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Bartonella bacilliformis and *Bartonella henselae*, the respective agents of Oroya fever and cat-scratch disease in humans, are known to produce bacteriophage-like particles (BLPs) that package 14 kbp segments of the host chromosome. Data from this study suggest that other *Bartonella* species including *Bartonella quintana*, *Bartonella doshiae* and *Bartonella grahamii* also contain similar BLPs, as evidenced by the presence of a 14 kbp extrachromosomal DNA element in their genomes, whereas *Bartonella elizabethae* and *Bartonella clarridgeiae* do not. A purification scheme utilizing chloroform, DNase I and centrifugation was devised to isolate BLPs from *B. bacilliformis*. Intact BLPs were observed by transmission electron microscopy and were round to icosahedral in shape and approximately 80 nm in diameter. RFLP and Southern blot analysis of BLP DNA from *B. bacilliformis* suggest that packaging, while non-selective, is less than the near-random packaging previously reported for the *B. henselae* phage. Data also suggest that the linear, double-stranded BLP DNA molecules have blunt ends with non-covalently closed termini. Packaging of the BLP DNA molecules into a protein coat appears to be closely related to nucleic acid synthesis, as unpackaged phage DNA is not detectable 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; values that closely correspond to proteins found in *B. henselae* BLPs. Western blot analysis performed with patient convalescent serum showed that BLP proteins are slightly immunogenic in humans. To determine if BLPs contribute to horizontal gene transfer, mutants of *B. bacilliformis* were generated by allelic exchange with an internal fragment of the 16S–23S rDNA intergenic spacer region and a suicide vector construct, termed pKB1. BLPs from one of the resultant strains were able to package the mutagenized region containing the kanamycin-resistance cassette; however, numerous approaches and attempts at intraspecies transduction using these BLPs were unsuccessful.

Keywords: *Bartonella*, bacteriophage, defective phage, transduction

INTRODUCTION

Bartonella bacilliformis is a Gram-negative, haemotrophic bacterium (Kreier & Ristic, 1981) that is capable of invading and replicating inside human erythrocytes and endothelial cells (Benson *et al.*, 1986; McGinnis-Hill *et al.*, 1992). The pathogen is transmitted to humans by the bite of a female sandfly (*Lutzomyia* spp.) (Hertig, 1942). The resulting infection manifests as a biphasic disease. The primary, or haematic phase, is characterized by fever, malaise, skin pallor and a severe haemolytic anaemia where nearly all of the circulating erythrocytes are infected (Reynafarje & Ramos, 1961) and nearly 80% are lysed (Hurtado *et al.*, 1938). Four to eight weeks following the haematic phase, patients generally proceed to a secondary, or tissue, phase in which the bacterium invades vascular endothelial cells (McGinnis-Hill *et al.*, 1992). This phase is characterized

by localized cutaneous eruptions, or haemangiomas, on the face and extremities and is termed verruga peruana (Garcia-Caceres & Garcia, 1991). The mechanisms by which *B. bacilliformis* gains entry into erythrocytes and endothelial cells, as well as additional virulence determinants, are being investigated (for a recent review, see Minnick, 1997).

Previous work showed that *Bartonella henselae*, an agent of cat-scratch disease and bacillary angiomatosis, produces bacteriophage-like particles (BLPs) that package random, 14 kbp fragments of *Bartonella* DNA (Anderson *et al.*, 1994). The heterogeneous mixture of double-stranded host DNA contained in the *B. henselae* BLPs is protected from chloroform/DNase I treatments by capsid proteins (Anderson *et al.*, 1994; Bowers *et al.*, 1998). Furthermore, BLPs are non-lytic even when the host cell is subjected to UV irradiation and/or the addition of mitomycin C. Little more is known about *Bartonella* BLPs or the mechanisms by which the DNA is packaged, although similarities with generalized defective phages suggest that headful packaging into a pre-formed capsid head may be taking place (Yarmolinsky & Sternberg, 1988; Birge, 1994).

Defective phages are unique when compared to true bacteriophages. Although they share some properties that are similar to true phages, most defective phages package host DNA into pre-formed capsid heads and do not contain any apparent genomic material from the phage (Garro & Marmur, 1970). Defective phages have been found in a variety of unrelated bacteria including the *Bacillus subtilis* PBSX phage (Yarmolinsky & Sternberg, 1988; Birge, 1994) and the *Serpulina hyodysenteriae* VSH-1 phage (Humphrey *et al.*, 1997), the latter of which has been shown to undergo generalized transduction. The purpose of this study was to characterize the molecular biology of the *B. bacilliformis* BLPs and begin to address our hypothesis that *Bartonella* BLPs participate in intraspecies (and possibly interspecies) horizontal gene transfer via transduction.

METHODS

Growth of bacterial strains. Bacterial species and strains used in this study are summarized in Table 1. *B. bacilliformis* strains were grown for 3–4 d at 30 °C in a water-saturated atmosphere on heart infusion agar–blood (HIAB), which consists of heart infusion agar plates (Difco) supplemented with 4% (v/v) defibrinated sheep erythrocytes and 2% (v/v) filter-sterilized sheep serum (Quad Five). Other *Bartonella* species were similarly cultured, but at 37 °C with 5% CO_2. *Escherichia coli* strains were grown overnight at 37 °C in Luria–Bertani medium with standard antibiotic supplements when required (Davis *et al.*, 1980). The antibiotic supplements used in this study included 25 µg kanamycin (Kan) ml^{-1} and 2 µg chloramphenicol (Cam) ml^{-1} (Sigma) and were used individually or combined depending upon experimental conditions.

Purification of BLPs. BLPs were prepared by inoculating *B. bacilliformis* strains onto HIAB. Approximately 40 plates were harvested into 15 ml SM phage buffer (Ausubel *et al.*, 1995). Chloroform (3%, v/v) was used to lyse the bacterial

cells, and cellular debris was subsequently removed by centrifuging for 5 min at 6000 **g**. The supernatant was collected, chloroform-treated again, and centrifuged a second time. DNase I was added to the resulting supernatant to a final concentration of 2 µg ml^{-1}, and incubated at 37 °C for 2 h to digest chromosomal DNA. The BLPs were then pelleted by ultracentrifugation for 2 h at 100 000 **g** in a SW60 rotor (Beckman), using a 15% sucrose cushion. The supernatant was discarded and the pellets were resuspended in 150 µl fresh SM buffer and stored at 4 °C until needed.

Preparation and manipulation of DNA. Nucleic acids for DNA hybridization or PCR were extracted from bacteria or BLP suspensions using a CTAB technique (cetyltrimethylammonium bromide) as described by Ausubel *et al.* (1995). Plasmids were extracted by either an alkaline lysis procedure (Birnboim & Doly, 1979), or a Qiagen Midi Prep kit as per the manufacturer's instructions. Restriction digestion, ligation, denaturation/renaturation (using 0·2 M NaOH and 2 M Tris/HCl, pH 8·0) and transformation of DNA fragments into *E. coli* DH5α were carried out using standard protocols (Ausubel *et al.*, 1995). DNA fragments for cloning or hybridization were extracted from ethidium-bromide-stained agarose gels by a GeneClean kit (Bio 101). Plasmids used or generated in this report are summarized in Table 1.

Agarose gel electrophoresis and Southern blot analysis. DNA was separated via electrophoresis through 0·8% (w/v) agarose gels containing ethidium bromide. DNA in the gel was transferred to nitrocellulose membranes (0·45 µm pore size; Schleicher & Schuell) by the method of Southern (1975) and subsequently baked for 1 h at 80 °C to fix the DNA. DNA probes were labelled by random primer extension with Klenow (Gibco-BRL) and [α-^{32}P]dCTP (New England Nuclear). Nitrocellulose blots were probed overnight at 60 °C, washed at high stringency (approx. 7% mismatch), and developed as previously described (Minnick *et al.*, 1990).

SDS-PAGE and immunoblotting. BLP proteins were resolved by electrophoresis through SDS-polyacrylamide (12·5%, w/v, acrylamide) gels using methods adapted from Laemmli (1970). 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 (Towbin *et al.*, 1979). Western blots were developed as previously described (Scherer *et al.*, 1993).

Transmission electron microscopy. Bacterial cells and/or purified BLPs were resuspended in 10% (v/v) glycerol/water. 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 5 min. Excess liquid was subsequently blotted away and the grids were allowed to air dry for 2 min. Grids were then stained for 3 min with 2% filter-sterile uranyl acetate (pH 7·0). After destaining with 1 M ammonium acetate (pH 7·0) for 4 min and washing with deionized H_2O for 1 min, the grids were air-dried and examined at 75 kV with a Hitachi 7100 transmission electron microscope.

Generation of Kan^R *B. bacilliformis* mutants. Kan^R mutants were generated via allelic exchange with a suicide vector, termed pKB1. pKB1 was constructed by cloning a 1·3 kbp BamHI–SalI fragment from pKRT3 containing the 16S–23S rDNA *ITS* region from *B. bacilliformis* into pUB1. After construction of pKB1, the plasmid was electroporated into *B. bacilliformis* JB584 as previously described by Battisti & Minnick (1999) and allowed to homologously recombine with

Table 1. Bacterial strains and plasmids

Strain/plasmid	Description	Reference
Strains		
E. coli		
DH5α	Host for cloning and plasmid propagation	Gibco-BRL
Bartonella		
B. bacilliformis KC583	Neotype strain	Brenner *et al*. (1991)
B. bacilliformis KC584	Peruvian isolate, 1963	Brenner *et al*. (1991)
B. bacilliformis JB584	HG584 cured of pEST (Kan^S Cam^S)	Battisti & Minnick (1999)
B. bacilliformis JB585	*fla* mutant of JB584 (Kan^R Cam^S *fla*)	Battisti & Minnick (1999)
B. bacilliformis KB484	JB584 containing pBBR1MCS (Kan^S Cam^R)	This study
B. bacilliformis KB584	One of three 16S–23S *ITS* regions of JB584 interrupted by insertion of pKB1 (Kan^R Cam^S)	This study
B. bacilliformis KB585	One of three 16S–23S *ITS* regions of JB584 interrupted by insertion of pKB1 (Kan^R Cam^S)	This study
B. bacilliformis KB686	Spontaneous Cam^R mutant of KB585 (Kan^R Cam^R)	This study
B. clarridgeiae	Isolate from cat with septicaemia	Lawson & Collins (1996)
B. elizabethae	Human endocarditis isolate	Daly *et al*. (1993)
B. doshiae R18	Type strain	Birtles *et al*. (1995)
B. grahamii V2	Type strain	Birtles *et al*. (1995)
B. henselae Houston R1302	Type strain	Regnery *et al*. (1992)
B. quintana Fuller	Type strain	Myers *et al*. (1979)
B. vinsonii Baker	Vole agent	Weiss & Dasch (1982)
Plasmids		
pKRT3	pUC19 containing 1·8 kbp *Bam*HI fragment of the 16S–23S *ITS* region from *B. bacilliformis*	Minnick *et al*. (1994)
pUB1	*B. bacilliformis* suicide vector containing the *nptI* gene (Kan^R)	Battisti & Minnick (1999)
pKB1	pUB1 containing 1·3 kbp *Bam*HI–*Sal*I fragment of the 16S–23S *ITS* region from pKRT3 (Kan^R)	This study
pBBR1MCS	*B. bacilliformis* shuttle vector (Cam^R)	Kovach *et al*. (1994)
pBBR1MCS-2	*B. bacilliformis* shuttle vector (Kan^R)	Kovach *et al*. (1995)
lp16	Linear plasmid from *Borrelia burgdorferi* with covalently closed ends	Barbour & Garon (1987)

one of the three 16S–23S rDNA operons within the *Bartonella* chromosome. Kan^R mutants were selected by plating the electroporated bacteria onto HIAB containing Kan. After 12 d, individual Kan^R colonies were harvested, grown and analysed via PCR and Southern blot analysis to verify allelic exchange.

Transduction experiments. Two plates of 2-d-old bacteria (KB484 and KB585) were harvested separately into 500 µl heart infusion broth (HIB), enumerated by plate counts ($1-2 \times 10^6$ cells ml^{-1}), and plated onto HIAB containing Kan and Cam to control for spontaneous mutations to either antibiotic. The two strains were combined into 350 µl HIB from which three 100 µl aliquots were plated onto HIAB to allow for interaction between the two strains. After 1 d, the resulting growth was harvested into 350 µl HIB and dispensed onto HIAB containing Kan, Cam, or a combination of the two antibiotics. Similar platings were performed after 2 and 3 d, respectively. After a 20 d incubation period, double antibiotic-resistant colonies were harvested, grown and characterized via Southern blot and PCR analysis.

In addition to co-incubating strains, purified BLPs from Kan^R *B. bacilliformis* mutants were also used in transduction experiments. Briefly, two plates of 2-d-old *B. bacilliformis* (KB484) were harvested into 500 µl sterile recovery broth. Bacteria were enumerated by plate counts to obtain approximate numbers being infected ($1-2 \times 10^6$ cells ml^{-1}). A 50 µl aliquot of purified BLPs from Kan^R *B. bacilliformis* mutants was then added to the harvested KB484 bacteria to yield an approximate m.o.i. of 10, and allowed to incubate at 30 °C for 1 h. To prolong interaction under optimal conditions, three 100 µl aliquots were plated onto HIAB for a period of 1–3 d. After the allotted time period, the resulting growth was harvested into 350 µl HIB and all subsequent actions were carried out exactly as stated above.

PCR analysis. PCR amplifications were done using a core kit and *Taq* polymerase (Perkin Elmer). Reactions were carried out as previously described (Minnick & Barbian, 1997). Oligonucleotide primers specific for the Kan- and Cam-resistance cassettes as well as primers specific for the flagellin, gyrase B and invasion associated locus B genes of *B. bacilliformis* were synthesized by The University of Montana Murdock Molecular Biology Facility.

RESULTS

BLP characteristics

Extrachromosomal DNA in *B. bacilliformis*. When total DNA was prepared from various *Bartonella* species and analysed by agarose gel electrophoresis, a band migrating at 14 kbp was observed in *Bartonella henselae*, *Bartonella quintana*, *Bartonella doshiae*, *Bartonella grahamii* and all strains of *B. bacilliformis* used in this study. However, *Bartonella clarridgeiae*, *Bartonella elizabethae* and *Bartonella vinsonii* did not contain a detectable extrachromosomal element. DNA isolated from *B. bacilliformis* (JB584) (the strain used for mutagenesis) clearly exhibited the 14 kbp extrachromosomal element (Fig. 1, lane 2). This 14 kbp DNA fragment was always present in DNA preparations from *B. bacilliformis* and is the same size as reported for *B. henselae* BLP DNA (Anderson *et al.*, 1994).

BLP ultrastructure. Transmission electron microscopy was performed to examine purified BLPs and BLPs in association with the *B. bacilliformis* cell. Particles of approximately 80 nm in diameter were found to exist freely as well as attached to the surface of the bacterium (Fig. 2a). The BLPs from *B. bacilliformis* appeared to be round to icosahedral; however, they were nearly twice
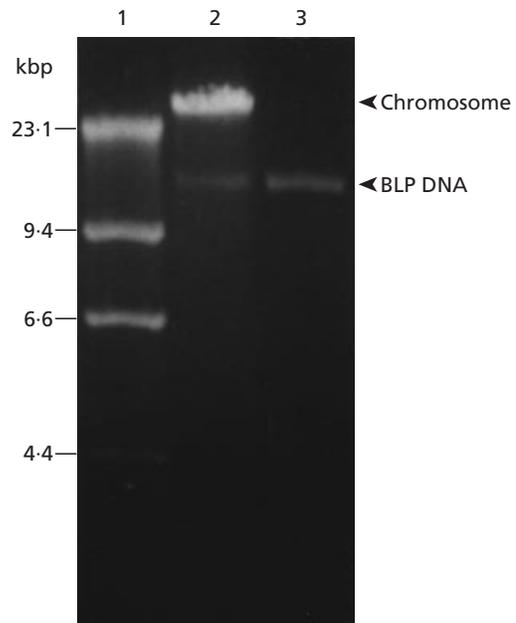
Fig. 1. Total DNA from *B. bacilliformis* (JB584) and analysis of BLP purity following the purification protocol described in the text. Lanes: 1, λ-*Hin*dIII DNA as a molecular size standard; 2, total DNA from *B. bacilliformis* (JB584) showing both chromosomal and BLP DNA; 3, DNA from purified BLPs showing a single DNA band at approximately 14 kbp.

the size reported for *B. henselae* BLPs (Anderson *et al.*, 1994). Transmission electron microscopy showed that purified BLPs (Fig. 2b) were indistinguishable from those observed in association with *B. bacilliformis* (Fig. 2a). Although a previous report concerning the ultrastructure of the *B. bacilliformis* phage reported tail structures (Umemori *et al.*, 1992), we never observed them. Likewise, tails were not found in BLPs from *B. henselae* (Anderson *et al.*, 1994).

Nucleic acid extraction of the purified BLP preparation followed by agarose gel electrophoresis revealed a single band migrating at 14 kbp (Fig. 1, lane 3), showing there is a direct correlation between the BLPs from *B. bacilliformis* and the 14 kbp extrachromosomal element.

BLP nucleic acid. To begin to characterize the nucleic acid harboured by *B. bacilliformis* BLPs, nucleic acid from *B. bacilliformis* (JB584) was treated with RNase-free DNase I and subjected to agarose gel electrophoresis. DNase I sensitivity of the 14 kbp element indicated that the BLP nucleic acid was DNA (e.g. see Fig. 5, lane 2). Furthermore, the BLP DNA was resistant to degradation if the particles were incubated with DNase I prior to nucleic acid extraction (e.g. see Fig. 5, lane 6), suggesting that a capsid or coat protects the enclosed DNA from degradation.

Isolated BLP DNA failed to renature when subjected to alkaline denaturation followed by rapid neutralization
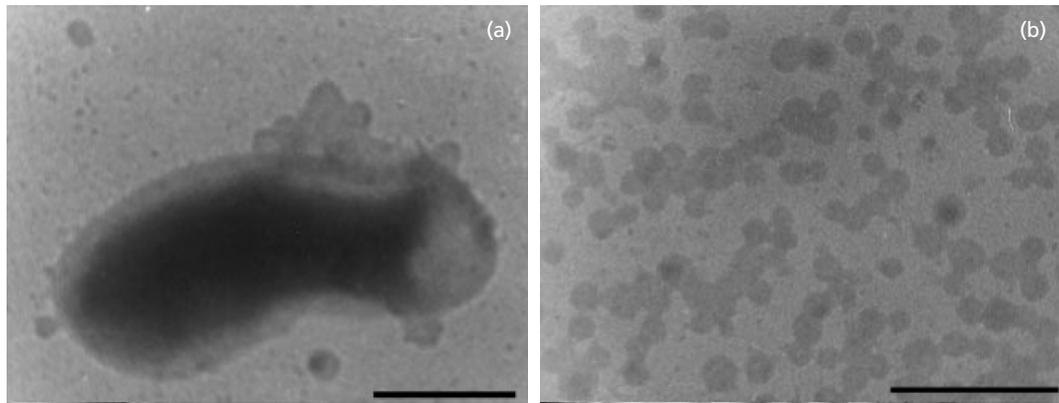
Fig. 2. Transmission electron micrographs showing *B. bacilliformis* BLP ultrastructure. (a) JB585 cells (Fla⁻ to eliminate contaminating flagella) showing attached BLPs. (b) Purified BLPs prepared from JB585. Bars, 250 nm.
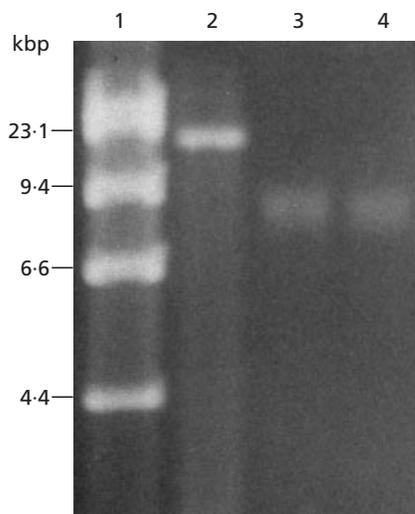
Fig. 3. Alkaline denaturation/renaturation analysis of BLP DNA on a 0·8% agarose gel showing that BLP DNA ends are not covalently closed. Lanes: 1, λ-*Hin*dIII DNA as a molecular size standard; 2, untreated BLP DNA; 3, BLP DNA treated with an equal volume of 0·2 M NaOH; 4, BLP DNA treated with an equal volume of 0·2 M NaOH followed by rapid renaturation with 2 M Tris/HCl (pH 8·0).

Fig. 4. Ligation analysis of BLP DNA on a 0·8% agarose gel showing that BLP DNA termini are blunt. (a) Ligation products produced when reaction conditions favour the ligation of cohesive ends (Ausubel *et al.*, 1995). Lanes: 1, λ-*Hin*dIII DNA as a molecular size standard; 2, purified BLP DNA ligated with a low concentration of T4 DNA ligase (note the absence of ligation products). (b) Ligation products produced when reaction conditions favour the ligation of blunt ends (Ausubel *et al.*, 1995). Lanes: 1, λ-*Hin*dIII DNA as a molecular size standard; 2, total DNA from *B. bacilliformis* (JB584) showing both chromosomal and BLP DNA; 3, purified BLP DNA treated as in lane 4 but without T4 DNA ligase; 4, purified BLP DNA with a high concentration of T4 DNA ligase (note the presence of multiple ligation products).

(Fig. 3, lane 4). Identical treatment of lp16, a linear plasmid from *Borrelia burgdorferi* with covalently closed hairpin ends, showed rapid renaturation as did circular plasmid controls (data not shown). These results suggest 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 conditions favouring the joining of either cohesive ends (1 mM ATP, 1 U T4 DNA ligase) or blunt ends (0·5 mM ATP, 20 U T4 DNA ligase) were employed to determine the nature of the BLP DNA termini. When the reaction mixture favoured cohesive-end ligation, no detectable ligation was ob-

served (Fig. 4a, lane 2). However, when the ligation reaction mixture favoured blunt-end ligation, ligation products were clearly visible (Fig. 4b, lane 4). Controls
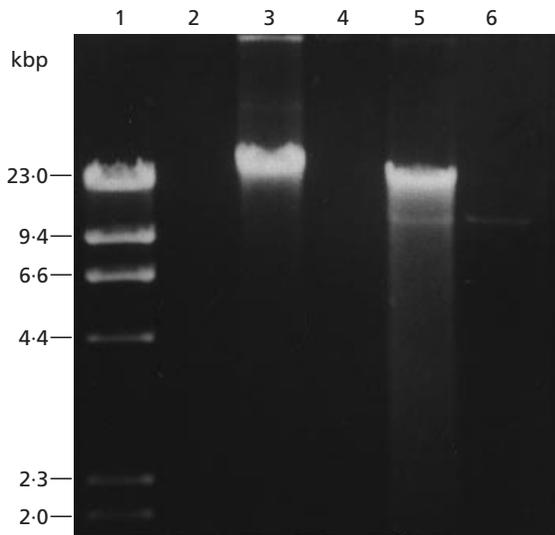
Fig. 5. A 0·8 % agarose gel showing that 'free' (unpackaged) BLP DNA molecules are not detectable within the *B. bacilliformis* cells. Lanes: 1, *λ-Hin*dIII DNA as a molecular size standard; 2, total DNA from *B. bacilliformis* (JB584) incubated with DNase I (2 μg ml^{-1}); 3, DNA from *B. bacilliformis* (JB584) treated with CHCl$_3$ (note the absence of the 14 kbp BLP DNA band); 4, DNA from purified BLPs treated with CHCl$_3$ (note the absence of 14 kbp BLP DNA); 5, DNA from *B. bacilliformis* (JB584) treated with 10 % SDS (note the presence of both chromosomal and BLP DNA); 6, DNA product when purified BLPs are incubated with DNase I (2 μg ml^{-1}) and treated with 10 % SDS immediately prior to electrophoresis.

Fig. 6. Coomassie-brilliant-blue-stained SDS-PAGE gel (12·5 %, w/v, acrylamide) showing *B. bacilliformis* (JB584) whole-cell lysate and BLP-associated proteins. Lanes: 1, molecular mass standards in kDa; 2, *B. bacilliformis* (JB584) whole-cell lysate showing the total protein profile; 3, protein profile of purified BLPs showing three major bands of approximately 32, 34 and 36 kDa and two minor bands of 47 and 49 kDa.

for the ligation experiments included *λ-Hin*dIII DNA fragments (cohesive ends) and pUC19 linearized with *Eco*RV (blunt ends). Under conditions favouring cohesive-end ligation, products were only observed with *λ-Hin*dIII DNA fragments and not with pUC19 linearized with *Eco*RV (data not shown). Additionally, under conditions that favoured blunt-end ligation, products were observed with both controls (data not shown). Taken as a whole, these results suggest that the BLP DNA has blunt ends.

To determine if BLP DNA exists as a naked extra-chromosomal element or if it is associated within a protein coat, chloroform (CHCl$_3$) treatment of *B. bacilliformis* followed by agarose gel electrophoresis analysis of the DNA was performed. Upon analysis, chromosomal DNA was clearly observed, but the 14 kbp BLP DNA could not be detected. However, a band possibly representing unlysed BLPs could be detected in the base of the well (Fig. 5, lane 3). When an equal aliquot of bacteria was treated with SDS to lyse both bacteria and BLPs then analysed by agarose gel electrophoresis, both chromosomal DNA and the 14 kbp BLP DNA were observed with concomitant loss of the band in the base of the well (Fig. 5, lane 5). These results suggest that, within the detection limits of ethidium-bromide staining, all BLP DNA is packaged in a protein coat and is protected from CHCl$_3$ extraction. Additional experiments performed on purified BLPs showed that

only SDS-treated BLPs yield a visible 14 kbp band (Fig. 5, lane 6). These data suggest that the BLPs encapsidate their DNA inside a chloroform-resistant, SDS-sensitive protein coat, or capsid, much like a true bacteriophage.

BLP proteins. To investigate the polypeptide composition of the BLP coat, purified BLP protein profiles were examined. Three major bands of approximately 32, 34 and 36 kDa and two minor bands of approximately 47 and 49 kDa were observed on Coomassie-blue-stained SDS-PAGE gels (Fig. 6, lane 3) and are also found within the total cell lysate at high concentration (Fig. 6, lane 2). These major proteins have a similar molecular mass to those analysed for *B. henselae* BLPs (Anderson *et al.*, 1994; Bowers *et al.*, 1998). A corresponding immunoblot was also performed to determine if the BLP proteins are immunogenic in humans. Although detection by patient convalescent serum occurred, the level of detection was very faint, suggesting that the BLP proteins are modestly immunogenic (data not shown).

Packaging of host DNA into BLPs. A previous report showed that BLPs from *B. henselae* package host DNA in a 'near-random' fashion (Anderson *et al.*, 1994). To
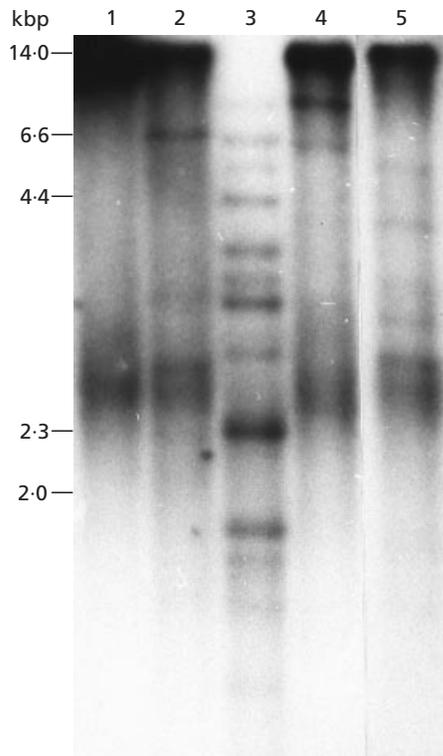
Fig. 7. Southern blot showing a RFLP analysis of BLP DNA using various restriction endonucleases. Electrophoretically separated BLP DNA fragments were transferred to nitrocellulose and probed using ^{32}P-labelled BLP DNA. Molecular size standards are indicated on the left. Lanes: 1, uncut BLP DNA migrating at 14 kbp; 2, *Bam*HI-digested BLP DNA; 3, *Hin*dIII-digested BLP DNA; 4, *Eco*RI-digested BLP DNA; 5, *Cla*I-digested BLP DNA.

assess the randomness of host DNA packaging by *B. bacilliformis* 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), distinct and repeatable banding patterns were observed on agarose gels (data not shown). In an effort to intensify the RFLP bands, Southern blots were done and probed with [32P]dCTP-labelled BLP DNA; a representative autoradiograph is shown in Fig. 7. Although the sum of the individual bands in each digest is greater than 14 kbp, a smear was never observed. These data suggest that the 14 kbp BLP DNA is heterogeneous but not completely random in nature. Southern blot analysis using *Hin*dIII-cut *B. bacilliformis* DNA and ^{32}P-labelled *B. bacilliformis* DNA as a probe showed multiple hybridization signals. In contrast, relatively few bands hybridized when ^{32}P-labelled BLP DNA was used as a probe. Taken as a whole, these data suggest that packaging is far less random in *B. bacilliformis* BLPs than previously reported for *B. henselae* BLPs (Anderson *et al.*, 1994).

To further analyse the specificity of packaging by *B. bacilliformis* BLPs, total genomic DNA preparations from *B. bacilliformis* (JB584) were Southern blotted and probed with various loci from the host chromosome, including the invasion associated locus B gene (*ialB*), the flagellin gene (*fla*), the gyrase B gene (*gyrB*) and the 16S–23S rDNA intergenic spacer region. An additional probe consisting of the plasmid pBBR1MCS was used on KB484 to determine if BLPs package plasmid DNA. The resulting blots (not shown) showed that the BLPs package *ialB* and one of the 16S–23S rDNA intergenic spacers, but do not package *fla* or *gyrB*. As predicted, the shuttle plasmid pBBR1MCS was not packaged. These data further confirm that packaging is non-random.

Transduction experiments

To explore the potential role that BLPs play in horizontal gene transfer, site-directed mutants of *Bartonella* were generated to obtain a selectable, antibiotic-resistance marker that could be used to track the BLP-mediated transfer of genes from one micro-organism to another. To accomplish this, we first needed to establish a site on the host chromosome that was not only packaged by BLPs, but also could be mutated without lethal effects on the bacterium. The 16S–23S *ITS* region was chosen for three reasons. First, three target loci exist on the *B. bacilliformis* chromosome. Second, inactivation of one of the rDNA operons would not likely be lethal since two remain. Finally, sequence conservation in this area exists across *Bartonella* species, facilitating future interspecies transduction experiments.

Development of Kan[R] *B. bacilliformis* mutants. An internal fragment of the 16S–23S rDNA operon was excised from pKRT3 (Minnick *et al.*, 1994) with *Bam*HI and *Sal*I restriction enzymes. The same enzymes were used to create compatible ends on the *Bartonella* suicide vector pUB1 (Battisti & Minnick, 1999). Ligation of the resulting fragments produced suicide vector pKB1, which was subsequently electroporated into JB584 and allowed to homologously recombine with the host chromosome. After 10 d, DNA from two Kan[R] *B. bacilliformis* mutants was analysed by agarose gel electrophoresis, PCR and Southern blotting. Both strains, KB584 and KB585 (Table 1), possessed the 14 kbp BLP DNA (Fig. 8a, lanes 3–4, respectively). Southern blot analysis of the two mutants using ^{32}P-labelled *nptI* as a probe showed that KB585 contained the Kan-resistance gene in both the chromosome and the 14 kbp BLP fragment (Fig. 8b, lane 4) whereas the other mutant, KB584, lacked BLP packaging of the locus (Fig. 8b, lane 3). These data indicated that allelic exchange had occurred and a trackable marker, *nptI*, was integrated into the *B. bacilliformis* chromosome. More importantly, BLPs from KB585 were packaging the mutagenized locus and its nested marker.

Coincubation of KB585 and KB484. Coincubation of KB585 and KB484 was performed to mimic a natural transduction event between two *B. bacilliformis* strains. This experiment led to five new *B. bacilliformis* strains, KB686 (A–E), which demonstrate both a Kan[R] and Cam[R] phenotype. However, PCR analysis (using both Kan and Cam primer sets) revealed the presence of only
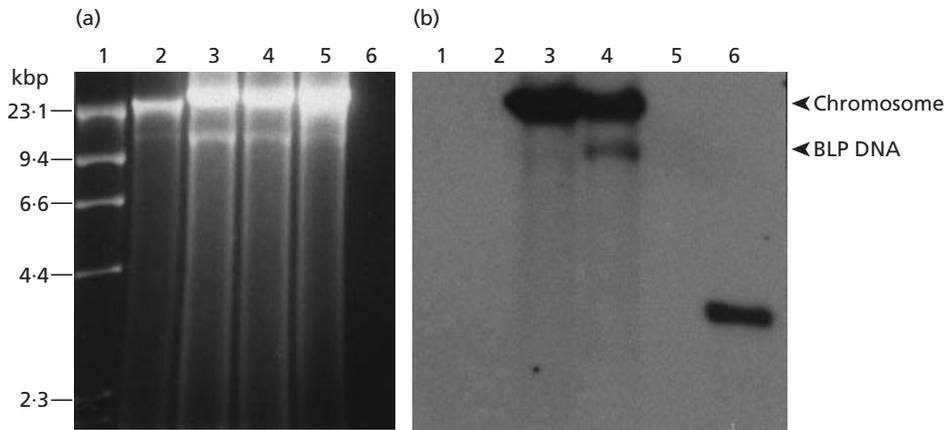
Fig. 8. DNA hybridization analysis showing BLP packaging of an integrated Kan-resistance gene. (a) Total DNA from *B. bacilliformis* showing both the chromosomal and BLP DNA, resolved on a 0·8 % agarose gel. Lanes: 1, λ-*Hind*III DNA as a molecular size standard; 2, total DNA from JB584; 3, total DNA from KB584 (mutant 1); 4, total DNA from KB585 (mutant 2); 5, same as lane 2; 6, linearized suicide plasmid used to generate the *B. bacilliformis* mutants and containing the Kan-resistance gene (*nptI*). (b) Corresponding Southern blot of the gel in (a) probed with [^{32}P]dCTP-labelled *nptI*. Note that while both mutants (KB584 and KB585) contain a chromosomal copy of *nptI* (lanes 3 and 4), only KB585 possesses BLPs that package the marker (lane 4).

the Kan-resistance gene, indicating that spontaneous antibiotic resistance to Cam produced the observed phenotype rather than transduction (data not shown).

Infection of JB584 or KB484 with purified BLPs from KB585. In an effort to rule out the possibility of conjugation or spontaneous Cam resistance, purified BLPs from strain KB585 were used in an attempt to transduce JB584 and KB484. Following an incubation period of approximately 28 d, no colonies were observed on HIAB containing either Kan or a combination of Kan and Cam. A modification of these experiments was also done wherein purified BLPs were added to the JB584 or KB484 bacteria, and the mixtures were incubated in the recovery broth for 24 h prior to plating. However, these experiments met with failure.

Electroporation of JB584 or KB484 with purified BLP DNA. In the event that BLPs are non-infectious, the attachment and injection stage of the virus life cycle was circumvented by direct introduction of BLP DNA into *B. bacilliformis* by electroporation. High concentrations of KB585 BLP DNA (1–3 µg) were electroporated into either JB584 or KB484, and the resulting electroporation mixture was plated on growth medium containing the appropriate antibiotic supplement(s). However, this technique also met with failure.

DISCUSSION

B. bacilliformis is a small, Gram-negative, intracellular parasite of human erythrocytes and endothelial cells. Although this haemotrophic bacterium is endemic to the Andes region of South America, 13 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. Horizontal transfer of genes encoding virulence deter-

minants may be a contributor to emergence. It is possible that transduction – an important means of gene transfer – may be facilitated by the BLPs found in association with many *Bartonella* species. BLPs have previously been described for *B. henselae* (Anderson *et al.*, 1994; Bowers *et al.*, 1998); however, little is known regarding the BLPs associated with *B. bacilliformis*. Thus the basis for this study 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 BLP DNA was detected in a variety of *Bartonella* species, this study focuses on the basic characteristics of the BLPs from *B. bacilliformis*. We describe an extracellular particle associated with *B. bacilliformis* that is round to icosahedral in shape and approximately 80 nm in diameter. The particle is similar to those observed before (Anderson *et al.*, 1994; Umemori *et al.*, 1992); however, it is approximately twice the previously reported diameter, probably due to swelling during the uranyl acetate staining procedure (Ackermann *et al.*, 1978). In contrast to observations made by Umemori *et al.* (1992), who reported tail structures in the *B. bacilliformis* phage, we observed no such structures in BLPs from any *B. bacilliformis* strain. Thus our results are more similar to those of Anderson *et al.* (1994), who found no tail structures in the *B. henselae* BLPs.

A 14 kbp extrachromosomal DNA element was observed in total DNA from five *Bartonella* species. No 14 kbp extrachromosomal DNA elements were seen in *B. clarridgeiae*, *B. elizabethae* or *B. vinsonii* DNA, suggesting that these *Bartonella* species are not infected or have been cured of BLPs. The 14 kbp extrachromosomal DNA was not detectable in CHCl₃-treated

B. bacilliformis cells, suggesting that it is not retained as a naked element, but rather it is packaged into a protein head or capsid. Additionally, BLP DNA was not digested with DNase I until the BLPs were disrupted with SDS, providing further evidence that BLP DNA is being sequestered into a protective protein head. In this respect, BLPs appear to be similar to other generalized transducing bacteriophages.

To characterize the ends of the BLP DNA molecules, alkaline denaturation followed by rapid neutralization as well as two ligation techniques were employed. First, BLP DNA was shown to poorly renature following alkaline denaturation (Fig. 3). In contrast, a covalently closed linear plasmid (lp16) from *Borrelia burgdorferi* or a covalently closed circular plasmid rapidly renatured following the same treatments. These observations suggest that the BLP DNA ends are not covalently closed. Secondly, ligation of BLP DNA molecules to one another (or to themselves) only occurred under reaction conditions that favoured blunt-end ligation (Fig. 4). These data suggest that the BLP DNA termini consist of blunt ends, an observation that coincides with that of Anderson *et al*. (1994), who reported that *B. henselae* BLP DNA was able to ligate to *Bam*HI linkers.

SDS-PAGE analysis of the BLP-associated proteins revealed three prominent bands with molecular masses of 32, 34 and 36 kDa and two minor bands with molecular masses of 47 and 49 kDa (Fig. 6). BLP-associated proteins of similar mass were also observed in two different strains of *B. henselae* and one strain of *B. bacilliformis* by Anderson *et al*. (1994). Recently, one of the capsid genes that encodes a protein from *B. henselae* BLPs, termed Pap31, was isolated and sequenced (Bowers *et al*., 1998). 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 a capsid. It is likely that synthesis of capsid proteins and a mechanism necessary for packaging the 14 kbp fragments of host DNA emanates from genes contained within the host chromosome as previously hypothesized (Anderson *et al*., 1994).

Near-random packaging of 14 kbp segments of host chromosome occurs in *B. henselae* BLPs (Anderson *et al*., 1994). However, RFLP and Southern blot analysis of BLP DNA from *B. bacilliformis* suggest that packaging by these BLPs is somewhat more selective. Although RFLP analysis revealed several BLP DNA fragments when subjected to various restriction endonucleases, the banding pattern was distinct and did not form a smear (Fig. 7), as observed in the heterogeneous mixture of chromosomal DNA packaged by the *B. henselae* BLPs (Anderson *et al*., 1994). Likewise, when *B. bacilliformis* chromosomal DNA was completely digested with *Hin*dIII, transferred to nitrocellulose and probed with [³²P]dCTP-labelled chromosomal DNA, numerous bands were detected on the resulting autoradiograph creating a smear. In contrast, when *B. bacilliformis* chromosomal DNA was probed with [³²P]dCTP-labelled BLP DNA, a smearing effect was not observed;

i.e. distinct banding was detected on the resulting autoradiograph. These data suggest that packaging of *B. bacilliformis* chromosomal DNA into BLPs is non-random and confined to certain loci on the host chromosome. Perhaps one of these fragments contains 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* to determine if virulence determinants, as well as other genes, were being packaged into the BLPs. In keeping with packaging data on *B. henselae* BLPs (Anderson *et al*., 1994), *B. bacilliformis* BLPs efficiently package at least one of the three rDNA operons in the *B. bacilliformis* chromosome. Furthermore, we discovered that at least one of the three 16S–23S *ITS* regions contained within the *B. bacilliformis* chromosome is packaged by BLPs from a mutagenized strain (KB585) (Fig. 8). Additional studies using DNA hybridization revealed that the *gyrB* gene and the *fla* gene were not packaged by the *B. bacilliformis* BLPs. However, the *ialB* gene, located in the middle of a known virulence gene cluster (Minnick *et al*., 1996), was shown to be packaged into the BLPs. Thus BLPs may contribute to horizontal gene transfer of virulence determinants via transduction; an activity that could facilitate the recent emergence of *Bartonella* species and bartonellosis.

Transduction has never been shown for any *Bartonella* species. Therefore, we completed a series of experiments designed to provide a foundation to investigate the potential role that BLPs play in generalized transduction. The first experiment was to insert an antibiotic-resistance marker into a BLP-packaged locus on the *B. bacilliformis* chromosome such that it could be used to track the transduction of antibiotic resistance to sensitive strains. However, several unsuccessful attempts were made to demonstrate transduction by *B. bacilliformis* BLPs. One possible explanation as to why we were unable to demonstrate transduction is superinfection immunity. This phenomenon occurs when a lysogen is exposed to a mature bacteriophage that is similar to the integrated prophage (Voyles, 1993), and has been thoroughly studied and documented for both λ and T4 phage infection of *E. coli* (Yarmolinsky & Sternberg, 1988; Voyles, 1993). For example, λ phage blocks expression of incoming virus DNA by utilizing cytosolic repressor proteins. T4 phage prevents further infection by degrading incoming virus DNA. Because all *B. bacilliformis* strains examined contained BLPs, these micro-organisms may have a BLP-mediated mechanism that prevents subsequent BLP infection. For this reason, future transduction studies will be directed at producing an indicator strain of *B. bacilliformis* (BLP⁻) or implementing the use of other naturally BLP⁻ *Bartonella* such as *B. vinsonii*, *B. elizabethae* or *B. clarridgeiae*. It is also possible that *B. bacilliformis* harbours nucleases designed for the degradation of linear DNA molecules, as previous attempts by our laboratory to mutagenize two loci with double-stranded or single-stranded linear DNA molecules were unsuccessful (Battisti *et al*., 1998;

Battisti & Minnick, 1999). Finally, the odds of a double-stranded crossover event would be low, especially considering the heterogeneity of the BLP DNA, i.e. only a minor fraction of the packaged BLP DNA would contain the Kan^R::*ITS* locus.

Horizontal gene transfer of virulence determinants has played a major role in the evolution of bacterial pathogens, and it is possible that this process may have contributed to the emergence of bartonellosis. Given the observation that BLPs can package at least one virulence gene, *ialB*, from the *B. bacilliformis* chromosome (Fig. 8), it is possible that they facilitate genetic exchange between *Bartonella*. We are currently investigating the role that BLPs play in mediating genetic exchange among members of the *Bartonella* genus as well as characterizing the packaging mechanism and the BLP genome in hopes of harnessing it as a potential tool in the genetic manipulation of *B. bacilliformis*.

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