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Free-Living *Rhizobium* Strain Able To Grow on N_2 as the Sole Nitrogen Source

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A *Rhizobium* strain isolated from stem nodules of the legume *Sesbania rostrata* was shown to grow on atmospheric nitrogen (N_2) as the sole nitrogen source. Non-N_2-fixing mutants isolated directly on agar plates formed nodules that did not fix N_2 when inoculated into the host plant.

Rhizobium spp. normally fix atmospheric nitrogen (N_2) only within nodules formed as a result of their symbiotic association with specific host plants of the family *Leguminosae*. Some strains belonging to the slow-growing *Rhizobium* group (*Rhizobium* spp. of the cowpea group and *R. japonicum*) are also able to express nitrogenase under free-living microaerobic conditions (4–6, 8, 9), but they require a source of combined nitrogen to support their growth. Therefore, the use of conventional bacteriological techniques to isolate symbiotic nitrogen fixation *Rhizobium* mutants has not yet been possible without screening each of the potential mutants on individual plants.

In previous papers (2, 3), Dreyfus and Dommergues indicated that a fast-growing *Rhizobium* strain (ORS 571) isolated from stem nodules of the tropical legume *Sesbania rostrata* forms nodules on both the stem and the roots of the host plant. We report here that this strain not only expresses nitrogenase activity in culture but also is able to grow on N_2 as the sole nitrogen source.

Preliminary experiments showed that the growth of strain ORS 571 in a defined medium was dependent on the addition of three vitamins: biotin, pantothenic acid, and nicotinic acid. We studied the growth of the strain in three media: a nitrogen-free medium (LO medium), LO medium), and a complete medium (YL medium, i.e., LN medium supplemented with 1 g of yeast extract per liter). The composition of LO medium was as follows (per 1000 ml): sodium lactate, 10 g; K_2HPO_4, 1.67 g; KH_2PO_4, 0.87 g; NaCl, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2$, 40 mg; $FeCl_3$, 4 mg; $MoO_4Na \cdot 2H_2O$, 5 mg; biotin, 10 mg; nicotinic acid, 20 mg; pantothenic acid, 10 mg; and trace elements. The pH was maintained constant at 6.8.

The strain was grown at 37°C in a 1.5-liter Biolafitte fermentor that contained 1 liter of medium and was inoculated with bacteria previously grown under aeration in YL medium and washed twice in LO medium. Internal agitation of the broth was at 400 rpm, and the gas mixture was constantly bubbled through at a rate of 1 liter/min.

Growth of strain ORS 571 on N_2 or NH_4 under different gas phase conditions is reported in Fig. 1. When grown in LN medium under air or 3% O_2–97% N_2, the strain exhibited a generation time of 3 h at 37°C. When grown in nitrogen-free LO medium under 3% O_2–97% N_2, strain ORS 571 showed exponential growth; the optical density at 570 nm increased from 0.15 to 2.30, and the generation time was 6 h at 37°C. It exhibited nitrogenase activity only 2 h after inoculation. Specific nitrogenase activity (not shown in Fig. 1) was maximum (29 nmol of C_2H_4 mg of protein^{-1} min^{-1}) for an optical density of 0.6. No ammonium accumulation could be detected in

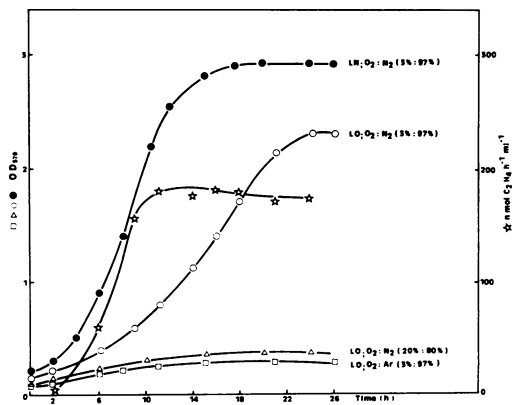

FIG. 1. Growth of strain ORS 571 on N_2 or NH_4.

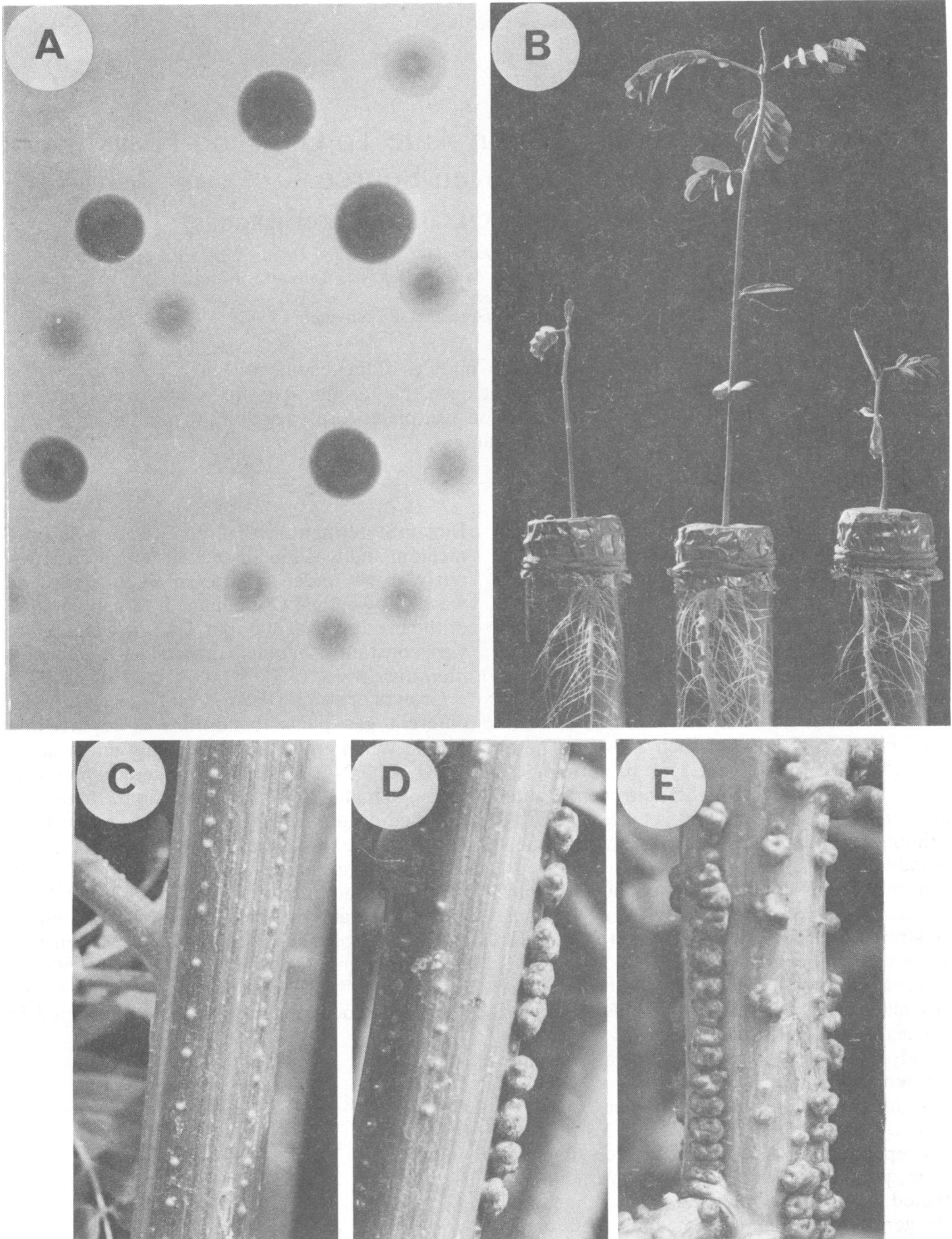

FIG. 2. (A) Nitrogen and non-nitrogen-fixing colonies on LO medium incubated for 5 days under 3% O_2–97% N_2. Colonies of the parent strain (ORS 571), which actively fixed N_2, appear as large dark spots; colonies of non-nitrogen-fixing mutant strain 5740, whose growth was very poor, appear as small pale spots. Previously tested strains had been mixed before plating. (B) Ability of non-nitrogen-fixing mutant 5740, isolated from a plate culture, to nodulate and fix nitrogen, as determined by the root inoculation test (2): left, uninoculated; center, inoculated with parent strain ORS 571; right, inoculated with mutant 5740. Root nodules formed by ORS 571 fixed nitrogen, but root nodules formed by 5740 did not. Roots were inoculated when plants were 3 days old, and the photograph was taken when the plants were 3 weeks old. (C, D, and E) Ability of mutant 5740 to nodulate and fix nitrogen, as determined by the stem inoculation test (2). (C) Absence of nodules on uninoculated stems, (D) nitrogen-fixing nodules on stems inoculated with parent strain ORS 571, (E) non-nitrogen-fixing nodules on stems inoculated with mutant 5740. Stems were inoculated when plants were 3 weeks old, and the photographs were taken 3 weeks later.

the medium. The total nitrogen content of the culture broth (which included nitrogen from the vitamin supplement) increased from 4 to 50 mg/liter. When the percentage of oxygen in the gas mixture was increased (20% O_2, 80% N_2) or when N_2 was replaced by argon (3% O_2, 97% Ar), the maximum observed optical density was ca. 0.3. These results clearly show that strain ORS 571 was able to grow in nitrogen-free LO medium with N_2 as the sole nitrogen source, provided that the O_2 tension was appropriate. Under the conditions which permitted the growth of ORS 571 on N_2, strain CB 756, a cowpea strain known to express nitrogenase activity in culture (1), neither grew nor exhibited any acetylene reducing activity.

Purity of the cultures was assured by repeated use of single colony isolates, and the identities of the isolates were checked by plant infection tests. Strains reisolated from nodules obtained under aseptic conditions exhibited the same characteristics as those shown by strain ORS 571.

Since strain ORS 571 in pure culture can grow on N_2, it was possible to isolate symbiotic nitrogen fixation mutants by standard bacteriological techniques. Ethyl methane sulfonate mutagenesis (200 μg of ethyl methane sulfonate ml^{-1}) was performed as described by Miller (7). Mutagenized bacteria (5% of the cells survived and a 5- to 10-fold-increase occurred in the number of streptomycin-resistant mutants) were grown overnight in LN medium, washed once with LO medium, and plated on solid LO medium. After 5 days of incubation under a 3% O_2–97% N_2 gas phase, 40,000 colonies from two mutagenized cultures were examined. Fifty small colonies were plated on LO, LN and YL media with toothpicks. Clones which exhibited the same growth on YL and LN media but which did not grow on LO medium were checked for nitrogenase activity. Of the 50 colonies tested, five mutants showed no or very little nitrogenase activity in culture. In liquid LN medium, the growth rate of these nitrogen fixation mutants was similar to that of the parent strain (ORS 571), but in liquid LO medium under 3% O_2–97% N_2, there was no significant growth, compared with strain ORS 571.

Under the same gas mixture but on solid LO medium, the growth of the mutants was very poor, compared with that of the parent strain (Fig. 2A). The colony size of the mutants grown on solid LO medium under 3% O_2–97% N_2 was similar to that of the parent strain grown on the same medium but under non-nitrogen fixation conditions (3% O_2, 97% Ar).

Each of the five mutants was then inoculated onto the roots or stems of *S. rostrata* to check nodulating and nitrogen fixing ability. Nodules appeared at the same time (4 to 5 days after inoculation), regardless of whether the plants had been inoculated with the parent strain or the mutants. Root and stem nodules formed by the parent strain fixed nitrogen actively (Fix^+). In contrast, nodules formed on both stem and roots by the mutants were ineffective (Fix^-): no acetylene reduction could be detected, and the plants inoculated with the mutants remained as yellow as the uninoculated control and quite different from the green healthy plants inoculated with the parent strain. Figure 2 (B, C, D, and E) shows the results of the infection tests performed with strain 5740, one of the five mutants.

Thus, the analysis of the *nif* (nitrogen fixation) genes of certain *Rhizobium* strains can be carried out as if these strains were classical free-living nitrogen-fixing bacteria, such as *Klebsiella* spp. The possibility of using plate screening for obtaining symbiotic nitrogen fixation *Rhizobium* mutants could accelerate investigations of the genetics of this agronomically important bacterial genus.

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