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 pSymb. 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 pSymb; a second, larger (1700 kb) plasmid, pSym, 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*, and

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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 activator protein. 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

A 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

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

Strain	Plasmid (description)	Units β -galactosidase ^a (fold induction)	
		– luteolin	+ luteolin
AL110	none	0.5	8.1 (16 $\times$)
B4	none	0.4	2.1 (5 $\times$)
AL110	pO57.33 (<i>nodD1</i> <i>nodABC'</i> - <i>lacZ</i>)	22	640 (29 $\times$)
B4	pO57.33	13	51 (4 $\times$)

^aCultures were grown in M9–sucrose liquid medium.

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, not 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

Table 2. Effect of B4 mutation on *nodD* expression and *NodD* activity

Strain (description)	Plasmid (description)	Enzyme activity ^a	
		– luteolin	+ luteolin
units β-galactosidase			
1. Rm1021	pM61 (<i>nodD1'–'lacZ</i>)	510	500
2. B4T1	pM61	620	620
3. AL110 (<i>nodABC'–'lacZ</i>)	pE65 (<i>P</i> <i>trp</i> → <i>nodD3</i>) ^b	490	480
4. B4T3 (<i>nodABC'–'lacZ</i>)	pE65	59	70
5. AL110	pJT5 (<i>syrM</i> , <i>nodD3</i> , . . .)	250	270
6. B4T3	pJT5	3	5
7. JAS301 (<i>nodD3–lacZ</i> 5-1 fusion)	pJT5	54	52
8. B4Sp2 <i>nodD3–lacZ</i> 5-1 fusion	pJT5	4	3
units β-glucuronidase × 10 ³			
9. AL110	pM142 (<i>syrM–gusA</i> fusion)	330	360
10. B4T3	pM142	650	660

^aCultures were grown in TY liquid medium.

^bTo confirm the validity of the expression vector, we tested the activity of the *trp* promoter with plasmid pF644 [R.F. Fisher (this laboratory), unpub.] and found that the same *trp* promoter drove similar levels of *lacZ* expression in the B4 background and in wild-type Rm1021 (data not shown).

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 (pJT5) (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 wild-type 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 in TY liquid medium). 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 pCHK57 (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-

tance did not cotransfer with either pSym (Finan et al. 1986), and subsequently by conjugation analysis (Glazebrook et al. 1992; Klein et al. 1992), finding that the B4 mutation lies between *fix-382* and *ura-501* (Fig. 1A). The mutation failed to show transductional linkage to loci mapped previously (*pho-1*, *fix-382*, *degP*).

We cloned the Tn5-containing region of mutant B4 on a *ClaI* fragment to produce pJO91 (Fig. 2A). Internal 1.6-kb *XhoI* fragment and 5.3-kb *BamHI* fragment probes (X and Y in Fig. 2A) consistently hybridized to two fragments of Rm1021 genomic DNA digested with enzymes that did not cut within the cloned *R. meliloti* sequence contained in pJO91 (data not shown; also, two nonidentical sets of much fainter bands also appeared on blots hybridized with probe X or probe Y). We concluded that there were at least two loci in the *R. meliloti* genome with DNA corresponding to the B4 open reading frame. We cloned strongly hybridizing 7.9- and 12-kb *BglII* fragments to yield a series of λ clones and the plasmids pJO101 and pJO32, respectively. We confirmed that in the B4 mutant, Tn5 is inserted into the sequence corresponding to the 7.9-kb *BglII* band.

The DNA sequence for pJO101 (GenBank accession number U19726) showed that it contains a 1635 base open-reading-frame (ORF) encoding a 57.7-kD polypeptide that bears a striking similarity to *E. coli* GroEL and other chaperonin-60 proteins (Ogawa 1993; data not shown). In mutant B4, the Tn5 interrupts this ORF about one-fifth of the way in from the 5'-end, after nucleotide 350 of the *groEL* ORF. Just upstream of the *groEL*-like sequence is a 294-base ORF encoding a 10.6-kD polypeptide that is similar to *E. coli* GroES and other chaperonin-10 proteins.

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 *SmaI* 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 GMI255 (Fig. 1C) but absent from GMI766 (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 *groES* and *groEL* 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 *groES*-like and *groEL*-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 groESL, groESLc, and groESLa

We tested function of broad host-range clones carrying the *R. meliloti groESLa* and *groESLc* and the *E. coli*

Figure 1. (See facing page for legend.)

groESL genes. The *R. meliloti* clones restored wild-type growth to B4 derivatives. Also, the *groESLc* and *groESLa*

clones both restore B4T3 luteolin-induced β -galactosidase activity to wild-type levels (Table 3). However, this

was not an exclusive property of the *R. meliloti* cloned genes: The *E. coli* groESL clone (pO43) increased luteolin-induced *nodC-lacZ* expression fourfold over wild type. The quantitative effect of the *E. coli* clone could be attributable at least in part to the high levels of expression supported by the *E. coli* groESL promoter seen in coupled transcription-translation reactions using *R. meliloti* S-30 extracts (Fig. 3, lanes 3,4), although it is possible that there are also functional differences between the *E. coli* and *R. meliloti* GroEL proteins.

We tested for complementation of the two other symbiotic defects, delayed nodulation and Fix^- nodules, by introducing the groESL clones into B4T1 and into a wild-type control; in each case, a parallel *recA::Tn5-233* derivative was also tested (data not shown; Ogawa 1993). The two *R. meliloti* clones successfully complemented both nodulation and nitrogen fixation phenotypes. Adding extra copies of groESLa or groESLc did not affect nodulation kinetics or Fix^+ phenotype of the wild-type control. A modest decrease in overall vigor of the Rec^- strains was evident in all cases but did not affect the conclusions: Clones of both groESLc and groESLa restored symbiotic phenotype to wild-type levels.

On the other hand, the *E. coli* groESL clone pO43 did not complement nodulation delay or the Fix^- phenotype of the B4 Tn5-insertion mutants. The apparent lack of function is probably attributable in part to plasmid instability: We observed a high frequency of loss of the *E. coli* groESL plasmid during nodulation (data not shown). Functional differences of the *E. coli* cloned groESL are nonetheless possible, as suggested by its failure to complement the slow growth in culture of B4 derivatives (data not shown).

We also tested the converse situation, asking whether the *R. meliloti* groESL genes would complement *E. coli* strains carrying mutations in groES and groEL, which fail to support λ growth and are temperature sensitive for growth at 43°C (Tilly and Georgeopoulos 1982). As shown in Table 4, pBluescript-based groESLc and groESLa clones were comparable to the *E. coli* groESL clones in ability to complement *E. coli* groES and groEL

mutants for ability to support λ growth. These clones were less effective at complementing the temperature-sensitive growth phenotype, in that colonies formed but they were very small. It is possible that *R. meliloti* GroEL does not function well at 43°C, because *R. meliloti* 1021 does not itself grow on LB agar at 43°C. We noted that when *E. coli* groESL were cloned into pBlue-script SK+ such that these genes were in the same orientation as the *lac* promoter, the resulting clone did a very poor job of complementing the mutants, whereas cloning these genes such that the groESL genes are transcribed opposite the *lac* promoter resulted in a clone that was very proficient at complementing the groES and groEL mutants. A lower copy number version of the *R. meliloti* groESLc, cloned downstream 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 AL110 (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* pSymb. (B) Diagram showing the portion of pSymb that is present in GMI255, but absent from GMI766. (C) Southern blot analysis of *A. tumefaciens* strain A136 carrying pSymb or pSymb shows that the second hybridizing (12-kb BglIII) band in *R. meliloti* DNA cotransfers with pSymb, and is present in *R. meliloti* strain GMI255 which carries a mapped deletion of pSymb. Total bacterial DNA from *R. meliloti* strain Rm1021, *A. tumefaciens* strain A136, and A136 carrying pSymb or pSymb, and *R. meliloti* deletion strain GMI255 was digested with BglIII, 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 BglIII bands in the Rm1021 DNA lane is indicated on the right. The 12-kb BglIII band which hybridized to the *R. meliloti* groELc probe was present in A136 carrying pSymb, but was absent from A136 carrying pSymb or no *R. meliloti* megaplasmid. The 7.9-kb BglIII band present in Rm1021 DNA was not present in A136 carrying pSymb 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 pSymb 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 GMI766, which carries a mapped deletion of pSymb shows that the second hybridizing (6.7-kb EcoRI, 10-kb ClaI, 12-kb BglIII) band is located in the region of pSymb that is deleted in GMI766, and also shows additional bands hybridizing to *R. meliloti* groES 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).

Figure 2. Map of the cloned *R. meliloti* *groES* and *groEL* genes. (A) Restriction map of pJO91 which contains the Tn5 and flanking DNA sequences from *R. meliloti* mutant B4. Probes used for Southern blot analysis and for isolating *groESL* clones from Rm1021 are indicated above the map. Restriction map of the corresponding region of the Rm1021 chromosome cloned in pJO101, and subclones used in this study. The locations of the ORFs corresponding to *groES* and *groEL* as determined by DNA sequencing are indicated with arrows just below the restriction map; subclones are shown below the restriction map. Restriction map of the region of pSyma cloned in pJO32 which hybridizes to *R. meliloti* *groES* and *groEL* probes, and subclones used in this study. Abbreviations: (B) *Bam*HI; (Bg) *Bgl*II; (C) *Cla*I; (E) *Eco*RI; (H) *Hind*III; (K) *Kpn*I; (S) *Sac*I; (X) *Xba*I. C^d denotes *Clal* sites that are not digested in plasmid DNA isolated from *dam*⁺ *E. coli*. For subclones, open arrows indicate the direction of transcription controlled by the *lac* promoter in the pBluescript or pTG10 vector; names of broad host-range subclones are italicized. (B) A more detailed restriction map of the region encoding the *groES* and *groEL* genes. The map of the 3.2-kb *Bam*HI fragment from pJO101, denoted by asterisks (*) in A, is expanded, and the locations of additional restriction sites are shown. Probes used for Southern blot analysis are indicated above the restriction map, and arrows below the map show the location of the *groES* and *groEL* ORFs. The map of the 2.9-kb *Xba*I fragment from pJO32, denoted by stars in A, is also expanded and shown in more detail. The two fragments have been positioned so that the restriction sites falling within the *groES* and *groEL* coding region are aligned. The DNA sequence is found under GenBank accession number U19726. A purine-rich region located ~8 nucleotides upstream of the initiation codon for the *groES*-like sequence, and another located ~9 nucleotides upstream of the initiation codon for the *groEL*-like sequence could serve as potential ribosome-binding sequences. 36 nucleotides downstream of the termination codon of the *groEL*-like sequence is a GC-rich sequence with dyad symmetry and other features of a rho-independent terminator. Abbreviations are as in A and (M) *Mlu*I; (N) *Nhe*I; (P) *Pst*I; (Sa) *Sal*I; (Sm) *Sma*I; (Sp) *Sph*I; (Xh) *Xho*I.

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*

Figure 3. In vitro expression of *R. meliloti* *groESc* and *groELc* protein products. Coupled transcription-translation analysis employed S-30 extracts from *R. meliloti* RCR2011 as described previously (Fisher et al. 1987). An autoradiograph of ³⁵S-labeled proteins separated on a 15% polyacrylamide-SDS gel displays proteins encoded by vector pBluescript KS+ (lane 2) and vector pBluescript SK+ (lane 11). Proteins encoded by clones of *E. coli* *groESL*, pKS+*groESL* and pSK+*groESL*, are shown in lanes 3 and 4, respectively. Clones of *R. meliloti* *groESLc* or *groESLa* were used to direct the synthesis of proteins shown in lanes 5–10 as follows: (lane 5) pJO112; (lane 6) pJO111; (lane 7) pJO131; (lane 8) pJO132; (lane 9) pJO47; (lane 10) pJO46. No template DNA was included in the reaction mixture for lane 1. The positions of both *E. coli* GroEL and *R. meliloti* GroEL are indicated by the top solid arrow. The position of *E. coli* GroES is indicated by the arrowhead, and the position of *R. meliloti* GroES is indicated by the bottom solid arrow. A novel 17-kD band that appeared in the pJO112 reaction (lane 5) could be attributable to transcription from the *lac* promoter into the region downstream of the *groEL*-like ORF, which is present in pJO112, but absent from pJO131.

Table 3. Complementation of the *B4* mutant phenotype

Strain (description)	Plasmid (description) ^a	Units β -galactosidase ^b	
		– luteolin	+ luteolin
AL110 (<i>nodABC'</i> –' <i>lacZ</i>)	pRK290 (vector)	0.7	5
B4T3 (<i>nodABC'</i> –' <i>lacZ</i>)	pRK290	0.6	1
AL110	pO113 (<i>Rm groESLc</i>)	0.7	5
B4T3	pO113	0.7	5
AL110	pO37 (<i>Rm groESLa</i>)	0.7	6
B4T3	pO37	0.7	5
AL110	pO43 (<i>Ec groESL</i>)	0.8	20
B4T3	pO43	0.8	16

^a*Ec* designates *E. coli* genes; *Rm* designates *R. meliloti* genes.^bCultures were grown in M9–sucrose liquid medium.

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 μ M 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 ex-

pression 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 4. Complementation of *E. coli groE* mutants

Plasmid	Description	Relative λ plating efficiency (colony formation at 43°C)		
		<i>E. coli</i> strain ^a		
		CG2245 (wt)	CG2244 (<i>groES</i> [–])	CG2241 (<i>groEL</i> [–])
pBluescript SK +	vector	1 (+)	<10 ^{–5} (±)	<10 ^{–5} (–)
pOF39	<i>Ec groESL</i> in pBR325	1 (+)	0.1 (+)	0.2 (+)
pKS + <i>groESL</i>	<i>Ec groESL</i> in pBluescript (oriented opposite <i>Plac</i>)	0.9 (+)	0.6 (+)	0.7 (+)
pSK + <i>groESL</i>	<i>Es groESL</i> in pBluescript (<i>Plac</i> → <i>Ec groESL</i>)	0.9 (+)	0.01 (±)	<10 ^{–5} (–)
pJO115	<i>Rm groESLc</i> in pBluescript (<i>Plac</i> → <i>Rm groESLc</i>)	1.4 (+)	0.7 (±)	0.8 (±)
pJO46	<i>Rm groESLa</i> in pBluescript (<i>Plac</i> → <i>Rm groESLa</i>)	1.5 (+)	0.7 (±)	1.3 (±)

^a(+) 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
R. meliloti		
AL110	Rm1021 <i>lac</i> ⁻ , <i>nodC</i> ⁻ – <i>lacZ</i> – <i>Sp</i> ^r 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 ^r Sp ^r	this work
GMI255	2011 Δ (<i>fix7064 nod nifHDK</i>)7125 (Tn5) Nal ^r	J. Dénarié
GMI766	2011 Δ (<i>nod nifA</i>)766 <i>Sp</i> ^r	Truchet et al. (1985)
JAS301	Rm1021 <i>nodD3::lacZ</i> 5-1 fusion, Nm ^r	Swanson et al. (1993)
Rm1021	SU47 <i>str-21</i>	H. Meade
Rm5300	Rm1021 pRmeSu47b <i>thi-502::Tn5–11</i>	Finan et al. (1986)
Rm5321	Rm1021 pRmeSU47a::Tn5–11	T. Finan
Rm6743	Rm1021 Ω 615::Tn5–Mob	S. Klein
Rm6761	Rm1021 Ω 611::Tn5–Mob	Klein et al. (1992)
Rm6826	SU47 <i>str-3 spc-1 rif-1 his-39 trp-33 aro-51 ura-501::Tn5</i>	Klein et al. (1992)
Rm6879	Rm5000 <i>recA::Tn5–Tp</i>	S. Klein
Rm8002	Rm1021 <i>pho-1</i>	Long et al. (1988)
Rm8382	Rm8002 <i>fix-382::TnpH</i>	Long et al. (1988)
Rm8620	Rm1021 Δ <i>degP65::Tn3HoKm</i>	Glazebrook et al. (1992)
E. coli		
CG2241	B178zjd::Tn10 <i>groEL44</i>	C. Georgopoulos
CG2244	B178 zjd::Tn10 <i>groES619</i>	C. Georgopoulos
CG2245	B178 zjd::Tn10 <i>groE</i> ⁺	C. Georgopoulos
NK5821	F ⁻ Δ (<i>lac-pro</i>)XIII Nal ^r Rif ^r <i>arg</i> ⁻ <i>recA56</i> su ⁻	N. Kleckner
A. tumefaciens		
A136	C58 derivative, cured of Ti plasmid, Nal ^r Rif ^r	E. Nester
Phage		
N3	<i>R. meliloti</i> transducing phage	Martin and Long (1984)
λ	wild type	A. Campbell
λ FIX II-4a	12 kb <i>Bgl</i> III fragment containing <i>R. meliloti groESLa</i> from Rm1021 in λ FIX II	this work
λ ZAP II-1	7.9-kb <i>Bgl</i> III fragment containing <i>groESLc</i> from Rm1021 in λ ZAP II	this work
Plasmids		
pMB1 or ColE1 replicon		
pBluescript SK +	phagemid vector	Stratagene
pBluescript KS +	phagemid vector	Stratagene
pAD10	pUC8 with <i>trp</i> promoter and <i>rpo C</i> terminator flanking the polylinker	A. Das
pE39	<i>nodD1</i> expressed under the control of <i>trp</i> promoter in pAD10	Egelhoff and Long (1985)
pOF39	<i>E. coli groESL</i> genes in pBR325	Fayet et al. (1986)
pSK + <i>GroESL</i>	<i>E. coli groESL</i> genes on a 2.2-kb <i>Eco</i> RI– <i>Hind</i> III fragment in pBluescript SK +	this work
pKS + <i>GroESL</i>	<i>E. coli groESL</i> genes oriented opposite <i>lac</i> promoter in pBluescript KS +	this work
pRK600	Cm ^r , pRK2013 Nm ^r ::Tn9 helper plasmid	Finan et al. (1986)
pRK607	pRK2013 containing Tn5–233, Gm ^r Sp ^r	De Vos et al. (1986)
pJOD3	<i>nodD3</i> expressed under the control of <i>trp</i> promoter in pAD10	this work
pJO32	12-kb insert from λ FIX II-4a cloned as a <i>Not</i> I fragment in pUC18	this work
pJO46	<i>R. meliloti groESLa</i> on a 2.9-kb <i>Xba</i> I fragment in pBluescript SK +	this work
pJO47	<i>R. meliloti groESLa</i> on a 2.9-kb <i>Xba</i> I fragment in pBluescript SK + (oriented opposite <i>lac</i> promoter)	this work
pJO91	10.5-kb Tn5-containing <i>Clal</i> fragment from B4T1 in pBluescript SK +	this work
pJO101	product of in vivo excision of λ ZAP II-1	this work
pJO111	<i>R. meliloti groESLc</i> on a 5-kb <i>Eco</i> RI fragment in pBluescript SK +	this work
pJO112	<i>R. meliloti groESLc</i> on a 5-kb <i>Eco</i> RI fragment in pBluescript SK + (oriented opposite <i>lac</i> promoter)	this work
pJO115	<i>R. meliloti groESLc</i> on a 3.2-kb <i>Eco</i> RI– <i>Hind</i> III fragment on pBluescript SK +	this work
pJO131	<i>R. meliloti groESLc</i> on a 2.9-kb <i>Clal</i> – <i>Bam</i> HI fragment in pBluescript SK + (oriented opposite <i>lac</i> promoter)	this work
pJO132	<i>R. meliloti groESLc</i> on a 2.9-kb <i>Clal</i> – <i>Bam</i> HI fragment in pBluescript KS +	this work
p15a replicon		
pTG10	pACYC184-based vector with <i>lac</i> promoter and multiple cloning site from pUC8, Cm ^r	Goloubinoff et al. (1989)
pGroESL	<i>E. coli groESL</i> cloned into pTG10	Goloubinoff et al. (1989)
pJO121	<i>R. meliloti groESLc</i> on a 3.2-kb <i>Bam</i> HI fragment cloned into pTG10, expressed under the control of <i>lac</i> promoter	this work
Broad host range		
pRK290	IncP, Tc ^r	G. Ditta
pTE3	pLAFR1 containing polylinker flanked by <i>trp</i> promoter and <i>rpoC</i> terminator	Egelhoff and Long (1985)
pCHK57	<i>PnifA</i> – <i>lacZ</i> fusion	Ditta et al. (1987)
pRK290.720::Tn5– <i>gusA2.774</i>	<i>nifH::Tn5–gusA</i> fusion	Sharma and Signer (1990)

Table 5. (Continued)

Strain or plasmid	Genotype or relevant characteristics	Source or reference
pE65	<i>nodD3</i> expressed under control of <i>trp</i> promoter in pTE3	Fisher et al. (1988)
pF644	<i>trp</i> promoter controlling transcription of <i>lacZ</i>	R. Fisher
pJT5	20.5-kb region of pRmeSU47a in pLAFR1, contains <i>syzM</i> , <i>nodD3</i> , <i>syra</i> , and other genes	Swanson et al (1987)
pM142	<i>nodD3-gusA-Sp^r</i>	Mulligan and Long (1989)
pO37	<i>R. meliloti</i> <i>groESLa</i> on a 6.7-kb <i>EcoRI</i> fragment in pRK290	this work
pO43	<i>E. coli</i> <i>groESL</i> on a 2.2-kb <i>EcoRI-HindIII</i> fragment in pLAFR3	this work
pO57.33	<i>nodD1, nodABC'-lacZ-Sp^r</i> from pM57 (Mulligan and Long 1985) on a 17-kb <i>EcoRI</i> fragment in pRK290	this work
pO113	<i>R. meliloti</i> <i>groESLc</i> on a 5-kb <i>EcoRI</i> fragment in pRK290	this work
pO130	<i>R. meliloti</i> <i>groESLc</i> on a 2.9-kb <i>ClaI-BamHI</i> fragment in pWB5a	this work

involvement 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). Tests using *groESL* on recombinant plasmids have provided indirect

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 μ g) 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 μ M, 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 $\times$ YT medium, because the *trp* promoter constructs were on high copy-number vectors that overcame repression of the *trp* promoter. Note that *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 pJO115; (lane 4) pE65 and pKS+*groESL*; (lane 5) pE65 and pJO115; (lane 6) pE65 and pBluescript SK+; (lane 7) pJOD3 and pTG10; (lane 8) pJOD3 and pJO121; (lane 9) pJOD3 and pGroESL; (lane 10) pAD10 and pGroESL; (lane 11) pE39 and pTG10; (lane 12) pE39 and pJO121; (lane 13) pE39 and pJO121; (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.

pE65 and pBluescript SK+; (lane 7) pJOD3 and pTG10; (lane 8) pJOD3 and pJO121; (lane 9) pJOD3 and pGroESL; (lane 10) pAD10 and pGroESL; (lane 11) pE39 and pTG10; (lane 12) pE39 and pJO121; (lane 13) pE39 and pJO121; (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.

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 partly 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 *fixJ*. Therefore, it appears that the function of the NodD proteins is unusually sensitive in its need for GroESL and/or requires exceptionally high levels of GroESL.

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 (Lehel 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 *groELc* and *groELa* 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 (*groEL₃*) is induced under anaerobic or symbiotic growth conditions; and the expression of *groEL₁* and *groEL₅* was very low, although the expression of *groEL₁* 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 *groESL* 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 groELc::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 coimmunoprecipitation 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, pRmE65, 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 pSymb 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 pSymb 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 ($\sim 10^{-8}$ per recipient), and to verify the A136 (pSymb) construct, Nal^r, Rif^r, Gm^r, Sp^r colonies from this mating were screened for resistance to *R. meliloti* phage N3, and DNA from these strains was probed in Southern hybridizations with pSymb-specific insert DNA from clone pJG56A (J. Glazebrook, unpubl.); mobilization of pSymb into A136 occurred at higher frequencies ($> 10^{-6}$), 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 Ω 611 and Ω 615 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 μ l 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 N 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.

pJOD3 was constructed by cloning a 1.6-kb *Pst*I-*Eco*RI fragment containing *nodD3* from pBGR1 (Rushing et al. 1991) into pAD10. pKS+groESL and pSK+groESL were constructed by cloning a 2.2-kb *Eco*RI-*Hind*III fragment containing *E. coli* *groESL* from pOF39 into pBluescript KS+ and pBluescript SK+, respectively. The same 2.2-kb *Eco*RI-*Hind*III 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 *Cla*I fragment, yielding pJO91 (Ogawa 1993). Bacteriophage λ clones of *R. meliloti* *groESLc* and *groESLa* were obtained by constructing libraries of size-selected *Bgl*II fragments. The 7.9- and 12-kb *Bgl*II fragments hybridizing to probe X and Y were cloned into λ ZAPII and λ FIXII vectors (purchased from Stratagene), yielding λ ZAPII-1 and λ FIXII-4a, respectively. The 12-kb insert in λ FIXII-4a was subsequently recloned into pBluescript SK+, yielding pJO32; in vivo excision of λ ZAPII-1 yielded pJO101. Southern blot hybridizations of DNA from B4T1 confirmed that the Tn5 is inserted into the 7.9-kb *Bgl*II fragment cloned as pJO101.

A faint 12-kb *Bgl*II band hybridizing to both *groEL* and *groES* probes is visible in the GMI766 Southern blot lanes (Fig. 1D, lanes 6 and 12), even though this strain lacks the strongly hybridizing 12-kb *groESLa* fragment cloned in pJO32. We isolated a faintly hybridizing clone from the same size-selected λ FIXII library from which we isolated λ FIXII-4a and verified that the insert faintly hybridized to both a *groESc* and *groELc* 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 NodD1-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 assayed by monitoring the specific shift in electrophoretic gel mobility of a labeled *nodA* promoter-containing fragment as described (Fisher et al. 1988). The *nodA* promoter-containing fragment used was the 246-bp *EcoRI*–*HindIII* fragment from pF528 (Fisher and Long 1989).

Cell-free extracts of *R. meliloti* and *E. coli* were prepared as described (Fisher 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 2× YT medium with appropriate antibiotics at 30°C, and harvested at an OD₆₀₀ reading of 0.8–1.0.

Enzyme assays

β-Galactosidase assays were carried out as previously described (Mulligan and Long 1985) using DMF 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 OD₆₀₀ 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 OD₆₀₀ 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 10⁶–10⁷ 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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References

- Adar, Y.Y., M. Simaan, and S. Ulitzur. 1992. Formation of the LuxR protein in the *Vibrio fischeri* lux system is controlled by HtpR through the GroESL proteins. *J. Bacteriol.* **174**: 7138–7143.
- Bae, Y.M., E. Holmgren, and I.P. Crawford. 1989. *Rhizobium meliloti* anthranilate synthase gene: Cloning, sequence, and expression in *Escherichia coli*. *J. Bacteriol.* **171**: 3471–3478.
- Barnett, M.J. and S.R. Long. 1990. DNA sequence and translational product of a new nodulation-regulatory locus: *SyrM* has sequence similarity to NodD proteins. *J. Bacteriol.* **172**: 3695–3700.
- Charles, T.C. and T.M. Finan. 1991. Analysis of a 1600-kilobase *Rhizobium meliloti* megaplasmid using defined deletions generated *in vivo*. *Genetics* **127**: 5–20.
- David, M., M.-L. Daveran, J. Batut, A. Dedieu, O. Domergue, J. Ghai, C. Hertig, P. Boistard, and D. Kahn. 1988. Cascade regulation of *nif* gene expression in *Rhizobium meliloti*. *Cell* **54**: 671–683.
- Dénarié, J. and J. Cullimore. 1993. Lipooligosaccharide nod factors, a new class of signaling molecules mediating recognition and morphogenesis. *Cell* **74**: 951–954.
- De Vos, G.F., G.C. Walker, and E.R. Signer. 1986. Genetic manipulations in *Rhizobium meliloti* utilizing two new transposon Tn5 derivatives. *Mol. & Gen. Genet.* **204**: 485–491.
- Ditta, G., E. Virts, A. Palomares, and C.-H. Kim. 1987. The *nifA* gene of *Rhizobium meliloti* is oxygen regulated. *J. Bacteriol.* **169**: 3217–3223.
- Dolan, K.M. and E.P. Greenberg. 1992. Evidence that GroEL, not σ³², is involved in transcription regulation of the *Vibrio fischeri* luminescence genes in *Escherichia coli*. *J. Bacteriol.* **174**: 5132–5135.
- Downie, J.A. 1994. Signalling strategies for nodulation of legumes by rhizobia. *Trends Microbiol.* **2**: 318–324.
- Egelhoff, T.T. and S.R. Long. 1985. *Rhizobium meliloti* nodulation genes: Identification of *nodDABC* gene products, purification of *nodA* protein, and expression of *nodA* in *Rhizobium meliloti*. *J. Bacteriol.* **164**: 591–599.
- Ehrhardt, D.W., E.M. Atkinson, and S.R. Long. 1992. Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. *Science* **256**: 998–1000.
- Ellis, R.J. and S.M. van der Vies. 1991. Molecular chaperones. *Annu. Rev. Biochem.* **60**: 321–347.

- Fayet, O., J.-M. Louarn, and C. Georgopoulos. 1986. Suppression of the *Escherichia coli* *dnaA46* mutation by amplification of the *groES* and *groEL* genes. *J. Bacteriol.* **202**: 435–445.
- Fayet, O., T. Ziegelhoffer, and C. Georgopoulos. 1989. The *groES* and *groEL* heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *J. Bacteriol.* **171**: 1379–1385.
- Finan, T.M., E. Hartwig, K. LeMieux, K. Bergman, G.C. Walker, and E.R. Signer. 1984. General transduction in *Rhizobium meliloti*. *J. Bacteriol.* **159**: 120–124.
- Finan, T.M., B. Kunkel, G.F. De Vos, and E.R. Signer. 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J. Bacteriol.* **167**: 66–72.
- Fischer, H.M., M. Babst, T. Kaspar, G. Acuña, F. Arigoni, and H. Hennecke. 1993. One member of a *groESL*-like chaperonin multigene family in *Bradyrhizobium japonicum* is coregulated with symbiotic nitrogen fixation genes. *EMBO J.* **12**: 2901–2912.
- Fisher, R.F. and S.R. Long. 1989. DNA footprint analysis of the transcriptional activator proteins NodD1 and NodD3 on inducible *nod* gene promoters. *J. Bacteriol.* **171**: 5492–5502.
- . 1992. *Rhizobium*-plant signal exchange. *Nature* **357**: 655–660.
- . 1993. Interactions of NodD at the *nod* box: NodD binds to two distinct sites on the same face of the helix and induces a bend in the DNA. *J. Mol. Biol.* **233**: 336–348.
- Fisher, R.F., J.A. Swanson, J.T. Mulligan, and S.R. Long. 1987. Extended region of nodulation genes in *Rhizobium meliloti* 1021. II. Nucleotide sequence, transcription start sites and protein products. *Genetics* **117**: 191–201.
- Fisher, R.F., T.T. Egelhoff, J.T. Mulligan, and S.R. Long. 1988. Specific binding of proteins from *Rhizobium meliloti* cell-free extracts containing NodD to DNA sequences upstream of inducible nodulation genes. *Genes & Dev.* **2**: 282–293.
- Geiger, O., T. Ritsema, A.A.N. Van Brussel, T. Tak, A.H.M. Wijfjes, G.V. Bloemberg, and H. Spaink. 1994. Role of rhizobial lipo-oligosaccharides in root nodule formation on leguminous plants. *Plant Soil* **161**: 81–89.
- Georgopoulos, C. 1992. The emergence of the chaperone machines. *Trends Biochem. Sci.* **17**: 295–299.
- Glazebrook, J., G. Meiri, and G.C. Walker. 1992. Genetic mapping of symbiotic loci on the *Rhizobium meliloti* chromosome. *Mol. Plant Microbe Interact.* **5**: 223–227.
- Goloubinoff, P., A.A. Gatenby, and G.H. Lorimer. 1989. GroE heat-shock proteins promote assembly of foreign prokaryotic ribulose biphosphate carboxylase oligomers in *Escherichia coli*. *Nature* **337**: 44–47.
- Govezensky, D., T. Greener, G. Segal, and A. Zamir. 1991. Involvement of GroEL in *nif* gene regulation and nitrogenase assembly. *J. Bacteriol.* **173**: 6339–6346.
- Guglielmi, G., P. Mazodier, C.J. Thompson, and J. Davies. 1991. A survey of heat shock response in four *Streptomyces* species reveals two *groEL*-like genes and three GroEL-like proteins in *Streptomyces albus*. *J. Bacteriol.* **173**: 7374–7381.
- Hemmingsen, S.M., C. Woolford, S. van der Vies, K. Tilly, D.T. Dennis, C.P. Georgopoulos, R.W. Hendrix, and R.J. Ellis. 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature* **333**: 330–334.
- Hendrick, J.P. and F.-U. Hartl. 1993. Molecular chaperone functions of heat-shock proteins. *Annu. Rev. Biochem.* **62**: 349–384.
- Henikoff, S., G.W. Haughn, J.M. Calvo, and J.C. Wallace. 1988. A large family of bacterial activator proteins. *Proc. Natl. Acad. Sci.* **85**: 6602–6606.
- Horwich, A.L., K.B. Low, W.A. Fenton, I.N. Hirschfield, and K. Furtak. 1993. Folding in vivo of bacterial cytoplasmic proteins: Role of GroEL. *Cell* **74**: 909–917.
- Hughes, A.L. 1993. Contrasting evolutionary rates in the duplicate chaperonin genes of *Mycobacterium tuberculosis* and *M. leprae*. *Mol. Biol. Evol.* **10**: 1343–1359.
- Jacobs, T.W., T.T. Egelhoff, and S.R. Long. 1985. Physical and genetic map of a *Rhizobium meliloti* nodulation gene region and nucleotide sequence of *nodC*. *J. Bacteriol.* **162**: 469–476.
- Jefferson, R.A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Mol. Biol. Rep.* **5**: 387–405.
- Klein, S., K. Lohman, R. Clover, G.C. Walker, and E.R. Signer. 1992. A directional, high-frequency chromosomal mobilization system for genetic mapping of *Rhizobium meliloti*. *J. Bacteriol.* **174**: 324–326.
- Kondorosi, A. 1992. Regulation of nodulation genes in *Rhizobia*. In *Molecular signals in plant microbe communications* (ed. D.P.S. Verma), pp. 325–340. CRC Press, Boca Raton, FL.
- Kondorosi, E., M. Buire, M. Cren, N. Iyer, B. Hoffman, and A. Kondorosi. 1991. Involvement of the *syzM* and *nodD3* genes of *Rhizobium meliloti* in nod gene activation and in optimal nodulation of the plant host. *5*: 3035–3048.
- Kong, T.H., A.R.M. Coates, P.D. Butcher, C.J. Hickman, and T.M. Shinnick. 1993. *Mycobacterium tuberculosis* expresses two chaperonin 60 homologs. *Proc. Natl. Acad. Sci.* **90**: 2608–2612.
- Lee, A.J. 1988. "Genetic circuits for *Rhizobium meliloti* nod gene regulation." B.Sc. honors thesis, Stanford University, CA.
- Lehel, C., D. Los, H. Wada, J. Györgyei, I. Horváth, E. Kovács, N. Murata, and L. Vigh. 1993. A second *groEL*-like gene, organized in a *groESL* operon is present in the genome of *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* **268**: 1799–1804.
- Leigh, J.A., E.R. Signer, and G.C. Walker. 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. *Proc. Natl. Acad. Sci.* **82**: 6231–6235.
- Long, S.R. 1989. *Rhizobium*-legume nodulation: Life together in the underground. *Cell* **56**: 203–214.
- Long, S., S. McCune, and G.C. Walker. 1988. Symbiotic loci of *Rhizobium meliloti* identified by random *TnphoA* mutagenesis. *J. Bacteriol.* **170**: 4257–4265.
- Martel, R., L.P. Cloney, L.E. Pelcher, and S.M. Hemmingsen. 1990. Unique composition of plastid chaperonin-60: α and β polypeptide-encoding genes are highly divergent. *Gene* **94**: 181–187.
- Martin, J., M. Mayhew, T. Langer, and F.U. Hartl. 1993. The reaction cycle of GroEL and GroES in chaperonin-assisted protein folding. *Nature* **366**: 228–233.
- Martin, M.O. and S.R. Long. 1984. Generalized transduction in *Rhizobium meliloti*. *J. Bacteriol.* **159**: 125–129.
- Mazodier, P., G. Guglielmi, J. Davies, and C.J. Thompson. 1991. Characterization of the *groEL*-like genes in *Streptomyces albus*. *J. Bacteriol.* **173**: 7382–7386.
- Mulligan, J.T. and S.R. Long. 1985. Induction of *Rhizobium meliloti* *nodC* expression by plant exudate requires *nodD*. *Proc. Natl. Acad. Sci.* **82**: 6609–6613.
- . 1989. A family of activator genes regulates expression of *Rhizobium meliloti* nodulation genes. *Genetics* **122**: 7–18.
- Ogawa, J. 1993. "Rhizobium meliloti nod gene regulation: A role for *groEL* in the activation of *nod* gene expression." Ph.D. thesis, Stanford University, CA.
- Ogawa, J., H.L. Brierley, and S.R. Long. 1991. Analysis of *Rhizobium meliloti* nodulation mutant WL131: Novel insertion sequence IS_{Rm3} in *nodG* and altered *nodH* protein product. *J. Bacteriol.* **173**: 3060–3065.
- Parkinson, A. 1993. Signal transduction schemes of bacteria. *Cell* **73**: 857–871.

- Peters, N.K., J.W. Frost, and S.R. Long. 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. *Science* **233**: 977–980.
- Phillips, D.A. 1992. Flavonoids: plant signals to soil microbes. In *Phenolic metabolism in plants* (ed. H. Stafford), pp. 201–231. Plenum Press, New York.
- Rinke de Wit, T.F., S. Bekelie, A. Osland, T.L. Miko, P.W.M. Hermans, D. van Soelingen, J.-W. Drijfhout, R. Schöningh, A.A.M. Janson, and J.E.R. Thole. 1992. *Mycobacteria* contain two *groEL* genes: The second *Mycobacterium leprae* *groEL* gene is arranged in an operon with *groES*. *Mol. Microbiol.* **6**: 1995–2007.
- Rusanganwa, E. and R.S. Gupta. 1993. Cloning and characterization of multiple *groEL* chaperonin-encoding genes in *Rhizobium meliloti*. *Gene* **126**: 67–75.
- Rushing, B.G., M.M. Yelton, and S.R. Long. 1991. Genetic and physical analysis of the *nodD3* region of *Rhizobium meliloti*. *Nucleic Acids Res.* **19**: 921–927.
- Schell, M. 1993. Molecular biology of the LysR family of transcriptional regulators. *Annu. Rev. Microbiol.* **47**: 597–626.
- Schlaman, H., B. Lugtenberg, and R. Okker. 1992. The NodD protein does not bind to the promoters of inducible nodulation genes in extracts of bacteroids of *Rhizobium leguminosarum* biovar *viciae*. *J. Bacteriol.* **174**: 6109–6116.
- Segal, G. and E.Z. Ron. 1993. Heat shock transcription of the *groESL* operon of *Agrobacterium tumefaciens* may involve a hairpin-loop structure. *J. Bacteriol.* **175**: 3083–3088.
- Sharma, S.B. and E.R. Signer. 1990. Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti* in planta revealed by transposon Tn5-*gusA*. *Genes & Dev.* **4**: 344–356.
- Swanson, J.A., J.K. Tu, J. Ogawa, R. Sanga, R.F. Fisher, and S.R. Long. 1987. Extended region of nodulation genes in *Rhizobium meliloti* 1021. I. Phenotypes of Tn5 insertion mutants. *Genetics* **117**: 181–189.
- Swanson, J.A., J.T. Mulligan, and S.R. Long. 1993. Regulation of *syrM* and *nodD3* in *Rhizobium meliloti*. *Genetics* **134**: 435–444.
- Tilly, K. and C. Georgopoulos. 1982. Evidence that the two *Escherichia coli* *groE* morphogenetic gene products interact *in vivo*. *J. Bacteriol.* **149**: 1082–1088.
- Tilly, K., H. Murialdo, and C. Georgopoulos. 1981. Identification of a second *E. coli* *groE* gene whose product is necessary for bacteriophage morphogenesis. *Proc. Natl. Acad. Sci.* **78**: 1629–1633.
- Truchet, G., F. Debellé, J. Vasse, B. Terzaghi, A.-M. Garnerone, C. Rosenberg, J. Batut, F. Maillat, and J. Dénarié. 1985. Identification of a *Rhizobium meliloti* pSym2011 region controlling the host specificity of root hair curling and nodulation. *J. Bacteriol.* **164**: 1200–1210.
- Turner, G.L. and A.H. Gibson. 1980. Measurement of nitrogen fixation by indirect means. In *Methods for evaluating biological nitrogen fixation* (ed. F.J. Bergersen), pp. 111–138. John Wiley & Sons Ltd., Chichester, UK.
- Wallington, E.J. and P.A. Lund. 1994. *Rhizobium leguminosarum* contains multiple chaperonin (cpn60) genes. *Microbiology* **140**: 113–122.
- Zeilstra-Ryalls, J., O. Fayet, L. Baird, and C. Georgopoulos. 1993. Sequence analysis and phenotypic characterization of *groEL* mutations that block λ and T4 bacteriophage growth. *J. Bacteriol.* **175**: 1134–1143.

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