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Development of a System for Genetic Manipulation of Bartonella bacilliformis

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Lack of a system for site-specific genetic manipulation has severely hindered studies on the molecular biology of all Bartonella species. We report the first site-specific mutagenesis and complementation for a Bartonella species. A highly transformable strain of B. bacilliformis, termed JB584, was isolated and found to exhibit a significant increase in transformation efficiency with the broad-host-range plasmid pBBRIMCS-2, relative to wild-type strains. Restriction analyses of genomic preparations with the methylation-sensitive restriction enzymes CiaI and Stul suggest that strain JB584 possesses a decm methylase mutation that contributes to its enhanced transformation efficiency. A suicide plasmid, pUB1, which contains a polylinker, a pMB1 replicon, and a nptI kanamycin resistance cassette, was constructed. An internal 508-bp fragment of the B. bacilliformis flagellin gene (fla) was cloned into pUB1 to generate pUB508, a fla-targeting suicide vector. Introduction of pUB508 into JB584 by electroporation generated eight KanR clones of B. bacilliformis. Characterization of one of these strains, termed JB585, indicated that allelic exchange between pUB508 and fla had occurred. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotting, and electron microscopy showed that synthesis of flagellin encoded by fla and secretion/assembly of flagella were abolished. Complementation of fla in trans was accomplished with a pBBRIMCS recombinant containing the entire wild-type fla gene (pBBRFLAG). These data conclusively show that inactivation of fla results in a bald, nonmotile phenotype and that pMB1 and REP replicons make suitable B. bacilliformis suicide and shuttle vectors, respectively. When used in conjunction with the highly transformable strain JB584, this system for site-specific genetic manipulation and complementation provides a new venue for studying the molecular biology of B. bacilliformis.

The Bartonella genus comprises a unique group of intracellular bacteria that employ arthropod-mediated transmission and hemotrophy as common parasitic strategies. Recent taxonomic reclassifications have expanded the number of Bartonella species from one, B. bacilliformis, to 11 based on sequence homology and genetic relatedness. Five of these species are presently considered agents of emerging infectious disease in humans (B. bacilliformis, B. claridgeiae, B. elizabethae, B. henselae, and B. quintana), and the diseases share the symptoms of bacteremia, hemolytic anemia, recurrent fever, and a variety of vascular lesions (for recent reviews, see references 5, 25, and 34).

B. bacilliformis is the etiologic agent of a biphasic disease that is indigenous to the Andes mountain region of South America. Oroya fever is commonly used to describe the first phase, which is characterized by an acute syndrome of fever, malaise, and severe hemolytic anemia (16, 37, 46). Humans exhibit the acute hemolytic phase of disease within 2 to 3 weeks following inoculation of Bartonella into the bloodstream by the bite of a nocturnal sandfly, Lutzomyia verrucarum (20). Subsequent erythrocyte invasions accompany a severe hemolytic anemia that is responsible for the high (40 to 80%) mortality rate observed in the absence of antibiotic therapy (20, 24, 28). The disease has killed over 10,000 humans in recorded time (20, 46). The chronic secondary phase of the disease, termed verruga peruana, develops approximately 4 weeks after the primary phase and is characterized by angiomatic cutaneous eruptions (20). During this phase, the bacteria invade vascular endothelial cells (14, 15, 32) and subsequently stimulate the formation of new blood vessels (14), a common sequela of bartonelloses. Recent reports of several atypical monophasic (verruga peruana) cases of B. bacilliformis in previously disease-free lowland elevations are cause for concern (2, 4).

Although a conjugative system for random Tn5-based mutagenesis has been reported for B. henselae (12), no means of site-directed mutagenesis exists for Bartonella species. This lack has been a major impediment to elucidating the molecular biology of this expanding group of emerging bacterial pathogens and was the impetus for the present study. Here we describe a system for site-specific mutagenesis and complementation of B. bacilliformis. This is the first report of site-specific mutagenesis and complementation for any of the Bartonella species.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Escherichia coli strains used for propagation of plasmids were grown overnight at 37°C in Luria-Bertani medium with antibiotic supplements when required (11). The strains of B. bacilliformis and E. coli used or generated in this study are summarized in Table 1. B. bacilliformis was routinely grown on heart infusion agar (Difco, Detroit, Mich.) supplemented with 5% defibrinated sheep erythrocytes and 2.5% filter-sterile sheep serum (Quad Five, Ryegate, Mont.) at 30°C in a water-saturated atmosphere. Antibiotic supplements for B. bacilliformis included kanamycin sulfate (25 μg/ml), and chloramphenicol (1 μg/ml) (both from Sigma Chemical Co., St. Louis, Mo.) and were used individually or combined depending upon the experimental conditions. Plates were routinely cultured by transferring growth from the surface of a 5-day culture plate to a fresh plate, using an initial plating density of approximately 4,000 CFU/plate. Since no suitable liquid growth medium is currently available for B. bacilliformis, the growth phase of the bacterium at harvest could not be determined. However, to ensure that the bartonelloses were actively growing, colonies were first observed at 3 days and subsequently harvested at 5 days postincubation.

For testing the motility of B. bacilliformis strains, a Bartonella motility medium was devised. The defibrinated sheep erythrocyte supplement of standard Bartonella growth medium was replaced with sheep erythrocyte lysate (8) at a 5%
TABLE 1. Bacterial strains and plasmids used in this study

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<td>This study</td>
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Final concentration to yield a transparent medium suitable for visual scoring, and the agar in the medium was reduced to 0.2% (wt/vol). Plates were poured and dried for 48 h at 22°C and dried for an additional 1 h at 45°C prior to inoculation, to reduce moisture on the surface of the agar.

Preparation and manipulation of DNA. Chromosomal DNA from B. bacilliformis was prepared with CTAB (cetyltrimethylammonium bromide) by the methods of Ausubel et al. (6). Plasmid DNA for cloning was isolated by the method of E. coli (27) by the alkali lysis procedure of Birnboim and Doly (9). Plasmid DNA for electroporation experiments was prepared with a MidipreP kit (Qiagen, Chatsworth, Calif.) or a QIAquick kit (Qiagen). Ligation and transformation of DNA into E. coli DH5\(\alpha\) was done by standard procedures (38). The plasmids used in this study are summarized in Table 1.

DNA hybridization analysis. Genomic DNA from B. bacilliformis strains and plasmid DNA were isolated, digested to completion with appropriate restriction enzymes, and resolved on ethidium bromide-stained agarose gels with either a GeneClean kit (Bio 101, Inc., La Jolla, Calif.) or a QIAquick kit (Qiagen). DNA probes were made by random primer extension with \([\text{32P}]\text{dCTP}\) (New England Nuclear, Boston, Mass.) and were used to probe blots overnight at 50°C as previously described (7). The blots were then washed at high stringency and exposed for 1 h to X-ray film (X-Omat XAR-5; Eastman Kodak Co., Rochester, N.Y.) as previously described (7) to visualize hybridized DNA fragments.

Electroporation. Approximately seven plates of 5-day-cultured B. bacilliformis cells were harvested into 1 ml of heart infusion broth at 4°C. The cells were subsequently washed four times with 1 ml of ice-cold 10% (vol/vol) glycerol in water with intermittent centrifugations at 2,090 \(\times\) g for 20 min at 4°C. The final bacterial concentration was measured with a Petroff-Hauser counter and adjusted to \(10^{10}\) cells/ml with 10% (vol/vol) glycerol. Electrottransformation was performed with a gene pulser with 0.2-cm cuvettes (Bio-Rad Laboratories, Hercules, Calif.) that had been chilled on ice for at least 15 min. In general, a 44-kd bacterial suspension (10\(^4\) cells/ml) was combined with 1 to 2 \(\mu\)l of DNA (1 to 44 \(\mu\)g/\(\mu\)l) and electroporated with an exponential-decay waveform set at a field strength of 125 kV/cm, a pulse time of 5 ms, and capacitance held constant at 25 \(\mu\)F as previously described for Bartonella (19, 36). Immediately following electroporation, cells were removed from the cuvette by being resuspended in 1 ml of ice-cold sterile recovery broth (heart infusion broth containing 0.5% [wt/vol] bovine serum albumin, 5% [vol/vol] sheep erythrocyte lysate [8] and 5 mM I-methionine). The suspension was then transferred to a 15-mL sterile tube and incubated for 14 h at 30°C in a water-saturated atmosphere. This incubation period corresponds to approximately two B. bacilliformis generation times (8) and was used to allow antibiotic resistance marker expression. Transforms were isolated by being plated on standard Bartonella growth medium supplemented with kanamycin and/or chloramphenicol, when required for selection. Antibiotic-resistant colonies usually appeared after 6 to 7 days of incubation at 32°C.

PCR and oligonucleotides. PCR amplification was achieved with a GeneAmp 2400 thermocycler (Perkin-Elmer, Norwalk, Conn.) by procedures developed by Mullis and Faloona (35). Reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 \(\mu\)M each deoxynucleoside triphosphate, 4 \(\mu\)M MgCl\(_2\), 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer), 1 to 100 ng of template DNA, and 0.1 \(\mu\)g of each primer. The reaction proceeded for 30 cycles of 1 min at 94°C, 1 min at 50 to 60°C (depending on the calculated primer melting temperature), and 1 min at 72°C with an initial 5-min denaturation at 94°C and a final 7-min extension at 72°C. Single-stranded oligonucleotide primers specific for the fla gene, FLA5\(_5\) (5'-AAGTCAGCGTAATGC-3') and FLA3\(_9\) (5'-AAATATTCTGGCTGCCCTGATTTGC-3'), were synthesized by The University of Montana Murdock Molecular Biology Facility. The “junction” amplifier set was designed to detect the integration of the pUBS58 suicide plasmid at the fla locus and consisted of primers NPTI\(_5\) and FLA3\(_9\). The target loci for each of the primers are illustrated in Fig. 1.

SDS-PAGE and immunoblotting. Whole-cell extracts of B. bacilliformis were prepared by boiling in sodium dodecyl sulfate (SDS) sample buffer for 10 min and were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (12.5% [wt/vol] acrylamide), using procedures adapted from those of Laemmli (30). Approximately 20 \(\mu\)g of total extract protein was added per lane. Protein bands were visualized by staining with Coomassie brilliant blue (38). For immunoblots, separated proteins were electrophoretically transferred from gels to supported nitrocellulose membranes (pore size, 0.45 \(\mu\)m; Schleicher & Schuell) by the methods of Towbin et al. (43). Immunoblots were developed by using the rabbit anti-flagellin antiserum and procedures described by Scherer et al. (39).

Transmission electron microscopy (TEM). B. bacilliformis was grown and harvested into 1 ml of heart infusion broth at 4°C. The cells were washed three times with 10% (vol/vol) glycerol at 4°C, with intermittent centrifugations at 2,090 \(\times\) g for 20 min at 4°C, and finally resuspended in 10% glycerol. Aliquots of this suspension (15 \(\mu\)l) were placed on Formvar-coated 300-mesh copper-palladium grids (Electron Microscopy Sciences, Fort Washington, Pa.) and incubated for 5 min at 22°C. The grids were then stained with 2% uranyl acetate (pH 7.0) for 3 min, destained with 1 M ammonium acetate (pH 7.0) for 3 min, and washed with deionized water for 1 min. They were then air dried and observed at 75 kV.
FIG. 1. Illustration of suicide plasmid and schematic representation of site-specific fla disruption. (A) The *B. bacilliformis* suicide plasmid pUB1 harbors a multiple cloning site, the kanamycin resistance cassette neomycin phosphotransferase I (*nptI*), and the pMB1 replicon. (B) The flagellin gene-targeting suicide plasmid, derived from pUB1, is shown with the 508-bp *BglII*-KpnI internal fragment of *fla* (*fla*\(_9\)). The transformable strain, JB584 (Kms, *fla*\(_{1}\)), containing the wild-type 1,127-bp *fla* ORF, is shown. Homologous recombination resulted in site-specific insertion of pUB508 at the *fla* locus, generating strain JB585 (Kmr, *fla*\(_9\)). Note the position of the flagellin (FLA\(_5^*\) and FLA\(_3^*\)) and kanamycin (NPTI5\(_{1}\) and NPTI3\(_{1}\)) amplimers, indicated by the small arrows. (Figure not drawn to scale.)
with a 7100 transmission electron microscope (Hitachi, Mountain View, Calif.) located at The University of Montana Electron Microscopy Center.

Cloning the B. bacilliformis flagellin gene (fla). The B. bacilliformis flagellin gene, fla, was chosen as the locus to develop a system of site-specific mutagenesis for two reasons. First, fla exists as a mapped, single-copy gene in B. bacilliformis (29), and mutations in fla are rarely lethal. Second, the phenotype associated with the gene is readily observable by TEM and easily scored by testing for motility.

fla was cloned, sequenced, and submitted to GenBank by another group (3). The fla gene was simultaneously isolated by our laboratory from a AZAP Express (Stratagene Cloning Systems, La Jolla, Calif.) expression library of B. bacilliformis by using rabbit anti-flagellin antisera. A pBR-CMV cosm id clone containing the entire fla gene in a 3,800-bp Sau3AI fragment was subsequently excised from the λ clone as specified by the manufacturer (Stratagene) and termed pAUL1.

Purified pAUL1 was digested with HindIII, and the 2,158-bp fragment containing fla was isolated by agarose gel electrophoresis (1% [wt/vol] agarose) and purified by using a GeneClean II kit (Bio 101). Ligation of this fragment into the HindIII site of pUC18 resulted in pFLA3, a source of fla DNA fragments for constructing the fla-targeting suicide plasmids.

Construction of suicide and shuttle/complementation plasmids. The suicide plasmid pUB1 (Fig. 1A) was constructed in several steps. Previous studies showed that the pUB1 replicon was not recognized by the replicational machinery of Bartonella species (19, 36). Therefore, we reasoned that this replicon could be used to construct a suicide vector for Bartonella. To construct the plasmid, a ~1,500-bp PsflI fragment containing the nptI gene, encoding neomycin-kanamycin resistance, was subcloned from pUCK18 into pUC19, resulting in pUCK19. Subsequently, the β-lactamase (bla) gene of pUCK19 was deleted by removing a 1,118-bp BglII fragment and religated to generate pUB1 (Fig. 1A).

To create a flagellin-specific suicide plasmid for insertional mutagenesis experiments, a 508-bp Kpol-BglII fragment from pFLAG3 containing an internal portion of fla was cloned into pUB1 to produce pUB508 (Fig. 1B).

A complementation shuttle plasmid, pBBRFLAG, was constructed by cloning the 2,158-bp HindIII fragment of pUB1 into the broad-host-range vector pBBR1MCS. The resulting plasmid, pBBRFLAG, contains a chloramphenicol resistance cassette, the entire wild-type fla gene, and a REP replicon which is recognized by the replicational machinery of B. bacilliformis (see below).

**RESULTS**

Isolation of a highly transformable strain. Grasseschi and Minnick previously reported mean transformation efficiencies of 7.8 × 10^3 following electroporation-mediated introduction of the cosmid pEST into B. bacilliformis KC584 (19). pEST is a cosmid harboring an RK2 origin of replication and a neomycin phosphotransferase I (nptI) gene encoding kanamycin resistance (36). We attempted to repeat these experiments with B. bacilliformis KC584 but were unable to isolate stable transformants. Electroporation-mediated introduction of a variety of constructs designed to disrupt genes in wild-type B. bacilliformis KC583 and KC584 were also unsuccessful. These trials included introduction of a variety of linear (double-stranded and single-stranded) and circular gene-targeting constructs designed to mutate genes at two separate loci, fla and gyrB (7). A number of methods designed to either alleviate restriction (13, 21–23, 40, 44) or promote homologous recombination (1, 18, 31) were used in conjunction with the various gene-targeting constructs without success (data not shown).

The discrepancy between the reported transformation efficiencies and those found at the onset of this study suggested that one or more spontaneous mutations probably altered the genetic background of the KC584 strain used by Grasseschi and Minnick (19), resulting in a highly transformable strain of B. bacilliformis. To test this hypothesis, we obtained a frozen stock of the Kan^r pEST-containing strain of B. bacilliformis (herein termed HG584) and cured this strain of the pEST cosmid by three passages, for a total of 15 days, in the absence of kanamycin sulfate. Six randomly selected clones were then subcultured independently and tested for sensitivity to kanamycin sulfate (25 μg/ml). All six clones exhibited wild-type sensitivities to kanamycin, and two of these Kan^r clones were cultivated and their genomic DNA was isolated. DNA hybridization analyses with a 32P-labeled pEST probe did not detect pEST in the genome (data not shown), suggesting that the cosmid was cured from these two strains. Further verification by using PCR and nptI amplifiers (NPTI5’ and NPTI3’) confirmed the absence of the Kan’ marker (see Fig. 2, lane 2).

One of these cured, Kan’ clones was termed JB584.

The transformation efficiency of JB584 was subsequently assessed by electroporation-mediated introduction of pBBR1MCS-2. This plasmid was chosen because it is more stably maintained than the cosmid pEST and the multiple cloning site of pBBR1MCS plasmids enabled efficient cloning for complementation analyses. JB584 demonstrated a transformation efficiency of 5.2 × 10^4 transformants per μg of pBBR1MCS-2, which corresponds to one transformant per 8.4 × 10^3 cells. This value is within the lower range of efficiency reported by Grasseschi and Minnick (19); however, the relatively higher DNA concentrations (1 μg) and the different replicon used in the present study may account for the lower relative efficiency. Electroporation lethality, previously estimated at 31% (19), was not considered here or in the previous study when calculating the transformation efficiency of B. bacilliformis. Here, our focus was not on optimizing pBBR1MCS-2 electroporation but simply on isolating a strain that efficiently serve as a host for allelic exchange experiments. Taken as a whole, these results demonstrated that the genetic background of the KC584 host strain used by Grasseschi and Minnick (19) was distinct from wild-type KC584 and suggested that JB584 would be an efficient host for allelic exchange experiments.

We hypothesized that the increased transformability of JB584 was due to one or more spontaneous mutations in the restriction-modification system, whereby restriction of introduced foreign DNA was reduced to a level that permitted plasmid replication and maintenance. To test this hypothesis, we compared genomic digests of KC583, KC584, JB584, HG584, JB85, and JB686 by using the methyl-sensitive restriction enzymes StuI (dcm sensitive) and CiaI (dam sensitive), with BamHI (methyl insensitive) as a positive control. Subsequent agarose gel electrophoresis revealed that StuI was able to digest genomic DNA from strains HG584, JB584, JB85, and JB686 but was unable to digest DNA from KC583 or KC584. These data strongly suggest that a dcm methylase is active in wild-type strains KC583 and KC584 and that the sites of methylation overlap the StuI recognition sequence. In contrast, CiaI and BamHI digestion of genomic DNA was evident in all of the strains mentioned and there was no apparent difference in activity among any of the strains. There was no significant difference in the growth rate, colony morphology, or overt phenotypes between strains KC584 and JB584, suggesting that JB584 was suitable for use as a host strain for mutagenesis experiments. These results, combined with the previously mentioned discrepancies between the transformation efficiencies, strongly suggest that loss of dcm methylase activity occurred during the multiple passages of the KC584 strain used in the Grasseschi and Minnick (19) study and also suggest that JB584 and HG584 have essentially the same restriction-modification system genetic alterations and the curing event itself did not alter this genetic background. Because this highly passaged KC584 strain was no longer viable, we were resigned to curing the previously transformed strain (HG584) and using the resulting strain (JB584) for our subsequent manipulation studies.

Characterization of Kan’ mutants by PCR. Eight kanamycin-resistant clones were isolated following electroporation-mediated introduction of pUB508 into JB584. Three amplifier sets, designated nptI, fla, and junction, were used to character-
bromide staining. DNA size markers (lane M) are shown to the right. Resolved by agarose gel electrophoresis (1% agarose) and visualized by ethidium (FLA5 and FLA3) were respectively used to detect the kanamycin cassette, the flagellin gene, and the junction between fla and the inserted pUB508. Bars below the gel indicate the amplimer set used in each reaction. Amplimer sets and the respective DNA templates used in this analysis are as follows: lane 1 (NPTI5 and NPTI3, Fla5 and Fla3, no template), lane 2 (NPTI5 and NPTI3; JB584), lane 3 (NPTI5 and NPTI3; JB585), lane 4 (FLA5 and FLA3; JB584), lane 5 (FLA5 and FLA3; JB585), lane 6 (NPTI5 and FLA3; JB584), lane 7 (NPTI5 and FLA3; JB585), lane 8 (NPTI5 and FLA3; pUB508 + JB584), lane 9 (NPTI5 and FLA3; JB585), lane 10 (FLA5 and FLA3; JB584), lane 11 (NPTI5 and FLA3; JB586). PCR products were resolved by agarose gel electrophoresis (1% agarose) and visualized by ethidium bromide staining. DNA size markers (lane M) are shown to the right.

The genotype of the eight Kan’ clones (refer to Fig. 1B for a schematic representation of amplifier target loci).

First, the nptI amplimer set (NPTI5 and NPTI3) generated a product in all eight Kan’ strains, consistent with the expected 983-bp size (data not shown). This verified that the Kan’ phenotype exhibited by these strains was not a result of natural mutation. The transformation efficiency was approximately 3.3 transformants per μg of pUB508, corresponding to one stable integrant per 1.6 × 10⁶ cells. Figure 2 shows the product generated by the nptI amplimer set from one of these Kan’ transformants, termed strain JB585 (Fig. 2, lane 3), and its absence from the parent strain, JB584 (lane 2).

Second, the fla amplimer set (FLA5 and FLA3) generated the expected 1,304-bp flagellin PCR product with the parent strain JB584 (Fig. 2, lane 4) and, in contrast, was absent from the Kan’ strain JB585 (lane 5). This showed that the homologous recombination event occurred at the fla locus in JB585. Of the eight Kan’ strains, two were analyzed with the fla amplimer set, and neither generated a product. We were unable to generate the expected ~4.2-kb product resulting from amplification of the entire mutagenized locus spanning the inserted pUB508, even when the elongation time was increased to 2.5 min.

Finally, the junction amplimer set (NPTI5 and FLA3) was used to verify the hypothesized chromosomal fusion between the inserted nptI and the mutagenized fla. The ~2,300-bp product generated from JB585 (Fig. 2, lane 9), as well as its absence from the parent strain (lane 6), confirms that the insertion of pUB508 occurred at fla as illustrated in Fig. 1B. In addition, as controls, pUB508 (lane 7) and a mixture of pUB508 and JB584 DNA (lane 8) were amplified with the junction amplimer set to further substantiate these results, since neither reaction produced an amplicon.

Characterization of the complemented mutant by PCR. To develop a Bartonella system for in trans complementation, we restored the wild-type flagellin phenotype to a fla mutant. Plasmid pBBRFLAG, containing fla, a chloramphenicol resistance cassette, and a REP origin, was introduced into JB585 by electroporation. Transformants were selected on medium containing kanamycin sulfate (25 μg/ml) and chloramphenicol (1 μg/ml) and resulted in the isolation of strain JB686. Initial confirmation of in trans complementation was accomplished by electrophoretic analysis of PCR products. The fla amplimer set (FLA5 and FLA3) was used to detect the presence of the plasmid-located fla gene, which was absent in the fla mutant strain JB585 (Fig. 2, lane 5) but was present in both JB584 and the complemented mutant JB686 (lanes 4 and 10, respectively). Finally, the ~2,300-bp product generated by the junction amplimer set (NPTI5 and FLA3) demonstrated that the chromosomal fla mutation was still present in the complemented mutant strain, JB686 (lane 11).

Characterization by DNA hybridization. To further substantiate the genotype of the mutant and trans-complemented strains, high-stringency DNA hybridizations were performed (Fig. 3). Southern blots probed with the 32P-labeled wild-type fla PCR product (generated with the FLA5 and FLA3 amplimers) produced a distinct two-band hybridization pattern in ClaI-digested genomic DNA in strains containing the disrupted fla gene, i.e., JB585 (Fig. 3, lane 4) and JB686 (lane 5). In addition, the pBBRFLAG complementation plasmid is clearly visible as a separate genetic element (Fig. 3A, lane 5).

Analysis of flagellin production in generated strains by SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting were used to determine the effect of the pUB508 insertion on the synthesis of flagellin in the mutant strain, JB585, and the complemented strain, JB686 (Fig. 4). The wild-type 1,127-bp B. bacilliformis fla open reading frame (ORF) encodes a 42-kDa polypeptide (39). When whole-cell lysates were visualized by SDS-PAGE, the flagellin polypeptide was clearly synthesized in strain JB584 (Fig. 4A, lane 1). In contrast, the fla mutant, JB585 (lane 2), lacked the 42-kDa flagel-
lin polypeptide, suggesting that flagellin synthesis had been disrupted. Finally, the 42-kDa flagellin polypeptide was clearly evident in the trans-complemented strain (lane 3), indicating that fla expression and synthesis was occurring from the plasmid locus. Immunoblot analysis was subsequently performed by reacting whole-cell lysates with rabbit anti-flagellin polyclonal antiserum. The immunoblot confirmed the presence of the flagellin polypeptide in strains JB584 and JB686 (Fig. 4B, lanes 1 and 3, respectively) and also demonstrated that flagellin synthesis was completely abolished in the mutant strain JB585 (lane 2). A truncated flagellin product was not observed by reacting whole-cell extracts of parent, flagellin mutant, and trans-complemented mutant strains were separated by SDS-PAGE (12.5% polyacrylamide) and stained with Coomassie brilliant blue. The 42-kDa flagellin polypeptide is present in the parent strain JB584 (lane 1) and is absent from the kanamycin-resistant flagellin mutant strain JB585 (lane 2). Flagellin synthesis is detected in trans from the complementation plasmid pBBRFLAG in strain JB686 (lane 3). (B) Corresponding immunoblot reacted with rabbit anti-flagellin polyclonal antiserum, indicating that flagellin synthesis is restored in the trans-complemented strain. Molecular mass standards are indicated to the left (in kilodaltons), and the 42-kDa flagellin polypeptide is marked by the arrow.

Motility phenotypes of generated strains. Initially, phase-contrast microscopy was used to examine wet mounts of each strain. By using this method, a loss of motility was observed for strain JB585, whereas strains KC584, JB584, and JB686 exhibited indistinguishable motility phenotypes. Motility was subsequently assessed by the ability of the bacterium to spread within Bartonella motility medium. To develop the assay, we tested a wild-type strain in motility medium with agar concentrations ranging from 0.2 to 0.8% by stabbing the medium with an inoculation needle from 5-day-old cultures. Incubation for 7 days at 30°C demonstrated that agarose concentrations above 0.6% inhibited motility. However, at agarose concentrations of 0.4 and 0.2%, motility produced a uniform halo of growth within the medium away from the site of inoculation. The strains generated in this study were subsequently tested in the same way by using Bartonella motility medium containing 0.2% agar. Multiple inoculations with each of the strains consistently generated indistinguishable and uniform halos of growth for strains KC584, JB584, and JB686, indicating that neither the modified genotype resulting in enhanced transformability (JB584) nor fla expression from an extrachromosomal locus (JB686) has a detectable effect on motility. In contrast, the mutant strain, JB585, was nonmotile and did not produce a halo (data not shown).

In conclusion, site-directed insertion of pUB508 at the fla locus of JB584 generated the fla mutant, JB585, which lacks flagellin expression and is nonmotile. Subsequent electroporation-mediated introduction of pBBRFLAG into strain JB585 generated the trans-complemented strain, JB686, which has a motility phenotype that is indistinguishable from KC584.
DISCUSSION

The ability to genetically manipulate an organism is essential for a better understanding of its molecular biology. In vivo genetic manipulation of a bacterium generally consists of two fundamental techniques, transformation and mutagenesis. These techniques utilize constructs consisting of circular plasmids or linear fragments of DNA that possess genetic elements specific to the manipulation desired. These DNA constructs can be introduced into the bacterium by natural (conjugation and transduction) or artificial (electroporation and chemical) methods. In this study, we used electroporation to introduce a variety of plasmids (both replicative and nonreplicative) and linear DNA fragments and were able to demonstrate plasmid transformation, site-directed mutagenesis, and complementation in trans within B. bacilliformis.

Plasmid transformation was first demonstrated in Bartonella by electrotransformation of the cosmid pEST into B. (previously Rochalimaea) quintana (36) and was subsequently accomplished in B. bacilliformis by Grasseschi and Minnick using the same cosmid (19). In both studies, the Bartonella species recognized the RK2 origin of replication but did not recognize pMB1, ColE1, or F origins. These observations, combined with our results, suggested that pUB1 (harboring the pMB1 origin) could be used as a suicide plasmid for all Bartonella species. In the course of our research, we determined that the most consistent replicon for high transformation efficiencies in B. bacil- liformis was the broad-host-range vector pBBR1MCS (27) and its derivatives (26), which contain the REP origin of replication.

Random mutagenesis by using chemical methods, UV light, or transposons is designed to generate nonspecific mutations that are subsequently selected by phenotypic or biochemical means. The major drawbacks to random mutagenesis are the difficulty in mutant isolation and the generation of secondary nonspecific mutations. These anomalous secondary mutations are of special concern when assessing the pathogenesis of an organism, since the virulence potential of a specific gene and gene product are in question relative to a wild-type background. Dehio and Meyer recently reported successful conjugation between E. coli and B. henselae as a means of plasmid transfer and delivery of Tn5 transposons on suicide plasmids for random gene inactivation (12).

Site-specific mutagenesis, the focus of this study, consists of two general methods, replacement recombination and insertional recombination, wherein single mutations are introduced at a specific genomic locus. Replacement recombination involves linear fragments of DNA designed so that during homologous recombination two crossover events result in the replacement of a target locus with the construct. We attempted replacement recombination with both single- and double-stranded linear DNA targeting two loci, gyrB (7) and fla, without success. Even when the highly transformable strain, JB584, was used as the host, replacement recombination was not achieved. However, insertional recombination with a circular segment of DNA, where a single homologous recombination event inserts the entire element into the chromosome, was successful in demonstrating site-specific genetic manipulation, as described in Results.

After several initial attempts to mutagenize the flagellin gene failed, we realized that there were in vivo barriers impeding homologous recombination in B. bacilliformis. This prompted us to try alternative methods for alleviating restriction-modification systems as well as biochemical and metabolic manipulations previously shown to increase the likelihood of homologous recombination. Although numerous manipula-
clones that are capable of maintaining a replicative plasmid, such as pBRB1MC-2. Subsequent curing of these potential methylase restriction mutants can result in a well-defined, transformable host strain for subsequent mutagenesis experiments.

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