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Agrobacterium tumefaciens Is a Diazotrophic Bacterium

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This is the first report that *Agrobacterium tumefaciens* can fix nitrogen in a free-living condition as shown by its abilities to grow on nitrogen-free medium, reduce acetylene to ethylene, and incorporate ^{15}N supplied as $^{15}N_2$. As with most other well-characterized diazotrophic bacteria, the presence of NH_4^+ in the medium and aerobic conditions repress nitrogen fixation by *A. tumefaciens*. The system requires molybdenum. No evidence for nodulation was found with pea, peanut, or soybean plants. Further understanding of the nitrogen-fixing ability of this bacterium, which has always been considered a pathogen, should cast new light on the evolution of a pathogenic versus symbiotic relationship.

Ever since the discovery that *Agrobacterium tumefaciens* is the causal organism of crown gall (20), it has been considered a plant pathogen (see reference 5). Consequently, no systematic investigation has ever been undertaken to determine whether any *Agrobacterium* strains can fix nitrogen, although the bacterium is closely related to the genus *Rhizobium* and belongs to the same family, *Rhizobiaceae* (8). Heumann (6), however, emphasized the close similarity of *Rhizobium meliloti* and *A. tumefaciens* and suggested the possibility of their interconversion. It is also true that several *Rhizobium* Sym plasmids have been transferred into Ti plasmid-cured *A. tumefaciens* (see, for example, reference 7), but in almost all cases the investigation was confined to the study of nodulation by the transconjugants.

In a brief study concerned with the expression of the *Klebsiella pneumoniae nif*$^+$ cluster (obtained in a P group plasmid, RP4) in different bacterial hosts, it was reported that *A. tumefaciens* transconjugants do not reduce acetylene, thereby indicating the absence of active nitrogenase (4). However, since the recipient strain used, 544, is neither well known nor available anymore and since no confirmatory tests (such as 3-ketolactose production and tumorigenicity [see next section]) were carried out, evaluation of these results remains difficult.

While investigating the expression of *K. pneumoniae nif-lac* fusions in *A. tumefaciens* (9), we discovered that several wild-type strains of the latter can grow well on minimal medium devoid of fixed nitrogen. In this report, we describe results from an investigation carried out with strains B6, C58, and NT1 which show that this property is a reflection of the diazotrophic nature of *A. tumefaciens*.

(A preliminary account of this work has appeared in abstract form [L. Kanvinde, M. H. Soliman, H. Wardhan, L. Nowell, D. Fox, and G. R. K. Sastry, III Int. Symp. Mol. Genet. Plant-Microbe Interactions, abstr., p. 309–312, 1986].)

MATERIALS AND METHODS

Bacterial strains. *A. tumefaciens* C58 and B6 were originally obtained from E. W. Nester and J. De Ley, respectively; NT1, a Ti plasmid-cured (and therefore nontumorigenic) C58 strain, was also provided by E. W. Nester. C58 strains with (i) an unknown position of Tn*5* (otherwise wild type), (ii) *pro-4*::Tn*5* Rif^r, and (iii) *pro-21*::Tn*5* Rif^r and B6 Sm^r Rif^r (spontaneous) were isolated in our laboratory. *K. pneumoniae* UNF722 [*lac* Δ(*his-nif*)] and UN1179 (*nif*$^+$) were from R. A. Dixon and V. Shah, respectively. *Escherichia coli* UNF514, obtained from R. A. Dixon, is a Mu lysogenic derivative of JC5466 (*his trp recA56 lacX74*).

Media. The nitrogen-free medium used (NFDM) (3) contained $MgSO_4 \cdot 7H_2O$ (0.1 g), $Na_2MoO_4 \cdot 2H_2O$ (25 mg), $FeSO_4 \cdot 7H_2O$ (25 mg), K_2HPO_4 (12.06 g), KH_2PO_4 (3.4 g), and glucose (20 g) in 1 liter H_2O. Phosphate solution was sterilized separately and subsequently added aseptically. No organic supplements, such as vitamins, were added. For complete medium LB, see reference 14. To make NFDM, chemicals of the highest quality available commercially (Aristar grade; BDH, Poole, England) were used without further purification; instead of bacterial agar, agarose (grade II, Sigma Chemical Co., Poole, England) was used to make NFDM plates. When required, filter-sterilized amino acids and antibiotics were added after autoclaving of the medium.

Assay for acetylene reduction. In most experiments, nitrogenase activity of bacterial cultures was measured by the ability to reduce acetylene to ethylene. Overnight cultures (5.0 ml) were grown from single colonies in ammonium sulfate-supplemented medium; 0.2-ml volume of such cultures were used to inoculate 3.0 ml of NFDM in 7.0 ml of bijou bottles supplemented with either serine (100 μg/ml; without NH_4^+) or ammonium sulfate (2.0 mg/ml; with NH_4^+). Bottles were closed tightly with suba seals (Freeman & Co., Barnsley, United Kingdom). After the cultures were grown to the mid-exponential phase (24 to 36 h), 0.5 ml of acetylene gas (produced from calcium carbide and routinely assayed for ethylene contamination) was injected into each bottle and incubated further on an orbital incubator (200 rpm at 30°C). Under these conditions of growth, the oxygen content of the headspace in the bottles was reduced to about 3% in 30 to 36 h; *A. tumefaciens* does not grow under completely anaerobic conditions. Actual oxygen levels in the culture medium were not measured. If continuation of the aerobic condition was required, the gas phase was replaced with fresh sterile air a few minutes before injection of acetylene. At appropriate intervals, 0.1 ml of the gas phase was withdrawn and analyzed by using a gas-liquid chromatograph (Pye Unicam PU4500) attached to an integrator (Pye Unicam PU4810). The quantity of ethylene produced was calculated on the basis of the area of its peak compared with that of a standard, or in some cases, the amount of ethylene was given as a percentage of the total gas phase. Readings taken immediately after injection of the acetylene gave 99.9

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to 99.99% values for the gas, the rest being impurities including (very rarely) ethylene.

Assay for $^{15}N_2$ incorporation. Overnight cultures grown in NFDM supplemented with ammonium sulfate (nitrogen source) were used (0.5 ml) to inoculate 60 ml (in 100-ml Erlenmeyer flasks) of NFDM or NFDM supplemented with different sources of nitrogen. After evacuation, each flask was injected with 5.0 ml of 99.9% $^{15}N_2$ (Amersham International plc, Amersham, England) and the remaining space was filled with a mixture of argon (95%) and oxygen (5%). Cultures were grown for 5 days on an orbital shaker (200 rpm at 30°C); at the end of that period, growth was stopped by adding a few drops of concentrated sulfuric acid to each flask, and rotoevaporated cultures were sent to R. H. Burris (Department of Biochemistry, University of Wisconsin, Madison), who completed the Kjeldahl digestion, distilled the digest, converted the NH_4^+ to N_2, and analyzed for ^{15}N by using a MAT250 isotope ratio mass spectrometer (2).

Protein assays. Protein assays were done as described by Lowry et al. (12).

Nitrogen analysis. Nitrogen analysis of the media was done by the Kjeldahl method (2).

Purity checks. Since the nature of the present project demands very stringent checks on the purity of the strains, extensive testing was undertaken at various stages of the work. Small samples of the cultures taken just before and at the end of the experiments were spread on LB and NFDM (auxotrophically supplemented when necessary) plates to obtain single colonies; these were tested for 3-ketolactose production and, with C58 and B6, for tumorigenicity. Procedural details of these two diagnostic tests for *A. tumefaciens* have already been described (1, 15). Colonies from LB and NFDM were cross-checked for the ability to grow on NFDM and LB, respectively. When appropriate, tests for additional markers, such as Tn*5* (kanamycin, 50 μg/ml), streptomycin (250 μg/ml), rifampin (100 μg/ml), and the auxotrophic requirement, proline (25 μg/ml), were also done at various stages.

Since *A. tumefaciens* produces considerable quantities of polysaccharides, especially when grown under less favorable conditions, experimental cultures were examined at various stages to make certain that no hidden diazotrophic contaminants were present (as described in reference 11). This was achieved by adding a nonionic detergent, Tween 40, to both cultures and plates to give a final concentration of 0.01% (vol/vol). Single colonies were obtained on LB and NFDM plates which were then tested for 3-ketolactose production and the ability to fix nitrogen.

RESULTS

Growth in nitrogen-free medium. *A. tumefaciens* B6, C58, and NT1 all grew well on NFDM plates; large single colonies appeared on this medium in 4 to 5 days versus 3 days on the medium supplemented with ammonium sulfate. This ability was not due to a carryover effect from rich medium, as these strains had been continuously subcultured for 2 years on NFDM plates with no loss of viability. Apart from wild types, B6 Sm^r Rif^r and C58::Tn*5* Rif^r were also included in these experiments; all of them grew equally well on NFDM. At the end of the experiments, all of the strains, when tested for diagnostic and various genetic features, gave positive results, thus ruling out any contaminants.

A. tumefaciens grew well on NFDM plates incubated in anaerobic jars under microaerobic conditions (Fig. 1). Apart from testing the ability to grow under reduced oxygen tension, these experiments were also intended to eliminate the unlikely possibility that NH_4^+ in the laboratory atmosphere might contribute fixed nitrogen. In several experiments designed to investigate acetylene reduction and $^{15}N_2$ incorporation, the growth pattern of *A. tumefaciens* in liquid NFDM was monitored concomitantly; all three strains did grow on this medium as well.

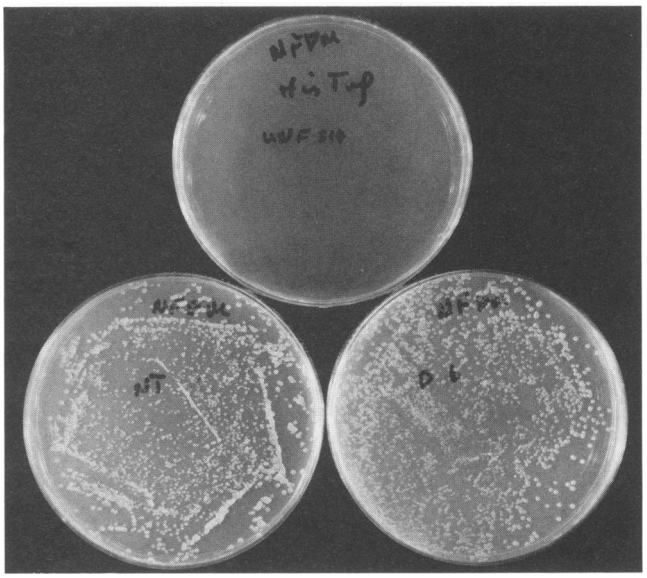

FIG. 1. Growth of B6 and NT1 on NFDM plates. Overnight cultures of *A. tumefaciens* and *E. coli* UNF514 grown in NFDM with ammonium sulfate were washed and diluted with nitrogen-free medium and then plated on NFDM; for *E. coli*, the media were supplemented with histidine and tryptophan (25 μg of each per ml). The illustrated growth was obtained after 4 days of incubation at 30°C in anaerobic jars filled with argon (85%), nitrogen (10%), and oxygen (5%). Negative controls UNF514 and UNF722 (not shown) did not grow, even under normal aerobic conditions, while *A. tumefaciens* grew. The top plate contained *E. coli* UNF514, the lower left contained *A. tumefaciens* NT1, and the lower right contained *A. tumefaciens* B6.

Kjeldahl analysis of several NFDM samples revealed no detectable levels of fixed nitrogen. Double-distilled and, in some cases, deionized water used to make the medium contained only insignificant concentrations of nitrogen (less than 0.5 μM), including nitrates. According to the manufacturers, glucose, arabinose, and agarose contain no detectable nitrogen.

Acetylene reduction. Most of the tests for acetylene reduction were designed primarily on the basis of the experience obtained with derepression of the nitrogen fixation system in *K. pneumoniae*. As with *K. pneumoniae*, the medium with no combined nitrogen was found to be inefficient in making *A. tumefaciens* cultures reduce acetylene; under these conditions, cultures produced excessive amounts of exopolysaccharides and flocculated. Addition of a nonpreferred source of nitrogen, such as serine or proline, helped the situation considerably.

Also, in the initial experiments, we increased the level of glucose from 2% (as in the recipe given in reference 3) to 4%, hoping that a high level of glucose would aid a high-energy-requiring process such as nitrogen fixation. However, contrary to this belief, reducing glucose to 1% (or even 0.5% [see Table 1, for an example]) helped the situation considerably, since the flocculation virtually disappeared.

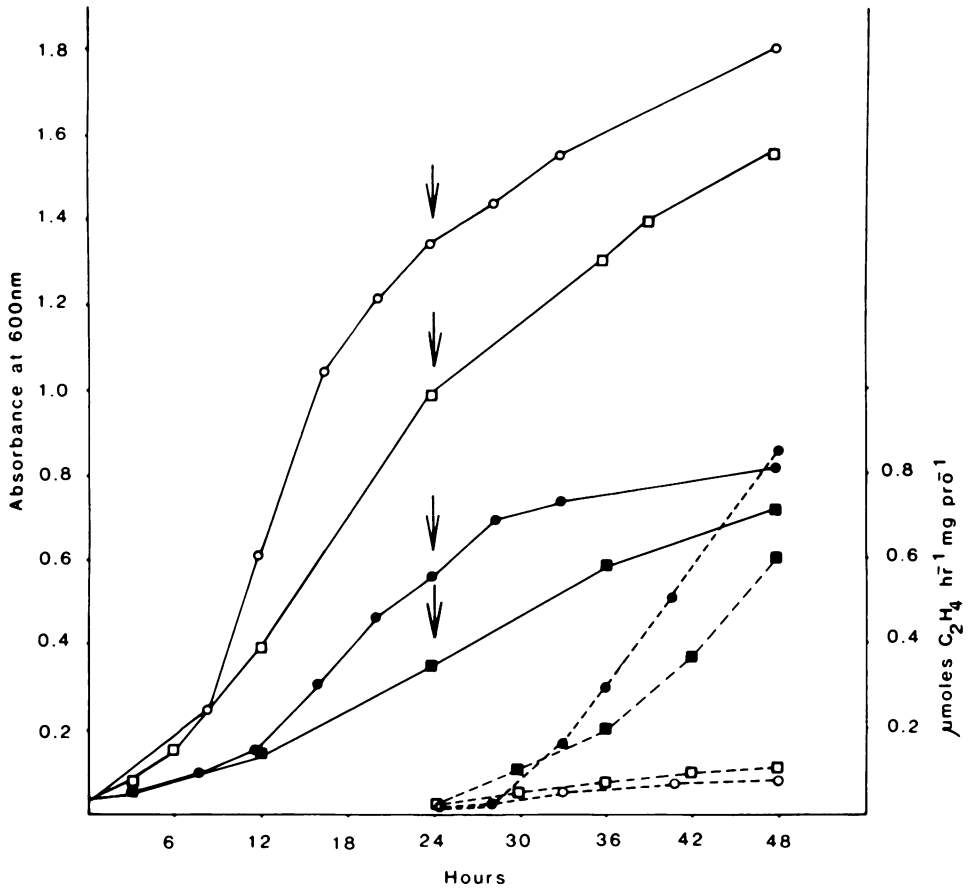

FIG. 2. Effects of proline (without NH_4^+) and ammonium sulfate (with NH_4^+) as nitrogen sources on acetylene reduction by C58 and B6; all media contained 1.0% glucose. The arrows indicate the time when acetylene was injected. Growth: C58 with proline (without NH_4^+) (●) or ammonium sulfate (with NH_4^+) (○); B6 with proline (without NH_4^+) (■) or ammonium sulfate (with NH_4^+) (□). Ethylene: in C58 with proline (without NH_4^+) (●) or ammonium sulfate (with NH_4^+) (○); in B6 with proline (without NH_4^+) (■) or ammonium sulfate (with NH_4^+) (□). Solid lines represent growth; broken lines represent ethylene production.

Bearing the above observations in mind, we carried out several experiments to study acetylene reduction by B6, C58, and NT1; in most cases, *K. pneumoniae* UN1179 (*nif*$^+$) and UNF722 Δ(*his-nif*) were used as positive and negative controls, respectively. During the early stages of the investigation, bacteria were tested for acetylene reduction when grown under microaerobic conditions in NFDM with serine as the nitrogen source. In one experiment, for example, the following values (accumulation of ethylene per milligram of protein) were obtained: C58, 13 μmol; NT1, 7 μmol; B6, 8 μmol. In the same experiment, *K. pneumoniae* UN1179 *nif*$^+$ and UNF722 Δ*nif* (positive and negative controls) showed 24 and 0.05 μmol, respectively.

At a later stage, however, serine was replaced with proline as the nitrogen source, since *A. tumefaciens* cultures grew better on the latter; the rate of acetylene reduction on this medium is illustrated in Fig. 2. As expected, the positive control, *K. pneumoniae* UN1179, when included, always reduced acetylene vigorously in medium without NH_4^+ under microaerobic conditions (see Fig. 3).

A summary of data obtained from four independent experiments carried out under conditions suitable for *A. tumefaciens* is presented in Fig. 3. Apart from establishing the diazotrophic nature of this bacterium, a highlight of the study was that, as in most diazotrophic bacteria, aerobic conditions (Fig. 3C) repressed the system significantly, as did NH_4^+ in the medium (Fig. 2 and 3A). The comparatively poor performance of C58 (Fig. 3B, 1) was improved considerably by using arabinose (1%) as the carbon source and addition of sodium succinate (20.0 mM). Under these conditions, both C58 and NT1 performed equally well; use of arabinose together with added succinate have been reported to improve the nitrogen fixation of some agar- and liquid-grown *Rhizobium* strains (see, for example, references 10 and 16).

Because molybdenum is an essential component of nitrogenase (see reference 19), an experiment was carried out to see what effect, if any, it has on acetylene reduction by *A. tumefaciens* C58; it was able to reduce acetylene very effectively in the medium containing a normal concentration (0.2 mM) of sodium molybdate. After 96 h of incubation, there was 5.5 μmol of ethylene per mg of protein in the gas phase of the culture grown with molybdenum versus 0.35 μmol/mg of protein when the element was omitted. This suggests that the enzyme responsible for nitrogen fixation in *A. tumefaciens* contains molybdenum, as in other diazotrophic bacteria.

Some activity, albeit low (0.35 μmol), shown by the cultures grown on NFDM without added molybdate may have been supported by contaminating traces of the metal; if

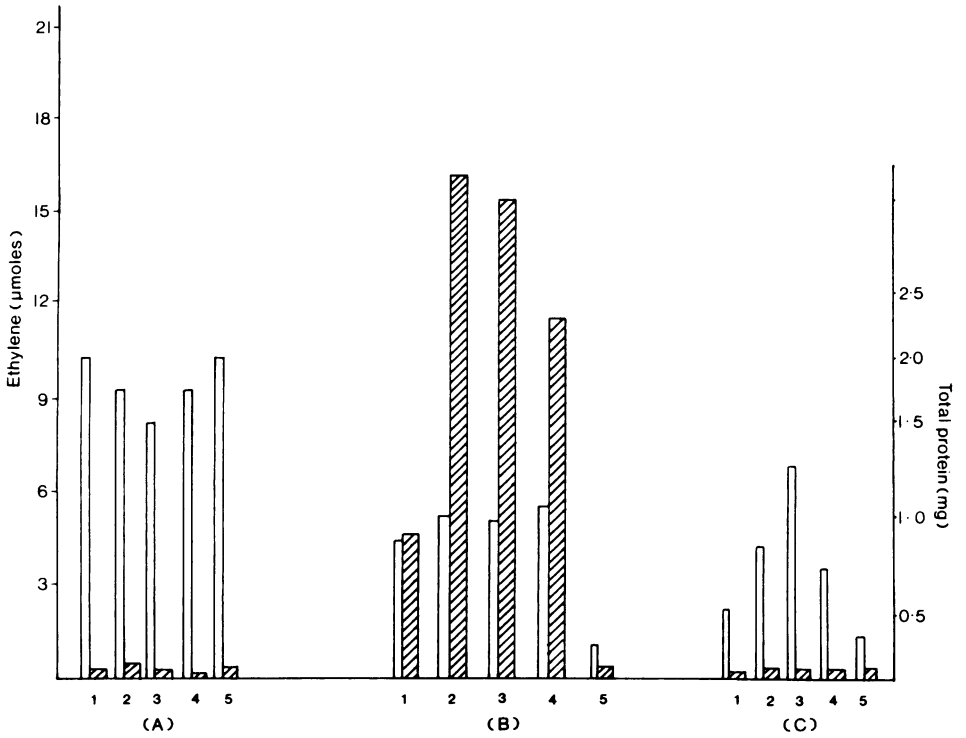

FIG. 3. Effect of NH_4^+ and aerobic conditions on acetylene reduction. Overnight starter cultures (grown in NFDM with ammonium sulfate) were used to initiate different treatments in bijou bottles: A, NFDM with ammonium sulfate (with NH_4^+); B, NFDM with proline (without NH_4^+); C, NFDM with proline (without NH_4^+). However, the aerobic conditions were maintained as described in Materials and Methods and 1% glucose was used as the carbon source in all cases. The effects of these treatments on *A. tumefaciens* C58 (column 1), NT1 (column 2), and B6 (column 3) were compared with those on *K. pneumoniae* UN1179 (column 4) and UNF722 (column 5). The amount of ethylene (▨) after 72 h of incubation from the acetylene injection is shown. *K. pneumoniae* UN1179 showed ethylene immediately after injection of acetylene and increased only up to 24 h; in contrast to this, ethylene appeared slowly in *A. tumefaciens* but increased for a longer period. Protein values (□) are for 3-ml cultures. The results shown are means from four experiments.

this is true, addition of tungstate to the medium should lower the activity further, since it is known to inhibit molybdenum uptake (21); very much lowered ethylene production (0.06 μmol/mg of protein) was indeed found in the medium in which sodium molybdate was substituted with sodium tungstate (2.0 mM), supporting that contention. Confirmation of the role of molybdenum in *A. tumefaciens* obviously requires more elaborate chemical procedures and stringent purification of the chemicals and glassware used.

Although most of the investigations were done in liquid medium, some experiments were also performed with media partially solidified with agarose (0.5%). Bottles (7.0 ml), each with 5.0 ml of medium, were used. After 4 days of growth, 0.5 ml of acetylene was injected into each bottle. Periodic analyses of the gas phase from these cultures produced results similar to those of cultures grown in liquid media. For example, in one experiment, the gas phase contained (total accumulation) 20 μmol of ethylene when C58 was grown microaerobically with proline as the nitrogen source, whereas ethylene was only 0.02 μmol with NH_4^+ and 0.176 μmol under aerobic conditions, respectively, even after incubation for 8 weeks. Tests for 3-ketolactose production and tumorigenicity carried out at the end of the experimental period gave positive results, as in previous experiments.

As described in Materials and Methods, several colonies from the cultures used for each experiment were checked before and at the end of the experiments; the results confirmed their purity. Also, in one set of experiments, acetylene reduction was studied with C58 *pro-4*::Tn*5* Rif^r and C58 *pro-21*::Tn*5* Rif^r grown in NFDM appropriately supplemented for the auxotrophic requirement; nutritional conditions for the absence or presence of NH_4^+ were produced by supplying additional amounts of proline and ammonium sulfate, respectively. As observed previously, cultures without NH_4^+ showed significant reduction of acetylene (data not shown). Colonies obtained from various experimental cultures showed all of the expected markers, resistance to kanamycin and rifampin and auxotrophic requirement of proline, thus once again ruling out contaminants as a cause of acetylene reduction.

$^{15}N_2$ incorporation. Two preliminary sets of experiments were carried out before initiating a definitive investigation with B6. In the first set, all three strains, C58, NT1, and B6, were grown in partially solidified NFDM in an atmosphere enriched with $^{15}N_2$. These gave variable but positive results; however, since the experiments were performed on a small scale, the total nitrogen obtained was not adequate to give results with good precision. In the second set of experiments, B6, which gave the best results in the previous study, was grown in a $^{15}N_2$-enriched atmosphere in NFDM liquid medium with 1.0 mg of ammonium sulfate per ml and in NFDM without fixed nitrogen. The amount of the culture grown in the nitrogen-free medium still did not give sufficient total nitrogen for good N^{15} analysis, and the culture produced a large quantity of polysaccharides. On the other hand, bacteria grown in NFDM supplemented with ammo-

TABLE 1. $^{15}N_2$ incorporation by *A. tumefaciens* B6 grown in NFDM supplemented with different levels of fixed nitrogen

Fixed nitrogen source and quantity (μg/ml)	^{15}N excess (atom%)[a]	C_2H_4 (μmol/mg of protein)[b]
None	1.8103	0.068
Proline		
100	0.9237	1.560
200	0.2765	0.224
500	0.0860	0.150
1,000	0.0582	0.021
Ammonium sulfate		
100	0.0699	0.004
200	0.0747	0.025
500	0.0122	0.007
1,000	0.0180	0.001
2,000	0.0239	0.000

[a] These are mean values obtained from an analysis of duplicate samples from each treatment.

[b] Single measurements were made 24 h after acetylene injection. Normally, acetylene reduction was not observed when the cultures were grown in NFDM with no fixed nitrogen, but when the glucose level was reduced to 0.5%, some ethylene did appear, as in this case; obviously, the process was still inefficient.

nium sulfate showed significant incorporation of the isotope (0.038 atom% excess ^{15}N); apparently, B6 fixed nitrogen (as indicated by $^{15}N_2$ incorporation) after exhausting the supply of fixed nitrogen in the medium.

On the basis of the above-described results, a large experiment was carried out in which B6 was grown in NFDM with or without fixed nitrogen; in the experiment with NFDM without fixed nitrogen, different levels of fixed nitrogen were provided as either proline or ammonium sulfate. One important difference from the preceding experiments was that the level of glucose was reduced from 1 to 0.5%. After 5 days of growth, 5.0 ml of acetylene was injected into one of the flasks from each treatment; cells from the remaining three flasks were pooled and analyzed for $^{15}N_2$ incorporation. Although 4 days of growth was adequate, 5 days was allowed to ensure incorporation of the isotope.

Reduction of glucose level to 0.5% visibly affected the cultures; even the one grown with no fixed nitrogen was less viscous and did not flocculate. The results presented in Table 1 show that *A. tumefaciens* was able to incorporate ^{15}N supplied as dinitrogen. Fixation ability was reduced by increased availability of fixed nitrogen; this result was corroborated by the findings on acetylene reduction by the same cultures.

DISCUSSION

A diazotrophic bacterium must, by definition, be able to grow well on a nitrogen-free minimal medium, be able to reduce acetylene to ethylene, and incorporate ^{15}N supplied as $^{15}N_2$. The data presented in the preceding sections satisfy all of these criteria, supporting the contention that *A. tumefaciens* is capable of fixing nitrogen in a free-living condition.

The demonstrated growth of *A. tumefaciens* on NFDM was not due to contaminating fixed nitrogen in the chemicals used to make up the medium. We know of no nondiazotrophic bacterium capable of growing on this type of medium. If sufficient quantities of fixed nitrogen were present in the medium, *E. coli* and *K. pneumoniae* deleted for *nif* genes also should have grown on it; they did not grow.

Three important points need to be emphasized in the present context. (i) In no case did *A. tumefaciens* cultures produce ethylene without injection of acetylene. (ii) The quantity of ethylene progressively increased in *A. tumefaciens* cultures grown under conditions of derepression until the gas phase was more or less completely filled by it. This rise in the quantity of ethylene was always associated with a concomitant, proportional decrease in the quantity of acetylene, showing that the ethylene was produced by acetylene reduction. (iii) The negative control, *K. pneumoniae* UNF722, which carries a deletion for the entire *nif* region, never showed any acetylene reduction, even after prolonged incubation.

The ability to reduce acetylene is specific for monitoring nitrogenase activity, and there are no reports to the contrary. However, it is only an indirect test. Therefore, we measured the incorporation of $^{15}N_2$ by *A. tumefaciens*. It might be noted that B6, even when grown in NFDM devoid of any combined nitrogen, incorporated some ^{15}N. Results from these experiments (Table 1) proved the diazotrophic nature of *A. tumefaciens*.

Throughout the present investigation, extreme care was taken to keep the cultures pure. Besides the purity checks described in the previous sections, all of the strains were tested on the root systems of pea, peanut, and soybean seedlings; none of them ever produced nodules. Similarly, Tween 40-treated cultures (see Purity Checks in Materials and Methods) showed neither morphologically variant colonies (see reference 11) nor any contaminants; all of the colonies tested were 3-ketolactose positive and fixed nitrogen. The treatment itself visibly reduced polysaccharide production. Also, in experiments (Fig. 2) in which the positive control, *K. pneumoniae* UN 1179, was not included to avoid any unlikely cross-contamination, *A. tumefaciens* still showed acetylene reduction.

The fact that the Ti-cured strain NT-1 can also fix nitrogen suggests that, at least in C58, the necessary genes are not located on the Ti plasmid; genetic analysis (data not shown) indicated that this gene complex is, in fact, closely linked to the chromosomal gene *his*$^+$ (13). We isolated R68.45 primes bearing *A. tumefaciens* C58 *his*$^+$ *nif*$^+$ in an *E. coli* recipient, UNF514; since these *E. coli* transconjugants are capable of growing on nitrogen-free medium, analysis of the R primes should allow us to learn details of the genetic organization of the nitrogen-fixing system in *A. tumefaciens*.

Several features of the nitrogen fixation system in *A. tumefaciens* resemble those of other well-investigated diazotrophs, such as *K. pneumoniae* (17). The DNA sequences of structural genes responsible for nitrogenase, *nifH*, *nifD*, and *nifK*, are highly conserved (18). Despite these facts, DNA from neither C58 nor B6 S3 (a derivative of B6) hybridized with a *nifHDK* probe from *K. pneumoniae* (18). Similar experiments in our laboratory with probes from *R. meliloti*, *Azorhizobium caulinodans* ORS571, and *K. pneumoniae* also produced negative results, even under low-stringency conditions of hybridization (H. Wardhan, unpublished data). Thus, while it is not surprising that *A. tumefaciens*, a close relative of the genus *Rhizobium*, is able to carry out nitrogen fixation, the fact that the systems are apparently dissimilar is puzzling.

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