Characterization and expression analysis of the Bartonella bacilliformis groES-EL operon

Julie A. Callison
The University of Montana

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Characterization and expression analysis of the *Bartonella bacilliformis* groES-EL operon

by

Julie A. Callison

B.S. The University of Montana, USA, 2002

presented in partial fulfillment of the requirements

for the degree of

Master of Science

The University of Montana

July 2004

Approved by:

[Signature]
Chairperson

[Signature]
Dean, Graduate School

7/30/04
Date
Characterization and expression analysis of the *Bartonella bacilliformis* groES-EL operon

The groESL operon from *Bartonella bacilliformis*, a facultative intracellular, Gram-negative bacterium and the etiologic agent of Oroya Fever, was characterized. Sequence analysis revealed three open reading frames (ORFs) of 75 nucleotides (ORF1; encoding a hypothetical protein related to the *hrcA* repressor for groESL), 297 nucleotides (groES) and 1635 nucleotides (groEL). The groES and groEL genes are separated by 55 nucleotides. The deduced amino acid sequence of *B. bacilliformis* GroES and GroEL revealed a high degree of identity with other bacterial GroES and GroEL proteins. The transcriptional start site was mapped 240 nucleotides upstream of the groES start codon by primer extension, at a site located immediately downstream of a σ^52^-like promoter. The transcript initiation site was identical regardless of whether RNA was isolated from heat-shocked or control cultures. Sequence analysis also revealed a potential controlling inverted repeat of chaperonin expression (CIRCE) element and a divergent fumarase C (*fumC*) gene upstream, plus a likely rho-independent transcriptional terminator downstream. Northern blot analysis indicated that ORF1, groES and groEL are transcribed as a single mRNA of ~2251 nucleotides and expression is upregulated in response to thermal stress. Immunoblot analysis revealed increased GroEL synthesis in response to thermal stress or the presence of coumermycin *A*₁ (a gyrase inhibitor) in the medium. *In vivo* transcription-translation analyses of the cloned operon revealed an insert-specific protein product of ~57 kDa, consistent with the predicted molecular mass for GroEL. Complementation assays with the cloned operon and temperature-sensitive *Escherichia coli* groES or groEL mutants restored growth at restrictive temperatures.
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INTRODUCTION

*Bartonella bacilliformis* is a facultative intracellular bacterial parasite of human erythrocytes and endothelial cells (Reynafarje & Ramos, 1961). The pathogen is spread between humans by the bite of phlebotamine sand flies (Hertig, 1942) and is responsible for Oroya fever and verruga peruana; life-threatening diseases endemic to Colombia, Ecuador, Chile and Peru (Brenner *et al.*, 1991). Oroya fever is a biphasic disease with remarkably disparate syndromes. During the primary phase, bacteria attach to and invade nearly all circulating erythrocytes (Benson *et al.*, 1986). Because approximately 80% of these infected cells are lysed, a life-threatening hemolytic anemia can result (Benson *et al.*, 1986). During the secondary phase, the bacterium invades endothelial cells of the vasculature (Benson *et al.*, 1986), and this stage of the disease is characterized by skin eruptions, termed verruga peruana (Schultz, 1968). *B. bacilliformis* produces a protein factor that triggers proliferation of human endothelial cells, and it is believed to enhance formation of the vascular lesions (Garcia *et al.*, 1990; Minnick *et al.*, 2003). The verruga tend to cluster on the head and limbs of the patient, and can last for months or years.

*B. bacilliformis*’ parasitic strategy relies on an insect vector and a subsequent intracellular location in the human host, conditions that are undoubtedly accompanied by a variety of environmental stressors including reactive oxygen intermediates, pH and temperature changes and ambient hemin fluctuations (Coleman & Minnick, 2003). One countermeasure used by all organisms against such stress is the heat-shock response (Lindquist & Craig, 1988). Two components of the bacterial heat-shock response include GroES and GroEL (Neidhardt *et al.*, 1996). Although referred to as heat shock components, *groES* and *groEL* genes are constitutively expressed and are essential for
growth at all temperatures (Neidhardt et al., 1996). GroES and GroEL are molecular chaperones responsible for the correct folding of polypeptides (Sigler et al., 1998). GroEL is composed of two rings consisting of seven subunits of approximately 57 kDA, and GroES is a heptamer of 10 kDA subunits (Sigler et al., 1998). Recent biophysical studies have shown that GroEL binds polypeptides after their release from the ribosome and hydrophobic forces help drive this interaction (Weissman et al., 1996). Hydrophobic interactions between the exposed side chains of the polypeptide and apical domain of GroEL mediate binding (Weissman et al., 1996). GroEL’s affinity for binding and folding of polypeptides requires ATP (Sigler et al., 1998). When the GroEL ring contacts GroES through a flexible, hydrophobic loop, the volume of the GroEL central channel doubles as the GroEL apical domains open upward and outward (Weissman et al., 1996). ATP and GroES bind to the GroEL ring containing the polypeptide to create a stable GroEL-GroES cis assembly (Sigler et al., 1998). Once GroES and ATP bind, the polypeptide is released into the central cavity to begin folding (Chaudhry et al., 2003).
Our laboratory recently showed that the *B. bacilliformis* GroEL protein participates in the proliferative response of cultured human vascular endothelial cells (HUVECs) to the soluble fraction of a *B. bacilliformis* cell lysate (Minnick *et al.*, 2003). Data showed that mitogenicity of the lysate was: a) augmented by up-shifting growth temperature of the source culture, b) inhibited by antiserum specific to GroEL, and c) that HUVEC numbers correlated with levels of GroEL in the bacterial cell lysate. Data also showed that GroEL is actively secreted by *B. bacilliformis* and is found in both the soluble and insoluble fractions of the bacterium (Minnick *et al.*, 2003).

To continue exploring GroEL’s involvement in human endothelial cell proliferation and its suspected role in pathogenesis, we characterized a *Bartonella groES-EL* operon and analyzed its expression in response to thermal stress; a biologically relevant environmental cue for this pathogen.
MATERIALS AND METHODS

Bacterial strains used in this study are listed in Table 1. *B. bacilliformis* strain KC583 was cultivated on heart infusion agar blood plates (HIAB; Carroll et al., 2000) at 30°C and 100% relative humidity, and was harvested at four days post-inoculation. *E. coli* TOP10F’ (Invitrogen) was used as a host for plasmid propagation and was grown in Luria Bertani (LB) medium for 16 hours at 37°C. *E. coli* strains CG2244 (Ardigo & Lipinska, 2003), CG2241 (Ardigo & Lipinska, 2003), CG2245 (Georgopoulos, 1971), and NRK117 (Kusukawa et al., 1989), were used in complementation studies. Antibiotics including kanamycin (25 μg ml⁻¹), ampicillin (100 μg ml⁻¹), tetracycline (10 μg ml⁻¹) or coumermycin A₁ (0.05 μg ml⁻¹) were added to the growth medium, as required.

Nucleic acid preparation and manipulation.

Plasmids used in this study are described in Table 1. The plasmid, pGROESL-CMV, contains a functional *groES-groEL* operon derived from a λ ZAP expression library of *B. bacilliformis* KC583 as previously described (Minnick et al., 2003). pGROESL-CMV served as a template to PCR amplify the operon using primers, BbGroESL_Operon-For-TTAAATCGACGTATAAAGAAATTTG and BbGroESL_Operon-Rev-CTTTTACAATGCAGCTCTTTCAATG (Sigma Genosys). The resulting amplicon was re-cloned into pCR2.1-TOPO (Invitrogen, Appendix A) to produce pGROESL-TOPO, a plasmid with reduced flanking sequences and the operon oriented in opposition to the lacZ’ promoter of the vector. Plasmids were routinely screened and purified using a Midi Prep Kit (Qiagen). DNA sequencing and in vivo transcription templates were purified with a Wizard Plasmid Prep kit (Promega). Messenger RNA was isolated using a RiboPure Bacteria kit per the manufacturer’s instructions (Ambion). For Northern blots,
rRNA was removed with a MicrobeExpress kit per the manufacturer's instructions (Ambion). Sequencing template concentrations were determined at 260 nm using a Spectronic Genesys 2 spectrophotometer (Milton Roy). DNA fragments and amplicons were isolated from ethidium bromide-stained gels using a GeneClean II Kit per the manufacturer’s instructions (QBIOgene). Chemical transformation of *E. coli* was done by the methods of Chung *et al.*, (1989).

**Automated DNA sequencing and analysis.**

Approximately 100 ng template was sequenced using oligodeoxynucleotide primers (Sigma Genosys), a BigDye Terminator Cycle Sequencing Ready Reaction kit (ABI/Roche) and an automated DNA sequencer (ABI; model 377). Resulting chromatograms were analyzed using Chromas software (Technelysium). Sequences were analyzed using a variety of software including BLAST (AltSchul *et al.*, 1990), the Sequence Manipulation Suite (Stothard, 2000) and the BCM Search Launcher (Smith *et al.*, 1996).

**Transcriptional start site determination.**

A Sequenase Version 2.0 kit (US Biochemical; USB) and deoxy/α-ATP $^{35}$S (NEN-Dupont) were used for manual sequencing. DNA template (~ 5 µg) was denatured following the manufacturer’s protocol (USB) and then annealed to a sequencing primer (BbGroESRev1- TCCCACCAGCTGTCTGG) for 2 min at 65°C, allowed to cool to 30°C and sequenced. Total RNA for primer extensions was obtained as above, from four culture plates of *B. bacilliformis* KC583; two plates were heat-shocked by a temperature upshift to 37°C for 15 min and two plates were maintained at 30°C as a control. Cultures were then harvested into 400 µl of filter-sterile PBS (pH 7.4) and centrifuged at 5000 x g
for 10 min at 4°C and RNA purified as above. A kinase reaction was done by standard protocol (USB) to end-label BbGroESRev1 primer (10 pmol/μl) with deoxy/γ-CTP ³²P (NEN-Dupont). The reaction was incubated for 30 min at 37°C and stopped by incubating for 20 min at 65°C. RNA (750 ng) was annealed to the primer by heating the reaction for 5 min at 70°C. A primer extension was done using MuMLV reverse transcriptase and 5X M-MLV buffer (Promega). The extension reaction was incubated for one hour at 42°C. Using the sequencing and primer extension reactions, a sequencing gel was run using both heat-shocked and control samples. The transcriptional start site of the operon was determined by comparing the primer extension to the sequencing reaction.

**Southern and Northern blotting.**

Southern blots were done at high stringency (~7% DNA mismatch) as previously described (Battisti & Minnick, 1999) using a gro^L-specific probe produced by PCR with primers BbGroelFor- ATGGCTGCTAAAGAAGTAAAATTTG and BbGroelRev- TTAGAAATCCATTCCGCCCCATTC.

For Northern blots, *B. bacilliformis* cultures were heat-shocked for 30 min by transferring culture plates from a 30°C to a 35°C incubator. Seven plates were then harvested into 350 μl of cold RNase-free heart infusion broth and centrifuged for 5 min at 5000 x g and 4°C. Pellets were resuspended in 350 μl cold RNAwiz (Ambion) to inhibit RNase. Cultures maintained at 30°C were harvested in parallel, as a control. A 255-bp gro^EL-specific probe was generated by PCR with primers, GroESLFor2/M13F3- CTATTTCTGCTAATGGCG and T7GroELR- TAATACGACTCACTATAGGGAGACAAAGATTGC. The T7GroELR primer has a
23 base T7 promoter sequence added to the 5' end for in vivo synthesis of a transcript. The resulting RNA probe was labeled with α-CTP\(^{32}\)P (PerkinsElmer) using a MAXIscript in vivo Transcription kit (Ambion). Unincorporated nucleotides were removed using a NucAway kit (Ambion). The isolated mRNA (1.1 µg per lane) and the labeled probe were used in a Northern Blot following the NorthernMax-Gly Kit protocol (Ambion).

**In vivo Transcription / Translation.**

Expression of the *B. bacilliformis* groES-EL operon was done in vivo using purified plasmid DNA, \([^{35}\text{S}]\text{Met} / \text{Cys}\) (EXPRESS; NEN-Dupont) and an in vivo transcription-translation system per the manufacturer’s instructions (Promega). \(^{35}\text{S}\)-labeled proteins were visualized by 24-hour autoradiography of a dried SDS-PAGE gradient gel, using X-Omat LS X-ray film (Kodak).

**SDS-PAGE and Western blots.**

*B. bacilliformis* cells were harvested at 0, 4, and 8-hour time points following transfer of plates from 30°C to 35°C. Cultures maintained at 30°C were harvested in parallel as a control. *B. bacilliformis* cultures grown on HIAB plates supplemented with coumermycin A₁ (0.05 µg ml\(^{-1}\)), were also done to determine whether the gyrase inhibitor would induce the operon as previously observed in spirochetes (Alverson & Samuels, 2002). Protein concentrations were determined by a BCA assay (Pierce), and SDS-PAGE was done by standard methods (Ausubel *et al.*, 1995) using 20 µg protein sample per lane and 12.5% (w/v) or 7-15% (w/v) linear gradient acrylamide gels. SDS-PAGE gels were either fixed and stained with Coomassie blue by standard protocol (Ausubel *et al.*, 1995) or transferred overnight to nitrocellulose filters (0.45 µm pore size) by the methods of Towbin *et al.* (1979). Resulting blots were probed 2 hours at 25°C with rabbit anti-*B.
*bacilliformis* GroEL polyclonal antiserum at a 1:1000 dilution, as previously described (Minnick *et al.*, 2003). Reactive bands were visualized as before (Scherer *et al.* 1993) using goat anti-rabbit IgG-horseradish peroxidase-conjugated antibody (Sigma) at a 1:1000 dilution, a hydrogen peroxide substrate and 4-chloronapthol as the chromogen. Densitometric analysis of immunoblots was performed using a UMAX Astra 1200S scanner and OneDscan software (Scanalytics).

**Complementation analysis.**

*E. coli* *groESL* mutants (CG2244 and CG2241), were complemented in *trans* with the cloned *B. bacilliformis* *groES-EL* operon by transforming with pGROESL-TOPO or pGroESL177, (high-copy number and low-copy number plasmids, respectively). Transformants were screened for plasmid content as above and subsequently tested on selective LB plates at both permissive and restrictive temperatures (determined to be 30°C and 47°C, respectively). A positive, and otherwise isogenic control strain (CG2245) was grown on LB plates containing tetracycline. *E. coli* NRK117 was used for complementation in *trans* in a similar fashion, at a restrictive temperature determined to be 44°C.
Table 1. Bacterial strains and plasmids

<table>
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<tr>
<th>Strain / plasmid</th>
<th>Relevant genotype / description</th>
<th>Source / Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Bartonella</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. bacilliformis</td>
<td>KC583 (neotype strain)</td>
<td>ATCC/Brenner et al., 1991</td>
</tr>
<tr>
<td>B. henselae</td>
<td>Houston-1 (type strain)</td>
<td>CDC/Regnery et al., 1992</td>
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<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOP10F</td>
<td>Host for plasmid propagation</td>
<td>Invitrogen</td>
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<tr>
<td>CG2244</td>
<td>groES619</td>
<td>Landry et al., 1993</td>
</tr>
<tr>
<td>CG2241</td>
<td>groEL44</td>
<td>Zeilstra-Ryalls et al., 1994</td>
</tr>
<tr>
<td>CG2245</td>
<td>Parental strain to CG2241 &amp; CG2244 (B178 Tn10)</td>
<td>Georgopoulos, 1971</td>
</tr>
<tr>
<td>NRK117</td>
<td>groEL44</td>
<td>Kusukawa et al., 1989</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pCR2.1-TOPO</td>
<td>TA cloning vector</td>
<td>Invitrogen</td>
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<tr>
<td>pACYC177</td>
<td>Low-copy number cloning vector</td>
<td>Chang &amp; Cohen, 1978</td>
</tr>
<tr>
<td>pGROESL-TOPO</td>
<td>B. bacilliformis groES-EL operon in pCR2.1-TOPO</td>
<td>Minnick et al., 2003</td>
</tr>
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<td>pGroESL177</td>
<td>B. bacilliformis groES-EL operon in pACYC177</td>
<td>This study</td>
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<td>pGroESL-CMV</td>
<td>B. bacilliformis groES-EL operon in pBKCMV</td>
<td>Minnick et al., 2003</td>
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</table>
RESULTS

Sequence analysis of the *B. bacilliformis* groES-groEL operon

The entire groES-EL operon of *B. bacilliformis* was sequenced, and its linkage map is shown in Figure 1B. The operon is unusual in that it is preceded by a small, 75-bp open reading frame (ORF1) encoding a hypothetical protein. A BLASTX search with ORF1 revealed a 71% identity to a 14-amino acid stretch of the heat shock gene repressor and negative regulator of CIRCE, (*HrcA*) from *Chlamydia muridarum* (Read et al., 2000; GenBank accession no: P54306). A divergent gene is located 282 bp upstream of groES and encodes a fumarase C gene (*fumC*), based on sequence homology. The groES gene is 297 nucleotides long and encodes a protein of 99 amino acids, with a predicted molecular mass of 10 kDa. A diagram showing the unusual region upstream of the operon including ORF1 and the groES gene sequences is shown in Figure 2. To our knowledge, this is the first sequence analysis of a *B. bacilliformis* groES gene and its flanking sequence. The *Bartonella henselae* data generated in this study (not shown), also revealed a divergent fumarase C gene, a CIRCE element (TTAGCACTC-N9-GAGCGCTAA) located 106 nucleotides upstream of the groES start codon (GenBank accession no. AJ749669) (See Appendix D). However, the groESL operon of *B. henselae* in this study was not preceded by a small open reading frame.

The predicted *B. bacilliformis* GroES protein is most closely related to the *B. henselae* homologue (GenBank accession no. AY664491) (See Appendix C), with 89.8% identity, and shares 49.0% identity to the *E. coli* GroES (Figure 3A).

The *B. bacilliformis* groEL gene is 1635 nucleotides long and encodes a predicted protein of 545 amino acid residues, with a predicted molecular mass of 57.5 kDa. The
**Fig. 1B.** Linkage map of the groESL operon of *B. bacilliformis*. Arrows indicate the positions of the open reading frames, including the partial, divergent *fumC* (*fumC*') ORF. The predicted *B. bacilliformis* GroEL protein is most closely related to homologues from *B. henselae* and *B. quintana* (This study; Haake *et al.*, 1997; Alsmark *et al.*, 2004), with 91.6% and 92% identity, respectively. The protein shares 65.8% identity to *E. coli* GroEL (Figure 3B). The GroEL protein from this study shares perfect identity to a previous submission for an immunoreactive HSP60 protein of *B. bacilliformis* (unpublished- GenBank accession No. M98257). Previous studies have observed a GMR motif (Gly-Gly-Met repeats) in the C-terminus of nearly all GroEL proteins (McLennan *et al.*, 1993), and this motif is conserved in the *Bartonella* homologues as well (Figure 3B).
Fig. 2. Nucleotide sequence containing the upstream flanking region of groES, the groES gene and the 5' end of groEL. The deduced amino acid sequence is shown below in one-letter code. A putative -35 and -10 σ^{32}-like promoter sequence for the operon is indicated above the start codon of groES. The potential ribosomal binding sites (RBS) are shown for each gene. The determined transcriptional start site is marked with a black diamond. The closed arrows indicate a conserved CIRCE element. The stop codon following each ORF is marked with an asterisk.
(A)

<table>
<thead>
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<th>Bb</th>
<th>Bh</th>
<th>Ml</th>
<th>Ec</th>
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<td></td>
<td>1 MANIKFRPLHDRVVRRVESENKTAGGIII PFDTEKEKFQEGEVIAVNGVULSNGOBVSL</td>
<td>1 MANIKFRPLHDRVVRRVESENKTAGGIII PFDTEKEKFQEGEVIAVNGGALDNGBRVPL</td>
<td>1 MASEKFRPLHDRVVRRVESENKTAGGIII PFDTEKEKFQEGEVIAVGSGARFESGGLVPL</td>
<td>1 MNRPLIRDBVIVKRKEVETKSAGGISLGSAAAKSTEGVVLAVGNRILENEQKPLDVK</td>
</tr>
<tr>
<td></td>
<td>61 EVKPGRDIFGKWSGTEVKGELLLIMKESDIMGILA</td>
<td>61 EVKTDGRDIFGKWSGTEVKGELLIMKESDIMGIG</td>
<td>61 EVKDGDRDIFGKWSGTEVKGELLIMKESDIMGIG</td>
<td>61 VGDIVIPNDDGYYKVKEKIDNEEVLGSLSESILCAYEA</td>
</tr>
</tbody>
</table>
Fig. 3. Comparison of the deduced amino acid sequence of: (A) GroES and (B) GroEL proteins from *B. bacilliformis* (AY664491) with homologues from *B. henselae* (AJ749669), *Mezorhizobium loti* (NP 108346), *B. quintana* (U78515), and *E. coli* (BAB38546, BAB38547).
Regulatory elements of the operon

The transcriptional start site of the *B. bacilliformis* operon was determined five times by primer extension analysis (Figure 4A), and located a distant 240 bases upstream of the start codon of *groES* with a $\sigma^{32}$–like promoter immediately upstream (Figure 2). The transcription initiation site was identical regardless of whether RNA was isolated from control or heat-shocked cultures (data not shown). Curiously, a potential promoter similar to an *E. coli* ($\sigma^{70}$) consensus promoter can be found 114 bp 5' to the *groES* gene, but a transcript corresponding to this start site was never observed. A potential CIRCE element of TTGGCGCTC-N9-GAGTGCTAA is located 78 base pairs before the *groES* initiation codon (Figure 2), and is nearly identical to CIRCE elements from other $\alpha$-Proteobacteria (Babst et al., 1996). Similar gene linkage and a CIRCE element were also observed in the region upstream of the *B. henselae* *groESL* operon (not shown). An inverted repeat representing a potential rho-independent transcriptional termination signal is located 25 nucleotides downstream of the *B. bacilliformis* *groEL* translational stop codon, and its predicted secondary structure has a $\Delta G$ of -11.5 (Figure 4B). Similarly, an inverted repeat, whose predicted secondary structure has a $\Delta G$ of -10.9, was discovered 21 nucleotides downstream of the *B. henselae* *groEL* translational stop codon (data not shown).

*B. bacilliformis* contains a single *groEL* gene

Previous studies have shown that bacteria that are closely related to *Bartonella*, e.g. *Rhizobium meliloti* and *Bradyrhizobium japonicum*, contain more than one copy of the *groEL* gene (Rusanganwa & Gupta, 1993; Babst et al., 1996). Further, there is apparent divergence between the two *B. bacilliformis* *groEL* sequences previously
Fig. 4A Transcriptional start site of the *B. bacilliformis* groESL operon by primer extension. RNA was prepared from *B. bacilliformis* under heat-shock or control temperature conditions and template DNA was sequenced as described in Methods. The transcriptional start site is marked with an asterisk and the corresponding nucleotide arrowed.
Fig 4B. Potential ρ-independent terminator of the *B. bacilliformis* groESL operon. The double-arrows indicate inverted repeats of the terminator, and its predicted secondary structure is shown (ΔG = -11.5).
deposited in GenBank (accession nos. Z15160 and M98257). To examine this possibility, we performed DNA hybridizations at high stringency. Southern blotting of *B. bacilliformis* KC583 chromosomal DNA restriction endonuclease-digested with *KpnI* or *XbaI* revealed single hybridization signal bands, whereas a band of 19.2 plus a minor band of 8.6 kbp were seen in a *ClaI* digest (Figure 5). The *groEL* gene was also PCR amplified from *B. bacilliformis* KC583 chromosomal and the resulting amplicons cloned into the pCR2.1-TOP0 vector (Invitrogen). Sequence analysis of the inserts from twenty random clones revealed perfect identity to the *groEL* sequence obtained in this study and to that previously submitted to GenBank for *B. bacilliformis* HSP60 (accession no. M98257) (data not shown). Taken as a whole, these data suggest that *B. bacilliformis* contains a single copy of the *groEL* gene. This observation is in agreement with data from the recent genome analysis of *B. henselae* and *B. quintana* (Alsmark et al., 2004).

**Thermal stress and its effect on *groEL* expression and GroEL synthesis**

To determine if expression of the *B. bacilliformis* operon is inducible by thermal stress, *in vivo* transcription was investigated using Northern blots. Total RNA was isolated from both heat-shocked and control *Bartonellae*. The Northern analysis of the *B. bacilliformis* mRNA revealed a single, and therefore polycistronic, transcript of approximately 2.2 kb. The *B. bacilliformis* heat-shocked culture showed an 18-fold increase in *groESL* mRNA transcript levels when compared to non-heat shocked controls (Figure 6). On Western blots, the *B. bacilliformis* GroEL has an estimated mass of 61 kDa, and heat-shocked cultures produced a 3 to 4-fold increase in GroEL protein levels as compared to controls at 4 to 8 hours (Figure 7A).
Fig. 5. Southern blot detection of *groEL* in *B. bacilliformis* chromosomal DNA digested with a variety of restriction endonucleases. Lane 1, λ *Hind*III DNA size standards; lane 2, *KpnI*-digest; lane 3, *XbaI*-digest; lane 4, *ClaI*-digest and lane 5, a *BamHI*-digest of pGroESL-TOPO as a positive control.
Fig. 6. Detection of thermal stress-induced upregulation of *groESL* mRNA by Northern blot analysis. The polycistronic transcript is ~2.2kbp in size and indicated. Northern blots from a 30°C control and a 35°C temperature upshifted culture are shown.
Fig. 7A. Immunoblot analysis of increased *B. bacilliformis* GroEL synthesis in response to stress. Typical Western blot reacted with rabbit anti-*B. bacilliformis* GroEL antiserum. Densitometry revealed a 3- and 4-fold increase for the 4 hour and 8 hour temperature-upshifted cultures, respectively. The GroEL protein is 61-kDa and marked by an arrow.
Immunoblots were used to follow relative GroEL protein levels in response to coumermycin A₁, a drug that impedes DNA replication by inhibiting ATP binding and hydrolysis catalyzed by the B subunit of gyrase (Maxwell, 1993). Previous studies have demonstrated that GroEL protein and groEL mRNA transcript levels are up-regulated in gyrB mutants of Borrelia burgdorferi (Alverson & Samuels, 2002). In mutant spirochetes possessing a coumermycin A₁-resistant DNA gyrase, supercoiling is more relaxed, and studies have shown that the groEL gene may be directly or indirectly regulated by DNA topology (Alverson & Samuels, 2002). In a similar fashion, cultures grown in the presence of coumermycin A₁ showed a slight, 1.5-fold increase in GroEL protein levels compared to controls when analyzed by Western blots (Figure 7B).

Complementation of *E. coli*- functional expression of *B. bacilliformis* groESL

To determine if the *B. bacilliformis* operon and its encoded chaperonins function in *E. coli*, complementation of temperature-sensitive mutants for groES and groEL, was employed. The cloned groESL operon in pGROESL-TOPO enabled NRK117 (groEL) to grow overnight at a restrictive temperature of 44°C, whereas NRK117 transformed with the pCR2.1-TOPO vector did not. However, pGROESL-TOPO was not able to complement *E. coli* CG2244 (groES) or *E. coli* CG2241 (groEL). The operon was therefore re-cloned into a low copy-number vector, pACYC177 (See Appendix B), to produce pGROESL177. *E. coli* CG2244 and CG2241 were transformed with pGROESL177, and the complementation assays repeated. This system enabled CG2241 and CG2244 to grow overnight at a restrictive temperature of 47°C, whereas strains transformed with the pACYC177 vector did not grow (see Table 2).
GroEL →

1

2
Fig 7B. Analysis of increased GroEL synthesis in response to growth on medium supplemented with coumermycin A$_1$. An immunoblot reacted with *B. bacilliformis*-specific rabbit anti-GroEL antiserum is shown. Lane 1, control sample grown on normal HIAB medium; Lane 2, sample from culture grown on HIAB containing coumermycin A$_1$ showing increased GroEL synthesis. Densitometry revealed a 1.5-fold increase compared to the control sample.
Table 2. Complementation of *E. coli* gro*ES* and gro*EL* mutations by cloned homologues from *B. bacilliformis*.

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<tr>
<td>NRK117 (gro<em>EL</em>)</td>
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<sup>a</sup> restrictive temperature of 44°C (NRK117) and 47°C (CG2244, CG2241, CG2245), (all strains grew at the permissive temperature of 30°C).

<sup>b</sup> ND- not done
In vivo transcription/translation of the operon

An *E. coli* S30 DNA expression kit (Promega) was used to visualize polypeptides encoded by the cloned *B. bacilliformis* groES/EL operon of pGROESL-TOPO. SDS-PAGE / autoradiography of the resulting reactions revealed an insert-specific protein of approximately 57 kDa, consistent with the predicted molecular mass for GroEL (57.5 kDa). Unfortunately, GroES and ORF1 proteins were indistinguishable from vector-specific bands of similar mass near the bottom of the autoradiograph. (Figure 8).

An autoradiograph is shown with reactions involving: lane 1, the cloning vector pCR2.1-TOPO and lane 2, pGroESL-TOPO. The insert-specific ~61-kDa protein corresponding to GroEL is marked with an arrow. The GroES and ORF1 proteins were indistinguishable from vector-specific bands of similar mass. C14 molecular mass standards are shown to the left in kDa.
DISCUSSION

Transmission of *B. bacilliformis* from sandflies to the human host is undoubtedly accompanied by several types of cellular stress including ambient temperature upshift, oxidative stress, pH change and hemin limitation (Coleman & Minnick, 2003), and the pathogen must be able to adapt if it is to survive. One means of adaptation used by all bacteria against such stress is the heat-shock response, which upregulates a group of highly-conserved genes encoding heat-shock proteins (Neidhardt *et al.*, 1996). Two components of the heat-shock response in bacteria include GroES and GroEL (Neidhardt *et al.*, 1996). GroEL proteins are major antigens for several pathogenic bacteria, including *Bartonella* (Knobloch & Schreiber, 1990; Chenoweth *et al.*, 2004). Our laboratory recently found that *B. bacilliformis* GroEL is also involved in the pathogen's mitogenicity for human endothelial cells *in vivo* (Minnick *et al.*, 2003). In this study, we describe the sequence, promoter analysis, regulation in response to temperature upshift and functional expression of the *B. bacilliformis* groES-EL operon.

Analysis of sequence data revealed a gene linkage of groES-groEL, in keeping with *E. coli* and the majority of eubacteria (Segal & Ron, 1996b). Surprisingly, a small ORF (ORF1) was discovered upstream of the groES gene of *B. bacilliformis* (Fig. 2). However, the small ORF is apparently not present in the *B. henselae* or the *B. quintana* genome sequence (Alsmark *et al.*, 2004; GenBank accession no. NC00595). This ORF1 encodes a hypothetical protein and based on the transcriptional start site mapping (Fig. 4A) and size of the transcript (2251 bases; Fig 6), the ORF1 would be included in the transcript. Unfortunately, the *in vivo* translation product of ORF1 could not be distinguished from the vector-encoded protein products (Fig. 8) and whether it is
synthesized is unknown. Interestingly, the predicted protein encoded by ORF1 has a short stretch of sequence identity to a heat shock gene repressor and negative regulator of CIRCE element, HrcA. Work is currently in progress to determine whether the ORF1 protein is involved in regulating this operon via CIRCE.

The GroES and GroEL from this study are closely related to homologues from *B. quintana* and *B. henselae* (>91% sequence identity) (Fig 3). This finding was fully expected, due to the fact that GroES and GroEL are among the most highly conserved proteins in nature and are essential for viability (Segal & Ron, 1996b). Interestingly, sequence identity to the *E. coli* homologue was only 49%, and it is possible that certain of these different amino acids may be involved in the unusual activities attributed to *B. bacilliformis* GroEL (Minnick *et al.*, 2003).

Previous studies have shown that *B. bacilliformis* GroEL is located in both soluble and insoluble fractions of the cell, including inner and outer membranes, and is actively secreted by the bacterium (Minnick *et al.*, 2003). Similarly, GroEL has been found in the outer membrane of *B. henselae* (Chenoweth *et al.*, 2004). However, sequence analysis in this study did not reveal a predicted signal sequence at the N-terminus of the *B. bacilliformis* GroEL protein (LeFranc, 2003). These data suggest that another secretory system is responsible for translocation. It is also interesting to note that all characterized *Bartonella* GroEL sequences to date (see Figure 3B) possess a C-terminal phenylalanine, a characteristic that is common to many secreted and integral membrane proteins (Struvye *et al.*, 1991).

The *groESL* operons from a variety of bacteria are frequently transcribed from two promoters; a σ$^{32}$ promoter for transcription under stressful conditions, and a σ$^{70}$
promoter for transcription in the absence of $\sigma^{32}$. However, primer extension results from this study revealed a transcriptional start site located 240 nucleotides upstream of the groES start codon, regardless of whether RNA was isolated from heat-shocked or control cultures (Fig 4A). As expected, a sequence similar to the consensus $\sigma^{32}$ heat-shock promoter of E. coli is found immediately upstream of the transcriptional start site (Fig 2). Interestingly, a sequence similar to an E. coli ($\sigma^{70}$) consensus promoter is located 114 bp upstream of the groES gene, but a transcript corresponding to this start site was not observed, regardless of ambient culture temperature.

Another system for regulation of groESL expression is present in Gram-positive and some Gram-negative bacteria, and consists of a highly-conserved inverted repeat called the CIRCE, which acts as a cis-acting negative control element (Segal & Ron, 1996a.). The B. bacilliformis CIRCE element is located 78 nucleotides upstream of the groES start codon and it is nearly identical to CIRCE described in other $\alpha$-Proteobacteria (Babst et al., 1996). The B. henselae operon also contains a similar element (this study).

Finally, a potential rho-independent transcriptional termination signal is located 25 nucleotides downstream of the groEL stop codon and consists of an inverted repeat, with a $\Delta G$ of -11.5, suggesting it would form spontaneously during transcription (Fig 4B). This type of terminator is typical for groESL operons in eubacteria. (Zeilstra-Ryalls et al., 1991).

To determine the effects of thermal stress on the groESL operon of B. bacilliformis, we performed temperature upshift experiments and analyzed them with Northern and Western blots. Northern blot analysis revealed a single polycistronic mRNA of approximately 2.2 kb (Fig 6); a transcript that could easily accommodate
ORF1, groES and groEL plus their respective intergenic spacer regions. A 1.9 kb fragment was also detected in our Northern blot, and other studies report similar cleavage products from the groESL mRNA of Agrobacterium tumefaciens, E. coli (Segal & Ron, 1995) and Rickettsia typhi (Radulovic et al., 2002). Comparison of groESL mRNA on Northern, by densitometry, revealed an 18-fold increase in mRNA levels in temperature-upshifted samples compared to controls, and these data undoubtedly reflect increased participation by the σ32-like promoter.

Western blot / densitometry analysis revealed a four-fold increase in GroEL protein levels from cultures upshifted for 8 hours at 35°C as compared to 30°C controls (Fig 7A). These findings were expected because the heat shock response consists of upregulated synthesis of these highly conserved proteins in response to thermal stress (Neidhardt et al., 1996). The apparent lag time needed to detect an increase in GroEL protein was previously observed in B. bacilliformis IalB synthesis in response to environmental cues (Coleman and Minnick, 2003), and is attributed to the 6 to 8-hour generation time for the bacterium (Benson et al., 1986).

Growth of B. bacilliformis on medium supplemented with the gyrase inhibitor, Coumermycin A1, increased GroEL protein levels 1.5-fold above cultures grown on standard medium (Fig 7B). These findings resemble previous studies showing that GroEL protein levels and groEL mRNA transcript levels were upregulated in gyrB coumermycin-resistant mutants of Borrelia burgdorferi (Alverson & Samuels, 2002) and suggest that DNA topology may also help regulate this locus in Bartonella.

The B. bacilliformis groES-EL genes were functionally analyzed by testing their ability to complement temperature-sensitive mutations in homologous genes of E. coli.
Complementation assays revealed that both *B. bacilliformis* genes were able to complement the *E. coli* mutants (Table 2), suggesting that they are transcribed and translated. *groES-EL* genes expressed from the high-copy number plasmid, pGroESL-TOPO, were able to complement the *E. coli* NRK117 mutant but not *E. coli* CG2244 or CG2241 (Table 2). These data are similar to those previously reported, where *groESL* complementation depended on copy number of the plasmids carrying the genes of interest (Ardigo & Lipinska, 2003). The mutants are apparently unable to be complemented by high-copy number plasmids (Ardigo & Lipinska, 2003). *B. bacilliformis* *groES-EL* genes cloned and expressed from a low-copy number pACYC177 construct (i.e., pGroESL177) were able to successfully complement the *E. coli* CG2244 and CG2241 mutants (Table 2).

Previous studies on the *B. bacilliformis* GroES-EL proteins have shown that GroEL is a major antigen and a participant in the bacterium’s mitogenicity for human endothelial cells (Knobloch & Schreiber, 1990; Minnick *et al.*, 2003). This study demonstrates that the GroES and GroEL sequences of *B. bacilliformis* contain signature sequences, like the CIRCE element, shared by members of the α-purple subdivision of bacteria. This study also demonstrates the *groES-EL* operon of *B. bacilliformis* is upregulated in response to the temperature upshift that would occur during transmission of the pathogen from its insect vector to human host. Further analysis of this locus is expected to yield clues on its suspected involvement in endothelial cell mitogenicity and production of angioproliferative lesions during bartonellosis.
ACKNOWLEDGMENTS

I would like to formally express my thanks and gratitude to the members of my committee (Drs. Mike Minnick, Scott Samuels, and Keith Parker) for their support and encouragement during my time here at the University of Montana. Dr. Mike Minnick has been patient, understanding and an amazing help to me, both in explaining protocols and answering my never ending questions. I am grateful to have been a student in his laboratory. I would also like to express my sincerest thanks to all of the people in the Minnick Lab. Laura Smitherman and Kate Sappington, two amazing technicians, have been invaluable resources and friends to me.

We gratefully acknowledge the contribution of *E. coli* strains CG2244 and CG2241 by Barbara Lipińska, CG2245 B178 Th10 by Costa Georgopoulos, and *E. coli* NRK117 by Christopher Coker. Automated sequence analyses were done by The Murdock Sequencing Facility at The University of Montana. This work was supported by American Heart Association Established Investigator Grant 9940002N to MFM. MFM was also supported by Public Health Service grants AI52101 and AI053111.
Appendix A. TA cloning vector

Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZa fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809
Appendix B. Low-copy number vector pACYC177
### Appendix C. GenBank Submission of the *B. bacilliformis* groESL operon.

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41
Appendix D. GenBank Submission of the B. henselae groESL operon.

LOCUS AJ749669 3905 bp DNA linear BCT 24-JUN-2004

DEFINITION Bartonella henselae groES gene, groEL gene and phnA gene.

ACCESSION AJ749669

VERSION AJ749669.1 GI:49203111

KEYWORDS alkylphosphonate uptake protein; groEL gene; groES gene; groES-EL operon; heat shock protein; phnA gene.

SOURCE Bartonella henselae str. Houston-1

ORGANISM Bartonella henselae str. Houston-1

Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bartonellaceae; Bartonella.

REFERENCE 1

AUTHORS Callison,J.A., Smitherman,L.S., Read,A.J., Birtles,R.J. and Minnick,M.F.

TITLE Characterization of the Bartonella bacilliformis groES-EL operon

REFERENCE 2 (bases 1 to 3905)

AUTHORS Birtles,R.J.

TITLE Direct Submission

JOURNAL Submitted (23-JUN-2004) Birtles R.J., Veterinary Pathology, University of Liverpool, Leahurst, Chester High Rd, Neston, Cheshire CH64 7TE, UNITED KINGDOM

FEATURES Location/Qualifiers

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/strain="Houston-1"
/isolation_source="Homo sapiens"
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/country="USA"

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/groES-EL

gene 271..567

/groES

CDS 271..567

/groES

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CDS 629..2272

/groEL

/operon="groES-EL"

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CDS 629..2272

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RQIATNAGEEAIIVKGVLVNNADTFGYNTATSEFGDLDIALGIVDPKVRSALQNA
SISLILITTEAMVAEVFDTPPVMPFGMGIAGMGMMDF"

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