

Rhizobium leguminosarum Biovar viciae Symbiotic Hydrogenase Activity and Processing Are Limited by the Level of Nickel in Agricultural Soils

Ana-Claudia Ureta,¹ Juan Imperial,^{1,2} Tomás Ruiz-Argüeso,¹ and Jose M. Palacios^{1*}

Department of Biotechnology, Universidad Politécnica de Madrid,¹ and C.S.I.C., Ciudad Universitaria s/n,² 28040 Madrid, Spain

Received 15 March 2005/Accepted 16 June 2005

Analysis of levels of hydrogenase processing and activity in *Rhizobium leguminosarum* biovar viciae bacteroids from pea (*Pisum sativum*) plants showed that the oxidation of nitrogenase-evolved hydrogen is limited by the availability of nickel in agricultural soils. This limitation was overcome by using an inoculant strain engineered for higher hydrogenase expression.

The efficiency of nitrogen fixation by the *Rhizobium*-legume symbiosis is affected by the hydrogen released by nitrogenase during this process (14). In bacteroids induced in pea (*Pisum sativum* L.) nodules by *Rhizobium leguminosarum* biovar viciae UPM791 the hydrogen generated in the nitrogen fixation process is recycled by a [NiFe] hydrogenase with two structural subunits (HupL and HupS). The biosynthesis of this hydrogenase is a complex process that involves at least 15 accessory proteins, some of which participate in the insertion of an essential nickel atom in the active site of the enzyme (16). This hydrogen recycling has been associated with significant increases of plant productivity in certain symbiotic systems such as soybean (2, 8).

Ni availability to pea bacteroids limits hydrogenase activity in plants grown under gnotobiotic (i.e., sterile vermiculite) conditions by affecting the Ni-dependent processing of hydrogenase subunits (5). Further work has demonstrated that this limitation also affects other symbiotic systems (4). In this work we analyze whether the available Ni levels in agricultural soils are sufficient for synthesis of adequate levels of hydrogenase for efficient hydrogen recycling.

Ni concentration in the soil depends on the parent material, the degree of contamination and the pedogenic process. The soil content of Ni is variable; values reported for total nickel typically range from 1 mg kg⁻¹ to over 400 mg kg⁻¹ in normal agricultural soils (1), with lower values usually associated with acid soil conditions. Ni is essential for some biological functions, such as urease synthesis in soybean leaves (11), and a critical deficiency of nickel has been defined for the growth of plants in urea nitrogen (9).

In order to ascertain whether Ni content in soils could indeed affect hydrogen oxidation by the *Rhizobium*-legume symbiosis, we decided to study hydrogen metabolism in pea bacteroids from plants grown in different soils. To this aim, six soils representative of the main agricultural areas in Madrid

Province were collected and analyzed for relevant physico-chemical traits by standard methods (15). The concentration of metallic elements Ni, Co, and Mn was determined by inductively coupled plasma-mass spectrometry (MS) following extraction with nitric acid (total content) or with ammonium oxalate (available content; 17). Inductively coupled plasma-MS analysis was performed using an Elan 6000 instrument (Perkin Elmer Sciex) at the Universidad Autónoma de Madrid core facility. The levels of Ni found in soils were variable (Table 1): the lowest value was associated with acid soil 3 (0.08 mg kg⁻¹ of total nickel), whereas the highest total nickel level was 3 mg kg⁻¹ (soil 5). The levels of available Ni ranged from 0.03 to 0.68 mg kg⁻¹ (soils 3 and 2, respectively). The concentration of Co and Mn showed essentially the same pattern as Ni in the soils analyzed.

The soil material was mixed (1:1) with quartz sand and used as the substrate for growing pea plants (*Pisum sativum* cv. Frisson) in a growth chamber (12). Seeds were surface sterilized and seedlings were inoculated with *Rhizobium* cultures as described previously (12). Plants were watered with a standard N-free nutrient solution (5) or with the same solution supplemented with different amounts of nickel, added as nickel chloride. Symbiotic H₂ metabolism was studied by determining the levels of H₂ evolution and acetylene reduction of nodules, and hydrogenase activity of bacteroids (4). In order to monitor the prevalence of the inoculated strain in the nonsterile substrate where plants were grown, we used *R. leguminosarum* biovar viciae strain UPM1156, a UPM791 (12) derivative carrying a constitutively expressed *gusA* gene inserted downstream of hydrogen oxidation genes obtained in our lab (C. Fernández et al., unpublished data), as inoculum. The percentage of nodule occupancy by the inoculated strain was determined by scoring for β-glucuronidase activity in intact nodules attached to root systems (19). In this analysis we found that most nodules (72 to 88%) stained blue (Table 2), indicating that the inoculated strain was highly prevalent. No blue nodules were found in plants from uninoculated pots.

Analysis of the acetylene reduction activity revealed normal levels of nitrogenase activity in all cases (Table 2), indicating that the conditions tested were compatible with the normal development of the symbiosis. In soils with no nickel addition,

* Corresponding author. Mailing address: Laboratory of Microbiology, Department of Biotechnology, E.T.S. Ingenieros Agrónomos-Universidad Politécnica de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain. Phone: 34-91-336 5759. Fax: 34-91-336 5757. E-mail: jose.palacios@upm.es.

TABLE 1. Physicochemical characteristics of soils

Soil ^a	Texture	OM ^b (%)	pH	EC ^c (μs)	Ni concn (mg kg ⁻¹)		Co concn (mg kg ⁻¹)		Mn concn (mg kg ⁻¹)	
					Total	Available	Total	Available	Total	Available
1	Loamy	1.9	7.51	263	1.15	0.48	1.97	0.99	123	49
2	Loamy-sandy	1.4	7.25	316	1.94	0.68	2.51	1.36	130	92
3	Loamy-sandy	0.68	5.83	30	0.08	0.03	0.31	0.20	99	64
4	Loamy-clayey	1.00	7.02	80	1.44	0.27	2.90	0.73	109	25
5	Loamy	1.54	7.74	312	3.00	0.53	3.20	0.75	247	55
6	Loamy-sandy	0.69	8.03	151	1.93	0.28	1.68	0.15	172	12

^a Three topsoil subsamples (0 to 15 cm) per site were collected from uniform areas of the field, mixed, and sieved. Collection sites: 1 and 2, El Encin experimental station (40°30'N 3°15'W); 3, Soto del Real (40°46,063'N 3°44,227'W); 4, Torrejón del Rey (40°35,608'N 3°21,940'W); 5, Aranjuez (40°04,657'N 3°36,860'W); 6, Pinto (40°14,184'N 3°39,220'W).

^b Organic matter content (OM) is expressed in %.

^c The electric conductivity (EC) is expressed in μs.

the levels of hydrogenase activity were very low in soils 2 and 3 (ca. 650 nmol H₂ h⁻¹ mg protein⁻¹). These low values resulted in the inability to completely recycle the hydrogen evolved by nitrogenase. Higher levels of hydrogenase activity, similar to those observed in unamended vermiculite (13), were observed for the remaining soils. With uninoculated plants no hydroge-

nase activity was detected, and the H₂ evolution values indicated that a large fraction (25 to 50%) of the electron flux through nitrogenase, estimated as the ratio between H₂ production and acetylene reduction activities, was diverted to proton reduction. We conclude from these results that most, if not all, *R. leguminosarum* biovar viciae strains resident in the soils

TABLE 2. Hydrogen metabolism in pea bacteroids and nodules induced by *R. leguminosarum* biovar viciae in plants grown under different Ni addition treatments

Soil	Strain	Ni added ^a	Hydrogenase activity ^b	Acetylene reduction ^c	H ₂ production ^d	Implantation ^e
1	UPM1156	0	1,890 ± 696	32 ± 3.8	<0.25	72
	UPM1156	10	2,580 ± 229	24.0 ± 0.4	<0.25	
	UPM1156	100	7,570 ± 2,368	11.8 ± 0.1	<0.25	
	SPF25	0	2,559 ± 727	29.9 ± 3.6	<0.25	
	n/i ^f	0	<50	32.0 ± 6.3	7.50 ± 2.80	
2	UPM1156	0	610 ± 42	28.4 ± 1.0	5.75 ± 0.01	80
	UPM1156	10	4,250 ± 1,956	19.4 ± 0.6	<0.25	
	UPM1156	100	5,230 ± 158	16.5 ± 0.4	<0.25	
	SPF25	0	1,336 ± 180	17.2 ± 1.8	<0.25	
	n/i	0	<50	18.0 ± 1.4	15 ± 1.20	
3	UPM1156	0	670 ± 143	38.9 ± 0.1	6.23 ± 0.31	81
	UPM1156	10	4,950 ± 692	39.6 ± 1.9	<0.25	
	UPM1156	100	6,210 ± 720	37.8 ± 0.3	<0.25	
	SPF25	0	1,151 ± 41	12.0 ± 0.4	<0.25	
	n/i	0	<50	22.0 ± 2.0	6.22 ± 1.48	
4	UPM1156	0	1,500 ± 169	30.0 ± 0.9	<0.25	85
	UPM1156	10	2,940 ± 11	19.2 ± 2.4	<0.25	
	UPM1156	100	4,370 ± 1,820	29.8 ± 0.4	<0.25	
	SPF25	0	1,834 ± 124	41.2 ± 3.5	<0.25	
	n/i	0	<50	27.0 ± 3.0	15.00 ± 1.80	
5	UPM1156	0	1,230 ± 366	42.2 ± 0.7	<0.25	79
	UPM1156	10	4,830 ± 435	44.0 ± 2.8	<0.25	
	UPM1156	100	6,800 ± 380	39.0 ± 4.9	<0.25	
	SPF25	0	2,255 ± 427	45.0 ± 5.0	<0.25	
	n/i	0	<50	22.0 ± 3.0	11.50 ± 0.27	
6	UPM1156	0	2,460 ± 59	20.5 ± 0.4	<0.25	88
	UPM1156	10	3,960 ± 465	22.2 ± 0.6	<0.25	
	UPM1156	100	5,130 ± 254	24.5 ± 0.3	<0.25	
	SPF25	0	2,321 ± 55	23.0 ± 4.6	<0.25	
	n/i	0	<50	15.5 ± 3.3	8.00 ± 0.50	

^a Ni added is expressed in mg per liter.

^b Hydrogenase activity is expressed as nanomoles of hydrogen per hour per milligram of protein, and are the averages of two independent bacteroid preparations ± S.E.

^c Acetylene reduction is expressed as micromoles of acetylene reduced per hour per gram of nodule fresh weight and are the averages of two independent determinations ± S.E.

^d Values of H₂ production are expressed as micromoles of H₂ produced per hour per gram of nodule fresh weight and are the averages of two independent determinations ± S.E.

^e Values of implantation are expressed as % of blue nodules.

^f n/i, plants not inoculated.

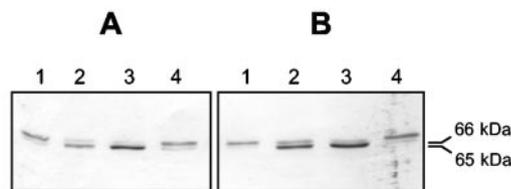


FIG. 1. Immunodetection of the hydrogenase large subunit (HupL) in bacteroids from pea plants grown in soil 1 (panel A) and soil 2 (panel B) with different Ni addition treatments. The immunoblot shows bands immunoreactive with an antiserum raised against *B. japonicum* HupL in bacteroid crude extracts resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) and transferred to polyvinylidene difluoride membranes. Numbers on the right side indicate the molecular masses of HupL protein in the unprocessed form (66 kDa) and the processed form (65 kDa) as deduced from comparison to molecular weight standards. Ni²⁺ addition treatments: lanes 1 and 4, no nickel added; lanes 2, 10 mg liter⁻¹ Ni²⁺ added; lanes 3, 100 mg liter⁻¹ Ni²⁺ added. Strains: lanes 1 to 3, UPM1156; lanes 4, SPF25.

tested were hydrogenase-negative, a situation that has been described previously in this species (16).

Addition of Ni to the nutrient solution caused a significant increase of H₂ uptake in all soils tested (Table 2) that was more evident for the highest level of nickel added (100 mg liter⁻¹). This increase led to the suppression of H₂ evolution where it was present. Since the Ni-dependent limitation of hydrogenase activity affects the processing of the hydrogenase large subunit HupL (5), we performed an immunoblot analysis of bacteroid crude extracts using an anti-HupL antiserum (a gift from R. J. Maier). Figure 1 shows representative results obtained for two of the soils tested. In this analysis we observed the presence of two bands (of 66 and 65 kDa) in crude extracts from UPM1156 (Fig. 1, lanes A1 and B1), with most of the specific signal associated with the larger unprocessed form of HupL (5). Addition of increasing concentrations of Ni²⁺ correlated with an increase in the relative amount of the fast-migrating band (Fig. 1, lanes 2 and 3). Similar results were obtained with the remaining soils (data not shown).

The largest amount of processed form was observed in extracts from bacteroids obtained from plants exposed to 100 mg liter⁻¹ Ni²⁺, and this correlated with the highest level of hydrogenase activity (Table 2). These data indicate that the level of hydrogenase activity is limited in agricultural soils due to low availability of Ni, and that this low availability of Ni affects the processing of the hydrogenase large subunit, thus extending to natural conditions the conclusions previously obtained in gnotobiotic systems (5). In our previous work the level of hydrogenase activity increased with higher amounts of Ni²⁺ added up to 10 mg liter⁻¹, but with 100 mg liter⁻¹ Ni²⁺ the level of hydrogenase activity was lower because this was a toxic concentration for the plants. In the current soil-based assays the overall pattern was similar, but the addition of 100 mg liter⁻¹ of Ni²⁺ resulted in higher levels of hydrogenase activity compared to those from 10 mg liter⁻¹ Ni²⁺. This difference is probably due to a lower Ni²⁺ availability for the plants in soils because Ni²⁺ forms stronger complexes with the soil particles that may act as buffer for this element (1).

The results presented in this paper contrast with previous work with soybean (7), where it was concluded that soil nickel

deficiency seemed unlikely to be agronomically important. These differences might be explained on the basis of the different abilities to provide nickel to bacteroids in pea versus soybean symbioses (5). In our case, a direct correlation between available Ni and hydrogenase activity cannot be deduced from the data obtained in this analysis. For instance in soil 2 we detected the maximal value of available Ni (0.68 mg liter⁻¹) but, at the same time, hydrogenase activity was very low and this situation could be reversed by nickel addition. This can be taken as another example of the fact that current chemical methods to predict the availability of nickel to the plants are unsatisfactory (1) and overestimate in most cases the amount of bioavailable nickel (3).

The need for efficient biological systems to determine the bioavailability of heavy metals has been pointed out (10), and a whole-cell biosensor for bioavailable Ni²⁺ has been developed (18). However this biosensor detects nickel available to bacteria in the free-living state, not to plants. The results presented in this paper indicate that bacteroid hydrogenase processing and activity could be used as a biosensor for soil Ni bioavailability to legume plants.

Toxicity associated with nickel implies that addition of this element to soil is not a feasible option to optimize the hydrogenase activity in nodules. Instead, we decided to use a modified *R. leguminosarum* strain (SPF25) engineered for higher hydrogenase expression by modification of the HupSL promoter (6). Pea plants inoculated with *R. leguminosarum* biovar viciae SPF25 were grown in the different soils without Ni addition, and hydrogen metabolism of nodules and bacteroids was analyzed (Table 2). In all cases, except for soil 6, SPF25 bacteroids exhibited significantly higher levels of hydrogenase activity. The increase compared to UPM1156 ranged between 6% (soil 4) and 117% (soil 2). Such activities were high enough to allow full recycling of the hydrogen evolved by nitrogenase. Immunoblot analysis of bacteroid crude extracts also revealed an increase in the levels of the processed form of the hydrogenase large subunit (Fig. 1, lanes A4 and B4). These results indicate that the promoter modification introduced into SPF25 has a beneficial impact on the energy efficiency of *R. leguminosarum* inoculants used in normal agricultural soils containing low nickel levels.

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