GENETICS OF NITROGEN FIXATION
(nitrogenase, molybdenum, ammonium excretion, legumes)

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SUMMARY

Recent advances in understanding the genetics of nitrogen fixation have provided ideas for novel applications of nitrogenase and nitrogen-fixing organisms. Mutant strains have been useful for identifying the active site of nitrogenase and for identifying other factors, besides nitrogenase, that are specifically required for an organism to fix nitrogen. These strains have been important tools for assaying such factors during their purification.

A fine-structure map of Klebsiella pneumoniae was obtained by deletion mapping of many nif (nitrogen fixation) mutations. Transformation between Nif- mutant strains of Azotobacter vinelandii has shown that the nif genes are scattered around the chromosome, unlike the situation in K. pneumoniae in which nif genes are clustered.

Regulatory mutations have been useful for constructing derepressed ammonium-excreting strains in A. vinelandii. Such strains can fertilize the roots of cereal plants. The regulation of nitrogen fixation seems to be quite complex since ammonium, oxygen, and molybdenum all play a role in nitrogenase synthesis in K. pneumoniae.

The Rhizobium-legume symbiosis has been studied with mutant strains of Rhizobium that are unable to form root nodules and mutant strains which form root nodules unable to fix nitrogen. Several strains that do not infect the host plant lack a surface polysaccharide that is present in wild-type cells. Some mutant strains of Rhizobium cause leghemoglobin-deficient nodules to be formed. Another mutant phenotype causes the plant to fix more nitrogen than the wild type. Such strains might have potential use in agriculture.
INTRODUCTION

Nitrogen fixation is the conversion of atmospheric N\textsubscript{2} to a compound that has N bonded to another element that is not N. Examples of fixed N compounds are nitrate, urea, and ammonia. This reaction is an essential part of the N cycle, and it offsets the loss of fixed N to N\textsubscript{2} by denitrifying bacteria. Most of the N\textsubscript{2} fixation is accomplished by a few species of bacteria. No eukaryote will fix N\textsubscript{2} by itself. Nitrogen-fixing bacteria are quite diverse in habitat and properties; some fix N\textsubscript{2} by themselves, and others require a eukaryote host in order to fix N\textsubscript{2}.

Catalysis of N\textsubscript{2} fixation occurs by the enzyme nitrogenase, which is composed of two proteins, components I and II. The biochemistry of nitrogenase has been reviewed by Winter and Burr is (1976) and Eady and Postgate (1974). Component I contains approximately 24 iron atoms and two molybdenum atoms. It has a molecular weight close to 250,000 daltons. Component II has a molecular weight of about 60,000 daltons and contains four iron atoms. Nitrogenases from all sources studied have quite similar properties (Eady and Postgate 1974). The reaction has a tremendous energy demand because 12-24 ATPs are required for each N\textsubscript{2} that is fixed (Burr is and Orme-Johnson 1974). This energy demand probably is the main reason that N\textsubscript{2} fixation is limited in nature.

An important property of nitrogenase is that both components are extremely labile to oxygen; therefore all biochemical studies with an active enzyme must be performed under strictly anaerobic conditions (Burr is and Orme-Johnson 1974). Bacteria that fix N\textsubscript{2} also must keep oxygen away from nitrogenase. Azotobacter vinelandii is one of the few bacteria that fix N\textsubscript{2} aerobically. Presumably, this organism keeps oxygen from inactivating nitrogenase because of a very high respiratory activity by which oxygen is quickly reduced to water (Phillips and Johnson 1961, Dalton and Postgate 1969). Klebsiella pneumoniae, on the other hand, will grow on fixed N either anaerobically or aerobically, but it will only grow on N\textsubscript{2} under anaerobic conditions (Pengra and Wilson 1958). This organism is unable to protect its nitrogenase from being oxygen-inactivated (St. John et al. 1974). Rhizobium japonicum, the bacterium that forms root nodules on soybean, somehow causes the plant to synthesize a hemoglobin-type protein called leghemoglobin which seems to keep free oxygen from inactivating nitrogenase in the bacteria but allows oxygen to be used for ATP synthesis by respiration (Berger sen et al. 1973). These three organisms are the ones that will be discussed in this paper.

Since N\textsubscript{2} fixation has such a great energy demand, cells use fixed N in preference to fixing their own N\textsubscript{2}. No nitrogenase is found in free-living bacteria when they are grown with excess ammonium, and no nodules form on soybean grown with sufficient fertilizer N. Therefore, regulation of nitrogenase synthesis in free-living bacteria and regulation of the infection process by Rhizobium are important mechanisms to understand.
It is much simpler to study bacteria that fix N₂ by themselves than to deal with the more complex *Rhizobium*-legume symbiosis. For this reason, results with *R. japonicum* will be discussed in a separate section.

**PHENOTYPES**

In order to make maximum use of genetic information, biochemical characteristics have to be monitored in mutant strains (Table 1). Component I and II activity were quantitated by titrating a crude extract of a mutant strain with purified components prepared from the wild type (SHAH and BRILL 1973). Inactive components were detected either by cross reaction with antisera prepared against native component or on polyacrylamide gels after electrophoresis. A specific staining procedure detects both components on gels by reacting with iron in the proteins to form pink bands on the gels (BRILL et al. 1974). Other iron proteins are present in much smaller amounts in the extracts so that they do not show up by this procedure.

**TABLE 1.** Phenotypes of Nif⁻ mutant strains of *Azotobacter vinelandii*

<table>
<thead>
<tr>
<th>Strains</th>
<th>Activity</th>
<th>Antigenic cross-reacting material</th>
<th>EPR signal</th>
<th>Fe stain on polyacrylamide gel</th>
<th>Activatable in vitro by FeMoCo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I II</td>
<td>I II g=3.65</td>
<td>I II</td>
<td>I II</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>+ +</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>UW1</td>
<td>- -</td>
<td>- -</td>
<td>-</td>
<td>- -</td>
<td>-</td>
</tr>
<tr>
<td>UW3</td>
<td>- -</td>
<td>+ -</td>
<td>-</td>
<td>- -</td>
<td>-</td>
</tr>
<tr>
<td>UW6</td>
<td>- +</td>
<td>- +</td>
<td>-</td>
<td>- +</td>
<td>-</td>
</tr>
<tr>
<td>UW10</td>
<td>- +</td>
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<td>-</td>
<td>+ +</td>
<td>-</td>
</tr>
<tr>
<td>UW38</td>
<td>- ++</td>
<td>- ++</td>
<td>-</td>
<td>- ++</td>
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</tr>
<tr>
<td>UW45</td>
<td>- -</td>
<td>- -</td>
<td>-</td>
<td>- -</td>
<td>+</td>
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<tr>
<td>UW91</td>
<td>+ -</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
<td>-</td>
</tr>
</tbody>
</table>

After BRILL et al. (1974), NAGATANI et al. (1974), SHAH et al. (1973), and SHAH et al. (1974).

A technique that has been very useful for understanding the mechanism of nitrogenase activity is electron paramagnetic resonance (EPR) spectroscopy. EPR spectroscopy of whole cells shows a unique signal having a spectroscopic splitting value.
(g) of 3.65 that is present only in cells that fix N₂ (DAVIS et al. 1972). This signal is caused by component I. When various mutant strains were examined for the g=3.65 signal, only those with active component I produced the signal (SHAH et al. 1973). Many mutant strains produce inactive component I with normal levels of iron and normal properties on acrylamide gels. None of these strains had this unique EPR signal. The tight correlation between the EPR signal and activity of component I was good evidence that the g=3.65 signal is caused by an active site on component I. The signal is induced by a transition metal, but it still is not known whether iron, molybdenum, or both are responsible for the signal.

Nitrate reductase, like component I of nitrogenase, is a molybdoprotein. A mutant strain of Neurospora crassa with a defective nitrate reductase could be reactivated in vitro when acid-treated nitrate reductase was added to the extract (KETCHUM et al. 1970, NASON et al. 1971). The exciting discovery was made that addition of any acid-treated molybdoprotein could reconstitute nitrate reductase activity in vitro when added to the extract of the mutant strain. This was strong evidence that all molybdoproteins contain a common cofactor. We therefore assumed that some mutant strains defective in nitrogenase might be activated in vitro when acid-treated component I is added. Such strains have been found in A. vinelandii and K. pneumoniae (NAGATANI et al. 1974, ST. JOHN et al. 1975) and have been very useful for assaying the molybdenum cofactor during purification. We have recently purified the cofactor (FeMoCo) and demonstrated that it is a small peptide containing both iron and molybdenum (SHAH, unpublished results). It is stable at room temperature but is inactivated immediately in the presence of small amounts of oxygen. All of the FeMoCo can be removed from component I by treatment with M-methylformamide, a solvent with a very high dielectric constant. All of the molybdenum is bound in FeMoCo. An interesting problem that has yet to be solved is the mechanism by which FeMoCo is synthesized. Many nif genes might be involved.

Another interesting phenotype (ST. JOHN et al. 1975) that was found in K. pneumoniae prevents the cell from growing on N₂, but the mutant strain has high levels of active nitrogenase when tested for activity in vitro (ST. JOHN et al. 1975). The mutation is not located in a structural gene for nitrogenase. A possible explanation for this phenotype is that the mutation is in a gene that specifies an electron transport factor that is required for N₂ fixation in vivo. The electron donor for in vitro assays is sodium dithionite, which directly reduces nitrogenase (BULEN et al. 1975). The in vitro assay, therefore, bypasses this factor.

REGULATION

Regulation of nitrogenase synthesis is under very tight control; however, there does not seem to be any type of feedback inhibition in these organisms (see BRILL 1975). When A. vinelandii is grown in the presence of excess ammonium, no de-
detectable nitrogenase activity was observed and no component I or II cross-reacting material was detected (DAVIS et al. 1972). Glutamine synthetase plays a role in the regulation of nitrogenase synthesis in *K. pneumoniae* since glutamine auxotrophs are unable to synthesize nitrogenase (TUBB 1974). The mechanism of regulation probably is similar to that found with histidase synthesis in *K. aerogenes*. In that system a particular form (unadenylylated) of glutamine synthetase is required for transcription of the gene specifying histidase (TYLER et al. 1974).

It is not certain how nitrogenase synthesis is regulated in *A. vinelandii*, but an analog of glutamate, methionine sulfoximine, will alter the regulation of nitrogenase synthesis in *A. vinelandii* as it does in *K. pneumoniae* (GORDON and BRILL 1974). When this analog is added to ammonium-grown cells, nitrogenase synthesis is derepressed. In the presence of methionine sulfoximine, *N₂*-growing cells form very high levels of nitrogenase and excrete into the medium additional ammonium that is formed. This proves that ammonium itself is not the effector of nitrogenase synthesis. The same results have been found for nitrogenase synthesis in a blue-green alga (STEWARD and ROWELL 1975).

Mutant strains were found that have altered regulatory properties. Strains lacking both components as well as FeMoCo are commonly found (SHAH et al. 1973, ST. JOHN et al. 1975). When some of these strains are reverted to the Nif* phenotype, derepressed levels of nitrogenase are observed in several (but not all) of the revertants (GORDON and BRILL 1972, GORDON et al. 1975). Such strains have a possibility of being agronomically important (GORDON and BRILL 1972, SHANMUGAM and VALENTINE 1975) since they continue to fix *N₂*, even in the presence of nitrogenous fertilizer. Perhaps *N₂*-fixing bacteria that normally grow on roots of a cereal plant can be isolated and mutated to the derepressed phenotype. Some of these strains should be able to excrete ammonium and therefore fertilize the plant. Obviously, the plant has to supply the carbon source for the bacterium.

One Nif* mutant strain of *A. vinelandii* (UWJ8) had no detectable component I, but had five-fold greater levels of component II than normally is seen in the wild type (SHAH et al. 1974). This phenotype was caused by a single mutation since all revertants of this strain produced equivalent but low levels of both components. A model was proposed which invokes an activator that exists in two forms—a different form is required to activate the synthesis of each component.

In *K. pneumoniae* excess ammonium is not the only way to specifically prevent nitrogenase synthesis. As stated previously, this organism is unable to fix *N₂* aerobically. It is possible that oxygen-denatured components are made when the cell is starved for N in the presence of air. However, neither component is synthesized, even in an inactive form, in the presence of air (ST. JOHN et al. 1974). Nothing is known about the nature of this regulation by oxygen.

When *K. pneumoniae* is grown in a medium deficient in molyb-
denum, it obviously is unable to fix N₂ since molybdenum is an essential part of component I of nitrogenase. Several molybdenum-deficient environments have been shown to limit N₂ fixation in nature (ANDERSON 1946, EVANS \textit{et al.} 1951). When the cell finds itself N-starved, but without molybdenum, the possible outcome is that active component II and inactive component I are synthesized. This would be a waste of energy because the organism will be unable to fix N₂. However, molybdenum seems to play a regulatory role because molybdenum starvation prevents either component from being synthesized (BRILL \textit{et al.} 1974). A different observation was made for molybdenum-starved \textit{A. vine­landii}. In that organism, active component II was present, but component I could not be detected serologically or on polyacrylamide gels (NAGATANI and BRILL 1974).

\textbf{GENETICS}

\textit{Klebsiella pneumoniae} is closely related to \textit{Escherichia coli}, and therefore the sophisticated genetic tools available for studies on \textit{E. coli} can be used with \textit{K. pneumoniae}. For instance, P1 has been used for transduction (STREICHER \textit{et al.} 1971). Fine-structure mapping by deletion analysis has ordered (see Figure 1) several hundred \textit{nif} mutations and has confirmed a previously published map (ST. JOHN \textit{et al.} 1975). The exact number of \textit{nif} genes is not yet known since detailed complementation analyses have not been completed, but two-dimensional polyacrylamide gel electrophoresis has shown that at least six polypeptides are coded for by the \textit{nif} region of the chromosome (ROBERTS, unpublished results).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Genetic map of the \textit{nif} region in \textit{Klebsiella pneumoniae}. After ST. JOHN \textit{et al.} (1975). The location, specified by X, is the region where phage Mu can integrate without preventing the strain from becoming Nif⁻ (MALAVICH, unpublished results).}
\end{figure}

Mutations that specifically eliminate component I, but not component II, activity cluster in three regions. A region closest to \textit{his} is \textit{nifB}, which codes for the synthesis of FeMoCo. The other two regions, \textit{nifD} and \textit{nifK}, are required for component I synthesis. A region on the chromosome that seems to have nothing to do with N₂ fixation separates \textit{nifD} from \textit{nifK}. Evidence for this comes from the finding that the phage Mu can be inserted in this region. The cell still is able to grow as well as the wild type on N₂. It is possible that each of these clusters codes for one of the two subunits of component I (KENNEDY \textit{et al.} 1976). However, the fact that the Mu insertion
does not alter component I synthesis is evidence that the two clusters are coded by different operons.

Mutations in *A. vinelandii* have been mapped by transformation (PAGE and SADOFF 1976). This is a rather crude mapping system since large pieces of DNA seem to be required and linkage can be demonstrated between a variety of different markers. Unlike the situation in *K. pneumoniae* where all of the *nif* mutations are closely clustered, the *nif* mutations in *A. vinelandii* are scattered on the chromosome (BISHOP and BRILL 1977, Figure 2). Mutations that are involved in the regulation of nitrogenase synthesis and synthesis of the molybdenum cofactor are not close to the structural genes specifying components I and II. Transformation will be useful for constructing strains with the desired properties for ammonium excretion.

**Figure 2.** Genetic map of *nif* genes in *Azotobacter vinelandii*. After BISHOP and BRILL (1977).

**RHIZOBIUM**

Unlike free-living N₂-fixing bacteria, *Rhizobium* normally fixes N₂ when it is packed in plant cells within the root nodule of its legume host (Figure 3). The plant supplies photosynthate to the *Rhizobium*, and nitrogenase within *Rhizobium* supplies fixed N to the legume. The plant codes for leghemoglobin (DILWORTH 1969), which is synthesized only in legume root nodules.

Very little information is available on the mechanism by which *Rhizobium* infects the plant or the basis for specificity of one *Rhizobium* species for its legume host. Since both bacteria and plant are required for normal expression of genes that code for N₂ fixation as well as genes specifically involved with the infection process, it is not very easy to obtain mutant strains with lesions in these genes.

No positive selection or enriching techniques are available for obtaining the desired mutant strains. A screening technique was used to detect mutant strains after mutagenesis. This screening method, called the effectiveness assay (WACEK and BRILL 1976), involved surface-sterilizing soybean seeds and placing the seeds in vials containing vermiculite with a N-free medium. A portion of a colony from a mutagenized culture was placed on a seed, and then a sterile plastic bag was placed over the vial. After the vial was incubated for two weeks in a plant-growth chamber, the root system was assayed for N₂-fixing ability by the acetylene-reduction technique (BURRIS 1975). Mutant strains of *R. japonicum* (see Table 2) that grew as well as the wild type on minimal and rich media but were unable to form N₂-
fixing root nodules on soybean were further analyzed (MAIER and BRILL 1976).

FIGURE 3. Scanning electron micrograph of *Rhizobium japonicum* cells inside a plant cell that is within a soybean root nodule. Magnification x 2,900.

One mutant strain (SM1) did not form any nodules and seemed to be missing a surface antigen that was found in the wild type (MAIER, unpublished results). Another strain (SM3) produced nodules that did not reduce acetylene. No leghemoglobin was found in those nodules. A third type of mutant strain (SM5) formed nodules containing leghemoglobin. These nodules had no component II activity, but component I still was active. Component II protein was detected serologically; therefore the lesion in this strain seemed to be in the structural gene for component II. This was the first example of a Nif− mutant strain in *Rhizobium*. No gene-transfer experiments have yet been performed with these strains.

The effectiveness assay also has been useful for detecting mutant strains of *R. japonicum* that reduce more acetylene than
TABLE 2. Mutant Strains of *Rhizobium japonicum*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ability to nodulate</th>
<th>Leghemoglobin in nodules</th>
<th>Active nitrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SM1</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SM3</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SM5</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

After MAIER and BRILL (1976).

the wild type (MAIER and BRILL 1977). Such "super" strains are currently being tested for their ability to improve yields of soybean. Another use of the effectiveness assay has been to screen different cultivars of soybean (WACEK and BRILL 1976). Table 3 shows the relationship between selected cultivars when tested by the effectiveness assay and when whole plants are dug up from the field and tested for acetylene reduction. Plants were tested from the field at three and eight weeks after planting. The most active cultivar (Ogemaw) determined by the effectiveness assay is the most active in the field trial as well. The least active cultivar, Hokkaido, does not have any activity at three weeks but is very active at eight weeks. This probably is an indication that this cultivar nodulates late. The remaining cultivars do not bear any similarity in field trials to results obtained by the effectiveness assay. It is possible that the effectiveness assay is useful for detecting N₂-fixing potential in plants and that this potential might not always be realized because of other limiting factors. This information could be of considerable value to crop breeders who are interested in developing plants with greater N input but with no dependence on nitrogenous fertilizer.

TABLE 3. Comparison between soybean cultivars tested by the effectiveness assay and field-grown plants

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Effectiveness assay</td>
</tr>
<tr>
<td>Ogemaw</td>
<td>202</td>
</tr>
<tr>
<td>Osaya</td>
<td>177</td>
</tr>
<tr>
<td>Norman</td>
<td>124</td>
</tr>
<tr>
<td>Corsoy</td>
<td>80</td>
</tr>
<tr>
<td>Wilson</td>
<td>17</td>
</tr>
<tr>
<td>Hokkaido</td>
<td>10</td>
</tr>
</tbody>
</table>

*Specific activity equals the amount of acetylene reduced per hour per plant. After WACEK and BRILL (1976).
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LITERATURE CITED


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