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In Vitro Nitrogen Fixation by Two Actinomycete Strains Isolated from *Casuarina* Nodules

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Acetylene reduction activity was demonstrated in pure cultures of two actinomycete strains isolated from nodules of *Casuarina equisetifolia*. This activity was comparable to that of free-living *Rhizobium* strains, but appeared to be less sensitive to pO_2 and more sensitive to the presence of combined nitrogen.

It is well established that some strains of *Rhizobium* can exhibit nitrogenase activity when grown in vitro on defined media (2, 7, 8, 13, 14), but such activity has not been reported for actinomycete strains isolated from root nodules of N_2-fixing nonlegumes. The present study was initiated to determine whether actinomycetal isolates from nodules of *Casuarina equisetifolia*, a nonleguminous tree widely introduced in tropical and semiarid countries, can express nitrogenase activity when grown in the absence of the plant host.

Two strains, designated D11 and G2, were isolated from nodules of *C. equisetifolia* sampled in Senegal (D11) and in Guadeloupe (G2). Seedlings of *C. equisetifolia* were grown axenically in test tubes (6) and inoculated at an age of 1 month with a suspension of crushed nodules. Nodules that developed after 2 months were used to obtain the isolates. Plates of agar QMOD medium (10) were inoculated with 1 ml of a 10^{-3} suspension of these nodules and incubated for 3 to 4 weeks. Since the actinomycete colonies are small (ca. 0.1 mm), the microscope had to be used to observe and isolate the strains. These colonies can be easily recognized since they exhibit a typical starfish-like appearance. Two features of isolates D11 and G2 (hyphae and sporangia with polyhedral spores) were very similar to those of actinomycete symbionts of the temperate nonlegume genera *Comptonia*, *Alnus*, and *Elaeagnus* as viewed both in culture (1, 4, 9, 11, 16) and in the nodule (17). But, in contrast with in vitro cultures of these temperate symbionts, our strains produced not only hyphae and sporangia but also vesicles (Fig. 1). Vesicles were formed on both Kalininskaya and QMOD media. Attempts to nodulate axenic seedlings of *Casuarina* sp. by inoculation with pure cultures of D11 and G2 have failed.

Isolates D11 and G2 were aerobically grown in a liquid QMOD medium at 30°C for 5 days by using a rotary shaker (108 rpm). The cultures were centrifuged, washed, suspended in a Kalininskaya saline medium supplemented with malate (20 mM) as a carbon source, and adjusted to pH 7.0 (3) to the same volume as that of the initial culture. A portion (25 ml) of this suspension was placed in 150-ml vials with serum stoppers. The vials were evacuated and filled with a gas mixture containing 10% C_2H_2, with increasing percentages of O_2 (2, 5, 10, 15, and 20%), and argon as the complementary gas. Incubation together with agitation was continued for 5 days at 30°C. During the incubation, there was no significant growth, but a profuse sporulation was observed together with the formation of vesicles. Specific acetylene reduction activity was measured daily by the method of Postgate (15) and was expressed as nanomoles of C_2H_4 produced per hour per milligram of protein, estimated by the method of Lowry et al. (12).

As shown in Fig. 2, the reduction of acetylene to ethylene by strain D11 occurred only in the presence of O_2. Specific activities were highest when pO_2 was 10 kPa. Comparison of the specific acetylene reduction activity of strains D11 and G2 at different levels of pO_2 showed that the behavior of those strains was similar, with pO_2 optimal at 5 to 15 kPa (Fig. 3).

To check the effect of different sources of nitrogen upon nitrogenase synthesis, strains D11 and G2, which had been previously grown on QMOD medium for 5 days, were incubated by the procedure mentioned above with Kalininskaya saline medium with different nitrogen sources. Table 1 shows that yeast extract slightly stimulated nitrogenase activity at a low concentration (25 μg/ml) with little effect at higher concentrations (50 to 100 μg/ml). Repression by glutamine occurred when the concentration was equal to or higher than 0.54 mM. Nitrate and ammonium drastically repressed nitrogenase synthesis even at the lowest concentrations (2 mM) used.

This is the first report of nitrogen fixation by free-living actinomycetes isolated from nodules of a nitrogen-fixing nonlegume. Acetylene reduc-

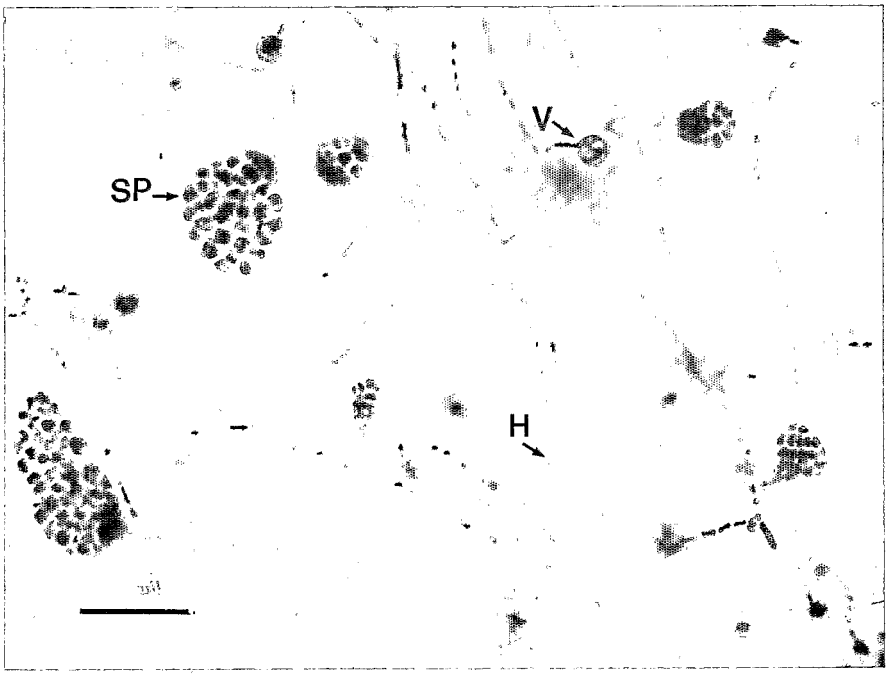

FIG. 1. *Photomicrograph of a portion of a 14-day-old colony of strain D11, grown in QMOD medium, showing hyphae (H), vesicles (V), and mature sporangia (SP) with disaggregating polyhedral spores. Bar, 10 μm.*

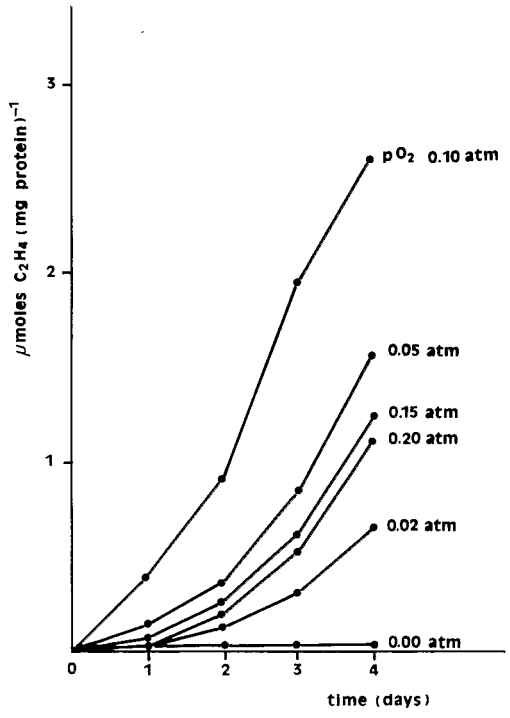

FIG. 2. *Time course of derepression of nitrogenase biosynthesis at different pO_2 (strain D11).*

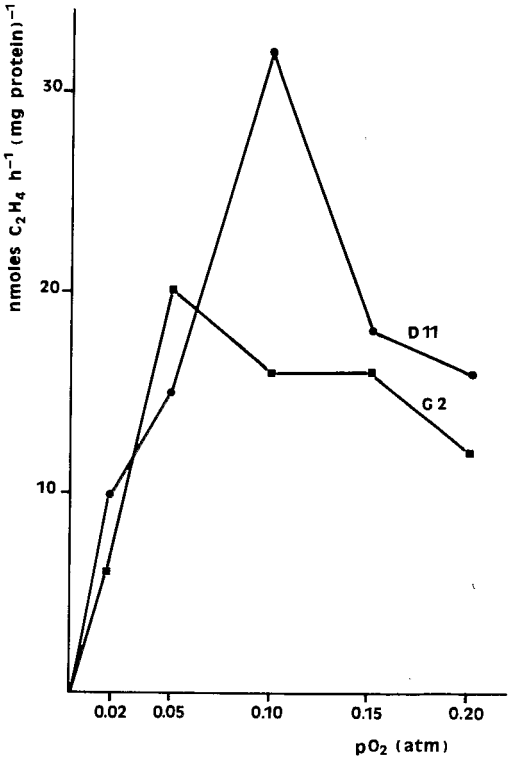

FIG. 3. *Specific nitrogenase activity at different pO_2 (strains D11 [●] and G2 [■]).*

TABLE 1. *Influence of different nitrogen sources on nitrogenase biosynthesis measured by the acetylene reduction method*[a]

Nitrogen source[b]	Specific acetylene reduction activity (nmol of C_2H_4 per h per mg of protein)	
	Strain D11	Strain G2
None (control)	34	14
Yeast extract (25 μg/ml)	42	20
Yeast extract (50 μg/ml)	30	12
Yeast extract (100 μg/ml)	17	12
L-Glutamine (0.25 mM)	32	16
L-Glutamine (0.54 mM)	3	1
L-Glutamine (1.1 mM)	3	1
KNO_3 (2 mM)	1	1
KNO_3 (5 mM)	0	0
KNO_3 (10 mM)	0	0
NH_4Cl (2 mM)	5	2
NH_4Cl (10 mM)	0	0
NH_4Cl (20 mM)	0	0

[a] Actinomycete strains were incubated in agitated vials filled with a gas mixture containing 10% O_2 and 90% argon at 30°C for 5 days. The specific acetylene reduction activity was calculated by using the slope of the C_2H_4 production curve.

[b] The medium contained malate (20 mM) and different amounts of combined nitrogen as indicated.

tion was ca. 10 to 30 nmol/h per mg of protein, which is comparable to values obtained for free-living *Rhizobium* strains which exhibited moderate acetylene reduction activities (2, 5). Since the incubation in the preliminary experiments reported here was carried out in agitated vials and the cell number was low, we assume that the concentration of dissolved O_2 in the culture was relatively high. Thus, it seems likely that our isolates fix nitrogen under more aerobic conditions than reported for free-living *Rhizobium* strains.

Nitrogenase synthesis by our isolates appeared to be more sensitive than *Rhizobium* strains to the presence of combined nitrogen, since *Rhizobium* strains are still able to form a significant amount of nitrogenase with an ammonium concentration of 40 mM (7).

Surprisingly, strains D11 and G2 did not initiate nodulation in the host plant. Failure to induce nodulation may indicate that these strains are noninfective in the pure culture condition as suggested by Knowlton et al. (7a) or that the requisite environmental conditions for nodulation were not achieved in our tests.

ADDENDUM IN PROOF

This note was in press when Tjepkema et al. (J. D. Tjepkema, W. Ormerod, and J. G. Torrey, Nature [London] **287**:633–635, 1980) reported that strains of *Frankia* isolated from *Comptonia peregrina* and *Alnus rubra* cultured in defined nutrient media could reduce acetylene.

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