Molecular Genetics of
Symbiotic Nitrogen Fixation
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Although most organisms, including all eucaryotes, cannot assimilate atmospheric dinitrogen (N₂), a group of evolutionarily divergent procaryotes are able to reduce N₂ directly to ammonia (nitrogen fixation) under a variety of physiological conditions. The molecular genetics of nitrogen fixation has been studied primarily in Klebsiella pneumoniae because of its close taxonomic relation to Escherichia coli. K. pneumoniae contains a cluster of 17 contiguous nif genes, which are all transcribed in the same direction and which are divided into six or eight operons. Three of these genes (nifK, nifD and nifH) encode nitrogenase, an enzyme complex composed of two characteristic components: a molybdenum–iron–containing protein, which reduces substrate, and an iron-containing protein, which transfers electrons to the molybdenum–iron–containing protein. The remaining nif gene products either synthesize an nif-specific iron–molybdenum cofactor or carry out regulatory, electron transport, protein maturation or unknown functions. The physical location of each nif gene has been mapped to within 100 bp with transposable genetic elements as mutagens (Riedel et al., PNAS 76, 2666–2670, 1979), and the regulatory circuitry controlling nif gene expression in K. pneumoniae has been deciphered (for a recent review, see Roberts and Brill, Ann. Rev. Microbiol. 35, 207–235, 1981).

In contrast with K. pneumoniae, which fixes nitrogen in a free-living form, bacteria of the genus Rhizobium normally fix nitrogen only in symbiotic association with plants of the family Leguminosae. Symbiotic nitrogen fixation occurs in root nodules—complex, highly differentiated structures formed by the interaction of bacteria and plant. Rhizobia usually gain entry into the legume root by penetrating root hairs. In response to bacterial invasion, the plant produces a cellulose tube called an infection thread, which surrounds the bacteria, penetrates root cells and ramifies. The bacteria within the thread proliferate, and cells in the inner root cortex, which are normally terminally differentiated, begin to divide, forming a meristematic region that produces a nodule ranging in size from 1 to 10 mm in different legume species. Finally, various branches of the infection thread release their bacteria intracellularly, and the bacteria differentiate into a new form called a bacteroid and derepress nitrogen-fixation genes. The host cells also show changes in protein composition (Legocki and Verma, Cell 20, 153–163, 1980), most prominently the synthesis of large quantities of an oxygen-binding protein, leghemoglobin, which protects the oxygen-sensitive nitrogenase within the bacteroids from oxygen inactivation.

To develop an overall experimental strategy designed to identify bacterial functions necessary for nodule formation, we must consider that at least some of the developmental steps involve the interaction of host plant and bacterial endosymbiont. This interactive model of nodule development predicts that we should be able to obtain Rhizobium mutants that would cause a spectrum of mutant root-nodule phenotypes, ranging from no nodule at all (Nod⁻) to nodules that appear morphologically normal but that fail to fix nitrogen (Fix⁻). Because Rhizobium strains can be grown and genetically manipulated in isolation from their hosts, several investigators have sought to isolate and characterize mutants that have no effect on free-living cells but that block specific stages in the nodulation process.

The isolation of symbiotic mutants of Rhizobium is hindered by the fact that symbiotic genes are not normally expressed in the free-living bacteria. To solve this problem, investigators have used transposon Tn5 to generate symbiotic mutants because approximately 0.1% of a randomly mutagenized population of cells contain Tn5 insertions in symbiotic genes (Beringer et al., Nature 276, 633–634, 1978; Buchanan-Wollaston et al., MGG 178, 185–190, 1980; Rolfe et al., Plant Sci. Lett. 19, 277–284, 1980; Meede et al., J. Bacteriol. 149, 114–122, 1982). Moreover, a transposon insertion in a symbiotic gene "marks" the symbiotic gene both genetically and physically so that it can be mapped genetically or cloned.

Although we can identify most symbiotic genes only by mutagenizing Rhizobium strains and then screening for symbiotic defects on plants, a much simpler procedure has been used to locate and study the genes that code for nitrogenase. Because the amino acid sequence of nitrogenase has been very highly conserved in evolution, it is possible to identify and clone the genes encoding this enzyme from other nitrogen-fixing species with cloned K. pneumoniae nif genes as hybridization probes. In this manner, nitrogenase structural genes from R. meliloti (Ruvkun and Ausubel, PNAS 77, 191–195, 1980), R. japonicum (Hennecke, Nature 297, 354–355, 1981) and Anaabaena strain 7120 (Mazur et al., PNAS 77, 186–190, 1980) have been cloned. As is the case in K. pneumoniae, nifH (iron-containing protein) and nifD (α subunit of molybdenum–iron–containing protein) are closely linked in all species tested, and in R. meliloti, nifHDK are arranged in the same transcriptional order as in K. pneumoniae (Ruvkun et al., Cell 29, in press).

To perform fine-structure genetic analysis on symbiotic genes and to prove that putative R. meliloti nif
genes cloned on the basis of DNA homology are essential for symbiotic nitrogen fixation, Ruvkun and Ausubel (Nature 289, 85–88, 1981) designed a strategy for replacing wild-type nif genes in the R. mellioti genome with homologous cloned sequences carrying Tn5 insertions. R. mellioti fragments contained on recombinant plasmids were mutagenized with Tn5 in E. coli, recloned into the low-copy-number mob + tra + broad-host-range vector pRK290 (Ditta et al., PNAS 77, 7347–7351, 1980) and mobilized into a wild-type (Fix' Nod') R. mellioti strain. Conjugation of a second R-group plasmid, incompatible with pRK290, into the R. mellioti strains containing the pRK290–Rmn rif:Tn5 plasmids allowed selection for those rare cells (about 0.1%) in which a double-homologous recombination event had occurred between the pRK290–Rmn rif:Tn5 plasmid and the R. mellioti genome, thereby transferring the Tn5 insertion from the plasmid to the genome.

A total of 31 R. mellioti strains containing Tn5 insertions in a 14 kb region of the R. mellioti genome that surrounds the nifHDK genes have been analyzed by Ruvkun et al. (op. cit.). The region examined contains at least two clusters of syngenic genes (approximately 6.3 kb and 5.0 kb). Using pRK290 derivatives, which contain selected Tn5 insertions in the 14 kb region, in genetic complementation studies with genomic Tn5 insertions, Ruvkun and coworkers deduced that the 6.3 kb cluster is a single transcription unit that contains the R. mellioti nifH gene at the 5' end.

One of the most interesting features of symbiotic nitrogen fixation is the clustering of syngenic genes, including nif genes, on large (100–500 kb) indigenous plasmids. A plasmid location for syngenic genes was first discovered by Johnston et al. (Nature 276, 634–636, 1978), who demonstrated that the host-range specificity of R. leguminosarum (peas) could be transferred to R. trifolii (clover) or to R. phaseoli (beans) by conjugal transfer of an H. leguminosarum plasmid carrying the kanamycin resistance gene of Tn5. In fact, Johnston and his colleagues have convincingly demonstrated that the chromosomes of these three Rhizobium species are highly homologous, and that host-range specificity of each species is determined by the plasmid.

Direct evidence that Rhizobium nif genes are plasmid-borne has been obtained by hybridization of cloned nitrogenase genes to purified plasmid DNA (Nutt et al., Nature 282, 533–535, 1979; Ruvkun and Ausubel, PNAS 77, 191–195, 1980; Prakash et al., J. Bacteriol. 145, 1129–1136, 1981). In several species, including R. leguminosarum and R. trifolii, the plasmids carrying nif genes are <200 kb and can be preparatively isolated. In R. mellioti, however, the plasmid containing the nif genes is very large (>800 kb) and can only be fractionated from the chromosome by an analytical "in-gel" lysis technique. The fractionated plasmid DNA can be transferred to nitrocellulose and hybridized with an nif probe. With this technique, it is possible to show that all R. mellioti strains contain a very large plasmid ("megaplasmid") that carries the nif genes (Banfalvi et al., MGG 184, 318–325, 1981; Rosenberg et al., MGG 184, 326–333, 1981; Buikema et al., J. Mol. Appl. Genet., in press).

The observation that both nif genes and genes involved in host-range specificity are located on plasmids suggested that many syngenic genes may be clustered on plasmids and coordinately regulated. To determine whether nif genes and other syngenic genes are closely linked on the R. mellioti megaplasmid, Long and Ausubel (Nature, in press) and Buikema et al. (op. cit.) used cosmid clones extending approximately 50 kb on each side of the R. mellioti nif genes to show that two mutations that result in a Nod" phenotype are located on an 8.7 kb Eco RI fragment approximately 20 kb away from the nif genes. Other investigators have also recently obtained evidence for the close linkage of nif and nodulation genes in R. mellioti, using spontaneous-deletion derivatives of the megaplasmid (Banfalvi et al., op. cit.; Rosenberg et al., op. cit.) that simultaneously cause a Nod" phenotype and loss of nif DNA hybridization.

Finally, it is important to consider whether it is only coincidental that syngenic genes in Rhizobium and oncogenic genes in Agrobacterium tumefaciens are both located on large crosshybridizing plasmids (Prakash et al., J. Bacteriol. 149, 1129–1134, 1982). Agrobacterium tumefaciens, which is taxonomically very similar to R. mellioti, causes the formation of crown gall tumors by transferring a 23 kb segment of DNA (T DNA), located on a large indigenous plasmid (Ti plasmid) to plant cells (for a recent review, see van Montagu and Schell, Curr. Topics Microbiol. Immunol. 96, 237–254, 1981). In the plant tumor cell, the T DNA becomes integrated into the chromosome, and appears to cause the transformation to a tumor phenotype by repressing the normal developmental signals involved in plant-cell differentiation. The remarkable similarity between Rhizobium and Agrobacterium has been convincingly demonstrated by Hooykas et al. (Nature 297, 351–353, 1981), who conjugated the 160 kb R. trifolii plasmid into an Agrobacterium tumefaciens strain (cured of its Ti plasmid), and demonstrated that the Agrobacterium tumefaciens strain containing the Rhizobium plasmid formed rudimentary nodules on clover, although the nodules were Fix".

There is no evidence at this time that Rhizobium plasmid DNA is transferred to plant root cells, where it could play a role in the differentiation of root cells into syngenic nodule cells. Obtaining experimental evidence for DNA transfer in the nodule will be difficult, because the nodule, in contrast with a crown gall tumor, is packed with bacterial cells. Nevertheless, the analogy between Agrobacterium and Rhizobium is a compelling one, and deserves careful examination in the future.