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Mutants That Produce Nitrogenase in the Presence of Ammonia

(soil fertility/*Azotobacter vinelandii*/pleiotropic-negative/2-methylalanine/derepressed)

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ABSTRACT **Mutants of *Azotobacter vinelandii* that fix N_2 in the presence of excess NH_4^+ have been isolated. A mutant that was unable to synthesize component I and component II of nitrogenase was spontaneously reverted to the N_2-fixing phenotype. Of 21 revertants picked, 7 revertants were not as sensitive as the wild type to repression. A derepressed mutant is as sensitive as the wild type to growth inhibition by 2-methylalanine in the presence of glucose.**

Rhizobium-legume symbionts and all N_2-fixing bacteria and N_2-fixing blue-green algae that have been examined exhibit a striking level of repression of nitrogenase synthesis when excess NH_4^+ is added to the growth medium (for example, refs. 1–4). This paper describes one technique that will successfully yield mutants of *Azotobacter vinelandii* that are derepressed for the synthesis of nitrogenase. The method is suitable for obtaining similar mutants with other nitrogen-fixing microorganisms. Normally, nitrogenase causes NH_4^+ to be formed by the organism (5), and this NH_4^+ consequently represses further nitrogenase synthesis until the NH_4^+ is depleted. Derepressed mutants, on the other hand, will keep synthesizing nitrogenase and produce an excess of NH_4^+. Such mutants might have potential use for increasing soil fertility.

There are several examples in which a mutation in a regulatory gene simultaneously causes more than one function in a biochemical pathway to be lost. Frequently, revertants of such mutants are derepressed for the synthesis of the structural gene products (6, 7). We isolated a mutant strain of *A. vinelandii* that did not synthesize either of the two nitrogenase components. Some revertants of this strain are derepressed for nitrogenase synthesis. One use of such a mutant is to determine whether 2-methylalanine, a reported corepressor of nitrogenase (8), affects the same site as does NH_4^+.

MATERIALS AND METHODS

Organism. *Azotobacter vinelandii* OP (9), referred to as strain UW, is the wild-type strain used in these studies. Mutant strains that are unable to fix N_2 were isolated as described (10).

Media. The N_2-free medium was a modified Burk's medium (11). Media that contain excess NH_4^+ include 400 μg of N per ml as ammonium acetate.

Preparation and Assay of Cell-Free Extracts. Genetically derepressed strains were grown in 200 ml of Burk's medium plus excess NH_4^+. Mutants unable to fix N_2 were derepressed by growth on limiting NH_4^+ followed by 3 hr of N-starvation (12). Cultures were grown in 1-liter baffled flasks to increase aeration. Growth was followed on a Klett–Summerson photoelectric colorimeter with a no. 64 filter. Cells were grown to a density of about 6×10^8 cells per ml and harvested in the manner described by Shah *et al.* (12). Preparation of extracts, acetylene and N_2 reduction assays, and chromatographic separation of component I and component II proteins of nitrogenase were performed with the modifications described (13). Protein concentrations were determined by the method of Lowry *et al.* (14). The procedure for detecting nitrogenase-specific crossreacting material was described (12). Specific activities of nitrogenase are defined as nmol ethylene produced/(min $\times$ mg protein).

RESULTS AND DISCUSSION

Extracts from cells of *A. vinelandii* that have been growing on N_2 have a specific activity (Table 1) of 52 nmol C_2H_4 produced/(min $\times$ mg protein). When the cells have been grown in a medium containing excess NH_4^+, the specific activity is less than 0.002. This 2.6×10^4-fold drop in activity is due to repression of nitrogenase synthesis by NH_4^+, rather than to inhibition of nitrogenase activity (15). Such a variation in enzyme activity is remarkable when compared to that of β-galactosidase in *Escherichia coli* (16) or the histidine biosynthetic enzymes in *Salmonella typhimurium* (17). Thus, it is of interest to determine how the synthesis of nitrogenase is controlled.

We have previously described methods for isolation and examination of mutants of *A. vinelandii* that are unable to fix N_2 (10, 12). One of these mutants, strain UW2, has the properties that might be expected of a regulatory mutant. Strain UW2 grows as well as the wild type (strain UW) in medium containing excess NH_4^+, but is unable to grow in the absence of fixed nitrogen. Strain UW2 grown under derepressed conditions has no component I or component II activity (Table 1), and also has no detectable crossreacting material for component I (Fig. 1) or component II (unpublished results).

Spontaneous revertants of strain UW2 were obtained by plating 1×10^8 cells on a medium containing no fixed nitrogen. Twenty-one independent revertants were picked and purified. Seven of these strains synthesized detectable quantities of nitrogenase in the presence of excess NH_4^+. Three of these seven derepressed mutant strains had about 25% of the amount of nitrogenase that is found in the derepressed wild type; the other four strains exhibited about 0.5% of the amount of derepressed wild type.

Strain UW59, obtained as a spontaneous revertant of strain UW2, has the properties described in Table 1. The specific activity of this mutant on N_2 is about half that found

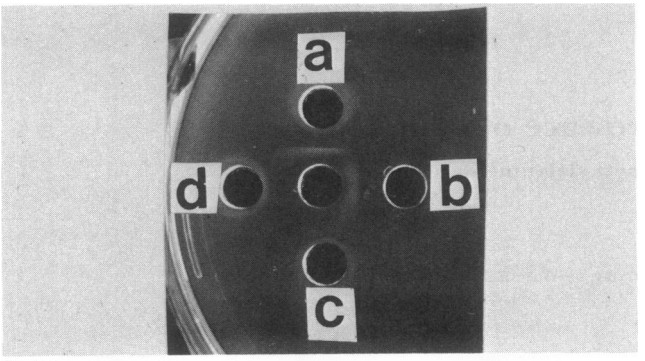

FIG. 1. Ouchterlony plate for testing antibody crossreactions against component I. The center well contains serum with antibody to component I (32 μg protein). The outer wells contain cell extracts (about 250 μg protein). *a* is from strain UW59 grown on NH_4^+; *b* is from strain UW grown on N_2; *c* is from strain UW2 grown under derepression conditions; *d* is from strain UW grown on NH_4^+.

in strain UW (wild type) that has been grown on N_2. The specific activity (by acetylene reduction) of nitrogenase from strain UW59 grown on excess NH_4^+ is about half that from strain UW59 grown on N_2. The same result was seen when nitrogenase activity was measured by N_2 conversion to NH_4^+. Titration of the crude extract of strain UW59 with purified components that have been isolated from the wild type indicates that both components in strain UW59 grown on excess NH_4^+ are synthesized coordinately. The assay for component II (addition of purified component I) usually yields lower specific activities (13), because component II is more labile than component I. Fig. 1 shows that an extract of strain UW59 grown with excess NH_4^+ crossreacts with antiserum prepared against purified component I, whereas strain UW under the same conditions of growth exhibits no such crossreaction.

The growth rates of strains UW and UW59, on a medium containing excess NH_4^+ as well as on a N-free medium, are compared in Fig. 2. The doubling times for both strains on NH_4^+ are the same (2 hr). In contrast, the growth rates of the strains on N_2 are markedly different, strain UW having a doubling time of 3 hr and strain UW59 having a doubling time of 8.6 hr. A likely explanation for the slow growth of strain UW59 is that the growth is limited by the amount of fixed N available to the bacteria, this in turn being limited by the amount of nitrogenase synthesized under N_2 growth conditions. The lower nitrogenase activity *in vitro* of strain UW59, therefore, might cause the growth of this strain on N_2 to be slower than that of strain UW.

TABLE 1. *Specific activities for acetylene reduction by strains UW, UW2, and UW59*

Strain	N-source		Specific activity*		
	N_2	NH_4^+	Extract	+I†	+II†
UW	+	−	52.1	57.7	61.5
UW	−	+	<0.002	—	—
UW2	+	−	<0.002	—	—
UW59	+	−	23.9	27.9	35.6
UW59	−	+	11.9	13.4	16.9

* Each number represents the average of at least 10 independent experiments. Data are given as nmol C_2H_4 formed/(min × mg protein).

† The specific activities represent the maximum amount of activity obtained by titration with purified component.

The coordinate synthesis of nitrogenase components I and II in strains UW (13) and UW59 supports the idea that a common regulatory gene is required for repression of the synthesis of both nitrogenase components. This gene might be one that codes for a repressor or activator that controls the expression of the structural genes for the components.

What causes strain UW59 to produce nitrogenase in the presence of NH_4^+? If the strain from which UW59 was derived had a mutation similar to the i^s-type of mutation observed in the *lac* operon of *E. coli* (6), almost all of the revertants of this strain would be expected to be derepressed. However, only a third of the revertants of strain UW2 are derepressed. One also would expect a revertant of a superrepressed mutant to have activities as great as those found in derepressed wild-type cells.

If nitrogenase were regulated by positive control (7) whereby an activator protein was converted into a repressor by the addition of NH_4^+, strain UW2 might have a mutation in the activator gene causing production of a nonfunctional product. The phenotype of strain UW59 then might be formed by a mutation restoring activity to that activator gene. If this modified activator no longer reacted efficiently with either the operator or the corepressor, enzyme synthesis would be derepressed.

If the phenotype of strain UW2 resulted from a mutation in a promoter region of an operon that codes for the nitrogenase genes, another mutation that formed a new promoter in the operator also might produce the derepressed phenotype. An example of this situation has been observed with the *lac* operon (18). Decreased synthesis of nitrogenase by strain UW59 might result if the new promoter were less efficient.

If exogenous NH_4^+ were not incorporated into the cell fast enough to maintain the intracellular pools of NH_4^+, this might lead to partial derepression of nitrogenase. However, the equivalence of the growth rates of strains UW and UW59 on NH_4^+ suggests that uptake of NH_4^+ in strain UW59 is not limiting. Derepressed enzyme synthesis may occur by inter-

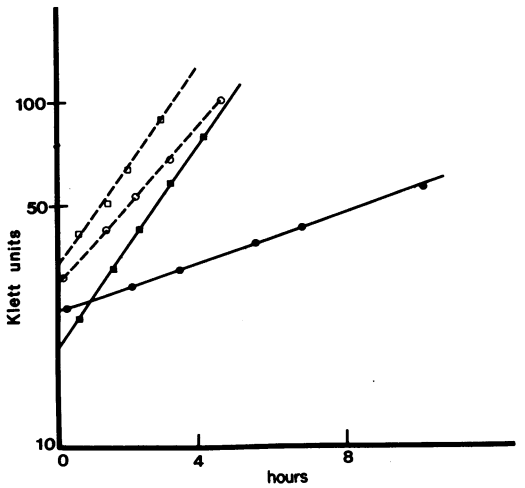

FIG. 2. Growth rates of strains UW and UW59 on N_2 and NH_4^+. Strain UW on NH_4^+, □- - -□; strain UW59 on NH_4^+, ■——■; strain UW on N_2, ○- - -○; strain UW59 on N_2, ●——●.

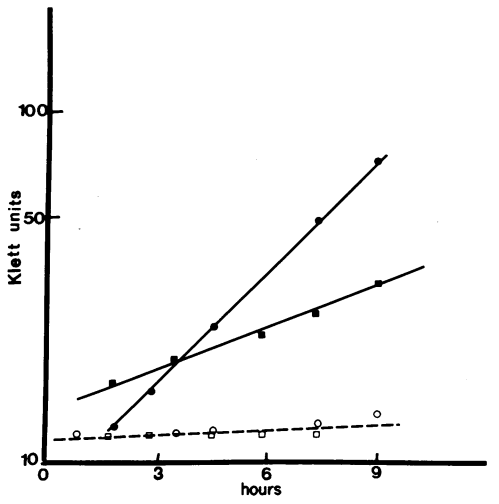

FIG. 3. Effect of methylalanine on the growth of strains UW and UW59. Strain UW on N_2, ●——●; strain UW59 on N_2, ■——■; strain UW on N_2 plus 0.1% methylalanine, ○- - -○; strain UW59 on N_2 plus 0.1% methylalanine, □- - -□.

ference with the synthesis of the effector (e.g., as has been found in the histidine-degrading system in *E. coli*, ref. 19). In this case derepression of nitrogenase might result from channeling an effector into other metabolic pathways, but this should give fully derepressed activity on N_2. Tests with Nessler's reagent of supernatant solutions of harvested cells indicated that NH_4^+ in the medium was not being depleted by rapid use. Genetic techniques such as transduction or transformation (20) should be useful for eliminating some of the above models as explanations for our results.

The derepressed strains we have isolated will be helpful, as controls, in deriving procedures for selecting derepressed strains directly from wild-type cultures. One potential method incorporates an alternative nitrogenase substrate, azide (21), into plates of Burk's medium plus excess NH_4^+. Derepressed strains will convert the N_3^- to NH_4^+, thereby detoxifying the medium; the wild-type cells will be killed. Cyanide, another alternative substrate (22), can be used similarly.

The derepressed strain UW59 was used to examine the involvement of 2-methylalanine in the repression of nitrogenase. Sorger (8) found that the nonmetabolizable compound 2-methylalanine inhibited growth of *A. vinelandii* on N_2, but had no effect on its growth on NH_4^+ or NO_3^-. He postulated that 2-methylalanine acted as a corepressor of nitrogenase synthesis. However, St. John and Brill (23) showed that the inhibitory effect of 2-methylalanine was evident only on cells grown on glucose or maltose and was much more pronounced with N_2-grown cultures than with NH_4^+-grown cultures.

The effect of 2-methylalanine on growth of strains UW and UW59 was observed under conditions described by St. John and Brill (23). Cultures were grown on excess NH_4^+, and a 1% inoculum was transferred to Burk's N-free glucose medium and shaken on a rotary shaker at 30°. The results are shown in Fig. 3. The derepressed strain, UW59, was inhibited by 2-methylalanine to the same extent as the wild-type strain, UW. The fact that nitrogenase formation in strain UW59 is not repressed by NH_4^+, but strain UW59 still is inhibited by 2-methylalanine, indicates that 2-methylalanine does not affect the regulatory site that interacts with NH_4^+.

We are also isolating derepressed strains of *Klebsiella pneumoniae* and several species of *Rhizobium*. All of these derepressed strains have a potential use for fertilizing soils because they should be able to fix N_2 even in the presence of NH_4^+ or NO_3^-.

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