

## Competitive Inhibition of an Energy-Dependent Nickel Transport System by Divalent Cations in *Bradyrhizobium japonicum* JH†

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Both nickel-specific transport and nickel transport by a magnesium transporter have been described previously for a variety of nickel-utilizing bacteria. The derepression of hydrogenase activity in *Bradyrhizobium japonicum* JH and in a gene-directed mutant of strain JH (in an intracellular Ni metabolism locus), strain JHK7, was inhibited by  $MgSO_4$ . For both strains,  $Ni^{2+}$  uptake was also markedly inhibited by  $Mg^{2+}$ , and the  $Mg^{2+}$ -mediated inhibition could be overcome by high levels of  $Ni^{2+}$  provided in the assay buffer. The results indicate that both *B. japonicum* strains transport  $Ni^{2+}$  via a high-affinity magnesium transport system. Dixon plots ( $1/V$  versus inhibitor) showed that the divalent cations  $Co^{2+}$ ,  $Mn^{2+}$ , and  $Zn^{2+}$ , like  $Mg^{2+}$ , were competitive inhibitors of  $Ni^{2+}$  uptake. The  $K_s$ s for nickel uptake inhibition by  $Mg^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$ , and  $Zn^{2+}$  were 48, 22, 12, and 8  $\mu M$ , respectively.  $Cu^{2+}$  strongly inhibited  $Ni^{2+}$  uptake, and molybdate inhibited it slightly. Respiratory inhibitors cyanide and azide, the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone, the ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide, and ionophores nigericin and valinomycin significantly inhibited short-term (5 min)  $Ni^{2+}$  uptake, showing that  $Ni^{2+}$  uptake in strain JH is energy dependent. Most of these conclusions are quite different from those reported previously for a different *B. japonicum* strain belonging to a different serogroup.

Nickel is an element essential for the growth of many microorganisms, because this element is a component of enzymes involved in ureolysis,  $H_2$  metabolism, methane production, and carbon monoxide utilization (12). Therefore, Ni-requiring microorganisms must somehow internalize nickel from their environment. Ni transport has been studied in many bacteria (5), such as *Escherichia coli* (16), *Methanobacterium bryantii* (15), *Alcaligenes eutrophus* (21), *Clostridium pasteurianum* (3), *Clostridium thermoaceticum* (22), and *Acetogenium kivui* (33), and in the cyanobacterium *Anabaena cylindrica* (4). In *E. coli*, which has three Ni-containing hydrogenases (26), Ni seems to be transported primarily via the magnesium uptake system (14, 16). In the best-studied system with regard to Ni transport, that of *A. eutrophus*, two Ni transport systems have been described (21)—a nickel-specific system and an uptake process which also transports magnesium. Subsequently, a nickel-specific transport gene (*hoxN*) from *A. eutrophus* was isolated and sequenced, and it encodes a predicted 33.1-kDa membrane-associated polypeptide (6, 7).

The  $N_2$ -fixing symbiont of soybeans, *Bradyrhizobium japonicum*, possesses a Ni-containing hydrogenase (1, 11, 30) that is expressed predominantly in the symbiotic bacteroids but also in free-living cultures under defined derepression conditions (23). Nickel not only serves as a required component of hydrogenase but also regulates hydrogenase synthesis in this bacterium (19, 20).  $Ni^{2+}$  transport and accumulation have been studied in *B. japonicum* SR and in the hydrogenase-constitutive strain derived from SR, strain SR470 (24, 29). Little of the accumulated Ni was exchange-

able with exogenously added  $NiCl_2$ , and the bulk of the Ni uptake seemed to be energy independent (29). Also,  $Mg^{2+}$  had little inhibitory effect on  $Ni^{2+}$  uptake (29). Hydrogenase is the major sink for nickel when cells are derepressed, but nickel incorporates into soluble proteins under non-hydrogenase-derepression conditions. This soluble pool can serve as a storage source for nickel until hydrogenase is later synthesized by incubation of cells under derepression conditions (24).

A nickel metabolism locus that encodes a factor responsible for insertion of Ni into hydrogenase has recently been identified within the 23-kb hydrogenase cluster of *B. japonicum* (9). A mutation at this locus (strain JHK7) causes the strain to require high Ni levels for hydrogenase activity as well as for hydrogenase synthesis at the transcriptional level (9). To understand Ni metabolism further, we have studied the transport characteristics of strains JH and JHK7. Specifically, we report here that (i)  $Mg^{2+}$  significantly inhibits Ni transport in both strains; (ii) this inhibition can be relieved by increasing the level of  $Ni^{2+}$  in the assay buffer; (iii)  $Ni^{2+}$  transport is competitively inhibited by the divalent cations  $Mg^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$ , and  $Zn^{2+}$ ; and (iv) cyanide, azide, *N,N'*-dicyclohexylcarbodiimide (DCCD), carbonyl cyanide *M*-chlorophenylhydrazone (CCCP), nigericin, and valinomycin all significantly inhibit  $Ni^{2+}$  transport, showing that  $Ni^{2+}$  transport in *B. japonicum* JH is energy dependent.

### MATERIALS AND METHODS

**Chemicals.** High-purity chemicals (Puratronic grade 1, certified to 99.99% purity) used for making nickel-free trace element stock and all other reagent-grade chemicals for media were described previously (9). 2-(*N*-Morpholino)-ethanesulfonic acid (MES), DCCP, CCCP, valinomycin,

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nigericin, NaCN, and  $\text{NaN}_3$  were purchased from Sigma Chemical Co., St. Louis, Mo. Radioactive  $^{63}\text{NiCl}_2$  (specific activity, 10.58 mCi/mg; supplied as 50  $\mu\text{Ci}/\mu\text{l}$ ) was purchased from New England Nuclear, Boston, Mass.  $^{63}\text{NiCl}_2$  was diluted to 5  $\mu\text{Ci}/\mu\text{l}$  with Ni-free deionized water before use as described previously (9). Controlled pore glass-8-hydroxyquinoline, used to remove contaminating trace divalent cations (8), was from Pierce Chemical Co. Gases were purchased from Linde Gases or Potomac Airgas, Baltimore, Md.

All glassware used for this study was soaked in 2 M nitric acid overnight and subsequently rinsed four times with deionized water as described previously (9).

**Bacteria and growth.** *B. japonicum* JH (10), derived from the parent strain USDA 110 (13), was used as the wild-type strain. Strain JHK7 is a gene-directed mutant (Ni metabolism locus was deleted) of strain JH (9). Both strains were grown in modified Bergersens (MB) medium as described before (2). Derepression medium containing no carbon (No-C) was described previously (9, 31). Cells were grown heterotrophically or derepressed at 30°C with shaking at 150 rpm. Cell numbers were routinely estimated by measuring the optical density at 540 nm and comparing it to standard curves of optical density versus viable cell number (9).

**Derepression of hydrogenase.** Ni-free mid-log-phase *B. japonicum* JH and JHK7 cells were harvested by centrifugation, washed twice in derepression medium containing no carbon, nickel, or magnesium (No-C, No- $\text{Ni}^{2+}$ , No- $\text{Mg}^{2+}$  medium) and resuspended in fresh No-C, No- $\text{Ni}^{2+}$ , No- $\text{Mg}^{2+}$  medium to  $3 \times 10^8$  to  $5 \times 10^8$  cells per ml. Cells were then dispensed (3 ml) into 150-ml serum bottles, and  $\text{NiCl}_2$  (0.5  $\mu\text{M}$ ) and different amounts of  $\text{MgSO}_4$  (as indicated in the figures) were added to the bottles by using a pipette. The bottles were tightly closed with butyl rubber stoppers and aluminum crimps and then flushed with an anaerobic gas mixture (10%  $\text{H}_2$ , 5%  $\text{CO}_2$ , 85%  $\text{N}_2$ ) for 30 min by use of inflow and outflow needles through the rubber stoppers as previously described (9).  $\text{O}_2$  at a 1% partial pressure (final concentration) was added by using a syringe, and the bottles were then incubated at 30°C at 150 rpm for 20 h for hydrogenase derepression (9). Hydrogenase activity ( $\text{H}_2$  uptake activity) was measured amperometrically as described previously (32) with  $\text{O}_2$  (air) as the electron acceptor (25, 27).

**Ni accumulation and Ni uptake assay.** For nickel accumulation assays, Ni-free mid-log-phase JH and JHK7 cells were harvested, washed twice in 50 mM MES buffer (pH 6.0) (29), and resuspended in fresh MES buffer, and then 50-ml portions of the cell suspension ( $3.5 \times 10^8$  cells per ml) were dispensed into 125-ml flasks. This buffer system is designed for optimized Ni uptake rates by *B. japonicum* (29).  $^{63}\text{NiCl}_2$  at 1  $\mu\text{M}$  (final concentration), which equals 0.67  $\mu\text{Ci}/\text{ml}$  of cell suspension, was added to each flask. The flasks were then incubated at 30°C with shaking at 150 rpm. Samples (3 ml) were removed by using a pipette at the indicated time (see the figures) for filter assays as described previously (9). The filters were placed in 5-ml scintillation vials to which 4 ml of Optifluor (Dupont, NEN Products) was then added for counting (9, 29). For  $\text{Ni}^{2+}$  uptake in the presence or absence of  $\text{MgSO}_4$  (see Fig. 3), strain JH cells were harvested, washed, and resuspended in 50 mM MES buffer (pH 6.0) as mentioned above and 5 ml of the cell suspension was dispensed into each 50-ml flask. A mixture of  $^{63}\text{NiCl}_2$  (1  $\mu\text{M}$ ), along with the required level of cold  $\text{NiCl}_2$  and other metal ions as indicated, was added to each flask. The flasks were immediately incubated for 5 min at 150°C with shaking

at 150 rpm, and samples were rapidly filtered through 25-mm-diameter type HA filters (1 ml for each filter) (pore size, 0.45  $\mu\text{m}$ ; Millipore Corp., Bedford, Mass.) as previously described (9).

**Effect of metal ions on Ni uptake.** Mid-log-phase cells were harvested, washed, and resuspended in 50 mM MES buffer (pH 6.0) as described above. Cell suspensions (5 ml at  $4.0 \times 10^8$  cells per ml) were dispensed into 50-ml flasks. A mixture of  $^{63}\text{NiCl}_2$  (1  $\mu\text{M}$ , or 0.67  $\mu\text{Ci}/\text{ml}$  of cell suspension) and the indicated divalent metal ion (see Table 1) was added to the flask. For the competition studies (see Fig. 4), a mixture of  $^{63}\text{NiCl}_2$  (1  $\mu\text{M}$ ) plus nonradioactive  $\text{NiCl}_2$  (to make concentrations of 10, 25, and 40  $\mu\text{M}$  nickel) and different amounts of the cation was added to the flask containing 5 ml of cell suspension. Flasks were immediately incubated for 5 min at 30°C with shaking at 150 rpm (see above), and filter assays were performed (9).

**Effect of inhibitors on Ni uptake.** Ni-free mid-log-phase cells were harvested, washed, and resuspended in 50 mM MES buffer as described above. The inhibitor was added to the 50-ml flask containing 5 ml of cell suspension ( $4 \times 10^8$  cells per ml), and flasks were incubated at 30°C for 30 min. Then 1.0  $\mu\text{M}$   $^{63}\text{Ni}$  (0.67  $\mu\text{Ci}/\text{ml}$  of cell suspension) was added, and the amount of  $^{63}\text{Ni}$  incorporated in the cells was determined after 5 min (30°C, with shaking at 150 rpm). The  $^{63}\text{Ni}$  assayed was the result of internalized Ni because the cells were washed extensively on the filters (9). Background counts of radioactive nickel binding to the filter (<1,000 cpm) were subtracted from the average of the samples with cells. The 1,000 cpm represents about 1.5 pmol of  $\text{Ni}^{2+}$  uptake. Data are means of duplicates for  $\text{H}_2$  uptake and triplicates for nickel uptake unless otherwise indicated.

## RESULTS

The previous studies on Ni transport utilized wild-type strain SR and a mutant derived from SR, strain SR470 (29). However, it has not been possible to obtain gene-directed mutations of strain SR (since it is Kan<sup>r</sup>) or of its derivative strain, LO (9), to permit definitive molecular genetics studies. Therefore, we have used strain JH, a strain in which we can obtain gene-directed mutations at loci involved in Ni metabolism (9). We have found the characteristics of  $\text{Ni}^{2+}$  uptake in strain JH to be different in many respects from those of strain SR and its derivative, SR470 (29).

**Effect of magnesium on hydrogenase activity.** A specific nickel transport gene is not present in *B. japonicum* JH on the basis of lack of homology to the *hoxN* gene from *A. eutrophus* (9). This result then raises the question of how nickel is transported into *B. japonicum* JH cells. One possibility was that the strain lacks an Ni-specific transporter and that nickel is taken up via a magnesium transport system, as has been seen in some other bacteria (14, 17). Initially, the effect of magnesium on derepression of hydrogenase activity with low (0.5  $\mu\text{M}$ )  $\text{Ni}^{2+}$  concentrations was studied (Fig. 1). *B. japonicum* JH (wild type) showed maximum hydrogenase activity (256 nmol of  $\text{H}_2$  oxidized per h per  $10^8$  cells) at 100  $\mu\text{M}$   $\text{MgSO}_4$ , and the activity decreased with increasing  $\text{Mg}^{2+}$  concentrations to 35 nmol of  $\text{H}_2$  per h per  $10^8$  cells at 10 mM  $\text{MgSO}_4$ . Hydrogenase activity in the Ni-metabolism-locus-deleted mutant strain (JHK7), which requires higher levels of nickel for hydrogenase expression than does strain JH (9), also showed inhibition by  $\text{MgSO}_4$  (data not shown), although all specific hydrogenase activities were lower in the mutant strain than in the wild type, as is expected for a mutant that is deficient in intracellular Ni metabolism into hydrogenase.

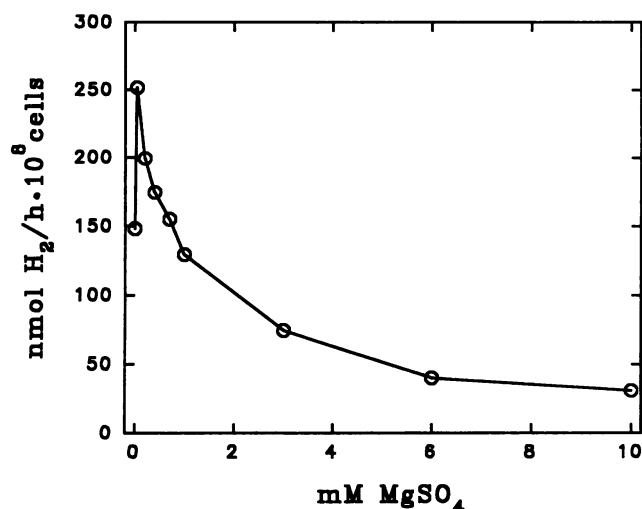


FIG. 1. Effect of  $\text{MgSO}_4$  on derepression of  $\text{H}_2$  uptake activity in *B. japonicum* JH (○).  $\text{NiCl}_2$  ( $0.5 \mu\text{M}$ ) was present in all samples. Experimental procedures and  $\text{H}_2$  uptake assays are described in the text.

**Effect of  $\text{MgSO}_4$  on Ni accumulation.** To determine whether the decreased hydrogenase activity was directly due to inhibition of  $\text{Ni}^{2+}$  uptake by magnesium, a 1-h time course of nickel uptake by strains JH and JHK7 in the presence and absence of  $\text{MgSO}_4$  ( $400 \mu\text{M}$ ) was conducted (Fig. 2). For both wild-type JH and mutant JHK7,  $\text{Ni}^{2+}$  accumulation was strongly inhibited by  $400 \mu\text{M}$   $\text{MgSO}_4$  in the assay buffer (Fig. 2), which was the concentration normally present in MB medium. The final internal Ni level accumulated in the presence of  $\text{MgSO}_4$  at 1 h was 18 and 15% of that in the absence of  $\text{MgSO}_4$  for strains JH and JHK7, respectively (Fig. 2). There was no significant difference in the  $\text{Ni}^{2+}$  uptake rate between strain JH and JHK7 (Ni-metabolism-locus-deleted mutant) in either the presence or absence of

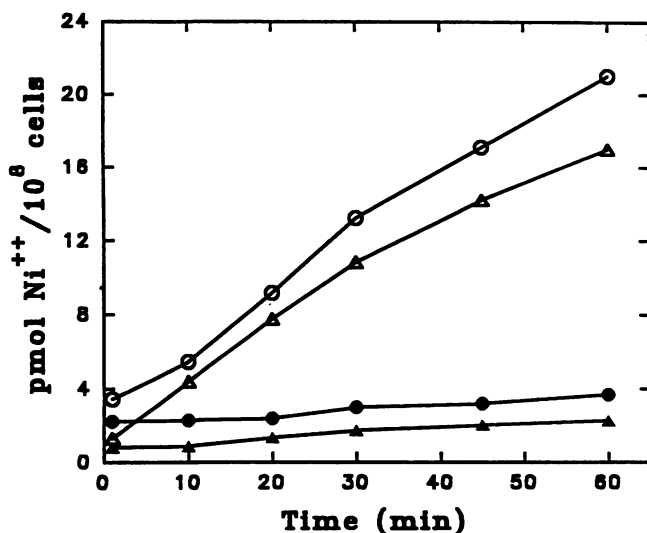


FIG. 2. Nickel accumulation by strains JH and JHK7. Symbols: ○, JH only; ●, JH +  $400 \mu\text{M}$   $\text{MgSO}_4$ ; △, JHK7 only; ▲, JHK7 +  $400 \mu\text{M}$   $\text{MgSO}_4$ . Each assay suspension initially contained  $1.0 \mu\text{M}$   $^{63}\text{NiCl}_2$ .

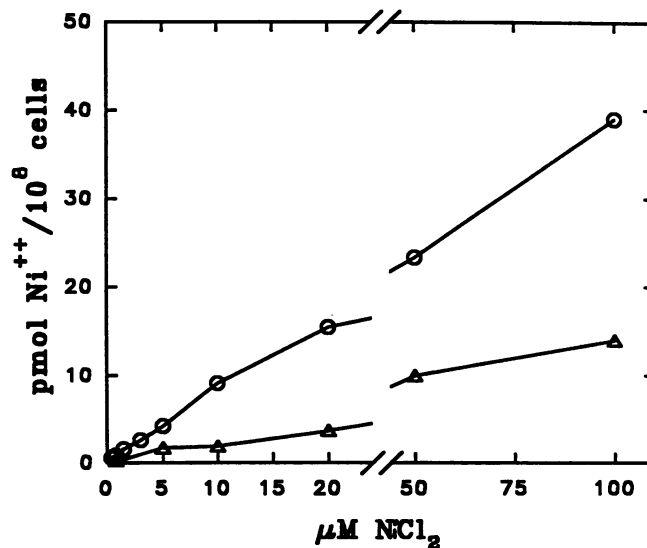


FIG. 3.  $\text{Ni}^{2+}$  uptake by strain JH at different concentrations of nickel in the presence (△) and absence (○) of  $\text{MgSO}_4$  ( $40 \mu\text{M}$ ).

$\text{MgSO}_4$  (Fig. 2); this finding is in agreement with our previous findings (9) that the  $\text{Ni}^{2+}$  metabolism gene product is involved not in Ni transport but in an intracellular Ni metabolism step into hydrogenase.

The strong inhibition of  $\text{Ni}^{2+}$  accumulation by magnesium in *B. japonicum* suggests that  $\text{Ni}^{2+}$  is mainly taken up via a magnesium transport system, and the two cations may compete for the same transport site. This competition obviously affects hydrogenase activity expression as well (Fig. 1).

$\text{Ni}^{2+}$  uptake (in short-term 5-min assays) was measured with increasing amounts of substrate (nickel) in the presence and absence of  $\text{MgSO}_4$  (Fig. 3). The inhibition of  $\text{Ni}^{2+}$  uptake by a constant magnesium concentration ( $40 \mu\text{M}$ ) was partially relieved by increasing nickel levels in the assay (Fig. 3). Similar results were obtained by using a higher  $\text{MgSO}_4$  concentration ( $3 \text{ mM}$ ) (data not shown). These results support the idea that  $\text{Ni}^{2+}$  and  $\text{Mg}^{2+}$  compete with each other at the (short-term) transport level rather than only at the (long-term [Fig. 2]) accumulation level.

**Effect of other metal ions on  $\text{Ni}^{2+}$  uptake.** The effect of other (potentially competing) metals on  $\text{Ni}^{2+}$  uptake was measured in strains JH and JHK7 in the presence of  $1 \mu\text{M}$   $^{63}\text{Ni}^{2+}$  (Table 1). In addition to  $\text{Mg}^{2+}$ , the divalent cations  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Zn}^{2+}$  at  $40 \mu\text{M}$  inhibited  $\text{Ni}^{2+}$  uptake by 74, 91, 62 and 85%, respectively; a similar inhibition pattern was observed for strain JHK7 (Table 1). These inhibitors were not toxic to the cells, because growth (on the basis of optical density) was not affected by these metals ( $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Zn}^{2+}$  at  $40 \mu\text{M}$  [data not shown]). Molybdate ( $40 \mu\text{M}$ ) inhibited strain JH  $\text{Ni}^{2+}$  uptake by 37%;  $\text{Fe}^{3+}$  and  $\text{Ca}^{2+}$  inhibited  $\text{Ni}^{2+}$  uptake slightly. Since no significant difference was found for  $\text{Ni}^{2+}$  uptake metal ion inhibition patterns between strains JH and JHK7, further studies focused on strain JH only. Nevertheless, the results presented for strain JHK7 reinforce our conclusion (9) that this mutant has Ni transport abilities similar to those of the wild type.

**Competition studies of  $\text{Ni}^{2+}$  uptake by  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Zn}^{2+}$ .** To determine whether inhibition of  $\text{Ni}^{2+}$  uptake

TABLE 1. Effect of metal ions on Ni uptake in JH and JHK7 in the presence of 1  $\mu\text{M}$   $^{63}\text{NiCl}_2^a$ 

Metal ion	Concn ( $\mu\text{M}$ )	Ni uptake <sup>b</sup> (% of control)	
		JH	JHK7
Control		100	100
CaCl <sub>2</sub>	40	95	89
	400	49	41
CoSO <sub>4</sub>	40	26	22
	400	7	4
CuSO <sub>4</sub>	40	9	11
	400	3	1
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	40	80	75
	400	73	64
MnCl <sub>2</sub>	40	38	28
	400	12	11
ZnSO <sub>4</sub>	40	15	18
	400	7	6
Na <sub>2</sub> MoO <sub>4</sub>	40	63	73
	400	47	64
MgSO <sub>4</sub>	40	32	43
	400	17	23

<sup>a</sup> The Ni uptake assay is described in Materials and Methods.

<sup>b</sup> The data represent the percentage of nickel uptake relative to that by control cells with  $^{63}\text{Ni}$  (1.0  $\mu\text{M}$ ) only. The specific Ni uptake activity of the control cells for JH and JHK7 was 1.51 and 1.20 pmol of Ni<sup>2+</sup> per min per 10<sup>8</sup> cells, respectively. 40  $\mu\text{M}$  Na<sub>2</sub>SO<sub>4</sub> did not affect Ni<sup>2+</sup> uptake.

by Mg<sup>2+</sup>, Co<sup>2+</sup>, Mn<sup>2+</sup>, Zn<sup>2+</sup>, and Cu<sup>2+</sup> is competitive or noncompetitive, Ni<sup>2+</sup> uptake was measured at three Ni concentrations each at a variety of inhibitor metal concentrations. The data are presented as 1/V-versus-I plots (Dixon plots) (Fig. 4). From these plots it is clear that Mg<sup>2+</sup>, Co<sup>2+</sup>, Mn<sup>2+</sup>, and Zn<sup>2+</sup> competitively inhibited Ni uptake in *B. japonicum* JH; the  $K_i$  for Ni<sup>2+</sup> uptake inhibition by Mg<sup>2+</sup>, Co<sup>2+</sup>, Mn<sup>2+</sup>, and Zn<sup>2+</sup> was 48, 22, 12, and 8  $\mu\text{M}$ , respectively. These  $K_i$ s are reasonably near the 62  $\mu\text{M}$   $K_m$  for nickel that we calculated previously for strains JH and JHK7 (9). Although Cu<sup>2+</sup> is an effective inhibitor (Table 1), the 1/V-versus-I plot showed that the inhibition was not competitive, but the type of inhibition (noncompetitive or uncompetitive) could not be clearly distinguished from our data (not shown).

**Effect of respiratory inhibitors on Ni uptake.** To determine whether Ni uptake in *B. japonicum* JH is an energy-dependent transport process, the effect of respiratory inhibitors was studied (Table 2). The cytochrome *c* oxidase inhibitors NaCN and NaN<sub>3</sub> significantly inhibited Ni uptake (68 and 75%, respectively). The ATPase inhibitor DCCD and the protonophore CCCP inhibited Ni<sup>2+</sup> uptake by 67 and 91%, respectively. The K<sup>+</sup>-H<sup>+</sup> antiporter nigericin and the electrogenic ionophore valinomycin, each with 50 mM KCl to dissipate the membrane potential, also inhibited Ni<sup>2+</sup> uptake (51 and 32%, respectively) compared with the control without inhibitor (Table 2). On the basis of these inhibitor results it seems that Ni<sup>2+</sup> uptake in *B. japonicum* JH is energy dependent. However, the addition of exogenous energy sources that stimulate respiratory activity, such as succinate or gluconate (20 mM), to the cell suspensions did not

stimulate Ni<sup>2+</sup> uptake. In fact, nickel uptake was significantly inhibited by these carbon sources (data not shown). The reason for this inhibition is unclear; perhaps succinate and/or gluconate can chelate the divalent nickel, rendering it unable to enter the cell. The inhibitors shown in Table 2 also inhibited the expression of hydrogenase activity when cells were subjected to derepression conditions (data not shown). Although other potential inhibitors and ionophores are available that mediate their effects in a manner similar to those listed in Table 2, the results obtained were quite clear, and the use of additional inhibitors was deemed to be unnecessary.

## DISCUSSION

Nickel is required by many bacteria, and its role has been studied most for the H<sub>2</sub>-oxidizing bacteria. Both Ni-specific transport and Ni transport by a magnesium transporter have been reported. Our recent study (9) showed that a Ni<sup>2+</sup>-specific transport gene was not found in *B. japonicum* by using *hoxN* (specific Ni<sup>2+</sup> transport gene) from *A. eutrophus* as a probe (6, 7). In support of the lack of such a transporter, our current results showed that hydrogenase activity decreases with the addition of MgSO<sub>4</sub> during derepression and that Ni<sup>2+</sup> transport is strongly inhibited by magnesium. The inhibition of Ni<sup>2+</sup> uptake by Mg<sup>2+</sup> was relieved by increasing the amount of Ni<sup>2+</sup> provided in the assay buffer. However, hydrogenase activity and Ni<sup>2+</sup> transport could not be inhibited completely by Mg<sup>2+</sup>, and so a low level of Ni-specific transport cannot be ruled out entirely. Nevertheless, all of the results reported here suggest that Ni<sup>2+</sup> is mainly transported in *B. japonicum* JH by a system that also transports Mg<sup>2+</sup>. In addition to Mg<sup>2+</sup>, the divalent cations Co<sup>2+</sup>, Mn<sup>2+</sup>, and Zn<sup>2+</sup> were highly inhibitory to Ni<sup>2+</sup> uptake in *B. japonicum* JH, and this inhibition is probably the result of binding of the cations to the Mg<sup>2+</sup> carrier that transports Mg<sup>2+</sup>/Ni<sup>2+</sup>. These results resemble those reported for the "major" Ni<sup>2+</sup> transport system of *A. eutrophus* (21) and for Ni<sup>2+</sup> transport in *E. coli* (16). Some inhibition of Ni<sup>2+</sup> uptake by some of these metal ions was reported for *B. japonicum* SR470 (29), although the extent of inhibition was not nearly that which we report here for strain JH. The competitive inhibition of Ni<sup>2+</sup> uptake by Co<sup>2+</sup>, Mg<sup>2+</sup>, and Mn<sup>2+</sup> in *B. japonicum* JH is similar to the metal inhibition effects reported for *E. coli* (16, 17) and (with the exception of Mn<sup>2+</sup>) *C. pasteurianum* (3) but unlike the inhibition described for *C. thermoaceticum* (22), *A. kivui* (33), and (with the exception of Co<sup>2+</sup>) *M. bryantii* (15). The competitive inhibition that we report for Zn<sup>2+</sup>-versus-Ni<sup>2+</sup> transport has been reported in only one other organism, *A. eutrophus* (21). Energy-dependent Zn<sup>2+</sup> transport systems have been reported for eucaryotic microbes; Ca<sup>2+</sup> and Mn<sup>2+</sup> are often competitive inhibitors of Zn<sup>2+</sup> uptake (14). It appears that different bacteria use quite different divalent metal-sequestering components and mechanisms, and our results suggest that highly significant differences can occur even within two *B. japonicum* strains. Strains JH and SR are derivatives of strains USDA 110 and USDA 122, respectively—strains that fall into different strain serogroups (18).

Cytochrome *c* oxidase inhibitors NaCN and NaN<sub>3</sub> strongly inhibited short-term Ni uptake. Therefore, the bulk of nickel taken up by *B. japonicum* JH is probably energy dependent. The ATPase inhibitor DCCD markedly inhibited

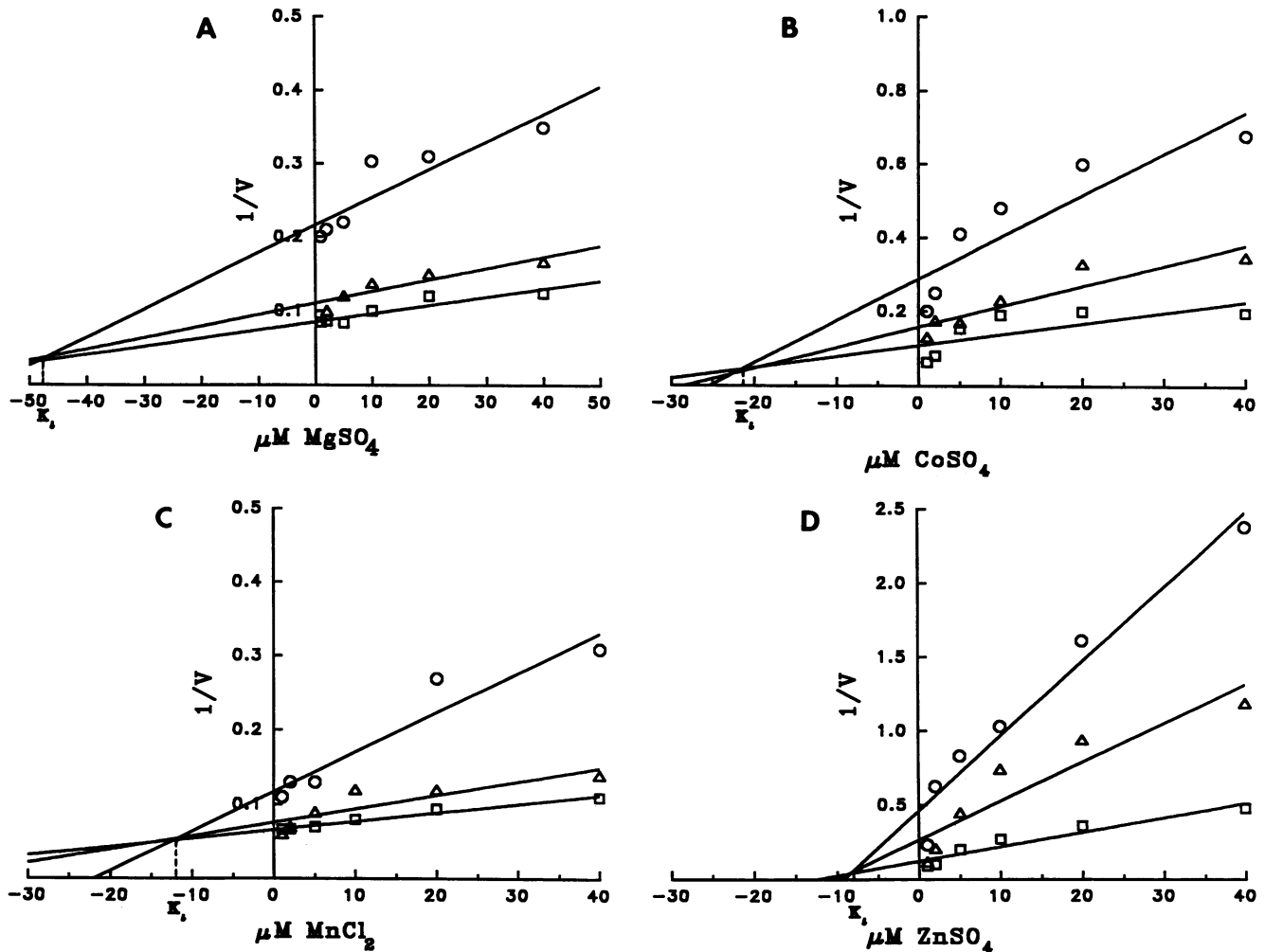


FIG. 4. Dixon plots of inhibition of Ni<sup>2+</sup> uptake by MgSO<sub>4</sub> (A), CoSO<sub>4</sub> (B), MnCl<sub>2</sub> (C), and ZnSO<sub>4</sub> (D) by strain JH with three nickel concentrations. Symbols: ○, 10 μM NiCl<sub>2</sub>; △, 25 μM NiCl<sub>2</sub>; □, 40 μM NiCl<sub>2</sub>. 1 μM <sup>63</sup>Ni (0.67 μCi/ml of cell suspension) was added to initiate each assay. The data were analyzed by linear regression and plotted by using Sigma-Plot (Sigma-Plot Scientific Graph System).

Ni uptake, suggesting that the proton motive force for ATP production via ATPase is required for Ni<sup>2+</sup> transport. The protonophore CCCP and ionophores valinomycin and nigericin also significantly inhibited Ni uptake, also suggesting that some of the nickel taken up is coupled to proton movement. These results are different from those in the report in which these inhibitors were used on *B. japonicum* SR470; it was concluded that most of the Ni<sup>2+</sup> transported was not energy generation dependent in that strain. Also, nickel uptake in *Azotobacter chroococcum* was apparently energy independent (28). On the basis of inhibitor studies, Jasper and Silver (16) demonstrated that Ni<sup>2+</sup> uptake via a high-affinity magnesium system was energy dependent. Bryson and Drake (3) also showed energy-dependent Ni<sup>2+</sup> transport (via a magnesium transporter system) in *C. pasteurianum*; DCCD, CCCP, valinomycin, and nigericin all inhibited transport of Ni<sup>2+</sup> in *C. pasteurianum*. In conclusion, Ni<sup>2+</sup> transport in *B. japonicum* JH is mainly via a magnesium transport system that also, evidently at the initial binding step(s), transports some other divalent cations. These ions are competitive inhibitors of Ni transport, and results of inhibitor studies strongly suggest that the uptake system is energy dependent.

TABLE 2. Effect of respiratory inhibitors and ionophores on Ni uptake<sup>a</sup>

Inhibitor	% Inhibition of Ni uptake <sup>b</sup>
None (control).....	0
Sodium cyanide (10 mM).....	68
Sodium azide (10 mM).....	75
DCCD (200 μM).....	67
CCCP (200 μM).....	91
Nigericin (20 μM) + KCl (50 mM).....	51 <sup>c</sup>
Valinomycin (20 μM) + KCl (50 mM).....	32 <sup>c</sup>

<sup>a</sup> Ni-free-grown cells (JH) were harvested, washed twice, and resuspended in 50 mM MES buffer (pH 6.0). The inhibitor was added to the flask containing 5 ml of cells, and the mixture was incubated for 30 min at 30°C, with shaking at 150 rpm; 1 μM <sup>63</sup>Ni (0.67 μCi/ml of cell suspension) was added, and the suspension was incubated again for 5 min. Samples were then taken out and filtered.

<sup>b</sup> The data represent the percent inhibition of nickel uptake on the basis of three or four replicates relative to the control cells without inhibitor. The nickel uptake rate of the control cells was 1.2 pmol of <sup>63</sup>Ni per min per 10<sup>8</sup> cells.

<sup>c</sup> The slight effect of KCl (50 mM) alone on Ni<sup>2+</sup> uptake was subtracted.

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