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Characterizing the molecular biology of a bacteriophage-like particle from Bartonella bacilliformis

Kent D. Barbian
The University of Montana
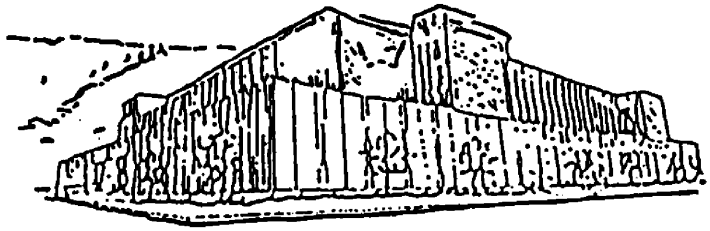
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Characterizing the Molecular Biology of

a Bacteriophage-Like Particle
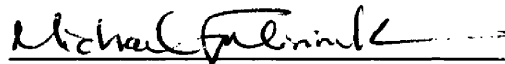
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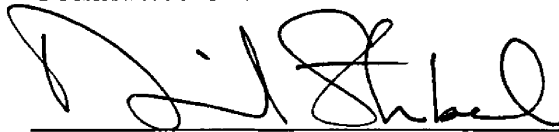
by

Kent D. Barbian

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Approved by:

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Committee Chair

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Characterizing the Molecular Biology of a Bacteriophage-Like Particle From
Bartonella bacilliformis

Director: Michael F. Minnick ᴍᏀᴛᴜ

B. bacilliformis and *B. henselae*, agents of Oroya fever and cat-scratch disease, respectively, are known to produce bacteriophage-like particles (BLPs) that package 14-kbp segments of the host chromosome. Data from this study suggest that a number of other *Bartonella* species including *B. quintana*, *B. doshiae*, and *B. grahamii* also contain similar BLPs for the 14-kbp extrachromosomal DNA fragment was present in their total genomic preparations. RFLP and southern blot analysis of the BLP DNA from *B. bacilliformis* suggest that packaging is much less random than observed in *B. henselae* phage. Data also suggest that these linear, double-stranded, BLP DNA molecules have non-covalently closed ends with 3′ overhangs. The 3′ overhangs are not complementary to one another, as ligation attempts were unsuccessful. Additionally, BLP DNA molecules appear to be packaged immediately upon synthesis as there is no unpackaged phage DNA present within the host cell. SDS-PAGE analysis of purified BLPs from *B. bacilliformis* showed three major proteins with apparent molecular masses of 32, 34, and 36 kDa. Intact BLPs were also observed by transmission electron microscopy and appeared to be round to icosahedral and approximately 80 nm in diameter.

To determine if BLPs contribute to horizontal gene transfer, mutants of *B. bacilliformis* were generated by allelic exchange with a suicide vector construct termed pKB1. pKB1 contains a pMB1 origin of replication, a kanamycin resistance cassette (*npt*I), and an internal fragment of the 16S-23SrDNA intergenic spacer region. Homologous recombination between pKB1 and one of the three rRNA operons present in the *B. bacilliformis* chromosome led to its disruption and produced a kanamycin-resistant phenotype. Southern blot analysis confirmed that allelic exchange had occurred. Furthermore, it was shown that BLPs from some of these strains were able to package the mutagenized region containing the kanamycin resistance cassette. However, numerous attempts at intraspecies transduction with BLPs from these strains were unsuccessful.

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Chapter I

Introduction

Bartonella bacilliformis is a small, motile, gram negative, hemotrophic bacterium (1) that is approximately 0.25 to 0.5 μm in width by 1.0 to 3.0 μm in length. Upon exposure, this intracellular pathogen is capable of invading and replicating inside human erythrocytes and endothelial cells (2,3). This parasitic pathogen, is transmitted to humans by the bite of a female sandfly of the *Phlebotomus* genus (4) and is endemic to the South American Andes regions of Peru, Ecuador, and Colombia (1). *B. bacilliformis* is the etiologic agent of Oroya fever, also termed Carrion's disease (5), and manifests itself with a biphasic disease process. The primary, or hematic, phase is characterized by fever, skin discoloration, and a severe hemolytic anemia (6) where nearly all of the circulating erythrocytes are infected (2) and close to 80% are lysed (7). Without antibiotic therapy, mortality rates can reach as high as 40% during this phase (1,8). Appearing one to two months following the hematic phase is the secondary, or tissue, phase in which the bacterium invades the vasculature. This phase, in which the bacterium invades human endothelial cells, is characterized by the appearance of small blood-filled warts, or hemangiomas, on the surface of the face and extremities and is termed verruga peruana (9). Often, the verruga stage presents with additional complications such as immunosuppression, hepatosplenomegaly, and lymphadenopathy (10). This stage of the *Bartonella* infection is rarely fatal and it usually resolves itself with time. The mechanisms by which *B. bacilliformis* gains entry into erythrocytes and endothelial cells, as well as additional virulence determinants, are currently being investigated (2,3,11,12,13).

Over the past decade, the *Bartonella* genus has undergone a dramatic re-organization with respect to its taxonomy. Since its initial classification by Barton in 1909 (14), *B. bacilliformis* was the only organism within this bacterial genus. However, bacteria once constituting the *Rochalimaea* and *Grahamella* genera were recently re-classified as *Bartonella* based upon 16S rRNA sequence similarities (15,16). Furthermore, there has been additional novel species added to the *Bartonella* genus with *B. clarridgeiae* being the most recent (17). The *Bartonella* genus has been growing at alarming rates over the last decade and is now composed of 13 species.

Currently, five *Bartonella* species pose a significant health threat to humans and cause a variety of emerging infectious diseases (18,19,20,21). Horizontal transfer of genes encoding virulence determinants and / or antibiotic-resistance markers may be contributing to this emergence. Horizontal gene transfer in bacteria is mediated via four mechanisms: 1) natural transformation, 2) conjugation, 3) transduction, or 4) transposition (22). However, bacterial conjugation between *Bartonella*, or transposition, has never been shown for any *Bartonella* species, and work by our lab suggests that *Bartonellae* cannot be naturally transformed (23).

Previous literature has shown that two *Bartonella* species, *B. bacilliformis* and *B. henselae*, agents of Oroya fever and cat-scratch disease, respectively, produce bacteriophage-like particles (BLPs) that package near-random, 14-kbp fragments of *Bartonella* DNA (24). The heterogeneous mixture of double-stranded host DNA contained in the *B. henselae* BLPs is protected from chloroform / DNase I treatments by capsid proteins (24,25). Electron microscopy has revealed round to icosahedral extracellular particles approximately 40 nm in diameter attached to the surface of *B.*

bacilliformis and *B. henselae* cells (24,26). BLPs are non-lytic even when *Bartonella* are subjected to induction agents such as UV irradiation and / or the addition of mitomycin C. Currently, little more is known of these BLPs or the mechanisms by which the DNA is packaged, although data available on generalized defective phages suggests that headful packaging into a pre-formed capsid head may be taking place (27,28).

These extracellular particles, or BLPs, are so termed because of their unusual characteristics when compared to true bacteriophages. A true bacteriophage typically exhibits one of two modes of replication, the first of which is the virulent or lytic cycle. During this cycle, a bacteriophage attaches and injects its nucleic acid into a host bacterium. This viral nucleic acid then requisitions the host cell's metabolic machinery to produce multiple progeny which are released upon cell lysis. These mature bacteriophage subsequently infect other host bacteria to continue the cycle. The second mode of replication, known as lysogeny, is characterized by the phage genome integrating with the host chromosome to produce a prophage. This prophage is replicated with the host chromosome and remains dormant until influenced by any number of DNA-damaging environmental factors. Under such environmental conditions, lysogens (a bacterium containing a prophage) can spontaneously produce mature phage that can re-establish the lytic cycle (22).

In bacterial transduction, DNA is transferred between cells via bacteriophages (27,28). This genetic transfer of host genes by phage can occur in one of two ways. In the first, called generalized transduction, random host DNA is incorporated into a few of the phage heads in place of the viral genome. In contrast, specialized transduction occurs when prophage DNA imperfectly separates from the host chromosome and some adjacent

bacterial genes are excised along with prophage DNA and are subsequently packaged into phage heads. In both cases, a few transducing virus particles are "defective" in that bacterial genes have replaced some, or all, necessary viral genes (22). These "defective" viral particles can attach to and inject their contents into another host bacterium, after which homologous recombination and allelic exchange with the transduced DNA and bacterial chromosome can occur, thus altering the recipient bacterium's genetic material. This alteration can have dramatic effects on both the evolution and survival of the newly transformed bacterium.

In summary, BLPs, or "defective" phages, are unique when compared to true bacteriophages. Although they have some properties which are similar to phage, most BLPs package host DNA into preformed capsid heads and do not contain any apparent genomic material from the phage. Similar BLPs have been found in a variety of unrelated bacterial species including PBSX phage from *Bacillus subtilis* (27,29) and VSH-1 phage from *Serpulina hyodysenteriae* (30), which has been shown to undergo generalized transduction. We hypothesize that *Bartonella* BLPs participate in intraspecies (and possibly interspecies) horizontal gene transfer via transduction-mediated genetic exchange.

Recent increases in the numbers of *Bartonella* species and human diseases associated with them is the basis for this research. Horizontal transfer of genetic material is one primary mechanism by which microorganisms evolve to survive in a variety of environmental habitats. The ability to acquire novel gene products by genetic transfer can confer tremendous selective advantages upon the recipient strain. These advantages, presented by phenotypic changes brought about by novel genes, can include any number

of alterations within the organism and may enable it to exploit new environments Given the widespread occurrence of defective phages in bacteria, a better understanding of their role in genetic exchange would greatly enhance our knowledge regarding the genetics and evolution of bacteria in general.

The purpose of this research was to provide a better understanding of the biology of the *B. bacilliformis* BLPs and their potential role in generalized transduction. BLP DNA was isolated and characterized along with BLP-associated proteins in order to gain a better understanding of BLP genetics and biology. Purified BLPs as well as BLPs associated with *B. bacilliformis* were observed by transmission electron microscopy. Finally, numerous transduction experiments were attempted to explore whether transduction-mediated genetic exchange plays a role in the virulence and emergence of *Bartonella* species and emerging diseases. This research has provided a better understanding of the molecular biology regarding the BLPs from *B. bacilliformis* and provides clues to investigate their potential role in mediating genetic exchange via generalized transduction. Although much additional work is needed to fully understand the mechanisms behind the BLPs, results from this study form a strong foundation on which to build.

Chapter II

Materials and Methods

2.1 Growth of bacterial strains

Bartonella species and stains, as well as *E. coli* strains, used in this report are

summarized in Table 2.1. The *B. bacilliformis* strains were grown on a standard medium,

termed HIBB, consisting of heart infusion agar plates (Difco, Detroit, MI) supplemented

with 4% defibrinated sheep erythrocytes (v / v) and 2% filter-sterilized sheep serum (v /

v) (Quad Five, Ryegate, MT) for 2-3 days at 30°C in a water-saturated atmosphere. Other

Bartonella species were similarly cultured, but at 37°C with 5% CO_2. *E. coli* strains used

for propagation of cloned genes were grown overnight at 37°C in Luria-Bertani medium

with standard antibiotic supplements when required (31). The antibiotic supplements

used in this study included 25 μg/ml kanamycin sulfate (Kan), and 2 μg / ml

chloramphenicol (Cam) (Sigma Chemical Co., St. Louis, Mo.) which were used

individually or combined depending upon experimental conditions.

2.2 Purification of BLPs

BLPs were prepared by inoculating either *B. bacilliformis* or *B. henselae* strains

onto *Bartonella* growth medium. After 4 days of incubation, 36 plates were harvested

into 15 ml of SM (32) phage buffer. Treatment with 3% (v / v) chloroform was used to

lyse the bacterial cells, whose cellular debris was subsequently removed by centrifugation

for 5 min at 6000 x g. The supernatant was collected, chloroform treated as before, and

centrifuged a second time to completely eliminate all viable bacteria. DNase I was added

to the remaining supernatant to a final concentration of 2 μg / ml, and incubated at 37°C

for 2 h to digest chromosomal DNA. The BLPs were then pelleted by ultracentrifugation

Table 2.1 Bacterial species and strains used in this study.

Species[*]/Strain	Relevant Characteristics	Reference
Bb/KC583	Neotype strain	Brenner et al., 1991 (33)
Bb/KC584	Peruvian isolate, 1963	Brenner et al., 1991 (33)
Bb/JB584	HG584 cured of pEST (Kan^S,Cam^S)	Battisti and Minnick, 1997 (13)
Bb/JB585	fla gene from JB584 interupted by insertion of pUB508 (Kan^R,Cam^S,fla⁻)	Battisti and Minnick, 1997 (13)
Bb/KB484	JB584 containing pBBRIMCS (Kan^S,Cam^R)	This study
Bb/KB584	One of three 16S-23S ITS regions of JB584 interrupted by insertion of pKB1 (Kan^R,Cam^S)	This study
Bb/KB585	One of three 16S-23S ITS regions of JB584 interrupted by insertion of pKB1 (Kan^R,Cam^S)	This study
Bb/KB686	Spontaneous chloramphenicol resistance of KB585 (Kan^R,Cam^R)	This study
Bc/	Isolate from cat with B. henselae septicemia	Lawson and Collins, 1996 (17)
Be/	Human endocarditis isolate	Daly et al., 1993 (34)
Bd/R18	Type strain	Birtles et al., 1995 (16)
Bg/V2	Type strain	Birtles et al., 1995 (16)
Bh/Houston R1302	Type strain	Regnery et al., 1992 (35)
Bq/Fuller	Type strain	Myers et al., 1979 (36)
Bv/Baker	Vole agent	Weiss and Dasch, 1982 (37)
Ec/DH5α	Used for cloning and propagation of relevant plasmids	Gibco - BRL (Gaithersburg, MD)

*Bb = B. bacilliformis; Bc = B. clarridgeiae; Be = B. elizabethae; Bd = B. doshiae;
Bg = B. grahamii; Bh = B. henselae; Bq = B. quintana; Bv = B. vinsonii; Ec = E. coli

for 2 h in a SW60 rotor, model # 1040 (Beckman, Palo Alto, California), at 100,000 x g

using a 15% sucrose cushion. The supernatant was discarded and the pellets were

resuspended in 150 μl of fresh SM buffer and stored at 4°C until needed.

2.3 Preparation and Manipulation of DNA

Nucleic acids for use in DNA hybridization or PCR were extracted from

suspensions of either bacteria or BLPs using a CTAB technique (cetyltrimethyl-

ammonium bromide) as described by Ausubel *et al.* (32). Plasmids were either extracted

by an alkaline lysis procedure outlined by Birnboim and Doly (38), or by a Qiagen Midi

Prep kit (Qiagen, Inc., Chatsworth, CA) as per the manufacturer's instructions.

Restriction digestion, Klenow and S1 nuclease treatment, ligation, denaturation /

renaturation, and transformation of DNA fragments into *E. coli* DH5α were all carried

out under standard conditions (32) utilizing enzymes from a variety of suppliers. DNA

fragments used for cloning purposes were extracted from ethidium bromide-stained

agarose gels by a GeneClean kit (Bio 101, Inc. La Jolla, CA). Plasmids used or generated

in this report are summarized in Table 2.2.

2.4 Agarose gel electrophoresis and Southern blot analysis

Nucleic acids extracted from bacterial cells or BLPs were separated via

electrophoresis through a 0.8% (wt / vol) agarose gel containing ethidium bromide using

TBE running buffer. The gels were photographed and/or the DNA transferred to

nitrocellulose membranes (0.45 μm-pore- size, Schleicher & Schuell, Keene, N.H.) by the

method of Southern (39) and subsequently baked for 1h at 80°C to fix the DNA. DNA

for probing was purified from agarose gels using a GeneClean kit following PCR

Table 2.2   Plasmids used or generated in this study.

Plasmid	Relevant Characteristics	Reference
pKRT3	pUC19 containing 1.8-kbp *Bam*HI fragment of the 16S-23S *ITS* region from *B. bacilliformis*	Minnick *et al.*, 1995 (40,41)
pUB1	*B. bacilliformis* suicide vecor containing the *nptI* gene, Kan^R	Battisti and Minnick, 1997 (13)
pKB1	pUB1 containing 1.33-kbp *Bam*HI/*Sal*I fragment of the 16S-23S *ITS* region from pKRT3, Kan^R	This study
pBBR1MCS	*B. bacilliformis* shuttle vector, Rep$_{Ec}$, Rep$_{Bb}$, Cam^R	Kovach *et al.*, 1994 (42)
pBBR1MCS-2	*B. bacilliformis* shuttle vector, Rep$_{Ec}$, Rep$_{Bb}$, Kan^R	Kovach *et al.*, 1995 (43)
lp16	a linear plasmid from *Borrelia burgdorferi* with covalently closed ends	Barbour and Garon 1987 (44)

amplification or restriction endonuclease digestion. Probes were labeled by random

primer extension with the Klenow fragment of *E. coli* polymerase I (Gibco-BRL) and [α-

^{32}P]dCTP (New England Nuclear, Boston, Mass.). Nitrocellulose blots were probed

overnight at 60°C and washed four times at high stringency (approximately 7%

mismatch) for 30 min at 60°C with 0.3 x SSC. The blots were subsequently exposed for

25 min to X-ray film (X-Omat XAR-5; Eastman Kodak Co., Rochester, N.Y.) to visualize

hybridized DNA fragments.

2.5 SDS-PAGE and Immunoblotting

Proteins from BLP preparations were resolved by electrophoresis through SDS-

polyacrylamide (12.5% acrylamide) gels using methods adapted from Laemmli (45). The

gels were then stained with Coomassie brilliant blue to visualize protein bands. For

immunoblots, separated proteins were transferred from gels to nitrocellulose membranes

(0.45 µm-pore size) via electrophoresis (46). The membranes were dried briefly, then

incubated in 0.3% Tween-20 and 2% non-fat, dry skim milk in phosphate-buffered saline

(PBS) pH 7.4 for 1 h at 25°C to block all non-specific binding sites. After rinsing in PBS,

the membranes were incubated in patient antiserum followed by a secondary horseradish

peroxidase-conjugated goat anti-human IgG. The membranes were finally developed

with PBS containing 4-chloro-1-napthol and H_2O_2 and observed to see if BLP capsid

proteins were detected.

2.6 Transmission Electron Microscopy

Intact bacterial cells and/or purified BLPs were concentrated by centrifugation and

were resuspended in a 10% glycerol-water (v / v) solution. Suspensions were prepared

for negative- stain electron microscopy on Silicon Monoxide Type-A support grids (300

mesh copper). Samples were allowed to electrostatically attach to the grids for a period

of 5 min. Excess liquid was subsequently blotted away and the grids were allowed to air

dry an additional 2 min. Grids were then stained with 2.0% filter-sterile uranyl acetate

(pH - 7.0) for 3 min. After destaining with 1 M ammonium acetate (pH - 7.0) for 4 min

and washing with deionized for H_2O 1 min, the grids were air dried and examined at 75

kV with a Hitachi 7100 Transmission Electron Microscope (Hitachi, Mtn. View, Calif)

2.7 Generation of Kan^R *B. bacilliformis* mutants

Kan^R mutants were obtained via allelic exchange and homologous recombination

with a suicide vector construct, termed pKB1, and the *Bartonella* chromosome. pKB1

was constructed by cloning a 1.33 kbp *Bam*HI / *Sal*I fragment of the 16S-23SrDNA *ITS*

region from *B. bacilliformis* into pUB1. After construction of pKB1, the plasmid was

electroporated into *B. bacilliformis* JB584 and allowed to homologously recombine with

one of the three 16S-23SrDNA operons within the *Bartonella* chromosome. The Kan^R

mutants were selected by plating the electroporated bacteria onto growth medium

containing kanamycin. Following an incubation of approximately 12 days, individual

Kan^R colonies were harvested, grown, and analyzed via PCR and Southern blot analysis

to verify recombination of the kanamycin resistance cassette into the host chromosome.

2.8 Electroporation of *B. bacilliformis*

Six to eight plates of *B. bacilliformis* were grown as stated above for 3 days and

harvested into 1 ml of 4°C heart infusion broth (HIB). The cells were subsequently

washed four times in 1 ml of ice-cold 10% glycerol water (v / v) with intermittent

centrifugations for 20 min at 4000 x g at 4°C. Following the final wash, the bacterial

pellet was resuspended in 400 µl of 10% glycerol water. Approximately 40 µl of the

bacterial suspension was combined with 0.25 to 3 µg of DNA in pre-chilled 0.2-cm disposable electroporation cuvettes and allowed to sit on ice for 5 min. Electroporation was performed using a Bio-Rad Gene Pulser (BioRad Laboratories, Hercules, CA) using an exponential decay waveform set at a field strength of 12.5 kV/cm, a pulse time of 5 msec, and capacitance held constant at 25 µF as previously described for *Bartonella* (23). Immediately following electroporation, cells were removed from the disposable cuvette and resuspended in 1 ml of filter-sterile recovery broth (HIB supplemented with 0.5% (w / v) BSA fraction V, 5% (v / v) sheep erythrocyte lysate, and 5 mM L-methionine) and transferred to a sterile culture tube. After incubation at 30°C for 16-20 h, the cell/recovery broth suspension was centrifuged for 10 min at 4000 x g at 4°C to pellet the bacteria. Following the removal of 900 µl of supernatant, the pellet was resuspended and plated onto standard *Bartonella* growth medium supplemented with Kan and/or Cam, where required for selection. Antibiotic-resistant strains appeared following 7-15 days of incubation.

2.9 Coincubation of two *B. bacilliformis* strains for transduction

Two plates of 2-day-old bacteria (KB484 and KB585) were harvested separately into 500 µl of HIB, enumerated by plate counts, and plated onto growth medium containing the antibiotic for which it was sensitive to control for spontaneous mutations. The two strains were combined into 350 µl of HIB from which three 100 µl aliquots were plated onto non-selective growth medium to allow for interaction between the two strains. After 1 day, the resulting growth was harvested into 350 µl of HIB and plated accordingly:

100 µl onto HIBB containing Kan and Cam

100 µl onto HIBB containing Kan and Cam

50 µl onto HIBB containing Kan only

50 µl onto HIBB containing Cam only

Similar platings were performed after 2 and 3 days, respectively. After a 20-day incubation period, double antibiotic-resistant colonies were harvested, grown, and characterized via Southern blot and PCR analysis.

2.10 Infection of *B. bacilliformis* strains with purified BLPs from Kan[R] *B. bacilliformis* mutants

Two plates of 2-day-old bacteria (KB484) were harvested into 500 µl of sterile recovery broth. Bacteria were enumerated by plate counts to obtain approximate numbers being infected. Additionally, approximate BLP numbers were determined by running the DNA obtained from 50 µl of BLPs out on an agarose gel stained with ethidium bromide. A 50 µl aliquot of purified BLPs from Kan[R] *B. bacilliformis* mutants was then added to the harvested KB484 bacteria and allowed to incubate at 30°C for 1 h. Following incubation, three 100 µl aliquots were plated onto non-selective growth medium and allowed to grow for 24, 48, and 72 h. After the allotted time period, the resulting growth was harvested into 350 µl of HIB and plated as above except the HIBB containing Kan was replaced with a third HIBB plate containing Kan and Cam. After a 20-day incubation period, double antibiotic-resistant colonies were harvested, subcultured, and characterized via Southern blot and PCR analysis.

2.11 PCR analysis

PCR amplifications were achieved using a core kit and Taq polymerase (Perkin Elmer) following procedures developed by Mullis *et al.* (47). Reaction mixtures (100 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM of each dNTP, 4 mM $MgCl_2$, approximately 100 ng of template DNA, 0.1 µg of each primer, and 2.5 U AmpliTaq DNA polymerase. Thirty cycles with a profile of 94°C for 1 min, 65°C for 1 min, and 72°C for 3 min, were run on a Gene Amp 2400 Thermocycler (Perkin Elmer, Norwalk, CT). Cycling was preceded by a hot start (95°C for 5 min) and followed by a final extension (72°C for 5 min). Ten microliters of each amplified product was analyzed by agarose (1.2% agarose) gel electrophoresis using standard protocol (32).

Single-stranded oligonucleotide primers specific for the kanamycin and chloramphenicol resistance cassettes were synthesized by The University of Montana Murdock Molecular Biology Facility. Primers for the identification of *B. bacilliformis* species were used as before (48).

Chapter III

Results

BLP Characteristics

3.1 Extrachromosomal DNA in *B. bacilliformis*

When total DNA was prepared from various *Bartonella* cultures and subjected to agarose gel electrophoresis, a band migrating at 14-kbp was observed in five species of *Bartonella*, including all strains of *B. bacilliformis* (Table 3.1). Species of *Bartonella* that did not exhibit the extrachromosomal element included *B. clarridgeiae*, *B. elizabethae*, and *B. vinsonii* (Table 3.1). DNA isolated from *B. bacilliformis* JB584 clearly exhibited the 14-kbp extrachromosomal element (Figure 3.1, Lane 2). This 14-kbp DNA fragment was always present in DNA preparations from *B. bacilliformis* and appears to co-migrate with the BLP DNA observed in *B. henselae* (24).

3.2 BLPs associated with *B. bacilliformis*

Transmission electron microscopy was performed to examine a) the BLP particle in association with the *B. bacilliformis* bacterium and b) purified BLPs. Particles of approximately 80 nm in diameter were found to exist freely as well as attached to the surface of the bacterial cells (Figure 3.2, Panel A). The BLPs from *B. bacilliformis* appeared to be round to icosahedral, however, they were nearly twice the size of the *B. henselae* BLPs (24). This size difference is also in contrast to BLPs that have been previously described for this microorganism by Umemori *et al.* (26). Also noted was the absence of tail structures as previously reported (26).

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Table 3.1 Presence of 14-kbp extrachromosomal elements in various *Bartonella* species and strains.

Bacterium	Strain	14-kbp DNA?
B. bacilliformis	KC583	YES
B. bacilliformis	KC584	YES
B. bacilliformis	JB584	YES
B. bacilliformis	KB584	YES
B. bacilliformis	KB585	YES
B. henselae	Houston R 1302	YES
B. quintana	Fuller	YES
B. doshiae	R18	YES
B. grahamii	V2	YES
B. clarridgeiae	Type strain	NO
B. elizabethae	Type strain	NO
B. vinsonii	Baker	NO
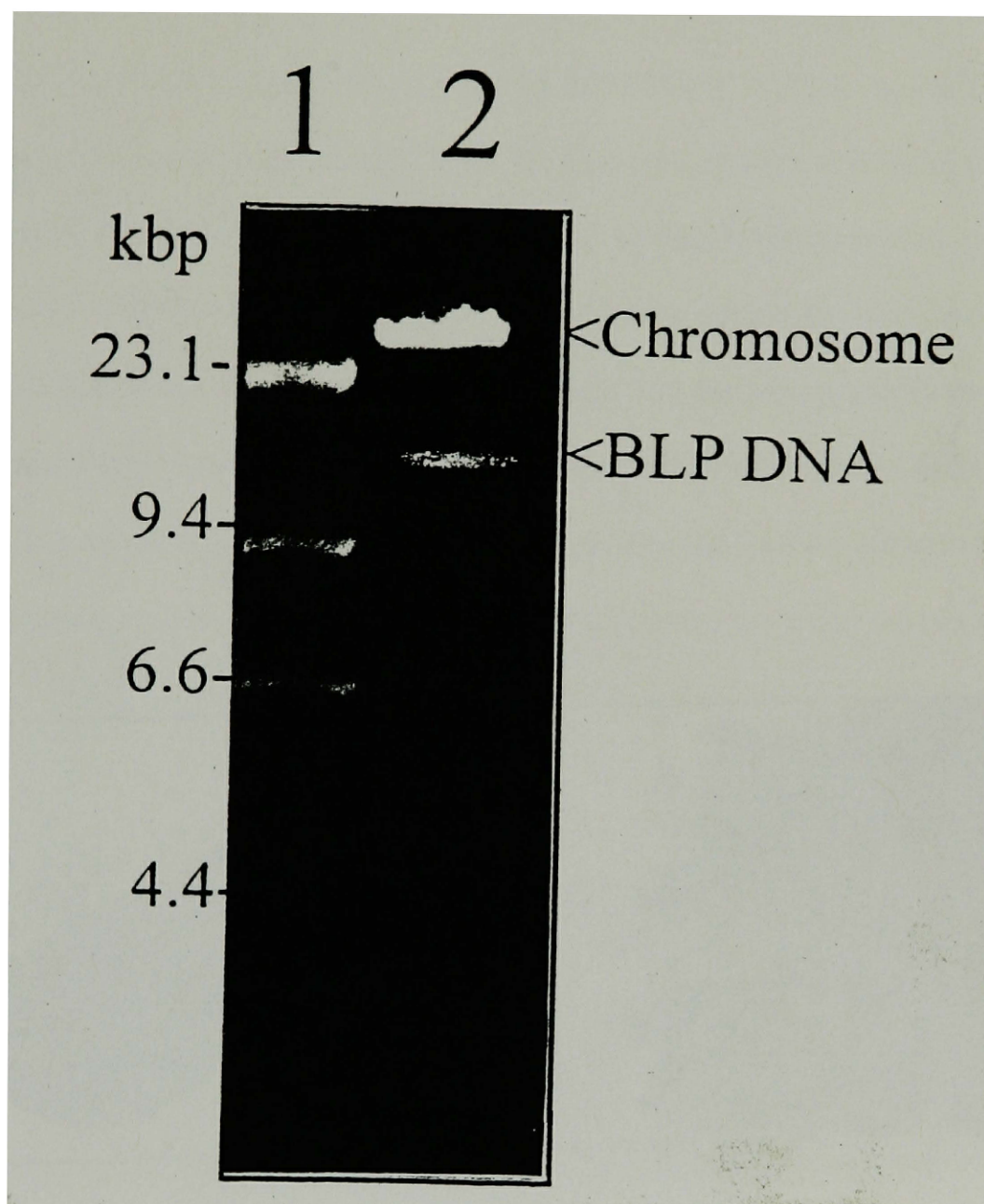
Figure 3.1 Total DNA preparation of *B. bacilliformis* JB584 cultures. Total DNA, showing the presence of a 14-kbp extrachromosomal element, was resolved on a 0.8% agarose gel. Lane 1, λ phage DNA cleaved with *Hin*dIII as a molecular size standard; lane 2, total DNA preparation from *B. bacilliformis* JB584.
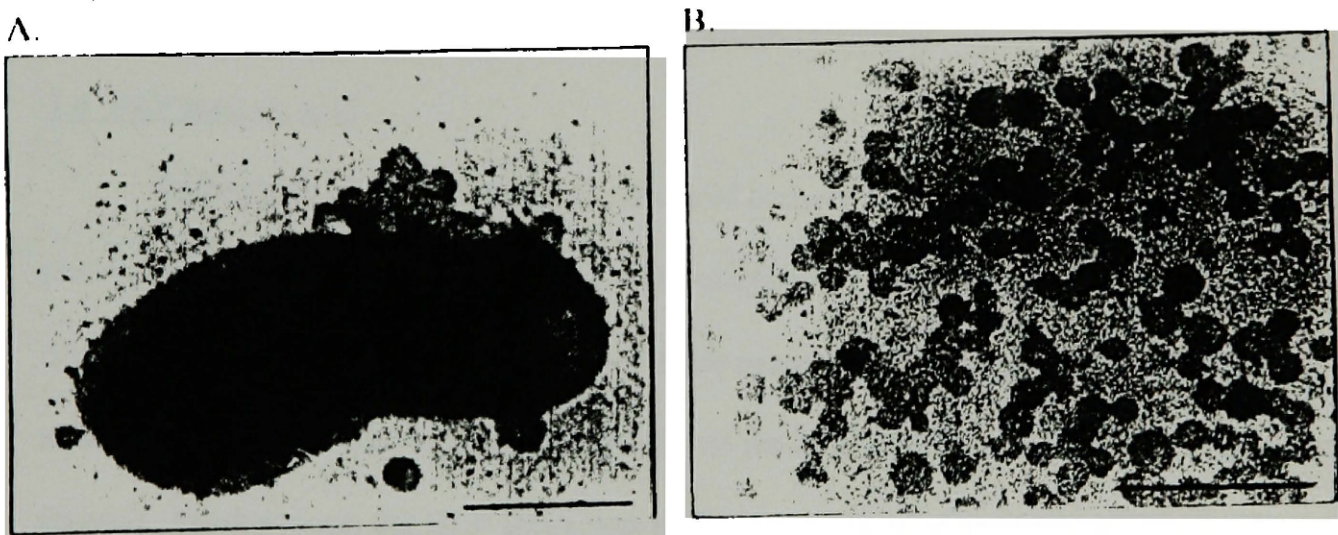
Figure 3.2 Transmission electron micrographs showing *fla⁻ B. bacilliformis* JB585 (to eliminate contaminating flagella) and associated BLPs. Panel A, whole *B. bacilliformis* JB585 cells showing attached BLPs (magnification 17,000 x). Panel B, purified BLPs from *B. bacilliformis* JB585 (magnification 20,000 x). The bars shown represent 250 nm.

3.3 Purification of BLPs and BLP nucleic acids

BLPs were purified from *B. bacilliformis* cultures following the procedures

outlined in Table 3.2. These relatively pure preparations of BLPs were examined by

transmission electron microscopy (Figure 3.2, Panel B). Nucleic acid extraction of the

purified BLP preparation followed by agarose gel electrophoresis, revealed a single band

migrating at 14-kbp (Figure 3.3, Lane 3). These results suggest that there is a direct

correlation between the BLPs from *B. bacilliformis* and the 14-kbp extrachromosomal

element present in total genomic preparations. The purification scheme described in

Table 3.2 was developed to optimize yield and purity of BLPs from *Bartonella* cultures.

Furthermore, nucleic acids obtained from these relatively pure preparations were used

extensively for many experiments described within this study.

3.4 BLP nucleic acid

To begin to characterize the nucleic acid harbored by *B. bacilliformis* BLPs,

purified nucleic acid preparations from *B. bacilliformis* JB584 were treated with

deoxyribonuclease I (DNase I) and subjected to agarose gel electrophoresis. DNase

sensitivity of the 14 kbp fragment indicated that the BLP nucleic acid was DNA (data not

shown). Furthermore, the BLP DNA was resistant to degradation if the particles were

incubated with DNase I prior to nucleic acid extraction (Figure 3.6, Lane 6), suggesting

that the particle protects the enclosed DNA from degradation. Isolated BLP DNA failed

to rapidly renature when subjected to alkaline denaturation followed by neutralization

(Figure 3.4, Lane 4). An identical treatment had minimal effects on a linear plasmid with

covalently-closed hairpin ends (lp16) from *Borrelia burgdorferi* which was seen to

Table 3.2 Flowchart outlining the protocol used in the purification of BLPs
from *B. bacilliformis*.

1) Harvest *Bartonella* from HIBB into SM phage buffer (Approx. 0.5 ml buffer / plate).

2) Add $CHCl_3$ to a final concentration of 3% (v / v).

3) Centrifuge at 4,000 x g for 5 min to remove cell debris.

4) Repeat steps 2) and 3).

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

6) Centrifuge at 100,000 x g for 2 hours 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 until needed.
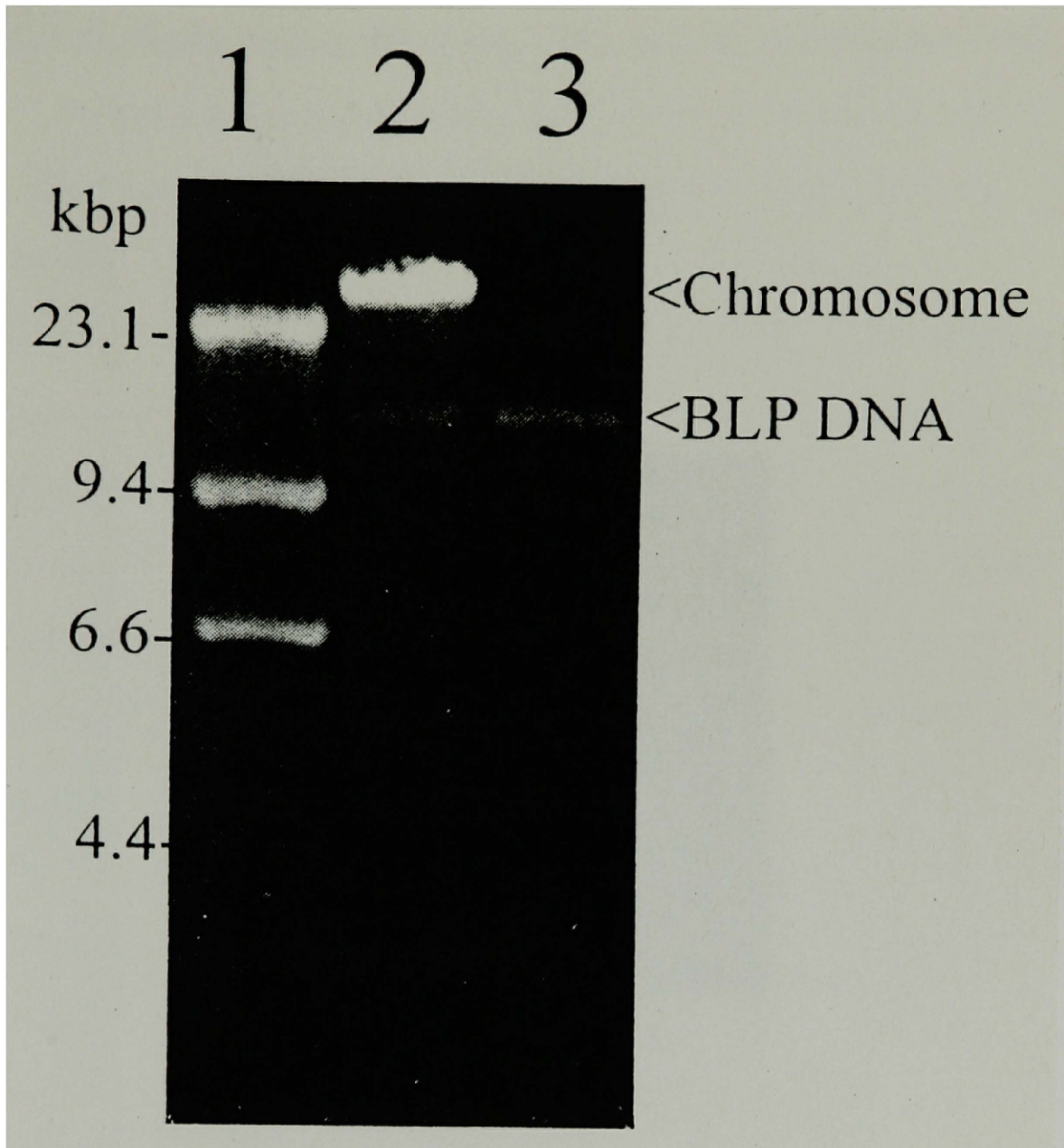
Figure 3.3 Analysis of BLP purity following the purification protocol. Total DNA, extracted from BLP preparations as prepared in Table 3.2, was resolved on a 0.8% agarose gel. Lane 1, λ phage DNA cleaved with *Hind*III as a molecular size standard; lane 2, total DNA preparation of *B. bacilliformis* JB584 showing both chromosomal and BLP DNA; lane 3, DNA profile of purified BLPs showing a DNA band at approximately 14 kbp.
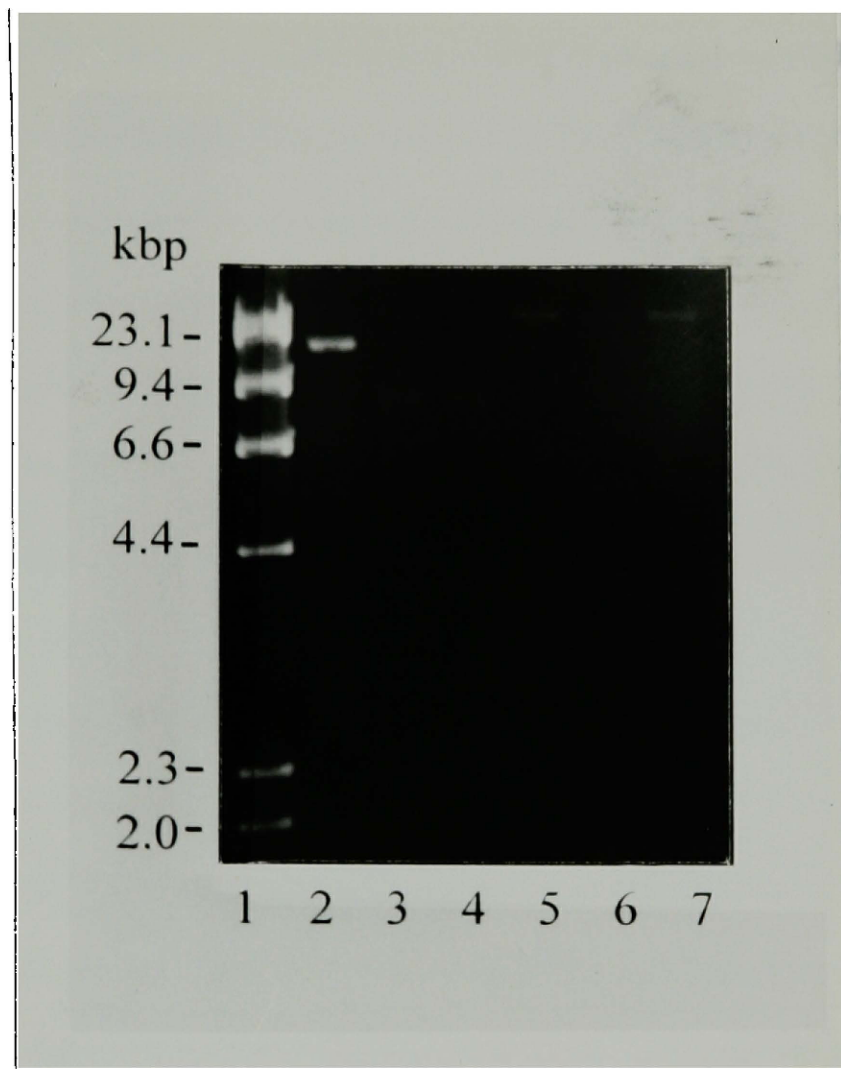
Figure 3.4 Alkaline denaturation / renaturation analysis of BLP DNA on a 0.8% agarose gel showing that BLP DNA ends are not covalently closed. Lane 1, λ phage cleaved with *Hin*dIII as a molecular size standard; lane 2, untreated BLP DNA; lane 3, BLP DNA treated with an equal volume of 0.2 N NaOH; lane 4, BLP DNA treated with an equal volume of 0.2 N NaOH followed by 1/10 th volume of 2 M Tris-HCl (pH 8.0) to renature the molecule; lanes 5, 6, and 7, are the same treatments as done in lanes 2-4 above except using a covalently-closed linear plasmid (lp16) from *Borrelia burgdorferi*.

rapidly renature (Figure 3.4, lane 7). These results suggested that the BLP nucleic acid consists of a 14-kbp, linear, double-stranded DNA with non-covalently closed ends.

To further characterize the termini of the BLP DNA molecules, ligation experiments were performed using T4 DNA ligase. Ligation experiments were performed at 15°C for 24 hours on untreated BLP DNA molecules (blunt or complementary overhangs), Klenow-treated BLP DNA molecules (5′ overhangs), and S1 nuclease-treated (5′ or 3′ overhangs) BLP DNA molecules. Upon separation of the products by agarose gel electrophoresis, only the S1 nuclease-treated BLP DNA molecules produced ligation products (Figure 3.5, Panel B, Lane 3). These results suggest that the BLP DNA molecules contain non-complementary 3′ overhangs. Attempts to ligate S1 nuclease-treated BLP DNA molecules into pBBR1MCS for sequencing were unsuccessful.

To determine if BLP DNA exists as a free extrachromosomal element or if it is only found enclosed within a protein coat, chloroform ($CHCl_3$) treatment of B. bacilliformis followed by agarose gel electrophoresis analysis of the DNA was performed. Upon analysis, only chromosomal DNA was observed and not the additional 14-kbp extrachromosomal DNA (Figure 3.6, Lane 3). Furthermore, an equal aliquot of bacteria were treated with sodium dodecyl sulfate (SDS) to lyse both bacteria and BLPs. When the contents of the SDS-treated mixture was separated by agarose gel electrophoresis, both chromosomal DNA and the associated 14-kbp BLP DNA were present (Figure 3.6, Lane 5). These results suggest that the BLP DNA molecules are being packaged immediately upon synthesis and are protected from $CHCl_3$ extraction. Additional experiments were performed by subjecting purified BLPs to either $CHCl_3$ or
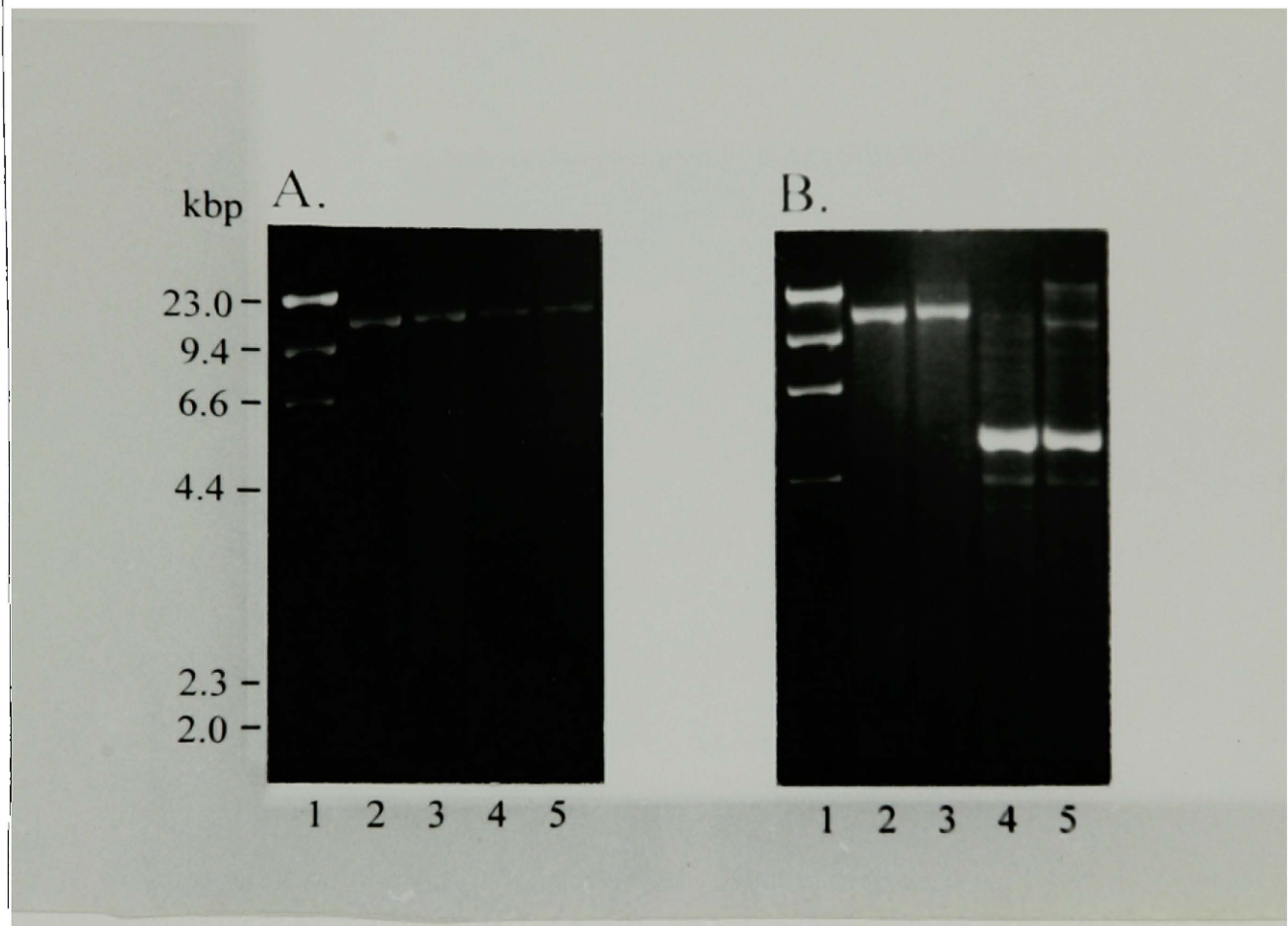
Figure 3.5 Ligation analysis of BLP DNA on a 0.8% agarose gel showing that BLP DNA ends consist of non-complementary 3′ overhangs. Panel A) Lane 1, λ phage DNA cleaved with *Hin*dIII as a molecular size standard; lane 2, untreated BLP DNA; lane 3, untreated BLP DNA subjected to T4 DNA ligase for 24 hr; lane 4, Klenow-treated BLP DNA; lane 5, Klenow-treated BLP DNA subjected to T4 DNA ligase for 24 hr. No ligation products were generated in lanes 2-5. Panel B) Lane 1, λ phage DNA cleaved by *Hin*dIII as a molecular size standard; lane 2, S1 nuclease-treated BLP DNA; lane 3, S1 nuclease-treated BLP DNA subjected to T4 DNA ligase for 24 hr; lane 4, pBBR1MCS linearized with *Eco*RV (a blunt-end endonuclease); lane 5, pBBR1MCS linearized with *Eco*RV subjected to T4 DNA ligase for 24 hr. as a control. Ligation products were generated in lanes 3 and 5.
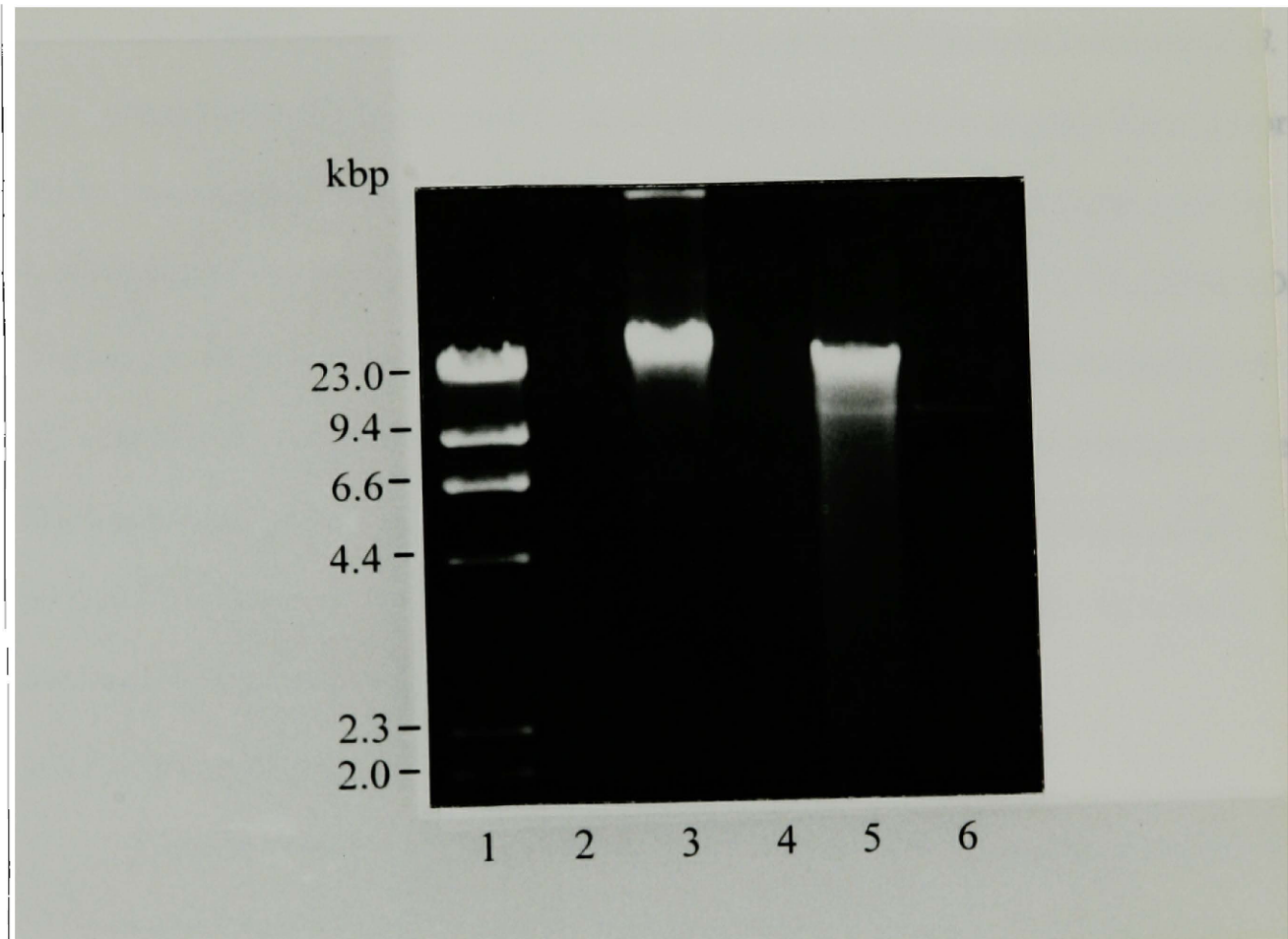
Figure 3.6 A 0.8% agarose gel showing that "free" (unpackaged) BLP DNA molecules are not present inside the *B. bacilliformis* bacterium. Lane 1, λ phage DNA cleaved with *Hind*III as a molecular size standard; lane 2, total DNA preparation of *B. bacilliformis* JB584 incubated with DNase I (2 μg / ml); lane 3, harvested *B. bacilliformis* JB584 bacteria treated with CHCl₃ (note the absence of the 14-kbp BLP DNA band); lane 4, purified BLPs subjected to CHCl₃ (note the absence of 14-kbp BLP DNA; lane 5, harvested *B. bacilliformis* JB584 bacteria treated with 10% SDS (note the presence of both chromosomal and BLP DNA); lane 6, purified BLPs incubated with DNase I (2 μg / ml) and treated with 10% SDS immediately prior to electrophoresis.

SDS. Only the SDS-treated BLPs yield a visible 14-kbp band after being subjected to

agarose gel electrophoresis (Figure 3.6, Lane 6). These data suggest that the BLPs

encapsulate their nucleic acid inside a chloroform-resistant protein coat, or capsid, much

like a true bacteriophage.

3.5 BLP proteins

To further investigate the BLP coat proteins, purified BLPs were compared to *B.*

bacilliformis total cell lysates on Coomassie blue-stained SDS-PAGE gels. Three major

bands were observed that bare remarkable similarities to those analyzed for *B. henselae*

BLPs (24,25). The sizes of the three major bands were approximately 32, 34, and 36 kDa

(Figure 3.7, Lane 3) and are consistent within the total cell lysate at high density(Figure

3.7, Lane 2). A corresponding immunoblot was performed four times to determine if the

BLP proteins were immunogenic. Although detection by human convalescent serum

occurred, the detection was very faint, suggesting poor immune reactivity. By contrast, it

has been shown that strong immune reactivity occurs in rabbits (49).

3.6 Packaging of host DNA into BLPs

Previous literature has stated that BLPs from *B. henselae* package host DNA in a

"near-random" fashion (24). To assess the "randomness of packaging" of host DNA from

B. bacilliformis into the associated BLPs, both Southern blot and RFLP analyses were

performed. When purified BLP DNA was digested separately with several restriction

endonucleases (*Bam*HI, *Hin*dIII, *Eco*RI, and *Cla*I) and then separated by agarose gel

electrophoresis, distinct banding patterns were observed (data not shown). In an effort to

intensify all bands, the DNA fragments from the agarose gel above were transferred to a

nitrocellulose membrane and probed with purified [^{32}P]dCTP-labeled BLP DNA. A
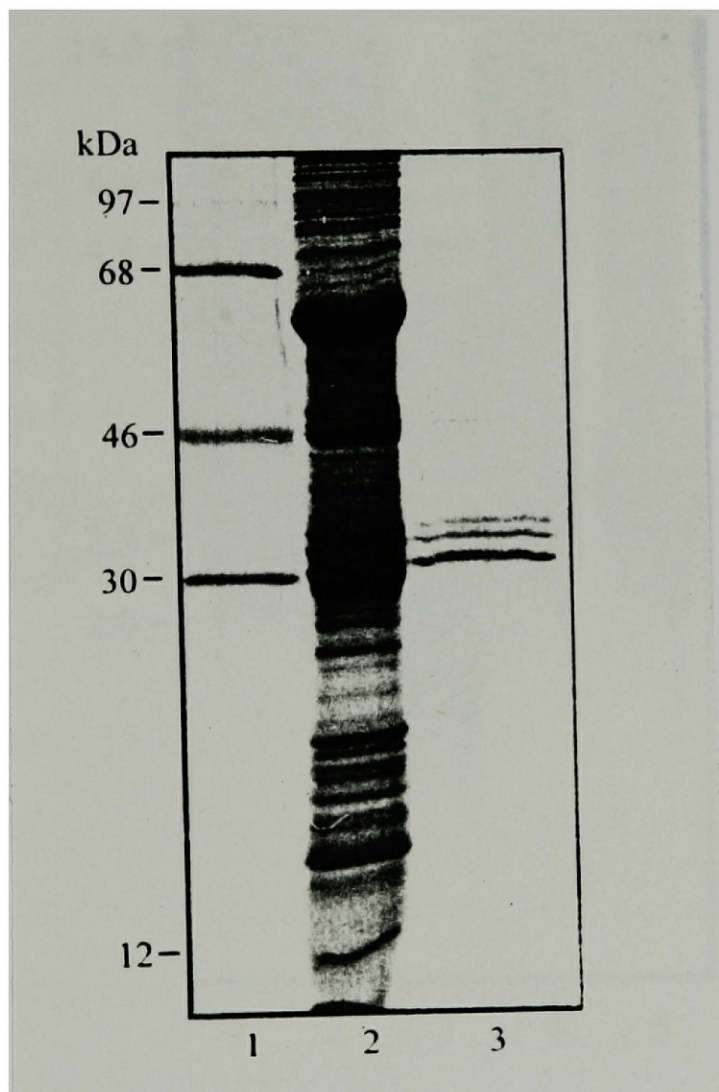
Figure 3.7 Coomassie brilliant blue-stained SDS-polyacrylamide gel (12.5% acrylamide) showing *B. bacilliformis* JB584 whole-cell lysate and BLP associated proteins. Lane 1, molecular mass standards in kilodaltons; lane 2, *B. bacilliformis* JB584 whole-cell lysate showing a total protein profile; lane 3, protein profile of purified BLPs showing 3 major bands at approximately 32, 34, and 36 kDa.
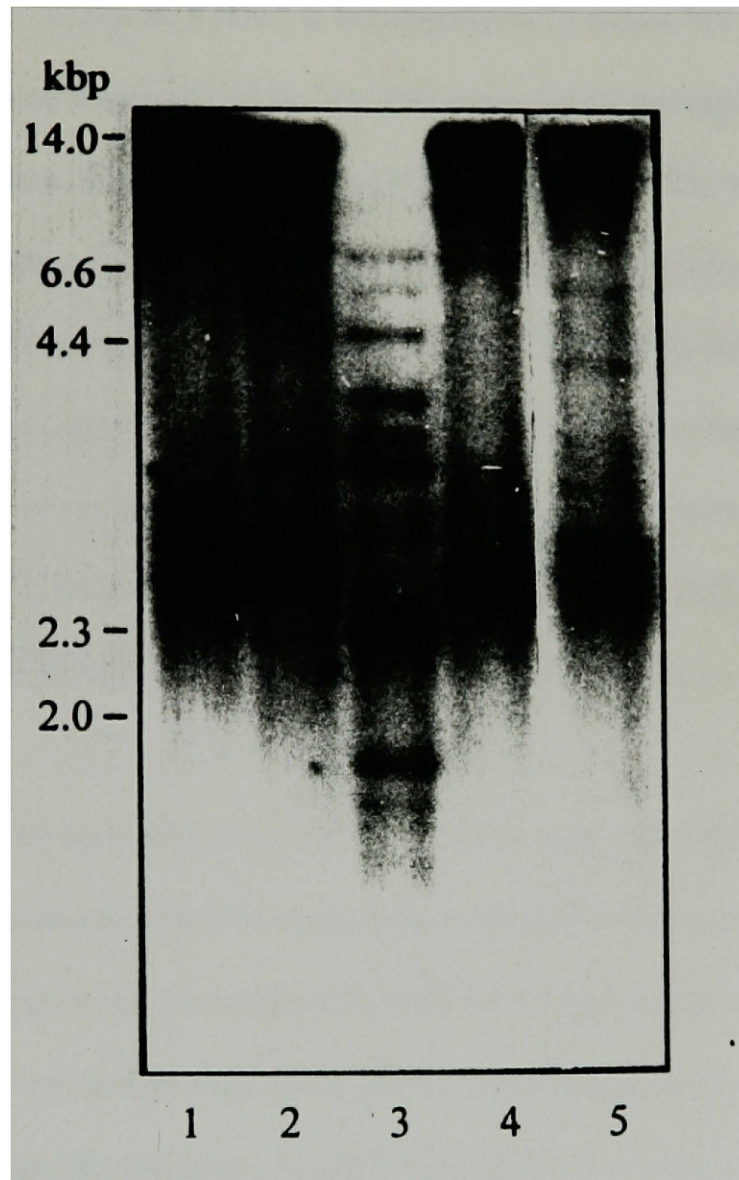
Figure 3.8 Southern blot showing the RFLP analysis of BLP DNA by various restriction endonucleases. Electrophoretically-separated BLP DNA fragments were transferred to nitrocellulose and probed using purified BLP DNA labeled with [^{32}P]dCTP. Approximate positions of molecular size standards are indicated to the left. Lane 1, uncut BLP DNA migrating at 14-kbp; lane 2, *Bam*HI-digested BLP DNA; lane 3, *Hind*III-digested BLP DNA; lane 4, *Eco*RI-digested BLP DNA; lane 5, *Cla*I-digested BLP DNA.

representative autoradiograph is shown in Figure 3.8. Although the sum of the individual

bands in each lane added to be greater than 14 kbp, a smear was not apparent. These data

suggest that the 14-kbp BLP DNA is heterogeneous in nature but not completely random.

In addition, not all fragments of the host chromosome are packaged into BLPs (Table

3.3). Furthermore, two aliquots of host DNA, cut with HindIII, were separated by

agarose gel electrophoresis, transferred to nitrocellulose membranes, and probed with

either $[^{32}P]dCTP$-labelled chromosomal DNA or $[^{32}P]dCTP$-labelled BLP DNA. The

resulting autoradiographs indicated the presence of many more bands when chromosomal

DNA was used as the probe (data not shown). This decrease in the number of resulting

bands when BLP DNA was used as a probe suggests that packaging is far less random in

B. bacilliformis BLPs than previously reported for B. henselae BLPs by Anderson et al

(24).

To further analyze the specificity of packaging by the BLPs associated with B.

bacilliformis, total genomic DNA preparations from B. bacilliformis strain JB584 were

separated by agarose gel electrophoresis much like those in Figure 1, Lane 2. The

resulting DNA was then transferred to nitrocellulose membranes and probed with specific

loci from the host chromosome. An additional probe consisting of the plasmid

pBBR1MCS was used on KB484 to determine if BLPs package plasmid DNA. The

resulting Southern blots were evaluated based on radioactivity at the site of the 14-kbp

DNA band (See Figure 3.10 for example). If radioactivity was present, as indicated on an

autoradiograph, then the BLP DNA was considered to contain that specific locus from the

host chromosome. The results are summarized in Table 3.3 and further confirm that

Table 3.3   BLP packaging of various loci from the *B. bacilliformis* JB584 chromosome.

Loci within host chromosome	Packaged within BLP
16S-23S *ITS* regions	Variable[1]
Invasion-Associated Locus B (*ialB*)	YES
Flagellin gene (*fla*)	NO
Gyrase B gene (*gyrB*)	NO
Extrachromosomal elements (pBBR1MCS)	NO

[1] Three 16S-23SrDNA loci exist; of those, one is packaged.

packaging is non-random. An explanation regarding the packaging results of the 16S-23S

ITS regions is provided in the next section.

Transduction Experiments

These experiments were designed to explore the potential role that BLPs play in

horizontal gene transfer between *Bartonella*. An essential component towards

accomplishing the proposed transduction experiments was the use of a system for site-

directed mutagenesis of *Bartonella* to obtain a selectable, antibiotic resistance marker that

could be used to track the transfer of genes from one microorganism to another. To

accomplish this, we first needed to establish a site on the host chromosome that not only

is packaged by BLPs but, also could be mutated without lethal effects to the bacterium.

The 16S-23S *ITS* region within the *Bartonella* chromosome was chosen for three reasons.

First, three target loci exist for the suicide vector to recombine with. Second, inactivation

of one of the 16S-23SrDNA operons would not be lethal since there would still be two

remaining. Finally, sequence similarities in this area exist across *Bartonella* species,

hence constructs could be utilized for interspecies transduction experiments.

3.7 Development of suicide vector pKB1

An internal fragment of the 16S-23SrDNA operon, previously cloned into pKRT3

(40), was excised out of pKRT3 with *Bam*H1 and *Sal*I restriction enzymes. The same

enzymes were used to create compatible ends on the suicide vector pUB1 (13). Upon

ligation and propagation in *E. coli*, the recombinant plasmids were extracted and analyzed

via restriction endonuclease digestion and PCR to verify the size as well as the various

components associated with the suicide vector pKB1 (Figure 3.9, A).
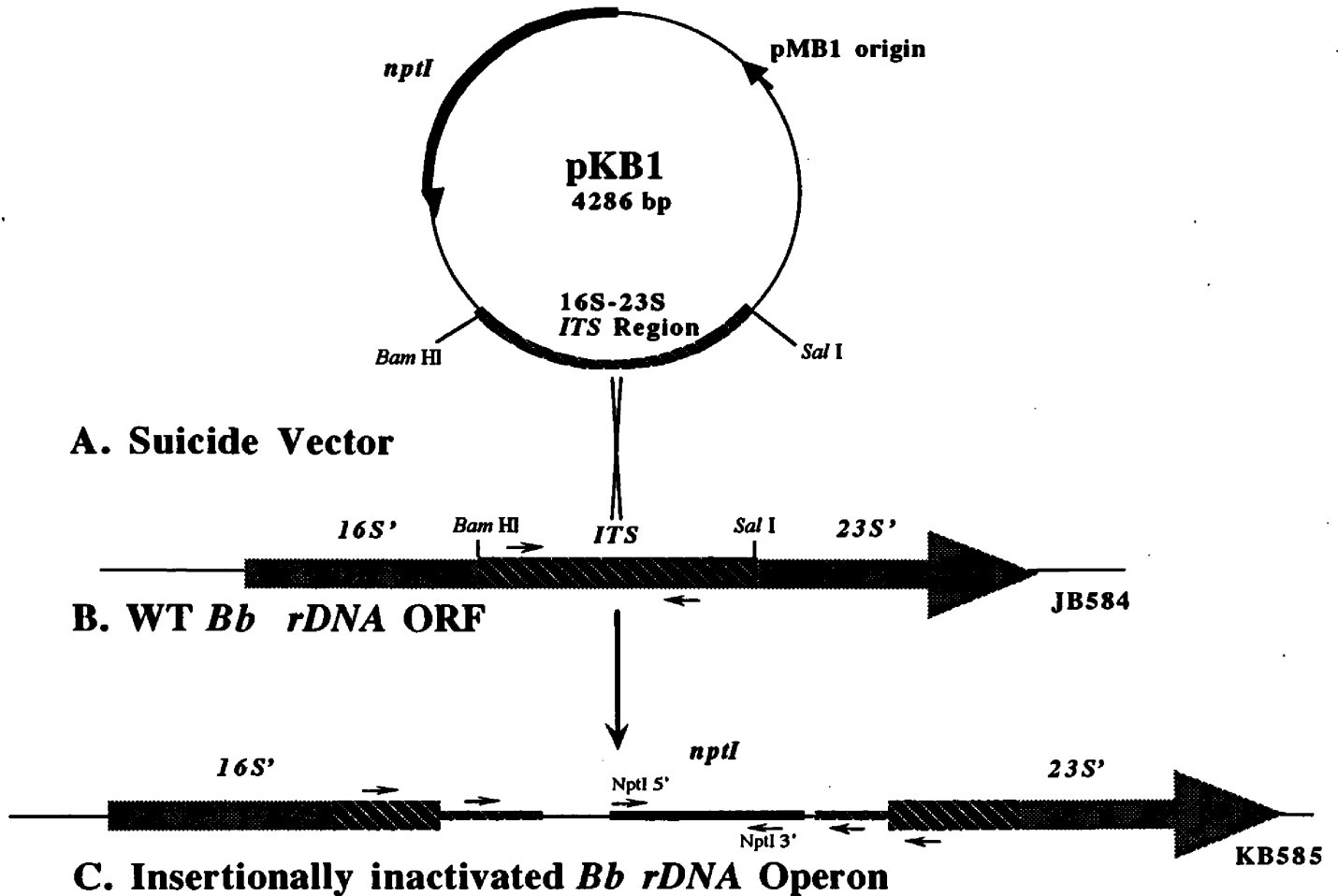
Figure 3.9 Successful strategy used for site-directed mutagenesis of *B. bacilliformis* JB584. Mutagenesis of one of the three 16S-23S *ITS* regions within the *B. bacilliformis* JB584 chromosome was accomplished using the pKB1 suicide vector containing a 1333 bp *Bam*HI / *Sal*I restriction fragment cloned from pKRT3. Following electroporation of pKB1 into JB584, a crossover event between pKB1 and one of the 16S-23SrDNA operons occured resulting in insertional inactivation of one of the three the targeted genes thus, confering a Kan[R] phenotype.

3.8 Development of Kan^R *B. bacilliformis* mutants

pKB1 was electroporated into JB584 and allowed to homologously recombine

with the host chromosome (Figure 3.9, C). After 10 days, two kanamycin-resistant

B. bacilliformis mutants were isolated, grown, and subjected to genomic DNA extraction.

The nucleic acid preparations were then subjected to agarose gel electrophoresis, PCR,

and Southern blot analysis. Both strains, KB584 and KB585 (Table 2.1), possessed a 14-

kbp fragment of BLP DNA that was consistent with total DNA preparations of all *B.*

bacilliformis and *B. henselae* strains (Figure 3.10, Panel A). Southern blot analysis of the

two kanamycin-resistant mutants with [^{32}P]dCTP-labeled *nptI* showed that one mutant,

KB585, contained the kanamycin-resistance cassette in both the chromosome and the 14-

kbp BLP fragment (Figure 3.10, Panel B) whereas the other mutant, KB584, did not.

These data suggested that allelic exchange had occurred and a trackable selection marker,

nptI, was integrated into the *B. bacilliformis* chromosome and that BLPs from KB585

were packaging it. This system provided an avenue to perform coincubation experiments,

using a chloramphenicol-resistant *B. bacilliformis* strain (KB484), to attempt

transduction.

3.9 Coincubation of KB585 and KB484

Coincubation of KB585 and KB484 was performed to mimic a natural

transduction event between two *Bartonella* strains. This experiment led to five new *B.*

bacilliformis strains, KB686 (strains A-E), that demonstrated both a kanamycin and

chloramphenicol resistant phenotype. Bacterial numbers were obtained by serial dilutions

followed by CFUs to give approximately 2 x 10^6 cells / ml of KB484 and 2.5 x 10^6 cells /

ml of KB585. However, spontaneous mutants giving chloramphenicol resistance were
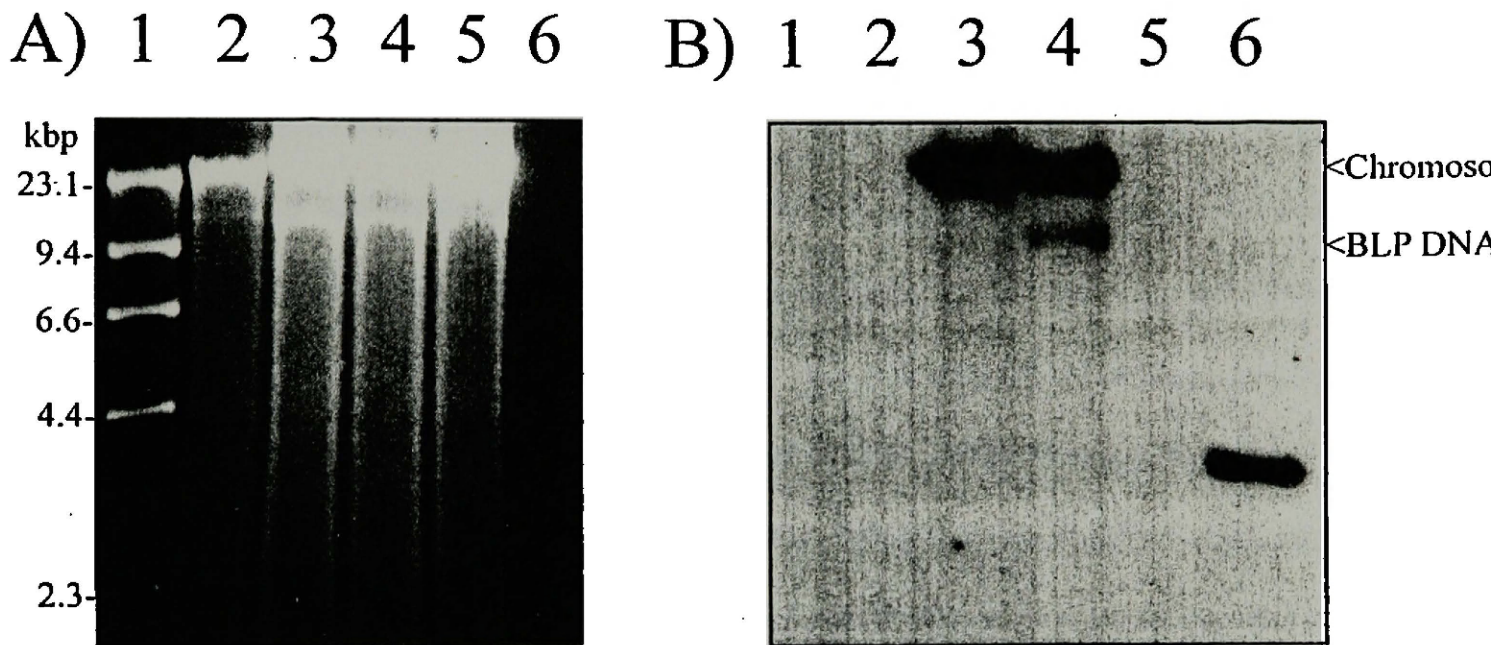
Figure 3.10 DNA hybridization analysis showing BLP packaging of the kanamycin resistance gene from KB585. Panel A) Total DNA, from *B. bacilliformis* strains JB584 and KB585 showing both the chromosomal and BLP DNA, resolved on a 0.8% agarose gel. Lane 1, λ phage DNA cleaved with *Hind*III as a molecular size standard; lane 2, total DNA preparations of *B. bacilliformis* JB584; lane 3, total DNA preparations from *B. bacilliformis* KB584 (mutant 1); lane 4, total DNA preparations from *B. bacilliformis* KB585 (mutant 2); lane 5, same as lane 2; lane 6, linearize suicide plasmid used to generate the *B. bacilliformis* mutants and which contains the kanamycin resistance cassette (*nptI*).
Panel B) Corresponding Southern blot analysis of DNA from gel in panel A. The agarose gel was blotted to nitrocellulose and probed using a [^{32}P]dCTP-labeled *nptI*. While *B. bacilliformis* JB584 does not contain a chromosomal copy of *nptI* (lanes 2 and 5), both *B. bacilliformis* mutants do (lanes 3 and 4). However, only *B. bacilliformis* mutant 2, termed KB585, shows packaging of the kanamycin resistance gene (*nptI*) by the BLPs (lane 4).

also observed on control plates. Furthermore, PCR analysis (using both Kan and Cam primer sets) of the KB686 strains revealed the presence of only the kanamycin-resistance cassette, indicating that spontaneous antibiotic resistance to chloramphenicol was generating the observed phenotype rather than transduction (data not shown).

3.10 Infection of JB584 and KB484 with purified BLPs from KB585

In an effort rule out the possibility of conjugation and spontaneous chloramphenicol resistance, purified BLPs from strain KB585 were used in an attempt to transduce JB584 and KB484. An MOI of approximately 10 BLPs / *Bartonella* cell was determined by agarose gel electrophoresis. JB584 and KB484 numbers were obtained by serial dilutions followed by CFUs to give approximately 1×10^6 cells / ml. Following an incubation period of approximately 28 days, no colonies were observed on growth media containing either Kan or a combination of Kan and Cam. These experiments were repeated a second time with the following change. When the purified BLPs were added to the JB584 and KB484 bacteria, the mixtures were incubated in the recovery broth for 24 hours before being plated. These experiments met with similar results; no colonies were observed on growth medium containing similar antibiotic supplements as above.

3.11 Electroporation of JB584 and KB484 with purified BLP DNA from KB585

In a mock transduction experiment, the attachment and injection stage of the viral lifecycle was surpassed by direct electroporation of BLP DNA into JB584 and KB484. This was done in an effort to determine if attachment of the BLPs to the host, or injection of their nucleic acids, were impeding BLP- mediated genetic exchange. Very high concentrations of BLP DNA from KB585 (1 - 3 µg) were electroporated into either JB584 or KB484, and the resulting electroporation mixture was plated on growth media

containing the appropriate antibiotic supplements. Although homologous recombination and allelic exchange may have occurred at other sites within the host chromosome, it did not occur at a 16S-23S *ITS* region as no kanamycin-resistant colonies were observed.

Chapter IV

Discussion

B. bacilliformis is a small, gram negative, intracellular parasite of human

erythrocytes and endothelial cells. Although this hemotrophic bacterium is endemic to

the Andes region of South America, twelve other species are found worldwide.

Currently, five *Bartonella* species pose a significant health threat to humans and are the

cause of a variety of emerging infectious diseases. One primary mechanism for this

emergence may stem from horizontal transfer of genes encoding virulence determinants.

One mechanism in particular, transduction, may be facilitated by the bacteriophage-like

particles (BLPs) found to be associated with many *Bartonella* species. These BLPs have

previously been described in detail for *B. henselae* (24,25), however, little is known

regarding the BLPs associated with *B. bacilliformis*. Thus, the basis for this research was

to provide a better understanding of the biology and genetics of the *B. bacilliformis* BLPs

and to build a foundation on which to further explore the potential role they play in

generalized transduction between and within *Bartonella* species.

Although BLPs were found to be present in a variety of *Bartonella* species, this

study explored the basic characteristics of the BLPs from *B. bacilliformis*. We describe

an extracellular particle associated with *B. bacilliformis* that appears to be round to

icosahedral and approximately 80 nm in diameter. The particle we observed is similar to

those described before (24,26), however, it is approximately twice the previously reported

diameter. Although we used a *fla⁻ B. bacilliformis* strain (JB585) to minimize flagella

contamination, we believe this size variation is due to swelling during the uranyl acetate

staining procedure (50). Similar to observations made by Anderson *et al.* (24), the

37

absence of tail structures was observed in *B. bacilliformis* BLPs. This is in contrast to observations made by Umemori *et al.* (26) in which tail structures were observed.

A 14-kbp extrachromosomal DNA element was observed in total DNA preparations of five *Bartonella* species including all strains of *B. bacilliformis* (Table 3.1). Likewise, the 14-kbp extrachromosomal DNA element was associated with purified BLP preparations from *B. bacilliformis* JB584 (Figure 3.3). No 14-kbp extrachromosomal DNA elements were seen in *B. clarridgeiae*, *B. elizabethae*, or *B. vinsonii* suggesting that these *Bartonella* species are not infected or have been cured of BLPs. The 14-kbp extrachromosomal DNA was not seen in $CHCl_3$-treated *B. bacilliformis* cells suggesting that it is immediately packaged into a protein head or capsid by following a headful packaging mechanisms (27,28). Additionally, BLP DNA was not digested with DNase I until the BLPs were disrupted with SDS, further suggesting that the BLP DNA is being sequestered into a protective protein head. In this respect, BLPs appear to be quite similar to other generalized transducing bacteriophages.

To characterize the ends of the BLP DNA molecules, alkaline denaturation followed by rapid neutralization was used, as well as various ligation techniques. First, the BLP DNA was shown to be alkaline-denaturable when subjected to basic conditions. The opposite was true when a covalently-closed linear plasmid (lp16) from *Borrelia burgdorferi* was treated with base followed by rapid neutralization. These observations suggest that the BLP DNA ends are not covalently closed (Figure 3.4). Secondly, ligation of BLP DNA molecules to one another only occurred after treatment with S1 nuclease to remove single-stranded overhangs (Figure 3.5) whereas ligation of untreated or Klenow-treated BLP DNA was not observed. These data suggest that the BLP DNA ends consist

of non-homologous 3' overhangs. Interestingly, it has been reported that homologous recombination and allelic exchange of genetic material occurs most readily if the invading DNA molecule contains a 3' overhang (51,52). This implies that the BLP DNA that is being packaged into the capsids would be optimally prepared to undergo homologous recombination and allelic exchange if injected into a suitable host, strengthening our hypothesis that *Bartonella* BLPs may engage in horizontal gene transfer via transduction-mediated genetic exchange.

SDS-PAGE analysis of the BLP-associated proteins revealed three distinct bands with molecular masses of 32, 34, and 36 kDa (Figure 3.7). Three similar-sized BLP-associated proteins were also observed in two different strains of *B. henselae* and one strain of *B. bacilliformis* by Anderson *et al.* (24). Recently, one of the capsid genes associated with *B. henselae* BLPs, termed Pap 31, has been isolated and sequenced (25). Although it is not known if the *B. bacilliformis* BLP proteins are unique to the particle, the results suggest that the BLPs are composed of a distinct set of proteins that constitute phage head proteins. This suggests a possible mechanism of synthesis for capsid protein from genes contained within the *B. bacilliformis* chromosome. Thus, it is speculated that some time ago, a prophage expanded its host range to include an ancestor of the *Bartonella* genus. This prophage, carrying on in its life cycle to include other *Bartonella* strains and / or species, had since lost its ability to continue the normal bacteriophage lytic cycle yet, somehow continues to code for capsid proteins and a mechanism necessary for packaging of the 14-kbp fragments of host DNA (24).

Random packaging of 14-kbp segments of host chromosome appears to be common for BLPs associated with *B. henselae* (24,53). However, RFLP and Southern

blot analysis of BLP DNA from *B. bacilliformis* suggests that packaging by these BLPs is

much less random. Although the RFLP analysis revealed several BLP DNA fragments

when subjected to various restriction endonucleases, the banding pattern was distinct and

did not form a smear (Figure 3.8), as would be observed in a truly heterogeneous mixture

of chromosomal DNA like that of the *B. henselae* BLP DNA (24). Furthermore, when

B. bacilliformis chromosomal DNA was completely digested by *Hind*III, transferred to

nitrocellulose, and probed with [^{32}P]dCTP-labeled chromosomal DNA, smearing was

seen as numerous bands were detected on the resulting autoradiograph. In contrast, when

a similar preparation of *B. bacilliformis* chromosomal DNA was probed with [^{32}P]dCTP-

labeled BLP DNA, a smearing effect was not observed; fewer bands were indicated on

the resulting autoradiograph. These data suggest that packaging of *B. bacilliformis*

chromosomal DNA into associated BLPs is non-random and confined to certain areas on

the host chromosome. Perhaps one of these fragments contain the "ancestral" prophage

genome.

To further investigate the non-random packaging event, specific loci from the *B.*

bacilliformis chromosome were used to probe total genomic DNA preparations of *B.*

bacilliformis JB584. We were also interested to determine if genes associated with

virulence were being packaged into the BLPs. As supported by packaging data on BLPs

from *B. henselae* (24), *B. bacilliformis* BLPs efficiently package at least one of the three

16S-23S *ITS* regions located within the *B. bacilliformis* chromosome. By generating two

Kan^R *B. bacilliformis* mutants, each with a different 16S-23SrRNA operon being

inactivated by a crossover event with a suicide plasmid, we have discovered that at least

one of the three 16S-23S *ITS* regions contained within the *B. bacilliformis* chromosome is

not packaged (Figure 3.10). Additional studies revealed that the *gyrB* gene and the *fla* gene are not packaged by the *B. bacilliformis* BLPs. However, the *ialB* gene, located in the middle of a known virulence gene cluster (11,12), was shown to be packaged into the BLPs. Thus, BLPs may contribute to the recent emergence of *Bartonella* species and associated infectious diseases in humans by horizontal gene transfer and spread of virulence determinants via transduction.

The existence of a transduction event has never been shown for any *Bartonella* species. We have completed a series of experiments designed to provide a foundation for discovering the potential role BLPs play in generalized transduction. The first experiment was to insert a selectable, antibiotic-resistance marker into the *B. bacilliformis* chromosome such that it could be used to track the transfer of antibiotic resistance to antibiotic-sensitive strains. This was accomplished by electroporating a suicide vector, termed pKB1 (Figure 3.9), into *B. bacilliformis* JB584 and allowing it to homologously recombine with one of the three 16S-23S *ITS* regions located within the *B. bacilliformis* chromosome, thus producing a kanamycin-resistant phenotype. Southern blot analysis confirmed that allelic exchange had occurred. The second experiment was to show packaging of this antibiotic-resistance marker into the *B. bacilliformis* BLPs. This was accomplished by probing total DNA preparations of the two Kan^R *B. bacilliformis* mutants with [^{32}P]dCTP-labeled *nptI*. It was shown that BLP DNA from Kan^R *B. bacilliformis* KB585, contained the *nptI* gene (Figure 3.10), providing the means to explore the potential role that BLPs may play in generalized transduction.

Several unsuccessful attempts were made to demonstrate transduction by BLPs associated with *B. bacilliformis*. One possible explanation as to why we were

unsuccessful in our transduction attempts stems from the natural phenomenon known as superinfection immunity. This phenomenon occurs when a lysogen is exposed to a mature bacteriophage similar to the integrated prophage (54). This phenomenon has been thoroughly studied and documented for both λ phage and T4 phage infection of *E. coli* (27,54). Each of these phages have their own mechanisms for controlling subsequent infections of an already infected host cell. For example, λ phage blocks expression of incoming viral DNA by utilizing repressor proteins already present in the cytoplasm. T4 phage prevents further infection by degrading incoming viral DNA. Because all *B. bacilliformis* strains contain BLPs, these microorganisms may have a BLP-mediated mechanism that prevents further BLP infection. For this reason, future transduction studies will be directed at producing a cured indicator strain of *B. bacilliformis* (BLP⁻) or implementing the use of other BLP⁻ *Bartonella* species such as *B. vinsonii or B. clarridgeiae*.

Another explanation as to why we were unsuccessful in our transduction attempts may be that *B. bacilliformis* harbors nucleases designed for the degradation of linear DNA molecules. Previous attempts by our lab to mutagenize with linear DNA molecules, either double-straded or single stranded, were unsuccessful (52).

Finally, the odds of a double-stranded crossover event are very low especially considering the heterogeneity of the BLP DNA. That is, only a minor fraction of the BLPs would contain the Kan::*ITS* locus.

Reasons for the recent increase in the incidence of Bartonellosis are unclear. It is posible that a contributing factor for this emergence is the horizontal transfer of genes encoding virulence determinants and / or antibiotic-resistance markers. BLPs may play

an essential role in mediating this genetic exchange since it has been shown that BLP

DNA has a 3′ overhang for optimal homologous recombination. Furthermore, BLPs were

found to package a known virulence gene, *ialB*, from the *B. bacilliformis* chromosome. It

may also be possible for BLPs to package genes containing the spontaneous

chloramphenicol resistance observed in the *B. bacilliformis* KB686 strains, thus

effectively transducing antibiotic resistance to previously Cam^S strains. We intend to

continue to investigate the role BLPs play in mediating genetic exchange among the

Bartonella genus as well as to characterize the BLP genome in hopes of harnessing it as a

potential tool in the genetic manipulation of *Bartonella bacilliformis*.

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