AEM
Journals.ASM.org

Positive Regulation of *Leptospira interrogans kdp* Expression by KdpE as Demonstrated with a Novel β-Galactosidase Reporter in *Leptospira biflexa*

James Matsunaga[a,b] and Mariana L. Coutinho[a,b,c]

Research Service, Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California, USA[a]; Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California, USA[b]; and Centro de Biotecnologia, Universidade Federal de Pelotas, Pelotas, Brazil[c]

Leptospirosis is a potentially deadly zoonotic disease that afflicts humans and animals. *Leptospira interrogans*, the predominant agent of leptospirosis, encounters diverse conditions as it proceeds through its life cycle, which includes stages inside and outside the host. Unfortunately, the number of genetic tools available for examining the regulation of gene expression in *L. interrogans* is limited. Consequently, little is known about the genetic circuits that control gene expression in *Leptospira*. To better understand the regulation of leptospiral gene expression, the *L. interrogans kdp* locus, encoding homologs of the P-type ATPase KdpABC potassium transporter with their KdpD sensors and KdpE response regulators, was selected for analysis. We showed that a *kdpE* mutation in *L. interrogans* prevented the increase in *kdpABC* mRNA levels observed in the wild-type *L. interrogans* strain when external potassium levels were low. To confirm that KdpE was a positive regulator of *kdpABC* transcription, we developed a novel approach for constructing chromosomal genetic fusions to the endogenous *bgaL* (β-galactosidase) gene of the nonpathogen *Leptospira biflexa*. We demonstrated positive regulation of a *kdpA′-bgaL* fusion in *L. biflexa* by the *L. interrogans* KdpE response regulator. A control *lipL32′-bgaL* fusion was not regulated by KdpE. These results demonstrate the utility of genetic fusions to the *bgaL* gene of *L. biflexa* for examining leptospiral gene regulation.

Pathogenic species of the spirochete *Leptospira* encounter a variety of environmental conditions throughout their life cycles (1, 18). The natural reservoirs for *Leptospira* are typically small rodents, which release the spirochetes from their renal tubules into the surrounding environment during urination. The leptospires contaminating the soil or water enter humans or animals through skin abrasions or mucous membranes and hematogenously disseminate to a number of organs, where they can cause severe systemic illness (1, 18). The genome sequences of four pathogenic and two nonpathogenic *Leptospira* strains revealed a large number of genes encoding potential transcriptional regulatory proteins, which are thought to regulate the expression of leptospiral genes as the spirochetes encounter these different environments (14). Whole-transcriptome studies and analyses of individual genes have identified a number of *Leptospira interrogans* genes that are regulated by culture conditions and the host environment (2). However, a true understanding of the genetic circuitry underlying the response of *Leptospira* to environmental stimuli cannot be obtained until the regulatory proteins or RNAs involved in the control of specific genes are identified.

Genetic approaches to identifying and examining *trans*-acting factors that regulate gene expression in pathogenic *Leptospira* are scant (18). Targeted gene disruption is extremely difficult and has been accomplished for only *ligB* and *fliY* (11, 21). Random insertional mutagenesis of pathogenic *Leptospira* can be performed by introduction of the *mariner*-based *Himar1* transposon by transformation or conjugation (31, 33). Some of the *Himar1* insertion mutations disrupted genes encoding potential transcriptional regulatory proteins and can aid the study of genetic regulatory systems in *L. interrogans* (31). For example, an *L. interrogans* mutation in a gene encoding a PerR homolog has revealed its role in the peroxide stress response and the genes controlled by the regulatory protein (23).

Many genetic studies have been performed with the nonpathogen *Leptospira biflexa*, since it is more amenable to genetic manipulation than *L. interrogans*. Targeted gene disruptions and high-frequency random *Himar1* mutagenesis have been performed on *L. biflexa* (24, 34, 39). *L. biflexa* is easily transformed with a plasmid carrying the origin of replication of the LE1 bacteriophage (37). The plasmid allows investigations of the functional properties of *L. interrogans* gene products in *L. biflexa*. In a recent study, expression of the *L. interrogans* adhesins LigA and LigB in *L. biflexa* enabled *L. biflexa* to serve as a surrogate host for studies of leptospiral binding to fibronectin and fibrinogen (9). Recently, a plasmid-based green fluorescent protein (GFP) reporter was developed to measure promoter activities of leptospiral genes in *L. biflexa* (3, 8). When the *L. biflexa hsp20* and *groES* heat shock promoters were fused to *gfp*, GFP levels increased with temperature (3). The *gfp* reporter was also used to assess the promoter activities of the *L. interrogans* genes *lig*, *sph2*, and *lipL41* in *L. biflexa* grown under different culture conditions (8).

Here, we describe a novel β-galactosidase reporter system for examining the regulation of expression of leptospiral genes in *L. biflexa* by *trans*-acting factors. We selected the *L. interrogans kdp* locus to test the reporter because of the well-understood role of the *E. coli* KdpE response regulator in activating transcription of the *kdp* operon encoding a P-type ATPase transporter in which translocation of the essential mineral potassium across the trans-

Received 6 March 2012 **Accepted** 25 May 2012
Published ahead of print 8 June 2012
Address correspondence to James Matsunaga, jamesm@ucla.edu.

doi:10.1128/AEM.00713-12

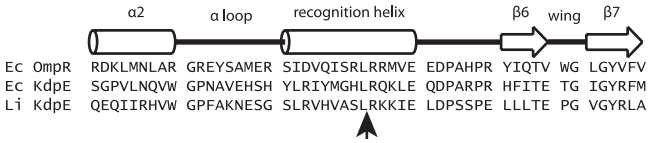

FIG 1 Location of the *Himar1* insertion in the predicted structure of *L. interrogans* KdpE. The alignment of the amino acid sequence of the carboxy terminus of *L. interrogans* KdpE with that of the winged helix-turn-helix of *E. coli* OmpR was aided by an earlier published alignment of *E. coli* KdpE and OmpR (26). The secondary structure of OmpR is depicted with cylinders (α helix) and open arrows (β strand). The location of the *Himar1* insertion in the *L. interrogans* M45 strain is marked by an arrow below the KdpE sequence.

membrane KdpA transporter subunit is coupled to the transient phosphorylation of an invariant aspartate residue in the KdpB ATPase subunit (5). When *E. coli* is starved for potassium, the KdpE response regulator is phosphorylated by the KdpD histidine kinase and activates transcription of the *kdpFABC* operon encoding the subunits of the high-affinity potassium uptake system, KdpFABC (15). We found that a *Himar1* insertion mutation in the *L. interrogans kdpE* gene prevented the increase in *kdpABC* mRNA levels observed in the wild-type *L. interrogans* strain in response to low potassium levels, indicating that KdpE was a positive regulator of *kdpABC* transcription when potassium is limiting. We next fused the *kdpE* promoter and translation initiation region from *L. interrogans kdpA* to the gene encoding the endogenous β-galactosidase on the chromosome of *L. biflexa*. Expression of *L. interrogans* KdpE in *trans* significantly increased β-galactosidase expression from the fusion, confirming that KdpE is a positive regulator of *kdp* expression. This reporter system should have broad applicability to the investigation of leptospiral gene expression.

MATERIALS AND METHODS

Strains and culture conditions. *L. interrogans* serovar Manilae strain L495, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, and *L. biflexa* servoar Patoc strain Patoc 1 (Paris) have been described previously (17, 35). The *L. interrogans* serovar Manilae mutant strain M45 has the transposon *Himar1* inserted into the *kdpE* gene (Fig. 1) (31). The *L. interrogans* L495 and M45 strains were generous gifts from Ben Adler and Gerald Murray. The leptospiral culture medium EMJH was assembled as described previously and included lactalbumin hydrolysate (Becton Dickinson), superoxide dismutase, sodium pyruvate, and 100 μg/ml 5-fluorouracil (43). *L. interrogans* Fiocruz L1-130 was cultivated in EMJH supplemented with 1% heat-inactivated rabbit serum. The serum was omitted when cultivating *L. biflexa* and *L. interrogans* L495 and M45. For experiments comparing the properties of the L495 and M45 strains, "low-K^+" EMJH was made by replacing KH_2PO_4 with NaH_2PO_4, and "standard" EMJH was made by adding potassium chloride to low-K^+ EMJH to achieve a final concentration of 2 mM. *Leptospira* cultures were placed in Erlenmeyer flasks and incubated on a rotary shaker at 30°C. The growth of cultures was monitored by counting the leptospires under a dark-field microscope.

Frozen competent *Escherichia coli* NEB5α was obtained from New England BioLabs. LB broth and LB agar powders were supplied by Genesee Scientific (San Diego, CA) and reconstituted with water as recommended by the manufacturer prior to autoclaving. Kanamycin and spectinomycin were added to LB medium to a final concentration of 40 μg/ml for selection of *E. coli* transformants.

Animals. To obtain low-passage-number spirochetes for the *in vitro* experiments, *L. interrogans* was passaged through hamsters (13). Female Golden Syrian hamsters were purchased from Harlan Laboratories (Indianapolis, IN), and 1,000 motile *L. interrogans* strain L495 and strain M45 spirochetes in 0.5 ml of EMJH were injected by the intraperitoneal route into the hamsters. The animals were euthanized when they displayed signs of illness. Their kidneys were removed, ground in phosphate-buffered saline (PBS), and inoculated into semisolid EMJH (0.2% agarose). The cultures were incubated at 30°C. All animal procedures were approved by the West Los Angeles Veterans Affairs Institutional Animal Care and Use Committee.

Reagents. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was supplied by Genesee Scientific. *ortho*-Nitrophenyl-β-D-galactopyranoside (ONPG), spectinomycin dihydrochloride, chloroform, isopropanol, and kanamycin monosulfate were obtained from Sigma. Nuclease-free water was obtained from Ambion. All other chemicals were purchased from Fisher Scientific.

RNA analysis. RNA was extracted from *L. interrogans* strains with TRIzol reagent (Invitrogen) as described previously (29). Absorbances were read with the NanoVue spectrophotometer (GE Healthcare). Two micrograms of each RNA was converted into cDNA with Superscript II reverse transcriptase (Invitrogen) and random nonamers (Sigma) following the manufacturer's instructions. The amounts of specific cDNA molecules were determined by quantitative PCR using the Bio-Rad iQ5 Real-time System. Each reaction mixture contained cDNA derived from 10 ng of RNA, 0.4 μM each primer, and 12.5 μl iQ SYBR green Supermix raised to a total volume of 25 μl with nuclease-free water. The assay was performed on three independent sets of samples, and each sample was assayed in triplicate. The ratio of *kdp* transcript to *flaA2* transcript was determined by the $2^{-\Delta\Delta CT}$ method (22).

To determine whether adjacent genes in *kdpABC* are cotranscribed, the cDNA was amplified with the primer pairs kdpA-4F/kdpB-3R and kdpB4F/kdpC-6R to amplify the *kdpA-kdpB* and *kdpB-kdpC* intergenic regions, respectively (Table 1). PCR was performed with the HotStarTaq Master Mix from Qiagen.

Plasmids. All plasmid purifications, DNA fragment purification from gel slices, and DNA purification from liquids were performed with the Zyppy Plasmid Miniprep Kit, Zyppy Gel Extraction Kit, and Zyppy DNA Clean & Concentrator-5 Kit, respectively, from Zymo Research (Irvine, CA). Genomic DNA was purified from *L. interrogans* Fiocruz L1-130 and *L. biflexa* with the Wizard genomic DNA purification kit from Promega and served as the PCR template for the plasmid constructions. Synthetic oligonucleotides were purchased from Invitrogen. Restriction enzymes, linkers, Klenow fragment, and Quick T4 DNA ligase were purchased from New England BioLabs. Phusion DNA polymerase was used for all PCRs done for plasmid constructions and was purchased from New England BioLabs. Ladder I DNA molecular weight standards were obtained from Genesee. All plasmid insertions were sequenced by Laragen (Culver City, CA).

pRAT562 harbors the 5′ fragment of *bgaL* (for beta-galactosidase of *Leptospira*) that targets the plasmid for integration into the *bgaL* gene of *L. biflexa*. To construct pRAT562, the first 293 codons of the *L. biflexa bgaL* gene were amplified from the genomic DNA of *L. biflexa* by PCR with the primers bgaL(Bm)-1F and bgaL(Sc)-2R (Table 1). The *bgaL* amplicon was then digested with BamHI and SacI. pGKlep4 was digested with BamHI and SacI to remove all leptophage sequences and attached to the digested *bgaL* DNA to create pRAT562, which encodes resistance to kanamycin (see Fig. 4A).

To fuse the promoters and translation initiation regions of various *L. interrogans* genes to the 5′ end of the *bgaL* sequence in pRAT562, the sequences upstream of the start codons of *kdpA* (LIC10990), *lipL32* (LIC11352), *tuf* (EF-Tu, LIC12875), and *flaB1* (LIC11890) were amplified from Fiocruz L1-130 genomic DNA by PCR with the oligonucleotide pairs kdpp(Xh)-1F/kdpp(Nd)-2R, lipL32p(Kp)-1F/lipL32p(Xh)-2R, tufp(Kp)-1F/tufp(Xh)-2R, and flaB1p(Kp)-1F/flaB1p(Xh)-2R, respectively (Table 1). The *kdp* PCR product was digested with XhoI and NdeI and inserted into pRAT562 that was digested with the same enzymes (see Fig. 4B). The *lipL32*, *tuf*, and *flaB1* amplicons were digested with KpnI and

TABLE 1 PCR primers

Oligonucleotide name[a]	Sequence (5′ to 3′)[b]	Coordinates[c]	Purpose[d]
bgaL(Bm)-1F	ATCggatccTCCTcatatgATCTTTGGAGCCTGTTATTACCCA	1, 27	Plasmid
bgaL(Sc)-2R	ACAgagctcGCGTGAGTATTGTTGCGTGGCAGTGAC	879, 853	Plasmid
kdpp(Xh)-1F	CTTATActcgagATCTTAACAATTTCTTAACTTCCTT	−214, −190	Plasmid
kdpp(Nd)-2R	CCTATCcatatgAATTCCTTAAAACTCTTCCGGTCTA	−4, −28	Plasmid
lipL32p(Kp)-1F	AGCGACggtaccTCAATTTGTGTCTGAGATTTGA	−231, −210	Plasmid
lipL32p(Xh)-2R	AGActcgagTTCcatatgCTCTCCTTAGTTAGGAAAATCACG	−4, −27	Plasmid
tufp(Kp)-1F	TCCAGGggtaccTAGTTGGTCCTATTTCACATTTG	−307, −285	Plasmid
tufp(Xh)-2R	ATActcgagTTCcatatgGACTTTTTAATCTCCTTACTTTTGAAC	−4, −30	Plasmid
flaB1p(Kp)-1F	CTCGCTggtaccGATCGAACCTAAGATTAGCTCA	−312, −291	Plasmid
flaB1p(Xh)-2R	GTCctcgagTTCcatatGTTTCCTCCTTGAAACTGATC	−4, −24	Plasmid
kdpE(Nd)-1F	CCTCTAcatatgAATCCTAAAATTCTGGTGGC	1, 23	Plasmid
kdpE(Xh)-4R	AATGctcgagTTATAAATGAATTGCAAGCCTATAACCTAC	684, 655	Plasmid
pGKlep4-2F	ATCGGTGCGGGCCTCT	5422, 5437	Verification
bgaL-4R	TCTACAATCTCTGGACCAAACATC	1430, 1407	Verification
kdpA-1F	TGCTGACCGTATTTTTAAGCGGGATA	1139, 1164	qRT-PCR
kdpA-2R	AAACCGTGAGGGCCTCGATTTGT	1334, 1302	qRT-PCR
kdpB-1F	TTGCCGCAGAAGCGGGAGTG	1457, 1476	qRT-PCR
kdpB-2R	TGCGCTAATGCAGGCGCATC	1598, 1579	qRT-PCR
kdpC-3F	TTTCCATTCGGCTTCTTTTG	26, 45	qRT-PCR
kdpC-4R	TACTTCCGCTGGATCGAAAC	133, 114	qRT-PCR
flaA2-5F	CATCTTACTTGCTGGACTGCTCTGC	15, 39	qRT-PCR
flaA2-6R	ATCTGGGTTTGCCCCTGTTTG	123, 103	qRT-PCR
kdpp-1F	ATCTTAACAATTTCTTAACTTCCTT	−214, −189	Cotranscription
kdpA-3R	TCGGAAAAAGTTTGTACAACTCCA	590, 567	Cotranscription
kdpA-4F	GGAAATAATGGAAGTGCTTTTGCT	1369, 1392	Cotranscription
kdpB-3R	CCCGGATAATAACATCGTAAGAGC	663, 640	Cotranscription
kdpB-4F	GCTTTGTTCGGAAGTTTTTATGCT	1792, 1815	Cotranscription
kdpC-6R	GCCTTCCGTTAGTTTACGTGACTT	447, 424	Cotranscription

[a] F, forward primer; R, reverse primer.
[b] Restriction sites are in lowercase; start and stop codons are underlined.
[c] Position of the 5′-most and 3′ nucleotide complementary to the target, relative to the first nucleotide in the start codon.
[d] Plasmid, for plasmid construction; Verification, for verification of proper integration of *bgaL* fusions into the *L. biflexa* chromosome; qRT-PCR, quantitative RT-PCR.

NdeI and similarly inserted into pRAT562. These plasmids were transformed into *L. biflexa* for integration into the *bgaL* gene.

pRAT575 was the vector used to express the *L. interrogans* KdpE protein from an autonomous plasmid in *L. biflexa*. pRAT575 was constructed by inserting a KpnI linker (5′-GGGTACCC-3′) and an XhoI linker (5′-CCTCGAGG-3′) into the PvuII site and the NgoMIV site of the shuttle plasmid pSLe94 (4) (a generous gift from Mathieu Picardeau), respectively. The NgoMIV 5′ overhangs were filled in with Klenow fragment prior to insertion of the XhoI linker. pRAT575 encodes resistance to spectinomycin.

The shuttle plasmids expressing *L. interrogans* KdpE from heterologous promoters were constructed in two steps. The promoters and translation initiation regions of *lipL32*, *tuf*, and *flaB1* were fused to *kdpE* (LIC10994) on pGKlep4, and the *kdpE* fusions were subsequently transferred to pRAT575 (4). For the first step, the *lipL32*, *tuf*, and *flaB1* promoter/translation initiation regions were amplified by PCR with the primer pairs listed above (Table 1). The PCR products and pGKlep4 were digested with KpnI and XhoI, and the promoter fragments were inserted into the pGKlep4 backbone. The reverse primers contained an NdeI recognition site near the XhoI site (Table 1). The *kdpE* protein-coding region was amplified by PCR with the primers kdpE(Nd)-1F and kdpE(Xh)-4R, digested with NdeI and XhoI, and inserted downstream of the promoters that were cloned into pGKlep4. Finally, the promoter-*kdpE* fusions were excised by digestion with KpnI and XhoI and transferred to pRAT575 to generate the plasmids used (see Fig. 5).

Integration of fusions into the *L. biflexa* chromosome. The plasmids harboring the *bgaL* fusions were denatured to promote homologous recombination with the *bgaL* gene in the chromosome (34), and 20 μl of each plasmid (4 to 5 μg) was denatured by adding 2 μl of 2 M NaOH and 2 mM EDTA and incubating the mixture for 30 min at 37°C. The mixtures were neutralized by addition of 2 μl 3 M sodium acetate (NaOAc), pH 5.2. To deplete the salt, the DNA was drop dialyzed against 40 ml water for 60 min (28). The denatured plasmid was then removed from the filter and transformed into *L. biflexa* by electroporation (37). Leptospires that had the plasmid integrated into the chromosome were selected by plating onto EMJH plates containing 30 μg/ml kanamycin. The plates also contained 20 μg/ml X-Gal to qualitatively assess the β-galactosidase expression of the transformants.

To verify integration of the fusion at the correct location, genomic DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen) and amplified with HotStarTaq (Qiagen) with the pGKlep4-2F and bgaL-4R primers (Table 1). The amplicons were digested with NdeI, and the digestion products were analyzed by electrophoresis in a 1.0% agarose gel (SeaKem LE agarose from Lonza).

β-Galactosidase assay. Three colonies from each *L. biflexa* transformation were incubated in EMJH with 30 μg/ml kanamycin and 40 μg/ml spectinomycin. *L. biflexa* carrying the *bgaL* fusions was grown to an optical density at 420 nm (OD_{420}) of 0.1 to 0.4. β-Galactosidase expression levels were determined essentially as described by Miller (30). In brief, cultures were chilled on ice for 20 min. The assay buffer comprised 9.6 ml of Z buffer (100 mM sodium phosphate, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0), 0.4 ml of 0.1% SDS, and 27 μl of β-mercaptoethanol. Five hundred microliters of the chilled cultures was mixed by vortexing with 500 μl assay buffer and 15 μl chloroform in 13- by 100-mm borosilicate glass tubes (VWR) for 10 s, and the mixtures were placed in a 28°C water bath for 5 min. Control reaction mixtures containing EMJH instead of the

culture were included for background subtraction. The reaction was initiated by adding 200 μl of 4 mg/ml *o*-nitrophenol-β-D-galactoside and allowed to proceed for at least 25 min. When the reaction mixtures turned yellow, the reactions were terminated by the addition of 500 μl of 1 M Na_2CO_3, and the reaction time was recorded. Slow reactions were terminated at 90 min. All reaction mixtures were clarified by centrifugation at 16,000 × *g* for 3 min in an Eppendorf 5424 microcentrifuge, and the absorbance of the supernatant fluid was read at 420 nm (A_{420}) with the Ultrospec 2000 (GE Healthcare). Miller units were calculated with the following equation (30): Miller units = $(1{,}000 \times A_{420})/(t \times v \times \mathrm{OD}_{420})$, where t is the reaction time in minutes, v is the volume of culture mixed with the assay buffer (0.5 ml), and OD_{420} is the optical density of the culture.

Statistical analysis. Statistical analysis was performed with GraphPad (La Jolla, CA) Prism 5. The statistical tests used are described in the figure legends. Growth rates during the logarithmic phase of growth were calculated with the Trendline function in Microsoft Excel 2000.

RESULTS

We first verified that KdpE was a regulator of *kdpABC* expression in *L. interrogans* before testing the *L. biflexa* reporter system with the *kdp* genes. An *L. interrogans kdpE* mutant designated M45 was one of the *Himar1* insertion mutants isolated by Murray et al. (31). The insertion point of the transposon in M45 follows codon 199 in *kdpE* (Fig. 1) (31). To predict which structural element of the KdpE response regulator was disrupted by the transposon, the amino acid sequence of LIC10994 was aligned with the DNA-binding domain of *E. coli* OmpR, a response regulator whose crystal structure had been solved (26). Alignment was aided by earlier published alignments of the DNA-binding domains of OmpR and *E. coli* KdpE (27). The alignment indicates that the transposon was inserted into the recognition helix of the putative winged helix-turn-helix of KdpE (Fig. 1).

To determine whether KdpE stimulated transcription of *kdpABC* during growth at a low concentration of potassium, the wild-type and M45 strains were cultivated in "low-K^+" EMJH, which was assembled by replacing potassium phosphate with sodium phosphate in the EMJH recipe, and "standard" EMJH, which was obtained by adding potassium to a final concentration of 2 mM (see Materials and Methods). Over three independent experiments, there were no significant differences in the growth rates among the four cultures (one-way analysis of variance [ANOVA]; $P = 0.7964$). RNA from the wild-type and M45 strains grown in standard and low-K^+ EMJH was extracted and analyzed by quantitative reverse transcriptase PCR (RT-PCR) with primers specific for *kdpA* (Table 1). A basal level of *kdpA* transcript was observed in the M45 strain under both growth conditions. When the wild-type strain was grown in low-K^+ EMJH, *kdpA* transcript levels were 3-fold higher than in standard EMJH, indicating that *kdp* transcription was increased in response to low potassium (Fig. 2A). In contrast, *kdpA* transcript levels failed to increase when strain M45 was grown in low-K^+ medium instead of standard EMJH. In low-K^+ medium, *kdpA* transcript levels were an average of 8-fold higher in the wild-type strain than in strain M45, while in standard EMJH medium there was no significant difference in *kdpA* transcript levels between the wild-type and M45 strains. These results are consistent with the notion that *L. interrogans* responds to limiting potassium levels by activating the KdpE response regulator, which then enhances transcription from the *kdp* promoter so that higher levels of the subunits for the high-affinity Kdp-ATPase potassium transporter can be expressed.

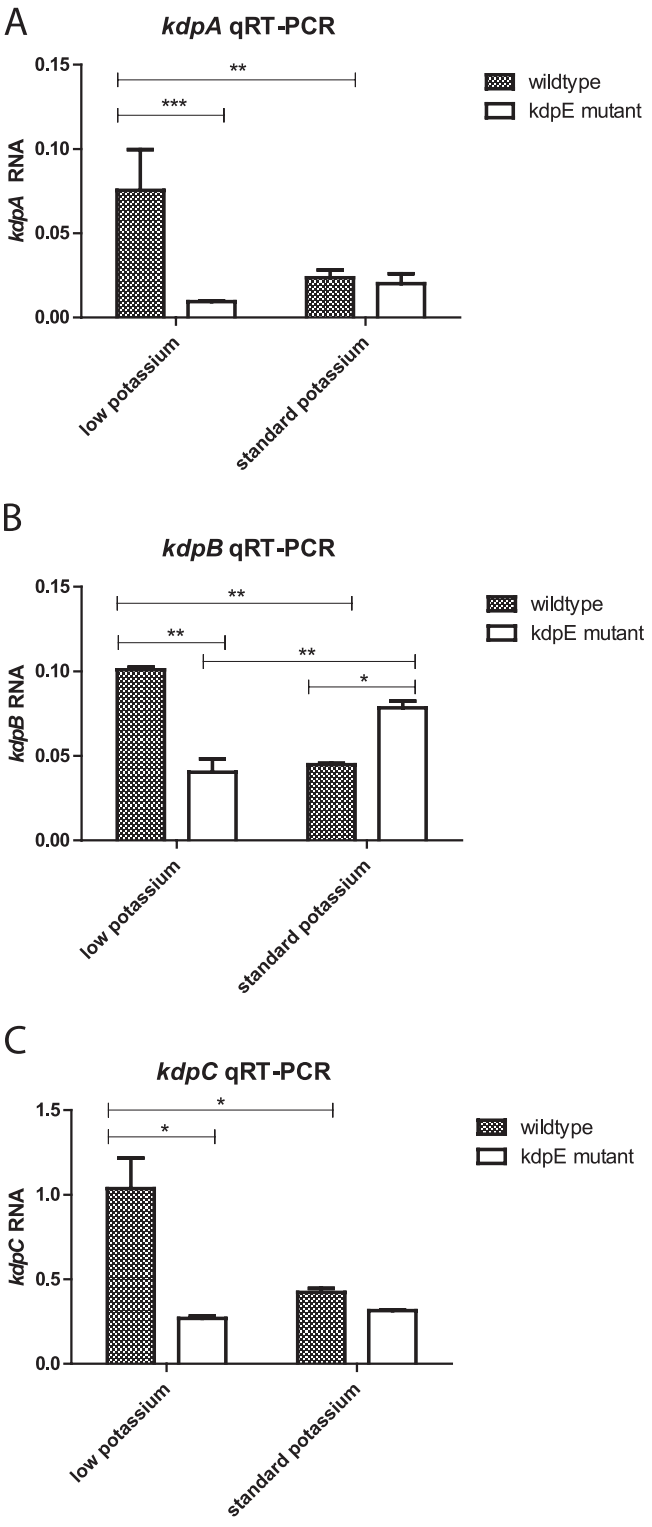

FIG 2 Effect of the *kdpE* mutation on *kdp* transcript levels in low-potassium medium. Transcript levels were determined by quantitative reverse transcriptase (qRT) PCR as described in Materials and Methods. Signals from the *kdpABC* mRNA were normalized to *flaA2* transcript levels. Representative results from three independent experiments are shown. The error bars denote the standard deviation. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$ (2-way ANOVA).

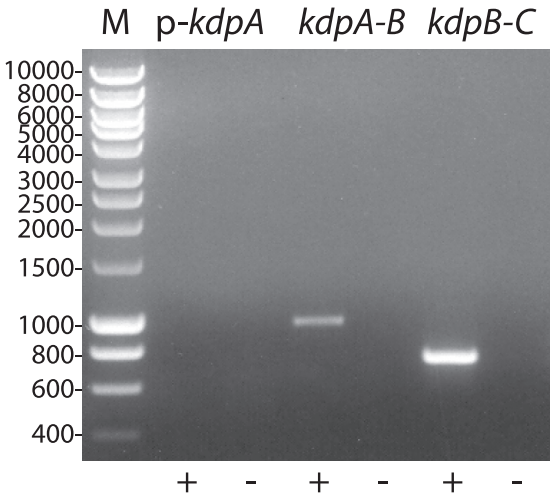

FIG 3 Cotranscription of adjacent genes in *kdpABC*. Total RNA was extracted from *L. interrogans* grown under low-potassium conditions. Primer pairs flanking the intergenic regions of *kdpABC* were used for reverse transcriptase PCR. The primer pairs that included the forward oligonucleotide targeting the *kdp* promoter region served as a negative control.

We also examined transcript segments encoding the other two subunits of the Kdp transporter. The transcript levels for the *kdpB* and *kdpC* sequences were significantly higher (2.3- and 3.0-fold, respectively) in the wild-type strain than in M45 during growth in low-K^+ EMJH (Fig. 2B and C). The *kdpE* mutation had no significant effect on *kdpC* transcript levels in standard EMJH. However, *kdpB* transcript levels were 1.7-fold higher in the *kdpE* mutant than in the wild-type strain (Fig. 2B) ($P < 0.01$; 2-way ANOVA). The biological significance of this modest effect, if any, is unclear. It is possible that KdpE functions as a negative regulator of *kdpB* when potassium is abundant. Nevertheless, the results suggest that KdpE is a positive regulator of *kdpABC* transcription in *L. interrogans* when the external potassium concentration is low.

The intergenic regions of the *kdpABC* sequence were analyzed by RT-PCR to determine whether the genes were cotranscribed. Figure 3 shows that primers flanking the *kdpAB* and *kdpBC* intergenic regions successfully generated amplicons by RT-PCR. The negative-control primer pair with forward and reverse primers annealing to the *kdp* promoter region and *kdpA*, repectively, failed to generate an amplicon by RT-PCR (Fig. 3, lane 2). All primer pairs tested generated amplicons from *L. interrogans* genomic DNA (data not shown). These results indicate that adjacent genes in *kdpABC* are cotranscribed.

We decided to monitor expression of *kdp* by fusing *kdpA* to a β-galactosidase reporter to examine the effects of expressing KdpE in *trans* on *kdp* expression. We selected the nonpathogen *L. biflexa* as the host strain for our experiments because of its ease of genetic manipulation compared to pathogenic strains of *Leptospira* and because it does not encode Kdp orthologs that may interfere with regulation of *kdpA* expression.

L. biflexa formed blue colonies on EMJH plates containing X-Gal, indicating that functional β-galactosidase was already being produced by the spirochete. A single gene in the *L. biflexa* genome (LEPBIa0024) is annotated as encoding a β-galactosidase. The *L. biflexa* and well-characterized *E. coli* β-galactosidases share little sequence identity and belong to different families of glycosyl hydrolases. *E. coli* β-galactosidase is a member of glycosyl hydrolase family 2, whereas *L. biflexa* β-galactosidase is a member of family 42. Despite their unrelated protein sequences, the catalytic domains of the two β-galactosidase variants share the TIM barrel superfold that includes the two glutamate residues that participate in catalysis (16).

To exploit the endogenous β-galactosidase gene of *L. biflexa*, we replaced the sequence upstream of the start codon of LEPBIa0024 with the corresponding region from various genes of *L. interrogans*. Since only a single plasmid capable of replicating in *L. biflexa* was available, the fusion was placed on the chromosome so that the plasmid remained available for expression of KdpE and other potential regulatory gene products. The first 293 of the 661 codons of LEPBIa0024 were cloned into a suicide plasmid unable to replicate in *L. biflexa*, creating the vector pRAT562 (Fig. 4A). The sequences upstream of the *kdpA*, *lipL32*, *tuf* (EF-Tu), and *flaB1* protein-coding regions were then inserted in front of the partial LEPBIa0024 coding sequence in pRAT562. The cloned segments comprised the sequence between the protein-coding region of the upstream gene and the start codon of the four genes. The five plasmids, including pRAT562, were next denatured with sodium hydroxide and transformed into *L. biflexa* by electroporation (Fig. 4B). The plasmid was treated with sodium hydroxide rather than UV light to minimize mutagenesis of the DNA. After a 24-hour period of outgrowth in liquid EMJH, transformants were selected on EMJH plates containing kanamycin and X-Gal. Because the plasmid DNA lacked an origin of replication that could function in *L. biflexa*, colonies could be recovered only if the plasmid integrated into the chromosome by homologous recombination at the 5′ end of LEPBIa0024. The colonies that grew following integration of pRAT562 were white on X-Gal plates, confirming that LEPBIa0024 was the sole source of β-galactosidase in *L. biflexa* during growth on EMJH. On the other hand, chromosomal integration of the plasmids harboring the *kdpA*, *lipL32*, *tuf*, and *flaB1* fusions resulted in blue colonies on X-Gal plates. Because LEPBIa0024 encoded β-galactosidase activity, the gene was named *bgaL* (for beta-galactosidase of *Leptospira*), following the convention adopted for naming β-galactosidase reporters belonging to glycosyl hydrolase family 42, including *bgaB* from *Bacillus stearothermophilus* and *bgaH* from *Haloferax lucentense* (12, 38).

Colonies were inoculated into EMJH medium containing kanamycin and grown to mid- to late log phase. PCR was employed to confirm that the plasmid properly integrated into the *bgaL* gene on the chromosome. Genomic DNA was purified from the spirochetes and analyzed by PCR with a forward primer annealing upstream of the *L. interrogans* sequence within the vector sequence and a reverse primer annealing downstream of the *bgaL* segment that was cloned into the plasmid (Fig. 4B). A PCR product would be observed only if the plasmid integrated into the chromosome at the expected location. Amplicons of the expected sizes were obtained (data not shown). Digestion of the amplicons with NdeI released fragments containing the upstream regions of *kdpA*, *lipL32*, *tuf*, and *flaB1* of the sizes anticipated (396 bp, 425 bp, 501 bp, and 506 bp, respectively) (Fig. 4B and C).

We measured the β-galactosidase activity expressed by the *L. biflexa bgaL* fusion strains essentially as described by Miller's standard protocol (see Materials and Methods). β-Galactosidase activity was not detected from a strain with the promoterless *bgaL* construct (Fig. 4D). A range of β-galactosidase activities was expressed by the strains that carried fusions of the 5′ flanking regions

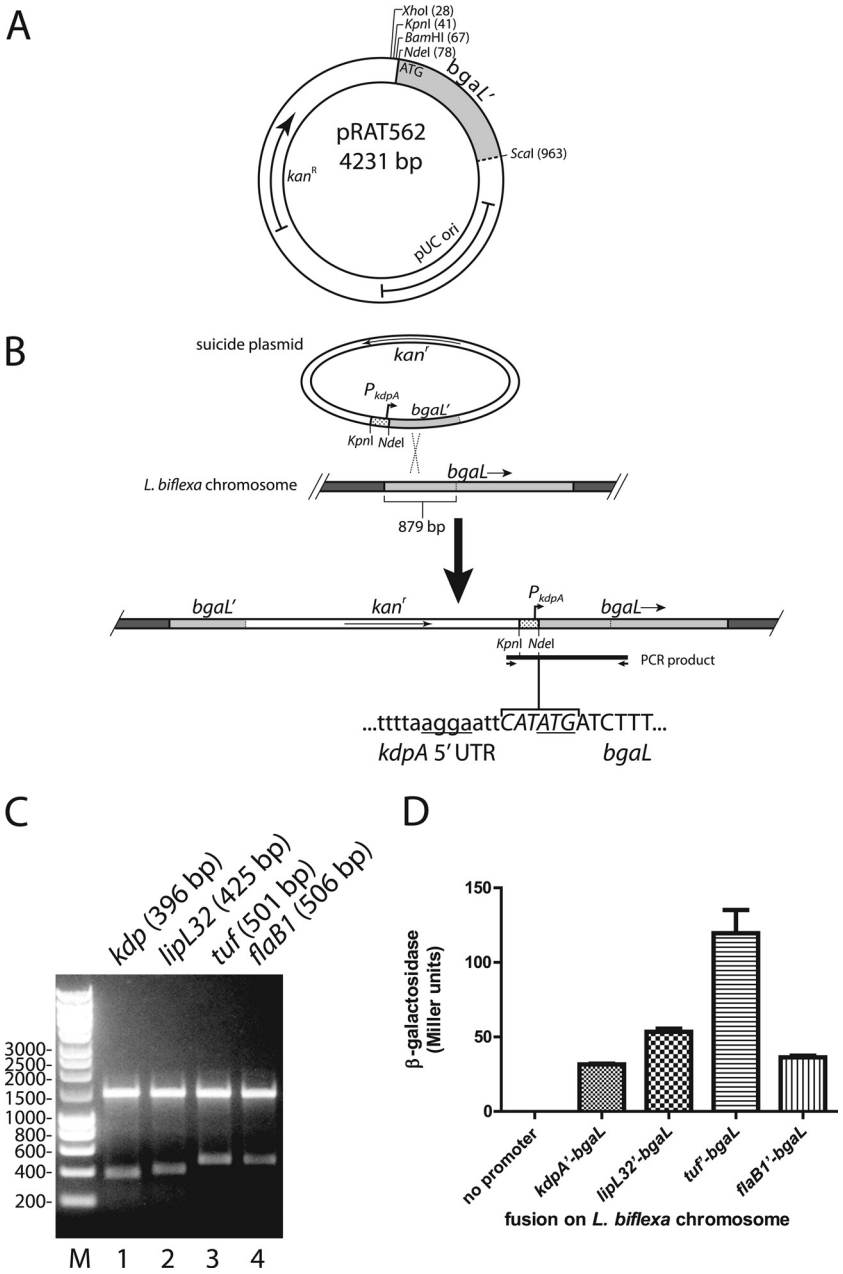

FIG 4 Integration of *bgaL* fusions into the chromosome of *L. biflexa*. (A) Sequence features of pRAT562. Restriction sites used for cloning are shown. The first 293 of the 661 codons of *L. biflexa bgaL* are present in pRAT562. (B) Scheme for integration of the *kdpA* promoter and translation initiation signals upstream of the *bgaL* coding region on the chromosome of *L. biflexa*. The *kdpA* promoter and 5′ untranslated region cloned into pRAT562 are depicted by the stippled segment. The *bgaL* sequences are shaded in light gray. Homologous recombination between the plasmid and chromosome occurred within the first 879 bp of *bgaL*. The integrated sequence was detected by PCR with a forward primer that annealed within the plasmid vector sequence and a reverse primer that annealed within the *bgaL* sequence downstream of the cloned segment. The nucleotide sequences of the NdeI recognition site and flanking regions are shown. The Shine-Dalgarno sequence and start codon are underlined. Lowercase, *kdpA* sequence; italics, NdeI recognition sequence; uppercase, second and third *bgaL* codons. (C) PCR products were digested with NdeI and subjected to electrophoresis in a 1.0% agarose gel. The expected sizes of the smaller fragments are indicated in parentheses. (D) β-Galactosidase activities measured from various chromosomal *bgaL* fusions. Each bar represents the mean β-galactosidase expression from three independent cultures. The error bars denote the standard deviations.

of *L. interrogans* genes to *bgaL*, with *tuf* producing the highest levels (Fig. 4D).

Following the successful demonstration of β-galactosidase expression driven by different *L. interrogans* promoters, we determined whether the *kdpA′-bgaL* fusion could be regulated by *L. interrogans* KdpE in *trans*. We cloned the protein-coding region of *kdpE* from *L. interrogans* immediately downstream of the *lipL32*, *tuf*, and *flaB1* promoters in the pSLe94 shuttle vector, which carries the LE1 bacteriophage replication origin and a gene encoding resistance to spectinomycin. The plasmids were then transformed into the *L. biflexa* strain carrying the *kdpA′-bgaL* fusion, and colonies were selected on EMJH plates containing kanamycin and

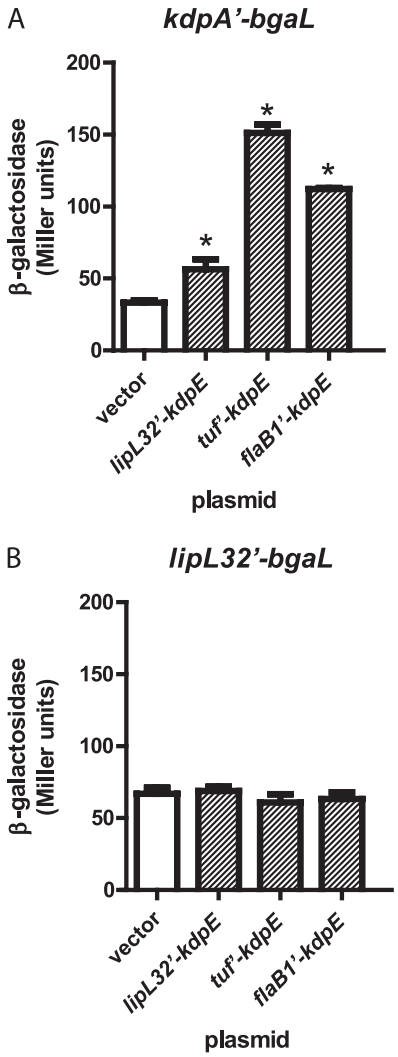

FIG 5 Positive regulation of the *kdpA′-bgaL* fusion by KdpE. (A) The *L. biflexa kdpA′-bgaL* strain was transformed with pSLe94 (vector) or a KdpE expression plasmid. (B) Same as panel A, except the *L. biflexa* strain harbored the *lipL32′-bgaL* fusion. Each bar represents the mean β-galactosidase expression from three independent cultures. The error bars denote the standard deviations. *, $P < 0.05$ (1-way ANOVA and Dunnett's multiple-comparison posttest).

spectinomycin. As a control, the pSLe94 vector was also transformed into *L. biflexa*. The colonies were transferred to standard liquid EMJH containing kanamycin and spectinomycin for growth.

Bar graphs of the results are shown in Fig. 5A. As expected, the expression levels of the *kdpA′-bgaL* strain transformed with pSLe94 were similar to those expressed without the plasmid (Fig. 4D and 5A). The plasmid expressing KdpE from the *lipL32* promoter and translation initiation region increased β-galactosidase expression 1.7-fold, from 33 to 56 Miller units. When KdpE expression was driven by *tuf*, β-galactosidase activity from the *kdpA′-bgaL* fusion increased 4.5-fold to 151 units. Similarly, when KdpE was expressed in *trans* from *flaB1* upstream signals, β-galactosidase expression increased 3.4-fold to 112 units. These results confirmed that KdpE of *L. interrogans* is a positive regulator of *kdp* expression. When the same *kdpE* expression plasmids were introduced into an *L. biflexa* strain harboring the control *lipL32′-bgaL* fusion, which was not expected to be regulated by KdpE, no effect of KdpE on fusion expression was discerned (Fig. 5B).

DISCUSSION

We developed a novel reporter gene system that enables translational fusion of leptospiral genes directly to the chromosomal copy of *bgaL* in *L. biflexa* (Fig. 4B). This system can be deployed to examine the regulation of leptospiral genes by *trans*-acting factors. Since complementation of *L. interrogans* mutations is difficult, this approach also provides an independent genetic approach to support conclusions derived from the study of *L. interrogans* mutations that disrupt *trans*-acting regulatory factors that control gene expression. To demonstrate an application of the *bgaL* reporter, we constructed a *kdpA′-bgaL* fusion to show that leptospiral KdpE positively regulates the expression of the P-type ATPase transporter Kdp (Fig. 4B and 5).

The chromosomal *bgaL* reporter has several advantages over the plasmid-based green fluorescent protein reporter described in earlier studies (3, 8). First, since only one plasmid capable of replicating stably in *L. biflexa* is available, placing the fusion on the chromosome allows genes encoding candidate regulatory factors to be cloned easily into the plasmid to test whether they can control fusion expression in *trans*. Second, background levels of β-galactosidase in *L. biflexa* with its *bgaL* gene disrupted is extremely low (Fig. 4D), permitting the study of weakly expressed genes. In contrast, the relatively high background fluorescence of *L. biflexa* may obscure changes in the expression of weak promoters fused to *gfp* (8). Third, the copy number of the *bgaL* fusion remains stable, since the fusion is located on the chromosome while the copy number of plasmid-borne fusions may be affected by culture conditions. Finally, the 5′ untranslated region of the fusion transcripts originated from the *L. interrogans* gene being tested. This should permit examination of small RNAs and other regulatory proteins that target the 5′ untranslated regions of mRNAs to control gene expression posttranscriptionally. *Leptospira* is likely to express small RNAs, since all the leptospiral genomes sequenced to date harbor a gene encoding a CsrA homolog, a protein that binds to specific regulatory small RNAs (19).

A minor limitation of our fusion constructs as currently designed is that translational initiation at the artificial fusion junction may be blocked by secondary structures, as would be the case with translational fusions to any reporter. This appears be the case with *flaB1*, which expressed low levels of β-galactosidase when fused to *bgaL* (Fig. 4D) yet was able to exert 4-fold positive control on the *kdpA′-bgaL* fusion when driving *kdpE* expression (Fig. 5A). When the 60 nucleotides encompassing the translation initiation region of *flaB1′-bgaL* was folded using Mfold (44), most of the Shine-Dalgarno sequence was located in double-stranded RNA, whereas the Shine-Dalgarno sequence remained single stranded when the corresponding regions flanking the start codons of *flaB1* and the *flaB1′-kdpE* transcripts were folded (data not shown). Moving the fusion junction downstream to include part of the coding region of the test gene might minimize problems with reporter sequences causing formation of double-stranded RNA within the translation initiation region.

LipL32 is the most abundant protein in *L. interrogans* (25), yet the β-galactosidase level expressed from the *lipL32′-bgaL* fusion was barely higher than that of the *kdpA′-bgaL* fusion in the absence of KdpE (Fig. 4D). Moreover, when its expression was driven by *lipL32*, KdpE barely enhanced β-galactosidase expres-

sion from the *kdpA′-bgaL* fusion (Fig. 5A). A recent study has shown that *lipL32* expression is altered during the interaction of *L. interrogans* with macrophage-derived cell lines *in vitro* (41). Strong expression of *lipL32′-bgaL* may require a transcriptional regulator that is not present in *L. biflexa*. Alternatively, some of the *lipL32* promoter elements may be missing from the fusion. Further studies with additional *lipL32′-bgaL* fusion constructs may allow us to distinguish between these possibilities.

Expression of the *L. interrogans kdpE* gene in *L. biflexa* growing in EMJH activated expression of the *kdpA′-bgaL* fusion despite the plentiful potassium in the culture medium and the absence of KdpD. Although KdpE must be phosphorylated to activate transcription from the *kdp* promoter in *E. coli* (15), phosphorylation of KdpE may not be necessary for the response regulator to enhance transcription of the *kdp* genes in *Leptospira*. Alternatively, *L. interrogans* KdpE may be phosphorylated somehow when expressed in *L. biflexa*. In other bacteria, a response regulator may be phosphorylated by a noncognate histidine kinase when the gene encoding the cognate sensor is deleted (20). In many cases, a phosphatase activity of the cognate sensor prevents cross talk under noninducing conditions by removing the phosphate from the response regulator. In *E. coli*, KdpD possesses a phosphatase activity that dominates over its kinase when potassium is plentiful in the culture medium (6). Therefore, the absence of KdpD in *L. biflexa* may permit stable phosphorylation of KdpE by aberrant cross talk from a noncognate histidine kinase. Although acetylphosphate can phosphorylate KdpE *in vitro*, it is unlikely to be the phosphate donor *in vivo*, since *L. biflexa* lacks the genes encoding phosphate acetyltransferase and acetate kinase, which are necessary to convert acetyl-coenzyme A (CoA) or acetate to acetylphosphate (40).

The role of the *kdp* gene products in potassium metabolism of *L. interrogans*, if any, remains unknown. The growth rate of the *L. interrogans kdpE* mutant was not affected even when the potassium concentration in the culture medium was minimized. Residual potassium in one or more of the EMJH components may have allowed growth of the *kdpE* mutant. It is also possible that the *kdp* genes are not required for growth when the potassium concentration is limited, as is the case for *Staphylococcus aureus* (42). Free-living bacteria with the Trk potassium transporter can lack Kdp orthologs (10). Neither of the two sequenced *L. biflexa* strains possesses the *kdp* genes, but they, along with the four sequenced pathogenic *Leptospira* strains, encode orthologs of the TrkA and TrkH subunits of Trk (7, 32, 35, 36), which may be capable of transporting enough potassium into the leptospires to support their growth even when potassium is limited. These observations suggest that the *kdp* gene products provide a function for *L. interrogans* beyond satisfying the spirochete's need for potassium. The absence of the *kdp* locus from the genomes of the two sequenced *Leptospira borgpetersenii* Hardjo strains, which lack a significant host-free environmental phase, suggest that their gene products are involved in the adaptation of *L. interrogans* following entry into or exit from the host (7, 29).

We are currently exploiting the *L. interrogans kdpE* mutant and the *kdpA′-bgaL* reporter strain to search for other *L. interrogans* genes that are regulated by KdpE. The *bgaL* reporter system described here can also be readily adapted to study the functions of other transcriptional or posttranscriptional regulators.

ACKNOWLEDGMENTS

We are grateful to Ben Adler and Gerald Murray for providing us with the *L. interrogans* L495 and M45 strains and the *kdp* sequences and to Mathieu Picardeau for providing us with the plasmid pSLe94. We thank David Haake, Henry Choy, and Jane Babbitt for helpful discussions. We also thank Alice Shapiro for technical support.

This study was supported by VA Medical Research Funds (to J.M.).

J.M conceived and designed the experiments, performed the experiments, analyzed the data, and wrote the paper. J.M. and M.L.C. contributed reagents, materials, and analysis tools.

REFERENCES

1. **Adler B, de la Peña Moctezuma A.** 2010. *Leptospira* and leptospirosis. Vet. Microbiol. **140**:287–296.
2. **Adler B, Lo M, Seemann T, Murray GL.** 2011. Pathogenesis of leptospirosis: the influence of genomics. Vet. Microbiol. **153**:73–81.
3. **Aviat F, Slamti L, Cerqueira GM, Lourdault K, Picardeau M.** 2010. Expanding the genetic toolbox for *Leptospira* species by generation of fluorescent bacteria. Appl. Environ. Microbiol. **76**:8135–8142.
4. **Bauby H, Saint Girons I, Picardeau M.** 2003. Construction and complementation of the first auxotrophic mutant in the spirochaete *Leptospira meyeri*. Microbiology **149**:689–693.
5. **Bramkamp M, Altendorf K, Greie JC.** 2007. Common patterns and unique features of P-type ATPases: a comparative view on the KdpFABC complex from *Escherichia coli* (review). Mol. Membr. Biol. **24**:375–386.
6. **Brandon L, Dorus S, Epstein W, Altendorf K, Jung K.** 2000. Modulation of KdpD phosphatase implicated in the physiological expression of the Kdp ATPase of *Escherichia coli*. Mol. Microbiol. **38**:1086–1092.
7. **Bulach DM, et al.** 2006. Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. Proc. Natl. Acad. Sci. U. S. A. **103**:14560–14565.
8. **Cerqueira GM, et al.** 2011. Development of transcriptional fusions to assess *Leptospira interrogans* promoter activity. PLoS One **6**:e17409. doi:10.1371/journal.pone.0017409.
9. **Choy HA, et al.** 2011. The multifunctional LigB adhesin binds homeostatic proteins with potential roles in cutaneous infection by pathogenic *Leptospira interrogans*. PLoS One **6**:e16879. doi:10.1371/journal.pone.0016879.
10. **Corratgé-Faillie C, et al.** 2010. Potassium and sodium transport in non-animal cells: the Trk/Ktr/HKT transporter family. Cell. Mol. Life Sci. **67**:2511–2532.
11. **Croda J, et al.** 2008. Targeted mutagenesis in pathogenic *Leptospira* species: disruption of the LigB gene does not affect virulence in animal models of leptospirosis. Infect. Immun. **76**:5826–5833.
12. **Gregor D, Pfeifer F.** 2001. Use of a halobacterial *bgaH* reporter gene to analyse the regulation of gene expression in halophilic archaea. Microbiology **147**:1745–1754.
13. **Haake DA.** 2006. Hamster model of leptospirosis. Curr. Protoc. Microbiol. **12**:12E.2. doi:10.1002/9780471729259.mc12e02s02.
14. **Haake DA, Matsunaga J.** 2010. *Leptospira*: a spirochaete with a hybrid outer membrane. Mol. Microbiol. **77**:805–814.
15. **Heermann R, Jung K.** 2010. The complexity of the 'simple' two-component system KdpD/KdpE in *Escherichia coli*. FEMS Microbiol. Lett. **304**:97–106.
16. **Hidaka M, et al.** 2002. Trimeric crystal structure of the glycoside hydrolase family 42 β-galactosidase from *Thermus thermophilus* A4 and the structure of its complex with galactose. J. Mol. Biol. **322**:79–91.
17. **Ko AI, et al.** 1999. Urban epidemic of severe leptospirosis in Brazil. Lancet **354**:820–825.
18. **Ko AI, Goarant C, Picardeau M.** 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat. Rev. Microbiol. **7**:736–747.
19. **Lapouge K, Schubert M, Allain FH, Haas D.** 2008. Gac/Rsm signal transduction pathway of γ-proteobacteria: from RNA recognition to regulation of social behaviour. Mol. Microbiol. **67**:241–253.
20. **Laub MT, Goulian M.** 2007. Specificity in two-component signal transduction pathways. Annu. Rev. Genet. **41**:121–145.
21. **Liao S, et al.** 2009. Inactivation of the *fliY* gene encoding a flagellar motor switch protein attenuates mobility and virulence of *Leptospira interrogans* strain Lai. BMC Microbiol. **9**:253.

22. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. Methods **25**: 402–408.
23. **Lo M, et al.** 2010. Transcriptional response of *Leptospira interrogans* to iron limitation and characterization of a PerR homolog. Infect. Immun. **78**:4850–4859.
24. **Louvel H, Saint Girons I, Picardeau M.** 2005. Isolation and characterization of FecA- and FeoB-mediated iron acquisition systems of the spirochete *Leptospira biflexa* by random insertional mutagenesis. J. Bacteriol. **187**:3249–3254.
25. **Malmstrom J, et al.** 2009. Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. Nature **460**:762–765.
26. **Martínez-Hackert E, Stock AM.** 1997. The DNA-binding domain of OmpR: crystal structures of a winged helix transcription factor. Structure **5**:109–124.
27. **Martínez-Hackert E, Stock AM.** 1997. Structural relationships in the OmpR family of winged-helix transcription factors. J. Mol. Biol. **269**:301–312.
28. **Marusyk R, Sergeant A.** 1980. A simple method for dialysis of small-volume samples. Anal. Biochem. **105**:403–404.
29. **Matsunaga J, et al.** 2007. Response of *Leptospira interrogans* to physiologic osmolarity: relevance in signaling the environment-to-host transition. Infect. Immun. **75**:2864–2874.
30. **Miller JH.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Plainview, NY.
31. **Murray GL, et al.** 2009. Genome-wide transposon mutagenesis in pathogenic *Leptospira* species. Infect. Immun. **77**:810–816.
32. **Nascimento AL, et al.** 2004. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. J. Bacteriol. **186**:2164–2172.
33. **Picardeau M.** 2008. Conjugative transfer between *Escherichia coli* and *Leptospira* spp. as a new genetic tool. Appl. Environ. Microbiol. **74**:319–322.
34. **Picardeau M, Brenot A, Saint Girons I.** 2001. First evidence for gene replacement in *Leptospira* spp. Inactivation of *L. biflexa flaB* results in non-motile mutants deficient in endoflagella. Mol. Microbiol. **40**:189–199.
35. **Picardeau M, et al.** 2008. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. PLoS One **3**:e1607. doi:10.1371/journal.pone.0001607.
36. **Ren SX, et al.** 2003. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. Nature **422**: 888–893.
37. **Saint Girons I, et al.** 2000. The LE1 bacteriophage replicates as a plasmid within *Leptospira biflexa*: construction of an *L. biflexa-Escherichia coli* shuttle vector. J. Bacteriol. **182**:5700–5705.
38. **Schrogel O, Allmansberger R.** 1997. Optimisation of the BgaB reporter system: determination of transcriptional regulation of stress responsive genes in *Bacillus subtilis*. FEMS Microbiol. Lett. **153**:237–243.
39. **Tchamedeu Kameni AP, Couture-Tosi E, Saint Girons I, Picardeau M.** 2002. Inactivation of the spirochete *recA* gene results in a mutant with low viability and irregular nucleoid morphology. J. Bacteriol. **184**: 452–458.
40. **Wolfe AJ.** 2005. The acetate switch. Microbiol. Mol. Biol. Rev. **69**:12–50.
41. **Xue F, et al.** 2010. Transcriptional responses of *Leptospira interrogans* to host innate immunity: significant changes in metabolism, oxygen tolerance, and outer membrane. PLoS Negl. Trop. Dis. **4**:e857. doi:10.1371/journal.pntd.0000857.
42. **Xue T, You Y, Hong D, Sun H, Sun B.** 2011. The *Staphylococcus aureus* KdpDE two-component system couples extracellular K^+ sensing and Agr signaling to infection programming. Infect. Immun. **79**:2154–2167.
43. **Zuerner RL.** 2005. Laboratory maintenance of pathogenic *Leptospira*. Curr. Protoc. Microbiol. **12**:12E.11. doi:10.1002/9780471729259.mc12e01s00.
44. **Zuker M.** 2003. Mfold Web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. **31**:3406–3415.