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Two of the three *groEL* homologues in *Rhizobium leguminosarum* are dispensable for normal growth

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Abstract Although many bacteria contain only a single *groE* operon encoding the essential chaperones GroES and GroEL, examples of bacteria containing more than one *groE* operon are common. The root-nodulating bacterium *Rhizobium leguminosarum* contains at least three operons encoding homologues to *Escherichia coli* GroEL, referred to as Cpn60.1, Cpn60.2 and Cpn60.3, respectively. We report here a detailed analysis of the requirement for and relative levels of these three proteins. Cpn60.1 is present at higher levels than Cpn60.2, and Cpn60.3 protein could not be detected under any conditions although the *cpn60.3* gene is transcribed under anaerobic conditions. Insertion mutations could not be constructed in *cpn60.1* unless a complementing copy was present, showing that this gene is essential for growth under the conditions used here. Both *cpn60.2*

and *cpn60.3* could be inactivated with no loss of viability, and a double *cpn60.2 cpn60.3* mutant was also constructed which was fully viable. Thus only Cpn60.1 is required for growth of this organism.

Keywords *Rhizobium leguminosarum* · GroEL · Chaperonins · Heat shock

Dedicated to the memory of Professor V. Javier Benedí, 1957–2002

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Introduction

The GroEL protein of *Escherichia coli* is the best-characterised example of the highly conserved Hsp60 family of molecular chaperones, which act by assisting the folding of a subset of cellular proteins under normal growth conditions (reviewed in Hartl 1996; Fenton and Horwich 1997; Ranson et al. 1998; Lund 2001). The GroEL and its co-chaperone GroES are essential for viability in *E. coli* (Fayet et al. 1989). Expression of these proteins is induced by heat shock and other stresses (Neidhardt and VanBogelen 1987), and they play a key role in protecting *E. coli* from the effects of high temperature (Kusukawa and Yura 1988). Together with other molecular chaperones, GroEL homologues reduce the aggregation which occurs through association of the hydrophobic regions of unfolded or partially folded proteins, a problem which becomes more acute when proteins partially unfold after heat shock (Hartl et al. 1994; Hartl 1996; Buchner 1996; Ellis 1997). Two basic modes of regulation of *groE* operons have been defined (reviewed in Bukau 1993; Narberhaus 1999; Yura and Nakahigashi 1999). In *E. coli*, the *groE* operon possesses both a σ^{70} - and a σ^{32} -dependent promoter, with transcription from the latter increasing under conditions when the normally unstable σ^{32} protein is stabilised, as happens after heat shock (Straus et al. 1987; Zeilstra-Ryalls et al. 1991). In many other eubacteria, the *groE* operon is regulated by the binding of a repressor (HrcA) to a conserved inverted repeat often referred to as a controlling inverted repeat for

chaperone expression (CIRCE) sequence (Zuber and Schumann 1994). A negative-feedback mechanism has been demonstrated that couples repression at CIRCE elements with the level of GroEL in the cell (Babst et al. 1996; Mogk et al. 1997). More complex aspects to the regulation of these genes are also known, including post-transcriptional regulation of mRNA stability (Segal and Ron 1995).

The GroEL in *E. coli* assists in the folding of between 5% and 15% of all proteins under normal growth conditions (Ellis and Hartl 1996; Lorimer 1996; Ewalt et al. 1997). A wide range of proteins of diverse sequence, size and structure has been identified as GroEL substrates by co-immunoprecipitation (Ewalt et al. 1997; Houry et al. 1999). It is thus unexpected to find that many bacteria contain two or more copies of the *groEL* gene, often though not invariably with an associated *groES* gene. Some examples of these are shown in Table 1. The investigation of the regulation and role of these genes has not answered the question of whether all the genes code for proteins that all carry out the same function, or whether some of them are more specialised. In the Rhizobiaceae, which to date appear to have the highest number of *groE* operons, the picture is particularly complex. *Sinorhizobium meliloti* has five *groEL* genes, three of which have *groES* homologues upstream (Rusanganwa and Gupta 1993). *Bradyrhizobium japonicum* has seven *groEL* genes, five of which have *groES* homologues upstream (Fischer et al. 1993; Kaneko et al. 2002). *Mesorhizobium loti* has five *groEL* genes, all with *groES* homologues upstream (Kaneko et al. 2000). We have previously shown that the pea and bean-nodulating bacterium *Rhizobium leguminosarum* contain at least three complete *groE* operons (i.e. with a *groES* and a *groEL* homologue), which we named *cpn1*, *cpn2* and *cpn3* (Wallington and Lund 1994). In order to further our understanding of the role of multiple *groE* operons

in eubacteria in general and the Rhizobiaceae in particular, we have undertaken a genetic and biochemical analysis of these three operons and their protein products. Here, we describe a genetic characterisation of the requirement for these three operons.

Materials and methods

Nomenclature

Following the system, which we used previously, and consistent with the nomenclature suggested by Coates et al. (1993), we refer to the three *groE* operons as *cpn1*, *cpn2* and *cpn3*. Each contains a *groES* and a *groEL* homologue, which are referred to as *cpn10.n* and *cpn60.n* respectively, where *n* is the number of the operon.

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Table 2. *R. leguminosarum* strains were grown at 28°C in TY (Berlinger 1974) or GGM (Rodríguez-Quiñones et al. 1989) media, unless stated otherwise. *E. coli* strains (DH5 α for routine manipulations, S17-1 for conjugation with *R. leguminosarum*) were routinely grown at 37°C in LB (Sambrook et al. 1989). Unless otherwise stated, heat shock was at 37°C for 30 min. When needed, sucrose (5%), tetracycline (10 μ g/ml), kanamycin (50 μ g/ml), gentamicin (20 μ g/ml), ampicillin (100 μ g/ml), chloramphenicol (50 μ g/ml) or spectinomycin (50 μ g/ml) were added to the media. To form root nodules, pea plants (variety Frisson) were grown and inoculated as described previously (Beynon et al. 1980). The plants formed similar numbers of nodules (80–120) in all cases.

Table 1 Examples of bacteria that possess multiple GroEL homologues

Species name	Number of <i>groEL</i> homologues	Number of <i>groES</i> homologues	Reference
<i>Bradyrhizobium japonicum</i>	7	5	Fischer et al. (1993); Kaneko et al. (2002)
<i>Chlamydia trachomatis</i>	3	1	Karunakaran et al. (2003)
<i>Mesorhizobium loti</i>	5	5	Kaneko et al. (2000)
<i>Mycobacterium leprae</i>	2	1	Rinke de Wit et al. (1992)
<i>M. tuberculosis</i>	2	1	Kong et al. (1993)
<i>Rhizobium leguminosarum</i>	3	3	Wallington and Lund (1994)
<i>Rhodobacter sphaeroides</i>	2	2	Lee et al. (1997)
<i>Sinorhizobium meliloti</i>	5	3	Rusanganwa and Gupta (1993); Galibert et al. (2001)
<i>Streptomyces albus</i>	2	1	Guglielmi et al. (1991); Mazodier et al. (1991)
<i>Streptomyces coelicolor</i>	2	1	Duchêne et al. (1994)
<i>Streptomyces lividans</i>	2	1	De Léon et al. (1997)
<i>Synechococcus</i> sp. PCC 6803	2	1	Chitnis and Nelson (1991); Lehel et al. (1993)
<i>Synechococcus vulcanus</i>	2	1	Furuki et al. (1996); Tanaka et al. (1997)

Table 2 Strains and plasmids

	Relevant details	Reference or source
<i>Escherichia coli</i> strains		
S17-1	<i>mob</i> , RP4 (<i>tra</i> ⁺), Spec ^R , Str ^R	Simon et al. (1983)
DH5 α	Δ (<i>lacZYA-argF</i>), F' ϕ 80 <i>dlacZ</i> Δ M15	Life Technologies
<i>R. leguminosarum</i> strains		
8002	Carries pRP2JI, determining bean nodulation.	Johnston and Beringer (1975)
78401	8002 cured of pRP2JI, Str ^R	Lamb et al. (1982)
A34	Originally referred to as 8401/RL1JI, with the plasmid determining pea nodulation; Str ^R	Downie et al. (1985)
A141	Derivative of A34 carrying <i>nifA137::Tn 5</i>	Ma et al. (1982)
MML2	A34 with p <i>cpn2-gusA</i> fusion on chromosome	This work
RQ2/RQL2	Derivative of 8002 or A34 respectively with <i>cpn60.2::kan</i>	This work
RQ3/RQL3	Derivative of 8002 or A34 respectively with <i>cpn60.3::kan</i>	This work
RQ23/RQL23	Derivative of 8002 or A34 respectively with <i>cpn60.2::spc</i> , <i>cpn60.3::kan</i>	This work
Plasmids		
pBI121	Source of <i>gus</i> gene for cloning	Clontech
pDW2	3.1-kb <i>Xba</i> I- <i>Bam</i> HI fragment carrying entire <i>cpn1</i> operon including promoter region, in pGB0	P.A. Lund and D. Winwood, unpublished
pFR545	<i>cpn60.3</i> with <i>kan</i> insert, in pJQ200SK	This work
pFR547	<i>cpn60.2</i> with <i>kan</i> insert in pJQ200SK	This work
pFR548	<i>cpn60.2</i> with <i>spc</i> ^R insert in, in pJQ200SK	This work
pFR551	<i>cpn60.1</i> with <i>kan</i> insert, in pJQ200SK	This work
pGB0	pMP220 with <i>gusA</i> gene replacing <i>lacZ</i> gene	This work
pGB1	680 bp upstream from <i>cpn1</i> operon, in pGB0	This work
pGB2	354 bp upstream from <i>cpn2</i> operon, in pGB0	This work
pGB3	894 bp upstream from <i>cpn3</i> operon, in pGB0	This work
pGEM7Zf(-)	T7 and SP6 RNA polymerase sites flanking the multiple cloning site.	Promega
pJQ200SK	<i>sacB</i> suicide broad host range plasmid	Quandt and Hynes (1993)
pMP220	Broad host range promoter probe plasmid with <i>lacZ</i> reporter gene	Spaink et al. (1987)
pTM1	pGB0 containing the <i>cpn3</i> operon under the control of p <i>cpn1</i>	P.A. Lund and T. Magnay, unpublished

Bacterial matings

Plasmids to be conjugated into *R. leguminosarum* were transformed into the mobilizing *E. coli* strain S17-1 (Simon et al. 1983). Bacterial conjugations between this strain and rhizobial recipient strains were done as described elsewhere (Kondorosi et al. 1982). Selection was on GGM medium plus the appropriate antibiotic for the transferred plasmid.

DNA procedures

Standard DNA procedures were used throughout (Sambrook et al. 1989). Genomic DNA was prepared as described elsewhere (Ausubel et al. 1987). DNA probes were labelled with [α -³²P]dCTP, using the Megaprime labelling kit (Amersham, UK). Oligonucleotide preparation and DNA sequencing were carried out by Alta Biosciences, Birmingham, UK, using standard methods. The nucleotide sequences of the *cpn2* and *cpn3* operons have been deposited in GenBank (accession numbers AF239163 and AF239164).

Plasmid construction

To analyse transcription from the promoters of the three *cpn* operons, we constructed a promoter probe plasmid pGB0, in which the *lacZ* gene present in the broad host range promoter probe plasmid pMP220 (Spaink et al. 1987) was replaced with a promoterless *gusA* gene, cloned from plasmid pBI121 (Clontech, Heidelberg, Germany). The *gusA* gene was cloned such that the first ATG was part of a unique *Sph*I site. All the promoter regions from the three *cpn* operons were amplified using PCR with an *Xba*I site in the forward primer and an *Sph*I site in the reverse primer, such that the ATG in the *Sph*I site was always the ATG of the respective *cpn10* gene. The amplified promoter regions from *cpn1*, *cpn2* and *cpn3*, were cloned into pGB0 to yield pGB1, pGB2 and pGB3, respectively. Thus the precise spacing of the promoter and ribosome-binding site from each *cpn* promoter to the ATG of the *cpn10* homologue was preserved in each case. The primers used are shown in Table 3. To integrate the promoter-*gusA* fusions onto the chromosome, the inserts were cloned as *Xba*I-*Dra*I fragments into *Xba*I-*Sma*I-cut pJQ200SK (Quandt and Hynes 1993). The resulting plasmids were then mobilised

Table 3 Oligonucleotide primers used for cloning, mutagenesis and expression analysis of the three *cpn* operons

Primer	Sequence (5'-3')	Restriction site (<i>underlined</i> in sequence)	Use
12601	GCATCTAGACCGTCTTCGGC	<i>Xba</i> I	Cloning of <i>cpn1</i> promoter
12602	GTTGGTGCTTGGCATGCTCTAATCC	<i>Sph</i> I	Cloning of <i>cpn1</i> promoter
D8116	GCCATTGTCTAATCCCTCGATCG	—	Primer extension of <i>cpn1</i>
D3656	GTTTGCACCCTTGACGG	—	RT-PCR of <i>cpn60.1</i>
7D3657	GGAAAACACCACGATCG	—	RT-PCR of <i>cpn60.1</i>
22207	GGATCTGGCTCTAGACATGTCGAG	<i>Xba</i> I	Cloning of <i>cpn2</i> promoter
22208	GGTCGGAACGGCATGCCTTCCTCC	<i>Sph</i> I	Cloning of <i>cpn2</i> promoter
12808	GAACGGAATTGCATGCAGGTCTTTC	<i>Sph</i> I	Cloning of <i>cpn 3</i> promoter
D8663	CCGGCAGCAGAAAGACCTCTATG	—	RT-PCR of <i>cpn60.3</i>
D8613	GATTTGACCTTCATCGCCC	—	RT-PCR of <i>cpn60.3</i>
gus2	GCGGTTTCTACAGGACGCAGC	—	Primer extension of promoter- <i>gus</i> fusions

into *R. leguminosarum* A34 as described above. Transconjugants were selected on gentamicin, and insertion of the plasmid by recombination on the chromosome was confirmed using Southern blots and PCR.

To construct knock-out mutations, the three *cpn60* genes containing inserted antibiotic-resistance markers were cloned into the vector pJQ200SK, conjugated into *R. leguminosarum* strains as described above, and single and double crossover events were selected in GGM and GGM-sucrose media, respectively, with the appropriate antibiotics added (kanamycin, spectinomycin, or both).

Fig. 1 The three *cpn* operons of *Rhizobium leguminosarum*. **a** Southern blot of *R. leguminosarum* genomic DNA showing the presence of three *cpn* operons. DNA from *R. leguminosarum* strain 8002 was digested to completion with *Bam*HI (B), *Eco*RI (E) or *Xho*I (X) and probed with a *Sma*I-*Pst*I fragment of the *cpn3* operon, containing approximately the 3' half of *cpn10.2* and the 5' half of *cpn60.3*. The position of the probe is shown by the solid bar beneath the map of the *cpn3* operon. Bands corresponding to the *cpn1* operon (filled circles), *cpn2* operon (filled triangles), and *cpn3* operon (filled squares) can be seen. **b** Map showing the positions of the insertions made in each operon for mutagenesis experiments. The insert into *cpn60.1* removed an internal fragment of 499 bp between the two *Bgl*II sites in this gene. Restriction enzyme abbreviations as above, together with S2 (*Sac*II)

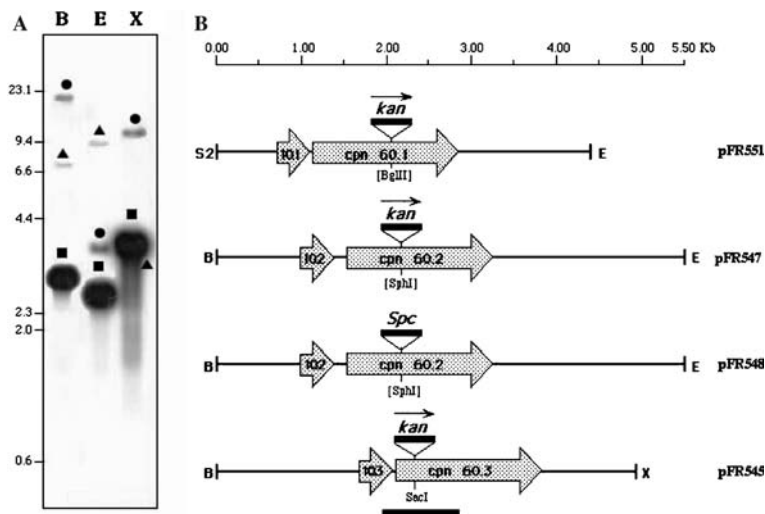
The positions of the inserted resistance markers in all three operons are shown in Fig. 1b.

To express the *cpn1* operon in *R. leguminosarum*, the entire *cpn1* operon including its own promoter was ligated into the plasmid pGB0 to give the plasmid pDW2. To over-express the Cpn60.3 protein, the *cpn3* operon was placed under the control of the *cpn1* promoter, maintaining the correct spacing from the promoter to the ATG of the *cpn10.3* gene as described above, and then the whole insert was ligated into plasmid pGB0 for expression in *R. leguminosarum*, yielding the plasmid pTM1.

For production of sense and anti-sense RNA for the RNase protection experiments, fragments of DNA overlapping the start point of the relevant *cpn10* genes and their upstream promoter regions were amplified by PCR and cloned into pGEM7Zf(-), so that appropriate transcripts could be synthesised in vitro using T7 or SP6 RNA polymerase.

RNA isolation

The RNA was isolated from cultures of *R. leguminosarum* by the hot acid phenol method (Aiba et al. 1981).



The DNaseI treatment of RNA was carried out for 1 h at 37°C using 10 units RNase-free DNaseI (Promega). The RNA samples were quantified by UV absorbance and checked on agarose gels for clear ribosomal RNA bands before use.

RNase protection

The RNase protection assays were performed by hybridising 10 µg RNA with ³²P-labelled antisense RNA probes produced by in vitro transcription from linearised plasmids. The RNA and excess probe were precipitated together and resuspended in hybridisation buffer [40 mM PIPES pH 6.4, 1 mM EDTA, 400 mM NaCl, 80% (v/v) formamide], denatured for 4 min at 85°C and incubated overnight at 45°C. Single-stranded RNA was digested with 100 u/ml RNase T₁ (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 300 mM NaCl) at 30°C for 30 min, and RNases were inactivated by treatment with SDS and proteinase K. Double-stranded protected RNA was precipitated, denatured and run on a 6% polyacrylamide sequencing gel. The DNA molecular-weight markers were produced by labelling a kilobase ladder (Life Technologies, Mississauga, Ont., Canada) with ³⁵S dATP and Klenow polymerase. Probes were chosen which overlapped upstream and putative promoter regions, so that transcription start sites could be roughly located. Control sense transcripts were also produced in vitro as size markers and to check the specificity of the labelled antisense probes. A mixture of all three sense transcripts was hybridised to each individual probe to check the specificity of the hybridisation and to confirm that any protected fragments were operon specific. No cross-reaction between probes and heterologous operons was seen in these experiments (data not shown). A calibration curve with which to size unknown transcript was also derived from these protected fragments, in conjunction with the ³⁵S-labelled DNA kilobase ladder.

Primer extension

Primer extension analysis was performed by hybridising the appropriate ³²P end-labelled primers to 10 µg RNA (see Table 3 for primers). Thirty picomoles of oligonucleotide primer were labelled with [γ -³²P] ATP (Amersham International) using T4 polynucleotide kinase, following the manufacturer's instructions. Ten micrograms RNA was coprecipitated with 3 µl labelled primer, resuspended in hybridisation buffer [20 mM HEPES (pH 6.5), 0.4 M NaCl 80% formamide] and denatured at 85°C for 5 min before hybridising overnight at 45°C. The hybridised nucleic acids were precipitated again before extension with 800 U Superscript reverse transcriptase (Gibco-BRL, Gaithersburg, Md., USA), used according to the manufacturer's instructions, for 1 h at 42°C. Reverse transcriptase was

inactivated at 72°C for 10 min before RNA templates were degraded by RNase. Extended products were then precipitated, resuspended in sequencing formamide dye mix and electrophoresed on 6% polyacrylamide sequencing gels. A ³³P-labelled DNA-sequencing ladder produced with the same primer, and a Sequenase sequencing kit (Amersham International) was used to size the products and determine the start point of transcription.

RT-PCR

RT-PCR was performed with MMLV Reverse Transcriptase (Gibco-BRL). After heating duplicate reactions to 75°C for 10 min and rapidly cooling to 37°C, either 200 U reverse transcriptase plus 20 U RNasin (Promega) or 10 µg RNase was added and the reactions allowed to proceed for 1 h. The cDNA products were then subjected to 30 cycles of PCR (94°C, 45 s; 66°C, 1 min 30 s; 72°C, 2 min). Products were analysed by agarose electrophoresis. Controls without reverse transcriptase were always done to ensure that the bands seen were not due to DNA contamination.

Protein methods

To analyse proteins, cell pellets from washed cultures of the same optical density were resuspended in SDS-PAGE loading buffer (Sambrook et al. 1989) and electrophoresed in 8% or 10% SDS-polyacrylamide gels. Gels were blotted onto PVDF membrane and, after washing and blocking, probed with a monoclonal antibody (4-3F) raised against *E. coli* GroEL that recognises all three Cpn60 proteins from *R. leguminosarum* equally well (George 2000). Bound antibody was detected with an anti-mouse antibody that was visualised with ECL developing solution (Roche, Penzberg, Germany), and the membrane was visualised in a Fluoro-S MultiImager (Bio-Rad).

Results

Cloning and sequence analysis of the *cpn2* and *cpn3* operons

Our earlier work (Wallington and Lund 1994) demonstrated the presence of three *cpn* operons in *R. leguminosarum*. To complete the analysis of all three operons, a 0.9-kb *SmaI*-*PstI* fragment, encompassing approximately the 3' half of *cpn10.3* and the 5' half of *cpn60.3*, was used to probe *R. leguminosarum* 8002 genomic DNA digested with *Bam*HI, *Eco*RI or *Xho*I (Fig. 1a). Identical results were obtained whether DNA from strains 8002, 8401 (which lacks a *sym* plasmid) or strain A34 was used. Thus, none of the *cpn* operons is

Table 4 Predicted properties of Cpn10 and Cpn60 proteins from *R. leguminosarum* and of GroES and GroEL from *E. coli*

Protein	Amino acids	Molecular weight (Da)	Per cent similarity ^a to:		
			Cpn10.1	Cpn10.2	Cpn10.3
Cpn10.1	98	10,517.69	100		
Cpn10.2	104	11,324.56	86.5	100	
Cpn10.3	105	11,270.58	78.1	74.3	100
GroES	97	10,386.46	58.3	62.5	57.3
			Cpn60.1	Cpn60.2	Cpn60.3
Cpn60.1	546	57,880.70	100		
Cpn60.2	542	57,512.77	87.5	100	
Cpn60.3	544	57,349.71	82.0	81.8	100
GroEL	548	57,265.65	76.9	75.6	73.7

^aThe per cent similarity scores are derived from pairwise comparisons using the GCG program GAP (Devereux et al. 1984)

on the *sym* plasmids (pRP2JI or pRL1JI), which are present in 8002 and A34, respectively. The 9.5-kb *EcoRI* band corresponding to *cpn2*, and the 3.5-kb *XhoI* band corresponding to *cpn3* were excised and cloned. Sequence analysis of these fragments confirmed that the *cpn2* and *cpn3* operons consisted of genes homologous to the *groES* and *groEL* genes. Features of the proteins predicted from these genes are summarised in Table 4, together with those of the *cpn1* and *E. coli groE* operons for comparison. Analysis of the three *cpn* operons revealed putative transcriptional terminators downstream of all three of the *cpn60* sequences, and a putative upstream inverted repeat regulator element (CIRCE; Zuber and Schuman 1994), in all operons, initially suggesting similar regulatory pathways for all three chaperonins. The three putative CIRCE sequences are compared to the CIRCE consensus sequence in Table 5.

Mutagenesis of the three *cpn* operons

We next attempted to construct mutations in each of the three *cpn60* genes. Kanamycin- or spectinomycin-resistance cassettes were introduced into each of the three *cpn60* genes cloned in plasmids in *E. coli*. These were then mobilised into *R. leguminosarum* strains using a non-replicable plasmid (pJQ200SK), which carries genes for gentamicin resistance and sucrose sensitivity. Merozygotes were selected as being resistant to gentamicin, and kanamycin or spectinomycin, and sensitive to sucrose. We then selected for homologous recombinants

by growing cells on sucrose and screening any colonies that arose for gentamicin sensitivity. Colonies where the wild-type *cpn60* gene had been replaced by the mutated gene would be predicted to be kanamycin-resistant or spectinomycin-resistant, sucrose-resistant and gentamicin-sensitive.

Mobilisation of the pJQ200SK derivatives into *R. leguminosarum* and selection for a single recombination event (see 'Materials and methods') occurred at frequencies ranging between 1.2×10^{-6} and 9.4×10^{-6} transconjugants per donor cell. Of these, approximately 1–3% were also sucrose-resistant and gentamicin-sensitive, indicative of successful insertional mutagenesis of the *cpn60* gene, except when plasmid pFR551 was used (*cpn60.1::kan*). If conjugations were selected directly on GGM medium containing sucrose, colonies arose at frequencies between 3.0×10^{-7} and 8.0×10^{-7} , but no sucrose-resistant gentamicin-sensitive colonies could be isolated on GGM-kanamycin when plasmid pFR551 was used. Identical results were obtained whether the recipient was *R. leguminosarum* strain 8002, 8401 or A34, confirming that the *cpn2* and *cpn3* operons are not on a *sym* plasmid (absent in 8401). Six sucrose-resistant, kanamycin-resistant, gentamicin-sensitive colonies derived from conjugation with plasmids pFR545 and pFR547 were examined by Southern blots, probing with the *kan* probe and the intra-*cpn3* (for pFR545) or intra-*cpn2* (for pFR547) probes. All the colonies analysed were found to be the result of bona fide double-recombination events (not shown). One of the colonies, which originated from pFR545 in strain 8002, and one of the colonies which originated from pFR547 in the same

Table 5 Alignments of the putative controlling inverted repeat for chaperone expression (CIRCE) regions of the three *cpn* operons

Putative CIRCE sequences ^a		Position of CIRCE sequence	Position of first base of cognate <i>cpn10</i> gene
TTAGCACTC ——— GAGTGCTAA	(Consensus)	—	—
CTAGCACTC ACCGCAAGG GAGTGCTAA	<i>cpn1</i>	+2 to +28	+87
TTGGCACTC ACGGGCAGG GAGTGCTAA	<i>cpn2</i>	–77 to –51	+98
TTGGCACTC CTATCAATC GAGTGCCAC	<i>cpn3</i>	+29 to +55	+109

^aThe sequences show the putative CIRCE sequence (in *boldface* where they match the consensus), and the position of these and the first base of the *cpn10* gene relative to the transcription start (+1)

recipient were chosen, and these were termed RQ3 and RQ2, respectively. Colonies containing insertions in *cpn60.2* and *cpn60.3* derived from strain A34, were named RQL2 and RQL3.

We next attempted to make strains mutated for both *cpn60.2* and *cpn60.3*. The *cpn2::spc* plasmid pFR548 was mobilised into strains RQ3 and RQL3 (both containing *cpn60.3::kan* insertions and no wild-type *cpn60.3* gene), and several of the sucrose-resistant, kanamycin-resistant, spectinomycin-resistant, gentamicin-sensitive exconjugants that arose were analysed by hybridisation against intra-*cpn2*, intra-*cpn3*, *kan* and *spc* probes (not shown). All the colonies tested were the result of double-recombination events into the *cpn60.2* gene, confirming that double *cpn60.2 cpn60.3* mutants could be constructed in both bean- and pea-nodulating strains. The double mutants were named RQ23 and RQL23, respectively.

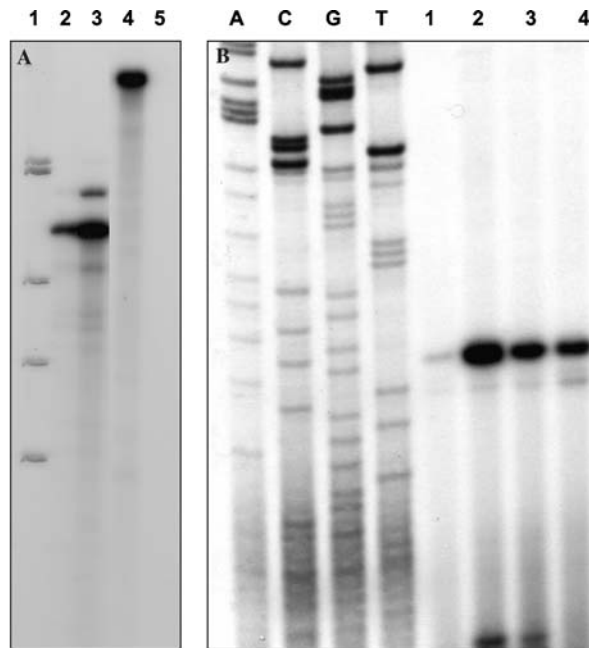
For the mating with plasmid pFR551 only single recombination events (leading to sucrose-sensitive, kanamycin-resistant, and gentamicin-resistant colonies) were obtained. Southern analysis confirmed that these had arisen by integration of the entire plasmid into the chromosome at the *cpn60.1* locus (data not shown). These strains thus contained an intact *cpn60.1* gene as well as a *cpn60.1::kan* insertion. These merodiploids were plated on medium with sucrose, and colonies consistently arose at a frequency of ca. 2.5×10^{-5} . However, nearly all these colonies were still gentamicin-resistant, thereby indicating that a mutation in either the *sacB* gene or its promoter had occurred to give sucrose resistance. After very extensive screening, four colonies were obtained that were sucrose-resistant and gentamicin-sensitive, but on Southern analysis these all proved to be derived from recombination that had occurred between the plasmid borne *cpn60.1::kan* gene and the *cpn60.3* gene, and all still carried an intact wild-type *cpn60.1* gene.

To confirm that failure to obtain inserts in the *cpn60.1* gene was not due to suppression of double crossover events in the vicinity of the *cpn1* operon, we made a construct with the *kan* gene immediately downstream from the *cpn60.1* gene. When this was mobilised into *R. leguminosarum* on pJQ200SK, homogenotes (kanamycin-resistant, gentamicin-sensitive, and sucrose-resistant) arose at a frequency of 5.7×10^{-6} . Finally, we showed that if a plasmid expressing the Cpn10.1 and Cpn60.1 proteins from the *cpn1* promoter (pDW2) were present in A34, then we were able to obtain *cpn60.1* knock-outs at approximately the same frequency as those in *cpn60.2* and *cpn60.3*. Taken together, these results show that the *cpn60.1* is essential for viability under the conditions used. We searched for differences in phenotype between the strains carrying the knock-out mutations in operons *cpn2* and *cpn3*, the double mutant, and the wild-type parents. No changes were found in rates of growth at normal or elevated temperatures, or rates of killing at a lethal temperature (data not shown).

Analysis of transcription of the three *cpn* operons

Previously, we demonstrated heat shock induction in *R. leguminosarum* of a 58-kDa protein, and confirmed this as the product of the *cpn60.1* gene by immunoprecipitation experiments and N-terminal sequence analysis (Wallington and Lund 1994). We therefore anticipated induction of the transcription of the *cpn1* operon by heat shock. Studies using RNase protection with operon-specific probes overlapping the predicted promoter regions indicated that the *cpn1* operon was expressed in *R. leguminosarum* A34 growing under non-stressed conditions, and that it was further induced on heat shock (Fig. 2a). The same result was seen with independent preparations of RNA from different cultures. A major protected fragment was seen in all experiments; various minor bands were also seen, but their position and intensity was not reproducible. Primer extension studies (Fig. 2b) using primer D8116 identified the start point for transcription of the *cpn1* operon as being immediately upstream from the conserved CIRCE sequence and at -87 with respect to the ATG of *cpn10.1*. The sequence TTGACT-N17-TAAATG is the expected distance upstream from this start site to be a good candidate for the promoter of this gene. The start point was identical in mRNA isolated from free living cells grown at 28°C and heat shocked at 37°C and 45°C, or incubated under anaerobic conditions, as shown in Fig. 2. No other bands were reproducibly seen in the primer extension experiments, which makes it likely that the minor bands seen in RNase protection experiments do not represent genuine transcription start points. The presence of a single transcription start site is a characteristic of bacteria with a CIRCE-regulated *groE* operon (e.g. *Bacillus subtilis*, Li and Wong 1992).

In several experiments we were unable to detect expression of *cpn2* from cells grown at 28°C by using RNase protection assays, but after exposure to heat shock, a transcript was reproducibly detectable, with the start site of transcription at approximately -100 (Fig. 3). We initially attempted to analyse expression from the cloned *cpn2* promoter using *gusA* as a reporter gene, but we found β -glucuronidase activity to be a poor reporter of heat shock-induced expression, even when the promoter-*gusA* fusion was integrated on the chromosome to reduce possible copy number artefacts (data not shown). We therefore used primer extension to determine the start point of transcription, but used a primer hybridising to the *gusA* reporter gene, rather than a *cpn2* specific primer, to eliminate artefacts due to cross-hybridisation with the highly homologous regions in *cpn1* and *cpn3*. The *cpn2* upstream region (354 bases upstream from the ATG of *cpn10.2*) was fused upstream of a promoterless *gusA* gene as described in 'Materials and methods', and recombined onto the chromosome to give the strain MML2. Primer extension with primer *gus2* revealed a unique transcript starting at position -98 relative to the ATG of *cpn10.2*, in close agreement with the RNase protection results. This places the transcrip-



C

CCCTAAATGCTCGTCTCCTAGCACTCACCGCAAGGGAGTGCTAACACCTATCCATGTGGGCCA
 CTCCGGTCCGCGAATGTCATTTCGATCGAGGGATTAGACAATG

Fig. 2 Analysis of *cpn1* expression. **a** RNase protection using a labelled probe covering the promoter and the start of the *cpn1* operon, with RNA from cultures grown at 28°C (lane 2) or from strains heat shocked at 37°C for 30 min (lane 3). Lane 1 shows molecular-weight markers (sizes in base pairs: 517, 506, 396, 344 and 298), lane 4 is undigested probe, and lane 5 is a negative control (probe hybridised with yeast tRNA and digested with RNase). **b** Primer extension using primer D8116 on RNA isolated from cultures grown at 28°C (lane 1), 37°C (lane 2) or 45°C (lane 3) for 30 min or incubated anaerobically for 1 h (lane 4). Sequencing tracks were produced using the same primer. **c** Sequence of region upstream from ATG of Cpn10.1 (shown in *italics*). The putative controlling inverted repeat for chaperone expression (CIRCE) sequence is *underlined*, and the transcription start site shown on a *black background*

tion start site over 60 bases downstream of the putative CIRCE region, which makes it less likely that the CIRCE acts to cause repression of this operon. The start site for transcription was unchanged on heat shock. The signal in both normally grown and heat-shock induced cells was much weaker than that obtained with the *cpn1* primer labelled to approximately the same specific activity, implying lower expression of the *cpn2* operon, a result that was consistent with our previous observations and was confirmed by analysis of protein levels (see below). No obvious promoter sequence could be found upstream of the transcription start site, consistent with this being a weak promoter (Fig. 4).

Expression of the *cpn3* operon was hard to detect. The RNase protection experiments with a probe homologous to the 5' end of the operon revealed a very faint band after prolonged exposure (data not shown), but attempts to detect transcription of the chromosomal locus using primer extension were unsuccessful. To increase the strength of the signal

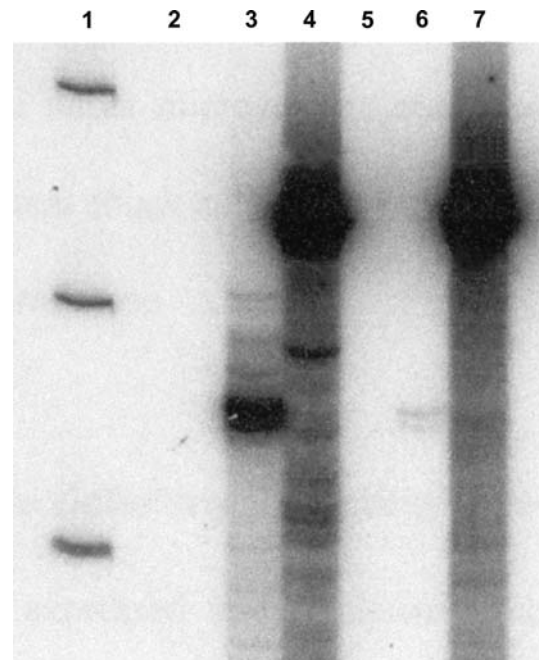
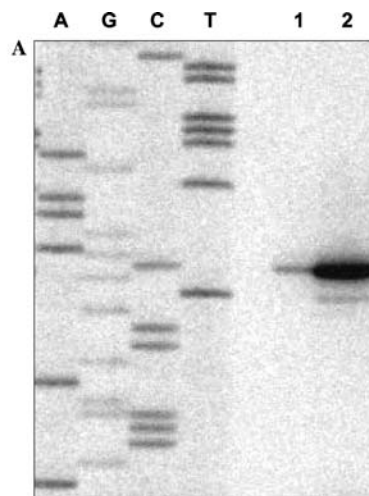


Fig. 3 Analysis of *cpn2* expression by RNase protection. The RNase protection using a labelled probe covering the promoter and the start of the *cpn2* operon, with RNA from cultures grown at 28°C (lane 5) or from strains heat shocked at 37°C for 30 min (lane 6). Lane 1 shows molecular-weight markers (sizes in base pairs 311, 249 and 200), lane 2 is a negative control (probe hybridised with yeast tRNA and digested with RNase), lane 3 is a positive control (probe hybridised with mRNA extracted from *E. coli* expressing the *cpn2* operon under the control of the pBAD promoter), lane 4 is a probe hybridised against a full-length sense mRNA synthesised *in vitro* before RNase digestion, and lane 7 is an undigested probe

Fig. 4 Analysis of *cpn2* expression by primer extension. **a** Primer extension using primer *gus2* on mRNA isolated from *R. leguminosarum* A34 MML2 (wild-type *cpn2* promoter controlling *gus* expression) grown without (*lane 1*) or with a heat shock (37°C for 30 min, *lane 2*). **b** Sequence of region upstream from ATG of *Cpn10.2* (shown in *italics*). The putative CIRCE sequence is underlined, and the transcription start site shown on a *black background*



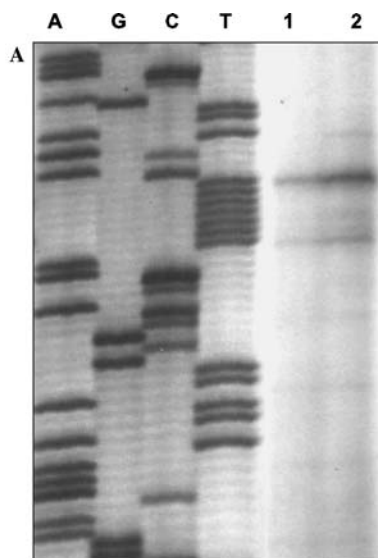
B
 CAGGCGTTGGCACTCACGGGCAGGGAGTGCTAATTTTCTTTTCATCCCTCTTGAATTGCAAA
 GCCGTCGAACCAAATCATTCTCCACGGCTCGCGGCTCGCGCGCTGCGCACCCGCCCGGATT
 GGTTCATCCGGTCCGGCTAGGGAACAACGTCATCATTGTTGTGCGATGGAGGAAGACATG

from the putative *cpn3* promoter, we increased its copy number by constructing the plasmid pGB3, which contains 894 bases of the *cpn3* upstream region fused to a promoterless *gusA* gene. Primer extension on mRNA extracted from cells containing this plasmid using primer *gus2* revealed a transcript, originating at -108 relative to the ATG for *cpn10.3* (Fig. 5). Faint bands may indicate other minor transcription start sites downstream, but there is no indication of the preferential use of different start sites under heat-shocked conditions. In contrast to *cpn1* and *cpn2*, transcription of *cpn3* was only detectable in cells which had been incubated anaerobically. A good consensus

CIRCE sequence was found shortly downstream from the transcription start site (see Table 5).

Because expression of the *cpn3* operon appeared to be restricted to anaerobically incubated cells, we speculated that the *cpn3* operon might be directly or indirectly under the control of the NifA transcriptional regulator, which regulates (together with the alternative sigma factor RpoN) expression of the nitrogen-fixation genes under anaerobic conditions. We tested this by looking for expression of the *cpn3* operon in wild-type and *nifA* cells, initially using primer extension. The transcript visible in wild-type cells was not seen in *nifA* cells (not shown). Because the faintness of the signal meant that

Fig. 5 Analysis of *cpn3* expression. **a** Primer extension was carried out using primer *gus2* on mRNA isolated from *R. leguminosarum* A34/pGB3 incubated anaerobically at 28°C (*lane 1*) or 37°C (*lane 2*). **b** Sequence of region upstream from ATG of *Cpn10.3* (shown in *italics*). The putative CIRCE sequence is underlined, and the transcription start site shown on a *black background*



B
 TCTCMAAAAAAATTCCTCCGGAATAATATTTCTTGGCACTCCTATCAATCGAGTGCCACGAG
 TGTATTATGAGTTGCAGCCGCATACGCGCCGCGAGCAGAAAGACCTCTATG

weak NifA-independent transcription might escape detection by this method, we used the more sensitive method of RT-PCR to detect transcription, using primers D8613 and D8663. The results (Fig. 6a) confirmed that expression of *cpn3* was only seen under anaerobic conditions and also showed that no expression occurred in a *nifA* strain. Under the same conditions and using the same assay, *cpn1* showed clear expression irrespective of the *nifA* mutation (Fig. 6b). We also looked using RT-PCR to see whether *cpn3* was expressed in bacteroid tissue. The result (Fig. 6a) confirmed that this was indeed the case. Expression appeared still to be weak, however, as analysis of mRNA from bacteroids using RNase protection still only detected a very faint signal (data not shown), and Cpn60.3 expression in bacteroids was undetectable by western blotting (see below; Fig. 7b, lane 2). We examined the upstream region of the *cpn3* operon for a σ^{54} consensus site, but were unable to detect one; the action of NifA may therefore be indirect.

Comparison of levels of the three Cpn60 proteins

Transcript levels do not necessarily correlate well with protein levels, and comparison of RNA analysis data obtained with different probes is not quantitative. We therefore analysed relative levels of Cpn60 protein in normally growing cells, using Western blotting with the monoclonal antibody 4-3F (Wallington and Lund

Fig. 6 RT-PCR analysis of expression of *cpn3* and *cpn1* in wild-type and *nifA* strains. **a** The RT-PCR using primers D8613 and D8663 (*cpn3*-specific) on mRNA isolated from *R. leguminosarum* A34 (lanes 2 and 3) or A141 (A34 *nifA*) (lanes 4 and 5) grown aerobically at 28°C (lanes 2 and 4) or incubated anaerobically for 1 h (lanes 3 and 5). Lane 6 shows the RT-PCR product with the *cpn3*-specific primers on mRNA isolated from bacteroids. The RT-PCR product is predicted to be 809 bp in length. Lane 1 and 7: DNA size markers (2,036, 1,636 and 1,018 bp). **b** Lanes 2–5 as for **a**, but using primers D3656 and D3657 (*cpn1*-specific). The RT-PCR product is predicted to be 326 bp in length. Lane 1 DNA size markers (1,018, 517, 506, 396, 344, 298, 220, 201, 154 and 134 bp)

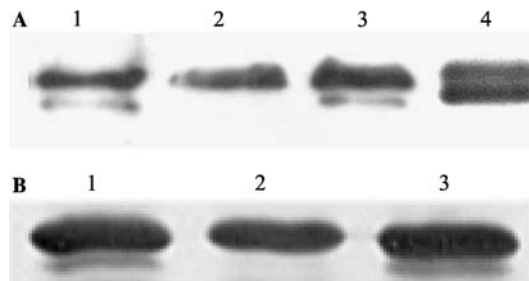
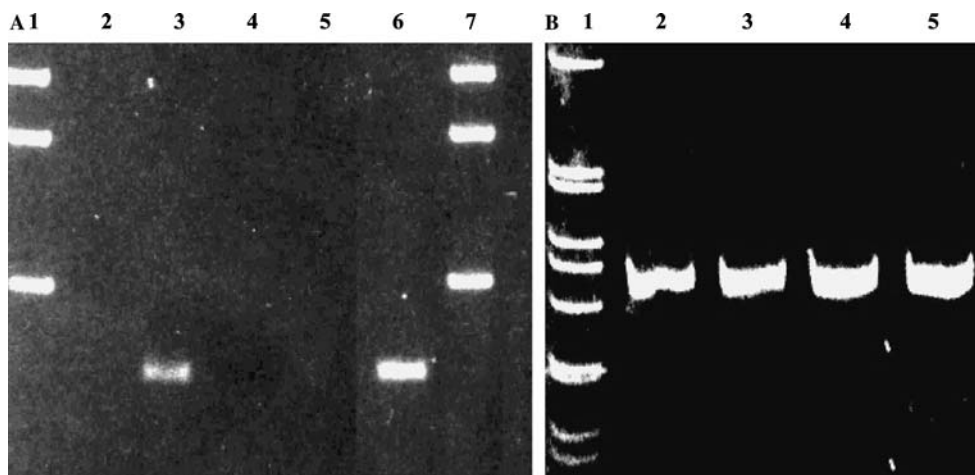


Fig. 7 Analysis of Cpn60 protein expression in free-living cultures and root nodules. **a** Western blot with 4-3F anti-GroEL antibody against total protein extracted from wild type *R. leguminosarum* A34 (lane 1), and *R. leguminosarum* carrying deletions of either *cpn60.2* (lane 2) or *cpn60.3* (lane 3), grown under non-heat shock conditions. Lane 4 contains extract from *R. leguminosarum* A34 expressing Cpn60.3 from the plasmid pTM1. **b** Western blot with 4-3F anti-GroEL antibody against total protein extracted from root nodules made with wild-type *R. leguminosarum* A34 (lane 1), and *R. leguminosarum* carrying deletions of either *cpn60.2* (lane 2) or *cpn60.3* (lane 3)

1994), which cross-reacts equally well with all three purified Cpn60 proteins (George 2000). Cpn60.1 runs slightly slower in polyacrylamide gels than do Cpn60.2 and Cpn60.3, which run at the same speed (George et al. 2004). The availability of strains carrying deletions of either *cpn60.2* or *cpn60.3* enabled us to confirm that the major cross-reacting band visible was Cpn60.1, and that Cpn60.2 was present at much lower relative abundance than Cpn60.1, both in free-living cultures grown under non-heat shock conditions and in bacteroids (Fig. 7a, b). We never observed a band corresponding to Cpn60.3 protein even in strains where the Cpn60.2 protein was not synthesised, including under heat shock conditions (data not shown) or in protein isolated from bacteroid tissue (Fig. 7b). Over-expression of Cpn60.3 from the *cpn1* promoter confirmed that, if present, it would run at the same position as Cpn60.2 (Fig. 7a, lane 4). Thus, the relative abundance of these three proteins in *R. leguminosarum* is in the order Cpn60.1 → Cpn60.2 → Cpn60.3, with Cpn60.1 easily the most abundant.

Discussion

In this study, we have demonstrated that the three *cpn* operons of *R. leguminosarum* are expressed at widely different levels and differ in the extent to which they are required for bacterial growth and function. How do these results compare with those for other bacteria, which possess multiple *groE* operons, and what do they imply about the function of the different homologues, which they encode?

Although GroE proteins are required for the viability of almost all bacteria, the reason why some bacterial species contain multiple *groE* operons remains obscure. The relatively few cases studied genetically to date do not reveal a consistent pattern other than that *groE* expression is always required at some level, which is as predicted given the essential nature of the *groE* operon in *E. coli* (Fayet et al. 1989), and the fact that a *groE* operon is a feature of nearly all bacterial genomes. In *Rhodobacter sphaeroides* for example, one of the two *groEL* genes (which is expressed at a very low level) can be deleted with no change in phenotype; the other is essential (Lee et al. 1997). In *Streptomyces albus*, one of the two *groEL* genes (both of which are expressed) is also essential (Servant et al. 1993). In *Bradyrhizobium japonicum*, all of the expressed *groE* operons can be individually inactivated without effect on cell viability (Fischer et al. 1999).

The results reported here are distinct from the only other root-nodulating organism where all the *groE* genes have been mutated (*B. japonicum*) in that one of the multiple *groE* operons of *R. leguminosarum* is essential for growth. Attempts to detect any differences in the viability of free-living strains lacking expression of either or both of the *cpn2* and *cpn3* operons were unsuccessful. Extensive analysis by Southern blotting at low stringency failed to reveal the presence of any further *cpn* operons in this organism. [Interestingly, a search of the *R. leguminosarum* genome (which is from a different strain of *R. leguminosarum* than that used here), currently available on <http://www.sanger.ac.uk>, reveals four *groEL* homologues, three of which are at least 97% identical to those described here. The fourth is on a plasmid which is not present in A43, with only around 50% identity to the genes found in A43 and with no cognate *groES* gene upstream]. Thus it appears likely that Cpn60.1 is the only essential GroEL homologue in strain A34. The essential nature of this gene was demonstrated by showing that null mutations in the *cpn60.1* gene could only be constructed if the *cpn60.1* gene were expressed from a plasmid. We are currently investigating the ability of the other two Cpn60 proteins to complement for loss of Cpn60.1.

The promiscuity of the GroEL protein for many different substrates (Viitanen et al. 1992; Ewalt et al. 1997; Houry et al. 1999) makes the occurrence of multiple *groE* operons somewhat surprising: why encode two or more proteins when one is capable of handling

such a range of substrates? Potentially, increased levels of GroEL proteins from more than one operon may represent one way in which the organism can respond to stresses, rather than up-regulating a single gene. Alternatively, some of the GroEL proteins may have evolved to have unique and specific functions not shared by the others. The data presented here do not rule out either of the above possibilities in the case of *R. leguminosarum*. However, the first explanation is unlikely given that the level of expression of the Cpn60.1 protein is much higher than that of Cpn60.2 and Cpn60.3, so neither of the latter two can contribute significantly to the total pool of Cpn60 (GroEL) protein in the organism. This leads to the second option as the most attractive possibility. Circumstantial evidence links GroE homologues in other rhizobia to the phenomenon of root nodulation and nitrogen fixation (Fischer et al. 1993; Rusanganwa and Gupta 1993; Ogawa and Long 1995; Fischer et al. 1999; Oke and Long 1999; Kaneko et al. 2000), but microarray analysis suggests reduction of expression of the *S. meliloti groEL* and *groES* genes during symbiosis (Ampe et al. 2003), although some of the homologues are induced by microaerobiosis (Puskas et al. 2004). The single and double *cpn60* knock-out *R. leguminosarum* strains constructed in this paper were all able to form root nodules and fix nitrogen on both peas and beans. Future careful examination of this process may reveal subtle differences in efficiency between the different knock-out strains.

Sequence analysis of the three operons in *R. leguminosarum* suggests that the *cpn1* and *cpn3* operons are likely to be regulated via the CIRCE sequence by an HrcA type repressor, such as has been found in many other bacteria, although NifA is also implicated in the regulation of the *cpn3* operon. A good CIRCE sequence is also seen upstream of the *cpn2* operon, but as it is a long distance upstream of the transcription start site, regulation by HrcA of this operon is less likely. Deletion analysis of the putative CIRCE sequences in the *cpn1* and *cpn2* operons, and of the *R. leguminosarum hrcA* gene, supports this prediction (P.S. Gould, M. Maguire and P.A. Lund, in preparation). The challenge now is to elucidate the details of the regulation of these three operons and relate it to the role of the respective gene products in both normal growth and stress survival, and in particular to uncover the specialised roles that the *cpn2* and *cpn3* operons may have.

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