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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 dcm 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 Kan^r 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. clarridgeiae, 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 hematic 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 invasion accompanies 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 angiomatous 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, Rye Gate, 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 bartonellose were actively growing, colonies were first observed at 3 days and subsequently harvested at 5 days postinoculation.

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%
Bovine serum albumin, 5% (vol/vol) sheep erythrocyte lysate and 5 mM ice-cold sterile recovery broth (heart infusion broth containing 0.5% (wt/vol) solution, cells were removed from the cuvette by being resuspended in 1 ml of previously described for

12.5 kV/cm, a pulse time of 5 ms, and capacitance held constant at 25 \( \mu \text{F} \), for 20 min at 4°C. The final concentration to yield a translucent 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 \( \textit{B. bacilliformis} \) was prepared with CTAB (cetyltrimethylammonium bromide) by the methods of Ausubel et al. (6). Plasmid DNA for cloning was isolated from \( \textit{E. coli} \) by the alkaline lysis procedure of Birnboim and Doly (9). Plasmid DNA for electroporation experimentation was prepared with a Midi-Prep kit (Qiagen, Chatsworth, Calif.) or a QiAQuick kit (Qiagen). Ligation and transformation of DNA into \( \textit{E. coli} \) DH5a was done by standard procedures (38). The plasmids used in this study are summarized in Table 1.

**DNA hybridization analysis.** Genomic DNA from \( \textit{B. bacilliformis} \) 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). Removal and transformation of DNA from \( \textit{E. coli} \) DH5a was done by standard procedures (38). The plasmids used in this study are summarized in Table 1.

**Electroporation.** Approximately seven plates of 5-day-cultured \( \textit{B. bacilliformis} \) 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 \( \times \text{g} \) 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. Electroporation was performed with a gene pulser (Bio-Rad Laboratories, Hercules, Calif.) that had been chilled on ice for at least 15 min. In general, a 44-\( \mu \text{F} \) capacitance held constant at 25 \( \mu \text{F} \) as previously described for \( \textit{Bartonella} \) (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 1-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 \( \textit{B. bacilliformis} \) generation times (8) and was used to allow antibiotic resistance marker expression. Transformants were isolated by being plated on standard \( \textit{Bartonella} \) 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 30°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 \( \mu \text{M} \) each deoxynucleoside triphosphate, 4 mM MgCl\(_2\), 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer), 1 to 100 ng of template DNA, and 0.1 \( \mu \text{g} \) 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 \( \textit{fla} \) gene, FLA-1 (5'-AAAGTTTAGAGATTTGTGTCGA-3') and FLA-2 (5'-AAATATCGGCTGCCTGACCTGTC-3'), and the kanamycin cassette, NPTI (5'-AGGCCACGTGTTGTCTCCTCAAAATCTC-3') and NPTII (5'-CGTCCGGGCAAGTAGCGCGGAATGTAATGGAC-3'), were synthesized by The University of Montana Murdock Molecular Biology Facility. The “junction” amplimer set was designed to detect the integration of the pUB508 suicide plasmid at the \( \textit{fla} \) locus and consisted of primers NPTI and FLA-3. The target loci for each of the primers are illustrated in Fig. 1.

**SDS-PAGE and immunoblotting.** Whole-cell extracts of \( \textit{B. bacilliformis} \) 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 \( \mu \text{g} \) 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 \( \mu \text{m} \); 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).** \( \textit{B. bacilliformis} \) cells were 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 \( \times \text{g} \) for 20 min at 4°C, and finally resuspended in 10% (vol/vol) glycerol. Aliquots of this suspension (15 \( \mu \text{l} \)) 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 (fla9). The transformable strain, JB584 (Kms, fla1), 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). Note the position of the flagellin (FLA5' and FLA3') and kanamycin (NPTI5' and NPTI3') amplifiers, 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 cloned as a 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 ZAP Express (Stratagene Cloning Systems, La Jolla, Calif.) expression library of *B. bacilliformis* by using rabbit anti-flagellin antiserum. A pBR-CMV cosmid 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 (Strategene) and termed pAIL1.

Purified pAIL1 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 pLAG3, a source of *fla* DNA fragments for constructing the *fla*-targeting suicide plasmid.

**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 *Pst*I fragment containing the *nptI* gene, encoding neomycin-kanamycin resistance, was subcloned from pUC18 into pUC19, resulting in pUC19. Subsequently, the β-lactamase (βl) gene of pUC19 was deleted by removing a 1,118-bp *Bgl*II fragment and religated to generate pUB1 (Fig. 1A). To create a flagellin-specific suicide plasmid for insertion mutagenesis experiments, a 508-bp *Kpn*1-BglI 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 pAIL1 into the broad-host-range vector pBBR1MCS-2. The resulting plasmid, pBBRFLAG, contains a chloramphenicol resistance cassette, the entire wild-type pBBR1MCS-2, which corresponds to one transformant among 103 transformants per μg of pBBR1MCS-2, which corresponds to one transformant per 8.4 × 106 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*.

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, JB585, and JB686 by using the methyl-sensitive restriction enzymes *Stu*I (*dem* sensitive) and *Cla*I (*dam* sensitive), with *Ban*HI (methyl insensitive) as a positive control. Subsequent agarose gel electrophoresis revealed that *Stu*I was able to digest genomic DNA from strains HG584, JB584, JB585, and JB686 but was unable to digest DNA from KC583 or KC584. These data strongly suggest that a *dem* methylase is active in wild-type strains KC583 and KC584 and that the sites of methylation overlap the *Stu*I recognition sequence. In contrast, *Cla*I and *Ban*HI 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 *dem* 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 Kanr 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 characterize...
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. 3B, 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-
flagellin 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 in the mutant strain analyzed by this procedure.

Ultrastructural characterization of strains by TEM. TEM was used to visualize the secretion and assembly of flagella in each of the strains (Fig. 5). Electron micrographs showed that the wild-type strain, KC584 (Fig. 5A), and the transformable strain, JB584 (Fig. 5B), maintained the normal synthesis, secretion, and assembly of flagellin polypeptides, resulting in the wild-type lophotrichous flagella. Second, as evidenced by the lack of flagellar filaments, the flagellin ORF of strain JB585 had been insertionally disrupted and the synthesis, secretion, and assembly of the flagellin polypeptide into filaments was abolished (Fig. 5C). Finally, not only did the complemented mutant, strain JB686, synthesize flagellin polypeptides from the plasmid locus, but also these polypeptides were secreted and assembled (Fig. 5D) as in the wild-type strain (Fig. 5A).

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. bacilliformis* 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. Delio 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 manipulations were attempted, the generation of strain JB584 was the critical step toward successful mutagenesis. It is likely that a spontaneous mutation occurred in a multiply-passaged KC584 strain used by Grasseschi and Minnick (19), allowing them to obtain exaggerated “wild-type” transformation efficiencies. By curing this *Kan ’HG584 strain of pEST*, which resulted in JB584, we obtained a strain with a significant increase in transformation efficiencies, which encouraged us to use this strain as the parent for successful mutagenesis experiments.

In addition to being sequence specific, restriction enzymes such as *StuI and CiaI* are methylation sensitive, where restriction occurs only in the absence of methylation, enabling the organism to recognize self and nonself. While the genotype of JB584 is not fully known, genomic DNA extracted from this strain differed from that of the wild-type strain in that it was *StuI* sensitive, indicating a different methylation pattern and suggesting that a *dem* methylase gene had been mutated. Since the altered methylation pattern did not result in lethal self-restriction of JB584, we speculate that a second mutation in the cognate restriction enzyme had occurred and that a lack of restriction enzyme activity explained the enhanced transformability of the strain. Alternatively, and more probably, a single, spontaneous mutation event involving the deletion of closely linked restriction and modification genes would also explain the phenotype of JB584. It is likely that wild-type strains of *B. bacilliformis* retain both an active *dem* methylase and the cognate restriction enzyme and that they digest foreign DNA, but strains such as JB584 have lost the cognate restriction enzyme to ensure survival and do not digest foreign DNA. However, a full characterization of the specific restriction endonuclease mutation(s) in strain JB584 enabling higher transformation efficiencies remains to be determined.

The hybridization data generated in this study suggest that the *B. bacilliformis* flagellar filament is encoded by a single flagellin gene, whereas many bacteria possess multisubunit flagella. Furthermore, the data produced in this study conclusively show that inactivation of a single *fla* gene completely abolishes synthesis of the flagellum and generates a nonmotile and nonflagellated (bald) strain. The lophotrichous flagella of *B. bacilliformis* and the high degree of motility that they impart have been implicated as virulence determinants in several reports (8, 33, 39, 45). The strains generated in this study provide the molecular means to assess Koch’s postulates and thus more fully define the role of flagella in the pathogenesis of *B. bacilliformis*. Using the highly transformable strain, JB584, the pBBR1MCS shuttle vectors, and the *fla*-specific suicide plasmid, we have demonstrated site-specific mutagenesis and complementation for the first time in any *Bartonella* species. Two additional loci (*ialB* and the 16S–23S rDNA intergenic spacer) have subsequently been manipulated in our laboratory by using this system.

The *Bartonella* species are notoriously difficult to manipulate genetically. We have developed a system for electroporation-mediated site-specific mutagenesis and complementation for *B. bacilliformis*. The pBBR1MCS series of broad-host-range vectors were shown to be useful shuttle vectors for *B. bacilliformis*, and recent work in our laboratory with *B. quintana* shows that the REP origin is functional in other *Bartonella* species. We have constructed a suicide vector (pUB1) with a polynucleotide, which is ideally suited for manipulating any desired target gene in *B. bacilliformis*. As stated above, a spontaneous or natural mutation was apparently present in the host strain used by Grasseschi and Minnick (HG584) (19) and resulted in the inflated transformation efficiencies that they reported. The data produced in this study suggest that a strain of *Bartonella* with enhanced transformability can be isolated by selecting...
clones that are capable of maintaining a replicative plasmid, such as pBBR1MCS-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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REFERENCES