Nickel resistance in *Escherichia coli* V38 isolated from city sewage sludge

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A nickel-resistant strain *Escherichia coli* V38 was isolated from Vilnius city sewage sludge. The strain could prow in the liquid mineral medium in the presence of 4 mM $NiCl_2$. Addition of 1 mM or 2 mM $NiCl_2$ at the beginning of logarithmic phase stopped the prowth, and the duration of lag perind was almost directly proportional to the Ni'' concentration used: the lag period was followed by logarithmic growth. The resistant strain V38 accumulated Ni^{2+} less than the sensitive strain JM101; pregrown overnight with nickel V38 cells did not accumulate nickel ions. At 0°C during the first 10 min induced or not induced resistant V38 and sensitive JM100 cells accumulate Ni^{2+} almost at the same rate. The data allow to suggest that resistance in V38 cells develops after a definite lag pcriod, is connected with loss of ability to accumulate Ni^{2+} and is inducible.

Key words: nickel, *Escherichia* *coli*, nickel pollution, nickel resistance

INTRODUCTION

In the environment polluted with heavy metals there can grow various microorganisms and higher organism which have evolved various mechanisms of tolerance. Plants, animals and numerous lower eukaryotes bind metals inside the cells with the help of special polypeptides – metallothioneins and phytochelatins [9] or metals arc bound to cell wall structures [1, 2, 15, 16]. Some other organisms secrete special glucoproteins to bind metals outside the cells [i] Many microorganisms have evolved a very complex mechanism of resistance: they actively efflux metal iom out of the cell [4].

Nickel-resistant bacteria were found in soil near the nickel-accumulating trees: the tree accumulated the metal in leaves which when falling down polluted soil near the tree. Both the amount of resistant bacteria and the level of resistance were in dependence on the pollution gradient near the tree [9].

Some peculiarities wcrc observed when bacterial cultures were grown in medium with metals. A prolonged lag phase was observed for *Alcaligenes* eutrophus in the presence of Ni^{2+} and Co^{2+} [7]. A 12–24 h long lag-phase was observed when the cells of *Alcaligenes* eutrophus N9A were grown in the presence of 3 mM $NiCl_2$. Pregrowth of the culture in the presence of subinhibitory concentration (0.5 mM) $NiCl_2$ enabled the culture lo grow at 3 mM $NiCl_2$ without a lag-period [13]. Similar results were obtained in the study of nickel resistance in the *Burkholderia* stain 32W-2 [14]. These results showed that the resistance to nickel was inducible. Unfortunately, there are no data in these studies about morphological changes of the cells in the lag-phase.

In this paper some characteristics of Ni-resistant *Escherichia* coli V38 isolated from city sewage sludge are described. This resistant strain showed a low level of Ni accumulation and prolongation of lag period during the growth in the presence of $NiCl_2$. The resistance was inducible.

MATERIAL AND METHODS

The following bacterial strains were used in this study: 1) *Escherichia coli* V38 – this strain was isolated from Vilnius city sewage sludge by pregrowing in liquid medium in the presence of 4 mM of $NiCl_2$ and plating on Ni-mineral agar medium. Selected colonies wcrc transferred into mineral liquid medium with the same nickel concentration and plated again on Ni-mineral agar medium and were stored on 2 mM Ni-agar medium. The selected strain was identified as E. *coli* and named as strain V38. This strain showed the resistance to Ni^{2+} (4 mM), Co^{2+} (1 mM), Zn^{2+} (1 mM) (this study). The growth experiments were carried out in the presence of 2 mM $NiCl_2$. 2) E. coli JM101 F' *traD36 proAB laclq* *Δ(lacZ)M15 / supE1 thi Δ(lac-proAB)* [17]; this strain was used as a Ni'' sensitive control. Both strains

Table 1. **Minimal** inhibitory **concentration of $NiCl_2$** for *E. coli* strains JM101 (sensitive) and V38 (resistant) (colony forming units)

Concentration of $NiCl_2$, mM	Strains	
	JM101	V38
Control	4.3×10^7	4.4×10^7
0.05	1.2 x 10'	
0.1	8.0×10^6	
0.2	5.6×10^5	
0.3	4.7×10^3	
0.4	6.0×10^1	
0.5	no growth	
1.0		4.5×10^7
3.0		4.0×10^7
4.0		6 7 x 10^6
5.0		2.3 x 10'
5.5		7.0×10^3
6.0		2.7×10^1
6.5		no growth

were grown at 37°C in mineral salt medium containing (in grams per litre of distilled water): Tris – 6.06; NaCl – 4.68; KCl – 1.49; NH_4Cl – 1.07; Na_2SO_4 – 0.48; $MgSO_4 \cdot 7H_2O$ – 0.23; $CaCl_2$ – 0.03; Na_2HPO_4 – 0.01; pH 7.1–7.2. Microelements and glucose (0.16%) were sterilized separately. To get an agar medium, 1.5% of Bacto-Agar (Ferak, Berlin) was added to the mineral medium before autoclaving. Nutrient Broth (Difco Laboratories) was used in control growth experiments. Optical density (OD) measurements were made with Perkin Elmer Spectrophotometer 550 at wave lenght 550 nm.

Sensitivity to nickel was estimated by growing E. *coli* strains in the presence of various $NiCl_2$ concentrations in mineral salt medium on agar plates and in liquid medium. To estimate the MIC values, the cells were grown overnight in mineral medium without nickel and were plated on mineral medium agar containing various $NiCl_2$ concentrations. The results were evaluated after 3 days of incubation and expressed as the number of colony forming units per 1 ml suspension (Table 1).

$^{63}NiCl_2$ (Izotop, Russia) was used for uptake experiments and added to bacterial suspension in trace concentration. The overnight bacterial culture was harvested by centrifugation and the cells were suspended in uptake-efflux medium containing (in grams per litre of distilled water) Tris – 6.06; NaCl – 7.6; glucose – 1.6 [11]. $^{63}Ni^{2+}$ uptake was determined by filtering 0.5 ml samples of bacterial suspension through 0.3–0.4 μm Synpor filters (Czech Republic) and washing with NaCl (130 mM) – Tris (5 mM) – EDTA (2 mM) buffer. The dried filters were immersed in scintillation liquid and radioactivity was measured (Counter SL30 Intertechnique).

RESULTS AND DISCUSSION

Determination of minimal inhibitory Ni^{2+} concentration for two *E. coli* strains – resistant V38 and sensitive JM101 was carried out by growing the cells in both liquid (for 20 h, data are not shown) and agar-mineral medium (for 3 days) in the presence of various $NiCl_2$ concentrations. The data are presented in Table 1. *E. coli* JM101 showed a slower growth in the presence of 0.1 mM and practically no growth in the presence of 0.4 mM $NiCl_2$. *E. coli* V38 showed decreased growth in the presence of 5 mM and practically no growth in the prescncc of 6 mM $NiCl_2$. All the following experiments were carried out in the presence of Ni^{2+} at a concentration not exceeding 4 mM to have the optimal growth conditions. This resistance of *E. coli* V38 is not very high in comparison with some strains of *Alcaligenes* resistant to 20–50 mM of $NiCl_2$ [10, 12] but it is about ten times higher than that of the laboratory strain JM101. It is important to note that nickel concentration in sewage sludge was about 317 mg/kg dw at the time of *E. coli* V38 isolation [3]; this sludge could be attributed to the V category of pollution [8]. According to analogous data [9], it is possible to suggest the dependence of Ni resistance in isolated *E. coli* V38 on the level of sewage sludge nickel pollution.

The growth of *E. coli* V38 culture in the Ni^{2+}-containing liquid mineral medium was characterized hy the presence of prolonged lag phase, the duration of which was dependent on nickel concentration in the medium. Addition of Ni^{2+} during the beginning of logarithmic phase stopped the growth, and the duration of this lag phase was in direct dependence on Ni^{2+} concentration used (Fig. 1).

A question could bc asked: What happened to the cells during this 2–4 h prolonged lag period when they did not divide or when the division period was stopped for 2–4 h by adding Ni^{2+}? We have no morphological data about possible changes in *E. coli* V38. Deep morphological changes in *E. coli* B cells – vacuolization and loss of colony-forming ability wcrc observed in the lag-phase caused by the presence of 3×10^{-6} M Cd^{2+}. After this lag-phase the cells restored their morphological structures and regained viability without chromosome replication [6].

It could be suggested that prolongation of the lag period was connected with the development of resistance, as the following logarithmic growth was almost of the same intensity as the control (Fig. 1).

In the medium with metal ions, bacteria accumulate them hy immediate sorbtion on their surface and by a comparitively slow uptake into the cell. Of biological importance is the latter process, as the adsorbed metal can he taken out by competitive ions or washed out by detergent agents. The development

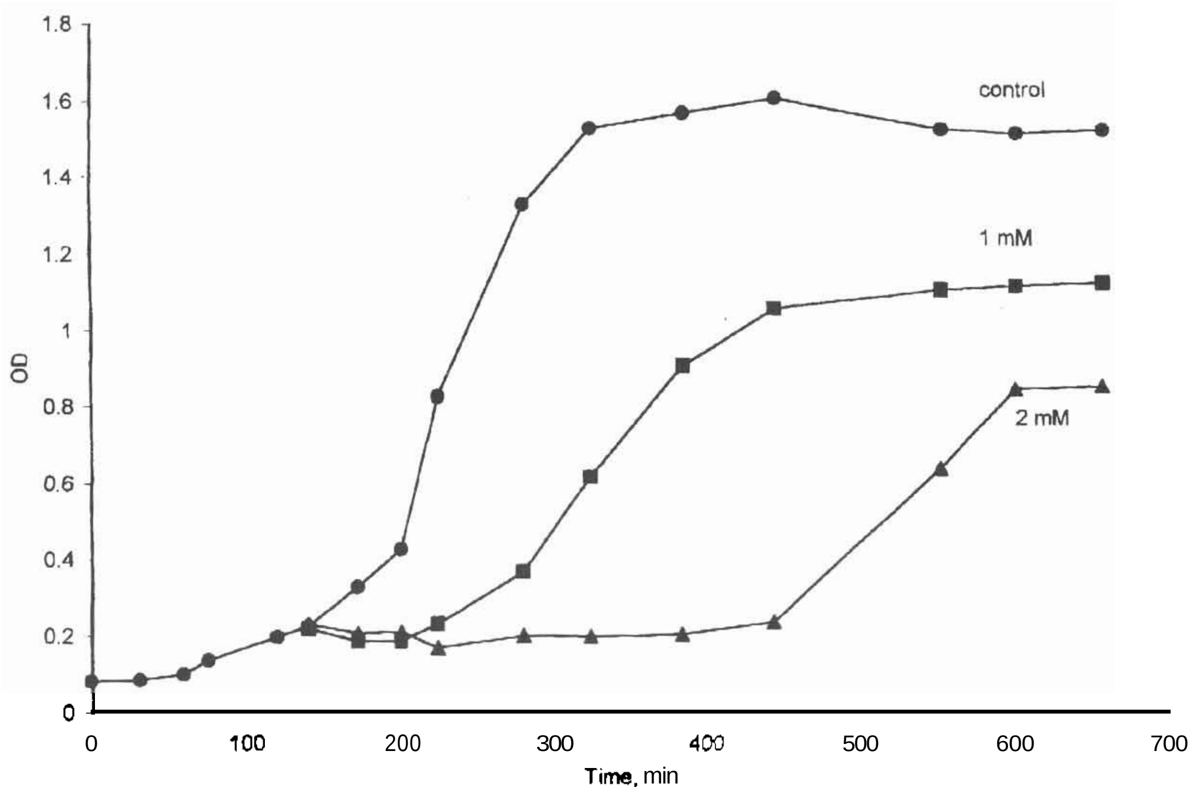

Fig. I. Growth of *Escherichia coli* V38 resistant strain in the absence (●) or in the presence of 1 mM (m) or 2 mM (A) $NiCl_2$. The culture was grown in mineral medium, nickel was added at the early logarithmic growth (f)

of resistance is connected with the decrease of the ability to accumulate metal ions [4].

Two conditions could be important in the metal ion uptake experiments: the temperature and

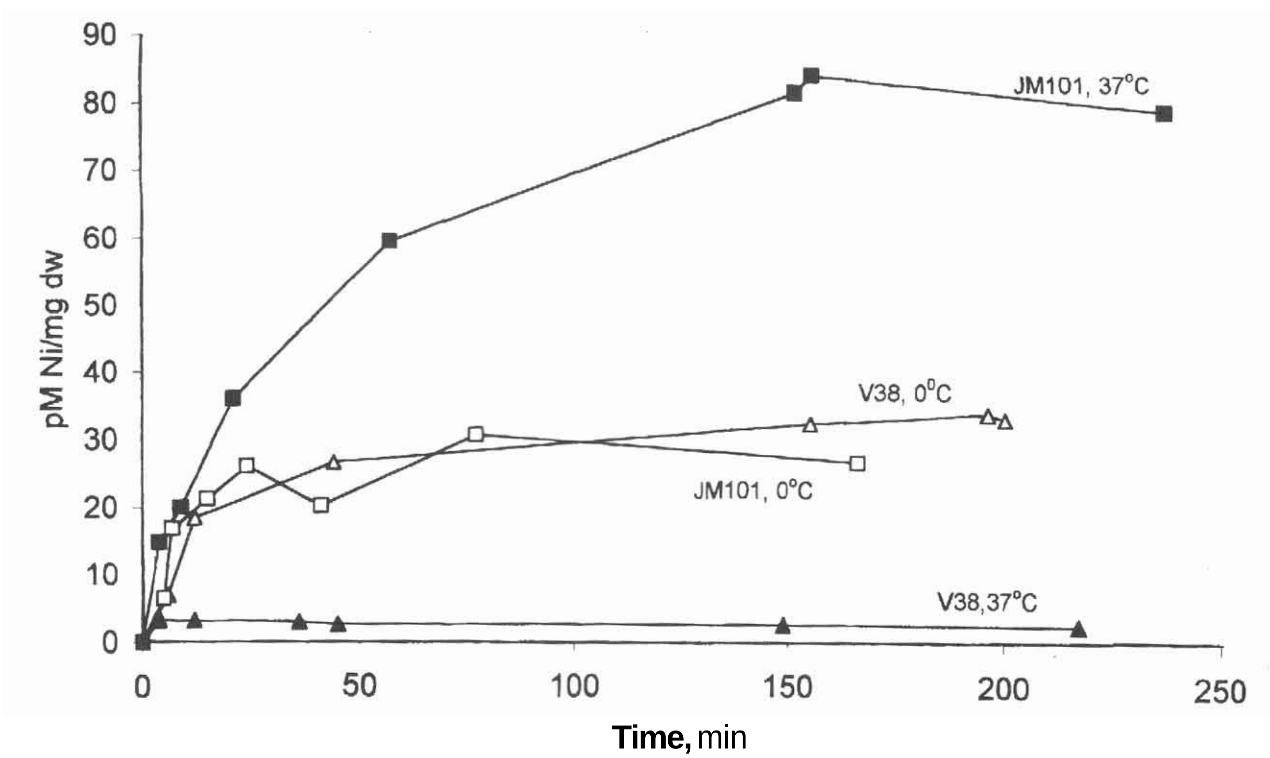

Fig 2. Nickel accumulation by the cells of *Escherichia coli* two strains: sensitive JM101 at 0°C (□) and at 37°C (m) and resistant V38 at 0°C (A) and at 37°C (A). 0.4 μM $NiCl_2$ and $^{63}Ni^{2+}$ were added at the beginning of incubation in uptake-efflux medium

growth of the cells, as the development of resistance is supposed to be inducible [13]. The overnight cultures of E. *coli* of both resistant V38 (induced in the presence of 0.1 mM $NiCl_2$ during overnight growth) and sensitive JM101 strains were washed and suspended in uptake-efflux medium in the presence of 0.5 μM $NiCl_2$ and $^{63}Ni^{2+}$, non-toxic concentration for strain JM101. The amount of accumulated nickel was calculated according to the radioactivity of samples. The incubation was at 37°C and 0°C. The data are presented in Fig. 2.

The sensitive JM1O1 cells at 37°C accumulated the highest level and more than at 0°C. V38 cells at 0°C accumulated almost at the same level as the sensitive JM101, and independently on whether V38 had been induced or not. The difference could be seen when the uptake at 37°C is compared between induced and not induced cells. Not induced cells during the first 20 min accumulated a definite amount of Ni^{2+}, but during the following incubation the amount of Ni^{2+} in the cells decreased (the data for not induced V38 in Fig. 2 are not shown). The induced cells showed practically no Ni^{2}+ accumulation.

Many data concerning Ni resistance were obtained in experiments with Gram negative bacterium *Ralstonia eutropha* CH34 (former name *Alcaligenes eutrophus*) [9]. Isolation of E. *coli* V38 naturally resistant to Ni^{2+} is rather new, and the physiological data were unknown. Three main features of this resistant strain V38 could be emphasized. First: the presence of lag period which was almost directly dependent on Ni^{2+} concentration in the medium; it is possible to speculate that during this lag period the elaboration of resistance takes place, as the following growth is like the logarithmic growth of control culture. Second: the resistant induced V38 cells at 37°C showed no Ni^{2}+ accumulation in comparison to sensitive JM1O1 cells; the cells of both strains at 0°C accumulate Ni^{2}+ at the same level (Fig. 2). This suggest an active role of energy supply in the development of resistance. Third: the absence of accumulation in induced cells allows to suggest that the development of resistance is inducible.

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Escherichia coli, IŠSKIRTOS IŠ MIESTO NUOTEKŲ DUMBLO, ATSPARUMAS NIKELIUI

S a n t r a u k a

Nikelio jonams atsparus *Escherichia* coli V38 kamienas buvo išskirtas iš Vilniaus miesto nuotekų dumblo. Ši bakterija auga skystoje mineralinėje terpėje esant 4 mM $NiCl_2$. Pridėjus 1 mM arba 2 mM $NiCl_2$ logaritminės fazės pradžioje sustabdomas

augimas ir tokio stabdymo trukmė yra beveik tiesiogiai priklausoma nuo panaudotos Ni koncentracijos; po to vyksta logaritminis augimas. Atsparus kamienas **V38** kaupia Ni^{2+} mažiau negu jautrus E. *coli* JM101 kamienas; paauginus per naktį terpėje su nikeliu V38 ląstelės visiškai nekaupia nikelio. Esant bakterijoms 0°C aplinkoje pirmąsias 10 min, V38 ir jautm JM101 Ni^{2+} kaupia vienodai. Duomenys leidžia daryti prielaidą, kad atsparumas V38 ląstelėse susidaro sustojus įvairios trukmės augimui; susidarius atsparumui, ląstelės nekaupia Ni^{2+}. Atsparumas nikeliui yra įjungiamas.

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УСТОЙЧИВОСТЬ К НИКЕЛЮ *Escherichia coli*, ВЫДЕЛЕННОЙ ИЗ ИЛА ГОРОДСКИХ СТОЧНЫХ ВОД

Резюме

Устойчивый к ионам никеля штамм *Escherichia coli* V38 был выделен из ила сточных вод г. Вильнюса. Клетки этой бактерии росли в жидкой минеральной среде npu наличии 4 мМ хлорида никеля. При добавлении к среде 1 мМ или 2 мМ хлорида никеля в начале логарифмической фазы отмечается задержка роста клеток, продолжительность которой находится в прямой зависимости от концентрации никеля. После задержки логарифмический рост продолжается. Установлено, что устойчивый штамм V38 накапливает никеля меньше, чем чувствительный штамм JM101. При выращивании клеток штамма V38 в течение 20 ч в среде с никелем накопления ими никеля не отмечалось. Выращивание клеток при 0°C показало, что устойчивый штамм V38 а чувствительный JM101 накапливают одинаковое количество ионов никеля. Полученные данные позволяют предположить, wo устойчивость к никелю в клетках штамма V38 вырабатывается nocne периода ингибирования роста. Устойчивость клеток штамма V38 к никелю является индуцируемым процессом.