Ni^{2+}-uptake in *Pseudomonas putida* strain S4: a possible role of Mg^{2+}-uptake pump

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Essential metal ion homeostasis is based on regulated uptake of metal ions, both during its scarcity and abundance. *Pseudomonas putida* strain S4, a multimetal resistant bacterium, was employed to investigate Ni^{2+} entry into cells. It was observed that Mg^{2+} regulates the entry of Ni^{2+} and by this plays a protective role to minimize Ni^{2+} toxicity in this strain. This protection was evident in both growth as well as viability. Intracellular accumulation of Ni^{2+} varied in accordance with Mg^{2+} concentrations in the medium. It was hypothesized that Ni^{2+} enters the cell using a broad Mg^{2+} pump, i.e. the CorA system, as the CorA inhibitor, i.e. Co(III) Hex, also inhibits Ni^{2+} uptake. This led to the inference that Mg^{2+}-based protection was basically due to competitive inhibition of Ni^{2+} uptake. We also show that Zn^{2+} can further regulate the entry of Ni^{2+}.

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1. Introduction

Metals are an integral part of all ecosystems, occurring in both elemental as well as locked form. Some of them are vital components of living systems known as essential metal ions. Based on the requirement, a cell may employ a specific and/or more broad-based non-specific uptake pathway to accumulate these ions from the external environment. We have studied the Ni^{2+}-resistance mechanism in *Pseudomonas putida* strain S4, a natural multimetal resistant isolate. The biological importance of nickel and magnesium is evident by the fact that they may be associated with many enzymes and thus play an important role in a wide variety of cellular functions (Smith and Maguire 1998; Mulrooney and Hausinger 2003).

While in natural environment, metal-microbe interactions are very important, metal-metal interactions can also be of great significance as metals generally occur in combinations. Since the cellular metal binding sites are never entirely specific for a single metal, metals with similar structures and charge can often bind competitively. They can therefore interfere with the metabolism of related metals. Due to this, the cells have developed broad-based metabolic pathways, like uptake or resistance machinery, to take care of more than one metal. These systems are more specific for major element and other less important metals can enter or thrown out of the cell with lower efficiency. In such situations, major ions play a crucial role in regulation of other metal ions entry. It is reported that major Mg^{2+} uptake pumps, such as, CorA, MgtA, and MgtB (Smith and Maguire 1998; Chamnongpol and Groisman 2002) can be utilized by other divalent ions like Ni^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+} along with Mg^{2+} with different efficiencies (Webb 1970a; Nelson and Kennedy 1971; Blackwell *et al* 1997). Thus, Mg^{2+} often regulates the entry of these divalent ions. This may get reflected in the better growth response of cells at toxic concentrations of some divalent ions, such as Ni^{2+} and Co^{2+} in the presence of higher concentration of Mg^{2+}, as reported in *Escherichia coli* (Webb 1970b). Magnesium transport by CorA system is virtually ubiquitous in bacteria and archaea and this pump is strongly inhibited by Cobalt (III) hexaammine [Co (III) Hex] (Kucharski *et al* 2000).

In this paper, we report the regulation of Ni^{2+} accumulation in *P. putida* strain S4. The maximum tolerable concentration (MTC) for Ni^{2+} in this organism is 2 mM.

Keywords. CorA; Co(III)Hex; metal uptake; Mg^{2+}; Ni^{2+}

2. Materials and Methods

2.1 *Organism, culture conditions and chemicals*

P. putida strain S4 was isolated from Khetri Copper Mines, Rajasthan and described earlier (Saxena and Srivastava 1998). *P. putida* strain S4 and its mutants (M3, M6, M9, M18 and M27) were maintained on gluconate minimal medium (GMM) (Gilotra and Srivastava 1997) without and with $NiCl_2$ (at their respective MTC) at 37°C. As per the requirement of an experiment, the media were supplemented with the appropriate concentration of autoclaved metal salt solutions and/or filter-sterilized inhibitor solution. Liquid cultures were raised on Controlled Environment Shaker Incubator (Kühner, Switzerland) at 200 rpm at 37ºC for the required period of time.

Metal induction (with Ni or Zn) was carried out by exposing the cells to 0.1 mM $NiCl_2$ or $ZnCl_2$ in GMM overnight and such cells were labelled as induced cells. If no pre-exposure was given, the cells were referred as uninduced.

$NiCl_2$ and $ZnCl_2$ were purchased from Merck (India) and Co(III)hexaammine was purchased from Sigma-Aldrich (USA). All other chemicals used were of analytical grade.

2.2 *Spheroplast isolation*

Spheroplast isolation was done by the method described by Wood (1978). For this purpose, cells (induced or uninduced wild type or mutants) exposed to metal for 14 h were used. Pellet of spheroplasts were resuspended in 0.1 M Tris-buffer (pH 8.0) and used for Ni^{2+} estimation.

2.3 *Metal estimations*

Metal-loaded cells/spheroplasts were harvested and washed with saline. One part of the culture was boiled with 1 M NaOH and protein estimation was done with the protocol of Lowry *et al* (1951). Rest of the biomass was used for metal estimations with atomic absorption spectrophotometer (Perkin Elmer Model 3110) at 232 nm and 213.9 nm for Ni^{2+} and Zn^{2+}, respectively as described by Bhagat and Srivastava (1993), and represented as μg metal. mg protein^{-1}.

2.4 *Statistical analysis*

All experiments were carried out as three independent sets and the values represent mean along with the standard errors.

3 Results

3.1 *Effect of Mg^{2+} on growth*

To begin with, the effect of Mg^{2+} on growth of S4 cells (induced as well as uninduced) in the presence of Ni^{2+} (1.0 mM) and different external concentrations of Mg^{2+} (0, 0.25, 0.5, 1.0 and 2.0 mM) was checked. It was earlier observed that lower (<1.0 mM) Mg^{2+} concentration did not affect the growth profile of strain S4 in the absence of Ni^{2+} within the experimental time frame. The results depicted in figure 1 show that while decreasing Mg^{2+} concentration reduced the growth of induced cells in the presence of Ni^{2+}, higher concentration of Mg^{2+} (2 mM) resulted in a better growth response. Uninduced cells responded the same way, but the extent of the effect at decreased Mg^{2+} concentrations was more severe. Thus, it could be concluded that at the same concentration of Ni^{2+}, Mg^{2+} could modulate the growth based on its external concentration.

Mg^{2+}-mediated protection of S4 cells towards Ni^{2+} toxicity was further supplemented by checking the cell viability under similar conditions. We observed that viability (in terms of CFU.ml^{-1}) showed similar trend, as there was a clear sign of improvement (~2-folds) with corresponding increase in Mg^{2+} concentration in the medium.

3.2 *Intracellular Ni^{2+} accumulation*

As the strain S4 is resistant to Ni^{2+}, we monitored the intracellular accumulation of Ni^{2+} in the spheroplast fractions of the induced cells and correlated the same with the different Mg^{2+} concentrations. Results showed that intracellular content of nickel varied with the varying concentrations of magnesium which also explains the growth response. It is evident from figure 2 that lowering the Mg^{2+} concentration in the medium led to increased intracellular Ni^{2+} accumulation, but at increased concentration a threshold appeared to have reached

3.3 *Effect of Mg^{2+} on Ni^{2+}-sensitive mutants*

Some Ni^{2+}-sensitive mutants (M3, M6, M9, M18 and M27 with MTC- 0.1 mM for each), isolated by UV mutagenesis (Tripathi and Srivastava 2006), were also employed to decipher the protective role of Mg^{2+}, both through growth responses and intracellular Ni^{2+} accumulation.

These mutants when exposed to an otherwise toxic concentration of Ni^{2+} (0.25 mM) along with 1.0 mM and 10 mM Mg^{2+}, showed an improved net growth at higher Mg^{2+} concentration. Under similar conditions, a clear reduction in intracellular Ni^{2+} content in each mutant was also seen at high Mg^{2+} (figure 3).

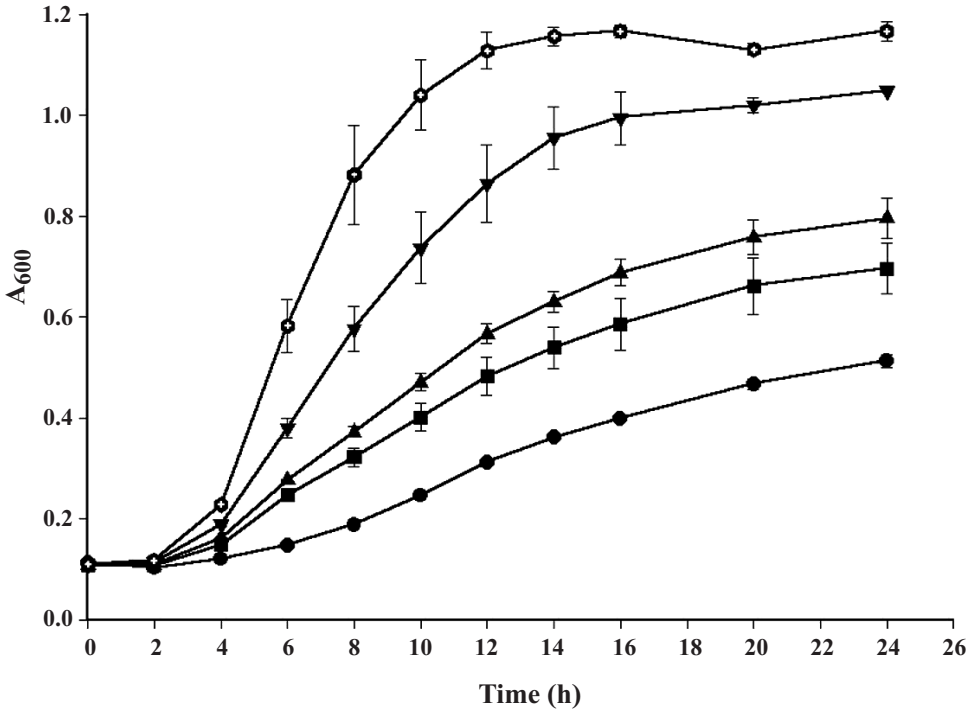

Figure 1. Growth response of strain S4 in presence of 1 mM Ni^{2+} with varying concentrations of Mg^{2+} [0.0 mM (●), 0.25 mM (■), 0.5 mM (▲) , 1.0 mM (▼) and 2. 0 mM (❁)] under induced conditions.

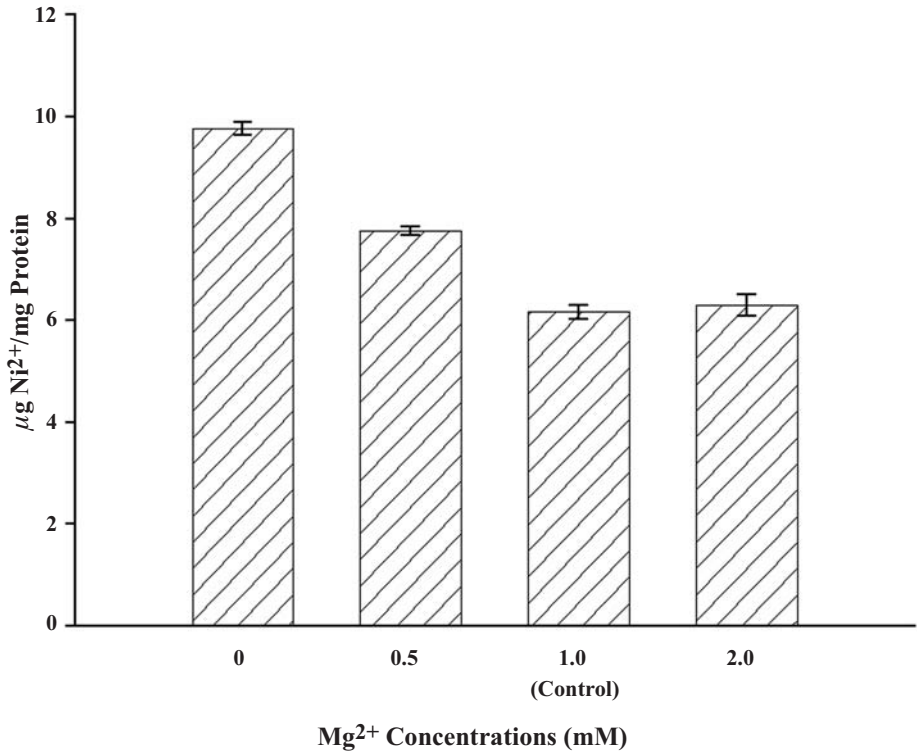

Figure 2. Effect of different Mg^{2+} concentrations (0, 0.5, 1.0 and 2.0 mM) on intracellular Ni^{2+} content of induced cells.

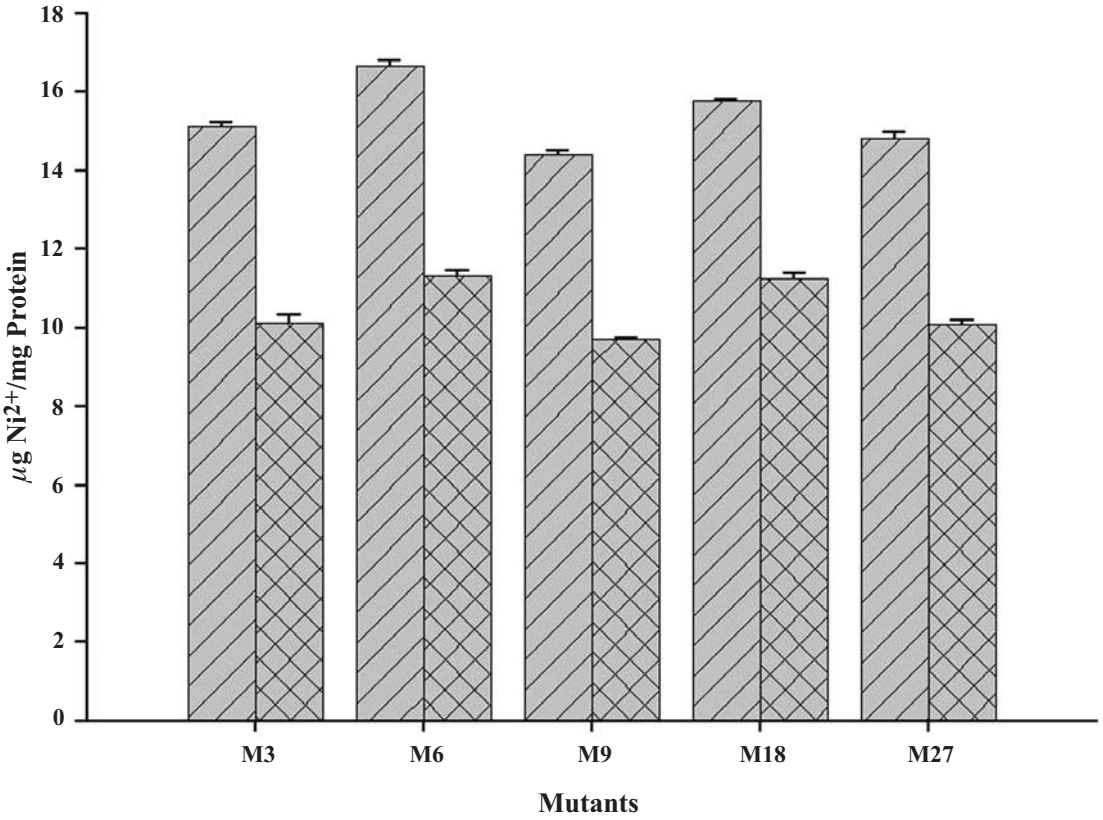

Figure 3. Modulation of intracellular level of Ni^{2+} by Mg^{2+} in different Ni-sensitive mutants in presence of 0.25 mM Ni^{2+} [1 mM Mg^{2+}(▨), and 10 mM Mg^{2+}(▩)].

3.4 *Effect of Mg^{2+} uptake inhibitor on Ni^{2+} uptake*

From these experiments, it was inferred that the protective effect of magnesium is due to competitive inhibition of nickel uptake in the cell. This also led us to assume that Ni^{2+} might share the Mg^{2+} uptake pathway.

To substantiate the above assumption, the CorA inhibitor, i.e. Co(III)Hex, was employed to study its effect on Ni^{2+} accumulation. The concentration of inhibitor (5 μM) where cells showed 50% reduction in intracellular accumulation of Ni^{2+} was chosen for further work.

In one experiment, accumulation of Ni^{2+} in the absence and presence of Mg^{2+} (1.0 mM) was studied along with inhibitor (5 μM) and nickel (1 mM) in the medium, keeping the sets without inhibitor as control. Results presented in figure 4 demonstrated that in contrast to the controls, where absence of Mg^{2+} led to increased levels of intracellular Ni^{2+}, in the presence of inhibitor intracellular Ni^{2+} level remained largely unaffected by Mg^{2+}. In the absence of magnesium, where Ni^{2+} accumulation was expected to be higher, the inhibitor was able to control its entry. This result strengthened our earlier proposition that Ni^{2+} may share the broad Mg^{2+}-pump. Moreover, Co(III)Hex functioned the same way as the higher Mg^{2+} concentration, in reducing Ni^{2+} uptake and protecting the cells from the toxic effects of Ni^{2+} accumulation.

3.5 *Effect of Zn^{2+} induction*

Metal-metal interaction in strain S4 was further investigated by inducing the cells with Zn^{2+}, as it was found that Ni^{2+}-mediated growth responses can be cross-induced by Zn^{2+} (V N Tripathi and S Srivastava, unpublished results). Intracellular levels of Ni^{2+} under Zn^{2+} induction with or without Mg^{2+} and/or Co(III)Hex, was studied. Results suggested that under Zn^{2+} induction, protective role of neither magnesium nor the inhibitor is apparent (figure 4). However, Ni^{2+} level did get substantially reduced almost down to the level of inhibitor exposed cells. As a control, one set was also exposed to Zn^{2+} (1 mM) and intracellular Zn^{2+} accumulation was measured, which showed similar levels under all conditions.

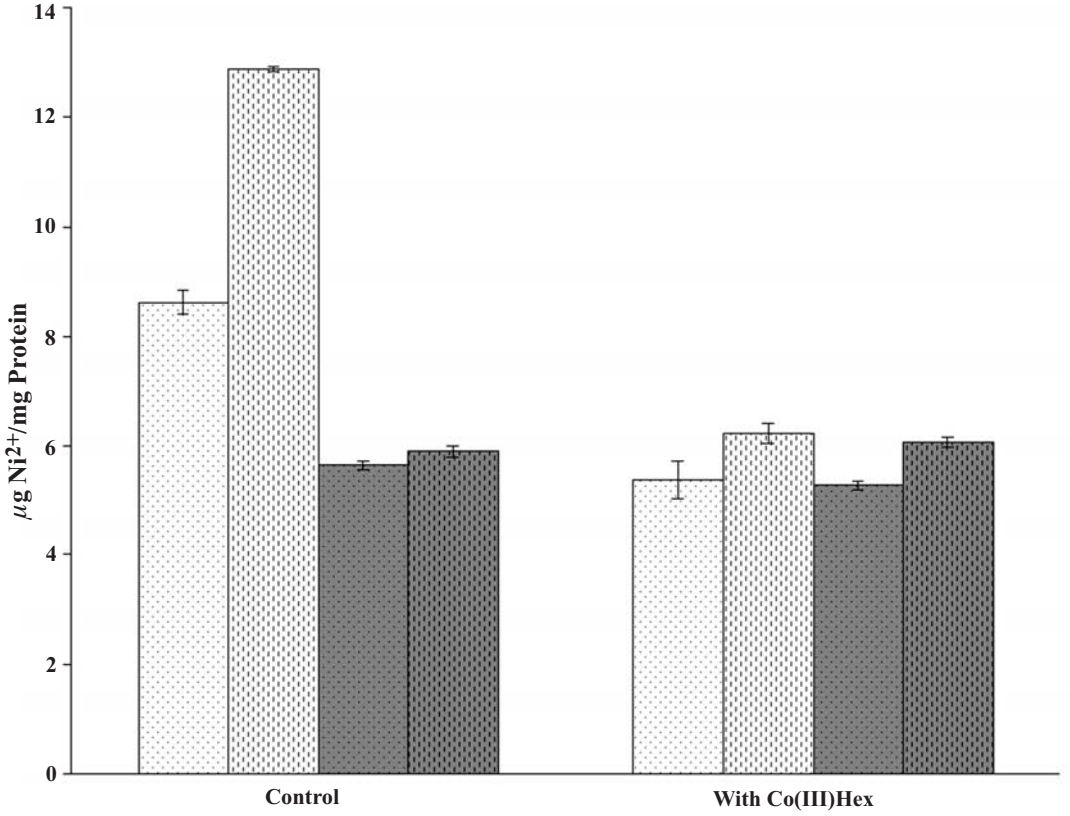

Figure 4. Intracellular accumulation of Ni^{2+} by strain S4 in presence of 1 mM Ni^{2+} with different Mg^{2+} and inhibitor [Co(III)Hex] conditions. Ni^{2+}-inducd cells [with Mg^{2+}(▨), without Mg^{2+}(□)] and Zn^{2+}-inducd cells [with Mg^{2+} (▩), without Mg^{2+}(▤)].

4. Discussions

Cellular requirements for various ions are highly variable; depending upon the role they play in metabolic functions (Gadd 1992). Cells, therefore, employ specific uptake pumps for macronutrients, while the micronutrients may be accumulated by one of the major pumps. Magnesium, an absolutely essential ion (Smith and Maguire 1998; Maguire and Cowan 2002) is accumulated by three uptake pumps, CorA, MgtA and MgtB (Smith and Maguire 1998; Tao *et al* 1998; Chamnongpol and Groisman 2002; Kehres and Maguire 2002; Maguire and Cowan 2002). CorA is a ubiquitous and constitutive transporter in bacteria and archaea, while the other two are inducible P-type ATPases (Snavely *et al* 1991). These pumps are also reported to mediate the entry of other ions, required in trace amounts, though with much lower efficiencies (Hmiel *et al* 1986; Snavely *et al* 1989; Fu and Maier 1991; Blackwell *et al* 1997; Kehres and Maguire 2002). This lower affinity suggests that uptake of these ions by this pump is unlikely to be important physiologically (Kehres and Maguire 2002) in normal environmental conditions, but in high metal stress conditions, uptake through this pump may play a crucial role.

While investigating a Ni-resistant (MTC 2.0 mM) *P. putida* strain S4, we observed that Mg^{2+} greatly influences the strain's Ni^{2+}-mediated growth response. This effect was more significant, when cells were not induced by Ni^{2+}. Improved cell viability in the presence of higher Mg^{2+} concentration further substantiated the role of Mg^{2+}. The resistant cells are expected to accumulate much higher amounts of metals than the normal homeostatic pathway (Krishnaswamy and Wilson 2000; Pradhan and Rai 2001). Protective effect of Mg^{2+}, attributed to a possible competitive inhibition of Ni^{2+} uptake by Mg^{2+} (Nies and Silver 1989; Fu and Maier 1991; Blackwell *et al* 1997), thus may serve a crucial purpose for cell survival. It was clear from our results also that the intracellular Ni^{2+} accumulation gets reduced in the presence of higher Mg^{2+} concentration. Blackwell *et al* (1997) showed that in *Saccharomyces cerevisiae*, cell viability and intracellular Mn^{2+} uptake varies according to the Mg^{2+} concentration in the medium in the presence of 5 mM Mn^{2+}. In case of *Bradyrhizobium japonicum* JH, Ni^{2+} entry inside the cell was inhibited significantly by Mg^{2+} and other ions like Zn^{2+}, Co^{2+} and Mn^{2+}, which can be relieved by increasing the Ni^{2+} concentration (Fu and Maier 1991).

Ni^{2+}-sensitive mutants of strain S4 further confirmed the role of Mg^{2+} as they responded much better to the otherwise toxic concentration of Ni^{2+} both in terms of growth (their MTC is increased) as well as competitive inhibition of Ni^{2+} uptake. Nelson and Kennedy (1971) also reported that in *E. coli* enhanced MTC for Co^{2+} was achieved when Mg^{2+} concentration was raised with corresponding decrease in the Co^{2+} uptake in the cell, whereas *cor*A mutant of *E. coli* showed Co^{2+} resistant phenotype (Park *et al* 1976). Similarly *cor*A over-expressing *S. cerevisiae* strains showed increased sensitivity to Co^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} (MacDiarmid and Gardner 1998).

These results pointed towards sharing of Mg^{2+} uptake pump by Ni^{2+}. The response of strain S4 to an inhibitor of CorA (Mg^{2+} uptake pump), Co(III)Hex, also suggested the same. Our premise was further substantiated as intracellular Ni^{2+} level remained low irrespective of Mg^{2+}, but when exposed to the inhibitor, in comparison to the controls. It is known that CorA has much higher affinity for Mg^{2+} than Ni^{2+}. Thus, in the presence of inhibitor, when CorA activity is expected to be low, it should preferentially transport Mg^{2+}, resulting in low accumulation of the related ions, as observed by us in the case of Ni^{2+}. This result is in agreement with the results published by Kucharski *et al* (2000)

When Zn^{2+} was introduced as inducing agent, growth responses of strain S4 in the presence of Ni^{2+} were better in comparison to the Ni^{2+}-induced cells, with lower intracellular accumulation of Ni^{2+} (V N Tripathi and S Srivastava, unpublished results). Ni^{2+} accumulation by Zn^{2+}-induced S4 cells in the presence of Co(III) Hex was expected to follow a pattern similar to that of Ni^{2+}-induced cells, but results showed a totally different response. While the effect of neither the inhibitor nor Mg^{2+} was apparent, Zn^{2+} was able to further regulate the entry of Ni^{2+}. Maintenance of intracellular level of metals may be due to the induction of the efflux pump, which is reported to be main mechanism of Zn^{2+} resistance in strain S4 (Choudhury and Srivastava 2001). Under such a condition, the role of Mg^{2+} as well as the inhibitor may become redundant. The similar pattern of Zn^{2+}-accumulation by the Zn^{2+} induced cells consolidated our view.

We thus, concluded that in strain S4, Ni^{2+} entry into the cytoplasm is mainly via CorA-Mg^{2+} uptake pump and improved growth of Zn^{2+}-induced, Ni^{2+}-exposed cells may be due to better regulation of the influx of intracellular Ni^{2+}. In resistance pathway, however, the role of Mg^{2+} in regulating the intracellular Ni^{2+} content may play a secondary role, as most of the Ni^{2+} gets sequestered in the periplasm in strain S4 (Tripathi and Srivastava 2006).

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