The *Rhizobium meliloti* groELc locus is required for regulation of early *nod* genes by the transcription activator NodD

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The molecular chaperones related to GroEL (hsp60, cpn60) interact with partially folded proteins and appear to assist them to attain active and correctly folded conformation. They are required for cell viability but are probably more important for some processes than for others. Through a random genetic search to find loci that are required for expression of the *Rhizobium meliloti* *nod* (nodulation) genes, we isolated a mutant (B4) defective in luteolin-dependent activation of *nod* gene expression, and found it carries a Tn5 insertion within a chromosomal groEL gene (groELc) located just downstream of a groESC gene. The groELc mutation affected activity of three related LysR-type activator proteins NodD1, NodD3, and SyrM; on plants, the mutants formed nodules late, and the nodules were Fix-. Hybridization and protein expression analysis show that a similar groESL locus (groESLa) maps to the Rm1021 megaplasmid pSyma. Southern blot analysis revealed additional, but less closely related sequences hybridizing to groELc and groESC probes elsewhere in the *R. meliloti* genome. Clones of groESLc and groESLa can each restore robust phage λ growth on an *Escherichia coli* groE mutant. Likewise each clone can complement all of the phenotypes observed for B4 mutants; thus, the two appear to be functionally equivalent if expression is controlled. We determined that groELc is required for normal DNA binding of the NodD target sequence in *R. meliloti*. GroEL coimmunopurifies with NodD1 from *R. meliloti*, which suggests a direct physical association between these proteins. GroEL is thus probably involved in the folding or assembly of transcriptionally active NodD.

[Key Words: *Rhizobium meliloti*; GroEL; *nod* genes; NodD; LysR; sym plasmid; transcriptional activation]

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*Rhizobium meliloti* is a Gram-negative bacterium that can either live as a soil scavenger, or can invade specific host plants including alfalfa (*Medicago sativa*) to initiate a symbiosis. The symbiotic process begins when the bacteria stimulate the plants to begin the development of root nodules (Long 1989). *Rhizobium* cells trigger nodule development by the action of their nodulation *nod* genes, which appear to encode enzymes that synthesize a family of morphogens active on plants [Fisher and Long 1992, Dénarié and Cullimore 1993; Downie 1994; Geiger et al. 1994]. The *nod* genes are located on a large [1400–1500 kb] plasmid termed pSyma; a second, larger [1700 kb] plasmid, pSymb, contains other genes for symbiosis including those for synthesis of exopolysaccharide [Charles and Finan 1991].

The correct expression of the early-acting *R. meliloti* *nod* genes requires induction from the host plant [for review, see Fisher and Long 1992, Kondorosi 1992]. We found that alfalfa seeds secrete a flavonoid, luteolin, that induces *Rhizobium nod* gene expression by a mechanism requiring an activator gene, *nodD* [Mulligan and Long 1985; Peters et al. 1986]. NodD is a member of the LysR family of prokaryotic transcription activator proteins [Henikoff et al. 1988; for review, see Schell 1993]. The LysR proteins are typically 30–35 kD, and often are transcribed divergently from an operon that they regulate. Some have both repressor and activator functions. Their molecular basis for action is not known.

Different *Rhizobium* strains have NodD proteins that function in common to activate expression of *nod* genes but do so in response to inducers that vary according to the host plant [for review, see Fisher and Long 1992, Kondorosi 1992; Schlaman et al. 1992]. *R. meliloti* has three *nodD* genes: *nodD1* is constitutively expressed [Mulligan and Long 1985], and the protein product NodD1 activates *nod* operons in the presence of luteolin, 4,4′-hydroxy-6-methoxy chalcone [HMC] and related compounds; *nodD2* requires compounds other than luteolin, including HMC and betaines such as trigonelline [for review, see Phillips 1992]. *nodD3* encodes a protein, NodD3, that is capable of transcriptional activation without any plant inducer [Mulligan and Long 1989, Swanson et al. 1993]. However, the expression of this locus is itself under complex control, so that there is little NodD3 protein present in cultured *Rhizobium*,
it is expressed later in the symbiosis under the control of syrM, another LysR-type activator (Barnett and Long 1990, for review, see Kondorosi 1992).

The action of NodD proteins has been studied by genetic and biochemical approaches. nod promoters are characterized by the presence of a nod box. NodD binds to the nod box (for review, see Kondorosi 1992), establishing a footprint of up to 55 bp, and bends the DNA at the nod box (Fisher and Long 1993). The presence of inducer is not necessary for DNA binding by purified NodD protein, and the exact biochemical role of inducer in transcription activation is not understood.

Some bacteria transduce external signals into gene expression by way of a two-component modular response regulatory system (for review, see Parkinson 1993). In the simplest cases, the regulatory properties of a DNA-binding protein (such as FixJ, CheY, DctD) are controlled by a partner histidine kinase (FixL, CheA, DctB), which senses an internal or external condition. We asked whether NodD required a comparable partner to respond to inducer, and searched genetically for such a partner by mutagenesis and screening. What we found instead was a locus that is necessary for the activity of NodD and SyrM, independent of inducer requirements. Analysis of this locus showed that it is a Rhizobium homolog of the Escherichia coli GroEL protein (hsp60, chaperonin-60), a molecular chaperone required for correct folding of some proteins, and for allowing their correct assembly with other polypeptides (Ellis and van der Vies 1991; Georgopoulos 1992; Hendrick and Hartl 1993). We established that GroEL affects the ability of NodD to bind promoters normally and that R. meliloti GroEL associates biochemically with the NodD protein activator. This is a novel direct genetic discovery in a homologous system of a role for GroEL in transcription activation. R. meliloti at least has two fully functional copies of groESL, and a chromosomal one is required for activation of nod genes through NodD; a second, borne on the sym plasmid pSyma, appears sufficient for all other growth functions.

Results

R. meliloti Tn5 mutant displays altered nod gene expression

To investigate how R. meliloti NodD acts with plant inducers to cause activation of nod gene promoters, we asked whether other genetic loci were required for correct induction by luteolin, the inducer isolated from alfalfa seeds (Peters et al. 1986). We examined nine mutants generated by Tn5 mutagenesis of R. meliloti strain AL110, which carries a nodC'–lacZ fusion. One mutant, designated B4, displayed reduced (−14–25% of normal) luteolin-mediated nodC'–lacZ expression, and this behavior was still displayed when extra copies of nodD1, nodABC'–lacZ were provided in trans (Table 1). NodA protein levels, as detected by antibody, were also markedly lower in a B4 mutant background (data not shown), confirming a direct and general effect of B4 on nod gene expression. Linkage of Tn5 to the phenotype was shown by phage N3 transduction.

Several lines of evidence indicated that the effect of the B4 mutant was on NodD activity, not on its expression or on the effect of luteolin. First, we found that expression of nodD1 was not reduced (Table 2, lines 1,2). Western blots probed with anti-NodD antibodies showed that NodD1 is present in B4T3 (pO57.33) at a lower level than that found in AL110 (pO57.33) (data not shown), however, the less than twofold difference in protein level could not account for the approximately sevenfold decrease in NodD function inferred from Table 1.

Second, we tested whether the B4 mutation affected cell reaction to inducer (luteolin) or whether it affected NodD action independent of inducer. We exploited the multigene nodD family of R. meliloti: One of the copies, nodD3, encodes an inducer-independent activator protein [Mulligan and Long 1989; Maillet et al. 1990; Kondorosi et al. 1991, Swanson et al. 1993]. Expression of nodD3 is normally low and is controlled by syrM, which, like nodD, encodes a member of the LysR protein activator family (Barnett and Long 1990; Kondorosi et al. 1991). We circumvented the requirement for syrM by using a plasmid (pE65) on which nodD3 is expressed from a trp promoter (Egelhoff and Long 1985; Fisher et al. 1988). The presence of the B4 mutation significantly decreased nodC'–lacZ expression (Table 2, cf. lines 3 and 4). This supported the hypothesis that the product of the B4 locus was required for NodD function, whether NodD1 or NodD3, for transducing the effect of the plant-derived inducer.

Examining the more complex effects of syrM and nodD3 combined suggested that SyrM activity itself is also affected by the B4 mutation. When both syrM and nodD3 are expressed in trans on plasmid pJT5, nodC'–lacZ gene expression is high in wild-type cells (Table 2, line 5). This effect was drastically reduced in the B4 mutant background (line 6). We tested for nodD3 expression by means of three independent nodD3–lacZ or nodD3–gusA transcriptional fusions (Table 2, lines 7 and 8; data not shown). We found that nodD3 expression was sensitive to the B4 mutant background. As syrM expression was not decreased (Table 2, lines 9 and 10), it is possible that SyrM protein loses its ability to activate nodD3 expression in the B4 background.

We used antibody to examine the relationship of

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**Table 1. Effect of B4 mutation on nod gene induction**

| Strain | Plasmid (description) | Units β-galactosidase* (fold induction) |
|--------|-----------------------|---------------------------------------|
| AL110  | none                  | 0.5  8.1 (16×)                        |
| B4     | none                  | 0.4  2.1 (5×)                         |
| AL110  | pO57.33 (nodD1 nodABC'–lacZ) | 22  640 (29×)                        |
| B4     | pO57.33               | 13  51 (4×)                           |

* Cultures were grown in M9–sucrose liquid medium.
NodD3 protein level to the inferred activity on downstream gene expression [data not shown]. When nodD3 is expressed from the trp promoter in the B4 mutant background (represented in Table 2, line 4), the level of NodD3 protein was decreased but not enough to account for the activity decrease. Moreover, this B4 strain containing trp-driven nodD3 (Table 2, line 4) has about twice the level of NodD3 protein detected in AL110 [pT15] (represented in Table 2, line 5), and yet the latter strain showed much higher NodD3-dependent nodC−lacZ expression. Overall, we conclude that the B4 mutation affects both the expression of nodD3 and the activity of the NodD3 that does get expressed.

**Free-living and symbiotic phenotypes of the B4 mutant**

Strain B4T1 [the Tn5 from B4 transduced into a wildtype Rm1021 background] grew slightly slower than wild type on both solid and liquid medium, either rich or minimal (e.g., 25% longer doubling time than wild type on both solid and liquid medium, either rich or minimal). Otherwise, we observed no effects of the mutation on growth of cultured cells. B4 mutants supported growth of phage N3, allowing transduction of markers between strains. B4 mutant colonies fluoresced under UV light when grown on Calcofluor-containing medium, indicating the normal presence of succinoglycan [Leigh et al. 1985]. Colony morphology was normal. However, the mucoid phenotype caused by multicopy syrM (Mulligan and Long 1989) was not seen in a B4 background [data not shown]. This phenotype was consistent with the parallel loss of SyrM-dependent nodD3 expression.

B4 mutants showed two levels of symbiotic defect: (1) B4T1-induced nodules formed 4–9 days later than wild type, and fewer plants became nodulated (40–60%, compared to almost 100% of plants nodulated by wild type after 22 days); and (2) B4T1-induced nodules failed to fix nitrogen (Fix−), resulting in stunted and chlorotic plants with white or gray nodules instead of the usual pink nodules formed by Rm1021. As judged by acetylene reduction assays, B4T1-induced nodules showed <1% of the nitrogen-fixing activity of wild-type Rm1021-induced nodules: 20–70 nmoles of ethylene were produced in 8 hr per B4T1-nodulated plant, ~10-fold over background, compared with 7000–10,000 nmoles for wild-type-nodulated plants. Uninoculated plants or plants inoculated with a nifA::Tn5 strain did not generate detectable levels of ethylene.

We suspected that the Fix− phenotype of the B4 mutants was not attributable to nod gene expression defects, because although nodD mutants form nodules late and in low number, these nodules are usually Fix+ once they form [Jacobs et al. 1985]. We tested for whether the Fix− phenotype correlated with reduced NifA-dependent activation of nif-gene expression. However, nif expression was not reduced. First, nifA-dependent activation of nifHDK expression was similar in wild type and in B4, as measured by a nifH−′-gusA fusion on a plasmid in these strains [Sharma and Signer 1990] cultured under microaerobic conditions that normally lead to activation of nifA expression and consequently to activation of nifHDK expression [Ditta et al. 1987; David et al. 1988] (data not shown). Second, expression of a nifA′−′lacZ fusion on pCHKS7 [Ditta et al. 1987] was comparable in mutant B4 and in Rm1021, grown under aerobic or microaerobic conditions. Thus, reduction of nifA-dependent activation of nif-gene expression is probably not the basis for the Fix− phenotype of the B4 mutant, and direct effects on nitrogen fixation seem more likely [see Discussion].

**The B4 Tn5 disrupts a groEL gene**

We mapped the Tn5 in the B4 mutant to the chromosome of R. meliloti, first by showing that the Tn5 resis-
R. meliloti groELc required for nod regulation

The second copy of the R. meliloti groESL is located on pSyma

The second B4-homologous locus (pJO32) lay on a 2.9-kb XbaI fragment, in a segment with a restriction map identical to the corresponding region of the pJO101 clone (Fig. 2). Preliminary DNA sequence was completely identical for at least 200 bp surrounding the Smal site of pJO32 and pJO101. Using R. meliloti S-30 extracts (Fisher et al. 1987), we found by coupled transcription-translation reactions that both clones encode a protein migrating at 60 kD [Fig. 3, lanes 5–10, top arrow], comparable to the upper band of the E. coli GroEL doublet (lanes 3, 4). Another band migrating at 13 kD [bottom arrow] corresponded to cloned R. meliloti groES genes. This band migrates faster than E. coli GroES [lanes 3, 4, open arrowhead], which displays an apparent mobility corresponding to 15 kD [Tilly et al. 1981], although its molecular size is inferred from DNA sequence to be 10 kD (Hemmingsen et al. 1988). The presence of the 60- and 13-kD bands in all reactions with plasmid containing cloned Rm1021 DNA, regardless of orientation, suggests the presence of active R. meliloti promoters on the cloned fragments.

The protein and partial sequence data of pJO32 suggested the existence of a second groESL locus. We showed that this was located on pSyma by probing DNA of megaplasmid-containing derivatives of Agrobacterium tumefaciens strain A136 (Fig. 1C). We further defined the location by analyzing R. meliloti strains with known deletions in pSyma: Analysis with Southern blots of restricted DNA revealed that the 12-kb BglII fragment was present in deletion GM1255 (Fig. 1C) but absent from GM1766 (Fig. 1D: cf. lane 3 with 6, and lane 9 with 12). Therefore, these genes are located within a region extending at least 70 kb from nifHDK, distal to the common nod genes (Truchet et al. 1985) (Fig. 1B). We have named the pSyma located genes groESLa; the chromosomal genes mutated in B4 have been designated groESLc.

Other related sequences are present in the R. meliloti genome: Hybridizations of Southern blots with small probes internal to the groELc ORF revealed two to three additional bands [Fig. 1D, lanes 7–9]. However, fewer, and different, genomic fragments hybridized to the groESc ORF compared with those hybridizing to the groELc probe [lanes 1–3 vs lanes 7–9]. We conclude that there are at least three additional copies of groEL-like sequences in the Rm1021 genome and at least two additional copies of groES-like sequences. Only one pair of the additional groESa and groELa copies are clearly linked to each other in the Rm1021 genome, but these hybridize only faintly to probes made from the pJO101 clone; if the other additional groESa-like and groELa-like copies are paired in operons, they do not share the same internal restriction pattern as the copies cloned in pJO32 and pJO101. In work to be published elsewhere (J. Ogawa and S.R. Long, in prep.), we have demonstrated that the presence of either groELc or of groELa is required to sustain cell viability. The function, if any, of the other hybridizing bands is not known.

Recently, Rusanganwa and Gupta [1993] reported the cloning of three groEL-like genes from R. meliloti Rm1021, which they obtained using a probe generated by polymerase chain reaction using degenerate oligonucleotide primers, and they reported that hybridizing bands were present on both pSyma and pSymb. Based on their reported restriction map and DNA sequence, we conclude that their clone RhzA corresponds to groELc. Our DNA sequence of groELc is nearly identical to the reported sequence of RhzA within the coding region. Outside of the coding region, there are several discrepancies between the two sequences; however, we think that these discrepancies are probably attributable to technical differences in the determination of the DNA sequence, and not the result of our having sequenced a different gene. One of the other two groEL-like clones described by Rusanganwa and Gupta [1993] is preceded by a groES-like sequence and the other is not; neither of these clones correspond to groELa or groELc.

Functional comparison of E. coli groEL, groESLa, and groESLc

We tested function of broad host-range clones carrying the R. meliloti groESLa and groESLc and the E. coli
groESL genes. The *R. meliloti* clones restored wild-type growth to B4 derivatives. Also, the groESLa and groESLc clones both restore B4T3 luteolin-induced β-galactosidase activity to wild-type levels (Table 3). However, this
DNA was digested with the restriction enzymes indicated, a Southern blot was prepared from a 0.8% agarose gel and hybridized to pSyma that is deleted in GMI766, and also shows additional bands hybridizing to present in A136 carrying pSyma or pSymb, consistent with the chromosomal location determined previously for this fragment. but was absent from A136 carrying pSymb or no mapped deletion of pSyma shows that the second hybridizing (6.7-kb band in R. meliloti groELc, cloned downstream of the lac promoter) resulted in a clone that was very proficient at complementing the groE and groEL mutants. A lower copy number version of the R. meliloti groELc, cloned upstream of the lac promoter in a pACYC184-based vector, was not as effective at complementing either of the phenotypes of the E. coli groE mutants (data not shown, see Table 5).

NodD–DNA binding requires GroEL

Cell-free extracts of B4T3 carrying the nodD3-over-expressing clone pE65 were tested in the gel-mobility shift assay side by side with extracts of ALl10 (pE65) (Fig. 4A). The nodA promoter-specific DNA-binding activity seen in an extract of the mutant (lanes 2, 4, 6, 8, 10) was reduced >32-fold compared with that seen for wild type (lanes 1, 3, 5, 7, 9). NodD1-dependent nodA promoter-specific DNA-binding activity was likewise found to be reduced in a B4 mutant strain (data not shown). This indicated that in the homologous system, GroEL expressed from groESLc is needed to achieve full DNA binding by NodD.

Although proteins from cell-free extracts of R. meliloti overproducing NodD1 or NodD3 are capable of binding to the nod promoter regions (Fisher et al. 1988), cell-free extracts of E. coli carrying the same NodD-overproducing plasmids have failed to show binding previously (R. Fisher, T. Egelhoff, S. Long, unpubl.). We found that overexpression of E. coli groESL enhances the nodABC promoter-specific DNA-binding activity of cell-free

**Figure 1.** Location of two groESL-like sequences in the R. meliloti genome, and location of two homologous copies. (A) Cartoon showing the location of the Tn5 in mutant B4 on the R. meliloti chromosome (groESLc), and the location of a second homologous copy of groESL on the R. meliloti pSyma. (B) Diagram showing the portion of pSyma that is present in GM1255, but absent from GM1766. (C) Southern blot analysis of A. tumefaciens strain A136 carrying pSyma or pSymb shows that the second hybridizing (12-kb BglII) band in R. meliloti DNA cotransfers with pSyma, and is present in R. meliloti strain GM1255 which carries a mapped deletion of pSyma. Total bacterial DNA from R. meliloti strain Rm1021, A. tumefaciens strain A136, and A136 carrying pSyma or pSymb, and R. meliloti deletion strain GM1255 was digested with BglII, separated by electrophoresis on a 0.7% agarose gel, blotted and hybridized to a 32P-labeled fragment corresponding to "probe L1" in Fig. 2B. The sizes of the two hybridizing BglII bands in the Rm1021 DNA lane is indicated on the right. The 12-kb BglII band which hybridized to the R. meliloti groELc probe was present in A136 carrying pSyma, but was absent from A136 carrying pSymb or no R. meliloti megaplasmid. The 7.9-kb BglII band present in Rm1021 DNA was not present in A136 carrying pSyma or pSymb, consistent with the chromosomal location determined previously for this fragment. Southern blot hybridizations of EcoRI-digested and HindIII-digested DNA from A. tumefaciens constructs [data not shown] revealed other pSyma and pSymb specific bands that weakly hybridized to our groELc probes, as was observed by Rusanganwa and Gupta (1993) but at slightly different calculated sizes. (D) Southern blot analysis of Rm1021 and of the derivative strain GM1766, which carries a mapped deletion of pSyma shows that the second hybridizing (6.7-kb EcoRI, 10-kb Clal, 12-kb BglII) band is located in the region of pSyma that is deleted in GM1766, and also shows additional bands hybridizing to R. meliloti groESL and groEL probes. Total bacterial DNA was digested with the restriction enzymes indicated, a Southern blot was prepared from a 0.8% agarose gel and hybridized to "probe S" or "probe L2" (shown in Fig. 2B).
extracts of *E. coli* carrying plasmids that overproduce NodD1 or NodD3 [Fig. 4B, lanes 4,9,14]. However, the *R. meliloti* groESLc clones were not as effective at conferring this property on *E. coli* (lanes 5,8,12). Overexpressing NodD3 from a high copy number plasmid gave low levels of nod box binding (lane 7), but again the *R. me-
liloti groESL had little effect (lane 8). The ability of extracts of E. coli carrying the nodD1-bearing plasmid pE39 to retard the migration of the nodA promoter fragment was not increased by the inclusion of 3 μM luteolin in the binding reaction (lanes 13, 15).

The improved nod box binding by NodD in E. coli (Fig. 4B) was not sufficient to confer nod promoter activity in vivo. We made triple constructs of Lac− E. coli containing a nodD plasmid (nodD1 or nodD3), a reporter plasmid with a nodC− LacZ fusion, and plasmids expressing E. coli groESL or R. meliloti groESLc. None of the resulting strains expressed detectable levels of β-galactosidase activity. Therefore, although pGroESL was able to enhance nod box DNA binding by E. coli overexpressing nodD3 or nodD1, it did not permit transcriptional activation of the nodABC genes in vivo by NodD1 or NodD3. Perhaps a further component of the transcription apparatus, such as a σ factor or other protein of R. meliloti RNA polymerase, also needs to be added to the system.

A 59-kD protein that coimmunopurifies with NodD1 is GroEL

The discovery of a groEL requirement for nod gene expression occurred through a genetic search, and in itself did not indicate whether the mechanism of its effect was indirect or direct. We tested for direct biochemical association of GroEL and NodD1 based on our previous report (Fisher et al. 1988) that a substantially purified preparation of NodD1 obtained from immunoaffinity chromatography of R. meliloti extracts contained two major proteins: One protein was NodD1, the other was an unidentified 59-kD protein. We prepared a Western blot of this same NodD1 preparation (Fig. 5) and probed it with anti-E. coli GroEL antibodies. We found that the 59-kD protein which coimmunopurified with NodD1 cross-reacts with the anti-GroEL antibodies. In trying to explain the presence of the 59-kD protein in immunopurified NodD1 fractions, Fisher et al. (1988) had suggested that the 59-kD protein independently associated with the immunoaffinity column matrix, because extracts from a R. meliloti triple mutant strain containing insertions in nodD1, nodD2, and nodD3 also yielded a small amount of 59-kD protein when passed over the column. However, we have since determined that this nodD mutant strain is leaky for nodD3 expression (Rushing et al. 1991) and produces a NodD1–LacZ fusion protein and possibly a truncated nodD2 gene product. Furthermore, the antibody to NodD1 fails to react with the 59-kD band on a Western blot. Thus, the “triple nodD” mutant probably presented some NodD3 and possibly partial NodD1 to the immunoaffinity column, resulting in enough protein to associate specifically with a small amount of GroEL. The biochemical evidence from the column affinity procedure thus suggests a direct binding of GroEL to NodD.

Discussion

GroEL is a molecular chaperone, a term describing proteins that are important for assembly of other proteins but do not themselves confer steric information nor constitute a part of the mature complex (Ellis and van der Vies 1991; Hendrick and Hartl 1993). GroEL is a bacterial example of the chaperonin-60 class, which also includes proteins in mitochondria, plastids, and eukaryotic cytoplasm (for review, see Ellis and van der Vies 1991). The groEL locus of E. coli was originally defined genetically as a host mutation that prevented successful infection by phage. Subsequent work has shown its in-

Table 3. Complementation of the B4 mutant phenotype

| Strain (description) | Plasmid (description) | Units β-galactosidase<sup>b</sup> | - luteolin | + luteolin |
|----------------------|-----------------------|---------------------------------|-----------|-----------|
| AL110 [nodABC−'lacZ] | pRK290 (vector)       | 0.7                             | 5         |           |
| B4T3 [nodABC−'lacZ]  | pRK290 (vector)       | 0.6                             | 1         |           |
| AL110                 | pO113 (Rm groESLc)    | 0.7                             | 5         |           |
| B4T3                  | pO113 (Rm groESLc)    | 0.7                             | 5         |           |
| AL110                 | pO37 (Rm groESLc)     | 0.7                             | 6         |           |
| B4T3                  | pO37 (Rm groESLc)     | 0.7                             | 5         |           |
| AL110                 | pO43 (Ec groESL)      | 0.8                             | 20        |           |
| B4T3                  | pO43 (Ec groESL)      | 0.8                             | 16        |           |

<sup>a</sup>E. coli designates E. coli genes; Rm designates R. meliloti genes.

<sup>b</sup>Cultures were grown in M9-sucrose liquid medium.

A 59-kD protein that coimmunopurifies with NodD1 is GroEL

The discovery of a groEL requirement for nod gene ex-

Table 4. Complementation of E. coli groESL mutants

| Plasmid     | Description                  | Relative λ plating efficiency (colony formation at 43°C) |
|-------------|------------------------------|----------------------------------------------------------|
|             |                              | E. coli strain<sup>a</sup>                               |
|             |                              | CG2245 (wt)    | CG2244 (groES<sup>−</sup>) | CG2241 (groEL<sup>−</sup>) |
| pBluescript SK + | vector                     | 1 [+]+        | <10<sup>−5</sup> (+)   | <10<sup>−5</sup> (-)   |
| pOF39       | Ec groESL in pBR325         | 1 [+]         | 0.1 (+)                  | 0.2 (+)                  |
| pKS + groESL | Ec groESL in pBluescript (oriented opposite Plac) | 0.9 (+) | 0.6 (+) | 0.7 (+) |
| pSK + groESL | Es groESL in pBluescript [Plac → Ec groESL] | 0.9 (+) | 0.01 (+) | <10<sup>−5</sup> (-) |
| pO115       | Rm groESLc in pBluescript [Plac → Rm groESLc] | 1.4 (+) | 0.7 (+) | 0.8 (+) |
| pO46        | Rm groESLc in pBluescript [Plac → Rm groESLc] | 1.5 (+) | 0.7 (+) | 1.3 (+) |

<sup>a</sup>[+]: Wild-type (wt) colony formation; (+): formation of tiny colonies; (-): no single colonies formed.
Table 5. Strains and plasmids

| Strain or plasmID | Genotype or relevant characteristics | Source or reference |
|------------------|--------------------------------------|---------------------|
| AL110            | Rm1021 lac-, nodC'-lacZ-Sp fusion     | Lee (1988)          |
| B4               | AL110::Tn5#B4                         | Lee (1988)          |
| B4T1             | Rm1021::Tn5#B4 (transductant)         | this work           |
| B4T3             | AL110::Tn5#B4 (transductant)          | this work           |
| B4Sp2            | Rm1021::Tn5-233#B4, Gm' Sp'         | J. Dénarié          |
| GM1255           | 2011 Δfix706a nod nifHDK/125 [Tn5] Na1' | Truchet et al. (1985) |
| GM1766           | 2011 Δnod nifA/766 Sp'               | Swanson et al. (1993) |
| JA5301           | Rm1021 nodD3::lacZ-5-1 fusion, Nm'   | this work           |
| Rm1021           | SU47 str-21                           | H. Meade            |
| Rm5300           | Rm5301 pRmsu47b thi-502::Tn5-11        | Finan et al. (1986) |
| Rm5321           | Rm5321 pRmsu47a::Tn5-11               | T. Finan            |
| Rm6743           | Rm6743 pRm615::Tn5-Mob                | Klein et al. (1992) |
| Rm6761           | Rm6761 pRm611::Tn5-Mob                | Klein et al. (1992) |
| Rm6826           | SU47 str-3 spec-1 rif-1 his-39 trp-33 aro-51::Tn5 | S. Klein |
| Rm6879           | Rm5000 recA::Tn5-Tp                    | Long et al. (1988)  |
| Rm8002           | Rm8002 fix-382:~TnphoA                | Long et al. (1988)  |
| Rm8620           | Rm8620 adegP65::Tn5HoKm               | Glazebrook et al. (1992) |
| E. coli          | CG2241 B1782j::Tn1 groEL4             | C. Georgopoulos     |
| CG2244           | CG2244 B1782j::Tn1 groES619           | C. Georgopoulos     |
| CG2245           | CG2245 B1782j::Tn1 groE+              | C. Georgopoulos     |
| NK5821           | F' Δlac-proXIII Na1' Rif'            | N. Kleckner         |
| A. tumefaciens    | CS8 derivative, cured of Ti plasmid, NaF/RIF' | E. Nester |
| N3               | R. meliloti transducing phage         | Martin and Long (1984) |
| λ                | wild type                            | A. Campbell         |
| øFIX II-4a       | 12 kb BglII fragment containing R. meliloti groELa from Rm1021 in øFIX II | this work |
| øZAP II-1        | 7.9-kb BglII fragment containing groESLc from Rm1021 in øZAP II | this work |
| pMB1 or          | phagemid vector                       | Stratagene          |
| pBluescript SK+  | pUC8 with trp promoter and rpo C terminator flanking the polylinker | Stratagene          |
| pBluescript KS+  | phagemid vector                       | A. Das              |
| pAD10            | nodD1 expressed under the control of trp promoter in pAD10 | Egelhoff and Long (1985) |
| pOE39            | E. coli groESL genes in pBR325        | Fayet et al. (1986) |
| pSK+ GroESL      | E. coli groESL genes on a 2.2-kb EcoRI-HindIII fragment in pBluescript SK + | this work |
| pKS+ GroESL      | E. coli groESL genes oriented opposite lac promoter in pBluescript KS + | this work |
| pRK600           | Cm', pRK2013 Nm':~Tn9 helper plasmid  | Finan et al. (1986) |
| pRK607           | pRK2013 containing Tn5-233, Gm' Sp' | De Vos et al. (1986) |
| pLOD3            | nodD1 expressed under the control of trp promoter in pAD10 | this work |
| pLO32            | 12-kb insert from øFIX II-4a cloned as a NsiI fragment in pUC18 | this work |
| pLO46            | R. meliloti groESLc on a 2.9-kb Xbal fragment in pBluescript SK + | this work |
| pLO47            | R. meliloti groESLc on a 2.9-kb Xbal fragment in pBluescript SK + (oriented opposite lac promoter) | this work |
| pLO91            | 10.5-kb Tn5-containing Clai fragment from B4T1 in pBluescript SK + | this work |
| pLO101           | a product of in vivo excision of øZAP II-1 | this work |
| pLO111           | R. meliloti groESLc on a 5-kb EcoRI fragment in pBluescript SK + | this work |
| pLO112           | R. meliloti groESLc on a 5-kb EcoRI fragment in pBluescript SK + (oriented opposite lac promoter) | this work |
| pLO115           | R. meliloti groESLc on a 3.2-kb EcoRI-HindIII fragment on pBluescript SK + | this work |
| pLO131           | R. meliloti groESLc on a 2.9-kb Clai-BamHI fragment in pBluescript SK + (oriented opposite lac promoter) | this work |
| pLO132           | R. meliloti groESLc on a 2.9-kb Clai-BamHI fragment in pBluescript SK + | this work |
| pLSα replicon    | pACYC184-based vector with lac promoter and multiple cloning site from pUC8, Cm' | Goloubinoff et al. (1989) |
| pTG10            | E. coli groESL cloned into pTG10, Cm' | Goloubinoff et al. (1989) |
| pGroESL          | R. meliloti groESLc on a 3.2-kb BamHI fragment cloned into pTG10, expressed under the control of lac promoter | this work |
| pTK290           | incP, Te' pLAFR1 containing polylinker flanked by trp promoter and rpo C terminator | G. Ditta |
| pTE3            | plAFR1 containing polylinker flanked by trp promoter and rpo C terminator | Egelhoff and Long (1985) |
| pCHK57          | pNhA-lacZ fusion                       | Ditta et al. (1987) |
| pTK290.720::Tn5-gusA2.774 | nifH::Tn5-gusA fusion                  | Sharma and Signer (1990) |
R. meliloti groESLa required for nod regulation

Table 5. (Continued)

| Strain or plasmid | Genotype or relevant characteristics | Source or reference |
|-------------------|-------------------------------------|---------------------|
| pE65              | nodD3 expressed under control of trp promoter in pTE3 | Fisher et al. (1988) |
| pF644             | trp promoter controlling transcription of lacZ | R. Fisher |
| pT5               | 20.5-kb region of prmeSU47a in pLAFR1, contains syrM, nodD3, syrA, and other genes | Swanson et al. (1987) |
| pM142             | nodD3-gusA-Sp' | Mulligan and Long (1989) |
| pO37              | R. meliloti groESLa on a 6.7-kb EcoRI fragment in pRK660 | this work |
| pO43              | E. coli groESL on a 2.2-kb EcoRI-HindIII fragment in pLAFR3 | this work |
| pO57.33           | nodD1, nodABC'-'lacZ-Sp' from pLM77 [Mulligan and Long 1985] on a 17-kb EcoRI fragment in pRK660 | this work |

Fisher et al. (1988)

R. Fisher

Swanson et al. (1987)

Mulligan and Long (1989)

Volvement in stress responses such as heat shock. In E. coli, groEL and groES are essential genes [Fayet et al. 1989]. Most of the characterized E. coli groEL mutants, such as the groEL44 mutant used in our study, carry missense mutations that limit GroEL function so as not to promote bacteriophage head assembly or not to allow bacterial growth at 43°C [Zeilstra-Ryalls et al. 1993]. Null mutants that completely lack functional GroEL are not isolable [Fayet et al. 1989]. Null mutations on recombinant plasmids have provided indirect evidence for the role of groESL in nodulation. Figure 4. Effect of groESL on NodD-DNA binding. (A) DNA-binding activity of NodD in extracts of wild-type or mutant B4 R. meliloti extracts. The nodA nod box promoter was mixed with cell-free extracts from R. meliloti strains AL110 or B4T3, both carrying the nodD3-overexpressing plasmid pE65, and both containing a nodC'-'lacZ fusion in pSyma. Extracts were prepared at the same time and under the same conditions. β-Galactosidase activity was determined for an aliquot of the same culture used for preparing the extracts: AL110 (pE65) gave 350 units and B4T3 (pE65) gave 90 units. The electrophoretically retarded complex is indicated by the top arrow. (B) DNA-binding activity of NodD in extracts of E. coli carrying plasmids that overexpress nodD and groESL. Extract protein (32 μg) was used in the reaction mixtures for lanes 2–15. The presence of nodD1 or nodD3 on a plasmid is indicated above the lanes, and the presence of E. coli groESL or R. meliloti groESLc on a plasmid is also indicated. For lanes 13 and 15, 3 μM, luteolin was included in the binding reaction mixture. For lanes 2–6, to increase expression from the trp promoter on the low copy-number vector, E. coli cultures were induced with indole-3-acrylic acid as described in Materials and methods. For lanes 7–15, E. coli were grown in 2× YT medium, because the trp promoter constructs were on high copy-number vectors that overcame repression of the trp promoter. Note that the trp promoter constructs lack the leader peptide-coding region and therefore are not subject to attenuation. E. coli strains used to prepare extracts were NK5821 containing the following plasmids: [lane 2] pTE3 and pKS+groESL; [lane 3] pTE3 and pFO115; [lane 4] pE65 and pKS+groESL; [lane 5] pE65 and pFO115; [lane 6] pE65 and pBluescript SK+; [lane 7] pJO121; [lane 8] pJO211; [lane 9] pJO121; [lane 10] pAD10 and pGroESL; [lane 11] pE39 and pFG10; [lane 12] pE39 and pFO121; [lane 13] pE39 and pFO121; [lane 14] pE39 and pGroESL; [lane 15] pE39 and pGroESL. No extract was included in the binding reaction for lane 1. Instead of extract, 160 ng of partially purified NodD3 [Fisher and Long 1989, 1993] was added to the binding reaction for lane 16. The electrophoretically retarded complex is indicated by the top arrow.
Ogawa and Long

Figure 5. *R. meliloti* GroEL coimmunopurifies with NodD1. (Lane 1) A silver-stained gel profile of a sample of NodD1 purified by immunoaffinity chromatography (Fisher et al. 1988); (lanes 2,3) Western blots of duplicate lanes of the same immunopurified NodD1 preparation. The Western blot in lane 2 was probed with anti-NodD1 antiserum; the blot in lane 3 was probed with anti-*E. coli* GroEL antiserum. Sample containing 670 ng of protein was loaded onto each of three lanes of a 12% polyacrylamide–SDS gel for separation by electrophoresis.

Evidence for GroEL (chaperonin-60) involvement in protein assembly in vivo. Overexpression of groEL can suppress temperature-sensitive mutations in numerous proteins and can assure correct formation of foreign proteins overexpressed in *E. coli* (for review, see Georgopoulos 1992), including heterologous transcription activators (Govezensky et al. 1991; Adar et al. 1992; Dolan and Greenberg 1992).

In vitro studies using well-characterized proteins as substrates have sought to establish the specific mechanism of GroES and GroEL action, along with other molecular chaperone proteins, in binding unfolded or partially folded proteins [for review, see Hendrick and Hartl 1993]. The biochemical studies, and observations of the fate of overexpressed genes in heterologous systems, do not specifically address the question of how much GroEL is required in vivo (Horwich et al. 1993). Do some proteins require it more than others? Genetic study in vivo is an important approach to answering this question.

Genetic requirement for GroEL in NodD-activated expression

Our discovery of a role for GroEL in *nod* gene expression had its origins in a genetic study similar to the original phage tests: The *R. meliloti* groESL locus emerged in a search for mutations important for gene expression in the *nod* gene induction system. Specifically, we discovered a transposon Tn5-induced mutant, B4, that decreased transcription resulting from NodD1–luteolin-mediated induction. We subsequently showed that this effect extends to NodD3 and to at least one other gene activator of the LysR family, SyrM.

The insertion in the groEL gene resulted in a null mutation. Although this would be lethal in *E. coli*, in which it has been shown that groESL is essential, the resulting *R. meliloti* B4 mutant was viable, showing only a slightly slower growth rate than wild type. This was explicable by the presence of two functional copies of groESL: one on the chromosome (groESLc) and one on the symbiotic plasmid pSyma (groESLa). The two *R. meliloti* groEL ORFs are functionally equivalent by two criteria: complementation of the original mutant phenotype, and complementation of the λ plating phenotype of an *E. coli* groEL mutant. These functional properties and the high sequence similarity (data not shown) suggest that the two ORFs are functionally redundant. However, we have shown in further work that the two are expressed at different levels, with the chromosomal locus, groESLc, being expressed more highly [J. Ogawa and S.R. Long, in prep.]. It appears that other essential cell functions can be sustained by relatively low expression of GroESL proteins. No comprehensive survey of functions was made, but we determined that two processes requiring function of two-component regulatory systems were normal in a B4 mutant strain: dicarboxylic acid transport, which requires dctB and dctD, and whose expression is necessary for growth on succinate as sole carbon

Figure 6. Model of the role of GroEL in *R. meliloti nod* gene regulation. GroEL is required for full NodD1-dependent activation of *nod* gene expression in the presence of inducer; there is evidence for a physical association between GroEL and NodD1. GroEL is also required for full NodD3-dependent inducer-independent activation of *nod* gene expression. In addition, GroEL is required for nodD3 expression, which is known to be dependent on the NodD-related protein SyrM.
source, and nifA expression, which requires fixL and fixF. Therefore, it appears that the function of the NodD proteins is unusually sensitive in its need for GroEL and/or requires exceptionally high levels of GroEL.

NodD and SyrM belong to the LysR family of prokaryotic transcriptional activators, a group whose relationship is noted mostly by sequence similarity (Henikoff et al. 1988; Schell 1993). NodD is a regulator first identified in R. meliloti (Egelhoff et al. 1985) and is apparently universal in all Rhizobium, Bradyrhizobium, and Azorhizobium (Kondorosi 1992). From our observations of gene fusion expression, we infer that GroEL is required for the activity of NodD1, NodD3, and SyrM (Fig. 6). This leads us to suggest that GroEL may be required for the activity of all members of the LysR-type activator protein family, whose molecular basis of action has not been established (Schell 1993). A central question about the involvement of GroEL and GroES with NodD will be whether the GroEL requirement is only to assist the folding of NodD itself, which is then fully active, or whether it is required for the correct assembly of NodD with other components of the active transcription apparatus.

The mechanism by which NodD affects gene expression is not known, but it involves binding of NodD to an upstream sequence, the nod box (Fisher and Long 1992; Kondorosi 1992; Schlaman et al. 1992). NodD binds to a 50- to 60-bp segment of DNA along one face of the helix and causes a bend (Fisher and Long 1993). We found that GroEL associates biochemically with NodD and that in the B4 mutant background, the NodD present in the cell binds less well to the nod box. In addition, we observed that an E. coli cell overexpressing groEL can produce NodD with a greatly improved ability to bind nod boxes, compared with NodD produced in E. coli expressing normal levels of GroEL. This suggests that, at minimum, the GroEL promotes the formation of an active NodD that can bind correctly to its target site. The presence of NodD capable of binding nod boxes did not suffice to permit expression of nod box promoters in E. coli, suggesting that other R. meliloti factors necessary for expression are absent from, or different in, E. coli. It is possible that E. coli RNA polymerase is not able to interact with NodD and the nod box promoter in a way that results in expression from the promoter. It has been observed previously that some constitutive Rhizobium promoters, such as trp, do not function in E. coli because of the failure of the E. coli RNA polymerase to recognize the upstream sequence (Bae et al. 1989). Whether the effect of GroEL on promoter-binding ability of NodD suffices to explain the whole effect of GroEL, will await complete in vitro reconstitution of NodD-induced gene expression using RNA polymerase from R. meliloti.

Multiple groEL loci

Our ability to study the role of GroEL in gene expression was made possible by the multiple, differentially expressed genes of R. meliloti. There have been several reports of multiple copies of groEL genes in other bacteria, including Streptomyces albus (Guglielmi et al. 1991; Mazodier et al. 1991], several species of Mycobacterium [Rinke de Wit et al. 1992; Hughes 1993; Kong et al. 1993], and Synechocystis sp. PCC6803 (Lehle et al. 1993). Purified plastid chaperonin 60 (Rubisco-binding protein) contains approximately equal amounts of two polypeptides [Hemmingsen and Ellis 1986] called α and β, which are both encoded by nuclear genes, and which show sequence divergence [50% identity] (Martel et al. 1990; Ellis and van der Vies 1991). The significance of such multiple GroEL loci—for example, differential function, or differential regulation—has not been established in most of these cases.

In the case of R. meliloti, there exist other hybridizing bands visible on Southern blots when groEL is used as a probe (Fig. 1, and Rusanganwa and Gupta 1993), although only one of these fragments also shows any hybridization to a groES probe. Whether these hybridizing sequences are functional is unknown, but we have found in other work (J. Ogawa and S.R. Long, in prep.) that a double null mutant in groEL and groES is apparently not viable, which suggests that no other copies can replace them, either for regulatory or functional reasons.

Our observation (Ogawa 1993) that multiple groEL copies seem to exist in other Rhizobium species and in Bradyrhizobium japonicum, but not in the closely related species A. tumefaciens (see also Fischer et al. 1993; Segal and Ron 1993; Wallington and Lund 1994), may point to a role for the additional copies of groEL in the nodulation process. This is reinforced by the studies of Fischer et al. [1993], who discovered five groEL genes in B. japonicum. Two of the copies [groEL2 and groEL4] seem to be expressed constitutively at significant levels, the expression of one copy [groEL5] is induced under anaerobic or symbiotic growth conditions, and the expression of groEL1 and groEL4 was very low, although the expression of groEL4 increased >25-fold after a shift in growth temperature from 28°C to 39°C [Fischer et al. 1993]. None of the mutant strains made by interrupting any single B. japonicum groEL gene displayed a detectable symbiotic phenotype, and, except for the groEL4 mutant whose growth in minimal medium under aerobic conditions was impaired, growth of the mutants was not affected [Fischer et al. 1993]; this points out at least one major difference in the functional organization of the groEL genes in B. japonicum compared with R. meliloti.

Given this discovery of NifA-dependent expression of one groEL in Bradyrhizobium, we were intrigued to see that the root nodules generated by our B4 groELc mutant were Fix−. NifA from Klebsiella pneumoniae has been reported to coimmunoprecipitate with GroEL, and genetic data suggest that K. pneumoniae NifA activity in E. coli may be decreased by a mutation in the host groEL (Govezensky et al. 1991). These data suggest a role for GroEL in NifA-related responses. However, we found that NifA-dependent nifH expression was normal in the R. meliloti groEL::Tn5 mutant; thus it is unlikely that the Fix− phenotype of this mutant is attributable to loss of NifA activity.

On the other hand, Govezensky et al. [1991] also re-
ported that newly synthesized *K. pneumoniae* NifH, NifD, and NifK proteins transiently associated with GroEL in communoprecipitation experiments of pulse-chased cells using anti-GroEL antibodies. If GroELc plays a role in nitrogenase component assembly, this could account for the Fix− phenotype of the *R. meliloti* groELc: Tn5 mutant, that is, structural genes such as nif−HDK may be expressed at high levels in the mutant, but the protein products may not assemble into functional nitrogenase enzyme. In addition, the groELc:: Tn5 mutation may affect the function of any of a number of fix gene products that are required for effective nodulation. Finally, as pointed out by Fischer et al. (1993), GroEL may be involved in the translocation of proteins from endosymbiotic bacteroids out into the peribacteroid space, or in the folding and assembly of host-plant polypeptides imported into bacteroids.

**Materials and methods**

**Strains, plasmids, and media**

Strains and plasmids are listed in Table 5. The “Rm” designation used previously for plasmids containing *R. meliloti* sequences, for example, pRM665, has been omitted from plasmid names listed here. *R. meliloti, Agrobacterium, E. coli,* and phage λ were grown by standard methods and as described previously (Ogawa et al. 1991; Ogawa 1993).

**Genetic techniques**

Transductions using *R. meliloti* phage N3 [Martin and Long 1984] were performed essentially as described [Finan et al. 1984], except that cells were washed once with 0.5 ml of LB following adsorption of phage, and the cell pellet was then resuspended in LB plus 5 mM sodium citrate for plating. Distances between markers were calculated (Martin and Long 1984) based on a phage N3 packaging size of 160 kb [Finan et al. 1984].

Mobilization of broad-host-range plasmids into *R. meliloti* was carried out in triparental matings using the helper plasmid pRK600 to provide mobilization functions in trans. Transposon replacements were performed as described [De Vos et al. 1986]. Mapping of antibiotic resistance markers to pSyma or pSymb was done by determining whether the marker cotransferred with either megaplasmid carrying the RK2 origin of transfer in Tn5− Tn11 when conjugated into Rm6879, as described by Long et al. (1988).

Mapping of unmarked sequences to the symbiotic megaplasmids was done by constructing strains of *A. tumefaciens* A136 into which pSyma or pSymb, marked with Tn5− Tn11, had been mobilized [Finan et al. 1986], followed by isolating DNA from these strains and preparing Southern blots, which were then hybridized with cloned probes. Mobilization of pSymb from Rm5300 into A136 occurred at very low frequency (≤10−8 per recipient), and to verify the A136 (pSymb) construct, Nal′, Rif′, Cm′, Sp′ colonies from this mating were screened for resistance to *R. meliloti* phage N3, and DNA from these strains was then probed in Southern hybridizations with pSymb-specific insert DNA from clone pSG56A (J. Glazebrook, unpubl.), mobilization of pSyma into A136 occurred at higher frequencies (≥10−4), and constructs were verified by antibiotic resistance, by screening for phage resistance, and by DNA hybridizations.

Mapping of the Tn5− Tn233 in B4Sp2 was done using approaches described by Klein et al. (1992) and Glazebrook et al. (1992). The B4Sp2 marker transferred with the highest efficiencies from Tn5-Mob f611 and f615 about equally (Ogawa 1993), indicating that the Tn5 insertion in mutant B4 lies between trp−33 and pyr−49 on the *R. meliloti* chromosome.

The plating ability of bacteriophage λ on *E. coli* hosts was initially determined by the spot titer method: Bacteriophage λ lysate was serially diluted in λ-dil and 5 µl of several dilutions was spotted onto lawns of appropriate *E. coli*. The efficiency of plating was subsequently confirmed by quantitative titration of the phage with preadsorption.

**DNA manipulations**

Total bacterial DNA was isolated from *R. meliloti* as described previously [Swanson et al. 1987]. Southern blots were prepared as described previously [Ogawa et al. 1991] or by blotting from agarose gels to Magna NT nylon membrane [Micron Separations, Inc.] in 0.25 M NaOH, 1.5 M NaCl. 32P-Labeled probes were prepared by primer extension using random hexanucleotide primers and gel-purified restriction fragment templates by standard methods. Hybridizations were carried out as described previously [Barnett and Long 1990; Ogawa et al. 1991]; final washes were done at 65°C unless otherwise noted.

pJO32 was constructed by cloning a 1.6-kb PstI− EcoRI fragment containing nodD3 from pBGR1 [Rushing et al. 1991] into pAD10. pKS+groESL and pSK+groESL were constructed by cloning a 2.2-kb EcoRI− HindIII fragment containing *E. coli* groESL from pOF39 into pBluescript KS+ and pBluescript SK+, respectively. The same 2.2-kb EcoRI− HindIII fragment was cloned into pLAFR3 to make pO43.

**Cloning of *R. meliloti* groESL**

The Tn5-containing region of B4T1 was cloned as a 10.5-kb ClaI fragment, yielding pIO91 [Ogawa 1993]. Bacteriophage λ clones of *R. meliloti* groESLc and groESLa were obtained by constructing libraries of size-selected BglII fragments. The 7.9- and 12-kb BglII fragments hybridizing to probe X and Y were cloned into pAZAP1 and pAFIXII vectors [Rushing et al. 1991]; the 7.9-kb BglII fragment cloned as pJO101. Southern blot hybridizations of DNA from B4T1 confirmed that the Tn5 is inserted into the 7.9-kb BglII fragment cloned as pJO101.

A faint 12-kb BglII band hybridizing to both groEL and groES probes is visible in the GM1766 Southern blot lanes [Fig. 1D, lanes 6 and 12], even though this strain lacks the strongly hybridizing 12-kb groESLs fragment cloned in pJO32. We isolated a faintly hybridizing clone from the same size-selected pAFIXII library from which we isolated pAFIXII-4a and verified that the insert faintly hybridized to both a groES and groEL probe; this clone was not characterized further.

**Protein analysis**

Coupled transcription–translation reactions using S-30 extracts from *R. meliloti* RCR2011 or *E. coli* HB101, and the separation and visualization of protein products, were carried out as described [Fisher et al. 1987]. Three micrograms of plasmid DNA purified by CsCl-banding was used as template in each reaction mixture. Western blots with NodA-specific antiserum or nodD-specific antiserum were prepared and probed as described [Egelhoff and Long 1985; Fisher et al. 1988]. Anti-*E. coli* GroEL antibodies were a gift from G. Lorimer (E.I. DuPont de Nemours and Co.).
DNA-binding assay

The ability of proteins in extracts of R. meliloti or E. coli to bind specifically to the nodA promoter was assessed by monitoring the specific shift in electrophoretic gel mobility of a labeled nodA promoter-containing fragment as described [Fishier et al. 1988]. The nodA promoter-containing fragment used was the 246-bp EcoRI-HindIII fragment from pF528 [Fishier and Long 1989].

Cell-free extracts of R. meliloti and E. coli were prepared as described [Fishier et al. 1988]. To induce expression from the trp promoter on the low-copy-number vector, E. coli carrying pTE3 or pE65 was grown overnight at 30°C in M9 minimal medium supplemented with 0.2% glycerol, 0.5% casamino acids, 20 μg tryptophan/ml, 40 μg arginine/ml, 1 μg thiamine/ml, and appropriate antibiotics, then subcultured into 9 volumes of the same medium without tryptophan, grown for another 3 hr, after which indole-3-acrylic acid was added to a final concentration of 10 μg/ml and the cells were grown for an additional 10 hr before being harvested. Other E. coli were grown in 2X YT medium with appropriate antibiotics at 30°C, and harvested at an OD 600 reading of 0.8–1.0.

Enzyme assays

β-Galactosidase assays were carried out as previously described [Mulligan and Long 1985] using DME stock dilutions of luteolin for 4 hr induction of early log phase cells [Ogawa 1993]. β-Glucuronidase (GUS) assays of nodD3 and syrM fusions were carried out as described [Swanson et al. 1993]. GUS assays of the nifH-gusA fusion were done using the fluorogenic assay described by Jefferson [1987]. One unit of GUS activity produces one nanomole of product per minute at 37°C and we have normalized this to the OD 600 reading of the bacterial culture. For microaerobic induction of nifA–lacZ and nifH–gusA fusions, the stoppered-tube assay [Ditta et al. 1987] was used and the cultures were assayed after 18 hr of growth in 1% oxygen, aerobically grown controls were shaken in well-aerated flasks and assayed at an OD 600 of ~0.2.

Assessing symbiotic phenotype

Nodulation assays were conducted using alfalfa seedlings on agar slants as described previously [Ogawa et al. 1991], except that buffered nodulation medium [Ehrhardt et al. 1992] solidified with 12 grams of Bacto-agar per liter was used. Plant tubes were inoculated with bacteria suspended in water from fresh M9-sucrose plates at a dosage of 10^6–10^7 bacteria per plant. To test for strain identity, bacteria were isolated from crushed nodules that had been surface-sterilized with 20% Clorox as described [Long et al. 1988]. Nodules formed late by B4 mutants were not attributable to revertants, as inferred from genetic markers.

The nitrogen-fixing activity of nodulated plants was measured indirectly using the acetylene reduction assay [Turner and Gibson 1980]. Four weeks after inoculation, test tubes containing nodulated plants were stoppered and injected with 1 ml of acetylene. Eight hours after the acetylene was added, 1 ml of gas was removed from the stoppered tube and analyzed on a Hewlett Packard 5890A gas chromatograph outfitted with a Porapak N column. Using the flame-ionization detector, we were able to detect 0.02% conversion of acetylene to ethylene.

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