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Increased divalent metal ion uptake associated with hydrogenase constitutive phenotypes of *Bradyrhizobium japonicum*

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Hydrogenase-constitutive (Hup^c) mutants of *Bradyrhizobium japonicum* were previously shown to accumulate more nickel than the wild-type strain. In a 2 h period Hup^c strains JH101 and JH103 also accumulated 2- to 3-fold more Mg^{2+}, Zn^{2+} and Cu^{2+}, and about 4-fold more Co^{2+} and Mn^{2+} than the wild-type strain JH. Initial uptake rates (first 10 min) by the Hup^c strains were also greater for all the metals. The mutation in the Hup^c strains affecting a *trans*-acting regulator of the *hup* structural genes appears to have also amplified a metal uptake/accumulation process common to many divalent metal ions. From efflux experiments (suspension of cells in metal-free medium after metal accumulation) to determine the degree of dissociation of each metal with the cells it was concluded that Zn^{2+}, like Ni^{2+}, was rapidly and tightly cell-associated. In contrast, about 50% of the accumulated Cu^{2+} and about 30% of the Mn^{2+} was effluxed within 2 h by both the Hup^c and wild-type strains. Cobalt was more tightly cell-associated than Mn^{2+} or Cu^{2+}, as the strains effluxed about 26% of the previously accumulated metal in 2 h. Even after accounting for 'effluxed metal', the Hup^c strains retained more of each metal than the wild-type. The increased metal accumulation by Hup^c strains could not be accounted for solely at the level of transport, as known metabolic inhibitors (carbonyl cyanide *m*-chlorophenylhydrazone and nigericin) of nickel transport partially inhibited (1 h) accumulation of only some (magnesium, zinc and copper) of the other metals. Hydrogenase-derepressed wild-type cells exhibited slightly higher (22–27% more) 2 h accumulation capacity for some of the metals (nickel, zinc and copper) than did non-derepressed cells, but not to the 2- to 4-fold greater level observed for Hup^c strains compared with the wild-type. The Hup^c strains JH101 and JH103 do not synthesize more capsular/cell wall carbohydrate than the wild-type strain.

Keywords: *Bradyrhizobium japonicum*, divalent cations, hydrogenase, metal accumulation, metal transport

Introduction

The N_2-fixing bacterial symbiont of soybeans, *Bradyrhizobium japonicum*, expresses a nickel-containing H_2-uptake hydrogenase that aids in the energetic efficiency of the symbosis (O'Brian & Maier 1988). To facilitate studies on H_2 oxidation, many types of hydrogenase mutants of *B. japonicum* have been isolated. Among these, some of the most interesting phenotypes are those of the hydrogenase constitutive mutants; these mutants (termed Hup^c) are not subject to the usual oxygen-mediated repression of hydrogenase (Merberg *et al.* 1983, Graham *et al.* 1984). In addition, in contrast to the wild-type strain, the Hup^c strains are not dependent on the addition of the transcriptional activators, nickel or H_2, in order to express hydrogenase. They transcriptionally express the *hup* structural genes constitutively (Kim *et al.* 1993).

Previously, it was shown that a wild-type *B. japonicum* strain and Hup^c mutant accumulated nickel into a form that was not exchangeable with exogenously-added nickel (Stults *et al.* 1987). Also, a Hup^c strain accumulated about 3-fold more nickel in a 2 h period than the wild-type (Stults *et al.* 1987). One of the major sinks for this nickel was undoubtedly the hydrogenase enzyme. Other phenotypes associated with Hup^c strains include expression of ribulose bisphosphate carboxylase activity in heterotrophic growth conditions (Maier & Merberg 1984) and increased H_2-oxidation activities when the bacterium is in symbiosis with soybean plants (Merberg & Maier 1983). More recently, it was shown that the uptake of nickel by *B. japonicum* wild-type strain JH was inhibited by the divalent metal cations Co^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}; four of these metal cations (the exception is Cu^{2+}) were competitive inhibitors of short-term Ni^{2+} transport by *B. japonicum*

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and the proposed Ni^{2+} transport system was energy-dependent (Fu & Maier 1991). Two potent inhibitors of nickel transport were CCCP and nigericin, which inhibited transport in a 5 min ^{63}Ni assay by 91 and 51%, respectively (Fu & Maier 1991). Here we address the ability of Hup^c strains to accumulate other divalent cations and the affects of hydrogenase derepression on cation accumulation by the wild-type. Furthermore, inhibitor studies have shown that the amplification of a common (short-term) transport process for these cations cannot fully explain the metal-accumulation phenotype associated with Hup^c strains. Rather than amplification of the transport process *per se*, the metal accumulation phenotype is probably due to the constitutive expression of a common metal sink(s).

Materials and methods

Metal accumulation assays

Ultrapure metal salts (Puratronic Grade 1; Johnson-Matthey Chemicals, Herts, UK) used were $NiCl_2$, $CoSO_4$, $CuSO_4$, $ZnSO_4$, $MgSO_4$ and $MnCl_2$. *B. japonicum* wild-type strain JH (Hup^+) or Hup^c mutants were grown in modified Bergersen's (MB) medium (Bishop *et al.* 1976) to a cell number of $5–7 \times 10^8$ cells ml^{-1}, harvested by centrifugation and washed in metal-free (Stults *et al.* 1987) 50 mM 2-(*N*-morpholino)ethane-sulfonic acid (MES) buffer (pH 6.0). When grown in Bergersen's medium, a heterotrophic growth condition, the wild-type does not express hydrogenase activity (Fu & Maier 1992). Cells were resuspended in the trace element-free MES to either 5×10^8 or 5×10^9 cells ml^{-1} (see below). HNO_3 was trace metal grade (Fisher Scientific Co.). Sufficient stock solutions of the metal cation in MES buffer were added to 25–30 ml volumes of the cells (at approximately 5×10^8 cells ml^{-1}) to achieve a 4 $mg\,l^{-1}$ concentration (approximately 0.08–0.15 mM) of the metal (not including the SO_4 or chloride portion of the chemical). The cell suspension was incubated in a sterile, capped 50 ml polypropylene centrifuge tube at room temperature on a rocking platform (slowly rocking). Portions of 4 ml were removed into sterile 15 ml polystyrene centrifuge tubes at the indicated assay times, the aliquot centrifuged in the cold room at $12\,000 \times g$ for 5 min and the supernatant solution carefully removed. The supernatant solution was acidified with 0.2 ml of 2 N HNO_3 to prevent metal ion adsorption to the walls of the test tube and stored frozen until atomic absorption spectroscopy (AAS) could be performed. Accumulation was determined by subtracting the amount remaining in the solution from the initial level of metal added. If dilution was necessary for AAS, samples were diluted in the metal-free MES. Control experiments without cells showed negligible loss of metal by the centrifugation or acidification treatment. Other control experiments were done to ensure that the balance of the metal not observed in the solution was indeed associated with the cells. After harvesting cells from accumulation experiments, cell pellets were digested with 3 ml of metal-free 2.5 N HNO_3 for at least 2 h, then the digested cells assayed for the metal by flame or graphite furnace AAS. In these controls the amount of metal associated with the cells (after accumulation) plus the amount determined as remaining in solution approximated the initial level (4 $mg\,l^{-1}$) for each metal. The exception to this was for magnesium, in which the amount of metal associated with cells even prior to exposure to magnesium (accumulation assay) was significant.

Metal efflux experiments

A cell suspension (100 ml) of each strain at 5×10^8 cells ml^{-1} in the MES buffer was allowed to accumulate the metal (4 $mg\,l^{-1}$ initial for zinc, cobalt and manganese; 5 $mg\,l^{-1}$ for copper) for 4 h in a loosely capped acid-rinsed 400 ml polycarbonate centrifuge bottle, slowly shaking at room temperature. A sample (80 ml) of cells was then harvested by centrifugation and the supernatant solution removed. All the residual supernatant solution was carefully removed by use of a pasteur pipette. Then the cells were suspended (by vortexing briefly) in 20 ml metal-free MES, and at the indicated times (10 min, 30 min, 60 min and 2 h) the suspension centrifuged and metal analysis of the cell-free supernatant solution was done. The amount of metal remaining cell-associated was calculated, on the same cell number basis at the 2, 4 and 6 h accumulation levels (5×10^8 cells ml^{-1}) for graphing. Separately, the metal accumulation at 2, 4 and 6 h time periods was determined on the same initial (100 ml) batch of cells in MES buffer, see above. For this, 4 ml samples were removed for centrifugation and metal analysis of the supernatant was done as described above. Duplicate 4 ml samples were taken for the 4 h time point.

AAS

Solutions were analyzed by Perkin-Elmer model 4000 (flame) or model 2380 (graphite furnace) AAS. Most samples were analyzed by the flame instrument, but some samples required the more sensitive graphite furnace instrument. The instruments were set to report an average of five readings on each sample and, unless otherwise indicated, two independent samples (an additional 25–30 ml cell suspension was set-up, see 'Metal accumulation assays') for each indicated time point (see figures) were assayed. Perkin-Elmer lamps specific for each metal were used. A standard curve of five points for each metal was used for calculation. To zero the instruments, metal-free 50 mM MES buffer (see above) was used, except for the cell-digested material which contained trace element grade 2.5 N HNO_3 in addition.

Derepression and inhibitor experiments

To compare metal accumulation in derepressed versus non-derepressed cultures, cells were grown in BM supplemented with 1 μM $NiCl_2$. This was necessary to enable expression of the hydrogenase gene during the derepression period, as nickel is a required transcriptional activator of hydrogenase expression (Kim & Maier 1990). Cells

were grown to 5–7 × 10^8 ml^{-1} and washed with metal-free derepression medium (Stults *et al.* 1986, Fu & Maier 1991). The mineral medium was as described (Stults *et al.* 1986) but contained added iron (0.45 μM $FeSO_4$) and a lowered level of magnesium (0.10 mM $MgSO_4$); no other trace elements were added. The cells were then suspended to a cell concentration of 5 × 10^8 cells ml^{-1} in the (above) modified derepression medium; H_2, CO_2 and O_2 were injected, and the cells derepressed as described previously (Fu & Maier 1991) for 24 h. Hydrogenase activities were then measured (Fu & Maier 1992) and metal accumulation assays performed on MES-washed and suspended cells as described above. Ultrapure metals were added at the initial level of 4 $mg\,l^{-1}$ as described above. Six independent replicates were done for each strain and metal uptake assay condition.

For inhibitor experiments, carbonyl cyanide *m*-chlorophenylhydrazone (dissolved in methanol) or nigericin (from Sigma Chemical Co.) plus KCl (50 mM) were added 20 min prior to addition of the metal (the latter being the start of the 1 h accumulation period). Strain JH103 was grown in BM (without nickel), washed and suspended in the MES buffer, and assays performed as described under 'Metal accumulation assays'. Five independent replicates for each metal and inhibitor were done, but all were from the same MB-grown batch of (JH103) cells.

Carbohydrate analysis

Total sugar content after acid hydrolysis of whole cells was performed on cell pellets containing approximately 6 × 10^9 cells by the phenol–sulfuric acid method of sugar estimation (Keleti & Lederer 1974). After growth of cells in BM, they were harvested and resuspended in the MES transport buffer to 6 × 10^8 cells ml^{-1}. Volumes of 10 ml were then harvested by centrifugation at 14000 × *g* for 8 min and the pellet analyzed after adding 1 ml of H_2SO_4 (Keleti & Lederer 1974).

Results

The hydrogenase-constitutive (Hup^c) mutant strains, JH101 and JH103, were tested for their abilities to accumulate divalent metal cations (Co^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Ni^{2+} and Mn^{2+}) in metal-free buffer within a 2 h period compared with the wild-type (Figure 1). Only the results for the wild-type (JH) and Hup^c strain JH103 are shown, as results for JH101 were essentially like those for JH103. The initial transport rate (first 10 min) as well as the overall accumulation (2 h) was consistently greater for the Hup^c mutant(s) for all the metals compared with the wild-type. In the experiments shown (Figure 1), the final accumulated level (at 2 h) by JH103 was 2- to 3-fold greater than the wild-type for the cations Mg^{2+}, Zn^{2+} and Cu^{2+}, and about 4-fold greater than the wild-type for the divalent cations Co^{2+} and Mn^{2+}. When accumulation of the metals was extended to 4 or 6 h, the fold differences between the Hup^c strains and the wild-type was about the same as for the 2 h period (data not shown). The picomoles accumulated in 2 h varied for each metal with cells incubated under identical accumulation conditions, with the most accumulation observed for manganese (about 1300 pmol per 10^8 cells for JH, 4500 for JH103) and magnesium (about 1600 pmol per 10^8 cells for JH, 4500 for JH103), and the least for cobalt (about 400 pmol per 10^8 cells for strain JH, about 1200 for JH103). These differences may likely reflect different (or additional) ultimate sinks for each metal.

Under the same conditions used for these cation accumulation experiments, in 2 h strain JH accumulated 1160 pmol Ni^{2+} per 10^8 cells and strain JH103 accumulated about 3000 pmol per 10^8 cells from a 0.08 mM Ni^{2+} solution (see Figure 1F). These results agree well with the previously-reported 3-fold increase in nickel accumulation by a different Hup^c strain (strain SR470) compared with its parent strain for nickel accumulation ability in 2 h (Stults *et al.* 1987). Using different procedures, we previously reported that strain JH (Fu & Maier 1991) accumulated 20–80 pmol ^{63}Ni per hour per 10^8 cells from a 1.0 μM ^{63}Ni containing buffer. Based on concentration-dependent comparisons of nickel accumulation in 1 h (and subsequent assays by use of AAS measurements) we now know this difference is due to non-saturation of the nickel-accumulation process by the lower Ni^{2+} (1.0 μM) levels previously used for strain JH (data not shown).

The nature of the sink(s) for each metal was of interest, to determine whether the metal ion was merely loosely associated with cells or tightly associated with cell components. Thus the efflux or release of the metal upon suspension of the cells in metal-free buffer was determined, after accumulation of each metal for 4 h (see Figure 2). The bulk of the effluxed material in these experiments is most likely due to metal accumulated in the 4 h period rather than that accumulated during growth, since cells were suspended and washed in metal-free MES initially. Most of the metal (for the four divalent metals tested) effluxed by both strains was lost in the first 10 min. Much of the accumulated copper (about 50%) was effluxed within 2 h for both strains, suggesting that much of the copper initially bound was either loosely bound or perhaps remained in the cell as an ionic (Cu^{2+}) form. Also, much of the accumulated manganese was rapidly lost within 2 h (27% effluxed for JH, 31% for JH103). Cobalt was similarly cell-associated, as less than 26% was lost by either strain in 2 h. Zinc was the most tightly associated metal for both strains, with about 15% of the accumulated metal lost within 2 h. The tight association of zinc is like that previously shown for nickel association with *B. japonicum* Hup^c strain SR470; only 15% of the radioactive nickel accumulated was 'exchanged' with a vast excess of non-radioactive nickel in 15 min (Stults *et al.* 1987). Similar efflux experiments with nickel for strains JH and JH103, performed as described here for the other metals, showed 13–19% efflux of the 4 h accumulated nickel within 2 h after the shift to metal-free medium (data not shown). All of these experiments (Figure 2) were repeated with Hup^c strain JH101; like the 2 h accumulation studies the results were nearly the same as for strain JH103.

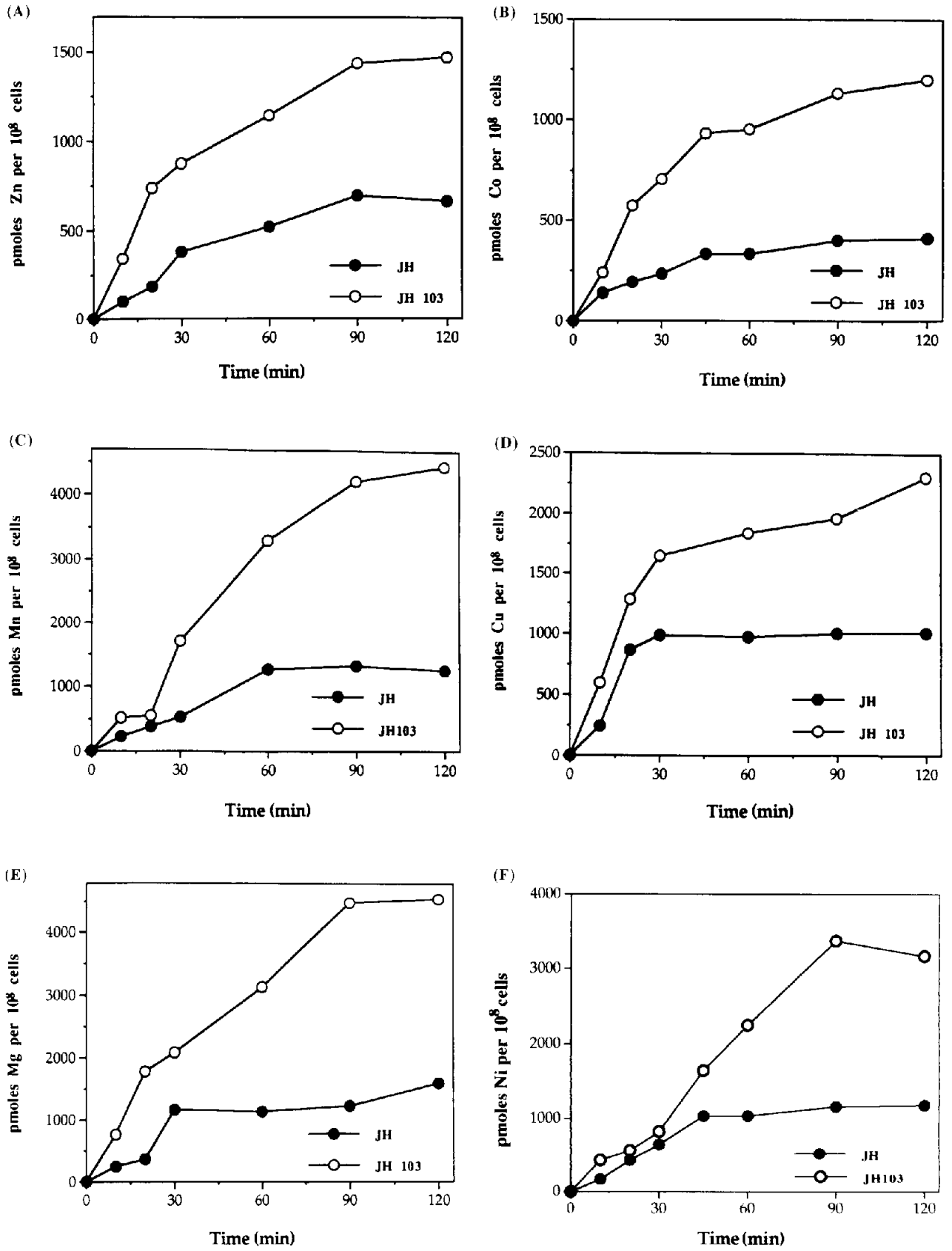

Figure 1. Metal accumulation over a 2 h period by Hup^c strain JH103 and wild-type JH. (**A**) Zn^{2+}, (**B**) Co^{2+}, (**C**) Mn^{2+}, (**D**) Cu^{2+}, (**E**) Mg^{2+} and (**F**) Ni^{2+}.

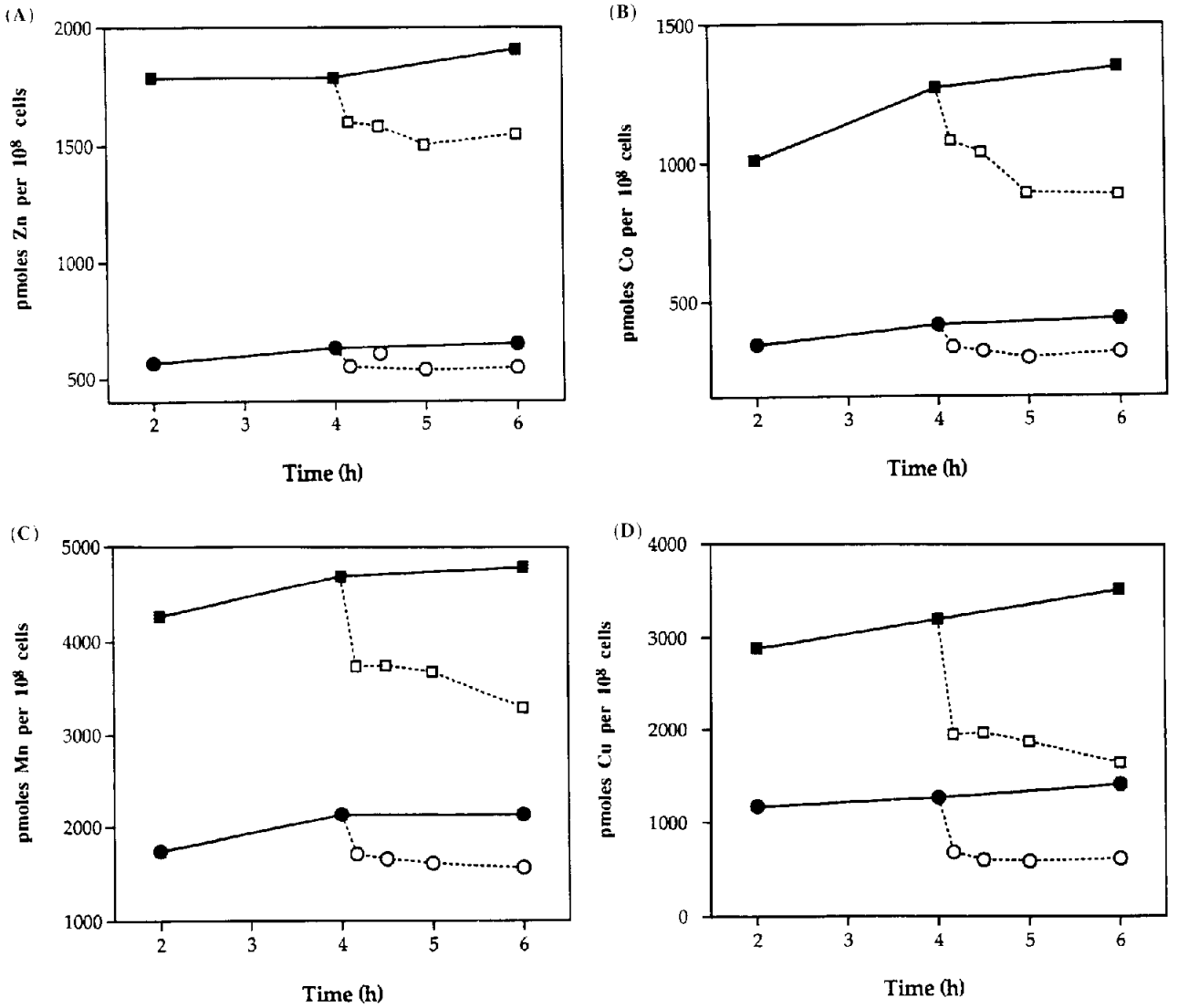

Figure 2. Efflux of the accumulated metal. The amount of calculated metal remaining with the cells at 10, 30, 60 and 120 min after the shift into metal-free medium (after 4 h of accumulation) was determined (– – –). The accumulated amount at 2, 4 and 6 h was determined separately on the same batch of cells (filled symbols). Strain JH (circles), strain JH103 (squares). (**A**) Zn^{2+}, (**B**) Co^{2+}, (**C**) Mn^{2+} and (**D**) Cu^{2+}.

Magnesium efflux was not studied as magnesium measurements on digested cells showed they contained a high level of magnesium even prior to accumulation (MB medium contains a high level of magnesium). Therefore efflux data could not be attributed to magnesium incorporated in the 4 h period, making comparisons to the other metals or conclusions on the degree of association useless.

The genetic lesion in the Hup^c strains affects transcriptional regulation of hydrogenase and other *Hup*-related characteristics. To address the possibility that hydrogenase derepression *per se* is the cause of increased metals accumulation, derepressed cultures were compared to non-derepressed cultures for metal accumulation abilities (Table 1). This was done for both the wild-type and strain JH103. With three of the metals (Zn^{2+}, Ni^{2+} and Co^{2+}), derepression of the wild-type did result in statistically significant increased accumulation although the affect(s) were modest. For zinc, nickel and cobalt, the affect of derepression of the wild-type on accumulation was an increase of about 22, 23 and 27%, respectively. For the other metals, little or no affect by hydrogenase derepression was noted. It must be remembered that in order to obtain hydrogenase derepression, nickel had to initially be added to cultures as transcription of that specific gene is nickel-dependent; such metal addition could result in the 'metal sinks' already having some nickel bound. Further, the derepression medium itself necessarily contained magnesium. Derepression of Hup^c strain JH103 does not increase its hydrogenase activity and metals accumulation for that strain was generally unaltered by the derepression incubation (Table 1). Still, the accumulation of all the metals was significantly greater for derepressed JH103

Table 1. Effect of hydrogenase derepression on 2 h metal accumulation by strain JH and Hup^c strain JH103

	Hydrogenase activity[a]	Metal accumulated[b]					
		zinc	nickel	cobalt	manganese	magnesium	copper
JH	0	603 ± 122	1325 ± 291	670 ± 81	984 ± 82	1018 ± 109	1143 ± 88
JH derepressed	109 ± 2	738 ± 119	1632 ± 184	854 ± 57	1089 ± 77	854 ± 79	986 ± 151
JH103	238 ± 19	1275 ± 375	3193 ± 364	1829 ± 340	3409 ± 393	2817 ± 343	2441 ± 310
JH103 derepressed	131 ± 10	1253 ± 268	2815 ± 373	1859 ± 416	3078 ± 398	2163 ± 316	2129 ± 464

[a]Hydrogenase activity in nmol H_2 oxidized per hour per 10^8 cells; mean ± SD of four replicates.
[b]Metal accumulation data are picomol accumulated per 10^8 cells in 2 h; mean ± SD of six replicates.
Based on the Student's *t*-distribution analyses, strain JH103 (whether derepressed or not) results are significantly greater than strain JH at greater than 95% confidence level for each of the metals. Zinc and nickel accumulation by strain JH derepressed is significantly greater than JH (not derepressed) at more than 90% confidence level, and for cobalt, results for JH derepressed is significantly greater than JH at greater than the 95% confidence level.

than for either of the two wild-type conditions and the level of accumulated metals for all strains was in good agreement with the results of Figure 1.

Previous studies showed that nickel transport, in a 5 min assay using ^{63}Ni, was markedly inhibited by the protonophone CCCP and the K^+–H^+ antiporter nigericin. To address the possible energy-dependence of an accumulation process common to all of the divalent metals, these inhibitors were used (Table 2). From the results, it seems that both inhibitors were effective of at least partial inhibition of nickel, zinc, cobalt and magnesium accumulation, with percent inhibitions of 23, 25, 24 and 35%, respectively, for CCCP and 15, 23, 28 and 29%, respectively, for nigericin. Thus, for three of the (non-nickel) metals the route for a portion of the metal accumulation is energy dependent, as it is for nickel. For Cu^{2+} and Mn^{2+}, no significant inhibition was observed: therefore those metals presumably can be accumulated via uptake pathways that are not energy-dependent, and that therefore differ from the route(s) of a portion of the accumulation exhibited by zinc, nickel, cobalt and magnesium.

Substantial binding of metals to bacterial cells can occur via capsular or outer membrane-associated polysaccharides (see Beveridge & Doyle 1989). For example, a purified capsular polysaccharide fraction from a sediment bacterium bound up to 253 nmol Cu mg^{-1} of carbohydrate (Mittleman & Geesey 1984). The *B. japonicum* parent strain (JH) synthesizes little capsular material, but it was possible strains JH101 and JH103 were high-capsule producers, as the parent strain of JH (USDA 110) is known to produce colony derivatives that vary greatly in their (capsular) polysaccharide quantity (Kuykendall & Elkan 1976). All three strains were tested for total carbohydrate levels after growth of cells in BM medium and then suspension in the (MES) transport buffer. All three strains had approximately the same level of total acid-hydrolyzed sugar (data not shown).

Discussion

The fact that spontaneous mutants can be easily isolated (Merberg *et al.* 1983, Graham *et al.* 1984) exhibiting a phenotype that simultaneously affects the sequestering characteristics of six divalent cations suggests the metals utilize common transport or accumulation component(s). Also, the fact that the Hup^c strains contain a mutation specifically affecting a *trans*-acting *hup* gene regulator (Kim *et al.* 1993) directly correlates hydrogenase expression with the multi-metal accumulation process. The results for *B. japonicum* strains reported here suggests that the Hup^c strains are constitutive or amplified in metal-uptake process(es). There is some information available on biological co-transport of these metals (Rosen & Silver 1987, Drake 1988); Co^{2+}, Mn^{2+} and Mg^{2+} apparently use the same transport system in at-least two bacteria, *Bacillus subtilis* and *Rhodobacter capsulata*. The major Ni^{2+} transport system of *Alcaligenes eutrophus* (Lohmeyer & Friedrich 1987), like the results for *B. japonicum* JH (Fu

Table 2. One hour metal accumulation of cations by JH103 in the presence and absence of nickel transport inhibitor

	Metal accumulation					
Inhibitor	nickel	zinc	cobalt	manganese	magnesium	copper
None	1902 ± 146	1207 ± 159	884 ± 69	1752 ± 232	3090 ± 640	1320 ± 129
CCCP (200 μM)	1469 ± 101	905 + 96	671 ± 72	1847 ± 410	1999 ± 471	1199 ± 202
Nigericin (50 μM)	1613 ± 121	938 ± 102	640 ± 114	1705 ± 328	2181 ± 391	1181 ± 187

All values are pmol per hour per 10^8 cells (mean ± SD) based on five replicates. KCl (50 mM) was added at the time of nigericin addition. The accumulation results with inhibitors were significantly less than the uninhibited samples at greater than 95% level of confidence (based on Students' *t*-distribution analyses) for nickel, zinc, cobalt and magnesium.

& Maier 1991), was inhibited by Co^{2+}, Zn^{2+}, Mn^{2+} and Mg^{2+}, and nickel uptake by various bacteria has been proposed to occur via a Mg^{2+} transport system (Drake 1988). On the other hand, examples of specific transport systems for Ni^{2-}, Co^{2+}, Mn^{2+} and Zn^{2+} exist (see Beveridge & Doyle 1989). Our results correlate the uptake of these metal ions to specific transcriptional expression of a metal-containing enzyme.

The lack of appreciable efflux is suggestive that the bulk of accumulated zinc and cobalt are tightly associated with cellular components, rather than loosely associated or accumulated merely as the ions. For copper (and to a lesser extent, manganese) much of the ion is released again within 2 h, for both wild-type and Hup^c strains. A significant proportion of the 2 h accumulated copper and manganese therefore appears not to be tightly bound to cell components. The efflux results here also suggest that zinc, like nickel, is the most tightly cell-associated metal. Tight binding (lack of exchange of intracellularly accumulated zinc with exogenously added zinc) was also observed in a study of zinc transport in *Escherichia coli* (Bucheder & Broda 1974). The low loss of accumulated zinc is like that seen for nickel previously for *B. japonicum* Hup^c strain SR470 (Stults *et al.* 1987), and for wild-type strain JH and strain JH103 described herein.

In contrast to Hup^c strains, the wild-type makes no detectable hydrogenase protein (by immunological criteria) unless subjected to specific derepression conditions (Stults *et al.* 1986). Derepression of the wild-type resulted in a slight increase in accumulation of zinc, nickel and cobalt compared with non-derepressed cells, thus a metal sink for these metals must be related to hydrogenase expression. However, the accumulation by wild-type expressing hydrogenase was still far less than that seen for Hup^c mutants. The sinks for metal accumulation must therefore be amplified in the Hup^c strains and these amplified components are apparently not strictly correlated with the level of hydrogenase expression. The Hup^c phenotype is certainly a result of an alteration in regulation of hydrogenase synthesis and metal accumulation is an ancillary phenotype related to the primary phenotype.

Even after the 'effluxed metal' is removed, the Hup^c strains retain more of each metal than the wild-type, thus the tightly cell-associated metal sequestering system in the Hup^c strains is amplified compared with the wild-type. The sinks for these metals are not known, but from the inhibitor studies we can conclude that energy-utilizing transport factors cannot be the major sink components that are amplified in Hup^c strains. Rather, an intracellular metal-binding component may have been amplified. Nevertheless, partial inhibition by the uncoupler CCCP, and the ionophore nigericin, of zinc, cobalt, magnesium and nickel accumulation was evident: the conclusion that these elements use a common uptake system (Fu & Maier 1991) is still valid. The (low) extent of inhibition means the metal-accumulation phenotype of Hup^c strains cannot be fully explained at the level of transport. The fact that copper and manganese were not significantly inhibited by the ionophores suggests that (1 h) accumulation of these metals can proceed via routes that do not require energy expenditure. This does not mean short-term transport of these metals occurs via an energy-independent process. Alternatively, even inhibitor-insensitive uptake may be sufficient to saturate the copper and manganese metal sinks in the (relatively long) 1 h period. From the shapes of the (Figure 1) time-dependent accumulation curves this could certainly be so. Short-term linear-rate transport assays (5 min) combined with use of inhibitors for all of the metals would be useful, but are not possible by use of the described procedures.

The Hup^c strains express hydrogenase under growth conditions in which the wild-type expresses no hydrogenase. In heterotrophic growth conditions, Hup^c strains synthesize at least six peptides not made by the wild-type (Merberg *et al.* 1983). Based on the (Hup^c) mutant isolation selection (Merberg *et al.* 1983, Graham *et al.* 1984), the increased metal accumulation must be related to sequestering nickel for hydrogenase expression, and it seems odd that in order for the organism to obtain nickel, in the presence of these other metals, it would accumulate the non-nickel metals as well. The metal-containing sink(s) expressed in Hup^c strains is related to proteins presumably co-regulated with hydrogenase and, based on gene sequencing of the *B. japonicum* hydrogenase gene cluster, one such protein contains 24 histidine residues within a 39 amino acid stretch (Fu & Maier 1994); this protein could therefore contain an excellent metal-binding area. However, there are no reports of a normally nickel-containing hydrogenase that has one of the other metals studied here as a nickel substitute. The results here relate hydrogenase expression and its obligate nickel-sequestering requirement to the accumulation of a number of other divalent cations. The specific proteins or other cell components that are co-regulated with hydrogenase and bind metals will be of interest.

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