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Hydrogenase in Actinorhizal Root Nodules and Root Nodule Homogenates

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Hydrogenases were measured in intact actinorhizal root nodules and from disrupted nodules of *Alnus glutinosa, Alnus rhombifolia, Alnus rubra,* and *Myrica pensylvanica.* Whole nodules took up H_2 in an O_2-dependent reaction. Endophyte preparations oxidized H_2 through the oxyhydrogen reaction, but rates were enhanced when hydrogen uptake was coupled to artificial electron acceptors. Oxygen inhibited artificial acceptor-dependent H_2 uptake. The hydrogenase system from *M. pensylvanica* had a different pattern of coupling to various electron acceptors than the hydrogenase systems from the alders; only the bayberry system evolved H_2 from reduced viologen dyes.

In N_2-fixing microorganisms, the nitrogenase enzyme system catalyzes the ATP-dependent reduction of N_2 to ammonia and protons to H_2 (31). Production of H_2 is wasteful, as it uses ATP that otherwise could be used in the transfer of electrons to N_2 (9). Even under optimal conditions in vitro, about 25% of the total electron flux is allocated to protons rather than to N_2 (12); in vivo the proportion may even be higher. Some N_2-fixing microorganisms recycle much of this H_2 with the enzyme hydrogenase. Roles proposed for such "uptake hydrogenases" include: (i) protection of the O_2-sensitive nitrogenase system by action of the oxyhydrogen reaction, (ii) prevention of H_2 inhibition of nitrogenase, or (iii) production of ATP for support of the nitrogenase system (8).

In actinorhizal associations, the roots of nonleguminous plants are invaded by actinomycetes that form N_2-fixing root nodules (26). Such plants often are pioneer species that occur in N-limited environments such as river and lake banks, sand dunes, glacial till, or dry chaparral (22, 23). They can be useful for land reclamation or forest restoration (20, 22). The systems have been particularly difficult to study because the plants grow slowly, plant phenolics inactivate the enzyme systems, cultures of the isolated endophyte have been unavailable until recently, and the plants apparently carry unidentified toxins. Progress has accelerated with the isolation of an infective microsymbiont (7), preparation of acetylene-reducing homogenates (3, 29), and extraction of cell-free nitrogenase and hydrogenase preparations (2).

Most intact actinorhizal root nodules studied have evolved little or no H_2 (15, 21), although cell-free extracts can be induced to evolve H_2 in the presence of Mg-ATP and dithionite (2). Whole legume nodules, in contrast, may divert to H_2 evolution a substantial fraction of the total electron flux generated in the nitrogenase system (14). The amount of H_2 lost by legume nodules is reduced when the particular *Rhizobium* strain in the nodules uses hydrogenase to recycle H_2 (10). We previously reported the presence of an uptake hydrogenase in cell-free extracts of *Alnus glutinosa* root nodules (2). Studies with whole nodules and fractionated homogenates of *A. glutinosa* nodules have shown that the hydrogenase can be detected with methylene blue or phenazine methosulfate as electron acceptors (17). This communication describes the hydrogenase system in intact nodules and the plant-free symbiotic form of the endophyte from four actinorhizal plants.

MATERIALS AND METHODS

Source of nodules. Nodulated red alder (*Alnus rubra*) seedlings were obtained from the base of Mary's Peak near Corvallis, Oreg., in the spring of 1979 with the aid of D. W. Emerich and H. J. Evans. The plants were transported to Madison, Wis., and transplanted into pots of sand outdoors. Bayberry (*Myrica pensylvanica*) was grown from seed obtained from Island Beach State Park in New Jersey as previously described (D. R. Benson, Ph.D. thesis, Rutgers University, New Brunswick, N. J., 1978). Seedlings were inoculated by the method of Bond et al. (5) with nodules from the same locale.

Black alder (*A. glutinosa*) and white alder (*Alnus rhombifolia*) were grown from seed in Seed-Pack growth pouches (Scientific Products, Chicago, Ill.) and inoculated with a suspension of an isolate from *Comptonia peregrina* that was designated CpI-1 and was generously supplied by J. G. Torrey. The plants were inoculated on day 14 after planting, and nodulation was evident after another week. The nodulated seedlings from the growth pouches were transferred after 4 weeks to a modified aeroponics chamber of twice the

capacity described by Zobel et al. (32). The plants were kept in a growth room at 23°C for their 18-h light cycle and at 20°C for their 6-h dark cycle. All plants were watered as needed with half-strength Hoagland N-free nutrient solution (11).

Preparation of nodular homogenates. Root nodules (1 to 5 g total weight) were excised from each plant type, assayed for H_2 production, H_2 uptake, and C_2H_2 reduction. They then were plunged into liquid N_2 and ground to a fine powder with a mortar and pestle while in liquid N_2. The frozen homogenate was transferred to centrifuge tubes, and the liquid N_2 was allowed to escape. The tubes were capped with serum stoppers, and the remaining N_2 was removed under vacuum. The gas phase was replaced with Ar. All subsequent operations were performed anaerobically at 5°C. The homogenates were suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 10 mM sodium dithionite. After centrifugation at 27 × *g* for 5 min, the cloudy supernatant was removed and the pellet was suspended in 20 mM Tris-hydrochloride buffer (pH 7.4) plus 2 mM dithionite. This second suspension was centrifuged as before, and the pellet was resuspended in fresh washing buffer. This washing procedure was repeated three times. Then after resuspending in washing buffer again, large particles of plant debris were allowed to settle in the centrifuge tubes for 3 min and the supernatant containing the majority of the endophyte was collected. The settling procedure was performed three times and the settled material was referred to as fraction I. To remove fine particles, the combined supernatants were centrifuged at 27 × *g* to pellet the endophyte and leave fine particles suspended. The pellet was resuspended, and the procedure was repeated twice. The resulting suspension (fraction II) consisted mainly of vesicle clusters from the endophyte. Supernatants from all the 27 × *g* washes were combined and centrifuged at 20,000 × *g* to pellet fine particles. These were suspended in a small amount of buffer and comprised fraction III.

C_2H_2 reduction (nitrogenase) assays. Nitrogenase activity was determined by the C_2H_2 reduction method (6). Whole nodules were assayed in 10% C_2H_2 and 90% air. Nodule homogenates were assayed under high Mg·ATP and dithionite concentrations as previously described (3, 29).

H_2 and O_2 determinations. Hydrogen and O_2 concentrations were measured simultaneously in a water-jacketed 1.8-ml cell modified to accept two Clark-type electrodes (Yellow Springs Instruments). One electrode was used to monitor O_2 and one to monitor H_2 concentration (30). Samples were added to the electrode chamber with a syringe and needle, and residual dithionite was oxidized by adding O_2-saturated buffer. Except where noted, all reactions of nodule homogenates were conducted at 30°C in 20 mM Tris-hydrochloride buffer (pH 7.4). Hydrogen or O_2 was added as a saturated solution of either gas in buffer.

For H_2-uptake experiments, artificial electron acceptors were degassed and added to the chamber with a syringe. ATP-dependent H_2 evolution was supported by adding 20 mM Mg-ATP and 50 mM dithionite. ATP-independent H_2 evolution was supported by adding dithionite-reduced methyl viologen or benzyl viologen to a final concentration of 6 mM. Hydrogen uptake assays in whole nodules were conducted in the same double-electrode cell, but whole nodules were incubated in a gas phase consisting of 2.8% H_2 in air or Ar; O_2 was added as indicated. Hydrogen evolution was determined by gas chromatography with a thermal conductivity detector or with the H_2 electrode cell.

Soybean nodules. Soybean plants (var. Hodgson) were grown as described earlier (1). The seeds were inoculated with *Rhizobium japonicum* 110 which has an active uptake hydrogenase. Bacteroids were prepared without anaerobic precautions by crushing fresh nodules in 20 mM Tris-hydrochloride buffer (pH 7.4), allowing the nodule debris to settle out and collecting the supernatant which contained bacteroids and plant cell cytosol.

RESULTS

Whole nodules. Intact actinorhizal nodules removed H_2 from the gas phase in an O_2-dependent reaction (Fig. 1). Rates obtained ranged from 50 nmol to 1 μmol × (min × g fresh weight of nodules)$^{-1}$. Figure 1 shows the absence of H_2 uptake under Ar and H_2 and the initiation of uptake when O_2 was added. When O_2 was depleted through nodular respiration, the H_2 uptake ceased. Occasionally, actinorhizal nodules with exceptionally high C_2H_2 reducing activity did not take up H_2. None of the nodules examined evolved H_2 gas. Intact soybean nodules containing *Rhizobium japonicum* 110 did not

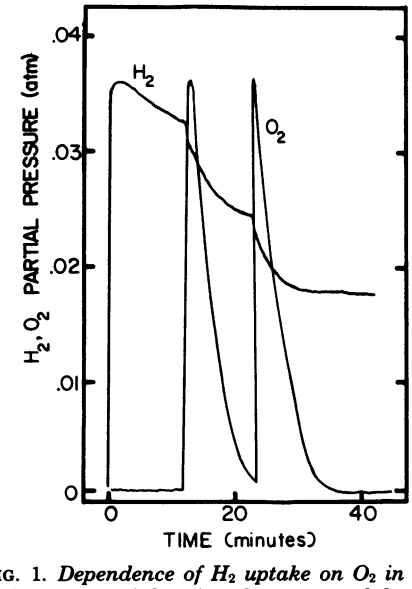

FIG. 1. *Dependence of H_2 uptake on O_2 in whole A. rubra root nodules. A. rubra root nodules (0.4 g fresh weight) were placed in the H_2-O_2 double-electrode chamber and sparged with Ar to remove O_2. Hydrogen gas was added at time 0, and O_2 was added as indicated. There was slow H_2 uptake before the addition of O_2.*

take up H_2 from the gas phase nor did they evolve H_2.

Characteristics of homogenates. Microscopic examination of the initial homogenate showed a complex mixture of plant cell debris and the actinomycete endophyte. Most of the endophyte was released from the host cells, although some remained enclosed in larger aggregates of plant cells. In all species, most of the endophyte population was in the vesicular form. This form originates in single host cells where a mycelial mass fills the cell and is surrounded by spherical (in alder) or club-shaped (in bayberry) hyphal distentions. Endophytes in this form are referred to as vesicle clusters.

Homogenates were separated into fractions by differential centrifugation and gravity sedimentation. Nitrogenase (C_2H_2-reducing) activity served as a marker for the endophyte, and Table 1 records the recovery of activity from a typical nodule preparation. All fractions exhibited ATP-dependent H_2 evolution. In general, unfractionated homogenates gave 5 to 55% recovery of the nitrogenase activity in whole nodules. Of the total recovered, 50 to 70% was located in fraction II, which consisted mostly of endophyte. Some plant tissue contaminated fraction II and consisted of cell walls, starch grains, and brown material originating in the cork layer of the nodules.

The large particles (fraction I) had 30 to 40% of the nitrogenase activity. This fraction consisted of fragments of plant tissue with three or more cells, some soil from field material, and larger pieces of undisrupted and uninfected root tissue. Fine particles (fraction III) consisted of contaminating bacteria, cellular constituents from the plant, and some broken hyphae, and the fraction contained 5 to 10% of the recovered nitrogenase activity. The 20,000 × *g* supernatant contained much colored phenolic material. Hydrogenase partitioning between the fractions paralleled nitrogenase activity (Table 1).

TABLE 1. *Distribution and recovery of activities in preparations from A. rubra nodules*[a]

Prepn	Nitrogenase		Hydrogenase
	C_2H_2 reduced[b]	H_2 evolved[c]	H_2 oxidized[d]
Whole nodules	6.4	0.0	0.26
Homogenates	0.93	0.88	1.6
Fraction I, large particles	0.25	0.21	0.69
Fraction II, vesicle clusters	0.31	0.25	0.90
Fraction III, fine particles	0.02	0.04	0.23

[a] See the text for preparation of fractions and assay methods. The results from *A. rubra* are typical of all actinorhizal plants examined. The electron acceptor for the hydrogenase assay was MB.

[b] Micromoles of C_2H_2 reduced × (hour × gram fresh weight)$^{-1}$.

[c] Micromoles of H_2 evolved × (hour × gram fresh weight)$^{-1}$.

[d] Micromoles of H_2 oxidized × (hour × gram fresh weight)$^{-1}$.

Fraction II took up O_2 (Fig. 2, 4, 5); the time course was not linear, and rates declined during the assay. When O_2 was present in the reaction chamber, fraction II preparations became brown because of spontaneous autoxidation of plant phenolics. Apparently, some O_2 was taken up through nonspecific interactions.

Hydrogen uptake by the endophyte. In all species studied, the vesicle cluster preparations (fraction II) were quite active in H_2 uptake when an artificial electron acceptor such as methylene blue (MB) was added. The activity was stable during storage in liquid N_2. Activity ranged from 0.91 nmol of H_2 taken up × (min × 10^6 vesicle clusters)$^{-1}$ for *A. glutinosa* (black alder with CpI-1) to 0.43 for *M. pensylvanica* (bayberry) (Table 2). The addition of O_2 to the reaction mixture containing MB immediately inhibited MB-dependent H_2 uptake. When O_2 was removed by endogenous respiration or nonspecific autoxidation of phenolics, the MB-dependent rate resumed (Fig. 2). The response to benzyl viologen (BV), phenazine methosulfate (PMS), and dichlorophenolindophenol (DCIP) was similar. The hydrogenase in the bayberry endophyte was more sensitive to O_2 inhibition than the hydrogenase from alder, and it did not return to full activity when O_2 was removed.

Preparations from *A. rubra* (red alder) had a broad pH optimum, between 7 and 8. Double-reciprocal plots of data from progress curves (Fig. 3) gave a K_m for H_2 of 2.7 μM when MB was used as the electron acceptor and a value of 12 μM with PMS as acceptor. Approximately 1%

TABLE 2. *Hydrogenase activity of fraction II (vesicle clusters)*[a]

Prepn	H_2 oxidized[b]	H_2 oxidized[c]
Alnus rubra (red alder)	10.2	0.61
Alnus glutinosa (black alder × CpI-1)	8.8	0.91
Alnus rhombifolia (white alder × CpI-1)	1.4	0.79
M. pensylvanica (bayberry)	1.6	0.43

[a] Fraction II was prepared and assayed as described in the text. The electron acceptor was 3 mM MB.

[b] Micromoles of H_2 oxidized × (hour × gram fresh weight)$^{-1}$.

[c] Micromoles of H_2 oxidized × (minute × 10^6 vesicle clusters)$^{-1}$.

of the activity was lost per minute, so sufficient fraction II was supplied to support a rate adequate to complete the progress curves in less than 5 min.

Acceptor specificity. Artificial electron acceptors with a wide range of midpoint potentials were tested for their ability to couple to the uptake hydrogenase of the endophyte. All the electron acceptors tested coupled to the actinorhizal hydrogenase in contrast to soybean nodule hydrogenase. The effectiveness of coupling in *A. rubra* nodule homogenates was as follows: PMS > MB > DCIP > BV > ferricyanide > methyl

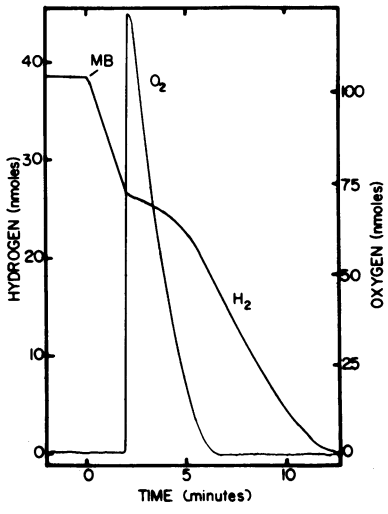

FIG. 2. *Inhibition by O_2 of the MB-dependent H_2 uptake by purified A. rubra endophyte. The reaction mixture contained 200 µl of fraction II from A. rubra with about 19 × 10^6 vesicle clusters in a final volume of 1.8 ml of 20 mM Tris-hydrochloride buffer (pH 7.4) with 3.3 mM MB. Residual $Na_2S_2O_4$ was oxidized by careful addition of O_2-saturated buffer before introducing MB and H_2.*

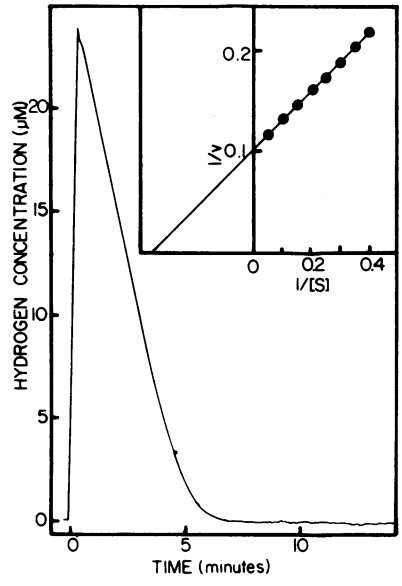

FIG. 3. *Progress curve of H_2 uptake by purified A. rubra endophyte. MB at 3.3 mM was used as the electron acceptor for determination of the apparent K_m for H_2. Sufficient fraction II was added to complete H_2 oxidation in less than 5 min. The inset shows a Lineweaver-Burk plot derived from the progess curve by measuring slopes of tangents in the lower portions of the curve. Substrate concentrations are in micromoles; velocities are expressed as nanomoles min^{-1}.*

TABLE 3. *Acceptor specificity of the hydrogenase activity from the root nodule homogenates of various plants*[a]

Plant	MV −440[b]	BV −360	PMS +8	MB +11	DCIP +217	FeCN +360	O_2 +820
A. rubra	1.4 (0.1)	14 (1.4)	100 (10.2)	66 (6.7)	32 (3.3)	11 (1.1)	<1 (<0.1)
A. rhombifolia	ND[c] ND	4 (<0.1)	100 (1.4)	62 (0.9)	<4 (<0.1)	ND ND	0 0
A. glutinosa	5.6 (0.5)	4.7 (0.4)	100 (8.8)	77 (6.7)	18 (1.6)	9.2 (0.80)	ND ND
M. pensylvanica	15 (0.2)	72 (1.2)	100 (1.6)	91 (1.5)	44 (0.8)	57 (0.9)	18 (0.3)
Soybean	0 0	0 0	100 (441)	44 (193)	20 (88)	5 (21)	52 (232)

[a] Values expressed as percent activity relative to PMS; actual rates are shown in parentheses as micromoles of H_2 oxidized × (hour × gram fresh weight)$^{-1}$.

[b] E°′ of each acceptor in millivolts.

[c] ND, Not determined.

viologen (MV) > O_2 (Table 3). The endophyte from *A. glutinosa* and *A. rhombifolia* (white alder) showed a similar pattern of hydrogenase acceptor specificity, whereas the bayberry endophyte exhibited a different pattern (Table 3). The bayberry hydrogenase was reversible with dithionite-reduced MV or BV, but the various alder hydrogenases were not reversible. Coupling of H_2 uptake to O_2 was observed, but the rates were very low (Fig. 4).

Bacteroids from soybean nodules took up H_2 when coupled to MB, PMS, O_2, or ferricyanide, but they did not couple to BV or MV. The most effective coupling of the bacteroid hydrogenase was to PMS, and O_2 was next most effective (Table 3).

All of the acceptors tested except ferricyanide enhanced O_2 uptake by the actinorhizal nodule endophyte over the endogenous rate (Fig. 5). The enhancement was not dependent upon H_2. Ferricyanide inhibited endogenous O_2 uptake. Relatively high concentrations of electron acceptor were needed to support maximal activity. For example, the alder and bayberry endophytes required 3 mM MB and 12 mM DCIP for optimal rates of H_2 uptake.

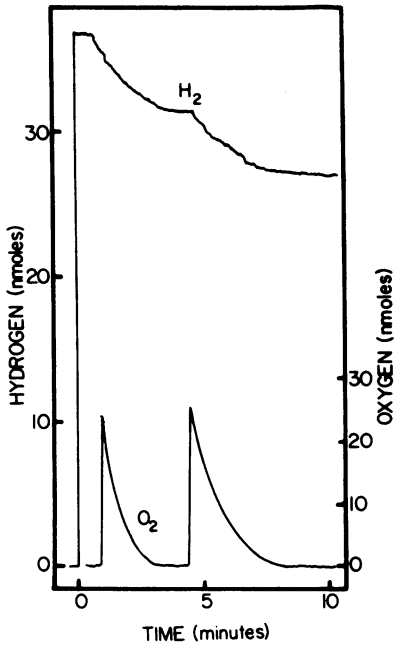

FIG. 4. *Oxyhydrogen reaction catalyzed by the hydrogenase system in purified A. rubra endophyte. Approximately 20 × 10^6 hyphal clusters were assayed in 1.8 ml of 20 mM Tris-hydrochloride buffer, pH 7.4. Residual dithionite was oxidized with O_2-saturated buffer.*

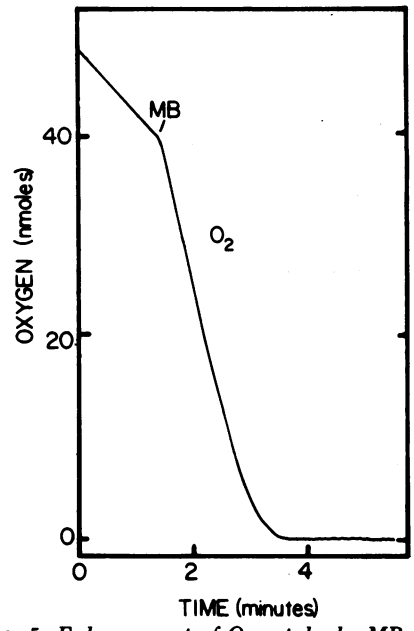

FIG. 5. *Enhancement of O_2 uptake by MB, in A. rubra endophyte. MB (100 mM) was added to a final concentration of 1.1 mM as indicated. The reaction mixture contained 15 × 10^6 hyphal clusters in 1.8 ml of 20 mM Tris-hydrochloride buffer, pH 7.4.*

DISCUSSION

Although we have demonstrated that cell-free nitrogenase from *A. glutinosa* root nodules can evolve H_2 in an ATP-dependent reaction (2), others have found little H_2 evolution from actinorhizal nodules (15, 21). This suggests that the actinorhizal nodules, like certain leguminous nodules, recycle nitrogenase-generated H_2 with an uptake hydrogenase. To resolve the issue, we have studied the H_2 metabolism of whole actinorhizal nodules and the H_2 uptake system in fractionated nodule homogenates.

Whole actinorhizal nodules from bayberry, red alder, and black alder and white alder inoculated with the endophyte isolated from *C. peregrina* took up H_2. The dependence of H_2 uptake on O_2 indicated the functioning of the oxyhydrogen reaction, much as in the *Rhizobium*-legume system (14).

Homogenates of the actinorhizal nodules consistently supported rapid uptake of H_2. The nodule homogenate was separated into three fractions by settling and centrifugation to provide a reasonably homogeneous preparation of the actinomycete endophyte (fraction II). The distribution of the hydrogenase activity among the fractions paralleled the distribution of nitrogenase activity as measured by C_2H_2 reduction and

ATP-dependent H_2 evolution. The hydrogenase apparently is located within the procaryotic symbiont. This agrees with the findings of Roelofsen and Akkermans (17). The difficulty in obtaining a completely homogeneous endophyte preparation reflects the filamentous nature of the endophyte as well as the fact that it is present in a variety of developmental stages (4, 13).

In bacteroids from legumes, H_2 uptake is coupled to O_2 more effectively than to most artificial electron acceptors (Table 3) (18). In actinorhizal endophyte preparations, the greatest activity was observed with electron acceptors such as MB.

Hydrogen uptake with O_2 as acceptor was very slow (Table 3); 2 μM O_2 inhibited coupling of H_2 to artificial acceptors. Hydrogenases often are inhibited by O_2 in vitro (16), and the inhibition by O_2 in endophyte preparations and the low rate of O_2-coupled H_2 uptake suggests one or more of the following explanations: (i) within the nodule the endophyte is maintained at a very low non-inhibitory PO_2; (ii) an essential link in the electron-transport chain between H_2 and O_2 has been damaged or lost during preparation; (iii) the hydrogenase has been damaged by removal from a protective environment. Conditions are aerobic within actinorhizal nodules, but the O_2 concentration is very low in cells occupied by endophyte (25). The presence of a hydrogenase system that relies on O_2 but also is inhibited by O_2 suggests that the endophyte exists in a microaerophilic environment.

As artificial electron acceptors enhance O_2 uptake by the endophyte, it seems logical that they may be substituting for natural electron carriers in increasing the rate of oxidation of endogenous substrates. Dyes may aid in bypassing damaged portions of the electron transport pathway and, thus, may increase the rate of oxidation of endogenous substrates, a proposal that can be tested by measuring CO_2 evolution and O_2 uptake both in the presence and absence of artificial electron acceptors.

Concentrations of acceptors (3 to 6 mM) required for maximal activity were high. These relatively high concentrations may reflect the barriers to uptake presented by intact microorganisms. Similarly, high concentrations of ATP and dithionite are needed for nitrogenase assays in comparable preparations (3, 29).

The actinorhizal hydrogenase systems observed were of two types. The systems from alder with a native endophyte or with the *C. peregrina* endophyte metabolize H_2 unidirectionally and have a broad acceptor specificity. The hydrogenase system from the bayberry endophyte also shows a broad acceptor specificity but is reversible when reduced dyes (e.g., BV or MV) of sufficiently low oxidation-reduction potential are utilized. The bayberry hydrogenase exhibits a relatively more effective coupling to BV and ferricyanide than does the hydrogenase from alder. Although the bayberry endophyte may contain one hydrogenase that is distinctly different from the hydrogenase present in the alders, our experiments cannot eliminate the possibility that the bayberry endophyte contains two hydrogenases, one of the reversible type and one of the H_2-oxidizing type (19). Although we saw no evidence to support the idea, it is possible that microorganisms other than the infecting actinomycetes were present in the bayberry nodules and that they contained the reversible hydrogenase observed. Uptake hydrogenases with similar properties are present in aerobic or microaerophilic nitrogen-fixing organisms such as *Azotobacter vinelandii* and *R. japonicum* (1, 28). Like these organisms, the actinorhizal system couples to O_2 in vivo via the oxyhydrogen reaction.

It is of special interest that the hydrogenase system from red alder (a member of the order Fagales) is similar to the hydrogenase from the *C. peregrina* endophyte. The red alder nodules came from plants grown in Oregon, whereas the *C. peregrina* endophyte was isolated in Massachusetts. *C. peregrina* is a member of the order Myricales to which bayberry also belongs. Thus, taxonomic similarity among host plants appears to have little relationship to the infecting microorganisms. It would be interesting to repeat the experiments with nodules from other actinorhizal plant species reported to have distinctly different endophyte forms, such as *Ceanothus* spp. or *Casuarina* spp. (24, 27).

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