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Screening of a *Leptospira biflexa* Mutant Library To Identify Genes Involved in Ethidium Bromide Tolerance

Helena Pětrošová,^{a,b} Mathieu Picardeau^b

Masaryk University, Faculty of Medicine, Department of Biology, Brno, Czech Republic^a; Institut Pasteur, Biology of Spirochetes Unit, Paris, France^b

Leptospira spp. are spirochete bacteria comprising both pathogenic and free-living species. The saprophyte *L. biflexa* is a model bacterium for studying leptospiral biology due to relative ease of culturing and genetic manipulation. In this study, we constructed a library of 4,996 random transposon mutants in *L. biflexa*. We screened the library for increased susceptibility to the DNA intercalating agent, ethidium bromide (EtBr), in order to identify genetic determinants that reduce *L. biflexa* susceptibility to antimicrobial agents. By phenotypic screening, using subinhibitory EtBr concentrations, we identified 29 genes that, when disrupted via transposon insertion, led to increased sensitivity of the bacteria to EtBr. At the functional level, these genes could be categorized by function as follows: regulation and signaling ($n = 11$), transport ($n = 6$), membrane structure ($n = 5$), stress response ($n = 2$), DNA damage repair ($n = 1$), and other processes ($n = 3$), while 1 gene had no predicted function. Genes involved in transport (including efflux pumps) and regulation (two-component systems, anti-sigma factor antagonists, etc.) were overrepresented, demonstrating that these genes are major contributors to EtBr tolerance. This finding suggests that transport genes which would prevent EtBr to enter the cell cytoplasm are critical for EtBr resistance. We identified genes required for the growth of *L. biflexa* in the presence of sublethal EtBr concentration and characterized their potential as antibiotic resistance determinants. This study will help to delineate mechanisms of adaptation to toxic compounds, as well as potential mechanisms of antibiotic resistance development in pathogenic *L. interrogans*.

Leptospira spp. are a group of spirochete bacteria that exist as free-living saprophytes or pathogens in association with a diverse range of animal hosts. Pathogenic *Leptospira* spp. are the causative agents of leptospirosis, a zoonotic disease with a broad spectrum of manifestations ranging from fever and jaundice to severe organ failure (1). Pathogenic strains infect wild and domestic animals and are maintained in rodents (1). Infected animals can shed leptospires in urine, which can lead to contamination of freshwater reservoirs with *Leptospira*. Transmission to another host can occur either through direct contact with *Leptospira*-positive urine or contaminated water (2).

The saprophyte *Leptospira biflexa* has proven to be an indispensable model for the study of *Leptospira* biology due to relative (compared to pathogenic species) ease of *in vitro* culturing and genetic manipulation. Genetic manipulation of *L. biflexa* can be achieved via random insertion mutagenesis using the *Himar1* transposon (3) and targeted homologous recombination (4), both with high efficiency. The genome sequence of *L. biflexa* has also been published (5), allowing identification of inactivated genes and their comparison to genes of pathogenic strains.

In the present study, we aimed to identify genes critical for ethidium bromide (EtBr) tolerance by exposing a library of *L. biflexa* transposon insertion mutants to a sublethal EtBr concentration. Ethidium bromide covalently binds to DNA and eventually causes DNA precipitation (6). It also elicits a bacterial SOS response by activating *lexA*, *recA* (7), and *umu* (8) genes. The proteins LexA and RecA are involved in the first line of the cell defense against DNA damage, and UmuD'2C is an error-prone polymerase expressed as the last effort to overcome severe DNA damage (9).

In addition to the SOS stress response (10), *L. biflexa* possess a mechanical barrier, the outer membrane (OM), as a basic defense against toxic compounds. Due to the presence of lipopolysaccharide (LPS) in the OM of *Leptospira*, hydrophilic agents, including EtBr and some antibiotics, cannot diffuse into the periplasm. Even so, the EtBr molecule enters into the periplasm, and this is thought

to occur through OM channels mainly formed by porins (11). In contrast, the inner membrane is permeable, and EtBr molecules may enter the cytoplasm from the periplasm via simple diffusion and bind to the DNA. Diffusion into the cytoplasm can be prevented by capturing EtBr molecules by multidrug efflux pumps spanning both the outer and the inner membranes (11). These membrane proteins are capable of active transport of a broad range of substrates from the cytoplasm to the exterior of the cell. EtBr is transported by the majority of multidrug efflux transporters and has been used to characterize the efflux activity in many bacterial species (12). In addition to efflux pumps, inner-membrane single-drug transporters can contribute to the efflux of EtBr back to the periplasm (11). The level of the EtBr sensitivity is therefore mainly determined by the ratio between influx through porins and the efflux through both types of efflux pumps (11). In general, efflux systems contribute to cell detoxification by exporting structurally unrelated drugs from the cell, either as specific single-drug or nonspecific multidrug pumps (13). It follows that overexpression of proteins involved in the process of drug efflux or mutational gain of function in the genes encoding these proteins contribute to antibiotic resistance in a number of bacterial species (14). It is important to note that constitutive expression of antibiotic resistance genes is not beneficial to the bacterial cell.

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Address correspondence to Mathieu Picardeau, mathieu.picardeau@pasteur.fr.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Source or reference ^b
Strains		
<i>L. biflexa</i> serovar Patoc strain Patoc I	Wild-type strain	NRCL
<i>L. interrogans</i> serovar Manilae strain L495	Wild-type strain	NRCL
<i>L. interrogans</i> serovar Manilae strain L495 m868	<i>ybiT</i> :: <i>Himar1</i> [Km ^r]	Monash (47)
<i>L. interrogans</i> serovar Manilae strain L495 m1133	<i>ybiT</i> :: <i>Himar1</i> [Km ^r]	Monash (47)
<i>E. coli</i> strain β2163	F [−] RP4-2-Tc::Mu Δ <i>dapA</i> ::(<i>erm</i> - <i>pir</i>) [Km ^r Em ^r]	85
<i>E. coli</i> strain π1	DH5α Δ <i>thyA</i> ::(<i>erm</i> - <i>pir</i> 116) [Em ^r]	85
<i>E. coli</i> strain S17-1	F [−] RP4-2-Tc::Mu <i>aph</i> ::Tn7 <i>recA</i> [Sm ^r]	86
Plasmids		
pCjTKS2	<i>E. coli</i> and <i>L. biflexa</i> shuttle vector (<i>Himar1</i> transposon) [Km ^r]	28
pSW29Le94	<i>E. coli</i> and <i>L. biflexa</i> shuttle vector [Spc ^r]	3
pMAT	<i>E. coli</i> and <i>L. biflexa</i> shuttle vector [Spc ^r]	This study
TnSC189	<i>E. coli</i> and <i>L. biflexa</i> shuttle vector [Spc ^r]	Monash (87)
pkefB	pSW29Le94::LEPBIa0085 [Spc ^r]	This study
p2382	pSW29Le94::LEPBIa2382 [Spc ^r]	This study
p0857	pMAT::[PflgB] LEPBIa0857 [Spc ^r]	This study
p0993	pMAT::LEPBIa0993 [Spc ^r]	This study
pybiT	pSW29Le94::LEPBIa2665 [Spc ^r]	This study
pybiT2	TnSC189::LEPBIa2665 [Spc ^r]	This study
parsB	pSW29Le94::LEPBIa2874 [Spc ^r]	This study
pfabGb3	TnSC189::LEPBIa0050 [Spc ^r]	This study

^a Em^r, erythromycin resistance; Spc^r, spectinomycin resistance; Km^r, kanamycin resistance.
^b NRCL, National Reference Center for *Leptospira*, Institut Pasteur, Paris, France; Monash, Monash University, Victoria, Australia.

The overexpression of efflux pump genes can lead to reduce nutrient accusation, leading to significantly reduced fitness (15). In addition to their role in cell detoxification, these channels are also used to transport LPS molecules across the periplasm and cell envelope (16) and to secrete proteins into the extracellular space, including siderophores, which are used to scavenge iron from the host (17). The numerous functions of these channels make them indispensable for many bacteria, as demonstrated in *Escherichia coli*, where AcrAB/TolC is essential for colonization of the host (18). Genes encoding transport related proteins account for 11.0% (i.e., 356) of all of the genes in the *L. biflexa* genome (19, 20), highlighting their importance for the survival of this bacterium. In pathogenic *L. interrogans* serovar Lai, transport-related genes represent 10.2% (i.e., 285) of all genes. In *E. coli*, the process of adaptation to toxic compounds involves changes in the OM, reduced expression of proteins forming porin channels, and/or increased expression of efflux pump-forming proteins (21). Adaptation in this manner can eventually contribute to the emergence of antibiotic-resistant strains (22). Adaptations can be driven by environmental sensing genes and successive modulation of gene expression. The *L. biflexa* genome encodes a broad repertoire of regulatory genes and environmental sensing genes, which can be further divided into three classes: two-component systems, GGDEF response regulator systems, and extracytoplasmic function sigma factors (ECFs) (2, 5). Rapid adaptation to environmental conditions is essential for survival, not only for the free-living *L. biflexa* but also for pathogenic species during the transmission cycle. Antibiotic-resistant *Leptospira* strains have yet to be identified in clinical isolates. However, in the related spirochetes *Treponema pallidum* (the causative agent of syphilis) and *Borrelia burgdorferi* (the causative agent of Lyme disease), macrolide-resistant clinical isolates have been reported (23, 24). Moreover, Criswell et al.

showed that *B. burgdorferi* has a high frequency of incidence of a spontaneous resistance to spectinomycin with a relatively low fitness cost (25). With the current overuse of antibiotics, the emergence of antibiotic-resistant *Leptospira* strains in humans or animals cannot be ruled out, and it is important to identify leptospiral genes with a potential to be involved in this process. In the only existing systematic study addressing *Leptospira* response to antibiotics, the DNA-gyrase inhibitor, ciprofloxacin, was used for screening (26). Malmström et al. observed an upregulation of recombinase A and defined a group of “Cipro-induced” hypothetical proteins that comprised ca. 20% of the entire proteome after the ciprofloxacin treatment (26). However, no precise mechanism for the ciprofloxacin response was suggested. Ethidium bromide is a convenient model compound with a broad range of antimicrobial effects, and it has been used for exploring antibiotic resistance for more than 2 decades (27). The present study contributes to a better understanding of toxic compound tolerance and resistance mechanisms in *Leptospira* species.

MATERIALS AND METHODS

Bacterial strains and plasmids. *L. biflexa* serovar Patoc strain Patoc I and *L. interrogans* serovar Manilae strain L495 were grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium or on solid medium containing EMJH supplemented with 1% agar at 30°C. *E. coli* strains β2163 and π1 were grown in Luria-Bertani (LB) medium, supplemented with diaminopimelic acid (DAP) (0.3 mM; Sigma-Aldrich, St. Louis, MO) or dT (0.3 mM; Sigma-Aldrich) at 37°C, respectively. *E. coli* S17-1 has no special growth requirements and was grown in LB medium at 37°C. When desirable, media were supplemented with spectinomycin, kanamycin, and/or carbenicillin (50 mg ml^{−1}). All strains and plasmids used in the present study are listed in Table 1.

Insertion mutant library preparation and screening. An *L. biflexa* mutant library was generated by random transposon insertion mutagenesis (28). Briefly, the shuttle vector pCjTKS2 carrying *Himar1* transposon

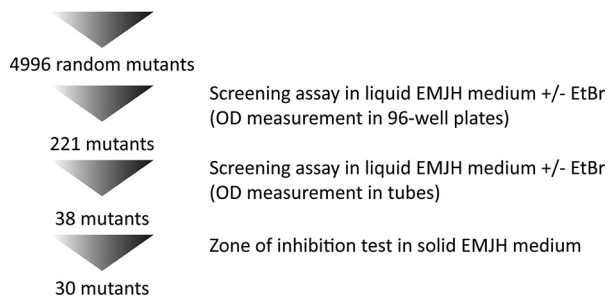
Transposon mutagenesis in *L. biflexa*
in solid EMJH medium

FIG 1 Schematic representation of library screening.

was introduced into *L. biflexa* strain Patoc by conjugation with *E. coli* strain β 2163. Kanamycin-resistant colonies were picked and recovered in liquid EMJH medium in 96-well microtiter plates. After 3 days of culturing at 30°C, 20 μ l of the culture was used to inoculate 180 μ l of EMJH medium in a 96-well microtiter plate. Two replicates for each mutant were prepared: one replicate in regular EMJH and another in EMJH supplemented with 1 μ M EtBr (Eurobio, Paris, France). Growth was monitored via measuring the optical density at 415 nm (OD_{415}) on days 3, 5, and 7. The library was subsequently screened in 3 ml of liquid EMJH, with the OD_{415} being measured every day for 7 days. A schematic of library screening is depicted in Fig. 1.

DNA isolation and identification of transposon insertion sites. DNA was isolated using a Maxwell 16 cell DNA purification kit and a Maxwell 16 instrument (Promega, Madison, WI), which was subsequently used for semirandom PCR to identify the transposon insertion site (28). The resulting PCR products were sequenced, and the SpiroScope database (19) was used for BLAST data analysis. Primers were designed using the Primer3 tool (29) for regions flanking potential insertion sites, and PCR was performed for transposon insertion confirmation.

Inhibition assays. Zone-of-inhibition tests were used to confirm the EtBr susceptibility of 38 mutants selected in the two-round library screening described above. A total of 2×10^7 cells (in a 200- μ l volume) were spread in duplicate on agar plates. A nonimpregnated paper disc (Bio-Rad, Hercules, CA) was placed in the center of the plate and supplemented with 5 μ l of 1.7 M EtBr (Eurobio) or 5 μ l of 17% bile salts aqueous solution (Sigma). The inhibition zone radius (IZR) was measured after 7 days, and each experiment was repeated at least twice. A parametric two-tailed *t* test ($P < 0.05$) was used to determine whether there was a significant difference between the IZR formed when using wild-type Patoc or mutant strains.

MIC was determined by broth microdilution assay (alarBlue [Life Technologies, Carlsbad, CA] assay; see Fig. S1 in the supplemental material) (30). Briefly, 2×10^6 cells (in a 20- μ l volume) were inoculated in 180 μ l of EMJH in a 96-well microtiter plate with decreasing concentrations of either compound (2-fold serial dilutions). Concentration ranges for tested compounds were set as follows: ethidium bromide at 270 to 0.26 μ M, erythromycin at 160 to 0.08 ng ml⁻¹, streptomycin at 24.00 to 0.02 μ g ml⁻¹, spectinomycin at 24.00 to 0.02 μ g ml⁻¹, chloramphenicol at 13.00 to 0.01 μ g ml⁻¹, tetracycline at 13.00 to 0.01 μ g ml⁻¹, bile salts at 5 to 0.01%, crystal violet at $(0.01 \text{ to } 9.80) \times 10^{-6}\%$, sodium dodecyl sulfate (SDS) at $(0.1 \text{ to } 9.80) \times 10^{-5}\%$, acridine orange at $(0.02 \text{ to } 2.00) \times 10^{-5}\%$, and NaCl at 0.9 M to 0.90 μ M (all from Sigma-Aldrich) and ampicillin at 26.00 to 0.03 μ g ml⁻¹ (Euromedex, Strasbourg, France). After 2 days of incubation at 30°C, 20 μ l of alarBlue was added into each well. For *L. interrogans* serovar Manilae, incubation time was extended to 4 days due to a slower doubling time. The MIC values were obtained after overnight incubation with alarBlue (see Fig. S1 in the supplemental material). A parametric two-tailed *t* test ($P < 0.05$) was used to determine whether there was a significant difference between the MICs of the wild-type Patoc and mutant strains.

Complementation. PCR products were first cloned into TOPO vector (Life Technologies), and the resulting constructs were used to transform XL10-Gold cells (Agilent Technologies, Santa Clara, CA). Plasmids were then isolated by the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) and ligated into the pSW29TLe24 shuttle vector (3). Because the conjugative *E. coli* β 2163 strain is transformed with a low efficiency, pSW29TLe24 containing the wild-type gene was first subcloned into *E. coli* π 1; the plasmid was isolated by the QIAprep Spin MiniPrep kit (Qiagen) and finally transformed into *E. coli* β 2163. Conjugation with mutant strains followed the protocol described above (28). One additional step was required for genes amplified without native promoters. Prior to cloning into the pSW29TLe24, *flgB* promoter from *Borrelia burgdorferi* was added to the construct. Alternatively, inserts recovered from the TOPO vector were cloned directly into the TnSC189 vector and maintained in *E. coli* S17-1 strain, which was used for the conjugation. This approach was used for the LEPBla0050 and LEPBla2665 genes. Genotypes of complements were confirmed by PCR and subsequently screened as described above to measure restoration of phenotype. All primers used for the amplification of wild-type genes are listed in Table 2.

Bioinformatic analyses. The NCBI Conserved Domain Search (NCBI CDS) (31), Pfam (32), ExPasy PROSITE (33), ABCISSE (34), and MEROPS (35) databases were used to confirm the annotation or to find potential function for genes annotated as hypothetical. Orthologues of disrupted genes in pathogenic species were identified using the MaGe database (19). The PSORTb database v3.0.2 (36) was used to predict protein subcellular localization, and the presence of signal peptides was predicted using SignalP v4.0 (37). The PePPER tool (38) was used for promoter predictions for designing the constructs for complementation.

qRT-PCR. To determine whether adjacent genes were cotranscribed and/or whether promoters of downstream genes resided in upstream genes, total RNA from the wild-type Patoc strain was isolated using TRIzol (Invitrogen, Carlsbad, CA) and prepared for reverse transcription-PCR as previously described (39, 40). Alternatively, total RNA was prepared from the LEPBla0073 mutant. cDNA was obtained using iSCRIPT kit (Bio-Rad). Primers for the real-time quantitative reverse transcription-PCR (qRT-PCR) were designed using the Primer3 tool (29) and are listed in Table 3. The following primers for the determination of a transcript level of the LEPBla0072 gene were also designed (29): 0072F (CAA TTGCTTGCTCCAGTGTG) and 0072R (TCGAGTTATCTGGCGAAA TCA). The real-time PCR mixture consisted of 10 μ l of Sso Fast Eva Supermix (Bio-Rad), 5 μ M forward primer, 5 μ M reverse primer, 2 μ l of cDNA, and 8 μ l of water. Real-time PCR was performed as follows: 3 min at 95°C, followed by 39 cycles of 10 s at 95°C and 30 s at 55°C, with a final 10 s at 95°C. A control melting curve was performed using a 65 to 95°C gradient with 0.5°C increments. The results were analyzed using Bio-Rad CFX Manager v3.1 software (Bio-Rad). The expression difference was determined using the $\Delta\Delta C_T$ comparative quantification algorithm with the housekeeping gene *flaB3* as a normalizer and the wild-type LEPBla0072 gene as a calibrator (41). An unpaired *t* test with Welch's correction was used to determine significant differences in expression.

RESULTS

Library screening revealed 30 mutants with increased susceptibility to EtBr. We first determined the MIC of EtBr for the wild-type Patoc strain by the broth microdilution assay ($MIC_{PatWT} = 30.4 \mu$ M). For the library screening, we used a sublethal EtBr concentration of 1 μ M (wild-type Patoc growth in liquid EMJH medium supplemented with 1 μ M EtBr was not affected) in order to avoid an extensive SOS response. Subinhibitory concentrations of antimicrobials has been shown to simplify discovery of drug targets by eliminating off-targets (42).

A library containing a total number of 4996 *L. biflexa* random mutants was constructed and screened. In the first round of the screening, we measured growth rates in EMJH supplemented with

TABLE 2 Primers used for the amplification of wild-type genes used for complementation of the mutant strains

Amplified gene	Primer	Sequence (5'–3') ^a
LEPBIa0050	fabG3b-F	CCGCTCGAGTCGAGGTCTTCTTTTGGGACT
	fabG3b-R	CCGCTCGAGCACTGTGCTTGCTAGTGTGA
LEPBIa0085	kefB-F	CCGCTCGAGGACCGGATGCCATGTATGTG
	kefB-R	CCGCTCGAGCGTGCTGTGACCGATTTTGA
LEPBIa0857	LEPBIa0857pl-F	GCTCATATGAAGCCACTGGAAGA
	LEPBIa0857pl-R	CCGCTCGAGCCCTCTCTTACATCTGCTGG
LEPBIa0993	LEPBIa0993-F	CCGCTCGAGCCAAACCGGGGAAAAGAGTAG
	LEPBIa0993-R	CCGCTCGAGTTGTCTGGCTGTTTTGCTGG
LEPBIa2328	LEPBIa2328-F	GCGTCGACGTAGTTCTTGGGCGCAGTTC
	LEPBIa2328-R	GCGTCGACATGAATCCCATCGCCTGAGT
LEPBIa2665	ybiTtn-F	GCGTCGACAGAACAGACTTCCCCTTGCA
	ybiTtn-R	GCGTCGACATAAACGAAGGTGCTCGGA
	ybiT-F	GCGTCGACCCGGGTACATTGGGGATTA
	ybiT-R	GCGTCGACAGGGAGAGATGCAATCCG
LEPBIa2874	arsBop-F	CCGCTCGAGCACAAACCAACGGCTTCTGA
	arsBop-R	CCGCTCGAGCGGTATAGGCGATGGATGG

^a Restriction enzyme recognition sites are indicated as follows: XhoI, underlining; NdeI, italics; and SalI, boldfacing.

1 μM EtBr and in regular EMJH. In total, 221 susceptible mutants were picked for a subsequent screening. In the second round of screening, these 221 mutants were monitored daily and growth curves were constructed for each mutant in the liquid EMJH medium with or without 1 μM EtBr. Based on the comparison of growth curves, 38 mutants displaying no/limited growth or a significant growth lag, were selected. Nested PCR was performed for each mutant, and PCR products were sequenced to determine the *Himar1* transposon insertion site (28). Insertion sites were identified in 36 distinct genes, and two mutants had insertions in two independent sites of the LEPBIa2328 gene (Table 4). All transposon insertion sites were further confirmed by PCR with primers flanking the insertion site. The scheme of the library screening is depicted in the Fig. 1.

To further confirm and quantify the susceptibility of each mutant, we cultured all 38 mutants on EMJH plates with an EtBr gradient (zone-of-inhibition test). This approach identified a significant difference in the sizes of the IZRs for 30 mutants compared to the wild-type Patoc strain (Table 4). The sizes of zones varied from 1.19 ± 0.07 cm to 2.7 ± 0.42 cm, while the zone formed by the wild-type Patoc strain was 0.3 ± 0.06 cm. Two independent LEPBIa2328 mutants displayed different IZRs in the zone-of-inhibition test (2.54 ± 0.3 cm and 1.65 ± 0.14 cm). The remaining strains, with insertions in LEPBIa0470, LEPBIa0681, LEPBIa1045, LEPBIa3204, LEPBIa3036, LEPBIb0001, LEPBIb0094, and LEPBIb0196, demonstrated IZRs comparable to that of the wild-type Patoc strain.

All mutants grew at the same rate as the wild-type Patoc strain in liquid EMJH medium at 30°C, with the exception of the LEPBIa2328 and LEPBIa2665 mutants. Both LEPBIa2328 and LEPBIa2665 mutants were able to reach stationary phase, but their growth was delayed in both solid and liquid media (see Fig. S2 in the supplemental material).

Of the 29 disrupted genes, 27 were located on the large chromosome CI, one on the small chromosome CII, and one on the

plasmid p74. It has been shown that the *Himar1* transposon integrates between TA nucleotides with no additional requirements (43). Nevertheless, we sequenced 104 additional mutants from the first round of the screening to evaluate the randomness of the library. We identified 141 unique insertion sites: 132 (93.6%) were located on CI, 8 (5.7%) on CII, and 1 (0.7%) on plasmid p74. One would expect similar distribution based on the share of individual elements on the whole genome size (91.1, 7.05, and 1.9% for CI, CII, and p74, respectively [5]). We therefore conclude that *Himar1* insertion was not biased toward any part of the *L. biflexa* genome and was thus truly random.

A high number of genes was part of putative operons. Of the 29 disrupted genes, 13 (44.8%) were organized in putative operons (Table 5). To assess whether genes downstream of the insertion site were deregulated and therefore could contribute to the observed phenotypes, we performed qRT-PCR. Cotranscription of genes was verified by using primers that amplified intergenic regions between genes in these putative operons. We detected PCR products for all tested intergenic regions, confirming that all tested genes were cotranscribed with their respective neighbors (see Table S1 in the supplemental material).

A polar effect of the *Himar1* insertion may elucidate the phenotype of two mutants, which we have designated the LEPBIa0073 and LEPBIa0275 mutants. LEPBIa0073 encodes a galactokinase, an enzyme that has not been shown to be involved in antibiotic tolerance or cell detoxification. However, the LEPBIa0073 gene is cotranscribed with the LEPBIa0072 gene, which encodes a putative anti-sigma factor antagonist. Gene LEPBIa0275 encodes a hypothetical protein without a predicted function, and the downstream LEPBIa0274 gene encodes a putative ABC2 ATPase; both are cotranscribed. Moreover, the *Himar1* insertion is localized at the 3' ends of both LEPBI0073 and LEPBIa0275 (Table 4). Since insertions in the 3' end of genes have a lower potential to affect the function of the encoded protein, this provides more evidence that the observed phenotype is the result of the polar effect on down-

TABLE 3 Primers used for qRT-PCR

Putative operon	Primers	Sequence (5'–3')
LEPBIa0072– LEPBIa0073	0072–0073F 0072–0073R	CTGATTTCAGGGAGTGGGACA AGGATTGGGAGTCATGGGTG
LEPBIa0146– LEPBIa0149	0146–0147F 0146–0147R 0147–0148F 0147–0148R 0148–0149F 0148–0149R	GGCAGATATTCCAAGACCCG GTTTTGATCCACGCTGACCA TGTCTGCCATCGAACTCACT CAAGATGCGTAACTCAGCGT GACATGGATGCTGCCGAAAT TAAACGCAAAAGTGGTCGCA
LEPBIa0274– LEPBIa0275	0274–0275F 0274–0275R	GGTTTGATGTGCCAGCTGAA CGCGAAAATGACGGTAGAGG
LEPBIa0674– LEPBIa0676	0674–0675F 0674–0675R 0675–0676F 0675–0676R	ATAAGCTCGGAATGCAAGGG TGGTGGCATTGGAGAGATGT TCTATCCCATTCACCTCGCA AATGGTAGACGAAGCTCCGG
LEPBIa0811– LEPBIa0812	0811–0812F 0811–0812R	GGAATTTCCGGGCAAGTTACC AATTGTGCCACCAATTCCTCC
LEPBIa0856– LEPBIa0857	0856–0857F 0856–0857R	ACAAGTGAGAACAGAAGCATCG CTTCAGGCGAAAATCCAGGG
LEPBIa0884– LEPBIa0885	0884–0885F 0884–0885R	TCCATGACCCGTGATGTTTG TGGCGGAAGAATACACTCGA
LEPBIa0993– LEPBIa0995	0993–0994F 0993–0994R 0994–0995F 0994–0995R	CCAGGTTCGGAGTTTGTTTCG TTGTCTGGCTGTTTTGCTGG ATCCTTGGAATGGTGGGGTT GGATTGGGCCAAGACGTTTC
LEPBIa1574– LEPBIa1576	1574–1575F 1574–1575R 1575–1576F 1575–1576R	GCGATGAAGTATGTCTGCC AACGTTTTGCTTGGCCTAAG TTTGAAAGGCGAACGGAAGG TGTGAACATCCAAATTCGCTTG
LEPBIa1579– LEPBIa1587	1579–1580F 1579–1580R 1580–1581F 1580–1581R 1581–1582F 1581–1582R 1582–1583F 1582–1583R 1583–1584F 1583–1584R 1584–1585F 1584–1585R 1585–1586F 1585–1586R 1586–1587F 1586–1587R	ACCACAGCAGAGTCATCGAT CGCACAAAACCCACGGAATA TGAAGGATCGGGTGCCATAA CGAAGAAGCACCTAGTTTTGGA ATTGATCACACCGGGGATGT CGCCTTCAAAACACCAATCG CCGGAAGTATACAACTGATCCA AAGGTGTCAGTGGGTCTAGC CGACATGAGCGAAGGAAACA CGTTACGGGACAAGGAAAGT TTGGTGTTTGGAGTGGCTTC CCAAACCGTTTCTCCCGAA CGAAACACTGCCGAGTCATC TCGAATACACCCACCTGTTGA CGTAAGTTGCTCCAATTCGC TCACAGGGATTGGCTGAAAA
LEPBIa2457– LEPBIa2459	2457–2458F 2457–2458R 2458–2459F 2458–2459R	CACTTTCTGCGGCCATCTTT GGCTGTGCAATGGATGTGAC ACCGAGAATGGCGATTAAGT CGGGAAAAGGAAATGTTCCGA
LEPBIa2871– LEPBIa2874	2871–2872F 2871–2872R 2872–2873F	CCAATCCACTGTGTCCCAAC CCCGCACTATACACATTCGGC ACAACCTCAGTGACCTTCCA

TABLE 3 (Continued)

Putative operon	Primer	Sequence (5'–3')
	2872–2873R 2873–2874F 2873–2874R	AATTCGCCGCCACAATCAACC CCGAATGCAAAGCCAAATCC TGTTCCACATTCAAGGCTTG
LEPBIa3230– LEPBIa3231	3230–3231F 3230–3231R	GGAAACCGCGAACAATCAGA CTTTCACAACCCCACTG

stream genes. We quantified the transcription of the LEPBIa0072 gene in the LEPBIa0073 mutant by qRT-PCR. The transcription of LEPBIa0072 was not abolished but was significantly reduced [(0.54 ± 0.02)-fold; $P = 0.0023$] compared to the wild-type Patoc strain.

Regulatory genes and transporters are the main determinants of EtBr tolerance. To ascertain biological processes that contribute to EtBr tolerance, we categorized genes into COG (Clusters of Orthologous Groups) according to the predicted function of the disrupted gene. Gene LEPBIa0073 was classified into two COG categories: carbohydrate transport and metabolism (G) and signal transduction (T) (Fig. 2 and Table 4). Similarly, gene LEPBIa0275 was classified as a gene with unknown function (S) and a gene with general function predicted (R) (Fig. 2 and Table 4).

We identified a significant bias toward genes involved in transport (P, Q, U, and V) and signaling (T) (Fig. 2) with six genes encoding transporters (LEPBIa0085 (*kefB*), LEPBIa0149 (*exbB1*), LEPBIa1312 (*czcA*), LEPBIa0857, LEPBIa0993, and LEPBIa2874) and 11 genes encoding signal transducers and regulators. Five genes were already annotated as signal transducers ((LEPBIa0072, LEPBIa1069, LEPBIa1587, LEPBIa2328, and LEPBIa2458), and we designated LEPBIa0274, LEPBIa0676, LEPBIa0811, LEPBIa1575, LEPBIa2665, and LEPBIa3231 as putative signal transducers and regulators based on identified domains and homology with signal transducing and regulatory genes of other bacteria. Genes LEPBIa0274 and LEPBIa2665 genes belong to the ART (Antibiotic Resistance and Translation regulation) ABC2 family. They possess two ATP-binding ABC domains with no transmembrane domain, and therefore they are unlikely to be involved in transport (34). Genes LEPBIa0274 and LEPBIa2665 are orthologues of the *ybiT* gene of *Erwinia chrysanthemi* (LEPBIa0275 identity [Id] = 27.3%, conserved protein [Pos] = 48.9%, and Id = 50.5%, Pos = 71.0%, respectively) (44). Gene LEPBIa1575 contains three GAF domains (cGMP-specific phosphodiesterases, Adenylyl cyclases and FhlA), SpoIIE (Stage II sporulation protein E) domain, and histidine kinase-like ATPase domain; all three domains are commonly present in signal transducers (31, 32). Gene LEPBIa1575 is a homologue of the RsbU phosphatase of *Bacillus subtilis* (Id = 31.4%, Pos = 56.73%). Genes LEPBIa0676, LEPBIa0811, and LEPBIa3231 were annotated based on the presence of functional domains. Gene LEPBIa0676 contains a histidine kinase-like ATPase domain, and it has homologues with sensing and regulatory functions in other *Leptospira* species (19, 31, 32). Gene LEPBIa0811 contains two GAF-3 domains and one GGDEF domain (named after the central motif GG[DE][DE]F) (32, 33). Both GAF and GGDEF domains are involved in signal transduction in numerous bacterial species (32, 33). Gene LEPBIa3231 contains a zinc binding HEXXH motif

TABLE 4 EtBr-susceptible mutants with corresponding IZR values and predicted functions of interrupted genes^a

Interrupted gene	Gene name	Tn insertion position (gene length) ^b	IZR ± SEM (cm)	P ^c	Homologue in <i>L. interrogans</i> (% identity) ^d	COG ^e	Annotation (putative proteins) ^f
LEPBla0050	<i>fabG3b</i>	373 (768)	2.70 ± 0.42	0.0016**	LIC10700 (79.2)	I, Q, R	3-Ketoacyl-acyl carrier protein reductase
LEPBla0073	<i>galK</i>	1093 (1,170)	1.19 ± 0.11	0.0014**	LIC13103 (44.1)	G, T	Anti-sigma factor antagonist
LEPBla0085	<i>kefB</i>	162 (2,109)	2.16 ± 0.14	<0.0001***	LIC12221 (45.0)	P, T	Glutathione-regulated potassium efflux system protein KefB; PMP
LEPBla0149	<i>exbB1</i>	118 (840)	1.98 ± 0.18	0.0002***	LIC10892 (58.8)	U	Biopolymer transport ExbB protein; PMP
LEPBla0275		137 (162)	1.24 ± 0.12	0.0014**	LIC10389 (57.3)	R	ABC ATPase
LEPBla0676		306 (630)	1.53 ± 0.03	0.0018**	LIC13344 (43.5)	T	Histidine kinase
LEPBla0711		215 (690)	1.73 ± 0.37	0.099**		T	Leptospiral OMP
LEPBla0811		717 (2,100)	1.64 ± 0.20	0.0011**	LIC11248 (43.3)	T	GGDEF protein
LEPBla0846	<i>lon</i>	1974 (2,373)	1.83 ± 0.45	0.0157*	LIC10608 (69.1)	O	DNA-binding ATP-dependent protease Lon
LEPBla0857		1660 (3,177)	1.95 ± 0.01	<0.0001***		V	RND efflux transporter; PMP
LEPBla0885		704 (1,050)	2.08 ± 0.23	0.0005***	LIC12555 (43.4)	L, D	Lipase chaperon
LEPBla0981		34 (1,464)	1.80 ± 0.07	<0.0001***		M	Glycosyltransferase; PMP
LEPBla0993		1585 (2,973)	1.90 ± 0.06	<0.0001***		V	RND efflux transporter; PMP
LEPBla1069		1614 (2,424)	1.65 ± 0.15	0.0004***		T	Hybrid histidine kinase; PMP
LEPBla1312	<i>czcA</i>	1283 (2,424)	1.65 ± 0.09	<0.0001***	LIC12224 (65.3)	P	Heavy metal efflux pump CzcA
LEPBla1575		1834 (2,748)	2.09 ± 0.28	0.0011**	LIC11515 (43.9)	K, T	Phosphatase RsbU
LEPBla1587		479 (1,623)	1.40 ± 0.04	<0.0001***	LIC11528 (33.3)	T	Histidine kinase
LEPBla1772	<i>typA</i>	180 (1,809)	1.19 ± 0.07	0.0005***	LIC12357 (78.2)	T	GTP-binding elongation factor family protein, TypA subfamily
LEPBla2066	<i>pcrA4</i>	1135 (2,037)	1.39 ± 0.07	0.0002***	LIC12588 (58.1)	L	UvrD recombinase
LEPBla2216		376 (639)	2.18 ± 0.97	<0.0001***		S	Hypothetical protein
LEPBla2328		256 (336)	2.54 ± 0.30	0.0004***	LIC11004 (90.1)	T	Anti-sigma factor antagonist
LEPBla2328		150 (336)	1.66 ± 0.14	0.0003***	LIC11004 (90.1)	S	Anti-sigma factor antagonist
LEPBla2458		728 (1,305)	1.93 ± 0.05	<0.0001***		T	Histidine kinase; PMP
LEPBla2480		513 (1,755)	1.81 ± 0.10	<0.0001***	LIC11350 (45.0)	I	Acyl coenzyme A dehydrogenase
LEPBla2489		577 (1,005)	2.20 ± 0.13	<0.0001***		C	Luciferase
LEPBla2665	<i>ybiT</i>	719 (1,641)	2.06 ± 0.07	<0.0001***	LIC10538 (73.4)	R	ABC ATPase
LEPBla2874	<i>arsB</i>	1004 (1,035)	1.90 ± 0.13	<0.0001***		P	Arsenite efflux pump ACR3; PMP
LEPBla3231		165 (876)	2.19 ± 0.59	0.0185*	LIC10044 (46.1)	R	M50 metalloendopeptidase; PMP
LEPBla0208	<i>deaD</i>	1125 (1,605)	1.65 ± 0.23	0.0017**		J, K, L	ATP-dependent RNA helicase, DEAD box family
pLEPBla0059		737 (966)	1.99 ± 0.04	<0.0001***		R	Hydrolase

^a The average IZR value for the wild-type Patoc strain was 0.30 ± 0.06 cm.
^b The coordinate of the transposon insertion is based on the gene coordinates. The numbers in parentheses represent the overall gene length in base pairs.
^c The number of asterisks reflects the relative level of significance, with one asterisk being the least significant.
^d *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genome sequence (84) was used to identify homologues in pathogenic species. Genes LIC10389 and LIC13103 are homologues of LEPBla0274 and LEPBla0072, respectively (see Results).
^e The COG (Clusters of Orthologous Groups) categories are indicated by single letters as follows: I, lipid transport and metabolism; Q, secondary metabolite biosynthesis, transport, and catabolism; R, general function prediction only; T, signal transduction; C, energy production and conversion; S, function unknown; P, inorganic ion transport and metabolism; K, transcription mechanisms; L, replication, recombination, and repair; D, cell cycle control, cell division, and chromosome partitioning; U, intracellular trafficking, secretion, and vesicular transport; V, defense mechanisms; O, posttranslational modification, protein turnover, and chaperones; M, cell wall/membrane/envelope biogenesis; J, translation, ribosomal structure, and biogenesis; G, carbohydrate transport and metabolism (20).
^f Annotation is based on the *L. biflexa* serovar Patoc strain Patoc I genome in the SpiroScope database (19), with new annotations as determined in the present study. PMP, putative membrane protein; PSP, putative signal peptide.

and belongs to the M50B family of bacterial metalloendopeptidases, which are involved in signal “partner-switching” cascades (35, 45, 46).
Complementation restores the wild-type phenotype. To confirm that the observed mutant phenotypes were caused by inactivation of the identified genes, we complemented a subset of mutants with the respective wild-type gene. We selected four mutants in transporter genes (LEPBla0085, LEPBla0857, LEPBla0993, and LEPBla2874 mutants), two mutants in signaling and regulatory genes (LEPBla2328 and LEPBla2665 mutants), and one mutant

(the LEPBla0050 [*fabG3b*] mutant), a gene involved in fatty acid synthesis.
Complementation of LEPBla0085 and LEPBla2328 mutants with the respective wild-type genes, restored the EtBr tolerance to levels comparable with the wild-type Patoc strain (Fig. 3 and see Fig. S3 in the supplemental material). Partial restoration of the wild-type phenotype was also obtained after the complementation of LEPBla0050, LEPBla0857, and LEPBla0993 mutants (Fig. 3).
We did not observe a restoration of the phenotype in the

TABLE 5 Operons identified in this study^a

Disrupted gene	Annotation of disrupted gene (putative proteins) ^b	Operon	Strand	Gene(s) downstream of the disrupted gene	Annotation of gene(s) localized downstream (putative proteins) ^c
LEPBIa0073	GalK	LEPBIa0072-LEPBIa0073	–	LEPBIa0072	Anti-sigma factor antagonist
LEPBIa0149	ExbB1	LEPBIa0146–LEPBIa0149	–	<i>exbB1</i> , <i>exbD2</i> , <i>tonB</i>	TBDT
LEPBIa0275	HP	LEPBIa0274-LEPBIa0275	–	LEPBIa0274	ABC ATPase
LEPBIa0676	HisK	LEPBIa0674–LEPBIa0676	–	LEPBIa0676-LEPBIa0675	Glycosyltransferase; PolA
LEPBIa0811	GGDEF protein	LEPBIa0811-LEPBIa0812	+	LEPBIa0812	Membrane-bound acetyltransferase
LEPBIa0857	RND efflux transporter	LEPBIa0856-LEPBIa0857	+	None	
LEPBIa0885	Lipase chaperon	LEPBIa0884-LEPBIa0885	–	LEPBIa0884	N-Acetyltransferase
LEPBIa0993	RND efflux transporter	LEPBIa0993–LEPBIa0995	+	LEPBIa0994-LEPBIa0995	RND efflux transporter; HP
LEPBIa1575	RsbU phosphatase	LEPBIa1574–LEPBIa1576	+	LEPBIa1576	Anti-sigma factor antagonist
LEPBIa1587	HisK	LEPBIa1579–LEPBIa1587	–	LEPBIa1579–LEPBIa1588	Chemotaxis operon
LEPBIa2458	HisK	LEPBIa2457–LEPBIa2459	–	LEPBIa2457	Thiodisulfide reductase
LEPBIa2874	ArsB	LEPBIa2871–LEPBIa2874	+	None	
LEPBIa3231	M50 metalloendopeptidase	LEPBIa3230-LEPBIa3231	+	None	

^a Quantitative PCR was positive for intergenic regions of all listed genes, confirming that they are cotranscribed.

^b HP, hypothetical protein; HisK, histidine kinase.

^c TBDT, TonB-dependent transporter; chemotaxis operon comprises the following putative genes (starting with LEPBIa1579): *cheB*, *cheD*, *cheW*, *mcp*, *cheA*, anti-sigma factor antagonist, *cheY*, and response regulator.

LEPBIa2665[–] mutant, even though two different complementation approaches were used (complementation via plasmid or direct insertion into the chromosome). We also did not succeed in complementing the LEPBIa2874 mutant with wild-type copy of the gene. However, we did succeed in transforming the LEPBIa2874 mutant with a construct comprising all genes in the predicted *ars* operon (LEPBIa2871 to LEPBIa2874).

Increased susceptibility to other antimicrobial compounds.

A subset of mutants selected for the complementation was tested for susceptibility to other compounds. We determined their MICs against antibiotics (erythromycin, streptomycin, spectinomycin, chloramphenicol, tetracycline, and ampicillin), dyes (acridine orange and crystal violet), and detergents (bile salts and SDS) with proven antimicrobial activity. With the exception of the LEPBIa2665 mutant, there were no differences between the wild-type Patoc strain and mutants in sensitivity to antibiotics (data not shown). The LEPBIa2665 mutant was found to be more suscep-

tible to chloramphenicol, erythromycin, and spectinomycin (Table 6).

The LEPBIa2665 (*ybiT*) gene is highly conserved among all sequenced pathogenic *Leptospira* strains (Id > 70%). Using two independent *ybiT* mutants (m868 and m1133), from the *L. interrogans* serovar Manilae transposon mutant collection (47), we performed the screening described above. The phenotypes of m868 and m1133 mutants were comparable to the phenotype of the LEPBIa2665 mutant strain. The MICs for both m868 and m1133 were the same, and both were found to be more susceptible to EtBr, chloramphenicol, erythromycin, and spectinomycin, compared to the wild-type Manilae strain (Table 6).

We did not find any significant difference in MICs for acridine orange, crystal violet, or SDS. We observed a slight increase in sensitivity to bile salts in LEPBIa0050, LEPBIa2328, LEPBIa2665, and LEPBIa2874 mutants (MIC_{wt} = 0.035% and MIC_{mutants} = 0.018%, respectively), and we subsequently confirmed this find-

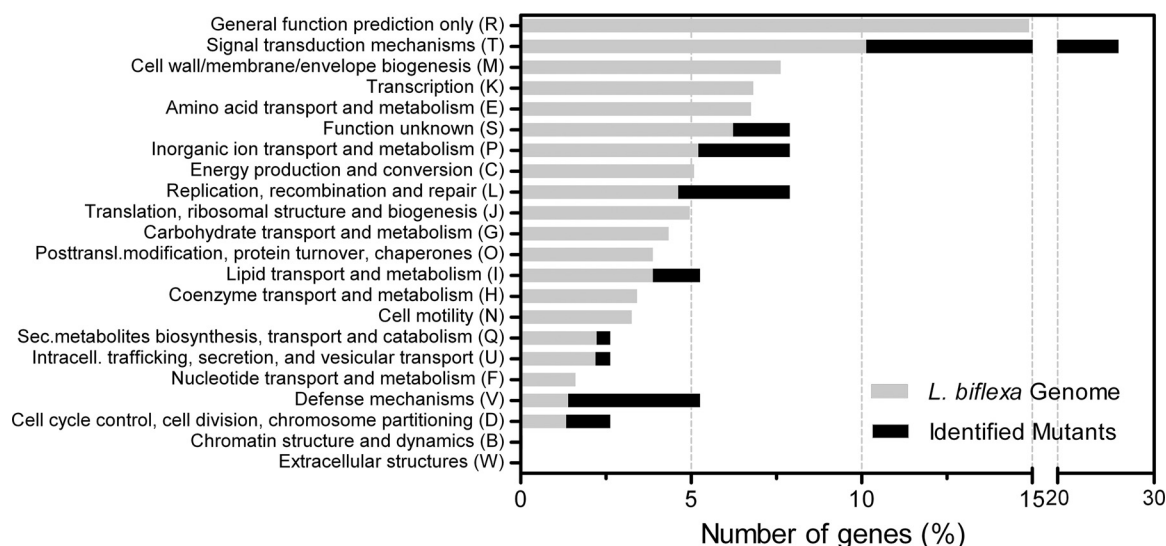


FIG 2 Classification of disrupted genes according to their predicted function. Genes were classified into COG (Clusters of Orthologous Groups) categories. Gray lines represent all genes in *L. biflexa* Patoc I genome, while black lines represent the *L. biflexa* mutants identified in the present study.

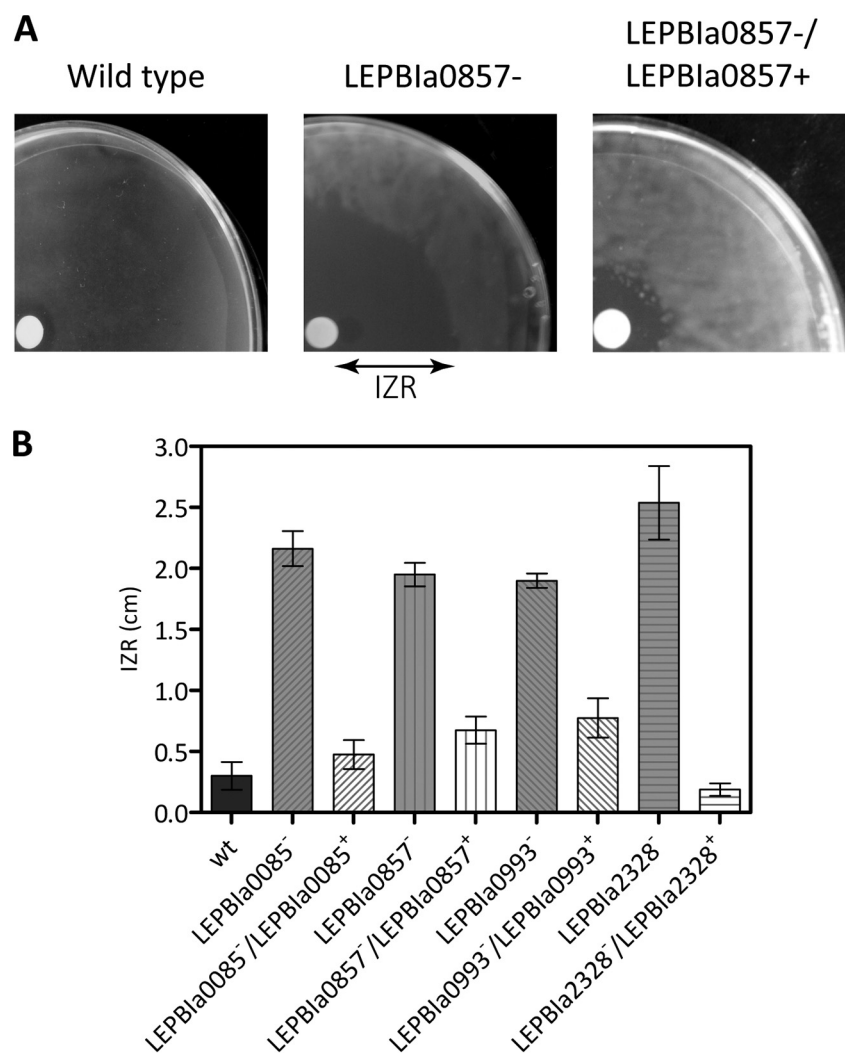


FIG 3 Complementation restores wild-type phenotype as confirmed by the zone of inhibition test. Strains were grown in the EtBr gradient. The size of the resulting inhibition zone is proportional to the level of sensitivity of an individual strain. (A) Complementation of the LEPBla0857 mutant. The inhibition zone radius (IZR) was defined as illustrated in the middle photograph. (B) The size of the bars represents the size of IZR. Mutant strains are indicated in gray, and their respective complements are indicated in white. The wild-type Patoc strain is shown in black.

TABLE 6 MICs of wild-type Patoc and Manilae strains and their respective *ybiT* mutants

Antibiotic	MIC ^a			
	<i>L. biflexa</i>		<i>L. interrogans</i>	
	Patoc	LEPBla2665 (<i>ybiT</i>) mutant	Manilae	m866/m1133 (<i>ybiT</i>) mutant
EtBr (μM)	30.4	1.0	30.4	15.2
Erythromycin (ng ml ⁻¹)	2.3	1.3	10.0	5.0
Chloramphenicol (μg ml ⁻¹)	1.6	0.4	3.2	0.1
Spectinomycin (μg ml ⁻¹)	2.0	1.0	2.0	1.0

^a MIC values were determined by using the alamarBlue assay (a broth microdilution assay) from two independent experiments, each containing two technical replicates. The MIC units are indicated in column 1. All differences between the wild-type and the *ybiT* mutant strains were statistically significant ($P < 0.001$).

ing by the zone-of-inhibition test (see Fig. S3 in the supplemental material). These experiments also further confirmed the complementation of the LEPBla2328 mutant strain, since the complemented LEPBla2328 mutant/LEPBla2328⁺ strain displayed susceptibility to bile salts comparable to the wild-type Patoc strain (see Fig. S3 in the supplemental material).

DISCUSSION

Very little is known about the leptospiral response upon exposure to antimicrobial agents. The purpose of the present study was to identify genes that contribute to tolerance to the DNA intercalating agent EtBr and identify possible resistance genes in *Leptospira* species. For the initial library screening, we used a subinhibitory concentration of 1 μM EtBr to avoid a nonspecific growth inhibition (wild-type Patoc strain growth is not impaired in the presence of 1 μM EtBr), to eliminate off-targets, and to avoid an extensive stress response (42).

Thirty-eight susceptible mutants in 36 genes were selected from 4,996 *L. biflexa* mutants in two rounds of the initial screening

performed in the liquid EMJH medium. The group of 38 mutants was further tested by the zone-of-inhibition test in a solid medium. Compared to the wild-type Patoc strain, we observed significant differences in susceptibility in 30 of 38 mutants. The lower EtBr susceptibility of the remaining eight mutants in a solid medium could be explained by a differential growth in liquid and solid media due to defects in membrane ultrastructure and/or altered osmotolerance. To avoid the distortion of results by a confounding effects in broth versus a solid medium, we continued with mutants displaying sensitivity in both liquid and solid media.

Signal transducers and regulators. Mutants in genes encoding signal transducers and regulators were the largest group identified in our study (11 genes in total [37.9%]). As an environmental bacterium, *L. biflexa* possesses a broad range of genes involved in signal transduction, sensing, and gene regulation (5). We showed here that these genes are as important for EtBr tolerance as genes encoding transporters.

Regulatory genes were represented by two genes, LEPBla2665 and its paralogue, LEPBla0275 (Id = 31.88%, Pos = 52.64%). Gene LEPBla2665 (*ybiT*) was originally annotated as an ABC transporter, but it lacks a necessary membrane-spanning domain (36). The YbiT protein is conserved among all sequenced pathogenic *Leptospira* strains, and it is among the 100 most highly expressed *L. interrogans* genes during growth within dialysis membrane chambers implanted in rats (19, 48), suggesting its importance in *Leptospira* biology. Gene LEPBla2665 has close orthologues in the ART ABC2 family, which is involved in cell regulatory processes and antibiotic resistance (34). The YbiT orthologue (YkpA) contributes to resistance to alkaline and osmotic stress in *Listeria monocytogenes* (49). In *E. coli*, its orthologue (Uup) assists DNA polymerase to correctly replicate areas between direct repeats, and its deletion reduces bacterial fitness (50, 51). Competitiveness is also reduced *in vivo* by the disruption of *ybiT* in *E. chrysanthemi* (44). The YbiT orthologues contribute to antibiotic resistance in *Streptomyces ambofaciens*, *Streptomyces lincolnensis*, and *Staphylococcus aureus* (52–54). Despite the number of described YbiT orthologues, no precise mechanism of YbiT function is known (34, 55).

Complementation did not restore the wild-type phenotype of the LEPBla2665 mutant. However, two independent *ybiT* mutants in the Manilae strain displayed sensitivity to the same range of compounds as the LEPBla2665 mutant. Since LEPBla2665 gene is not part of an operon, a polar effect on downstream genes is unlikely. Altogether, these observations suggests that disruption of the *ybiT* gene is indeed responsible for the susceptible phenotype.

The first group of signal transducers identified in the present study is represented by genes involved in “partner-switching,” a phosphorylation cascade leading to activation of ECF in response to membrane stress (56). The ECF sigma factors control expression of genes involved in extracytoplasmic functions (e.g., iron uptake, efflux, secretion, or synthesis of exopolysaccharides) (56). In the absence of stimuli, ECFs are blocked by anti-sigma factor kinases. Activation of ECFs occurs through inactivation of anti-sigma factors by STAS (sulfate transporter and anti-sigma factor antagonists) (57). In our study, we identified two STAS anti-sigma factor antagonists, LEPBla2328 and its paralogue LEPBla0072 (Id = 39.4%, Pos = 65.7%) (31–33, 58). The importance of LEPBla2328 in tolerance to EtBr is supported by the fact that we identified two independent mutants in this gene. The

probability that such event occurred by a pure chance is extremely low ($P = 6.42 \times 10^{-5}$, as determined by the Clarke and Carbon equation [59]). In addition, the LEPBla2328 mutant exhibited an increased susceptibility to bile salts. Gene LEPBla2328 displays homology to anti-sigma factor antagonists in pathogenic *Leptospira* strains (Id > 90%), which in turn colonize the kidneys and livers of their host animals. Compared to other organs, the liver produces the highest concentration of bile salts, and it may be that these anti-sigma factor antagonists are required for bile salt resistance when colonizing the liver.

It is also possible that ECF activation is facilitated by the stressosome (protein complex involved in stress response activation); in *B. subtilis*, the σ^B partner-switching activation is initiated by phosphorylation of signaling RsbU phosphatase by the environmental sensing kinase RsbT (60, 61). The library screening revealed one putative RsbU phosphatase, LEPBla1575, which forms a putative sigma factor regulon with a STAS (LEPBla1576). In addition, we identified LEPBla2458, a putative kinase, which was paired with a putative RsbU phosphatase (LEPBla2459).

Stress-responsive sigma factors can also be activated by a M50 zinc-metalloendopeptidase-mediated cleavage of anti-sigma factors (45, 46). We identified one putative M50 zinc-metalloendopeptidase, LEPBla3231, organized in an operon downstream of a putative RsbU phosphatase (LEPBla3230) (35). Altogether, four different partner-switching cascades and one metalloendopeptidase are probably involved in activation of *L. biflexa* ECFs in a response to EtBr exposure.

The second group of signaling genes identified by library screening comprises bacterial two-component systems. Bacterial two-component systems receive signals from the environment through histidine kinases (HisKs) and transfer this signal to downstream proteins, response regulators (RRs), that eventually induce a cell response by regulation of gene expression. In *E. coli*, overexpression of HisKs and RR can lead to an increased expression of efflux transporters and therefore to drug resistance phenotypes (62, 63). Based on a number of identified HisKs among the susceptible mutants, a similar mechanism could be involved in a response to EtBr exposure in *L. biflexa*.

Gene LEPBla1069 encodes a putative hybrid histidine kinase, a protein that comprises both HisK and RR domains, and it is located upstream of a putative σ^{70} factor (LEPBla1070). Gene LEPBla1587 is a putative HisK also paired with an RR (LEPBla1586). This two-component system probably controls a cluster of chemotaxis genes (LEPBla1585, LEPBla1583, LEPBla1582, and LEPBla1581), including an anti-sigma factor antagonist (LEPBla1584), located downstream from LEPBla1587. Based on predicted HisK domain, we re-annotated the LEPBla0676 protein as a putative HisK (31, 32).

The third and last group of signaling proteins is represented by diguanylate cyclase proteins (GGDEF) that synthesize the bacterial intracellular secondary messenger bis-(3',5')-cyclic dimeric GMP (c-di-GMP) (64). In other bacteria, these proteins regulate fatty acid synthesis genes and genes involved in antibiotic resistance (65). Based on the identification of LEPBla0811 as a putative GGDEF protein, a similar mechanism is most likely present in *L. biflexa*.

Transport and membrane structure. A malfunction of efflux pumps or an increased influx caused by a change in the OM or porin permeability can lead to elevated accumulation of EtBr in *Leptospira* cells and a consequent increase in susceptibility. In the

present study, we identified 11 genes involved in these processes: 6 transporter coding genes and 5 genes contributing on the OM structure.

Proteins LEPBla0857, LEPBla0993, and LEPBla1312 (CzcA) contain a multidrug efflux ACR domain that is typically present in proteins belonging to the resistance-nodulation-cell division (RND) family of transporters (31, 32). The RND family consists of promiscuous tripartite efflux systems with a wide spectrum of transported compounds, including antibiotics, detergents, and biocides (14). The main efflux pump of *E. coli*, AcrAB/TolC, is involved in tolerance to EtBr; deletion of *acrAB* genes increases sensitivity (66), and their overexpression leads to EtBr resistance (67). Because both LEPBla0857 and LEPBla0993 mutants showed no increased sensitivity to any other tested compound, they probably represent minor RND transporters of *L. biflexa*. Protein LEPBla1312 (CzcA) is a homologue of a heavy metal efflux pump.

Gene LEPBla0857 partially overlaps (23 bp) the upstream LEPBla0856 gene, which encodes a protein with a predicted signal peptide and an OM efflux domain. These two genes may therefore constitute an efflux pump. Complementation of the LEPBla0857 mutant by cloning the LEPBla0857 gene under the control of constitutive promoter (*P_flgB*) resulted in partial restoration of the phenotype. It is therefore possible that a strong constitutive expression of LEPBla0857 is not optimal for the synthesis of the efflux pump. Gene LEPBla0993 is cotranscribed with another putative multidrug resistance protein with an ACR domain (LEPBla0994), and *Himar1* insertion probably disrupted expression of both genes. For the complementation, we used a construct consisting of LEPBla0993 under the control of its native promoter. The resulting restoration of phenotype was only partial, suggesting that both LEPBla0993 and LEPBla0994 are important for EtBr tolerance.

Gene LEPBla0149 (*ExbB1*) is a part of the TonB-dependent transporter (TBDT) system utilized for iron and vitamin B uptake (68). In the *L. biflexa* and *L. interrogans* genomes, five copies of *exbB* are present in five distinct loci (69). However, only *exbB1* and *exbB2* are located in the immediate vicinity of *exbD* and *tonB* genes and therefore part of a putative TBDT operon. In general, TBDTs are involved in uptake, but TonB is also required for proper function of MexAB-OprM antibiotic efflux system in *Pseudomonas aeruginosa* (70). Insertion in *exbB1* is likely causing disruption of the entire operon, including *tonB*, thus preventing TonB regulation of other putative efflux systems.

Gene LEPBla2874 (*ArsB*) contains an ACR3 domain, present in arsenic resistance proteins, and belongs to the family of sodium-bile acid symporter family (31, 32). Indeed, we observed an increased sensitivity to bile salts compared to the wild-type Patoc strain. LEPBla2874 is the last gene of the predicted *ars* operon (LEPBla2871 to LEPBla2874). The *Himar1* insertion therefore did not disrupt the whole operon, which could explain why the increase in sensitivity to bile salts was minor. Also, the *Himar1* insertion affects only a small portion of the 3' end of the LEPBla2874 gene. We failed to complement the LEPBla2874 mutant, suggesting that the whole operon is tightly regulated and reintroduction of the operon in *trans* was not sufficient for restoration of the wild-type phenotype.

The last transporter identified by the library screening was encoded by the LEPBla0085 gene. Protein LEPBla0085 (*KefB*) is a putative glutathione-regulated potassium efflux transporter. Since it does not contain a multidrug efflux domain, it probably

belongs to the group of single-drug transporters of the inner membrane (13).

In addition to transporters, the proper functioning of proteins contributing to native OM ultrastructure is essential for the toxic compound tolerance. It follows that disruption of the LEPBla0050 (*fabG3b*) gene, encoding a putative β -ketoacyl-acyl carrier protein reductase that catalyzes the first reduction step during the fatty acid elongation cycle (71), led to the most sensitive phenotype. Protein FabG belongs to the large short-chain alcohol dehydrogenase/reductase (SDR) family, which is involved in numerous different pathways, and it is essential for the growth of enterobacteria (72–74). To confirm the LEPBla0050 gene function, we tested sensitivity of the LEPBla0050 mutant to osmolarity, SDS, and bile salts (i.e., compounds targeting the OM), and only sensitivity to bile salts was increased (see Fig. S3 in the supplemental material). Temperature-dependent inactivation of *fabG* in *E. coli* has been shown to suppress synthesis of fatty acids longer than four carbons (72). The length of fatty acid carbon chains is extremely important for the LPS structure; the lipid A molecule is composed of fatty acids longer than 10 carbons in the majority of Gram-negative bacteria (75). Therefore, the relatively minor phenotype change observed in the LEPBla0050 mutant (no increased SDS and NaCl sensitivity) suggests that its function as fatty acid elongation initiator is supplemented from one of its paralogues. Nevertheless, the LEPBla0050 mutant showed the highest EtBr sensitivity and increased susceptibility to bile salts, which suggests that LEPBla0050 is a true *fabG* gene. Since the complementation in *trans* only partially restored the sensitivity comparable to wild-type and LEPBla0050 is not part of an operon, we conclude that its expression is likely tightly regulated.

Gene LEPBla2480 (*fadE*) encodes a putative acyl coenzyme A (acyl-CoA) dehydrogenase, an enzyme initiating the first step of the fatty acid degradation pathway (FAD). The role of the LEPBla2480 in OM ultrastructure in *L. biflexa* is also demonstrated by the increased sensitivity of the LEPBla2480 mutant to bile salts (see Fig. S3 in the supplemental material). In *L. interrogans* *fad* genes (FAD pathway) are downregulated, whereas *fab* genes (FASII pathway) are upregulated after a shift to physiologic osmolarity (76). In conclusion, *Leptospira* OM permeability is most likely altered by changes in fatty acid composition in response to osmotic and toxic stress, which is well documented in other Gram-negative bacteria (77).

In addition, three hypothetical genes may be involved in OM biogenesis. Gene LEPBla0885 contains a lipase chaperon domain (31, 32), and it is located upstream of a putative apolipoprotein *N*-acyltransferase (LEPBla0884), an enzyme transferring a fatty acid group to membrane lipoproteins (31). A polar effect of *Himar1* insertion on the downstream gene LEPBla0884 could hinder the membrane lipoprotein synthesis. Membrane structure could also be affected by disruption of the LEPBla0981 gene, encoding a putative glycosyltransferase contributing to bacterial OM biogenesis (31, 32). Gene LEPBla0711 is a putative *Leptospira* OMP, and its role in the OM ultrastructure remain to be revealed.

Stress response and DNA damage repair enzymes. Only three genes involved in a stress response and DNA damage repair were identified, suggesting that they represent a minor second line of a defense. Both LEPBla0846 (*Lon*), a putative ATP-protease, which degrades misfolded proteins, and LEPBla1772 (*TypA*), a putative GTP-binding elongation factor, are involved in the bacterial stress response (78–81). Gene LEPBla2066 (*prcA4*) belongs to the family

of UvrD/Rep helicases and shares 36.35% identity with the UvrD helicase of *E. coli*, which contributes to UV lesion and DNA mismatch repair (82).

We also sequenced 104 mutants randomly selected from the first round of screening to determine randomness of the library, and among others we identified a mutant in the LEPBla2446 (*recA*) gene. Protein RecA is part of the bacterial SOS response; in *L. biflexa* it contributes to DNA damage repair caused by UV and mitomycin C (83), and it is upregulated in the presence of EtBr in *S. aureus* (7). The LEPBla2446 mutant displayed a significantly larger inhibition zone compared to the wild-type Patoc strain (IZR = 1.41 ± 0.1 cm versus 0.30 ± 0.06 cm for the wild-type strain; $P < 0.001$). We may have failed to identify this mutant in the first round of the screening due to the possibility of 1 μ M EtBr being too low of a concentration to inhibit LEPBla2446 mutant growth. Alternatively, the LEPBla2446 mutant can exhibit different levels of susceptibility in agar and broth due to the altered osmotolerance and/or altered membrane ultrastructure.

Hypothetical genes and other enzymes. We identified four genes that could not be classified into any of the above-mentioned categories. Also, a polar effect on genes in their vicinity could not explain the EtBr-susceptible phenotype. Therefore, the mechanisms where genes LEPBla0208 (DEAD box RNA helicase), LEPBla2489 (luciferase), pLEPBI0059 (alpha-beta hydrolase), and LEPBla2216 (no predicted function) contribute to *L. biflexa* EtBr tolerance remain to be revealed.

Conclusions and significance. In the presence of subinhibitory EtBr concentrations, the main defense mechanism of *L. biflexa* is the prevention of EtBr entering the cell. Our results suggest that this response is specific, since we identified a minimal number of genes typically involved in stress response (2) and DNA damage repair (1). The EtBr concentration used in the present study was 30 times lower than the MIC of the wild-type Patoc strain. Using higher EtBr concentration in future studies is desirable to assess whether *L. biflexa* has a greater repertoire of genes involved in EtBr resistance (e.g., a higher proportion of stress response and DNA damage repair genes). Alternatively, the gain-of-function screening for more resistant mutants could help to identify functional leptospiral proteins.

The majority of the identified genes (62%) have homologues in the pathogenic *L. interrogans* (84). We therefore hypothesize that response to EtBr exposure is fairly conserved among *Leptospira* species. We identified at least three genes that warrant further future characterization: LEPBla0050, LEPBla2328, and LEPBla2665. All three genes can be found in pathogenic species with high homology at the protein level (79, 90, and 73%, respectively). In the future we would like to determine whether LEPBla0050 disruption influences the length of fatty acid carbon chains produced by *L. biflexa* and, consequently, whether LEPBla0050 knockout alters the lipid A structure, since the acyl chain length of the lipid A could alter outer membrane permeability. Further studies are also warranted on the gene expression profile of the LEPBla2328 mutant in order to identify its respective sigma factor and genes under the control of its regulon. Protein YbiT (LEPBI2665) is involved in tolerance to three antibiotics with different mechanisms of action, not only in *L. biflexa*, but also in pathogenic *L. interrogans*. Determination of its precise function would be of a great interest, since its homologue, LIC10538, is highly expressed in animal models of infection (48).

To the best of our knowledge, this is the first study addressing

leptospiral response to EtBr and cell detoxification in *Leptospira* species. Our results improve our understanding of cell adaptation and antibiotic resistance mechanisms and confirm that *L. biflexa* is a suitable biological model. The results presented here also identified targets that could be exploited to enhance antimicrobial activity.

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