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Isolation and Characterization of FecA- and FeoB-Mediated Iron Acquisition Systems of the Spirochete *Leptospira biflexa* by Random Insertional Mutagenesis

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The specific mechanisms by which *Leptospira* spp. acquire iron from their ecological niches are unknown. A major factor contributing to our ignorance of spirochetal biology is the lack of methods for genetic analysis of these organisms. In this study, we have developed a system for random transposon mutagenesis of *Leptospira biflexa* using a *mariner* transposon, *Himar1*. To demonstrate the validity of *Himar1* in vivo transposon mutagenesis in *L. biflexa*, a screen of mutants for clones impaired in amino acid biosynthesis was first performed, enabling the identification of tryptophan and glutamate auxotrophs. To investigate iron transporters, 2,000 *L. biflexa* transposon mutants were screened onto media with and without hemin, thus allowing the identification of five hemin-requiring mutants, and the putative genes responsible for this phenotype were identified. Three mutants had distinct insertions in a gene encoding a protein which shares homology with the TonB-dependent receptor FecA, involved in ferric citrate transport. We also identified two mutants with a *Himar1* insertion into a *feoB*-like gene, the product of which is required for ferrous iron uptake in many bacterial organisms. Interestingly, the growth inhibition exhibited by the *fecA* and *feoB* mutants was relieved by deferoxamine, suggesting the presence of a ferric hydroxamate transporter. These results confirm the importance of iron for the growth of *Leptospira* and its ability to use multiple iron sources.

Iron is a cofactor in a wide range of biological reactions in both eukaryotes and prokaryotes. This element is therefore an essential nutrient to support the growth of most organisms. However, despite the relative abundance of iron in nature, oxidation creates insoluble iron complexes that are unavailable to bacteria. To satisfy their iron needs, bacteria have evolved different strategies, such as the direct binding of iron-containing proteins encountered in their ecological niches to specific outer membrane receptors. The ability to acquire iron and iron complexes has also long been recognized as an important determinant of bacterial virulence (5).

The genus *Leptospira* belongs to the order *Spirochaetales* and is composed of both saprophytic and pathogenic members, such as *Leptospira biflexa* and *Leptospira interrogans*, respectively (18). We lack a fundamental understanding of most aspects of the biology of *Leptospira* spp. Surprisingly, the genome sequences of the pathogenic spirochetes *Borrelia burgdorferi* and *Treponema pallidum* were found to contain no identifiable iron-protein-encoding genes. In addition, *B. burgdorferi* is able to grow normally under severe iron-limited conditions (24). These obligate parasites have overcome host iron limitation by eliminating genes that encode proteins requiring iron as a cofactor. In contrast, iron is an essential nutrient for *Leptospira* (10), and several iron-protein-encoding genes were found in the genome sequences of the pathogen *L. interrogans* (20, 26). In addition, the genome sequences suggest that this bacterium possesses several iron acquisition systems (20, 26). A better knowledge of the iron uptake systems in this intriguing group of organisms is essential to understand the basis of pathogenic mechanisms (5).

Analysis of gene function in spirochetes is hampered not only by their slow growth, but mainly by a paucity of genetic tools. This problem has been alleviated somewhat in recent years by the development of genetic tools for use with *Leptospira* saprophyte members (2, 12, 22, 23, 27, 31). However, to our knowledge, genetic transfer attempts in pathogenic *Leptospira* spp. have failed to date. An important approach for investigating metabolism processes is the generation of large numbers of mutant bacteria. We chose to develop a mutagenesis system that used the transposable element *Himar1*, a member of the *mariner* family of transposons, originally isolated from the horn fly. *Himar1* (i) has no apparent need for host accessory factors (17), (ii) has been demonstrated to transpose in both eukaryotes and prokaryotes (14), and (iii) apart from the dinucleotide TA, has no specific sequence requirements for its insertion (14). We therefore reasoned that *Himar1* would likewise insert randomly within the genome of the saprophyte *L. biflexa*, and here we demonstrate that to be the case. To validate this random-mutagenesis approach, we first used a screen for amino acid auxotrophs. We have characterized some of these auxotrophs as insertions in genes involved in tryptophan and glutamate biosynthesis. Finally, we addressed the issue of iron acquisition systems by screening a transposon mutant library of *L. biflexa* and identifying hemin-requiring mutants. We recently demonstrated that *L. biflexa* is able to use exogenous heme proteins (12), an important source of iron for bacteria. The use of hemin as a phenotypic screen should therefore reveal mutants that are affected in other iron assimilation systems, such as the ones identified in the genome of the pathogen *L. interrogans*. Genetic analysis of hemin-requiring mutants showed that they were defective in either FecA- or

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FeoB-mediated iron acquisition systems. Functional analysis confirmed this prediction and revealed fundamental insights about iron acquisition in *Leptospira* spp.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. biflexa* serovar Patoc strain Patoc I (National Reference Center for Leptospira, Paris, France) was grown at 30°C in EMJH (9, 15) medium. Kanamycin, when required, was added to the culture medium or plates at 40 μg/ml.

Construction of *L. biflexa* transposon libraries. *L. biflexa* was grown in liquid EMJH to logarithmic phase, a density of 5×10^8 bacteria/ml (optical density at 420 nm, >0.25). Bacterial cells were pelleted at 4,000 × *g* for 20 min at 20°C, washed in water, and then concentrated to 10^{11} bacteria/ml. To deliver the transposon *Himar1* to *L. biflexa*, between 100 and 500 ng of plasmid pSC189 (7) was added to 50 μl of freshly prepared competent cells and transferred into a prechilled 0.2-cm-diameter cuvette. Electroporation was done in a Gene Pulser (Bio-Rad Laboratories, Richmond, CA) as previously described (27), and then 1 milliliter of EMJH liquid medium was immediately added to the cuvette and the cells were incubated at 30°C for 24 h with shaking.

Isolation of *L. biflexa* auxotrophs and complementation studies. After the recovery period following electroporation with *Himar1*, transformants were spread on EMJH containing kanamycin and either 50 μM hemin or 0.5% Casamino Acids solution (Becton Dickinson and Company, Sparks, MD). Since Casamino Acids contain low concentrations of tryptophan, glutamine, cysteine, and asparagine, these amino acids were added at a final concentration of 0.2 mM. Colonies appearing on these plates after 7 to 10 days were replica plated to Casamino Acids- or hemin-EMJH and EMJH plates. Those colonies which failed to grow on EMJH plates but did grow on the same medium supplemented with Casamino Acids or hemin were restreaked on EMJH plates to confirm their auxotrophy. When necessary, additional tryptophan (1 mM), glutamate (1 mM), hemin (10 to 100 μM), deferoxamine (10 to 100 μM; Desferal; Novartis), and ferric dicitrate (100 to 500 μM) were added to EMJH solid and liquid media. To reduce the available iron in EMJH medium, $FeSO_4$ was omitted during the preparation of the EMJH medium or bacteria were grown in EMJH medium that had been preincubated overnight with 0.1 mM 2,2′-dipyridyl (Sigma-Aldrich, Saint Louis, MO).

For complementation studies, the *trpC* (1,605-bp), *fecA* (3,302-bp), and *feoB* (2,954-bp) loci of *L. biflexa* were amplified by PCR (primer nucleotide sequences are available on request) and ligated into the SmaI blunt-ended site of the spectinomycin-resistant *L. biflexa-E. coli* shuttle vector pGSLe24 (M. Picardeau, unpublished results). Complementation of the *L. biflexa trpE* mutant was also performed with plasmid pGSLtrpE (2).

Identification of transposon insertion sites in the *L. biflexa* genome. The flanking DNA of the *Himar1* insertion site was deduced by ligation-mediated PCR (25) using EcoRI (5′-AATTGCTCGTGC-3′), BamHI (5′-GATCGCTCG TGC-3′), AseI (5′-TAGCTCGTGC-3′), and ClaI (5′-CGGCTCGTGC-3′) adaptors as described previously (23). Amplification was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) using primer LKgd (5′-TAGAGTATTCCTCAAGGCACGAGC-3′), which is sequence specific to adaptors, and primer LMR1 (5′-TTTATAATCACCGTCATGGTC-3′) or LMR2 (5′-GCGTCTAGGCGGCCGCGAAG-3′), directed upstream and downstream from *Himar1*, respectively. Amplification was achieved using one cycle of denaturation (94°C; 5 min), followed by 35 cycles of amplification consisting of denaturation (94°C; 30 s), annealing (51°C; 30 s), and primer extension (72°C; 1 min), and a final cycle of extension of 10 min at 72°C. PCR products were directly sequenced with primer LKgd at Genome Express (Meylan, France).

A genomic library of *L. biflexa* was prepared in pCC1FOS according to the manufacturer's instructions (Epicentre, Madison, WI). Briefly, *L. biflexa* genomic DNA was randomly sheared by nebulization to ~40-kb fragments, and the insert DNAs were end repaired and ligated into pCC1FOS as described by the manufacturer. The ligated DNA was packaged with MaxPlax Lambda Packaging Extracts to form the fosmid library. The library was transduced into *Escherichia coli* EPI300 and spread on Luria-Bertani agar plates containing 12.5 μg/ml chloramphenicol and 0.01% arabinose. Recombinant colonies were transferred onto a nylon filter (N^+ Hybond; Amersham Biosciences, Little Chalfont, England) as previously described (28). The filters were hybridized overnight at 60°C in Rapid Hybridization buffer (Amersham Biosciences) with radiolabeled probes (the nucleotide sequences of primers used for the amplification of probes are available on request) and then washed at 60°C as previously described (23). Fosmid DNAs of candidate clones were purified using the Plasmid Miniprep kit (QIAGEN GmbH, Hilden, Germany). For sequencing, fosmid DNAs were sequenced with an ABI PRISM BigDye Terminator cycle-sequencing ready-reaction kit and a 3700 or a 3730 Xl Genetic Analyzer (Applied Biosystems) at the Genomics Platform (Pasteur Genopole Île-de-France, Technological Platforms). The DNA sequence data were analyzed with the BLAST program (1) at the National Center for Biotechnology Information.

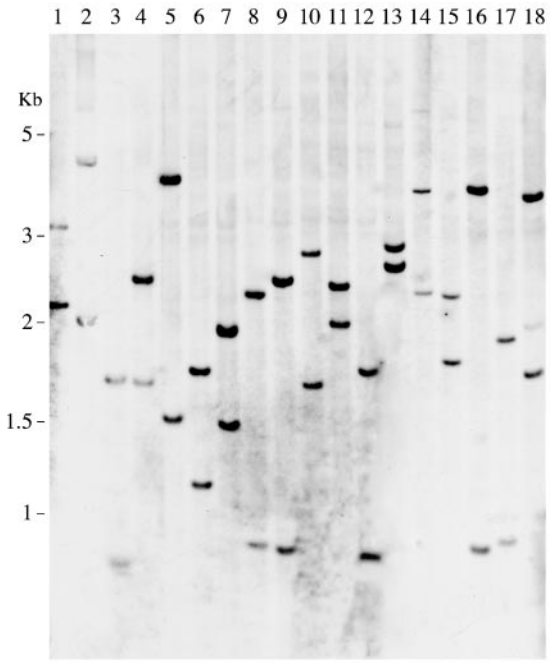

FIG. 1. Southern blot analysis of kanamycin-resistant clones of *L. biflexa* transformed with pSC189. Lanes 1 to 18, DraI-digested genomic DNAs of randomly chosen clones. The Southern blot was hybridized with the radiolabeled pSC189 plasmid. Sizes are indicated on the left.

Nucleotide sequence accession numbers. The nucleotide sequences of loci containing the *L. biflexa* genes *hkl*, *trpC*, *trpE*, and *gltB* were deposited under accession numbers AY828564, AY828565, AY828566, and AY828568, respectively. The sequences of the *L. biflexa fecA* and *feoB* genes were deposited into GenBank under accession numbers AY744288 and AY744289, respectively.

RESULTS

Transposition of *Himar1* in the *L. biflexa* genome is efficient, random, and stable. Plasmid pSC189 (7) was used to deliver the kanamycin-resistant *Himar1* transposon in *L. biflexa*. Among several independent experiments, ~5×10^3 kanamycin-resistant colonies per μg of plasmid DNA were obtained in *L. biflexa*, suggesting the feasibility of *Himar1* transposition in *Leptospira* spp. Southern blot hybridization of 50 randomly chosen kanamycin-resistant colonies showed the transposition to be random. Because of one DraI site in *Himar1*, there are two variable fragments, indicating that a single random-transposition event had occurred (Fig. 1). To further confirm the randomness of insertion, the *Himar1* flanking sequences of 40 kanamycin-resistant colonies, distinct from colonies selected for Southern blotting, were sequenced. Again, the results showed no apparent consensus sequence beyond the required TA dinucleotide. Southern blot analysis of two insertion mutants subcultured with and without antibiotic selection for 6 months (corresponding to ~500 generations) showed that

TABLE 1. *L. biflexa* mutants isolated by random insertional mutagenesis

Clone (mutant)	*Himar1* insertion site (TA)[a]	BLAST results (% identity)[b]	Putative interrupted ORF[c]	Auxotrophic phenotype
Casamino Acids screen				
B	tgacgtcttTAtttgatatt	*L. interrogans* indole-3-glycerol phosphate synthase (54)	*trpC* (Prom) AY828565	Tryptophan
C	taggttatgaTAttgaggaatt	*L. interrogans* signal transduction histidine kinase (43)	*hkl*[d] (5′) AY828564	Glutamate
D	aacaaagagTAtgcgggcgt	*Bradyrhizobium japonicum* glutamate synthase (53)	*gltB* (5′) AY828568	Glutamate
E	caaggtatgTAtgatcctgc	*B. japonicum* glutamate synthase (53)	*gltB* (5′) AY828568	Glutamate
G	catagtacaTAtgaggtgaa	*L. interrogans* anthranilate synthase component I (85)	*trpE* (3′) AY828566	Tryptophan
Hemin screen				
L	tgatgaacgTAggtctgcaa	*L. interrogans* iron(III) dicitrate TonB-dependent receptor (49)	*fecA* (3′) AY744288	Hemin
P	gacagacaaTAtggaaatct	*L. interrogans* iron(III) dicitrate TonB-dependent receptor (49)	*fecA* (5′) AY744288	Hemin
Q	tggaatgttTAataacaatc	*L. interrogans* iron(III) dicitrate TonB-dependent receptor (49)	*fecA* (5′) AY744288	Hemin
V	agtaaaaaaTAaacttttga	*L. interrogans* ferrous iron transport protein B (52)	*feoB* (3′) AY744289	Hemin
W	ctttgcattTAcaagaacaa	*L. interrogans* ferrous iron transport protein B (52)	*feoB* (5′) AY744289	Hemin

[a] *Himar1* flanking sequences (lowercase) were deduced by ligation-mediated PCR as described in Materials and Methods. Each clone contains a unique inserted transposon, as demonstrated by Southern analysis.
[b] Best BLAST score of the putative ORF interrupted by the *Himar1* insertion.
[c] In parentheses, location of *Himar1* in the coding sequence; Prom, location of *Himar1* in the *trpC* promoter region. The GenBank accession number of the *L. biflexa* locus is indicated.
[d] *hkl*, histidine kinase from *L. biflexa*.

Himar1 insertions were stable in the *L. biflexa* genome (data not shown).

Genetic and biochemical investigations in *Leptospira* spp. demonstrated that these bacteria have the potential to synthesize the essential amino acids (2, 6, 20, 22, 26). As an initial test of the utility of this random-mutagenesis system, we screened 1,000 *L. biflexa* transposon mutants for amino acid auxotrophy. Table 1 summarizes the genotypes and growth characteristics of five auxotrophic mutants. In mutant B, *Himar1* was found in a putative gene encoding a protein with similarities to an anti-sigma factor antagonist 55 bp upstream from the start codon of *trpC*. Transposon insertion into this unusual system for regulating tryptophan synthesis, or into the *trpC* promoter region, may be responsible for this auxotrophic phenotype. Another mutant exhibited *Himar1* insertion into *trpE*. Growth restriction of the *trpE* and *tprC* mutants on EMJH was relieved by tryptophan or by complementation with the *L. biflexa trpE* and *trpC* wild-type genes (data not shown). In mutant C, the gene encoding a putative histidine kinase was interrupted by *Himar1*, resulting in glutamate auxotrophy as with the *gltB* mutants (Table 1). Interestingly, a previous study showed that the disruption of a histidine kinase from *Corynebacterium glutamicum* significantly reduced the level of glutamate synthase (GOGAT; glutamine 2-oxoglutarate amidotransferase; EC 1.4.1.13) activity (29). In bacteria, GOGAT, the subunits of which are encoded by the genes *gltB* and *gltD*, usually has a major role in ammonium turnover. In *E. coli*, *gltB* mutants were unable to utilize a number of amino acids as sources for nitrogen (21). Further study of the *L. biflexa gltB* mutants should allow analysis of the physiological role of GOGAT activity in *Leptospira* spp.

Isolation of hemin-requiring mutants. A total of 2,000 mutants from an *L. biflexa* random-transposon mutant library was screened for auxotrophy by replica plating them onto medium with or without added hemin. This yielded five mutants that required the hemin supplement for growth (Table 1). Sequence analysis of insertion sites revealed genes that had been implicated in iron acquisition systems in other bacteria. In three cases, the transposon inserted into the *fecA* homologue, which encodes the outer membrane ferric citrate transporter. Screening of a genomic library of *L. biflexa* allowed the identification of the complete open reading frame (ORF) encoding 821 amino acids (the mature protein consists of 797 amino acids). *L. biflexa* FecA shows similarities to putative outer membrane receptor proteins of *L. interrogans* (49% identity; 69% similarity), *Bdellovibrio bacteriovorus* (28% identity; 50% similarity), *Rubrivivax gelatinosus* (30% identity; 47% similarity), and *Pseudomonas aeruginosa* (23% identity; 39% similarity). *L. biflexa* FecA also exhibits 22% identity (165/740) and 40% similarity (296/740) to *E. coli* FecA, an Fe^{3+}-dicitrate transporter requiring an energy transduction complex constituted by the cytoplasmic proteins TonB, ExbB, and ExbD (4). Three regions of the amino acid sequence of *L. biflexa* FecA exhibit extensive sequence similarities to conserved motifs of TonB-dependent receptors (motifs I, II, and III in Fig. 2): PGA (positions 104 to 106), FRG (positions 122 to 124), and KGSGSILFGPSTIGGVVN (positions 168 to 185) (33), suggesting that this protein is a TonB-dependent outer membrane receptor. The structure of TonB-dependent receptors usually consists of a monomeric β-barrel made of 22 β-strands derived from the C terminus and a plug domain from the N terminus (33). Most of the 26 conserved residues found in the structurally described TonB-dependent receptors (33) were also found in *L. biflexa* FecA, i.e., 12/14 in the plug domain and 10/12 in the barrel domain.

Mutants V and W showed inactivation of a gene whose product shows homologies with FeoB, involved in ferrous iron acquisition (Fig. 3); the highest BLAST scores were found for *L. interrogans* (52% identity; 69% similarity), *Cytophaga hutchinsonii* (38% identity; 59% similarity), *Porphyromonas*

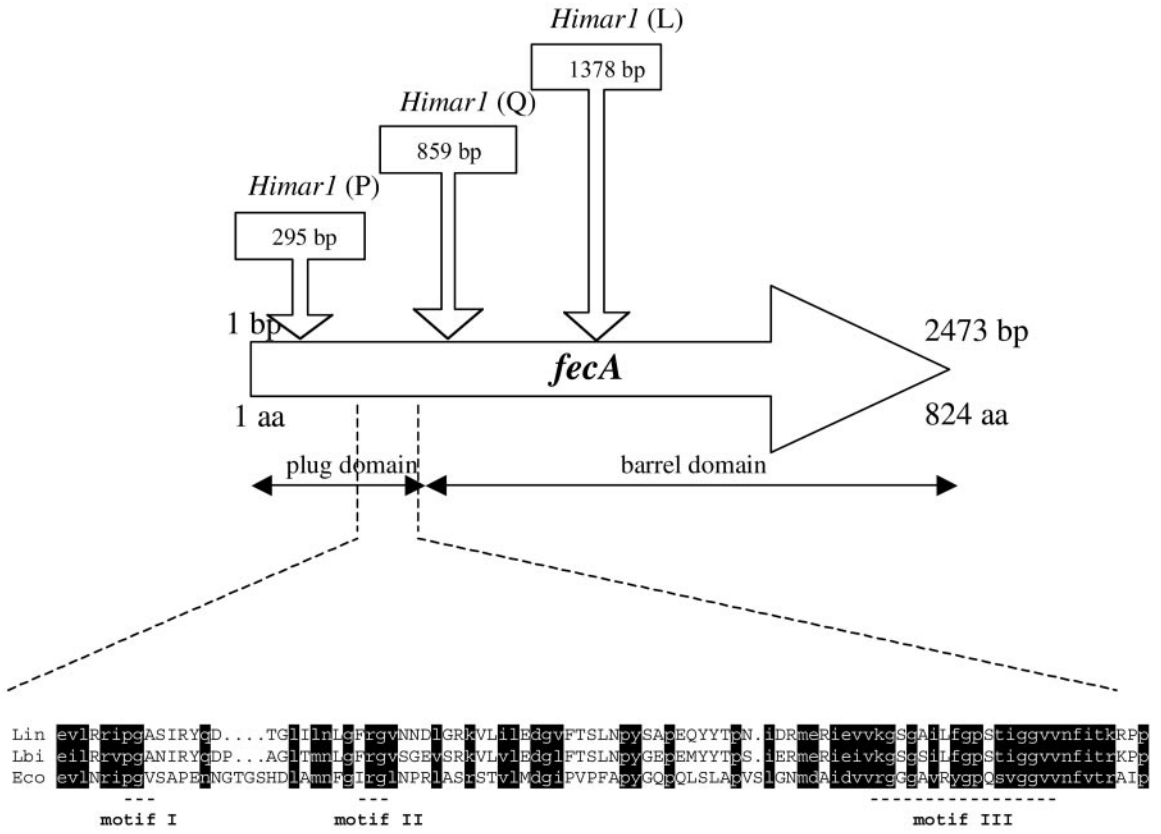

FIG. 2. Schematic representation of the *L. biflexa fecA* gene and its encoded protein. The locations of the *Himar1* insertion site in the mutants L, P, and Q are indicated. A sequence alignment of the N-terminal region in FecAs of *E. coli* (Eco), *L. biflexa* (Lbi), and *L. interrogans* (Lin) containing the three conserved motifs (I, II, and III) common to most of the TonB-dependent receptors is also shown. Residues conserved in the three homologous proteins are shaded and shown in lowercase. aa, amino acids.

gingivalis (35% identity; 56% similarity), and *Azotobacter vinelandii* (35% identity; 52% similarity). *L. biflexa* and *E. coli* FeoB proteins show 32% identity and 51% similarity. The cytoplasmic membrane protein FeoB of *E. coli* is a GTPase with sequence similarities to eukaryotic G proteins (19). The G protein-like motifs are necessary for ferrous uptake, but their functions remain to be elucidated. Similar to *E. coli* FeoB, *L. biflexa* FeoB consists of a hydrophilic domain at the N terminus that contains G protein-like motifs: motif G1, GNPNCGKT (positions 10 to 17); motif G2, VTV (positions 36 to 38); and motif G3, DLPG (positions 56 to 59) (Fig. 3). In *L. biflexa*, this hydrophilic domain is followed by a hydrophobic C-terminal domain consisting of at least eight α-helical membrane-spanning segments (Fig. 3).

The EMJH medium used for growth of leptospira is extremely complex and includes undefined components. Since iron is essential for the growth of these bacteria, regular EMJH medium contains iron sulfate (9, 15). The iron chelator dipyridyl or iron sulfate-deficient EMJH medium was used to produce iron-limited conditions that inhibited the growth of wild-type and mutant strains. Addition of 10 μM hemin, 10 μM deferoxamine, and 100 μM ferric dicitrate restored the ability of the wild-type strain to grow under iron starvation conditions, suggesting that *L. biflexa* is able to use these compounds as sole iron sources. Similarly, addition of exogenous hemin or deferoxamine in EMJH and iron depleted EMJH restored growth of *feoB* and *fecA* mutants. In contrast, ferric citrate was not able to restore growth of *feoB* and *fecA* mutants at the wild-type rate, suggesting that these mutants are not able to acquire iron supplied as ferric dicitrate under conditions of iron deficiency. Finally, the *L. biflexa-E. coli* shuttle vector containing the wild-type *fecA* and *feoB* genes allowed the functional complementation of the *L. biflexa fecA* and *feoB* mutants, respectively (data not shown).

DISCUSSION

We have developed a system for in vivo transposition of the *L. biflexa* genome with a *mariner*-based transposable element and constructed mutant libraries. The utility of the *Himar1* mutagenesis system is illustrated by the ease with which we obtained auxotroph mutants (Table 1). Surprisingly, a limited range of amino acid biosynthetic pathways have been affected by this transposon mutagenesis system, suggesting that the use of Casamino Acids selects a subset of amino acid auxotrophs. This may reflect an artifact of the screening system due to EMJH and Casamino Acids constituents.

It has been known for decades that iron is an essential nutrient requirement for *Leptospira* (10). Here, we explore how this bacterium acquires the iron that it needs for growth.

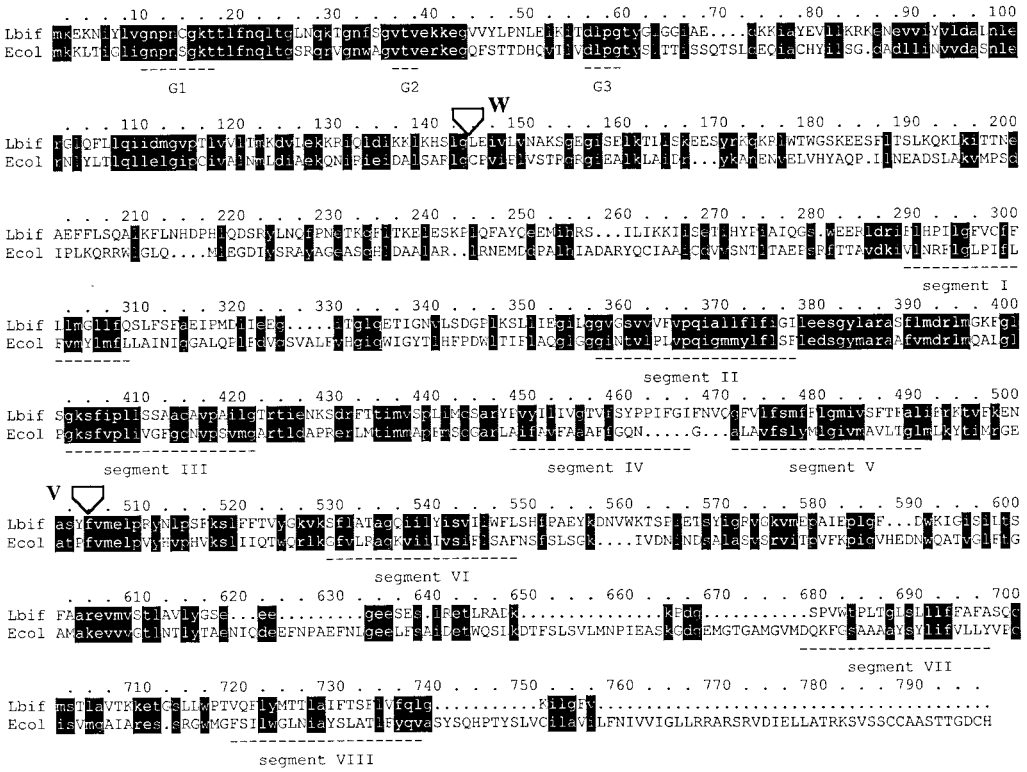

FIG. 3. Sequence alignment of FeoB proteins of *E. coli* and *L. biflexa*. The three conserved G motifs in the N-terminal region are indicated. Candidate membrane-spanning segments of *L. biflexa* FeoB are also indicated. Arrows map the *Himar1* insertion site in mutants V and W. Residues conserved in the two homologous proteins are shaded and shown in lowercase.

The order *Spirochaetales* constitutes one phylum of bacteria, and thus, they are different from either gram-positive or gram-negative bacteria. Although the membrane composition differs substantially from that of gram-negative bacteria, spirochetes possess a double-membrane structure composed of the peptidoglycan-cytoplasmic membrane complex, the periplasm, and the outer membrane (13). Gram-negative bacteria take up various iron sources into the periplasm against their concentration gradients. The energy is provided by an energy-transducing complex consisting of the TonB, ExbB, and ExbD proteins. Similarly, the *L. interrogans* genome harbors genes encoding two distinct TonB systems (one locus where *exbB*, *exbD*, and *tonB*-like genes are contiguous on each chromosome) that can interact with seven putative TonB-dependent receptors (20, 26). In this study, we show that *L. biflexa* is able to take up deferoxamine, which belongs to the group of hydroxamate siderophores that are produced by various bacteria, including enterobacteria. The *L. interrogans* genome exhibits an ORF (LA2641) displaying similarities to receptors for hydroxamate-type siderophores, such as *E. coli* FhuA (25% identity and 42% similarity on 720 residues) and *Yersinia enterocolitica* FoxA (25% identity and 42% similarity on 673 residues). The three conserved motifs found in TonB-dependent receptors are also found in this protein. Although no siderophore-encoding genes were found in the *L. interrogans* genome, saprophytic strains may be able to take up siderophores produced by other bacterial species.

Another *L. interrogans* TonB-dependent receptor (LA3149) exhibits similarities (37% to 39%) to the heme receptors HmuR, HemR, and HutA of *Yersinia pestis*, *Y. enterocolitica*, and *Vibrio cholerae*, respectively. The *L. interrogans* HemR-type receptor contains the amino acid motif FRA $P(X_{10})H(X_9)N(X_2)L(X_2)E$, containing the His461 residue specific to heme receptors (3). The presence of a putative heme oxygenase in the *L. interrogans* genome (LB186), which is currently an uncommon feature of bacteria, may serve to catabolize and detoxify heme.

In this study, we identified *L. biflexa* mutants with *Himar1* insertion into a *fecA*-like gene. The ferric dicitrate iron transport system Fec has been well characterized in enterobacteria (4). The *E. coli* FecA protein has been crystallized, revealing a monomeric transmembrane β-barrel that forms a pore, which is plugged from the periplasm by a globular N-terminal domain (11). Direct physical interaction between TonB and FecA occurs via TonB boxes located at the N termini of all TonB-dependent receptors. However, the N-terminal extension of FecA, which is involved in the regulation of the *fec* transport genes in *E. coli* (4), was not found in *Leptospira*. In addition, there is no evidence of *fecBCDE* homologues, suggesting an alternative pathway for ferric dicitrate transport.

We also identified a TonB-independent receptor for iron acquisition, called FeoB. The inability of *feoB* mutants to transport ferric dicitrate and iron sulfate suggests that FeoB is involved in the uptake of both Fe^{2+} and Fe^{3+} which could imply an Fe^{3+}-reductase as previously demonstrated in *Helicobacter pylori* (32). Amino acid comparison of the conserved

domains of FeoB receptors revealed domains containing invariant residues (19). These residues were also conserved in the *L. biflexa* FeoB receptor (Fig. 3), further suggesting that these domains are essential for iron uptake.

Several different routes for the translocation of iron across the cytoplasmic membrane are possible in bacteria (16). These distinct iron acquisition systems must be tightly regulated in *Leptospira* spp. In many bacteria, genes involved in iron acquisition are transcriptionally regulated by the availability of iron (5). Although we identified Fur homologues in the *L. interrogans* genome, we were not able to find putative Fur boxes in the upstream region of the start codons of genes that could be involved in iron uptake. Recently, Cullen et al. showed that several *L. interrogans* genes encoding outer membrane proteins were regulated in response to growth with iron (8).

In conclusion, the ability to use multiple sources of iron attests to the importance of iron for the survival of *Leptospira*. We have also demonstrated that it is possible to generate and identify large numbers of *L. biflexa* mutants using transposon mutagenesis. Similarly to a recent study on the spirochete *B. burgdorferi* (30), we showed that this mutagenesis approach yields a randomly distributed set of insertion mutations throughout the genome, which can be screened for phenotypes affecting diverse aspects of metabolism and physiology.

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