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Nickel resistance in *Escherichia coli* V38 is dependent on the concentration used for induction

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Abstract

Strain *Escherichia coli* V38 resistant to 4 mM $NiCl_2$ was isolated from the city sewage sludge. It showed low nickel accumulation by cells and nickel ion efflux. Cells were pregrown (induced) overnight in the presence of Ni^{2+}, then the culture was kept on ice for 20–30 min and transferred to 37°C for further incubation. When the Ni^{2+} concentration during growth was the same as during incubation, there was no noticeable accumulation of Ni^{2+}. When the Ni^{2+} concentration during incubation was higher than that used for induction, uptake of $^{63}Ni^{2+}$ and delayed efflux were seen. The uptake and delay of both efflux and growth were directly proportional to the difference between the concentrations used for induction and incubation. Active nickel ion uptake was seen in cells taken from cultures in the delayed efflux period.

Keywords: Metal resistance; Nickel; *Escherichia coli*

1. Introduction

Microorganisms and higher organisms evolved different mechanisms of tolerance in environments polluted with heavy metals. Some microorganisms evolved active efflux that leads to decreased intracellular accumulation of a respective metal ion [1–3]. This process, energy dependent efflux, may be determined by plasmid encoded proteins, as was shown for cadmium in *Staphylococcus aureus* [4]. Metal cation resistance in the Gram-negative bacterium *Alcaligenes eutrophus* CH 34 [5–7] involves a prolonged lag phase in the presence of Ni^{2+} and Co^{2+} [8]. A 12–24 h long lag phase was observed when *A. eutrophus* N9A was grown in the presence of 3 mM $NiCl_2$. Growth in the presence of subinhibitory concentrations (O.5 mM) of $NiCl_2$ enabled the culture to grow at 3 mM $NiCl_2$ concentration without a lag period [5]. Similar results were obtained with *Burkholderia* strain 32W-2 [7]. Resistance to nickel was inducible.

This type of inducible, energy dependent resistance, based on ion efflux, was demonstrated for Cd^{2+}, Co^{2+}, Zn^{2+} and Ni^{2+} in *A. eutrophus*. The genes coding for resistance determinants were found on large plasmids pMOL28 and pMOL30 [8–14]. However in a strain of *A. denitrificans*, nickel resistance was chromosomally coded [15].

In this paper Ni^{2+} resistance in *Escherichia coli* V38 is reported. The level of nickel resistance was

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dependent on concentration used for induction, as measured by the delay of both the cell growth and nickel ion efflux.

2. Material and methods

E. coli V38 is a strain isolated from Vilnius city sewage sludge by growing on mineral salt agar medium and then in liquid mineral salt medium; the strain showed resistance to 4 mM Ni^{2+}, 0.5 mM Co^{2+} and 0.5 mM Zn^{2+}. The strain was stored on mineral salt medium containing 4 mM $NiCl_2$ in plates or test tubes under sterile glycerin. *E. coli* JM101 *F′ traD36 proAB lacI*^{q} *ΔlacZ M15/supE1 thi Δ(lac-proAB)* [16] was used as Ni^{2+} sensitive control. Cells were grown at 37°C in mineral salt medium containing (in grams per liter of distilled water): 6.06 Tris-HCl, 4.68 NaCl, 1.49 KCl, 1.07 NH_4Cl, 0.48 Na_2SO_4, 0.23 $MgSO_4$, 0.7 H_2O, 0.03 $CaCl_2$, 0.01 Na_2HPO_4, pH 7.1–7.2. Microelements and glucose (1.6 g/l) were sterilized separately. Bacto-Agar (1.5%) was added to the mineral medium when needed. Nutrient Broth was used in control growth experiments. Optical density measurements were made with Perkin Elmer Spectrophotometer 550 at wavelength 550 nm.

Sensitivity to nickel was estimated by growing *E. coli* strains in the presence of various concentrations

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	4.3×107	4.4×107
0.05	1.2×10^7	
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		6.7×10^6
5.0		2.3×10^5
5.5		3.0×10^3
6.0		2.7×10^1
6.5		no growth

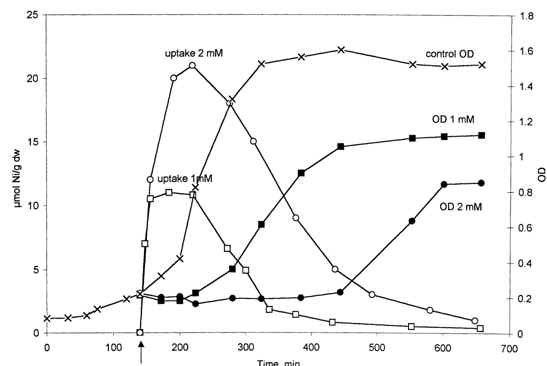

Fig. 1. Growth of the resistant strain V38 control (×) or in the presence of 1 mM (■) or 2 mM (●) $NiCl_2$ and $^{63}Ni^{2+}$ accumulation by the cells with 1 mM (□) or 2 mM (○). $^{63}Ni^{2+}$ was added (↑) at the early logarithmic growth.

of $NiCl_2$. $^{63}NiCl_2$ (Izotop, Russia) was used for uptake and efflux experiments. Overnight bacterial cultures were harvested by centrifugation and the cells were suspended in efflux medium containing (in grams per liter of distilled water) 6.06 Tris-HCl, 7.6 NaCl and 1.6 glucose [17].

$^{63}Ni^{2+}$ uptake and efflux were determined by filtering 0.5 ml samples of the cell suspension through filters (pore size 0.4 µm, Synpor, Czech Republic) and then washed with buffer (5 mM Tris-HCl, 0.13 M NaCl and 2 mM EDTA). The dried filters were immersed in scintillation liquid and radioactivity was measured (Counter SL30 Intertechnique).

3. Results

Determination of minimal inhibitory Ni^{2+} concentration (MIC) for *E. coli* resistant strain V38 and laboratory strain 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 (Table 1). The MIC for *E. coli* JM101 was 0.1 mM and for *E. coli* V38 5 mM $NiCl_2$. The growth of *E. coli* V38 in the Ni^{2+} containing liquid mineral medium was characterized by a prolonged lag phase, the duration of which was dependent on nickel concentration in the medium (Fig. 1).

A culture of *E. coli* V38 was grown overnight in a mineral salt medium in the presence of non-toxic 0.4

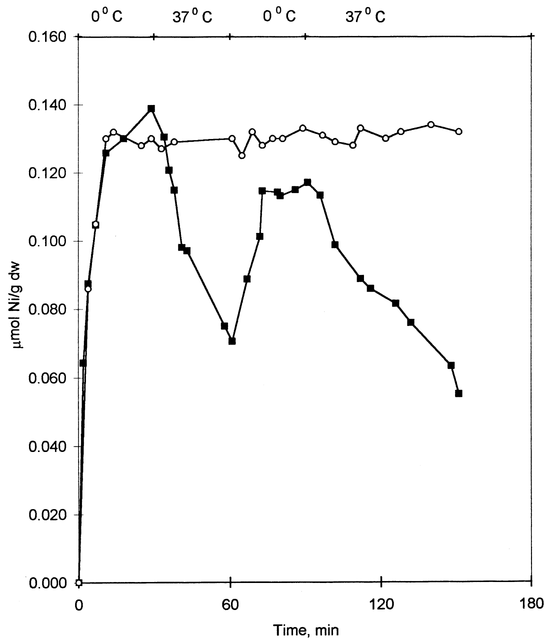

Fig. 2. Uptake at 0°C and efflux at 37°C of 0.4 µM $^{63}Ni^{2+}$ by *E. coli* V38 (■) or JM101 (○) cells. The temperature was changed every 30 min as indicated at top. Samples were filtered and washed.

µM $NiCl_2$. The Ni^{2+} sensitive strain *E. coli* JM101 was also grown under the same conditions. Cells were centrifuged and suspended at 0°C. The suspensions were kept on ice and 0.4 µM $^{63}NiCl_2$ was added. After 30 min the suspensions were transferred to 37°C (Fig. 2). At 0°C, the cells accumulated $^{63}Ni^{2+}$; and after they were transferred to 37°C, the level of radioactivity in the resistant cells sharply decreased. This cycle was repeated (in Fig. 2) twice and accumulation at 0°C and the efflux at 37°C were observed. The cells of sensitive control culture *E. coli* JM101 accumulated $^{63}Ni^{2+}$ in cold at the same level as did the resistant cells, but after transfer to 37°C the level of $^{63}Ni^{2+}$ in the cells did not change (Fig. 2).

E. coli V38 cells were grown overnight at low (0.05 mM) $NiCl_2$ and were suspended in efflux medium with various concentrations of $^{63}NiCl_2$ (0.1 mM; 0.3 mM; 1 mM and 3 mM). The cell suspensions were kept at 0°C for 20 min, and then transferred to 37°C (Fig. 3). There was accumulation of nickel at 0°C. When the cells were transferred to 37°C a sharp increase in uptake during about 10 min followed by Ni^{2+} ion efflux. The level of uptake increase and the efflux delay were dependent on nickel concentration: at high (1 and 3 mM) concentrations, high uptake was followed by a slow and delayed efflux (Fig. 3). At 0°C there was uptake into the cells which was dependent on the concentration used. In the experiment in Fig. 2, a low non-toxic Ni^{2+} concentration was used and the ion uptake reached equilibrium and did not increase at 37°C but was followed by efflux. When higher Ni^{2+} concentrations were used, the uptake was higher and continued at 37°C.

The role of Ni^{2+} concentration used for induction on the nickel uptake and efflux was further investigated. *E. coli* V38 cells were pregrown in medium containing 0.2 mM, 0.5 mM or 1 mM $NiCl_2$. The cells were washed and suspended in efflux medium containing 1 mM $^{63}Ni^{2+}$. The suspensions were kept at 0°C for 20 min and then transferred to 37°C (Fig. 4). When the nickel concentrations used for

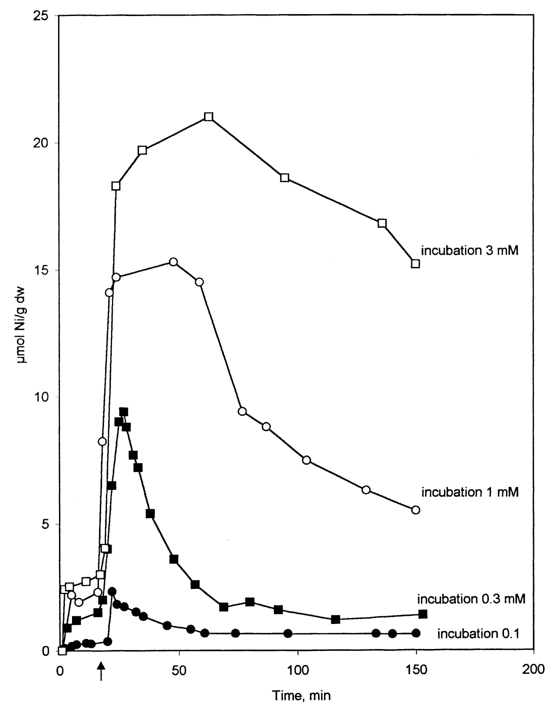

Fig. 3. Uptake and following efflux of $^{63}Ni^{2+}$ by V38 cells induced by overnight growth with 0.05 mM $NiCl_2$. The cells were harvested and suspended in efflux medium; after adding $^{63}Ni^{2+}$ the cells were kept at 0°C for 20 min and transferred to 37° (↑). Curves: incubation in the presence of 0.1 mM (●); 0.3 mM (■); 1 mM (○) or 3 mM (□).

both induction and incubation were equal, i.e. 1 mM, there was no noticeable accumulation of nickel in cold, nor at 37°C: uptake and efflux were perhaps in equilibrium. When concentration used for induction was lower than that of incubation, additional accumulation in the cold was followed by sharp uptake increase at 37°C, and then slow efflux. The level of uptake increase was higher when the difference between the concentrations used for induction and incubation was bigger.

To measure cation/cation exchange in non-induced cells during various phases of resistance development, parallel *E. coli* V38 overnight cultures were washed and suspended in efflux medium in the presence of 1 mM $NiCl_2$. After 20 min at 0°C $^{63}Ni^{2+}$ was added to one culture and radioactivity in the cells was measured. From the parallel non-radioactive culture 10 ml samples were taken at the times corresponding to the uptake, delay and efflux periods. Trace $^{63}Ni^{2+}$ was added to the samples and radioactivity in the cells was measured five times during subsequent 7 min (Fig. 5). Three phases were seen:

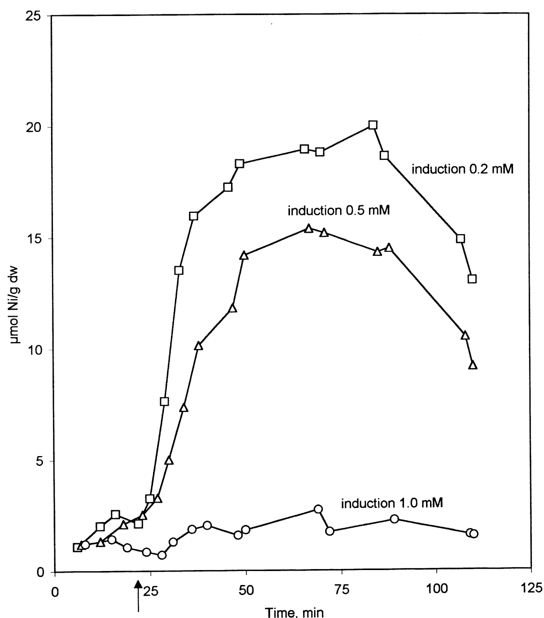

Fig. 4. The uptake and efflux of 1 mM $^{63}Ni^{2+}$ by the resistant strain V38. After adding $^{63}Ni^{2+}$, the cells were kept at 0°C for 20 min and transferred to 37°C (↑). The cells were induced during pregrowth in mineral medium with: 0.2 mM (□), 0.5 mM (△) or 1 mM (○) $^{63}Ni^{2+}$.

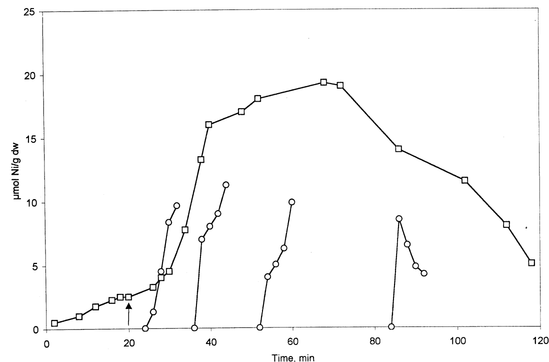

Fig. 5. Uptake and efflux with uninduced *E. coli* V38 cultures. Both cultures were incubated in efflux medium with 1 mM $NiCl_2$ for 20 min at 0°C and transferred to 37°C (↑). $^{63}Ni^{2+}$ was added to one culture (□). From the second culture 10 ml samples were taken after 23, 35, 50 and 85 min of incubation, and trace $^{63}Ni^{2+}$ was added. Over 7 min at 37°C, 5 samples were taken to measure the amount of $^{63}Ni^{2+}$ in the cells (○).

the upgoing phase corresponds to net $^{63}Ni^{2+}$ uptake; the delay phase corresponds to the relative plateau; and the downgoing phase with net efflux of nickel. The uptake curves which began at 23, 35, 50 and 85 min of incubation at 37°C showed $^{63}Ni^{2+}$ uptake at those times. At 23 min a very active uptake of $^{63}Ni^{2+}$ took place. Active uptake was seen at 35 and 50 min. At 85 min, when nickel efflux process dominated, an immediate uptake was followed by Ni^{2+} efflux.

4. Discussion

Some heavy metals are essential at trace concentrations and are toxic at higher concentrations [20]. In *A. eutrophus* CH34 the resistance to Co^{2+}, Zn^{2+} and Cd^{2+} is determined by system encoded by *czc* operon located on plasmid pMOL30 [13]. The encoded efflux system CzcABC functions as a cation-proton antiporter [21]. The nickel and cobalt resistance in *A. eutrophus* is determined by similar *cnr* operon located on plasmid pMOL28 [14].

E. coli strain V38 was isolated from Vilnius city sewage sludge and grows in the presence of 4 mM $NiCl_2$. In comparison with some strains of *Alcaligenes* resistant to 20–50 mM of $NiCl_2$, this level of resistance was not very high [18,19], but it was more than ten times higher than that of *E. coli* laboratory strain JM101 (Table 1).

The development of nickel resistance in *E. coli* V38 could be divided into two phases: the induction of resistance and the expression of resistance. When the culture grew in nickel containing medium, these two phases corresponded to the delay of growth and further logarithmic growth (Fig. 1). In the pregrowth cold incubation experiments where different nickel concentrations were used, a two phase resistance development was clearly seen: during pregrowth at low Ni concentration, there is an induction phase during which a definite level of resistance was elaborated. The following phase depended on Ni concentration used in medium: if the concentration is the same as during induction, the cells started growing immediately after incubation. If the nickel concentration in the incubation medium was higher, the cells stopped growing and the delay of growth depends on the difference between Ni concentrations used for both induction and incubation (Fig. 1). Inducible efflux as Co^{2+}, Zn^{2+} and Cd^{2+} resistance mechanism was observed in *A. eutrophus* as was metal ion accumulation at 4°C and ion efflux when the culture was transferred to 37°C [9]. Our results using *E. coli* V38 and Ni^{2+} (Fig. 2) are similar to those in [9].

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 in rate as the control. This suggestion was supported by comparatively high uptake of $^{63}Ni^{2+}$ during the prolonged lag phase which changed to net efflux during logarithmic growth (Figs. 1 and 3). Similar data were obtained in *Bacillus subtilis* manganese transport studies: when cells were exposed again to high toxic levels of manganese, they rapidly accumulate high levels of Mn^{2+} and subsequently release back into medium up to 90% of the accumulated manganese [22,23]. At high manganese intracellular levels inhibition of RNA and protein synthesis occurs; the cells began to grow when the concentration declines to nontoxic.

However the delay in lag phase is not necessary, at least not in all microorganisms [5,7].

In our experiments the development of resistance in *E. coli* V38 had some peculiarities. First, during induction the level of resistance was dependent on the concentration used (Fig. 4). Second, during further incubation, the lag in growth, the rate of Ni^{2+} accumulation and the delay of Ni^{2+} efflux were interconnected and were dependent on the difference between concentrations used for both induction and incubation (Figs. 1 and 3). It could be supposed that net Ni^{2+} accumulation took place until it reached a level of saturation and elaboration of corresponding level of resistance, and then net Ni^{2+} efflux began. The duration of the uptake efflux period was almost the same as the duration of the prolonged lag phase (Fig. 1). The delay of both growth and efflux was connected with elaboration of a definite level of Ni resistance. It was shown in *A. eutrophus* CH34 that induction of *czc* proton-cation antiporter efflux system required mRNA synthesis and the amount of *czc* mRNA increased with duration of induction [24].

When the culture was incubated in the presence of 1 mM $NiCl_2$ and the uptake of $^{63}Ni^{2+}$ in the cells was measured (Fig. 5), there was almost constant Ni^{2+}/Ni^{2+} ion exchange during incubation. Similar to Co^{2+}, Zn^{2+} and Cd^{2+} in *A. eutrophus* [9], Ni^{2+} in *E. coli* V38, may be proposed, enter the cell by magnesium uptake system and could be effluxed by an cation-proton antiporter system [9,21].

Our data show (no efflux in cold and very small efflux in glucose exhausted media [these data are not shown]) that the main mechanism of resistance is based on energy dependent metal ion efflux. The mechanisms of induction and elaboration of the resistance remain for further work.

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