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Rhizobium phaseoli: A molecular genetics view

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Abstract

We have used molecular genetics techniques to analyze the structural and functional organization of genetic information of *Rhizobium phaseoli*, the symbiont of the common bean plant *Phaseolus vulgaris*. As in other *Rhizobium* species, the genome consists of the chromosome and plasmids of high molecular weight. Symbiotic determinants, nitrogen fixation genes as well as nodulation genes, are localized on a single replicon, the symbiotic (*sym*) plasmid. The *sym* plasmid of different *R. phaseoli* strains was transferred to an *Agrobacterium tumefaciens* strain cured of its native plasmids. In all cases, Agrobacterium transconjugants able to nodulate bean plants were obtained. Some of the transconjugants had the capacity to elicit an effective symbiosis. The genome of *R. phaseoli* is complex, containing a large amount of reiterated DNA sequences. In most *R. phaseoli* strains one of such reiterated DNA families corresponds to the nitrogenase structural genes (*nif* genes). A functional analysis of these genes suggested that the presence of reiterated *nif* genes is related to the capacity of fixing atmospheric nitrogen in the symbiotic state. The presence of several repeated sequences in the genome might provide sites for recombination, resulting in genomic rearrangements. By analyzing direct descendants of a single cell in the laboratory, evidence of frequent genomic rearrangements in *R. phaseoli* was found. We propose that genomic rearrangements constitute the molecular basis of the frequent variability and loss of symbiotic properties in different Rhizobium strains.

Introduction

Bacteria of the genus Rhizobium have the capacity to establish a symbiosis with the roots of legumes. The microsymbiont reduces atmospheric nitrogen to ammonia, which in turn is assimilated by the plant host. In addition to the actual scientific interest of the plant-microbe interactions that comprises the essence of symbiosis, the process of symbiotic fixation itself has great potentiality for application in agriculture.

Recent advances in molecular genetics have greatly contributed to our understanding of Rhizobium-legume interactions. Several symbiotic genes have been identified, isolated and characterized and their expression under different conditions has been studied. Among others, the early nodulation genes (*nod*), host specificity genes (*hsn*), and nitrogen fixation (*nif*) genes have been analyzed. In the near future a comprehensive scheme of bacterial genes that participate in the symbiotic process will certainly emerge. Moreover, laboratory constructions will result in bacteria harboring recombinant genomes, with potentially improved symbiotic capabilities. On the other hand, few studies have focussed on the general characteristics of the Rhizobium genome. It is our view that a general knowledge of the organization, dynamics and ecology of the Rhizobium genome is essential, in particular for a rational genetic manipulation in Rhizobium and the use of altered organisms in agriculture. From this viewpoint, the present paper discusses results from our laboratory and proposes future avenues of research.

Molecular approach to the *Rhizobium phaseoli* population

Previous reports indicated that *R. phaseoli* is a very heterogeneous group of bacteria on the basis of protein patterns (Roberts *et al.*, 1980), plasmid profiles (Martínez and Palacios, 1984), antibiotic resistance (Beynon and Josey, 1980) and DNA hybridization patterns (Gibbins and Gregory, 1982). The analysis of strains isolated from bean nodules in nature using the tools of molecular genetics led us to establish the existence of two symbiotypes of *R. phaseoli*. One comprises the majority of *R. phaseoli* strains (symbiotype I) and is characterized by the presence of multiple copies of the nitrogenase structural genes, a limited host range for nodulation (Martínez *et al.*, 1985), and the production of melanin-like pigment (unpublished). Symbiotype II is characterized by having a single copy of the *nif* genes, an extended host range that includes *Leucaena* species as well as beans (Martínez *et al.*, 1985), and no pigmentation (Martínez *et al.*, 1987). We have used the presence of *nif* gene reiteration as a marker for *R. phaseoli* symbiotype I.

In regard to the host range, our results show that *Phaseolus vulgaris* (bean) is nodulated under laboratory conditions by a wide range of Rhizobium strains isolated from legumes not closely related to *P. vulgaris*. Moreover, under natural conditions in soils where *R. phaseoli* is diluted and other *Rhizobium* species are enriched, atypical symbionts may be isolated from *P. vulgaris* nodules (Martínez *et al.*, 1985).

These studies suggest that a particular symbiotic phenotype, in this case the capacity to nodulate *Phaseolus vulgaris* effectively, is present in different types of strains (some examples are shown in Table 1). Further molecular characterization of these strains will shed light on the taxonomical status of *R. phaseoli*.

Nitrogenase genes organization

By structural homology to the *K. pneumoniae* nitrogenase genes (*nif*HDK) the corresponding sequences of *R. phaseoli* were identified (Quinto *et al.*, 1982), cloned and sequenced (Quinto *et al.*, 1985). In contrast to other *Rhizobium* species analyzed (Ruvkun and Ausubel, 1980) a striking characteristic of *R. phaseoli* is the existence of multiple copies of these genes in the *sym* plasmid. Structurally *nif* genes are organized in defined and distinctive patterns (Martínez *et al.*, 1985) and functionally they are organized in operons; two *nifHDK* operons and one *nifH* gene (unpublished).

To elucidate the biological functions of various copies of nitrogenase structural genes, mutants in single or both nitrogenase operons have been constructed. Nodules formed by single mutants have a diminished acetylene reduction activity and contribute less nitrogen to the plant (unpublished). In *R. fredii*, the bacteria that nodulate 'Peking' soybean, repeated *nif* gene sequences have also been described (Prakash and Atherly, 1984). Loss of some *nif* regions results in reduced nitrogen fixation of the nodules formed by such altered bacteria (Mathis *et al.*, 1985). From the point of view of evolution, it would be interesting to determine whether *nif* gene reiteration in *R. fredii* and *R. phaseoli* is a case of convergence. This would mean that in both genomes, reiterations were generated independently and fixed because they conferred some advantage. The alternative position to evolutionary convergence is the postulation of a common origin of these *nif* reiterations. Structural analysis of both types of *sym* plasmids will provide support to one of the two alternative hypotheses.

Genome organization and dynamics

Nitrogenase genes are also repeated in *Rhizobium* sp. broad host range ANU240 (also called NGR 234, Morrison *et al.*, 1983), and in bacteria other than rhizobia (Chen *et al.*, 1986; Jacobson *et al.*, 1986; Kallas *et al.*, 1983; Rice *et al.*, 1984). Other examples of repeated DNA sequences have been found in Rhizobium and Bradyrhizobium strains, such as insertion sequences (Kaluza *et al.*, 1985; Ruvkun *et al.*, 1982) an early nodulation gene (*nodD*) (Appelbaum *et al.*, 1985; Göttfert *et al.*, 1986; Honma *et al.*, 1986; Long *et al.*, 1986) and regulatory regions (Better *et al.*, 1983; Rostas *et al.*, 1986).

Repetitive DNA is found extensively in eukaryotic genomes, and few examples of repeated DNA in prokaryotes have been described (Fliss *et al.*, 1986; Furano *et al.*, 1978; Sapienza and Doolittle, 1982).

We have analyzed the entire genome of *R. phaseoli* for the presence of other repeated DNA sequences. Our results show (Flores *et al.*, submitted; Palacios *et al.*, 1985) that there are several repeated DNA sequences in *R. phaseoli*, as well as in the other two strains analyzed: *i.e.* one *R. meliloti* strain (the endosymbiont of alfalfa) and one *Agrobacterium tumefaciens* strain (which induces tumors in various dicotyledoneous plants). The copy number per genome of these repeated sequences usually varies from two to five, and some sequences may be present in more than ten copies.

Both *Rhizobium* and *Agrobacterium* species have various high molecular weight plasmids. As to the location of the repeated sequences, they are not restricted to the chromosome or to specific plasmids, rather they are widespread in the genome (Flores *et al.*, submitted).

The search for repetitive DNA sequences was extended to cover other *R. phaseoli* strains of symbiotype I from different geographical origins. DNA sequences were found to be reiterated in all nine strains analyzed. However these repeated sequences were organized differently in the various strains, as revealed by the hybridization bands observed (Flores *et al.*, submitted). To explain this extensive polymorphism, mechanisms of rearrangement of repeated sequences must be argued.

It has been suggested that repeated elements are targets for recombination processes. Following the pioneer work of Sapienza *et al.* (1982) in Archaebacteria, we examined the possibility of the generation of rearrangements in Rhizobium cells which were grown and maintained for several generations under normal laboratory conditions. To reveal genetic variation of individuals, single colonies from these subcultures were selected and the patterns of different repetitive sequences were analyzed. We detected rearrangements that occur at high frequency for some reiterated DNA families (Flores *et al.*, submitted; Palacios *et al.*, 1986). The generation of rearrangements is a reproducible phenomenon and interestingly there is a limited number of rearrangement patterns that can be generated. The various controls to identify the strains exclude the possibility of any contaminants.

We are presently evaluating whether some of these rearrangements may alter symbiotic performance of Rhizobium strains. One such rearrangement is particularly interesting because symbiotic ability is lost concominant with a large deletion of genetic material containing the different *nif* gene copies (Soberón-Chávez *et al.*, 1986). Genomic rearrangements also may explain the frequently found variability and loss of symbiotic properties of different Rhizobium strains.

Rhizobium genetic manipulation

We have transferred plasmids from some *Rhizobium phaseoli* strains of the two symbiotypes to a plasmid-free *Agrobacterium tumefaciens* strain (Brom *et al.*, unpublished; Martínez *et al.*, 1987). This allowed us to identify the *sym* plasmids from these strains and to evaluate the symbiotic performance of these plasmids in another bacterial background. We found that *A. tumefaciens* with *R. phaseoli* plasmids acquire the ability to nodulate bean. The host range of infection is maintained in the Agrobacterium transconjugants. Agrobacterium harboring *sym* plasmids derived from symbiotype I strains only nodulate bean, while those with plasmids from symbiotype II present the broad host range.

Sym plasmids from *R. phaseoli* symbiotype II are particularly suitable to direct an effective symbiosis in an *A. tumefaciens* background. The strains CFN299 and CIAT899 nodulate bean and the tropical tree *Leucaena esculenta* (Table 1). *A. tumefaciens* with the *sym* plasmid from either of these strains nodulate and fix nitrogen in both hosts. We have managed to construct an *A. tumefaciens* transconjugant that carries the whole set of plasmids (three plasmids) from CFN299. This transconjugant performed better in symbiosis than the *A. tumefaciens* transconjugant that harbors only the *sym* plasmid (Martínez *et al.*, 1987), suggesting that plasmids other than the *sym* plasmid may help in the symbiotic process. However this construction is less effective than the original *R. phaseoli* CFN299 pinpointing the importance of the bacterial chromosome in symbiosis.

A. tumefaciens transconjugants are one of the many possibilities of strains construction and our results show that *R. phaseoli* plasmids may be expressed in this heterologous background.

Table 1. Structural and functional properties of different Rhizobium strains[a]

Strain	Original host legume	Geographical origin	Nodulation of bean	Nitrogen fixation on bean	Nodulation of *Leucaena* spp.	Number of hybridization bands with a *nif* gene probe	Production of pigment	Classification	Reference
CFN42	*P. vulgaris*	México	+	+	−	3	+	*R. phaseoli* symbiotype I	Quinto *et al.*, 1982
CFN273	*P. vulgaris*	México	+	+	−	3	+	*R. phaseoli* symbiotype I	Martínez *et al.*, 1985
CFN275	*P. vulgaris*	México	+	+	−	3	+	*R. phaseoli* symbiotype I	Martínez *et al.*, 1985
CFN277	*P. vulgaris*	México	+	+	−	3	+	*R. phaseoli* symbiotype I	Martínez *et al.*, 1985
CFN285	*P. vulgaris*	México	+	+	−	3	+	*R. phaseoli* symbiotype I	Martínez *et al.*, 1985
CIAT 894d	*P. vulgaris*	Colombia	+	+	−	3	+	*R. phaseoli* symbiotype I	Dr Peter Graham[b]
281	*P. vulgaris*	Brazil	+	+	−	3	+	*R. phaseoli* symbiotype I	Dr Johanna Döbereiner[c]
CIAT 899d	*P. vulgaris*	Colombia	+	+	+	1	−	*R. phaseoli* symbiotype II	Dr Peter Graham
CFN299[d] (UMR1026 = CENA183)	*P. vulgaris*	Brazil	+	+	+	1	−	*R. phaseoli* symbiotype II	Martínez *et al.*, 1985
CFN249	*Dalea leporina*	México	+	+	+	1	−	*R. spp*	Martínez *et al.*, 1985
CFN234	*Leucaena leucocephala*	México	+	+	+	1	−	*R. spp.*	Martínez *et al.*, 1985
CFN401	*Pachyrhizus erosus*	México	+	+	−	1	−	*R. spp.*	Dr E Martínez CFN collection

[a] Only some strains are listed as examples. Of 50 isolates we have analyzed from *P. vulgaris* nodules, 48 strains correspond to symbiotype I and 2 to symbiotype II.
[b] Dr Peter Graham, Dept. of Soil Science, University of Minnesota St. Paul, Minn., USA.
[c] Dr Johanna Döbereiner, EMBRAPÀ, SNLCS, Rio de Janeiro, Brazil.
[d] These strains are acid and aluminium resistant.

Conclusions and perspectives

Our laboratory has studied *R. phaseoli* as a model to better understand the Rhizobium genome. Some of the findings such as the presence of reiterated DNA sequences can be generalized to other *Rhizobium* species as well as to *Agrobacterium tumefaciens*. Analysis of strains belonging to different taxonomic groups will indicate how general this characteristic is in eubacteria. It seems that the Rhizobium genome can generate and tolerate repeated DNA and that some repeated sequences get fixed during evolution in relation to specific physiological demands in particular strains. This is probably the case of reiterated *nif* genes in *R. phaseoli* and other species.

The results with reiterated *nif* genes have important implications. The fact that nitrogen fixation is greater in a wild type strain containing two functional operons as compared to a mutant with only one functional operon suggests an avenue of future research: *i.e.* genetic manipulation of Rhizobium to amplify nitrogen fixation genes.

Direct experiments have given evidence of internal dynamics in the *Rhizobium phaseoli* genome. We propose that genomic rearrangments are the molecular basis for the variability and instability of Rhizobium strains. Because this is certainly an important problem in handling and preserving the properties of Rhizobium strains, in particular when using them as inoculants, research efforts should be focussed on understanding the mechanisms of such rearrangements and in trying to stabilize relevant genetic information.

From another point of view, we could ask what is the biological significance of frequent genomic rearrangements. Genomic plasticity could give the organism an increased capacity to adapt to soil conditions or to interact with specific plants.

Not only internal dynamics, but also exchange of genetic information among strains could increase the adaptability of the Rhizobium genome, in regard to survival under certain soil conditions or interaction with particular plant hosts. Numerous examples of exchange of genetic material between Rhizobium strains in the laboratory have been reported. However, it is not known at what frequency transfer of genetic information occurs in nature. Understanding the degree and limits of genetic exchange in nature will have very important implications in view of the interest in introducing strains harboring recombinant genomes in the soil. Hopefully future research will give insight into such problems.

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