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Molecular Biology of Nitrogen Fixation

Manipulation of nitrogen fixation genes may lead to increased production of high-quality protein.

K. T. Shanmugam and Raymond C. Valentine

The nitrogen in our protein foodstuffs is derived from the vast reservoir of atmospheric nitrogen by nitrogen fixation. Nitrogen-fixing organisms, such as the familiar root nodule bacteria of leguminous plants (soybeans, for example) probably account for about one-half of the total amount of nitrogen fixed annually. Large amounts of nitrogen are also fixed by chemical means, during the manufacture of nitrogenous fertilizers, for example. The great demand for these fertilizers is indicated by the more than 580 chemical fertilizer plants now in operation or under construction throughout the world, representing an investment of more than $10 billion (*1*). The manufacture of fertilizers, however, requires vast inputs of energy. Sweeney estimates that the total amount of energy required for the production of ammonium fertilizers is equivalent to 2 million (2×10^6) barrels of oil per day, worldwide (*1*). In North America, the consumption of nitrogenous fertilizers exceeded 8 million tons (metric) in 1973, more than 22 percent of the total world consumption (*2*). For corn production alone, U.S. farmers today apply nitrogenous fertilizers representing an energy input of nearly 900,000 kilocalories per acre (2,430 megacalories per hectare) (*3*). This energy input itself is almost as large as the total energy input for the 1945 corn crop.

The basic commercial process for manufacturing ammonia, which consists of catalytically reacting hydrogen with nitrogen under high pressure and temperature to form ammonia ($3H_2 + N_2 \rightleftharpoons 2NH_3$), has not changed since it was inaugurated in 1913. The dramatically rising price of petroleum as a source of hydrogen, and the many ecological and economic limitations to the heavy use of chemical fertilizers, have stimulated much interest in nitrogen fixation by microorganisms. A better understanding of the nature and manipulation of the biological system might lead to cheaper and more efficient means of producing high quality plant protein.

The only organisms known to fix nitrogen are certain bacteria that harbor the nitrogen fixation (*nif*) genes. Nitrogenase genes code for nitrogenase proteins that catalyze biological nitrogen fixation. One objective of particular significance in the application of molecular biology to the study of nitrogen fixation in bacteria is eventual "infection" of nonleguminous plants such as the cereals with *nif* genes.

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Discovery and Mapping of the Genes for Nitrogen Fixation

At Berkeley in 1971 we identified several crucial genes for nitrogen fixation which were clustered on a small segment of the chromosome of the nitrogen-fixing bacterium, *Klebsiella pneumoniae* (*4*). These genes were discovered at about the same time by Dixon and Postgate who were working in England (*5, 6*). Although the biochemistry of nitrogen fixation was not as well understood in *K. pneumoniae* as it was in other better known nitrogen-fixing bacteria, such as *Azotobacter* and some of the clostridia, we chose to work with the *Klebsiella* species because of its close genetic (evolutionary) relationship to *Escherichia coli*, which is generally acknowledged as the best understood of all living cells. We have been able to exploit the close genetic relationship between these two bacteria in a number of ways.

The finding that the bacteriophage P1 of *E. coli* would mediate the transduction of the *nif* genes (*4*) provided the foundation for studies of the molecular biology of nitrogen fixation. Transductional analysis of *K. pneumoniae* with coliphage P1 yielded several important facts; the most important was that the *nif* genes formed a cluster near the histidine operon on the *K. pneumoniae* linkage map, as shown by cotransductional mapping (*4, 7*). Most chemically induced mutations of the *nif* genes, in which the ability to synthesize nitrogenase was lost (*nif*$^-$), were found to be cotransduced with the *his* D gene (*8*) at a frequency between 30 and 80 percent. As calculated from

the cotransduction frequencies, this quantity of DNA would be more than sufficient to code for nitrogenase as well as other ancillary *nif* genes (*7*).

Although the majority of *nif*$^-$ lesions were located near the histidine operon, occasional non–nitrogen-fixing phenotypes were found in which the lesions did not map in this region (*7*). It is possible that the biochemical functions of these genes correlate with various regulatory as well as ancillary enzymes and proteins essential for nitrogen fixation.

There is much indirect evidence that the cluster of *nif* genes that comprise the *nif* operon and map near the histidine operon on the genetic linkage map of *K. pneumoniae*, code for nitrogenase. Most mutations of the *nif* genes in this cluster result in strains that are devoid of nitrogenase activity, although some mutants are still capable of synthesizing one or both of the two different subunit proteins comprising the nitrogenase complex (*9*). Proof of the importance of this cluster of *nif* genes has been provided recently by Dixon and Postgate (*6*) who constructed nitrogen-fixing recombinants of *E. coli* by transferring the *nif* segment of DNA from *K. pneumoniae* to *E. coli*.

By studying deletion mutants (*10*) we have been able to define the location of some *nif* genes between the histidine operon and the *shi* A marker (Fig. 1) on the *K. pneumoniae* linkage map (*8*). We have isolated approximately 100 mutants carrying deletions in this region. About 70 percent of such mutants have deletions extending through the histidine operon and into (through) the *nif* segment of the DNA. Of the remaining 30 percent, the majority have deletions extending beyond the *nif* region into the shikimic acid region, while the rest terminate within the histidine operon. As shown in Fig. 1, *E. coli* apparently lacks *nif* genes.

It has been brought to our attention by Cannon *et al.* (*11*), that *nif* genes mapping near the histidine operon may, in fact, occur as two separate clusters (operons?), one on each side of the histidine operon. Our finding that one or more *his* genes may play a role in the regulation of the synthesis of nitrogenase has added increasing complexity to the *nif* map in this region (*7*). Clearly more experiments are necessary before we can be sure of the exact location of the *nif* genes near the histidine operon.

It is of interest to compare the map position of the *nif* genes in *K. pneumoniae* with the location of the *nif* cluster on the genetic linkage map of a stable nitrogen-fixing hybrid of *E. coli*. A map of such a hybrid was constructed by Dixon and Postgate (*6*). Genetic analysis indicated that the *nif* genes were integrated near the histidine operon on the *E. coli* genome, and that the gene order was probably: *rfb, nif, gnd, his* (*8, 12*), in contrast to the order *rfb, gnd, his, nif* in *K. pneumoniae* (*10*). Such shuffling of gene order could be the result of a complex series of rearrangements of chromosome segments being required for insertion of the heterologous *K. pneumoniae* DNA carrying the *nif* genes into the *E. coli* chromosome.

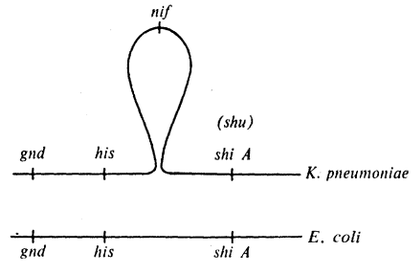

Fig. 1. Genetic map of the *nif* gene region of *K. pneumoniae*, compared with a map of the same region in *E. coli*, which lacks *nif* genes. See text for details. Abbreviations: *gnd*, 6-phosphogluconate dehydrogenase; *his*, histidine enzymes responsible for biosyntheses; *shi* A, shikimate permease (this area is also involved in auxotrophy for shikimate utilization). [From (*39*); courtesy of Cambridge University Press]

The locations of the *nif* genes on the chromosomes of other nitrogen-fixing bacteria are not yet known. It is possible that in some bacteria extrachromosomal DNA, such as the DNA of plasmids, may be responsible for nitrogen fixation.

Nitrogen Fixation Plasmids

Plasmids that code for the synthesis of nitrogenase have recently been reported from two laboratories. Cannon *et al.* (*13*) have found that heterologous matings between *K. pneumoniae* nitrogen-fixing donors and *E. coli* C non–nitrogen-fixing (*nif*$^-$) recipients often yield genetically unstable *E. coli* hybrids that fix nitrogen. The *nif* genes in two such hybrids are present as covalently closed circular molecules of DNA plasmids. A plasmid with *nif* genes, which also carries *his* genes of one of the parent strains and has a molecular weight of 9.5×10^6, does not promote its own transfer, presumably because it lacks genes for sex pili, the male specific appendages required for mating. However, the *nif* plasmids are readily transferred to another cell if that cell carries a drug-resistant (R) factor, or "helper," capable of producing sex pili. The R-factor mediated transfer of *nif* plasmids occurs at a much higher frequency than the mobilization of the *nif* genes on the chromosome, a finding that can be utilized in the construction of new strains of *E. coli* K12 that fix nitrogen. The maintenance of the *nif* genes as plasmids in most *E. coli* hybrids may indicate that the recombination enzymes of *E. coli* are not effective for promoting the recombination of the *nif* genes from *K. pneumoniae* with the recipient chromosome.

Cannon *et al.* (*11*) have recently described the genetic construction of an *E. coli* hybrid with an infectious (transferrable) *nif* episome—that is, an F′ factor that carries the *his* and *nif* genes from *K. pneumoniae* to recipients lacking *nif* genes. This large episome, with an approximate size of 10^8 daltons, also carries genetic markers for *met* G, *rfb, gnd,* and *shi* A (*8*). Because it carries the genes of a complete sex factor (including sex pili), the *nif* genes can be transferred during cell to cell contact, from an F′ nitrogen-fixing donor to a *nif*$^-$ recipient. Such transfers occur at a high frequency; for example, in sexual crosses in which both the donors and recipients were *E. coli* strains, 20 percent of the donor cells were able to transfer the *nif* episome to *nif*$^-$ recipients. Episomes of this type represent interesting new tools for the genetic analysis of nitrogen-fixing organisms. For instance, the introduction of F′ *nif* DNA into Gln C$^-$ strains of *K. pneumoniae* has suggested a new method for studying the regulation of *nif* genes.

Recent studies in Ireland (*14, 15*) show that intrageneric transfer of *nif* genes occurs in matings between the root nodule bacterium, *Rhizobium trifolii*, and *Klebsiella aerogenes*. Because *K. aerogenes* does not naturally fix nitrogen, it is evident that its ability to do so must depend on the transfer of the appropriate DNA from the root nodule bacterium. Whether root nodule bacteria routinely depend on *nif* plasmids to fix nitrogen, or whether they also contain *nif* genes in their chromosomes, is not known. DNA extracted from nitrogen-fixing hybrids of root nodule bacteria and *K. aerogenes* contains extrachromosomal DNA which

is absent in the *K. aerogenes* recipient and also in *nif*$^-$ segregants. Dunican and his co-workers have concluded that *nif* genes are carried on a plasmid in the hybrids. A plasmid of 9×10^6 daltons was implicated as a carrier of *nif* DNA.

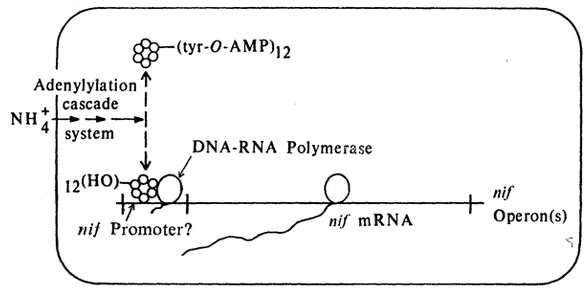

Fig. 2. Working model for genetic regulation of nitrogen fixation (*21*). Tyr-*O*-AMP refers to adenosine monophosphate moieties covalently attached to tyrosine residues of glutamine synthetase. Growing chains of *nif* messenger RNA (mRNA) are shown as wavy lines attached to polymerase molecules. See text for further details.

Regulation of Nitrogen Fixation

Klebsiella pneumoniae appears to have evolved an elaborate control system for modulating nitrogen fixation—a regulatory system which may be one of the most sophisticated yet encountered in bacteria. It has long been known that the ammonium ion, NH_4^+, the product of nitrogen fixation, shuts off or represses the genes that direct the synthesis of nitrogenase. A working model of the regulation of *nif* genes by NH_4^+, based on current concepts, is illustrated in Fig. 2. The model is adapted from a proposed mechanism for genetic regulation of the *hut* (histidine utilization) genes of *K. aerogenes* (*16*) summarized in a recent review by Magasanik *et al.* (*17*).

Experiments conducted at the laboratories of Stadtman (see *18*) and Holzer (see *19*) revealed the existence of an enzyme system known as the "adenylylation enzyme cascade" (Fig. 2). In essence, the addition of NH_4^+ (or one of a variety of other nitrogenous compounds that repress nitrogenase synthesis) is thought to trigger the enzyme cascade system that catalyzes the covalent modification of glutamine synthetase by attachment (or removal) of adenyl moieties on specific tyrosine residues of the protein; such modification has a marked effect on the catalytic properties of glutamine synthetase. The cascade might be regarded as the "biochemical" part of the model in contrast to the "genetic" part dealing with the activation of expression of the *nif* genes. The simplest notion is that adenylylation blocks the binding of glutamine synthetase to the *nif* promoter or, conversely, deadenylylation leads to the binding of glutamine synthetase and the subsequent activation of *nif* transcription. Interplay between glutamine synthetase and DNA-RNA polymerase bound to the *nif* promoter region is envisioned.

There is some experimental evidence that implicates glutamine synthetase as a direct participant in the genetic control of nitrogen fixation (*20, 21*). We have found that glutamine-requiring auxotrophs (*gln* A$^-$) that lack catalytically active glutamine synthetase (*20, 21*) cannot synthesize nitrogenase. These *gln* A$^-$ mutants fail to synthesize nitrogenase under a variety of growth conditions tested, all of which indicates that the wild-type *K. pneumoniae* is able to synthesize nitrogenase. One may speculate that the mutation that blocks the catalytic activity of glutamine synthetase simultaneously destroys the regulatory properties of the (glutamine synthetase) protein.

When *gln* A$^-$ mutants reacquire the glutamine synthetase genes as a result of conjugation with a strain of *E. coli* carrying the episome F′133, they can again make nitrogenase. *Klebsiella pneumoniae gln* A$^-$-F′133 hybrids are characterized by their genetic instability: as many as 85 percent of the clones lose the episome when they are cultured in glutamine-supplemented broth. It thus seems probable that F′133 functions as an episome in the *K. pneumoniae* mutant, coding for *E. coli* glutamine synthetase which activates expression of *nif* genes.

Mutants of *K. pneumoniae* in which glutamine synthetase is constitutive (Gln C$^-$), continue to synthesize glutamine synthetase under conditions in which such synthesis would normally be repressed. These regulatory Gln C$^-$ mutants are characterized by their high specific activities of glutamine synthetase (about 600 to 1000 nanomoles of glutamyl hydroxymate produced per minute per milligram of protein) in the presence of NH_4^+ in the growth medium, in contrast to wild-type strains which produce about 300 to 400 units of glutamine synthetase under similar conditions. One of these strains has been found to continue to synthesize nitrogenase, in the presence of NH_4^+, in amounts up to 30 percent of those produced in the absence of NH_4^+ (*20, 21*).

Cannon *et al.* (*11*) have provided additional evidence for the role of glutamine synthetase as the activator of *nif* gene expression. These workers introduced the F′ *nif* DNA into a Gln C$^-$ (constitutive) mutant of *K. aerogenes* and observed that nitrogenase activity was partially derepressed on a medium containing NH_4^+ (10 to 20 percent of derepressed activity).

There are still many questions to answer regarding the mechanism of activation of *nif* expression by glutamine synthetase. One of the most interesting questions concerns the make-up of the postulated operator-promoter region of the *nif* DNA. Gordon and Brill (*22*) have described mutant strains of *Azotobacter vinelandii* that synthesize nitrogenase in the presence of NH_4^+. These mutants were isolated as revertants of *nif*$^-$ strains and they may contain operator-promoter mutations. In line with current concepts of bacterial operons (*23*) the *nif* operon would be expected to be composed of structural genes and a regulatory element (operator), or elements, presumably located adjacent to structural genes. The *nif* operator-promoter region would presumably provide binding sites along its length for DNA-RNA polymerase to initiate transcription of the *nif* message, as well as other sites for attaching various regulatory proteins such as glutamine synthetase. This completes the hypothesized regulatory circuit (Fig. 2) which might allow *K. pneumoniae* to turn on or off the expression of *nif* genes, depending on the availability of exogenous supplies of nitrogen. This picture of the *nif* operator may be overly simplified. Additional binding sites on the operator may be necessary for other unidentified regulatory proteins. For instance, expression of *nif* genes by *K. pneumoniae* in an aerobic environment would, in all likelihood, represent a futile cycle of synthesis and degradation of nitrogenase since the enzyme is extremely oxygen-labile. A mechanism for repression of *nif* genes in the presence of oxygen (such as a regula-

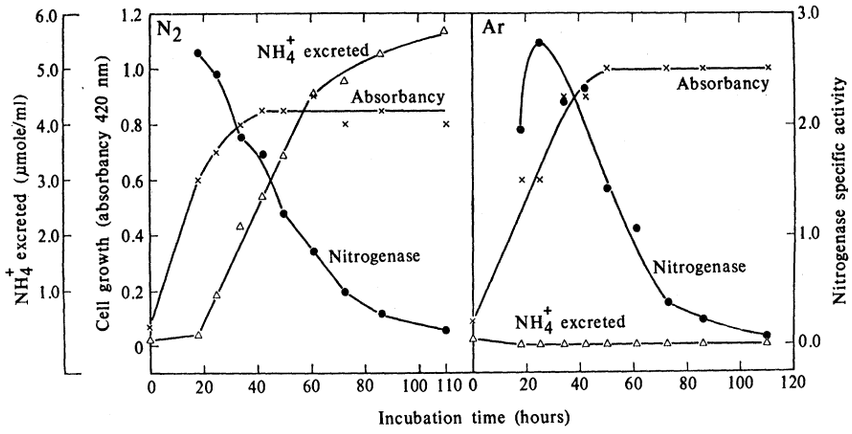

Fig. 3. Time course of NH_4^+ excretion by a *K. pneumoniae* mutant in which the *nif* genes are derepressed and NH_4^+ assimilation is blocked (*asm*$^-$). The concentration of NH_4^+ was determined colorimetrically by the Conway-diffusion procedure, with Nessler's reagent (*40*). *Klebsiella pneumoniae* M5A1 (laboratory strain SK-24) was grown anaerobically, with constant sparging with nitrogen (left) or argon (right), in a glucose-salts medium (*10*) supplemented with 100 micrograms of glutamate per milliliter. Incubation was at room temperature (about 22°C). Nitrogenase activity (measured as micromoles of ethylene formed per hour per milligram of protein) of whole cells was determined by means of the acetylene procedure (*10*).

tory protein capable of responding to the redox state of the cell?) might be of considerable survival value (*24*).

As already mentioned, Dunican and Tierney (*15*) have constructed hybrid strains of *K. aerogenes* harboring *nif* genes donated by a root nodule bacterium, *R. trifolii*. Nitrogenase synthesis in these hybrids is repressed by NH_4^+, raising the possibility that the two parent organisms have a common system of *nif* gene regulation. Further studies of the mechanism of regulation of nitrogen fixation in *K. pneumoniae* may contribute to the construction of root nodule bacteria that catalyze more efficiently the symbiotic fixation of nitrogen.

Further Applications

As a result of our increasing knowledge of the molecular biology of nitrogen fixation it might eventually be possible to increase the biological production of nitrogenous fertilizer from atmospheric nitrogen. We have recently isolated a strain of *Klebsiella* (*K. pneumoniae* M5A1, laboratory strain SK-24) in which the *nif* genes are constitutive or derepressed (Nif C$^-$) (*25*). This strain excretes large amounts of fixed nitrogen as NH_4^+: up to 20.2 micromoles of NH_4^+ are produced per milligram of cell protein during an incubation period of 24 hours at room temperature, when nitrogen gas is supplied by sparging (see Fig. 3). The biosynthesis of NH_4^+ proceeds at the expense of a variety of fermentable sugars, glucose being used as the energy source in this particular experiment. It seems possible to account for the pattern of NH_4^+ excretion in strain SK-24 on the basis of the following perturbations in crucial enzyme levels. Biochemical analysis reveals that the strain SK-24 continues to synthesize nitrogenase, in the presence of NH_4^+, in amounts up to 65 percent of those produced in the absence of NH_4^+. Glutamine synthetase is produced constitutively (Gln C$^-$), whereas glutamic dehydrogenase activity is missing. As expected, the Nif C$^-$ strain does not produce glutamate synthase, a property shared with its parents (lacking glutamate synthase *asm*$^-$) (*8, 26*).

Although these mutations have not been mapped, it appears that at least two genetic lesions are responsible for the NH_4^+-excreting phenotype: (i) a constitutive mutation (probably Gln C$^-$) which releases nitrogenase from repression by NH_4^+ (also glutamic dehydrogenase is somehow repressed in such strains, accounting for the absence of this enzyme); and (ii) an *asm*$^-$ mutation blocking glutamate synthase activity (*26*). In the absence of both of the glutamate-forming enzymes the strain must be supplemented with glutamate for growth; and this provides a convenient way for controlling cell densities during the experiment. It is interesting to note from Fig. 3 that once growth has ceased, production of NH_4^+ continues for several days. Exhaustion of glutamate needed for maintaining protein synthesis may account for the gradual decrease in nitrogenase activity which leads to cessation of NH_4^+ production. Ways to prolong the duration or increase the efficiency of the reaction, or both, are being explored. In essence it appears relatively easy, by genetic manipulation, to disrupt the *nif* gene regulatory system that integrates nitrogen fixation with cell growth, thereby channeling fixed nitrogen as NH_4^+ into the environment. These findings might well be applied to other nitrogen-fixing bacteria. For example, mutants of the versatile blue-green algae, if they had derepressed *nif* genes, might be ideal organisms for producing ammonium fertilizer because these microbes utilize an unlimited energy source—solar energy—directly for nitrogen fixation. Microbial production of NH_4^+ at the expense of other cheaply available energy sources, such as cellulose or molasses, might also prove feasible.

There is currently considerable interest in creating new nitrogen-fixing species. In choosing new strains of bacteria or higher organisms as potential recipients for the *nif* genes it is important to keep in mind the basic biochemical requirements for fixation (*27*). These requirements, as summarized in Table 1, are: (i) nitrogenase; (ii) a supply of strong reductant, usually as reduced ferredoxin or flavodoxin; (iii) adenosine triphosphate (ATP) as an energy source, which can be provided by fermentation, substrate level phosphorylation, oxidative phosphorylation, or photophosphorylation; (iv) a specialized NH_4^+ assimilation pathway; (v) a regulatory system for the *nif* genes; and (vi) a method of protection of the nitrogen-fixation system from oxygen denaturation and competition for reductant (in aerobic bacteria only). There may also be a need for enzymes that catalyze the transformation of the trace element molybdenum to a form suitable for insertion into nitrogenase (*28*), and that catalyze various membrane-associated reactions, such as active transport systems (permeases), that effect the entry of substrates and the exit of products of nitrogen fixation. The role of the membrane seems especially pertinent in the case of root nodule symbiosis where, for example, nitrogenous products must be transported from the root nodules. In turn, photosynthetic products must be moved to the nodule and across the bacteroid membrane for consumption as the carbon and energy source for nitrogen

fixation. The part played by the cell membrane in nitrogen fixation is still virtually unknown.

An organism that fixes nitrogen has a high energy demand, and this needs to be remembered in the search for new recipients of *nif* genes. Nitrogenase ranks as one of the most unusual enzymes ever encountered, with the purified enzyme consuming as much as 15 moles of ATP per mole of nitrogenous substrate reduced to NH_4^+ (*29*): there are no other examples of an enzyme requiring such a high concentration of ATP. In addition to the high-energy phosphate from ATP nitrogenase also requires as substrate powerful reducing agents, such as reduced ferredoxin, an energy form which is equivalent to large numbers of additional high-energy phosphates. This is by no means a complete energy balance for fixation; one must add to this list the high-energy phosphate equivalents for the synthesis of the nitrogenase.

Even the higher plants, such as soybeans, which utilize solar energy indirectly for supporting nitrogen fixation in their root nodules (*30*), probably must invest a large proportion of their net energy to produce NH_4^+. For example, an energy balance calculated for pea plants by Minchin and Pate (*31*) shows that 32 percent of the total carbon fixed by the plant is translocated to the nodules, and the nodules in turn return 45 percent of this carbon as amino compounds for the plant growth while using the remaining 55 percent for their own growth and to supply energy for the fixation of nitrogen. The high energy cost of this process may explain why most higher plants have not evolved symbiotic relationships with nitrogen-fixing bacteria—they simply may not be able to afford the energy. Bacteria in association with plants may have a great advantage over most free-living bacteria with respect to the vast and constant supply of energy needed for supporting nitrogen fixation (the blue-green algae are an exception to this rule since they can photosynthetically convert solar energy to energy available for biosynthetic processes). Certain species of plants, such as sugar cane, which are relatively efficient because of their low rates of photorespiration, may transfer enough photosynthate to nitrogen-fixing bacteria in or surrounding the root system to sustain annual rates of nitrogen fixation (*32*) as high as 100 kilograms per hectare.

In spite of the relatively stringent requirements for biological nitrogen fixation it would seem that a large number of free-living bacteria possess the basic biochemistry needed to support nitrogen fixation. A point by point check of the biochemical requirements for nitrogen fixation by the wild-type *E. coli*, which does not naturally fix nitrogen, reveals that most, if not all, ancillary reactions are present and functional in this organism (Table 1). (This suggests that such ancillary reactions may have other functions besides nitrogen fixation.) For instance, it is well known that during the fermentation of carbohydrates, *E. coli* produces a powerful reductant—equivalent in reducing power to hydrogen gas. This reductant could be utilized for nitrogen fixation. It is also known that the synthesis of at least one crucial enzyme in *E. coli*, glutamine synthetase, is repressed by NH_4^+ (*33*), suggesting that a system of NH_4^+ repression is operative. The NH_4^+ assimilatory system involving glutamate synthase is also functional in wild-type *E. coli* (*34*). In addition, the presence of nitrate reductase, a molybdenum-containing enzyme (*35*), indicates that *E. coli* possesses the necessary metabolic machinery for interconversion of molybdenum into a catalytically active form. Thus, in retrospect, *E. coli* was a good choice as recipient for the *nif* genes since it already possessed the necessary supporting enzymes.

The successful construction of *E. coli* strains capable of nitrogen fixation has stimulated many workers to think about similar experiments with plant and animal systems. Perhaps more attention

Table 1. Biochemical requirements for nitrogen fixation in different potential recipients of *nif* genes (+, observed; −, not observed).

Requirements	Potential recipient		
	E. coli (microbe)	Chloroplast (plant)	Mitochondrion (animal)
Nitrogenase	−	−	−
Reduced ferredoxin or equivalent	+	+	−
ATP	+	+	+
Glutamate synthase pathway of NH_4^+ assimilation	+	−	−
Genetic activator	+	−	−
Oxygen protection	+ (anaerobic only)	−	−
Molybdenum	+	+	+

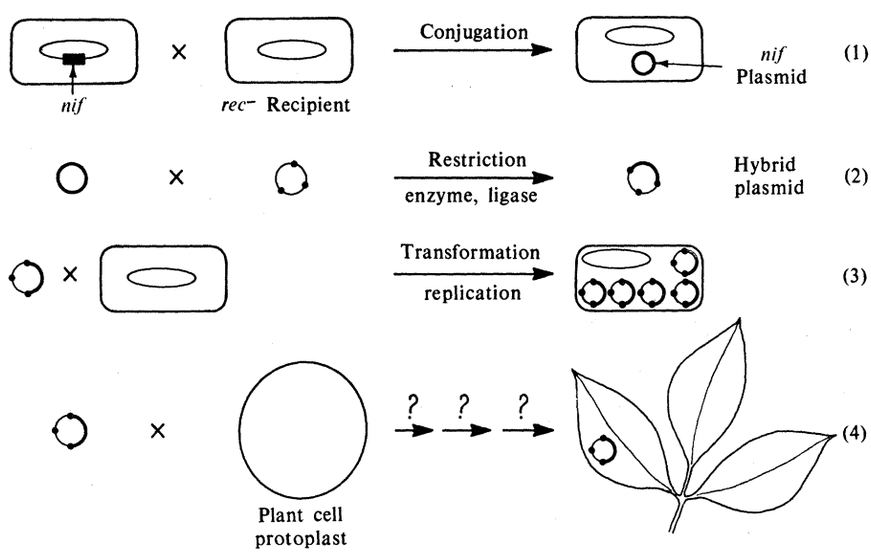

Fig. 4. Hypothetical scheme for the production of bulk quantities of *nif* plasmids, based on recent studies of the molecular biology of plasmid replication (*37*, *38*). In step 2, a *nif* plasmid (a circular DNA molecule) is specifically cleaved with a restriction enzyme and nonenzymatically hybridized in vitro with Col E1 (a fast-replicating plasmid), yielding a new circular hybrid molecule (nif-Col E1). This hybrid molecule is treated with the enzyme DNA ligase to obtain a covalently closed circular DNA molecule. Step 3 represents the transformation and replication of the hybrid plasmid in *E. coli* yielding a large number of copies of the *nif* genes per cell as was recently achieved for *trp* genes (*37*). Step 4 represents one of several ways molecular biologists may utilize *nif* plasmids, in this instance for "infecting" higher plant cells directly with *nif* DNA.

has been given to plants because they seem to possess more of the required biochemical (genetic) capacity for fixation (Table 1). For example, during photosynthesis plants produce reduced ferredoxin, a powerful reductant required for fixation. However, there is the severe handicap of oxygen protection of nitrogenase to be dealt with in plants that evolve oxygen. Finally, permanent "storage" and replication of the *nif* genes in the plant cell poses an enormous problem.

Plasmids may play (or be genetically engineered to play) an important role in the synthesis of the large quantities of nitrogenase needed for nitrogen fixation. The rapidly increasing numbers of plasmids being encountered in bacteria may be separated into two categories, those such as the drug-resistant plasmids (carrying the R-factors) (*36*) which are "infectious" in the sense that they are able to promote their own spread from cell to cell, and those which apparently have no sex pili for infecting a new host. Bacteria with infectious plasmids carrying *nif* DNA can readily donate the nitrogen-fixing ability to other bacteria permitting construction of new hybrid strains.

There also may be advantages in selecting bacteria with noninfectious *nif* plasmids. One possibility is that many copies of small *nif* plasmids, for example, in a single root nodule bacterium, might function as multiple templates for the synthesis of the large quantities of nitrogenase required for nitrogen fixation.

Recent advances by several investigators (*37, 38*) concerned with the "amplification" of genes fused with bacterial plasmids have led us (in collaboration with Helinski, Meyer, and co-workers) to consider the possibility of enzymatically inserting *nif* DNA into a fast-replicating plasmid from *E. coli* (Col E1) (Fig. 4). One object of this experiment is to increase or prolong the synthesis of nitrogenase and thus to enhance the production of NH_4^+. Helinski and co-workers (*37*) have previously shown that insertion of the tryptophan operon (*8*) into a fast-replicating bacterial plasmid (Col E1) (see step 2 of Fig. 4), followed by transformation of the hybrid plasmid into *E. coli*, led to a great amplification of tryptophan biosynthetic enzymes (step 3 of Fig. 4). Under conditions of maximum plasmid replication about 560 copies of the plasmid associated tryptophan operon were present per cell, corresponding to about 50 percent of the total cellular DNA. Large quantities of tryptophan messenger RNA were transcribed from the plasmids carrying the *trp* genes, and this led to the production of enormous quantities of tryptophan enzymes—about 50 percent of the total protein of the cell. These findings provide a theoretical basis for the amplification and isolation of bulk quantities of *nif* plasmids suitable for a variety of experiments including the transcription of *nif* genes in vitro and the transfer of *nif* genes to higher plants (Fig. 4).

We hope that by applying molecular biology to problems in agriculture, mankind will ultimately benefit. Although sustained and expensive experimentation will be required before it will be possible to improve the productivity of biological nitrogen fixation, the goals fully warrant long-range cooperative efforts by scientists from a wide spectrum of the biological and chemical disciplines.

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8. The gene symbols are defined as follows: *asm*-, glutamate synthase· *gln* A, glutamine synthetase; *gnd*, gluconate-6-phosphate dehydrogenase; *his* D, histidinol dehydrogenase; *met* G, methionyl-transfer RNA synthetase; *rfb*, a gene cluster coding for biosynthesis of cell-wall lipopolysaccharide; *shi* A, shikimic acid permease; *trp*, tryptophan biosynthetic operon.
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