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Nickel Availability to Pea (*Pisum sativum* L.) Plants Limits Hydrogenase Activity of *Rhizobium leguminosarum* bv. Viciae Bacteroids by Affecting the Processing of the Hydrogenase Structural Subunits

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***Rhizobium leguminosarum* bv. viciae UPM791 induces the synthesis of an [NiFe] hydrogenase in pea (*Pisum sativum* L.) bacteroids which oxidizes the H_2 generated by the nitrogenase complex inside the root nodules. The synthesis of this hydrogenase requires the genes for the small and large hydrogenase subunits (*hupS* and *hupL*, respectively) and 15 accessory genes clustered in a complex locus in the symbiotic plasmid. We show here that the bacteroid hydrogenase activity is limited by the availability of nickel to pea plants. Addition of Ni^{2+} to plant nutrient solutions (up to 10 mg/liter) resulted in sharp increases (up to 15-fold) in hydrogenase activity. This effect was not detected when other divalent cations (Zn^{2+}, Co^{2+}, Fe^{2+}, and Mn^{2+}) were added at the same concentrations. Determinations of the steady-state levels of *hupSL*-specific mRNA indicated that this increase in hydrogenase activity was not due to stimulation of transcription of structural genes. Immunoblot analysis with antibodies raised against the large and small subunits of the hydrogenase enzyme demonstrated that in the low-nickel situation, both subunits are mainly present in slow-migrating, unprocessed forms. Supplementation of the plant nutrient solution with increasing nickel concentrations caused the conversion of the slow-migrating forms of both subunits into fast-moving, mature forms. This nickel-dependent maturation process of the hydrogenase subunits is mediated by accessory gene products, since bacteroids from H_2 uptake-deficient mutants carrying Tn*5* insertions in *hupG* and *hupK* and in *hypB* and *hypE* accumulated the immature forms of both hydrogenase subunits even in the presence of high nickel levels.**

Most hydrogen uptake hydrogenases are membrane-bound, heterodimeric iron-sulfur proteins containing nickel ([NiFe] hydrogenases) (for reviews, see references 9, 37, and 46). Bacteria forming nodules on legume roots synthesize uptake hydrogenases which recycle the H_2 evolved by nitrogenase in the nodules (5, 27) and contribute to the overall efficiency of the N_2 fixation process (6). These bacteria include *Rhizobium leguminosarum* bv. viciae and *Bradyrhizobium japonicum*, the microsymbionts of peas and soybeans, respectively. The genetic determinants for H_2 uptake (*hup* genes) in *R. leguminosarum* bv. viciae UPM791 are clustered in a 20-kb DNA region of the symbiotic plasmid and have been isolated in cosmid pAL618 (24). This cosmid has the capacity to confer H_2 uptake activity on Hup^- strains of *R. leguminosarum* bv. viciae and *Rhizobium etli* in symbiosis with peas and beans, respectively (25). The DNA region spanning the H_2 uptake gene cluster has been sequenced, and 17 genes, closely linked and oriented in the same direction, were identified (15, 38, 40). The first two genes, *hupS* and *hupL*, encode the hydrogenase structural polypeptides (14). The predicted proteins for the small (360 amino acid residues, including a leader peptide of 45 residues) and the large (596 amino acid residues) subunits are homologous to the corresponding hydrogenase structural proteins from *B. japonicum* (over 87% identical) and other bacteria. The precise molecular functions of the remaining 15 genes, named *hupCDEFGHIJK* and *hypABFCDE*, are unknown, although analysis of Tn*5* insertion mutants indicates that they are required for the synthesis of an active hydrogenase (14, 38, 40). Besides the hydrogenase structural genes, accessory genes homologous to the *R. leguminosarum hup* and *hyp* genes have also been identified in other bacteria (see references 9 and 46 for reviews).

The analysis of purified, active [NiFe] hydrogenases from different bacteria has revealed that both hydrogenase subunits undergo posttranslational modifications during the synthesis of the active enzyme. It has been shown in *Escherichia coli* that the processing of the small subunit involves the removal of the N-terminal signal peptide (7). Processing of the hydrogenase large subunit has been studied in *Azotobacter vinelandii* (13) and *Desulfovibrio gigas* (31). These studies demonstrate that the large subunit undergoes proteolytic cleavage that removes a 15- to 16-amino-acid-residue peptide from the C terminus. The occurrence of these two types of processing in hydrogenases from other bacteria has been shown by immunoblot analysis of the hydrogenase subunits (11, 21, 32).

Nickel is an essential constituent of the active center of [NiFe] hydrogenases. It is apparently bound to the large hydrogenase subunit, which contains the site for H_2 activation (37). A requirement for nickel for hydrogenase activity has been shown for different bacteria (8, 20, 35, 45). In *B. japonicum* symbiotic cells, small increases in hydrogenase activity were observed in response to nickel additions to soybean plants (2, 19). On the other hand, very little is known about how nickel is incorporated into hydrogenase. Two different

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TABLE 1. *R. leguminosarum* bv. viciae strains used in this study

Strain	Relevant characteristics[a]	Reference
128C53	Wild type, Hup^+ on peas	41
UPM791	128C53 Str^r	24
AL1	UPM791 *hypE*::Tn*5*	40
AL18	UPM791 *hypB*::Tn*5*	40
AL25	UPM791 *hupG*::Tn*5*	38
AL26	UPM791 *hupK*::Tn*5*	38
AL51	UPM791 *hupS*::Tn*5*	15

[a] Str^r denotes resistance to streptomycin.

mechanisms have been described for nickel uptake in aerobic H_2-oxidizing bacteria: a high-affinity system specific for Ni^{2+}, and a nonspecific, low-affinity Mg^{2+} transporter (9). In heterotrophically grown *B. japonicum* cells, nickel accumulates in soluble proteins, and it is not exchangeable with nickel in the medium (28). Several loci have been shown to be involved in internal nickel metabolism in *B. japonicum* (10), *Alcaligenes eutrophus* (22), and *E. coli* (17, 29, 32), and mutations in these loci apparently affect the processing of hydrogenase subunits.

We show in this work that nickel availability to pea plants grown on standard, unpurified nutrient solution limits the hydrogenase activity of *R. leguminosarum* bv. viciae bacteroids and that the nickel deficiency blocks the maturation of both hydrogenase subunits by affecting a process which involves the participation of several *hup* and *hyp* gene products.

MATERIALS AND METHODS

Chemicals and enzymes. The chemicals used as constituents in culture media and plant nutrient solutions were of analytical grade. Radioactive [α-^{32}P]dATP (specific activity, 3,000 Ci/mmol) used for DNA labeling was obtained from Amersham International, Amersham, United Kingdom. Reagents and enzymes used for immunological detections were purchased from Bio-Rad Laboratories Inc., Hercules, Calif.

Bacterial strains, plasmids, and growth conditions. The *R. leguminosarum* bv. viciae strains used in this work are listed in Table 1. Cosmid pAL618 is a pLAFR1 derivative containing the hydrogenase gene cluster from strain UPM791 in a DNA insert of 20 kb (24). This cosmid was introduced into UPM791 by triparental mating as previously described (25). Strains were routinely grown on yeast mannitol medium (25) at 28°C.

Plant tests and hydrogenase activities. The conditions for inoculation of pea (*Pisum sativum* L. cv. Frisson) seedlings with *R. leguminosarum* bv. viciae strains have been described previously (24). Plants were grown in Leonard-type assemblies placed in bacteriologically controlled conditions as described by Ruiz-Argüeso et al. (41). The nitrogen-free nutrient solution used contained (per liter) KCl, 74 mg; K_2HPO_4, 175 mg; $MgSO_4 \cdot 7H_2O$, 246 mg; $CaSO_4$, 345 mg; Fe(III) citrate, 1.8 mg; $MnSO_4 \cdot 7H_2O$, 1 mg; H_3BO_3, 700 μg; $ZnSO_4 \cdot 5H_2O$, 110 μg; $CuSO_4 \cdot 5H_2O$, 39 μg; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 5 μg. The level of contaminating nickel in the solution was shown to be less than 0.1 mg/liter, as determined with a Perkin Elmer 2380 atomic absorption spectrophotometer equipped with a nickel-specific Intensitron lamp (Perkin Elmer Co., Norwalk, Conn.). When indicated, Ni^{2+} or other cations were incorporated into the plant nutrient solution as chloride salts 10 days after inoculation of the seedlings.

Hydrogen evolution, acetylene reduction, and relative efficiency (relative efficiency = 1 − [H_2 evolved/C_2H_2 reduced]) were determined in pea nodules as previously described (41). Pea bacteroids were prepared from 24-day-old nodulated plants and analyzed amperometrically for H_2 uptake hydrogenase activity (41). The protein contents of bacteroid suspensions were determined by the bicinchoninic acid method (43) after alkaline digestion of cells at 90°C in NaOH for 10 min, with bovine serum albumin as the standard.

Purification of RNA and dot blot analysis. The conditions for RNA extraction from bacteroids and dot blot hybridization with radioactively labeled DNA probes have been described previously (34). The DNA fragment used as the probe was a 0.9-kb *Sal*I-*Eco*RI restriction fragment internal to *hupL* (14).

Western (immunoblot) analysis. Proteins from bacteroid suspensions were denatured and resolved by standard discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (23) performed on 10% acrylamide gels. Proteins were then electrophoretically transferred into polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, Mass.) with 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11, as the transfer buffer and assayed for immunoreactivity against hydrogenase large- and small-subunit antibodies by standard protocols (42). Filters were developed with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) and nitro blue tetrazolium reagents as recommended by the manufacturer. The molecular masses of immunoreactive bands were estimated by comparison with low-range prestained SDS-PAGE size standards purchased from Bio-Rad, including phosphorylase B (apparent molecular mass, 106,000 Da), bovine serum albumin (80,000 Da), ovalbumin (49,500 Da), carbonic anhydrase (32,500 Da), soybean trypsin inhibitor (27,500 Da), and lysozyme (18,500 Da).

For the generation of antibodies against *R. leguminosarum* bv. viciae hydrogenase small subunit, an *Xmn*I restriction fragment internal to *hupS* (corresponding to the DNA encoding amino acid residues 51 to 225) was cloned in frame into the expression vector pUEX2 (1). The resulting plasmid, designated pUES69, was used to overproduce the β-galactosidase-ΔHupS chimeric protein in *E. coli*. This protein was subsequently resolved by SDS-PAGE, extracted from the gel, and used for rabbit immunization. The final antiserum obtained was used at a 1:1,000 dilution. Antibodies against the large subunit of *B. japonicum* hydrogenase were a gift from R. J. Maier (Johns Hopkins University, Baltimore, Md.) and used at a 1:2,000 dilution.

RESULTS

Hydrogenase activity of pea bacteroids depends on nickel availability to the plants. Since nickel is required for hydrogenase activity in free-living cells of different species, we decided to investigate whether this element could be limiting the hydrogenase activity of pea bacteroids. To this aim, pea plants inoculated with *R. leguminosarum* bv. viciae Hup^+ strain UPM791 were exposed to increasing Ni^{2+} concentrations (up to 100 mg/liter) in the nutrient solutions, and the hydrogenase activities of bacteroids prepared from plants grown under these conditions were determined (Table 2). A clear response of H_2 uptake rates to nickel addition was observed when either O_2 or methylene blue (MB) was used as the terminal electron acceptor. The addition of 10 mg of Ni^{2+} per liter caused a sevenfold increase in O_2-dependent hydrogenase activity and a 15-fold increase in hydrogenase activity with MB. These activity levels were maintained even in bacteroids from plants grown with 100 mg of Ni^{2+} per liter, which severely affected plant growth (data not shown). Since MB is supposed to receive electrons directly from the hydrogenase enzyme, the much higher rates of H_2 uptake with this electron acceptor

TABLE 2. Hydrogenase activities of *R. leguminosarum* bv. viciae from nodulated pea plants grown on nutrient solutions supplemented with different amounts of nickel[a]

Ni added (mg/liter)	Hydrogenase activity (nmol of hydrogen/h/mg of protein)			
	UPM791		UPM791(pAL618)	
	O_2	MB	O_2	MB
0	1,620	1,920	4,500	7,500
0.1	1,700	2,020	5,280	16,080
1	5,140	12,000	8,690	31,034
10	11,300	28,800	13,330	66,670
100	7,580	20,500	9,900	40,800

[a] Hydrogenase activities were determined with oxygen (O_2) or MB as the terminal electron acceptor. Values are the averages for at least two determinations from independent bacteroid preparations.

indicate that at high nickel concentrations, the electron transfer to oxygen, not the amount of active hydrogenase enzyme, is the limiting factor for H_2 oxidation in bacteroids.

Nodules induced by strain UPM791 in plants grown with no added nickel still evolved low amounts of hydrogen, resulting in a relative efficiency of approximately 0.8. In contrast, the supplementation of plant nutrient solution with 10 ppm of Ni^{2+} resulted in nodules which evolved no detectable amounts of hydrogen (relative efficiency = 1), indicating that under such conditions, all the hydrogen evolved by nitrogenase was recycled.

The effect of nickel on the hydrogenase activity of pea bacteroids was higher when the number of copies of the *hup* and *hyp* genes was increased, as is the case in the merodiploid strain UPM791(pAL618) (Table 2). This strain contains about 15 copies of cosmid pAL618 (34). Interestingly, the presence of pAL618 enhanced hydrogenase activity even at the lowest nickel concentrations, suggesting that extra copies of *hup* and *hyp* genes increase the efficiency of nickel utilization in the bacteroids.

We also investigated whether other metals chemically related to Ni^{2+} could substitute for nickel in hydrogenase processing. The addition of Fe^{2+}, Co^{2+}, Zn^{2+}, or Mn^{2+} to the plant nutrient solutions at concentrations equivalent to 1 and 10 mg/liter (17 and 170 μM) caused no significant increases in the hydrogenase activity of pea bacteroids (Table 3). These results indicate that the observed effect of Ni^{2+} on the hydrogenase activity of pea bacteroids was specific for this cation. The addition of Co^{2+} led to reduced hydrogenase activity (ca. 50% reduction), suggesting an interference of this cation with nickel metabolism for hydrogenase.

TABLE 3. Relative hydrogenase activities of *R. leguminosarum* bv. viciae UPM791 bacteroids as a function of divalent cations added to the plant nutrient solution[a]

Cation	Hydrogenase activity (% of control) at cation concn:	
	17 μM	170 μM
Ni^{2+}	317	697
Zn^{2+}	123	156
Mn^{2+}	108	122
Co^{2+}	60	56
Fe^{2+}	121	121

[a] Hydrogenase activities were measured with oxygen as the terminal electron acceptor. Values are expressed as the percentage of the hydrogenase activity of bacteroids from pea plants grown on standard nutrient solution (1,600 nmol/h/mg of protein).

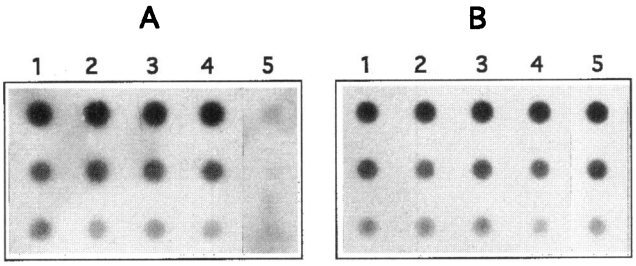

FIG. 1. Dot blot analysis of transcription of hydrogenase structural genes. (A) Autoradiogram of a dot blot filter containing serial 1:1 dilutions (1 μg on top row) of total RNA from *R. leguminosarum* bv. viciae UPM791 pea bacteroids prepared from plants exposed to different amounts of Ni^{2+} in the plant nutrient solution. A 0.9-kb *Eco*RI-*Sal*I DNA fragment internal to *hupL* was used as the probe. Lanes 1 to 4, bacteroids from plants supplemented with 0, 1, 10, and 100 mg of Ni^{2+} per liter, respectively; lane 5, RNA from mutant AL51, included as a negative control. (B) Same filter hybridized to UPM791 total DNA.

Transcription of hydrogenase structural genes is not affected by nickel addition to the plants. To test whether the observed effect of nickel addition was due to stimulation of *hupSL* transcription in pea bacteroids, the steady-state level of *hupL*-specific mRNA was measured semiquantitatively by dot blot analysis of total RNA from UPM791 bacteroids with a 0.9-kb DNA fragment internal to *hupL* as the probe. Bacteroids were obtained from plants grown with nutrient solutions containing increasing concentrations of nickel. As shown in Fig. 1, no significant changes in *hupL*-specific mRNA levels were observed in bacteroids from plants grown with no nickel added compared with those from plants grown in solutions containing 1, 10, or 100 mg of Ni^{2+} per liter. These results indicate that the increase in hydrogenase activity in response to the addition of Ni^{2+} to plants was not due to stimulation of transcription of hydrogenase structural genes.

Nickel is involved in processing of both hydrogenase subunits in pea bacteroids. Since no effect of nickel addition on *hupSL* transcription was detected, we investigated whether the observed increases in hydrogenase activity corresponded to variations in the levels of hydrogenase subunit proteins. For this purpose, we performed an immunoblot analysis of bacteroids with antibodies generated against the *R. leguminosarum* bv. viciae hydrogenase small subunit (see Materials and Methods) and antibodies raised against the *B. japonicum* hydrogenase large subunit, which were previously shown to be cross-reactive with the homologous polypeptide from *R. leguminosarum* bv. viciae (25).

Bacteroids from nodules produced by strain UPM791 in pea plants grown with no nickel added yielded two prominent specific bands of ca. 66 and 65 kDa that were immunoreactive against the hydrogenase large-subunit antiserum (Fig. 2A, lane 1), the larger band containing most of the reactive material. The intensities of both bands were greatly amplified in bacteroids from the merodiploid strain UPM791(pAL618), containing extra copies of the structural genes (Fig. 2A, lane 3). Again, most of the reactive material was associated with the larger band. Two additional immunoreactive bands of ca. 40 and 27 kDa were clearly observed in bacteroids from this strain. These bands were hardly seen in bacteroids from strain UPM791 (Fig. 2A, lane 1) and may represent degradation products of the larger 66-kDa form (see below). Finally, another band of

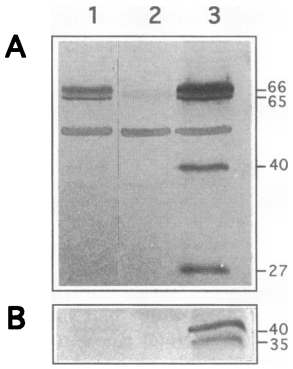

FIG. 2. Immunological detection of hydrogenase structural subunits. Western blots of crude extracts from pea bacteroids (20 to 30 μg of total protein) were developed with antibodies raised against the large (A) and small (B) hydrogenase subunits. *R. leguminosarum* bv. viciae strains: UPM791 (lane 1), AL51 (lane 2), and UPM791 (pAL618) (lane 3). The molecular masses (in kilodaltons) of relevant bands are shown to the right.

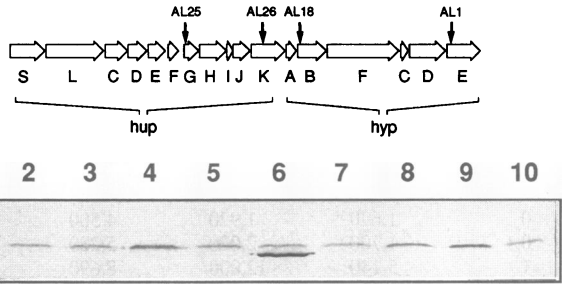

FIG. 4. Western blot analysis of hydrogenase large subunit from pea bacteroids produced by *R. leguminosarum* bv. viciae *hup* and *hyp* mutants. The location of the Tn*5* insertion in each mutant is shown over the genetic map of the *hup/hyp* cluster. Pea plants were grown on standard nutrient solutions (lanes 1 to 5) or on nutrient solutions supplemented with 10 mg of Ni^{2+} per liter (lanes 6 to 10). Strains: wild-type UPM791 (lanes 1 and 6) and mutants AL25 (lanes 2 and 7), AL26 (lanes 3 and 8), AL18 (lanes 4 and 9), and AL1 (lanes 5 and 10).

ca. 50 kDa was observed, although it was considered nonspecific because it was also present in the hydrogenase-deficient mutant AL51 used as a negative control (Fig. 2A, lane 2).

When similar immunoblots were developed with antibodies against the small subunit, two specific bands of about 40 and 35 kDa were identified in the UPM791 bacteroids, although the signal obtained was much weaker than those obtained with the large-subunit antiserum (Fig. 2B, lane 1). These two bands were more evident in the merodiploid strain (Fig. 2B, lane 3), for which most of the signal was associated with the larger band. The lower level of immunoreactive material detected with the small-subunit antibodies may be due to differences in the degree of sensitivity between the two antisera or to a greater susceptibility to proteolysis of this polypeptide, as has been observed by others (33).

The addition of Ni^{2+} to plant nutrient solutions caused a similar pattern of response in the major proteins recognized by each of the antisera used (Fig. 3): a gradual increase in the intensity of the smaller bands (65 and 35 kDa), along with elimination of the larger ones (66 and 40 kDa). This suggests a nickel-dependent conversion of the large into the small forms of both subunits. In bacteroids from pea nodules exposed to 10 mg/liter or higher concentrations of Ni^{2+}, which exhibited maximal hydrogenase activities (Table 2), the fastest forms of both subunits accounted for most of the signal. From the correlation between the accumulation of these forms and hydrogenase activity in pea nodules, we conclude that the fast-migrating peptides of 65 and 35 kDa correspond to mature forms of the large and small subunits, respectively, of the *R. leguminosarum* bv. viciae active hydrogenase enzyme and that these forms arise from larger, unprocessed forms by nickel-dependent proteolytic processing.

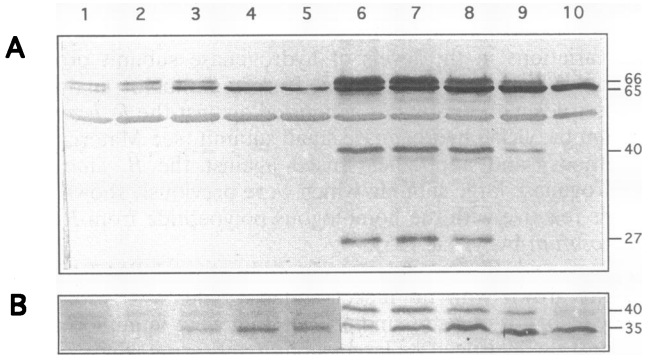

FIG. 3. Effect of nickel on the processing of hydrogenase structural subunits. Crude extracts of pea bacteroids (20 to 30 μg of total protein) from plants grown on nutrient solutions containing different nickel concentrations were subjected to Western blot analysis with antibodies raised against the large (A) and small (B) hydrogenase subunits. *R. leguminosarum* bv. viciae strains UPM791 (lanes 1 to 5) and UPM791 (pAL618) (lanes 6 to 10) were used as inocula for pea plants. Nickel additions (in milligrams of Ni^{2+} per liter): 0 (lanes 1 and 6), 0.1 (lanes 2 and 7), 1 (lanes 3 and 8), 10 (lanes 4 and 9), and 100 (lanes 5 and 10). The molecular masses (in kilodaltons) of relevant bands are shown to the right.

Pea bacteroids from merodiploid strain UPM791(pAL618) contained in all cases higher amounts of total hydrogenase proteins and, remarkably, higher amounts of the processed forms of both subunits than bacteroids from strain UPM791 (Fig. 3). Furthermore, this increase in processing of the hydrogenase polypeptides occurred even in bacteroids from plants grown at the lowest nickel concentrations, suggesting a dosage effect of one or more of the *hup* and *hyp* genes on the efficiency of nickel utilization.

The intensities of the bands of 27 and 40 kDa that were reactive with the large-subunit antiserum decreased in response to the addition of nickel, as did the unprocessed form of the large subunit, and such bands were not detected when this form was not observed (Fig. 3A, lanes 6 to 10). These results, together with the fact that the sum of their molecular masses roughly equals the molecular mass of the unprocessed large subunit, strongly support the idea that the 27- and 40-kDa bands are the result of proteolytic cleavage of this unprocessed form.

Accessory gene products are required for hydrogenase processing. Pea bacteroids produced by *R. leguminosarum* bv. viciae UPM791 derivative mutants AL25, AL26, AL18, and AL1, carrying genomic Tn*5* insertions in *hupG*, *hupK*, *hypB*, and *hypE*, respectively (38, 40), lack hydrogenase activity (25). When bacteroids from pea plants nodulated by these mutants under nickel-limited conditions were examined for the presence of the hydrogenase large subunit by immunoblot analysis, only a single immunoreactive band was detected (Fig. 4, lanes 2 to 5). This band comigrated with the larger, unprocessed polypeptide observed in bacteroids from the wild-type strain

(Fig. 4, lane 1). Furthermore, bacteroids produced by these mutants in plants grown in the presence of high Ni^{2+} concentration also yielded only the unprocessed forms of the hydrogenase large subunit (Fig. 4, lanes 7 to 10). Similarly, no processed forms of the small subunit were detected when bacteroids from these mutants were analyzed with the corresponding antiserum (data not shown). These results imply that the *R. leguminosarum* bv. viciae mutant strains examined are able to transcribe and translate the hydrogenase structural genes at levels at least similar to those found in the wild-type strain but are unable to process the hydrogenase subunits even when supplied with large amounts of nickel. Taking into account that the Tn*5* insertions in AL25, AL26, and AL18 and AL1 map in different transcriptional units (25), it can be concluded that several accessory genes, at least one from each of the clusters *hupGHIJ*, *hupK hypA*, and *hypBFCDE*, are involved in the nickel-dependent processing of pea bacteroid hydrogenase subunits.

DISCUSSION

The data presented in this work show that nickel availability to pea plants grown in nitrogen-free nutrient solutions limits the hydrogenase activity of pea bacteroids from *R. leguminosarum* Hup^+ strains. The incorporation of up to 10 mg of Ni^{2+} per liter into the plant nutrient solution increased the rate of MB-dependent H_2 oxidation of the bacteroids up to 15-fold. These results imply that bacteroids from pea plants not supplemented with Ni^{2+} receive inadequate amounts of this element to support maximal hydrogenase activities. This is remarkable since the plant nutrient solutions were not purified to minimize the nickel contamination in the chemicals used to prepare the solutions. Under these conditions, additional sources of nickel might come from the plant growth support material (vermiculite) and from the seeds. Our observation of the stimulating effect of nickel on the hydrogenase activity of pea bacteroids is relevant because nickel availability in soils may be severely limiting the hydrogenase activity of pea plants in field conditions. This possibility is currently being investigated in our laboratory.

The levels of hydrogenase activity observed in conditions of no Ni^{2+} supplementation correspond well with the levels of hydrogenase activity previously reported for Hup^+ strains of *R. leguminosarum* bv. viciae (25, 36), indicating that the potential for hydrogenase activity of this species has been underestimated in the past. Nickel-dependent stimulation of the activity of the nickel-containing enzymes urease and hydrogenase has been reported previously for soybeans grown in low-nickel soil (2). However, in this case, the greatest increase in hydrogenase activity in response to nickel addition was only 45%. Similarly, the addition of nickel to soybean plants grown hydroponically in nutrient solutions purified to minimize nickel contamination led to small increases in hydrogenase activity (19). These data suggest that the availability of nickel to plants is less limiting for hydrogenase activity in soybeans than in peas. Variations in the nickel requirement for hydrogenase activity among different legume-bacteria combinations must be due either to differences in the ability of plants to provide nickel to the bacteroids or to differences in the nickel transport capacity of bacteroids. Metal metabolism in plants is a poorly understood area of plant physiology. Particularly, it is not known how nickel is taken up by the plant roots and transported into the nodules or how nickel crosses the peribacteroid membrane. Studies on xylem fluids suggest that in plants, nickel is transported complexed with organic acids like citrate or malate (4). Plant proteins may be specifically involved in a mechanism of nickel provision to the bacteroids, and plants may differ in the efficiency of this mechanism. In this regard, it is relevant that soybean mutant plants that are unable to synthesize active urease have been suggested to be deficient in the provision of nickel for hydrogenase synthesis to *Methylobacterium mesophilum*, an inhabitant of their phyllosphere (16).

Regarding nickel metabolism in bacteria, the limitation of hydrogenase activity by nickel in standard (i.e., unpurified for nickel) media is not a general rule. The demonstration of nickel-mediated stimulation of hydrogenase activity in free-living cells has always required prior removal of contaminating nickel, either by passing the culture media through CPG-8-hydroxyquinoline to remove divalent cations (3, 45) or by using metal chelators (8, 30, 35). With the former system, the recovery of full hydrogenase activity was observed at Ni^{2+} concentrations in the nanomolar range. This suggests that these systems are much more efficient than the *R. leguminosarum* bv. viciae bacteroids for the provision of nickel to hydrogenase. Although the nickel transport systems of different bacteria have been studied in detail (26, 35, 44), no specific studies have been carried out with either free-living or symbiotic cells of *R. leguminosarum* bv. viciae.

Our data support the idea that the effect of nickel on the hydrogenase activity of pea bacteroids is mainly due to the role of this cation in the processing of hydrogenase subunits, and this constitutes the first report on nickel-dependent hydrogenase processing in endosymbiotic cells. By using antibodies raised against both hydrogenase subunits, it was shown that bacteroids from plants grown with no nickel added contained two differently migrating forms of both the large and small subunits (Fig. 2). Two lines of evidence suggest that these forms correspond to the precursor and mature states of each subunit. First, an N-terminal signal peptide was predicted from the sequence analysis of *R. leguminosarum* bv. viciae HupS (14), and HupL from this species (14) contains the consensus DPCxxCxxHV motif present in cases where C-terminal processing has been demonstrated (30). The molecular masses of the slow-migrating forms (ca. 40 and 66 kDa) correspond well with the predicted sizes of gene products from *hupS* and *hupL* (39,153 Da and 66,115 Da, respectively), whereas the molecular masses of the fast-migrating forms are consistent with the predicted sizes of products arising from removal of the signal peptide from the small subunit (34,369 Da) and from the cleavage of a 15-amino-acid-residue peptide from the C terminus of large-subunit precursor (64,382 Da). Second, the nickel-dependent conversion of the slower into the faster forms parallels the increase in hydrogenase activity of the bacteroids. Two forms of both subunits have also been described for the membrane-bound hydrogenases of *Alcaligenes eutrophus* (21), *E. coli* (32), free-living cells of *B. japonicum* (11), and *Azotobacter vinelandii* (30). We also observed here (Fig. 3A) that pea bacteroids containing high levels of the unprocessed form of the large subunit also contain two immunologically related bands of 40 and 27 kDa, which have been interpreted as degradation products. It is tempting to speculate that these two polypeptides are originated by a specific proteolytic activity acting at low efficiency on the unprocessed form of the hydrogenase large subunit. This activity could be due to the same protease which processes the large subunit by recognizing a different site in the protein under nickel-deficient conditions, which could lead to abnormal folding of the protein.

The lack of significant differences in the amount of *hupL*-specific mRNA in response to nickel supplementation indicates that the observed stimulation of hydrogenase activity in pea bacteroids is not due to an effect on transcription of

hydrogenase structural genes. Similarly, in *Azotobacter vinelandii*, the triggering of nickel-dependent stimulation of hydrogenase activity is not inhibited by the addition of rifampin (30). An increase in hydrogenase activity in response to nickel additions to culture media has been observed for *B. japonicum* (45), *Azotobacter vinelandii* (30), *Alcaligenes eutrophus* (8), *Alcaligenes latus* (3), and *Rhodospirillum rubrum* (20). In most cases, these authors concluded that nickel had a stimulatory effect on protein synthesis. We also observed an increase in hydrogenase protein levels in response to nickel addition to pea plants (Fig. 3). However, the effect does not seem to be due to an increase in hydrogenase protein synthesis but rather to stabilization of hydrogenase proteins mediated by nickel. This conclusion is based on the observation that in pea bacteroids, the presence of degradation products from the large subunit is inversely correlated with the availability of nickel to the pea plants. No such degradation products are observed when the large subunit is fully processed, suggesting that the unprocessed form is less stable than the mature form. Degradation of abnormal proteins is a well-documented phenomenon in *E. coli* (12), and in *B. japonicum*, the small subunit has been shown to be sensitive to proteolysis (33). A rapid degradation of hydrogenase subunits, rather than an effect on hydrogenase synthesis, has also been suggested to explain the effect of nickel on hydrogenase from *Alcaligenes latus* (3). If that were the case, then the dependence of nickel-mediated stimulation of hydrogenase activity on protein synthesis observed by different authors (8, 20, 30) is likely due to replacement of degraded immature protein rather than to an increase in the rate of protein synthesis. Nevertheless, an effect of nickel on the transcription of hydrogenase genes in pea bacteroids at lower levels than those usually available to bacteroids cannot be discarded. In fact, low levels of nickel have been shown to activate transcription of hydrogenase structural genes in free-living *B. japonicum* cells in media severely depleted of nickel (18).

The nickel-dependent maturation process of the hydrogenase structural polypeptides observed in pea bacteroids involves several *hup* and *hyp* gene products, since hydrogenase-defective mutants carrying polar Tn*5* insertions in *hupG*, *hupK*, *hypB*, and *hypE* did not process the large subunit and accumulated the precursors in the cell (Fig. 4). Several observations point to a role for the *hyp* genes in nickel metabolism. The product of *hypB* has been shown to catalyze some step in nickel metabolism in *E. coli* (17), and *hypB* mutants are unable to process the *hycE* gene product (hydrogenase 3) (29). Moreover, *hyp* mutants of *Alcaligenes eutrophus* synthesize an inactive hydrogenase enzyme lacking nickel (21). In *B. japonicum*, a locus involved in nickel metabolism, which may correspond to the homologous *hyp* genes in this bacterium, has been identified (10). Finally, the HypB protein from *R. leguminosarum* bv. viciae has been purified and shown to bind nickel (39).

The incorporation of nickel into the apoenzyme complex may not be the only requirement for processing of the subunits. The incorporation of other metal clusters into the enzyme and the assembly of the enzyme into the membrane are candidate functions for some of the *hup* and *hyp* proteins (9). A complete model describing the different steps leading to an active hydrogenase enzyme may not emerge until the specific molecular role of each of the accessory gene products is understood.

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