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The Leeuwenhoek Lecture, 1992: Bacterial Evolution and the Nitrogen-Fixing Plant

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Phil. Trans. R. Soc. Lond. B 1992 **338**, 409-416
doi: 10.1098/rstb.1992.0158

The Leeuwenhoek Lecture, 1992
Bacterial evolution and the nitrogen-fixing plant

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SUMMARY

Biological nitrogen fixation is fundamental to the economy of the biosphere, yet it is restricted to a few dozen bacterial species. Why have plants not acquired it during evolution? No serious physiological or genetic obstacles seem to exist. Has a relatively late emergence, among genomically flexible prokaryotes, effectively precluded appropriate seletion pressure?

December 26 1676 was the birthday of Bacteriology. It was the day on which Antoni van Leeuwenhoek first saw what we now know to be bacteria, inhabiting water in which pepper had been steeped overnight (Schierbeek 1959; Hall 1989). It is rare for the date of a seminal discovery to be known so precisely, but van Leeuwenhoek was in the habit of including such details in his letters to the Royal Society. Some of the bacteria which he observed can be recognized from his drawings. Nitrogen-fixing bacteria were among them: at the beginning of this century one of these was identified by another great Dutch microbiologist, M. W. Beijerinck (see Schierbeek 1959, p. 77) as 'undoubtedly' *Azotobacter*, and other examples could hardly fail to have been present in his canal-water samples. But the features that are central to my present theme would have passed unremarked by van Leeuwenhoek; neither the concept of nitrogen fixation, nor that of biological evolution, was to arise for another century and a half.

I am concerned with the question of why there is no such thing as a nitrogen-fixing plant. Clover, peas and the like are sometimes described, casually, as able to fix nitrogen but, of course, they do not: they form symbiotic associations with nitrogen-fixing bacteria. The ability to fix nitrogen is exclusive to bacteria; indeed, it is restricted to some 50 of the many thousands of named species, and even when it is found within a species, it is often present in only a proportion of strains. As a genetic character it has a limited and haphazard distribution, yet it is a text-book truism that the few types of nitrogen fixer which exist have, until this century, sustained the nitrogen economy of the biosphere for some half a billion years, compensating for the loss of fixed nitrogen brought about by denitrifying bacteria. Why has the property not spread to higher organisms, notably to plants, which are the primary recipients of newly fixed nitrogen?

A clue to the answer lies in the remarkable genetic fluidity of bacteria. For several decades we have known that bacteria, collectively, display a variety of means of gene exchange. Reminders of the best-studied processes – conjugation, transformation and transduction – are given in table 1. They were originally discovered in a few bacterial groups – pneumococci, bacilli and coliforms – and were long regarded as rare. But in the last two decades it has become apparent that processes of this kind are the rule rather than the exception. The widespread incidence of conjugative gene transfer was first indicated by plasmid-mediated spread of antibiotic resistance in post-war medical and veterinary practice, and the contemporary recognition of the wide host ranges of promiscuous plasmids. Twenty years ago, the late Dr R. Hedges suggested, from a consideration of the ecology of drug resistance plasmids, that much of the bacterial genome might be a communal thing: that mobile plasmids represented a bank or reserve of genetic information within the bacterial world, on which a variety of genera might draw (Hedges 1972).

In the intervening years, much has been discovered to substantiate views of that kind. We have learned that self-transmissible plasmids can sometimes co-transfer other plasmids into the recipient organism (the mobilization phenomenon), and that almost any bacterial species can be rendered competent for transformation, at least using raw plasmid DNA, by stresses such as freeze-thawing or ion (e.g. calcium) imbalance. And new processes resembling transduction have been discovered, such as the enigmatic 'Gene Transfer Agent' of photosynthetic bacteria. Chater & Hopwood (1989) have recently summarized the many mechanisms of gene transfer and modification in bacteria. Equally significant, plasmids were once thought to be rare, but DNA electrophoresis has revealed that the reverse is true: they are very common, both in laboratory strains and in isolates from the natural environment. It is not unusual for two to six to be present in *Azotobacter chroococcum*, for example (Robson *et al.* 1984). Some plasmids are extremely large, approaching a quarter of the size of the chromosome, such as the megaplasmids of rhizo-

Phil. Trans. R. Soc. Lond. B (1992) **338,** 409–416
Printed in Great Britain

Table 1. *Gene transfer processes in bacteria*

Transduction Bacterial virus (bacteriophage) particles, while infecting and destroying cells, acquire some of the victim's DNA and, in due course, carry that DNA into a new host. Most of the new cells being attacked succumb to the virus infection, but a few resistant cells integrate the alien DNA into their own genomes, altering their genotypes.

Transformation Some bacteria at a certain stage of their growth cycle (when they are termed 'competent') can take up raw DNA and incorporate stretches into their own genomes. Stresses, such as treatment with calcium chloride or freeze-thawing, sometimes induce competence in bacteria that are not naturally transformable.

Conjugation Bacteria may conjugate when one of the cells possesses fertility genes, often extra-chromosomally on a plasmid, and the other does not. The two organisms come into physical contact and the fertile strain donates fertility genes and associated DNA to the recipient, which then becomes fertile itself.

bia (Dénarié *et al.* 1981); others, such as colicin plasmids, are small, but numerous within a single cell (Sheratt 1974). Most electrophoretically observed plasmids are cryptic, which means that the characters encoded by their DNA are not known (among the few exceptions are the Ti plasmids of agrobacteria and plasmids encoding host specificity and nitrogen fixation in rhizobia). But crypticity does not imply triviality: they represent a mass of potentially mobile packages of DNA distributed throughout the bacterial world.

At a subcellular level, bacteria have a remarkable capacity for gene rearrangement. Whole plasmids may integrate into the chromosome and later re-emerge bearing chromosomal DNA. Packages of DNA called transposons can move from one plasmid to another, and in and out of the chomosome; some carry fertility genes, rendering the recipient DNA capable of conjugation. Stretches of DNA called insertion sequences can move around bacterial chromosomes, cotransferring or silencing genes where they insert themselves.

Bacteria clearly have an immense capacity for gene rearrangement and gene exchange. The latter, with its consequent hybrid formation, operates not just within species as in the sexual hybridization of eukaryotes, but among species, genera and groupings of higher taxonomic rank. Intraspecific lateral transfer of chromosomal DNA seems probable in 'wild' *E. coli*, and transfer of a stretch of *trp* DNA to *Salmonella typhimurium* has been proposed (Milkman & Crawford 1983; Stoltzfus *et al.* 1988). Especially interesting in this context is the work of Spratt and his colleagues (see Spratt *et al.* 1991) demonstrating lateral interspecific DNA transfer in the evolution of streptococci and neisserias: stretches of DNA conferring penicillin resistance have been recruited by sensitive species from different, naturally resistant, species, presumptively by transformation. Recent ecological studies, stimulated by anxieties about the release of genetically engineered bacteria, suggest that gene transfer between species and genera takes place in nature. Therefore saltatory evolution – involving sudden and substantial changes of genotype – is likely to have been frequent among bacteria.

Here a note of caution is necessary. Some microbiologists, deeply impressed by such revelations of the flexibility of bacterial genomes, have advanced the view that the bacterial world is a super-organism: a single planetary entity, one to which ordinary concepts of evolution do not apply (Sonea & Paniset 1983). Such thinking has semantic rather than scientific overtones; I raise the matter to emphasize that, despite the genetic flexibility of bacteria as a whole, independent, autonomous bacterial species are the norm. This is not to deny that, in bacterial nomenclature, specific and generic names may have historical or functional rather than biological bases, but most of the strains and groups which microbiologists call species and genera behave as stable biological hierarchies and possess physiological and genetic equipment enabling them to sustain their specific integrity. Notably among these are DNAses, restriction enzymes and the *mut* gene products (Rayssiguier *et al.* 1989), all of which serve to reject alien DNA.

Additional to their capacity for gene exchange and rearrangement, and highly relevant to considerations of their evolution, is the remarkable capacity of bacteria for mutation. Although the actual mutation rate within bacterial DNA may be no greater than that in cells of animals and plants, their small genomes and huge numbers make mutation a significant part of their everyday biology. The point is illustrated by the elementary calculation shown in table 2, which shows the astronomical number of mutations that take place daily in mankind's commensal *E. coli*. Yet the number in table 2 represents but a minimum mutation rate, for a variety of reasons. First, it does not take into

Table 2. *Mutations in mankind's commensal* Escherichia coli

An average human discharges 200 g faeces a day. A typical *E. coli* count of human faeces is about 10^8 cells per gram, equivalent to an output of about 2×10^{10} *E. coli* per day, almost all freshly grown since the last defaecation.

There are now over 5×10^9 humans. The global growth rate of our intestinal *E. coli* is thus about 10^{20} cells per day.

The *E. coli* genome comprises about 4×10^6 base pairs of DNA. Gene lengths average about 10^3 base pairs. So *E. coli* has about 4×10^3 genes.

Spontaneous non-lethal mutations (e.g. to drug resistance, to a new nutritional requirement, etc.) occur in the genomes of multiplying *E. coli* at frequencies in the range 1 per 10^4 to 1 per 10^9 new progeny. For the sake of argument, say 1 in 10^7. Each one signifies an altered gene.

Therefore more than 10^{13} *E. coli* genes mutate daily inside humanity. Which means that, on average, every gene of the *E. coli* genome traversing mankind's intestines mutates at least 2.5×10^9 times daily.

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account the *E. coli* which inhabit non-human guts, which probably greatly outnumber ours. Nor does it take account of the secondary habitat of *E. coli*, which it occupies transiently after discharge from the mammalian gut: soil and water. Half of the world's population of *E. coli* occupies this niche (Savageau 1983), where it is gradually starved out by competing indigenous microflora. Recent work by Cairns *et al.* (1988) and Hall (1990, 1991) has established that a burst of mutations takes place in *E. coli* as the terminal stages of starvation are approached. Clearly the mutation rate, sometimes thought of as a sort of Planck's constant of bacterial genetics, can increase abruptly under stress. Cairns-Hall mutations will multiply the global total substantially, and some of the other stresses *E. coli* encounters outside the primary habitat are likely to be additionally mutagenic, too (see Walker 1984). Among these millions of mutations will be those, such as *mut*, concerned with rejecting alien DNA and sustaining species and genus barriers. One wonders how many of the global output of *E. coli* achieve, by lateral gene transfer, a genome enabling them to survive in their new habitat, albeit no longer recognizable as *E. coli*? That question might well become answerable as molecular taxonomy advances.

What is true of *E. coli* may not be true of the elephant, but it seems generally to be true of other bacteria. The calculation in table 2 refers to a fast-growing bacterium which is not especially good at DNA repair compared with, for example, a methanotroph or an azotobacter. But if the numbers are changed to refer to, say, soil arthrobacters, good at DNA repair, doubling only every week or so, their collective capacity for mutation in this planet's soils is still astronomical.

All this potential for gene exchange and mutation in no way challenges the role of natural selection in bacterial evolution, but it implies that evolutionary change, sometimes saltatory, can be expected, given appropriate selection pressure, in matters of days, weeks or months, rather than the tens of millenia observed among animals and plants. Moreover, seemingly improbable co-operative events, such as truly alien gene acquisition associated with specific mutations, move into the realms of plausibility. Evidence has been offered that one of the rhizobial glutamine synthetases was acquired from a plant source (Carlson & Chelm 1986).

I ought not to leave this discussion of the genetic flexibility of bacteria without addressing the question which then arises: are there any features of bacterial genomes which can be taken as definitive of species and genera? Conventional wisdom has it that the coding for ribosomal RNA, in particular the 16S-rRNA, are definitive. The reason, presumably, is that there is no obvious selection pressure which would favour exchanges of protein-synthesizing machinery; there is also the pragmatic reason that the analysis of 16S-rRNA from over 500 species of bacteria, and its cataloguing in hierarchies of relatedness, has provided microbiologists, for the first time, with a natural systematics (see Woese 1987), a tree of relationships which seems to have phylogenetic significance. That tree led to the recognition of the Archaebacteria as a third domain of living things additional to the Eubacteria and Eukaryotes (Archaea, Bacteria and Eucarya are more recent names for these domains (Woese *et al.* 1990)). It has contributed critically to evolutionary theory by substantiating the prokaryotic origin of organelles, and is still revolutionizing prokaryotic taxonomy. But I emphasize a point about which Woese (1987) was very clear: the tree reflects ancestral relationships but says little about absolute chronology. In itself, it does not say whether the bacterial world that we encounter today emerged a couple of billion years ago, a few million years ago, or in the last few millenia. Any of these three timescales would be consistent with present knowledge of the flexibility of bacterial genomes; their evolutionary chronology has to be set by reference to other events in the history of the biosphere.

Yet a tentative view can be reached from first principles. The flexibility of bacterial genomes is such that the distribution of genotypes in the bacterial world is rapidly and completely determined by the state of the biosphere. Species and genera will have been stable in stable ecosystems; they will become extinct and be replaced rapidly in changing ones. The mammalian gut has been stable for about 1.3×10^8 years, when *E. coli* and its 'cousin' *Salmonella* are thought to have diverged from a common ancestor (Ochman & Wilson 1987*a*). This is why *E. coli*, despite its flexible genome, is stable in the sense that isolates from all over the world, although showing a scatter of presumptively neutral mutations, cluster within three clonal types (see Milkman & Crawford 1983; Duyhuizen & Green 1986; Young 1989). To generalize, bacteria whose primary habitat is a commensal or symbiotic association with a plant or an animal can be expected to have coevolved in a linear manner with their hosts, in directions substantially imposed by their hosts. A substantial proportion of the world's prokaryotes live in such relationships with members of the four eukaryotic Kingdoms, and their evolutionary chronologies can be expected to have followed those of their hosts. The chronology chosen by Ochman & Wilson (1987*b*) rests largely on such associations and assumes that they were formed early in the history of the hosts.

The evolutionary chronology of pathogens, too, will be host determined, although their relationships with their reservoir, and effects on their victims, lead to more complex selection pressures.

When one considers terrestrial and aquatic ecosystems, the autochthonous bacteria of soil, sea, lakes and so on, a different picture emerges. Present-day speculations on the early geochemical history of the earth seem to agree that most of the geochemical evolution of its surface took place before the multicellular eukaryotes appeared, when the dominant flora comprised bacteria of bizarre habits and trophic types, corresponding to today's thermoacidophiles, sulphate-reducing bacteria, methanogens, bacteroides and, later but by no means least, photosynthetic anaerobes. It was during the first 2+ billion years of life on this planet, when pristine prokaryotes were in a sense

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creating, and certainly transforming, the biosphere, that what one could call an autonomous evolution of bacteria took place, as such microbes came into being and colonized new zones of the geosphere, acquiring the extraordinary chemical versatility familiar to microbiologists today. In return, so to speak, the changing geosphere would have conditioned the directions of bacterial evolution.

The oxygenic cyanobacteria duly emerged, diverging from non-oxygenic photosynthetic bacteria. Oxygen had long been a transient component of the atmosphere, despite the atmosphere's overall reducing or neutral character, but oxygenic photosynthesis ultimately led to persistently oxic conditions, probably later than a billion years ago, although the timing is much debated. This was a drastic transformation of the geosphere; it would have eliminated many anaerobes and brought about a population explosion among the bacteria that learned to respire aerobically, and it permitted the emergence of aerobic eukaryotes. Thereafter, the geosphere became gradually more oxic, albeit in a fluctuating manner. The question arises, was there sufficient linear continuity of chemical microenvironments for the original trophic types of bacterial species to persist and evolve in a linear manner? Only if microenvironments resembling today's, in different relative proportions, of course, not only existed but persisted despite repeated physical stresses, such as glaciation, vulcanism, drought and inundation, sustaining reservoirs of trophic types of bacteria to recolonize devastated areas. Is this likely? One also has to take seriously the possibility that, throughout geological time, terrestrial and aquatic bacteria were subject to repeated extinctions and replacements, perhaps even reinventions of trophic abilities, leading to a reticulate pattern of evolution, with many discontinuities.

However, since half a billion years ago, when the first eukaryotic fossils began to form, the geosphere has been chemically relatively stable, and most evolutionary change among bacteria as a group has been conditioned by the evolutionary development of higher organisms; changes in the chemistry of the geosphere, mainly brought about by bacteria, have had a minor influence.

It is a sobering thought that many of the really new microbial ecosystems that one can point to in the biosphere are man-made, mostly during this century. How many new prokaryotes have we unwittingly created? This is another question which I doubt that anyone can answer at present, but it is pertinent to mention that 60 years ago another distinguished Dutch microbiologist in the lineage of van Leeuwenhoek, A. J. Kluyver, pointed out that laboratory cultures of bacteria are necessarily laboratory artefacts (Kluyver & Baars 1932), a truth which microbiologists have widely disregarded ever since, perhaps because the illustrative example chosen proved, a quarter of a century later, to be mistaken.

The temporal remoteness of the major evolutionary changes among bacteria, taken with their genetic flexibility, means that there is very little that one can say today about the relationships of primitive species and genera to those observed today. Traces have been detected in pre-Cambrian cherts which recall today's flexibacteria and cyanobacteria, but morphology is so unreliable a prokaryotic character that one simply has to suspend judgement. In effect, one cannot speak realistically of the evolutionary emergence of early bacterial species and genera, only of recent ones. But one can say something of the emergence of specifically prokaryotic functions, such as methanogenesis, sulphate reduction, oxygenic photosynthesis, aerobic respiration, and, to rejoin my theme, nitrogen fixation.

The evolution of nitrogen fixation has been discussed often in recent decades (see Postgate 1982 and, more recently, Hennecke *et al.* 1985; Sprent & Raven 1985; Postgate & Eady 1988; Normand & Bousquet 1989; Young 1992). There are two principal views on the topic: the classical view, that it is an ancient property which has been widely lost during the course of evolution, and the dissident view, which suggests that it originated late among the trophic properties of prokaryotes, after oxygenic photosynthesis had become widespread, and spread by lateral gene transfer. I shall summarize and update the contributory arguments very briefly.

The restriction of nitrogen fixation to bacteria

The classical view, accepting present-day bacteria as directly descended from comparable pre-Cambrian ancestors, regards today's nitrogen-fixing microflora as the residual repositories of a once widespread metabolic trait; the dissident view regards bacteria as the only creatures with a genome flexible enough to have allowed the process to originate.

Its association with 'ancient' metabolic properties

A few decades ago most of the known nitrogen fixers were anaerobes, organisms held to be primitive and possessing enzymes such as hydrogenase, ferredoxins and the pyruvic phosphoroclastic system, also deemed to be primitive. Never a strong point, its force has waned with the discovery of nitrogen fixation in relatively highly evolved bacteria, and the elucidation of positive roles in nitrogen fixation for hydrogenase, ferre- or flavodoxins, and pyruvate oxido-reductase.

The haphazard distribution of nitrogen fixation among bacteria

The classical view is that the property was lost in a random manner; the dissident view is that it emerged late and spread by lateral gene transfer as appropriate selection pressure directed.

The matter of lateral transfer of nitrogen fixation (*nif*) genes needs amplification. Nitrogen fixation requires the regulated operation of some 20 *nif* genes (Kennedy 1989; Elmerich 1991; Robson 1991). Their transfer across species and genus barriers in bacteria has been a routine laboratory operation for over 20 years (see Postgate *et al.* 1987). Conjugative plasmids have generally been used, and instances of chromosomal integration of transferred *nif* have been few. Lateral transfer of so substantial a package of DNA in evolution would need either a contiguous gene cluster such as is found in *Klebsiella*, for a transformational

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event, or a natural transferable plasmid which included all essential *nif* genes among its gene complement in the donor. Natural *nif* plasmids have been found in several species of bacteria, and lateral *nif* transfer in nature was proposed, without firm evidence, by several authorities (see Postgate & Eady 1988). Evidence bearing upon lateral transfer can be deduced from comparisons of the base sequences of particular *nif* genes in various bacteria: a pattern of relationships congruent with the rRNA phylogeny would be inconsistent with lateral gene transfer during the period since the species studied came into being (for example, as reported for eight species by Hennecke *et al.* 1985). The cogency of such evidence has been somewhat diminished by the discovery that the particular *nif* gene most studied, *nifH* (which codes for dinitrogenase reductase; see next paragraph), can sometimes be multiple, and divergent, within a single strain of nitrogen-fixing bacteria. However, a survey of 22 *nifH* sequences from 16 species (Normand & Bousquet 1989) revealed a high degree of congruence with appropriate rRNA dendrograms among most of the species studied, but also two major discrepancies, suggesting two lateral *nif* transfer events during the evolution of those particular species (but see Young (1992) for disagreement).

Its oxygen sensitivity

Nitrogenase is a functional complex of two proteins. Dinitrogenase (which is primarily concerned with substrate binding) is generally a molybdo-ferro-protein, and dinitrogenase reductase (concerned with ATP-activated electron donation) is a ferro-protein (see Miller 1991). In the mid 1980s, alternative nitrogenases were discovered, so far mainly among azotobacters, coded for by different structural genes which are none the less present in the same genome as genes specifying the Mo enzyme. There are two classes of alternative nitrogenase: in one V replaces Mo in the dinitrogenase; in the other it is probably replaced by Fe; both have their own ferro-protein (see Pau 1991). All nitrogenase proteins, conventional and alternative, are rapidly and irreversibly destroyed by exposure to oxygen. This dramatic oxygen sensitivity can be considered, in the classical view, to be a primitive character which reflects the anoxic biosphere of the enzyme's early emergence; on the dissident view, it merely suggests that the property originated in an anaerobe, perhaps out of chemical necessity, at any time.

The highly conserved structures of nitrogenase proteins and DNA

The two component metallo-proteins of nitrogenase show such pronounced structural similarities between species and genera that, *in vitro*, the dinitrogenase reductase from one organism will generally form a functional enzyme with complementary dinitrogenase extracted from a taxonomically very different organism. The corresponding DNA sequences reflect these similarities, which extend to the alternative nitrogenases. Sometimes adduced as evidence for a relatively short period of evolution, compatible with lateral gene transfer, these high levels of conservation could equally reflect rigid functional constraints on the two proteins' structures.

Nitrogen fixation fossils

The traces resembling cyanobacteria in pre-Cambrian cherts, dating from some 2.3 billion years ago, include bodies which look like heterocysts. This observation has been held to be direct evidence that nitrogen fixation is truly ancient, because heterocysts are today the loci of nitrogen fixation in filamentous cyanobacteria, acting as sophisticated compartments for the protection of nitrogenase from oxygen damage (see Gallon & Chaplin 1987). However, assuming that the traces are indeed of heterocysts and not artefacts (see Zhang Yun 1984), it seems unlikely that they were then the loci of nitrogen fixation, because the ambient pO_2 was still vanishingly low and they would not have been necessary. A function as stress-resistant bodies resembling today's akinetes seems more plausible.

Nif gene arrangement

Postgate & Eady (1988) pointed out that the *nif* genes of oxygen-consuming nitrogen fixers such as *Azotobacter* and *Rhizobium* are dispersed about the chromosome, unlike the contiguous *nif* cluster of *Klebsiella pneumoniae*, which can only fix nitrogen in the absence of dissolved oxygen, although it grows readily in air when supplied with fixed nitrogen. The rhizobia are micro-aerobic nitrogen fixers which form symbioses with legumes, so they are unlikely to be more than 200 million years old, when the first legumes appeared (Norris 1956); *Azotobacter* is an obligate aerobe with highly developed methods of screening its nitrogenase from oxygen (respiratory and 'conformational' protection (see Postgate 1982); note, however, that its alternative nitrogenases are not conformationally protected). Both genera may thus be regarded as highly evolved among nitrogen fixers, suggesting that a contiguous structure was ancestral to the more dispersed arrangement.

The selection pressure

This is a central question, but the least tractable. The classical view did not deal satisfactorily with the point that plentiful fixed nitrogen as ammonia was long believed to be present among the methane, hydrogen, water vapour and CO_2, which were then thought to have made up this planet's early, highly reducing atmosphere. Plentiful fixed nitrogen is wholly incompatible with natural selection for nitrogen fixation, and therefore inconsistent with so ancient an origin. The discovery that nitrogenase reduces small molecules such as cyanides and acetylene lead to Silver & Postgate's (1973) proposal that detoxification might have been its pristine function, an idea which periodically resurfaces (see Postgate & Eady 1988; Normand & Bousquet 1989). The paradox also provoked the dissident view (Postgate 1974). Today the basis of the argument has shifted: ammonia, like methane and hydrogen, is no longer widely believed to have been abundant well into the pre-Cambrian era (Schopf 1983; Mason 1991). Nevertheless, the problem remains in principle because, even in the more neutral

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yet anoxic atmospheres now postulated, lightning, light (visible and UV), and locally intense heating, acting upon N_2, CO_2, H_2O and nascent O, would generate fixed N as hydroxylamine, nitrite, nitrate and organic N.

When did the biosphere become sufficiently limited in non-biological fixed nitrogen for sustained selection pressure to generate so complex a process as biological nitrogen fixation? There appears to be no clear geochemical answer to this question. Given that even today spontaneous chemical processes acount for some 15% of the global annual input of newly fixed N (Svensson & Soderlund 1976), I am compelled to the view that it post-dated the spread of oxygenic photosynthesis, with its consequential population explosion of aerobes, probably after 1 billion years ago. It would also have post-dated the emergence of other types of bacterial assimilatory nitrogen metabolism, such as nitrate, nitrite and hydroxylamine reduction.

Little new can be said about the nature of the immediate ancestors, genetic or biochemical, of nitrogenase and its processing and ancillary enzymes. Some of the 20 *nif* genes needed by today's *K. pneumoniae*, for example those coding for enzymes concerned with uptake of metals such as Fe and Mo or V, those coding for electron donors such as flavodoxins, or for pyruvate mobilization, probably existed already in many anaerobes, but more than half of the 20, those concerned with forming and processing nitrogenase itself, are likely to have been developed specifically.

A new problem has arisen with the discovery of the alternative nitrogenases and the consequent further question of their ancestral relationships (Robson 1991). Postgate & Eady (1988) suggested that the pristine nitrogenase might have had an optional prosthetic metal; they also deduced that the Mo class was ancestral to the V and Fe classes, a scenario favoured by Kennedy & Dean (1992). Alternative nitrogenases are widespread among azotobacters and crop up only rarely among other prokaryotes, being found in substantially unrelated genera such as *Anabena*, *Clostridium* and the archaebacterium *Methanosarcina* (Fallik *et al.* 1991). The *nifH* gene of the Fe nitrogenase of *Azotobacter vinelandii* (now correctly termed *anfH*) is much more closely related to *nifH* genes from *Methanosarcina* and *Clostridium* than it is to the two other *nifH* genes in *A. vinelandii*'s own genome (Normand & Bousquet 1989). I am tempted to imagine that the alternative nitrogenases originated recently in azotobacters (whose multigenomic character (Robson *et al.* 1984) would have facilitated the extensive mutational changes necessary), probably as an evolutionary adaptation to nitrogen fixation at low temperatures (Miller & Eady 1989), and that the first to arise, *anf* coding for the Fe-nitrogenase system, moved by lateral transfer into hosts able either to avoid oxygen or to exclude it efficiently.

I now return to the question of plants. An age for nitrogen fixation of 0.5–1 billion years may be young in terms of prokaryotic evolution, but it remains ancient in terms of the evolution of plants and animals. Why did plants not adopt the process, during their half a billion years of existence? Several reasons have been proposed, most concerned with the rather special physiological pre-requisites of nitrogenase function. Again I shall summarize and discuss the arguments briefly.

ATP requirement

Nitrogenase consumes 8 molecules of ATP for every $\frac{1}{2}N_2$ molecule reduced to NH_3. This ATP requirement is unexpected in thermodynamic terms. It represents something of a metabolic burden to an organism, compared with assimilating ammonia, especially as another 6 ATP molecules are deflected from general metabolism to generate reductant, making 14 ATP per NH_3 in all. However, plants normally assimilate their N from nitrate, for which purpose 12 molecules of ATP are deflected from general metabolism to provide one molecule of NH_3. Thus nitrogen fixation is only marginally more demanding a process than nitrate reduction in terms of energy consumption. Ancillary processes such as ammonia assimilation, hydrogen recycling, relative amounts of enzyme needed and so on affect the energy balances of the two processes in different directions (see Postgate 1982), but in terms of energetics, nitrogen fixation should present no evolutionary obstacle to plants.

Oxygen sensitivity

The notorious oxygen sensitivity of nitrogenase proteins has clearly been a serious problem to nitrogen-fixing bacteria, influencing their evolution and determining much of the present day physiology of nitrogen fixation, as any text book on the subject makes clear. However, bacteria solved the problem in a variety of ways, ranging from respiratory and conformational protection of nitrogenase in *Azotobacter* to the sequestering of nitrogenase into low-oxygen compartments, as in root nodules. And cyanobacteria have reconciled nitrogen fixation with oxygenic photosynthesis in several ways, including the well-known heterocyst (see Gallon & Chaplin 1987). Plants could have done something of the sort, for localized anaerobic compartments exist within plant cells. Mitochondria, for example, with their low redox potential, ATP supply and near-prokaryotic genetic apparatus, ought to be especially suitable recipients of bacterial *nif*. Physiologically, oxygen sensitivity ought not to have presented a serious obstacle to plants.

Repression by ammonium

The ammonium ion, which represses nitrogenase synthesis and function, is a normal component of eukaryotic cytoplasmic fluid. Even if its concentration approached repressive levels, it ought not to have presented a problem: some cyanobacteria, some rhizobia and several types of Nif^- mutant escape ammonium repression.

Other prerequisites

Other physiological obstacles can be envisaged, such as the need to process Fe and Mo or V, the need to dispose of H_2 (a functional by-product), and the need to make a lot of enzyme because of nitrogenase's slow

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turnover. None presents serious difficulty to bacteria; none would seem to be an important obstacle to plants, given appropriate selection pressure. Equally, there seems to be no mechanistic obstacle to the expression of *nif* in plants, as a recent report by Dowson-Day *et al.* (1991) suggests: in subcellular preparations of plants *in vitro*, they demonstrated expression of cloned *nifH* and *nifM* from *K. pneumoniae*, as well as import of their gene products into chloroplasts.

In summary, present-day understanding of both the physiology and the genetics of nitrogen fixation does not imply any serious obstacle to the evolution of an autonomous nitrogen-fixing plant (cf. Merrick & Dixon 1984). If I am right in my view of the comparative youth of nitrogen fixation, it could well have arrived on the evolutionary scene too late for plants to acquire the property by direct linear inheritance from prokaryotic ancestors. They would certainly have had difficulty in reinventing the process, if only because of the multiplicity of *nif* genes. But acquisition of *nif* by lateral transfer from bacteria into an organelle genome – into that of the mitochondrion or a modified chloroplast – seems feasible (Merrick & Dixon 1984). And it remains surprising that no plant followed what seems to be the easiest path to independence of bacteria: to exploit bacterial solutions to both the genetic and physiological problems of nitrogen fixation by way of a 'diazoplast', a new organelle analogous to a chloroplast, acquired in a like manner by accretion of an endosymbiotic prokaryote into the plant's genome. The genetic obstacles to the emergence of autonomous nitrogen-fixing plants seem, like the physiological obstacles, to be minor. So why has none appeared?

It must be a matter of inadequate selection pressure. Ecologists know that, in all the climatically benign areas of this planet's land mass, climax vegetation is not normally N-limited: that nitrogen-fixing bacteria provide plants with an adequate trickle of fixed nitrogen, and other parameters (P, K, S, H_2O etc.) limit biological productivity. The situation changes in nature only as a result of gross disturbances, such as fire, glaciation, drought, inundation, vulcanism: disturbances which lead to abrupt recycling of other elements. Then N becomes limiting, and nitrogen-fixing bacteria and their plant symbioses are the pioneers of recolonization. Such disturbances have undoubtedly been frequent during the half billion or so years since plants appeared, but the flexibility of bacterial genomes is such that bacteria could adjust much more rapidly than plants to the prerequisites of recolonization, either by exploiting their existing nitrogen-fixing capacity or by acquiring it by lateral gene transfer from bacteria less able in other ways to recolonize. So nitrogen fixation has been, and remains, an evolutionary option for plants, but, as Sprent *et al.* (1987) pointed out, with their high C:N ratio, plants can make do with rather little N. This feature, together with the rapidity with which bacteria could exploit suitably N-limited niches as they arose, has effectively precluded that option for the duration of plant's evolution.

There is a corollary for today. Since the early 1900s, large areas of this planet's climax vegetation have been subject to catastrophic disturbance as a result of mankind's agriculture and forestry. This ought rapidly to have lead to N-limitation, but the widespread selection pressure in favour of extended nitrogen fixation which would once have been the consequence has been neutralized by substantial applications of N-fertilizer, manufactured from the atmosphere by the Haber process. Today Haber nitrogen accounts for about a quarter of the annual global input of newly fixed N (Svensson & Soderlund 1976): anthropogenic nitrogen fixation is supplementing the bacterial process. Otherwise protracted and widespread selection pressure might already have led to a nitrogen-fixing plant, a thought which may give encouragement to scientists, such as my erstwhile colleagues, who seek to create such a plant by genetic manipulation. They are only giving evolution a push in a direction in which it is already poised to go.

I thank Dr R. A. Dixon, Professor J. Maynard Smith, F.R.S., and my wife, who kindly read and commented helpfully on drafts of this article.

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Lecture delivered 11 June 1992

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