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Comparative and Functional Genomic Analyses of Iron Transport and Regulation in *Leptospira* spp.▽†

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The spirochetes of the *Leptospira* genus contain saprophytic and pathogenic members, the latter being responsible for leptospirosis. Despite the recent sequencing of the genome of the pathogen *L. interrogans*, the slow growth of these bacteria, their virulence in humans, and a lack of genetic tools make it difficult to work with these pathogens. In contrast, the development of numerous genetic tools for the saprophyte *L. biflexa* enables its use as a model bacterium. *Leptospira* spp. require iron for growth. In this work, we show that *Leptospira* spp. can acquire iron from different sources, including siderophores. A comparative genome analysis of iron uptake systems and their regulation in the saprophyte *L. biflexa* and the pathogen *L. interrogans* is presented in this study. Our data indicated that, for instance, *L. biflexa* and *L. interrogans* contain 8 and 12 genes, respectively, whose products share homology with proteins that have been shown to be TonB-dependent receptors. We show that some genes involved in iron uptake were differentially expressed in response to iron. In addition, we were able to disrupt several putative genes involved in iron acquisition systems or iron regulation in *L. biflexa*. Comparative genomics, in combination with gene inactivation, gives us significant functional information on iron homeostasis in *Leptospira* spp.

Leptospira belongs to the bacterial phylum of spirochetes, which has a deep branching lineage in *Bacteria*, as indicated by 16S rRNA analysis (42). The genus *Leptospira* was initially divided into two groups: the pathogenic *Leptospira* referred to as *Leptospira interrogans* sensu lato and the saprophytic *Leptospira* referred to as *L. biflexa* sensu lato (9). Saprophytic and pathogenic *Leptospira* spp. were first classified into serovars, with more than 220 serovars defining the pathogens. More recently, DNA-DNA hybridization studies separated *Leptospira* species into 17 genomospecies, including 7 pathogenic species (9).

In the past decade, leptospirosis has emerged as a widespread zoonosis, and its incidence is high in tropical countries. Leptospirosis is acquired by direct or indirect contact with the urine of infected animals such as rodents (9). Virulence mechanisms and more generally the fundamental understanding of the biology of the causative agent of leptospirosis remain largely unknown. Recently, the genome sequencing of two serovars of *L. interrogans* sensu stricto, the main species associated with human leptospirosis, has been achieved (38, 48). However, the lack of genetic tools in pathogenic *Leptospira* does not allow the full characterization of genes of interest. Only recently the first evidence of gene transfer has been demonstrated in *L. interrogans* by transposition of *Himar1*, a transposon of eukaryotic origin (13). In contrast, numerous tools for genetic manipulation of saprophytic *Leptospira* species have been developed in recent years (7, 27, 35, 43, 44, 51, 56). These studies enable the use of the saprophyte *L. biflexa* as a model spirochete. The availability of the genome sequence of the saprophyte *L. biflexa* (unpublished data) and its comparison with the genomes of pathogenic species give us functional information on the lifestyles of *Leptospira* spp. in the environment and the infected host.

Iron plays a central role in many major biological processes, such as the electron transport chains for most living cells, including *Leptospira* spp. However, a few organisms, such as the spirochete *Borrelia burgdorferi*, do not require iron for growth (46). Spirochetes possess a double-membrane structure composed of a cytoplasmic membrane that differs substantially from that of gram-negative bacteria, the periplasm, and the outer membrane (9), which may constitute a barrier for molecules that could be used as an iron source. In gram-negative bacteria, iron sources can be recognized by specific outer membrane receptors, called TonB-dependent receptors, and then transported across the inner membrane by periplasmic binding protein-dependent ABC permeases (38, 48). We recently characterized the *fecA*- and *feoB*-like genes by random transposon mutagenesis in *L. biflexa* (35). Genes involved in iron acquisition are usually transcriptionally regulated by the availability of iron through regulators such as the ferric uptake regulator protein Fur. Cullen et al. have shown that the expressions of some genes from *L. interrogans* were regulated by iron (19), and *fur*-like genes are present in the *L. interrogans* genomes (38, 48).

In this study, the analysis of the genome sequence of *L. biflexa* allowed us to identify putative genes involved in iron transport and regulation. We showed that some of these pu-

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tative genes modulate their expression in response to iron. To study the function of these genes, we generated several mutants in *L. biflexa*, and their phenotypes were characterized. Since pathogenic *Leptospira* spp. as well as saprophytic species need to obtain iron to grow in vitro, and probably in vivo in the host, better knowledge of the iron uptake systems and their regulation is essential to understand the pathogenesis of this intriguing group of organisms.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. biflexa* serovar Patoc strain Patoc1 and *L. interrogans* serovar Lai strain Lai (National Reference Center for Leptospira, Paris, France) were grown at 30°C in EMJH (22, 31) medium. When necessary, kanamycin and spectinomycin were added at 40 μg/ml. Minimal iron EMJH medium was prepared by either omitting iron sulfate (normally 330 μM) or, prior to inoculation, treating normal EMJH medium with 50 μM 2,2′-dipyridyl (Sigma-Aldrich, St. Louis, MO) overnight. Media for testing the ability of *Leptospira* to use specific sources of iron was prepared by supplementing the dipyridyl-treated EMJH medium. The siderophores ferrichrome (final concentration, 20 μM), enterobactin (final concentrations, 10 to 100 μM), and aerobactin (final concentration, 50 μM) were purchased from EMC Microcollections GmbH (Germany). Desferrioxamine (final concentration, 10 μM), also called ferrioxamine B, refers to deferoxamine mesylate (Sigma-Aldrich, St. Louis, MO). Bovine hemin (final concentration, 10 μM) and lactoferrin (final concentration, 10 μM) were obtained from the Sigma-Aldrich Company (St. Louis, Mo). Iron citrate and iron chloride were used at 100 μM.

DNA and RNA manipulations. Genomic DNA of *Leptospira* was isolated as previously described (44). Plasmid DNA was purified using the Plasmid Miniprep kit (QIAGEN GmbH, Hilden, Germany). Total RNA was isolated by using RNAwiz (Ambion Inc.) and treated with DNaseI. For transcription studies, RNA was isolated from exponential-phase cultures of *L. biflexa* grown in EMJH or in dipyridyl-treated EMJH. The absence of DNA contamination was confirmed by PCR. RNA concentration was measured by spectrophotometry at 260 nm. Reverse transcription-PCR (RT-PCR) of RNA was performed using conditions recommended by the manufacturer (SuperScript One Step RT-PCR with Platinum Taq; Invitrogen) with primer pairs (primer nucleotide sequences are available on request) corresponding to selected open reading frames as previously described (12). The semiquantitative determination of transcript levels by RT-PCR was performed with 1, 10, and 100 ng of total RNA from *L. biflexa*. The amplified products were analyzed by agarose gel electrophoresis. The assays were performed in triplicate. For real-time quantitative reverse transcription-PCR (qRT-PCR), RNA (800 to 1,500 ng) was reverse transcribed using random primers and reverse transcriptase as described in the manufacturer's instructions (Roche Diagnostics, GmbH, Mannheim, Germany). The cDNA was used as template for gene-specific primer pairs with a LightCycler FastStart DNA Master^{PLUS} SYBR Green (Roche Diagnostics) in a LightCycler apparatus (Roche Diagnostics). The thermal cycling conditions were as follows: 10 min at 95°C, followed by 45 cycles of 10 s at 95°C, 5 s at 60°C, and 13 s at 72°C, then 1 cycle of 5 s at 95°C, 15 s at 65°C, and 30 s at 40°C. Data were analyzed using RelQuant (Roche Diagnostics). In all cases, transcript levels were determined in duplicate and at least two independent RNA samples were used for each condition tested. The relative expression of target genes was normalized to the level of 23S rRNA as an endogenous control.

Mutagenesis in *L. biflexa*. To mediate allelic exchange, a pGEM7Z-f$^+$ (Promega) derivative plasmid was used for the construction of plasmids containing insertional inactivated genes. The process was as follows: PCR primers for the amplification of the kanamycin or spectinomycin resistance cassette and the left and right arms of the target gene were designed, and in each instance a restriction endonuclease site was introduced at each end of each PCR product. The resulting three PCR products were digested with the appropriate restriction endonucleases and ligated into the pGEM7Z-f$^+$ derivative plasmid. The plasmid constructs delivering the inactivated allele were formed by insertion of a resistance cassette between the right and left arms (~0.5 kb in length) of the target gene; this introduces a partial gene deletion. The plasmids, which are not replicative in *Leptospira* spp., were then subjected to UV irradiation and used to deliver the inactivated alleles in *L. biflexa* as previously described (44). Kanamycin- or spectinomycin-resistant colonies were picked and tested for the insertion of the resistance cassette in the target gene by PCR as previously described (44). Random insertion mutagenesis using *Himar1* was carried out in medium with or without hemin as previously described (35). We used RT-PCR assays to check that the mutations did not prevent transcription of genes downstream of the inactivated genes.

Sequencing and annotation of the genome of *L. biflexa*. The sequenced strain, *L. biflexa* serovar Patoc strain Patoc1, was initially isolated from stream water (3) and maintained in the collection of the National Reference Center of *Leptospira* (Institut Pasteur, Paris, France). *L. biflexa* genomic DNA was randomly sheared by nebulization (hydroShear; GeneMachines) to short (1.5 to 2.5 kb) and long (35 to 45 kb) DNA fragments, and the insert DNAs were end repaired and ligated into a derivative of plasmid pGEM7-Zf$^+$ (Promega) and fosmid pCC1FOS (Epicenter, Madison, WI), respectively. Sequencing reactions were performed, from both ends of DNA template, using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit and run on a 3700 or a 3730 xl Genetic Analyzer (Applied Biosystems) at the Genomics Platform (Pasteur Genopole Île-de-France). Phred (23), Phrap (26), and in-house software (cover and coverparse; unpublished data) were used for genome assembly. The complete genome sequence was obtained from 58,663 end sequences (giving >8× coverage). Annotation was done using MaGe (http://www.genoscope.cns.fr/agc/mage/) (59), which allows graphic visualization of the *L. biflexa* annotations enhanced by a synchronized representation of synteny groups in other genomes chosen for comparisons. Coding sequences (CDSs) likely to encode proteins were predicted with the AMIGene (10) and MICheck (18) software. Putative orthologs showed at least 30% identity and a minimum ratio of 0.8 to the length of the smallest protein. Each predicted gene was assigned a unique identifier prefixed with "LEPBIa" for the large chromosome, "LEPBIb" for the small chromosome, and "pLEPBI" for the 74-kb plasmid. TMHMM, version 2.0 (34), and PRED-TMBB (4) were used to identify putative transmembrane domains and β-barrel domains, respectively. Deduced amino acid sequences were also analyzed using the databases of Pfam protein families (6), membrane transport systems (http://www.membranetransport.org/) (47), and ABC systems (ABSCISSE v3.0 database; http://www.pasteur.fr/recherche/unites/pmtg/abc/database.iphtml) (11). The complete genomic sequence of *L. biflexa* serovar Patoc strain Patoc1 analyzed in this work will be published in another study.

Nucleotide sequence accession numbers. Nucleotide sequences of predicted CDSs involved in iron uptake and regulation (Table 1) were deposited into EMBL under accession numbers AM162599 to AM162646 (see the supplemental material).

RESULTS AND DISCUSSION

Iron is an essential nutrient for *Leptospira* spp. Iron is essential for the growth of both saprophytic and pathogenic *Leptospira* spp. (24). In biological systems, iron is typically complexed to other molecules, and free iron is virtually absent. *Leptospira* spp. are usually cultivated in a standard albumin Tween 80 medium, the EMJH medium, containing iron sulfate as an iron source. To test for other iron sources, iron sulfate was omitted during the preparation of the EMJH medium. We find that whereas *L. biflexa* was not able to grow in iron sulfate-free EMJH, *L. interrogans* retained a wild-type growth. It is postulated that iron traces in iron sulfate-free EMJH were sufficient for the observed growth of *L. interrogans*. Further experiments were therefore done in iron-depleted EMJH that had been preincubated with 2,2′-dipyridyl. In these conditions, growth tests demonstrated that both *L. biflexa* and *L. interrogans* were able to use iron chloride, iron sulfate, and iron citrate. The most abundant source of iron in the host is heme and heme-containing proteins (62). *Leptospira* spp. were able to use exogenous hemin/hemoglobin as an iron source. Together with our previous study on an *L. biflexa hemH* mutant (27), this suggests that *L. biflexa* uses heme/hemoglobin as an iron as well as a heme source. Lactoferrin is another host protein that binds iron with high affinity, but neither *L. biflexa* nor *L. interrogans* was able to use lactoferrin as an iron source. Siderophores are high-affinity ferric chelators which are generally low-molecular-weight compounds synthesized and secreted by microorganisms in response to iron restriction. The

TABLE 1. Putative genes discussed in this study

Classification	*L. biflexa*[a,b,c] serovar Patoc	*L. interrogans* serovar Lai	*L. interrogans* serovar Copenhageni	E value[d] (% identity)	Putative function[e]	Comment(s)[f]
1. Iron transport systems						
1.1. TonB-dependent receptors and biopolymer transport proteins	LEPBIa0146	LA3247	LIC10889	e-63 (60)	TonB	1 TMS
	LEPBIa0147	LA3246	LIC10890	e-55 (76)	ExbD	1 TMS
	LEPBIa0148	LA3245	LIC10891	e-51 (69)	ExbD	1 TMS
	LEPBIa0149	LA3244	LIC10892	e-79 (59)	ExbB	4 TMS
	LEPBIa0500	LA1356	LIC12374	e-199 (51)	TB-DR	β-barrel OMP/plug domain; F/YRIP-NP/TN/KL motif
	LEPBIa1631	LA2320	LIC11621	e-59 (55)	ExbB	4 TMS
	LEPBIa1632	LA2319	LIC11622	e-53 (72)	ExbD	1 TMS
	LEPBIa1883	LA3468	LIC10714	e-229 (48)	TB-DR	β-barrel OMP/plug domain
	LEPBIa2760	LA2242	LIC11694	e-277 (60)	TB-DR	β-barrel OMP/plug domain
	LEPBIa3017	LB279	LIC20214	e-270 (52)	TB-DR	β-barrel OMP/plug domain
	LEPBIa3019	LB281	LIC20216	e-56 (59)	ExbB	3 TMS
	LEPBIa3020	LB282	LIC20217	e-53 (78)	ExbD	1 TMS
	LEPBIa3021	LB283	LIC20218	e-71 (57)	TonB	1 TMS
	LEPBIa3354	LA0706	LIC12898	e-233 (46)	TB-DR	β-barrel OMP/plug domain; YNAP-NPNL motif
	LEPBIa3362	LA3242	LIC10896	e-268 (52)	TB-DR	β-barrel OMP/plug domain
	LEPBIa3432	LB191	LIC20151	e-238 (59)	TB-DR	β-barrel OMP/plug domain; FRPP-NDKL motif
	LEPBIb0153	LB213	LIC20169	e-12 (28)	ExbD	1 TMS
	LEPBIb0154	LB214	LIC20170	e-17 (54)	ExbB	4 TMS
	LEPBIb0083	LB283	LIC20218	e-71 (62)	TonB	1 TMS
	LEPBIb0084	LB281	LIC20216	e-60 (62)	ExbB	4 TMS
	pLEPBI0018	LB191	LIC20151	e-118 (34)	TB-DR	β-barrel OMP/plug domain; Y/FRA/PP-NP/SN/DL motif
	NH	LA0572	LIC12998	e-09 (22); *E. coli* P76115	TB-DR	β-barrel OMP/plug domain
	NH	LA2641	LIC11345	e-41 (26); *Salmonella enterica* Q56145	TB-DR	β-barrel OMP/plug domain
	NH	LA3102	LIC10998	e-11 (20); *B. thetaiotaomicron* Q8ABG0	TB-DR	β-barrel OMP/plug domain
	NH	LA3149	LIC10964	e-17 (23); *Yersinia pestis* Q56989	TB-DR	β-barrel OMP/plug domain; FRAP-NPNL motif
	NH	LA3258	LIC10881	e-22 (20); *Colwellia psychrerythraea* AAZ27059	TB-DR	β-barrel OMP/plug domain; FQAP-NPNL motif
1.2. Divalent metal transporters of the NRAMP family	LEPBIa0902	NH	NH	e-72 (37); *Vibrio parahaemolyticus* Q87LD1	Mn(II) and Fe(II) transporters of the NRAMP family	11 TMS
1.3. Ferrous iron transport systems of the Feo type	LEPBIa0879	LA2579	LIC11402	e-203 (53)	Ferrous iron transport protein B (FeoB)	8 TMS
	LEPBIa0880	LA2578	NH	0.0007 (25)	Ferrous iron transport protein A (FeoA)	
1.4. ABC transport systems of the siderophore/heme/vitamin B_{12} type	pLEPBIa0012	NH	NH	e-66 (42); *Yersinia pestis* Q56990	HmuS hemin transport protein	Similar to *Bordetella* sp. BhuS, *Shigella dysenteriae* ShuS, and *Y. enterocolitica* HemS
	pLEPBI0013	NH	NH	e-39 (37); *Y. pestis* Q56993	HmuV hemin transport system ATP-binding protein	Similar to *Bordetella* sp. BhuV, *Shigella dysenteriae* ShuV, and *Y. enterocolitica* HemV
	pLEPBI0014	NH	NH	e-63 (39); *Y. pestis* Q56992	HmuU hemin transport system permease protein	Similar to *Bordetella* sp. BhuU, *Shigella dysenteriae* ShuU, and *Y. enterocolitica* HemU
	pLEPBI0015	NH	NH	e-47 (38); *Y. pestis* Q56994	HmuT hemin-binding periplasmic protein	Similar to *Bordetella* sp. BhuT, *Shigella dysenteriae* ShuT, and *Y. enterocolitica* HemT

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secretion of siderophores by *L. interrogans* could explain its ability to grow in iron-depleted medium. However, analysis of the *Leptospira* genomes did not allow the identification of any genes that encoded proteins related to proteins involved in siderophore synthesis or siderophore secretion. In addition, *L. biflexa* failed to grow in the supernatant derived from an iron-depleted culture of *L. interrogans*. This result suggests that *L. interrogans* does not produce siderophore or that this sid-

TABLE 1—*Continued*

Classification	*L. biflexa*[a,b,c] serovar Patoc	*L. interrogans* serovar Lai	*L. interrogans* serovar Copenhageni	E value[d] (% identity)	Putative function[e]	Comment(s)[f]
1.5. ABC transport systems of the iron/metal type	LEPBIa1264	NH	NH	e-40 (34); *Aquifex aeolicus* O67917	ABC-type metal ion transport system, periplasmic component	1 TMS
	LEPBIa1265	NH	NH	e-25 (36); *Bacillus subtilis* O34348	ATP-binding protein	Putative Fe(III)-transporting ATPase
	LEPBIa1266	NH	NH	e-15 (25); *Streptococcus pyogenes* Q8K8F0	Permease protein	ABC transport system (8 TMS)
2. Hemolysins and hemolysin secretion systems						
2.1. Hemolysins	LEPBIa0082	LA0327	LIC10284	e-51 (48)	Hemolysin A	Hemolytic activity (68)
	LEPBIa0717	LA3937	LIC13143	e-148 (58)	Hemolytic protein-like protein	Hemolytic activity (68) (4 TMS)
	LEPBIa2015	LA1650	LIC12134	e-86 (50)	Hemolysin hemolytic protein HlpA	Hemolytic activity (68)
	LEPBIa2375	LA0378	LIC10325	e-104 (48)	HlyX hemolysin	Hemolytic activity (68) (1 TMS)
	LEPBIa2477	NH	NH	e-53 (47); *Bacillus cereus* P54176	Predicted membrane protein, Hly-III	6 TMS
	NH	LA1029	LIC12631	e-61 (42); *S. aureus* P09978	Phospholipase C precursor	Sphingomyelinase activity (68)
	NH	LA3050	LIC11040	e-49 (51); LA3540/ LIC13198	Hemolytic protein-like protein	Sphingomyelinase activity (68)
	NH	LA3540	LIC13198	e-54 (38); *S. aureus* P09978	Sphingomyelinase	Sphingomyelinase activity(68)
	NH	LA4004	LIC13198	e-58 (42); *Bacillus cereus* P09599	Sphingomyelinase	Sphingomyelinase activity (68)
2.2. ABC transporter complex involved in hemolysin export	LEPBIa0357	NH	NH	e-87 (34); *Xyllela fastidiosa* Q87BN6	Toxin secretion ABC transporter ATP-binding protein	RTX toxin transporter(6 TMS)
	LEPBIa0358	NH	NH	e-18 (22); *E. coli* P09986	Hemolysin secretion protein D	RTX toxin transporter(1 TMS)
	LEPBIa0359	LA0150	LIC10136	e-67 (32) e-77 (28); *E. coli* Q46717/HlyB	Hemolysin secretion protein B ATP-binding protein	RTX toxin transporter 4 TMS
	LEPBIa0360	NH	NH	e-13 (23); *Bacteroides fragilis* Q64Y57	Outer membrane efflux protein TolC	1 TMS
	NH	LA3926	LIC13134	e-265 (44); *Bdellovibrio bacteriovorus* Q6MPG6	Multidrug efflux transporter AcrB	
	NH	LA3927	LIC13135	e-15 (25); *Vibrio cholerae* Q9K2Y1	Outer membrane efflux protein TolC	
3. Fur-like proteins	LEPBIa2152	LB183	LIC20147	e-14 (30)	FUR family	PerR "CXXC" motifs
	LEPBIa2330	LA3094	LIC11006	e-60 (71)	FUR family	
	LEPBIa2461	LA1857	LIC12034	e-45 (62)	FUR family	
	LEPBIa2849	NH	NH	e-07 (25); *Borrelia burgdorferi* F70180/ BosR	FUR family	PerR "CXXC" motifs
	NH	LA2887	LIC11158	e-11 (37); *Rhizobium leguminosarum* Q8KLU1	FUR family	
4. Iron storage	LEPBIa1791	LA2690	LIC11310	e-63 (71)	Bacterioferritin	
	LEPBIa2540	LA3598	LIC10606	e-54 (65)	DNA-binding stress protein Dps	

[a] EMBL accession numbers are indicated in the supplemental material.
[b] NH, no homolog.
[c] Underlined gene names indicate that a mutant is available (see Table 2).
[d] Shown is the E value (% identity) between *L. biflexa* and *L. interrogans* orthologs, except where otherwise stated.
[e] TB-DR, TonB-dependent receptor.
[f] Protein sequences were analyzed for the prediction of β-barrel/plug domains (PRED-TMBB) and transmembrane helices (TMHMM). OMP, outer membrane protein; TMS, transmembrane segments (the number of putative TMS is indicated).

erophore is not utilized by *L. biflexa*. *Leptospira* spp. do not appear to synthesize siderophores; however, they could use exogenous siderophores of other microorganisms as an iron source. Among the hydroxamate-type siderophores, aerobactin and ferrichrome were used by both *L. biflexa* and *L. interrogans*, while desferrioxamine was only used by *L. biflexa*. Desferrioxamine is produced by the gram-positive *Streptomyces pilosus* (37) and is utilized by some gram-negative bacteria,

such as *Yersinia enterocolitica* (8) or *Vibrio vulnificus* (65), and aerobactin is synthesized by *Shigella* spp. as well as some *Escherichia coli* clinical isolates, while the siderophore ferrichrome is synthesized by various fungal species. The catechol siderophore enterobactin is also produced by enterobacteria but was not utilized as an iron source by *Leptospira* spp.

The in vitro utilization of exogenous siderophores suggests that *Leptospira* spp. encounter the corresponding siderophores in their environment. While it is not surprising that *Leptospira* would use exogenous siderophores as an expedient way to acquire iron, it is not clear why saprophytes have the ability to use hemin and hemoglobin as an iron source.

Genomic sequences of *Leptospira* spp. Recently, the complete genome sequences of *L. interrogans* serovar Lai (48) and *L. interrogans* serovar Copenhageni (38) have been determined. The two genomes exhibit 95% identity at the nucleotide level and a further 99% identity for predicted protein-coding genes that are orthologs. The original annotations found that *L. interrogans* serovar Lai has nearly 1,000 more genes (4,727 open reading frames versus 3,658 CDSs) than *L. interrogans* serovar Copenhageni (38). However, this discrepancy may not reflect the reality and rather may be due to the annotation criteria used by the two genome projects (58). Using the MICheck software (18) on the two genomes, we predicted a total number of 3,798 and 3,651 CDSs for *L. interrogans* serovar Lai and *L. interrogans* serovar Copenhageni, respectively. Since all the homologous gene products of serovars Lai and Copenhageni share more than 95% identity, the term *L. interrogans* will therefore refer to both of the serovars. The complete genome sequence of the saprophyte *L. biflexa* serovar Patoc strain Patoc1 is also available and consists of approximately 3,790 predicted coding genes (M. Picardeau et al., unpublished data).

Iron participates as a metabolic cofactor in a variety of biochemical processes involving electron transfer. Genes encoding proteins that typically require iron as a cofactor, such as cytochromes, catalase, and tricarboxylic acid enzymes, were detected in both *L. biflexa* and *L. interrogans*. In contrast, a putative superoxide dismutase gene (LEPBIa0027) was only detected in *L. biflexa*. As previously shown, *Leptospira* spp. possess a complete heme biosynthesis pathway, including a ferrochelatase (27). A putative heme oxygenase was also found in the genomes of *L. biflexa* (LEPBIa0669) and *L. interrogans* (LB186/LIC20148). We have used comparative genomics to identify putative genes involved in iron acquisition systems and iron regulation in the genomes of *L. biflexa* and *L. interrogans*. We used the BLAST program and queried all *L. biflexa* protein sequences exhibiting significant similarities with iron transporters and iron regulators against the *L. interrogans* databases and vice versa. Genes encoding putative hemolysins, hemolysin excretion apparatus, and iron storage proteins were also included in this study (Table 1).

Transport across the outer membrane via TonB uptake systems. We have shown that *Leptospira* spp. were able to utilize various iron sources such as siderophores, but the uptake system mechanisms are not known. The acquisition of molecules by bacteria often relies on the active transport through dedicated outer membrane receptors. In gram-negative bacteria, transport of iron sources is mediated by outer membrane receptors, also called TonB-dependent receptors, which utilize the energy produced by the inner membrane complex of TonB, ExbB, and ExbD.

Bacteria often possess multiple TonB-dependent receptors, each providing the bacterium with specificity for different iron sources. For example, *E. coli* has one set of TonB, ExbB, and ExbD as well as eight TonB-dependent receptors (29). Other gram-negative organisms may have two or more TonB-ExbB-ExbD-like systems with distinct specificities for the TonB-dependent receptors (36). Analysis of the predicted proteins encoded by the *L. biflexa* and *L. interrogans* genomes revealed five putative ExbB and ExbD proteins and three putative TonB proteins. These genes are grouped in five distinct loci, and their arrangements are similar in both *L. biflexa* and *L. interrogans*. Consistent with an inner membrane localization, the putative TonB and ExbD proteins possess a single transmembrane segment (TMS) near the N-terminal end, and the putative ExbB proteins have three TMS (Table 1). Further analysis of the genomes reveals a total of 8 *L. biflexa* and 12 *L. interrogans* genes encoding proteins related to TonB-dependent receptors (Table 1), and it is notable that only one gene encoding a protein related to a TonB-dependent receptor (LEP BIa3017/LB279/LIC20214) is genetically linked to an ExbB-ExbD-TonB system. Despite low primary sequence similarity, the structure of TonB-dependent receptors is usually well-conserved in bacteria; all share a 22-stranded transmembrane β-barrel that forms a pore, which is plugged from the periplasm by a globular N-terminal domain (67). The 19 putative TonB-dependent receptors of *L. biflexa* and *L. interrogans* are 684 to 991 amino acids in length. Protein analysis using the Pfam database showed that they all exhibit a putative plug domain (E value from e-03 for LA3102 to e-24 for LA3021) and a TonB-dependent receptor domain (E value from e-03 for LEPBIa3354 to e-31 for LEPBIa3432). Further analysis predicts that these proteins are hydrophobic and have a putative large β-barrel domain typical of a TonB-dependent receptor. None of the putative TonB-dependent receptors of *Leptospira* possesses an N-terminal extension that could interact with anti-sigma factor, as in the *E. coli fecA-fecIR* system (15).

TonB-dependent receptors usually have high affinity and specificity for their substrates. In *E. coli*, the well-characterized TonB-dependent receptors FecA, FhuA, FepA, and BtuB are required for the active transport of ferric citrate, ferrichrome, enterobactin, and vitamin B_{12}, respectively. However, substrate specificity is difficult to identify by sequence analysis of the receptors.

The construction or identification of mutants in each of the genes encoding TonB-dependent receptors and subsequent characterization of substrate specificity of the mutants is the preferred approach to the characterization of these receptors. Such an approach would not have been possible until the recent development of some key genetic manipulation techniques for *Leptospira* (35). By random transposon mutagenesis in *L. biflexa*, we recently identified mutants with insertions in a gene (LEPBIa1883) encoding a protein that shares homology with FecA (35). By screening a total of 6,000 *L. biflexa* transposon mutants for hemin auxotrophy, 11 out of 14 auxotrophic mutants exhibited the transposon inserted in LEPBIa1883 at distinct locations (data not shown). These strains were impaired in their ability to use iron citrate, iron chloride, iron

TABLE 2. Effect of iron sources on growth of *L. biflexa* mutant strains[d]

Interrupted gene in *L. biflexa*	Mutagenesis method	Putative function[b]	Growth phenotype[a]						
			Iron sulfate	Iron chloride	Iron citrate	Hemin	Dfrx	Aerobact	Ferrichr
LEPBIa1883	*Himar1*[c]	TonB-DR	0	0	0	+	+	0	+
LEPBIa2760	Allelic exchange	TonB-DR	+	+	+	+	0	+	+
LEPBIa3354	Allelic exchange	TonB-DR	+	+	+	+	+	+	+
LEPBIa3362	Allelic exchange	TonB-DR	+	+	+	+	+	+	+
LEPBIa3432	Allelic exchange	TonB-DR	+	+	+	+	+	+	+
pLEPBI0018	Allelic exchange	TonB-DR	+	+	+	+	+	+	+
LEPBIa0902	Allelic exchange	MntH	+	+	+	+	+	+	+
LEPBIa0879	*Himar1*	FeoB	0	0	0	+	0	0	0
LEPBIa0880	*Himar1*	FeoA	0	0	0	+	0	0	0
LEPBIa1265	Allelic exchange	ATP-ABC	+	0	0	0	0	0	0
pLEPBI0012	Allelic exchange	HmuS	+	+	+	+	+	ND	ND
pLEPBI0013	Allelic exchange	HmuV	+	+	+	+	+	ND	ND
LEPBIa2152	Allelic exchange	Fur	+	+	+	+	+	ND	ND
LEPBIa2461	Allelic exchange	Fur	+	+	+	+	+	ND	ND

[a] For growth tests, iron-depleted EMJH medium was supplemented with iron sulfate (100 μM), iron chloride (100 μM), iron citrate (100 μM), hemin (10 μM), desferrioxamine (Dfrx; 10 μM), aerobactin (Aerobact; 50 μM) and ferrichrome (Ferrichr; 20 μM). "+" indicates wild-type growth in liquid media (see legend of Table 2), and "0" indicates no growth or poor growth after 1 week in liquid media. The growth curve of *L. biflexa* in iron-depleted EMJH liquid medium supplemented with distinct iron sources (at the concentration indicated above) was similar to the growth curve obtained in EMJH liquid medium. ND, not determined.

[b] TonB-DR, TonB-dependent receptor; ATP-ABC, ATP-binding protein of an ABC transport system.

[c] See reference 35.

[d] Late-exponential-phase cultures (optical density at 420 nm [OD_{420}], 0.5) were diluted 1:2,000 in fresh iron-depleted culture medium containing distinct iron sources and incubated at 30°C with shaking for 1 week. At each time point (48, 72, 96, 120, and 146 h), aliquots were removed from the liquid medium, and growth was determined by measuring the OD_{420}. For each mutant, growth was compared to the *L. biflexa* wild-type strain in similar conditions.

sulfate, and aerobactin as an iron source (Table 2). Interestingly, aerobactin-like siderophores are derived from citrate (39), and therefore aerobactin and iron citrate share a similar structure that is evidently recognized by the same receptor. The gene product of LEPBIa1883 (642 amino acids in length) has 48% identity (E value, e-229) with the *L. interrogans* LA3468/LIC10714 product (650 amino acids in length). The reciprocal best BLAST hit test indicates that these proteins are orthologous and strongly suggests that they share the same function.

Besides *fecA*, we have attempted to disrupt the other putative genes encoding TonB-dependent receptors by allelic exchange in *L. biflexa*. Gene inactivation of pLEPBI0018, LEPBIa3432, LEPBIa3362, and LEPBIa3354 resulted in a wild-type phenotype in iron-depleted medium supplemented with different iron sources (Table 2). The lack of a phenotype in these mutants could be due to functional redundancy with another iron uptake system.

Disruption of LEPBIa2760 resulted in a mutant that was impaired in its ability to use desferrioxamine as an iron source (Table 2). Introduction of a recombinant plasmid harboring the LEPBIa2760 locus restored the ability of the mutant to use desferrioxamine (data not shown). Desferrioxamine is also utilized via a TonB-dependent receptor, called FoxA, in *Yersinia enterocolitica* (8). These results are evidence that LEPBIa2760 encodes the receptor protein for ferrioxamines in *L. biflexa*.

Finally, we failed to obtain double-crossover events in LEPBIa0500 and LEPBIa3017. This may indicate that these genes are essential for the survival of *L. biflexa*. *Leptospira* spp. have an absolute requirement for vitamin B_{12}, which is usually transported via TonB-like systems in other gram-negative bacteria. Since vitamin B_{12} is a cofactor for enzymes of major biological processes, inactivation of its receptor should result in nonviable mutants. Amino acid comparisons of TonB-dependent receptors of heme, hemoglobin, siderophores, and vitamin B_{12} revealed a highly conserved domain containing the FRAP and NPNL amino acid box (14, 53). This conserved domain was found in some TonB-dependent receptors from *Leptospira*, including LEPBIa0500 (Table 1; Fig. 1).

It is important to note that the current understanding about the mechanism of TonB-dependent transport across the outer membrane comes from studies in *E. coli*. Further studies are required to understand the precise physiological role of the TonB-dependent receptors of *Leptospira* spp. in iron uptake.

Transport across the cytoplasmic membrane. The TonB systems release Fe(III)-containing complexes into the periplasm, and these complexes are transported across the inner membrane via ATP-binding cassette (ABC) transport systems. In bacteria, the passive diffusion of Fe(II) through the

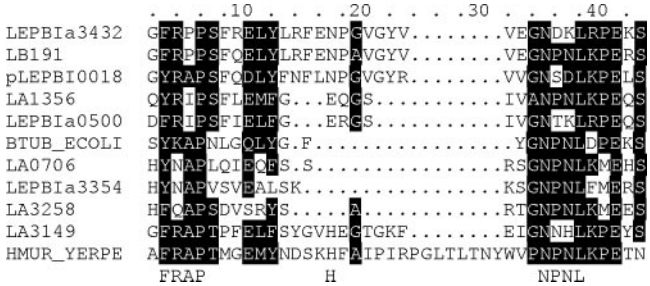

FIG. 1. Alignment of the FRAP/NPNL motif from several predicted TonB-dependent receptors. Similar residues are shaded in black. The His461 marked by an "H" has been shown to be essential for the *Y. enterocolitica* HemR function (14). The TonB-dependent receptors from *L. biflexa* (pLEPBI0018, LEPBIa0500, LEPBIa3354, and LEPBIa3432), *L. interrogans* (LA0706, LA1356, LA3149, LA3258, and LB191), *E. coli* (BTUB_ECOLI), and *Yersinia pestis* (HmuR_YERPE) are shown.

outer membrane can represent a second source of iron; active transport of ferrous irons across the cytoplasmic membrane is normally distinct from the transport of ferric iron. In *E. coli*, uptake of Fe(II) across the cytoplasmic membrane is performed by the energy-driven high-affinity transporter FeoAB (32) and by the proton-dependent MntH transporter from the NRAMP (natural resistance-associated macrophage proteins) family (17).

The NRAMP family of membrane metal transporters was originally identified in eukaryotes and then in prokaryotes. Phylogenetic analyses of the NRAMP family of proteins suggested horizontal transfer from eukaryotes to bacteria (17). Characterized eukaryotic NRAMP proteins transport divalent cations such as iron, manganese, and zinc through the cytoplasmic membrane. In bacteria, these proteins have been most extensively characterized in enterobacteria where they act as manganese transporters, hence the designation MntH proteins. The *Mycobacterium tuberculosis* MntH also transports significant amounts of both iron and zinc (1). *L. biflexa* LEP BIa0902 displays a small but significant similarity to MntH proteins (Table 1). This protein contains 11 putative TMS, which is consistent with the predicted topology model of MntH proteins (28). However, most of the conserved residues found essential for the function of *E. coli* MntH (28), i.e., Asp-34, Glu-102, Asp-109, Glu-112, and Asp-238, were not found in LEPBIa0902. Disruption of LEPBIa0902 resulted in a wild-type phenotype in iron-depleted medium supplemented with different iron sources (Table 2), manganese, or zinc (data not shown). In conclusion, we do not have evidence on the role of LEPBIa0902 as a member of the NRAMP family.

In an earlier study, random transposon mutagenesis allowed us to identify an *feoB*-like gene in *L. biflexa* (35). By using the same methodology, we also identified an *L. biflexa feoA* mutant (Table 2). FeoA, a protein of approximately 75 amino acids in length, and FeoB were shown to be the only ferrous ion transport systems in both *E. coli* and *Legionella pneumophila* (32, 49). FeoB proteins are cytoplasmic membrane proteins that have two main regions: a hydrophilic N-terminal domain with GTPase activity and a hydrophobic C-terminal region with 7 to 12 transmembrane-spanning α-helices (FeoB in *L. biflexa* has 8 predicted TMS). *L. biflexa feoA* and *feoB* mutants were impaired in the uptake of ferrous/ferric iron, iron citrate, and the siderophores desferrioxamine, aerobactin, and ferrichrome (Table 2). This suggests that iron is released from the siderophores in the periplasm and then transported into the cytoplasm via FeoAB. Previous studies have demonstrated that FeoB was important for the infectivity of pathogenic bacteria such as *Legionella pneumophila*, *Helicobacter pylori*, and *Shigella flexneri* (49, 50, 60). The *L. interrogans feoA* and *feoB* genes (Table 1) may also have a major role in the acquisition of ferrous iron while in the host.

Besides the NRAMP-type and the FeoAB-type iron transporters, four distinct families of ABC transporters related to iron uptake are known (33). Their components can mediate the transfer of ferric iron, siderophores, heme, and vitamin B_{12} into the cytosol of prokaryotes. The ABC transporters are typically composed of periplasmic binding proteins, one or two identical or homologous permeases, and one or two ATPases located on the inner surface of the cytoplasmic membrane and supplying the system with energy. The ATPases are the most conserved modules among the ABC transporters, and the permeases are characterized by their overall hydrophobicity. Only a few ABC transport systems of the iron/metals type has been described in spirochetes (21, 30). By similarity searching against a database of characterized ABC transporters (ABSCISSE v3.0 database), the *L. biflexa* locus containing genes LEPBIa1264 to LEPBIa1266 is predicted to encode a MET (metallic cation uptake) family ABC transporter (11). No orthologous locus of this putative ABC transporter was detected in *L. interrogans* (Table 1). Inactivation of the gene encoding the ATP-binding protein, LEPBIa1265, had pleiotropic effects on growth in the presence of an Fe(III) source but not with Fe(II), i.e., iron sulfate (Table 2).

The characterization of an *L. biflexa hemH* mutant suggested that *Leptospira* can transport the entire heme molecule into the cell (27). Transport of heme across the cytoplasmic membrane is usually mediated via ABC transporters (62). In *L. biflexa*, but not in *L. interrogans*, four genes encoding a putative ABC transport system (genes pLEPBI0012 to pLEPBI0015; Fig. 2) showed significant similarity to the corresponding proteins encoded by a well-defined hemin uptake system operon, *hmuSTUV*. Similar operons have been described in numerous pathogenic bacteria (57). Interestingly, pLEPBI0018, encoding a putative TonB-dependent receptor, is located immediately downstream of the putative *hmuSTUV* operon in the *L. biflexa* circular plasmid p74 (Fig. 2). In *Yersinia pestis*, the gene encoding the hemin receptor HmuR is also linked to the operon *hmuSTUV*. This genetic organization suggests that *L. biflexa* pLEPBI0018 encodes the hemin receptor. However, hemin uptake was not affected in the pLEPBI0018 mutant (Table 2). In addition, the alignment of putative leptospiral TonB-dependent receptor sequences (Fig. 1) reveals that a His residue, thought to be essential for heme binding in the HemR receptor from *Y. enterocolitica* (14), was not found in pLEPBI0018 and was only in the protein encoded by LA3149 (Fig. 1).

The ABC transporter proteins of the *L. biflexa hmu* locus exhibit similarities to *Y. pestis* HmuS, HmuT, HmuU, and HmuV proteins (Table 1). It was previously proposed that HmuS was a heme oxygenase (55), hence the designation of heme-degrading protein. However, the inactivation of either *Y. pestis hmuS* or *Shigella dysenteriae shuS* showed that these mutants were still able to use hemin and all hemoproteins as an iron source (57, 66). In addition, no heme oxygenase activity was detected for the HmuS homolog protein, ShuS, of *S. dysenteriae* (63). Similarly, an *L. biflexa* mutant of the *hmuS*-like gene grew like the wild-type strain when hemin was provided as the sole source of iron (Table 2). It has to be noted that the *L. biflexa* genome also contains another putative gene, LEP BIa0669, encoding a protein with 62% and 55% similarity to the heme oxygenase from *Synechocystis* sp. strain PCC6803 and human, respectively. Another hypothesis is that HmuS is involved in heme storage and/or the oxidative stress. Two types of iron storage proteins are also found in the genomes of *Leptospira* spp.: the heme-containing bacterioferritin and the Dps protein (Table 1). In *E. coli*, Dps does not have a strict function in iron storage, and it also protects DNA against the combined action of ferrous iron and hydrogen peroxide in the production of a hydroxy radical. In *Y. pestis*, HmuT is proposed to be a periplasmic binding protein which specifically binds

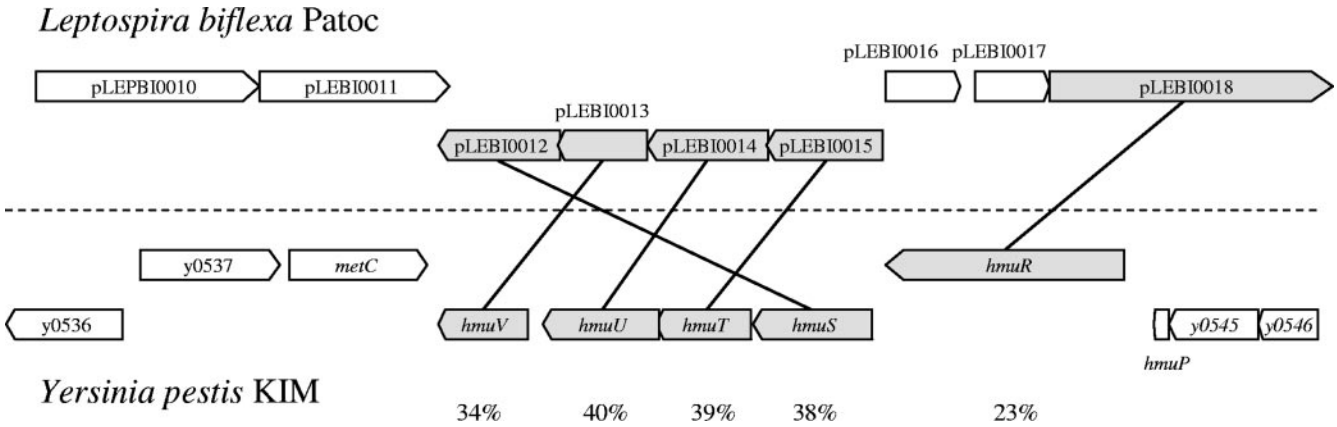

FIG. 2. Schematic representation of the *L. biflexa hmuSTUV* locus and the corresponding locus in *Y. pestis*. Orthologous proteins are shaded in gray and joined by a line, and the percentage of identity is indicated below.

heme and acts as a receptor for the active uptake of heme into the cytoplasm. The HmuU and HmuV proteins have been proposed to comprise the cytoplasmic membrane permease and ATPase, respectively. Transcriptional analysis by RT-PCR with a set of primers for the genes pLEPBI0012, pLEPBI0013, pLEPBI0014, and pLEPBI0015 revealed that these genes form an operon (data not shown). Inactivation of the putative *hmuV* (pLEPBI0013) by allelic exchange had no effect on hemin utilization (Table 2). This hemin uptake system was also found not to be essential for hemin utilization in *Vibrio cholerae* and *Bradyrhizobium japonicum* (40, 41). This might be explained by the presence of alternative mechanisms or low-affinity systems for transporting hemin across the cytoplasmic membrane.

Secretion of putative hemolysins. Extracellular bacteria such as *L. interrogans* could release heme and hemoglobin from host red blood cells by the secretion of hemolysins, constituting a mechanism by which bacteria can gain access to ready sources of iron in the host. Once released, hemin may be rapidly bound to host proteins but may also be directly transported by bacteria or by binding of hemin or hemin complexes to TonB-dependent receptors (62). The genomes of *Leptospira* spp. contain several genes encoding putative hemolysins, even in the saprophyte *L. biflexa* (Table 1). There are at least five *L. biflexa* and eight *L. interrogans* genes that encode products that exhibit similarities to hemolysins (Table 1). In a recent study, Zhang et al. (68) demonstrated that the recombinant proteins encoded by the hemolysin genes of *L. interrogans* have hemolytic activities in *E. coli* (Table 1). In addition, a putative hemolysin secretion system similar to the *E. coli*-hemolysin (HlyA) secretion system (16) was identified in *L. biflexa* (LEP BIa0357-LEPBIa0360), but no orthologous system was found in *L. interrogans* (Table 1). The putative *L. biflexa* hemolysin secretion system comprises HlyB-, HlyD-, and TolC-related proteins. Feasibly, *L. interrogans* could use an alternative TolC-based secretion system, given the presence of a gene encoding a TolC-related protein (LA3927/LIC13135). Other genes encoding putative proteases may also be involved in degrading heme-containing compounds like in *Porphyromonas gingivalis* (54).

Fur and other regulatory proteins. Bacteria typically regulate their metabolism in response to iron availability. In *E. coli*, most of the genes involved in iron acquisition are transcriptionally regulated by the ferric uptake regulator protein Fur (29). The expression of seven *L. biflexa* genes of interest, including four *fur*-related genes, the ferrous iron transporter gene (*feoB*), and two TonB-dependent receptors involved in iron transport (*fecA* and *tbr3*), were analyzed by quantitative reverse transcription-PCR (qRT-PCR). Our results were supported by the semiquantitative RT-PCR method (data not shown). No significant change in the relative expression of *fur1* (LEPBIa2461) was determined in response to iron availability. Iron depletion led to a threefold increase in transcript levels of *tbr3* (LEPBIa2760) encoding the receptor for desferrioxamine, whereas the other genes (*fecA*, *feoB*, *fur2*, *fur3*, and *fur4*) showed more than a 10-fold decrease in expression (Fig. 3). Interestingly, *fecA* and *tbr3* have different transcription profiles. Under iron limitation, the expression of *tbr3* is induced to utilize siderophores, which are high-affinity iron-chelating molecules. Conversely, the expression of *fecA*, which encodes a receptor for a relatively large number of iron sources (i.e., iron citrate, iron sulfate, iron chloride, and aerobactin), is more important in the presence of higher iron concentrations (Fig. 3). The expression of these genes involved in iron uptake is therefore sensitive to iron level, but expression is likely to be regulated by a complex regulatory mechanism in which Fur may not be the exclusive regulatory protein.

In the genomes of both *L. biflexa* and *L. interrogans*, four *fur*-like genes were identified (Table 1). On chelation of iron from the growth medium, the level of transcripts from the *L. biflexa fur*-like genes LEPBIa2152 (*fur4*), LEPBIa2330 (*fur2*), and LEPBIa2849 (*fur3*) decreased at least 10-fold, while the expression of LEPBIa2461 (*fur1*) was independent of iron concentration (Fig. 3). Among the Fur family of proteins, there are three types of proteins (Fur, Zur, and PerR) sharing high sequence identity. In *E. coli*, Fur regulates iron uptake and siderophore biosynthesis, Zur regulates zinc uptake systems, and PerR regulates oxidative stress response genes (29). We should find Fur, Zur, and PerR proteins in the genomes of *Leptospira* spp. Indeed, zinc and iron are important nutrients for *Leptospira*, and high concentrations of these metals are usually toxic for bacteria. A leptospiral PerR protein may also play an important role when *Leptospira* spp. encounter envi-

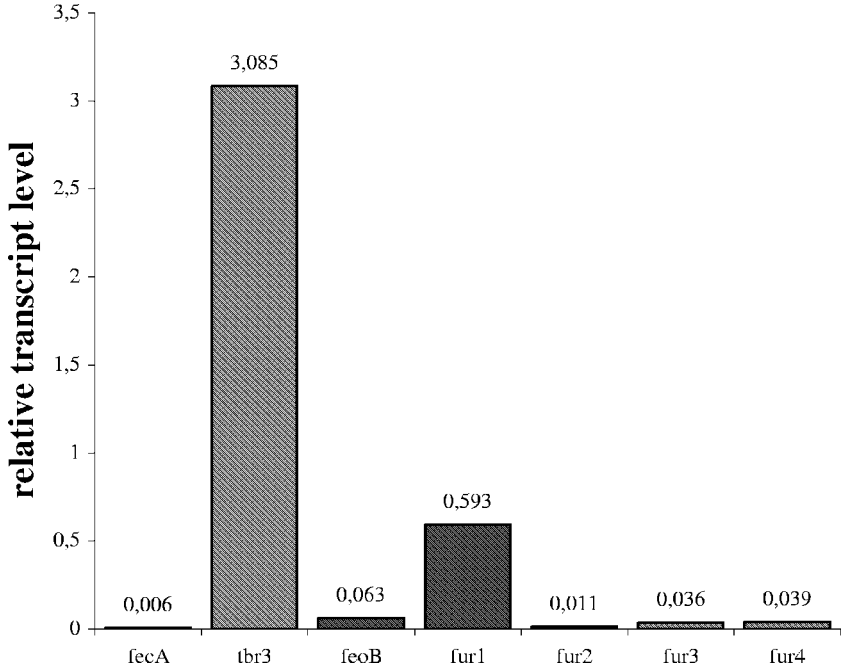

FIG. 3. Effects of iron depletion on mRNA levels of *feoB* (LEPBIa0880), *tbr3* (LEPBIa2760), *fecA* (LEPBIa1883), and *fur*-like homologs (*fur1*, LEPBIa2461; *fur2*, LEPBIa2330; *fur3*, LEPBIa2849; and *fur4*, LEPBIa2152) in *L. biflexa*. For qRT-PCR, RNA was isolated from *L. biflexa* cells grown in EMJH (high-iron conditions) and iron-depleted EMJH media (low-iron conditions). The amount of a specific mRNA transcript in low-iron conditions is relative to the quantity of that particular mRNA transcript in high-iron conditions. As an endogenous control, the 23S rRNA was used for normalization of transcript levels (i.e., 23S rRNA = 1.0).

ronmental oxidative stresses. All Fur family proteins share an N-terminal DNA-binding domain with a helix-turn-helix motif and conserved metal-binding sites (45). PerR regulators typically have two separate CXXC motifs in the C terminus (29). Such motifs were found in the leptospiral LB183, LEPBIa2152, and LEPBIa2849 Fur-like proteins. LEPBIa2849 also presents significant similarities with an oxidative stress regulator from *Borrelia burgdorferi* (52). In addition to iron-binding sites, *E. coli* Fur possesses one zinc-binding site composed of Cys93 and Cys96, which are also perfectly conserved in Zur proteins (25). Analysis of the Fur-like proteins from *Leptospira* spp. indicates that LEPBIa2461/LA1857 on one hand and LA2887 on the other hand do not contain this motif and therefore may not function as Fur or Zur proteins (Fig. 4). Since it is not possible to distinguish between Zur and Fur regulators on the basis of sequence, we have attempted to disrupt each gene by allelic exchange in *L. biflexa* to determine their biological role. Inactivation of LEPBIa2152 and LEPBIa2461 resulted in a wild-type phenotype in EMJH liquid medium (Table 2) as well as a wild-type peroxide sensitivity (data not shown). The lack of obvious phenotype in the LEPBIa2461 and LEPBIa2152 mutants could be due to functional redundancy with another member of the Fur family. The *fur*-like genes LEPBIa2330 and LEPBIa2849 showed different expression under iron-replete and -deplete conditions, and in the absence of allelic exchange mutants they appeared essential, as is the case in some other *fur* genes (20).

Fur-binding sites, known as the Fur boxes, were originally identified as a 19-bp inverted repeat sequence in the promoter region of iron-regulated genes. A 150-bp region preceding the start codon of putative iron-regulated genes from *L. biflexa* and *L. interrogans* such as *fecA*, *feoAB*, and *fur*-like genes were analyzed for the presence of putative Fur-binding sites (5). Although these genes are likely members of the Fur regulon, we were not able to identify a leptospiral Fur box. The DtxR protein family is another family of iron regulators that were first found in gram-positive organisms with a high-GC content and also in the spirochete *Treponema pallidum* (30). No DtxR-like proteins were detected in the genomes of *Leptospira* spp. Despite the large number of putative extracytoplasmic function sigma factors in both *L. biflexa* and *L. interrogans*, no FecI-related proteins, which are referred to as iron starvation sigma factors (15, 61), were identified. This suggests the absence of this type of signal transduction cascade in *Leptospira*. A high concentration of extracellular iron, which can be toxic for bacteria, can be detected by two-component systems (64). At least 47 potential response regulator genes were identified in the *L. interrogans* genome (2). This indicates that *Leptospira* spp. have developed a vast array of detection systems that enable them to respond to environmental signals, one of which could be iron.

Comparative genomics between saprophytes and pathogens. The possession of specialized iron transport systems for the saprophyte *L. biflexa* and the pathogen *L. interrogans* may reflect the various iron sources they may encounter in their diverse habitats. A more detailed comparative genomic analysis should provide clues to the lifestyle of *Leptospira* in the environment and in the infected host, increasing our understanding of the transition from environmental bacterium to major human and animal pathogen (unpublished data).

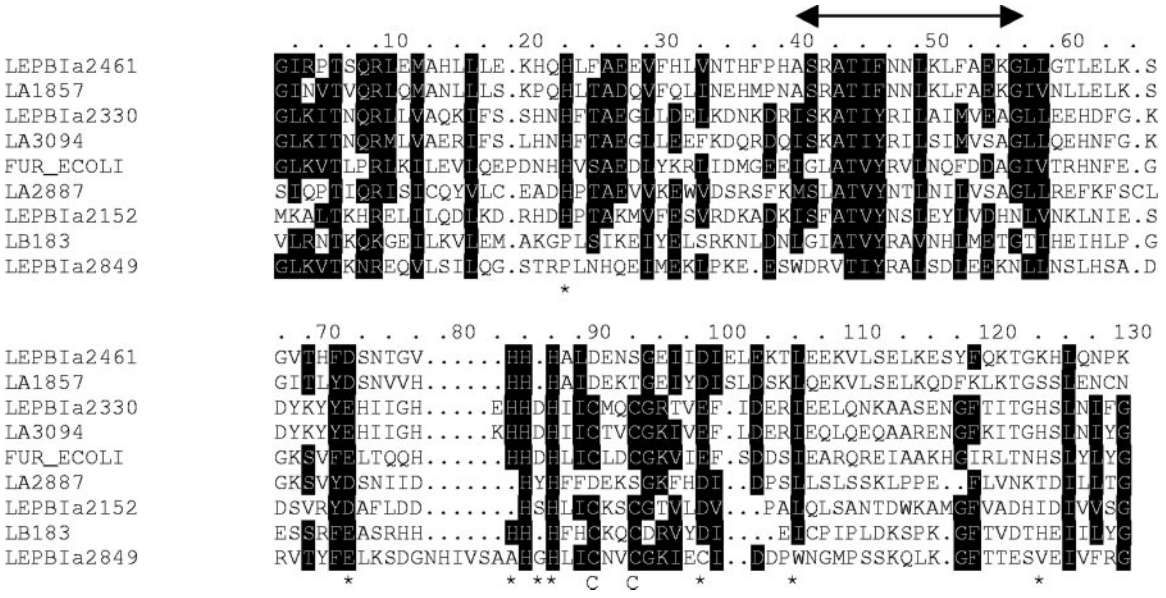

FIG. 4. Alignment of Fur-like proteins from *L. interrogans* and *L. biflexa*. Similar residues are shaded in black. Metal-binding sites defined by Pohl et al. (45) are indicated by asterisks. Cysteine residues corresponding to Cys93 and Cys96 of the *E. coli* Fur were shown to be the zinc-binding site by Gonzales de Peredo et al. (25). The putative DNA-binding α-helix is indicated by arrows. LB183, LA2887, LA3094, and LA1857 are *L. interrogans* Fur homologs; LEPBIa2152, LEPBIa2330, LEPBIa2461, and LEPBIa2849 are *L. biflexa* Fur-like proteins; Fur_Ecol indicates *E. coli* Fur.

Based on our findings, a model for iron uptake in *Leptospira* can reasonably be proposed (Fig. 5). The analysis of the genome of the pathogen *L. interrogans* has allowed the identification of 12 putative TonB-dependent receptors, while *L. biflexa* possesses 8 putative TonB-dependent receptors. This difference suggests that pathogenic species are able to use a wider range of iron sources than saprophytes. Alternatively, the pathogens may also present redundancy in their genome content. As in gram-negative bacteria, periplasmic binding proteins may shuttle iron-containing complexes from TonB-

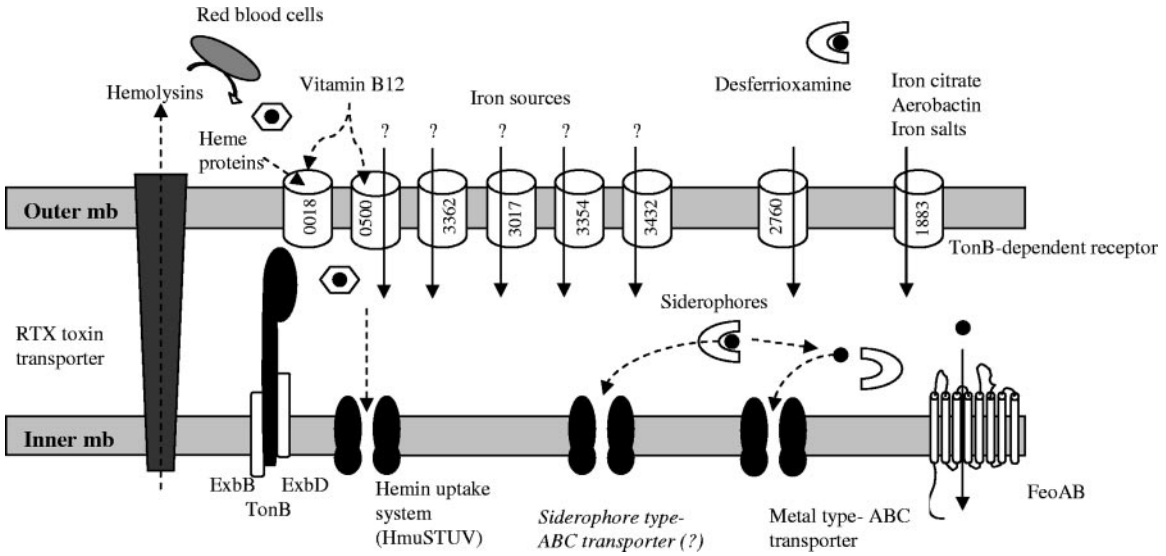

FIG. 5. Diagram showing an overview of iron acquisition systems in *L. biflexa*. Eight putative TonB-dependent receptors were identified in the *L. biflexa* genome. The five TonB loci identified could be involved in the formation of the ExbB-ExbD-TonB complex (for simplicity, only one ExbB-ExbD-TonB system is indicated). By mutagenesis in *L. biflexa*, we characterized the function of two TonB-dependent receptors (LEPBIa1883 and LEPBIa2760), the FeoAB system, and the metal-type ABC transporter (LEPBIa1265). The absence of knockout mutants, suggesting that the product is essential, indicates that pLEPBI0018 or LEPBIa0500 is the TonB-dependent receptor for either vitamin B_{12} or hemin. The HmuSTUV transport proteins may be involved in the periplasmic transport of hemin. An unidentified system may also exist for the periplasmic transport of siderophores (indicated in italics). *Leptospira* spp. could also release heme and hemoglobin from host red blood cells by the secretion of hemolysins. There is no evidence of siderophore synthesis in *Leptospira* spp. All the molecules participating in each step of the transport process have not been identified; other proteins such as reductases and other periplasmic proteins may also be involved. mb, membrane.

dependent receptors to cytoplasmic membrane ABC transporters that in turn deliver them in the cytoplasm. The pathogen *L. interrogans* may obtain iron from heme by secreting hemolysins to lyse red blood cells and thereby making the iron available for uptake. Surprisingly, *L. biflexa*, a nonpathogenic species, has putative hemin uptake and hemolysin secretion systems. Notably, the *L. biflexa* genome does not contain orthologs of the *L. interrogans* sphingomyelinases, which may be involved in the typical vascular damage seen in acute leptospirosis. *Leptospira* spp. also possess uptake systems that use siderophores produced by other bacteria or fungi. This ability to utilize xenosiderophores broadens the available sources of iron and allows the bacterium to occupy extended ecological niches. Bacterial iron homeostasis is best understood in *E. coli*, a bacterium phylogenetically distant from *Leptospira*. This study is a first step towards understanding iron acquisition systems in *Leptospira*. This study highlights the importance of mutagenesis tools, such as random transposon mutagenesis systems, to the molecular analysis of *Leptospira*.

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