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Cloning and characterization of a gene encoding a protein associated with the bacteriophage-like particle of *Bartonella bacilliformis*

by

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Presented in partial fulfillment of the requirements for the degree of

Master of Science in Microbiology

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November 2002

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Date: 11-1-02

Committee Chair
Dean, Graduate School
Cloning and Characterization of a Gene Encoding Proteins Associated with the Bacteriophage-like Particle of *Bartonella bacilliformis*

Chair: Michael F. Minnick, Ph.D.

*Baronella bacilliformis*, the etiologic agent of Oroya fever, has been shown to package 14-kbp DNA fragments of host chromosome into bacteriophage-like particles (BLPs). These BLPs were isolated and purified using chloroform, DNase I, and ultracentrifugation. Polyclonal antiserum was raised against BLPs in a New Zealand white rabbit, and seroreactivity was tested on a whole cell lysate of *B. bacilliformis* screened with the antiserum. A χZAP II genomic library of *B. bacilliformis* DNA was screened with collected antiserum, and multiple rounds of plaque purification were done to isolate recombinant clones. The DNA insert of interest was recovered from the library as a recombinant phagemid, termed pLC1, and used to infect the XLOLR strain of *E. coli*. The size of the insert in pLC1 was determined to be approx. 2,800 bp, and the sequence of the insert was determined at the Murdock Molecular Facility. Multiple BLAST searches of the sequenced insert revealed genes encoding homologues of three translational machinery proteins found in other bacteria, including elongation factor TS (*tsf*), ribosome recycling factor (*frr*), and uridylate kinase (*pyrH*). The *E. coli* XL-1 Blue containing the pLC1 was found to synthesize a protein of 39 kDa in size. While the insert did not contain nucleotide sequences encoding the 32, 34, or 36 kDa capsid proteins initially sought, this work nonetheless characterized three novel genes of the pathogen that are essential to translation.
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Chapter I

Introduction

*Bartonella bacilliformis* is a Gram negative, hemotrophic bacterium (1) that is roughly 0.25 to 0.50 μm in width and 1.0 to 3.0 μm in length. *B. bacilliformis* parasitizes human erythrocytes and endothelial cells (2, 3). Transmission of the pathogen to humans occurs through the bite of the phlebotomus sand fly (9), a vector present only in high altitudes of the Andes regions of Colombia, Peru, and Ecuador (1). *B. bacilliformis* is the etiologic agent of Oroya fever (Carrion’s disease) (4); a disease that exhibits two unique forms. The primary form is characterized by infection of nearly all circulating erythrocytes. Resulting symptoms include a severe hemolytic anemia, along with fever, malaise and skin pallor (2). Lysis of almost 80% of the circulating erythrocytes is not uncommon (5). Mortality rates associated with this phase are nearly 40% without the administration of proper antimicrobials (1, 6). Progression of the disease into the secondary (tissue) phase involves invasion of vascular endothelial cells and commonly manifests 1-2 months after the initial infection. The presence of cutaneous hemangiomas (termed verruga peruana by the natives) on the face and extremities is characteristic of the tissue phase, and is a direct result of the bacterium invading the vasculature (7). Hepatosplenomegaly, lymphadenopathy, and severe immunosuppression are additional complications frequently encountered (8). In addition, masses of bartonellae can fill the cytoplasm of host cells lining the blood vessels, leading to endothelial swelling and thrombosis (42). The verruga stage is rarely fatal and will resolve over time. Investigation into
the pathogenic mechanisms and virulence factors instrumental in *B. bacilliformis*

invasion of erythrocytes and endothelial cells is currently underway (2, 3, 33, 35, 36).

For over 80 years, *B. bacilliformis* was the only known member in the genus *Bartonella* (10). Recent advances in molecular biology have shown that the 16S rRNA sequences of *Rochalimaea* and *Grahamella* are homologous to *B. bacilliformis* (11, 12). Thus, a taxonomic reclassification was done to include these members. In addition, several novel species have since been added, the most recent being *B. clarridgeiae* (13).

Among the 14 species of *Bartonella* currently recognized, five are known to be pathogenic to humans, and are the causative agents of several emerging diseases (14, 37, 38, 39). Two examples, *B. henselae* and *B. bacilliformis*, are unique in that they produce bacteriophage-like particles (BLPs) (15). Previous electron microscopy studies revealed the presence of round to icosahedral extracellular particles of 80 nm in diameter associated with the outer surface of *B. bacilliformis* and *B. henselae* (15, 16). These particles package near-random, 14-kbp *Bartonella* genomic DNA fragments (15). The double-stranded extrachromosomal elements are well-protected from chloroform/DNase I treatment by the external capsid proteins (15, 18). Bacteriophage-like particles are similar but not identical to true bacteriophages in that they package host DNA into pre-formed capsid heads, but apparently contain no obvious genetic material from the phage (15).

BLPs are not unique to certain species of *Bartonella*. Several other unrelated bacteria have been found to possess them (19, 21). The PBSX phage of *Bacillus subtilis* (30), and the VSH-1 phage of *Serpulina hyodysenteriae* (31) are prime
examples, and the latter has been shown to undergo generalized transduction.

*Rhodobacter capsulatus*, a purple non-sulfur bacterium, utilizes a small bacteriophage-like particle (GTA) that packages random 4.5-kbp fragments of the host cell’s genome and transfers it to a recipient cell (40). With the presence of these BLPs across a broad range of different bacterial species, one could assume they share some common functions. Horizontal gene transfer of virulence determinants is one mechanism contributing to the development of emerging infections (14), and some BLPs are thought to aid in this process via transduction (18).

Bacteria can exchange DNA by utilizing three possible mechanisms including transduction, transformation, and conjugation (34). During bacterial transduction, bacterial DNA is abnormally encapsidated into a bacteriophage and transferred to a recipient cell. These phage particles are composed of a core of nucleic acid surrounded by a protein coat. A phage capable of infecting bacterial cells binds to a receptor site on the host cell. A phage enzyme then weakens the host cell wall, allowing the viral nucleic acid to be “injected” into the host. After penetration of viral DNA, one of two possibilities can occur, depending on whether the phage is temperate or lytic (41).

Lytic phages are capable of causing the destruction and death of a bacterial cell. Once viral nucleic acid has entered the host bacterium, the phage genes direct the host’s machinery to synthesize phage proteins and nucleic acids. Some of these proteins may degrade the host cell DNA, while others are structural components of future phages. Eventually, the intracellular viral titer reaches a critical point, and
specific enzymes are released from the phages, causing destruction of the bacterial cell in a process termed lysis. These new progeny phage can now infect other cells. The temperate phage utilizes a different mechanism. Instead of lysing the host cell, a temperate phage will undergo a stage analogous to transposition, whereby its nucleic acid integrates into the genome of the host bacterial cell. This unique characteristic of temperate phages allows it to reproduce along with the host DNA. This quiescent phage nucleic acid insert is referred to as a prophage. Temperate phages can replicate as a prophage in a bacterial chromosome. Temperate phages are able to undergo two forms of transduction: generalized and specialized.

Specialized transduction occurs when a certain gene or group of genes is transferred via the bacteriophage to another bacterium. Specifically, the prophage nucleic acid is incorrectly excised from the host chromosome, allowing a portion of the bacterial DNA to be carried along with it. Thus, subsequent packaging of this novel host DNA occurs, allowing some portion of the bacterial DNA to be included in the bacteriophage particle. Generalized transduction occurs when non-specific portions of the bacterial chromosome are incorporated into phage heads in place of phage DNA. Some phage enzymes are able to degrade host DNA into small fragments. Since headful packaging is usually a random event (although previous studies with *B. bacilliformis* have shown the specificity to be much less random than previously thought) (16), random pieces of host DNA are incorporated into the capsid head. In some cases, the phage DNA is replaced almost entirely by host DNA. These "defective" phages can now attach to another bacterial cell and inject the packaged host DNA into the new bacterial cell. Homologous recombination and
allelic exchange between the transduced DNA and host bacterial chromosome can now occur, leading to alteration of the bacterial chromosome of the recipient. Transfer of antibiotic resistance genes is one possibility that can have a dramatic effect on the viability of the recipient bacterium. To date, transfer of genetic material via other methods such as transformation and conjugation have not been demonstrated in the Bartonella genus (22). While it was hypothesized that B. bacilliformis BLPs participate in intraspecies horizontal gene transfer via transduction-mediated genetic exchange, previous research was unable to definitively demonstrate this activity (16). Research on the structure of the BLPs of B. bacilliformis has revealed the presence of three major proteins in the capsid coat with molecular weights of 32, 34, and 36 kDa (16). The nucleotide sequences for these genes has not been elucidated to date.

Due to its nature as an emerging pathogen with newly classified species and subspecies, the genus Bartonella is a constant threat to human and other mammalian hosts. While Oroya fever was once thought to be contained within a narrow location around 10,000 ft, recent evidence has shown an increase in cases affecting coastal populations at the base of the Andes (43). In addition, the frequency of international travel gives this isolated pathogen more opportunity to spread to non-endemic areas. Thus, the mechanisms of invasion and genetic transfer of this bacterium must be studied in detail.

While the initial purpose of this research was to identify the nucleic acid sequences encoding at least one of the 32, 34, or 36 kDa proteins of the BLP capsid, genomic library screening with anti-BLP antibodies resulted in recovery of a plasmid
containing DNA sequences coding for three translational machinery proteins of the host. These encoded proteins were identified via homology using a BLAST search, and include Uridylate kinase, Elongation factor TS, and Ribosome recycling factor.
Chapter II
Materials and Methods

A. Growth of bacterial strains

The primary *B. bacilliformis* strain used in this study was JB585, a flagellin\(^{-}\) (*fla*) mutant, kan\(^{+}\) (Battisti and Minnick, 1999) grown on heart infusion agar plates supplemented with 4% defibrinated sheep erythrocytes (v/v) and 2% filter-sterilized sheep serum (v/v) (Quad Five, Ryegate, MT). Cultures were incubated at 30°C for 4 to 5 days at 5% CO\(_2\). The major antibiotic used in this study was kanamycin (25 \(\mu\)g/ml). All *E. coli* strains required for propagation of cloned genes were grown overnight at 37°C in Luria-Bertani medium containing kanamycin (25 \(\mu\)g/ml) (Sigma Chemical Co., St. Louis, MO) when required (25).

B. Purification of BLPs

Forty plates of *B. bacilliformis* were grown as above, and harvested into 15 ml SM phage buffer (28). Chloroform treatment (3% vol/vol) was then employed to lyse the bacterial cells. This was followed by centrifugation at 6,000 x g for 5 min to remove cellular debris. Removal of any remaining viable bacteria was done by collecting supernatant, chloroform treating as before, and centrifuging a second time. DNase I was added to the resultant supernatant at a final concentration of 2 \(\mu\)g/ml, and incubated at 37°C for 2 h. After digestion of chromosomal DNA, BLPs were
pelleted via ultracentrifugation at 100,000 x g for 2 h using a SW60 rotor, model 1040 (Beckman, Palo Alto, California). The supernatant was decanted, and remaining pellets were resuspended in 150 μl of fresh SM buffer. BLP preparations were stored at 4°C until needed.

C. SDS-Page Analysis/Western Blotting

Proteins from the BLP preparations were visualized using SDS-polyacrylamide gels (12.5% acrylamide) by the methods from Laemmli (29). Gels were stained with Coomassie brilliant blue (0.05%) for visualization of protein bands. Immunoblotting was accomplished by first transferring the separated proteins from the acrylamide gel to a nitrocellulose membrane (26). Membranes were dried, then blocked in 0.3% Tween-20 and 2% non-fat skim milk in 1X phosphate-buffered saline (PBS) at pH 7.4 for 1 h at 25°C. Membranes were then rinsed in 1X PBS, and incubated overnight with polyclonal antiserum raised against the 32, 34, and 36 kDa BLP proteins. Incubation with a secondary antibody (horseradish peroxidase-goat anti-rabbit IgG) was then done for 1 h. Development was accomplished using 1X PBS, H₂O₂, 4-chloro-1-napthol, and methanol.

D. Inoculation of rabbit

The 32, 34, and 36-kDa capsid proteins in the BLP preparation were verified by SDS-PAGE analysis. Protein concentrations were measured using a bicinchoninic assay kit (Sigma Chemical, St. Louis, Mo.) and spectrophotometric analysis at 562 nm. A 4 ml pre-inoculation sample of rabbit blood was obtained prior to injection.
1) Harvest *B. bacilliformis* from HIBB into SM phage buffer (Approx. 0.5 ml buffer/plate).

2) Add CHCl₃ to a final concentration of 3% (v/v).

3) Centrifuge at 4,000 x g for 5 minutes to remove cellular debris.

4) Repeat step 3).

5) Incubate the resulting supernatant with DNase I (2 μg/ml) for 2 h at 37°C.

6) Centrifuge at 100,000 x g for 2 h at 37°C.

7) Discard the supernatant and resuspend the pellet in SM phage buffer.

8) Store at 4°C for up to 2 weeks.

Fig 1 Flowchart illustrating the protocol used for the purification of BLPs from *B. bacilliformis*. 
A vaccine consisting of 1.5 ml Complete Freund’s Adjuvant (CFA) (Sigma), 1.4 ml 1X phosphate-buffered saline (0.01 M phosphate, 0.15 M NaCl) (150 µl 10X PBS, 1.35 ml DIH2O), and 0.1 ml BLP (0.61 mg/ml) was injected into a rabbit at 3 subcutaneous depots above the shoulders. The rabbit was then boosted after one month with Incomplete Freund’s Adjuvant (IFA) (Sigma), 1X PBS, and BLP as above. Generated antiserum was then collected in 10cc volumes for 2 weeks after the booster.

E. Genomic Library Screening

A previously-constructed *B. bacilliformis* ZAP Express II genomic library was screened with the rabbit anti-BLP antiserum. Nitrocellulose circles were laid over infected lawns of *E. coli*, removed and incubated overnight with rabbit anti-BLP antiserum. Positive plaques were identified through successive rounds of plaque purification.

Library Titer:

The host strain XL-1 Blue MRF” was grown overnight from a frozen stock in 2 ml LB Mag/Mal (LB supplemented with 20 µl 20% maltose and 20 µl 1M magnesium sulfate) at 30°C with shaking at 200 RPM. Expansion was accomplished by adding 0.5 ml of the culture to 50 ml LB Mag/Mal. The culture was incubated for 2.5 h at 37°C with shaking at 200 RPM. The phage library was 10-fold serially diluted in SM buffer. Each dilution (1 µl) was then added to 100 µl of the host strain (XL-1 Blue). All samples were incubated in a 37°C water bath for 30 min. Overlays were then prepared by melting 3 ml of top agar in a tube and cooling to 55°C. The
phage/host mixture (100 µl) was added to the overlay, and poured over each LB plate. Plates were allowed to harden for 10 min., incubated at 37°C overnight, then counted the next day.
Table 2.1 Table showing all vaccine injections and serum extractions listed with dates and quantities.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Amount</th>
<th>Type</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-bleed</td>
<td>4 ml</td>
<td>Blood</td>
<td>5/17/00</td>
</tr>
<tr>
<td>1&lt;sup&gt;o&lt;/sup&gt; injection</td>
<td>3 ml</td>
<td>CFA/JB585</td>
<td>5/17/00</td>
</tr>
<tr>
<td>2&lt;sup&gt;o&lt;/sup&gt; injection</td>
<td>3 ml</td>
<td>IFA/JB585</td>
<td>6/14/00</td>
</tr>
<tr>
<td>1&lt;sup&gt;o&lt;/sup&gt; serum col</td>
<td>12 ml</td>
<td>Blood</td>
<td>7/03/00</td>
</tr>
<tr>
<td>2&lt;sup&gt;o&lt;/sup&gt; serum col</td>
<td>6 ml</td>
<td>Blood</td>
<td>7/11/00</td>
</tr>
<tr>
<td>3&lt;sup&gt;o&lt;/sup&gt; serum col</td>
<td>10 ml</td>
<td>Blood</td>
<td>7/14/00</td>
</tr>
<tr>
<td>4&lt;sup&gt;o&lt;/sup&gt; serum col</td>
<td>10 ml</td>
<td>Blood</td>
<td>8/09/00</td>
</tr>
<tr>
<td>Booster</td>
<td></td>
<td>IFA/JB585</td>
<td>9/14/00</td>
</tr>
<tr>
<td>(w/excised bands)</td>
<td>4 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;o&lt;/sup&gt; serum col</td>
<td>10 ml</td>
<td>Blood</td>
<td>9/30/00</td>
</tr>
<tr>
<td>2&lt;sup&gt;o&lt;/sup&gt; serum col</td>
<td>12 ml</td>
<td>Blood</td>
<td>10/05/00</td>
</tr>
<tr>
<td>3&lt;sup&gt;o&lt;/sup&gt; serum col</td>
<td>10 ml</td>
<td>Blood</td>
<td>10/12/00</td>
</tr>
<tr>
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<td>12 ml</td>
<td>Blood</td>
<td>10/24/00</td>
</tr>
</tbody>
</table>
Library screen

The phage library was diluted to 15,000 pfu per plate. The diluted phage (1 μl) was added to 200 μl of host XL-1 Blue and incubated at 37°C for 4 hr. Nitrocellulose disks were soaked briefly in 10 mM IPTG, and stored dry in empty petri dishes until needed. Disks were then carefully laid onto agar overlays and the plates incubated upside down at 37°C overnight. The next day, the plates were dried for 30 min. at 37°C with lids open. The plates were then cooled by inversion at 4°C for 30 min. Orientation of the filters was recorded by marking distinct spots through the circles into the agar using india ink and a probe. The nitrocellulose circles were carefully pulled from the agar using forceps and allowed to dry protein side up for 10 min. Probing with anti-BLP antisera was accomplished via the immunoblot protocol described above.

F. Excision

Positive plaques identified from the library were excised in vivo using E. coli XLOLR and XL-1 Blue MRF' strains, and rescued using the exassist helper phage per manufacturer’s instructions (Stratagene). The resulting recombinant phagemids were used to subsequently infect the XLOLR strain. Plaques that bound the primary antibody were cored out of the agar and transferred to a sterile microfuge tube containing 500 μl SM buffer and 20 μl chloroform. The microfuge tube was then vortexed in order to elute the phage particles. The tube was then incubated for 2 h at room temperature. Overnight cultures of XL-1 Blue in LB Mag/Mal and XLOLR in LB were prepared at 30°C. The following day, a 1:100 dilution of the overnight
culture was prepared by adding 0.5 ml of overnight culture to 50 ml LB. The culture was then grown for 3 h at 37°C to mid-log phase. This was repeated for the XLOLR strain. 200 μl of the XL-1 Blue culture was then added to a 15 ml conical tube, along with 100 μl of phage stock, and 1 μl of ExAssist helper phage. The contents of the tube were incubated in a 37°C hot water bath for 20 minutes. Three ml of LB media was added to the conical tube, followed by incubation for 2.5 hrs. at 37°C with shaking at 200 RPM. The tube was then heated at 70°C for 15 min. and centrifuged for an additional 15 min. at 4,000 x g (7,000 RPM). The supernatant was decanted into a sterile tube and stored at 4°C. Phagemids were then plated by adding 200 μl of XLOLR cells to two 1.5 ml tubes. 100 μl of phage stock was added to one tube, while 10 μl of stock was added to the second tube. These tubes were incubated at 37°C for 15 min. 300 μl of LB was added to each tube and incubation was completed at 37°C for 45 min. with no shaking. Finally, 100 μl from each tube was plated on LB/Kan plates (50 μg/ml), and incubated overnight at 37°C.

G. Manipulation of DNA

Recovery of pure plasmid DNA from *E. coli* was accomplished using a Perfect Prep kit (Eppendorf). Protocol was followed as per manufacturer’s instructions.
H. Ligation of restricted DNA

All ligations were accomplished by standard protocol. DNA (6 µl) at a concentration of 0.4 µg was added to a microfuge tube with 1 µl of 10X ligation salts (40 mM Tris-Cl, 10 mM MgCl₂). This mixture was heated at 65°C for 2 minutes. The mixture was then placed on ice, and 1 µl each of the following was added: 10X DTT (10 mM), 10X ATP (0.5 mM), and T4 DNA ligase (1 “weiss” U/µl). Following a quick “spin” at 14,000 RPM, the ligation was incubated at room temperature for 2 hrs.

I. Transformation

A culture of E. coli strain XL-1 Blue was grown overnight at 30°C with shaking (200 RPM). This culture was then expanded the following day by transferring 1 ml into an Erlenmeyer flask containing 20 ml LB. This mixture was incubated for 2 hrs. at 37°C with 200 RPM shaking. The culture was then centrifuged for 5 min. at 6,000 RPM. The supernatant was decanted, and the resulting pellet was resuspended in 2 ml of ice cold TSS (a chemical agent useful in opening channels in the bacterium). Competent cells were then separated into 100 µl aliquots in sterile Eppendorf tubes. 5 µl of DNA of interest (recovered insert) was added to one aliquot. These preparations were then incubated on ice for 45 min. Finally, 900 µl of TSS was added to each aliquot, and samples were incubated for 75 min. at 37°C with shaking at 250 RPM. In order to achieve equal concentration of the samples, the following procedure was used. Samples of 500 µl, 250 µl, and 100
μl were centrifuged in a tabletop centrifuge for 30 sec. at 14,000 RPM. The volume of the supernatant decanted was 400 μl, 150 μl, and 0 μl respectively. The resulting pellet was then resuspended in the remaining volume (100μl). This volume of bacterial culture was plated on LB/Kan25 plates, and incubated overnight at 37°C.

J. Nucleotide sequencing and analysis

Both strands of DNA from the cloned DNA insert were sequenced at the Murdock Molecular Biology Facility at the University of Montana. T3 and T7 sites on the pBK/CMV phagemid located outside the insert were used as initial primers for the sequence reactions. Blast 2.0 was used for database searches for homologies, and all sequence alignments were completed using FASTA (44), Clustal W 1.6 (45), and BOXSHADE 3.21 (K. Hofmann, and M.D. Baron, http://www.ch.embnet.org/software/BOX_form.html, 1998).

K. Induction of E. coli XL-1 Blue cultures

All E. coli XL-1 Blue strains were grown overnight at 37°C, with shaking (200 RPM) using a “seed” culture of 2 ml LB, and kanamycin to a concentration of 25 μg/ml. Cultures were then expanded on day 2 by adding 1.25 ml of seed to 8.75 ml of LB, and 21.9 μl of Kanamycin stock (10 mg/ml). This preparation was grown for 1 hr. at 37°C with shaking at 200 RPM. IPTG (10 mg/ml) was then added to cultures at a final concentration of 2 mM., and incubated for 2, 3, and 4 hrs each. Cultures were then stored in 1 volume of glycerol and frozen at –80°C until needed.
Chapter III

Results

A. Purification of BLPs in *B. bacilliformis*

Figure 2 (next page) shows purified BLPs alongside a molecular weight marker and a total cell lysate of *B. bacilliformis*. The presence of the 32, 34, and 36 kDa bands in both the first and second lane clearly show that these proteins are normally present in *B. bacilliformis*, and are major constituents of the BLP.

B. Generation of antiserum against *B. bacilliformis* BLPs

Following recovery of purified BLPs, the protein content was quantified via bicinchoninic acid protein kit (Sigma Chemical, St. Louis, Mo.) plus spectrophotometry at 562 nm. Data were graphically arranged using computer analysis (QuatroPro for Windows). The quantified proteins were then added to a vaccine preparation, as described previously, and injected into a New Zealand white rabbit. All antiserum recovered from the rabbit was tested for immunogenicity against the 32, 34, and 36-kDa proteins in the *B. bacilliformis* BLP. Figure 3 shows the corresponding immunoblot to Figure 2, and contains a molecular weight marker, total protein profile for JB585, and the BLP preparation. Incubation with recovered anti-BLP antibodies from serum as primary antibody, followed by subsequent development led to the observed pattern. The anti-BLP antibody probe recognized the three bands corresponding to the 32, 34, and 36 kDa proteins in the total protein profile and the BLP prep. This result indicates that the
Fig 2. Coomassie brilliant blue-stained SDS PAGE gel (12.5% acrylamide) showing *B. bacilliformis* JB585 whole-cell lysate and BLP associated proteins. Lane 1, *B. bacilliformis* JB585 whole-cell lysate showing a total protein profile; lane 2, protein profile of purified BLP proteins from *B. bacilliformis*, showing the three large bands at 32, 34, and 36 kDa (marked with >); lane 3, molecular mass standard in kilodaltons.
Fig 3. Corresponding Western blot to Figure 2. Blotted proteins were probed with anti-BLP antiserum. Lane 1, 32, 34, and 36-kDa BLP capsid proteins (marked with >) from B. bacilliformis JB585 whole-cell lysate; lane 2, protein profile of purified B. bacilliformis BLPs showing the 32, 34, and 36-kDa major bands; lane 3, molecular weight standards (rainbow) in kilodaltons.
recovered antiserum from the rabbit is highly specific and immunoreactive with respect to the three BLP proteins.

C. Cloning the genes located on pLC1

A λZAP II *B. bacilliformis* genomic library was screened using the anti-BLP antisera as the primary antibody probe, as described in materials and methods. Several positive plaques were isolated and the purified, and recombinant PBK/CMV plasmids were recovered from each *E. coli* clone. In addition, an SDS-PAGE gel (Fig 4) shows several proteins from pLC1 that are not found in the lane corresponding to *E. coli* containing the control plasmid (PBK/CMV with no insert). The corresponding immunoblot clearly shows the position of these immunogenic proteins as existing outside the 32-36 kDa range. Thus, these are not the 32, 34, or 36 kDa proteins initially pursued in this study.
Fig 4. Coomassie brilliant blue-stained SDS-polyacrylamide gel (12.5% acrylamide) of selected clones. Lane 1, molecular mass standards in kilodaltons; lane 2, *B. bacilliformis* whole-cell lysate showing total protein profile; lane 3, total protein profile of pBK/CMV phagemid with no insert; lane 4, protein profile of pLC1; lane 5, total protein profile of pLC4. Marked band (<) in lane 4 corresponds to 39 kDa protein encoded by recovered DNA insert.
D. Analysis of nucleotide sequences in the insert of pLC1

Figure 5 shows the map of the sequenced insert which contains 3 complete genes, 4 intergenic spacers (ITS), and part of a fourth gene. Orientation of the genes was surmised from homologues identified by BLAST. Thus, the 5' end contains roughly 168 bp corresponding to a gene (*rpsB*) with homology to the 30S ribosomal protein S2 of *Mesorhizobium loti*. The first uninterrupted gene lies 122 bp downstream from the S2 gene, and has 76% identity with the elongation factor-Ts gene from *Bartonella quintana* (*tsf*). This gene is by far the largest in the insert, measuring almost a full kilobase in size (942 bp). The *B. bacilliformis tsf* gene was found to have a 42.0 mol% G+C. The third gene is located 142 bp downstream of *tsf*, and is 720 bp long. This gene shares 64% identity with the uridylate kinase (*pyrH*) gene of *M. loti*, and has a 39.1 mol% G+C. The final gene in this locus is located 34 bp downstream from *pyrH*, and is 558 bp long with a 41.0 mol% G+C. This gene (*frr*) encodes the ribosome recycling factor and shares homology with a *B. melitensis* homologue (72% identity). Transmembrane computer predictions (TMPred program; ISREC server) identified two potential transmembrane helices in mature *pyrH*, found at amino acids 49 to 65 and 120 to 144. The immediate function of these helices is unknown, and the lack of a C-terminal phenylalanine would tend to discourage a role as an integral outer membrane protein (51).

Analysis for inverted repeat sequences in the four intergenic spacers was accomplished using PC/GENE software. Inverted repeats in these regions would be characteristic of potential Rho-independent transcriptional terminators. Non-
palindromic repeats were found in three of the four spacers, including ITS1, ITS2, and ITS4. The ITS3 spacer consisted of only 34 bp, and had 2 palindromic repeats. Analysis of the ITS regions for potential promoter sequences by neural network prediction yielded expected results. The 34 bp ITS3 contained no potential promoters. ITS2, which seemed the most likely candidate for containing a promoter region due to its relative position in the locus, had two hits. The second hit had a score of 0.99 by prokaryotic promoter neural network prediction due to the presence of a −TATAAT region. Finally, ITS1 had 3 hits. The importance of these findings will be discussed in the next chapter.

E. Generation of a deletion mutant in E. coli

Several attempts were made at generating a truncated form of the insert by deletional mutagenesis. HindIII was used to cut at the internal restriction site lying 800 bp inside (see fig. 5). The 750-bp internal HindIII fragment was excised from the gel, gene cleaned, religated, and transformed into DH5α cells. While no results were generated, this experiment would have allowed us to map which of the three complete genes on the insert encoded the gene product present as the 39-kDa band in the total protein profile of B. bacilliformis.

F. Nucleotide and predicted translation of sequence from insert

Figures 6, 8, and 10 contain the complete nucleotide sequence and translation of all three genes from the pLC1 clone. These figures also display the nucleotide sequence. In addition, the molecular weight of each protein encoded by these genes
was determined using Paul Stothard's home page
(http://www.ualberta.ca/~stothard/javascript/download.html).

Four-Gene Locus in *Bartonella bacilliformis*

![Diagram of the rpsB-tsf-pyrH-frr locus in pLC1](image)

Fig 5. (above) Map of the *rpsB-tsf-pyrH-frr* locus in pLC1 showing the relative positions of each gene, the intergenic spacers, and the *HindIII* site located in *tsf*. (Following page) Map of the pBK/CMV phagemid used in the excision of the insert.
Fig 6 Sequence of tsf and its encoded protein. Nucleotides within the tsf ORF are indicated with uppercase letters. The deduced amino acid sequence of tsf is shown below the first base of its respective codon triplet. The stop codon is indicated by an asterisk. Inverted repeats constituting a potential ρ-independent terminator are underlined. The downward arrow indicates the position of the HindIII site.
Fig 8 Sequence of \textit{pyrH} and its encoded protein. Nucleotides within the \textit{pyrH} ORF are indicated with uppercase letters. The deduced amino acid sequence of PyrH is shown below the first base of its respective codon triplet. Amino acids in boldface indicate those identified by TMPred to be part of a transmembrane helix.
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</tr>
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</table>

Fig 10 Sequence of frr and its encoded protein. Nucleotides within the frr ORF are indicated with uppercase letters. The deduced amino acid sequence of Frr is shown below the first base of its respective codon triplet.
The EF-Ts gene (tsf) was shown to encode a protein of molecular weight 32.4 kDa. The other two genes encoded protein products of smaller molecular weights. Uridylate kinase gene (pyrH) generated a protein product of 25.4 kDa, and the protein translated from the ribosome recycling factor gene (frr) was 20.8 kDa. The elongation factor-Ts is actually a subunit of the larger Elongation factor-T.

During the elongation phase of translation, the EF-T combines with GTP to form the EF-Tu-GTP complex. The EF-Ts subunit is subsequently released, leaving the EF-Tu-GTP complex to bind the aminoacyl-tRNA (47). Uridylate kinase functions primarily as a nucleotide kinase. This enzyme is required for the generation of UDP from UMP. The ribosome recycling factor is instrumental in the fourth step of protein biosynthesis. The primary step in this post-termination disassembly centers around the ribosome recycling factor. Simply, the ribosome recycling factor catalyzes the disassembly of the post-termination complex. This complex consists of the bound mRNA containing the termination codon, the deacylated tRNA bound to the P-site, and the ribosome itself. In sum, the recycling of ribosomes for another round of protein synthesis is accomplished (48). Figures 7, 9, and 11 are multiple sequence alignments generated using Boxshade and Clustal showing the closest homologues to these proteins in GenBank.
Fig 7. Multiple sequence alignment of *B. bacilliformis* Tsf (ef-Ts) with *B. quintana* Tsf and *M. loti* Tsf. Identical amino acid residues are noted in black, conserved residues in gray, and white indicates no homology. The GenBank accession numbers for the *B. quintana* and *M. loti* homologues are Q9XCMS and NP102414, respectively.
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</table>

Fig 9. Multiple sequence alignment of *B. bacilliformis* PyrH with *M. loti* PyrH and *C. crescentus* PyrH. Identical amino acid residues are noted in black, conserved residues in gray, and white indicates no homology. The GenBank accession numbers for the *M. loti* and *C. crescentus* homologues are NP102410 and AAK23896, respectively.
Fig 11. Multiple sequence alignment of *B. bacilliformis* Frr with *B. melitensis* Frr and *M. loti* Frr. Identical amino acid residues are noted in black, conserved residues in gray, and white indicates no homology. The GenBank accession numbers for the *B. melitensis* and *M. loti* homologues are ACCP94340 and NP102409, respectively.
Fig. 7 contains the amino acid sequence of the protein product from the tsf gene in B. bacilliformis, and its homologues from B. quintana and Mesorhizobium loti. B. quintana shares 76% amino acid identity with the TSF protein from B. bacilliformis, while there is 71% identity between M. loti and B. bacilliformis homologues. The amino acid sequence for PyrH and nearest homologues is given in Fig. 8. Considerable identity (75%) was found to exist between B. bacilliformis and the M. loti homologue. The Caulobacter crescentus homologue also shared identity with B. bacilliformis (54%). Figure 10 shows that the Frr protein in B. bacilliformis has significant homology with Brucella melitensis (72%), and M. loti (65%).

G. Induction/expression of proteins encoded on the pLC1 insert

An induction experiment involving E. coli strain XL-1 Blue harboring pLC1 (termed B111-10) was carried out as indicated in materials/methods. Cultures were grown for 2, 3, and 4 hrs. respectively, and protein profiles from B. bacilliformis and E. coli XL-1 Blue B111-10 at each time interval were separated on a 12.5% SDS-PAGE gel. In addition, a negative control consisting of E. coli containing the mother phagemid (pBK/CMV) without DNA insert was included for comparison. Figures 11 and 12 clearly show a 39-kDa band in lanes 4-6 that is strongly produced when the B111-10 strain is grown in the presence of IPTG. This protein band is not present in the mother phagemid, but is part of the total protein profile for B. bacilliformis (lanes 2). These results indicate that the 39-kDa protein is a product of one of the genes in the cloned insert of pLC1.
Figure 12 Coomassie brilliant blue-stained SDS-polyacrylamide gel (12.5% acrylamide). Lane 1, molecular weight standards in kilodaltons; lane 2, *B. bacilliformis* JB585 whole-cell lysate showing a total protein profile; lane 3, total protein profile of pBK/CMV phagemid; lanes 4-6, total protein profile of pLC1 after induction with IPTG 2, 3 and 4 hours, respectively. Marked band indicates an insert-specific protein at approx. 39 kDa.
Figure 13 Corresponding Western blot to Figure 12. Blotted proteins were probed with anti-BLP antiserum. The 39 kDa bands in lanes 4-6 are clearly recognized by the antibody, as are the 32, 34, and 36 kDa proteins in lane 2.
Chapter IV
Discussion

*Bartonella bacilliformis* is an invasive, Gram negative intracellular parasite of human erythrocytes and endothelial cells. While this bacterium has been traditionally limited to a narrow range of altitude in the Andes mountains, there are two dangers. First, at least twelve species of *Bartonella* are found worldwide. In addition, at least one case of Oroya fever has been recorded along the coastal regions of Peru, far removed from its endemic region (43). Of the twelve aforementioned species, five pose a significant health threat to humans and are the cause of a number of emerging diseases. Horizontal transfer of virulence genes via transduction-mediated exchange is one possible mechanism contributing to the rise in emergent infectious bartonellosis.

While the initial purpose of this study was to characterize genes encoding the 32, 34, or 36-kDa BLP capsid proteins, genomic screening instead recovered a 2.8-kbp DNA insert containing part of a locus of genes encoding translational machinery proteins of the host bacterium. This locus consisted of three complete genes, three complete intergenic spacers, one incomplete gene, and part of a fourth intergenic spacer region. Multiple BLAST searches of the sequenced DNA identified genes with homology to elongation factor Ts from *Bartonella quintana*, 30S ribosomal protein from *Mesorhizobium loti*, and both uridylylate kinase and ribosome recycling factor from *Brucella melitensis*. 

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Based upon the many attempts at screening the same genomic library (~ a
dozen times), and the discovery of a locus of genes rather than the BLP capsid genes,
it is safe to theorize that the particular 32, 34, and 36 kDa proteins in question
cannot be recovered in an *E. coli* XLOLR strain. One potential reason for this
problem could be that the recombinant capsid proteins are somehow toxic to *E. coli*,
thus preventing bacterial growth.

The BLPs from *B. bacilliformis* were initially purified with the intent of
producing polyclonal antiserum to be used for screening genomic libraries. The
purification scheme, discussed earlier, was accomplished using a kan\(^r\) strain, JB585.
This strain was chosen for two reasons. First, JB585 contains the neomycin
phosphotransferase gene. This is useful as it confers kanamycin resistance. In
addition, JB585 is also a flagellin (\(\text{fla}^+\)) strain, which prevents flagella contamination
during BLP preparation. Figure 2 clearly shows the presence of the 32, 34, and 36
kDa bands in lanes 2 and 3. These data suggest that these proteins are present in the
BLP and that the BLP exists in the *B. bacilliformis* lysate. The vaccine containing
purified BLPs was administered to the rabbit via the schedule shown in table 2.1. In
total, more than 25 ml of antiserum were recovered over a 6-month span. Figure 3 is
a Western blot showing the specificity of this antiserum and its reactivity to both a
purified preparation of BLPs, and a total cell lysate of *B. bacilliformis*. In addition
to being a cornerstone of this project, the generated antisera will prove to be an
important research tool in future library screenings.

Multiple attempts were made at screening the \(\lambda\)ZAPII genomic library with
the polyclonal antisera generated above. Recovered plaque DNA was screened by
initial restriction digestion with \textit{Hind}III, and those with inserts retained. One clone
contained two fragments of roughly 5,800 bp and 1,900 bp. Thus, we concluded
from these data that since the pBK/CMV phagemid has a size of 4,512 bp, the
recovered clone (termed pLC1) consisted of the mother plasmid and a 2,800-bp
insert. Adding the approximate sizes of the fragments yields a value of 7,700 bp.
While this doesn't exactly match the value of 4,512 bp, there is actually a third
fragment generated when pLC1 is cut with \textit{Hind}III. An analysis of the restriction
sites on the insert (http://www.ualberta.ca/~stothard/javascript/rest_trans_map.html)
shows that in addition to the \textit{Hind}III site at nucleotide 784 of the insert, there is a
second site at nucleotide 1,076. Thus, a 300-bp fragment is being generated that is
not within resolution of the 1% agarose gel.

An SDS-PAGE gel/immunoblot of pBK/CMV and pLC1 screened with anti-
BLP antiserum is shown in Figure 4. There is at least one band at approximately 39
kDa which is recognized by the antibody. This band is also present in the total
protein profile for \textit{B. bacilliformis}, but is difficult to see on the accompanying
immunoblot (not shown). In addition, the band is not in the pBK/CMV control lane,
suggesting that it is insert-specific.

The insert of pLC1 was sequenced at the Murdock Molecular Biology facility
at the University of Montana using the T3 and T7 promoters on the pBK/CMV
phagemid as initial primer with growing sequence used to walk the insert DNA.
Returned sequence was then converted to FASTA format, and BLAST searches
were accomplished using (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).
The EF-Ts gene (tsf) was shown to encode a protein of molecular weight 32.4 kDa. The other two genes encoded protein products of smaller molecular weights.

Uridylate kinase gene (pyrH) generated a protein product of 25.4 kDa, and the protein translated from the ribosome recycling factor gene (frr) was 20.8 kDa. The elongation factor-Ts is actually a subunit of the larger Elongation factor-T. During the elongation phase of translation, the EF-T combines with GTP to form the EF-Tu-GTP complex (see Fig. 13). The EF-Ts subunit is subsequently released, leaving the EF-Tu-GTP complex to bind the aminoacyl-tRNA (47).

![Figure 14 The interaction between EF-Ts and EF-Tu during translation.](image)

Uridylate kinase functions primarily as a nucleotide kinase. This enzyme is required for the generation of UDP from UMP. The ribosome recycling factor is instrumental in the fourth step of protein biosynthesis. The primary step in this post-termination disassembly centers around the ribosome recycling factor. Simply, the
riboseome recycling factor catalyzes the disassembly of the post-termination complex. This complex consists of the bound mRNA containing the termination codon, the deacylated tRNA bound to the P-site, and the ribosome itself. In sum, the recycling of ribosomes for another round of protein synthesis is accomplished (48).

The orientation of the genes found on the locus (see Fig 5) for *B. bacilliformis* appears to be unique. Four genes are present in the sequence, including *rpsB* (encoding the 30S protein S2), *tsf* (EF-Ts), *pyrH* (UK), and *frr* (RRF). Each of these genes has an intergenic spacer of variable length set between them. When compared to the same gene cluster in other bacteria, there are some interesting similarities. First, it has been shown that an evolutionarily conserved locus of genes exists in *Lactococcus lactis* (46), which consists of *pyrH* (UK) and *frr* (RRF). The promoter for this cluster lies upstream of an open reading frame (orfA) of unknown function. The two genes lie downstream of orfA, in the same orientation as they appear in *B. bacilliformis*. Another common gene cluster that has been identified in *E. coli* contains the *rpsB* gene and the *tsf* gene. A promoter that lies upstream of *rpsB* serves both of these genes. Again, the orientation of this cluster is identical to the four-gene cluster in *B. bacilliformis*. Since the two separate gene clusters just discussed are evolutionarily highly conserved, it is safe to theorize that there is one promoter for the *pyrH*/frf genes, and another for the *rpsB* and *tsf* genes. Another possibility is that only one promoter exists for all four genes. Analysis of the nucleotide base sequence of the insert in pLC1 was accomplished using PC/GENE software. The three intergenic spacer regions were entered, and the amount of non-palindromic repeats (nprs) was determined. Non-palindromic repeats are
characteristic of rho-independent termination sequences. These sequences contain multiple repeats that interact to form a secondary structure resembling a stem-loop. It is theorized that RNA polymerase reaches this stem-loop and is slowed to the point of cessation (47). ITS 3, which was only 34 bp long, had no nprs. Thus, the \( pyrH \) and \( frr \) genes may be transcribed from the same promoter.

The ITS 1 region was analyzed for both non-palindromic repeats and likelihood of promoter regions. PC/GENE software determined that ITS 1 had six nprs of sizes 6-8 bp. In addition, there were three possible promoter predictions with scores of 0.94 or above by neural network prediction. The ITS 2 region, which lies between \( tsf \) and \( pyrH \), seemed to be the most logical place for a promoter region. Both gene clusters in \( E. coli \) and \( L. lactis \) terminated at some point downstream from \( tsf \), or upstream of the \( pyrH/frr \) genes. The cluster in \( E. coli \) contained only the \( rpsB \) and \( tsf \) genes, while the \( L. lactis \) cluster had a separate promoter, controlling transcription of the \( pyrH \) and \( frr \) genes. Regardless, I theorized that there should be both a strong promoter for the \( pyrH \) and \( frr \) genes in ITS 2. PC/GENE results showed nine nprs in this region, characteristic of some strong termination. Second, there were two possible promoter sequences discerned. One had a relative score of 0.92, but the second region was much more impressive. This possible promoter had a score of 0.99, making ITS 2 the most likely place in all three intergenic regions where a promoter was to be found. This 0.99 sequence contained the standard TATA box about 75 bp upstream from the start of \( pyrH \). Thus, it is likely that there are two separate promoters in this four-gene cluster from \( B. bacilliformis \). An unidentified promoter controls transcription of the \( rpsB \) and \( tsf \) genes (as well as
other genes upstream), and a second promoter in the ITS 2 region is responsible for

\textit{pyrH} and \textit{frr}.

From the data presented, it is not possible to accept this explanation completely. Since we are dealing with computer-generated algorithms, and not experimental data, the strength of the argument is tepid at best.

Translation and alignment with nearest relatives constitutes the brunt of Figures 6-11. The \textit{frr} gene was shown to code for a protein of 239 amino acids and molecular weight of 25.4 kDa. This protein had greatest similarity (77.6%) and identity (72%) to \textit{B. melitensis}. The \textit{pyrH} gene encoded a protein of 186 amino acids and molecular weight of 20.8 kDa, with greatest similarity (76%) and identity (64%) with \textit{M. loti}. For the \textit{tsf} gene, a polypeptide containing 307 amino acids and having a molecular weight of 32.5 kDa was translated from the nucleotide sequence. The most closely related protein was the Tsf found in \textit{B. quintana} (84.6% similarity). Tsf in \textit{B. bacilliformis} had 260 matches out of a total 307 amino acids with \textit{B. quintana} (76% identity). This would most likely be the band that was visible at 39 kDa via SDS-PAGE analysis. As stated earlier, attempts at deleting part of the pLC1 insert, and re-introducing it into \textit{E. coli} via transformation ended in failure. Since this deletion would have inactivated \textit{tsf}, it would have proven beyond a doubt the relative position of the protein product of \textit{tsf} in the protein profile.

The intensity of the insert-specific protein bands in the induction experiment (Fig.12) was strong, demonstrating that the genes on the insert were both in the correct frame and direction relative to the \textit{lacZ} gene. This result is important since it
helps us to visualize which bands on the SDS-PAGE gel/immunoblot are representative of the insert-specific genes.

In conclusion, the initial intent of this research was to identify and characterize at least one of the genes encoding the 32, 34, and 36 kDa BLP proteins in \textit{B. bacilliformis}. While we were unable to correctly locate the appropriate insert from genomic library screenings corresponding to these proteins we did, however, identify a novel operon locus from \textit{B. bacilliformis}. The arrangement of the genes, \textit{rpsB-tsf-pyrH-frr}, is similar to that reported in \textit{E.coli} and \textit{P. aeruginosa} (50). This locus contained four genes essential for protein translation in the pathogen. Possible future work may involve a more careful study of the genes in the locus (i.e. repeat generation of the deletion mutants-also chromosomal walking upstream of the \textit{rpsB} gene) in order to better characterize the genes and their protein products. In addition, over 25 ml of polyclonal antiserum was generated against the purified BLPs. This useful tool can be utilized for future attempts at locating an insert from the genomic library that may contain the genes initially sought after.
Acknowledgements

I would like to thank the members of my thesis committee (Drs. Michael Minnick, D. Scott Samuels, and Keith Parker). Dr. Michael Minnick, my mentor, has taught me more about scientific research than he will ever know. His patience with my many mistakes and mishaps is greatly appreciated, and I am surely indebted to him. Thanks to Laura Smitherman, our talented Senior Research Assistant, for answering all my questions, no matter how mundane. I would also like to thank our other graduate student, Sherry Coleman, for all the time she took in sharing her vast knowledge with me. Finally, thanks to Patty McIntyre (Murdock Molecular Facility) for sequencing the pLC1 insert.

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References


