Heat shock response and *groEL* sequence of *Bartonella henselae* and *Bartonella quintana*

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Transmission of *Bartonella* species from ectoparasites to the mammalian host involves adaptation to thermal and other forms of stress. In order to better understand this process, the heat shock response of *Bartonella henselae* and *Bartonella quintana* was studied. Cellular proteins synthesized after shift to higher temperatures were intrinsically labelled with [³⁵S]methionine and analysed by gel electrophoresis and fluorography. The apparent molecular masses of three of the major heat shock proteins produced by the two *Bartonella* species were virtually identical, migrating at 70, 60 and 10 kDa. A fourth major heat shock protein was larger in *B. quintana* (20 kDa) than in *B. henselae* (17 kDa). The maximum heat shock response in *B. quintana* and *B. henselae* was observed at 39 °C and 42 °C, respectively. The *groEL* genes of both *Bartonella* species were amplified, sequenced and compared to other known *groEL* genes. The phylogenetic tree based on the *groEL* alignment places *B. quintana* and *B. henselae* in a monophyletic group with *Bartonella bacilliformis*. The deduced amino acid sequences of *Bartonella* GroEL homologues contain signature sequences that are uniquely shared by members of the Gram-negative α -purple subdivision of bacteria, which live within eukaryotic cells. Recombinant His₆-GroEL fusion proteins were expressed in *Escherichia coli* to generate specific rabbit antisera. The GroEL antisera were used to confirm the identity of the 60 kDa *Bartonella* heat shock protein. These studies provide a foundation for evaluating the role of the heat shock response in the pathogenesis of *Bartonella* infection.

Keywords: heat shock, *Bartonella henselae*, *Bartonella quintana*, GroEL

INTRODUCTION

The genus *Bartonella* includes organisms formerly classified in the genera *Grahamella* and *Rochalimaea* (Brenner *et al.*, 1993; Birtles *et al.*, 1995). Common features of *Bartonella* species include transmission by an ectoparasitic vector and survival within mammalian reservoirs, usually exploiting an intracellular refuge (Schwartzman, 1996). For example, *Bartonella bacilliformis*, the causative agent of Oroya fever, occurs in persons in the Andes mountains exposed to sandfly bites. Human disease caused by *B. bacilliformis* is

characterized by invasion of erythrocytes and endothelial cells (Reynafarje & Ramos, 1961). *Bartonella henselae*, the agent of cat scratch disease, appears to be transmitted from infected cats to humans largely by the cat flea (Higgins *et al.*, 1996; Chomel *et al.*, 1996). The cat serves as a reservoir host for *B. henselae*, and the organism has been found within feline erythrocytes (Kordick & Breitschwerdt, 1995). *Bartonella quintana*, the agent of trench fever, is transmitted by the human body louse and has also been reported to be haemotrophic (Weiss & Moulder, 1984).

Transmission from ectoparasites to a mammalian host requires bacterial mechanisms of adaptation to various stress factors. The bacterial heat shock response consists of upregulated synthesis of a highly conserved group of proteins which presumably enable pathogenic bacteria to adapt to thermal and other forms of stress associated

The GenBank accession numbers for the *groEL* nucleotide sequences from *Bartonella henselae* and *Bartonella quintana* reported in this paper are U78514 and U78515, respectively.

with life within a mammalian host. In the case of intraerythrocytic pathogens such as *Bartonella* species, these additional stresses would include adverse nutritional, pH and oxidative environments. Two of the components of the heat shock response, GroES (Hsp10) and GroEL (Hsp60), form an oligomeric complex which plays an essential role as a molecular chaperonin in protein-folding, assembly and secretion (Mayhew & Hartl, 1996). The GroEL family of proteins are also of interest because they are major antigens of a number of pathogenic bacteria, and because of their potential role in an autoimmune reactivity to Tcp-1 (T-complex polypeptide 1), the eukaryotic homologue of GroEL. The antibody response to bacterial heat shock proteins is predominantly directed towards non-conserved epitopes (Young *et al.*, 1990). However, antibody and T-cell responses towards conserved epitopes have been reported (Lamb *et al.*, 1989; van Eden *et al.*, 1988). In humans with *B. bacilliformis* infection, GroEL is one of the major antigens recognized by the humoral immune response (Knobloch & Schreiber, 1990). For these reasons, we have characterized the heat shock response of two *Bartonella* species, *B. henselae* and *B. quintana*. In order to characterize the 60 kDa *Bartonella* heat shock protein we isolated the *groEL* genes of these organisms. We found that the deduced amino acid sequences of the *Bartonella* GroEL proteins contain signature sequences shared by other intracellular bacteria.

METHODS

Bacterial strains. The type strain of *B. henselae*, ATCC 49882, was provided by the American Type Culture Collection, Rockville, MD, USA (Brenner *et al.*, 1993; Regnery *et al.*, 1992). A human blood isolate of *B. quintana*, designated ATCC 51694, was generously provided by David Welch, Oklahoma City, OK, USA (Welch *et al.*, 1992). Stocks were maintained in trypticase soy broth with glycerol at -70°C and cultured at 35°C on brucella agar containing 5% (v/v) defibrinated rabbit blood in a 5% (v/v) CO_2 environment. *Escherichia coli* DH5 α [*supE44* Δ *lacU169* (ϕ 80 *lacZ* Δ M15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*] was used as the host strain for transformations of recombinant DNA. *E. coli* strain JM109 [*recA1* *supE44* *endA1* *hsdR17* *gyrA96* *relA1* *thiA*(*lac-proAB*) *F'*(*traD36* *proAB*⁺ *lacI*^q *lacZ* Δ M15)] was used as the host strain for the pRSET expression vector. *E. coli* cells were routinely grown in Luria-Bertani (LB) broth or LB agar, unless otherwise noted (Sambrook *et al.*, 1989).

Gel electrophoresis and immunoblotting. Samples for SDS-PAGE were solubilized in final sample buffer (FSB) composed of 62.5 mM Tris/HCl pH 6.8, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 2% (w/v) SDS. Samples for immunoblots to be probed with GroEL antisera were solubilized in FSB containing 8 M urea. Proteins were separated on a 10% (w/v) gel with a discontinuous buffer system (Laemmli, 1970) and stained with Coomassie brilliant blue or were transferred to nitrocellulose (Schleicher and Schuell) for immunoblotting. For antigenic detection on immunoblots, the nitrocellulose was blocked with 5% (w/v) nonfat dry milk in 0.1 M PBS (0.145 M sodium chloride, 0.10 M sodium phosphate) pH 7.4/0.1% (v/v) Tween 20 (PBS-T), incubated for 1 h with antiserum diluted 1:5000 (unless otherwise noted) in PBS-T

and probed with donkey anti-rabbit antiserum conjugated to horseradish peroxidase (Amersham). Immunoblots containing immunoprecipitated antigens were probed with protein A conjugated to horseradish peroxidase. Antigen-antibody binding was detected using the Enhanced Chemiluminescence system (ECL, Amersham). Blots were incubated in ECL reagents for 1 min and then exposed to XAR-5 film (Kodak).

Heat shock procedure and radioactive labelling of cell proteins. Bacteria used for heat shock experiments were harvested from solid media and incubated overnight at 35°C in 5% CO_2 in liquid medium (Wong *et al.*, 1995). In brief, the liquid medium was prepared with RPMI 1640 (Sigma) and 1% (v/v) each of L-glutamine (Sigma), HEPES buffer (Sigma), sodium pyruvate (Sigma) and nonessential amino acids (Sigma). Haemin (Sigma) was added to a final concentration of 15 mg l^{-1} . The pH was adjusted to 7.0 and the medium was filter-sterilized by filtration through a 0.2 μm filter. After overnight incubation, bacteria were enumerated by serial tenfold dilution and plating on solid media. Bacteria were concentrated by centrifugation at 10000 g for 10 min and suspended in labelling medium prepared as described above using RPMI 1640 without methionine. Sets of 1 ml samples of each organism in sterile Eppendorf tubes were incubated at 35°C , 37°C , 39°C or 42°C . Fifteen minutes after the temperature shift, $100\text{ }\mu\text{Ci}$ [^{35}S]methionine [$10\text{ }\mu\text{Ci ml}^{-1}$ (370 kBq ml^{-1} ; Amersham)] was added to the samples and incubation was continued at the same temperature for another 60 min. The samples were chilled by submersion in an ice water bath, and the cells were then pelleted by centrifugation and washed once in cold 0.1 M PBS pH 7.4.

Immunoprecipitation of radiolabelled proteins. Samples of radiolabelled *B. henselae* or *B. quintana* cells were resuspended in 1.25 ml 10 mM Tris/HCl pH 8.0, 10 mM EDTA, 1 mM PMSF. To this suspension was added 12.5 μl 10% (v/v) protein-grade Triton X-100 (Calbiochem), followed by gentle agitation for 30 min at 4°C . The insoluble material was removed by centrifugation at 16000 g for 10 min. To the supernatant was added 0.2 ml GroEL antiserum and 0.25 ml of a slurry of staphylococcal protein A-Sepharose CL-4B (Sepharose-SpA; Sigma). The suspension was gently agitated for 1 h. The Sepharose-SpA-antibody-antigen complexes were washed twice in 0.01% Triton X-100 in 10 mM Tris/HCl pH 8.0, and resuspended in FSB.

PCR amplification of *groEL* genes. PCR was used to amplify *groEL* genes. Synthetic oligonucleotides were prepared using an automated oligonucleotide synthesizer (380B, Applied Biosystems). The 5'-oligonucleotide contained the nucleotide sequence encoding the amino-terminus of *B. bacilliformis* GroEL (GenBank accession number Z15160), including a *Bam*HI restriction endonuclease site (underlined): 5'-AAA ATG GGA TCC AAA GAA GTN AAR TTY GGN-3'. The 3'-oligonucleotide contained the nucleotide sequence encoding the carboxyl-terminus of *B. bacilliformis* GroEL, including an *Eco*RI restriction endonuclease site (underlined): 5'-TTT ATG GAA TTC TTA RAA RTC CAT NCC NCC CAT-3'. The *groEL* gene was amplified in a Programmable Thermal Controller (PTC-100, M. J. Research) with amplification for 30 cycles under standard conditions, using the above synthetic oligonucleotides and *Thermus aquaticus* polymerase (Promega). Template DNA was prepared by incubating a suspension of bacteria at 100°C for 5 min, followed by centrifugation in a microfuge at top speed for 5 min.

DNA sequencing. Standard recombinant DNA procedures were performed as described by Sambrook *et al.* (1989).

Fig. 1. Fluorogram of [^{35}S]methionine-labelled proteins of *B. henselae* (a), and *B. quintana* (b) at various temperatures. To determine the identity of the 60 kDa heat shock protein, radiolabelled proteins of *B. henselae* (not shown) and *B. quintana* (c) were immunoprecipitated with GroEL antiserum. Lanes: 1, 35 °C; 2, 37 °C; 3, 39 °C; 4, 42 °C. Proteins synthesized at a higher level following temperature shift are indicated by arrows. Locations of molecular mass standards are indicated on the left in kDa.

Restriction endonuclease digests were performed as recommended by the suppliers (New England Biolabs and Promega). Based upon statistical considerations, direct sequencing of PCR-amplified DNA should not result in errors (Rao, 1994). Therefore, the amplified DNA was directly sequenced using as sequencing primers the two degenerate oligonucleotide PCR primers (described above) and the following three oligonucleotides: GroEL-1, 5'-ATGAACCCAATGGATCTC-3'; GroEL-2, 5'-TCGCTGGAAGATGTGGAAGGTG-3'; GroEL-3, 5'-TTCAAGGCATCATCAAC-3'. The amplified *groEL* genes were also sequenced after cloning into pBluescript KS(+) and transformation into *E. coli* DH5 α . DNA sequencing was performed at the UCLA Core DNA Sequencing Facility by the dideoxy chain-termination method with fluorescein-labelled dideoxy nucleotides (Applied Biosystems). DNA sequence information was analysed by the DNA Strider program (Marck, 1988).

Generation of GroEL antisera. The amplified *groEL* gene was ligated into pRSET (Invitrogen) after digestion with *Bam*HI and *Eco*RI and transformed into *E. coli* JM109. The resulting construct, pRSET-*groEL*, was transformed into *E. coli* JM109. Expression of the His6-GroEL fusion protein was achieved by IPTG (Sigma) induction followed by infection

with M13/T7 phage containing the T7 polymerase gene driven by the *E. coli lac* promoter. The His6-GroEL fusion protein was purified by affinity chromatography using Ni-NTA-Agarose (Qiagen) and analysed by SDS-PAGE. The His6-GroEL band containing 50 μg protein was cut out of the acrylamide gel, dessicated, ground to powder, mixed with Freund's complete adjuvant and inoculated subcutaneously and intramuscularly into a New Zealand White male rabbit. The secondary immunization was given 4 weeks after the primary immunization, using His6-GroEL protein prepared in the same way, mixed with incomplete Freund's adjuvant. The rabbit was bled 2 weeks after the secondary immunization.

RESULTS

Characterization of the heat shock response of *Bartonella* species

Under the conditions used in these experiments, active protein synthesis was found to be occurring in both *B. henselae* and *B. quintana* with incorporation of [^{35}S]methionine into a large number of different proteins.

Bh	ATGGCTGCTAAAGAAGTGAAGTTTGGCCGTGAAGCGCGTGAGCGTTTGTTCGCGGCGTTGATATCCTTGCTAACGCTGTTAAAGTAACACTCGGCCCTA	100
Bq	ATGGCTGCTAAAGAAGTCAAGTTTCGGCCGTGAAGCGCGTGAGCGTTTGTTCGCGGCGTTGATATCCTTGCTAACGCTGTTAAAGTAACGCTCGGCCCTA	
	----- * *	
Bh	AAGGGCGCAATGTGGTGATCGATAAATCATTTGGTGCGCCTCGCATTACAAAAGATGGTGTATCCGTTGCAAAAAGAAATCGAACTGGAAGATAAGTTTCGA	200
Bq	AAGGTCGCAATGTGTAAATTGATAAATCGTTTGGTGCGCCTCGCATCACAAAAGATGGTGTATCTGTTGCAAAAAGAAATCGAACTGGAAGATAAGTTTCGA	
	* * * *	
Bh	AAATATGGGTGCGCAATGTTCGCTGAAGTTGCTTCTAAACCAATGACATTGCTGGTGATGGAACAACAACGGCAACTGTTTTAGGACAGGCTATTGTGTC	300
Bq	AAATATGGGTGCGCAATGTTCGCGGAAGTTGCTTCTAAACCAATGACATTGCTGGGGATGGAACAACAACGCAACTGTTTTAGGACAGGCTATTGTGTA	
	* *	
Bh	CAAGAAGGTGTAAAGCCGTGCTGCAGGCATGAACCCAATGGATCTCAAACGTGGTATCGATGCTGCTGTTGATGAAGTGGTGCAAACTCTTTTCAAAA	400
Bq	CAAGAAGGGGTGAAAGCCGTGCTGCTGGCATGAACCCAATGGATCTCAAACGCGGGATTGATGCTGCTGTTGAAGAAGTGGTGGGTAATCTTTTCAAAA	
	* * *	
Bh	AAGCGAAAAAATCCAACTTCAGCAGAAATTGCACAAGTGGGAACAATTTCCGCTAATGGTGTGCTGAAATCGGCAAAATGATCGCTGATGCCATGGA	500
Bq	AAGCGAAAAAATCCAGACTTCAGCAGAAATTGCACAAGTGGGAACAATTTCCGCCAATGGTGTGCAAAATCGGTAAATGATCGCTGATGCTATGGA	
	* *	
Bh	AAAAGTTGGCAATGAAGGTGTCATTACCGTTGAAGAAGCAAAAACCTGCTGAAACGGAATTAGAAGTCGTTGAAGGAATGCAGTTTGATCGTGGATATCTT	600
Bq	AAAAGTTGGCAATGAAGGCATTATCACCGTGGAAGCAAGCAAAAACCTGCTGAAACGGAATTGGAAGTCGTTGAAGGGATGCAATTCGACCGTGGATATCTT	
	* * *	
Bh	TCCCCCTACTTTGTACCAATGCTGAGAAAAATGGTGGCTGATCTTGATGATCCTTACATTCTTATTCACGAAAAAGAACTCTCTAATCTCCAATCCTTGC	700
Bq	TCCCCCTATTTTCGTACCAATGCTGATAAAATGGTGGCTGATCTCGACCATCTTACATCCTCATTCACGAAAAAGAAATTTGTCTAATTTGCAATCCTTGC	
	* * *	
Bh	TTCCAGTACTTGAAGCTGTTGTTGCTGCTGAAAGCTCCAGGATTTGGTGATCGCCGTAAAGCCATGTTAGAAGATATTGCAATCTTAACATCCGGACAGGTT	800
Bq	TTCCAGTCTCGAAGCTGTTGTTGCTGCTGAAAGCTCCAGGATTTGGTGATCGCCGTAAAGCCATGTTGGAAGATATTGCAATCTTGAATCTTAACATCCGGT	
	* * *	
Bh	GCGTGGTGGTTTGAAGATGCTGCTGTAAGCTCCAGGATTTGGTGATCGCCGTAAAGCCATGTTAGAAGATATTGCAATCTTAACATCCGGACAGGTT	900
Bq	GCGGTTGGTTTGAAGATGCTGCTGTAAGCTCCAGGATTTGGTGATCGCCGTAAAGCCATGTTGGAAGATATTGCAATCTTGAATCTTAACATCCGGT	
	* *	
Bh	ATTTCTGAAGATGTTGGCATTAATTTGGAAGATGTCACCTTTGGATATGCTTGGTCGTGCAAGAAAGTCAATATTTCTAAAGAAATACACGATTATTG	1000
Bq	ATTTCTGAAGATGTTGGCATTAATTTGGAAGATGTTACTTTGGATATGCTTGGTCGTGCAAGAAAGTCAATATTTCTAAAGAAATACACGATTATTG	
	* *	
Bh	ATGGTGTGGACAAAAGAGCGAAATTAATGCGCGCGTCAACCAATCAAGGTACAGATCGAAGAAACAACCTCTGACTATGACCGTGAAAAATTGCAAGA	1100
Bq	ATGGTGTGGGAAAAAGCTGAAATTAATGCACGCGTCAACCAATCAAGGTACAGATCGAAGAAACAACCTCTGACTATGACCGTGAAAAATTGCAAGA	
	* * *	
Bh	AAGACTTGCTAAACTCGCTGGAGGTGTTGCTGTTATCCGTGTTGGTGGAGCAACAGAGTTGAAGTGAAAGAAAAGAAAGATCGTGTGATGATGCCTTG	1200
Bq	AAGACTTGCTAAACTCGCTGGTGTGTTGCTGTTATCCGTGTTGGTGGAGCAACAGAGTTGAAGTGAAAGAAAAGAAAGATCGGTTGATGATGCCTTG	
	* *	
Bh	AACGCAACACGTGCAGCTGTGGAAGAAGGTATTGTTGCCGGTGGTGAACCGCATTTGTTGCGTGCAAGAAATGCCCTGACTGTTAAAGGAAGCAATCCTG	1300
Bq	AATGCAACACGTGCAGCTGTGGAAGAAGGTATTGTTGCCGGTGGTGAACCGCATTTGTTGCGTGCAAGAAATGCCCTGACTGTTAAAGGAAGCAATCCTG	
	* *	
Bh	ATCAGGAAGCTGGTATCAATATTGTTGCTGCTGCACTCCAAGCGCCAGCACGCCAAATTTGCTACCAATGCCGGTGAAGAAGCAGCGATTATCGTTGGCAA	1400
Bq	ATCAAGAAGCTGGTATCAATATTGTTGCTGCTGCACTACAGGCACAGCACGCCAAATTTGCAACTAATGCAGGTGAAGAAGCAGCGATTATCGTTGGCAA	
	* *	
Bh	AGTGCTTGAAAAACAATGCAGATACATTTGGTTACAACACCGCAACCGGTGAATTTGGTGATTGATTGCTTTGGGAATCGTTGATCCTGTGAAGGTTGTG	1500
Bq	AGTGCTTGAAAAACAATGCAGATACATTTGGTTACAATACCGCAACTGGTCAATTTGGTGATTGATTGCTTTAGGAATCGTTGATCCTGTGAAGGTTGTG	
	* *	
Bh	CGCTCTGCTCTCAGAATGCTGCCTCAATTGCTAGCCTTCTCATTACAACAGAAAGCAATGGTTGCTGAAAGTTCCAAAGAAAGACACACAGTGCCTCCAA	1600
Bq	CGTTCTGCTCTCAGAATGCTGCATCGATTGCCAGCCTTCTCATACAACAGAAAGCAATGGTTGCTGAAAGTTCCAAAGAAAGACACTCCAATGCCTCCAA	
	* *	
Bh	TGCCTGGTGGCGGAATGGGTGGAATGGGCGGCATGGACTTCTAA	1644
Bq	TGCCTGGTGGCGGAATGGGTGGAATGGGCGGAATGGACTTCTAA	
	* *	

Fig. 2. Nucleotide sequences of the *groEL* genes of *B. henselae* (Bh) and *B. quintana* (Bq). Underlined sections of the sequences indicate locations of the degenerate oligonucleotide primers used to amplify the *groEL* genes. Asterisks indicate locations of silent nucleotide differences. Triangles indicate location of nucleotide differences that result in amino acid substitutions. Dashes indicate location of sequence data predicted from *groEL* genes of related organisms.

Fig. 3. Phylogenetic tree based on nucleotide sequence differences of the *groEL* sequences of *B. henselae* (this work), *B. quintana* (this work), *B. bacilliformis* (GenBank accession number Z15160), *Brucella abortus* (Gor & Mayfield, 1992; GenBank accession number M83930) and *Agrobacterium tumefaciens* (Segal & Ron, 1993; GenBank accession number X68263). The tree places *B. quintana* and *B. henselae* in a monophyletic group with *B. bacilliformis*. Sequences were analysed using the PAUP software package (version 3.1; D. Swafford, Smithsonian Institution, Washington, DC, USA). Horizontal lengths are proportional to nucleotide step differences (indicated above lines).

Both organisms exhibited a heat shock response, with a subset of proteins that were synthesized at a distinctly higher level at temperatures above 37 °C. In *B. henselae* there was an increase in synthesis of proteins with apparent molecular masses of 70, 60, 17 and 10 kDa (Fig. 1a). While there were numerous differences between the protein synthesis profiles of the two organisms, the heat shock response of *B. quintana* was very similar to that of *B. henselae*, except that instead of a 17 kDa protein, a 20 kDa major heat shock protein was synthesized (Fig. 1b). The molecular masses of all four major heat shock proteins of *B. quintana* are similar to those of *Brucella abortus* (Lin *et al.*, 1992). We confirmed the identity of the 60 kDa proteins of *B. henselae* (data not shown) and *B. quintana* (Fig. 1c) by selective recovery of the 60 kDa proteins by immunoprecipitation with our

rabbit polyclonal GroEL antisera. Based on their apparent molecular masses, it is possible to tentatively identify two of the other *Bartonella* species heat shock proteins as DnaK (70 kDa) and Hsp10 (i.e. GroES) (10 kDa).

Sequence analysis of *groEL* genes from *Bartonella* species

Use of either *B. henselae* or *B. quintana* DNA in PCR with the *groEL* primers resulted in a single amplicon. Comparison of the two amplified *groEL* DNA sequences revealed 93.7% DNA sequence identity (103/1644 nucleotide differences). As shown in Fig. 2, three of these nucleotide differences were found at degenerate positions of the oligonucleotide PCR primers. Most of the DNA sequence differences were silent: as shown in Fig. 2, only 10 out of 107 nucleotide differences would result in amino acid substitutions. Several regions of the *groEL* sequence had a high concentration of nucleotide differences allowing identification of variable regions potentially useful in *Bartonella* species identification. The *groEL* sequences of *B. henselae* and *B. quintana* were aligned with those of several related bacteria to perform phylogenetic analysis. The resulting phylogenetic tree shown in Fig. 3 was very similar to that based on rRNA sequence alignment (Brenner *et al.*, 1993).

The deduced amino acid sequences of the GroEL proteins of *B. henselae* and *B. quintana* were aligned with those of the GroEL proteins of several related bacteria (Fig. 4). There was a high degree of GroEL sequence conservation within the *Bartonella* genus. The sequence of *B. quintana* GroEL was more similar to that of *B. henselae* than to the only other *Bartonella* GroEL sequence known, that of *B. bacilliformis*. *B. quintana* GroEL exhibited 98.4% sequence identity with *B. henselae* GroEL and 90.9% sequence identity with *B. bacilliformis* GroEL. There was also considerable similarity with GroEL sequences of other α -purple bacteria, such as *Brucella abortus* (86.3% identical) and *Agrobacterium tumefaciens* (81.4%). This pattern of GroEL amino acid sequence relatedness is reflected in the phylogenetic tree constructed using *groEL* sequences (Fig. 3).

The *groEL* sequences reported here are two of the first transcribed genes to be sequenced in these two *Bartonella* species; others include the *B. henselae* genes encoding citrate synthase (Norman *et al.*, 1995) and the 17 kDa antigen (Anderson *et al.*, 1995). Since GroEL is highly expressed, it is reasonable to assume that the *groEL* genes are representative of the preferred codon usage for optimal translation in these bacteria. The G + C content of the *groEL* genes of *B. henselae* and *B. quintana* was found to be 44.3 mol% and 44.5 mol%, respectively, reflecting the low G + C content of the genomes of these bacteria (41.0 mol% and 40.3 mol%, respectively) (Daly *et al.*, 1993). As expected, *B. henselae*

Bq	MAAKEVKFGREARERLLRGVDILANAVKVTLGPKGRNVVIDKSFGAPRITKDGVSVAKEIELEDKFENMG	70
Bh	.....D.....D.....N.....	
Bb	.....D.....T.....KM.....D.....A-.....E.....V.....	
Ba	.....P.....S.....KM.K.....D.....P.....	
At	.....P.....S.....KM.K.....D.....P.....	
Bq	AHMLREVASKTNDIAGDGTATVLGHAIVHEGVKAVAAGMNPMDLKRGIIDAAVEEVVGNLFKKAKKIHT	140
Bh	.....D.....A.....	
Bb	.....S.....A.....AD.....	
Ba	.....T.....A.....L.....N.....AE.L.....N.....	
At	..LV.....A.....R.....A.....L.....A.....KD.HAD.DD.N.....	
Bq	SAEIAHVGTTISANGAAEIGKMIADAMEKVGNEGVIITVEEAKTAETELEVVEGMHFDRLGYLSPYFVTNADK	210
Bh	.....E.....	
Bb	.E.....A.....ED.....SE.....	
Ba	.E.V.....-KH.....E.H.....PE.....	
At	.E.V.....ERH..LD..E..HR.....PE.....	
Bq	MVADLDDPYILIQEKKLSNLHSLLPVLEAVVHSGKPLLIIAEDVEGEALATLVVNKLRGGLKIAAVKAPG	280
Bh	.....	
Bb	.MV.....A.....	
Ba	.....E.A..L.....A.....TS.....R.....R.....	
At	.....E.A..L.....AM.....T.....V.....R.....	
Bq	FGDRRKAMLEDIAILTSGHVISEDVGIKLENTLDMLGRAKKVNISKENTTIIDGAGKKAIEINARVNHK	350
Bh	.....H.S.....	
Bb	.....V.....E.....QV..T..V..H.S.....S.....	
Ba	.....R.....G.....L.....S.....S.....V..H..D..G...	
At	.....G.T.....L.....S.....KS..S.....V..H.SD.EG..A...	
Bq	VHIEETTSYDREKLHERLAKLAGGVAVIRVGGATEVEVKEKKDRVDDALNATRAAVEEGIVAGGGTALL	420
Bh	.....	
Bb	A.....S.....P.....P.....	
Ba	H.....	
At	A.....S.....I.....H.....P.....V.....	
Bq	RAANALAIKGSNPDHEAGINIVRRALHAPARHIATNAGEEAAIIVGKVLNENADTFGYNTATGHFGDLIA	490
Bh	.....TV.....E.....	
Bb	..K..S..K.....G.I.....Q.....V.....CS.....A..R...S	
Ba	..STKITA..V.A.....I.....T.....SV...I..TSE.....N.EY...S	
At	..SSTKITV..V.D.....K...LSV...E...D..S.V...I.DK.E.NY...AH..EY.....	
Bq	LGIVDPVKVRSALHNAASIASLLITTEAMVAEVPKKDTPMPPMPGGGMGGMGMD	548
Bh	.....V.....	
Bb	F.....EAAA.A.....---	
Ba	.....T.....V.G.....I..L...AAPAG.....-	
At	L.....T.....V..V.....I..L...ESA..H.....---	

Fig. 4. Alignment of the predicted GroEL amino acid sequences using the CLUSTAL program (Higgins & Sharp, 1988). Sequences aligned were those of *B. quintana* (Bq), *B. henselae* (Bh), *B. bacilliformis* (Bb), *Brucella abortus* (Ba) and *Agrobacterium tumefaciens* (At). GenBank accession numbers and references for the sequences are given in the legend to Fig. 3. Dots indicate residues identical to those present in the top row. Dashes indicate gaps introduced to maximize alignment.

and *B. quintana* have a preference for codons with an A or T in the wobble position. However, as shown in Fig. 5, a number of exceptions were found where codons were used either more or less frequently than would have been expected, presumably reflecting the tRNA populations in these species. Knowledge of the preferred

codon usage is useful for a variety of practical molecular biology applications.

Further analysis of the GroEL amino acid sequences of *B. henselae* and *B. quintana* was performed, comparing their intracellular signature sequences with those of *E.*

Amino acid	Codon	Bq	Bh	Amino acid	Codon	Bq	Bh
Phe	TTT	5	7	Tyr	TAT	3	2
Phe	<u>TTC</u>	5	3	Tyr	<u>TAC</u>	2	3
Leu	TTA	3	4	Och	TAA	1	1
Leu	TTG	18	14	Amb	TAG	0	0
Leu	CTT	11	13	His	CAT	0	0
Leu	CTC	9	9	His	<u>CAC</u>	1	1
Leu	CTA	1	0	Gln	CAA	10	10
Leu	<u>CTG</u>	1	3	Gln	<u>CAG</u>	7	7
Ile	ATT	23	24	Asn	AAT	20	18
Ile	<u>ATC</u>	17	15	Asn	<u>AAC</u>	5	7
Ile	ATA	0	0	Lys	<u>AAA</u>	33	33
Met	ATG	17	16	Lys	AAG	10	9
Val	<u>GTT</u>	30	28	Asp	GAT	24	26
Val	GTC	3	8	Asp	GAC	7	5
Val	<u>GTA</u>	6	5	Glu	<u>GAA</u>	43	45
Val	GTG	15	15	Glu	GAG	3	2
Ser	<u>TCT</u>	8	7	Cys	TGT	0	0
Ser	<u>TCC</u>	3	5	Cys	TGC	0	0
Ser	TCA	1	3	Opa	TGA	0	0
Ser	TCG	3	-	Trp	TGG	0	0
Pro	CCT	9	8	Arg	CGT	13	16
Pro	CCC	-	-	Arg	<u>CGC</u>	9	7
Pro	CCA	7	8	Arg	CGA	0	0
Pro	<u>CCG</u>	-	-	Arg	CGG	1	0
Thr	<u>ACT</u>	10	6	Ser	AGT	0	0
Thr	<u>ACC</u>	4	8	Ser	<u>AGC</u>	2	3
Thr	ACA	11	13	Arg	AGA	1	1
Thr	ACG	5	4	Arg	AGG	0	0
Ala	<u>GCT</u>	35	35	Gly	<u>GGT</u>	28	27
Ala	GCC	7	8	Gly	<u>GGC</u>	11	11
Ala	<u>GCA</u>	23	22	Gly	GGA	11	15
Ala	GCG	8	7	Gly	GGG	5	1

Fig. 5. Codon usage in the *groEL* genes of *B. quintana* (Bq) and *B. henselae* (Bh). Underlined and emboldened letters are the preferred codons in *E. coli* (Grosjean & Fiers, 1982; de Boer & Kastelein, 1986). The arrows indicate codons that are used more (↑) or less (↓) frequently than anticipated given the low G+C content of the genomes (41.0 mol% and 40.3 mol%, respectively) and *groEL* genes (44.3 mol% and 44.5 mol%, respectively) of *B. henselae* and *B. quintana* (Daly et al., 1993).

	145	165	380	393	495	511					
Ec	AQVG	TISATS	DETVG	KLIAEA	KVGA	AATEVEM	KEKK	DPTKV	TRSAL	QYAAS	VA
Bq	.....	NGAAEI..	M..D.	R..G.....	V....	..V..V.....	N...I.				
Bh	.....	NGAAEI..	M..D.	R..G.....	V....	..V..V.....	N...I.				
Bb	...A....	NGA.DI..	M..D.	R..GS....	V....	..V..V.....	N...I.				
Ba	.....	N-AKQI..	M....	R..G.....	V....	..V..V.T...	N...I.				
At	.....	NGERQI..	LD....	R..GS....	V....	..V..V.T...	N...I.				
Hm	...A....	NG.KEI.NI.	SD.	..GTSD..	VN...	....V.T...	LD..G..				
		**		*	*	*					

Fig. 6. Partial alignment of Hsp60 sequences from prokaryotic and eukaryotic species depicting signature sequences described by Gupta (1995). Asterisks identify sequence features that are uniquely shared between α -purple bacteria and mitochondria. Dots indicate residues identical to those present in the top row. The dash at position 154 in the alignment identifies a gap introduced to maximize alignment. Ec, *E. coli*; Bq, *B. quintana*; Bh, *B. henselae*; Bb, *B. bacilliformis*; Ba, *Brucella abortus*; At, *Agrobacterium tumefaciens*; Hm, human mitochondria.

coli, other α -purple bacteria and human mitochondria (Fig. 6). These signature sequences are found exclusively in mitochondrial GroEL homologues and GroEL proteins of α -purple bacteria (Gupta, 1995). Unlike *E. coli* GroEL, the GroEL sequences of *B. quintana* and *B. henselae* contain these intracellular signature sequences.

DISCUSSION

This study presents the first characterization of the heat shock response that we are aware of in *Bartonella* species. The finding that *B. henselae* and *B. quintana* exhibit a heat shock response was expected and is

consistent with their respective life cycles, which involve both mammalian and ectoparasitic hosts. A significant heat shock response was not observed until the incubation temperature was raised above 37 °C, the normal mammalian core body temperature. A similar finding has been observed in other bacteria, including *Brucella abortus* (Lin *et al.*, 1992) and *Borrelia burgdorferi* (Stamm *et al.*, 1991), suggesting that factors in addition to temperature may be involved in regulating heat shock protein expression in *Bartonella* species.

The heat shock response is highly conserved in prokaryotes and is thought to represent adaptation to the stressful environment of the mammalian host. The heat shock response of *B. henselae* and *B. quintana* involved expression upregulation of GroEL, a 10 kDa protein (presumed to be GroES), and a 70 kDa protein (presumed to be the molecular chaperonin DnaK). Both *Bartonella* species also upregulated expression of a fourth major heat shock protein (Mhsp4), with an apparent molecular mass in the range 17–20 kDa, a pattern similar to that seen in *Brucella abortus* (Lin *et al.*, 1992). The discrepancy in molecular masses of Mhsp4 suggests that elucidation of its identity and function may be important in understanding differences in the pathobiology of these two *Bartonella* species. In this regard, it is interesting to note that immunoblots of *B. henselae* and *B. quintana* probed with *B. henselae*-specific antiserum detect a prominent 17 kDa antigen which is unique to *B. henselae* (data not shown). This immunoreactive 17 kDa antigen, described by Anderson *et al.* (1995), may be the same protein as the *B. henselae* Mhsp4 we describe in this study.

After identification of *B. henselae* DNA (Relman *et al.*, 1991) and the subsequent isolation of the organism from patients with bacillary angiomatosis (Regnery *et al.*, 1992; Welch *et al.*, 1992), *B. henselae* was initially assigned to the genus *Rochalimaea*. At that time, only two other members of the *Rochalimaea* genus had been described, *R. quintana* (the agent of trench fever) and *R. vinsonii* (Weiss & Moulder, 1984). Subsequent phylogenetic analysis based on rRNA sequences resulted in unification of the genera *Bartonella* and *Rochalimaea* (Brenner *et al.*, 1993). In this study we report the phylogenetic analysis of alignments of the *groEL* gene sequences of *B. quintana*, *B. henselae* and other related bacteria. We found that *B. quintana* and *B. henselae* form a monophyletic group with *B. bacilliformis*, confirming their taxonomic assignment to the genus *Bartonella*. These results suggest that *groEL* sequences would serve as an alternative to rRNA for determining the relatedness of the other *Bartonella* species. Another potential use of comparative analysis of *Bartonella* *groEL* sequences is to identify variable regions which would allow design of species- and/or genus-specific oligonucleotide probes. Oligonucleotide probes based upon *groEL* gene sequences have been found to be useful for species identification of staphylococci (Goh *et al.*, 1996).

Intracellularity is an essential aspect of the interaction of *Bartonella* species with their mammalian hosts

(Reynafarje & Ramos, 1961; Kordick & Breitschwerdt, 1995). *Bartonella* species have also been shown to invade a number of cultured human cell types (McGinnis Hill *et al.*, 1992; Batterman *et al.*, 1995). Recently, the *B. bacilliformis* genes involved in mediating erythrocyte invasion have been identified (Mitchell & Minnick, 1995). These phenotypic and molecular characterizations are consistent with the phylogenetic analysis of rRNA and *groEL* sequences which indicate that *Bartonella* species are members of the α -purple subdivision of Gram-negative bacteria, organisms found to live intracellularly within eukaryotic plant or animal cells. Analysis of bacterial *groEL* gene sequences has been of particular interest because, unlike rRNA genes, homologues are present in eukaryotic organelles such as mitochondria and chloroplasts. Global alignment of prokaryotic and eukaryotic GroEL sequences revealed that GroEL sequences of α -purple bacteria, including *B. bacilliformis*, are more closely related to those of mitochondrial homologues than they are to GroEL sequences of other bacteria, such as *E. coli* (Gupta, 1995). A number of GroEL signature sequences have been identified that are uniquely shared by members of α -purple bacteria and their mitochondrial homologues (Gupta, 1995). The present study demonstrates that the GroEL sequences of *B. quintana* and *B. henselae* also contain these intracellular signature sequences (Fig. 6). This evidence for the genetic relatedness of *B. quintana* and *B. henselae* to intracellular bacteria and eukaryotic organelles confirms the importance of intracellularity in the biology of these organisms.

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