YebC regulates variable surface antigen VlsE expression and is required for host immune evasion in *Borrelia burgdorferi*

Yan Zhang, Tong Chen, Sajith Raghunandan, Xuwu Xiang, Jing Yang, Qiang Liu, Diane G. Edmondson, Steven J. Norris, X. Frank Yang, Yongliang Lou

1 Wenzhou Key Laboratory of Sanitary Microbiology, Key Laboratory of Laboratory Medicine, Ministry of Education, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou, China, 2 Optometry and Eye Hospital and School of Ophthalmology, School of Biomedical Engineering, Wenzhou Medical University, Wenzhou, China, 3 Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana, United States of America, 4 Department of Pediatrics, Division of Medical Genetics, Duke University, Durham, North Carolina, United States of America, 5 Department of Anesthesiology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China, 6 Department of Pathology and Laboratory Medicine, UTHealth Medical School, Houston, Texas, United States of America

☯ These authors contributed equally to this work.

* xfyang@iu.edu (XFY); lyl@wmu.edu.cn (YL)

Abstract

*Borrelia burgdorferi*, the Lyme disease pathogen causes persistent infection by evading the host immune response. Differential expression of the surface-exposed lipoprotein VlsE that undergoes antigenic variation is a key immune evasion strategy employed by *B. burgdorferi*. Most studies focused on the mechanism of VlsE antigen variation, but little is known about VlsE regulation and factor(s) that regulates differential *vlsE* expression. In this study, we investigated BB0025, a putative YebC family transcriptional regulator (and hence designated BB0025 as YebC of *B. burgdorferi* herein). We constructed yebC mutant and complemented strain in an infectious strain of *B. burgdorferi*. The yebC mutant could infect immunocompromised SCID mice but not immunocompetent mice, suggesting that YebC plays an important role in evading host adaptive immunity. RNA-seq analyses identified *vlsE* as one of the genes whose expression was most affected by YebC. Quantitative RT-PCR and Western blot analyses confirmed that *vlsE* expression was dependent on YebC. *In vitro*, YeBC and VlsE were co-regulated in response to growth temperature. In mice, both *yebC* and *vlsE* were inversely expressed with *ospC* in response to the host adaptive immune response. Furthermore, EMSA proved that YebC directly binds to the *vlsE* promoter, suggesting a direct transcriptional control. These data demonstrate that YebC is a new regulator that modulates expression of *vlsE* and other genes important for spirochetal infection and immune evasion in the mammalian host.
Author summary

The Lyme disease pathogen evolves strategies to subvert host immune responses to cause persistent infection. Antigen variation is a common strategy employed by some pathogens to escape immune recognition and clearance. The VlsE antigen variation system, discovered in 1997, is one such mechanism by which *Borrelia burgdorferi*, evades host immune responses. *vlsE* expression increases concomitantly with downregulation of the major immunodominant surface lipoprotein OspC in response to host adaptive immune response activation. But factors that regulate the differential expression of *vlsE* have not been well studied. In present study, we identified a key transcription factor, YebC (BB0025), that regulates *vlsE* expression. A mutant lacking YebC has decreased levels of VlsE and was attenuated in immunocompetent mice but was virulent in immunocompromised mice. Identification of YebC as a regulator for *vlsE* sets the foundation to further study how the pathogen senses the immune pressure to activate the VlsE antigenic variation system and provides a potential therapeutic target to combat persistent Lyme disease.

Introduction

Lyme disease is the most commonly reported arthropod-borne infection in the United States and Europe, and is also found in Asia [1]. The causative agents of Lyme disease are members of the genus *Borrelia* (including *B. burgdorferi*, *B. garinii*, and *B. afzelii*), which are transmitted to mammals via the bite of *Ixodes* ticks. During the transmission and colonization in both tick vectors and mammalian hosts, *B. burgdorferi* dramatically regulates its gene expression [2–6]. In the past two decades, several key regulators/pathways have been identified that govern differential expression of *B. burgdorferi* during the tick-mammal transmission. These include two sets of two-component systems, with each modulating the adaptation to each of the two hosts [5]. Hk1/Rrp1, a c-di-GMP producing system, controls spirochete’s adaptation to the tick vector [7–13], whereas Hk2/Rrp2 is essential for *B. burgdorferi* to establish infection in the mammalian host [14–17]. Rrp2, along with transcriptional activator BosR and repressor BadR, activates the RpoN-RpoS (σ^54-σ^S) sigma cascade, which in turn controls the production of OspC and several virulence factors [14–28]. Several additional regulators have been identified that differentially regulate gene expression during the tick-mammal transmission including DsrA [29], Hfq [30], Hbb [31,32], CsrA [33–35], BpuR [36], EbfC [37], BpaB [38], SpoVG [39], BBD18 [40], LptA [41], BadP [42] and DksA [43,44].

Differential gene expression of *B. burgdorferi* is vital to its transition from the early phase to the persistent phase of mammalian infection. In the first week of infection, expression of *ospC*, which is essential for the early stage of spirochetal infection, is high [45–47]. As a highly immunogenic surface lipoprotein, OspC levels become downregulated as the host adaptative immune response is activated [48, 49]. Meanwhile, another surface-exposed lipoprotein, VlsE, which is structurally similar to OspC but antigenically variable, is upregulated [47,49,50]. Antigen variation of VlsE is achieved via gene conversion that occurs between the expressing *vlsE* locus and the adjacent 15 silent cassettes, each of which contains six VlsE antigenic variable regions [50–52]. Spirochetes lacking *vlsE* are able to maintain persistent infection in immunocompromised mice, but are unable to sustain infection in immunocompetent mice [53–57]. Despite the importance of VlsE in immune evasion, very little is known about the mechanism of differential expression of *vlsE* [51,52].

*B. burgdorferi* has a reduced genome with relatively few known or predicted transcriptional regulators [58]. BB0025, originally assigned as a hypothetical protein, recently was annotated...
as a putative YebC/PmpR family DNA-binding transcriptional regulator (TACO1 family, pfam PF01709) in the UniProt database (Fig 1). Herein, we designated BB0025 as YebC of *B. burgdorferi*. Although functions of this group of proteins are largely unknown, several studies suggest that they play important roles in gene regulation and pathogenesis. In *Pseudomonas aeruginosa*, PmpR is involved in regulation of the quinolone signal (PQS) system and of pyocyanin production [59]. Disruption of yebC in *Escherichia coli* resulted in reduced survival upon on exposure to extreme ionizing radiations [60]. In *Lactobacillus*, YebC was shown to regulate proteolytic activity by binding to the promoter region of genes involved in proteolysis [61]. More recently, YebC of *Edwardsiella piscicida* was shown to control virulence by directly binding to the promoter and activating its Type III secretion system [62]. In current study, we discovered that YebC of *B. burgdorferi* is a new regulator that controls expression of *vlsE* and other genes important for mammalian infection of *B. burgdorferi*.

Fig 1. Alignment of YebC of *B. burgdorferi* (BB0025) with other members of YebC/PmpR family transcriptional regulators. (A), YebC of *B. burgdorferi* was aligned with three other YebC/PmpR family proteins (TACO1 family, pfam PF01709), YebC from *E. coli* (Ec), PmpR from *P. aeruginosa* and YebC from *Edwardsiella piscicida* (Ep). Multiple alignment was generated with CLUSTALW (https://www.genome.jp/tools-bin/clustalw) and the figure was draw with BoxShade (https://embnet.vital-it.ch/software/BOX_form.html). (B), Homology model for *B. burgdorferi* YebC. The model was generated using the crystal structure of *E. coli* YebC (1KON) as template and SWISS-MODEL program (https://swissmodel.expasy.org/interactive/HvXRyP).
Results

Construction of a yebC mutant and the complemented strain

Sequence analyses revealed the BB0025 belongs to YebC/RmpR transcriptional regulator family (Fig 1A). Structural modeling of B. burgdorferi YebC based on the crystal structure of E.coli YebC demonstrated strong structural similarity to E.coli YebC (Fig 1B). To investigate the function of YebC, we constructed a yebC mutant by allelic exchange in the low-passage, infectious strain of B. burgdorferi strain B31 clone 5A4NP1 [63]. A suicide vector pCT007 was constructed with an aaddA gene (which confers streptomycin-resistance) with flanking upstream and downstream regions of yebC (Fig 2A), and transformed into 5A4NP1. Streptomycin-resistant Borrelia transformants were analyzed by PCR for confirming yebC deletion (Fig 2B). One of the yebC mutant clones that had plasmid profiles identical to that of 5A4NP1 was chosen for complementation with pCT016, a pBSV2-derived shuttle vector with a gentamicin-resistance cassette carrying a wild-type copy of yebC with its native promoter (Fig 2C). A complemented clone that had an identical endogenous plasmid profiles to that of the yebC mutant was selected for further study (Fig 2C). The RT-PCR result confirmed that the yebC mutant no longer expressed yebC, and the complemented strain restored yebC expression (Fig 3). In addition, RT-PCR confirmed that deletion of yebC did not affect expression of the adjacent genes bb0024 and bb0026 (Fig 3).

The yebC mutant was not able to infect immunocompetent mice

During in vitro cultivation, the yebC mutant did not show any significant difference in growth when compared to parental strain. To examine the yebC mutant’s phenotype in vivo, groups of

Fig 2. Construction of the yebC mutant and the complemented strain. (A) Strategy for constructing the yebC mutant. WT: genomic context of yebC in the parent strain, 5A4NP1 (referred to here as WT). pCT007: the suicide vector used for inactivation of yebC. ΔyebC: the yebC mutant. Arrows indicate the primers used for PCR analyses. (B) PCR analyses of the wild-type (W) and the yebC mutant (M) strains. The specific primer pairs used are indicated at the top. (C) Endogenous plasmid profiles of each strain by multiplex PCR analyses as previously described [82]. cp, circular plasmid; lp, linear plasmid. Letters on the left indicate the bands corresponding to each endogenous plasmid that was defined previously for the B. burgdorferi strain B31 genome [58, 84]. ★ indicates the band corresponding to plasmid C (cp9) that is missing in all three strains.

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C3H/HeN mice were needle inoculated with the 5A4NP1 parent strain (hereafter referred to as wild-type strain), the yebC mutant, or the complemented strain. Ear punch biopsies were collected at 2, 3, 4 weeks post-infection and cultured in BSK-II medium for the presence of spirochetes. At 4-weeks post-infection, all mice were sacrificed and several mouse tissues including ear, joint, heart, skin, and bladder were collected and cultured. As shown in Table 1, mice inoculated with the wild type or the complemented strain were culture-positive at each time point of post-infection with two different doses (1×10^5 or 1×10^6 spirochetes/mouse). No mice were infected with 1×10^5 of the yebC mutant (Table 1). However, at high dose of infection (1×10^6), after a prolonged culture of mouse tissues in BSK-II medium (>2 weeks), two mice infected with the yebC mutant showed culture positive for some tissues, while remaining eight were culture negative (Table 1). These data indicate that the yebC mutant has a defect in establishing infection in immunocompetent mice.

We further examined whether the yebC mutant could infect immunocompromised mice. Groups of SCID mice were needle inoculated with wild-type strain, the yebC mutant, or complemented strain with a dose of 1×10^6 spirochetes per mouse. All mice infected with wild type or the complemented strain were culture positive at all time points of post infection (Table 2). No mice yielded positive cultures at 2 weeks post-infection with the yebC mutant, 3 out of 6 mice were culture positive at 3 weeks post infection, and all mice were culture positive at 4 weeks post infection. These observations suggest that the yebC mutant was able to establish infection in immunocompromised mice despite a delayed infectious course.

**Transcriptome analyses of the yebC mutant**

To investigate the molecular mechanisms underlying the requirement of YebC for mammalian infection, we sought to identify YebC-regulated genes by RNA sequencing analyses. Wild-type 5A4NP1 and the yebC mutant were cultivated in BSK-II at 37˚C and harvested at the mid-logarithmic growth. Comparison of the transcriptomes of the wild type and yebC mutant revealed a total of 33 genes whose expressions were either up- or down-regulated by YebC (>2.5-fold) under
standard *in vitro* culture conditions. Among these, 21 genes were positively regulated by YebC (Table 3), whereas 11 genes were negatively regulated by YebC (Table 4). Most of differentially regulated genes are located on various endogenous plasmids, especially on the redundant cp32 circular plasmids. Genes having >2.5-fold changes of expression were highlighted in the volcano plot, and genes with > 3-fold changes were highlighted and labeled (Fig 4). One of the genes most positively regulated by YebC was *vlsE*, which showed 6.43-fold reduction in the *yebC* mutant.

### Verification of genes positively or negatively regulated by YebC

qRT-PCR analyses were performed to validate the expression of YebC-regulated genes identified by RNA-seq. Two positively regulated genes that are important for infection, *vlsE* and

### Table 1. Infection of the *yebC* mutant in immunocompetent mice (C3H/HeN).

| Strains and infection duration | No. cultures positive/No. tested | No. infected/Total No. of mice |
|-------------------------------|---------------------------------|-------------------------------|
|                               | Ear | Joint | Heart | Skin | Bladder |                               |
| 5A4NP1 (1 × 10^5, 1 × 10^6)  |     |       |       |       |         |                               |
| 2 weeks                       | 5/5 |       |       |       | 5/5     |                               |
| 3 weeks                       | 5/5 |       |       |       | 5/5     |                               |
| 4 weeks                       | 5/5 | 5/5   | 5/5   | 5/5   | 5/5     | 5/5                           |
| △*yebC* (1 × 10^5)           |     |       |       |       |         |                               |
| 2 weeks                       | 0/5 |       |       |       | 0/5     |                               |
| 3 weeks                       | 0/5 |       |       |       | 0/5     |                               |
| 4 weeks                       | 0/5 | 0/5   | 0/5   | 0/5   | 0/5     | 0/5                           |
| △*yebC* (1 × 10^6)           |     |       |       |       |         |                               |
| 2 weeks                       | 0/10|       |       |       | 0/10    |                               |
| 3 weeks                       | 0/10|       |       |       | 0/10    |                               |
| 4 weeks                       | 0/10| 0/10  | 1/10  | 2/10  | 2/10    | 2/10                          |
| *yebC*Δ*com* (1 × 10^5, 1 × 10^6) |     |       |       |       |         |                               |
| 2 weeks                       | 3/5 |       |       |       | 3/5     |                               |
| 3 weeks                       | 5/5 |       |       |       | 5/5     |                               |
| 4 weeks                       | 5/5 | 5/5   | 5/5   | 5/5   | 5/5     | 5/5                           |

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### Table 2. Infection of the *yebC* mutant in immunodeficient mice (SCID).

| Strains and infection duration | No. cultures positive/No. tested | No. infected/Total No. of mice |
|-------------------------------|---------------------------------|-------------------------------|
|                               | Ear | Joint | Heart | Skin | Bladder |                               |
| 5A4NP1 (parent strain)        |     |       |       |       |         |                               |
| 2 weeks                       | 6/6 |       |       |       | 6/6     |                               |
| 3 weeks                       | 6/6 |       |       |       | 6/6     |                               |
| 4 weeks                       | 6/6 | 6/6   | 6/6   | 6/6   | 6/6     | 6/6                           |
| △*yebC*                      |     |       |       |       |         |                               |
| 2 weeks                       | 0/6 |       |       |       | 0/6     |                               |
| 3 weeks                       | 3/6 |       |       |       | 3/6     |                               |
| 4 weeks                       | 6/6 | 6/6   | 6/6   | 6/6   | 6/6     | 6/6                           |
| *yebC*Δ*com*                 |     |       |       |       |         |                               |
| 2 weeks                       | 3/3 |       |       |       | 3/3     |                               |
| 3 weeks                       | 3/3 |       |       |       | 3/3     |                               |
| 4 weeks                       | 3/3 | 3/3   | 3/3   | 3/3   | 3/3     | 3/3                           |

* Dose of infection: 1 × 10^6 spirochetes per mouse

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rpoS (bb0771), were examined. Consistent with RNA-seq results, qRT-PCR analyses also showed that vlsE expression was under the control of YebC (Fig 5). Even though rpoS showed 3.14-fold decrease of expression in the yebC mutant in RNA-seq, no significant difference was observed in qRT-PCR analysis. Three top negatively regulated genes, bbi16, bba49, and bba51, were assessed by qRT-PCR. The result confirmed that all the three genes were negatively regulated by YebC (Fig 5).

| Locus | Common Name | Location     | WT/ΔyebC |
|-------|-------------|--------------|----------|
| BB_Q37 | hypothetical protein | lp56        | 7.36     |
| BB_F0041 | outer surface protein VlsE1 | lp28-1 | 6.43     |
| BB_P29 | hypothetical protein | cp32-1 | 4.64     |
| BB_L36 | protein BppA | cp32-8 | 3.92     |
| BB_R32 | hypothetical protein | cp32-4 | 3.70     |
| BB_0844 | lipoprotein | chromosome | 3.39     |
| BB_0771 | RNA polymerase sigma factor RpoS | chromosome | 3.14     |
| BB_L31 | hypothetical protein | cp32-8 | 3.08     |
| BB_0799 | hypothetical protein | chromosome | 3.01     |
| BB_L32 | PF-32 protein | cp32-8 | 2.99     |
| BB_K07 | lipoprotein | lp36 | 2.96     |
| BB_N02 | hypothetical protein | cp32-9 | 2.85     |
| BB_L29 | hypothetical protein | cp32-8 | 2.81     |
| BB_0438 | DNA polymerase III subunit beta | chromosome | 2.77     |
| BB_0800 | transcription termination/antitermination protein NusA | chromosome | 2.74     |
| BB_0801 | translation initiation factor IF-2 | chromosome | 2.72     |
| BB_F01 | hypothetical protein | lp28-1 | 2.71     |
| BB_F20 | BBF20 | lp28-1 | 2.67     |
| BB_L27 | protein BdrP | cp32-8 | 2.61     |
| BB_S34 | hypothetical protein | cp32-3 | 2.59     |
| BB_R33 | plasmid partition protein, putative | cp32-4 | 2.57     |
| BB_N03 | hypothetical protein | cp32-9 | 2.52     |

*Fold changes of gene expression of the wild-type strain with respect to the yebC mutant (the genes with fold change > 2.50). q (adjusted p value) < 0.05.

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Table 4. RNA-seq analysis identification of genes that are negatively regulated by YebC.

| Locus | Common Name | Location    | WT/ΔyebC |
|-------|-------------|-------------|----------|
| BB_I16 | repetitive antigen A, VraA | lp28-4 | -6.17    |
| BB_R13 | hypothetical protein BB_R13 | cp32-4 | -3.85    |
| BB_A51 | hypothetical protein BB_A51 | lp54 | -3.47    |
| BB_A49 | hypothetical protein BB_A49 | lp54 | -2.91    |
| BB_N14 | hypothetical protein BB_N14 | cp32-9 | -2.90    |
| BB_0603 | integral outer membrane protein p66 | chromosome | -2.70    |
| BB_S17 | hypothetical protein BB_S17 | cp32-3 | -2.70    |
| BB_0240 | glycerol uptake facilitator | chromosome | -2.68    |
| BB_G02 | hypothetical protein | lp28-2 | -2.67    |
| BB_t31 | NA          |         | -2.65    |
| BB_0001 | hypothetical protein BB_0001 | chromosome | -2.57    |

*Fold changes of gene expression of the wild-type strain with respect to the yebC mutant (the genes with fold change < -2.50). q (adjusted p value) < 0.05.

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YebC controls VlsE production in vitro

To determine whether YebC regulates VlsE protein levels, immunoblot analysis was performed with cells lysates from wild type, the yebC mutant, the complemented strain, and a B. burgdorferi strain lacking the entire plasmid lp28-1 that harbors vlsE. The result showed that the VlsE protein was dramatically reduced in the yebC mutant and levels were restored in the complemented strain (Fig 6A). Previous reports showed that environmental cues can influence vlsE expression, although disparate results were observed [64–66]. We thus further investigated VlsE and YebC levels under various growth conditions. The result showed that cell density did not dramatically affect VlsE expression at the protein level, and elevated pH (pH 8.0) moderately increased VlsE production (Fig 6B). Consistent with previous reports,
ambient temperature (23°C) dramatically increased the level of VlsE, which is inversely correlated with the level of OspC (Fig 6C) [64,65]. The level of YebC displayed a similar pattern of temperature-dependent regulation as that of VlsE, showing an increased production when grown at ambient temperature (Fig 6C).

**Correlative expression of yebC and vlsE in mice**

vlsE is differentially expressed during mammalian infection: at the early stage of spirochetal infection, vlsE level is low whereas ospC is high. As the host adaptive immune response is
activated, expression of vlsE increases when ospC becomes downregulated [45–50,53]. To gather further evidence that YebC modulates vlsE expression, vlsE, ospC, and yebC transcript levels were examined during the course of mammalian infection. Similar to what was reported previously, ospC expression was high at 1 week post-infection in skin at the site of inoculation, and then became undetectable in heart and joint tissues during persistent infection (1, 2, 3 months post infection) (Fig 7A). On the other hand, vlsE expression was low in skin at the site of inoculation at 1 week post-infection, and then dramatically increased (5 to 10-fold increase) at 1 to 3 months of post-infection (Fig 7B). The result showed that yebC displayed a similar pattern of differential expression as vlsE during infection (Fig 7C), supporting the hypothesis that YebC modulates vlsE expression during spirochete’ transition from early to persistent infection in mammals.

YebC directly binds to the vlsE promoter

Since YebC is predicted to be a transcriptional regulator, we postulate that YebC may directly bind to vlsE promoter to regulate its expression. Accordingly, recombinant YebC proteins were purified and electrophoretic mobility shift assays (EMSA) were performed using various concentrations of YebC proteins (ranging from 50 nM to 750 nM) and with 50 nM of 200-bp DNA fragment of the vlsE promoter region (Fig 8A). There is a 100-bp inverted repeat sequence overlapping the -35 region of the vlsE promoter in B. burgdorferi B31 [67] (Fig 8A). The inverted repeat DNA may form cruciform and higher form structures [68]. Interestingly, in the absence of YebC, two DNA bands were observed with the purified PCR DNA fragment from vlsE promoter under EMSA conditions (Fig 8B, first lane), but not with the control DNA fragment from the bosR promoter (Fig 8C, first lane). The lower band corresponded to the 200
bp linear form of the DNA fragment (Lin-DNA). We labeled the higher DNA band as High-DNA. Whether it is cruciform remains to be determined. A protein-DNA interaction was observed at 50 nM of protein concentration (1:1 of protein/DNA ratio) (Fig 8B). A larger protein-DNA complex was formed when protein concentrations increased (100 nM to 750 nM). No binding was evident when YebC protein was incubated with a DNA fragment from the \textit{bosR} promoter (Fig 8C), nor when BosR protein incubated with the \textit{vlsE} promoter (Fig 8D), indicating specific binding of YebC to \textit{vlsE} promoter.

\section*{Discussion}

One of the unique features of \textit{B. burgdorferi} infection is the ability of the spirochete to evade immune responses and maintain a persistent infection in the mammalian host [1,52]. Antigen variation and differential expression of \textit{VlsE} plays a critical role in this process [46,50–52]. Over the past two decades, while much information regarding \textit{Borrelia} gene regulation has been revealed, limited is known about regulation of \textit{vlsE} expression [2,6,49,51,65–67]. In this study, we provide several lines of evidences to support the notion that YebC, a member of YebC/PmpR transcription factor, modulates differential expression of \textit{vlsE}. First, genome-wide RNA-seq analysis showed that \textit{vlsE} is one of the most regulated genes and its expression was dramatically reduced upon inactivation of \textit{yebC} (Fig 4). Second, both \textit{vlsE} and \textit{yebC} expression were induced by ambient temperature when grown in vitro (Fig 6). YebC directly binds to the \textit{vlsE} promoter (Fig 7). Third, the expression of \textit{vlsE} and \textit{yebC} correlated well during the course of infection in mice, consistent with the hypothesis that YebC regulates \textit{vlsE} expression (Fig 7). Forth, YebC directly binds to the \textit{vlsE} promoter (Fig 8). Lastly, the infection data showed that the \textit{yebC} mutant could not infect immunocompetent mice efficiently but was still able to infect immunocompromised mice (Tables 1 and 2), indicating the essential role of \textit{yebC} in evading the host adaptive immune system.
The YebC/PmpR family of proteins are widespread in all prokaryotes and eukaryotes [69]. Several recent structural and functional studies indicate that these proteins function as transcriptional regulators [69,70]. Bioinformatic analyses reveal that YebC family proteins can be divided into two subgroups [69]. Subgroup I yebC genes are adjacent to ruvABC, which encode for Holliday junction branch migrases and resolvase, suggesting that this subgroup of YebC proteins may be involved in regulating the process of Holliday junction resolution during DNA recombination. B. burgdorferi has a subgroup I YebC, and the yebC gene (bb0025) is adjacent to ruvAB (bb0022-bb0023). Interestingly, two groups independently reported that the mechanism underlying VlsE antigen variation is unique: unlike other bacteria, B. burgdorferi does not require RecA or other proteins generally involved in DNA recombination, repair, or replication, rather, it requires RuvAB for efficient vlsE recombination [71, 72]. Thus, along with the finding on YebC in present study, we postulate that the ruvAB-yebC gene cluster of B. burgdorferi plays a key role in modulating both processes of gene recombination and differential expression of vlsE.

Limited knowledge is known about the mechanism underlying differential expression of vlsE. Jutras et al. reported that SpoVG, a DNA-binding protein, binds to a sequence in the vlsE coding region of B. burgdorferi B31 [39]. As the binding sequence is located near the recombination site within the coding region of the vlsE gene, it may be involved in vlsE recombination rather than vlsE expression [39]. Drecketrah et al. performed RNA-seq analysis and showed that RelBbu, a bifunctional synthetase/hydrolase (RelA/SpoT homolog) that is responsible to synthesize and hydrolyze the alarmones guanosine tetraphosphate and guanosine pentaphosphate ([p] ppGpp), upregulates vlsE expression. They also demonstrated that vlsE was upregulated upon starvation [66]. However, how RelBbu regulates vlsE expression remains to be elucidated. In this study, we showed that YebC directly binds to the vlsE promoter (Fig 7B). Hudson et al. first reported the presence of a long inverted repeat sequence overlapping with the -35 sequence of the promoter in B. burgdorferi B31 genome [67]. Recently, more IR sequences are reported in other B. burgdorferi strains [52]. The Function of this IR in vlsE expression remains unknown. These inverted repeats can form cruciform structures that have been recognized to play important biological roles [73]. Interestingly, we observed two bands of the vlsE promoter DNA under native conditions, and a single band on an agarose gel for the purified PCR product of the vlsE promoter fragment. Given that the lower band ran at 200-bp position and was the linear form of DNA, we postulate that the higher band (High-DNA) observed under the native condition could be cruciform DNA. Obviously, further direct structural evidence such as electron microscopy is needed to demonstrate that it is indeed the cruciform DNA. Nevertheless, one important observation is that both linear form and higher form of the DNA fragment were diminished when incubated with YebC (Fig 7B). We envision three scenarios based on this observation. 1, YebC is capable of binding to both linear form and cruciform of the vlsE promoter; 2, YebC can only bind to the linear form but stabilize it such that the equilibrium between linear and cruciform structures favors the linear form; 3, YebC can only bind to the cruciform structure and the linear form is eventually converted to cruciform. Given that YebC is a putative transcriptional activator that positively regulates vlsE expression, the second scenario makes sense: YebC binds and stabilizes the linear form of the vlsE promoter, drives conversion of cruciform to linear form, releases the -35 promoter region available for transcriptional activation. Further biochemical analysis is warranted to determine whether YebC has the activity to alter the thermodynamics of between linear and cruciform DNA structure.

Several factors, such as anti-OspC antibody, have been implied involving in regulation of vlsE expression [51]. The expression of vlsE increases as B. burgdorferi transitions from the early to persistent stages of mammalian infection, coinciding with the downregulation of ospC [47]. However, in SCID mice or B-cell-deficient mice, the transcript levels of both vlsE and ospC are high [47]. Furthermore, treatment of SCID mice with monoclonal antibodies against
OspC resulted in dramatic decreases in ospC mRNA levels and increases in vlsE transcripts. These observations suggest that the induction of antibody responses against OspC may lead to the reciprocal regulation of ospC and vlsE observed in mice [47]. Co-culture of B. burgdorferi with endothelial cells was also shown to increase vlsE expression [67]. In addition, interferon γ expression was also reported to promote vlsE recombination, and possibly vlsE expression [74]. Whether yebC expression is in some manner linked to these host factors remain to be determined. In present study, RNA-seq analysis showed no influence of ospC in vivo with the fact that a yebC mutant's infection phenotype. The role of VlsE of B. burgdorferi in evading host innate immune response was evidenced with the fact that a B. burgdorferi strain lacking the entire lp28-1 plasmid or an intact vls locus cannot sustain infection in wild-type mice at 2 week post-infection, but can maintain persistent infection in SCID mice [55–57,75,76]. The present study also showed that the yebC mutant had similar difference in infectivity in wild-type mice and SCID mice as that of the vlsE mutant (Tables 1 and 2), suggesting that an defect in vlsE expression is the key contributor to the yebC mutant's infection phenotype. The yebC mutant could not establish infection in immunocompetent mice even at a dose of 1 X 10⁵ spirochetes/mouse, suggesting that YebC is essential for mammalian infection. We did observe that very few tissue samples became positive in mice inoculated with 1 X 10⁶ spirochetes/mouse, likely due to that fact that yebC deletion does not completely abolish vlsE expression and recombination during infection, as shown in vitro (Fig 6). The avirulent phenotype prevents us to examine vlsE expression in the yebC mutant in immunocompetent mice. The yebC mutant was infectious in SCID mice. Of note, the yebC mutant had delayed infection in SCID mice (Table 2). It is possible at lower dose of inoculation that the yebC mutant may show avirulent phenotype even in SCID mice since YebC may other functions in addition to VlsE. Regardless, because differential expression of vlsE and ospC does not occur in SCID mice, these mice are not suited for studying vlsE regulation. Therefore, we relied on the correlation of yebC and vlsE expression in wild-type spirochetes during the course of infection, which supports the hypothesis that YebC regulates vlsE expression (Fig 7).

It is reported that the vlsE-lacking spirochetes are capable of establishing infection in the early stage of infection in immunocompetent mice, and spirochetes can be detected in blood samples in first 1–2 weeks of infection [45–50,53]. One caveat of present study is that the infectivity of the yebC mutant was not examined at early time point of post-infection. We speculate that the yebC mutant may be deficient in establishing infection in mice even at this early time point, given that YebC regulates additional functions important for infection. In addition to vlsE, RNA-seq results showed that YebC regulates expression more than 33 genes (Tables 3 and 4). Many of these genes are located on the plasmids and have unknown functions. One gene whose function is known to be important for pathogenesis is rpoS, which showed over three-fold reduction in the yebC mutant. RpoS controls expression of ospC, dbpBA and several other genes important for mammalian infection [2,6,17,18]. However, this result could not be confirmed by qRT-PCR analysis (Fig 4). RNA-seq also did not show that the yebC deletion affected ospC and dbpBA expression. Thus, it is unlikely that YebC involves in regulation of the RpoS regulon. Other top positively regulated genes by YebC, including bbq37, bbp29, bbl36 and bbr32, are members of paralogous families whose sequences are highly identical and could not be verified by qRT-PCR. Three top negatively regulated genes, bbi16, bba49, and bba51, were confirmed by qRT-PCR to be negatively regulated genes by YebC (Fig 5). bbi16 is located on lp28-4, encoding VraA (virulent strain-associated repetitive antigen A), a surface antigen that shows partial protection with active immunization [77]. bba49 and bba51 are located on linear plasmid lp54, encoding conserved hypothetical proteins with unknown function [58].
Whether increased expressions of bbi16, bba49 and bba51 in the yebC mutant contributed to the avirulence phenotype remain to be determined.

Recently, Ramsey et al. used transposon insertion sequencing (Tn-seq) for a genome-wide screen and identified several factors important for resistance to NO, H$_2$O$_2$, and TBHP in vitro and YebC (BB0025) was identified as one of the genes that may be involved in H$_2$O$_2$ resistance [78]. Ramsey et al. also performed animal study with groups of pooled Tn mutants and found that bb0025 Tn mutant was attenuated in both mouse strains. Our results using yebC deletion mutant was consistent with previous infection data using pooled Tn mutants. YebC’s role in defending host ROS may also explain why the yebC mutant had a delayed infection in SCID mice observed in this study (Table 2), as SCID mice have both macrophages and neutrophils that produce ROS. In addition, Ramsey et al., also showed that the gp91phox-/- mice lacking phagocyte superoxide production could not rescue the infectivity of the bb0025 Tn mutant [78]. Given that YebC controls vlsE expression discovered herein, the phenotype of the yebC Tn mutant in the gp91phox-/- mice becomes evident.

In summary, this work identified YebC as the transcriptional factor that regulates vlsE gene expression, and it plays a vital role in mammalian infection of B. burgdorferi. This finding opens many exciting opportunities to study the mechanism of host immune evasion by B. burgdorferi. For example, how is YebC itself regulated, and what activates YebC during mammalian infection? How does IR influence YebC to regulate vlsE expression? Does YebC also modulate vlsE antigenic variation and contribute to the avirulent phenotype observed in the yebC mutant? In addition, given that YebC is also important for ROS response and that spirochetes encounter ROS during tick feeding in tick gut and salivary gland, it will be interesting to investigate whether YebC is important for the tick part of enzootic cycle of B. burgdorferi.

Materials and methods

Ethics statement

All animal experiments were approved by the IACUC committee of Indiana University School of Medicine under the protocol number # 11339. All experiments were in accordance with the institutional guidelines.

B. burgdorferi strains and culture conditions

Low-passage, virulent B. burgdorferi strain 5A4NP1 (a gift from Drs. H. Kawabata and S. Norris, University of Texas Health Science Center at Houston) was used in this study. It was derived from wild-type B. burgdorferi strain B31 by inserting a kanamycin resistance marker in a restriction modification gene bbe02 on plasmid lp25 [63]. Spirochetes were cultivated in Barbour-Stoenner-Kelly (BSK-II) medium supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR) [79] at 37°C with 5% CO$_2$. At the time of growth, appropriate antibiotics were added to the cultures with the following final concentrations: 300 μg/ml for kanamycin, 50 μg/ml for streptomycin, and 50 μg/ml for gentamicin. The constructed suicide vector (pCT007) and shuttle vector (pCT016) were maintained in Escherichia coli strain DH5α. The antibiotic concentrations used for E. coli selection were as follows: kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; and gentamicin, 10 μg/ml.

Immunoblot analysis

Spirochetes from mid-log cultures were harvested by centrifuging at 8,000 × g for 10 min and followed by three time washing with PBS (pH 7.4) at 4°C. Pellets were suspended in SDS buffer containing 50 mM Tris-HCl (pH 8.0), 0.3% sodium dodecyl sulfate (SDS) and 10 mM
dithiothreitol (DTT). Cell lysates (10^8 cells per lane) were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (GE-Healthcare, Milwaukee, WI). Membranes were blotted with mouse polyclonal antibody against VlsE (1:1,000 dilution) [76] and monoclonal antibody against FlaB (1:1,000 dilution) [41], and then incubated with goat anti-mouse IgG-HRP secondary antibody (1:1,000; Santa Cruz Biotechnology). Detection of horseradish peroxidase activity was determined by the enhanced chemiluminescence method (Thermo Pierce ECL Western Blotting Substrate) with subsequent exposure to X-ray film.

Generation of the yebC mutant and the complemented strain

To inactivate the yebC in parent strain 5A4NP1, a suicide vector pCT007 was constructed for homologous recombination as following: the regions of DNA corresponding to 1.4 kb upstream and 1.4 kb downstream regions of yebC (Fig 2A) were PCR amplified from 5A4NP1 genomic DNA with primer pairs PRCT017/PRCT018 and PRCT019/PRCT020 (Supplemental S1 Table), respectively. The resulting PCR fragments were then cloned into upstream and downstream of an aadA streptomycin-resistant marker within the suicide vector pMP025 to generate the suicide vector pCT007. pCT007 was confirmed by both restriction enzyme digestion and sequencing, and then was transformed into 5A4NP1 as described previously [80,81]. Streptomycin-resistant Borrelia transformants were analyzed by PCR to confirm the correct yebC deletion (Fig 2B). Plasmid profiles of the confirmed yebC mutant clones were determined by multiplex PCR analyses with twenty pairs of primers specific for each of the endogenous plasmids as reported by Bunikis et al.[82]. One of the yebC mutant clones (BbCT006) that had plasmid profiles identical to those of 5A4NP1 was selected for complementation (Fig 2C). For trans complementation, a shuttle plasmid carrying a native promoter-driven yebC was generated as follows. The yebC regions flanked by BamHI and PstI restriction sites were amplified using primers PRCT092 and PRCT096 and then cloned into the shuttle vector pBSVG [83], resulting in in complementation plasmid named as pCT016. One complementation clone (BbCT009) that had an identical endogenous plasmid profiles to that of 5A4NP1 was selected for further study (Fig 2C).

Mouse infection studies

Four-week-old C3H/HeN mice and C3H/SCID mice (Harlan, Indianapolis, IN) were subcutaneously inoculated with two doses of spirochetes (1×10^5 and 1×10^6) respectively. Ear punch biopsy samples were taken at 2 and 3 weeks post-injection. At 4 weeks post-injection, mice were euthanized, and multiple tissues (i.e. ear, joint, heart, skin and bladder tissues from each mouse) were harvested. All tissues were cultivated in 2 ml of the BSK-II medium (Sigma-Aldrich, St. Louis, MO) containing an antibiotic mixture of phosphomycin (2 mg/ml), rifampin (5 mg/ml), and amphotericin B (250 mg/ml) (Sigma-Aldrich) to inhibit bacterial and fungal contamination. All cultures were maintained at 37˚C and examined for the presence of spirochetes by dark-field microscopy beginning from 5 days after inoculation. A single growth-positive culture was used as the criterion to determine positive mouse infection.

qRT-PCR analyses

For verification of differentially expressed genes by YebC, wild-type B. burgdorferi strain 5A4NP1 (WT), the yebC mutant (Mut) and the complemented strain (Com) were cultured in BSK-II medium at pH 7.5 and harvested at the mid-log phase. RNAs were extracted and subjected to qRT-PCR analyses for expression of vlsE, rpoS, bbi16, bba49, bba51. Primers used were listed in Supplemental S1 Table. For yebC, vlsE and ospC expression in infected mice,
four-week-old C3H/HeN mice were injected with wild-type strain 5A4NP1 at a dose of $1 \times 10^6$ spirochetes per mouse. Mice were euthanized at different time points as indicated and mouse tissues were harvested and homogenized using the FastPrep-24 (MP Biomedicals). Total RNA was isolated using the TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. To reduce trace amounts of DNA contamination, samples were further digested with RNase-free DNaseI (Qiagen), purified using the RNeasy mini kit (Qiagen) and analyzed with NanoDrop Spectrophotometer (Thermo Fisher Scientific). DNA-free RNA was confirmed by PCR amplification for the \textit{B. burgdorferi flaB} gene. cDNA was synthesized using the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa). Given the low levels of bacterial RNA in mouse tissues, the specific primers for each gene target were used for cDNA synthesis instead of random primers. To quantify the transcript levels of genes of interest, an absolute quantitation method was used to create a standard curve for the qPCR assay according to the manufacturer’s protocol (Strategene, La Jolla, CA). Briefly, the PCR product of the \textit{flaB} gene served as a standard template. A series of tenfold dilutions ($10^2$-$10^7$ copies/ml) of the standard template was prepared, and qPCR was performed to generate a standard curve by plotting the initial template quantity against the Ct values for the standards. The quantity of the targeted genes in the cDNA samples was calculated using their Ct values and the standard curve. The samples were assayed in triplicate using a ABI 7000 Sequence Detection System and PowerUp SYBR Green Master Mix (Applied Biosystems). The levels of the target gene transcript were reported as per 100 copies of \textit{flaB}.

\textbf{RNA Sequencing (RNA-seq)}

Wild-type 5A4NP1 and the \textit{yebC} mutant were cultivated in BSK-II at 37°C and harvested at the mid-logarithmic growth. RNA samples were extracted using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Three independent culture samples were used for each strain. Removal of contaminated genomic DNA in the RNA samples was performed using RNase-free DNase I (Promega), and then confirmed by PCR amplification for the \textit{flaB} gene. The concentration and quality of total RNA samples were first assessed using Agilent 2100 Bioanalyzer. A RIN (RNA Integrity Number) of 5 or higher was required to pass the quality control. rRNA removal was performed for each RNA sample (2 μg/sample) using Ribo-Zero rRNA Removal Kit (Bacterial, Illumina), and the RNA samples were then subjected to dual-indexed strand-specific cDNA library synthesis using TruSeq Stranded Total RNA Library Prep Kit (Illumina). Synthesized libraries were assessed for the quantity and size distribution using Qubit and Agilent 2100 Bioanalyzer. Two hundred picomolar pooled libraries were utilized per flow cell for clustering amplification on cBot using HiSeq 3000/4000 PE Cluster Kit and sequenced with 2×75bp paired-end configuration on HiSeq4000 (Illumina) using HiSeq 3000/4000 PE SBS Kit. A Phred quality score (Q score) was used to measure the quality of sequencing. More than 90% of the sequencing reads reached Q30 (99.9% base call accuracy). The sequencing data were first assessed using FastQC (Babraham Bioinformatics, Cambridge, UK) for quality control. Then all sequenced libraries were mapped to the \textit{Borrelia burgdorferi} B31 genome (NCBI, GCA_000008685.2) using STAR RNA-seq aligner with the following parameter: “—outSAMmapqUnique 60”. The reads distribution across the genome was assessed using bamUtils software (from ngsutils). Uniquely mapped sequencing reads were assigned to \textit{Borrelia burgdorferi} B31 refSeq genes using featureCounts (from subread) with the following parameters: “-s 2 -p -Q 10”. Quality control of sequencing and mapping results was summarized using MultiQC. Genes with read count per million (CPM) $> 0.4$ in more than 4 of the samples were kept. The data was normalized using TMM (trimmed mean of M values) method. Differential expression analysis was performed using edgeR. False discovery rate
(FDR) was computed from \( p \) values using the Benjamini-Hochberg procedure. Genes with fold changes greater than 2.5 are listed in Tables 1 and 2. All genes are listed in supplemental file S1 Data. The RNA-seq data set was plotted on a volcano plot with the negative log of the \( q \) value on the y axis and the \( \log_2 \) of the fold change between wild type and the \( yebC \) mutant on the x axis using GraphPad Prism software (Fig 4).

**Electrophoretic mobility shift analyses (EMSA)**

200-bp upstream to \( vlsE \) ORF and 350-bp region upstream to \( bosR \) were PCR amplified using specific sets of primers as listed in Supplementary Table 1. All the EMSA reactions were carried out in a 20 \( \mu L \) reaction mixture containing PCR amplified promoter DNA (50 nM), and various concentrations of recombinant \( YebC \) or \( BosR \) proteins (50 nM to 750 nM). Final 1 X EMSA buffer used for the reaction contained 10 mM Tris (pH 7.5), 1 mM EDTA, 100 mM KCl, 5% glycerol, 0.1 mM dithiothreitol and 0.01mg/ml BSA. Binding reactions were carried out for 30 min at 25˚C. The reaction mix was then loaded on a 5% polyacrylamide gel and electrophoresis was carried out at 35 V at 4˚C and later stained using ethidium bromide. Image was captured using a DigiDoc- Imaging System by UVP.

**Protein modeling**

For molecular modeling of \( B. burgdorferi \) \( YebC \), online tools Iterative Threading Assessment Server9 and SWISS MODEL were used. The pair-wise sequence alignment threshold was set to 70% by default. The template for generating model, \( E. coli \) \( YebC \) (PDB ID: 1KON) was taken from RCSB PDB (https://www.rcsb.org/). Further validation of the model was carried out employing RAMPAGE (http://mordred.bioc.cam.ac.uk/ ~rapper/rampage.php) and ERRAT (http://services.mbi.ucla.edu/ERRAT/). The model was further tested for all structural parameters which includes: MolProbity Score, 2.06; Clash score, 10.47; Ramachandran favored, 94.14%; Ramachandran outliers, 1.46%; Rotamer outliers, 1.46%; ERAT score, 7.

**Supporting information**

S1 Table. Strains, plasmids, and primers used in this study.

(DOCX)

S1 Data. Complete RNA-seq data.

(XLSX)

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**Author Contributions**

**Conceptualization:** X. Frank Yang, Yongliang Lou.

**Data curation:** Yan Zhang, Tong Chen, Sajith Raghunandanan, Xuwu Xiang, Jing Yang, Qiang Liu.

**Formal analysis:** Yan Zhang, Tong Chen, Sajith Raghunandanan, Xuwu Xiang, Jing Yang, Qiang Liu, Diane G. Edmondson, Steven J. Norris.

**Funding acquisition:** X. Frank Yang, Yongliang Lou.

**Investigation:** Yan Zhang, Tong Chen, Sajith Raghunandanan, Xuwu Xiang, Jing Yang, Qiang Liu.
Methodology: Yan Zhang, Tong Chen, Sajith Raghunandanan, Xuwu Xiang, Jing Yang, Qiang Liu.

Project administration: X. Frank Yang, Yongliang Lou.

Supervision: X. Frank Yang, Yongliang Lou.

Writing – original draft: Yan Zhang, Tong Chen, Sajith Raghunandanan, X. Frank Yang, Yongliang Lou.

Writing – review & editing: Sajith Raghunandanan, Diane G. Edmondson, Steven J. Norris, X. Frank Yang.

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