***Rhizobium leguminosarum* contains multiple chaperonin (*cpn60*) genes**

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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_r 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_r 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 or 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 M_r 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 meliloti*, *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 biphosphate carboxylase (Goloubinoff *et al.*, 1989), pre- β -lactamase (Laminet *et al.*, 1990), nitrogenase (Govezensky *et al.*, 1991), citrate synthase (Buchner *et al.*, 1991), rhodanese (Mendoza *et al.*, 1991), dihydrofolate reductase (Viitanen *et al.*, 1991), α -glucosidase (Holl-Neugebauer *et al.*, 1991), lactate dehydrogenase (Badcoe *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 (Viitanen *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

The GenBank accession number for the sequence data reported in this paper is L20775.

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 8401 (*sym*⁻) 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* *ts* mutation and was used in the complementation experiments. This strain was constructed by P1 transduction of the *cpn60* 44 mutation from CG2241 (C. Georgopoulos) into TG1 [*supE* *hsdΔ5* *thi* *Δ(lac-proAB)* F' (*traD* *proAB*⁺ *lacI*^a *lacZΔM15*); S. Fowell, pers. comm.]. *E. coli* TG2 [TG1 containing *Δ(srl-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* Cpn60 and the *R. leguminosarum* C15 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 µCi (185 kBq) [³⁵S]methionine (trans-label, ICN) was added for 20 min, before pelleting the cells by centrifugation (13000 g, 5 min) in a microfuge, and washing excess label away with non-radioactive medium. Cells were resuspended in 50 µl SDS-PAGE sample buffer, and boiled briefly. Equal volumes were electrophoresed on SDS-PAGE gels, using a Bio-Rad Mini Protean II system. Gels were dried before autoradiography, and autoradiographs were scanned using an Ultrascan laser densitometer (LKB).

Protein N-terminal analysis. This was carried out by Alta Bioscience, University of Birmingham. Proteins were separated by SDS-PAGE, electroblotted onto PVDF membrane and

stained with PAGE Blue 83. The band of interest was excised and subjected to Edman degradation.

Immunoblotting. Proteins were detected, following separation by SDS-PAGE and electroblotting onto nitrocellulose, with a monoclonal antibody (IA1) raised against *M. bovis*, which cross-reacts well with *E. coli* Cpn60 (J. de Bruyn pers. comm.) or a monoclonal antibody (4-3F) raised against *E. coli* Cpn60 (A. Ivic, pers. comm.). Bound antibody was detected using 4-chloro-1-naphthol via an anti-mouse horseradish peroxidase conjugate.

Immunoprecipitation. Cells were grown up, heat shocked and pulse chased with [³⁵S]methionine as described above. The labelled cells were then centrifuged, the pellet washed with PBS, and resuspended in 125 µl TES (50 mM Tris/HCl, pH 8.0, 5 mM EDTA, 20%, w/v, sucrose plus 600 µg lysozyme ml⁻¹), and incubated on ice for 30 min. Equilibration buffer (375 µl) (50 mM Tris/HCl, pH 8.0, 66 mM EDTA, 200 mM KCl) was added, and the cells were lysed by three cycles of freeze-thawing in liquid nitrogen. Cell debris was pelleted by centrifugation at 13000 g for 10 min at 4 °C. A 100 µl vol. of the supernatant was precleared for 1 h on ice with 100 µl GF6 (a monoclonal antibody raised against *Helicobacter pylori* flagella sheath, a gift from C. Luke and C. Penn) to remove proteins which bound non-specifically to IgG molecules, the immune complex being removed by incubation with Protein A as described below. A 100 µl vol. of 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/cetyltrimethylammonium 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 × SSPE, 0.1% SDS, 42 °C). ³²P-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). Subcloning the 3.5 kb *EcoRI* fragment into the vector pGEM7zf(+) 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 *PstI*-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.

Fig. 1

Fig. 2

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 M_r 60000 and 58000 are indicated by single and double arrows respectively in (b). Gels were 12.5% (a) or 7.5% (b) polyacrylamide. Positions of M_r weight markers are indicated.

Fig. 2. Densitometric scan of the band at M_r 60000 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.

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 [35 S]methionine. A 10 min heat shock at 37 °C resulted in the increased synthesis of proteins with M_r about 90000, 79000, 60000, 58000, 19000, 17000 and 13000 (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 [35 S]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 M_r 60000 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 (1A1) against the *M. bovis* Cpn60 protein cross-reacted with a protein with M_r 60000 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 M_r 58000 and 60000 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 58000 as well as the 60000 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 [35 S]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 M_r 60000 was precipitated from cells grown at 28 °C, and more was precipitated after the cells were heat shocked at 37 °C. The 58000 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 M_r 60000 and 58000, 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. 3

Fig. 4

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, ^{14}C -labelled M_r 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.

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 ($0.1 \times \text{SSPE}$, 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. 5. Southern blot of digested genomic DNA from 8401/pRL1 probed with the *cpn60* fragment from pC15. The enzymes used were *Bam*HI (lane 1), *Eco*RI (lane 2), *Hind*III (lane 3), *Pst*I (lane 4) and *Sma*I (lane 5). Strong, medium and weakly hybridizing bands are indicated with a ■, ► and ●, respectively.

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      10          30          50          70          90
GAATTCAGGAGTTAAGAATATGGCATCTAAAGAAATCAAGTTTGGCCGCACCGGCCGCGAAAAGATGCTGCGTGCGCTCGACATTCTCGCCGATGCAGTGG
      M A S K E I K F G R T G R E K M L R G V D I L A D A V

     110          130          150          170          190
AAGGTCACGCTCGGCCCGAAGGGCCGTAACGTTCATCATCGACAAGTCCTTCGGCGCTCCGCGCATCACCAGGACGGCGTTTCGGTTCGCCAAGGAGATCG
K V T L G P K G R N V I I D K S F G A P R I T K D G V S V A K E I E

     210          230          250          270          290
AACTCGAAGACAAGTTTCGAAAACATGGGCGCCCGATGGTCCGCGAAGTTGCTTCGAAGACCAACGACATCGCCGCGCAGCGGCACCACGACTGCAACCGT
L E D K F E N M G A Q M V R E V A S K T N D I A G D G T T T A T V

     310          330          350          370          390
TCTTGCCCGAGGCGATCGTTTCGCGAAGGCAACAGGCCGTTGCGCGCGGCATGAACCCGATGGACCTGAAGCGCGGCATCGATCTTGCTGTTGCTGACGTC
L A Q A I V R E G N K A V A A G M N P M D L K R G I D L A V A D V

     410          430          450          470          490
GTGAAGGATCTCCAGGCCAAGGCCAAGAAGATCTCCACCTCGGAAGAAGTTGCACAGGTCGGCACGATTTTCGGCCAATGGCGACAAGCAGGTCGGTCTCG
V K D L Q A K A K K I S T S E E V A Q V G T I S A N G D K Q V G L D

     510          530          550          570          590
ACATTGCTGAAGCCATGCGAAGGTTCGGCAACGAAGGCGTCATCACGGTTGAAGAAGCCAGACCGCCGAAAACCGAACTCGAAGTCGTCGAAGGCATGCA
I A E A M Q K V G N E G V I T V E E A K T A E T E L E V V E G M Q

     610          630          650          670          690
GTTTCGACCGCGGCTACCTCAGCCCTACTTCGTGACCAACCCGGAAGAAGATGATCGCAGACCTCGAAGATGCTTTCATTCTCCTTCACGAGAAGAAGCTC
F D R G Y L S P Y F V T N P E K M I A D L E D V F I L L H E K K L

     710          730          750          770          790
TCGAACCTCGAGTCGATGCTCCCGGTTCTCGAAGCTGTCGTCAGACCGGCAAGCCGCTCTCATCGTCGCTGAAGACGTAGAAGGCCAAGCCCTCGCAA
S N L Q S M L P V L E A V V Q T G K P L L I V A E D V E G E A L A T

     810          830          850          870          890
CGCTCGTCGTCGAACAGCTGCGCGGTGGCCCTCAAGATCGCTGCCGTCAAGGCGCCCGGCTTCGGCGATCGCCGCAAGCGCATGCTCGAAGACATCGCCAT
L V V N K L R G G L K I A A V K A P G F G D R R K R M L E D I A I

     910          930          950          970          990
CCTGACGGGCGGCACGTGTCATCTCTGAAGATCTCGGCATCAAGCTCGAATCCGTCACGCTCGACATGCTCGGCGGTGCCAAGAAGGTTTCGATCTCCAAG
L T G G T V I S E D L G I K L E S V T L D M L G R A K K V S I S K

    1010          1030          1050          1070          1090
GAAAACACCACGATCGTCGACGGTTTCGGGCGCCAGACCGACATCGAAGGCGGTGTTGCCAGATCAAGGCTCAGATCGAAGAAACACCTCCGACTACG
E N T T I V D G S G A K T D I E G R V A Q I K A Q I E E T T S D Y D

    1110          1130          1150          1170          1190
ACCGGAGAAAGCTGCAGGAACGCCTTGCCAAAGCTCGCTGGCGGCGTTGCCGTCATCCGCGTCGGCGGCTCGACGGAAGTTGAAGTGAAGGAAAAGAAGGA
R E K L Q E R L A K L A G G V A V I R V G G S T E V E V K E K K D

    1210          1230          1250          1270          1290
CCGCATCGACGACGCGCTCAACGCGACCCGCGCTGCTGTTTCAGGAAGGCATCGTCCCGGCGGCGGTATCGCTCTCGCTCGCTCCTCCACGAAGATCACC
R I D D A L N A T R A A V Q E G I V P G G G I A L A R S S T K I T

    1310          1330          1350          1370          1390
GTCAAGGGTGCAAACGACGACCGGAAGCCCGGCATCAACATCGTTCGCGCGCGCCCTGCGAGTCGCTCGTTTCGTCAGATCGCTGAAAACGCGAGCGACGAAG
V K G A N D D Q E A G I N I V R R A L Q S L V R Q I A E N A G D E A

    1410          1430          1450          1470          1490
CCTCGATCGTTGTCGGCAAGGTCCTCGACAAGAACGAAGACAACCTTCGGCTACAACGCCAGACATCCGAATATGGCGACATGATCGCCATGGGTATCGT
S I V V G K V L D K N E D N F G Y N A Q T S E Y G D M I A M G I V

    1510          1530          1550          1570          1590
CGACCCGCTCAAGGTCGTTTCGCACGGCGCTGCAGAACGCCGCTTCTGTGCGATCGCTGTCGATCACCACCGAAGCCATGATCGCCGAACCTGCCGAAGAAG
D P L K V V R T A L Q N A A S V A S L L I T T E A M I A E L P K K

    1610          1630          1650
GATGCCCGAGCTGGCATGCCGGGCGGCATGGGCGGCATGGGCGGTATGGACATGATGTGATCGG
D A P A G M P G G M G G M G G M D M M *

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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.

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.

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 57 865 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 *Pst*I 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 *Pst*I 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 *Pst*I 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. 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.

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 Cpn60 migrated as an oligomer of approximately the same M_r as the native *E. coli* protein (data not shown). Over-expression of C15 from this plasmid failed to restore growth at 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. comm.) 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 Cpn60

proteins. Pulse labelling experiments showed the induction of novel proteins in heat shocked *R. leguminosarum*. Enhanced synthesis of proteins at approximate M_r 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 M_r values were seen in *R. leguminosarum*; in addition, a species of M_r 58000 was also observed. We believe both the 58000 and 60000 proteins to be Cpn60 homologues due to their cross-reactivity with both polyclonal and monoclonal antisera, although we cannot completely rule out the possibility that the 58000 band represents a truncated or slightly degraded form of the 60000 protein. The identity of some of the other strongly heat shock induced proteins seen here (M_r 19000 and 17000) are unknown; the possibility that they might be processed forms of Cpn60 as is seen in *Streptomyces albus* (Guglielmi *et al.*, 1991) is rendered less likely by the failure of anti-Cpn60 serum to cross-react with them. The heat shock induced protein with an apparent M_r of 13000 may be one of the Cpn10

Fig. 9. Expression of *R. leguminosarum* *cpn60* in *E. coli*. Coomassie blue stained SDS-PAGE gel of protein extracted from *E. coli* (lane 1), *E. coli* containing pC15 without IPTG (lane 2), and plus IPTG (lane 3), or *R. leguminosarum* (lane 4).

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_r 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_r 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 (Rusanganwa *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 *cpn10* gene (as is invariably seen when only one *cpn60* gene is present). In the current study, two of the three *cpn60* genes identified have *cpn10* 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 λ 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 *Acyrtosiphon 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 not due 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

functional, whether they are differentially regulated, and 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

- Ausubel, F. M. (1987). *Current Protocols In Molecular Biology*. New York: Wiley Interscience.
- Badcoe, I. G. V., Smith, C. J., Wood, S., Halsall, D. J., Holbrook, J. J., Lund, P. & Clarke, A. R. (1991). Binding of a chaperonin to the folding intermediates of lactate dehydrogenase. *Biochemistry* **30**, 9195–9200.
- Beringer, J. E. (1974). R-Factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* **84**, 188–198.
- Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F. X. & Kiefhaber, T. (1991). GroE facilitates refolding of citrate synthase by suppressing aggregation. *Biochemistry* **30**, 1586–1591.
- Del Sal, G., Manfioletti, G. & Schneider, C. (1989). The CTAB-DNA precipitation method – a common mini-scale preparation of template DNA from phagemids, phages or plasmids suitable for sequencing. *Biotechniques* **7**, 514–519.
- De Wit, T. F. R., Bekelie, S., Osland, A., Miko, T. L., Hermans, P. W. M., van Soolingen, P., Drijfhout, J.-W., Schoeninger, R., Janson, A. A. M. & Thole, J. E. R. (1992). Mycobacteria contain two *groEL* genes: the second *Mycobacterium leprae* *groEL* gene is arranged in an operon with *groES*. *Mol Microbiol* **6**, 1995–2007.
- Ellis, R. J. & van de Vies, S. M. (1991). Molecular chaperones. *Annu Rev Biochem* **60**, 321–347.
- Ferreira, R. G., Soncini, F. C. & Viale, A. M. (1993). Cloning, characterization, and functional expression in *Escherichia coli* of chaperonin (*groESL*) genes from the phototrophic sulphur bacterium *Chromatium vinosum*. *J Bacteriol* **175**, 1514–1523.
- Fischer, H. M., Babst, M., Kaspar, T., Acuna, G., Arigoni, F. & Hennecke, H. (1993). One member of a *groESL*-like chaperonin multigene family in *Bradyrhizobium japonicum* is co-regulated with symbiotic nitrogen fixation genes. *EMBO J* **12**, 2901–2912.
- Fisher, M. T. (1992). Promotion of the *in vitro* renaturation of dodecameric glutamine synthetase from *Escherichia coli* in the presence of Cpn60 (chaperonin-60) and ATP. *Biochemistry* **31**, 3955–3963.
- Gething, M.-J. & Sambrook, J. (1992). Protein folding in the cell. *Nature* **355**, 33–45.
- Goloubinoff, P., Gatenby, A. A. & Lorimer, G. H. (1989). GroE heat-shock proteins promote assembly of foreign prokaryotic ribulose biphosphate carboxylase oligomers in *Escherichia coli*. *Nature* **337**, 44–47.
- Gor, D. & Mayfield, J. E. (1992). Cloning and nucleotide sequence of the *Brucella abortus* *groE* operon. *Biochim Biophys Acta* **1130**, 120–122.
- Govezensky, D., Greener, T., Segal, G. & Zamir, A. (1991). Involvement of GroEL in *nif* gene regulation and nitrogenase assembly. *J Bacteriol* **173**, 6339–6346.
- Guglielmi, G., Mazodier, P., Thompson, C. J. & Davies, J. (1991). A survey of the 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., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C., Hendrix, R. W. & Ellis, R. J. (1988). Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature* **333**, 330–334.
- Holl-Neugebauer, B., Rudolph, R., Schmidt, H. & Buchner, J. (1991). Reconstitution of a heat-shock effect *in vitro*: influence of GroE on the thermal aggregation of α -glucosidase from yeast. *Biochemistry* **30**, 11609–11614.
- Kong, T. H., Coates, A. R. M., Butcher, P. D., Hickman, C. J. & Shinnick, T. M. (1993). Mycobacterium-tuberculosis expresses two chaperonin-60 homologs. *Proc Natl Acad Sci USA* **90**, 2608–2612.
- Kundig, C., Hennecke, H. & Gottfert, M. (1993). Correlated physical and genetic map of the *Bradyrhizobium japonicum* 110 genome. *J Bacteriol* **175**, 613–622.
- Lamiet, A. A., Ziegelhoffer, T., Georgopoulos, C. & Pluckthun, A. (1990). The *E. coli* heat-shock proteins GroEL and GroES modulate the folding of β -lactamase precursor. *EMBO J* **9**, 2315–2319.
- Lehel, C., Los, D., Wada, H., Gyorgyei, J., Horvath, I., Kovacs, E., Murata, N. & Vigh, L. (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**, 1901–1907.
- Lindquist, S. & Craig, E. A. (1988). The heat-shock proteins. *Annu Rev Genet* **22**, 631–677.
- Martinez, E., Bartolome, B. & de la Cruz, F. (1988). pACYC184-derived cloning vectors containing the multiple cloning site and *lacZ* alpha reporter gene of pUC8/9 and pUC18/19 plasmids. *Gene* **68**, 159–162.
- Mendoza, J. A., Rogers, E., Lorimer, G. H. & Horowitz, P. M. (1991). Chaperonins facilitate the *in vitro* folding of monomeric mitochondrial rhodanese. *J Biol Chem* **266**, 13044–13049.
- Ohtaka, C., Nakamura, H. & Ishikawa, H. (1992). Structures of chaperonins from an intracellular symbiont and their functional expression in *Escherichia coli* *groE* mutants. *J Bacteriol* **174**, 1869–1874.
- Rusanganwa, E. & Gupta, R. S. (1993). Cloning and characterization of multiple *groEL* chaperonin-encoding genes in *Rhizobium meliloti*. *Gene* **126**, 67–75.
- Saibil, H. & Wood, S. (1993). Chaperonins. *Curr Opin Struct Biol* **3**, 207–213.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning*:

A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schoen, U. & Schumann, W. (1993). Molecular cloning, sequencing, and transcriptional analysis of the *groESL* operon from *Bacillus stearothermophilus*. *J Bacteriol* **175**, 2465–2469.

Segal, G. & Ron, E. Z. (1993). Heat shock transcription of the *groESL* operon of *Agrobacterium tumefaciens* may involve a hairpin-loop structure. *J Bacteriol* **175**, 3083–3088.

Sherwood, M. T. (1970). Improved synthetic medium for the growth of *Rhizobium*. *J Appl Bacteriol* **33**, 708–713.

Silhavy, T. J., Berman, M. L. & Enquist, L. W. (1984). *Experiments with Gene Fusions*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Taguchi, H., Konishi, J., Ishii, N. & Yoshida, M. (1991). A chaperonin from a thermophilic bacterium *Thermus thermophilus* that

controls refoldings of several thermophilic enzymes. *J Biol Chem* **266**, 22411–22418.

Viitanen, P. V., Donaldson, G. K., Lorimer, G. H., Lubben, T. H. & Gatenby, A. A. (1991). Complex interactions between the chaperonin 60 molecular chaperone and dihydrofolate reductase. *Biochemistry* **30**, 9716–9723.

Viitanen, P. V., Gatenby, A. A. & Lorimer, G. H. (1992). Purified chaperonin 60 (Cpn60) interacts with the non-native states of a multitude of *Escherichia coli* proteins. *Protein Sci* **1**, 363–369.

Zeilstra-Ryalls, J., Fayet, O. & Georgopoulos, C. (1991). The universally conserved GroE (Hsp60) chaperonins. *Annu Rev Microbiol* **45**, 301–325.

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