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The Sigma Factor σ^{54} is Required for the Long-Term Survival of *Leptospira biflexa* in Water

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Abstract

Leptospira spp. comprise both pathogenic and free-living saprophytic species. Little is known about the environmental adaptation and survival mechanisms of *Leptospira*. Alternative sigma factor, σ^{54} (RpoN) is known to play an important role in environmental and host adaptation in many bacteria. In this study, we constructed an *rpoN* mutant by allele exchange, and the complemented strain in saprophytic *L. biflexa*. Transcriptome analysis revealed that expression of several genes involved in nitrogen uptake and metabolism, including *amtB1, glnB-amtB2, ntrX*, and *narK*, were controlled by σ^{54}. While wild-type *L. biflexa* could not grow under nitrogen-limiting conditions but was able to survive under such conditions and recover rapidly, the *rpoN* mutant was not. The *rpoN* mutant also had dramatically reduced ability to survive long-term in water. σ^{54} appears to regulate expression of *amtB1, glnK-amtB2, ntrX*, and *narK* in an indirect manner. However, we identified a novel nitrogen-related gene, *LEPBI_I1011*, whose expression was directly under the control of σ^{54} (herein renamed as *rcfA* for RpoN-controlled factor A). Taken together, our data reveal that the σ^{54} regulatory network plays an important role in the long-term environmental survival of *Leptospira* spp.

Abbreviated Summary

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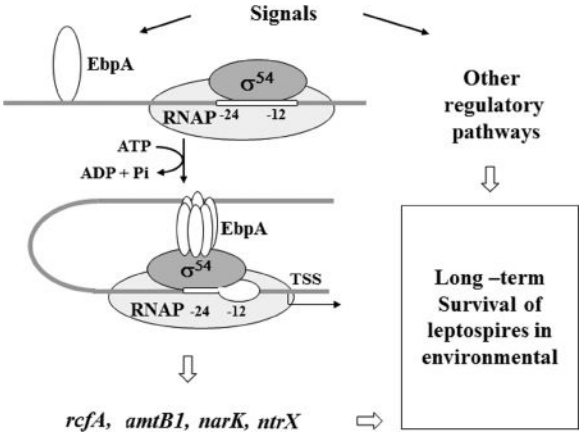
Primers

The primers used in this study were synthesized by Integrated DNA Technologies Inc. The purposes and sequences of the primers are shown in Table 3.

Author Contributions

JJZ and XFY designed the study and wrote the paper. JJZ performed most of the experiments and data analysis. WLH, YY, and HL helped with the experiments. MP and JY provided valuable suggestions for the study and the manuscript. All authors read and approved the final manuscript.



Little is known about the environmental adaptation and survival mechanisms of *Leptospira*. This study reports inactivation and complementation of *rpoN* encoding the alternative sigma factor σ^{54} in *Leptospira biflexa*, and demonstrates that σ^{54} is required for the long term survival in water of leptospires.

Keywords

Sigma 54; Regulation; *Leptospira*; Environment survival

Introduction

The genus *Leptospira* belongs to the phylum *Spirochaetes* and is comprised of saprophytic and pathogenic species (Ko *et al.*, 2009, Picardeau, 2017, Adler & de la Peña Moctezuma, 2010). Pathogenic *Leptospira* are the causative agents of leptospirosis, a re-emerging globally important zoonotic disease (Costa *et al.*, 2015). Despite of the development of genetic tools, genetic manipulation of *Leptospira*, especially for pathogenic *Leptospira* species, remains to be a major challenge and has greatly hampered the characterization of the pathogenesis as well as the general biology of *Leptospira*. *L. biflexa* is a free-living saprophytic spirochete that survives exclusively in the external environment. It has extensive genetic and structural similarities with infectious species of *Leptospira* (Picardeau *et al.*, 2008). *L. biflexa* has a faster growth rate and is more amenable to genetic manipulation than pathogenic *Leptospira*, which makes it a useful system for genetically elucidation of gene functions of *Leptospira* (Guegan *et al.*, 2003, Louvel *et al.*, 2008, Louvel *et al.*, 2006b, Picardeau *et al.*, 2001, Picardeau *et al.*, 2008).

One feature of *Leptospira* is its ability to survival in a vast array of environments that range from soil and water to the tissues of mammalian hosts (Barragan *et al.*, 2017). Transcriptomic and proteomic analyses of *Leptospira* spp. have identified global changes in gene expression in response to diverse changes of environmental factors, such as innate immunity (Xue *et al.*, 2010), iron limitation (Lo *et al.*, 2010a), temperature (Lo *et al.*, 2006, Qin *et al.*, 2006), osmolality (Matsunaga *et al.*, 2007), growth phase (Stewart *et al.*, 2016), serum exposure (Patarakul *et al.*, 2010), host-adaption (Caimano *et al.*, 2014, Nally *et al.*, 2017), and chronic infection (Monahan *et al.*, 2008). On the other hand, our understanding

of the mechanisms underlying gene regulation in *Leptospira* is limited. Genome analyses reveal a large number of genes encoding proteins involved in signal transduction and gene regulation in *Leptospira* species. For example, they have a large number of genes encoding the two- component systems (102 in *L. biflexa* and 76 in *L. interrogans*) (Fouts *et al.*, 2016). So far, only a response regulator, HemR, has been characterized, showing that it affects transcriptional activation and repression of genes involved in heme metabolism (Morero *et al.*, 2014). Few other regulators have been studied in *Leptospira*, including PerR, which regulates oxidative stress response (Lo *et al.*, 2010a), and KdpE, which activates the KdpABC potassium transporter (Matsunaga & Coutinho, 2012). Elegant in-depth work has been conducted on the regulator LexA in *L. interrogans* (Schons-Fonseca *et al.*, 2016, Fonseca *et al.*, 2013, Cuñé *et al.*, 2005). *L. interrogans* Copenhageni serovar has two copies of *lex*A genes: *lexA1* and *lexA2*. LexA activates an SOS response following DNA damage. ChIP-seq data identified 24 LexA1 binding sites in the genome upstream of the genes whose expression increases upon DNA damage (Schons-Fonseca *et al.*, 2016).Alternative sigma factors are a common strategy for bacteria to regulate gene expression in response to environmental and physiological cues (Kazmierczak *et al.*, 2005, Feklistov *et al.*, 2014). In addition to a housekeeping σ^{70}, all leptospiral species have alternative sigma factor 54 (σ^{54} or RpoN) and extracytoplasmic function (ECF) sigma factors (σ^{E}) (Fouts *et al.*, 2016). σ^{54} is a unique sigma factor that is phylogenetically distinct from other sigma factors and is widely distributed among bacteria (Bush & Dixon, 2012, Yang *et al.*, 2015, Bonocora *et al.*, 2015, Zhang *et al.*, 2016, Siegel & Wemmer, 2016). It recognizes a unique −24/−12 promoter motif instead of the −35/−10 motif recognized by σ^{70}. Its activation absolutely requires the input of free energy (ATP) from an associated activator, referred as enhancer-like binding protein (EBP), to initiate transcription. Each EBP-σ^{54} pair activates a set of genes in response to different signals (Bush & Dixon, 2012).

σ^{54} is a well-recognized factor that plays important roles in environmental and host adaptation in many bacteria (Francke *et al.*, 2011). The function of σ^{54} in any *Leptospira* species remains unknown heretofore. We recently reported that a transposon mutant lacking production of one of the EBP, EbpA in *L. interrogans*, is incapable of surviving in environmental water (Hu *et al.*, 2016). Since EbpA activates σ^{54}-dependent genes, such finding suggests that σ^{54} is important for leptospires to survive in water. However, inactivation of the *rpoN* gene encoding σ^{54} in pathogenic leptospires has not been successful (Hu *et al.*, 2016). In fact, no mutant has been generated for any of predicted alternative sigma factors or global regulators in *Leptospira* species.

In this study, we reported the successful inactivation and complementation of the σ^{54}-coding gene (*rpoN*) in *L. biflexa* by allelic exchange. We performed transcriptome analysis and identified several putative σ^{54}-dependent genes, including a novel nitrogen-responsive gene, *LEPBI_I1011*, whose expression is under the direct control by σ^{54}. We showed that σ^{54} is important for leptospiral adaptation to the natural environment outside of a host.

Results

Comparison of EBP and σ^{54} regulatory pathways among *Leptospira* species

Both saprophytic and pathogenic *Leptospira* encode a single copy of *rpoN* gene (encodes for σ^{54}), which is located on the large chromosome of the genome. *L. biflexa* σ^{54} sequence shares ~60% identity at the amino acid level with that of pathogenic and intermediate leptospiral species. Phylogenetic analysis showed that the *Leptospira* σ^{54} was evolutionary separated from other bacteria, even from other *Spirochaetaceae* genera including *Borrelia* and *Treponema* (Fig. 1). *Leptospira* σ^{54} has all three conserved functional domains, Region I, II, and III (Fig. 2A), despite that the overall identities of *Leptospira* σ^{54} at the amino acid level are less than 30% with a typical σ^{54} such as in *Enterobacteriaceae*.

Activation of σ^{54}–controlled genes requires a prokaryotic enhancer binding protein (EBP. Both pathogenic and saprophytic *Leptospira* have the activator EbpA for activation of σ^{54}-controlled transcription initiation (Hu *et al.*, 2016, Fouts *et al.*, 2016) (Fig. 2B). EbpA is an FhlA (formate hydrogen lyase activator)-like EBP (group III), which includes two GAF (cGMP-specific phosphodiesterases, Adenylyl cyclases and FhlA) domains (Hopper & Bock, 1995). In addition, pathogenic *Leptospira* species have an activator EbpB that is not present in saprophytic *Leptospira* (Hu *et al.*, 2016, Fouts *et al.*, 2016). EbpB is an NtrC-like EBP (group I), which is activated by phosphorylation at the RR domain by a cognate histidine kinase (Doucleff *et al.*, 2005). Thus, pathogenic and saprophytic *Leptospira* share an EbpA-σ^{54} regulatory network, whereas pathogenic *Leptospira* have an additional EbpB-σ^{54} network.

Targeted inactivation and complementation of *rpoN* in *L. biflexia*

We first attempted to inactivate *rpoN* in pathogenic *Leptospira*. Despite multiple attempts, no mutant could be obtained in. *L. interrogans* strain Lai 56601, Fiocruz L1-130, or L495. We thus focused on saprophytic *Leptospira*, and successfully inactivated the *rpoN* gene (*LEPBI_I1650*) by allelic exchange in *L. biflexa* serovar Patoc strain Patoc1 (Fig. 3A). The lack of *rpoN* expression and the product σ^{54} in the *rpoN* mutant were demonstrated by quantitative reverse transcription-PCR (qRT-PCR) (Fig. 3B) and immunoblotting analyses (Fig. 3C). A complemented strain for the *rpoN* mutant was also achieved by transforming a shuttle vector containing a wild-type *rpoN* gene driven under the *hsp10* promoter of *L. biflexa* (Fig. 3A), and restoration of σ^{54} was confirmed in the complementary strain (*rpoN*/*pJJ003*) (Fig. 3B & 3C).

Transcriptome analysis for the identification of σ^{54}-regulated genes

To identify genes influenced by σ^{54}, we performed whole-transcriptome analysis for wild-type *L. biflexa* and the *rpoN* mutant. The results revealed that expression of 20 genes was lower in the *rpoN* mutant than that in the wild-type strain (with a cut-off of over 2-fold of changes in expression), suggesting that these genes were positively regulated by σ^{54} (Table 1). The analysis also identified 36 genes whose expression was negatively regulated by σ^{54} (Table 2). Among those genes that were σ^{54}-dependent, several genes are predicted involving in nitrogen transport and metabolism, including genes encoding ammonia transporter AmtB1 (LEPBI_I1767), AmtB2 (LEPBI_I2408), nitrogen regulatory protein P-II

(LEPBI_I2407), Nark-like nitrate/nitrite antiporter (LEPBI_I2771), and NtrX family nitrogen assimilation regulator (LEPBI_I1654). Further qRT-PCR analysis confirmed that expressions of these genes were significantly downregulated in the *rpoN* mutant, while the complemented strain could fully restore their expression (Fig. 4). Note that we also examined the patter of gene regulation in the *rpoN* mutant at both 30°C and room temperature, and a similar pattern was observed.

The σ^{54} regulon is important for leptospiral survival under the nitrogen starvation or environmental water conditions

To investigate whether σ^{54} is important for nitrogen utilization of *L. biflexa*, wild-type, the *rpoN* mutant and the complemented strains were cultured either under the nitrogen-rich conditions (regular Ellinghausen-McCullough-Johnson-Harris (EMJH) medium, containing 5 mM NH_4Cl) (Fig. 5A), or nitrogen-poor conditions in which NH_4Cl in the EMJH medium was replaced with 5 mM of glutamine (Fig. 5B), arginine (Fig. 5C), or alanine (Fig. 5D). The result showed that all *L. biflexa* strains grew well when glutamine, a known major amide-donor amino acid, was used as a nitrogen source. However, both wild-type and the mutant grew poorly when arginine or alanine was used as a nitrogen source, suggesting that *L. biflexa* cannot utilize these poor nitrogen sources (Fig. 5C–D).

We then examined whether there is a difference in survival between wild-type and the *rpoN* mutant strains under the long-term nitrogen starvation conditions. After spirochetes were incubated under the nitrogen-starvation conditions for one week, cells were plated on regular EMJH solid media for colony count. As shown in Fig. 5E, wild-type cells recovered well, whereas the *rpoN* mutant could no longer recover and only few tiny colonies were found. This data suggests that the *rpoN* mutant has a defect in survive under the long-term nitrogen starvation conditions.

Leptospira is known to be capable of surviving in environmental water for a long time. Since the *rpoN* mutant could not survive during nitrogen starvation, we argued that it should be incapable to survive in water. To this end, wild-type, the *rpoN* mutant and the complemented strain of *L. biflexa* were incubated in spring water, and samples were collected weekly and plated on the solid EMJH media for quantitation of cell viability. The *rpoN* mutant formed tiny colonies, and became no longer detectable at week 4 after incubation in water (Fig. 6), whereas wild-type and the complementary strains grew normally and remained detectable at week 6 (Fig. 6). This result indicates that σ^{54} is also important to the viability of *Leptospira* in water.

amtB1, amtB2, and narK are induced under the nitrogen starvation condition, and are controlled by σ^{54} indirectly

To begin elucidating the mechanisms underlying how the RpoN regulon governs the survival of *L. biflexa* in the nitrogen-limiting mediums and in water, we focused on σ^{54}-regulated genes, *amtB1, amtB2*, and *narK*. We examined if the expression of these genes is induced under the nitrogen-limiting conditions (Fig. 7A–C). In wild-type *L. biflexa*, their expressions were significantly upregulated under the nitrogen limiting conditions. The expression of these genes in the rpoN mutant could not be detected under any of the conditions tested,

further supporting the notion that the expression of these genes is absolutely dependent on σ^{54}. $^{\infty}$-dependent.

σ^{54} recognizes a highly conserved −24/−12 promoter sequence (TGGCA<6bp>TTGCT/A). Further analysis of the upstream sequences of *amtB1, amtB2*, and *narK* (including the upstream sequences of the putative operons in which these genes may reside) did not identify a putative σ^{54}–type promoter, a result that is consistent with recent reports of genome-wide analysis of σ^{54}–type promoters in published bacteria genomes (Francke *et al.*, 2011, Bonocora *et al.*, 2015). These data suggest that the expression of *amtB1, amtB2*, and *narK* are not directly under the control of σ^{54}.

rcfA encoding a tetratricopeptide repeat protein is induced under the nitrogen starvation condition, and is under the direct control of σ^{54}

Transcriptome analysis revealed that the expression of *LEPBI_I1011*, which encodes a hypothetical protein containing a tetratricopeptide repeat (TPR, pfam13424) domain, was dramatically downregulated in the *rpoN* mutant (Table 1). Further qRT-PCR analysis confirmed this finding (Fig. 4). Similar to other predicted nitrogen utilization-related genes, expression of *LEPBI_1011* was significantly upregulated in ammonia starvation but downregulated when ammonia is in excess, and such regulation is dependent on σ^{54} (Fig. 7D). These data suggest that LEPBI_I1011 is a novel protein involved in nitrogen regulation of *L. biflexa*. We hereby rename it as RpoN-controlled factor A (RcfA).

Promoter analysis of the upstream sequence of *rcfA* revealed that there is a putative σ^{54}-type promoter sequence (TGGCA<6bp>TTGCA) that is virtually identical to the consensus sequence of the σ^{54}-type promoter, located 33bp upstream of the ATG translational start codon of RcfA (Fig. 8A). Based on the predicted transcriptional start site, transcription from this promoter will yield a *rcfA* mRNA containing a 21 bases of untranslated sequence, which includes a putative CGGAGG ribosomal binding site (Shine-Dalgarno sequence).

To examine if σ^{54} of *L. biflexa* binds to the putative *rcfA* promoter, we purified recombinant RpoN protein (σ^{54}) of *L. biflexa* and performed the *in vitro* electrophoretic mobility shift assays (EMSAs). To this end, a 40 bp oligonucleotide encoding the predicted −24/−12 region of the promoter of *rcfA* (Fig. 8A) was end-labeled with ^{32}P and incubated with varying amounts of purified RpoN. The results showed that σ^{54} bound to the *rcfA* promoter region in a dose-dependent fashion (Fig. 8B). Moreover, the binding of RpoN to labeled probe was inhibited by the addition of 200-fold excess of specific cold competitor DNA (Fig. 8C), and was not affected by the addition of 100-fold excess of non-specific salmon sperm DNA. Noted that no DNA shift was observed in the EMSAs using promoter fragments of *LEPBI_I2408*, *LEPBI_I1767* and *LEPBI_I2771* (data not shown). These results suggest that *rcfA* is directly under the control of σ^{54}.

Since EbpA is the essential activator for genes with σ^{54}-type promoter, we examined EbpA binding to the upstream region of *rcfA* (−450-50 relative to the ATG start code). The EMSA results demonstrated that EbpA was capable of binding to the upstream region of *rcfA* in a dose-dependent manner (Fig. 9A). The binding of EbpA to *rcfA* was specific, as 1) the

binding was not affected with addition of nonspecific competitor poly(dI-dC) (Fig. 9B), and 2) EbpA did not bind to the upstream region of *glpF* that has a σ^{70}-type promoter (Fig. 9C).

Discussion

Pathogenic leptospiras colonize the renal tubules of wild and domestic animals, and are shed by urine into soil and water. Their ability to survive in the aquatic environment for months is one of the key contributing factors to leptospirosis outbreak (Smith & Turner, 1961, Andre-Fontaine *et al.*, 2015, Trueba *et al.*, 2004, Ganoza *et al.*, 2006, Hagan *et al.*, 2016). However, the molecular mechanisms underlying the leptospiral survival in the environment are largely unexplored. In this study, we showed that the σ^{54} regulon is required for *L. biflexa* to survive in mineral water environments. This function of σ^{54} is through modulation of nitrogen uptake and metabolism, as the σ^{54}–defective mutant is incapable of survival under the nitrogen starvation conditions as well as in water. The gene targets regulated by σ^{54} identified in this study, either directly or indirectly controlled by σ^{54}, set the foundation for further investigation how the σ^{54} regulon responds to environmental signals and maintains survival under the nutrient-deprived conditions.

This study was carried out in the saprophytic strain *L. biflexa*, but the finding also has implication for the function of σ^{54} in pathogenic *Leptospira*. First, σ^{54} is highly conserved among all *Leptospira*, including saprophytic and pathogenic species. Second, we recently showed that EbpA, one of the EBPs that activate σ^{54}, is also important for environmental survival of *L. interrogans* (Hu *et al.*, 2016). Third, σ^{54} of *L. biflexa* and EbpA of *L. interrogans* both control the expressions of several nitrogen uptake/metabolism genes (Hu *et al.*, 2016). Obviously, constructing a σ^{54}–defective mutant in pathogenic *Leptospira* is needed to have a complete understanding of σ^{54} in *Leptospira* species. Unfortunately, constructing an *rpoN* mutant has not been successful despite multiple attempts in several strains of *L. interrogans* (data not shown). Nevertheless, based on the finding in this study and the previous finding on EbpA of *L. interrogans*, we postulate that the EbpA-σ^{54} pathway in both *L. biflexa* and *L. interrogans* has a similar role, i.e., modulating genes that are important for *Leptospira* to survive in the environment.

σ^{54} is known to be involved in a variety of processes in bacteria, such as nitrogen assimilation, carbon source utilization, certain fermentation pathways, flagellar synthesis and bacterial virulence (Francke *et al.*, 2011, Reitzer & Schneider, 2001). In response to nitrogen limitation, σ^{54} regulates expression of genes involved in ammonia transporters and assimilation via the NtrB-NtrC system in *E.coli* (Reitzer & Schneider, 2001). Based on our bioinformatics analysis, no NtrB-NtrC system is present in *Leptospira* species (data not shown). However, we showed that the expressions of many nitrogen-utilization related genes of *L. biflexa* including *amtB1, amtB2*, *narK*, and *narX*, are also σ^{54}-dependent and are induced under nitrogen starvation. Membrane-bound AmtB belongs to the Ammonia Channel Transporter (Amt) family, which facilitates the uptake of ammonium/ammonia and is important for bacteria growth at low external ammonium concentrations in *E. coli* and other bacteria (Javelle *et al.*, 2004, van Heeswijk *et al.*, 2013). In ammonia-sufficient conditions, the diffusion of ammonia across the cytoplasmic membrane is sufficient to support bacterial growth, as demonstrated in *E. coli* (Soupene *et al.*, 1998), *Bacillus subtilis*

(Detsch & Stulke, 2003), and *Corynebacterium glutamicum* (Meier-Wagner *et al.*, 2001, Siewe *et al.*, 1996). *L. biflexa* has three copies of *amtB* genes, *LEPBI_I1767* (*amtB1*), *LEPBI_I2408* (*amtB2*) and *LEPBI_I0794* (*amtB3*). The latter two genes are genetically linked with the gene encoding GlnK-like signal transduction protein. The AmtB-GlnK physical interaction is highly conserved, and the two genes form the conserved gene pair in a diverse range of Eubacteria and Archaea (Thomas *et al.*, 2000). They are responsible for ammonia uptake and modulating the activity of glutamine synthetase (GlnA) for ammonia assimilation. Of note, our transcriptome analysis showed that the expression level of *amtB2* is 10-fold higher than *amtB1* and *amtB3* (data not shown), indicating that AmtB2 may play a major role in ammonia uptake in *L. biflexa*.

The finding from this study suggests that the reason that the lack of nitrogen source is one of the reasons why the σ^{54} mutant of *L. biflexa* cannot sustain viability in the environmental water, as the σ^{54} mutant is unable to survive in the growth medium that lacks ammonia. This is in contrast to wild-type *L. biflexa* which remains viable for months in such medium. How does σ^{54} contribute to the survival of *L. biflexa* under the nitrogen starvation conditions? One of the possible mechanisms is via regulation of genes involved in nitrogen assimilation, in particular, the *glnK-amtB* operon. GlnK of *E. coli* has been shown to play many functions during nitrogen assimilation (Atkinson & Ninfa, 1999, Atkinson *et al.*, 2002, Blauwkamp & Ninfa, 2002). It has a dramatic effect on the ability of *E. coli* cells to survive during nitrogen starvation (Blauwkamp & Ninfa, 2002). Given that the expression of the *glnK-amtB2* operon is drastically downregulated in the σ^{54} mutant of *L. biflexa*, we hypothesize that σ^{54} contributes to the survival of *L. biflexa* in nitrogen starvation and environmental water conditions via regulation of *glnK-amtB* operon. Further studies to construct the *glnK* mutants in *L. biflexa* and *L. interrogans* and test their survival ability in environmental water are warranted to test this hypothesis.

We demonstrated in this study that σ^{54} regulates nitrogen assimilation genes (*amtB1, amtB2, narK*, and *ntrX*) indirectly. How σ^{54} regulates expression of these genes remains to be elucidated. One of the genes we identified whose expression is directly controlled by σ^{54} is *rcfA* (*LEPBI_I1011*). Although the function of this 146 amino acid long, TPR domain-containing protein remains undefined, the level of *rcfA* expression is dramatically upregulated under the nitrogen starvation condition (Fig. 7D), suggesting that RcfA is involved in nitrogen assimilation. TPR-containing proteins often function as a module for protein-protein interactions, and can involve in a variety of cellular functions including gene regulation, signaling, transport, as well as involved in virulence (Cerveny *et al.*, 2013). Whether σ^{54} regulates other nitrogen-regulated genes via RcfA remains to be tested. Of note, RcfA is highly conserved among saprophytic *Leptospira* species including *L. meyeri*, *L. terpstrae*, *L. vanthielii*, *L. wolbachii*, and *L. yanagawae*. Interestingly, we showed that a gene encoding a TPR domain-containing protein, *LMANv2_200027*, in *L. interrogans* serovar Manilae strain L495, is also controlled by EbpA directly (Hu *et al.*, 2016). We postulate that these two σ^{54}-controlled TRP proteins may function similarly in response to nitrogen deprivation. In fact, most of the genes identified in this study to be differentially expressed in the *rpoN* mutant are not unique to saprophytic leptospires, and have homologous in pathogenic leptospiral species (Tables 1 and 2). These results indicate that EbpA-σ^{54} regulation is similar between saprophytic and pathogenic leptospires, and are

important for environmental survival for *Leptospira* species. Pathogenic leptospires have addition pathway EbpB-σ^{54}, which is lacking in saprophytic leptospires and likely plays a role in mammalian infection (Hu *et al.*, 2016).

In addition to the genes positively regulated by σ^{54}, transcriptome analysis in this study uncovered 36 genes whose expression was negatively regulated by σ^{54}. Interestingly, several genes relate to iron-acquisition such as hemin binding/transport/degradation (*LEPBI_p0012, LEPBI_p0013, LEPBI_p0014, LEPBI_pa0017*,), TonB-dependent outer membrane receptor (*LEPBI_p0018, LEPBI_I2760*), ABC-type Fe3+-hydroxamate transport system (*LEPBI_p0015*), and putative iron-regulated membrane protein; *LEPBI_I2762*). Iron is essential for the growth of both saprophytic and pathogenic *Leptospira* spp (Lo *et al.*, 2010b, Murray *et al.*, 2009, Asuthkar *et al.*, 2007, Louvel *et al.*, 2005, Cullen *et al.*, 2002). Iron limitation upregulates many genes including genes predicted or demonstrated to be involved in hemin uptake or TonB-dependent membrane receptor (Cullen *et al.*, 2002, Lo *et al.*, 2010b). *LEPBI_I2760* was experimentally showed to encode a TonB-dependent membrane receptor protein for ferrioxamines in *L. biflexa*, and the disruption of *LEPBIa2760* resulted in an impaired utilization of desferrioxamine as an iron source (Louvel *et al.*, 2006a). Our data suggest that inactivation of σ^{54} upregulates hemin or other iron-related transport system as a general response to nutrient deprivation. How σ^{54} indirectly influences these gene expressions remains unclear. In this regard, *L. biflexa* and *L. interrogans* genomes have four *fur*-like genes encoding Fur, Zur, and PerR family proteins that are likely involved in modulating expression of genes involved in iron acquisition (Louvel *et al.*, 2005, Lo *et al.*, 2010b). Expression levels of these genes were not affected by σ^{54} deletion. One possibility is that function, not the level of these proteins, were altered in the deficient mutant, subsequently, led to upregulation of these iron-related genes.

In summary, although a number of reports showing that leptospiras regulate their gene expression in responding to a wide range of environmental and host signals, genetic networks modulating gene expression in response to environmental cues have not been uncovered heretofore. This study identified the EbpA-σ^{54} regulatory network that modulates expression of genes associated with nitrogen uptake and metabolism, and that plays a key role in the environmental survival of *Leptospira*. Based on this and our previous finding, we propose the following model: EbpA likely senses environmental stress signals via its GAF motifs, and along with σ^{54}, activates transcription of *rcfA* and other genes. σ^{54} indirectly regulates the expression of nitrogen-utilization related genes including *amtB1*, *glnK-amtB2*, *narK*, and *ntrX* (Fig. 10). Expression of these and other σ^{54}–dependent genes is one of the key factors that allow *Leptospira* to survive in the environment. Given the complex environmental conditions leptospires encounter and the presence of many putative regulatory proteins in Leptospira genomes, further identification of other regulatory systems modulating environmental adaptation of *Leptospira* are warranted.

Experimental Procedures

Bacterial strains and culture conditions

L. biflexa serovar Patoc strain Patoc 1 (Paris) was maintained and grown in EMJH medium at 30°C. For the *rpoN* mutant and its complemented strains, spectinomycin and gentamycin

were added to a final concentration of 50 μg/ml, respectively. For the modified EMJH, NH_4Cl (5 mM) in the standard EMJH medium was replaced with the same concentration of glutamine, arginine, or alanine, with the pH adjusted to 7.4. The constructed suicide and shuttle vectors were maintained in *E. coli* strain DH5α, and *E. coli* strain β2163 was used as the donor for *E. coli- Leptospira* conjugation.

Construction of the *rpoN* mutant and the complementation strains

The RpoN (LEPBI_I1650)-coding gene disruption mutant was created by allelic exchange in *L. biflexa* by transforming with the suicide vector pLb-RpoN-KO (Fig. 3A). To construct the knockout plasmid, both the upstream and downstream 1,700-bp fragments of *rpoN* were PCR amplified from *L .biflexa* genomic DNA. The resulting DNA fragments were then cloned upstream and downstream of a spectinomycin-resistance marker (*aadA*) driven by a constitutively expressed *Borrelia* promoter *flaB* via HindIII/MluI and NheI/XmaI restriction sites, respectively. The resulting suicide plasmid was confirmed by both restriction enzyme digestion and sequencing followed by electroporation. Positive *Leptospira* transformants were selected via proper antibiotic resistance, and *rpoN* inactivation was verified by qRT-PCR and immunoblot analysis.

A shuttle vector, pJJ003, was constructed for the complementation of *rpoN* (Fig. 3A) based on the *E. coli-Leptospira* shuttle vector pCJSpLe94 (Picardeau, 2008). To this end, the spectinomycin-resistance marker in pCJSpLe94 was first replaced by a gentamycin-resistance marker originated from plasmid pSL94PfGenta (Poggi *et al.*, 2010) to construct pJJ002. Then, an *hsp10*-promoter driving *rpoN* cassette was inserted into plasmid pJJ002. The resulting plasmid, pJJ003, was sequenced before it was transformed into the *L. biflexa rpoN* mutant by conjugation as described (Picardeau, 2008). The transformants with both gentamycin and spectinomycin resistance were selected and subjected to qRT-PCR and immunoblot analysis to confirm the restoration of RpoN expression.

RNA extraction, microarray construction, scanning and data analysis

The annotation of the *L. biflexa* strain Patoc 1 (Paris) genome (http://www.ncbi.nlm.nih.gov/genome/?term=NC_010602) was used to design 60-mer oligonucleotides using the web-based application eArray (Agilent). Three different probes were designed for each open read frame, based on the optimization of melting temperature (Tm), secondary structure, and homology to other sites in the whole genome. In total, 11,166 oligonucleotides were designed, representing 3,722 (out of 3,730) putative open reading frames of the *L. biflexa* genome. They were printed in triplicate on each subarray, which were in turn printed in quadruplicate on the slide. The oligonucleotide synthesis and microarray print, as well as the scanning and data analysis, were completed by MOgene.

For the RNA extraction, both the wild-type and the *rpoN* mutant strains were cultivated in EMJH medium at room temperature and harvested at the mid-logarithmic growth. Total RNA was extracted from two biological replicates using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Digestion of contaminating genomic DNA in the RNA samples was performed using RNase-Free DNase I (New

England Biolabs), and removal of DNA was confirmed by PCR amplification using primers specific for *flaB* of *L. biflexa*.

cDNA was synthesized and labeled with Cy3 or Cy5 by use of the Amersham Post-Labeling Kit according to the manufacturer's instructions, with minor modifications. Briefly, 5 μg of total RNA was converted to cDNA by use of CyScript RT in the presence of 1 μl of random nonanucleotides (Amersham Biosciences). Each cDNA sample was labeled with Cy3 and Cy5 separately. Cy3- or Cy5-labeled cDNA from the parental strain was then combined with the Cy5- or Cy3-labeled cDNA from the mutants. Labeled probes were purified and then used in the microarray experiment. With two pairs of samples plus dye switching, we made a total of four hybridized slides. Hybridized slides were then scanned on an Axon 4000B microarray scanner using GenePix Pro 6.1 (Molecular Devices). The image was analyzed using the GenePix program, and data were then analyzed with Acuity 4.0 (Molecular Devices) by using the ratio-based normalization method. A 2-fold change cutoff value was used to select candidate genes.

qRT-PCR

RNA samples were extracted from leptospiral cultures grown to middle log phase (around 1×10^8 cells per ml) at 30 °C using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. The cDNA was synthesized using the SuperScript III Reverse Transcriptase with random primers (Invitrogen) from 1 μg RNA. qRT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7000 sequence detection system. The *flaB* gene of *L. biflexa* was used as a reference. The relative transcription level was determined by the threshold cycle ($2^{-\Delta\Delta Ct}$) method (Livak & Schmittgen, 2001). All samples were run in triplicate, and the student *t* test was performed to determine the statistical significance between the expression levels of different groups.

Measurement of cell growth and viability on solid medium

To investigate the growth abilities of *Leptospira* strains in the mediums with different nitrogen sources, the tested strains were first grown in regular EMJH medium to late log phase. The cells were harvested by centrifugation and washed once with ammonia-free EMJH to avoid carryover of residual nitrogen sources. Then cells were subcultured in the modified fresh EMJH with different nitrogen sources at an initial concentration of 10^6 spirochetes per ml. Every medium was independently analyzed two times. Those nitrogen sources that supported growth were further analyzed with three independent replicates each time. All cultures were incubated at 30°C and the cell numbers were numerated every 24 hours under a dark-field microscope. The viabilities of leptospires were enumerated by plating diluted culture samples on solid EMJH medium.

Water survival test

The long-term water survival abilities of *Leptospira* strains were investigated by a modified method (Smith & Turner, 1961, Trueba *et al.*, 2004). Since *Leptospira* cannot survive in distilled water, the spring waters used in the study were commercially available drinking water in North American (pH 7.2, Kirkland Signature, USA) with trace mineral contents. The waters were filter sterilized to avoid modifying the chemical equilibrium. In brief,

leptospiral cells grown to logarithmic phase (3×10^8 cells per ml) were harvested by centrifugation. The supernatants were thoroughly drained, and the cells were resuspended in the original volume (15 ml) of water. All cultures were incubated at 30°C, and the viabilities of leptospires were enumerated every week by plating 100 μl diluted culture samples on solid EMJH medium. The plates were incubated at 30°C for two weeks, and the efficiency of plating was determined by dividing the total number of colonies, irrespective of size, by the number of colonies expected based on the cell counting via dark-field microscopy. Culturable cells were also detected by inoculating 0.5 ml culture into 5 ml fresh EMJH and incubating for 4 weeks. The typical gyrations in liquid media were used to identify live leptospires.

EMSA

EMSAs were performed to determine protein: DNA interactions as described previously (Hellman & Fried, 2007). For RpoN binding, a 50-bp putative promoter fragment containing an engineered *EcoR*I site was PCR amplified. The resulting fragment was purified and digested with *EcoR*I, and then labeled with ^{32}P using Taq DNA polymerase by filling in the *EcoR*I sites with [α-^{32}P]dATP. For EbpA binding, a 500-bp DNA fragment upstream of the gene *LEBI_I1011* (−450 and +50 bp relative to the ATG start codon) was PCR amplified from the genome of *L. biflexa* with primers with containing an engineered EcoRI site, and then labeled as above. Labelled fragments (0.2 pmol) and various amounts of purified RpoN or EbpA were mixed in 10 μl binding reactions in the presence of 100 μg ml^{-1} salmon sperm DNA for 30 minutes at room temperature. Some reactions included unlabeled promoter competitors or non-specific competitors for competition studies. The reactions were analyzed on non-denatured polyacrylamide gels, and then the gels were dried and exposed in a cassette using an X-ray film for autoradiography.

Protein expression and antisera preparation

For expression and purification of RpoN of L. *biflexa*, the *rpoN* gene was PCR amplified from genomic DNA of *L. biflexa* and cloned into the expression vector pET100 (Invitrogen). The resulting plasmid, pJJ032, was transformed into *E. coli* BL21DE3 (Novagen), and the C terminal His_6 tagged RpoN was expressed with the induction of 0.4 mM IPTG. The recombinant RpoN protein was further purified using Ni-NTA affinity chromatographic column (NEB) according to the manual. To obtain the recombinant protein EbpA, the full-length *ebpA* gene was PCR amplified by PCR application. The PCR product was cloned into pGEX-4T-2 vectors (Amersham Pharmacia Biotech) via *BamH*I and *Not*I (NEB) digestion. The resulting plasmid, pGEX-4T-2ebpA, was transformed into *E. coli* BL21(DE3) for the expression of glutathione S-transferase (GST) fused recombinant EbpA protein. The protein was expressed under the induction of 0.5 mM IPTG, and purified by glutathione superflow agarose (Pierce) according to the manual. To increase the solubility of the recombinant proteins, all induction was conducted at 16°C. The expressed and purification of recombinant proteins were monitored by SDS polyacrylamide gel electrophoresis.

To obtain the recombinant protein GroEL, the full-length *groEL* gene was PCR amplified from *L. interrogans* strain Lai genomic DNA. The product was digested with NdeI and XhoI endonucleases before it was inserted into pET42a and transformed into *E. coli* BL21DE3

(Novagen). The His_6 tagged recombinant GroEL protein was expressed under the induction of 1mM isopropy-β-Dthiogalactoside (IPTG), and the soluble GroEL was purified using Ni-NTA affinity chromatographic column (NEB) according to the manual.

For antisera production, New Zealand rabbits were immunized intradermally on days 1, 14, 21 and 28 with 2 mg *leptospiral* RpoN and GroEL respectively, which were pre-mixed with Freund's adjuvant. Fifteen days after the last immunization, the sera were collected to separate the IgGs by ammonium sulfate precipitation plus a DEAE-52 column (Sigma) purification. Phosphate buffer (10 mM, pH 7.4) was used for elution and the titer of the IgGs binding was detected by immunodiffusion test.

Immunoblot analysis

Leptospira strains were inoculated into EMJH medium and grown to late logarithmic phase before they were harvested by centrifugation at 7,000 g and washed twice with phosphate buffered saline (PBS, 50 mM, pH7.4). The pellets were collected for immunoblotting using the SuperSignal West Pico chemiluminescent substrate with home-made antibodies according to the manufacturer's instructions (Pierce).

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Plain Language Summary

This study reports inactivation and complementation of *rpoN* encoding the alternative sigma factor σ^{54} in *Leptospira biflexa*, and demonstrates that σ^{54} is required for the long term survival in water of leptospires.

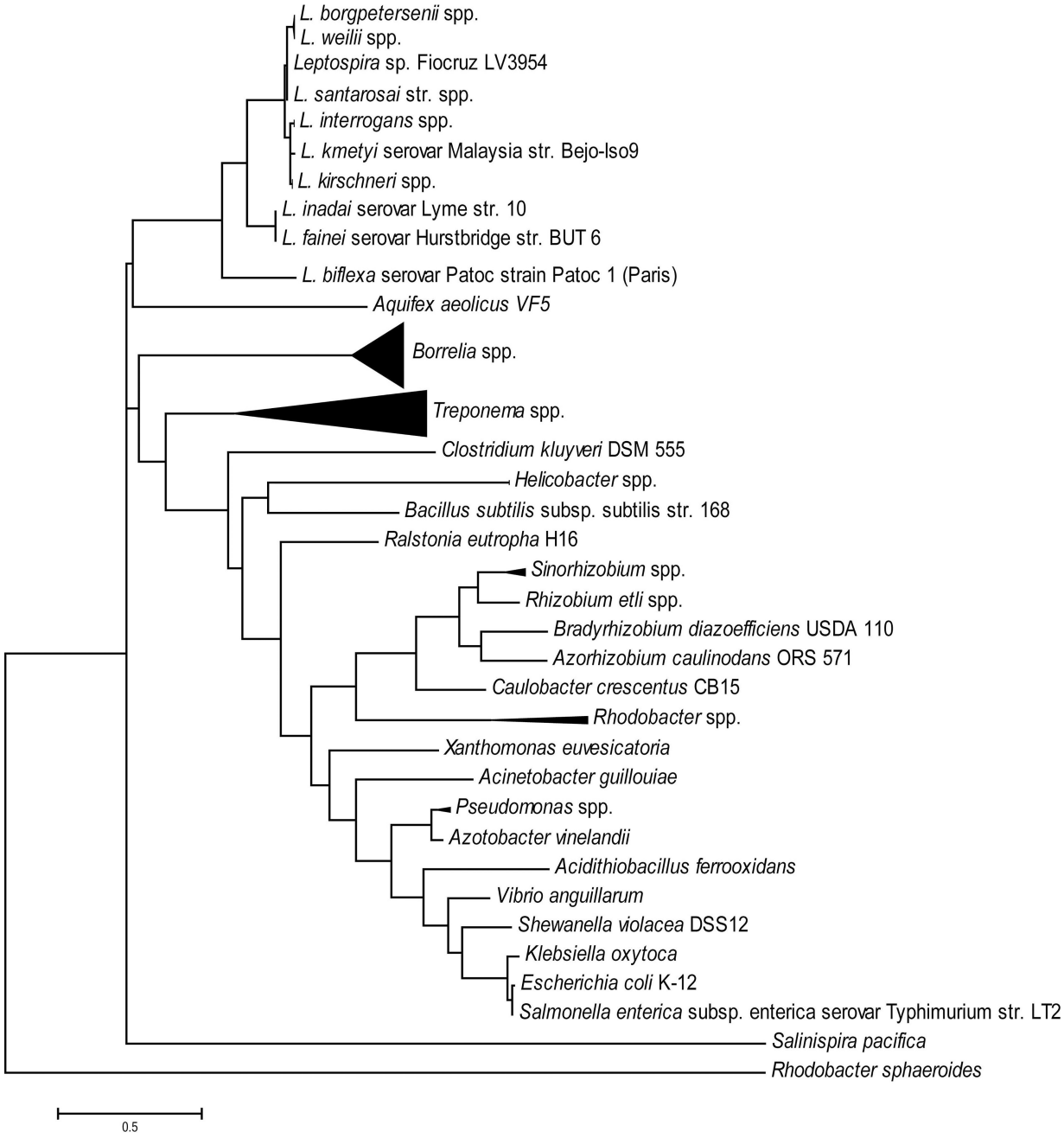

Fig. 1. Molecular phylogenetic analysis of *Leptospira* σ^{54}

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones *et al.*, 1992). The tree with the highest log likelihood (−10238.1361) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 101 amino acid sequences. Multiple items were selected for the genera of *Leptospira*, *Borrelia* and *Treponema*, while only function characterized RpoN

items were selected for other genera. All positions containing gaps and missing data were eliminated. There were a total of 142 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016). Triangles represent the diversity within genospecies.

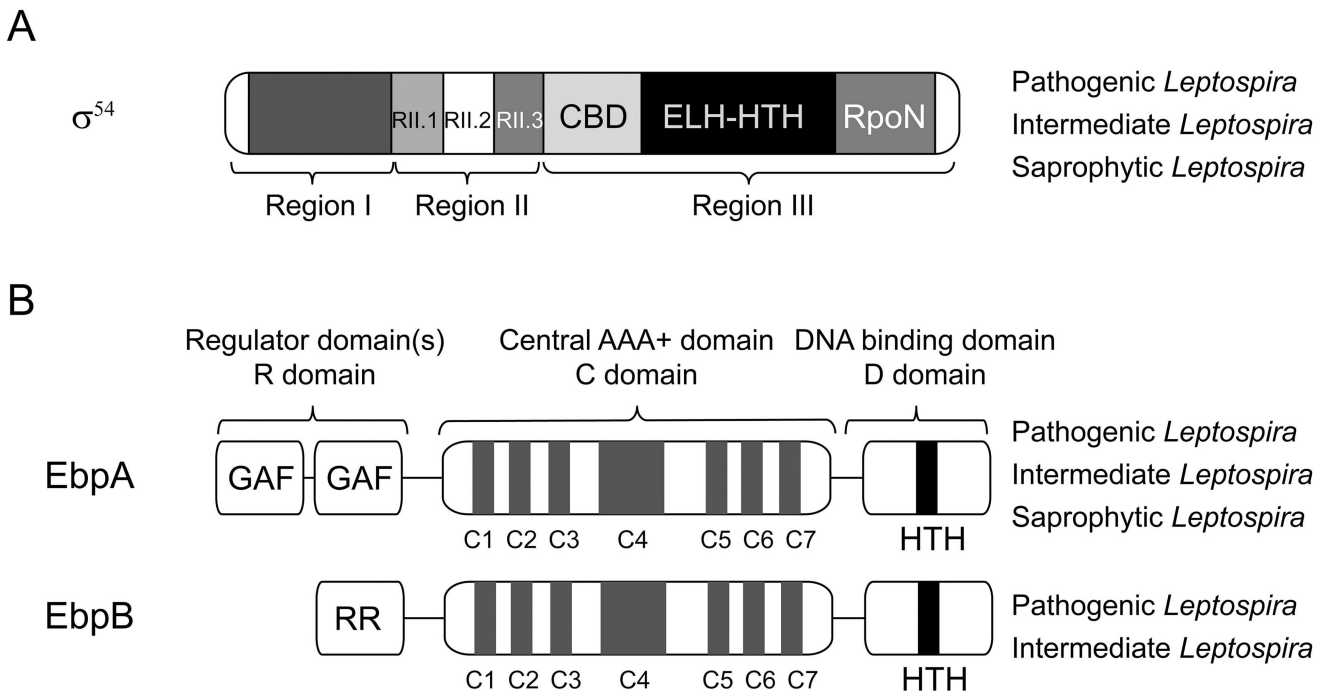

Fig. 2. Domain architectures of σ^{54} and their activators, enhancer-like binding proteins (EBPs) in the *Leptospira* genus

(**A**) The *Leptospira* σ^{54} contains all three conserved regions (I to III) found in other σ^{54} (Yang *et al.*, 2015). Region I plays an inhibitory role and contains the contact sites for its cognate activator proteins. Region II is the most variable region among σ^{54} proteins with high abundance of acidic amino acids, and it is proposed to facilitate template loading (RII. 2) and RNA separation from the DNA template (RII.3). The subdomains of Region III, including the RNAP core-binding domain (CBD) and the following extra-long α-helix-helix-turn-helix motif (ELH-HTH) domain, are involved in interacting with the −12 promoter element. The RpoN box, located in the C-terminal of Region III, is responsible for −24 promoter element recognition. DNA cross-linking region [ELH] and helix-turn-helix [HTH] are involved in DNA-binding. (**B**) The pathogenic *Leptospira* have two EBPs, EbpA and EbpB, while the saprophytic *Leptospira* only has EbpA. Both EbpA and EbpB consist of three functional domains: an N-terminal regulatory domain (R domain), a central AAA+ (ATPases Associated with a wide variety of cellular Activities) domain (C domain), and a C-terminal DNA-binding domain (D domain). The R domain does not share a common homolog with other members of the EBP family, and different sensory domains are present depending on the signal to be detected. EbpA is an FhlA (formate hydrogen lyase activator)-like EBP, which includes two GAF (cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA) motifs in the R domain. EbpB is an NtrC-like EBP, which has a conserved response regulator-type R-domain. The central C domain of EbpA and EbpB contain seven C1 to C7 are seven conserved subdomains that are essential for σ^{54}-dependent transcription. The HTH motif in D domain directs the EBP to a specific binding site.

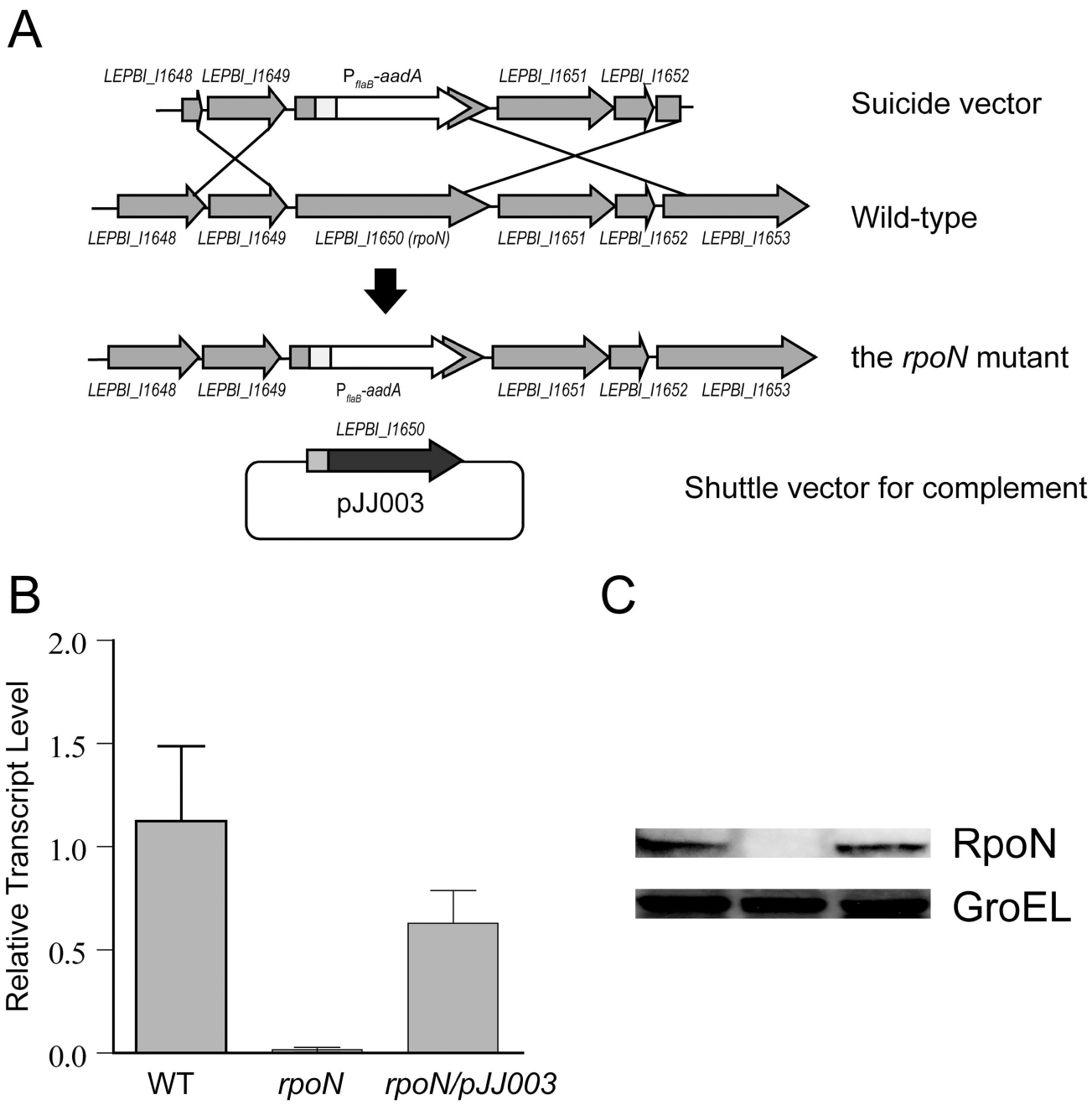

Fig. 3. Inactivation and complementation of *rpoN* (*LEBI_1650*) in *L. biflexa* by allele exchange

(A). Schematic representation of *rpoN* and its neighboring genes in the chromosome of wild-type *L. biflexa*. The middle 793 bp of the *rpoN* gene was disrupted by insertion of a 1,197 bp *flaB* driven streptomycin-resistance gene (*aadA*) cassette from *Myxococcus xanthus* (Magrini *et al.*, 1998) by homologous recombination. The shuttle plasmid pJJ003 containing the *hsp10* promoter that drives the expression of the *rpoN* gene was used to complement the *rpoN* mutant (bottom panel). (B). Confirmation of the *rpoN* disruption and complementation by qRT-PCR analysis of *rpoN* transcriptional levels. (C). Immunoblot analysis of wild type, *rpoN* mutant and complemented strains. Whole-cell lysates of *L.*

biflexa equivalent to 10^8 cells were separated on 12% Tris-glycine gel and then immunoblotted with antisera directed against RpoN and cytoplasmic heat shock protein, GroEL (loading control). The blotted membrane was developed by chemical luminescence.

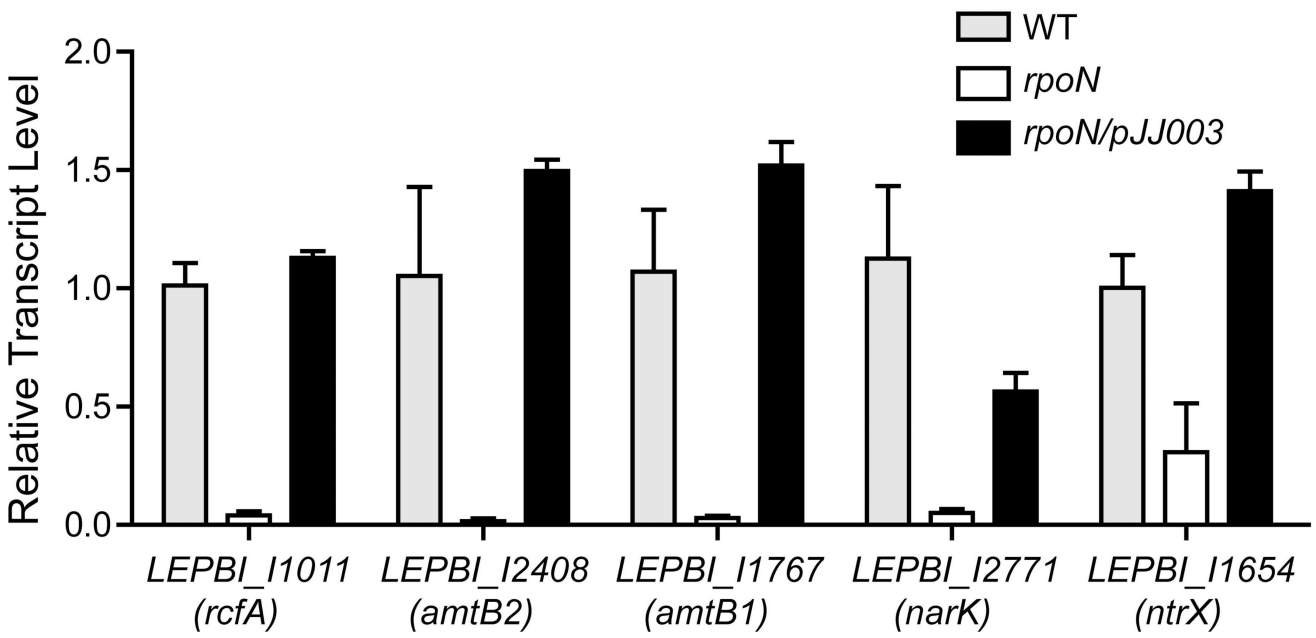

Fig. 4. qRT-PCR analyses of σ^{54} dependent genes in *L. biflexa*

The *L. biflexa* wild-type (WT), *rpoN* mutant (*rpoN*) and complemented strain (*rpoN*/*pJJ0003*) were grown in regular EMJH medium to mid-logarithmic phase. RNA samples were then isolated and subjected to qRT-PCR analysis. The levels of gene expression in each sample were normalized to *flaB*, and the relative levels to that in wild-type strain (set as value of 1) were reported. Error bars indicate standard deviation from three replicates. The expression levels of all tested candidates in the mutant demonstrated statistical differences ($P < 0.05$) in student *t* test compared to expression in wild-type.

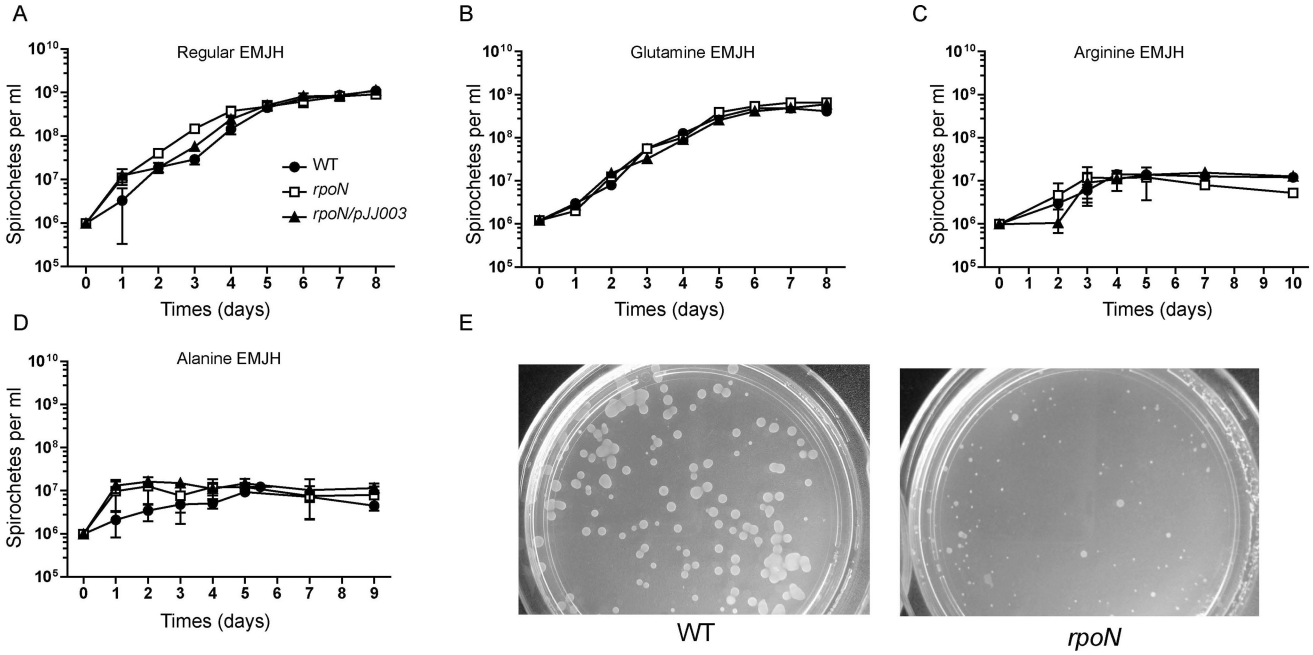

Fig. 5. Growth phenotypes of the *rpoN* mutant under various nitrogen conditions

The *L. biflexa* wild-type (WT), *rpoN* mutant (*rpoN*) and complemented strains (*rpoN*/*pJJ0003*) were grown in regular EMJH medium to mid-logarithmic phase and then subcultured into EMJH (A) or modified EMJH with 5 mM glutamine (B), arginine (C), or alanine (D) replacing NH_4Cl. All cultures were incubated at 30°C, and the growth was monitored by enumeration under dark-field microscopy daily. The experiment was performed three times with a representative result shown. Error bars indicate standard error from triplicate cultures. (E), σ^{54} is required to recover from nitrogen starvation. The strains of *L. biflexa* wild-type and *rpoN* mutant were first starved for a week in EMJH media without NH_4Cl and then plated on solid EMJH media. Plates were incubated for 7 days at 30°C before the images were taken.

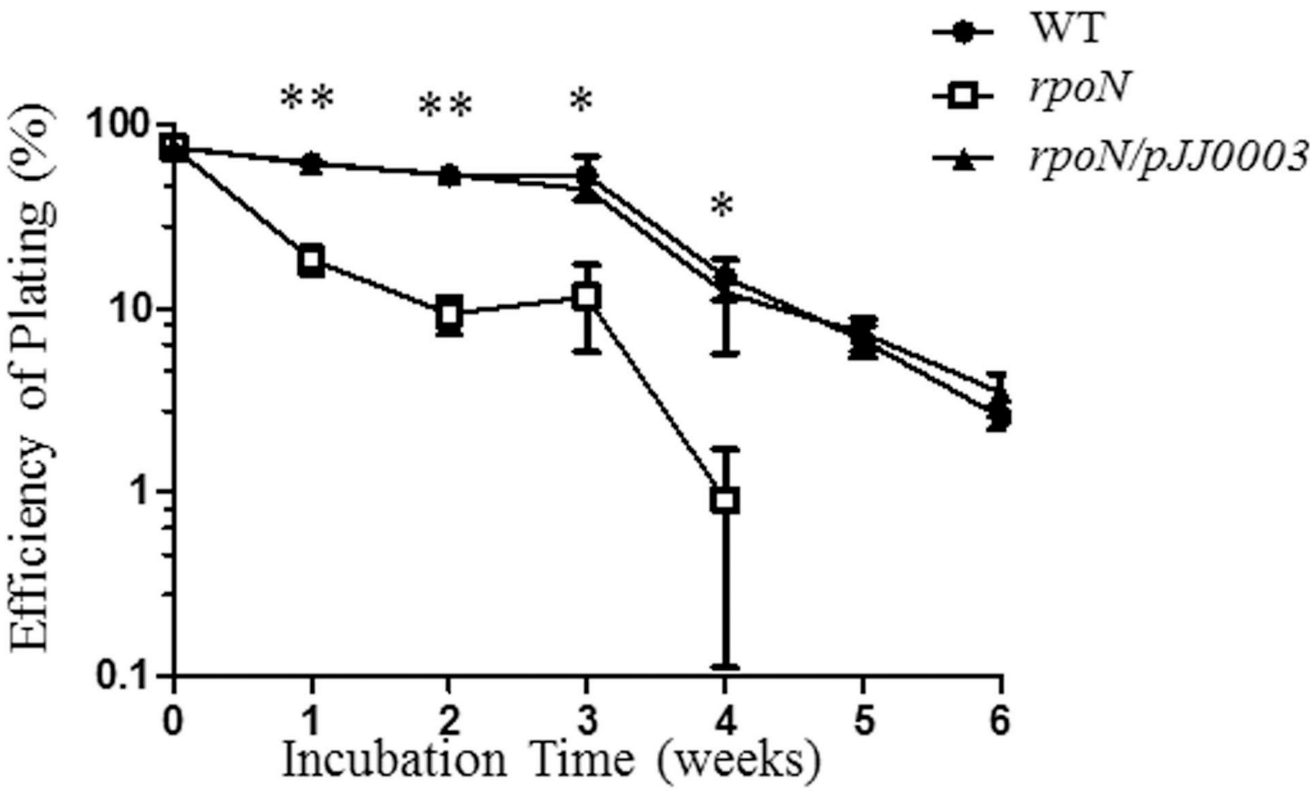

Fig. 6. σ^{54} is required for the long-term survival of *L. biflexa* in mineral water

The *L. biflexa* wild-type (WT), *rpoN* mutant (*rpoN*) and complemented strains (*rpoN*/*pJJ0003*) were grown in regular EMJH medium to mid-logarithmic phase and then harvested by centrifugation. The cells were resuspended in spring waters and incubated at 30°C. Viable cells were counted weekly by plating a defined volume of diluted cultures on solid EMJH media and counting the number of colonies that arose. The experiment was performed three times with a representative result shown. Error bars indicate standard error from triplicate cultures. Student *t* test was used for the statistical comparisons between wild-type and the *rpoN* mutant. * P<0.05, **P<0.01.

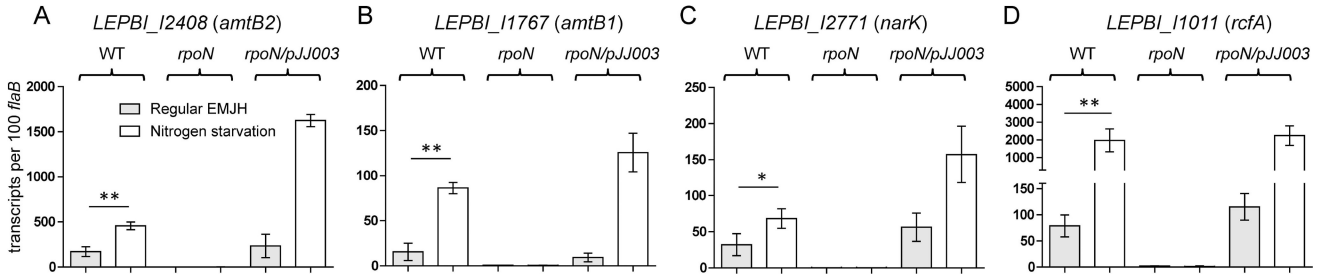

Fig. 7. Several σ^{54}-dependent genes are highly regulated in response to nitrogen limitation by qRT-PCR analysis

Wild-type (WT), the *rpoN* mutant (*rpoN*), and the complemented (*rpoN*/*pJJ0003*) strains were grown in regular EMJH medium to mid-logarithmic phase, harvested and resuspended into standard EMJH or nitrogen-limited (ammonia-free EMJH) medium. All treatments were incubated at 30°C for one week, and RNA samples were then isolated and subjected to qRT-PCR analyses for the expression levels of *LEPBI_I2408* (*amtB2*) (**A**), *LEPBI_I1767* (*amtB1*) (**B**), *LEPBI_I2771* (*narK*) (**C**) and *LEPBI_I1011* (*rcfA*) (D). Values represent the average numbers of each transcript per 100 copies of *flaB* from three biological replicates. Student *t* test was used for the statistical analysis. * P<0.05, **P<0.01.

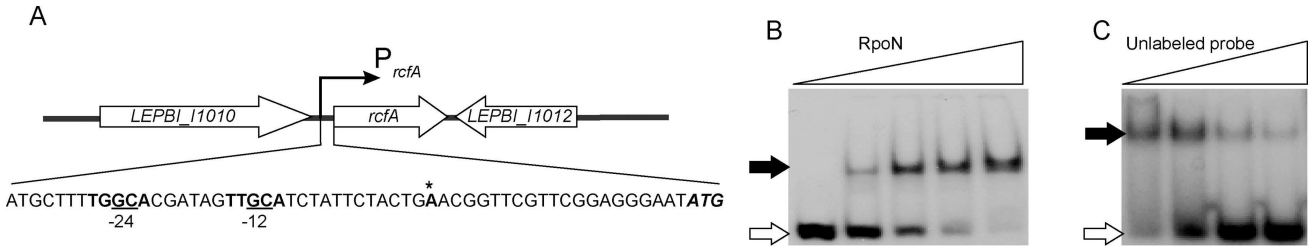

Fig. 8. *LEPBI_I1011* (*rcfA*) was directly controlled by σ^{54} in *L. biflexa*

(**A**) Schematic of the gene organization of *rcfA* and its promoter sequence. The −24 and −12 motifs of the typical σ^{54} promoter are labeled and underlined. The translational start site, ATG, is indicated in italics. The bold letter with a star indicates the predicted transcriptional start site and the boxed sequence indicates a putative ribosomal binding site (Shine-Dalgarno sequence). (**B**) The electrophoretic mobility shift assays (EMSAs) were conducted using purified RpoN and DNA fragment containing the promoter region of gene *rcfA* labeled with ^{32}P. Various amounts of purified RpoN (0, 5, 10, 15, and 25 pmol) were incubated with 0.25 pmol of labeled DNA in a 10 μl reaction. For the competition reaction (**C**), unlabeled probes (0, 20, 50, and 200-fold) were added to the reaction that contained 0.25 pmol labeled DNA and 15 pmol purified RpoN. All reactions contain 0.5 μg salmon sperm DNA, and were incubated for 30 min at 23°C and then analyzed on non-denatured polyacrylamide gels. Gels were dried, and radioactive signals were visualized by exposure to X-ray film. The free probes are indicated by open arrows and the retarded DNA fragments by solid arrows.

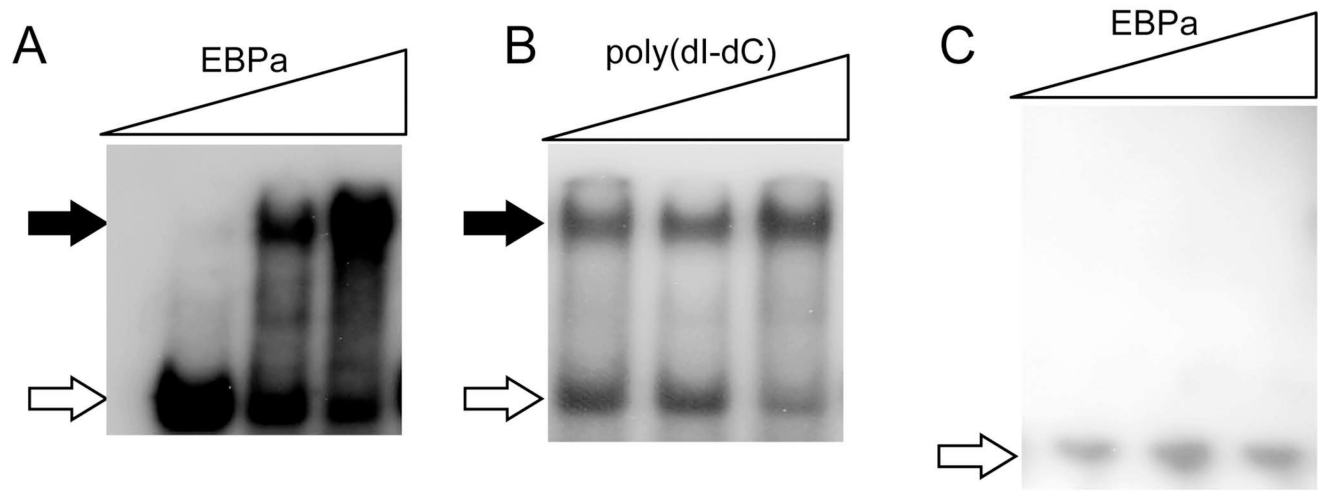

Fig 9. The enhancer-like binding protein EbpA binds to the upstream region of *LEPBI_I1011* (*rcfA*)

EMSA was conducted as described in Fig 8. (**A**)Various amount of recombinant EbpA (0, 5, 10 pmol) were incubated with ^{32}P labelled DNA fragment containing 450bp upstream of *rcfA* (0.05 pmol) in the 10 μl reaction. (**B**) EbpA binding to upstream of *rcfA* in the presence of non-specific competitor poly dI:dC (0, 50, 100 ng). (**C**) EbpA binding to upstream of a σ^{70} promoter-driven gene, *glpF*. The free probes are indicated by open arrows and the retarded DNA fragments by solid arrows.

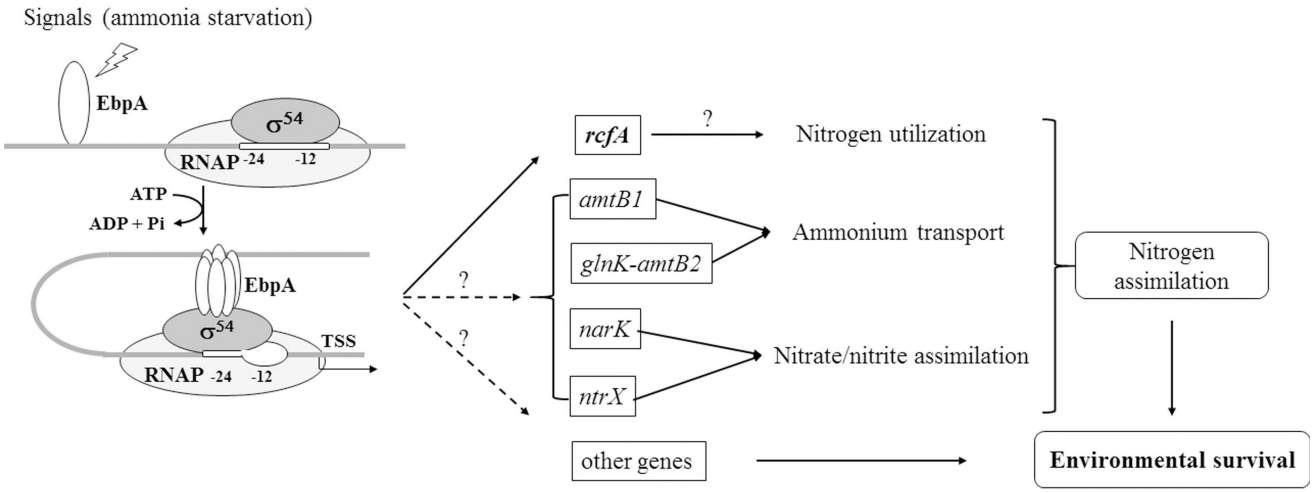

Fig. 10. Model for the σ^{54} regulon in *L. biflexia*

EbpA, the sole EBP in *L. biflexa*, senses environmental stress signals, and activates transcription of genes from a σ^{54}-type promoter (−24/−12), in an ATP-dependent manner (upon oligomerization). EbpA-σ^{54} directly regulates expression of *rcfA* from a σ^{54}–type promoter, and indirectly regulates expression of nitrogen-utilization related genes including *amtB1*, *glnK-amtB2*, *nark*, and *ntrX*. Expression of these and other σ^{54}–dependent genes allows *Leptospira* to adapt and survive in nutrient-limiting environmental conditions such as environmental water. RNAP, RNA polymerase; TSS, transcriptional start site.

Table 1

Genes positively regulated by σ^{54} in *L. biflexa*

Locus tag	Mean fold change[a]	COG[b]	Description of gene product	Orthologous in *L. interrogans* serovar Lai 56601	Orthologous in *L. borgpetersenii* serovar Hardjo-bovis str. L550
LEPBI_I1650	176.64	COG1508K	RNA polymerase sigma-54 factor	*LA_2404*	*LBL_1670*
LEPBI_I2408	10.19	COG0004P	Ammonium transporter, AmtB2	*LA_3622, LA_3806*	*LBL_2706*
LEPBI_I2407	3.25	COG0347E	Nitrogen regulatory protein P-II (GlnB)	*LA_3807*	*LBL_2705*
LEPBI_I1011	2.92	-	hypothetical protein	No hit	No hit
LEPBI_I1767	2.51	COG0004P	Ammonium ABC transporter permease, AmtB1	*LA_3622, LA_3806*	*LBL_2706*
LEPBI_I1174	2.49	-	Hypothetical protein	No hit	No hit
LEPBI_I3124	2.49	COG2208TK	Serine phosphatase	No hit	No hit
LEPBI_I0440	2.48	-	Hypothetical protein	No hit	No hit
LEPBI_I1657	2.30	COG0223J	Methionyl-tRNA formyltransferase	*LA2396*	No hit
LEPBI_I2653	2.29	COG0687E	Putative spermidine/putrescine-binding periplasmic protein PotF/PotD; putative signal peptide	No hit	No hit
LEPBI_I2502	2.25	-	Hypothetical protein	No hit	No hit
LEPBI_I1652	2.21	COG1925G	Phosphocarrier protein HPr (histidine-containing protein)	*LA_2402*	*LBL_1668*
LEPBI_I1653	2.20	COG5000T	Histidine kinase sensor protein; putative membrane protein	*LA_2401*	*LBL_1667*
LEPBI_II0220	2.17	-	Hypothetical protein	*LA_2746, LA_4046*	*LBL_1948, LBL_2853*
LEPBI_II0102	2.14	-	Hypothetical protein	No hit	No hit
LEPBI_I1651	2.08	COG1493T	HPr kinase/phosphorylase	*LA_2403*	*LBL_1669*
LEPBI_I0858	2.07	-	Hypothetical protein	No hit	No hit
LEPBI_I2771	2.04	COG2223P	Nitrite extrusion protein 1, NarK	No hit	No hit
LEPBI_p0010	2.02	COG2208TK	Putative phosphoserine phosphatase; putative membrane protein	No hit	No hit
LEPBI_I1654	2.02	COG2204T	Putative nitrogen assimilation regulatory protein NtrX	*LA_2400*	*LBL_1666*

[a]Mean values of two replicates are shown for three oligonucleotides on microarray with a cut-off value of 2.

[b]COG (clusters of orthologous groups) categories.

Table 2

Genes negatively regulated by σ^{54} in *L. biflexa*

Locus tag	Mean fold change [a]	COG[b]	Description of gene product	Homologues in *L. interrogans* serovar Lai	Homologues in *L. borgpetersenii* serovar Hardjo-bovis str. L550
LEPBI_p0016	7.27	-	Hypothetical protein	No hit	No hit
LEPBI_II0270	6.09	COG3329R	Putative sodium bicarbonate cotransporter; putative membrane protein	*LA_4270*	*LBL_0122*
LEPBI_pa0017	5.79	-	Heme-binding protein HmuY	*LB_192*	*LBL_4177*
LEPBI_p0015	5.73	COG4558P	ABC-type Fe3+-hydroxamate transport system, periplasmic component	No hit	No hit
LEPBI_II0269	5.70	COG0288P	Putative beta-type carbonic anhydrase	No hit	*LBL_2497*
LEPBI_p0018	4.44	COG4206H	Putative TonB-dependent outer membrane receptor	*LB_191*	*LBL_4178*
LEPBI_p0013	4.09	COG4559P	ABC-type hemin transport system, ATPase	*LA_0969*	*LBL_1280*
LEPBI_p0012	3.93	COG3720P	Hemin degradation protein HemS	No hit	No hit
LEPBI_p0014	3.93	COG0609P	ABC-type hemin transport system, permease; putative membrane protein	No hit	No hit
LEPBI_II0279	3.90	-	Hypothetical protein; putative signal peptide	No hit	No hit
LEPBI_I2760	3.32	COG1629P	Putative TonB-dependent receptor protein	*LA_2242*	*LBL_1447*
LEPBI_I2761	3.17	-	Putative signal peptide	*LA_2241*	*LBL_1445*
LEPBI_II0185	3.00	COG0499H	S-adenosyl-L-homocysteine hydrolase	*LB_106*	*LBL_4105*
LEPBI_I2678	2.99	-	Hypothetical protein	No hit	No hit
LEPBI_I2246	2.97	-	Hypothetical protein	No hit	No hit
LEPBI_I2762	2.66	COG3182S	Putative iron-regulated membrane protein; putative membrane protein	*LA_2440*	No hit
LEPBI_I2257	2.59	-	Hypothetical protein	No hit	No hit
LEPBI_I0683	2.58	-	Putative lipoprotein; putative signal peptide	No hit	No hit
LEPBI_I2431	2.54	-	Hypothetical protein	No hit	No hit
LEPBI_II0183	2.54	COG1410E	B12-dependent methionine synthase	*LB_108*	*LBL_4107*
LEPBI_I3034	2.45	COG3329R	Putative permease; putative membrane protein	*LA_4270*	*LBL_0122*
LEPBI_I2424	2.42	COG1136V	ABC-type transport system, ATP binding protein	*LA_0274, LA_2982, LA_3713*	*LBL_0572, LBL_0299, LBL_2006, LBL_2140*
LEPBI_I3255	2.32	COG0300R	SDR family dehydrogenase/reductase	*LA_2621*	*LBL_1854*
LEPBI_I2672	2.27	-	Hypothetical protein	*LA_1273*	*LBL_2068*

Locus tag	Mean fold change [a]	COG[b]	Description of gene product	Homologues in *L. interrogans* serovar Lai	Homologues in *L. borgpetersenii* serovar Hardjo-bovis str. L550
LEPBI_I0141	2.17	-	Hypothetical protein	No hit	No hit
LEPBI_I1418	2.16	COG0174E	Glutamine synthetase (glutamate--ammonia ligase)	*LA_1313*	*LBL_1991*
LEPBI_I3269	2.16	COG4974L	Putative integrase	No hit	No hit
LEPBI_I1664	2.14	COG0335J	50S ribosomal protein L19	*LA_2387*	*LBL_1656*
LEPBI_I0682	2.08	-	Hypothetical protein	No hit	No hit
LEPBI_I1089	2.07	COG4270S	Hypothetical protein	No hit	No hit
LEPBI_I3149	2.06	COG2885M	OmpA-family lipoprotein	*LA_0222*	*LBL_2925*
LEPBI_II0159	2.06	-	Hypothetical protein	No hit	No hit
LEPBI_II0050	2.05	-	Hypothetical protein	No hit	No hit
LEPBI_I1195	2.05	COG0623I	Putative enoyl-(Acyl-carrier-protein) reductase	No hit	No hit
LEPBI_I2927	2.03	-	Hypothetical protein	*LA_0268*	*LBL_0294*
LEPBI_I0937	2.01	COG1004M	UDP-glucose 6-dehydrogenase (UDP-Glc dehydrogenase; UDP-GlcDH) (UDPGDH)	*LA_1459*	*LBL_2074*

[a] Mean values of two replicates are shown for three oligonucleotides on microarray with a cut-off value of 2.

[b] COG (clusters of orthologous groups) categories.

Table 3

Primers used in this study.

Purpose	Oligo name	Sequence (5′–3′)
L. biflexa rpoN upstream amplification for knockout	rpoN upstream F	CGGAAGCTTATTTAAAAGGGGAA
	rpoN upstream R	GAAACGCGTTTTCTGTCACTCGC
L. biflexa rpoN downstream amplification for knockout	rpoN downstream F	CGGGCTAGCCGAATCGACAATTT
	rpoN downstream R	ATTCCCGGGCAAGCGTTAGTCGG
Streptomycin-resistance cassette (*flaB-aadA*) amplification	flaB-aadA F	CGGACGCGTCTTCAAGGAAGATT
	flaB-aadA R	CGGGCTAGCCTAATTGAGAGAAG
Gentamycin-resistance gene cassette (*hsp10-Gen*) amplification	Hsp10-Gen F:	CCCGGGCCATGGAATTCTCTAAAAGTATGAATTCC
	Hsp10-Gen R:	CCCGGGCAGCTGCGTAAGCCGATCTCGGCTTGAACG
rpoN ORF application for complementation	Lb-I1650-F	AAGGCATATGAAACTCGGGGCTTC
	Lb-I1650-R	GATTGGTACCTTACCCCTTGAGCGAACTG
hsp10 promoter amplification	L.in-pHsp10-F	TTATGGGCCCGAATTCCTACAATTTTAGAATTTG
	L.in-pHsp10-R	GGTACCATATGGTGATGGTGATGGTGATGAATC
GroEL overexpression in *E. coli*	pET42a–groEL F	CGCCATATGGCGAAAGATATTGAA
	pET42a–groEL R	CGCCTCGAGCATCATTCCGCCCATTCC
EBPa overexpression in *E. coli*	pGEX-4T-2-ebpA F	CGCGGATCCATGTCAGGATATGTGAAG
	pGEX-4T-2-ebpA R	ATAAGAATGCGGCCGCATAATCGATTTT
RpoN overexpression in *E. coli*	pET100 Lb rpoN F	CACCATGAAACTCGGGGCTTCACTTTCAC
		TTACCCCTTGAGCGAACTGATTCGC
rpoN qRT-PCR	lebi-rpoN-qPCR-F1	ACTGGTGATGACCCAGGACT
	lebi-rpoN-qPCR-R1	CGCCTAATTCATCGAGAAGAGGAT
flaB qRT-PCR	lebi-flaB-qPCR-F	ACACTGCGGCATTGGGATTA
	lebi-flaB-qPCR-R	AGCATGCTCCATACGGTTGT
rcfA qRT-PCR	lebi-I1011-qPCR-F	CCCTCATTGGCCAGTACGAT
	lebi-I1011-qPCR-R	ACGGATTTTGATGGCTCGGT
amtB1 qRT-PCR	lebi-I1767-qPCR-F	CCTGGGTTAAATCCAAACCAACC
	lebi-I1767-qPCR-R	GGCAAATTCCAATCCGATGGTAG
amtB2 qRT-PCR	lebi-I2408-qPCR-F	ACATACCTGCAACTAACACCCAT
	lebi-I2408-qPCR-R	AAATCAATCGCCTTCCTTCTCCT
narK qRT-PCR	lebi-I2771-qPCR-F	CTGATCTTCGGATTCTTCGTTGC
	lebi-I2771-qPCR-R	CAGGTGTATCTTGCGTTCCAAAG
ntrX qRT-PCR	lebi-I1654-qPCR-F	GCGGTCAATGCGACGAAAAA
	lebi-I1654-qPCR-R	CGGCCTGGAAAACAGCAAAC
rcfA promoter region for EMSA	I1011 promoter	TAAGCCCAATATGCTTTTGGCACGATAGTTGCATCTATTCTACTG
	I1011 promoter C	TTCAGTAGAATAGATGCAACTATCGTGCCAAAAGCATATTGGGCTT
rcfA upstream region for EMSA	I1011UP F	ATGGAATTCCAATTGCCCTCTCACGGGAA
	I1011UP R	CGTGAATTCAGACTCACCTGCTTTCACAGA