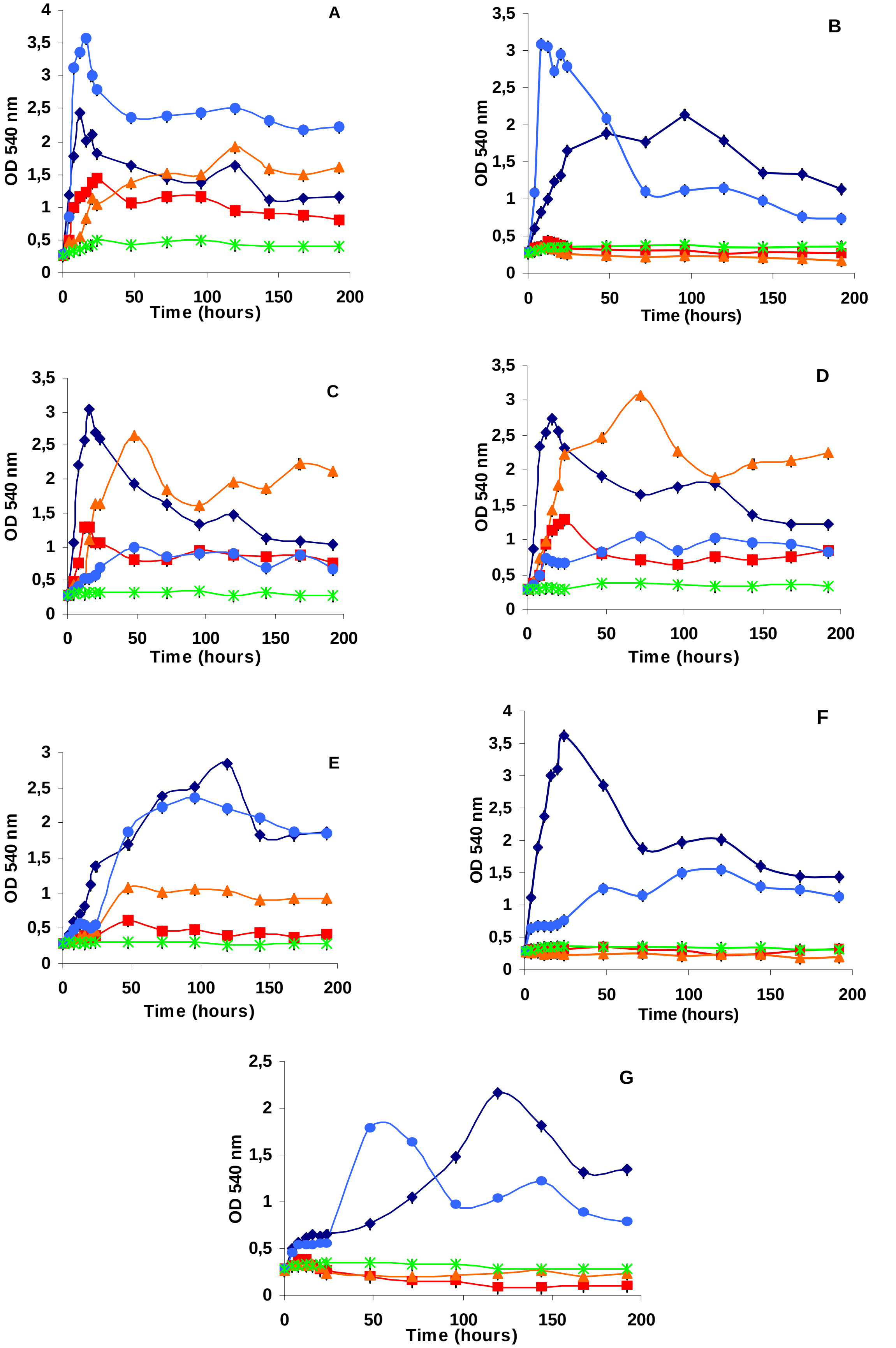

Fig.1. Effect of heavy metal ions on the growth of multiresistant strains A17 (A), 35 (B), A03 (C), C25a (D), C25b (E), OC11a (F) and 044 (G). Cells were grown in liquid mineral medium supplemented with 1 g/L of Cu^{2+} (■), 0.5 g/L of Cd^{2+} (▲), 1 g/L of V^{5+} (●) and 0.1 g/L of Ag^{+} (⁎) and in metal-free medium (♦).

Table 2. Growth of multiresistant bacterial isolates in the presence of heavy metals

Strain	Generation time (hours) in the medium supplemented with				
	No metal	Copper	Cadmium	Vanadium	Silver
A17	3.86	9.99	21.37	4.42	-
35	9.57	19.34	-	2.35	-
A03	4.7	5.48	15.06	26.7	-
C25a	4.95	10.8	21.1	9.05	-
C25b	23.31	43.3	24.7	24.04	-
OC11a	6.6	-	-	22.78	-
044	40	-	-	18.52	-

The study of growth of the selected strains in a liquid medium supplemented with heavy metals showed that strains A17, A03 and C25a were most resistant among the studied isolates by being resistant to cadmium, copper and vanadium followed by the strain C25b, while strains 35, OC11a and 044 were most sensitive by showing a noticeable increase in optical density only in medium containing vanadium. In most cases heavy metal addition resulted in a longer generation time which is in agreement with findings of other authors [6] and in some cases in a longer lag phase [8, 22]. Though a slight increase in optical density was shown by some strains in medium with silver and the microscopic examination revealed the maintenance of motility by cells, no visible growth was detected after seeding the cultures onto a solid medium either supplemented with metal or with no added metal. Ivanova et al. noticed the change in size and motility by the strains of *Pseudomonas citrea* and *Marimonas* sp. after incubation in medium supplemented with cadmium [9]. Future studies will provide more data, which could explain this phenomenon.

The strains A03 and C25a showed an increase in optical density when grown in the medium with cadmium even greater than that at the control. The peak was observed at 48-72 h with subsequent continuous decrease in optical density. Wang et al. observed the similar picture when growing the strain CW-96-1 of *Pseudomonas aeruginosa* in medium containing up to 5 mM cadmium [20]. In these cases optical density was influenced not only by cell density but also by precipitation of cadmium ions and possible flocculation. It was hypothesized that such an increase in optical density could be determined by production of sulfide. The growth of the isolates on Kligler's agar confirmed production of sulfide by the strains A03 and C25a and to a lesser extent by A17 (Table 3), which was consistent with the data that the final optical density in cadmium-supplemented medium produced by these strains was higher than in metal-free medium. Though most microorganisms are known to precipitate cadmium as sulfide under anaerobic conditions [17], recently some authors demonstrated the possibility of cadmium precipitation as cadmium sulfide in aerobic culture by *P. aeruginosa* [20] and *Escherichia coli* [2].

Table 3. Sulfide production and vanadium reduction by the selected strains

Strain	A17	35	A03	C25a	C25b	OC11a	044
Sulfide production	+/-	-	+	+	-	-	-
Vanadium reduction	+	-	+	+	+	-	+

Note: vanadium (V) reduction was observed by the change of the colour of the medium to blue

As all the isolates were able to grow in the liquid medium supplemented with vanadium under aerobic conditions, we studied the ability of multiresistant strains to reduce vanadate (V) ions under semi-anaerobic conditions. Among 7 isolates five changed the colour of the medium to blue, thus indicating the formation of the vanadyl ion (Table 3). Several microorganisms have been reported to reduce vanadium under anaerobic conditions [5, 15].

The multiresistant strains showed different patterns of growth in a liquid medium in the presence of copper, cadmium, vanadium and silver ions. For further study of heavy metal resistance and possible mechanisms of metal tolerance in multiresistant bacteria we selected 3 strains A17, A03 and C25a based on the growth rates and the profile of heavy metal resistance of the studied isolates.

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