Characterization of Carbon Storage Regulator A (CsrA) homolog as a global gene regulator in *Leptospira* spp.

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บทบาทของ Carbon Storage Regulator A (CsrA) ในการควบคุมการแสดงออกของยีนของเชื้อเลปโตสไปรา

นายธีรภัทร์ โภคา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ธีรภัทร์ โภคา : บทบาทของ Carbon Storage Regulator A (CsrA) ในการควบคุมการแสดงออกของยีนของเชื้อเลปโตสไปรา. (Characterization of Carbon Storage Regulator A (CsrA) homolog as a global gene regulator in *Leptospira* spp.) อ.ที่ปรึกษาหลัก : รศ. ดร. พญ. กนิษฐา ภัทรกุล

เชื้อเลปโตสไปราถูกจำแนกเป็นสายพันธุ์ที่ไม่ก่อโรคและสายพันธุ์ก่อโรค เชื้อเลปโตสไปราสามารถปรับตัวให้ดำรงชีวิตอยู่ได้ในสภาพแวดล้อมที่หลากหลาย ดังนั้น แบคทีเรียชนิดนี้จึงต้องมีกลไกที่สามารถตอบสนองต่อการเปลี่ยนแปลงของสิ่งแวดล้อมได้อย่างรวดเร็ว อย่างไรก็ตาม ความรู้และความเข้าใจเกี่ยวกับกลไกการตอบสนองของเชื้อเลปโตสไปรายังมีอยู่อย่างจำกัด การศึกษาในแบคทีเรียชนิดอื่นพบว่าโปรตีน Carbon Storage Regulator A หรือ CsrA มีความอนุรักษ์สูง ทำหน้าที่ควบคุมการแสดงออกของยีนในระดับ post-transcription หลายยีนได้พร้อมกัน และมีบทบาทสำคัญในการตอบสนองต่อสิ่งแวดล้อมที่เปลี่ยนแปลงในแบคทีเรียหลายชนิด CsrA ควบคุมการแสดงออกได้หลายแบบ เช่น เมทาบอลิซึมของไกลโคเจน การเคลื่อนที่ของเซลล์ การสร้างไบโอฟิล์ม และยีนที่เกี่ยวข้องกับปัจจัยก่อโรค การสำรวจจากฐานข้อมูลจีโนมของเชื้อเลปโตสไปราพบว่า เชื้อเลปโตสไปราทุกสายพันธุ์มียีนที่อาจเป็น *csrA* โดยมีความคล้ายคลึงของลำดับกรดอะมิโนประมาณร้อยละ 50 กับ CsrA ของเชื้อ *E. coli* แต่ยังไม่เคยมีการศึกษา *csrA* ในเชื้อเลปโตสไปรามาก่อน วัตถุประสงค์ของงานวิจัยนี้คือเพื่อศึกษาบทบาทของ CsrA ในการควบคุมการแสดงออกของยีนต่างๆในเชื้อเลปโตสไปรา งานวิจัยนี้ได้สร้างเชื้อกลายพันธุ์ของยีน *csrA* (*csrA* mutant) *csrA* complementation และ *csrA* overexpression ในเชื้อเลปโตสไปราสายพันธุ์ไม่ก่อโรค *Leptospira biflexa* serovar Patoc และสร้าง *csrA* overexpression ในเชื้อสายพันธุ์ก่อโรค *L. interrogans* serovar Manilae ผลการศึกษาพบว่า *csrA* mutant ของ *L. biflexa* serovar Patoc เจริญเติบโตได้ช้าในสภาวะขาดแคลนอาหารเมื่อเปรียบเทียบกับสายพันธุ์ wild type แต่ *csrA* complementation ไม่สามารถทำให้การแสดงออกกลับมาเหมือน wild type ได้ นอกจากนี้ *csrA* mutant ยังเพิ่มความไวต่อ H_2O_2 และ ethidium bromide แต่ไม่พบการเปลี่ยนแปลงของการเคลื่อนที่ทั้งใน *csrA* mutant และ *csrA* overexpression ของ *L. biflexa* serovar Patoc นอกจากนั้น ยังพบว่า *flaB2* และ *flaB3* อาจจะเป็นยีนเป้าหมายของ *csrA* ในเชื้อสายพันธุ์ไม่ก่อโรคเนื่องจากมีการแสดงออกของยีนดังกล่าวมากขึ้นใน *csrA* mutant และแสดงออกลดลงเมื่อ *csrA* overexpression การศึกษาในเชื้อสายพันธุ์ก่อโรค *L. interrogans* serovar Manilae พบว่า *csrA* overexpression สูญเสียความสามารถในการเคลื่อนที่บน soft agar โดยมีการแสดงออกของยีน *flaB1 flaB2* และ *flaB4* เพิ่มขึ้น การศึกษานี้พบว่ารีคอมบิแนนท์ CsrA ของเชื้อสายพันธุ์ก่อโรคสามารถจับกับ 5' untranslated region ของ mRNA ของยีน *flaB4* ได้อย่างจำเพาะ จากผลการทดลองทั้งหมดทำให้สรุปได้ว่า *CsrA* อาจทำหน้าที่ควบคุมการแสดงออกของยีนในเชื้อเลปโตสไปรา นอกจากนี้ เป็นที่น่าสนใจว่า *CsrA* อาจมีกลไกการทำงานต่างกันในเชื้อเลปโตสไปราสายพันธุ์ไม่ก่อโรคและสายพันธุ์ก่อโรค

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2563 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5887779820 : MAJOR MEDICAL MICROBIOLOGY
KEYWORD: Carbon Storage Regulator A (CsrA), Gene regulation, Leptospira

Theerapat Phoka : Characterization of Carbon Storage Regulator A (CsrA) homolog as a global gene regulator in *Leptospira* spp.. Advisor: Assoc. Prof. KANITHA PATARAKUL, M.D. Ph.D.

Leptospira spp. consist of non-pathogenic and pathogenic species. The bacteria can survive in diverse environments and therefore need mechanisms of rapid adaptation to environmental changes. However, molecular mechanisms of stress response in *Leptospira* are not fully understood. Carbon Storage Regulator A (CsrA) is a well-characterized, conserved, post-transcriptional global regulator that plays a critical role in response to environmental changes in many bacteria. CsrA has been reported to regulate several pathways such as glycogen metabolism, motility, biofilm formation, and virulence-associated genes. Based on available genomics data of *Leptospira* spp., we found putative *csrA* homolog, approximately 50% similarity with *csrA* of *E. coli.* However, *csrA* and its function have not been characterized in *Leptospira*. To investigate the role of *csrA* as a global gene regulator in *Leptospira*, *csrA* mutant, *csrA* complemented mutant, and *csrA* overexpressing strains of *Leptospira. biflexa* serovar Patoc, as well as *csrA* overexpressing strain of *L. interrogans* serovar Manilae, were constructed in this study. In comparison to wild type, *csrA* mutant of *L. biflexa* serovar Patoc displayed poor growth under starvation condition. However, complementation of *csrA* could only partially restore the phenotype. The *csrA* mutant also increased susceptibility to stress caused by H_2O_2 and ethidium bromide. Although no alteration in motility was observed in both *csrA* mutant and *csrA* overexpressing *L. biflexa* serovar Patoc, *flaB2* and *flaB3* showed their potential target of CsrA due to significant upregulation in the *csrA* mutant but significant downregulation in *csrA* overexpressing strain. In contrast, *csrA* overexpressing strain of *L. interrogans* serovar Manilae had poor motility on soft agar and significant upregulation of *flaB1*, *flaB2*, and *flaB4*. We also showed the specific binding of recombinant CsrA of *L. interrogans* serovar Manilae to the 5' untranslated region of *flaB4* transcript. In conclusion, our data suggested the role of *CsrA* as a global regulator in *Leptospira* spp. Interestingly, the mechanisms of action and gene targets of CsrA might be different between non-pathogenic and pathogenic *Leptospira* strains.

Field of Study:	Medical Microbiology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....

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Finally, I am indebted to my parents for their open-mindedness that allows me to do anything I want to do.

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Theerapat Phoka

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csrA homolog may be involved in regulation of genes required for adaptation to environmental changes in *Leptospira* spp.

Part II: Identification of In Vivo Expressed Protein relevant to Cross-Serovar Protection against *Leptospira.*

In vivo induced (*ivi*) proteins identified by *In vivo* induced antigen technology (IVIAT) may be potential gene targets of leptospiral CsrA homolog.

Two manuscripts in this thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Medical Microbiology.

Background and Rationales

Part I:

Leptospirosis remains one of public health problems worldwide including Thailand. This disease is caused by spirochetal bacteria, *Leptospira*. The bacteria can live in different lifestyles ranging from free-living in water or soil to pathogens in the susceptible hosts [1]. In addition, non-pathogenic *Leptospira* are saprophytic in natural environments [2]. Therefore, both pathogenic and non-pathogenic leptospires should have mechanisms for alteration of gene expression to respond to environmental changes. However, gene regulation in *Leptospira* is not well understood.

Carbon Storage Regulator A (CsrA) is a conserved, well-characterized, post-transcriptional regulator. CsrA is a small RNA binding protein that binds to its target genes leading to either activation or suppression of their translation [3, 4]. This protein plays a role in the regulation of carbon metabolism, stress response, motility, biofilm formation, and virulence-associated genes in other bacteria [5]. A study of CsrA in Lyme disease spirochete, *Borrelia burgdorferi,* has reported the association of

CsrA on various phenotypes and showed the importance of this protein on virulence [6-8]. While common gene regulators presented in other bacteria are absent in leptospiral genomes [9], we found that putative *csrA* homolog is present in all available leptospiral genomes [10-12]. However, the function of *csrA* homolog and its role in gene regulation has not been characterized in *Leptospira* spp. Therefore, the mechanism of leptospiral CsrA and its gene targets should be investigated to understand the role of this protein in the biology and pathogenesis of *Leptospira*.

Part II:

Currently available bacterin vaccines have some drawbacks including serovar specificity, short-term immunity, and reactogenicity [13]. Recombinant protein vaccines against *Leptospira* have been developed to overcome the limitations of the killed vaccines. Most studies focus on the outer membrane/surface-exposed antigens that are accessible to antibodies, the main protective immunity against *Leptospira* [14, 15]. To obtain broad protection against various serovars of *Leptospira*, the identification of cross-protective antigens is required. Previous reports have shown the cross-protection provided by live attenuated LPS mutant of *L. interrogans* serovar Manilae M1352 (Live M1352). Moreover, Live M1352 had superior protection to heat-killed M1352 (HK M1352) against heterologous challenge in hamsters [16, 17]. *In vivo* induced antigen technology (IVIAT) is a powerful technique to identify *in vivo* induced (*ivi*) genes using antibodies that are pre-adsorbed with *in vitro* grown pathogens [18]. Therefore, the *ivi* proteins specifically found in Live M1352 in addition to HK M1352 may be involved in cross-protection and can be used as novel vaccine antigens against *Leptospira*.

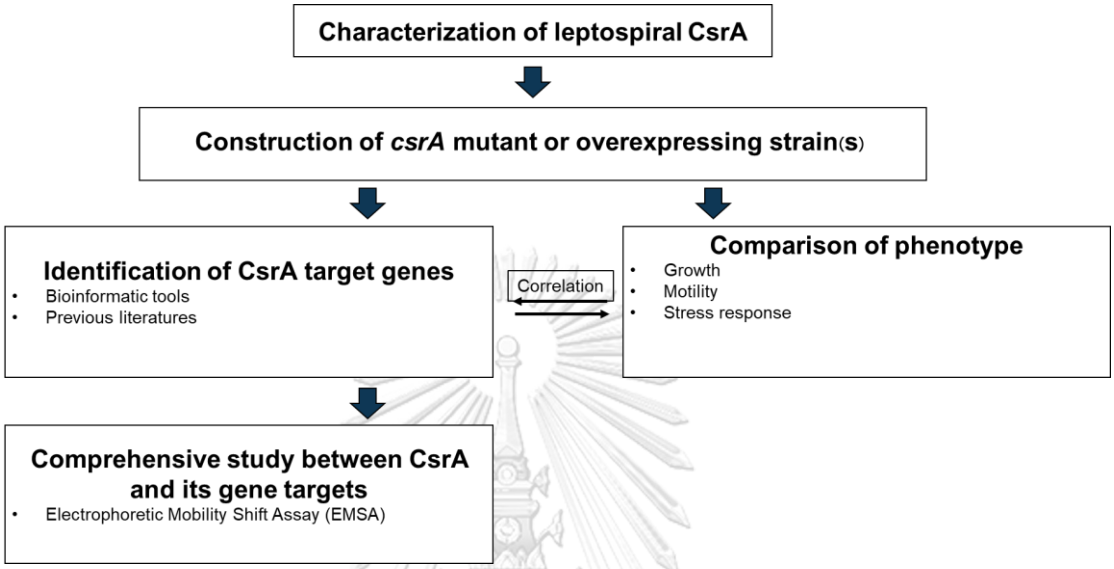
Objectives

Part I: To investigate the role of CsrA homolog as a global gene regulator in *Leptospira* and identify gene targets of CsrA.

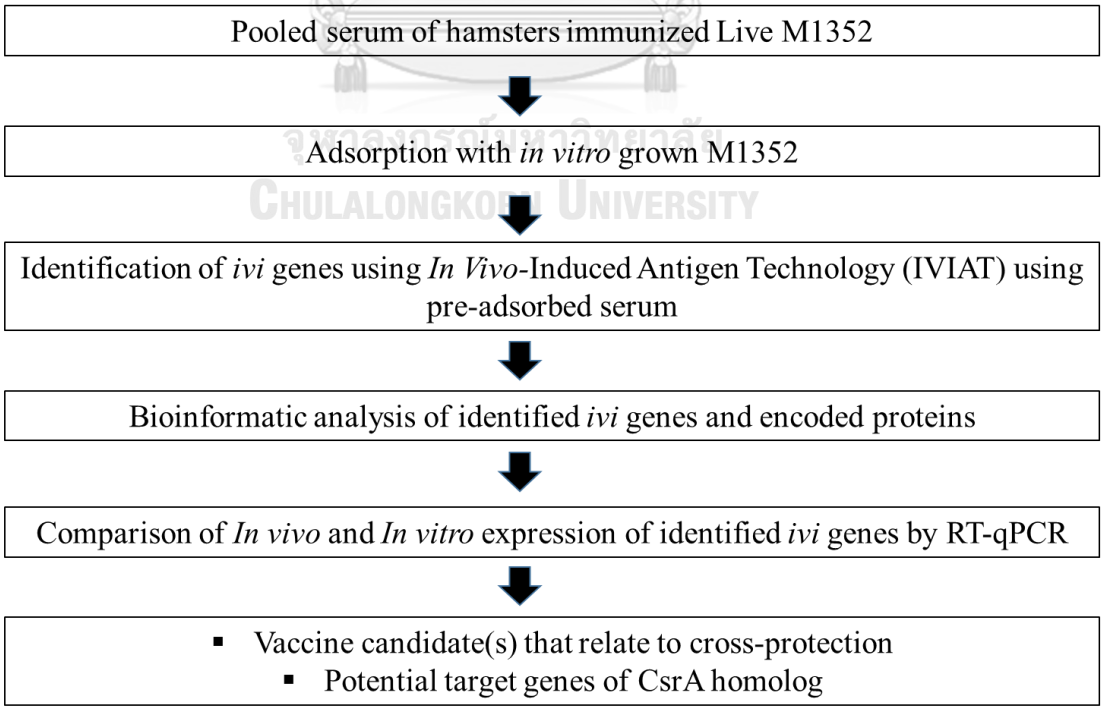
Part II: To identify *in vivo* expressed proteins of an LPS mutant of *Leptospira interrogans* serovar Manilae M1352 that are involved in cross-protection.

Conceptual framework

Part I:



Part II:



Benefits of the Study

Part I: The investigation of the role of CsrA homolog as a global gene regulator can provide better understanding about *Leptospira* including their biology, gene regulation, and pathogenicity. This study could provide a great opportunity to identify novel genes regulated by CsrA that may be essential or virulence genes crucial for response to environmental changes in *Leptospira*. The knowledge will lead us to identify new targets for development of diagnostic tests and effective vaccines.

Part II: The identification of *in vivo* expressed proteins that are involved in cross-protection could provide novel antigens for vaccine development. Furthermore, the antigens identified by IVIAT may be novel virulence factors that are important for pathogenesis of leptospirosis.

SECTION II

Part I: Characterization of Carbon Storage Regulator A (CsrA) homolog as a global gene regulator in *Leptospira* spp.

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ABSTRACT

Carbon Storage Regulator A (CsrA) is a well-characterized, conserved, post-transcriptional global regulator that plays a critical role in response to environmental changes in many bacteria. CsrA has been reported to regulate several pathways such as glycogen metabolism, motility, biofilm formation, and virulence-associated genes. The role of *csrA* in *Leptospira* spp., bacteria that are able to survive in different environmental niches and infect a wide variety of reservoir hosts, has not been characterized. To investigate the role of *csrA* as a global gene regulator in Leptospira, we generated csrA mutant and/ or csrA overexpressing strains in the saprophyte L. *biflexa* and the pathogen *L. interrogans*. Surprisingly, the *csrA* mutant in *L. biflexa* exhibited no alteration in both motility and *in vitro* growth in rich medium. The *csrA* mutant only displayed poor growth under starvation condition. Additionally, despite several attempts, we were not able to generate a *csrA* mutant in *L. interrogans*, suggesting this gene to be essential in the pathogen. We therefore decided to overexpress *csrA* in *L. interrogans*. The overexpression strain had poor motility on soft agar and significant upregulation of *flaB1, flaB2,* and *flaB4*. Interestingly, the mechanisms of action and gene targets of CsrA might be different between non-pathogenic and pathogenic *Leptospira* strains.

INTRODUCTION

Leptospira spp. are gram-negative spiral-shaped bacteria categorized into nonpathogenic and pathogenic strains. Nonpathogenic *Leptospira* are saprophytic in the natural environment. Pathogenic leptospires can cause leptospirosis in susceptible hosts including humans. Leptospirosis is an important zoonotic disease that spreads worldwide [2].

The ability to survive in a wide range of environments is crucial for both pathogenic and nonpathogenic *Leptospira* spp. Pathogenic strains have to complete the zoonotic cycle to live in distinct habitats including free living in aqueous or terrestrial environment [19], renal persistence in reservoir hosts [1], or hematogenous spreading and invasion of target organs in susceptible hosts [20]. A few mechanisms underlying this survival ability have been reported such as the role of biofilm formation in long-term persistence [21]. Furthermore, omics studies revealed the changes in gene expression profiles in response to different environmental signals, such as temperature shift [22], physiologic osmolarity [23], serum exposure [24], iron limitation [25], *in vivo* cultivation on dialysis membrane chamber [26], and biofilm formation [27]. These evidences highlight the role of global gene regulation, a crucial process employed by bacteria to deal with the changes in the environmental signals. However, due to the lack of suitable genetic manipulation, knowledge of gene regulation is not well understood in *Leptospira* spp. Some regulators have been characterized, PerR homolog of *L. interrogans* responds to oxidative stress **[**25**].** KdpE sensor has been reported as an activator of potassium transport **[** 28**].** LexA is involved in DNA repair **[**29**].** An alternative sigma factor RpoN plays a role in nitrogen metabolism and is also important for long term persistence in the water of *L. biflexa* **[** 30, 31**].** Recently, leptospiral virulence regulator (Lvr), a pathogen-specific two-component system composed of *lvrA* and *lvrB*, has been reported. Mutation of either *lvrB* alone or *lvrA* and *LvrB* affected transcriptomic changes of several hundred genes involving motility and virulence [32]. Besides these regulators, *csrA* homolog encoding putative CsrA, a well-characterized post-transcriptional global regulator, is found in all available leptospiral genomes [10-12].

Carbon Storage Regulator A (CsrA) (or its homolog RsmA) is one of the most studied RNA binding proteins in bacteria. This protein is widely conserved and is annotated in more than 1,500 bacterial species [33]. According to the first report by T. Romeo, transposon mutagenesis of *csrA* in *Escherichia coli* displayed pleiotropic phenotypes including alteration in glycogen accumulation, adhesion ability, and cell size compared with those of wild type strain [34]. Due to substantial pleiotropic effects, several omics studies have reported the effect of CsrA on global transcriptomic changes [35-38] and therefore, CsrA has been recognized as a global regulator. CsrA regulates gene expression at the post-transcriptional regulation by binding to mRNA targets and affects mRNA stability and translation [39]. This protein could negatively or positively regulate mRNA expression. For negative regulation, CsrA binds to 5' untranslated region on mRNA targets overlapping the Shine-Dalgarno sequence to prevent ribosome access resulting in translation block [4, 40, 41]. In addition, CsrA may bind to mRNA targets overlapping the start codon [42]. For positive regulation, CsrA binds to mRNA target and prevents the target from RNase cleavage [3, 43]. The consensus sequence for *E. coli* CsrA binding site identified by Systematic evolution of ligands by exponential enrichment (SELEX) experiment is 5'RUACARGGAUGU'3 which GGA motif located in hairpin loop is a critical binding site [44]. The involvement of CsrA in the regulation of various bacterial processes has been reported including carbon metabolism [45], motility [3], biofilm formation [46], quorum sensing [47], stress response [48], as well as virulence-associated traits such as iron acquisition [49], invasion [50], and type III secretion system [51].

CsrA was also found in *Borrelia burgdorferi*, a Lyme disease spirochete which is a closely related genus to *Leptospira*. The mutant decreased in the protein level of some virulence determinants and became attenuated in mice [7]. Another work reported that CsrA affected cell morphology and flagellin protein FlaB was a target of *B. burgdorferi* CsrA [6]. These results suggest a pivotal role of CsrA as a global gene regulator in a spirochete. However, its function as a gene regulator as well as gene targets have not been characterized in *Leptospira* spp.

In this study, the *csrA* mutant, complemented mutant, and *csrA* overexpressing strains were constructed in non-pathogenic strain as well as *csrA*

overexpressing stains in pathogenic *Leptospira* to determine the role of CsrA in gene regulation in *Leptospira*. We found the effects of *csrA* on *Leptospira* and one of putative CsrA targets was identified in this study.

Material and Method

Bacterial strains and growth conditions.

All *Leptospira* spp. were cultured in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) [52] medium at 30^{o}C with 100 rpm shaking or 1% agar of solid EMJH at 30^{o}C. *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae L495 were used in all experiments. All *Escherichia coli* strains were cultured in Luria-Bertani medium (LB) at 37^{o}C with 200 rpm shaking. When needed, appropriable antibiotic was added to the culture medium. All bacterial strains were listed in Table 1.

Table 1 Bacterial Strains used in this study

Strain	Antibiotic Selection	Description
WT *L. biflexa* serovar Patoc + pMaoRi	Spc 50 mg/mL	Control strain
WT *L. biflexa* serovar Patoc + *csrA*_promo_Patoc	Spc 50 mg/mL	Overexpressing strain with native *csrA* promoter
WT *L. biflexa* serovar Patoc + *csrA*_promo_Manilae	Spc 50 mg/mL	Overexpressing strain with native *csrA* promoter
WT *L. biflexa* serovar Patoc + *LocP*	Spc 50 mg/mL	Overexpressing strain with native *csrA* promoter
WT *L. biflexa* serovar Patoc + *LocM*	Spc 50 mg/mL	Overexpressing strain with native *csrA* promoter
csrA- *L. biflexa* serovar Patoc	No (for selection: Kan 100 mg/mL)	Knock out
csrA- *L. biflexa* serovar Patoc + CsrA_promo_Patoc	Spc 50 mg/mL	Complemented strain with *csrA* _native promoter
csrA- *L. biflexa* serovar	Spc 50 mg/mL	Complemented strain with

Patoc + CsrA_promo_Manilae		*csrA* _native promoter
csrA- *L. biflexa* serovar Patoc + LocP	Spc 50 mg/mL	Complemented strain with *csrA* loci containing *csrA*
csrA- *L. biflexa* serovar Patoc +LocM	Spc 50 mg/mL	Complemented strain with csrA loci excluding *csrA*
csrA- *L. biflexa* serovar Patoc + pMaori	Spc 50 mg/mL	Control strain
L. interrogans serovar Manilae WT	No	Control strain
L Manilae + *csrA*_promo_Manilae	Spc 50 mg/mL	Overexpressing strain with native *csrA* promoter
L Manilae + csrA_promo_Patoc	Spc 50 mg/mL	Overexpressing strain with native *csrA* promoter
L. interrogans serovar Manilae WT + pMaori	Spc 50 mg/mL	Control strain
E. coli DH5α	No	Strain for cloning and plasmid amplification
E. coli TOP10 thermo	No	Strain for cloning and plasmid amplification
E. coli Bl-21	No	Strain for recombinant protein production
E. coli β2163	No	Donor strain for Conjugation with *Leptospira* spp.
E. coli P1	No	Strain for plasmid amplification

Suicide plasmid construction

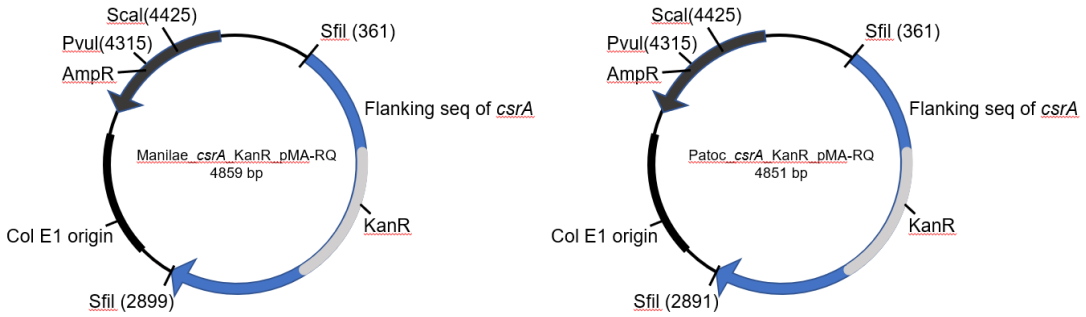


Figure 1 Map of suicide vector for *L. interrogans* serovar Manilae and *L. bilfexa* serovar Patoc which Kan^R located between flanking sequence of *csrA*.

Suicide plasmids for *L. interrogans* serovar Manilae and *L. biflexa* serovar Patoc were synthesized by replacing the csrA gene with kanamycin resistance cassette (Kan^R) using pMA-RQ (Amp^R) as a backbone vector. Kan^R with ~1100 bp upstream and downstream of csrA flanking sequences was inserted into pMA-RQ. The plasmids were transformed into TOP-10 *E. coli* to be maintained. For plasmid preparation, TOP-10 *E. coli* containing suicide vector was grown overnight at 37 ^{o}C in LB supplemented with kanamycin. Then, plasmids were extracted using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen™, Carlsbad, CA, USA). For electroporation, plasmid was treated with UV for 10s to 30s before use.

Allelic exchange mutagenesis of leptospiral *csrA* by electroporation with suicide plasmid

According to a previous study [53], briefly, *Leptospira* spp. were cultured in 36 mL of EMJH medium with 100-rpm shaking at 30^{o}C to obtain the exponential culture (OD_{420} ~ 0.1 to 0.2). Bacteria were centrifuged at 4000 xg for 20 mins, washed with equal volume of sterile water, centrifuged again and resuspended in 1 mL of sterile water. Then, 200 µL of bacteria were transferred to 1.5-mL sterile tube containing 100 nanograms of salt-free suicide plasmid. The mixture was transferred to a pre-chilled

electroporation cuvette. Plasmids were introduced into bacterial cells by electroporation using 18 kV, 25uF and 200 Ω. One mL of EMJH medium was added to the cuvette immediately after electroporation. The mixture was transferred to a 15-mL sterile tube and incubated with shaking overnight. Bacteria were plated on EMJH agar containing appropriable antibiotics then plates were incubated at 30 ^{o}C until leptospiral colony appeared, about 1 and 4 weeks for *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae, respectively. Transformant colonies were picked up and sub-cultured in EMJH medium with appropriable antibiotics for further experiment.

Construction of plasmid for complementation and overexpression.

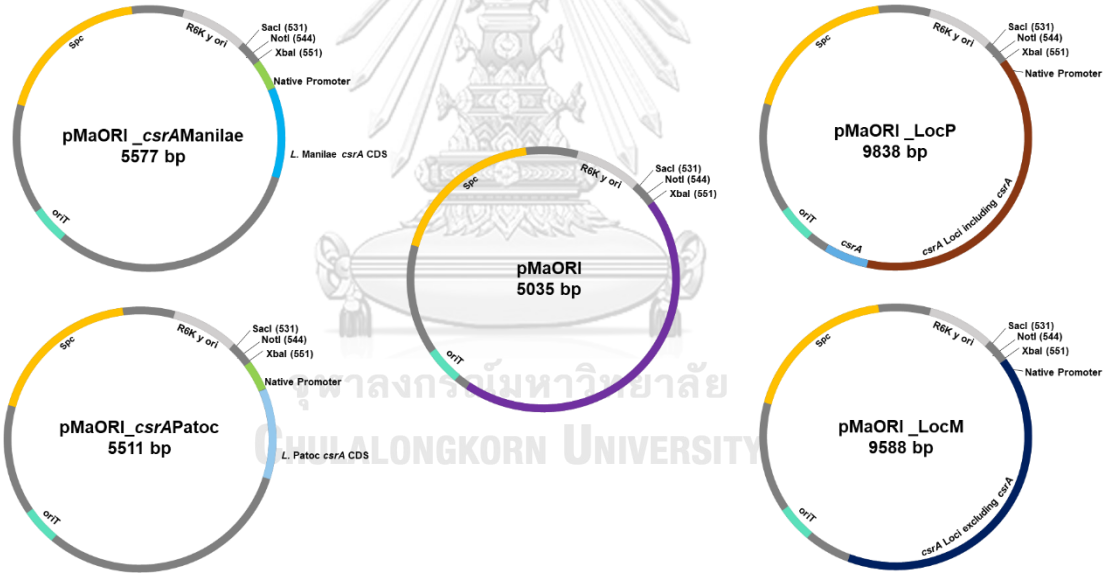


Figure 2 Map of pMaORI empty vector. pMaORI containing *csrA* of *L. interrogans* serovar Manilae and *L. biflexa* serovar Patoc with its native promoter or *csrA* locus either including *csrA* (LocP) or excluding *csrA* (LocM).

To construct plasmids for complementation and overexpression with a native promoter of *csrA*, pMA-RQ containing *csrA* ORF of *L. biflexa* serovar Patoc or *L. interrogans* serovar Manilae fusion with its native promoter was synthesis

(Invitrogen™). The plasmids were transformed into TOP-10 *E. coli* for amplification. Plasmids were digested with SacI and XbaI, ligated with SacI and XbaI digested pMaORI [54], transformed into *E. coli* P1 strain, and plated on LB agar supplemented with 100 µg/mL Ampicillin and 300 mM deoxythymidine (dT). Positive clones were confirmed by restriction enzyme digestion. pMaORI containing *csrA* and its native promoter was transformed into β2163 *E. coli*, the donor strain for conjugation, and were confirmed by restriction enzyme digestion. β2163 *E. coli* containing plasmid of interest were prepared as a DMSO stock for use in conjugation experiment.

To confirm the efficacy of overexpression, *csrA* gene locus was amplified by PFU polymerase either a locus including *csrA* defined as LocP (Locus plus *csrA*) or a locus excluding *csrA* defined as LocM (Locus minus *csrA*). PCR product of LocP (~ 4.8 Kb) and LocM (~ 4.5 Kb) was purified and incubated with Taq polymerase in order to add the 3'A overhang. PCR product containing 3'A overhang was ligated with pTOPO TA-cloning vector (Invitrogen™), transformed into TOP10 *E. coli,* plated on LB agar supplemented with 100 µg/ mL Kanamycin. Products were released from TOPO vector by XbaI digestion, ligated with XbaI digested and alkaline phosphatase (AP) treated pMaORI. pMaORI containing LocP or LocM was transformed into *E. coli* P1 strain and selected for the positive clones. The plasmid was extracted and transformed into β2163 *E. coli* as described above.

All the pMaORI constructed in this study were used for conjugation and complementation.

Conjugation Experiment

β2163 *E. coli* containing a plasmid of interest were grown in EMJH supplemented with 0.3 mM Diaminopimelic acid (DAP) at 37 °C until bacteria reached the exponential phase. β2163 *E. coli* was incubated with log-phase *Leptospira* on a membrane filter at a ratio of 1:10 and allowed all media flow through the membrane filter by vacuum pump. The membrane filter containing *E. coli* and *Leptospira* was placed on EMJH plate supplemented with 0.3 mM DAP and incubated for 16-20 hours at 30 °C. The membrane filter was put into a 15 mL tube containing 1 mL of

EMJH medium, *E. coli* and *Leptospira* were removed from the membrane filter by using 1 mL serological pipette to scratch the membrane. 15 mL tube containing *E. coli* and *Leptospira* was incubated for 1 hour at 30 ^{o}C. 200 µL of culture was plated on EMJH supplemented with 50 µg/ mL spectinomycin. Plate was incubated until leptospiral colonies were observed, approximately 5 to 7 days for *L. biflexa* serovar Patoc and 14 days for *L. interrogans* serovar Manilae.

Genomic analysis of wild type and *csrA* mutant *Leptospira*

Genomic DNA of *Leptospira* spp. was prepared from *Leptospira* grown in EMJH medium. To check the double crossing over event, Flk_L (5' TGGTGGTCGTGATTTAGGA '3) and Flk_R (5' ATCGGCTGGTTCATCGGCA '3) binding specifically to franking sequences of *csrA* were used as primers for PCR. To confirm *csrA* knockout, ORF_L (5' GCGAGGAGAAGTAACCAATCC '3) and ORF_R (5' ACTTGCCTAAATCTTCCGGTT '3) binding specifically to ORF of *csrA* were used as primers for PCR.

RNA extraction and RT-qPCR

RNA isolation was performed as described previously [55]. Briefly, *Leptospira* spp. were grown until the growth reached exponential phase (OD_{420} ~ 0.1 to 0.2) or (~ 2.5 × 10^8 cell/mL). Cells were harvested and RNA was prepared using TRIZOL reagent (Thermo Fisher Scientific) following the instructions. RNA pellets were resuspended in UltraPure DNase/RNase Free Distilled Water (Thermo Fisher Scientific, Vantaa, Finland). 500 ng of RNA was used for cDNA synthesis using iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (BIO-RAD). Quantitative reverse transcription-PCR (RT-qPCR) was performed using SYBR® Green Master Mix (BIO-RAD). The results were expressed as the normalized difference of the threshold cycle (ΔΔCT), using *cysK* and *lipL32* as a reference gene for *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae, respectively. All primers were listed in **Table 2**.

Table 2 Primers used in this study

PRIMER	Sequence	Specific target
csrAPatocNdeI	AACATATGTTAGTTTTAGCGAGG	*csrA* cloning for overexpression strain
csrAPatocXbaI	TTTTCTAGACTCCAGAGAATTCTGAG	*csrA* cloning for overexpression strain
ORF_L	GCGAGGAGAAGTAACCAATCC	inside *csrA* ORF
ORF_R	ACTTGCCTAAATCTTCCGGTT	inside *csrA* ORF
Flk_L	TGGTGGTCGTGATTTAGGA	franking seq of *csrA* in Patoc
Flk_R	ATCGGCTGGTTCATCGGCA	franking seq of *csrA* in Patoc
CsrAManilaeNdeI	AACATATGCTGGTTTTAGCGAGAAG	*csrA* cloning for overexpression strain
CsrAManilaeXbaI	TTTCTAGACAAACTACAAGAACTTCCAG	*csrA* cloning for overexpression strain
LEPBIa1589a	CTTCCGCCGTTTGAATGAGG	*flaB1*
LEPBIa1589b	AGTCTAACGACGCGAACCTG	*flaB1*
Locp1	TCTAGAAAGATGAATTTACTCCC	REVERSE PRIMER FOR *csrA* OPERON INCLUDING *csrA*
Loc2	AATCTAGATAACCTATTGATTGG	FORWAED PRIMER *csrA* OPERON
Locm1	AATCTAGATCGCCAATCATAATG	REVERSE PRIMER FOR *csrA* OPERON EXCLUDING *csrA*
csrAMaorf1	GTTTTAGCGAGAAGAACGAACGA	INSIDE *csrA* ORF
csrAMaorf2	TTATTTTGGTTTCCGCGGCTTT	INSIDE *csrA* ORF
flaB1 A	TGCTGCCCTTACGAAGATCA	*flaB1 L. interrogans* serovar Manilae qPCR
flaB1 B	TATTTGCCTGCGCCAACATT	*flaB1 L. interrogans* serovar Manilae qPCR

flaB2 A	GCCAATGACTCTATCGGGGT	*flaB2 L. interrogans* serovar Manilae qPCR
flaB2 B	CATTGCTGTTCCCGACTGAA	*flaB2 L. interrogans* serovar Manilae qPCR
flaB3 A	AACAAGCCCTCTCTTCTCCC	*flaB3 L. interrogans* serovar Manilae qPCR
flaB3 B	CGGCGACCATGTTTACGTAG	*flaB3 L. interrogans* serovar Manilae qPCR
flaB4 A	TGAAACGATGAACACTGCGG	*flaB4 L. interrogans* serovar Manilae qPCR
flaB4 B	ATGAGACCTTTTGCAGCGTG	*flaB4 L. interrogans* serovar Manilae qPCR
cysKa	TCATCGTGGAGCCAACTTCT	Normalization for *L. biflexa* serovar Patoc
cysKb	TGCCGCTACCATTTCTTGTG	Normalization for *L. biflexa* serovar Patoc
LEPBIa1872F	CACCAGTCGAGACATGGACA	*flaB2 L. biflexa* serovar Patoc qPCR
LEPBIa1872R	CTGGGTATTTCGCTCCGCTT	*flaB2 L. biflexa* serovar Patoc qPCR
LEPBIa2132F	TCAGGCGGAAAGGAATACGG	*flaB3 L. biflexa* serovar Patoc qPCR
LEPBIa2132R	TCCACCAGCGCAGATACTTC	*flaB3 L. biflexa* serovar Patoc qPCR
LEPBIa2133F	TTAGCCGCGATCAACTCACA	*flaB4 L. biflexa* serovar Patoc q PCR
LEPBIa2133R	CAGGCTCATACCGTCTTCGG	*flaB4 L. biflexa* serovar Patoc q PCR
1589F	AGTCTAACGACGCGAACCTG	*flaB1 L. biflexa* serovar Patoc q PCR

1589R	CTTCCGCCGTTTGAATGAGG	*flaB1 L. biflexa* serovar Patoc q PCR
lIPL32 A	AAGCATTACCGCTTGTGGTG	Normalization for *L.* Manilae
lIPL32 B	GAACTCCCATTTCAGCGATT	Normalization for *L.* Manilae
Patoc_csrA_XhoI	CTCGAGTAGTGTTAGTTTTAGC	*L. biflexa* serovar Patoc *csrA* recombinant protein production
Patoc_csrA_HindIII	AAGCTTGAAAGAAAGATCAAG	*L. biflexa* serovar Patoc *csrA* recombinant protein production
Manilae_csrA_BamHI	GGATCCTAGTGCTGGTTTTAGC	*L. interrogans* serovar Manilae *csrA* recombinant protein production
Manilae_csrA_HindIII	GGCAAGCTTTTATTCTTTTTTTCC	*L. interrogans* serovar Manilae *csrA* recombinant protein production

Measurement of growth rate

Bacteria were grown in EMJH medium until the culture reached exponential phase (OD_{420} ~ 0.1 to 0.2 or 2.5×10^8 cell/mL for *L. biflexa* serovar Patoc). Then 2×10^6 cells of bacteria were added into 10 mL of EMJH medium. The cultures were incubated at 30 ^{o}C or at 30 ^{o}C with 100 rpm shaking or at 37 ^{o}C with 200 rpm shaking. One ml of each culture was taken for OD_{420} measurement every 24 hours. In order to perform a growth curve in diluted EMJH, *Leptospira* cells were prepared as described above before inoculation into 1/5 or 1/10 EMJH medium diluted in water.

Stress response by Alamar blue assay

2×10^5 cells of exponential-phase *Leptospira* in 100 µL of EMJH medium were prepared and added into a 96-well plate containing 100 µL of 2-flow serial dilution of each chemical substance as follows: 1M of NaCl, 2 mg/mL of Ethidium bromide (EtBr), 10^{-4} dilution of 10% sodium dodecyl sulfate (SDS), and 8 mM of H_2O_2. All chemical substances were diluted in EMJH medium. The plate was incubated at 30 ^{o}C for 48 hours and then 20 µL of Alamar blue (Thermo Fisher Scientific) was added into each well. After 48-hour incubation, the growth of bacteria was checked by the change of Alamar blue color. Bacteria in 200 µL EMJH alone was used as a control.

Measurement of motility, cell length, and velocity

The motility of exponential-phase *Leptospira* was checked by dark-field microscopy. The motility was also checked in 0.6% semisolid EMJH medium. Exponential-phase *Leptospira* were diluted in EMJH to obtain OD_{420} = 0.1 as a starter culture. The starter culture was inoculated either 2 µL or 5 µL on semisolid EMJH plates. The plates were incubated for 1 weeks for *L. biflexa* serovar Patoc and 2 weeks for *L. interrogans* serovar Manilae. For cell length and velocity measurement, late exponential phase of *Leptospira* spp. (OD_{420} ~ 0.5) were diluted in EMJH to obtain an appropriate number of cells per field under a dark-field microscope. Cell length and velocity of bacteria were measured by cellSens software (OLYMPUS).

***In silico* prediction for possible leptospiral CsrA gene target**

In order to identify possible leptospiral CsrA gene target(s), Virtual Footprint/ PRODORIC [56] was used as a database. 5' RUACARGGATGT' 3 a known consensus sequences of CsrA in *E. coli* and *P. aeruginosa* was searched against *L. interrogans* and *L. biflexa* genomes. Due to limited leptospiral genome available on the Virtual Footprint/ PRODORIC platform, we performed searching against *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni, strain Fiocruz L-130 and *L. biflexa* serovar Patoc strain Patoc-1/Ames chromosome. To obtain most likely specific target(s), search option was restricted to intergenic regions which contained GGA, had the same orientation to the gene target, and located between 1

and 100 nucleotides of the start codon. The most possible specific RNA sequences were performed using BLAST [57] and predicted for their secondary structure using MFOLD [58].

Recombinant protein production

PCR products of full sequences of *csrA* amplified from *L. biflexa* serovar Patoc or *L. interrogans* serovar Manilae genomic DNA were cloned into pRSET-C (Invitrogen™). The recombinant plasmids were transformed into *E. coli* DH5α and verified by DNA sequencing (Macrogen., South Korea). Recombinant proteins with N-terminus 6x His tag was induced in *E. coli* BL21 (DE3) pLysS by 1 mM IPTG at 37 ^{o}C for 4 hours. Bacteria were pelleted, resuspended in phosphate buffered saline (PBS) pH 7.4, and disrupted using a high-pressure homogenizer (Constant System Ltd., Northants, UK). The soluble fraction was isolated by centrifugation at 15000 xg at 4 ^{o}C for 30 mins. Protein samples were purified using Ni Sepharose columns (GE Healthcare, Buckinghamshire, UK) and dialysis with PBS pH 7.4. To check the purity of purified recombinant proteins, the proteins were subjected to 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with blocking buffer (PBS pH 7.4 plus 0.05% tween), incubated 1 hour with anti-His tag monoclonal antibody (1:5000; KPL, MD, USA), incubated 1 hour with HRP-conjugated goat anti-mouse IgG (secondary antibody). Amersham ECL (GE Healthcare), HRP substrate, was added and incubated for 1 min. The membrane was exposed to CCD camera (Bio-Rad) for chemiluminescent signal reading.

Electrophoretic Mobility Shift Assay (EMSA)

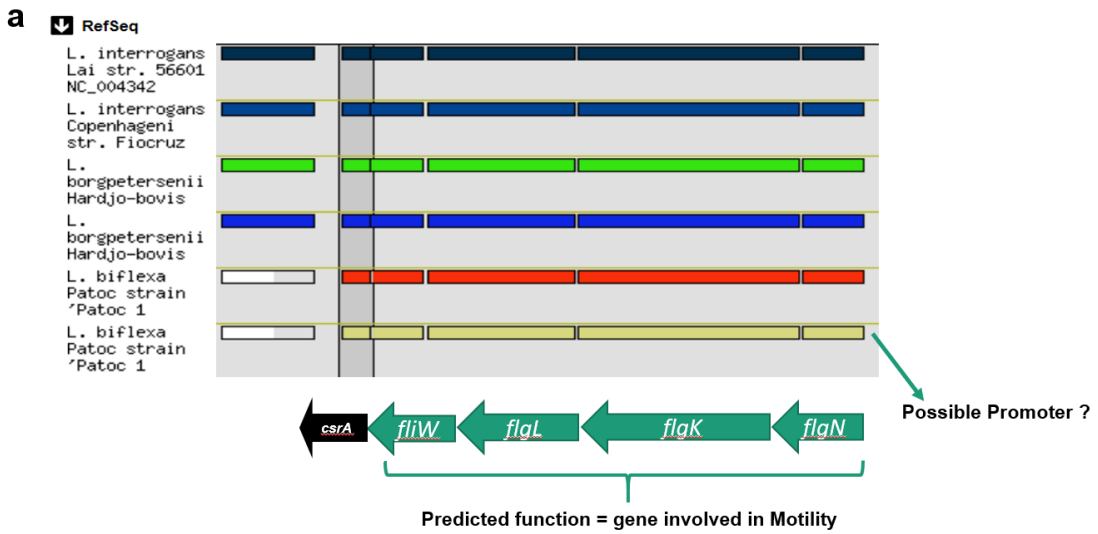
EMSA was performed according to the manual of LightShift™ EMSA Chemiluminescent RNA Kit (Thermo Fisher Scientific) using all reagents included with the kit. Briefly, the binding reaction was prepared, each binding reaction consisted of 1X binding buffer (10mM HEPES pH 7.3, 20 mM KCL, 1 mM MgCl2, and 1 mM DTT) 2 nM of biotinylated-RNA, 7.5% glycerol, 10 mM DTT, 0.2 µg/µL Yeast tRNA, and 0.25 µM to 16 µM of rCsrA in a total volume of 20 µL. The binding reaction was incubated

at 37^{o}C for 30 mins. After incubation, loading buffer was added into each reaction, separated on 10% native PAGE for 1 hour at 100 V. The reaction was transferred onto a nylon membrane, crosslinked with UV for 1 min, blocked 15 mins with a blocking buffer, washed once with washing buffer. 1: 300 stabilized Streptavidin-HRP in a blocking buffer was added and incubated for 15 mins. Membrane was washed 5 times, incubated with a substrate equilibration buffer for 5 mins. Amersham ECL was added and incubated for 5 mins. The membrane was exposed to CCD camera (Bio-Rad) for chemiluminescent signal reading. For competitive EMSA, the binding reaction was prepared as described above except CsrA concentration was fixed at 8 μM and unlabeled RNA was added at concentrations ranging from 0.016 μM to 10 μM.

RESULTS

Feature of leptospiral *csrA* and *csrA* locus

a



b

Strain/ % Similarity	*B. burgdorferi*	*T. pallidum*	*E. coli*	*C. jejuni*	*B. subtilis*	*L.* Patoc	*L.* Manilae
L. Patoc	58.33	58.33	50	50.59	54.76	100	88.1
L. Manilae	62.65	62.03	53.16	56.79	60.76	88.1	100

c

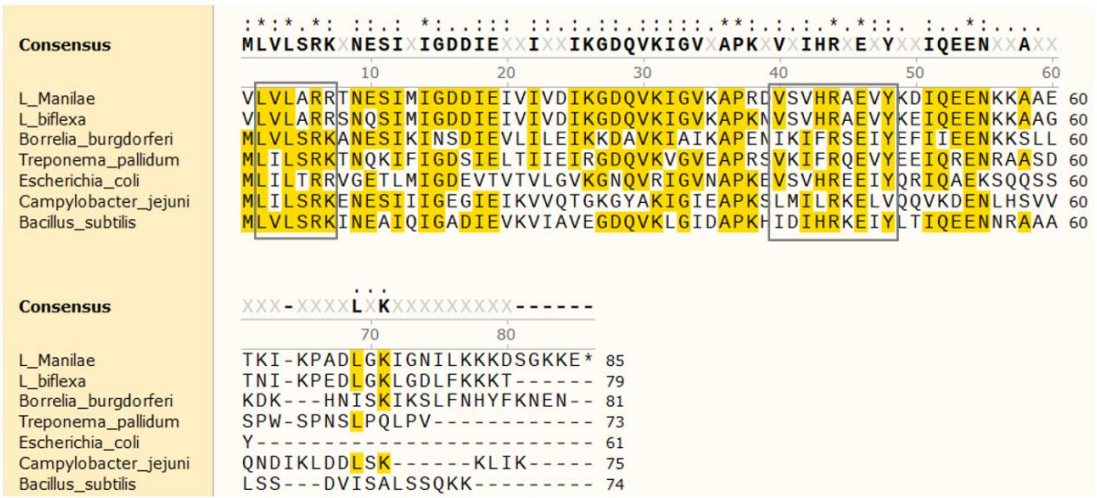


Figure 3 csrA operon in *Leptospira* spp.

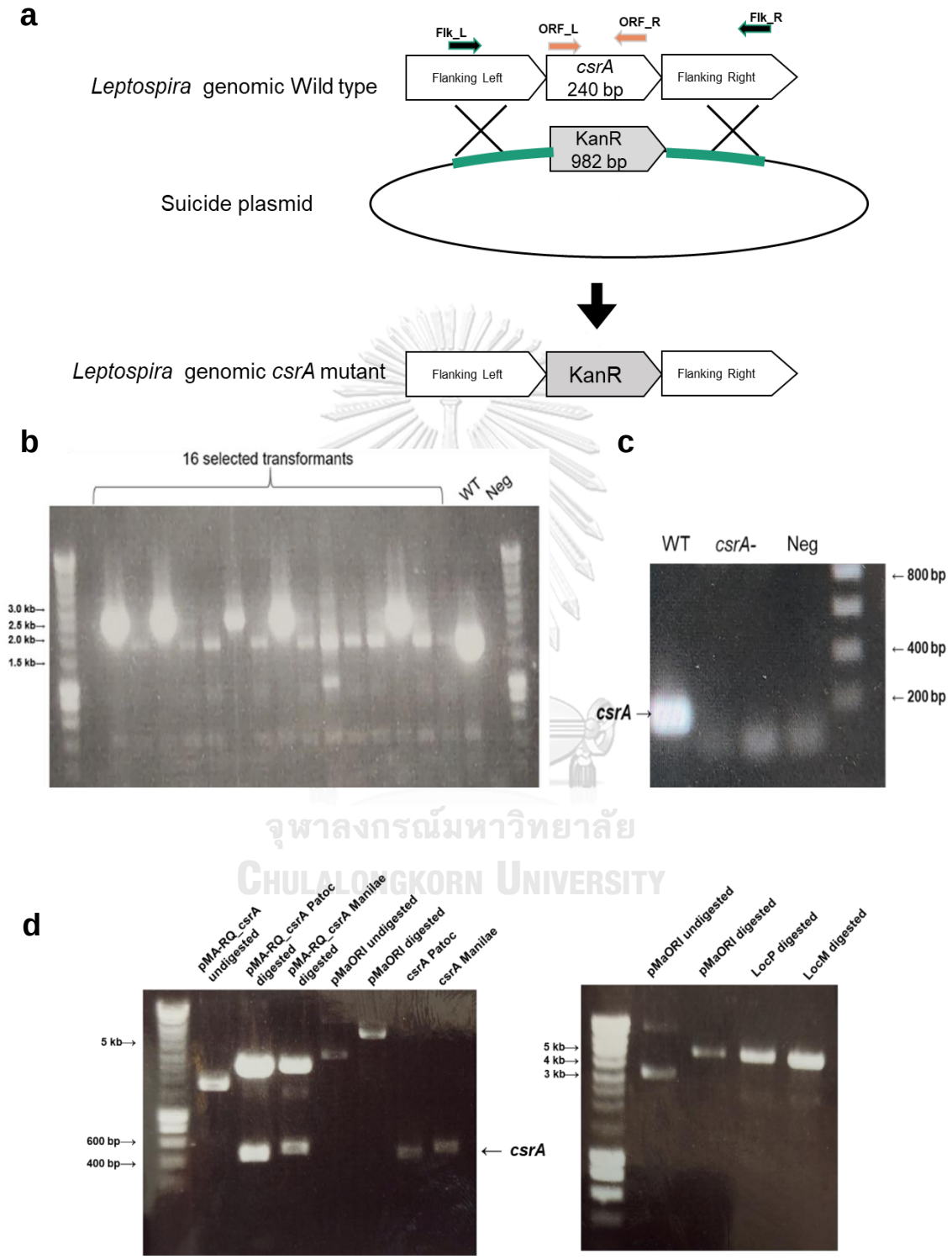
(**a**) Syntenic loci encoding *csrA* in *Leptospira* spp. The arrangement of genes in csrA operon in *L. interrogans* serovar Lai, *L. interrogans* serovar Copenhageni, *L. interrogans* serovar Hardjo-bovis and *L. biflexa* serovar Patoc were shown, information was obtained from MicroScope platform: v3.14.2 [59],https://mage.genoscope.cns.fr/microscope/mage/viewer.php?submit=MoveTo&label=25032433_

(**b**) Amino acid similarity among CsrA of *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae to CsrA of other bacteria was calculated using Sequence Manipulation Suite [60], http://www.bioinfo.ba/tools/sms2/ident_sim.html.

(**c**) Alignment of amino acid sequences of CsrA in *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae strains used in this study were performed with CsrA from other bacteria. (*) represents conserved amino acid and two black boxes indicate conserved residues that are important for RNA binding in *E. coli*.

The gene locus containing *csrA* of *L. interrogans* serovar Lai, *L. interrogans* serovar Copenhageni, *L. interrogans* serovar Hardjo-bovis and *L. biflexa* serovar Patoc is shown (**Fig.3a**). It was observed that *csrA* is most likely to be a part of an operon. Furthermore, the operon containing *csrA* is synteny in *Leptospira*, composed of the same set of genes inside the operon in the same direction. This operon consists of 5 consecutive genes that are *flgN*, *flgK*, *flgL*, *fliW* and *csrA*. Predicted function for *flgN*, *flgK*, *flgL*, *fliW* are genes involved in motility. There is a 200-bp intergenic region locating upstream of *flgN*, the first gene of the operon. Amino acid similarity between *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae is 88.10 %, while CsrA of these 2 leptospiral strains is about 50-60% similar to that of other bacteria (**Fig.3b**). Amino acid alignment of CsrA showed that leptospiral CsrA consists of 85 and 79 amino acid residues in *L. interrogans* serovar Manilae and *L. biflexa* serovar Patoc, respectively (**Fig.3c**), which was slightly longer than CsrA of other bacteria. It was also found that leptospiral CsrA has high similarity in the 2 conserved domains (black box) that are important for RNA binding in *E. coli* [61].

Allelic exchange mutagenesis and complementation of *csrA*



e

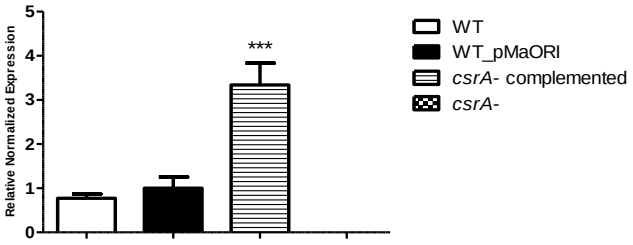


Figure 4 Allelic exchange of *csrA* in *L. biflexa* serovar Patoc.

(**a**) To construct *csrA* mutant strain, *L. biflexa* serovar Patoc was electroporated with a suicide vector. *csrA* was replaced with Kan^R by double crossing over event resulting in *csrA* mutation. (**b**) and (**c**) PCR confirmation of *csrA* mutant strain in *L. biflexa* serovar Patoc. (**b**) Genomic DNA of wild type and 16 selected transformants was prepared and amplified by PCR using primers specific to franking sequences of *csrA* (Flk-L and Flk-R). (**c**) To confirm the absence of *csrA*, genomic DNA from 2 transformants which positive for double crossing over event was amplified by PCR using primers specific to the coding sequence of *csrA* (ORF-L and ORF-R). (**d**) Plasmids construction for complementation and overexpression. Agarose gel electrophoresis gel 1, Lane 1: 1kb smart DNA ladder, lane 2: pMA-RQ containing leptospiral *csrA* fragment, lane 3: *csrA* of *L. biflexa* serovar Patoc released from pMA-RQ by restriction enzyme digestion, lane 4: *csrA* of *L. interrogans* serovar Manilae released from pMA-RQ by restriction enzyme digestion, lane 5: pMaORI undigested vector, lane 6: digested pMaORI, lane 7: purified released fragments of *csrA* from *L. biflexa* serovar Patoc, lane 8 ; purified released fragments of *csrA* from *L. interrogans* serovar Manilae. Agarose gel electrophoresis gel 2, Lane 1: 1kb smart DNA ladder, lane 2: pMaORI undigested vector, lane 3, digested pMaORI vector, lane 4: purified digested fragments of LocP, lane 5: purified digested of LocM. (**e**) To confirm complementation of *csrA*, RNA was extracted from each *Leptospira* strain and then used for RT-qPCR. Results obtained from 3 independent cultures were presented as relative fold changes using *cysK* gene for normalization.

In this study, suicide plasmids were treated with UV three times for 10 s, 20 s, and 30 s. After electroporation and plating, only transformant colonies of *L. biflexa* serovar Patoc were obtained. However, we were unable to obtain a transformant colony of *L. interrogans* serovar Manilae after electroporation was performed at least 5 times. Sixteen colonies on EMJH plate supplemented with 50 µg/mL of Kanamycin were randomly selected to inoculate into EMJH broth. After the culture reached the exponential phase, genomic DNA was isolated and used for PCR to confirm the allelic exchange of *csrA* by Kan^R. As shown in **Fig.4a**, two primer sets were used for PCR, Flk primers bind to the franking sequences of *csrA* whereas ORF primers bind to inside coding sequences of *csrA*. In comparison to 1.86 kb PCR product of wild type (WT), 5 of 16 selected transformants produced a ~2.6 kb PCR product, the expected size if *csrA* was successfully replaced with Kan^R by double crossing over event (**Fig.4b**). To ensure that *csrA* was absent in the transformants, genomic DNA of 2 transformants that gave a ~2.6 kb PCR product, was used for PCR amplification with primers specific to *csrA* coding region. While the WT produced the expected size of 199-bp PCR product, approximately 1-kb PCR products were obtained from 2 transformants. The unpredicted 1-kpb products were most likely the result of non-specific amplification occurred when primers bind to Kan^R that replaced *csrA* ORF in those 2 transformants but was not present in WT genome (**Fig.4c**).

In order to complement the *csrA* mutant, pMa-RQ containing *csrA* fusion with its native promoter, ~240 bp upstream of the operon, were synthesized. The fragments were released by restriction enzyme and ligated with pMaORI. The constructed pMaORI were used for conjugation. This study also complemented the *csrA* mutant with *csrA* from *L. interrogans* serovar Manilae, heterologous expression as well as *csrA* locus of *L. biflexa* serovar Patoc either containing (LocP) or excluding *csrA* (LocM) (**Fig.4d**). All plasmid constructs were shown in (**Fig.2**). To confirm the success of complementation, RT-qPCR was performed to detect the expression of *csrA* in the complemented strain. Because pMaORI vector containing spectinomycin as a selection marker was used for complementation and overexpression in this study, WT *Leptospira* containing pMaORI empty vector (WT_pMaORI) was used as a control strain in all experiments to eliminate the effect of the pMaORI vector on

gene expression and phenotype. The relative fold change of *csrA* in the complemented strain was significantly higher, 4.33-fold, than WT_pMaORI (**Fig 4e**). RT-qPCR was unable to detect the expression of *csrA* in *csrA* mutant, indicating successful allelic exchange of *csrA* in *L. biflexa* serovar Patoc. In addition, *csrA* expression level of WT was slightly higher than but not significantly different from that of WT_pMaORI, suggesting the effect of pMaORI vector on gene expression.

Construction of *csrA* overexpressing strain in *Leptospira* spp.

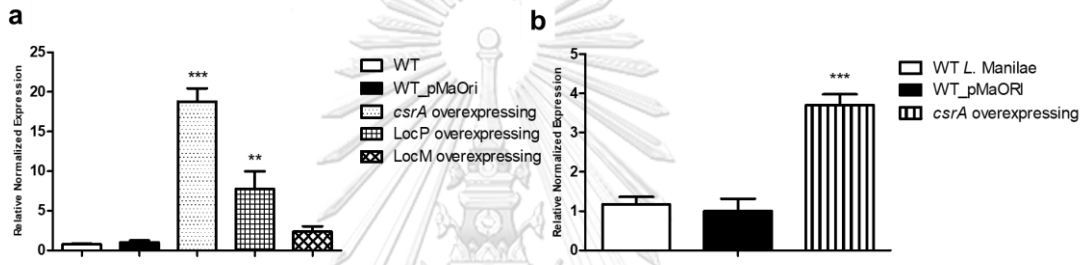


Figure 5 Overexpression of *csrA* in *Leptospira* spp.
Overexpression of *csrA* in *L. biflexa* serovar Patoc (**a**) and *L. interrogans* serovar Manilae (**b**). WT Leptospira was conjugated with *E. coli* containing pMaORI and gene of interests. Spectinomycin resistant colonies were selected, RT-qPCR was performed to confirm expression level of csrA in *L. biflexa* serovar Patoc (**a**) and *L. interrogans* serovar Manilae (**b**) *cysK* and *lipL32* were used as a normalization *for L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae, respectively.

pMaORI containing gene or locus of interest was constructed (**Fig.2**) and plasmids were transformed into *E. coli* β2163 conjugation donor. WT *Leptospira* spp. were conjugated with *E. coli* β2163 containing the plasmid of interest before spectinomycin resistance colonies were selected and gene expression was determined by RT-qPCR. In *L. biflexa* serovar Patoc, relative expression level of *csrA* in *csrA* overexpressing strain and LocP overexpressing strain were significantly 18.77-fold and 7.74-fold higher than WT_pMaORI, respectively (**Fig.5a**). The relative

expression level of *csrA* in LocM overexpressing strain and WT_pMaORI was 2.35-fold higher but not significant, suggesting the effect of other gene in the operon on *csrA* expression. LocP and LocM overexpressing strains were not examined further because they contained more than 1 gene in the plasmid (**Fig.2**) that might affect gene expression profile and phenotype. In *L. interrogans* serovar Manilae, relative expression level of *csrA* in *csrA* overexpressing strain was significantly 3.70-fold higher than WT_pMaORI (**Fig.5b**).

csrA was not essential for growth in _Leptospira_ spp.

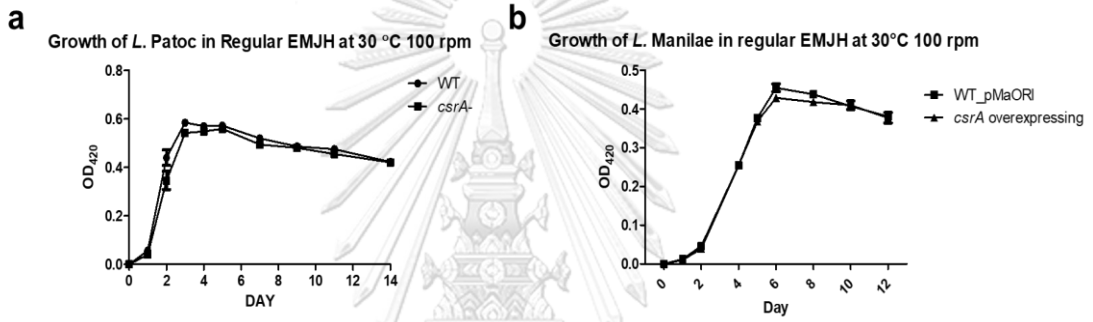


Figure 6 Growth curve of *Leptospira spp.* in regular EMJH.
2 × 106 cells of bacteria were grown in 10 mL of regular EMJH medium under 30 ^{o}C with 100 rpm shaking. OD_{420} measurement for growth was measured every 24 hours

To determine if *csrA* can affect the growth of *Leptospira* spp., the growth curve of *csrA* mutant *L. biflexa* serovar Patoc, as well as *csrA* overexpressing strain *L. interrogans* serovar Manilae, was performed in EMJH medium at 30 ^{o}C with 100 rpm shaking. We observed a similar growth curve between WT and *csrA* mutant *L. biflexa* serovar Patoc (**Fig.6a**) similar to *csrA* overexpressing *L. interrogans* serovar Manilae (**Fig. 6b**). In addition, the growth curve of *csrA* overexpressing *L. biflexa* serovar Patoc was not different from WT_pMaORI, suggesting that *csrA* is not required for the growth of *Leptospira* spp. *in vitro*.

csrA mutant showed poor growth under starvation

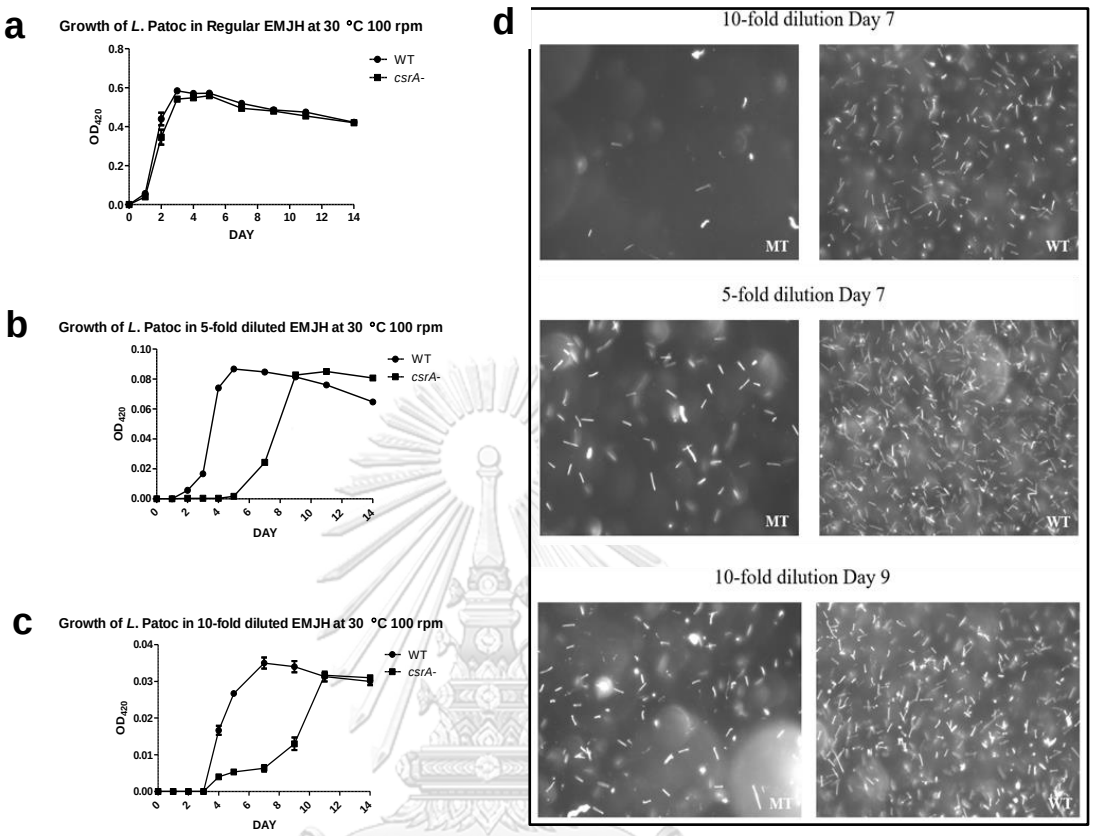


Figure 7 Growth curve of the wild type and the *csrA* mutant *L. biflexa* serovar Patoc under starvation.

2×10^6 cells of bacteria were grown in 10 mL of regular EMJH medium (***a***), 5-fold (**b**) or 10-fold diluted EMJH medium (**c**) in water under 30 ^{o}C with 100 rpm shaking. OD_{420} measurement for growth was performed every 48 hours and visualized under dark-field microscopy.

To examine if *csrA* is related to starvation, a growth curve under starvation condition was performed using either 5-fold or 10-fold EMJH medium as a stress and regular EMJH medium as a control condition. We found that *csrA* mutant *L. biflexa* serovar Patoc grew slower in both 5-fold and 10-fold diluted EMJH than wild type strain (**Fig.7b and c**). There was no difference in *csrA* overexpressing strains in both *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae, suggesting that the absence of *csrA* affects growth under starvation of *L. biflexa* serovar Patoc. For 5-fold EMJH medium, the mutant reached the exponential phase at day 7 and reached the stationary phase at day 11, while the wild type took 3 days to reach the exponential phase and 7 days to reach the stationary phase (**Fig.7b**). For 10-fold diluted EMJH, growth of the mutant was observed since day 4 but they grew slowly and reached the exponential phase after day 7 and then reached the stationary phase at day 10. Wild type took 4 days to reach the exponential phase and 7 days to reach the stationary phase (**Fig.7b**). There was no difference in the stationary phase of these 2 strains in any treatment.

Complementation partially restored growth under starvation

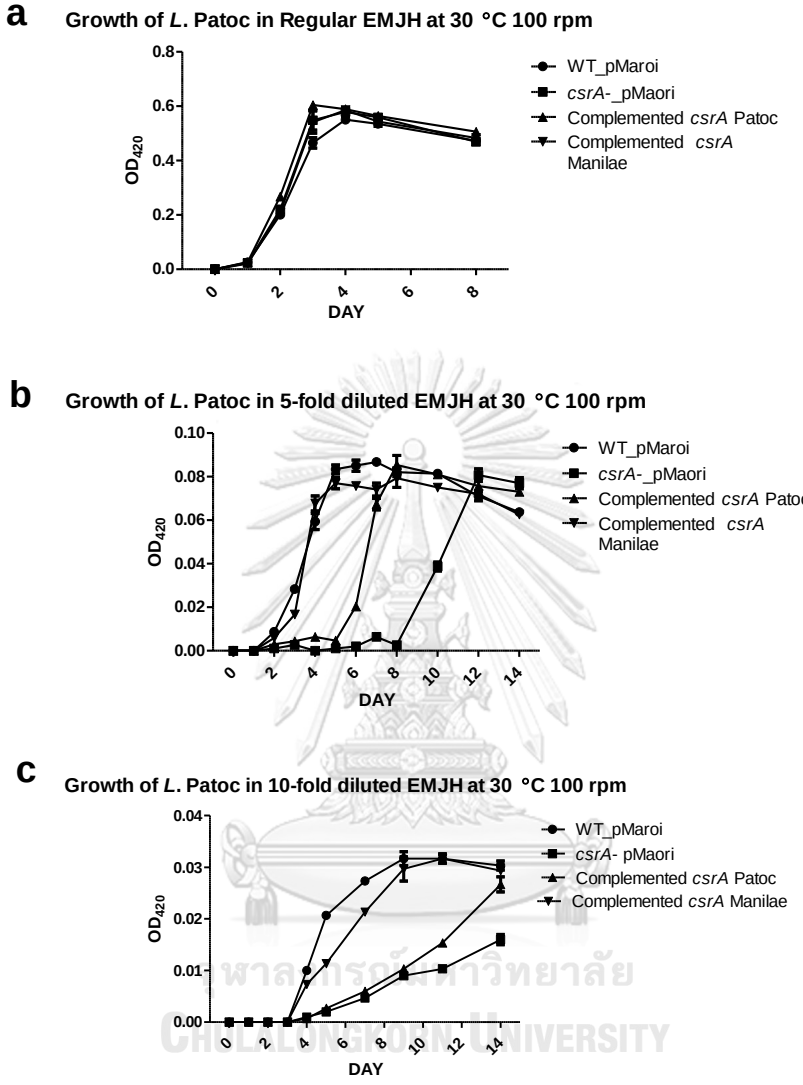


Figure 8 Growth curve of the wild type containing empty pMaORI (WT_pMaORI), *csrA* mutant containing empty pMaORI (*csrA*- pMaORI), csrA of *L. biflexa* serovar Patoc complemented strain, and *csrA* of *L. interrogans* serovar Manilae complemented strain under starvation.

2×10^6 cells of bacteria were grown in 10 mL of regular EMJH medium (***a***), 5-fold (**b**) or 10-fold (**c**) diluted EMJH medium in water under 30 ^{o}C with 100 rpm shaking. OD_{420} measurement for growth was performed every 24 h.

To confirm poor growth under starvation in *csrA* mutant *L. biflexa* serovar Patoc, a growth curve of *csrA* complemented strains either *csrA* of *L. biflexa* serovar Patoc or *csrA* of *L. interrogans* serovar Manilae as heterologous expression, was performed. We found that complemented with *csrA* from *L. biflexa* serovar Patoc could partially restore the growth under starvation condition in both 5-fold or 10-fold diluted EMJH. Surprisingly, heterologous complementation with *csrA* from *L. interrogans* serovar Manilae could fully restore the growth by showing an almost similar growth curve compared with WT_pMaORI (**Fig.8b and c**). For 5-fold dilution (**Fig.8b)**, WT_pMaORI and complemented *csrA* of *L. interrogans* serovar Manilae reached the exponential phase at day 3 and reached the stationary phase at day 5, whereas complemented *csrA* of *L. biflexa* serovar Patoc reached the exponential phase at day 6 and reached the stationary phase at day 7. While the *csrA* mutant containing empty pMaORI (*csrA*-pMaORI) took 9 and 12 days to reach the exponential phase and stationary phase, respectively. For 10-fold (**Fig. 8c)** dilution, WT_pMaORI and complemented *csrA* of *L. interrogans* serovar Manilae reached the exponential phase at day 4 and reached the stationary phase at day 9. The growth of complemented *csrA* of *L. biflexa* serovar Patoc and *csrA*-pMaORI was observed at day 5. However, while the complemented strain reached the stationary phase at day 14, *csrA*-pMaORI did not reach the stationary phase.

Mutation of *csrA* increased sensitivity to H_2O_2 and Ethidium bromide

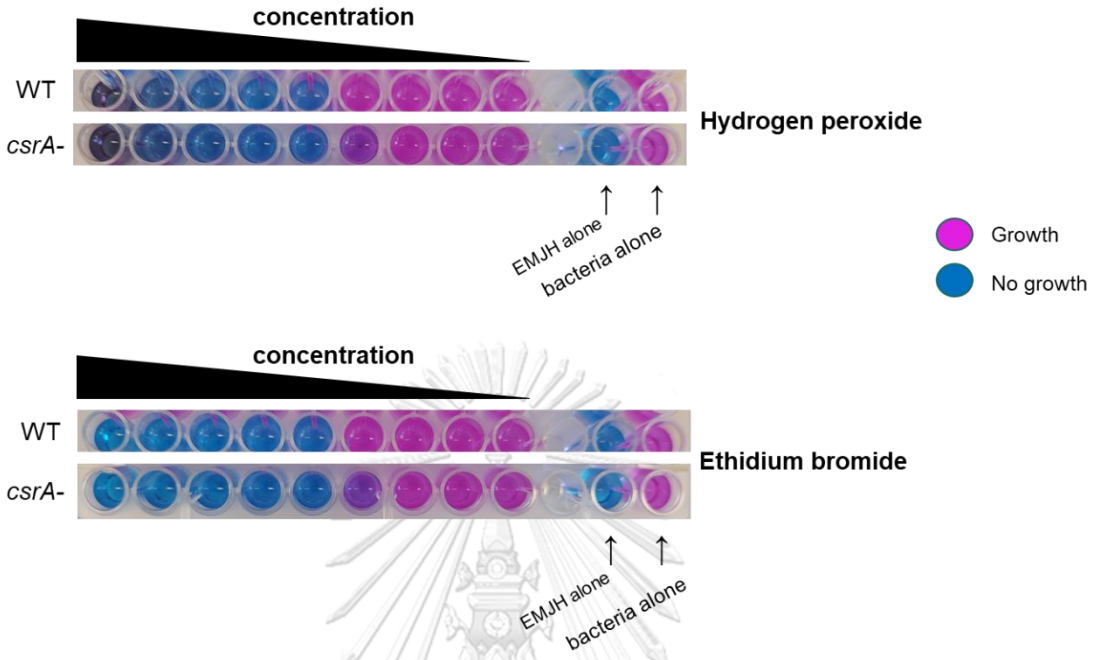


Figure 9 Stress response of *Leptospira*.

2×10^5 cells of wild type and *csrA* mutants *L. biflexa* serovar Patoc was add into 96-well plate containing a dilution of hydrogen peroxide (**a**) or ethidium bromide (**b**). Plates were incubated for 48 hours, 20 µL of Alamar blue was added to each well and continue incubated for 48 hours to observe the change of Alamar blue color. Bacteria and EMJH alone was used as control.

To determine if *csrA* involved in stress response, Alamar blue assay was performed with *csrA* mutant *L. biflexa* serovar Patoc. The chemical substances used for stress response were NaCl, EtBr, SDS, and H_2O_2. We found that *csrA* mutant was slightly more sensitive to EtBr (**Fig.9a**) and H_2O_2 (**Fig.9b**) than wild type strain. This indicated that *csrA* might be involved in these stress responses to a limited extent. However, there was no difference in the responses to NaCl and SDS between *csrA* mutant and wild type strain (data not shown).

CsrA was involved in motility regulation in pathogenic *Leptospira.*

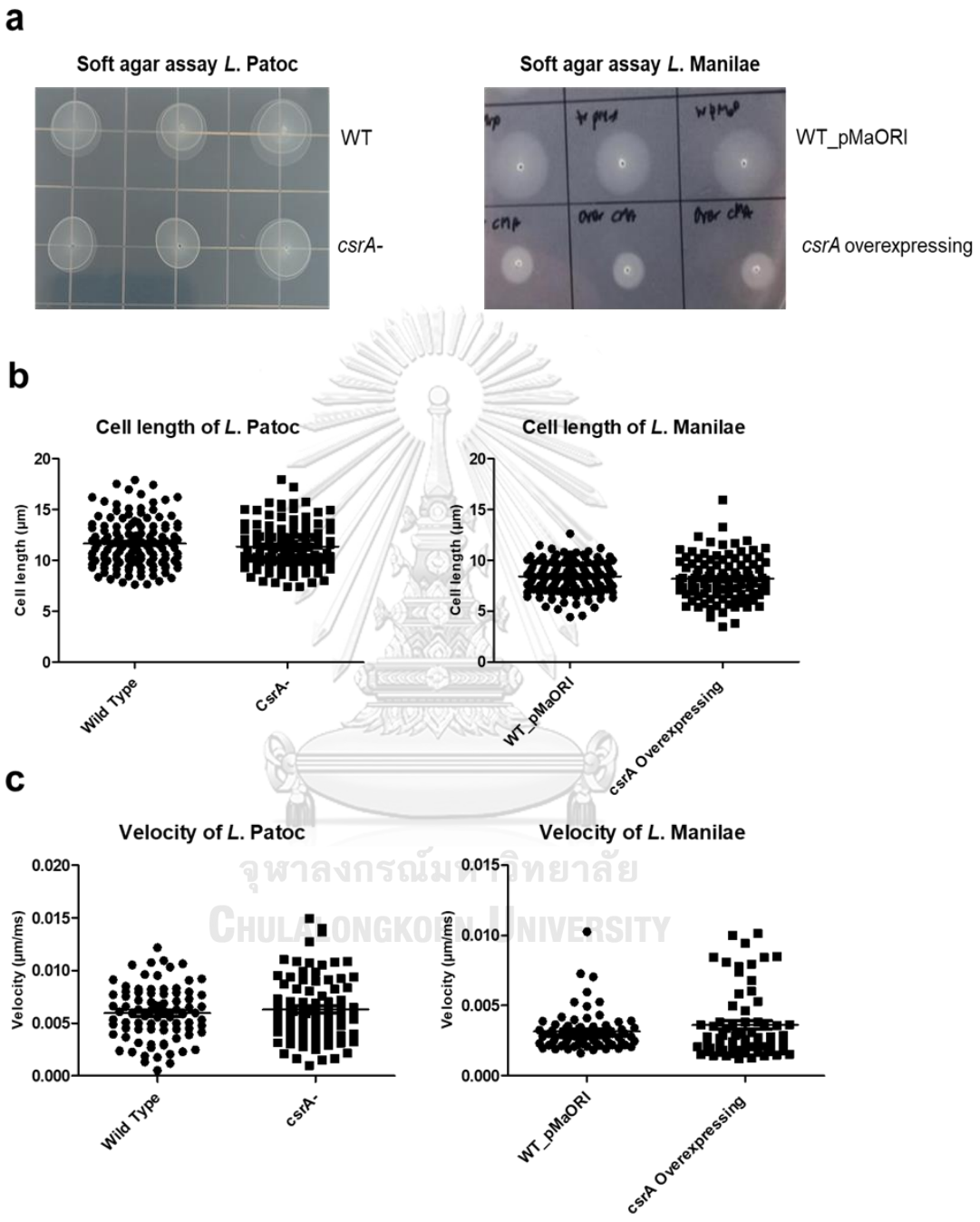


Figure 10 Motility of *Leptospira* spp.

(**a**) Soft agar assay of *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae. *Leptospira* OD_{420} = 0.1 were inoculated onto 0.6% semisolid EMJH plate and

incubated at 30 ^{o}C. (**b**) Measurement of cell length and (**c**) Measurement of velocity, late exponential phase of *Leptospira* spp. grown in EMJH medium were measured for cell length and velocity under dark-field microscope using cellSens software (OLYMPUS).

To determine if *csrA* was involved in motility, a soft agar assay of *Leptospira* spp. were performed on 0.6 % EMJH agar. We found that there was no difference between WT and csrA mutant *L. biflexa* serovar Patoc (**Fig. 10a**) or *csrA* overexpressing *L. biflexa* serovar Patoc and WT_pMaORI (data not shown), indicating that *csrA* was not essential in motility in *L. biflexa* serovar Patoc. In contrast, in *L. interrogans* serovar Manilae, *csrA* overexpressing strain showed poor motility compared with WT_pMaORI (**Fig. 10a**), suggesting that *csrA* was involved motility in pathogenic strain. There was no difference in cell length (**Fig.10b**) or motility (**Fig.10c**) between *csrA* mutant or *csrA* overexpressing strains compared to their parental strains. In addition, the motility behavior of all *Leptospira* strains observed under dark-field microscopy was no difference both in *csrA* mutant and *csrA* overexpressing strains, compared to their parental strains.

csrA could regulate leptospiral *flaB*

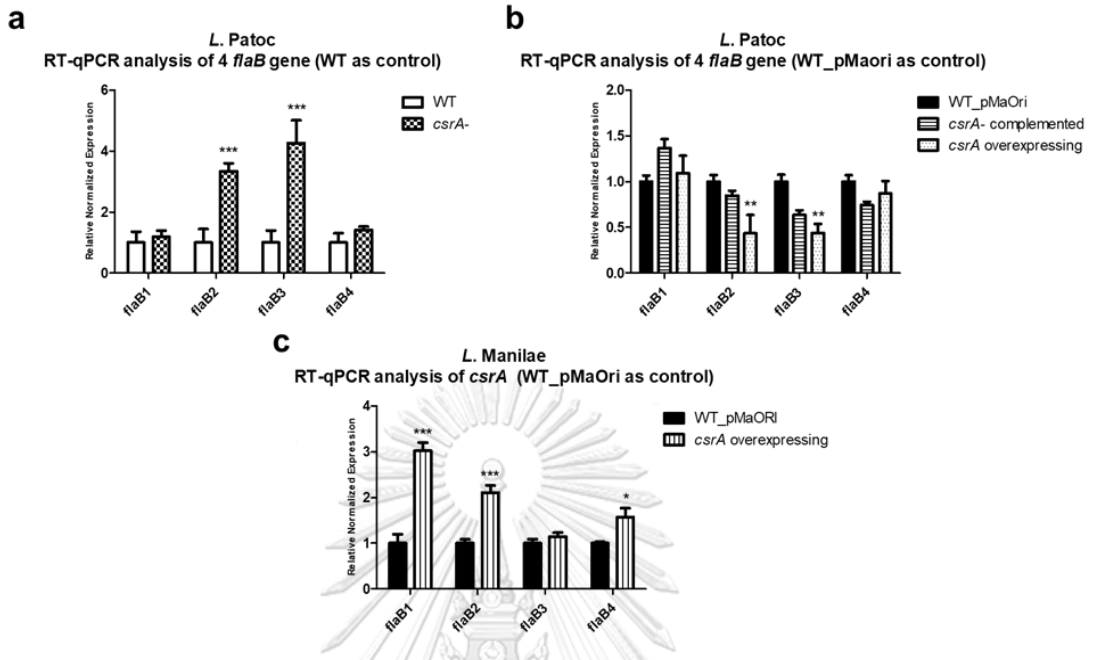


Figure 11 RT-qPCR analysis of 4 *flaB* expression.

RNAs were extracted from log phase culture of either *L. biflexa* serovar Patoc or *L. interrogans* serovar Manilae strains, treated with DNase, and converted to cDNA. qPCR was performed to detect 4 *flaB* expression with gene specific primers. cDNAs were prepared from three dependent experiments. *cysK* and *lipL 32* were used for normalization in *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae, respectively. (**a**)**.** Comparison between *csrA* mutant and WT L. biflexa serovar Patoc. (**b**) Comparison between either *csrA*- complementation strain or *csrA* overexpressing strain to WT_pMaORI *L. biflexa* serovar Patoc. (**c**) Comparison between *csrA* overexpressing and WT_pMaORI *L. interrogans* serovar Manilae.

To investigate if *csrA* relates to motility regulation, RT-qPCR was performed to detect all 4 *flaB* genes encoding core proteins of the flagella. *flaB2* and *flaB3* were significantly upregulated 4.26 and 3.33-fold compared with WT strain, respectively (**Fig. 11a**). To confirm the observation, RT-qPCR was performed to determine the expression level of these *flaB* genes in the complemented strain. The result showed

that the complemented strain could restore the level of both *flaB* genes since the relative expression level was not different compared with WT_pMaORI, suggesting that *csrA* regulated these two genes. Consistently, in *csrA* overexpressing strain *L. biflexa* serovar Patoc, the level of *flaB2* and *flaB3* were significantly down regulated compared with WT_pMaORI, indicating that *L. biflexa* serovar Patoc *csrA* could regulate these 2 *flaB* genes (**Fig. 11b**). In contrast, in *L. interrogans* serovar Manilae, *csrA* overexpressing strain showed significantly higher level of *flaB1*, *flaB2*, and *flaB4* at 3.02, 2.10, and 1.57-fold compared with WT_pMaORI, respectively (**Fig. 11c**), suggesting different mechanisms were employed by *csrA* in non-pathogenic and pathogenic *Leptospira* to regulate *flaB*.

Leptospiral *flaB* genes were putative CsrA targets.

Table 3 Analysis of 5'-untranslated region of *flaB* transcripts in *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae.

Gene	Strain	Sequences (5'-RUACARGGAUGU-'3)	Mismatch	Distance to start codon
flaB1	*L. biflexa*	TTTCAAGGAGGA	4	6
	L. interrogans	TTTCAAGGAGGA	4	5
flaB2	*L. biflexa*	ACACAAAGGAGT	4	8
	L. interrogans	ACAAAGGAGTGT	4	3
flaB3	*L. biflexa*	ACACAGGAGGGT	4	6
	L. interrogans	AAACAGGGAGGT	2	7
flaB4	*L. biflexa*	AUUCAAGGAGGA	3	7
	L. interrogans	AUUCAAGGAGGA	3	6

Underline letters represent mismatched nucleotides.

Yellow highlighted letters represent Shine-Dalgarno sequence.

Table 4 Identification of potential target genes of leptospiral CsrA using Virtual Footprint/PRODORIC.

Strain	Gene	Sequences (RUACARGGAUGU)	Mismatch	Distance to start codon	Predicted Function
L biflexa	*lEPBIa2414*	GAAUAAGGAGGG	4	5	Conserved hypothetical protein
L.biflexa	*flaB4 lEPBIa1589*	AUUCAAGGAGGA	3	7	Flagellar filament core protein FlaB
L. interrogans	*flaB4 lic11531*	AUUCAAGGAGGA	3	6	Flagellar filament core protein FlaB
L. interrogans	*lic10996*	GAAAAAGGAGGU	3	9	Signal transduction protein

Underline letters represent mismatched nucleotides.

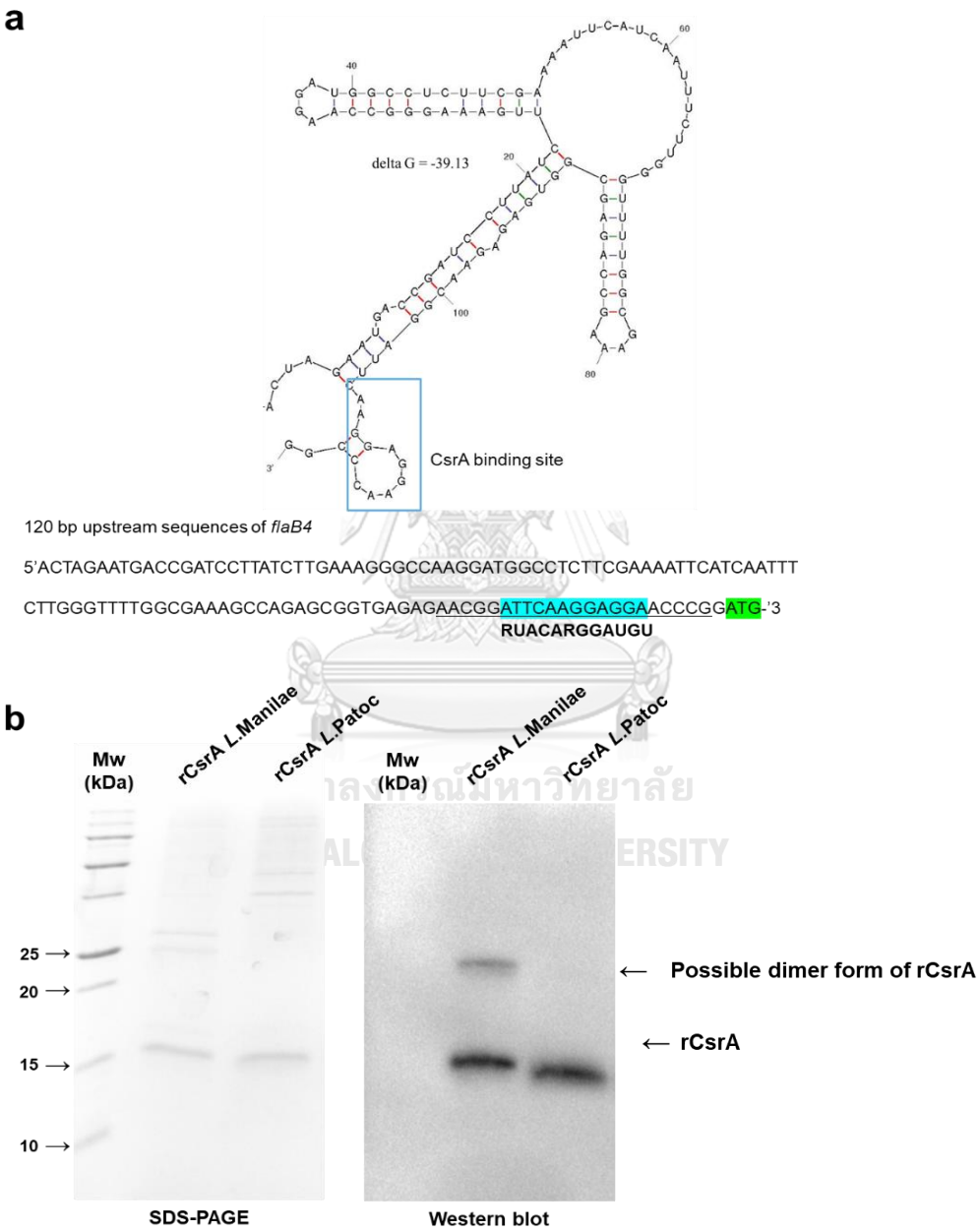
Necleotides highlighted yellow represent Shine-Dalgarno sequence.

According to the difference in *flaB* transcript in either *csrA* mutant or *csrA* overexpressing strain of *L. biflexa* serovar Patoc, as well as *L. interrogans* serovar Manilae, we analyzed 5' untranslated region of these *4 flaB* genes. The consensus sequence of CsrA binding site in *E. coli*, 5'RUACARGGAUGU'3, [44] was employed for searching against both *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae upstream sequences of *flaB*. Consistent with RT-qPCR results, all *flaB* genes contain the putative binding site for CsrA with few mismatches compared with consensus sequences, GGA motif, and overlapping Shine-Dalgarno sequence (SD), as shown in **Table 3**. *flaB2* and *flaB4* of *L. biflexa* serovar Patoc that showed significant difference in gene transcript in *csrA* mutant and *csrA* overexpressing strains contain CsrA binding site with 4 nucleotide mismatches compared with the consensus sequence. In *L. interrogans* serovar Manilae, the 5' untranslated regions of *flaB1*, *flaB2*, and *flaB3*, which showed upregulation in *csrA* overexpressing strain, contain a CsrA binding site with 4, 2, and 3 nucleotide mismatches compared with the consensus sequence, respectively.

In addition, we also employed Virtual Footprint/PRODORIC prediction previously used to identify CsrA gene target in *E. coli* and *P. aeruginosa* [62, 63] as a bioinformatic tool to identify gene targets of leptospiral CsrA (**Table.4**). Neither *L. interrogans* nor *L. biflexa* genomes showed 100 % match or 1 nucleotide mismatch to the consensus sequence, indicating that leptospiral CsrA may recognize different consensus sequence for RNA binding. The most promising gene targets obtained by prediction for *L. biflexa* serovar Patoc were *lEPBIa2414* encoding conserve hypothetical protein and *flaB4* encoding flagella filament core protein. Both had SD sequence overlapping and localized closely to the start codon. However, *flaB4* was most likely to has high affinity to CsrA since it has fewer mismatch nucleotides compared with *lEPBIa2414* (**Table.4**). In *L. interrogans* serovar Manilae, the most potential CsrA targets were determined to be *flaB4* encoding flagella filament core protein and *lic10996* encoding signal transduction protein. Both had 3 mismatch nucleotides, SD sequence over lapping, and closely localized to the start codon. These findings suggested the potential of *flaB4* as a leptospiral CsrA gene target bioinformatic prediction results

were correlated with promoter analysis (**Table. 3**) and RT-qPCR results of csrA overexpressing strain *L. interrogans* serovar Manilae (**Fig.11c**).

Recombinant CsrA bound to upstream sequences of *flaB4*



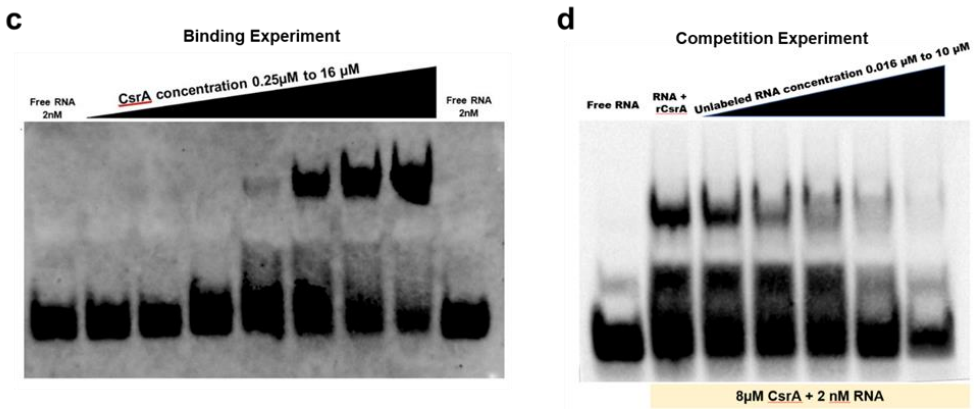


Figure 12 Recombinant CsrA bonded to upstream sequences of *flaB4.*
(**a**) Secondary structure prediction of 120 nucleotides 5'-untranslated region of *flaB4* performed by MFOLD. The putative CsrA binding site is highlighted in blue, start codon (ATG) is highlighted in green. (**b**) Production of recombinant CsrA. PCR products of complete sequences of csrA either from *L. biflexa* serovar Patoc or *L. interrogans* serovar Manilae were purified, digested, cloned into pRSET-C expression vector, transformed into E. coli BL21 (DE3) pLysS, and was induced expression with IPTG. Purified N-terminal 6x His tag recombinant CsrA was subjected to 15% SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Separated recombinant proteins were blotted onto a nitrocellulose membrane, detected with mouse monoclonal antibody against 6x His tag (primary antibody) and HRP-conjugated anti-mouse IgG (secondary antibody) using Amersham ECL Western Blotting Detection Reagent. (**c**) Electrophoretic Mobility Shift Assay (EMSA), 2 nM biotinylated 5' untranslated region of *flaB* 4 transcript was incubated with different concentrations of rCsrA of *L. interrogans* serovar Manilae. The reaction solution was subjected to 10% native PAGE, transferred to nylon membrane, probed with HRP-conjugated streptavidin, and were detected for chemiluminescent signal after addition of Amersham ECL Western Blotting Detection Reagent. (**d**) Competitive EMSA, 2 nM biotinylated 5' untranslated region of *flaB* 4 transcript was incubated with 8 μM rCsrA

of *L. interrogans* serovar Manilae with the addition of increasing concentration of unlabeled biotinylated of 5' untranslated region of *flaB*.

CsrA has been reported as an RNA binding protein that binds specifically to consensus sequences of its gene targets to regulate gene expression [64, 65]. To examine this characteristic, leptospiral rCsrA was produced to determine its interaction with 5' untranslated region of *flaB4*, which is the most promising putative leptospiral CsrA target obtained from promoter analysis experiment (**Table. 3**), *in silico* prediction (**Table.4**), and the upregulation of this gene in overexpressing strain *L. interrogans* serovar Manilae (**Fig. 11c**). Predicted secondary structure of 120-bp 5' untranslated region upstream of the start codon of *flaB4* was shown in **Fig. 12a.** Interestingly, the putative CsrA binding site, 5'AUUCAAGGAGGA'3, forms a hexaloop that contains GGA motif (**Fig.12a**, blue box), which is critical for CsrA binding in *E. coli* [44], suggesting the potential gene target for leptospiral CsrA. We hypothesized that leptospiral CsrA can regulate *flaB4* by binding to the 5' untranslated region of *flaB4* transcript. To test this hypothesis, N-terminal 6xHIS-tag leptospiral recombinant CsrA protein (rCsrA) of *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae was produced in *E.* coli system. As, expected, we observed the corresponding size of rCsrA, approximately 14 kDa including 6xHis-tag, on SDS-PAGE and Western blot for both strains (**Fig. 12b**). Moreover, an approximately 25-kDa band was observed in rCsrA of *L. interrogans* serovar Manilae on SDS-PAGE and Western blot, suggesting a dimer formation of rCsrA. To examine the interaction between rCsrA and the upstream sequence of *flaB4* transcript, EMSA was performed using rCsrA and synthetic RNA of 23-bp 5' untranslated region before the start codon of *flaB4*. In the binding experiment (**Fig. 12c**), rCsrA of *L. interrogans* serovar Manilae bound to the upstream sequence of *flaB4* in a dose dependent manner, which was consistent with the relative fold change of *flaB4* expression in *csrA* overexpressing strain *L. interrogans* serovar Manilae. To ensure the specific binding, competition experiment was performed using an unlabeled RNA probe. The results showed that unlabeled RNA could inhibit the binding reaction in a dose dependent manner (**Fig.12b**). These results indicated that rCsrA of pathogenic strain specifically interacted with the

upstream sequence of *flaB4*. In contrast, rCsrA of *L. biflexa* serovar Patoc did not interact with the *flaB4* transcript when the same molar ratio and incubation condition was used (data not shown) which was consistent with no relative fold change of *flaB4* in both *csrA* mutant and *csrA* overexpressing strains of *L. biflexa* serovar Patoc.

Discussion

Leptospira spp. are spirochetal bacteria that cause leptospirosis with worldwide distribution. The bacteria can live in different lifestyles ranging from free-living in water or soil to pathogens in the susceptible hosts. Therefore, they should have mechanisms for alteration of gene expression to respond to environmental changes leading to their ability in living, colonization, competition as well as pathogenicity. Several publications reported transcriptomic changes in leptospires in response to different signals such as temperature shift [22], isotonic osmolarity [23], and serum exposure [24]. Therefore, global gene regulators are required for their rapid and effective adaptation. However, the knowledge of gene regulation in *Leptospira* remains unclear. We found that *csrA* homolog, a well-characterized post transcriptional global regulator, is present in all available leptospiral genomes. CsrA was previously shown to play a role in regulation of motility, morphology, virulence associated genes, and virulence in *Borrelia burgdorferi*, a Lyme disease spirochete [6-8]. Thus, we hypothesized that leptospiral *csrA* homolog might have a crucial function in global gene regulation.

Firstly, we performed a bioinformatic search to investigate leptospiral *csrA* gene loci. We found that putative leptospiral *csrA* is located in the operon related to flagellum biosynthesis (**Fig. 3a**) alike other bacteria [66]. Moreover, *csrA* operon of both *L. biflexa* serovar Patoc *and L. interrogans* serovar Manilae are synteny with *csrA* operon of other spirochete bacteria including *B. burgdorferi* and *T. pallidum* [8]. It is possible that leptospiral CsrA homolog may use similar mechanism to *B. burgdorferi* CsrA to control gene expression. In gamma-proteobacteria, non-coding RNA (ncRNA) such as *csrB* [67] and *csrC* [68] modulates CsrA function. In epsilon-proteobacteria and firmicutes that have no gene encoding ncRNA antagonist, FliW

was reported as the protein antagonist of CsrA [69, 70]. In *Leptospira*, CsrA-like ncRNA, which could regulate leptospiral CsrA activity, was identified in *L. biflexa* serovar Patoc [27]. Our analysis of *csrA* loci in *Leptospira* revealed that *fliW* is located adjacent to *csrA* (**Fig. 3a**). Thus, this protein may function as the leptospiral CsrA antagonist. Although the amino acid sequences of putative leptospiral CsrA proteins have 50% to 60% similarity to other bacterial CsrA protein (**Fig. 3b**), two residues critical for RNA binding in *E. coli* [61] are conserved (**Fig. 3c**). We suspected that these 2 residues in leptospiral CsrA homolog might be used for binding its gene targets. CsrA proteins of *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae showed additional amino acid residues at C-terminus resulting in relatively longer than CsrA in other bacteria. In *B. burgdorferi*, the additional C-terminal residues are important for RNA binding [71]. Therefore, these residues in leptospiral CsrA protein may also have a role in RNA binding. However, the mechanism of RNA binding and the candidate gene targets of leptospiral CsrA protein may be different from other bacteria because there are some unknown genes in leptospiral genomes that are not present in other bacteria as a result of evolutionary change [2].

To investigate the role of *csrA* in *Leptospira*, we attempted to construct *csrA* mutant, complemented mutant, and *csrA* overexpressing strains. While mutant construction was successful in a nonpathogenic strain of *L. biflexa* serovar Patoc, we were unable to obtain a *csrA* mutant in a pathogenic strain of *L. interrogans* serovar Manilae, suggesting that this protein might have essential role or directly regulates growth in pathogenic leptospires. This observation was previously reported in *csrA* deletion of *Salmonella* Typhimurium. The *csrA* mutant showed severe growth defect compared with its parental strain [50]. Instead of a *csrA* deletion mutant, several works of CsrA in *S.* Typhimurium used a partial *csrA* disruption mutation with diminished CsrA function in their studies [72, 73]. In addition, the relative expression level of *csrA* in *csrA* overexpressing *L. biflexa* serovar Patoc was extremely high, almost 20-fold, while it is only ~ 4-fold higher than that of WT_pMaORI in *csrA* overexpressing *L. interrogans* serovar Manilae (**Fig. 5a** and **b**). Presumably, *csrA* had a stronger effect on the pathogenic strain than that on the non-pathogenic strain. Moreover, it seems that *csrA* is toxic to *Leptospira* when its expression level is excess

because no *csrA* overexpressing strains was obtained in both *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae when *csrA* was expressed under P_{gRoEL}, which is a strong promoter (data not shown). These findings highlight crucial function of *csrA* in *Leptospira* spp. It is obvious that the plasmids constructed in this study can express *csrA* since the level of *csrA* was higher in *csrA* overexpressing strain than that in WT containing pMaORI empty vector in both *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae (**Fig. 5a** and **b**). Furthermore, in LocP strain containing *csrA* locus including *csrA* ORF showed higher expression level of *csrA* than LocM strain that contains *csrA* locus excluding *csrA* (**Fig. 5a**). Hence, these findings ensured the overexpression of *csrA* in the plasmid.

To investigate phenotypes regulated by *csrA*, we firstly examined growth under *in vitro* conditions. Growth curves of *csrA* mutants in regular EMJH medium were not different from those of their parental strains (**Fig.6a** and **b**), suggesting that *csrA* was not essential for growth in all leptospiral strains in this study. Therefore, any phenotypic changes observed in this study did not result from the effect on growth. Metabolism is one of the common phenotypes regulated by CsrA in many bacteria [74-78]. For instance, *E. coli* CsrA regulates carbon starvation gene, *cstA*, which plays a role in peptide transport during carbon starvation [78]. Another work reported a strong activity of CsrA in exponential-phase growth to support growth during iron-limited condition [49]. To further investigate the role of *csrA* in *Leptospira*, we performed a growth curve under starvation condition. We found that the growth of *csrA* mutant of *L. biflexa* serovar Patoc was defective under starvation condition compared with its parental WT strain. As shown in **Fig.7b and c**, the growth curve of *csrA* mutant was relatively slow in lag phase, between day 0 and day 5 in 5-fold diluted EMJH (**Fig.7b**) or between day 0 and day 8 in 10-fold diluted EMJH (**Fig.7c**). However, the complemented mutant strain could only partially restore the phenotype (**Fig.8b and c**). It was unlikely a result of polar effect because *csrA* is the last gene of the operon (**Fig.3a**). It was noticed that the relative fold change of *csrA* in complemented strain was significantly higher than that of WT containing empty vector (WT_pMaORI) even when *csrA* was fused with its native promoter (**Fig. 4e**). We assume that high level of *csrA* might affect several gene expression and

phenotypes because CsrA protein has been recognized as a global regulator [79] leading to incomplete phenotype restoration. It seems that *csrA* of *L. biflexa* serovar Patoc has robust activity on exponential phase to regulate genes involved in metabolism or stress response. Unlike *E. coli* [80], *cstA* homolog, which responds to starvation, was not found in the genome of *L. biflexa* [81], suggesting that *L. biflexa* have unique genes in response to starvation. There may be multiple pathways involved in poor growth under starvation because EMJH is a complex medium. In contrast, complementation with *csrA* of *L. interrogans* serovar Manilae completely restored growth under starvation condition (**Fig. 8**). The data suggest that the mechanisms of *csrA* are different between *Leptospira* strains. Therefore, *Leptospira* spp. require *csrA* during starvation so that they can survive in the environment where nutrient is limited. Further investigations are required to identify genes or pathways regulated by *csrA* that are involved in growth under such condition.

Reactive oxygen species (ROS), *i.e.* superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$) are harmful to numerous bacteria. These substances can be produced by aerobic respiration and environmental redox reactions in competing microbes and immune response [82]. To deal with the oxidative stress, bacteria have detoxification enzymes such as superoxide dismutase and catalase [82]. Compared to other bacteria, *Leptospira* were more sensitive to oxidative stress due to limited genes involving in oxidative stress response [83, 84]. *L. biflexa* and *L. interrogans* were reported to express catalase that was in different enzyme groups. *L. biflexa* expressed *katG* gene [9], whereas *L. interrogans* produced KatE required for virulence in hamsters [85]. In addition, peroxide stress regulator (PerR) of *L. interrogans* had a crucial role in H_2O_2 resistance [86]. Association of CsrA with oxidative stress has been reported in other bacteria. The *csrA* mutant strains of enteric pathogens, *Campylobacter jejuni* and *Helicobacter Pylori,* were more sensitive to H_2O_2 compared to their parental WT [48, 87]. Our study found that *csrA* mutant of *L. biflexa* slightly increased sensitivity to H_2O_2 (**Fig. 9a**), indicating that leptospiral CsrA might have indirect or partial effect on oxidative stress response. Our data suggests the role of CsrA in survival fitness in the environment which may link to ROS resistance during infection.

Efflux pump is a common mechanism that bacteria use for eliminating unfavorable substances so that bacteria can survive in a harsh environment [88]. Efflux pumps belonged to members of resistance nodulation-division (RND) is the most clinically significant [88]. RND family consists of tripartite efflux systems that have a broad range of substrates and relates to multiple drug resistance [89]. It has been reported that CsrA activates the expression of AcrAB which is the most extensively studied RND efflux pump [90]. EtBr has been used as a common substrate to measure the efflux pump efficacy in a number of bacteria [91, 92]. EtBr enters the cells causing DNA precipitation [93]. To eliminate EtBr and protect their DNA, bacteria employ an efflux pump system and SOS stress response [91, 94]. In *L. biflexa*, several genes involved in EtBr tolerance have been reported including RND efflux pump homolog [95]. We found that *csrA* mutant of *L. biflexa* serovar Patoc increased sensitivity to EtBr (**Fig. 9b**). It is possible that *csrA* indirectly or partially regulated efflux pump or SOS stress response since there was only slight difference between WT and *csrA* mutant (**Fig.9b**).

Motility is one of the common traits regulated by CsrA. The alteration in motility affected by CsrA as well as the molecular mechanisms of CsrA that act on motility genes have been well documented in many bacteria [3, 96-101]. However, the mechanism of CsrA on motility regulation can be distinct in different bacteria and sometimes may not correlate with the present phenotype. For example, mutation of *csrA* in *E. coli* and *S.* Typhimurium displayed non-motile bacteria [3, 100]. It was found that CsrA positively regulated the master operon in flagellum biosynthesis, *flhDC* [3, 102]. In contrast, RsmA, a CsrA homolog, of *Erwinia carotovora* negatively regulated *flhDC*, and the *rsmA* mutant showed hypermotility [99]. In addition, CsrA in *Salmonella* regulated different motility genes from that in *E. coli* [42]. These results indicate that CsrA in different bacteria may have distinct effects on motility i.e., different mechanisms of action on the same gene or controlling different gene targets.

In spirochete *B. borgdorferi*, overexpression of *csrA* resulted in the loss of spiral shape [8]. Moreover, CsrA repressed the major flagellin protein FlaB by binding to the two consensus CsrA binding sites localized on *flaB* transcript [6]. Additionally, Hag

protein, a FlaB homolog, in *Bacillus subtilis* was also regulated by CsrA with a similar mechanism observed in *B. burgdorferi* [103]. These findings suggest the strong involvement of CsrA in motility. The motility of *Leptospira* is a complicated network compared with other bacteria. While most bacteria generally have one flagellin component [104], *Leptospira* have two flagellin homologs including FlaA and FlaB as well as 2 flagellar sheath components, FcpA and FcpB, which are unique to genus *Leptospira* [105]. Protein copy number of FlaB was reported previously in *L. interrogans*. There are approximately 14000, 2000, 300, and 3500 copies of FlaB1, FlaB2, FlaB3, and FlaB4, respectively [106]. Motility has been reported as a putative virulence factor in pathogenic strains [107]. Soft agar assay was performed to determine the role of leptospiral *csrA* in motility. We found that *csrA* was not essential for motility in *L. biflexa* serovar Patoc in both *csrA* mutant (**Fig.10**) and *csrA* overexpressing strain (data not shown) because no difference in motility was observed on soft agar assay, which was consistent with no difference in both cell length and velocity. In contrast, *csrA* overexpressing *L. interrogans* serovar Manilae showed poor motility compared with WT_pMaORI in spite of no difference in cell length and velocity (**Fig. 10**). The results indicated that *csrA* might regulate the motility of *L. interrogans* serovar Manilae. Moreover, these findings also suggested the distinct mechanism of leptospiral *csrA* in non-pathogenic and pathogenic *Leptospira* spp. Since *B. burgdorferi* CsrA bound to *flaB* transcript to regulate motility [6], we postulated that leptospiral CsrA also interacted with *flaB* transcripts. To better understand the underlying mechanism of leptospiral *csrA* on motility, RT-qPCR was performed to detect *flaB* expression. We found that *flaB2* and *flaB3* might be specific targets of CsrA in *L. biflexa* serovar Patoc. In *csrA* mutant strain, these two *flaB* genes were upregulated, while they were downregulated in *csrA* overexpressing strain (**Fig.11a and b**). Because FlaB2 and FlaB3 proteins are the minor Fla proteins expressed in *Leptospira* [106], changes at the transcriptional level of these two *flaB* genes did not affect motility phenotype. In contrast, the level of *flaB1*, *flaB2*, and *flaB4* were significantly upregulated in *csrA* overexpressing *L. interrogans* serovar Manilae (**Fig.11c**). The results of RT-qPCR were not correlated with the soft agar assay. Although *csrA* of *L. interrogans* serovar Manilae positively regulated these 3

flaB genes as shown by RT-qPCR, it might also negatively regulate other motility genes, resulting in poor motility of *csrA* overexpressing *L. interrogans* serovar Manilae as shown in the soft agar assay.

CsrA regulates gene targets by binding to the consensus sequence localized at 5' untranslated region which sometimes overlaps the Shine-Dalgarno (SD) sequence [4, 78, 103]. SELEX delivered consensus sequence of CsrA binding site in *E. coli* has been determined to be 5'RUACARGGAUGU'3 [44]. The GGA motif localized in a hairpin loop is a critical binding site for CsrA [44]. Several works have been reported that this consensus sequence can be employed for CsrA of other bacteria including *B. burgdorferi* [6, 103, 108]. According to RT-qPCR results, we hypothesized that leptospiral CsrA protein could regulate *flaB* by binding to the consensus sequence localized at 5' untranslated region of *flaB*. We performed an analysis of 5' untranslated region of 4 *flaB* genes of both *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae. Interestingly, the 5'untraslated region of all *flaB* genes were potential CsrA targets characterized by a few nucleotide mismatches compared to the consensus sequences and closely located to the start codon (**Table.3**). The promoter analysis results of *flaB2* and *flaB3* were correlated with the RT-qPCR results of *L. biflexa* serovar Patoc (**Fig.11a and b**). However, *flaB1* and *flaB4* which were also analyzed as potential gene targets did not show any transcriptional changes in RT-qPCR results (**Fig.11a and b**). For *L. interrogans* serovar Manilae, promoter analysis of all *flaB* genes (**Table.3**) were correlated with the RT-qPCR results, except *flaB3* (**Fig.11c**). We speculated that the consensus sequence of leptospiral CsrA may be different from other bacteria. There are at least two possible explanations. First, the consensus sequence was obtained from *E. coli* CsrA, which is only 50% amino acid similarity compared with leptospiral CsrA. The second explanation is that SELEX experiment constructed previously [44] used the random RNA library to identify the high-affinity RNA targets for CsrA. Therefore, the consensus sequence obtained from that study might not be present in the real bacterial genome. We also used the bioinformatic tools, which had been previously used for prediction of CsrA gene targets [62, 63], to search for potential leptospiral CsrA targets. Based on the consensus sequence of *E. coli*, we did not find any sequences with 100% identity in

leptospiral genome, suggesting the distinct consensus sequences used by leptospiral CsrA. Consistent with RT-qPCR results of *L. interrogans* serovar Manilae (**Fig.11c**), bioinformatic prediction identified *flaB4* as the most likely leptospiral CsrA targets (**Table.4**). Thus, we hypothesized that CsrA of *L. interrogans* serovar Manilae could bind to 5'untranslated region of *flaB4* to control its gene expression. To examine this hypothesis, the recombinant CsrA (rCsrA) was produced. As observed in *E. coli* [61], rCsrA of *L. interrogans* serovar Manilae appeared as a homodimer on SDS-PAGE and Western blot (**Fig.12b**), while it was not observed in rCsrA of *L. biflexa* serovar Patoc. The binding of rCsrA of pathogenic strain and 5'untraslated region of *flaB4* transcript was confirmed by EMSA (**Fig.12c and d**). It seems that the molar ratio of rCsrA to *flaB4* transcript was quite high, indicating that rCsrA might have low affinity to this consensus sequence. These findings suggest the involvement of CsrA in pathogenesis because motility is one of leptospiral virulence factors [107].

In conclusion, we characterized the role of CsrA as a global post-transcriptional regulator in *Leptospira* spp. We found that *csrA* of non-pathogenic *L. biflexa* serovar Patoc involved in response to starvation, oxidative stress, EtBr exposure and might repress expression of *flaB2* and *flaB3* without any alteration in motility phenotype. In contrast, *csrA* of pathogenic *L. interrogans* serovar Manilae was involved in motility and might be an activator of *flaB1, flaB2*, and *flaB4* genes. The specific binding of rCsrA of the pathogenic strain to *flaB4* transcript was demonstrated. This study showed distinct mechanisms employed by CsrA even in bacteria belonged to the same genus i.e., pathogenic and non-pathogenic *Leptospira* spp. In addition, the consensus sequence of CsrA binding site of *Leptospira* spp. might be different from that of other bacteria.

Part II: Identification of *In Vivo* Expressed Protein relevant to Cross-Serovar Protection against *Leptospira.*

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ABSTRACT

Development of subunit protein vaccines that have broad protection against numerous *Leptospira* strains should overcome the drawback of currently licensed bacterin vaccines. *In Vivo*-Induced Antigen Technology (IVIAT) is a powerful tool to identify *in vivo*-induced (*ivi*) genes which are the genes that particularly express *in vivo* during infection. These genes may be related to pathogenesis and suitable for vaccine and diagnostic development. Similar to the findings of a previous study, this study showed that a live attenuated vaccine derived from an LPS mutant strain of *Leptospira interrogans* serovar Manilae M1352 (Live M1352) had better protective efficacy than heat killed M1352 (HK M1352) against a heterologous challenge. IVIAT was employed to identify *ivi* genes of M1352 that may be responsible for cross protection. Pooled sera from hamsters vaccinated with Live M1352 were sequentially adsorbed with various preparations of *in vitro* grown M1352. The remaining sera were used to screen genomic expression library of M1352. Finally, 19 strongly reactive clones were identified and sequenced. These genes were involved in multiple pathways and conserved in most *Leptospira* strains. The *in vivo* expression of 4 screened *ivi* genes including *tolB*, *araC*, *fliI*, and *Lman_3156* was confirmed by RT-qPCR. Further studies should investigate the protective efficacy of these *ivi* genes as well as their roles in pathogenesis.

Introduction

Leptospirosis is one of the most widespread zoonosis caused by pathogenic *Leptospira*. Humans are considered as accidental hosts by acquiring the pathogen via direct contact with urine excreted from the reservoir hosts or indirect contact with contaminated environment [1]. Approximately one million of leptospirosis cases with sixty thousand deaths per year were estimated around the world [109].

Due to the lack of understanding in pathogenesis of leptospirosis and limitations of genetic manipulation for *Leptospira*, currently licensed leptospirosis vaccines rely only on killed whole cell vaccines. The protective efficacy of this type of vaccine has some limitations such as short-term immunity, incomplete protection, no broad protection against all serovars, and reactogenicity. Subunit vaccines have been developed to overcome the disadvantages of killed vaccines. There are various strategies to select vaccine antigens. Most focused on conserved surface exposed proteins that could induce humoral immunity with broad protection. Many leptospiral subunit vaccines have been reported to protect against homologous challenge [110-112]. However, identification of protective antigens that confer cross protection is still challenging.

A previous study reported that a lipopolysaccharide (LPS) mutant of *L. interrogans* serovar Manilae M1352 (M1352) was attenuated because infected hamsters, a susceptible animal model, had no clinical sign of leptospirosis and no renal colonization [113]. Furthermore, the protective efficacy of live attenuated vaccine (Live M1352) against a heterologous strain, *L. interrogans* serovar Pomona, was significantly higher than that of the heat killed vaccine derived from wild type *L. interrogans* serovar Manilae (HK WT). There was no microscopic agglutination test (MAT) titers against LPS of *L. interrogans* serovar Pomona suggesting the role of proteins for cross protection. Beside superior protection to HK WT, Live M1352 showed higher protection than HK M1352 [16]. The cross-protection provided by Live M1352 was subsequently confirmed [114]. Use of single dose or two doses of Live M1352 showed significant protection against 5 heterologous serovars including Pomona, Grippotyphosa, Canicola, Autumnalis, and Australis. Consistently, Live M1352 had superior protection to HK M1352 [114]. Those results indicated that

proteins that were indeed present in Live M1352 but not in HK M1352 might be responsible for additional protection.

In vivo-induced (*ivi*) genes are particularly expressed during infection when the interaction between host and pathogen occurs but are suppressed during *in vitro* growth [115]. Therefore, these genes might be related to pathogenicity and may be suitable targets for development of vaccines or diagnostic tests. Several methods have been developed to identify *ivi*-genes, such as *in vivo* expression technology (IVET) [116], signature-tagged mutagenesis (STM) [117], RNA sequencing [118], Differential fluorescence induction (DFI) [119], and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [120]. A number of leptospiral proteins have been identified by those approaches including LigA, the most promising vaccine antigen, which was identified by expression library screening using convalescent mare's serum [121].

In Vivo-Induced Antigen Technology (IVIAT) is a potential tool to identify *ivi* gene by screening expression library with convalescent serum that have been pre-adsorbed with *in vitro* grown pathogens. This method is simple and suitable for screening of proteins specifically expressed during infection and does not require animal models. Proteins identified by IVIAT do not only take part in pathogenesis, but they are also immunogenic. Thus, these proteins can be candidates for vaccine development and serodiagnosis [18]. IVIAT has been widely used in bacteria such as *Escherichia coli* [122], *Mycobacterium* [123], *Vibrio cholera* [124], *Campylobacter* [125] and recently in pathogenic *Leptospira* in order to identify antigens for serodiagnosis of acute phase leptospirosis [126]. Consistent with a previous studies [16, 114], we found that protective efficacy of Live M1352 was better than HK M1352 against heterologous challenge. Therefore, we hypothesized that *ivi* genes particularly expressed in Live M1352 were involved in cross-protection. In this study, IVIAT was employed to identify *ivi* genes. Genomic library of M1352 was constructed and screened with convalescent hamster sera in order to identify *ivi* genes that conferred cross-protection. Nineteen *ivi* genes were identified, four of which were confirmed for *in vivo* expression by RT-qPCR.

Materials and Methods

Bacterial strains and Culture condition

Leptospira interrogans serovar Manilae strain M1352 (M1352) [113], a mutant of lipopolysaccharide biosynthesis gene was used for immunization and construction of expression library. Virulent low passage (< 5 times *in vitro* passage) *L. interrogans* serovar Pomona was used for hamster challenge. All *Leptospira* strains were cultured in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) [52] medium at 30^{o} C without shaking. *E. coli* XL-1 Blue MRF' and SOLR (Agilent technologies, Wilmington, DE) were used as host strains for phage manipulation and excision, respectively. All *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar supplemented with appropriate antibiotics following manufacturer's instructions.

Preparation of Vaccines

Log-phase *Leptospira* were counted by Petroff-Hausser counting chamber using a dark-field microscope. After centrifugation, bacterial pellets were resuspended in Phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.4) to a desired concentration. For heat killed (HK) preparation, bacteria were incubated at 56 ^{o}C for 30 mins and stored at -20 ^{o}C until use. Killed bacteria were confirmed by no growth observed after inoculation and incubation in EMJH broth.

Immunization and Challenge

Each hamster (five per group) was subcutaneously immunized with a total volume of 250 µL of different vaccine formulations as follows; Live 10^7 cells of *L. interrogans* serovar Manilae M1352 (Live M1352) in PBS, 10^8 cells of HK M1352 in PBS and alum (Thermo Fisher Scientific, Vantaa, Finland) at a ratio of 1:1 (v/v), and PBS mixed with alum (1:1, v/v) used as an adjuvant control group (**Table. 5**). Each hamster was immunized 3 times at 2-week interval except 2 times at 2-week interval for Live M1352. Blood was collected at 7 days after the second immunization and 7 days

prior to challenge. All hamsters were challenged with 20x LD50 (200 cells) of low passage virulent *L*. Pomona and monitored for 4 weeks. All hamsters were euthanized with isoflurane at the end of the fourth week. All procedures involving manipulations of golden Syrian hamsters were approved by the IACUC of the Armed Forces Research Institute of Medical Sciences, Thailand (approved No. ARAC 1/60).

Table 5 Vaccine formulation

Group	Vaccine formulation	Immunization			Volume of injection	Route of injection
		1st	2nd	3rd		
1	10^7 cells Live M1352	☑	☑	☒	250 µL	SC*
2	10^8 cells HK M1352 + Alum (1:1)	☑	☑	☑	250 µL	SC*
3	PBS + Alum (1:1)	☑	☑	☑	250 µL	SC*

*Subcutaneous

Antibody adsorption

Sera from 5 hamsters collected one week after the second immunization with Live M1352 were pooled and adsorbed with *in vitro* grown M1352 in order to remove antibodies that recognized *in vitro* expressed antigens.

For antigen preparation used for adsorption, *Leptospira* were grown in EMJH medium until reaching log phase, cells were washed twice with EMJH base, and resuspended in PBS/ 0.02% sodium azide. *Leptospira* obtained at this step were defined as intact whole cells. For whole cell lysate preparation, *Leptospira* were lysed by sonication using 30% output for 15s pulse and 45s rest for 30 mins. To prepare heat denatured cell lysates, supernatant obtained after ultrasonication and centrifugation at 12000x g was heated at 95 ^{o}C for 10 mins. *E. coli* lambda phage lysates were prepared as described previously [127]. Protein concentration was measured by Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific). One mg of each antigen was coated onto a nitrocellulose membrane and kept at 4 ^{o}C before use.

Each adsorption step was performed on a rocking platform at 4 ^{o}C for 6 h as previously described [128]. Briefly, pooled serum was sequentially adsorbed 6 times with intact M1352, 3 times with whole cell lysate of M1352, once with heat denatured whole cell lysate of M1352, and once with *E. coli* XL-1 Blue MRF' lambda phage lysates to eliminate background. Indirect Enzyme-Linked Immunosorbent Assay (Indirect ELISA) against whole cell lysate of M1352 was performed after each adsorption to confirm the efficacy of adsorption.

Expression library construction and screening

A Lambda ZAPII Vector Kit (Agilent technologies) was used for expression library construction as the manufacturer's instruction. Briefly, genomic DNAs of M1352 were prepared and partially digested with Sau3AI (NEB) at 37^{o}C for 30 mins. DNA fragment size between 0.5 – 3 Kb was purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific) and ligated with XhoI (NEB) digested Lambda ZAII Vector. Recombinant Lambda Vectors were packed using Gigapack III gold packing extracts (Agilent technologies). Packaged mixture containing recombinant bacteriophage was amplified and measured the titer until the desired concentration was achieved. Recombinant bacteriophages were plated on NZY agar. Plaques were transferred onto the nitrocellulose membrane, denatured with 1.5 M NaCl and 0.5 M NaOH, and neutralized with 1.5 M NaCl and 0.5 M Tris-HCl. Pre-adsorbed pooled hamster serum obtained from the last adsorption step was added at a dilution of 1:500, and then 1:2000 horseradish peroxidase (HRP)-labeled anti-hamster IgG (Sigma) was added. Chemiluminescence signal was detected under CCD camera (BioRad). Positive plaques on the agar plug were selected, transferred to SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, and 0.01% w/v gelatin) and eluted. Plasmids containing inserts were rescued using ExAssist helper phage and *E. coli* SOLR strain. Plasmids were prepared using HiYield™ Plasmid Mini Ki (RBC Bioscience) and sequenced using T3 and T7 primers. DNA sequences were searched against *L. Interrogans* serovar Manilae genome using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Protein localization prediction was performed

using SignalP-5.1 (http://www.cbs.dtu.dk/services/SignalP/). Cluster of Orthologous Groups (COG) was used for protein functional classification prediction [129].

Gene Expression Analysis by Quantitative Real-Time PCR

Relative fold change of screened *ivi* genes was tested by comparing the RNA transcripts between *in vivo* during infection and *in vitro* culture. RNA of *in vitro* grown *L. interrogans* serovar Pomona was prepared using TRIZOL as manufacturer's instruction (Thermo Fisher Scientific) [55]. For *in vivo* expression, total RNA was extracted from kidneys of 3 hamsters infected with *L. interrogans* serovar Pomona obtained from our previous experiment [130], using Monarch® Total RNA Miniprep Kit (NEB). RNA was treated with DNase I and reverse transcribed to cDNA using iScript™ Reverse Transcription Supermix (BioRad). cDNA was used for qPCR with gene specific primers. Relative gene transcript was calculated using *flaB* for normalization [131]. All primer sequences are shown in **Table 6**.

Table 6 Primers used in this study

Name	Sequences	Description	Reference
Manilae *tolB* F	GGGTTTGTGGGCGGATAAGA		
Manilae *tolB* R	TTTCATCGGCTCCTTGCCTT	RT-qPCR	This study
Lman3156F	GCAGTGGAGGCTCAGACTTT		
Lman3156R	ACCGTAGCTTTTGTATGCCCA	RT-qPCR	This study
Lman3446 *araC* F	AGCCGAGATCTGCGAGTTTG		
Lman3446 *araC* R	ACCGAATCCAGACCGATTCT	RT-qPCR	This study
Lman2296 fliI F	CTATTCAGAAGGACCGCCCG		
Lman2296 fliI R	TTTTCCGACTGGGATTGCGA	RT-qPCR	This study
flaB F	AGCGAGACAACTTCTTCCGCCATA		
flaB R	ATGAAGCAGAGAGCGGATATGGGA	Normalization	[131]
T7 promoter	TAATACGACTCACTATAGGG	Sequencing	Universal primer
T3 promoter	GCAATTAACCCTCACTAAAGG	Sequencing	Universal primer

RESULTS

Immunization with live attenuated M1352 conferred 100% protection against heterologous *L. interrogans* serovar Pomona in hamsters

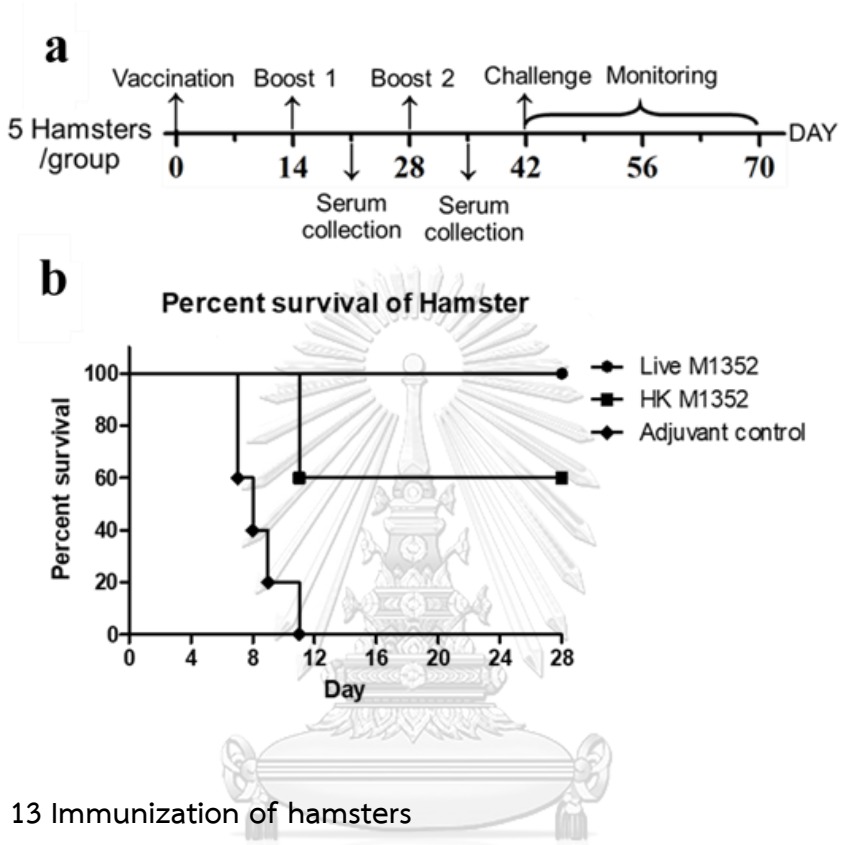

Figure 13 Immunization of hamsters

(**a**). Immunization schedule. Hamsters were divided into 3 groups (n = 5 per group). The hamsters were subcutaneously immunized with different vaccine formulations three times at 2-week interval, except 2 times at 2-week interval for Live M1352. Sera were collected 7 days after the second and the third immunizations. All hamsters were challenged with 20x LD50 of virulent *L. interrogans* serovar Pomona and were monitored for 28 days. (**b**) Percent survival of hamsters after challenged with *L. interrogans* serovar Pomona.

Protective efficacy on survival of live attenuated *L. interrogans* serovar Manilae M1352 (Live M1352) and HK *L. interrogans* serovar Manilae M1352 (HK M1352) on survival of hamsters were tested. Live M1352 vaccinated hamsters showed 100% survival compared with 0% in the adjuvant control group, whereas 60% of hamsters

vaccinated with HK M1352 survived (**Fig. 13b**). Superior protection conferred by Live M1352 suggests the role of *in vivo* expressed proteins in additional protection.

Preparation of pre-adsorbed serum for IVIAT

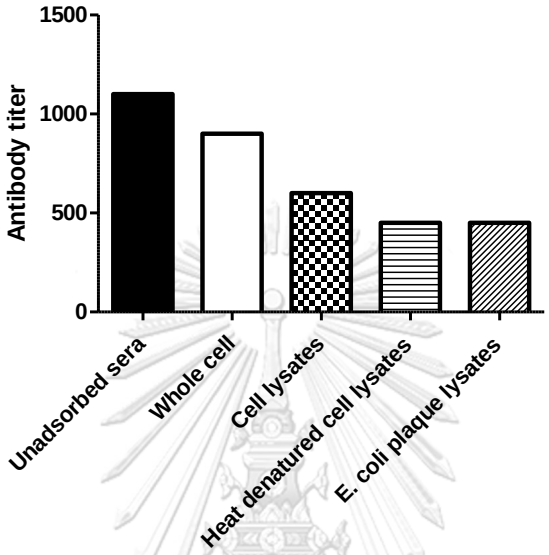

Figure 14 Antibody titers against whole cell lysates of M1352 measured by ELISA after each step of adsorption of pooled hamster serum.

Hamster sera from the second immunization were pooled and adsorbed with intact whole cell of M1352 for 5 times, cell lysates of M1352 for 3 times, heat denatured cell lysates once, and lambda phage E. coli plaque lysates once. Data are shown as antibody titers and corrected for background after each step of adsorption.

To eliminate antibodies that bind specifically to the *in vitro* expressed proteins of M1352, pooled sera obtained from hamsters vaccinated with Live M1352 were adsorbed sequentially with various preparations of *in vitro* grown M1352 as follows: intact whole cells of M1352 in order to adsorb antibodies against *in vitro* expressed surface/ outer membrane antigens; whole cell lysates of M1352 to eliminate antibodies against *in vitro* expressed periplasmic/cytoplasmic antigens; heat denatured whole cell lysates of M1352 to eliminate antibodies against heat-stable

antigens, which were likely to be non-proteins in nature, and antigens of HK 1352; and *E. coli* lambda phage plaque lysates used for expression library construction to remove antibodies against the *E. coli* background proteins. Finally, pre-adsorbed pooled sera obtained at the last adsorption step most likely contained antibodies against *in vivo* expressed surface/ outer membrane proteins conferring protection in addition to HK1352.

As shown in figure **14**, the antibody titers against M1352 gradually decreased after each step of adsorption suggesting successful removal of antibodies against undesirable *in vitro* expressed antigens. There was no further reduction of antibody titers after adsorption with *E. coli* plaque lysates indicated that the antibodies were specific to *Leptospira* proteins, but not *E. coli* or lambda phage. Thus, these antibodies were suitable for screening *ivi* genes.

Construction of an expression library of M1352 and screening of *in vivo* induced genes (*ivi*-genes)

Table 7 Results of screening *ivi* genes

Gene locus tag / Gene name	**Predicted funtion**	**Localization**[a]	**COG**[b]
Lman_1470	Sugar pyridoxal-phosphate-dependent aminotransferase	other	M
Lman_1941	Hypothetical protein	other	unknown
Lman_2562	Dehydrogenase	other	I, Q, R
Lman_3774	Metalloendopeptidase	other	M
Lman_0616	hypothetical protein	other	unknown
Lman_2698/tolB	Tol transport system component	Outer membrane/Signal peptide	U
Lman_2401	Sensor histidine kinase of a two-component response regulator	Other	T
Lman_2296/fliI	Endoflagellar biosynthesis/type III secretory pathway ATPase	Other	N, U
Lman_0491	hypothetical protein	Other	unknown
Lman_3156	hypothetical lipoprotein	Lipoprotein signal peptide	unknown
Lman_3680	DNA mismatch repair protein ATPase component	Other/IM	L
Lman_2523	Hydrolase or acyltransferase	Other	R
Lman_0662/tufB	Protein-synthesizing GTPase complex, EF-Tu component	Other	J
Lman_1799/metY	O-acetylhomoserine aminocarboxypropyltransferase	Other	E

Lman_1920	hypothetical protein	Other	unknown
Lman_3303/pth-2	Aminoacyl-tRNA hydrolase	Other	J
Lman_0879	ATP-dependent DNA ligase	Other	L
Lman_3446	Transcriptional regulator, AraC family	Signal peptide	T
Lman_1449/galE	Glucose galactose epimerase	Other	M, G

Expression library of M1352 was constructed using Lambda ZapII vectors. Library was plated on NZY agar, plaques were transferred on nitrocellulose membrane and probed with adsorbed hamster sera obtained from Live M1352 vaccination.

[a] Protein localization prediction by SignalP-5.1

[b] Protein functional classification based on COG category

M = cell wall/membrane/envelope biogenesis, I = Lipid transport and metabolism, Q = Secondary metabolites transport biogenesis, transport, and catabolism, R = General function prediction only, U = intracellular trafficking, secretion and vesicular transport, T = Signal transduction mechanisms, N = Cell motility, L = replication, recombinant and repair, J = Translation, ribosomal structure and biogenesis, E = Amino acid transport and metabolism, G = Carbohydrate transport and metabolism.

In order to identify *ivi* genes, approximately $1x10^5$ plaques of M1352 expression library were screened using pre-adsorbed pooled hamster serum. Ninety-eight positive plaques were obtained from primary screening, nineteen of which showed strongly positive reaction with pre-adsorbed hamster serum and subsequently selected for DNA sequencing. Based on COG category, the identified *ivi* genes were involved in several pathways including metabolism, cellular processes and signaling, and information storage and processing (**Table. 7**). Localization prediction showed that 3 genes including Lman_2698, Lman_3156, and Lman_3446 have a signal peptide, suggesting possible outer membrane proteins. Five of 19 genes were predicted to encode proteins of unknown function.

Conservation of screened *ivi* genes among *Leptospira* strains

Table 8 The presence of screened *ivi* genes among *Leptospira* strains performed by BLAST search

Gene locus tag / Gene name	*L.* Autumnalis	*L.* Bataviae	*L.* Bratislava	*L.* Copenhageni	*L.* Lai	*L.* Manilae	*L.* Pomona	*L. borgpetersenii*	*L. weilii*	*L. biflexa*
Lman_1470	+	+	+	+	+	+	+	+	+	+
Lman_1941	+	+	+	+	+	+	+		+	
Lman_2562	+	+	+	+	+	+	+	+	+	+
Lman_3774	+	+	+	+	+	+	+	+	+	+
Lman_0616	+	+	+	+	+	+	+		+	
Lman_2698/tolB	+	+	+	+	+	+	+	+	+	+
Lman_2401	+	+	+	+	+	+	+	+	+	+
Lman_2296/flil	+	+	+	+	+	+	+	+	+	+
Lman_0491	+	+	+	+	+	+	+	+	+	+
Lman_3156	+	+	+	+	+	+	+		+	+
Lman_3680	+	+	+	+	+	+	+	+	+	+
Lman_2523	+	+	+	+	+	+	+	+	+	+
Lman_0662/tufB	+	+	+	+	+	+	+	+	+	+
Lman_1799/metY	+	+	+	+	+	+	+		+	+
Lman_1920	+	+	+	+	+	+	+	+	+	+
Lman_3303/pth-2	+	+	+	+	+	+	+	+	+	+
Lman_0879	+	+	+	+	+	+	+	+	+	
Lman_3446	+	+	+	+	+	+	+	+	+	
Lman_1449/galE	+	+	+	+	+	+	+	+	+	+

To obtain *ivi* proteins that conferred cross protection in this study, BLAST search was performed to identify *ivi* genes encoding conserved proteins in *Leptospira* strains. Nine pathogenic strains including 7 different serovars of *L. interrogans*, *L. borgpetersenii* and *L weilii*, and one non-pathogenic strain, *L. biflexa* serovar Patoc, were selected as representative strains. As shown in **Table. 8**, All 19 identified *ivi* proteins were present in the genome of both *L. interrogans* serovar Manilae and *L. interrogans* serovar Pomona, suggesting that these proteins may have cross-protection potential. In addition, 14 of 19 genes are conserved in all *Leptospira* strains. Two of 19 genes, Lman_0879 and Lman_3446, are conserved in all pathogenic strains, suggesting their possible association in pathogenesis. Lman_1941, Lman_0616 are present in *L. interrogans* and *L. weilii* but not in *L. borgpetersenii* and *L. biflexa*. Lman_3156 and Lman_1799 are present in all representative strains except *L. borgpetersenii*.

Validation of selected *ivi* genes by RT-qPCR

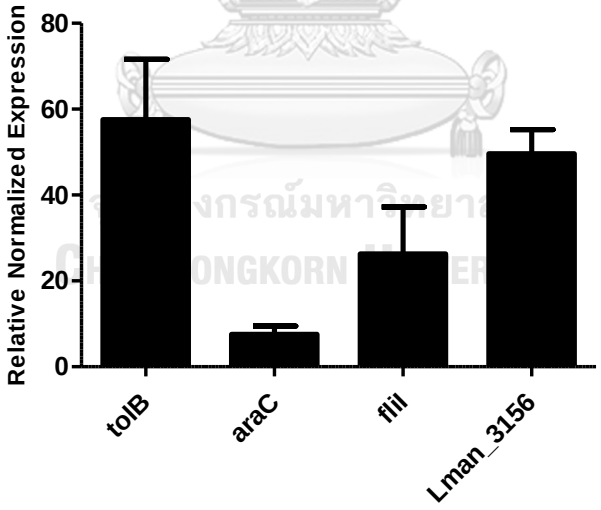

Figure 15 *In vivo* expression of selected *ivi* genes relative to in vitro expression.

RNA was prepared from 3 kidneys of different hamsters infected with *L. interrogans* serovar Pomona, treated with DNase, and converted to cDNA. RT-qPCR was performed to detect the expression of 4 selected *ivi* genes compared to their expression level *in vitro*. *flaB* was used for normalization.

To evaluate the *in vivo* expression of the *ivi* genes identified in this study, four identified *ivi* genes were selected for RT-qPCR analysis. Since antibody is the protective immunity against *Leptospira* [14, 15, 132] , we selected possible membrane proteins including Lman_2698 (*tolB*), Lman_3446 (*araC*), and *Lman_3156* based on their predicted localization. Lman_2296 (*flil*) was also selected based on the involvement of this protein in pathogenesis of Lyme disease spirochete, *Borrelia burgdorferi* [133]. Using RNA prepared from the kidneys of 3 hamsters infected with *L. interrogans* serovar Pomona, we found that all four selected *ivi* genes were upregulated during infection (**Table.15**), 57.55-fold, 7.57-fold, 26.31-fold, and 49.60-fold for Lman_2698 (*tolB*), Lman_3446 (*araC*), Lman_2296 (*flil*), and *Lman_3156*, respectively, compared with the expression level in *in vitro* grown *L. interrogans* serovar Pomona. The finding ensured the accurate results of the IVIAT screening procedure.

Discussion

To date, the commercially available vaccines for leptospirosis are killed whole-cell vaccines or bacterins. However, there are some drawbacks such as short-term immunity, reactogenicity, and restricted cross-protection against heterologous serovars excluded in vaccine formulations [13]. To overcome these disadvantages, recombinant protein-based vaccines using leptospiral outer membrane proteins (OMPs) as vaccine antigens have been developed against leptospirosis [134-136]. However, no vaccine antigens conferred complete and broad protection against leptospirosis in animal models.

A previous study showed that immunization with Live M1352, an LPS mutant strain of *L. interrogans* serovar Manilae, showed higher protection against heterologous serovar than HK M1352, even slightly higher against homologous serovar [16]. The protective efficacy of Live M1352 was higher than that of HK M1352 (**Fig.13b**) consistent with the previous work [16, 114]. These results indicate that certain *in vivo* expressed proteins of Live M1352 were responsible for efficacious protection. Therefore, this study focused on identification of proteins that Live M1352 particularly expressed *in vivo* after immunization to obtain new protective antigens that conferred protection in addition to HK M1352.

Based on transcriptomics results using microarray, there were different RNA profiles between *in vitro* grown *Leptospira* and *in vivo*-mimicking conditions such as temperature shift to 37 ºC [22], isotonic osmolarity [23], and serum exposure [24]. These results suggest that there are specific genes required for infection in the hosts. In addition, cross-protective antigens of Live M1352 were identified previously by using two-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D-PAGE) [16]. Three proteins including LA3961, Loa22, and LA2372 were identified. Loa22 was one of the putative virulence factors of *Leptospira* [137] and its partial protection has been investigated [138]. However, these approaches have limitations such as the genes expression profiles identified by microarray were specific to one condition used in the study that may not be similar to natural infection because bacteria simultaneously encounter various host signals *in vivo*. Using 2D-PAGE may miss to identify some *ivi*-proteins because *in vitro* grown pathogens were used in the

experiment. IVIAT is a valuable tools to identify *ivi* proteins because this immunological screening technique can overcome the limitations of other methods. Several virulence associated proteins of pathogens have been identified by IVIAT. For example, mutation of *pyrH, pyrH*, and *hlyU* genes identified by IVIAT in *Vibrio vulnificus* decreased the virulence of bacteria in animal model [139]. Furthermore, leptospiral recombinase A (RecA) identified by IVIAT was able to induce sterile immunity against heterologous challenge when it was used as a DNA-prime protein-boost vaccine [17, 126]. This evidence emphasizes the importance of *ivi* genes and corresponding proteins as new vaccine candidates.

Humoral immune response is the major protective immunity against *Leptospira* [14, 15, 132]. Therefore, outer membrane proteins that are accessible to antibodies should be suitable for vaccine antigens. In this study, IVIAT was modified to identify protective antigens of Live M1352 that were not only *in vivo* expressed proteins, but also surface/outer membrane proteins by using pooled serum of hamsters vaccinated with Live M1352 that were adsorbed in sequential steps in order to remove antibodies against cytoplasmic proteins of *in vitro* grown M1352 (**Fig.14**) before use. Because all 19 *ivi* proteins are conserved in both M1352 and *L. interrogans* serovar Pomona genomes (**Table.8**), they might be associated with cross-protection conferred by Live M1352 vaccine.

Our IVIAT results revealed 3 potential putative membrane proteins; Lman_3156, Lman_3446, and Lman_2698; according to localization prediction (**Table.7**). Lman_3156, a hypothetical lipoprotein also known as OmpL31, was upregulated after temperature shift and reacted to sera of leptospirosis patients [22, 140], suggesting to be a putative virulence factor. Lman_3446 encodes a transcriptional regulator in AraC family, a conserved regulator involved in metabolism, stress response, and virulence regulation [141]. In *Vibrio chorelae*, ToxT, the most studied protein in AraC family, was induced by bicarbonate leading to chorela toxin production [142]. AraC is presumably associated with virulence of *Leptospira* since this gene is present only in pathogenic strains. Lman_2698 encodes TolB which is a part of Tol-Pal system. This system is involved in cell integrity in *E. coli* [143]. TolB is one of the most abundant periplasmic proteins in *P. aerugionosa*

and a mutant of this gene showed poor growth and more susceptibility to antibiotic and human serum compared to WT strain [144]. This protein was shown to be a potential vaccine antigen in *Acinetobacter baumannii* [145]. According to a strong upregulation in RT-qPCR results in this study (**Fig.15**), leptospiral TolB should have a crucial role in pathogenesis.

Proteins identified by IVIAT may be related to virulence of bacteria. Lman_2401 encoding sensor histidine kinase was identified in this study, indicating that at least one TCS might be involved in pathogenesis or survival during infection. Sensor histidine kinase is a general component of two-component signal transduction system (TCS), an essential system used by bacteria to repond to the environmental changes. After sensing an enviromental signal, sensor histidine kinase is autophosphorylated, transferring the phosphoryl group to a specific gene regulator that controls gene expression [146]. It was reported that mutation of *lvr* (leptospira virulence regulator) encoding TCS caused *Leptospira* to become attenuated [32]. Lman_3774 encoding metalloendopeptidase was identified in this study. Metalloendoproteases play a role in pathogenesis of *Pseudomonas aeruginosa* and *Helicobacter pylori* [147, 148]. One putative leptospiral metalloendoprotease, Leptallo I, was reported to have enzymatic activity to elastin and showed significant protection in hamsters [149]. The result indicates the involvement of leptospiral metalloendoprotease in organ damage, especially in lung where elastin is abundant [150]. Thus, Lman_3774 may also have a pivotal role in pathogenesis of leptospirosis. Lman_2296 encoding FliI, an important enzyme for flagellum biosynthesis, was identified. Motility is related to virulence of *Leptospira* [107, 151]. FliI interacts with FliH and FliJ to export flagllar proteins [152]. Mutation of *fliI* in Lyme disease *Borrelia burgdorferi* affects flagellum assembly, morphology as well as motility and infectivity [133]. The upregulation of leptospiral *fliI* was confirmed by RT-qPCR (**Fig.15**), ensuring the essential role of motility for *Leptospira* pathogenesis.

Our study indentified a number of proteins involved in metabolism indicating that *Leptospira* might need multiple metabolic pathways to survive and cause infection *in vivo*. Lman_1470 may encode sugar pyridoxal phosphate-dependent aminotransferase, which is similar to NtdA involving in biosynthesis of kanosamine, a

sugar antibiotic synthesized by *Bacillus subtilis* [153]. Although Lman_1941 is predicted to have unknown function in pathogenic strains, it shares 43% amino acid similarity to Acyl-CoA dehydrogenase of nonpathogenic *L. biflexa* serovar Patoc. This enzyme is a major player in lipid catabolism that is an important pathway of *Leptospira* especially during limited nutrients [154]. Mutations of this gene in mycobacteria leaded to attenuation [155]. MutS, a DNA mismatch repair protein, encoded by Lman_3680 was upregulated during infection in rats [156]. MutS maintains genome stability by recognition of mismatched bases and cooperation with MutL and other components in mismatch repairing [157]. In *Neisseria gonorrhoeae,* this protein links to pathogenesis [158]. Glucose galactose epimerase (GalE) encoded by Lman_1449 is one of major enzymes in galactose metabolism and is involved in O-antigen and biofilm synthesis [159-161]. These results suggest the significant role of galactose metablism during infection.

IVIAT is an efficient method to identify genes that specifically express *in vivo*. Therefore, virulence associated genes that constitutively express both *in vitro* and *in vivo* are excluded by this method. For example, LA3961, Loa22, and LA2372 which have been previously identified by 2D-PAGE [16] were not identified in this study. Moreover, LipL32, the most abundant protein of pathogenic *Leptospira* that express during infection [162, 163], and have been used as an antigen for vaccine as well as diagnostic tests [111, 164, 165], could not be detected in this study. It is possible that antibodies against these proteins were removed from pooled serum by *in vitro* expressed antigens during the antibody adsorption step. Moreover, LigA, the most promising vaccine candidate [112, 166], was upregulated under *in vivo* conditions [121, 167] but was not detected by IVIAT. We found that *in vitro* expression of LigA was strain specific. LigA was expressed *in vitro* in *L. interrogans* serovar Manilae as shown previously by 2D-PAGE [16], whereas we could not detect LigA in *in vitro* grown *L. interrogans* serovar Pomona (data not shown). Therefore, antibodies against LigA might be removed by *L. interrogans* serovar Manilae M1352 used for adsorption.

Although we modified IVIAT to detect surface/outer membrane proteins, some cytoplasmic proteins, such as Lman_2562 encoding dehydrogenase, Lman_2523 encoding hydrolase or acryltransferase, and Lman_0879 encoding ATP-

dependent DNA ligase, were identified. Presumably, these proteins might be released during cell lysis in the hosts to induce antibody production. In addition, although these proteins are likeky expressed during *in vitro* grown condition, they might be higly upregulated *in vivo* or highly immunogenic. Thus, antibodies against these proteins were not completely adsorbed by cell lysates of *in vitro* grown M1352.

This study provided a great opportunity to identify novel vaccine candidates that have not been studied in *Leptospira* before. We identified 19 screen *ivi* proteins of M1352 that might not only induce humoral immune response, but also involve in cross-potection against heterologous serovars.

In conclusion, we identified 19 *ivi* proteins of Live M1352 that may play a role in cross-serovar protection in this study. The *in vivo* upregulation of 4 genes including *tolB*, *araC*, *fliI*, and *Lman_3156* were confirmed by RT-qPCR. Further investigations are required to select and test these *ivi* proteins as potential vaccine candidates for protective efficacy against leptospirosis as well as their role in pathogenesis of leptospirosis.

SECTION III

Conclusion

Part I:

To investigate the role of *csrA* as a global gene regulator in *Leptospira*, *csrA* mutant, *csrA* complemented mutant, and *csrA* overexpressing strains of *L. biflexa* serovar Patoc, as well as *csrA* overexpressing strain of *L. interrogans* serovar Manilae, were constructed in this study. In comparison to wild type, *csrA* mutant of *L. biflexa* serovar Patoc displayed poor growth under starvation condition. However, complementation of *csrA* could only partially restore the phenotype. The *csrA* mutant also increased susceptibility to stress caused by H_2O_2 and EtBr. Although no alteration in motility was observed in both *csrA* mutant and *csrA* overexpressing *L. biflexa* serovar Patoc, *flaB2* and *flaB3* showed their potential target of CsrA due to significant upregulation in the *csrA* mutant but significant downregulation in *csrA* overexpressing strain. In contrast, *csrA* overexpressing strain of *L. interrogans* serovar Manilae had poor motility on soft agar and significant upregulation of *flaB1*, *flaB2*, and *flaB4*. We also showed the specific binding of recombinant CsrA of *L. interrogans* serovar Manilae to the 5' untranslated region of *flaB4* transcript. In conclusion, our data suggested the role of *CsrA* as a global regulator in *Leptospira* spp. Interestingly, the mechanisms of action and gene targets of CsrA might be different between non-pathogenic and pathogenic *Leptospira* strains.

Part II:

To identify cross-protective antigens against *Leptospira* using IVIAT, nineteen *ivi* genes of an LPS mutant *L. interrogans* serovar Manilae strain M1352 (M1352) were identified. All *ivi* genes are conserved in M1352 and *L. interrogans* serovar Pomona, which was used for heterologous challenge. Thus, these proteins might be responsible for cross-protection. Three identified genes including *tolB*, *araC*, and *Lman_3156* were predicted to encode putative outer membrane proteins. *In vivo* upregulation of 4 genes including *tolB*, *araC*, *flil*, and *Lman_3156* was confirmed by

RT-qPCR. This work reported novel proteins that may be the targets for vaccine development or virulence factors of *Leptospira*.

Limitations

1. Growth under starvation is most likely to be the specific phenotype regulated by CsrA of *L. biflexa* serovar Patoc because the mutant showed poor growth under such condition and the homologous complemented strain could partially restore this phenotype. However, specific metabolism pathways regulated by CsrA have not been identified in this study. Additionally, heterologous complementation with *csrA* of *L. interrogans* serovar Manilae fully restored the phenotype. Presumably, *csrA* of different *Leptospira* strains uses different mechanism to control gene expression. However, this question remains to be examined.

2. We were unable to obtain the *csrA* mutant in pathogenic strain of *L. interrogans* serovar Manilae. In this study, only *csrA* overexpressing strain was constructed. Therefore, direct effect of csrA and gene targets might not be fully investigated in the pathogenic strain.

3. Using IVIAT to identify vaccine antigens may lose some *in vitro* expressed antigens that are responsible for protection. In addition, the involvement of identified *ivi* proteins in cross protection has not been confirmed.

Further studies

1. To determine the role of *csrA* as a global regulator by transcriptomics or proteomics study and possible targets of *csrA* will be identified.

2. To investigate the association of *csrA* in other aspects of pathogenesis in pathogenic *Leptospira* that have not been examined in this study.

3. To investigate the cross-protective efficacy of identified *ivi* proteins as well as their roles in pathogenesis in animal models.

4. To investigate if *csrA* can regulate *ivi* genes obtain from our study.

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