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Biochemical Genetics of Nitrogen Fixation†

WINSTON J. BRILL

Department of Bacteriology and Center for Studies of Nitrogen Fixation, University of Wisconsin, Madison, Wisconsin 53706

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INTRODUCTION

Procaryotes capable of N_2 fixation have representatives in many genera. Free-living N_2-fixing bacteria (those not intimately associated with a specific plant) include members of *Klebsiella*, *Azotobacter*, *Clostridium*, *Rhodospirillum*, *Aspirillum*, and various cyanobacteria. Included among bacteria that normally fix N_2 only when they are symbiotically associated with a plant are *Rhizobium* spp. (which nodulate legumes [78, 241]), certain actinomycetes (which nodulate *Comptonia* and alder [225]), and *Anabaena azollae* (which fixes N_2 within the leaf pores of the water fern *Azolla* [169]). No eucaryotic organisms have been shown to fix N_2.

The biology, biochemistry, regulation, and genetics of N_2 fixation are quite complex. However, the production of active cell-free extracts (40), the purification of nitrogenase (31, 33, 72, 146, 194, 231), and the use of the acetylene reduction assay for nitrogenase activity (60, 91; R. Schöllhorn and R. H. Burris, Fed. Proc. **25**:710, 1966) have led to rapid advances in the past decade in our understanding of this economically important system.

Several books on N_2 fixation have been published recently (32, 67, 90, 101, 154, 178), and a variety of specialized topics concerning N_2 fixation have been reviewed, including biochemistry (62, 71, 126, 147, 249), the *Rhizobium* infection process (16, 27, 53, 190), nonlegume symbioses (1), cyanobacteria (95, 250), and genetics and regulation (14, 25, 201).

This article focuses on concepts that have been elucidated by analyzing mutants defective in their ability to fix N_2 (Nif^- mutants). In some cases, studies with mutants have confirmed ideas already indicated by other approaches, and in other cases work with mutants has led to new information. One purpose of this review is to acquaint biochemists with the wide range of Nif mutants available and with the potential value of these mutants. The need for certain mutants, not yet described, is discussed with regard to their value for biochemical studies.

Klebsiella pneumoniae, which is closely related to *Escherichia coli*, is the bacterium that has been used for detailed genetic analyses of the genes involved in N_2 fixation (*nif* genes). Phage P1 transduction (213), phage Mu mutagenesis (8, 74, 133, 179), transposon mutagenesis (142), and complementation analysis (63, 134) have been extremely helpful for studying the biochemical complexity and regulation of *nif* expression. Therefore, in this review I focus particularly on genetic work with this organism.

BIOCHEMISTRY

Nitrogenase

Nitrogenase is made up of two soluble proteins (31, 72, 146, 194, 231), which are called component I and component II. Component I also is known as MoFe protein or nitrogenase; component II is known as Fe protein or nitrogenase reductase (88). It is remarkable that the nitrogenases isolated from a wide variety of organisms are very similar to each other (44, 71). For instance, component I from one genus can interact with component II from another to yield

† Based on the Eli Lilly Award Address (1979).

active enzyme (11, 20, 59, 112, 223, 227). Antiserum prepared against a component from a member of one genus can react with that component isolated from a bacterium of a different genus (137). A question which has yet to be answered is whether nitrogenases have evolved from a common source or whether the evolution of nitrogenases has been convergent, due to the stringent demands required to reduce the very stable triple bond in N_2.

Component I has a molecular weight of 200,000 to 250,000 and contains 2 Mo atoms, 28 to 34 nonheme Fe atoms, and 26 to 28 acid-labile sulfides (71). Polyacrylamide gel electrophoresis of component I in sodium dodecyl sulfate has indicated that component I is composed of two copies each of two subunits ($\alpha_2 \beta_2$), each subunit having a molecular weight of approximately 60,000 (152, 228). In fact, sodium dodecyl sulfate gels do not separate the two subunits from each other unless certain preparations of sodium dodecyl sulfate are used (114). Amino acid analyses of the subunits have also demonstrated that they are very similar to each other (71).

Even though the carboxyl- and N-terminal amino acids of the two subunits of *Azotobacter vinelandii* component I are different (130), it seemed possible that the two subunits could have resulted from modifications of a single polypeptide. However, different tryptic digestion patterns argue against common amino acid sequences of the two subunits (114, 217). In the case of *K. pneumoniae*, two genes code for the structural proteins of component I. Assignment of the *nifK* gene to one subunit and of the *nifD* gene to the other came from two-dimensional polyacrylamide gel electrophoresis of extracts from mutants with lesions in these two *nif* genes (184). Several mutations in *nifD* specifically alter the electrophoretic mobility of only one subunit, and some mutations in *nifK* alter the gel position of only the other subunit. Thus, the two subunits are distinct proteins rather than alterations of one protein. Perhaps the similarities between the two subunits of component I are the result of a gene duplication that may have occurred during the evolution of the nitrogenase (26).

Each subunit of component I requires the other for stability in vivo. When a mutation in either gene makes one gene product unstable, the other gene product also is rapidly degraded (184).

Component II has a molecular weight of 55,000 to 65,000 and is composed of two copies of a single subunit (71). This protein has four nonheme Fe atoms and four acid-labile sulfides. The amino acid sequence of component II from *Clostridium pasteurianum* has been determined (218–220). In *K. pneumoniae*, the *nifM* gene (and possibly the *nifV* and *nifS* genes) codes for a protein that is required to process (184) component II (encoded by *nifH*). Nothing is known about the biochemistry of this modification.

Substrate binding and reduction take place on component I (161, 207, 208). The role of component II is to supply electrons, one at a time (128, 149), to component I. No free intermediates of N_2 reduction have been found; however, there is some evidence that an enzyme-bound N_2-hydride is an intermediate (222). The sequence of reductive steps has not been ascertained, and it might be valuable to examine mutants with lesions in component I to determine whether trapped enzyme-bound intermediates are formed on the defective component.

Kinetic data support the idea that the two components dissociate after only one electron is transferred from component II to component I (88). However, ultracentrifugation of mixtures of the components has indicated that the two components can form rather stable complexes (70). As discussed above, mutations in *nifD* or *nifK* that drastically alter one of the subunits prevent the other subunit from accumulating. However, strains with Mu insertions of *nifK* or *nifD* still allow accumulation of component II, although the amount of component II is decreased (184). This is not due to polarity or regulation of transcription (see below). These data support the hypothesis that the two components somehow interact as a complex in vivo and that one subunit is required for stability of the other.

The turnover number (50 mol/min per mol of Mo in the nitrogenase [32]) is quite low. Because of this, N_2-fixing bacteria synthesize about 2 to 5% of the total cell protein as nitrogenase when they grow on N_2 as the sole N source (unpublished data). Nif^- mutants of *A. vinelandii* that lack both components of nitrogenase grow faster (doubling time, 6 h) than Nif^- strains that synthesize an inactive component (doubling time, 12 h) when asparagine is the N source (unpublished data). This may be caused by the extra energy and amino acid demand for nitrogenase synthesis.

Energetics

Even though the reduction of N_2 to NH_4^+ is exothermic (210), N_2 fixation requires energy in the form of adenosine 5′-triphosphate (ATP) (93, 144) due to a high activation energy. ATP binds to component II and lowers its redox potential (261). ATP is not hydrolyzed to adenosine 5′-diphosphate until component II transfers an electron to component I (161). There seem to be two ATPs hydrolyzed for each electron transferred (127). However, ATP hydrolysis can be uncoupled from electron transfer. For instance,

an excess of component I leads to a higher ratio of ATP to electrons transferred (127). In vivo, a constant ratio of these two components is maintained by having the three structural genes (in *K. pneumoniae*, at least) being part of a single operon, *nifHDK* (134, 142). An interesting physiological study might be to examine the effect on cell growth and yield in strains having different copy numbers (e.g., in merodiploids) of the structural genes.

In vitro the ATP requirement for N_2 fixation does not necessarily reflect the requirement in vivo. Data from growth yield experiments indicate that only 4 or 5 ATPs are required for each N_2 fixed by *Azotobacter* (50), whereas 29 ATPs are required in *K. pneumoniae* (97) and 20 ATPs are required in *C. pasteurianum* (49). The relationship between NH_4^+ formation and carbon substrate used was studied with a derepressed mutant of *K. pneumoniae* that was not able to assimilate the NH_4^+ produced by N_2 fixation (4). The conclusion from this experiment was that 21 to 25 ATPs are required for each N_2 fixed.

Oxygen Lability

Each of the two nitrogenase components is rapidly and irreversibly inactivated by a short exposure to O_2 (31). Thus, anaerobic conditions must be maintained during purification of the components. Organisms that fix N_2 have mechanisms to protect their nitrogenases from being inactivated by O_2. In the case of strict anaerobes (e.g., *C. pasteurianum*), this presents no special problem. Facultative aerobes (e.g., *K. pneumoniae* and *Bacillus polymyxa*) are not able to fix N_2 aerobically.These organisms only grow on N_2 in the absence of air (see below for the role of O_2 in regulating *nif* expression), but they grow on fixed N sources both aerobically and anaerobically (99, 135). Mutants with an O_2-tolerant nitrogenase have not been isolated even after prolonged mutagenesis of *K. pneumoniae* (R. T. St. John and W. J. Brill, unpublished data). *Azospirillum* fixes N_2 only under microaerophilic conditions, but grows on fixed N aerobically (158). Presumably, sufficient O_2 must be available for oxidative phosphorylation, but too much intracellular O_2 denatures the nitrogenase.

In the case of the strict aerobe *Azotobacter*, a very high respiratory activity rapidly reduces O_2 (173). This has a net result of lowering the internal O_2 concentration, thereby protecting the nitrogenase. To maintain this high respiratory activity, adequate carbon substrate must be available for reducing power. If the flow of electrons is sufficiently limited, *Azotobacter* protects its nitrogenase (87, 185, 232) by complexing the two components of the enzyme with another Fe-S protein (known as the Shethna protein or Fe-S protein II). This complex of component I, component II, and the Shethna protein in a 1:1:1 ratio has increased stability in the presence of O_2. The complex in its O_2-stable form seems to be unable to fix N_2 until sufficient electron flow is restored (thereby lowering intracellular O_2 levels), at which point the complex dissociates to restore nitrogenase activity. Some spontaneously revertible Nif$^-$ mutants of *A. vinelandii* synthesize both components of the nitrogenase, but these components are inactive in vitro (198). Such mutants should be analyzed for the presence of the Shethna protein. If that protein is found in the mutants, whether it will complex and protect the nitrogenase from O_2 should be determined. An alternative possible defect in these mutants is that they are less effective in their ability to reduce O_2.

Most aerobic N_2-fixing bacteria produce gummy colonies on agar medium (98). Thus, the capsule may play a role in protecting the nitrogenase from O_2. However, a nongummy mutant of *A. vinelandii* retains its ability to fix N_2 aerobically (34).

In the case of heterocystous cyanobacteria, nitrogenase seems to be found only in the heterocysts (77, 170), specialized thick-walled cells lacking the O_2-producing reactions of photosynthesis (221). Some mutants unable to fix N_2 have defective heterocysts (46); therefore, the heterocysts play an essential role in N_2 fixation. Mutants of *Anabaena* deficient in heterocyst envelope glycolipids fix N_2 only under very low O_2 levels (96). Of the several classes of O_2-sensitive mutants, one class can be incubated aerobically for 10 h, and mere removal of O_2 reverses this inhibition (85). Perhaps a nitrogenase protection protein similar to the Shethna protein in *Azotobacter* exists in some cyanobacteria. The developmental regulation of heterocyst formation might be understood from studies of mutants having altered heterocyst spacing along their filaments of cells (248). There may be other protection mechanisms, such as respiratory protection or reduction of O_2 by an uptake hydrogenase (see below). An uptake hydrogenase is localized in the heterocysts (171). Mutants defective in this hydrogenase should be isolated to test whether the uptake hydrogenase actually protects nitrogenase from O_2.

Membrane envelopes compartmentalize rhizobia in plant cell cytoplasm (12, 237). Surrounding the packets of bacteria are large quantities of leghemoglobin (111, 122, 242). The function of leghemoglobin is to bind O_2 and prevent free O_2 from reaching the nitrogenase within the bacteria (13, 247). The plant codes for this globin (28, 48, 61, 202, 237), and the bacteria seem to produce the heme moiety (47, 150) of leghemo-

globin. Leghemoglobin is not found in legume tissues other than root nodules. We still do not understand how rhizobia induce plants to synthesize globin messenger ribonucleic acid, which is found in nodule plant cytosol (6). Mutants of *Rhizobium* that produce nodules lacking leghemoglobin (137; W. T. Leps, H. Setatou, D. Noel, and W. J. Brill, unpublished data) should be very useful for understanding the induction of plant globin synthesis and the production and transfer of bacterial heme into globin.

The cytosol of a nodule plant cell contains more than one species of leghemoglobin; these species are separable on the basis of charge differences (79, 224). The amino acid differences between two of these species (leghemoglobin *a* and leghemoglobin c_2 of soybean) indicate that they are probably encoded by independent genes, but that they have extremely similar tertiary structures (103). Because leghemoglobin is produced in large quantities in nodules, duplication of leghemoglobin genes may have occurred due to selective pressure for increased leghemoglobin synthesis. Independent mutations within these duplicated genes may accumulate as long as the biochemical function of leghemoglobin is not altered. If this is true, then soybean varieties not closely related should have variable amounts of the different leghemoglobins. The possibility that different leghemoglobins have different functions comes from work indicating that the ratio of leghemoglobin *c* to leghemoglobin *a* changes during soybean development (80, 235). Perhaps plant mutants that lack one of the leghemoglobin proteins will be found. If *Rhizobium* mutants capable of inducing only one of the leghemoglobin species can be found, such strains will be very important for understanding the roles of the different leghemoglobins.

An exception to the specificity of *Rhizobium* for legumes is that certain rhizobia form N_2-fixing nodules on the nonlegume *Parasponia* (2, 226). In this case, the nodules do not contain leghemoglobin (45).

Nonlegumes which are nodulated by certain actinomycetes (36), such as alder and *Comptonia*, do not contain leghemoglobin in their N_2-fixing nodules. It will be interesting to determine how nitrogenase is protected in such nodules. The nitrogenase from alder nodules has properties, including O_2 lability, which are similar to the properties of nitrogenases from other sources (11). Respiration or O_2 barriers may play a protective role in this system.

Hydrogen Evolution

Many N_2-fixing bacteria evolve H_2 when they fix N_2 (172) but not when they are grown on NH_4^+ (a condition that represses nitrogenase synthesis). Nitrogenase itself can be responsible for H_2 evolution (30, 94). When electrons and ATP are available to nitrogenase but N_2 is not, the electrons combine with protons to yield H_2 (30). Both components of nitrogenase are required, and ATP is hydrolyzed during H_2 evolution (145). However, even when N_2 is in the gas phase, some of the electrons are evolved as H_2, and the remaining electrons are used to reduce N_2 to NH_4^+ (30). This is a property common to all nitrogenases that have been examined. In vitro, the partitioning of electrons to N_2 or to protons depends on the component ratio, the supply of electrons, and the ATP concentration (88, 127, 203).

In vivo, about 50 to 65% of the electrons are lost as H_2 (182) under normal growing conditions (ambient N_2 concentrations). Nif^- mutants also are not able to evolve H_2 (76, 245). Thus, H_2 evolution seems to share active sites common to N_2 fixation. However, it is interesting that CO inhibits N_2 fixation but not H_2 evolution (129) and that H_2 is a competitive inhibitor of N_2 fixation (246). Thus, the active sites for N_2 fixation and H_2 evolution do not seem to be identical. It should be possible to find rare mutants that are Nif^- but still evolve nitrogenase-catalyzed H_2. Biochemical analyses of such mutants should yield important information regarding the mechanisms of these two reactions because a part of the N_2 reactive site would be defective.

H_2 evolution by nitrogenases seems to be detrimental to cells since ATP and electrons are wasted. It has been proposed that the H_2 evolved serves to protect the nitrogenases from inhibition by O_2 (66, 187); however, *C. pasteurianum* (a strict anaerobe) also evolves H_2 from hydrogenase (94, 145).

Mutants of *K. pneumoniae* that are derepressed for nitrogenase synthesis (synthesize nitrogenase in the presence of NH_4^+) seem to use H_2 evolution by nitrogenase to remove excess electrons. Under conditions of anaerobic growth with sucrose as the carbon source, the derepressed cells grow faster than the wild type in NH_4^+-containing medium (D. MacNeil and W. J. Brill, J. Bacteriol., in press). The derepressed mutants do not grow faster than the wild type when fumarate is included in the medium. Presumably, fumarate acts as an acceptor of excess electrons, and the energy drain from ATP-driven nitrogenase activity is not as important to the cells as the pressure to remove electrons.

Another explanation for H_2 evolution by nitrogenases is that H_2 formation is a consequence (along with O_2 sensitivity) of the nature of the active site. In other words, the nitrogenase active site has not evolved to eliminate this potentially

wasteful side reaction. One would predict that naturally occurring strains having an active nitrogenase unable to produce H_2 will not be found since natural selection should already have removed this detrimental reaction. The selection for such a mutant would be to demand more efficient growth than wild-type growth under N_2-fixing conditions. The selection would be greater if a mutant lacking the uptake hydrogenase (see below) were used as the parent strain.

Some N_2-fixing bacteria recycle the electrons from the H_2 evolved back to the nitrogenase (66). This is done by a hydrogenase that oxidizes H_2 (uptake hydrogenase). The electrons produced by uptake hydrogenase can support ATP synthesis by oxidative phosphorylation, or the electrons can reduce N_2 via the nitrogenase (66, 75, 244). The ATP and electrons wasted through nitrogenase-catalyzed H_2 evolution are partially recouped by the action of the uptake hydrogenase. Mutants of *K. pneumoniae* that lack the uptake hydrogenase activity but retain the active nitrogenase evolve more H_2 than the wild type (4); therefore, the uptake hydrogenase does indeed recycle nitrogenase-produced H_2 in vivo. Nif^- mutants of *Azotobacter* retain the ability to oxidize H_2 (86). H_2 uptake also occurs with NH_4^+-grown cells; therefore, N_2 fixation and H_2 oxidation do not seem to be coregulated.

Some wild-type *Rhizobium* strains oxidize H_2 via an uptake hydrogenase (41, 191). These strains seem to be more efficient with respect to N_2 fixation than strains without an uptake hydrogenase (192, 193). Mutants of *Rhizobium japonicum* defective in the uptake hydrogenase have been isolated (139), and these also seem to be less efficient than the wild type (3). This uptake hydrogenase is particulate and also is O_2 sensitive (5).

Alternative Substrates

Protons (for H_2 evolution) and N_2 are not the only substrates that can be reduced by nitrogenase. Many low-molecular-weight compounds with triple bonds between two N atoms (e.g., azide reduced to NH_4^+), between two C atoms (e.g., acetylene reduced to ethylene), or between C and N atoms (e.g., cyanide reduced to methane and NH_4^+) can be reduced by nitrogenase (92). Both nitrogenase components, as well as ATP, are required for these reactions. Except for H_2 evolution, carbon monoxide inhibits all of the reactions of the nitrogenase (129, 182). However, H_2 is an inhibitor only for N_2 fixation (104). No mutants that contain a nitrogenase with the ability to reduce one substrate but not another have been described. Of the many hundreds of independently isolated Nif^- mutants of *K. pneumoniae*, those that are unable to fix N_2 in vivo also are unable to reduce acetylene in vivo (76, 184, 198, 204, 211). This is evidence that the same (or overlapping) enzyme sites are responsible for acetylene reduction as well as N_2 fixation. It also is interesting that nitrogenases from a wide variety of sources have approximately the same reactivity with the different substrates (32).

The acetylene reduction assay for nitrogenase activity (60, 91; Schöllhorn and Burris, Fed. Proc. **25**:710, 1966) has been widely used by researchers; however, data obtained by acetylene reduction cannot be extrapolated quantitatively to N_2 fixation. Since only two electrons reduce acetylene to ethylene and six electrons are required to reduce N_2 to $2NH_4^+$, it would seem that three times as many acetylene molecules would be reduced for each N_2 fixed. However, in vitro this (acetylene reduced to N_2 fixed) is dependent on the concentration of reductant, the ratio of the two components, and the ATP concentration (51, 197). Mutants leaky in electron transport or leaky for activity of one of the two components will be useful for determining the effect of the above-mentioned parameters in vivo.

Nitrogenase substrates (e.g., cyanide and azide) that are toxic to cells have been tested as possible selective reagents to isolate Nif^- mutants or mutants derepressed for nitrogenase synthesis. In the selection of derepressed mutants, the wild type would be killed on cyanide-containing medium with NH_4^+ as the N source. A derepressed mutant might detoxify cyanide (by reducing it to harmless compounds), and the mutant would grow in the medium. Unfortunately, this technique has not yet been successful for isolating Nif mutants. Too much cyanide in the medium kills all of the cells, and it has been impossible to keep the correct cyanide (or azide) level that just inhibits growth (J. K. Gordon, R. T. St. John, and W. J. Brill, unpublished data). A problem may be that these inhibitors encounter sensitive membrane sites before they encounter nitrogenase.

Electron Donors

N_2 fixation by nitrogenases occurs readily in vitro with a chemical reducing agent, such as hydrosulfite (30, 94). Photosynthetically derived electrons (in the case of cyanobacteria) or electrons from carbon substrates drive nitrogenase activity in vivo. Ferredoxin (10, 148, 255, 257, 259) and flavodoxin (9, 10, 118, 119, 253, 256) have been shown to donate electrons in vitro to nitrogenase. In some cases, there are two or more of these electron carriers in the same cell (255, 257, 258). Therefore, it is difficult to ascertain which of these proteins actually donates

electrons to nitrogenase in vivo.

Certain mutants of *K. pneumoniae* seem to lack the natural electron-donating protein because they fix N_2 in vitro (upon addition of hydrosulfite) but not in vivo (184, 211). One of these mutants, which has a lesion in the *nifF* gene, has been used to assay and purify the *nifF*-encoded protein from a strain that synthesizes an active *nifF* product (155). The protein that has been purified is a flavin mononucleotide-containing flavoprotein which is synthesized only when nitrogenase is synthesized. It is not made when cells are grown on NH_4^+ (184). Previous work showed that a flavoprotein (flavodoxin) is required for electron transport in vitro to the nitrogenase from *K. pneumoniae* (256). In that system, extracts were frozen to remove electron-donating activity from the wild type. Electron flow was restored to the nitrogenase by adding the flavodoxin to a system that was coupled to spinach chloroplast-driven electron transport. The flavoprotein assayed with the Nif^- mutant seems to be different than the protein isolated from the same organism (*K. pneumoniae*) with the photosynthetically coupled assay (155, 256). The fact that a $NifF^-$ mutant is not able to grow on N_2 supports the hypothesis that the *nifF*-encoded flavoprotein is the actual electron-donating protein for N_2 fixation and that this is the sole role of the flavoprotein. The other flavodoxin must play roles in vivo other than transferring electrons to the nitrogenase. These data illustrate some of the problems caused by extrapolating electron transport data in vitro to what actually occurs in vivo.

Another protein in *K. pneumoniae* also seems to be involved in specific electron transport to nitrogenase, as judged by in vitro hydrosulfite reduction of nitrogenase. This protein, encoded by *nifJ*, contains a subunit monomer of 120,000 molecular weight (184). Presumably, this *nifJ*-encoded protein is the reduced nicotinamide adenine dinucleotide phosphate flavoprotein reductase (87, 109, 252). Neither the *nifF* nor the *nifJ* proteins are membrane bound (D. Nieva-Gomez, G. P. Roberts, and W. J. Brill, unpublished data).

Molybdenum

Work with other molybdoenzymes has indicated that Mo resides in some type of cofactor (116, 153). A mutant of *A. vinelandii* (strain UW45) produces an inactive component I and an active component II. Cell-free extracts of this mutant can be activated for nitrogenase activity when acid-treated component I is added (151). Acid treatment completely inactivates component I. This is evidence that there is a Mo cofactor in component I.

The Mo cofactor of component I has been isolated (195) and contains eight Fe atoms and six acid-labile sulfides for each atom of Mo. This cofactor is called the iron-molybdenum cofactor (FeMo-co), and it seems to have a molecular weight of less than 5,000.

Another method used to produce cell-free extracts of *A. vinelandii* capable of being activated by FeMo-co is to grow cells on WO_4^{2-} instead of MoO_4^{2-} (151, 195). Activation of component I from WO_4^{2-}-grown cells is caused by activation of an inactive cofactor species. ATP is required for activation of this inactive cofactor in vitro by MoO_4^{2-} (P. T. Pienkos and W. J. Brill, manuscript in preparation). A mutant of *Nostoc* that utilizes tungsten instead of molybdenum to fix N_2 by nitrogenase has been described (205), but this unusual result has yet to be confirmed.

Purified component I has a unique electron paramagnetic resonance signal with a spectroscopic splitting constant (g value) of 3.65 (33). This signal can be detected in whole cells (52). Some mutants synthesize inactive component I with normal levels of Fe and Mo (V. K. Shah, P. T. Pienkos, and W. J. Brill, unpublished data). These mutants do not exhibit the signal with a g value of 3.65. Because of defects in component I, leaky mutants have less of this signal than the wild type (198). The correlation of the signal having a g value of 3.65 with component I activity is quite remarkable, and this supports the hypothesis that this electron paramagnetic resonance signal is caused by an active site on component I. FeMo-co also has this electron paramagnetic resonance signal (180); therefore, FeMo-co is an active site of nitrogenase.

Further support of FeMo-co as the active site comes from the observation that in *N*-methylformamide FeMo-co catalyzes acetylene reduction to ethylene when borohydride is the reducing agent (196). ATP, component II, and apocomponent I are not required for this reaction, as they are in enzymatic acetylene reduction. Like the enzymatic process, carbon monoxide is a potent inhibitor of FeMo-co-catalyzed acetylene reduction. However, FeMo-co alone does not catalyze N_2 fixation.

Lesions in any one of three genes (*nifB*, *nifN*, and *nifE*) of *K. pneumoniae* cause the cells to produce an inactive component I that can be activated in vitro by FeMo-co (184, 211). A fourth gene, *nifQ*, is not absolutely essential for N_2 fixation since Mu insertions in this gene only reduce N_2-fixing ability to 50% of that normally observed with the wild type (184). This activity also can be increased in vitro by adding FeMo-co. Mutants defective in these gene products will be important for understanding FeMo-co biosynthesis and insertion into component I. For

instance, it is not known whether FeMo-co is synthesized and then inserted into component I or whether FeMo-co synthesis occurs on component I. Also, FeMo-co intermediates might accumulate in these mutants.

Both nitrogenase and nitrate reductase are involved in N metabolism, and both are Mo-containing enzymes. The Mo in nitrate reductase is in a cofactor that is different than the cofactor in component I (174). The two cofactors do not seem to share common pathways since deletions of the entire *nif* region still allow *K. pneumoniae* to utilize nitrate (P. T. Pienkos, unpublished data). However, certain nitrate reductase-deficient mutants of *E. coli* ($NarD^-$) that are able to regain activity in the presence of high levels of MoO_4^{2-} are not able to fix N_2 when they carry a *nif*-containing plasmid unless high levels of MoO_4^{2-} are added (115). Therefore, it is possible that both molybdoenzymes may rely on a common MoO_4^{2-} permease or some other early Mo-processing step.

Mutants of a "cowpea" *Rhizobium* unable to reduce nitrate retain the ability to fix N_2 (162). Mutants of *Rhizobium meliloti* unable to utilize nitrate have lesions that are located at four different chromosomal sites (117), and some of these strains also lack xanthine oxidase (a molybdoenzyme) activity. All of these mutants produce N_2-fixing nodules; thus, no genes common to N_2 fixation and the other Mo-containing enzymes have been found for this system.

KLEBSIELLA PNEUMONIAE

Organization of *nif* Genes

All *nif*$^-$ mutations cluster near the *his* operon on the chromosome of *K. pneumoniae* (65, 102, 113, 134, 199, 214). At least 17 *nif* loci make up this cluster, and no non-*nif* genes seem to be interspersed within the *nif* region (134; G. P. Roberts, D. MacNeil, and W. J. Brill, manuscripts in preparation). Complementation analyses with polar mutations (74, 134, 142) and studies of fusions of *lac* to each gene (MacNeil and Brill, in press) indicate that the genes are organized into seven distinct operons and that the transcription of all of the operons is in the same direction, namely, toward the *his* genes (Fig. 1).

The *nif* region is approximately 24 kilobases long (39). *nif* deoxyribonucleic acid (DNA) is now easy to obtain in large quantities since the entire *nif* region (177) and portions of the *nif* region (38, 39) have been ligated onto amplifiable plasmids. Genetic techniques have been used to produce specialized transducing lambda phage containing *nif* (132). A physical map of a large part of the *nif* region has been produced by restriction enzyme analysis of cloned DNA (181).

Most of the gene functions are discussed above and are summarized in Fig. 1. Three genes do not seem to be essential for N_2 fixation under the conditions normally used for growing the cells in the laboratory. Mutations in these genes (*nifL*, *nifW*, and *nifU*) are detected only when they have a polar effect on an essential *nif* gene (184; G. P. Roberts and W. J. Brill, J. Bacteriol., in press). Strains with Mu insertions in these genes readily revert to Nif^+. It will be challenging to determine conditions that demand functioning of these "nonessential" *nif* gene products. For instance, if one of these genes codes for an Mo permease that functions only at very low Mo concentrations, then N_2 fixation in a medium with high Mo levels would not require that the permease be present.

Two-dimensional polyacrylamide gel electrophoresis has been useful for determining gene-product relationships in this system (184). A polypeptide product is assigned to a specific gene after it has been determined that some mutations in that gene alter the charge or molecular weight of that polypeptide. The products of most of the *nif* genes have been identified in this way (184; Roberts and Brill, in press).

The *nif* genes of *K. pneumoniae* have been transferred by conjugation to other bacteria, including *E. coli* (65), *Salmonella typhimurium*

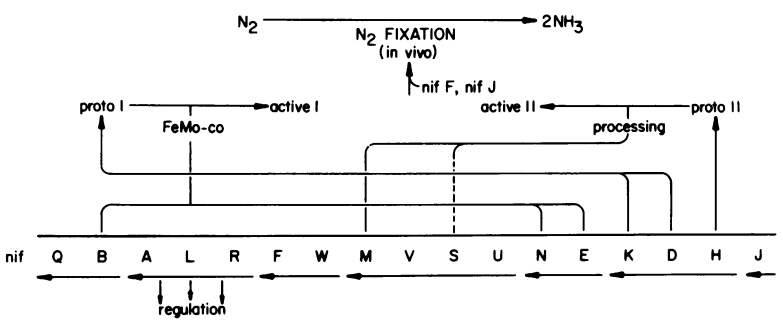

FIG. 1. *Order, transcriptional organization, and function of the K. pneumoniae nif genes.*

(176), Nif⁻ mutants of *A. vinelandii* (37), *Rhizobium* (64), and *Agrobacterium tumefaciens* (64). In the hybrid enteric bacteria, N_2 fixation is expressed anaerobically only under N-limiting conditions. *Azotobacter* expresses the *Klebsiella nif* genes aerobically. Presumably, high respiratory activity protects the nitrogenase. *Agrobacterium* containing *nif* seems to synthesize an inactive (O_2-denatured?) nitrogenase.

Cloned segments of *nif* from *K. pneumoniae* have been hybridized with the DNAs from a wide variety of N_2-fixing bacteria (140, 188). Homologies are exclusive within the *nifK,D,H* structural genes, and no homology has been detected with the other *nif* genes of *Klebsiella*. Considering the diversity of the N_2-fixing organisms tested, it seems remarkable that such a close degree of relatedness is found at the DNA sequence level of only the structural genes. One explanation is that these bacteria are all relatively recent recipients of *nif* DNA, which has been passed around by gene transfer. In fact, certain *Rhizobium* strains contain the *nif* structural genes on large plasmids (156). However, if promiscuous transfer of *nif* has occurred, *nif* genes other than the structural genes also should have maintained close relatedness. Another explanation is that nitrogenase activity has such stringent constraints with regard to the secondary and tertiary structures of the components that very few variations in amino acid composition can be tolerated. Therefore, it is possible that large runs of common amino acid sequences may be found in the nitrogenases derived from independent (convergent) evolution. The result of such unique primary structures would be DNA sequences having close codon relationships and thus a high degree of ability to be hybridized to each other. The finding that the *nif* structural genes seem to be clustered together in other organisms besides *K. pneumoniae* is not surprising in light of the fact that regulation of the components via operon control insures that optimum ratios of the components are produced.

Regulation of *nif* Expression

In the presence of excess NH_4^+ in the medium, *K. pneumoniae* contains no detectable *nif*-encoded proteins or activity (184). Other N sources also repress nitrogenase synthesis. Generally, the better the N source for growth, the greater the degree of repression (168, 260). Certain mutations in the genes responsible for the expression of glutamine synthetase prevent expression of the nitrogenase (215, 229). Inhibitors of glutamine synthetase (methionine sulfoximine and methionine sulfone) cause high levels of nitrogenase to be synthesized even in the presence of excess NH_4^+ (84). Some mutations in genes involved in NH_4^+ assimilation via glutamine synthetase also result in a derepressed phenotype (215). This is evidence that NH_4^+ is not the actual effector of repression. The regulation of *nif* seems to be similar to the regulation of histidine degradation (Hut) in enteric bacteria (230) in that glutamine synthetase and/or another gene product of the glutamine synthetase system is required for expression (123, 166). These *gln* genes are not phage P1 cotransducible with *nif* or *hut*. Regulation seems to be independent of the biosynthetic activity of glutamine synthetase (7). The mechanism of regulation by glutamine synthetase is not yet understood. There also may be a level of regulation by amino acids that is independent of glutamine synthetase (200).

None of the known *nif*-encoded proteins have been detected in extracts of cells grown on NH_4^+ (184). *nifA* codes for a protein required for expression of all of the operons with the exception of *nifRLA*. Certain Nif⁺ revertants of Mu insertions of *nifL* regain the ability to produce the *nifA* product and are simultaneously derepressed for nitrogenase synthesis (Roberts et al., manuscript in preparation). Therefore, control by fixed N (via glutamine synthetase) must occur only on the *nifRLA* operon. Once that operon is expressed, expression by the other *nif* operons is turned on (Fig. 1).

An interesting but as-yet-unexplained observation is that mutants with a functional *nifA* product convert the colorless adenine analog 6-cyanopurine to a purple pigment (131). No other *nif* gene product is required for this reaction. The purpling of 6-cyanopurine has been useful for isolating derepressed mutants in the presence of NH_4^+, as well as for testing whether deletions in *nif* have removed *nifA* (131; MacNeil and Brill, in press). It seems that the *nifA* product itself catalyzes the color change, or else the *nifA* product regulates the synthesis of a protein encoded by a gene unlinked to *nif* and this protein catalyzes the purpling reaction.

When *K. pneumoniae* is grown aerobically, no nitrogenase is synthesized (212). It seems that O_2 rapidly turns off all of the known *nif*-encoded proteins except those produced by the *nifRLA* operon (Roberts and Brill, in press). Some Nif⁺ revertants of Mu insertions in *nifL* overcome repression by O_2. These strains synthesize all *nif*-encoded proteins under aerobic conditions; however, O_2 inactivates the nitrogenase. These data indicate that *nifL* codes for a protein that plays a role in regulation by O_2. Perhaps the *nifL* protein interacts with O_2 to produce an altered protein that reacts with the *nifA* protein and prevents the *nifA* protein from turning on

the expression of the remaining *nif* operons.

Certain strains that lack the *nifL* protein, but have the *nifA* protein, are insensitive to repression by O_2 but are still sensitive to repression by NH_4^+ (Roberts and Brill, in press). The wild type does not synthesize either regulatory protein in the presence of NH_4^+. Thus, there must be a site between *nifL* and the promoter for the *nifRLA* operon, at which NH_4^+ regulation occurs. This locus is defined as *nifR*, and it is not yet known whether *nifR* codes for a protein.

NODULATION BY *RHIZOBIUM*

Rhizobium species exhibit some specificity with regard to the legume hosts that they nodulate (78). *R. meliloti* strains nodulate only alfalfa, whereas *Rhizobium leguminosarum* (nodulating pea) and *Rhizobium trifolii* (nodulating clover) each have strains that are able to nodulate both pea and clover. A lot of data now support the hypothesis that lectins are involved in the initial stages in the infection process and thus in *Rhizobium*-legume specificity (24, 56, 57, 89, 110, 175, 251). Lectins isolated from host plants seem to bind preferentially to the *Rhizobium* strains that normally infect the plants. For instance, soybean hemagglutinin binds to *R. japonicum* strains but not to strains from other *Rhizobium* species (19, 24).

A problem with the idea that lectin binding is necessary for infection comes from evidence that certain soybean varieties which are capable of being nodulated by *Rhizobium* actually lack soybean hemagglutinin (159). Perhaps another lectin serves to bind *Rhizobium* in these varieties. Support for the lectin binding hypothesis comes from studies with mutants unable to nodulate. Mutants of *R. meliloti* that are not able to nodulate alfalfa have been isolated, and these mutants also have lost the ability to bind to an alfalfa lectin that is found on the surfaces of alfalfa roots (A. Paau, W. Leps, and W. J. Brill, unpublished data).

The nature of the lectin binding site on the bacterium has not yet been resolved. A mutant of *R. leguminosarum* that produces nongummy colonies on plates is not able to nodulate its host (189). This mutant produces less carbohydrate material in cell-free filtrates of the growth medium. This supports the hypothesis that exopolysaccharide is the lectin binding site. On the other hand, lectins seem to bind specifically to the lipopolysaccharide from the infecting bacteria (251). Mutants of *R. japonicum* that take much longer than the wild type to nodulate soybeans (G. Stacey and W. J. Brill, unpublished data) seem to be defective in a portion of the O antigen (137, 138). Similarly, *R. trifolii* contains a polysaccharide, very similar to the O antigen, that binds to the clover lectin (55).

There are many reports of nonnodulating *Rhizobium* mutants (18); however, some of these putative mutants have turned out to be non-*Rhizobium* contaminants (Paau et al., unpublished data). This emphasizes the problem of the identification of *Rhizobium* strains. *Rhizobium* classification (108, 241) is dependent on the ability of a strain to nodulate certain legumes. If a strain is not able to nodulate, other means of identification are necessary. Perhaps two-dimensional polyacrylamide gel electrophoresis will be used more frequently to identify strains because this technique is not prejudiced by the ability of the strains to nodulate plants (183).

Because the legume-*Rhizobium* interaction involves an intimate symbiosis, the plant contribution also must be taken into account. As mentioned above, the globin part of leghemoglobin is encoded by the plant. Another protein in the nodule plant cytosol from soybeans also may be involved specifically in this symbiosis (124). To understand the interaction between bacterium and plant in more detail, nonnodulating or ineffective plant mutants (35, 81, 83, 100, 125, 238–240) should receive more attention.

DNA from *Rhizobium* can transform mutants of *A. vinelandii* (163). Some of the transformed *Azotobacter* strains gain the infection-specific polysaccharide from the *Rhizobium* donor strain (22, 136). These transformed *Azotobacter* strains bind to clover roots when *R. trifolii* is the DNA donor. Wild-type *A. vinelandii* does not bind to clover roots. These intergeneric hybrids could be useful for understanding the biochemistry of the lectin binding reaction.

It seems that the nodulating ability of *Rhizobium* is encoded by plasmid-borne genes. With *R. leguminosarum* (nodulates pea) as the donor, conjugation to *R. trifolii* (nodulates clover) or *Rhizobium phaseoli* (nodulates bean) has yielded *R. trifolii* or *R. phaseoli* transconjugants with the ability to nodulate pea (106). Also, a strain of *R. leguminosarum* that has lost a 100 $\times$ 10^6-molecular-weight plasmid is not able to nodulate its normal host (156). This plasmid is different than the plasmid containing the *nif* structural genes.

Legumes fix N_2 only when there is insufficient fixed N in the soil (78). In fact, excess NH_4^+ or NO_3^- completely prevents nodulation (54, 243). Regulation of N_2 fixation in legumes by fixed N does not seem to be at the level of nitrogenase synthesis (23; D. Noel and W. J. Brill, manuscript in preparation), but rather is at the level of nodulation. In the case of *R. trifolii* and clover, fixed N seems to prevent the lectin from being available to the bacteria (54).

Mutants of *R. japonicum* lacking nitrate re-

ductase are not able to nodulate soybeans in the presence of high levels of nitrate (82); therefore, nitrate or plant products from nitrate metabolism must be responsible for inhibition of nodulation. No one has yet found *Rhizobium* mutants that overcome depression of nodulation by fixed N. Some varieties of soybeans are much more sensitive to fixed N control of nodulation than other varieties (H. Setatou and W. J. Brill, unpublished data).

Strains of *Rhizobium* unable to fix N_2 but still able to nodulate generally produce more nodules on a host than wild-type bacteria (78). A possible explanation for this is that fixed N produced by N_2-fixing *Rhizobium* in the process of nodule formation is transported to neighboring cells to form a gradient of fixed N. For some reason, areas of the root having a certain level of fixed N cannot be nodulated (perhaps by inactivating the *Rhizobium*-binding lectin). This would cause nodules to be separated from each other. In the case of a Nif$^-$ mutant, no N_2 is fixed, and the roots contain more nodules, spaced more closely together, than roots inoculated with the wild type (W. T. Leps, R. J. Maier, and W. J. Brill, unpublished data).

Perhaps motility of *Rhizobium* is important for infection and nodulation because the bacteria are motile within infection threads (78). However, nonmotile mutants still are able to nodulate their hosts (C. Napoli and P. Albersheim, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, N43, p. 169). Motility and chemotaxis probably play important roles in the competitive environment of the soil.

Within a nodule, rhizobia presumably utilize carbon compounds supplied through plant photosynthesis. Even though sucrose is the main sugar in plants, sucrose does not seem to be the compound used by the bacteria since mutants of *Rhizobium* defective in glucose or fructose metabolism effectively nodulate their host plants (186). A mutant of *R. meliloti* with a defective α-ketoglutarate dehydrogenase forms ineffective nodules (69). This indicates that tricarboxylic acid cycle intermediates may be the actual energy and electron sources that drive N_2 fixation within the nodules. Plant-produced metabolites clearly get to the *Rhizobium* cells since a variety of amino acid auxotrophs still form effective nodules (167).

CONCLUDING REMARKS

Our knowledge of N_2 fixation has been expanding rapidly in the past decade because of certain key discoveries and because world wide many more investigators are becoming involved in N_2 fixation research. Integration of genetics, biochemistry, and physiological regulation is only beginning in the system studied in the most detail, *K. pneumoniae*. Other N_2-fixing bacteria that are much more important in current agriculture (e.g., *Rhizobium*) still require a great deal more effort to be at the level of understanding that we now have for *K. pneumoniae*. The future promises a great deal of excitement as our knowledge expands. How is leghemoglobin synthesis induced in a legume nodule? How does the actinomycete in alder nodules protect nitrogenase from being inactivated by O_2? How does the *nifA*-encoded protein interact with the different operons? What is the reason that *nif* and infection genes of *Rhizobium* are located on plasmids? How are heterocysts formed? What reactions synthesize FeMo-co? These questions should be answered soon. As more systems become amenable to genetic studies (e.g., *A. vinelandii* [21, 164, 165], *Rhodopseudomonas capsulata* [245, 254], *Azospirillum lipoferum* [143], *Anacystis nidulans* [160], *Nostoc muscorum* [209], *R. meliloti* [42, 43, 58, 120, 121, 141, 216], *R. leguminosarum* [15, 17, 29, 105–107], *R. trifolii* [73, 206, 233, 234], and *R. japonicum* [68]), the rate of important discoveries is certain to increase.

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