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# Isolation and Characterization of *Rhizobium* (IC3342) Genes that Determine Leaf Curl Induction in Pigeon Pea

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**Nodulation by the *Rhizobium* strain IC3342 causes a leaf curl syndrome in certain tropical legumes such as pigeon pea (*Cajanus cajan*) (N. M. Upadhyaya, J. V. D. K. Kumar Rao, D. S. Letham, and P. J. Dart, Physiological and Molecular Plant Pathology 39:357-373, 1991). Transposon (Tn*5*) mutagenesis of this leaf curl-inducing (Curl$^+$) *Rhizobium* strain yielded two Curl$^-$ Fix$^-$ and three Curl$^-$ Fix$^+$ mutants. Plasmid visualization and subsequent Southern blot hybridization analyses with Tn*5*, *nif* and *nod* gene probes showed that the Tn*5* element had inserted into the symbiotic (Sym) plasmid in three of the mutants. Restriction endonuclease analyses indicated that none of the Tn*5* insertions were closely linked. Tn*5*-containing *Eco*RI fragments were cloned from each mutant and used as probes to isolate the corresponding wild-type DNA fragments from a cosmid (pLAFR3) genomic library. Fix$^+$ and/or Curl$^+$ phenotypes were restored in each mutant by the introduction of cosmids containing the corresponding wild-type DNA. A closely related but Curl$^-$ *Rhizobium* strain ANU240 was shown, by Southern hybridization, to contain conserved DNA sequences of all but one of the identified genetic regions of the Curl$^+$ *Rhizobium* strain IC3342. Cosmids containing the genetic region unique to the strain IC3342, designated *lcr1*, conferred a Curl$^+$ phenotype on the strain ANU240. DNA sequence analysis of the cloned *lcr1* region revealed five open reading frames (ORFs). The ORF2 showed homology with the *Escherichia coli* regulatory gene *ompR*, and ORF4 showed homology with *E. coli* and *Rhizobium meliloti* regulatory genes *fnr* and *fixK*, respectively.**

*Additional keywords*: cytokinin, radioimmunoassay, siratro, Tn*5* mutagenesis.

The best studied examples of plant-bacterial interactions, from the point of view of their biology and genetics, are the pathological *Agrobacterium*-crown gall interaction and the symbiotic nitrogen-fixing *Rhizobium*-legume interaction. On infection of legume roots, rhizobia normally induce nodules and fix atmospheric nitrogen, which is ultimately made available for plant growth. However, certain specific bacteria-plant interactions become either parasitic, as in the case of nodulation by ineffective strains, or pathogenic due to the production of toxins, as in the case of certain *Bradyrhizobium japonicum* (Kirchner) Jordan-soybean interactions (Owen and Wright 1965). A recently discovered leaf-curling syndrome (hyponasty, release from apical dominance, lateral bud development, and stunted growth) of pigeon pea (*Cajanus cajan* (L) Huth) and several other tropical legumes was reported to be due to nodulation induced by *Rhizobium* strains IC3342 and IC3324 (Kumar Rao *et al.* 1984).

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Nucleotide and/or amino acid sequence data have been submitted to GenBank, EMBL, and DDBJ as accession number M38698.



Grafting and sap feeding experiments indicate that the leaf curling induced by the *Rhizobium* strain IC3342 is mediated via a curl-inducing principle, produced in the roots or nodules, and translocated to the leaves (Kumar Rao *et al.* 1984; Upadhyaya *et al.* 1991a). Studies on the nodulation host range, the effect of inorganic combined nitrogen, and plasmid cured non-nodulating derivatives of the strain IC3342 demonstrate that effective nodulation is necessary for the development of the leaf curl symptoms (Upadhyaya *et al.* 1991a). We have also provided evidence for the involvement of cytokinins in this leaf curl syndrome (Upadhyaya *et al.* 1991b, 1991c). By radioimmunoassays and high-performance liquid chromatography analyses, we have confirmed over-production of cytokinins zeatin (Z) and isopentenyladenine (iP) in culture medium of the *Rhizobium* strain IC3342 and increased levels of zeatin riboside (ZR) and dihydrozeatin riboside (DZR) in xylem exudates of IC3342-nodulated, leaf-curled plants compared to those in xylem exudates of non-nodulated plants, or of plants nodulated by a normal strain or by a Curl$^-$ mutant of the strain IC3342.

Phytohormones in general, and cytokinins in particular, are known to be involved in several leaf-curling diseases including: fasciation caused by *Corynebacterium fascians* (Roussaux 1965) and peach leaf curl caused by the fungus *Taphrina deformans* (Sziraki *et al.* 1975). Hormone action has also been postulated to be involved in the *Rhizobium*-legume symbiosis, especially in nodule compartmentalization (Vance 1983). *Rhizobium* genes may have a role in the regulation of growth hormone activity in nodulated plants, and *Rhizobium*-induced leaf curling may be due to an altered hormone regulation culminating in the over-production of cytokinins. Therefore, we believe that genetic analysis of the *Rhizobium* strain IC3342 with respect to the leaf curl induction may help in understanding hormone action at the molecular level in plant-microbe interactions.

In this paper, we report the isolation and characterization of Tn*5*-induced Curl$^-$ mutants of the leaf curl-inducing (Curl$^+$) *Rhizobium* strain IC3342 and the cloning of DNA fragments from five bacterial genetic regions determining this phenotype. We also present the DNA nucleotide sequence data of one of these regions. The transfer of this region to a Curl$^-$ *Rhizobium* strain (ANU240) confers a Curl$^+$ phenotype. The DNA sequences of two of the putative genes from this region show homologies with regulatory genes from *Escherichia coli* (Migula) Castellani and Chalmers (*ompR* and *fnr*) and from *Rhizobium meliloti* Dangeard (*fixK*) that are responsive to environmental stimuli.

## MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. Constructed plasmids, cosmids, and transconjugant strains are listed in Table 2. *E. coli* strains were grown on Luria (L) broth or L agar medium (Miller 1972) with ampicillin (Amp) (50 μg/ml), kanamycin (Km) (50 μg/ml), or tetracycline (Tc) (25 μg/ml) added for plasmid-containing strains. *Rhizobium* strains and transconjugants were routinely grown at 29° C on tryptone yeast (TY) (Beringer 1974) or yeast extract mannitol (YEM) agar medium (Vincent 1970) supplemented with appropriate antibiotics (i.e., streptomycin [Sm] [100 μg/ml], rifampicin [Rif] [100 μg/ml], Km [100 μg/ml], or Tc [25 μg/ml]). Tn*5*-induced mutants were purified and maintained in Bergersen's defined medium (BDM) (Bergersen 1961) with antibiotic selection.

**Tn5 mutagenesis.** Strain ANU1298, a spontaneous Sm$^r$ (streptomycin resistant) Rif$^r$ (rifampicin resistant) mutant of the strain IC3342 was used to generate transposon-induced mutants by conjugal transfer of the Tn*5*-containing suicide vector pSUP1011 (Simon *et al.* 1984) from *E. coli* SM10 as described by Kondorosi *et al.* (1977).

**Plant assays.** Tn*5*-induced mutants and transconjugants were tested for their phenotype on *Macroptilium atropurpureum* (Moc. & Sessé ex DC.) Urb. (siratro) grown on a mixture of sand and vermiculite (60:40, v/v) in test tubes (50 × 200 mm) using Fahraeus nutrient solution (Vincent 1970) under growth chamber conditions (14-hr day at 28° C and 10-hr night at 20° C) as described previously (Upadhyaya *et al.* 1991a). Mutants exhibiting a Curl$^-$ phenotype were further tested on pigeon pea to verify the stability of the phenotype. The experiment was conducted using a completely randomized block design, and statistical analysis was performed using GENSTAT (GENSTAT release 4.04, 1983, Rothamsted Experimental Station, UK). Nitrogenase activities in plants nodulated by the wild-type and mutant bacteria were determined as described previously (Upadhyaya *et al.* 1991a).

**Molecular techniques.** Total genomic DNA from the wild-type and mutant *Rhizobium* strains and recombinant plasmid DNA were isolated using established techniques (Maniatis *et al.* 1982). Restriction analysis, molecular cloning, DNA labeling (using random primers), Southern hybridization, cosmid genomic library construction, isolation

**Table 1.** Bacterial strains and plasmids used in this study

| Strain | Characteristics | Reference or source |
|---|---|---|
| *Rhizobium* | | |
| IC3342 | Leaf curl-inducing, fast-growing cowpea group, nodulates pigeon pea and siratro | Kumar Rao *et al.* 1984 |
| ANU240 | Sm$^r$ spontaneous mutant of fast-growing *Rhizobium* NGR234 | Trinick 1980 |
| ANU1298 | Sm$^r$ Rif$^r$ spontaneous mutant of IC3342 | This work |
| ANU3000 | Tn*5*-induced mutant of ANU1298 Nod$^+$ Fix$^-$ Curl$^-$ | This work |
| ANU3001 | Same as for ANU3000 | This work |
| ANU3002 | Tn*5*-induced mutant of ANU1298 Nod$^+$ Fix$^+$ Curl$^-$ | This work |
| ANU3003 | Same as for ANU3002 | This work |
| ANU3004 | Same as for ANU3002 | This work |
| IHP100 | Effective (Fix$^+$) fast-growing cowpea strain | Kumar Rao *et al.* 1984 |
| *E. coli* | | |
| RR1 | *hsdS20 ara14 proA2 lacY1 galK2*, *rpsL20* xyl-5 mtl-1 *supE44* lambda$^-$ F$^-$ | Bolivar *et al.* 1984 |
| SM10 | *thi thr leu tonA lacY supE recA* [RP4.2 Tc::Mu] Ap$^s$ Tc$^s$ Mu2$^+$ Km$^r$ Tra$^+$ | Simon *et al.* 1984 |
| HB101 | *pro leu thi lacY* Sm$^r$ *recA hsdS* | Ditta *et al.* 1980 |
| DH5 | F$^-$ *endA1 hsdR17* ($r_k^-$,$m_k^+$) *supE44 thi-1* lambda$^-$ *recA1 gyrA96 relA1* | Hanahan 1983 |
| JM107 | Δ(*lac-proAB*), *thi*, *supE44*, *relA1* [F' *traD36*, *proAB*, *lacIq*,*z*^ M15 | Yannisch-Perron *et al.* 1985 |
| Plasmids | | |
| pSUP1011 | Mobilizable (Ori-T$^+$) suicide vector containing Tn*5*; Km$^r$ Cm$^r$ | Simon *et al.* 1984 |
| pRK2013 | ColE1::pKK2 used as a helper in cosmid transfer from *E. coli* to *Rhizobium*, Km$^r$ Tra$^+$ | Ditta *et al.* 1980 |
| p5a | 3.2-kb *Eco*RI fragment from ANU240 containing *nifH* and *nifD* in pUC8 | Badenoch Jones *et al.* 1989 |
| pANU1 | 1.9-kb *Hin*dIII-*Bam*HI fragment from Tn*5* in pUC8 | J. M. Watson |
| pRt587 | 14-kb *Hin*dIII fragment from ANU843 *nod* region, in pBR328 | Schofield *et al.* 1984 |
| pLAFR3 | pRK290 (Tc$^r$) having lambda *cos*, *lacZ*, and multiple cloning site | Staskawicz *et al.* 1987 |
| pUC18/19 | Amp$^r$ *lacZ* multi-copy cloning vector | Yannisch-Perron *et al.* 1985 |
| M13mp18/19 | Bacteriophage M13 sequencing vector | Norrander *et al.* 1983 |

**Table 2.** Plasmids, cosmids, and transconjugant strains used in this study

| Mutant strain | Tn*5*-containing *Eco*RI fragment (kb) | Recombinant plasmid (in pUC19) | Homologous cosmids[a] | Transconjugants (mutants with homologous cosmids) | Transconjugants (ANU240 with cosmids) |
|---|---|---|---|---|---|
| ANU3000 | 6.9 | pMNU1 | pMNU36 (25) | ANU3009 | ANU3020 |
| ANU3001 | 7.2 | pMNU2 | pMNU27 (22) | ANU3010 | ANU3021 |
| ANU3002 | 8.2 | pMNU3 | pMNU31 (20) | ANU3011 | ANU3022 |
| ANU3003 | 6.7 | pMNU4 | pMNU15 (27) | ANU3012 | ANU3023 |
| | | | pMNU16 (22) | | ANU3024 |
| | | | pMNU17 (17) | | ANU3025 |
| | | | pMNU18 (19) | | ANU3026 |
| ANU3004 | 9.6 | pMNU5 | pMNU8 (27) | ANU3005 | ANU3016 |

[a] Insert size in kilobases in cosmid pLAFR3.

**Table 3.** Acetylene reduction activity (ARA), nodule number, nodule weight, and dry matter production of plants inoculated with strain ANU1298 and its Tn*5*-induced mutant derivatives[a]

| Strain | Phenotype Fix | Curl | ARA[b] | Nodule number | Nodule dry weight (mg) | Shoot dry weight (g) |
|---|---|---|---|---|---|---|
| ANU1298 | + | + | 177 | 49 | 54 | 0.44 |
| ANU3000 | − | − | 2 | 34 | 27 | 0.23 |
| ANU3001 | − | − | 3 | 40 | 38 | 0.25 |
| ANU3002 | + | − | 187 | 50 | 57 | 0.49 |
| ANU3003 | + | − | 195 | 48 | 55 | 0.47 |
| ANU3004 | + | − | 190 | 48 | 58 | 0.48 |
| ESE[c] | | | 17.2 | 3.8 | 3.0 | 0.036 |
| CV %[d] | | | 23 | 15 | 11 | 16 |

[a] Mean of three replicate plants.
[b] Measured as n moles $C_2H_4$ per hour by excised root assay in 500-ml bottles with 15% acetylene.
[c] Effective standard error.
[d] Coefficient of variation.

of homologous cosmid clones, cosmid mobilization, complementation, and subcloning into M13mp18/19 were performed using standard techniques (Maniatis *et al.* 1982). Plasmid profiles were visualized by the Eckhardt method as described by Plazinski *et al.* (1985).

**DNA sequence analysis.** DNA sequencing was carried out by the Sanger chain-termination method (Sanger *et al.* 1977) using M13mp18/19 (Norrander *et al.* 1983) and established techniques. To locate the position of the Tn*5* insertion in the *lcr1* region, sequencing of the *Rhizobium* DNA flanking the Tn*5* insertion was carried out using a Tn*5* primer (Schofield and Watson 1986). Further sequencing of the *lcr1* region was carried out with wild-type DNA sequences subcloned from the cosmid pMNU15 (Table 2). Three subclones, a 0.9-kb *Eco*RI fragment (pMNU22), a 1.7-kb *Hin*dIII fragment (pMNU44A and pMNU44B), and a 2.2-kb *Sal*I-*Bam*HI fragment (pMNU26) were used. Random *Sau*3A subclones were used to obtain sequence data for both strands and to resolve any ambiguities in the sequence. Sequence data were analyzed on a VAX computer using programs developed and compiled by the computer staff of the Research School of Biological Sciences, The Australian National University, A.C.T., Australia or the GCG sequence analysis software package (Devereux *et al.* 1984).

## RESULTS

**Transposon mutagenesis and isolation of Curl⁻ mutants.** Mating of the Sm$^r$ Rif$^r$ derivative strain ANU1298 with *E. coli* strain SM10 containing Tn*5* in a mobilizable suicide vector (pSUP1011) produced Km$^r$ transconjugants at a frequency of $1.6 \times 10^{-4}$ per recipient cell. Among 1,000 such transconjugants, 50% were Cm$^s$ (chloramphenicol sensitive), indicating loss of pSUP1011. Auxotrophic mutants were avoided by further purification on minimal medium. Screening of ~400 such Cm$^s$ transconjugants on siratro plants in test tubes under growth chamber conditions yielded two Fix$^-$ Curl$^-$ and three Fix$^+$ Curl$^-$ mutants. These five mutants, when tested on pigeon pea, showed the same phenotypes (Fig. 1). The nitrogenase activities of the nodules formed by the wild-type strain ANU1298 and the mutants are presented in Table 3. Nodule numbers did not vary significantly among plants inoculated with wild-type and Fix$^+$ Curl$^-$ mutants, whereas nodule numbers on plants inoculated with Fix$^-$ Curl$^-$ mutants were significantly lower than on plants inoculated with wild-type strain ANU1298.

**Genetic analysis of Curl$^-$ mutants.** We have previously reported the presence of three large plasmids in the strain IC3342 and have also shown by hybridization that the second largest plasmid is a Sym (symbiotic) plasmid (Upadhyaya *et al.* 1991a). Plasmid profiles of the five Curl$^-$ mutants were found to be similar to the wild-type strain (IC3342) in having three large plasmids (Fig. 2A). Hybridization of Southern blots of these plasmids with radioactively labeled Tn*5* sequences revealed that, in three of the Curl$^-$ mutants (ANU3001, ANU3003, ANU3004), Tn*5* was located on one of the plasmids (Fig. 2B). Probes for the *nod* (pRt587) and *nifHD* (p5A) genes hybridized to the same plasmid as the Tn*5* gene probe (data not shown). These data therefore indicate that the Tn*5* insertions in ANU3001, ANU3003, and ANU3004 are located on the Sym plasmid and that the Tn*5* insertions in strains ANU3000 and ANU3002 are chromosomally located.

Southern blot hybridization analysis of *Eco*RI-digested DNA from the five Tn*5*-induced mutants with radioactively labeled Tn*5* sequences showed that each possessed a distinctly sized, Tn*5*-containing *Eco*RI fragment in the size range of 6.7–9.7 kb (Fig. 3). To identify restriction sites around each Tn*5* insertion, the *Bgl*II-*Sma*I and *Bgl*II-*Bam*HI subfragments of Tn*5* (which specifically recognize the left side of Tn*5*) (Jorgensen *et al.* 1979) were used as hybridization probes. Southern blots of genomic DNA from each mutant, digested with a variety of restriction enzymes (for which Tn*5* had a single or no recognition site), were hybridized with one of these probes. From the hybridization data, the position of the first recognition site for each of the enzymes, on either side of the Tn5 insertion, was located for each of the mutants. These data indicate that the different regions, identified by Tn5 insertions, are not tightly clustered. The largest Tn5-containing fragments of the mutants were a 35.5-kb *Sma*I fragment from ANU3000, a 24-kb *Sma*I fragment from ANU3001, a 25-kb *Bam*HI fragment from ANU3002, a 19.5-kb *Bam*HI fragment from ANU3003, and a 25-kb *Sma*I fragment from ANU3004. Each of these had a distinctly different restriction pattern (Fig. 4). These data also indicate that the minimum possible distance between any of the three Sym plasmid-located Tn5 insertions is 16 kb, and that between the two chromosomally located Tn5 insertions is 21 kb.

To demonstrate whether any of the Tn5-containing fragments of the mutants carried *nif* structural genes, the Southern blots used for Tn5 probing were deprobed and rehybridized with radioactive-labeled sequences from the *nifH* gene and part of the *nifD* gene of the *Rhizobium* strain ANU240. None of the *nif*-hybridizing bands corresponded to those that hybridized to the Tn5 probe (data not shown), indicating that none of the Tn5 insertions in these mutants are closely linked to the *nifHD* structural genes.

**Molecular cloning of Tn5-containing genomic fragments.** Genomic *Eco*RI fragments containing the intact Tn5 transposon and flanking *Rhizobium* sequences were cloned from each of the five mutants into pUC19. Transformants con-

A B C D E

F G H I J K

**Fig. 1.** Phenotype of Tn5-induced mutants. Pigeon pea plants inoculated with **A** and **J,** wild-type *Rhizobium* strain IHP100; **E** and **K,** wild-type leaf curl-inducing *Rhizobium* strain ANU1298 (leaf-curling symptoms are indicated by arrows); **C,** the Fix$^-$ Curl$^-$ mutants ANU3000 and **D,** ANU3001; and **G,** the Fix$^+$ Curl$^-$ mutants ANU3002, **H,** ANU3003, and **I,** ANU3004. **B** and **F** are uninoculated plants.

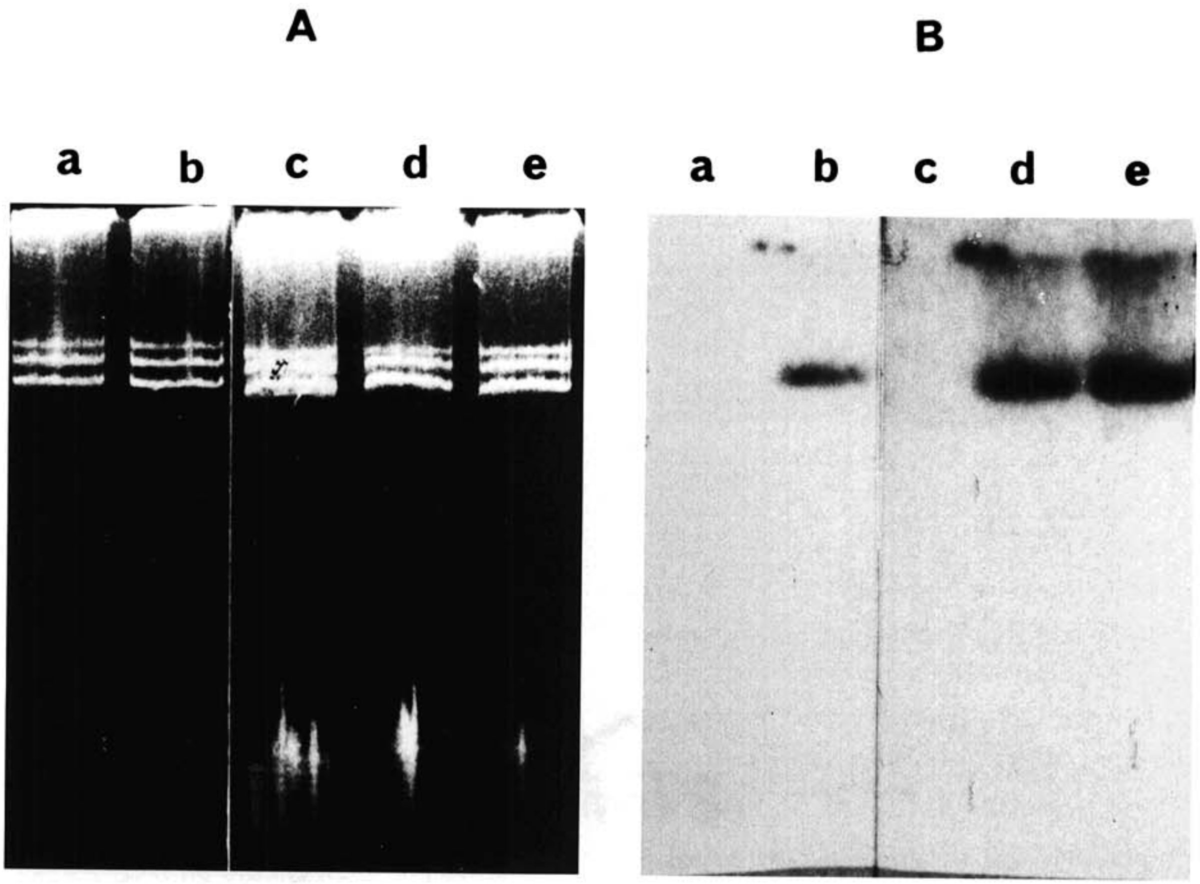


**Fig. 2.** Plasmid profiles of Tn*5*-induced mutants and hybridization with a Tn*5* probe. **A,** mega-plasmids of Tn*5*-induced mutant strains (a) ANU3000, (b) ANU3001, (c) ANU3002, (d) ANU3003, and (e) ANU3004 were visualized by a modified Eckhardt method (Plazinski *et al.* 1985). **B,** $^{32}$P-labeled plasmid pANU1 (Table 1) was used as a Tn*5* hybridization probe on the Southern blots of plasmids from (a) ANU3000, (b) ANU3001, (c) ANU3002, (d) ANU3003, and (e) ANU3004 (see text for details).

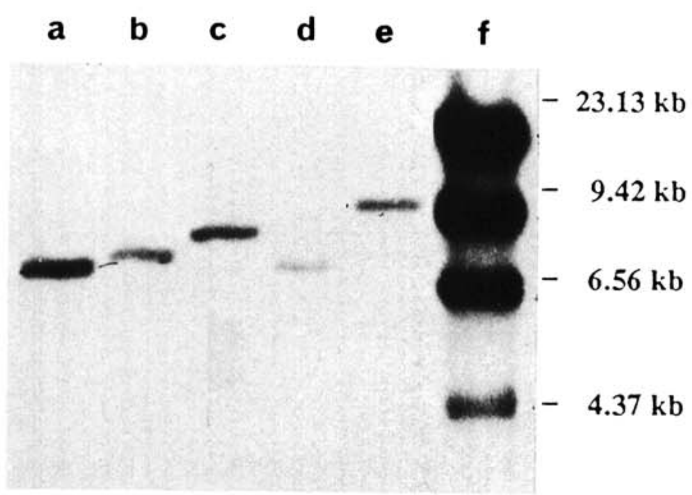


**Fig. 3.** Identification of Tn*5* insertions in Curl$^-$ mutants and molecular cloning of mutant fragments. Plasmid pANU1 (Table 1) sequences were used as a Tn*5* hybridization probe on Southern blots of *Eco*RI-digested genomic DNA from **A,** ANU3000; **B,** ANU3001; **C,** ANU3002; **D,** ANU3003; and **E,** ANU3004. A $^{32}$P-labeled, *Hin*dIII digest of bacteriophage lambda DNA (F) was used as a size marker.

taining these recombinant plasmids were selected on L agar plates containing Amp and Km. The cloned Tn*5*-containing *Eco*RI fragment in each of the recombinant plasmids and the Tn*5*-hybridizing *Eco*RI fragment in the genomic DNA of the corresponding mutant had the same size (data not shown). The recombinant plasmids are detailed in Table 2.

**Isolation of wild-type gene sequences and complementation.** A genomic library of the curl-inducing *Rhizobium* strain ANU1298, using *Sau*3A partially digested DNA, was constructed in the cosmid vector pLAFR3. Cosmids containing wild-type DNA sequences homologous to the mutant fragments were selected by colony hybridization using the corresponding Tn*5*-containing *Eco*RI fragments as probes. Selected cosmids were digested with *Eco*RI, Southern blotted, and probed with the corresponding Tn*5*-containing fragment to confirm the presence of the homologous wild-type sequences. Using the restriction enzyme digestion data, these overlapping cosmids were mapped with respect to the location of the Tn*5* insertions in each of the mutants (Fig. 4). One representative cosmid for each of the five identified genetic regions was transformed into *E. coli* strain DH5 and then plate-mated with the respective mutant strain in the presence of *E. coli* strain HB101 containing the mobilizing plasmid pRK2013. Purified transconjugants were inoculated onto siratro and pigeon pea plants. In each case, the Tn*5*-induced mutation was corrected by the introduction of the corresponding wild-type DNA into the mutant, as indicated by the Curl$^+$ Fix$^+$ phenotype of the transconjugants. Bacteria re-isolated from the nodules of these plants were found to be Sm$^r$ Rif$^r$ Km$^r$ Tc$^r$.

**Identification of structural homologues of IC3342 genetic loci in *Rhizobium* strain ANU240.** We have previously reported that the strain IC3342 resembles the *Rhizobium* strain ANU240 in its growth rate, plasmid profile, nodulation host range, and conserved *nif* and *nod* gene sequences (Upadhyaya *et al.* 1991a). However, strain ANU240 does not induce leaf curling. To examine whether strain ANU240 contains structural homologues of any of the IC3342 genetic loci, identified to be involved in leaf curling, *Eco*RI-digested genomic DNA of the strain ANU240 was probed with radioactive-labeled DNA sequences flanking the Tn*5* insertions of each of the mutants. The sizes of these flanking DNA were small except those from ANU3004 (see Table 2). Homologies to each of the loci except that of ANU3003 were found in the ANU240 genome (Fig. 5). These data

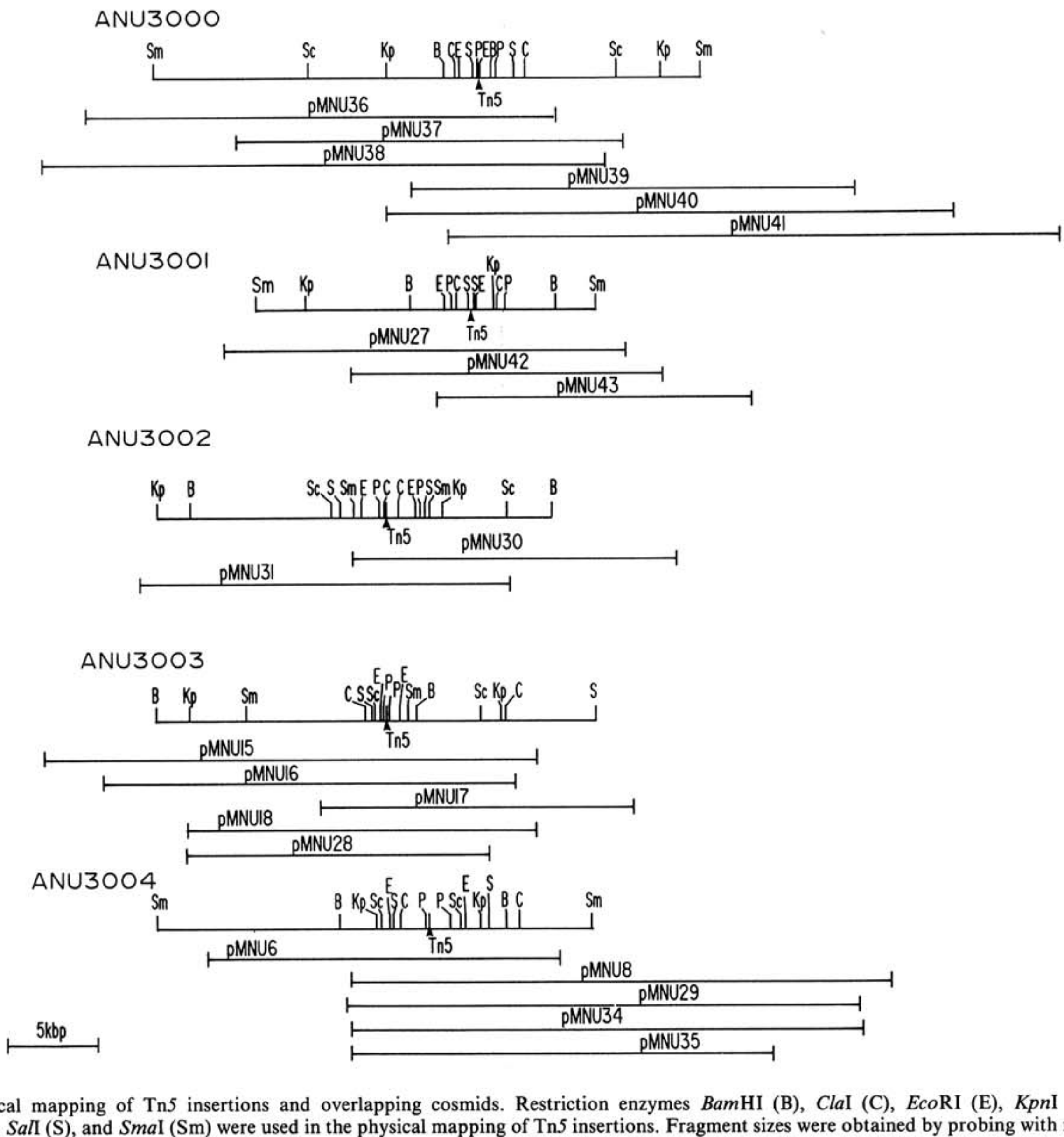


**Fig. 4.** Physical mapping of Tn*5* insertions and overlapping cosmids. Restriction enzymes *Bam*HI (B), *Cla*I (C), *Eco*RI (E), *Kpn*I (Kp), *Pvu*II (P), *Sac*I (Sc), *Sal*I (S), and *Sma*I (Sm) were used in the physical mapping of Tn*5* insertions. Fragment sizes were obtained by probing with radioactively labeled Tn*5* sequences from the central *Bgl*II fragment (Jorgensen *et al.* 1979). The relative position of the restriction site to the left side of each Tn*5* insertion, for each of the enzymes used, was located by hybridization of genomic DNA restricted singly (in the case of *Bam*HI, *Sma*I, or *Sal*I) or in combination with *Sma*I or *Bam*HI (in the case of other enzymes) as required, to radioactively labeled sequences from the lefthand side of Tn*5* (*Bgl*II-*Sma*I or *Bgl*II-*Bam*HI). The positions of the restriction sites on the other side of the Tn*5* insertion were then inferred from the hybridization data of single digests. Arrowheads indicate the mapped location of Tn*5* in each mutant. Isolated cosmids were digested with various restriction enzymes, and the fragment sizes were used to orient the overlapping cosmids in relation to the location of the Tn*5* insertion in each mutant.

suggest that the strain IC3342 harbours gene(s) unique and/or central to leaf curl induction that are not present in the strain ANU240. They also suggest that some of the genes required for leaf curling are also present in other *Rhizobium* strains.

**Cosmid-mediated transfer of the Curl$^+$ phenotype to *Rhizobium* strain ANU240.** To further test the abovementioned predictions, one representative cosmid containing the wild-type homologue of the mutated region of each of the mutants was introduced into the Curl$^-$ *Rhizobium* strain ANU240 by triparental mating. Purified transconjugants were then tested for their phenotype on siratro plants. The transconjugant (ANU3023), with a cosmid containing wild-type sequences homologous to the mutant region of ANU3003, produced leaf curling similar to that induced by the wild-type strain ANU1298, whereas the others did not confer this phenotype (data not shown). The four cosmids overlapping this region in ANU3003 (Fig. 4) were then transferred to ANU240, and transconjugants were tested for leaf curl induction. Three of these transconjugant strains produced complete leaf curl symptoms, whereas the fourth (pMNU16) produced intermediate symptoms (Fig. 6).

This particular genetic region was designated as the leaf curl response (*lcr1*) region, because it contains gene(s) required for leaf curl induction. A restriction map of the

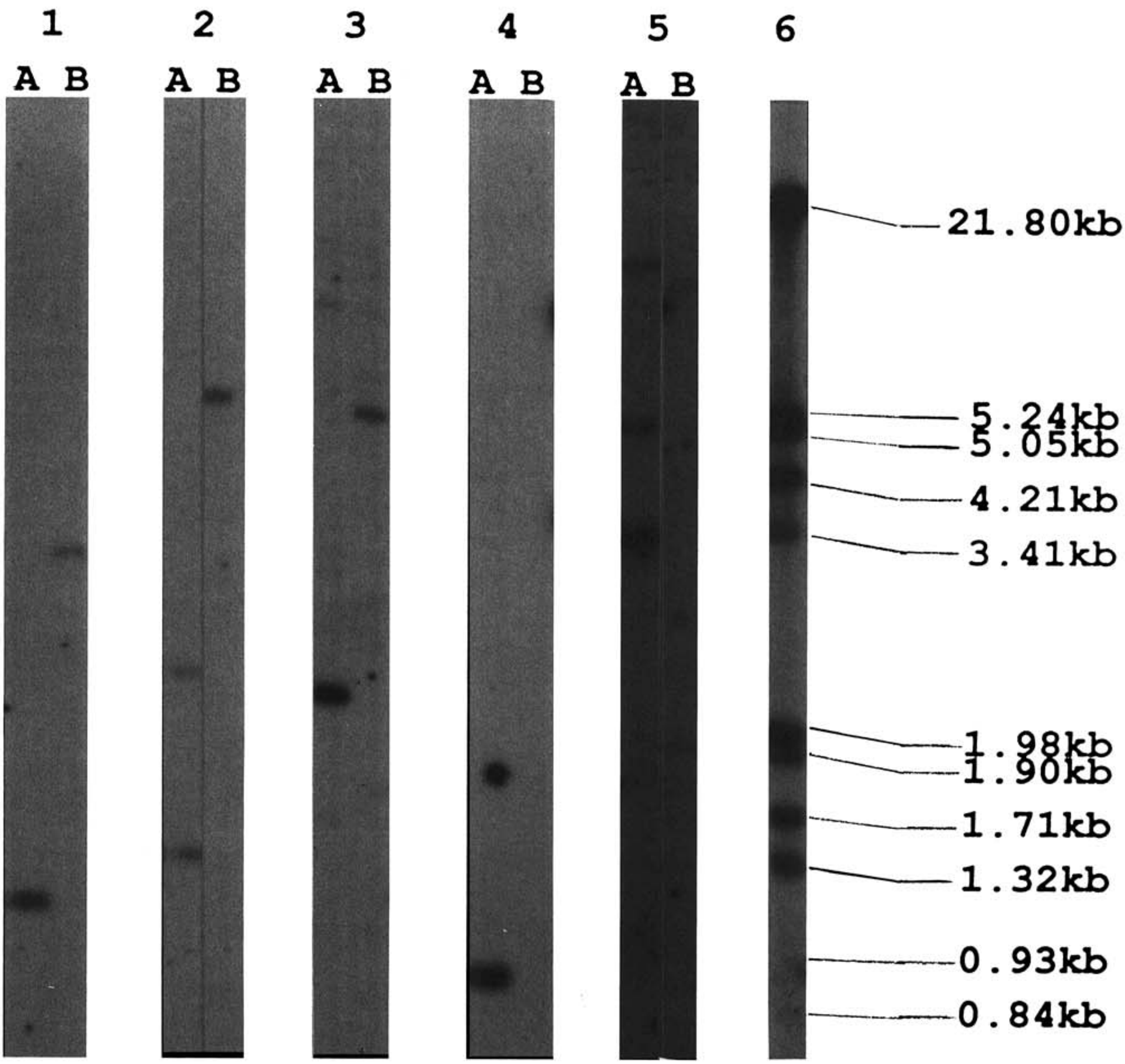


**Fig. 5.** Identification of structural homologues of *Rhizobium* strain IC3342 genetic loci in *Rhizobium* strain ANU240. Autoradiogram of Southern blots with *Eco*RI-digested genomic DNA fragments of **A,** leaf curl-inducing strain ANU1298 and **B,** the nonleaf curl strain ANU240 hybridized with $^{32}P$-labeled DNA sequences flanking Tn*5* insertion in (1) pMNU1, (2) pMNU2, (3) pMNU3, (4) pMNU4, and (5) pMNU5. Lane 6 is *Eco*RI-*Hin*dIII digested bacteriophage lambda DNA hybridized with $^{32}P$-labeled lambda DNA as size markers.

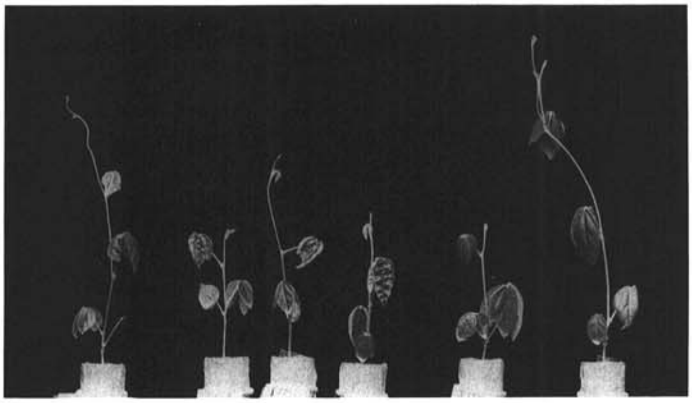


**Fig. 6.** Cosmid-mediated transfer of leaf curl-inducing ability to strain ANU240. Cosmids containing wild-type sequences homologous to mutant DNA were mobilized to the strain ANU240 by conjugal transfer in presence of a helper plasmid (*Escherichia coli* strain HB101 containing pRK2013). The transconjugants were screened on siratro plants for their phenotype. Siratro plants inoculated with **A,** leaf curl-inducing strain ANU1298; transconjugant strains **B,** ANU3023 (ANU240/pMNU15); **C,** ANU3025 (ANU240/pMNU17); **D,** ANU3026 (ANU240/pMNU18); **E,** ANU3024 (ANU240/pMNU16); and **F,** recipient strain ANU240.

region around *lcr1*, together with overlapping cosmids, are shown in Figure 7. The position of the Tn*5* insertion in the mutant strain ANU3003 was mapped within the region of overlap. Based on plant phenotypes produced by these overlapping cosmids, a maximum of 11 kb of the *lcr1* region could be involved in conferring the leaf-curling phenotype (Fig. 7).

**DNA sequence of the *lcr1* region.** Computer analysis of a 3.29-kb sequence of DNA in the *lcr1* region revealed the presence of three open reading frames (ORFs) longer than 500 bp in the upper strand sequence and two in the complementary lower strand sequence. The sequence data for both strands, along with the deduced amino acid sequences of the coding regions of the ORFs, are presented in Figure 8.

ORF1 has an ATG codon at position 33 downstream from the *Hin*dIII site, with a termination codon preceding it. No obvious ribosome-binding sequence (Shine and Dalgarno 1975) was detected in the sequence preceding the ATG codon.

The 264-bp region between the termination codon of ORF1 and the initiation codon of ORF2 has several interesting features. Fifteen basepairs upstream from the ATG

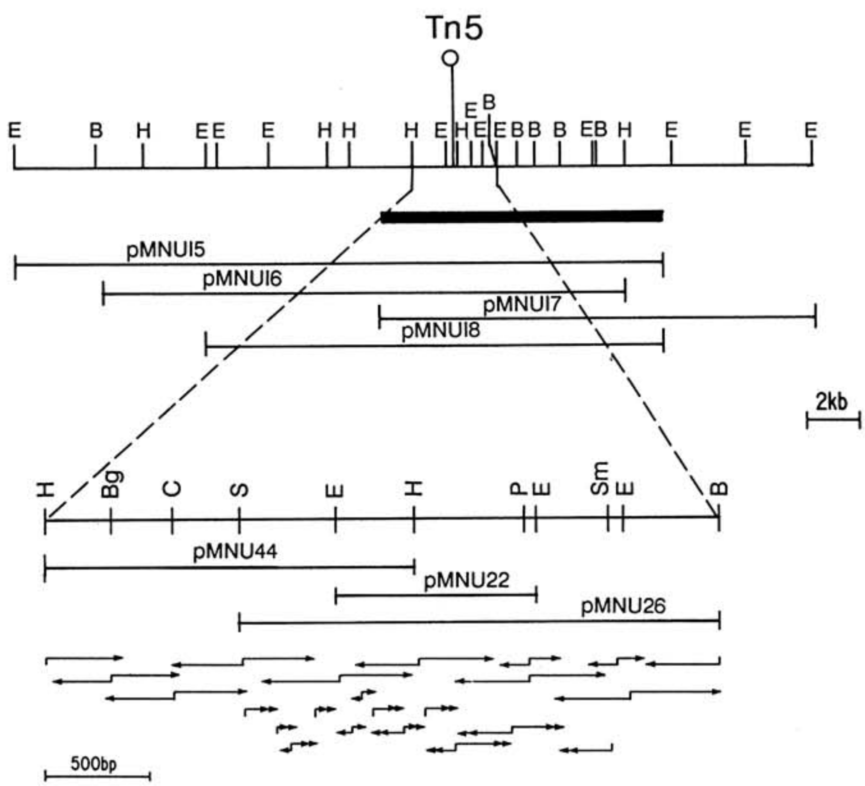


**Fig. 7.** Physical map of *lcr1* showing overlapping cosmids and sequencing strategy. Restriction endonucleases used in the physical mapping of the *lcr1* region were *Bam*HI (B), *Bgl*II (Bg), *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), *Sal*I (S), and *Sma*I (Sm). The cosmids with overlapping sequences that were isolated from a genomic library by hybridization were pMNU15, pMNU16, pMNU17, and pMNU18. The position of the Tn*5* insertion in ANU3003 is indicated by ⚲. The maximum region of DNA from strain ANU1298 that was essential for transfer of the leaf curl phenotype to strain ANU240 is indicated by the thick line. Recombinant plasmids, pMNU22 (0.9-kb *Eco*RI fragment), pMNU26 (2.2-kb *Bam*HI-*Sal*I fragment), and pMNU44 (1.7-kb *Hin*dIII fragment), each subcloned from pMNU15, were used in physical mapping and further subcloning for DNA sequencing by the chain termination method. The direction and extent of sequencing is indicated by horizontal arrows. *Sau*3A subclones (indicated by →→) were used to resolve ambiguities.

codon of ORF2 lies a sequence (boxed in Fig. 8) that resembles the consensus *E. coli* ribosome-binding site. There are sequences resembling the −35 and −10 promoter elements of *E. coli* in the further upstream region (underlined in Fig. 8).

ORF3 overlaps with the stop codon of ORF2 at the sequence GTGA. The N-terminal end of the coding region of ORF3 contains cysteine clusters: C-2-C-4-C-3-C. The position of the Tn*5* insertion (indicated by the arrowhead in Fig. 8) in the mutant ANU3003 is within this cysteine cluster and thus would be expected to disrupt the function of the gene product of ORF3. The first 25 amino acid residues are highly hydrophobic, and the hydropathy profile of this region resembles that of a signal peptide (Sjostrom *et al.* 1987).

There are two overlapping ORFs in the sequences of the complementary strand. ORF4 has its initiation codon at position 2403 because this is the first ATG codon in this phase following a stop codon. The region preceding the ATG codon has a sequence 5′ GGAAAGACGGAG 3′ (boxed in Fig. 8) that might function as a ribosome-binding site. Sequences resembling the consensus *E. coli*

```
                      *                     ORF1  M  R  R  E  H  A  K  L  D  E  L  R  L  S  T
(5')..  AAGCTTTGTAGACCGCCTCACTCCTCGACAGTATGCGGCGAGAACACGCAAAGTTAGACGAGCTTCGCCTCAGCACA     77
(3')..  TTCGAAACATCTGGCGGAGTGAGGAGCTGTCATACGCCGCTCTTGTGCGTTTCAATCTGCTCGAAGCGGAGTCGTGT

 W  N  D  H  A  D  H  Q  R  F  C  N  G  P  T  K  A  L  V  S  T  G  F  S  Q  Q  N  L
TGGAACGATCACGCCGATCATCAACGCTTTTGCAACGGGCCCACGAAGGCACTTGTCAGCACGGGTTTCTCTCAGCAAAACCTT   161
ACCTTGCTAGTGCGGCTAGTAGTTGCGAAAACGTTGCCCGGGTGCTTCCGTGAACAGTCGTGCCCAAAGAGAGTCGTTTTGGAA

 L  L  K  A  Q  H  L  R  F  R  D  R  Q  R  V  P  Q  G  T  G  G  I  E  E  C  A  V  Q
CTGCTCAAAGCTCAGCATCTCCGGTTTCGCGACCGGCAGCGCGTTCCCCAAGGTACGGGAGGTATTGAGGAGTGCGCGGTTCAG   245
GACGAGTTTCGAGTCGTAGAGGCCAAAGCGCTGGCCGTCGCGCAAGGGGTTCCATGCCCTCCATAACTCCTCACGCGCCAAGTC

 G  S  A  N  D  D  G  D  A  E  R  T  G  N  L  R  G  G  D  V  E  R  F  R  S  P  R  C
GGGAGCGCGAACGACGATGGTGACGCCGAGCGTACGGGCAATCTTCGAGGCGGTGACGTGGAACGCTTCAGATCTCCCCGTTGC   329
CCCTCGCGCTTGCTGCTACCACTGCGGCTCGCATGCCCGTTAGAAGCTCCGCCACTGCACCTTGCGAAGTCTAGAGGGGCAACG

 S  S  S  E  V  A  S  A  S  S  T  S  L  G  P  A  I  S  P  S  T  A  T  D  I  S  R  A
AGTTCGTCGGAGGTCGCAAGCGCTTCCAGCACGAGCCTAGGTCCCGCCATTTCGCCATCGACTGCGACCGACATTTCACGTGCC   413
TCAAGCAGCCTCCAGCGTTCGCGAAGGTCGTGCTCGGATCCAGGGCGGTAAAGCGGTAGCTGACGCTGGCTGTAAAGTGCACGG

 S  D  S  R  P  L  D  A  R  C  S  P  R  H  A  I  Q  A  R  N  R  S  H  R  V  A  P  A
AGCGATTCGCGCCCACTCGACGCGCGCTGCTCACCTCGACATGCCATCCAGGCCAGGAACCGAAGCCACAGAGTAGCACCGGCA   497
TCGCTAAGCGCGGGTGAGCTGCGCGCGACGAGTGGAGCTGTACGGTAGGTCCGGTCCTTGGCTTCGGTGTCTCATCGTGGCCGT

 D  E  G  S  T  K  T  R  K  K  V  N  G  A  R  R  V  R  D  R  P  R  A  R  G  R  E  V
GACGAGGGCAGCACAAAGACCAGGAAGAAGGTCAACGGCGCTCGCCGCGTCAGGGACAGACCGCGTGCACGGGGGCGAGAGGTC   581
CTGCTCCCGTCGTGTTTCTGGTCCTTCTTCCAGTTGCCGCGAGCGGCGCAGTCCCTGTCTGGCGCACGTGCCCCCGCTCTCCAG

 P  S  A  T  V  G  V  D  G  D  L  L  I  D  G  L  P  A  L  T  A  A  A  G  Q  Q  W  *
CCGTCCGCGACAGTGGGTGTTGACGGAGATTTGCTCATCGATGGTCTTCCCGCGCTGACGGCAGCCGCTGGCCAACAGTGGTGA   665
GGCAGGCGCTGTCACCCACAACTGCCTCTAAACGAGTAGCTACCAGAAGGGCGCGACTGCCGTCGGCGACCGGTTGTCACCACT

AAGACATTGCTCTAGCCCAAAGACAGCTCTTCGAAATTGATCCAACGCAAGATCGGGGCTCTTCTTTTGGGTACAACGCAAAG   748
TTCTGTAACGAGATCGGGTTTCTGTCGAGAAGCTTTAACTAGGTTGCGTTCTAGCCCCGAGAAGAAAACCCATGTTGCGTTTC
                                                                                  *
CAAAAACGACAATGAGCTCGTGGCCGGGAAGCGCCCCCCGGAACGCACGCAAAAGAGCACCGGAAAAATTGCCCTCGAGTTTGA   832
GTTTTTGCTGTTACTCGAGCACCGGCCCTTCGCGGGGGGCCTTGCGTGCGTTTTCTCGTGGCCTTTTTAACGGGAGCTCAAACT

CGCCGGACAAAGTTCAATAATGAGTACGTGGTATCGGCTGTTCGGGAACGAGCTGCAAGAAGACGGGCTAAGTCTTTGGCTGGA   916
GCGGCCTGTTTCAAGTTATTACTCATGCACCATAGCCGACAAGCCCTTGCTCGACGTTCTTCTGCCCGATTCAGAAACCGACCT

      ORF2  M  P  H  L  I  I  V  D  D  D  P  R  I  R  S  M  L  S  R  Y  L  E  D  E
AATGGATTATCAATGCCGCACCTGATTATCGTCGACGATGATCCGCGGATTCGCTCCATGCTTTCACGTTATCTCGAAGACGAG  1000
TTACCTAATAGTTACGGCGTGGACTAATAGCAGCTGCTACTAGGCGCCTAAGCGAGGTACGAAAGTGCAATAGAGCTTCTGCTC

 G  F  R  V  R  L  A  E  N  I  S  Q  L  R  R  V  L  S  P  S  V  D  L  V  L  L  D  I
GGCTTTCGCGTGAGGCTGGCGGAGAATATTTCGCAGTTGCGCCGGGTGCTATCTCCGTCTGTTGACCTGGTGCTGCTCGACATC  1084
CCGAAAGCGCACTCCGACCGCCTCTTATAAAGCGTCAACGCGGCCCACGATAGAGGCAGACAACTGGACCACGACGAGCTGTAG
                                                 *  R  R  R  N  V  Q  H  Q  E  V  D

 G  L  P  D  G  N  G  L  E  L  A  R  E  I  D  A  N  F  R  V  P  T  I  I  V  S  G  R
GGACTTCCCGACGGCAACGGATTGGAGCTCGCACGCGAGATCGACGCGAATTTTCGCGTTCCAACCATAATCGTCTCCGGGCGC  1168
CCTGAAGGGCTGCCGTTGCCTAACCTCGAGCGTGCGCTCTAGCTGCGCTTAAAAGCGCAAGGTTGGTATTAGCAGAGGCCCGCG
 S  K  G  V  A  V  S  Q  L  E  C  A  L  D  V  R  I  K  A  N  W  G  Y  D  D  G  P  A
```

(*continued on next page*)

**Fig. 8.** Nucleotide sequence of the 3.29-kb *Rhizobium* strain IC3342 (ANU1298) *lcr1* region. Deduced amino acid sequences of five open reading frames (ORFs) (three in the upper strand and two in the lower complementary strand), possible ribosome-binding sites (boxed), putative promoter sequences (underlined), translation stop codons (*), and possible transcription terminator sequences (arrow underline) are indicated. The Tn*5* insertion site in the mutant ANU3003 is indicated by the arrowhead. Cysteine residues in ORF3 are boxed.

−35 promoter elements are also present further upstream (underlined in Fig. 8).

ORF5 overlaps with ORF4 in the sequence ATGA. The Tn*5* insertion in the mutant strain ANU3003 was 20 bp upstream of the C-terminal end of ORF4 (indicated by the arrowhead in Fig. 8).

In the sequences downstream from the termination codon of ORF1, ORF3, and ORF5, there are sequences potentially capable of forming stable secondary (stem and loop) structures (indicated by arrowed underlines in Fig. 8) that may be involved in transcriptional termination (Steitz *et al.* 1982).

**Sequence homologies of ORF2 and ORF4 with regulatory genes from *E. coli* and *R. meliloti*.** To predict the possible functions encoded by the putative genes from the *lcr1* region, a search for sequence homology was carried out against the GENBANK/EMBL database using the algorithm of Wilbur and Lipman (1983). Those database sequences showing homology were identified, and sequence comparisons were made between the deduced amino acid sequences of the coding regions using the GCG program GAP (Devereux *et al.* 1984). These searches showed that ORF2 has substantial homology to the outer membrane protein (*ompR*) gene from *E. coli,* whereas ORF4 has homology to the transcriptional activator protein gene (*fnr*) of the anaerobic respiratory pathways in *E. coli* (Spiro and Guest 1987) and the regulatory gene *fixK* from *R. meliloti* (Batut *et al.* 1989).

Amino acid sequence comparison data between the ORF2 and *ompR* gene products is presented in Figure

(*continued from preceding page*)

```
 D  D  D  V  D  R  I  I  G  L  E  M  G  A  D  D  Y  V  S  K  P  F  N  L  R  E  L  L
GACGACGACGTCGATCGTATCATCGGTCTGGAGATGGGGGCCGATGACTATGTGTCGAAGCCATTCAACCTTCGCGAACTGTTG  1252
CTGCTGCTGCAGCTAGCATAGTAGCCAGACCTCTACCCCCGGCTACTGATACACAGCTTCGGTAAGTTGGAAGCGCTTGACAAC
 V  V  V  D  I  T  D  D  T  Q  L  H  P  G  I  V  I  H  R  L  W  E  V  K  A  F  Q  Q

 A  R  V  R  S  V  L  R  R  S  Q  R  A  M  P  L  G  P  A  Q  K  A  T  A  G  I  F  H
GCGCGCGTAAGAAGCGTATTGAGACGATCGCAGCGGGCAATGCCGCTCGGACCGGCGCAGAAGGCCACAGCCGGTATCTTTCAT  1336
CGCGCGCATTCTTCGCATAACTCTGCTAGCGTCGCCCGTTACGGCGAGCCTGGCCGCGTCTTCCGGTGTCGGCCATAGAAAGTA
 R  A  Y  S  A  Y  Q  S  S  R  L  P  C  H  R  E  S  R  R  L  L  G  C  G  T  D  K  M

 F  D  G  W  M  L  D  A  D  R  R  Q  L  T  S  T  A  G  Q  T  I  E  L  T  T  G  E  F
TTCGACGGTTGGATGCTCGACGCCGACCGGCGTCAACTGACGTCCACCGCAGGGCAGACGATCGAACTCACTACCGGCGAATTC  1420
AAGCTGCCAACCTACGAGCTGCGGCTGGCCGCAGTTGACTGCAGGTGGCGTCCCGTCTGCTAGCTTGAGTGATGGCCGCTTAAG
 E  V  T  P  H  E  V  G  V  P  T  L  Q  R  G  G  C  P  L  R  D  F  E  S  G  A  F  E

 D  L  L  M  V  F  V  T  H  P  G  R  V  L  T  R  D  F  L  L  D  Q  T  R  G  R  T  R
GACCTGCTCATGGTTTTCGTCACGCATCCGGGCCGCGTCCTGACGCGCGACTTTCTTCTGGATCAGACACGCGGCCGCACGCGA  1504
CTGGACGAGTACCAAAAGCAGTGCGTAGGCCCGGCGCAGGACTGCGCGCTGAAAGAAGACCTAGTCTGTGCGCCGGCGTGCGCT
 V  Q  E  H  N  E  D  R  M  R  A  A  D  Q  R  A  V  K  K  Q  I  L  C  A  A  A  R  S

 E  A  F  D  R  A  I  D  V  Q  V  T  R  L  R  A  K  V  E  D  D  P  G  D  P  R  R  I
GAGGCATTCGACCGCGCGATCGACGTGCAGGTCACGCGCCTTAGGGCAAAGGTGGAAGACGATCCGGGAGATCCGCGACGGATA  1588
CTCCGTAAGCTGGCGCGCTAGCTGCACGTCCAGTGCGCGGAATCCCGTTTCCACCTTCTGCTAGGCCCTCTAGGCGCTGCCTAT
 L  C  E  V  A  R  D  V  H  L  D  R  A  K  P  C  L  H  F  V  I  R  S  I  R  S  P  Y

                                                  ORF3   V  T  I  S  A  R  R  N [C] A
 K  S  V  R  G  A  G  Y  V  F  A  A  K  V  F  R  P  T  *
AAGTCCGTGCGCGGAGCCGGCTACGTATTTGCGGCGAAGGTCTTCCGCCCAACGTGACGATATCCGCGCGACGCAATTGCGCC  1671
TTCAGGCACGCGCCTCGGCCGATGCATAAACGCCGCTTCCAGAAGGCGGGTTGCACTGCTATAGGCGCGCTGCGTTAACGCGG
 L  G  H  A  S  G  A  V  Y  K  R  R  L  D  E  A  W  R  S  S  I  R  A  V  C  N  R  R
                      ▼
 S [C] L  G  I  S [C] T  A  L [C] A  V  I  A  L  P  V  R  K  S  D  Q  R  V  H  F  Q
TCATGCCTTGGCATATCTTGCACTGCGCTGTGCGCCGTCATCGCACTCCCCGTCCGCAAGAGCGATCAGCGCGTCCATTTTCAG  1755
AGTACGGAACCGTATAGAACGTGACGCGACACGCGGCAGTAGCGTGAGGGGCAGGCGTTCTCGCTAGTCGCGCAGGTAAAAGTC
 * A  K  A  Y  R  A  S  R  Q  A  G  D  D  C  E  G  D  A  L  A  I  L  A  D  M  K  L
  M   ORF5

 D  G  D  G  V  T  P  D  N  R  Y  D  P  A  A  G  K  L  G  D  S  P  R  D  G  F  D  R
GATGGCGATGGCGTGACGCCCGACAATCGCTATGACCCCGCTGCCGGCAAGCTTGGTGATAGTCCGCGAGACGGTTTCGATCGT  1839
CTACCGCTACCGCACTGCGGGCTGTTAGCGATACTGGGGCGACGGCCGTTCGAACCACTATCAGGCGCTCTGCCAAAGCTAGCA
 I  A  I  A  H  R  G  V  I  A  I  V  G  S  G  A  L  K  T  I  T  R  S  V  T  E  I  T

 H  A  Q  I  V  G  N  I  E  P  C  H  R  Q  F  D  D  R  A  P  V  L  R  L  A  A  C  H
CATGCCCAGATAGTCGGCAACATCGAGCCGTGTCATCGGCAGTTCGATGACCGGGCGCCGGTTCTCCGATTGGCCGCGTGCCAT  1923
GTACGGGTCTATCAGCCGTTGTAGCTCGGCACAGTAGCCGTCAAGCTACTGGCCCGCGGCCAAGAGGCTAACCGGCGCACGGTA
 M  G  L  Y  D  A  V  D  L  R  T  M  P  L  E  I  V  P  R  R  N  E  S  Q  G  R  A  M

 H  Q  Q  E  T  G  H  F  F  L  G  T  P  T  R  Q  K  N  H  L  I  L  G  G  R  H  L  V
CATCAGCAGGAAACCGGCCACTTTTTCCTCGGCACTCCTACGCGACAGAAGAACCATCTGATCCTGGGCGGCCGCCATCTCGTC  2007
GTAGTCGTCCTTTGGCCGGTGAAAAAGGAGCCGTGAGGATGCGCTGTCTTCTTGGTAGACTAGGACCCGCCGGCGGTAGAGCAG
 M  L  L  F  G  A  V  K  E  E  A  S  R  R  S  L  L  V  M  Q  D  Q  A  A  A  M  E  D
```

(*continued on next page*)

9. Forty-three percent of the deduced amino acid residues of ORF2 are identical to those of *ompR* and another 22% have conserved amino acid changes.

The predicted amino acid sequence of ORF4 has strong homology with that of the *fnr* (also called *nirR* or *nirA*) gene product of *E. coli* and the *fixK* gene product of *R. meliloti* (Fig. 10). Thirty-three percent of the deduced amino acid residues of the ORF4 gene product are identical to those of the *fixK* gene product with another 28% having conserved changes. The amino acid sequence identity and similarity between the ORF4 and *fnr* gene products were 29 and 58%, respectively. Interestingly, the ORF4 gene product has the conserved N-terminal glycine residues and the possible helix-turn-helix motif (a potential DNA-binding motif) in the C-terminal end (boxed in Fig. 10) that are conserved among the gene products of *fixK* and *fnr* (Batut *et al.* 1989).

The database searches failed to identify significant homology of ORF1, ORF3, or ORF5 with any other known gene sequences.

## DISCUSSION

The data presented in this paper indicate that the induction of the leaf-curling syndrome requires the expression of a number of bacterial genes, possibly including some of the nitrogen fixation genes, and that the genetic region *lcr1* contains putative regulatory genes required for leaf curl induction.

(*continued from preceding page*)

```
 A  E  A  G  E  K  L  L  P  Q  M  W  R  S  R  A  F  A  F  E  T  T  P  R  K  A  P  Q
GCAGAGGCGGGAGAAAAGTTGCTCCCTCAGATGTGGCGCTCGCGCGCTTTCGCTTTCGAAACGACGCCGCGAAAAGCGCCGCAG   2091
CGTCTCCGCCCTCTTTTCAACGAGGGAGTCTACACCGCGAGCGCGCGAAAGCGAAAGCTTTGCTGCGGCGCTTTTCGCGGCGTC
 C  L  R  S  F  L  Q  E  R  L  H  P  A  R  A  S  E  S  E  F  R  R  R  S  F  R  R  L

 F  D  V  G  D  G  F  H  R  V  E  V  V  L  L  D  G  D  A  E  *
TTCGACGTGGGTGATGGCTTCCACCGTGTAGAGGTAGTGCTCCTTGACGGAGACGCCGAGTAAATCTCCGGGCCGCAGGAAGCC   2175
AAGCTGCACCCACTACCGAAGGTGGCACATCTCCATCACGAGGAACTGCCTCTGCGGCTCATTTAGAGGCCCGGCGTCCTTCGG
 E  V  H  T  I  A  E  V  T  Y  L  Y  H  E  K  V  S  V  G  L  L  D  G  P  R  L  F  G

GATGATGACACGACGGCCATCATTCAGAATCCTGACTGCCCGCAAGGTGCCTTCAACGACCTCAAAGATATGCCTCGCCTGATC   2259
CTACTACTGTGCTGCCGGTAGTAAGTCTTAGGACTGACGGGCGTTCCACGGAAGTTGCTGGAGTTTCTATACGGAGCGGACTAG
 I  I  V  R  R  G  D  N  L  I  R  V  A  R  L  T  G  E  V  V  E  F  I  H  R  A  Q  D

CCCTTCCCAGAAGACCGCGGCAGCCGGGGTGAAACGCTCGACCGGCTGCGCGTCGAAAAGCGACGAAAGGTCCTGCTTCTCGGC   2343
GGGAAGGGTCTTCTGGCGCCGTCGGCCCCACTTTGCGAGCTGGCCGACGCGCAGCTTTTCGCTGCTTTCCAGGACGAAGAGCCG
 G  E  W  F  V  A  A  A  P  T  F  R  E  V  P  Q  A  D  F  L  S  S  L  D  Q  K  E  A

AGCGAACGGCACTGCAGCAGCGACACGTGATTGATGTCCGGTGACGAGCTGAAGGCTCATGAGAATTCTCCGTCTTTCCAAATT   2427
TCGCTTGCCGTGACGTCGTCGCTGTGCACTAACTACAGGCCACTGCTCGACTTCCGAGTACTCTTAAGAGGCAGAAAGGTTTAA
 A  F  P  V  A  A  A  V  R  S  Q  H  G  T  V  L  Q  L  S  M   ORF4

TGACGGAAAGATTTGGCCGGCGCTGCGTGACTCCAGGATGACTTTTGCGTCAACATATTCGCGAATTTGTTACAGTTTGTAACA   2511
ACTGCCTTTCTAAACCGGCCGCGACGCACTGAGGTCCTACTGAAAACGCAGTTGTATAAGCGCTTAAACAATGTCAAACATTGT
                                                                             *
GCTCCAAAGCAAACGGTGCGCCGTCATCTATAGTAATGGAGGTTTTCTCATGCGGTTGAGGCGACAGTAATATTAACAGTCGCC   2595
CGAGGTTTCGTTTGCCACGCGGCAGTAGATATCATTACCTCCAAAAGAGTACGCCAACTCCGCTGTCATTATAATTGTCAGCGG
                            *
GATGAATAACCTCCCCGTTACCAGGGTGGTGGTATTTTATTCGCGATTATTTTGACTCGCCTCAATGACCGATGCCCGCCCGTG   2679
CTACTTATTGGAGGGGCAATGGTCCCACCACCATAAAATAAGCGCTAATAAAACTGAGCGGAGTTACTGGCTACGGGCGGGCAC

CGCAATCAAACTCGCGCACAACTTCCGCATATTCGAGGTCTTGCATGCCAGAAAAAAGATCACGACACGACACAGCCCGGGCGA   2763
GCGTTAGTTTGAGCGCGTGTTGAAGGCGTATAAGCTCCAGAACGTACGGTCTTTTTTCTAGTGCTGTGCTGTGTCGGGCCCGCT

CTCATGGGGCAAGATTGGACCTAGATGCGATTGGACGCCGAACGGCACGCAAACGCCGTGGAGCGGAATTCATTGGGTTTCGCG   2847
GAGTACCCCGTTCTAACCTGGATCTACGCTAACCTGCGGCTTGCCGTGCGTTTGCGGCACCTCGCCTTAAGTAACCCAAAGCGC

TTCCGGAAGCGCACGCCGGGCCTCCAGATCGCCGATCACCAAGAAACACGAGGAGTGTAATTTATGGCCGAGTCTGTTGCAGTT   2931
AAGGCCTTCGCGTGCGGCCCGGAGGTCTAGCGGCTAGTGGTTCTTTGTGCTCCTCACATTAAATACCGGCTCAGACAACGTCAA

TCCGCGCTTACAACCCCTAACCATTTGACCTCTCCCGATCCCGATTTCCGCGTCGAGAAAAAGCAGGAGCGGAGCTGGAGGATC   3015
AGGCGCGAATGTTGGGGATTGGTAAACTGGAGAGGGCTAGGGCTAAAGGCGCAGCTCTTTTTCGTCCTCGCCTCGACCTCCTAG

AGCCTACTGTCACCACGGGCAAGGCACTGGTTCGAAGCAAACTTCGCCGAGGCTAGCCGGGAGGCACGTGTGCTCGAAACAAAT   3099
TCGGATGACAGTGGTGCCCGTTCCGTGACCAAGCTTCGTTTGAAGCGGCTCCGATCGGCCCTCCGTGCACACGAGCTTTGTTTA

CTTGCGGGGGCGAATGCCTTTATTCGCAAGGCGCGAAACTTCGGCTTCACGACGGAATATATCGGCCCGATTGCGAAGTCCTAT   3183
GAACGCCCCCGCTTACGGAAATAAGCGTTCCGCGCTTTGAAGCCGAAGTGCTGCCTTATATAGCCGGGCTAACGCTTCAGGATA

TTTTGAGCACTTGACCTCAACGAACCGAACGCGACGGCATTATCATCATGGTCGCGACAATCACGGTAACTGCCACATCGCCCG   3267
AAAACTCGTGAACTGGAGTTGCTTGGCTTGCGCTGCCGTAATAGTAGTACCAGCGCTGTTAGTGCCATTGACGGTGTAGCGGGC

AAAGGCGCCCAATCTCGGATCC ..(3')                                                          3289
TTTCCGCGGGTTAGAGCCTAGG ..(5')
```

Transposon Tn*5* mutagenesis of the leaf curl-inducing *Rhizobium* strain IC3342 and subsequent screening by a test tube plant assay system was successful in identifying Curl⁻ mutants. The frequency of Curl⁻ mutants was very high (greater than 1%) suggesting that the leaf curl induction phenomenon is genetically complex. Because *Rhizobium* may contain about 3,000 genes, this mutation frequency suggests that some 30 genes may be involved in leaf curl induction. However, screening of a large number of mutants would be required to establish this prediction. Each of the five mutants had a single Tn*5* insertion in different *Eco*RI fragments. This was confirmed by complementation with cloned wild-type DNA fragments. Physical mapping of Tn*5* insertions in these mutants showed that neither the two chromosomally located mutant loci nor the three Sym plasmid-located mutant loci were linked within 20 kb. Furthermore, none of these mutant genes appear to correspond to, or to be closely linked to, the *nifH* or *nifD* genes.

Interestingly, two of the Curl⁻ mutants also had a Fix⁻ phenotype. Fix⁻ mutations, other than those involving the *nif* structural genes, have been mapped both on indigenous plasmids and on the chromosome in *R. leguminosarum* (Beringer *et al.* 1977) and in *R. meliloti* (Ruvkun *et al.* 1982; Kahn *et al.* 1987). Two possible ways by which a gene could be involved in both nitrogen fixation and leaf curl induction are: the expression of both phenotypes may require common metabolic pathways such as an electron transport system, the expression of which is controlled by symbiotically-specific regulators such as *fixL* and *fixJ* (David *et al.* 1988); or leaf curl induction may be a late event relative to bacteroid development and occur only after bacteroid formation. Thus, mutations affecting both Fix and Curl phenotypes may not be directly involved in the leaf curl induction.

In the three Curl⁻ Fix⁺ isolates, mutations could be in genes involved in the biosynthesis or transport of the curl-inducing principle(s). This suggestion is supported by our recent findings that *Rhizobium* strain IC3342 over-produces cytokinins (Z and iP), that nodulation by this strain results in increased cytokinin ZR and DZR levels in the xylem exudate compared to the normal plant, and that the ZR and DZR levels in the xylem exudates from plants nodulated by the Tn*5*-induced Curl⁻ Fix⁺ mutant ANU3003 are similar to those in the xylem exudate from nonleaf-curled plants (Upadhyaya *et al.* 1991c). In this paper, we show that the wild-type sequences corresponding to the mutated genetic region of ANU3003 confer a Curl⁺ phenotype on a closely related, nonleaf curl-inducing *Rhizobium* strain ANU240. Wild-type sequences corresponding to the other four mutant loci did not confer a Curl⁺ phenotype on the recipient strain ANU240. We designate this genetic region, unique to strain IC3342, as *lcrl*. By Southern blot hybridization, we also show the presence of conserved sequences of the other four identified genetic regions of IC3342 in the strain ANU240 and more importantly, the

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ORF2    ...MPHLIIVDDDPRIRSMLSRYLEDEGFRVRLAENISQLRRVLSP.SV    45
             . ::|||| |:|.:|.|||.::||.|| ..| .|: |:|.. |.
ompR    MQENYKNLVVDDDMRLRALLERYLTEQGFQVRSVANAEQMDRLLTRESF    49

ORF2    DLVLLDIGLPDGNGLELAREIDANF.RVPTIIVSGRDDDVDRIIGLEMG    93
        .|::||: ||:::||.:.| : ..  .:|.|:|.:::::||||:|||:|
ompR    HLMVLDLMLPGEDGLSICRRLRSQSNPMPIIMVTAKGEEVDRIVGLEIG    98

ORF2    ADDYVSKPFNLRELLARVRSVLRRSQRAMPLGPAQKATAGIFHFDGWML   142
        ||||:.|||| ||||||:|.|||| ...:| :|.|.  .::: |: :.|
ompR    ADDYIPKPFNPRELLARIRAVLRRQANELPGAPSQE..EAVIAFGKFKL   145

ORF2    DADRRQLTSTAGQTIELTTGEFDLLMVFVTHPGRVLTRDFLLDQTRGRT   195
        : : |:: . .::.:.||.|||.:|..:|.||  .|.|| |:: .|||.
ompR    NLGTREMFR.EDEPMPLTSGEFAVLKALVSHPREPLSRDKLMNLARGRE   197

ORF2    REAFDRAIDVQVTRLRAKVEDDPGDPRRIKSVRGAGYVFAAKVFRPT     238
         .|::|.||||:.||| .||:||:.|| |..|:| ||||.:.. ::
ompR    YSAMERSIDVQISRLRRMVEEDPAHPRYIQTVWGLGYVFVPDGSKA.     240
```

**Fig. 9.** Sequence homology of the *lcrl* ORF2 (open reading frame) with the regulatory gene *ompR* of *Escherichia coli.* The deduced amino acid sequences of ORF2 (IC3342) and *ompR* (*E. coli*) were compared using the GCG "GAP" program, which uses the algorithm of Needleman and Wunsch (Devereux *et al.* 1984). The following parameters were used for comparison: gap weight = 3, length weight = 0.1. Gaps were adjusted to maximize homology. Identical amino acids (|) and conservative amino acid changes (Gribskow and Burgess 1986) with score >0.50 (:) and 0.1–0.4 (.) are indicated. Single letter abbreviations are used for the amino acids.

absence of an *lcr1* homologue in strain ANU240. Possibly, *lcr1* gene product(s) have a role in the expression of genes at other loci under symbiotic conditions.

Evidence for a possible regulatory role of gene(s) located in the *lcr1* region comes from observed homologies of deduced amino acid sequences of two ORFs, identified in this region, with regulatory genes from *E. coli* and from *R. meliloti.* The deduced amino acid sequence of ORF2 showed strong homology with that of the *ompR* gene of *E. coli.* The OmpR and EnvZ proteins (co-transcribed genes) are both implicated in osmotic sensing and in regulation of the biosynthesis of the outer membrane matrix proteins (porins) OmpF and OmpC in *E. coli* (Comeau *et al.* 1985). In addition, this regulatory gene pair has been shown to exert pleiotropic regulatory effects on at least four genes in *Salmonella typhimurium* (Loeffler) Castellani and Chalmers (Gibson *et al.* 1987). Recently, the *R. meliloti* genes *fixL* and *fixJ* were shown to be positive regulators of the symbiotic expression of diverse nitrogen fixation genes (David *et al.* 1988). Based on sequence homology, this gene pair is also suggested to be a member of the family of two-component regulatory systems. By analogy, the protein encoded by ORF2 may be a positive regulator of other genes involved in leaf curl induction.

The observed homologies of the deduced amino acid sequence of ORF4 with those of the *fnr* gene product of

```
fixK    .................MYAAAQ...AKPQSIEVEHLGPAPMSGPRLVA    29
                         : .: |    | : .:..|. : ..: :.. |.
ORF4    .............MSLQLVTGHQSRVAAAVPFAAEKQDLSSLFDAQPVE    36
                     : .: ... |   . .:||. :.::|. |   .. :|
fnr     MIPEKRIIRRIQSGGCAIHCQDCSISQLCIPFTLNEHELDQL..DNIIE    47

fixK    TYKP...GREIYAQGDLNDKCYQVSTGAVRIYRLLSDGRRQVVSFHLPG    75
         :.|   : .:: :||  . ..::|..||.:|  |:|.|||| :::|  ||
ORF4    RFTP...AAAVFWEGDQARHIFEVVEGTLRAVRILNDGRRVIIGFLRPG    82
        | .|   :..:|..|||: : ::.: .|||::.  | ::||   |.|| :|
fnr     RKKPIQKGQTLFKAGDELKSLYAIRSGTIKSYTITEQGDEQITGFHLAG    96

fixK    EMFGFE..AGSNHSFFAEAITETTLAIFGRRNMQERS......RELLAL   116
        :::||..  . .:. : .||||...|  |:||.::. |     ||:|
ORF4    DLLGVS..VKEHYLYTVEAITHVELRRFSRRRFESESARAPHLREQLFS   129
        ||:||.   ..:|.   .:|:.   :  :.   ::. |:: |:||:|::
fnr     DLVGFDAIGSGHHPSFAQALETSMVCEIPFETLDDLSGKMPNLRQQMM.   144

fixK    ALT.GMARAQQHLLVIGRQCAVERI.AAFLVDLCER..QGGGRQ..LRL   159
         |. :|| ||::::::::|.:| |:: |:||: :. |  |::.|.  : |
ORF4    RLCDEMAAAQDQMVLLSRRSAEEKV.AGFLLMMA.RG.QSENRRPVIEL   175
        || .:    ::::|:|| .:...|.  |:|:. :. |  |.: ... : |
fnr     RLMSGEIKGDQDMILLLSKKNAEERLAAFIYNLSRRFAQRGFSPREFRL   193

fixK    PMSRQDIADYLGLTIETVSRVVTKLKERSLIALRDARTIDIMKPEALRS   208
        ||.| |:||||:||||||.:|||:.::||: : :.|.|:| :|| .
ORF4    PMTRLDVADYLGMTIETVSRTITKLAGSGVIAIVGRHAIAILKMDALIA   224
        .||| |:::|||:|:|||:|| :.::. ||::|: |:. |.| . ||| .
fnr     TMTRGDIGNYLGLTVETISRLLGRFQKSGMLAVKGKY.ITIENNDALAQ   241

ixK     LCN...............    211
        |.:
ORF4    LADGECDDGAQRSARYAKA   243
        ||: . :
fnr     LAGHTRNVA..........   250
```

**Fig. 10.** Sequence homologies of the *lcr1* ORF4 (open reading frame) with the regulatory genes fnr of *Escherichia coli* and *fixK* of *Rhizobium meliloti.* Deduced amino acid sequences of the ORF4 gene product was compared with those of the *fnr* gene of *E. coli* and the *fixK* gene of *R. meliloti* by the GCG "GAP/OUT" and "PRETTY" programs. The following parameters were used for comparison: gap weight = 3, length weight = 0.1. Gaps were adjusted to maximize homology. Identical amino acids (|) and conservative amino acid changes (Gribskow and Burgess 1986) with score >0.50 (:) and 0.1–0.4 (.) are indicated. Single letter abbreviations are used for the amino acids.

*E. coli* and the *fixK* gene product of *R. meliloti* also suggest a regulatory role for the ORF4 gene product. Like Fnr and FixK (Batut *et al.* 1989), the ORF4 gene product may act both as an activator and as a negative regulator of genes involved in leaf curl induction.

As reported previously, leaf curl induction occurs only when there is effective nodulation by the strain IC3342 (Upadhyaya *et al.* 1991a), and the *lcr1* mutation (Fix$^+$ Curl$^-$ mutant strain ANU3003) results in a reduction of endogenous cytokinin level in the xylem sap of plants nodulated by this mutant strain (Upadhyaya *et al.* 1991b). Thus, it is reasonable to predict a role for *lcr1* genes in the production of a curl-inducing principle (presumably cytokinins) or an effector molecule (signal) involved in the induction of other genes required for the synthesis of this curl-inducing principle. However, specific probing failed to detect a structural homologue of the *Agrobacterium* cytokinin biosynthesis gene (*ipt*) in the genome or in cloned regions of the strain IC3342. Also, putative *lcr1* genes showed no sequence homology with any of the identified cytokinin biosynthetic genes from other microorganisms (Upadhyaya *et al.* 1991b).

Thus, *lcr1* warrants further characterization as an important genetic region that may determine hormonal balances associated with the *Rhizobium*-legume symbiosis. Because *Rhizobium* is amenable to genetic manipulation, we believe that this system could serve as a model for the study of hormone action at the molecular level in leaf curling-type diseases, such as fasciation caused by *C. fascians* (Roussaux 1965) and peach leaf curl caused by *T. deformans* (Sziraki *et al.* 1975), where phytohormones are known to be involved.

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