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Host-specific nodulation is encoded on a 14kb DNA fragment in *Rhizobium trifolii*

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Summary

The *Rhizobium trifolii* genes necessary for nodule induction and development have been isolated on a 14.0kb fragment of symbiotic (Sym) plasmid DNA. When cloned into a broad-host-range plasmid vector, these sequences confer a clover nodulation phenotype on a derivative of *R. trifolii* which has been cured of its endogenous Sym plasmid. Furthermore, these sequences encode both host specificity and nodulation functions since they confer the ability to recognize and nodulate clover plants on *Agrobacterium* and a fast-growing cowpea *Rhizobium*. This indicates that the bacterial genes essential for the initial, highly-specific interaction with plants are closely linked.

Introduction

Members of the bacterial genus *Rhizobium* form a specific symbiotic interaction with plants of the family *Leguminosae* (legumes) (37). Bacteria invade the emerging root hairs of the host plant and elicit formation of the characteristic root nodules in which atmospheric nitrogen is reduced to ammonia, a form which the plant can assimilate and use in protein biosynthesis (38, 32). For the fast-growing species of *Rhizobium,* which infect the temperate legumes, this interaction is highly specific e.g. *Rhizobium trifolii* only nodulates species of clover *(Trifolium)* (37). The genetic basis of this interaction, especially with respect to host specificity and nodulation responses, is poorly understood.

The use of transposons as mutagens (6) and the development of a rapid plant assay system to screen rhizobia for defective symbiotic phenotypes (27) has facilitated the analysis and manipulation of symbiotic genes. It has recently been demonstrated that symbiotic genes are located on large plasmids in many *Rhizobium* species (25, 30, 13, 3). Therefore, this class of plasmid has been termed a Sym (*sym*biotic) plasmid (14).

The linkage of nitrogen fixation *(nif)* and nodulation *(nod)* genes on Sym plasmids has been demonstrated in *R. leguminosarum* (13), *R. phaseoli* (19), *R. meliloti* (2, 29, 20), *R. trifolii* (14, 11, 31), and a fast-growing cowpea *Rhizobium* (23). In *R. trifolii, nod* genes are located some 16kb from the nitrogenase structural genes, *nif*HDK, (31) whereas in *R. meliloti* this distance is about 25kb. (20). In *R. leguminosarum,* a 45kb region of DNA has been shown to carry two clusters of genes encoding nitrogen-fixation functions separated by a cluster of nodulation genes (10). The close linkage of these two symbiotic gene regions has led to the postulation that there are a limited number of bacterial genes involved in the symbiosis. The low frequency of symbiotic lesions in mutagenesis experiments is consistent with this postulate, especially with regard to the low numbers of nodulation-deficient mutants observed (8, 22, 33).

The ability to cure endogenous plasmids from *Rhizobium* has previously been demonstrated and Sym plasmid-cured strains of *R. trifolii* have been isolated (39; Djordjevic *et al.*, submitted for publi-

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cation). We have previously suggested the use of Sym plasmid-cured strains of *Rhizobium* to assay for Sym plasmid-encoded functions (31). This report describes the results of such experiments using a Sym plasmid-cured strain of *R. trifolii*. The *R. trifolii* (strain ANU843) genes involved in host specificity and the initiation and development of nodules on clover plants were isolated by molecular cloning. These nodulation functions are encoded on a 14.0 kilobase (kb) *Hind*III restriction fragment. Expression of these cloned nodulation genes in *Agrobacterium* and a fast-growing cowpea *Rhizobium* was also studied.

Materials and methods

Bacterial strains

Rhizobium trifolii strain ANU843 (27) nodulates and fixes nitrogen on white and subterranean clover. ANU851 is a transposon Tn5-induced derivative of ANU843 which is unable to nodulate or effect clover root *ha*ir *c*urling (Hac$^-$ phenotype) (33). ANU845 is a derivative of ANU843 which has been cured of its symbiotic (Sym) plasmid by growth at elevated temperatures (39). The isolation of ANU845 is described in detail by Djordjevic *et al.* (submitted for publication). Strain ANU240 is a streptomycin-resistant derivative of the fast-growing, broad-host-range cowpea *Rhizobium* strain NGR234 (36, 23) which was originally isolated from *Dolichos lablab*. This strain nodulates a large variety of tropical legumes and the non-legume *Parasponia* (36).

Agrobacterium tumefaciens strain A136 is a Ti plasmid-cured derivative of the wild-type strain C58 (Hooykaas, personal communication).

Escherichia coli strain RR1 (7) was used as the transformation recipient for all recombinant plasmids and as a donor strain in mobilization experiments.

DNA isolation and analysis

Preparation of total *R. trifolii* DNA and recombinant plasmid DNA, restriction endonuclease digestion, agarose gel electrophoresis and hybridization analysis were all as previously described (31).

Cloning of the Rhizobium *nodulation genes*

Recombinant plasmids containing overlapping DNA sequences had previously been obtained in establishing the molecular linkage map of *nod* and *nif* genetic regions (31). The inserts of the plasmids pRt587 and pRt572 were subcloned into the IncQ group vectors pKT240 and pKT231 (1) yielding the recombinant plasmids pRt032 and pRt033, respectively (see Fig. 1).

Conjugation and plant nodulation assays

E. coli RR1, carrying recombinant derivatives (pRt032 and pRt033) of IncQ group plasmid vectors (1) were mated for 6 h to an *E. coli* strain carrying an IncP group mobilizing plasmid (RP4) (4). The cells were resuspended in L broth and mated with the respective recipient strain on solid TY media (5). After 24 h incubation at 29 °C, transconjugants were selected by replica plating to TM agar (27) containing carbenicillin (100 μg/ml) or kanamycin (150 μg/ml), respectively.

Purified bacterial colonies were inoculated onto nitrogen-free medium for rapid plate plant assay (27) with seedlings of either *Trifolium repens* (N.Z. white clover 5826), *T. subterranean* (subterranean clover, Mt. Barker variety), *Macroptilium atropurpureum* (Siratro), *Pisum sativum* (peas, Green Feast variety) or *Medicago sativa* (alfalfa, Hunter River Red variety). Bacteria were isolated from nodules in osmotically-protective media after surface sterilization and lysis (12).

Light and electron microscopy

Nodules were excised and fixed in glutaraldehyde, block-stained in uranyl acetate, post-fixed in osmium tetroxide, and embedded in Spurr's resin (35) after dehydration through an acetone series. Light sections were microtomed to 0.5 μm thickness and stained with toluidine blue. Silver EM sections (approx. 0.07 μm thick) were stained with uranyl acetate and lead citrate.

Results

We have previously identified genetic regions of the Sym plasmid of *R. trifolii* strain ANU843 that are involved in both nodulation and nitrogen fixation. The *nod* genetic region was characterized by the analysis of a Tn5-induced nodulation-defective mutant, ANU851 (33, 31). This mutant is unable to induce clover root *ha*ir *c*urling (Hac$^-$ phenotype),

an early stage in nodule development (38, 28), and has previously been mapped on the *R. trifolii* Sym plasmid (31) (see Fig. 1).

14kb of DNA encodes nodulation functions

Definition of the limits of the nodulation gene region was facilitated by the availability of a derivative of *R. trifolii* ANU843 which has been cured of its Sym plasmid. *R. trifolii* strain ANU845 is a Sym plasmid-cured, Nod⁻ derivative of ANU843, isolated after prolonged incubation at elevated temperature (39; Djordjevic *et al.*, submitted for publication). As essential symbiotic functions are encoded on the Sym plasmid, one can use such Sym plasmid-cured strains to determine which symbiotic functions are encoded on various cloned DNA fragments derived from the Sym plasmid (31).

The observation that an *in vitro*-generated mutation, (constructed as previously described (16, 34)), located 8kb to the right of the site of Tn5 insertion in ANU851 (see Fig. 1), did not affect nodulation, (P. R. Schofield, unpublished), suggested that the nodulation genes may not extend a great distance beyond the Nod⁻ mutation of ANU851. This hypothesis was tested by cloning the 14kb *Hind*III fragment of wild-type ANU843 DNA, which spans the Nod⁻ mutation, into the multicopy, broad-host-range vector pKT240 (Bagdasarian, personal communication). The recombinant plasmid (pRt032) was mobilized into the Sym plasmid-cured strain ANU845.

Transconjugants were then inoculated onto seedlings of white clover *(Trifolium repens)* and subterranean clover *(T. subterraneum)* using the plate plant assay. After 10 days incubation, the ANU845 (pRt032) transconjugants nodulated both species of clover (Fig. 2c). The nodules induced by these transconjugants were ineffective due to the absence of *nif* genes. Nodule development was similar to that observed with wild-type bacteria up until twelve days after inoculation, however, further nodule development was not observed. The average number of nodules per plant induced by ANU845 (pRt032) transconjugants was consistently higher than that induced by the wild-type strain ANU843. Such a phenomenon is commonly seen with other Fix⁻ mutants of this and other strains (26). The Sym plasmid-cured strain ANU845 failed to induce nodule formation (Fig. 2).

Bacteria were isolated from two individual nodules induced by ANU845 (pRt032) transconjugants and total DNA was extracted. Hybridization analysis of this DNA confirmed the presence of both the 14kb *Hind*III fragment and the vector plasmid (Fig. 3). The lack of hybridization of this DNA to the *nif*HD probe and a restriction fragment profile similar to that of the wild-type DNA (33) confirmed that the bacteria isolated from these nodules were derivatives of the Sym plasmid-cured strain ANU845.

Thus, sequences contained on the 14kb *Hind*III fragment are sufficient to replace the clover nodulation capacity of the Sym plasmid. Since the ANU845 (pRt032) transconjugants lack the *nif* genes (and perhaps other symbiotic genes), the nodules are blocked in later stages of the symbiosis and do not fix nitrogen.

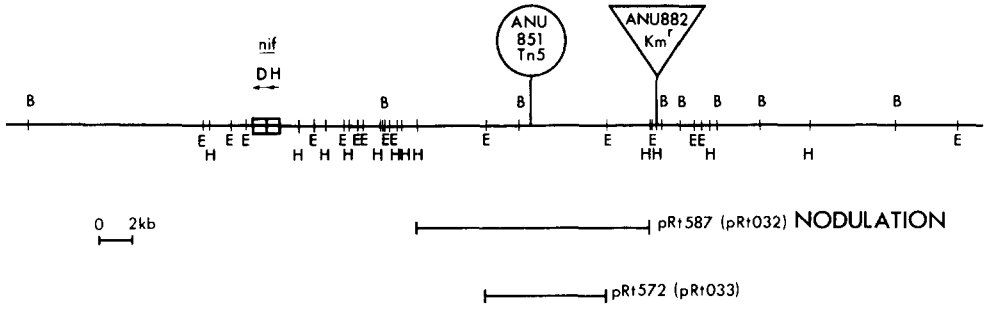

Fig. 1. Molecular linkage map of the nodulation and nitrogen fixation regions on the Sym plasmid of *R. trifolii* strain ANU843. For details regarding the construction of this map refer to Schofield *et al.* (31). The circle indicates the insertion point of Tn5 in the Nod⁻ mutant ANU851. The triangle indicates the point of insertion of a Tn5-derived, Km^r fragment in strain ANU882 (see text). The cloned fragments used in the Sym plasmid-cured replacement experiments are indicated. E = *Eco*RI, H = *Hin*dIII, B = *Bam*HI.

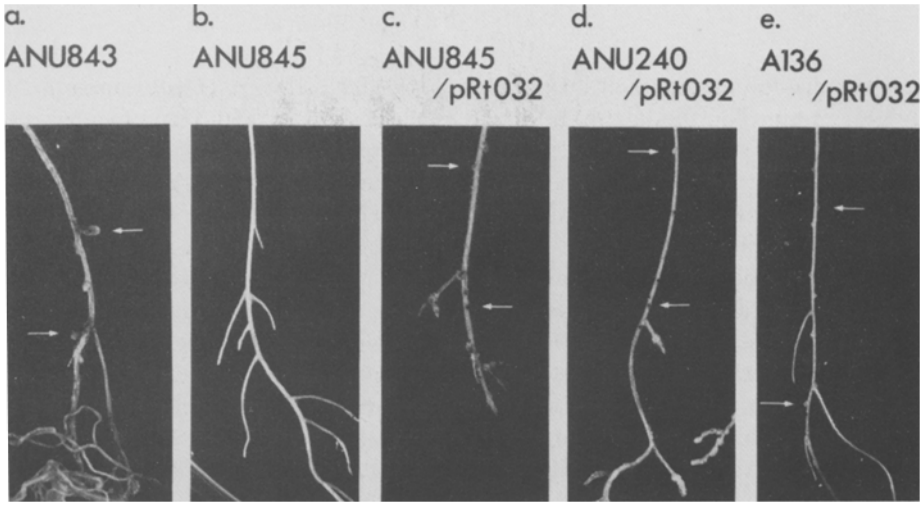

Fig. 2. Demonstration that 14kb of *R. trifolii* strain ANU843 DNA encodes nodulation functions. Nodules induced on white clover were photographed five weeks after inoculation.
Panel a: Wild-type ANU843. Large, cylindrical, indeterminate nodules are present (arrowed) and are actively fixing nitrogen. Plant growth (not shown) is luxuriant.
Panel b: ANU845, the Sym plasmid-cured derivative, does not induce nodule formation. Seedling growth is limited by seed reserves and the plant dies within 5 weeks of inoculation.
Panel c: ANU845 (pRt032) induces the development of small, rounded nodules (arrowed), as seen initially with the wild-type (ANU843). However, these nodules do not develop to the indeterminate (cylindrical) form. Nitrogen is not fixed, and the plant eventually dies.
Panel d: *Rhizobium* ANU240 (pRt032) showing a similar degree of nodule development (arrowed) as with ANU845 (pRt032) transconjugants.
Panel e: *A. tumefaciens* A136 (pRt032) transconjugants also induce small, rounded nodules (arrowed) as seen in panel c.

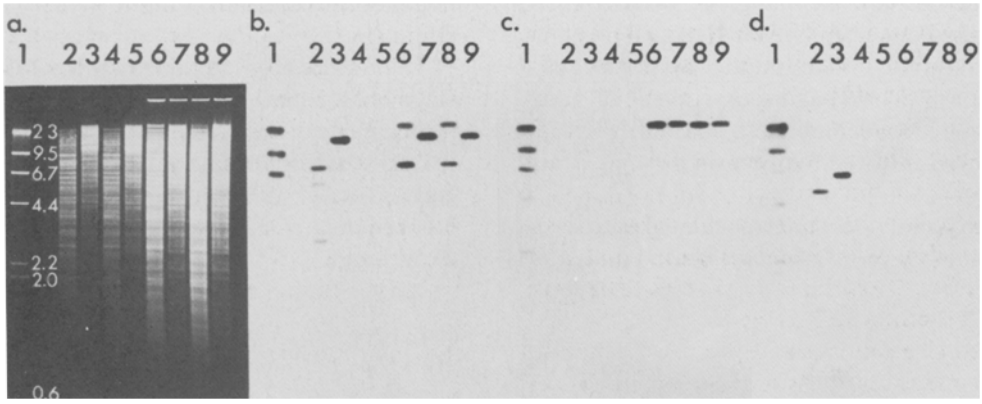

Fig. 3. Confirmation of the nodulation ability of ANU845 (pRt032) transconjugants by hybridization analysis. Southern blots were prepared from parallel restriction digests of DNA derived from ANU845 (pRt032) transconjugants and hybridized to various radioactively-labelled probes: panel b: pRt587, (Nod region); panel c: pKT240, (Vector); and panel d: pRt585, (Nif region) (31).
(a) Photograph of the ethidium bromide-stained gel and (b, c and d) autoradiographs:
Lane 1: Size standards (kb) from *Hin*dIII-restricted λcI857 DNA.
Lane 2: ANU843 restricted with *Eco*RI.
Lane 3: ANU843 restricted with *Hin*dIII.
Lane 4: ANU845 (Sym plasmid-cured) restricted with *Eco*RI.
Lane 5: ANU845 (Sym plasmid-cured) restricted with *Hin*dIII.
Lanes 6 & 8: Two separate ANU845 (pRt032) transconjugants restricted with *Eco*RI.
Lanes 7 & 9: The same ANU845 (pRt032) transconjugants restricted with *Hin*dIII.
The two smallest positively-hybridizing *Eco*RI fragments (panel b, lane 2), when fused to vector fragments, appear as a doublet of approximately 25kb (lanes 6 and 8). Very faint hybridization can be observed (not visible in this figure) between the ampicillin resistance genes of the vector pKT240 and pBR328 sequences present in the plasmids used as hybridization probes.

To determine the minimum amount of information required for infection and nodule initiation, the 7.2kb *Eco*RI restriction fragment (see Fig. 1) was cloned into the multicopy, broad-host-range vector pKT231 (1). The recombinant plasmid (pRt033) was mobilized into the Sym plasmid-cured strain ANU845. The resultant transconjugants failed to nodulate and did not curl clover root hairs, although bacteria appeared to enter most of the root hairs in an unregulated fashion (P. R. Schofield, unpublished).

Nodule morphology induced by the 14kb DNA fragment

Normal nodule development induced by fast-growing strains of *Rhizobium* such as *R. trifolii* leads to an indeterminate, cylindrical nodule morphology (24) (see Fig. 2a). The genetic information encoded on pRt032 does not allow complete manifestation of this indeterminate nodule morphology. In this case, nodule development terminates at an earlier developmental stage than in the wild-type situation. The nodules are spherical in shape and appear morphologically similar to determinate nodules (Fig. 3c) (24).

During the formation of wild-type nodules, bacteria are released from the branching infection thread into a host-elaborated peribacteroid membrane. They subsequently undergo division, enlarge into bacteroids, and are individually packaged into peribacteroid membranes (see Fig. 4c and g). This is the differentiated form of *Rhizobium* which actively fixes nitrogen. A distinct meristematic zone is formed (see Fig. 4a) and the nodule extends into an elongated, cylindrical form (38, 28, 24).

Light and electron microscopic examination of spherically-shaped nodules induced on white and subterranean clover plants by ANU845 (pRt032), revealed that the meristematic zones were not fully developed (Fig. 4, compare panels a and d). Moreover, differences were observed in the extent of nodule morphogenesis on the two species of clover. The extent of phenotypic expression of the genes cloned in pRt032 thus depends significantly upon the plant host background. In white clover nodules, induced by ANU845 (pRt032), bacterial release did not appear to synchronize with host-initiated peribacteroid membrane formation and the few bacterial cells present in the nodules were not packaged in these membranes (Fig. 4e and f). In contrast, formation of bacteroids was observed in a few of the nodule cells in subterranean clover. These bacteroids were mostly half the normal size and had not reached the stage of individual packaging, remaining packaged in groups of as many as 16 bacteroids (Fig. 4h). A few bacteroids had developed to wild-type size.

Host specificity determinants are linked to nodulation genes

To confirm that the genes carried on the 14kb *Hin*dIII insert of pRt032 encode the functions necessary for host specificity and nodule morphogenesis, the plasmid was mobilized into *Agrobacterium tumefaciens* strain A136 (a Ti plasmid-cured derivative of strain C58) and into a fast-growing cowpea *Rhizobium*, strain ANU240. The latter is a fast-growing, broad-host-range *Rhizobium* which nodulates a range of cowpea group plants, as well as alfalfa *(Medicago sativa), Leucaena, Acacia farnesiana* and the non-legume *Paraspoeia* (36, 23). Neither of these two baterial species are normally capable of nodulating white clover.

The nodulation ability of the *A. tumefaciens* and *Rhizobium* ANU240 transconjugants carrying pRt032 was assayed on white clover seedlings. Nodules similar to those induced by ANU845 (pRt032) were observed in each case (Fig. 2). Nodule development was almost as rapid as that seen with wild-type bacteria, in contrast to the observation of Hooykaas *et al.* (14), when *A. tumefaciens* carrying an entire *R. trifolii* Sym plasmid (pRtr5*a*) took from five to six weeks, instead of ten to fourteen days, to induce nodule formation. The nodules induced by transconjugants of *A. tumefaciens* carrying pRt032 all appeared normal until twelve days after inoculation when further development ceased. No 'pseudo nodules', swellings or aborted lateral roots were observed as was reported by Kondorosi *et al.* (18). This was confirmed by the ability to isolate, after nodule surface sterilization, *A. tumefaciens* A136 (pRt032) transconjugants from all nodules examined. Furthermore, these transconjugants were unable to induce lateral root distortions or swellings on either peas *(Pisum sativum)* or alfalfa *(Medicago sativa),* thus indicating the fidelity of recognition of the specific *R. trifolii* host, clover *(Trifolium).*

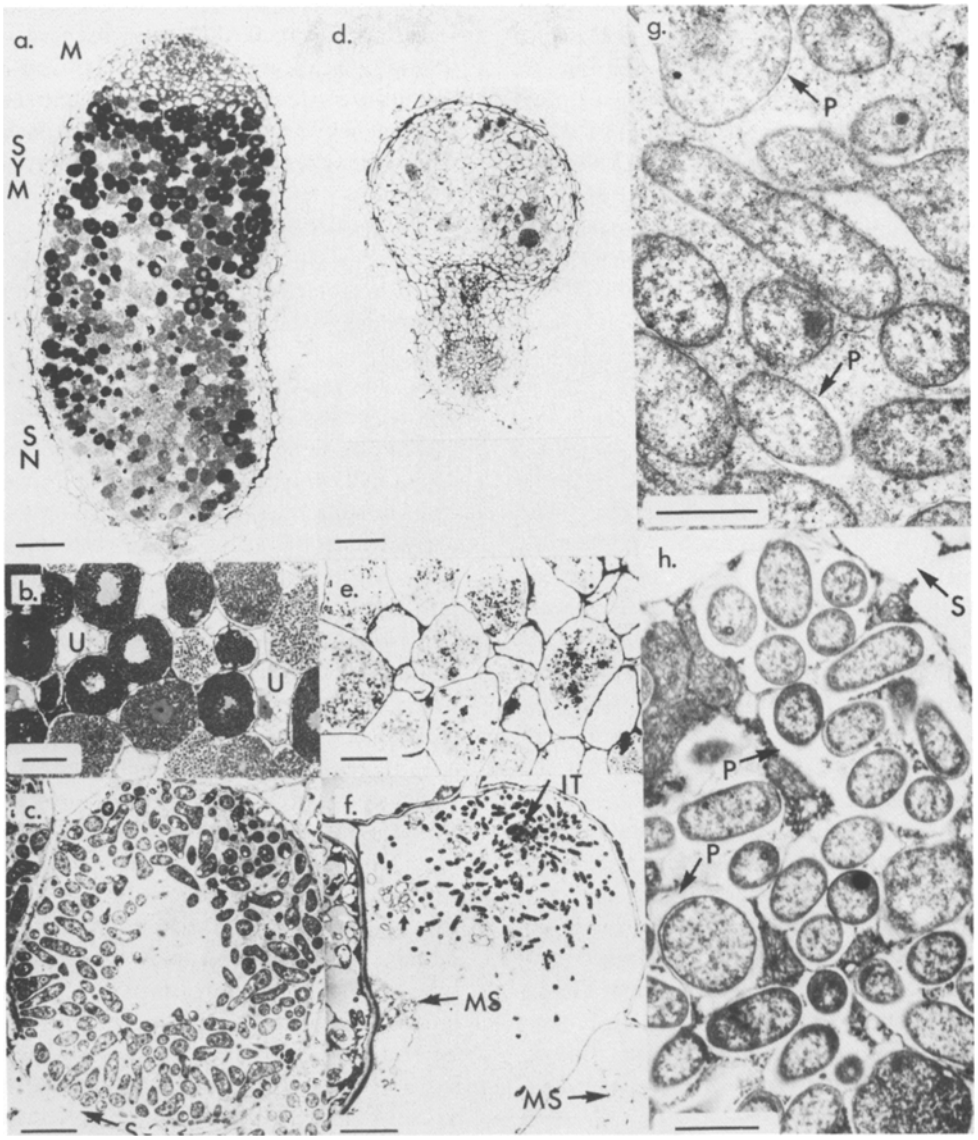

Fig. 4. Microscopic examination of sections of nodules induced by ANU845 (pRt032) transconjugants on white and subterranean clover. Light and electron micrographs showing the phenotypic expression of ANU845 (pRt032) and the wild-type ANU843, 18 days after inoculation onto white and subterranean clover. Panels a, b, c, white clover infected with ANU843 (wild-type); d, e, f, white clover infected with ANU845 (pRt032); g & h, subterranean clover infected with ANU843 and ANU845 (pRt032), respectively.

At low magnification the wild-type nodule of white clover (panel a) (subterranean clover is similar) has the shape characteristic of an indeterminate nodule. It has a prominent meristem (M), a main symbiotic zone (SYM) and a senescent zone (SN), with no well-defined boundary between these latter two zones. The nodule induced on white clover by ANU845 (pRt032) (panel d) (subterranean clover is similar) has not developed a meristematic zone and remains the same shape as that induced by ANU843 after twelve days, just before meristematic growth commenced. No distinct zones are visible in the nodule tissue. At higher magnification (compare panels b & c to e & f, respectively), it is clear that normal bacteroid development is blocked in ANU845 (pRt032) transconjugants. There appears to be a disfunction in peribacteroid membrane synthesis (MS) and the bacteria are released naked into the host cytoplasm, where they quickly degenerate. However, in subterranean clover, bacteroid development continues in a few cells (panel h), although these bacteroids fail to become individually packaged after initial division inside the peribacteroid membrane (P). They generally reach only half the size of wild-type bacteroids (panel g) although some manage to become packaged individually and attain wild-type size.

IT, infection thread; U, uninfected cells; S, starch granules. The bar in each micrograph indicates the size in microns: a, d: 100 μm; b, e: 20 μm; c, f: 5 μm; g, h: 1 μm.

E. coli strain RR1, containing pRt032, was unable to induce clover nodulation. This result was, however, expected on the basis of previous reports of lack of expression of *Rhizobium* genes in *E. coli* (17).

To test for a broadened host range of the ANU240 transconjugants, bacteria were isolated from the nodules induced by ANU240 (pRt032), and inoculated onto both the tropical legume Siratro (*Macroptilium atropurpureum,* a host of *Rhizobium* ANU240) and white clover seedlings. The normal nodulation response was observed on the Siratro plants, and the expected small, noncylindrical, ineffective nodules were observed on the white clover seedlings. The introduction of pRt032 into *Rhizobium* ANU240 thus extends the host range of this strain to include clover plants.

Discussion

The data presented in this paper demonstrate that the 14kb *Hin*dIII fragment, derived from the Sym plasmid of *R. trifolii* strain ANU843, encodes (directly or indirectly) at least those functions required for host specificity, infection, nodule initiation, nodule development and bacterial release (38).

To date, direct analysis of nodulation functions encoded on Sym plasmids has been accomplished by transfer of the entire Sym plasmid from one species of *Rhizobium* to another, or to other genera, e.g. *Agrobacterium* (15, 14, 18; Djordjevic *et al.,* submitted for publication). Banfalvi *et al.* (3) performed similar experiments using R-primes derived from the *R. meliloti* Sym plasmid. However, the precise location of nodulation determinants on these R-primes (128–240kb) has not yet been achieved.

Plasmid-curing techniques permit the isolation of *Rhizobium* strains which have lost one or more of their endogenous plasmids, including the Sym plasmid (39). This has been achieved in *R. trifolii* (Djordjevic *et al.,* submitted for publication), *R. leguminosarum* (9) and a cowpea *Rhizobium* (23). We have used a Sym plasmid-cured *R. trifolii* to assay various symbiotic functions involved in early functions (29) of the symbiosis. Furthermore, use of this approach allows identification of Sym plasmid genes necessary for the establishment of bacterial infection, nodule initiation, nodule development and nitrogen fixation itself. In addition, transfer of cloned segments of the Sym plasmid to other nonsymbiotic genera may also provide a valuable means of assaying for symbiotic functions, as demonstrated by the localization of host-specific functions encoded on the cloned 14kb fragment.

The time required for nodule development, induced by bacteria harbouring Sym plasmids or cloned Sym plasmid genes, has been reported to vary considerably. *R. trifolii* ANU845 (pRt032) transconjugants developed nodules as rapidly as wild-type bacteria. However, *A. tumefaciens* carrying an entire *R. trifolii* Sym plasmid (pRtr5*a*) took five to six weeks to induce nodules on clovers as opposed to the normal period of ten to fourteen days (14). Nodulation of alfalfa was observed when the *R. meliloti* Sym plasmid (pRme41*b*) was mobilized into a cowpea *Rhizobium* (equivalent to ANU240), a *Lotus Rhizobium* and a Ti plasmid-cured derivative of *A. tumefaciens* C58 (equivalent to A136) (18). In each instance, the onset of nodulation occurred within twelve to fourteen days. However, cosmid clones containing *R. meliloti* nodulation genes in *A. tumefaciens* took five to six weeks to induce nodule formation (F. M. Ausubel, personal communication). There is currently no data available to suggest why nodule development is retarded in some instances.

The 7.2kb *Eco*RI restriction fragment (in pRt033), contained within the 14kb *Hin*dIII fragment (see Fig. 1), has also been analysed. This subclone cannot induce nodule formation and therefore does not contain all the determinants for nodulation. However, the observation that this Sym plasmid subfragment enabled *Rhizobium* to enter clover root hairs, without root hair curling, would indicate that some early nodulation genes are located on this fragment.

Previous attempts to identify nodulation genes by Tn5 mutagenesis have yielded few nodulation-deficient mutants (8, 22, 33). Originally, this was attributed to Tn5 reversion (reversion of auxotrophic mutants has been reported to range from 10^{-5} to less than 10^{-8}), coupled with plant selection of rare Nod^+ bacteria (6, 33). More recently, results have been presented which indicate that there may be a small number of nodulation genes (21).

However, since 14kb of DNA encodes the functions necessary for nodule development and host specificity, only a small number of genes may be

required for the nodulation of clovers and this may account for the low numbers of nodulation-defective mutants isolated during random transposon mutagenesis experiments.

Alternatively, the small number of Sym plasmid genes that appear essential for nodule initiation and development may in fact be due to bacterial induction of host plant responses. This is demonstrated by the differential development of bacteroids in white and subterranean clovers.

The use of the *in planta,* Sym plasmid-cured assay system, coupled with specific mutagenesis of the symbiotic region will facilitate fine structure mapping of the genetic determinants of both host specificity and nodulation as well as nitrogen fixation itself. Furthermore, the ability to introduce specific cloned symbiotic genes from *Rhizobium* to other bacterial species (as recombinant or synthetic Sym plasmids, such as pRt032) should extend our understanding of the nodulation process and facilitate extension of the host range of this symbiosis.

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References

1. Bagdasarian M, Lurz R, Ruckert B, Franklin FCH, Bagdasarian MM, Frey J, Timmis KN: Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene 16:237–247, 1981.
2. Banfalvi Z, Sakanyan V, Koncz C, Kiss A, Dush I, Kondorosi A: Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *Rhizobium meliloti*. Mol Gen Genet 184:318–325, 1981.
3. Banfalvi Z, Randhawa GS, Kondorosi E, Kiss A, Kondorosi A: Construction and characterization of R-prime plasmids carrying symbiotic genes of *R. meliloti*. Mol Gen Genet 189:129–135, 1983.
4. Barth PT, Grinter NJ: Map of plasmid RP4 by insertion of transposon C. J Mol Biol 113:455–474, 1977.
5. Beringer JE: R factor transfer in *Rhizobium leguminosarum*. J Gen Microbiol 84:188–198, 1974.
6. Beringer JE, Beynon JL, Buchanan-Wollaston AV, Johnston AWB: Transfer of drug-resistance transposon Tn5 to *Rhizobium*. Nature 276:633–634, 1978.
7. Bolivar F, Rodriguez R, Greene PJ, Betlach M, Heyneker HL, Boyer HW, Crosa J, Falkow S: Construction and characterisation of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113, 1977.
8. Buchanan-Wollaston AV, Beringer JE, Brewin NJ, Hirsch PR, Johnston AWB: Isolation of symbiotically defective mutants in *Rhizobium leguminosarum* by insertion of the transposon Tn5 into a transmissible plasmid. Mol Gen Genet 178:185–190, 1980.
9. Brewin NJ, Wood EA, Johnston AWB, Dibb NJ, Hombrecher G: Recombinant nodulation plasmids in *Rhizobium leguminosarum*. J Gen Microbiol 128:1817–1827, 1982.
10. Downie JA, Ma Q-S, Knight CD, Hombrecher G, Johnston AWB: Cloning of the symbiotic region of *Rhizobium leguminosarum:* the nodulation genes are between the nitrogenase genes and a *nif*A-like gene. EMBO J 2:947–952, 1983.
11. Djordjevic MA, Zurkowski W, Rolfe BG: Plasmids and stability of symbiotic properties of *Rhizobium trifolii*. J Bacteriol 151:560–568, 1982.
12. Gresshoff PM, Skotnicki ML, Eadie JF, Rolfe BG: Viability of *Rhizobium trifolii* bacteroids from clover root nodules. Plant Sci Lett 10:299–304, 1977.
13. Hombrecher G, Brewin NJ, Johnston AWB: Linkage of genes for nitrogenase and nodulation ability on plasmids in *Rhizobium leguminosarum* and *R. phaseoli*. Mol Gen Genet 182:133–136, 1981.
14. Hooykaas PJJ, van Brussel AAN, den Dulk-Ras H, van Slogteren GMS, Schilperoort RA: Sym plasmid of *Rhizobium trifolii* expressed in different rhizobial species and *Agrobacterium tumefaciens*. Nature 291:351–353, 1981.
15. Johnston AWB, Beynon JL, Buchanan-Wollaston AV, Setchell SM, Hirsch PR, Beringer JE: High frequency transfer of nodulating ability between strains and species of *Rhizobium*. Nature 276: 634–36, 1978.
16. Jorgensen RA, Rothstein SJ, Reznikoff WS: A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. Mol Gen Genet 177:65–72, 1979.
17. Kondorosi A, Johnston AWB: The genetics of *Rhizobium*. Int. Rev. Cyto., Sup. 13. Giles K & Atherly A (eds) Biology of the Rhizobiaceae. Academic Press, New York, 1981, pp 191–224.
18. Kondorosi A, Kondorosi E, Pankhurst CE, Broughton WJ, Banfalvi Z: Mobilization of a *Rhizobium meliloti* megaplasmid carrying nodulation and nitrogen fixation genes into other rhizobia and *Agrobacterium*. Mol Gen Genet 188: 433–439, 1982.
19. Lamb JW, Hombrecher G, Johnston AWB: Plasmid-determined nodulation and nitrogen-fixation abilities in *Rhizobium phaseoli*. Mol Gen Genet 186:449–452, 1982.
20. Long SR, Buikema WJ, Ausubel FM: Cloning of *Rhizobium meliloti* nodulation genes by direct complementation of Nod$^-$ mutants. Nature 298:485–488, 1982.

21. Ma Q-S, Johnston AWB, Hombrecher G, Downie JA: Molecular genetics of mutants of *Rhizobium leguminosarum* which fail to fix nitrogen. Mol Gen Genet 187:166–171, 1982.
22. Meade HM, Long SR, Ruvkun GB, Brown SE, Ausubel FM: Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by Tn5 mutagenesis. J Bacteriol 149:114–122, 1982.
23. Morrison NA, Hau CY, Trinick MJ, Shine J, Rolfe BG: Heat curing of a sym plasmid in a fast-growing *Rhizobium* sp. that is able to nodulate legumes and the non-legume *Parasponia* sp. J Bacteriol 153:527–531, 1983.
24. Newcomb W: A correlated light and electron microscopic study of symbiotic growth and differentiation in *Pisum sativum* root nodules. Can J Bot 54:2163–2168, 1976.
25. Nuti MP, Lepidi AA, Prakash RK, Schilperoort RA, Cannon FC: Evidence for nitrogen fixing *(nif)* genes on indigenous *Rhizobium* plasmids. Nature 282:533–535, 1979.
26. Nutman PS: Physiological studies on nodule formation II. The influence of delayed inoculation on the rate of nodulation in red clover. Ann Bot 13:261–283, 1949.
27. Rolfe BG, Gresshoff PM, Shine J: Rapid screening for symbiotic mutants of *Rhizobium* and white clover. Plant Sci Lett 19:277–284, 1980.
28. Rolfe BG, Djordjevic MA, Scott KF, Hughes JE, Badenoch-Jones J, Gresshoff PM, Cen Y, Dudman WF, Zurkowski W, Shine J: Analysis of nodule-forming ability of fast-growing *Rhizobium* strains. In: Gibson A & Newton W (eds) Current Perspective in Nitrogen Fixation. Australian Academy of Science, Canberra, 1981, pp 142–145.
29. Rosenberg C, Boistard P, Dénarié J, Casse-Delbart FL: Genes controlling early and late functions in symbiosis are located on a megaplasmid in *Rhizobium meliloti*. Mol Gen Genet 184:326–333, 1981.
30. Ruvkun GB, Ausubel FM: Interspecies homology of nitrogenase genes. Proc Natl Acad Sci USA 77:191–195, 1980.
31. Schofield PR, Djordjevic MA, Rolfe BG, Shine J, Watson JM: A molecular linkage map of nitrogenase and nodulation genes in *Rhizobium trifolii*. Mol Gen Genet, 1983 (in press).
32. Scott DB, Farnden KJF, Robertson JG: Ammonia assimilation in lupin nodules. Nature 263:703–705, 1976.
33. Scott KF, Hughes JE, Gresshoff PM, Beringer JE, Rolfe BG, Shine J: Molecular cloning of *Rhizobium trifolii* genes involved in symbiotic nitrogen fixation. J Mol Appl Genet 1:315–326, 1982.
34. Simon R, Weber G, Arnold W, Puhler A: Analysis of plasmid borne genes in *Rhizobium meliloti* by Tn5 mutagenesis. In: Clark K & Stephens J (eds) Proceedings of the 8th North American Rhizobium Conference. University of Manitoba Press, Winnipeg, 1982, pp 67–89.
35. Spurr AR: A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43, 1969.
36. Trinick MJ: Relationships amongst the fast-growing rhizobia of *Lablab purpureus, Leucaena leucocephala, Mimosa* spp. *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. J Appl Bacteriol 49:39–53, 1980.
37. Vincent JM: Root-nodule symbiosis with *Rhizobium*. In: Quispel A (ed) Biology of Nitrogen Fixation. North Holland Publ. Co., Amsterdam, 1974, pp 265–341.
38. Vincent JM: Factors controlling the legume *Rhizobium* symbiosis. In: Newton W & Orme-Johnson W (eds) Nitrogen Fixation 2. University Park Press, Baltimore, 1980, pp 103–129.
39. Zurkowski W, Lorkiewicz Z: Effective method for the isolation of non-nodulating mutants of *Rhizobium trifolii*. Genet Res 32:311–314, 1978.

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