

Proton Gradient-Driven Nickel Uptake by Vacuolar Membrane Vesicles of *Saccharomyces cerevisiae*

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A vacuolar H⁺-ATPase-negative mutant of *Saccharomyces cerevisiae* was highly sensitive to nickel ion. Accumulation of nickel ion in the cells of this mutant of less than 60% of the value for the parent strain arrested growth, suggesting a role for this ATPase in sequestering nickel ion into vacuoles. An artificially imposed pH gradient (interior acid) induced transient nickel ion uptake by vacuolar membrane vesicles, which was inhibited by collapse of the pH difference but not of the membrane potential. Nickel ion transport into vacuoles in a pH gradient-dependent manner is thus important for its detoxification in yeast.

Nickel ion is a heavy metal ubiquitously distributed in nature and constitutes a trace element in most living cells (3); it is reported that this metal acts as a cofactor of several enzymes, such as hydrogenase, methyl coenzyme M reductase, CO dehydrogenase, and urease, in various organisms (7). At higher concentrations, nickel ion can be very toxic to both eukaryotic and prokaryotic cells. Nickel ion potentially inhibits synthesis of macromolecules such as RNA and protein (5). Toxicity is also exhibited as an alteration in the metabolism of carbohydrate and organic ions by interfering with the roles of other trace elements, such as Mg²⁺ and Fe³⁺, and excretion of pyruvate or potassium ions from the cell probably because of damage to membrane integrity (10). A series of homeostatic mechanisms has evolved to balance the internal metal contents with the need to acquire essential metals at low levels. In bacteria, homeostasis is exhibited as reduction of the internal ion levels by energy-dependent, metal-specific extrusion systems (21). In eukaryotes, metal detoxification is exhibited as metal-conjugate formation by binding of the ion not only internally with specific molecules such as metallothionein (6) but also externally with excreted small organic metabolites (14). In *Saccharomyces cerevisiae*, it has been reported that nickel ion detoxification was acquired as a decrease in free nickel ion by conjugation with excreted glutathione (16). Recently, Joho et al. (11) isolated a nickel ion-resistant mutant of *S. cerevisiae*, which could grow without any decrease in nickel ion uptake in nickel ion-supplemented medium. In this mutant, more than 70% of the internal nickel ion is distributed in the vacuolar fractions, suggesting a role of this organelle as the detoxifying compartment. Since it was estimated that the vacuoles of this mutant also contained large amounts of histidine (9), these authors suggested a possible role of histidine-nickel ion complex formation in sequestering excess amounts of the internal nickel ion into vacuoles. Various metabolites and ions are stored in vacuoles (17, 20). Active accumulation of most of these is driven by the electrochemical potential of protons across the vacuolar membranes, which is established by electrogenic proton influxes via the vacuolar H⁺-ATPase (13). Accumulation of histidine in vacuoles is likely mediated by a proton-linked transport system (20), but the route of nickel ion

transport into vacuoles is still unknown. In this paper, we propose the pH gradient-driven system as the pathway for penetration of nickel ion into the vacuoles of *S. cerevisiae*.

S. cerevisiae YPH499 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*), RH104 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 Δvma1::TRP1*), or DV3T-A (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 Δvma3::TRP1*) was grown in a complex medium (yeast-peptone-dextrose [YPD]) or a completely synthetic medium (CSD) (15) containing 0.4 mM MgCl₂; the medium pH was adjusted to 5.5 with 10 mM morpholinoethanesulfonic acid (MES)-NaOH (pH 5.5), since the growth of these vacuolar ATPase mutants was optimal at lower pH (8, 22). Cell growth was monitored by measuring the optical density at 540 nm with a spectrophotometer. Internal Ni²⁺ content was measured in cells grown in medium containing ⁶³NiCl₂ (67.8 MBq/mmol); the cells were washed twice with 50 mM potassium phosphate buffer (pH 5.8), and the radioactivity of the cells was counted. The uptake of ⁶³Ni²⁺ by whole cells was assayed as described previously (15). Right-side-out vacuolar membrane vesicles were prepared from the cells grown in YPD medium as described previously (12, 17) and were suspended in 10 mM MES-Tris(hydroxymethyl)aminomethane (pH 6.9) containing 5 mM MgCl₂ and 25 mM KCl, and the suspension was stored at -80°C. Artificially pH gradient (interior acidic)-dependent ⁶³Ni²⁺ uptake by the vesicles was assayed as follows. The vesicles were washed four times with 5 mM MES-Tris (pH 6.9) containing 25 mM KCl and suspended in the same buffer (0.3 mg of protein/ml). After the addition of 50 μM ⁶³NiCl₂ (169.5 MBq/mmol) to the suspension, the medium pH was shifted from 6.9 to 9.0 by the addition of KOH. At intervals, the vesicles (100 μl of the suspension) were collected on the filter (Millipore [pore size, 0.4 μm]) and washed twice with 5 ml of the same buffer, and the radioactivity of the filters was counted. The intravesicular water space was estimated to be 5 μl per mg of protein as described previously (13) by the use of [³H]water and [¹⁴C]inulin.

Figure 1 shows the effect of nickel ion on the growth of *S. cerevisiae* in CSD medium (pH 5.5) containing 0.4 mM MgCl₂. The growth of strain YPH499 was nearly arrested by the addition of 0.5 mM NiCl₂ (Fig. 1A). The growth of a vacuolar H⁺-ATPase mutant (strain RH104), in which the *VMA1* gene was disrupted (8), was highly sensitive to nickel ion; 0.1 mM NiCl₂ was enough for growth arrest (Fig. 1B). In the same medium, the growth of another vacuolar ATPase

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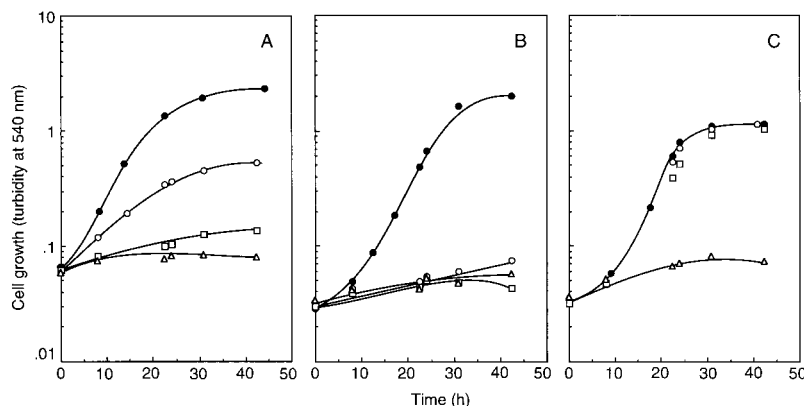


FIG. 1. Effect of nickel chloride on the growth of *S. cerevisiae*. *S. cerevisiae* YPH499 (A) or RH104 (B and C) was cultured in CSD medium containing 0.4 mM MgCl_2 (A and B) or 10 mM MgCl_2 (C). Nickel chloride was added to the medium at various concentrations before starting the culture, and cell growth was monitored by measuring the optical density at 540 nm. ●, control; ○, 0.1 mM NiCl_2 ; □, 0.5 mM NiCl_2 ; △, 5 mM NiCl_2 .

mutant (strain DV3T-A), in which the *VMA3* gene was disrupted (22), was also inhibited by 0.1 mM NiCl_2 (data not shown). Since it has been observed that the sizes and abundance of vacuoles in these vacuolar ATPase mutants did not differ so much from those of the parent strain (8, 22), the function of the vacuolar ATPase as a proton pump is linked to the sensitivity of yeast to nickel ion. Figure 2 shows the time course of $^{63}\text{Ni}^{2+}$ uptake by whole cells. In YPH499, $^{63}\text{Ni}^{2+}$ was taken up with a K_m value of 13 μM and a V_{max} value of 0.5 nmol/min/mg (dry weight). Defect of the vacuolar ATPase was exhibited by much slower velocity and a reduced level of $^{63}\text{Ni}^{2+}$ accumulation; accumulation of $^{63}\text{Ni}^{2+}$ by RH104 was about 50% of the value for YPH499. Nickel ion accumulation in these strains in different media was also examined. In the cells cultured in CSD medium containing 0.1 mM $^{63}\text{NiCl}_2$, in which YPH499 grew but RH104 did not (Fig. 1), the amounts of the internal nickel ion of YPH499 and RH104 were 19.9 and 11.5 nmol/mg (dry weight), respectively. The vacuolar ATPase thus plays a role in vacuolar compartmentation of nickel ion; a role for the vacuole in toxic metal ion detoxification has been recently proposed by using other vacuolar mutants (19). The absolute requirement of this metal for the growth of yeast is obscure; nickel ion is usually omitted from the standard culture medium (Fig. 1). Although the significance of nickel ion in the cellular metabolism of yeast is unknown, vacuolar compartmentation of nickel ion is likely to be the method of removing the toxic metal from the cytoplasm.

Several roles of the H^+ -ATPase in vacuolar compartmentation of various metabolites and ions have been reported (1). First, ATPase generates the electrochemical gradient of protons across the vacuolar membranes, which is utilized as the driving force of various proton-coupled secondary transport systems; for instance, the $\text{Ca}^{2+}/\text{H}^+$ antiporter plays an important role in calcium homeostasis in *S. cerevisiae* (1). Histidine probably taken up via the antiporter system participates in complex formation with nickel ion in vacuoles (20). Second, it is reported that several cationic substances are accumulated in vacuoles by binding with polyphosphate (4); acidification of intravacuolar space by the ATPase may be important for polyphosphate synthesis (23). In our work, these possibilities with regard to the role of the ATPase in the sequestering of nickel ion into vacuoles were discriminated with the vacuolar membrane vesicles; the intravacuolar constituents were removed by disintegration of the intact vacuoles. First, we attempted to investigate Mg^{2+} -ATP-driven $^{63}\text{Ni}^{2+}$ uptake by

vacuolar membrane vesicles; however, this was unsuccessful (data not shown). The following are several explanations for this failure: (i) free nickel ions could be decreased by chelating with ATP, (ii) nickel ion could inhibit the activity of ATPase, or (iii) magnesium ion, which is required as the substrate chelated with ATP, may interfere with Ni^{2+} transport. Instead, $^{63}\text{Ni}^{2+}$ uptake of the vacuolar membrane vesicles was examined by imposing artificially a pH gradient (Fig. 3). Transient $^{63}\text{Ni}^{2+}$ uptake was observed by shifting the external pH from 6.9 to 9.0 (Fig. 3A [closed circles]) with 3 mM KOH; the uptake was not observed by the addition of 3 mM KCl (Fig. 3A [open circles]). The membrane-permeative cation dibenzylidimethylammonium (DDA) (Fig. 3A [open squares]), collapsing the membrane potential, did not inhibit $^{63}\text{Ni}^{2+}$ uptake, suggesting that nickel ion uptake did not result from passive uptake by the membrane potential generated by proton move-

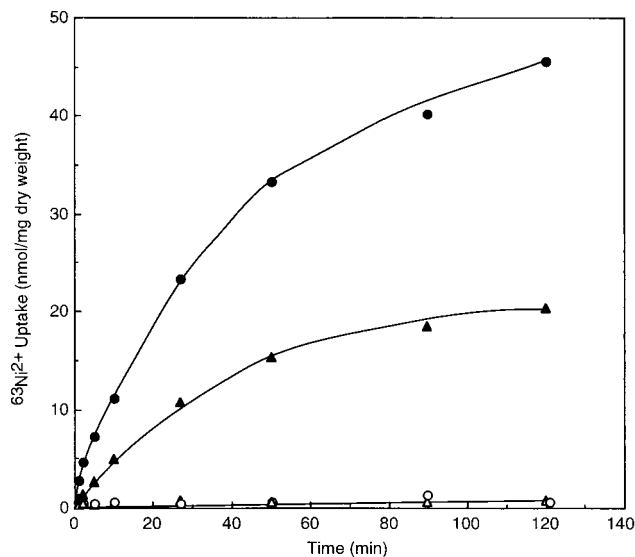


FIG. 2. Nickel ion uptake by whole cells. *S. cerevisiae* cells grown in YPD medium (pH 5.5) were harvested at late logarithmic phase, washed, and suspended in 20 mM MES-NaOH (pH 5.5) containing 10 mM glucose at 0.3 mg (dry weight) per ml. $^{63}\text{NiCl}_2$ uptake (67.8 MBq/mmol) by YPH499 (circles) or RH104 (triangles) was assayed at 30°C (closed symbols) or 0°C (open symbols) as described in Materials and Methods.

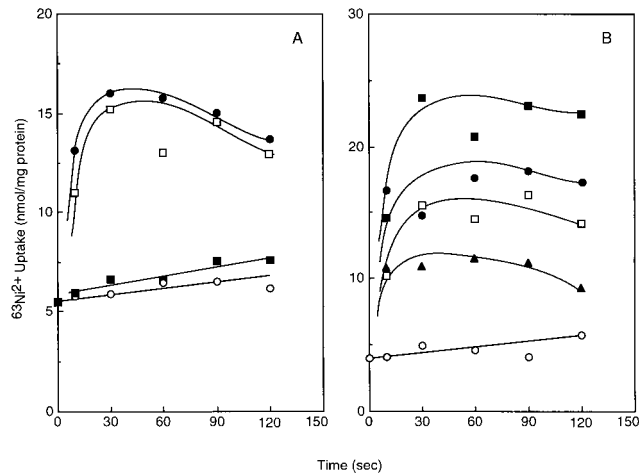


FIG. 3. Nickel ion uptake by vacuolar membrane vesicles. Vacuolar vesicles were prepared from cells of YPH499 grown in YPD medium as described by Ohsumi and Anraku (17). The vesicles were suspended in a buffer (5 mM MES-Tris [pH 6.9], 25 mM KCl) at 0.3 mg of protein/ml. After the addition of 50 μM $^{63}\text{NiCl}_2$ (169.5 MBq/mmol) to the suspension, the external pH was shifted from 6.9 to 9.0 by the addition of 3 mM KOH. As a control, 3 mM KCl (\circ) instead of KOH was added. Several reagents and cations were added 30 s before the pH shift. (A) Effect of ammonia and DDA (\bullet , control; \square , 25 mM DDA; \blacksquare , 50 mM ammonium chloride); (B) effect of divalent cations at 250 μM (\bullet , control; \square , MgCl_2 ; \blacksquare , CaCl_2 ; \blacktriangle , ZnCl_2).

ments. Nickel ion uptake by a shift in pH was completely inhibited by the addition of 50 mM ammonium chloride, which collapses the pH gradient (Fig. 3A [closed squares]), suggesting that the nickel ion uptake is driven by the pH gradient across the membranes. Under experimental conditions, the established gradient of Ni^{2+} was calculated to be about 20 by imposing a pH gradient of 2. Assumption of an electroneutral $\text{Ni}^{2+}/2\text{H}^+$ antiport mechanism does not fit the data; explanation of these data and the mechanism needs further investigation.

When considering the toxicity of nickel ion in yeast, it is important to understand the pathway of nickel ion accumulation. The presence of a nickel ion-preferential transport system in yeast may be dubious, since nickel ion is not essential for the growth of yeast under standard culture conditions. It is reported that $^{63}\text{Ni}^{2+}$ uptake by the intact cells of *S. cerevisiae* was competitively inhibited by Mg^{2+} (2, 15). $^{63}\text{Ni}^{2+}$ uptake by whole cells in this work was also preferentially inhibited by Mg^{2+} but not as much by other metal ions such as Zn^{2+} , Co^{2+} , and Ca^{2+} (data not shown). In addition, the toxicity of nickel ion to *S. cerevisiae* cells was rescued by supplementation with higher concentrations of magnesium ion (Fig. 1C); the internal Ni^{2+} amount was negligible in the presence of 10 mM Mg^{2+} . Nickel ion is probably taken up into the cells via the putative Mg^{2+} transport system in the plasma membrane. It is noteworthy that uptake of $^{63}\text{Ni}^{2+}$ in the vacuolar membrane vesicles by the pH gradient was inhibited more intensely by Zn^{2+} than by Mg^{2+} and Ca^{2+} (Fig. 3B). Inhibition by Zn^{2+} was reduced at the higher concentrations of external Ni^{2+} . Although no zinc-specific transport systems in the vacuolar membranes of *S. cerevisiae* cells have been identified, the presence of zinc is quite probable, since a zinc transporter in the endosomal-lysosomal compartment in mammalian cells has been reported (18). We plan to investigate this possibility.

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