Mutants That Produce Nitrogenase in the Presence of Ammonia

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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).

Medium. The \(N_2\)-free medium was a modified Burk's medium (11). Media that contain excess \(NH_4^+\) include 400 \(\mu g\) 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 \(6 \times 10^8\) 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 × 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 × 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 \(\times 10^4\)-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 \(\beta\)-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 \(\times 10^8\) 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 mutants 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
in strain UW (wild type) that has been grown on N₂. The specific activity (by acetylene reduction) of nitrogenase from strain UW grown on excess NH₄⁺ is about half that from strain UW grown on N₂. The same result was seen when nitrogenase activity was measured by N₂ conversion to NH₃. Titrations of the crude extract of strain UW59 with purified components that have been isolated from the wild type indicate that both components in strain UW59 grown on excess NH₄⁺ 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 NH₄⁺ 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 NH₄⁺ as well as on a N-free medium, are compared in Fig. 2. The doubling times for both strains on NH₄⁺ are the same (2 hr). In contrast, the growth rates of the strains on N₂ 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 N₂ growth conditions. The lower nitrogenase activity in vitro of strain UW59, therefore, might cause the growth of this strain on N₂ to be slower than that of strain UW.

The coordinate synthesis of nitrogenase components I and II in strains UW (13) and UW59 suggests 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 NH₄⁺? If the strain from which UW59 was derived had a mutation similar to the i⁻-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 super-repressed 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 NH₄⁺, 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 NH₄⁺ were not incorporated into the cell fast enough to maintain the intracellular pools of NH₄⁺, this might lead to partial derepression of nitrogenase. However, the equivalence of the growth rates of strains UW and UW59 on NH₄⁺ suggests that uptake of NH₄⁺ in strain UW59 is not limiting. Derepressed enzyme synthesis may occur by inter-

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>N-source</th>
<th>Specific activity*</th>
<th>Extract</th>
<th>+I†</th>
<th>+II†</th>
</tr>
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<tr>
<td>UW</td>
<td>N₂</td>
<td></td>
<td>52.1</td>
<td>57.7</td>
<td>61.5</td>
</tr>
<tr>
<td>UW</td>
<td>NH₄⁺</td>
<td></td>
<td>&lt;0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UW2</td>
<td>NH₄⁺</td>
<td></td>
<td>23.9</td>
<td>27.9</td>
<td>35.6</td>
</tr>
<tr>
<td>UW59</td>
<td>N₂</td>
<td></td>
<td>11.9</td>
<td>13.4</td>
<td>16.9</td>
</tr>
</tbody>
</table>

* Each number represents the average of at least 10 independent experiments. Data are given as nmol C₂H₂ formed/(min × mg protein).
† The specific activities represent the maximum amount of activity obtained by titration with purified component.
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 N₂. Tests with Nessler's reagent of supernatant solutions of harvested cells indicated that NH₄⁺ 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 NH₄⁺. Derepressed strains will convert the N₂⁻ to NH₄⁺, 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 N₂, but had no effect on its growth on NH₄⁺ or NO₃⁻. 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 N₂-grown cultures than with NH₄⁺-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 NH₄⁺, 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 NH₄⁺, but strain UW59 still is inhibited by 2-methylalanine, indicates that 2-methylalanine does not affect the regulatory site that interacts with NH₄⁺.

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 N₂ even in the presence of NH₄⁺ or NO₃⁻.

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