Rhizobium leguminosarum contains multiple chaperonin (cpn60) genes

Emma J. Wallington and Peter A. Lund

We have examined the heat shock response of Rhizobium leguminosarum. After normal growth at 28 °C, a 10 min heat shock at 37 °C induced the synthesis of proteins with approximate M, values of 90 000, 70 000, 60 000, 58 000, 19 000, 17 000 and 13 000. A monoclonal antibody raised against the E. coli Cpn60 cross-reacted with proteins of M, 60 000 and 58 000 in R. leguminosarum, suggesting that both were Cpn60 homologues. Hybridization of an E. coli cpn60 probe to total DNA from Rhizobium leguminosarum also showed evidence for at least two cpn60 homologues. One of these was cloned and completely sequenced, and showed close homology to cpn60 sequences from other prokaryotes. The expression of this gene in E. coli failed to complement a cpn60 mutation, either for growth at high temperature or for growth of bacteriophage λ. Hybridization of total R. leguminosarum DNA with a probe from this gene revealed the presence of a third putative cpn60 gene. Two further hybridizing clones were analysed and found to consist of two additional cpn60 sequences plus upstream regions containing putative cpn10 genes.

Keywords: Rhizobium leguminosarum, molecular chaperones, heat-shock response, cpn60, groEL.

INTRODUCTION

All organisms show a conserved response to stresses such as heat shock, characterized by the induction of elevated synthesis of a set of specific proteins (Lindquist & Craig, 1988). Much interest is currently centred on the properties and roles of these proteins, several of which appear to act as molecular chaperones, assisting other proteins to attain their final correctly folded and active structure (Ellis & van der Vies, 1991; Gething & Sambrook, 1992). A subclass of the molecular chaperone proteins is typified by the essential GroEL protein of E. coli, which is present in the cell as a complex of 14 subunits, each of Mr 57 000 (Zeilstra-Ryalls et al., 1991). Proteins which are in the same family as the GroEL protein are referred to as chaperonins, or Cpn60 proteins. Cpn60 protein interacts in vivo and in vitro with the Cpn10 (GroES) protein (Ellis & van der Vies, 1991; Zeilstra-Ryalls et al., 1991). Of the 33 bacterial species for which complete cpn60 sequences have been reported in the GenBank and EMBL databases (up to July 1993), 28 are reported as having only one copy of this gene. However, several Streptomyces species, Mycobacterium tuberculosis and M. leprae, Rhizobium mellitor, Bradyrhizobium japonicum and Synechocystis sp. PCC 6803 have been shown to have two or more copies of the cpn60 genes (Guglielmi et al., 1991; De Wit et al., 1992; Kong et al., 1993; Rusanganwa & Gupta, 1993; Fischer et al., 1993; Lehel et al., 1993). The functional significance of these multiple copies is unknown. In vitro, Cpn60 can bind to a wide variety of proteins, including bacterial ribulose bisphosphate carboxylase (Goloubinoff et al., 1989), preβ-lactamase (Lamnet et al., 1990), nitrogenase (Govezensky et al., 1991), citrate synthase (Buchner et al., 1991), rhodanese (Mendoza et al., 1991), dihydrofolate reductase (Vitiannen et al., 1991), x-glucosidase (Holl-Neugebauer et al., 1991), lactate dehydrogenase (Baceoe et al., 1991), glutamine synthetase (Fisher, 1992), and several thermophilic enzymes (Taguchi et al., 1991). Polypeptides which vary greatly in their final size, shape, function or cellular location interact with Cpn60; about half of the proteins in E. coli have been shown to be capable of interacting with E. coli Cpn60 (Vitiannen et al., 1992). The details of the precise role of Cpn60 in vivo are not clear but it appears to act in enabling proteins to fold correctly into their final structure.
active conformation, either after de novo synthesis or following damage by heat shock or similar stresses (Zeilstra-Ryalls et al., 1991; Saibil & Wood, 1993). As this function and wide range of binding specificity is provided by a single gene product in most organisms, the presence of multiple cpn60 genes in a few bacteria is unexpected.

In the current study we present evidence suggesting that *R. leguminosarum* possesses at least three copies of the *cpn60* gene, and that at least two Cpn60 proteins are expressed. The possible significance of this with respect to nodulation and nitrogen fixation is discussed.

**METHODS**

**Bacterial strains and culture conditions.** *R. leguminosarum* isogenic strains 8401/pRL1 (bv. *viciae*), 8002 (bv. *phaseoli*) and of multiple *cpn60* were grown at 28 °C in a complete medium (Beringer, 1974). For [35S]methionine pulse labelling experiments, the cells were grown in minimal Y media (Sherwood, 1970). In both cases streptomycin was added to a final concentration of 100 μg ml⁻¹. *E. coli* SF103 contains a *cpn60* mutation and was used in the complementation experiments. This strain was constructed by P1 transduction of the *cpn60* mutation from CG2241 (C. Georgopoulos) into TG1 [SUPE lysd5 thi Δ(lac–pro-AB) F’ (traD pro-AB’ lacB lacZΔM15); S. Fowell, pers. comm.]. *E. coli* TG2 [TG1 containing Δ(sol–recA)306; Tn10; T. Gibson pers. comm.] was used as the host strain for cloning and sequencing experiments. *E. coli* strains were grown in Luria broth (Sambrook et al., 1989). The plasmid vector pSU9 (Martinez et al., 1988) was used in cloning experiments, and was maintained in cultures by chloramphenicol selection (50 μg ml⁻¹). IPTG was added to a final concentration of 0.1 mM in complementation experiments. The lysogenic *E. coli* strain ATCC 10798 was used to prepare a λ lysate for the phage growth experiments. Preparation methods for the λ-lysate and TB media were as described by Silhavy et al. (1984).

**Assessment of λ growth.** *E. coli* strains TG1, or SF103 harbouring various plasmids were mixed with dilutions of the λ lysate and plated onto TB agar in TB overlay. Both the *E. coli* CG2241 and the *R. leguminosarum* C13 gene were expressed from the lac promoter in pSU-based plasmids. λ growth was assessed both in the presence and absence of IPTG by scoring plaques after overnight incubation.

**Protein labelling in heat shocked cells.** Cultures were grown to early exponential phase in a methionine free medium (minimal Y) at 28 °C. Aliquots (0.5 ml) were heat shocked at 37 °C or 45 °C for 10 min and 5 min (185 kBq) [35S]methionine (trans-label, ICN) was added for 20 min, before pelleting the cells by centrifugation (13000 g, 10 min, 4 °C). The pellet was washed three times in PBS and resuspended in 50 μl SDS-PAGE buffer. labelled cells were then centrifuged, the pellet washed with PBS, and resuspended in 4-3F monoclonal antisera was then added, and incubation was continued on ice for 1 h; 100 μl protein A (BRL) was then added and the immune complex removed by centrifugation (13000 g, 10 min, 4 °C). The pellet was washed three times in PBS and resuspended in 50 μl SDS-PAGE buffer.

**Analysis of total DNA.** Total DNA was prepared using a proteinase K/ctytrimethylammonium bromide method (Ausubel, 1987). Southern blots were prepared and hybridized as described (Sambrook et al., 1989), with washing steps being carried out to high stringency (1 x SSPE, 0.1% SDS, 42 °C). P-labelled probes were prepared using random oligonucleotide primed synthesis of isolated restriction fragments (Sambrook et al., 1989).

**Cloning strategy and sequence determination of R. leguminosarum cpn60 homologues.** Total DNA from *R. leguminosarum* strain 8401/pRL1 was digested with EcoRI, and size fractionated on a 0.6% agarose gel. The 2.5-4 kb region was purified from a gel, ligated into the vector pSU9 and transformed into TG2 cells. Plasmids containing inserts that hybridized with a probe containing the *E. coli* cpn60 gene were identified by digestion with EcoRI and Southern blotting. Sequence determination of the insert DNA was carried out using a Sequenase kit (United States Biochemicals), using double-stranded DNA and the M13 universal and reverse primers, as described by Del Sal et al. (1989). Subeloning the 3-5 kb EcoRI fragment into the vector pGEM7zef (+) allowed the creation of nested deletion series in both orientations using the Erase-a-Base system (Promega), such that both strands of the *cpn60* ORF could be sequenced. Specific oligonucleotide primers were synthesized (Alta Bioscience) to complete the sequence.

Similarly, a 4-6 kb PstI library was produced from PRL-digested size fractionated *R. leguminosarum* total DNA and analysed for hybridizing sequences using the EcoRI fragment cloned as described above. Two hybridizing plasmids were identified by hybridization, and the DNA sequence of the inserts was partially determined.
R. leguminosarum multiple chaperonin (cpn60) genes

RESULTS

Effect of temperature on protein synthesis in R. leguminosarum

The effect of heat shock on protein synthesis in R. leguminosarum was determined by labelling proteins with [35S]methionine. A 10 min heat shock at 37 °C resulted in the increased synthesis of proteins with Mr about 90 000, 79 000, 60 000, 58 000, 19 000, 17 000 and 13 000 (Fig. 1). A similar but less marked response was also seen after temperature shift to 34 °C (data not shown). Increasing the temperature of the heat shock to 45 °C resulted in the inhibition of most protein synthesis (Fig. 1).

The kinetics of the heat shock response was examined by labelling cultures of R. leguminosarum with [35S]methionine for 10 min at various times after heat shock at 37 °C. A final point was also taken 6 min after returning the cultures to 28 °C. The labelled protein profiles were analysed by densitometric scanning of the autoradiographs. This showed that the levels of synthesis of the heat shock proteins rose rapidly, and remained high 120 min after heat shock. The scan of the band corresponding to a protein with Mr 60 000 is shown as an example (Fig. 2). Shortly after temperature shift-down, the levels of heat shock protein synthesis also dropped (last point on Fig. 2).

A monoclonal antibody (11A1) against the M. bovis Cpn60 protein cross-reacted with a protein with Mr 60 000 in R. leguminosarum. The cross-reactivity with this protein was markedly less strong than with the E. coli homologue (data not shown). However, two proteins of Mr 58 000 and 60 000 cross-reacted with both polyclonal and monoclonal antisera (4-3F) raised against E. coli Cpn60 in this laboratory (data not shown and Fig. 3). One other monoclonal antibody (4-10H) raised against the E. coli Cpn60 also weakly cross-reacted with the 58 000 as well as the 60 000 species. No other cross-reactivity was detected between these reagents and the smaller R. leguminosarum heat shock proteins, making it unlikely that these proteins are processed forms of Cpn60 as are seen in Streptomyces spp. (Guglielmi et al., 1991).

Immunoprecipitation experiments were carried out to determine whether the [35S]methionine-labelled proteins which were induced upon heat shock were identical to those which cross-reacted with the monoclonal antisera. As seen in Fig. 4, a protein of Mr 60 000 was precipitated from cells grown at 28 °C, and more was precipitated after the cells were heat shocked at 37 °C. The 58 000 protein was not precipitated under these conditions. As it was possible that the cells were not completely lysed by the gentle freeze-thaw method, we modified the lysis procedure by the addition of SDS (1 % final concentration) prior to immunoprecipitation. This resulted in the immunoprecipitation of proteins of Mr 60 000 and 58 000, in addition to other unidentified proteins (Fig. 4).

Identification of cpn60 homologues in R. leguminosarum

Hybridization of an E. coli cpn60 probe to a Southern blot

Fig. 1. Autoradiograph of the protein profile at 28 °C (lane 1), and after a 10 min heat shock at 37 °C (lane 2) and 45 °C (lane 3). Newly induced proteins are indicated by dots. The proteins with Mr 60 000 and 58 000 are indicated by single and double arrows respectively in (b). Gels were 12.5 % (a) or 7.5 % (b) polyacrylamide. Positions of Mr weight markers are indicated.

Fig. 2. Densitometric scan of the band at Mr 60 000 in SDS-PAGE gels of heat shocked R. leguminosarum. The bacteria were pulse-labelled for 10 min at the indicated times after heat shock (37 °C). The final time point was taken 6 min after shifting the culture back to 28 °C.
containing total EcoRI-digested DNA from *R. leguminosarum* showed two bands, suggesting the presence of at least two *cpn60* homologues in this organism (data not shown). The cloning (see below) and restriction mapping of one of these homologues ruled out the possibility that the two bands were due to the presence of an EcoRI site in a single *cpn60* homologue.

A *R. leguminosarum* *cpn60* specific probe was obtained by restriction digestion of pC15, the plasmid containing the cloned *cpn60* homologue (see below). The use of this probe on *R. leguminosarum* DNA revealed the presence of a third *cpn60* homologue which had not previously been identified (Fig. 5). The three *cpn60* homologues which were detected with this probe consistently exhibited different levels of intensity, indicative of varying degrees of homology, but hybridization was seen to all three bands even at very high stringency (0.1 × SSPE, 0.1 % SDS, 42 °C). None of the three genes were on the *sym* plasmid, as identical hybridization patterns were observed with three strains which contained either the *viciae, phaseoli* or no *sym* plasmid (data not shown) but were otherwise isogenic.

**Isolation and characterization of a *R. leguminosarum* *cpn60* homologue**

A plasmid (designated pC15) containing one of the *R. leguminosarum* *cpn60* homologues was isolated as described. This plasmid was shown to contain a 3.5 kb insert. Sequence analysis revealed that an ORF with high homology to other prokaryotic *cpn60* genes was immediately downstream of one of the EcoRI sites at the end containing total EcoRI-digested DNA from *R. leguminosarum* showed two bands, suggesting the presence of at least two *cpn60* homologues in this organism (data not shown). The cloning (see below) and restriction mapping of one of these homologues ruled out the possibility that the two bands were due to the presence of an EcoRI site in a single *cpn60* homologue.

A *R. leguminosarum* *cpn60* specific probe was obtained by restriction digestion of pC15, the plasmid containing the cloned *cpn60* homologue (see below). The use of this probe on *R. leguminosarum* DNA revealed the presence of a third *cpn60* homologue which had not previously been identified (Fig. 5). The three *cpn60* homologues which were detected with this probe consistently exhibited different levels of intensity, indicative of varying degrees of homology, but hybridization was seen to all three bands even at very high stringency (0.1 × SSPE, 0.1 % SDS, 42 °C). None of the three genes were on the *sym* plasmid, as identical hybridization patterns were observed with three strains which contained either the *viciae, phaseoli* or no *sym* plasmid (data not shown) but were otherwise isogenic.

**Isolation and characterization of a *R. leguminosarum* *cpn60* homologue**

A plasmid (designated pC15) containing one of the *R. leguminosarum* *cpn60* homologues was isolated as described. This plasmid was shown to contain a 3.5 kb insert. Sequence analysis revealed that an ORF with high homology to other prokaryotic *cpn60* genes was immediately downstream of one of the EcoRI sites at the end

**Fig. 3.** Immunoblot with monoclonal antibody 4-3F against total protein from *E. coli* (lane 1) and *R. leguminosarum* grown at 28 °C (lane 2), then heat shocked at 37 °C (lane 3) or 45 °C (lane 4).

**Fig. 4.** Autoradiograph of immunoprecipitated *R. leguminosarum* proteins. Lanes 1 and 10, 14C-labelled *M* markers, sizes indicated. Protein extracts in lanes 2, 4, 6 and 8 were from cells grown at 28 °C, whereas extracts in lanes 3, 5, 7 and 9 were from cells grown at 28 °C and heat shocked at 37 °C. Proteins in lanes 2 and 3 were extracted from cells lysed with SDS and immunoprecipitated with 4-3F. Lanes 4-9 were from the standard immunoprecipitation protocol: lanes 4 and 5, total cell protein extract; lanes 6 and 7 cell lysate (soluble fraction); and lanes 8 and 9, proteins immunoprecipitated with 4-3F.

**Fig. 5.** Southern blot of digested genomic DNA from 8401/pRL1 probed with the *cpn60* fragment from pC15. The enzymes used were *BamH*1 (lane 1), *EcoR*1 (lane 2), *HindIII* (lane 3), *PstI* (lane 4) and *Smal* (lane 5). Strong, medium and weakly hybridizing bands are indicated with a ■, ▽ and ●, respectively.
Fig. 6. Nucleotide and deduced amino acid sequences of the *R. leguminosarum* *cpn60* homologue C15. The nucleotide sequence is numbered from the first base of the upstream EcoRI site used to clone the fragment.
of the cloned fragment. The insert was subcloned as described, and the DNA sequence determined on both strands (Fig. 6). Translation of the largest ORF would give a protein of 546 amino acids, with an $M_r$ of 57865 and a pI of 4.97. The predicted amino acid sequence showed high homology with other bacterial Cpn60 proteins in the GenBank database, confirming that this was a $cpn60$ homologue. We refer to this gene as C15 throughout this paper, pending a complete characterization of all the $cpn60$ and $cpn10$ genes in this organism. The highest degree of identity was seen with one of the Cpn60 proteins of $Rhizobium$ meliloti (RhzA). Other related proteins, including the $E. coli$ Cpn60 protein, are shown in the dendrogram (Fig. 7) with their relative identities and similarities to C15, and are aligned with the sequence described here in Fig. 8.

Two further clones which hybridized to the cloned $cpn60$ homologue were isolated from a size fractionated $PstI$ library by probing with the pC15 insert. The first (pB45) contained a 5 kb insert; preliminary sequence data from one end showed that it includes the 5' region of a $cpn60$ gene distinct from that on C15, being closer in homology to $R. meliloti$ RhzC (data not shown). A deletion was constructed in this plasmid that enabled the determination of the sequence upstream of the $cpn60$ gene, and this revealed the presence of sequences homologous to $cpn10$. The second $PstI$ clone (pD98) contained a 4-7 kb insert. Preliminary sequence data (obtained using internal primers designed for the C15 homologue) showed that this contained the 5' region of a third $cpn60$ gene, again with a $cpn10$ gene upstream. Sequence derived from the termini of this cloned insert showed that unrelated sequences flank the $cpn60/10$ genes. It is therefore likely that a complete operon has been isolated on this fragment. The $cpn60$ gene in this clone is closely related to $R. meliloti$ RhzA, but restriction mapping and DNA sequence analysis of the insert shows that it is distinct from the C15 homologue (data not shown). A third clone which hybridized with the C15 probe was isolated from the $PstI$ library. It contained a 2 kb insert, and DNA sequence analysis showed that it contained the downstream end of the C15 gene.

**Expression of C15 in E. coli**

The $R. leguminosarum$ $cpn60$ homologue C15 was expressed in $E. coli$ under the control of the $lac$ promoter on the plasmid pSU9, induced by the addition of IPTG to the medium (Fig. 9). N-Terminal sequence analysis of the first

---

**Fig. 7.** Dendrogram showing the relationship between $R. leguminosarum$ Cpn60 C15 and selected Cpn60 homologues, based on a multiple sequence alignment (University of Wisconsin GCG package 'Pileup'). Cpn60 sequences with the closest homology are shown: $R. meliloti$ RhzA, B and C (Rusanganwa & Gupta, 1993), $A. tumefaciens$ (Segal & Ron, 1993), $B. abortus$ (Gor & Mayfield, 1992), $B. bacilliformis$ (Y. Xu and others, unpublished results; GCG accession number Gb-Ba:BaBaobb63a) and $B. japonicum$ (Fischer et al., 1993). The $E. coli$ Cpn60 sequence is also included (Hemmingsen et al., 1988). The percentage identity and similarity of the Cpn60 proteins to $R. leguminosarum$ C15 are shown.
nine amino acids of the over-expressed protein confirmed its identity as the R. leguminosarum cpn60 gene product, initiated at the correct methionine. Native gradient gel electrophoresis also demonstrated that the foreign Cpn6O migrated as an oligomer of approximately the same apparent Mr of 13000 may be one of the Cpn6O homologues (as Fig. 7). Only amino acids which are non-identical with the 60000 protein. The identity of some of the other strongly heat shock induced proteins seen here (Mr 19000 and 17000) are unknown; the possibility of the heat shock response of many organisms (Lindquist & Craig, 1988). Proteins with these enhanced synthesis of proteins at approximate 42 °C in an E. coli strain carrying a ts mutation in the cpn60 gene (SF103), whereas expression of the E. coli cpn60 cloned into pSU18 (D. Olden, pers. commun.) restored growth at the non-permissive temperature. The temperature-sensitive mutation in this strain also blocks bacteriophage λ growth even at 37 °C. Bacteriophage λ growth on SF103 was not restored by the expression of the R. leguminosarum gene, but was restored by the plasmid-borne E. coli cpn60 after induction with IPTG.

**DISCUSSION**

The results presented here provide evidence that R. leguminosarum contains and expresses multiple Cpnn60 proteins. Pulse labelling experiments showed the induction of novel proteins in heat shocked R. leguminosarum. Enhanced synthesis of proteins at approximate M, 90000, 70000 and 60000 (corresponding to stress proteins Hsp90, DnaK and Cpn60) is a conserved feature of the heat shock response of many organisms (Lindquist & Craig, 1988). Proteins with these Mr values were seen in R. leguminosarum; in addition, a species of M, 58000 was also observed. We believe both the 58000 and 60000 proteins to be Cpn6O homologues due to their cross-reactivity with both polyclonal and monoclonal antisera, likely by the failure of anti-Cpn60 serum to cross-react with them. The heat shock induced protein with an apparent Mr of 13000 may be one of the Cpnn60 genes.

---

**Fig. 8.** Amino acid sequence alignment of R. leguminosarum Cpn60 C15 with selected Cpn60 homologues (as Fig. 7). Only amino acids which are non-identical with the R. leguminosarum sequence are shown.
homologues which we have identified by sequence analysis.

Our data indicate that R. leguminosarum contains at least three cpn60 genes, and we have completely sequenced one of these. We have demonstrated that none of these genes are located on the sym plasmid, but we have not addressed the possibility that one or more copies may be located on a mega plasmid, as is seen with R. meliloti (Rusanganwa & Gupta, 1993). Although we cannot currently rule out the possibility that one or more of these copies may be pseudogenes, evidence for the expression of two Cpn60 homologues has been demonstrated by Western blotting and by immunoprecipitation. It is not clear whether the proteins of M, 60000 and 58000 seen in R. leguminosarum each represents a gene product with the third gene either not expressed or expressed at undetectable levels, or whether two Cpn60 proteins of very similar M, are superimposed in one of the bands. Attempts to resolve the bands into more than one spot using two-dimensional electrophoresis have not been successful (data not shown).

These results are similar to those reported recently for other members of the Rhizobiaceae: Bradyrhizobium japonicum and R. meliloti have both been shown to possess multiple cpn60 genes (Fischer et al., 1993; Rusanganwa & Gupta, 1993). Three cpn60 homologues from R. meliloti have been cloned and sequenced, and two copies have been mapped to the mega plasmids (Rusungaw et al., 1993). In B. japonicum, two of the five homologues have been mapped to the chromosome, one being close to symbiotic cluster II and under NifA regulation (Kundig et al., 1993; Fischer et al., 1993). In all the examples so far reported of bacteria where multiple cpn60 genes are found, not all the cpn60 genes are organized with an upstream cpnl0 gene (as is invariably seen when only one cpn60 gene is present). In the current study, two of the three cpn60 genes identified have cpnl0 genes upstream; the upstream region of the third cpn60 gene (C15) has not yet been cloned.

Expression of the cloned R. leguminosarum Cpn60 from the lac promoter in E. coli did not allow complementation of the cpn60 ts mutation in SF103 or permit growth in this strain, despite the induced polypeptide initiating at the correct methionine and appearing to oligomerize. Complementation was seen when the E. coli cpn60 gene was expressed from the same plasmid under plac control. Complementation of defective cpn60 alleles in E. coli is generally only seen with Cpn60 proteins with a high level of identity to the E. coli protein (for example Chromatium vinosum, 75-5% identical; Ferreyra et al., 1993), or the bacterial endosymbiont of Acyrthosiphon pisum (85-8% identical; Ohtaka et al., 1992), although it is interesting to note that the cpn60 gene from Bacillus stearothermophilus can complement a cpn60 ts mutation in E. coli despite only having 61% identity to the E. coli gene (Schoen & Schumann, 1993). Failure to complement in the case of the R. leguminosarum gene is due not to poor protein expression or failure to assemble into an oligomer, and may be due to the inability of the R. leguminosarum protein to interact with the E. coli Cpn10 protein. We are currently investigating this further.

Cpn60 proteins have been shown to interact with a wide variety of proteins, which they appear to bind in an unfolded or partially folded state (Ellis & van der Vies, 1991; Zeilstra-Ryalls et al., 1991; Saibil & Wood, 1993). Because of this low specificity, the presence of multiple copies of cpn60 genes in several bacteria is somewhat unexpected. It may be the case that the existence of multiple genes is simply one way in which the organism can increase the total levels of Cpn60 protein in the cell at times of stress, or under other conditions when the protein is needed at high levels. Alternatively, different Cpn60 proteins may serve different functions. For example Rhizobium species, in addition to having a free-living existence, are also endosymbiotic. The formation of root nodules is a complex process involving the synthesis of many new bacterial proteins, both for the formation of the bacteroid and subsequently the enzymic fixation of nitrogen. In connection with this, it has been shown that the production of nitrogenase components either in Klebsiella pneumoniae or E. coli is much enhanced by (and may require) the presence of Cpn60 (Govezensky et al., 1991). The possibility exists that one or more of the Cpn60 protein complexes are specialized for particular functions in the development of the endosymbiont. It is not yet known for any of the organisms which express more than one Cpn60 protein whether the proteins form mixed multimers or homo-oligomeric complexes; this is also not known for chloroplasts which are the first organelles in which the function of these proteins were recognized (Hemmingsen et al., 1988) and which contain two cpn60 homologues. It will be of great interest to follow the expression of the diverse cpn60 genes as the endosymbiotic bacteria differentiate. Complete characterization, including mutagenesis, of the three R. leguminosarum cpn60 genes will be necessary to determine whether they are all
whether they have different roles within the bacterial cell.

Note added in proof

We have now completed the cloning of the region upstream of the C15 gene and confirmed that this region contains a cpn10 gene. We thus suggest the following nomenclature for R. leguminosarum chaperone genes: cpn60-1 (for pC15); cpn60-2 (for pD98) and cpn60-3 (for B45), with the cognate cpn10 genes being referred to as cpn10-1, cpn10-2 and cpn10-3 respectively.

ACKNOWLEDGEMENTS

We are grateful to Dr A. Downie (John Innes Institute, Norwich) for provision of bacterial strains and helpful advice and discussions, to Dr J. de Bruyn (Institut Pasteur du Brabant, Brussels) for monoclonal antibody against M. bovis Cpn60, to A. Ivic (University of Birmingham) for monoclonal antibodies against E. coli Cpn60, and to Professor C. Georgopoulos (Centre Medical Universitaire, Geneva) for the provision of a cpn60 ts strain. E. J. W. is an Agricultural and Food Research Council postdoctoral fellow.

REFERENCES


IS. J. WALLINGTON and P. A. LUND


Received 15 July 1993; revised 27 August 1993; accepted 31 August 1993.