Nitrogen Fixation: Prospects for Genetic Manipulation

In almost every account of the pros and cons of recombinant DNA research, the pros include the possibility that the gene-splicing techniques may lead to the development of new ways to increase biological nitrogen fixation; that is, the production by living organisms of ammonia, which is needed for plant growth, from atmospheric nitrogen. Investigators hope that such an achievement will increase crop productivity while decreasing the farmer's reliance on synthetic ammonia fertilizers that are costly in terms of both money and energy.

The problem is that recombinant DNA techniques and other forms of genetic engineering are unlikely to pay off in greater biological nitrogen fixation and crop productivity in the near future. At least that was the nearly unanimous consensus of a group of investigators participating in a recent symposium titled "Genetic Engineering for Nitrogen Fixation." * They cited numerous problems that will hinder attainment of these goals. Among them is the prospect that increasing biological nitrogen fixation, for which the plant supplies the energy, may actually decrease crop yields by taking energy that would otherwise go into producing the useful parts of the plant. The extremely rapid inactivation by oxygen of the nitrogen-fixing machinery is another obstacle to attempts to confer the capacity to produce ammonia on organisms that normally lack it. (The naturally occurring nitrogen-fixing systems have evolved ways to prevent the inactivation.)

The problems with genetic engineering do not mean that the outlook for increased biological nitrogen fixation is bleak, however. Many of the symposium participants predicted that the rapidly expanding body of knowledge gleaned from conventional biochemical and agricultural studies would provide a quicker route to the desired goals. One possibility that received considerable attention at the symposium was the use of a nitrogen-fixing blue-green algae living in symbiotic association with a small aquatic fern for fertilization of rice paddies.

Even though the conventional studies are more advanced in terms of practical applications the researchers do think that genetic manipulations provide valuable tools for answering some of the fundamental questions about the *nif* (nitrogen fixation) genes that occur only in certain bacteria and blue-green algae. (See the accompanying box for a discussion of the safety issues involving genetic manipulation of nitrogen fixation.) Recently, a group of investigators, including Frederick Ausubel of Harvard University and Frank Cannon of the University of Sussex, England, used recombinant DNA techniques to clone a portion of the DNA molecule containing the *nif* genes of the bacterium *Klebsiella pneumoniae*.

Molecular cloning (*Science*, 19 March 1976) is a valuable technique because it provides a means of obtaining many exact copies of a particular DNA segment. More material can be produced—and in a pure form—than can be obtained by trying to isolate the genes directly from the cells carrying them. This facilitates analysis of the organization and structure of the genes and may permit the study of their function in simpler conditions than those found in nature.

In order to clone DNA molecules, specific enzymes are used to produce fragments of the DNA under investigation. The fragments are then inserted into a vehicle, either a plasmid or a virus, that reproduces in bacterial cells. (Plasmids are small circular pieces of DNA found in many bacteria; they replicate independently of the bacterial chromosome and may produce multiple copies in each bacterial cell.) After the hybrid plasmid or virus has reproduced, the desired DNA segment can be recovered by digesting the hybrid molecules with the same enzyme used to produce the DNA fragments in the first place.

Ausubel and his colleagues used the plasmid designated pMB9 as the cloning vehicle. This plasmid, which originated in the bacterium *Escherichia coli* and was developed as a vehicle by Herbert Boyer and his colleagues at the University of California in San Francisco, reproduces to form multiple copies of itself in several different types of bacteria. After forming hybrid plasmids containing *K. pneumoniae* DNA, the investigators used them to transform a *K. pneumoniae* mutant that lacks the capacity to fix nitrogen.

Some of the bacteria transformed by the hybrid plasmids regained the capacity to reduce nitrogen to ammonia. Structural and genetic analysis of the largest of these plasmids indicated that it contains one of the two clusters into which the *nif* genes are divided on the *K. pneumoniae* chromosome. The two clusters are separated by a DNA sequence that does not appear to function in nitrogen fixation.

The segment of the *K. pneumoniae* chromosome that contains the *nif* gene clusters is quite large. Investigators, including Winston Brill of the University of Wisconsin, Ray Dixon of the University of Sussex, England, and Raymond Valentine of the University of California at Davis, have thus far identified eight genes required for nitrogen fixation and mapped their positions on the bacterial chromosome. Three of them are structural genes that code for the three proteins that combine to form the nitrogenase enzyme. This is the enzyme that catalyzes the reduction of nitrogen to ammonia. The remaining genes either code for additional proteins needed to help the nitrogenase do its job or function in regulating the enzyme activity.

The three structural genes and one of the regulatory sites are in one cluster and the remaining four are in the other. It is the latter cluster that Ausubel and his colleagues have now cloned. The DNA segment containing both *nif* gene clusters is too large to clone on one vehicle, but Ausubel hopes to eventually clone the cluster encompassing the structural genes in another plasmid that can replicate together with pMB9 in bacterial cells. In this way all of the known *nif* genes could be transferred into the same bacterial cell where they could reproduce and express their nitrogen-fixing function. Thus, the cloning techniques may provide a way to introduce *nif* genes into bacteria that now lack them. The hope is that these new forms of bacteria could be used to develop novel symbiotic relationships with crop plants, including corn and wheat, that do not have their own nitrogen-fixing symbionts.

However, techniques involving recombinant DNA and molecular cloning are not the only route by which *nif* genes may be introduced into bacterial cells. Dixon and his colleagues have produced by standard genetic techniques a hybrid *E. coli* plasmid that carries the *nif* genes of *K. pneumoniae*. The plasmid is "promiscuous" in that it can transform a wide range of bacterial species. The Dixon group has shown that the *Klebsiella* genes are expressed in bacteria of different species, including *E. coli* and

*The symposium was held at Brookhaven National Laboratory on 13 to 17 March and was sponsored by the Research Applied to National Needs Program of the National Science Foundation.

Azotobacter vinelandii, that have been transformed by the hybrid plasmid.

There is also the possibility that the *nif* genes could be introduced directly into plant cells to produce a plant that needs no nitrogen fertilizer. A nitrogen-fixing corn or wheat plant could be considered the Holy Grail for investigators of nitrogen fixation, but even they do not think that they will find it in the near future.

A number of formidable problems will first have to be overcome. These can be divided into two areas; the first is development of a suitable and safe means of introducing new genes into plants, and

Increased Biological Nitrogen Fixation and the Environment

Investigators who do research on nitrogen fixation have always considered improvement of the biological process to be a worthwhile goal. They include among the potential benefits increased crop productivity, conservation of energy, and economic savings for the farmer. The benefits could be just as important—if not more so—in the developing countries as in highly industrialized nations. The former usually have a greater need than the latter to increase crop yields to provide for their rapidly expanding populations; they may also lack both the capital to pay for artificial fertilizer and an efficient system to distribute it.

Recently, however, the investigators, impelled to a great extent by the continuing controversy regarding the desirability of recombinant DNA research, have been considering what, if any, hazards might be associated with genetic manipulation of nitrogen-fixing organisms. Although public attention has been focused largely on the recombinant DNA issue, the participants in the Brookhaven symposium made it clear that alternative methods can be used to alter the nitrogen-fixing capabilities of organisms. For example, workers in the laboratories of Raymond Valentine and Winston Brill have developed mutant strains of bacteria that continue to reduce nitrogen even in the presence of ammonia concentrations that normally halt the synthesis. Production of the mutant bacteria did not require the use of recombinant DNA techniques. Nor did that of the hybrid *E. coli* plasmid carrying the *nif* genes of *K. pneumoniae* recently described by Ray Dixon and his colleagues. The plasmid can transmit the *nif* genes to bacteria that would not normally contain them. The same result can be achieved with recombinant DNA techniques.

Since there is more than one route to the genetic manipulation of nitrogen-fixing organisms, the question that needs to be answered is: Will increasing ammonia production by organisms that live in the soil and water—no matter how this is accomplished—somehow damage the environment? A number of possible, if not necessarily probable, scenarios have been suggested.

Ammonia is toxic, and overproduction could be hazardous if the gas escaped into the atmosphere. Too much ammonia could also increase the alkalinity of the soil and water. Or if the oceans contained excess ammonia, they might trap more than normal quantities of carbon dioxide and contribute to a temperature decrease that could lead to shorter growing seasons and a decline in food production.

Another possibility is that overproduction of ammonia will indirectly accelerate the depletion of ozone in the stratosphere. Since the ozone layer screens out a great deal of the ultraviolet radiation coming from the sun, its depletion might result in a rise in the number of cases of skin cancer and in the mutation rates of many organisms. Soil bacteria are known to convert an unknown but probably substantial amount of ammonia to nitrous oxide, which is, in turn, converted to nitric oxide in the upper atmosphere. This compound catalyzes ozone breakdown.

Although the issue is still controversial, some investigators think that synthetic nitrogen fertilizers are a major source of nitrogen oxides and thus of ozone breakdown. There is a chance that development of mutant strains of bacteria that continuously produce ammonia, even in the presence of high ammonia concentrations, might exacerbate the problem. The normal strains stop producing ammonia when the supply is adequate but the mutants do not and excess production might occur.

However, because of the high energy requirement of biological nitrogen fixation, Valentine thinks that the chances of overproduction of ammonia are slim. Valentine says that the mutant bacterial strain developed in his laboratory excretes ammonia into the incubation medium under laboratory conditions when it is supplied with sufficient glucose. But in the field, nitrogen fixation by these bacteria is likely to be limited by the scarcity of needed nutrients. In fact, this limitation is one of the major problems that must be solved before the altered bacteria can be used as a practical source of fixed nitrogen for crops.

Valentine also points out that nitrogen fixation by symbiotic bacteria, such as the rhizobia, which live in close symbiosis with plants, is much "cleaner" with respect to production of oxides of nitrogen than is the addition of large quantities of artificial nitrogen fertilizer to the fields. The bacteria produce the ammonia relatively slowly and it is used by the plant as it is formed. However, this may not be true for the free-living bacteria and blue-green algae.

Introduction of *nif* genes into bacteria that do not have them in the hope that they can be used to generate new symbiotic associations with plants is another strategy for genetic manipulation now being explored. In a letter to the participants at the Brookhaven symposium, A. H. Gibson, of the Division of Plant Industry of the Commonwealth Scientific and Industrial Research Organization of Australia, said: "The dangers of indiscriminate transfer of nitrogen-fixing genes to new material, especially to microorganisms that are pathogens or potential pathogens (using their infective capabilities), are very considerable." The danger to which he refers is the unplanned spread of nitrogen-fixing capabilities to additional plants, including weeds. Several investigators thought this would be unlikely because the known symbiotic relationships are highly evolved and intricate. For example, a particular rhizobial species usually infects only one kind of legume. But Gibson thinks that attention should be focused on altering the plants, which are easier to control than microorganisms, so that they gain the capacity to form symbiotic relationships with nitrogen-fixing bacteria.

Most investigators emphasize that although research has shed a great deal of light on the biology and biochemistry of nitrogen fixation during the last few years, the exploitation of this knowledge is still far in the future. Thus, at present, the benefits and hazards of genetic manipulation of nitrogen fixation are equally hypothetical.—J.L.M.

the second revolves around the question of whether the genes will be expressed and whether the products will actually function and produce ammonia.

Participants in the symposium described a number of potential means for introducing new genetic material into plant cells; all of them, however, are in a very early stage of development. One possibility is the use of viruses. Richard Meagher of the University of Georgia is currently studying cauliflower mosaic virus, one of the rare plant viruses that contains a DNA, rather than an RNA, genome. At present he is concerned mainly with such basic questions as the structure of the viral genome. These questions need to be answered before considering whether the virus can be altered to make it a nonpathogenic vehicle for introducing genes into plants. Meagher has used recombinant techniques to clone the viral DNA in *E. coli* to obtain sufficient quantities of the material for analysis.

At least one kind of bacterium, *Agrobacterium tumefaciens*, can transfer genes directly into plant cells by means of a plasmid. This bacterium causes crown gall tumors in many plant species. Eugene Nester of the University of Washington and J. Schell of the University of Ghent described work from their laboratories that indicates that plasmid transfer from the bacterium to the plant cells results from infection and causes transformation of normal cells to tumor cells.

Schell has incorporated the *nif* genes of *K. pneumoniae* into an *A. tumefaciens* plasmid but has not yet determined what happens when bacteria carrying the plasmid infect plant cells. Because of the pathogenic nature of viruses and the *A. tumefaciens* plasmid, investigators who wish to develop them as vehicles for introducing new genetic material into plants will have to exercise a great deal of caution.

Another way to intermix genes from different species is protoplast fusion. Protoplasts are formed by treating bacterial or plant cells with enzymes that digest cell walls and expose the cell membranes. When brought together under appropriate conditions, protoplasts of different species will fuse. Attempts to fuse plant protoplasts with those of nitrogen-fixing bacteria or blue-green algae are already under way. A slightly different tack would be fusion of legume protoplasts with those of nonlegumes in the hope of transferring the genes that make the legumes good hosts for nitrogen-fixing symbiotic bacteria.

After the protoplasts are fused, it is still necessary to grow whole plants from the hybrids in order to have a useful product. Plants of several species, including carrot, tobacco, petunia, and asparagus, have been grown from protoplasts, according to Oluf Gamborg of the Prairie Regional Laboratory in Saskatoon. Gamborg described some of the protoplast research from his laboratory and those of other investigators at the symposium. He points out that as yet no one has been able to grow any of the cereal plants from protoplasts, although they have been grown from cultured plant cells. Whole plants have been grown from hybrids formed by fusing protoplasts of two strains of petunia or tobacco, but this has not yet been achieved for hybrid protoplasts formed from two different species.

Will *Nif* Genes Function in Plants?

Introducing the *nif* genes into plant cells is only the first part of the problem; they have to produce functional products once they get there if they are to be of any value to the plant. Investigators studying the biochemistry of nitrogen fixation are skeptical that plant cells carrying the *nif* genes would ever synthesize any ammonia. On the third day of the symposium, one of these investigators, William Orme-Johnson of the University of Wisconsin, ticked off 11 reasons why such a result is unlikely. He says that the extreme sensitivity of the nitrogenase enzyme to oxygen is by itself sufficient reason to think that introduction of *nif* genes into plant cells will be of little value. The enzyme is irreversibly inactivated within seconds of exposure to oxygen, a necessary product of photosynthesis.

All of the naturally occurring systems that fix nitrogen have evolved mechanisms to protect their nitrogenase from oxygen. For example, the rhizobia, the nitrogen-fixing bacteria that infect the roots of legumes, are sequestered in nodules that keep out most of the oxygen. The nodules also contain leghemoglobin, a form of hemoglobin with extremely high affinity for oxygen. The leghemoglobin picks up what little oxygen that does enter the nodules and transfers it to the parts of the plant and bacterial cells that require it without exposing the nitrogenase to free oxygen. Some of the blue-green algae carry their nitrogen-fixing machinery in special heavy-walled cells called heterocysts that do not perform photosynthesis; thus, these algae physically separate the two processes.

The goal of the nitrogen-fixing plant may be unattainable but investigators think that it may be possible to manipulate the bacteria or blue-green algae that already have this capacity to increase their ammonia production. All the nitrogen-fixing organisms suffer from the same limitation: high ammonia concentrations, whether produced by the organism itself or by addition of exogenous ammonia, shut down further ammonia synthesis.

Investigators would like to develop strains that continue to fix nitrogen even in the presence of ammonia. Mutant strains of soil bacteria (not symbiotic) with this characteristic have already been identified in the laboratories of Brill and Valentine. It is too soon to tell whether such strains can make a significant contribution to nitrogen fixation in the field.

Investigators have been learning a great deal about the energy requirements of biological nitrogen fixation and how they are met. It turns out that a great deal of energy is used by the process; more than 20 moles of adenosine triphosphate (ATP), the cell's energy currency, are needed to fix 1 mole of nitrogen. In fact, this high requirement is another major biochemical obstacle in the path of investigators who would like to develop a nitrogen-fixing plant. Since the plant would have to supply the energy, a reduction of crop yields might ensue. Moreover, several investigators pointed out that yield reductions might result from any attempt to increase biological nitrogen fixation, even that carried out by the normal soil and symbiotic bacteria, unless there were means of either increasing photosynthesis or of decreasing the energy requirement of nitrogen fixation. Recent research provides indications that both of these manipulations might be possible.

A number of investigators have shown that nitrogen fixation by rhizobia depends on photosynthesis by the plant and that increasing photosynthesis increases both the amount of nitrogen fixed and crop yields. One way to do this, although it is not practical for widespread use, is increasing the carbon dioxide concentration in the air over legume crops, according to Ralph Hardy and U. D. Havelka of E. I. du Pont de Nemours & Company. Investigators in other laboratories have also shown that increasing the carbon dioxide concentration in air over nonlegumes, including wheat, rice, and barley, produces some enhancement of yields although not as great as that observed with legumes.

Hardy says that this treatment probably works by decreasing photorespiration by the plant. Photorespiration is a wasteful process in which some of the

carbon dioxide trapped during photosynthesis is diverted from the photosynthetic pathway into another series of reactions that produce no energy, although some amino acids needed by the plant are synthesized. Other investigators have shown that photorespiration is favored by a high ratio of atmospheric oxygen to carbon dioxide (like that in normal air) and that decreasing the ratio enhances photosynthesis.

Some plants, such as corn and sugarcane, are naturally twice as efficient as others, including legumes and cereals, in photosynthetic fixation of carbon dioxide. The more efficient plants have low rates of photorespiration and lose little of the carbon dioxide fixed during photosynthesis. Moreover, some strains of even the low-efficiency plants may have lower rates of photorespiration and higher photosynthetic efficiencies than others. Thus, Hardy and other investigators think that it may be possible to select strains of plants that are more effective photosynthesizers. This could help to provide more energy for nitrogen fixation and generally improve crop yields.

Biochemical studies also indicate that nitrogen fixation by rhizobia is not as efficient as it could be. Harold Evans and his colleagues at Oregon State University have found that 30 percent or more of the energy used by the nitrogenase of most rhizobial species is wasted because the enzyme produces hydrogen in addition to ammonia. The investigators estimate that every year the rhizobial species associated with the U.S. soybean crop produces a volume of hydrogen with energy equivalent to that of 300 billion cubic feet of natural gas. However, not all species of rhizobia waste energy by producing hydrogen. Evans says that the one associated with the cowpea loses little energy in this way. The host range of each rhizobial species is limited; one infects cowpeas, another soybeans, and so on. The cowpea is not economically important, but recently workers in Evans' laboratory have identified strains of the species that infect soybeans that produce little hydrogen. Evans says that it is important to provide farmers with similar varieties of rhizobia that infect economically important legumes such as soybeans, and are more efficient nitrogen-fixers than the strains that are currently used.

The nitrogen-fixing blue-green algae can provide their own photosynthetic energy for ammonia production. But even some of these may establish symbiotic relationships. One especially efficient alga, *Anabaena azollae*, lives symbiotically with a small aquatic fern of the *Azolla* genus. Each leaf of the fern contains a cavity that holds the algae.

Fig. 1. The fern *Azolla* forms a mat on the water as it grows in a rice paddy. [Source: Stephen Talley and D. William Rains, University of California at Davis]

The alga-fern system has been used for centuries to fertilize rice paddies in countries of Southeast Asia, including Vietnam. Investigators, including Stephen Talley and D. William Rains of the University of California at Davis, are now trying to find out how to manage the *Azolla* system to supply fixed nitrogen for rice grown in more advanced agricultural conditions. The high-yielding rice strains used in these situations require added fertilizer to produce well.

The investigators find that the growth of the fern-alga system in the paddy water must be carefully synchronized with the growth of the rice if *Azolla* is to work as a supplier of fixed nitrogen. For example, if the fern multiplies too fast, it may cover the surface of the paddy water, shade the rice seedlings, and prevent their growth (Fig. 1). However, it is desirable for the fern cover to shade weeds.

In some of their experimental plots, Talley and Rains plowed one crop of *Azolla* into the soil as a "green manure" before planting the rice. They later grew another fern crop in the paddy water. The yield of these plots was more than 200 percent better than that of controls. The yield of plots with *Azolla* in the paddy water but not plowed into the soil was almost 25 percent greater than that of unfertilized controls. The investigators think that the fern must die and decay in order to release fixed nitrogen into the paddy water and that transfer of the fertilizer from water to the plants is less efficient than if the fern decomposes in the soil.

Investigators at the International Rice Research Institute in the Philippines are also attempting to find the right cultural conditions for fertilizing rice with *Azolla* in tropical climates. In addition, Rains says that the *Azolla* system might be useful for fertilization of any irrigated crop and need not be limited to rice fields. Since *Azolla* fertilization of rice works in Vietnam, the investigators think it should be possible to find the right methods for applying *Azolla* in different circumstances and climates.

A significant trend in nitrogen fixation research is the growing body of evidence that the organisms that perform this function and the plants that benefit from it are more diverse than was once thought. Investigators have learned that a rhizobial species infects at least one nonlegume. In addition, bacteria not of the genus *Rhizobium* may form important symbiotic relationships. Alder trees, for example, have such a symbiotic bacterium living in root nodules. The bacterium is an efficient nitrogen-fixer and may make a significant contribution of fixed nitrogen to forests.

However, other newly discovered relationships between plants and nitrogen-fixing bacteria are not as close as the one with alder trees. Johanna Dobereiner of the Universidade Federal Rural do Rio de Janeiro has discovered that the nitrogen-fixing bacterium *Spirillum lipoferum* lives in and around the roots of tropical grasses and maize (*Science*, 1 August 1975). The discovery is important because maize is a very close relative of corn. Efforts are under way to determine whether inoculation of corn plants with the bacterium can increase yields by enhancing nitrogen fixation. So far the results have been inconclusive. But the work of Dobereiner and other investigators around the world indicates that the genetic base underlying nitrogen fixation is a good deal broader than it once appeared.

Although the investigators at the symposium are among the first to admit that realization of the goal of increased biological nitrogen fixation is still in the future, they think that studies of the genetics and biochemistry of nitrogen-fixing organisms and of plants are providing the information needed to achieve greater crop productivity. The techniques of genetic engineering as applied to nitrogen fixation may be in their infancy but other areas of investigation are much nearer to maturity.—JEAN L. MARX