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Rhizobitoxine Inhibition of Hydrogenase Synthesis in Free-Living *Bradyrhizobium japonicum*

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Rhizobitoxine produced by *Bradyrhizobium* species strongly prevented derepression of hydrogenase expression in free-living *Bradyrhizobium japonicum*, although the toxin had no effect on the activity of cells which had already synthesized hydrogenase protein. Dihydrorhizobitoxine, a structural analog of rhizobitoxine, proved to be a less potent inhibitor of hydrogenase derepression. Rhizobitoxine did not cause cell death at a concentration sufficient to eliminate hydrogenase expression. The large subunit of hydrogenase was not detectable with antibody after derepression in the presence of rhizobitoxine. The general pattern of proteins synthesized from ^{14}C-labeled amino acids during derepression was not significantly different in the presence or absence of rhizobitoxine. These results indicated that rhizobitoxine inhibited hydrogenase synthesis in free-living *B. japonicum*. Cystathionine and methionine strongly prevented the inhibition of hydrogenase derepression by rhizobitoxine, suggesting that the inhibition involves the level of sulfur-containing amino acids in the cell.

Rhizobitoxine (2-amino-4-(2-amino-3-hydropropoxy)-*trans*-but-3-enoic acid) is a phytotoxin produced by some strains of *Bradyrhizobium* species (22–24). Rhizobitoxine-producing strains of *B. japonicum* induce chlorosis in new leaves of soybean as a result of synthesis of the toxin in nodules (7, 9, 23). In terms of biochemical and physiological functions, rhizobitoxine is known to inhibit β-cystathionase in the methionine biosynthetic pathway and ethylene formation (4, 11, 19, 22). *B. japonicum* bacteroids possessing the hydrogen uptake (Hup) system are capable of ATP production by recycling H_2 evolved from nitrogenase (2). Free-living cells of *B. japonicum* can express hydrogenase activity (13) and are able to use H_2 as the sole energy source for chemolithotrophic growth (5, 10).

Adequate uptake hydrogenase activity in soybean bacteroids often increases plant growth, as well as the efficiency of energy utilization during nitrogen fixation (3). In contrast, rhizobitoxine production in soybean bacteroids often causes abnormal growth of nodulated soybeans (6).

In a recent survey of *B. japonicum* strains for the presence of uptake hydrogenase or rhizobitoxine production, no strain was found that exhibited both traits (15). These findings suggested that there is a negative relationship between hydrogenase activity and rhizobitoxine-producing ability in bacteroids or free-living cells of *B. japonicum*. To examine this possibility, we used two different approaches. (i) The genetic relationship of Hup-positive and rhizobitoxine-producing strains of *B. japonicum* was examined, and (ii) the effect of rhizobitoxine on hydrogenase derepression was tested (15). Among the strains tested, a good correlation was found between sequence divergence in and around the *nifDKE* genes, rhizobitoxine production, Hup phenotype, and extracellular polysaccharide composition (16, 16a). The sequence divergence in and around *nifDKE* clearly divided *B. japonicum* strains into two different groups, designated genotypes I and II. Genotype I strains produced no rhizobitoxine and an extracellular polysaccharide composed of glucose, mannose, galactose, 4-*O*-methylgalactose, and galacturonic acid, whereas genotype II strains produced rhizobitoxine and an extracellular polysaccharide composed of rhamnose and 4-*O*-methylglucuronic acid. Hup-positive strains, possessing the *hup* structural genes, were confined exclusively to genotype I. Therefore, it was demonstrated that Hup-positive and rhizobitoxine-producing strains fall into two separate evolutionary lines, which explains the absence of strains that exhibit both hydrogen uptake ability and rhizobitoxine production.

Nevertheless, the lack of hydrogenase activity was also observed when free-living Hup^+ cells of *B. japonicum* were derepressed for hydrogenase in the presence of rhizobitoxine (15). In this work, we report the effect of rhizobitoxine on the activity and synthesis of hydrogenase in free-living *B. japonicum*. Rhizobitoxin had no effect on hydrogenase activity but inhibited derepression of hydrogenase.

MATERIALS AND METHODS

Bacterial strains. *B. japonicum* strains USDA 122 and USDA 94 were obtained from H. H. Keyser of the U.S. Department of Agriculture, Beltsville, Md. Strain USDA 122 was used for all experiments on hydrogenase derepression. Stock cultures were maintained on yeast extract-mannitol (YEM) agar medium (16). YEM medium supplemented with 1 g of Casamino Acids (Difco Laboratories, Detroit, Mich.) per liter was used for rhizobitoxine purification (24).

Chemicals. Rhizobitoxine and dihydrorhizobitoxine were isolated and purified from cultures of *B. japonicum* USDA 94 by the modified method of Giovanelli et al. (4). The concentrate from 3.8 liters of culture was passed through a column (3.8 by 18 cm) of Dowex 50 resin (50/100 mesh) in the NH_4^+ form. The column was washed with 10 bed volumes of distilled water and eluted with 2.5 bed volumes of 0.1 M NH_4OH to obtain a basic amino acid fraction. The eluate fraction was subjected to chromatography on a Dowex 50 column (pyridine form, 200/400 mesh, 1.8 by 110 cm). The column was eluted with a linear gradient from 1,000 ml of 0.2 M pyridine buffer (pH 4.4) to 1,000 ml of 1.0 M pyridine buffer (pH 5.1). The pH of the buffer was adjusted with acetic acid. Flow rate and fraction size were 1.6 ml/min and 15 ml, respectively. Samples (0.2 ml) of eluate fractions were evaporated to dryness and assayed for rhizobitoxine and dihydrorhizobitoxine by ascending chromatography on paper (Whatman no. 1). The developing solvent was 1-butanol–

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acetic acid–water (12:3:5, vol/vol/vol) and the detection reagent was 2% ninhydrin in 1-butanol. Rhizobitoxine (R_f = 0.17) formed a yellow reaction product with ninhydrin on paper, while dihydrorhizobitoxine (R_f = 0.24) appeared purple. Fractions containing rhizobitoxine (fractions 60 to 67) and dihydrorhizobitoxine (fractions 62 to 68) were combined, evaporated to dryness, and rechromatographed on a Dowex 50 column (NH_4^+ form, 200/400 mesh, 1.8 by 110 cm). The column was eluted with a linear gradient from 1,000 ml of distilled water to 1,000 ml of 0.08 M NH_4OH. The flow rate and fraction size were 1.6 ml/min and 15 ml, respectively. Samples of the eluate fraction were assayed by paper chromatography as described above. Rhizobitoxine fractions (47 to 52) and dihydrorhizobitoxine fractions (66 to 77) were evaporated separately to dryness. The purity and concentration of rhizobitoxine and dihydrorhizobitoxine obtained were determined with an amino acid analyzer and mass spectrometer (14). Other reagents were special-grade products from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ^{14}C-labeled amino acids with a specific activity of 50 μCi/mmol were purchased from Amersham Japan Ltd. (Tokyo, Japan).

Hydrogenase derepression. *B. japonicum* USDA 122 was derepressed for hydrogenase as described by Maier et al. (12, 13). Approximately 0.2 ml of an exponential liquid culture (YEM medium) was distributed over the surface of H_2-uptake agar medium (12). After 5 days of growth at 30°C in air, the cells were washed off the agar with magnesium-phosphate buffer (0.05 M potassium phosphate, 2.5 mM $MgCl_2$), the pH of which was adjusted to 6.0 unless otherwise indicated. The cells were centrifuged aseptically at 8,000 × *g* for 10 min and resuspended in the magnesium-phosphate buffer to a density of 6×10^8 cells per ml. Samples (5 ml) of the cell suspensions were transferred aseptically to a 33- or 122-ml sterile bottle to achieve gas/liquid ratios of 5.6 and 23.4, respectively. The bottles were then sealed with sterile rubber stoppers and flushed with N_2. Sufficient H_2, CO_2, and air were added to each bottle to obtain an atmosphere composed of 84% N_2, 10% H_2, 5% CO_2, and 1% O_2. The derepression bottles were incubated at 26°C at a shaking speed of 106 cycles/min. Various inhibitors and nitrogen sources as indicated were added to the cell suspensions at the start of derepressing conditions unless otherwise indicated. When cells were to be labeled, 2 μCi (per 5 ml of cells) of ^{14}C-labeled amino acids was added 6 h after derepressing conditions to eliminate labeling preexisting stable messages or polypeptides that existed before derepression. At the end of the derepression period, the cells were broken in SDS sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Hydrogenase assays. The cell suspensions were sparged with N_2 to remove excess H_2 and tested for hydrogenase activity in a 1.2-ml amperometric chamber (27) in the presence of adequate O_2 (>20 μM).

Protein determination. Cells were removed from the suspension by centrifugation (15,000 × *g*, 5 min). The pellet obtained was digested with 3% NaOH, and protein was determined by the method of Stickland (25), using bovine serum albumin as a standard.

Determination of rhizobitoxine concentration. The concentration of rhizobitoxine was measured with an amino acid analyzer (14). The cell suspensions were centrifuged at 15,000 × *g* for 5 min. Aliquots of the supernatant were passed through a column (0.6 by 4 cm) of Dowex 50 resin (50/100 mesh) in the H^+ form. The column was washed with distilled water and eluted with 5 bed volumes of 2 M NH_4OH. The eluate fraction was evaporated to dryness and dissolved in dilution buffer (14).

Determination of O_2 tension. The O_2 tension in the gas phase of the derepression bottles was measured by gas chromatography (Shimadzu GC-7A gas chromatograph) with a thermal conductivity detector and a column (3 mm by 2.0 m) containing Molecular Sieve 5A (80/100 mesh).

Cell viability. Cells were assayed for viability by serial dilution in 0.85% saline and plating on YEM agar medium.

Gel electrphoresis. The discontinuous PAGE system described by Laemmli (8) was used with a 9% acrylamide resolving gel. Proteins of derepressed cells were prepared by the method of Novak and Maier (17) with some modifications. The 5-ml samples of derepressed cells were harvested, washed once in 62.5 mM Tris hydrochloride (pH 6.8), and suspended by vortexing in 100 μl of SDS sample buffer (8). Samples were alternately boiled and quick-frozen in dry ice-ethanol through four successive cycles and centrifuged to pellet cell debris. A 10-μl sample of each resultant supernatant was loaded per gel lane to detect total and labeled proteins. For Western blotting (immunoblotting), 20 μl of each supernatant was loaded per gel lane. Gels were run at 15 mA for 4 h.

Fluorography. After electrophoresis, the gel was fixed in 50% (vol/vol) methanol–10% (vol/vol) acetic acid, treated with En^{3}Hance (Dupont, NEN Research Products, Boston, Mass.), washed with distilled water, and dried on a paper filter (Whatman 3MM). The gel was exposed to Fuji X-ray film at −80°C for 30 h.

Immunoblotting and antigen detection. Proteins were electrophoretically transferred onto nitrocellulose (Nihon Millipore Kogyo) as described by Towbin et al. (26). The blot was blocked with a solution containing 20 mM Tris hydrochloride (pH 7.5), 0.5 M NaCl, and 2% (wt/vol) skim milk and treated overnight at room temperature with a 1:500 dilution of the antibody prepared against the 65-kilodalton subunit of hydrogenase from *B. japonicum* (obtained from H. J. Evans, Oregon State University, Corvallis). The blot was then treated with a 1:500 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G for 6 h at room temperature.

RESULTS

Inhibition of hydrogenase derepression. Cells of *B. japonicum* USDA 122 were derepressed for hydrogenase in the presence of rhizobitoxine or dihydrorhizobitoxine. The effects of these compounds on the expression of hydrogenase activity are summarized in Table 1. Rhizobitoxine strongly inhibited derepression of hydrogenase activity in a 30-h period. Dihydrorhizobitoxine, a structural analog of rhizobitoxine, is found with rhizobitoxine in soybean nodules and has been identified as *O*-(2-amino-3-hydroxypropyl)homoserine (21). Dihydrorhizobitoxine proved much less potent than rhizobitoxine in inhibiting hydrogenase derepression (Table 1). The apparent inhibition of hydrogenase derepression at higher concentrations of dihydrorhizobitoxine may be due to very low (0.01%) rhizobitoxine contamination, since both were isolated from the same USDA 94 culture. Aminoethoxyvinyl glycine (L-2-amino-4-(2-aminoethoxy) *trans*-3-butenoic acid), an ethoxy analog of rhizobitoxine (11), had no inhibitory effect on hydrogenase derepression (data not shown).

To investigate whether rhizobitoxine inhibits hydrogenase activity, we added rhizobitoxine to cell suspensions at the beginning of hydrogenase derepression (Fig. 1). Addition of rhizobitoxine after 14 h of derepressing conditions led to a

TABLE 1. Effects of rhizobitoxine and dihydrorhizobitoxine on derepression of hydrogenase activity[a]

Addition	Concn (μM)	% Hydrogenase activity[b]
Rhizobitoxine	0.30	27
	0.95	9
	3.0	1
	9.5	0
	30	0
	95	0
	300	0
Dihydrorhizobitoxine	3	91
	30	69
	300	51

[a] Cells were derepressed for 30 h in the presence of various concentrations of rhizobitoxine or dihydrorhizobitoxine. A gas/liquid ratio of 5.6 was used. Molecular weights of rhizobitoxine and dihydrorhizobitoxine are 190 and 192, respectively (21, 22).

[b] Hydrogenase activities are expressed as percentages of the control. The activity of the control was 3.03 μmol/h per mg of protein. Results are means for three replicates.

cessation in the increase of hydrogenase activity within 4 h. In contrast, the activity of control cells continued to increase at a rapid rate until 26 h. This result suggested that rhizobitoxine did not have any effect on the activity of synthesized hydrogenase protein but affected the derepression of hydrogenase.

To examine whether rhizobitoxine caused cell death, we plated cells for viability after treatment with increasing concentration of rhizobitoxine (Fig. 2). Cell viability at the end of derepression did not decrease even in the presence of 100 μM of rhizobitoxine, indicating that the toxin did not cause cell death at concentrations sufficient to eliminate

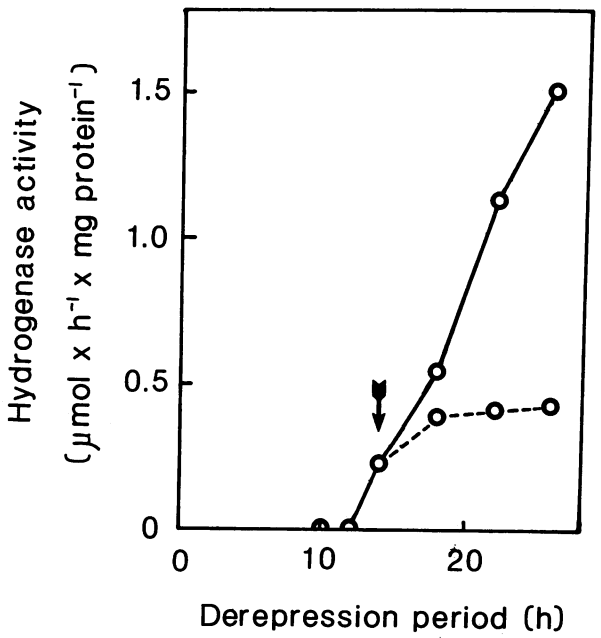

FIG. 1. Effect of addition of rhizobitoxine on hydrogenase derepression. Derepression of hydrogenase was started in magnesium-phosphate buffer without rhizobitoxine. After 14 h, rhizobitoxine was added (arrow) and amperometric assays were conducted at 18, 22, and 26 h. A gas/liquid ratio of 23.4 was used. Each point represents the means of duplicate determinations. Symbols, —, no addition at 14 h; - - -, addition of 100 μM rhizobitoxine at 14 h.

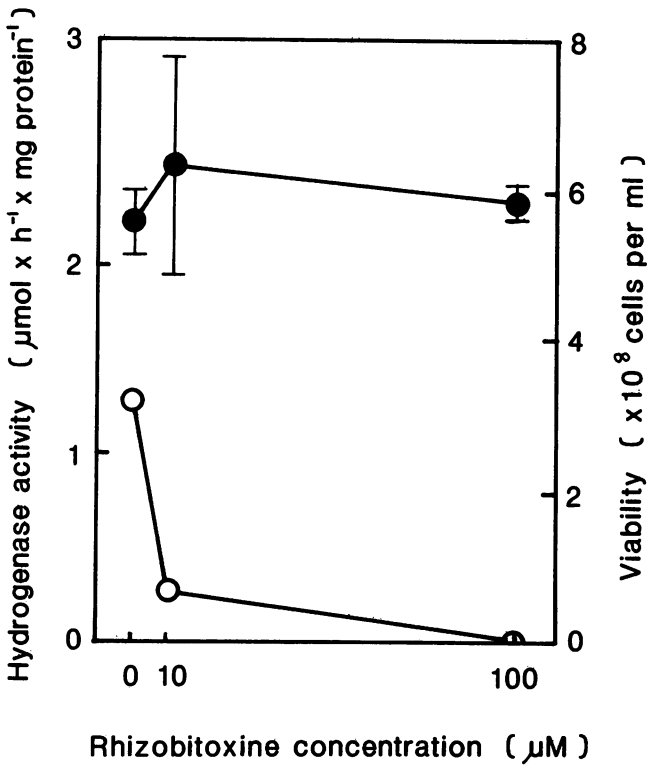

FIG. 2. Effect of rhizobitoxine on cell viability and hydrogenase activity. Cells were derepressed for 20 h in the presence or absence of rhizobitoxine and then assayed for viability (●) and hydrogenase activity (○). A gas/liquid ratio of 23.4 was used. Values of cell viability are means with standard error for four independent cultures, while values of hydrogenase activity are means of duplicate determinations. Initial cell number was (6.4 ± 0.6) × 10^8 cells per ml at the start of derepression.

hydrogenase activity. Recently, the expression of hydrogenase activity in *B. japonicum* was reported to be influenced by pH in the cell suspensions and pO_2 in the gas phase after the addition of carbon substrates (1). To test whether added rhizobitoxine caused changes of pH or pO_2, we measured these parameters after the addition of increasing concentrations of rhizobitoxine (Table 2). Rhizobitoxine concentrations in the cell suspensions did not change greatly during the derepression period. Moreover, pH in the cell suspension and pO_2 of the gas phase did not vary as a result of rhizobitoxine additions. Thus, added rhizobitoxine apparently was not consumed by the cells as a substrate. These results indicated that the inhibition of hydrogenase derepression was not due to cell death, pH, or O_2 tension.

TABLE 2. Rhizobitoxine concentration, O_2 tension, and pH during hydrogenase derepression in the presence or absence of rhizobitoxine[a]

Rhizobitoxine concn (μM)		Final pO_2[b]	Final pH[c]
Initial	Final		
0.00	0.00	0.5	6.7
7.85	7.58	0.5	6.7
91.1	85.6	0.5	6.7

[a] Cells were derepressed for 30 h. A gas/liquid ratio of 5.6 was used.

[b] The initial pO_2 was 1%.

[c] The initial pH was 6.8.

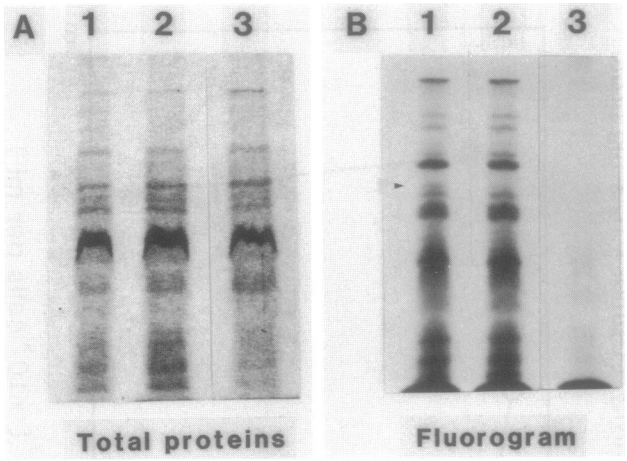

FIG. 3. SDS-PAGE of cell extracts after derepression in the presence of rhizobitoxine or chloramphenicol. Cells were derepressed for 20 h in the presence of the following: lane 1, no inhibitor; lane 2, 50 μM rhizobitoxine; lane 3, 200 μg of chloramphenicol per ml. A gas/liquid ratio of 23.4 was used. (A) Total proteins stained with Coomassie brilliant blue R-250. (B) Fluorogram of proteins labeled with ^{14}C-amino acids during derepression. ^{14}C-amino acids were added 6 h after the start of derepression. Hydrogenase activities (micromoles per hour per milligram of protein) of cells derepressed in the presence of inhibitors were as follows: control cells (lane 1), 2.8; rhizobitoxine (lane 2), <0.1; chloramphenicol (lane 3), <0.1.

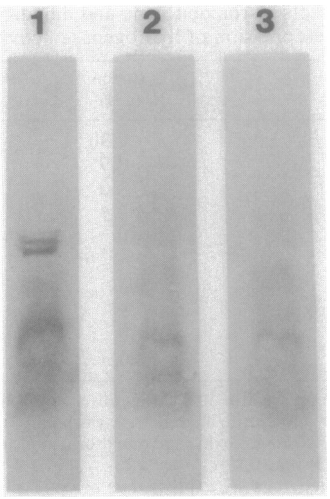

FIG. 4. Western blot of an SDS-polyacrylamide gel containing the same samples as those in Fig. 3 and probed with antibody to the 65-kilodalton subunit of hydrogenase. Cells were derepressed in the presence of the following: lane 1, no inhibitor; lane 2, 50 μM rhizobitoxine; lane 3, 200 μg of chloramphenicol per ml. Hydrogenase activities of cells were the same as described in the legend to Fig. 3.

Protein synthesis. To examine the effect of rhizobitoxine on protein synthesis, we labeled cells with a ^{14}C-amino acid mixture in the presence of rhizobitoxine or chloramphenicol during derepression. Hydrogenase activity was not detected in cells derepressed in the presence of rhizobitoxine or chloramphenicol, whereas the hydrogenase activity of control cells was 2.8 μmol/h per mg of protein.

SDS-PAGE of proteins from rhizobitoxine- or chloramphenicol-treated cells showed no difference when stained by Coomassie brilliant blue (Fig. 3A). Moreover, SDS-PAGE of ^{14}C-labeled proteins clearly showed that rhizobitoxine did not affect the general pattern of labeling (Fig. 3B). When compared with labeled proteins from control cells, only a single band (Fig. 3B, lane 1, arrowhead) seemed to disappear upon addition of rhizobitoxine. This band may correspond to the subunit of hydrogenase. This indicated that general protein synthesis continued normally in the presence of rhizobitoxine. When chloramphenicol was added during derepression, there was little or no ^{14}C labeling of proteins (Fig. 3B, lane 3). These results clearly indicate that the effect of rhizobitoxine on hydrogenase derepression is specific and not related to a general reduction in protein synthesis. Western blotting of proteins after derepression showed that the 65-kilodalton subunit of hydrogenase was not synthesized in the presence of rhizobitoxine (Fig. 4). These results agreed with the data obtained from amperometric assays of hydrogenase activity.

Effects of nitrogen compounds. Rhizobitoxine has been reported to be a potent inhibitor of β-cystathionase in the methionine biosynthetic pathway (4, 19). Thus, we investigated the effects of nitrogen compounds, including cystathionine and methionine, on the inhibition of hydrogenase derepression by rhizobitoxine. Addition of any of the nitrogen compounds tested reduced the inhibition of hydrogenase derepression by rhizobitoxine (Table 3). These effects of nitrogen compounds were not due to changes in pH or pO_2, since these were unaltered at the end of the derepression period (data not shown). Among nitrogen compounds tested, the sulfur-containing amino acids (cystathionine and methionine) completely relieved the inhibitory effect of rhizobitoxine on hydrogenase derepression. These results suggest that sulfur-containing amino acids are involved in the inhibition of hydrogenase derepression by rhizobitoxine.

DISCUSSION

The results clearly demonstrated that rhizobitoxine inhibits its hydrogenase synthesis in free-living *B. japonicum*. Novak and Maier (17, 18) reported that DNA gyrase inhibitors, such as novobiocin and coumermycin, specifically prevent expression of hydrogenase in *B. japonicum*. They suggested that gyrase activity, which controls the tertiary structure of

TABLE 3. Effects of nitrogen compounds on rhizobitoxine inhibition of hydrogenase derepression[a]

Nitrogen compound added (mM)	Hydrogenase activity[b]		% Inhibition[c]
	−RT	+RT	
None	1.25	0	100
Ammonium chloride (1)	4.45	0.71	84
Sodium glutamate (0.5)	6.07	1.08	82
Leucine (0.5)	2.91	1.16	60
Cystathionine (0.25)	4.27	4.27	0
Methionine (0.5)	2.91	3.04	−4

[a] Cells were derepressed for 20 h in the presence of various nitrogen sources. At the start of derepression, rhizobitoxine was added to a final concentration of 30 μM. A gas/liquid ratio of 23.4 was used. Values are the means of duplicate determinations.

[b] Hydrogenase activity is shown as micromoles of H_2 uptake per hour per milligram of cell protein. −RT, No addition of rhizobitoxine; +RT, addition of rhizobitoxine.

[c] Percent inhibition is expressed as the percentage that hydrogenase activity is reduced by the addition of rhizobitoxine.

DNA, is an important element in the control of hydrogenase synthesis. However, this is unlikely to explain the effect of rhizobitoxine, since rhizobitoxine and gyrase inhibitors differ in chemical structure. In addition, the patterns of proteins labeled by ^{14}C-amino acids during derepression in the presence of rhizobitoxine were different from those labeled in the presence of novobiocin (data not shown).

The extent of rhizobitoxine inhibition of hydrogenase derepression decreased when nitrogen compounds were added (Table 3), suggesting that rhizobitoxine did not directly repress *hup* gene expression. Among the nitrogen compounds tested, cystathionine and methionine strongly prevented the inhibition of hydrogenase derepression by rhizobitoxine. In terms of biochemical function, rhizobitoxine is a potent inhibitor of β-cystathionase, which catalyzes the cleavage of cystathionine to form homocysteine and pyruvate during the process of methionine biosynthesis (19). Therefore, it is suggested that cystathionine and rhizobitoxine compete for a site of β-cystathionase and that this inhibition is due to a decrease in the level of methionine or its later metabolites such as *S*-adenosylmethionine. The hydrogenase of *B. japonicum* is an iron-sulfur protein (3) which may be involved in this phenomenon.

Previous hybridization experiments showed that rhizobitoxine-producing strains of *B. japonicum* apparently lack the structural genes for hydrogenase (16a). The inhibitory effect of rhizobitoxine on hydrogenase expression likely accounts for selective pressure for the absence of the *hup* genes in these bacteria.

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LITERATURE CITED

1. **Berkum P. V., and R. J. Maier.** 1988. Lack of carbon substrate repression of uptake hydrogenase activity in *Bradyrhizobium japonicum* SR. J. Bacteriol. **170**:1962–1964.
2. **Emerich, D. W., T. Ruiz-Argüeso, T. M. Ching, and H. J. Evans.** 1979. Hydrogen-dependent nitrogenase activity and ATP formation in *Rhizobium japonicum* bacteroids. J. Bacteriol. **137**: 153–160.
3. **Evans, H. J., A. R. Harker, H. Papen, S. A. Russell, F. J. Hanus, and M. Zuber.** 1987. Physiology, biochemistry, and genetics of the uptake hydrogenase in rhizobia. Annu. Rev. Microbiol. **41**:335–361.
4. **Giovanelli, J., L. D. Owens, and S. H. Mudd.** 1971. Mechanism of inhibition of spinach β-cystathionase by rhizobitoxine. Biochim. Biophys. Acta **227**:671–684.
5. **Hanus, F. J., R. J. Maier, and H. J. Evans.** 1979. Autotrophic growth of H_2-uptake-positive strains of *Rhizobium japonicum* in an atmosphere supplied with hydrogen gas. Proc. Natl. Acad. Sci. USA **76**:1788–1792.
6. **Johnson, H. W., U. M. Means, and F. E. Clark.** 1958. Factors affecting the expression of bacterial-induced chlorosis of soybeans. Agron. J. **50**:571–574.
7. **Johnson, H. W., U. M. Means, and F. E. Clark.** 1959. Responses of seedlings to extracts of soybean nodules bearing selected strains of *Rhizobium japonicum*. Nature (London) **183**:308–309.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
9. **La Favre, J. S., and A. R. J. Eaglesham.** 1986. Rhizobitoxine: a phytotoxin of unknown function which is commonly produced by bradyrhizobia. Plant Soil **92**:443–452.
10. **Lepo, J. E., F. J. Hanus, and H. J. Evans.** 1980. Chemoautotrophic growth of hydrogen-uptake-positive strains of *Rhizobium japonicum*. J. Bacteriol. **141**:664–670.
11. **Lieberman, M.** 1979. Biosynthesis and action of ethylene. Annu. Rev. Plant Physiol. **30**:533–539.
12. **Maier, R. J., N. E. R. Campbell, F. J. Hanus, F. B. Simpson, S. A. Russell, and H. J. Evans.** 1978. Expression of hydrogenase activity in free-living *Rhizobium japonicum*. Proc. Natl. Acad. Sci. USA **75**:3258–3262.
13. **Maier, R. J., F. J. Hanus, and H. J. Evans.** 1979. Regulation of hydrogenase in *Rhizobium japonicum*. J. Bacteriol. **137**:824–829.
14. **Minamisawa, K., and N. Kume.** 1987. Determination of rhizobitoxine and dihydrorhizobitoxine in soybean plants by amino acid analyzer. Soil. Sci. Plant Nutr. **33**:645–649.
15. **Minamisawa, K.** 1988. Hydrogenase phenotype, rhizobitoxine-producing ability and serinol content in soybean nodules formed with various strains of *Bradyrhizobium japonicum*, p. 586. *In* H. Bothe, F. J. de Bruijn, and W. E. Newton (ed.), Nitrogen fixation: hundred years after. Gustav Fisher Verlag, Stuttgart.
16. **Minamisawa, K.** 1989. Comparison of extracellular polysaccharide composition, rhizobitoxine production, and hydrogenase phenotype among various strains of *Bradyrhizobium japonicum*. Plant Cell Physiol. **30**:877–884.
16a. **Minamisawa, K.** 1990. Division of rhibotoxine-producing and hydrogen-uptake positive strains of *Bradyrhizobium japonicum* by *nifDKE* sequence divergence. Plant Cell Physiol **31**:81–89.
17. **Novak, P. D., and R. J. Maier.** 1987. Inhibition of hydrogenase synthesis by DNA gyrase inhibitors in *Bradyrhizobium japonicum*. J. Bacteriol. **169**:2708–2712.
18. **Novak, P. D., and R. J. Maier.** 1989. Hydrogenase synthesis in *Bradyrhizobium japonicum* Hup^c mutants is altered in sensitivity to DNA gyrase inhibitors. Appl. Environ. Microbiol. **55**: 1157–1164.
19. **Owens, L. D., S. Guggenheim, and J. L. Hilton.** 1968. An inhibitor of β-cystathionase in *Salmonella typhimurium*. Biochim. Biophys. Acta **158**:219–225.
20. **Owens, L. D., M. Lieberman, and A. Kunishi.** 1971. Inhibition of ethylene production by rhizobitoxine. Plant Physiol. **48**:1–4.
21. **Owens, L. D., J. F. Thompson, and P. V. Fennessy.** 1972. Dihydrorhizobitoxine, a new ether amino-acid from *Rhizobium japonicum*. J. Chem. Soc. Chem. Commun. **1972**:715.
22. **Owens, L. D., J. F. Thompson, R. G. Pitcher, and T. Williams.** 1972. Structure of rhizobitoxine, an antimetabolic enol-ether amino-acid from *Rhizobium japonicum*. J. Chem. Soc. Chem. Commun. **1972**:714.
23. **Owens, L. D., and D. A. Wright.** 1964. Rhizobial-induced chlorosis in soybeans: isolation, production in nodules, and varietal specificity of the toxin. Plant Physiol. **40**:927–930.
24. **Owens, L. D., and D. A. Wright.** 1964. Production of the soybean-chlorosis toxin by *Rhizobium japonicum* in pure culture. Plant Physiol. **40**:931–933.
25. **Stickland, L. H.** 1951. The determination of small quantities of bacteria by means of the biuret reaction. J. Gen. Microbiol. **5**:698–703.
26. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
27. **Wang, R. T.** 1980. Amperometric hydrogen electrode. Methods Enzymol. **69**:409–413.