Development of an alkaline phosphatase reporter system for use in the lyme disease spirochete borrelia burgdorferi

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DEVELOPMENT OF AN ALKALINE PHOSPHATASE REPORTER SYSTEM
FOR USE IN THE LYME DISEASE SPIROCHETE BORRELIA BURGDORFERI

by

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A thesis submitted in partial fulfillment of requirements for the Honors in the Major Program in Molecular Biology and Microbiology in the College of Medicine and in the Burnett Honors College at the University of Central Florida Orlando, Florida

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Thesis Chair: Dr. Mollie W. Jewett
Abstract

The use of the periplasmic alkaline phosphatase (PhoA) reporter protein from *E. coli* has been critical for definition of the topology of transmembrane proteins of multiple bacterial species. This report demonstrates development of a PhoA reporter system in *B. burgdorferi*. Codon usage of the *E. coli phoA* in *B. burgdorferi* was analyzed and an optimized version of the gene was obtained. In order to assess the differential activity of the reporter system, two optimized PhoA-fusion construct using *B. burgdorferi* proteins were engineered: one using the periplasmic protein OppAIV and one using the cytoplasmic protein PncA. The activity of PhoA requires periplasmic localization. The periplasmic OppAIV-PhoA fusion as well as the cytoplasmic PncA-PhoA fusion produced detectable PhoA protein in *E. coli* and in *B. burgdorferi*. The periplasmic fusion construct, but not the cytoplasmic fusion construct, resulted in functional alkaline phosphatase (AP) activity in *E. coli*, as observed by blue colonies on agar plates containing a chromogenic substrate for AP. In contrast, both of the fusion constructs produced limited detectable levels of functional alkaline phosphatase activity in *B. burgdorferi*, as observed by yellow color change in liquid protein lysate containing a chromogenic substrate for AP. Development of a PhoA fusion reporter system for use in *B. burgdorferi* will provide a new molecular genetics tool for analyzing the topology of *B. burgdorferi* transmembrane proteins. These types of studies are critical for understanding the function of *B. burgdorferi* transport systems and may identify novel molecular approaches for the treatment of Lyme disease.
Dedications

I dedicate this thesis to all the people who stood by me and supported me during this project and writing process including my parents, my friends, and my lab groupmates. Without you this thesis would never have been written. I also want to dedicate this to my mentor and thesis Chair Dr. Mollie Jewett, without whose help and guidance none of this would have been possible.
Acknowledgements

This work was supported by UCF Laboratory Start-Up funds to MWJ. I acknowledge Dr. Jewett, Dr. Teter, and Dr. Miles for serving on my committee and providing invaluable feedback. I thank lab members Sunny Jain, Tisha Choudhury, and Micah Halpern for their continuing support on this project. I also thank the members of Dr. Travis Jewett’s laboratory for feedback and input during joint lab meetings.
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Introduction

Lyme disease is the leading vector-borne bacterial disease in the world. Early symptoms include fatigue, headache, and fever, but if left untreated the disease can slowly progress into arthritis, meningitis, carditis, and a host of other serious symptoms (20). This disease is caused by tick bite transmission of the spirochete *Borrelia burgdorferi*. As this bacterium moves through the environments of the arthropod and the mammalian tissue, different genes are expressed in order to adapt to the differing environments. Little is known about the genetic components of the bacteria that are required for infection and pathogenesis (20).

Molecular genetics tools have been powerful resources for understanding the molecular basis of disease in other bacterial pathogens. Recently there have been great strides made in the ability to genetically manipulate *B. burgdorferi* including development of selectable markers, shuttle vectors, reverse genetics by targeted homologous recombination, forward genetics using transposon mutagenesis, and inducible promoter systems (20). Other reporter systems currently in use include chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), luciferase (20) and most recently LacZ (11).

*Borrelia burgdorferi* has a reduced genome, lacking the ability to synthesize vital nutrients such as nucleotides, amino acids, enzyme cofactors and fatty acids (3, 9). It relies on salvage of these nutrients from its host environments. Little is known about the transport systems critical for *Borrelia burgdorferi* to take in these essential components. In general bacterial transport systems include proteins harboring multiple transmembrane domains. Bioinformatic analysis of the amino acid sequence of putative transmembrane proteins provides a means to predict the number and topology of the transmembrane domains within these
proteins. Nonetheless experimental analyses are required to determine these characteristics definitely. Topological analysis of the transmembrane domains of putative transport proteins provides important biochemical information about which amino acid residues in the protein are exposed in the periplasmic space versus those that are oriented toward the cytoplasm (4). Those residues oriented toward the periplasmic space likely are involved in the interaction with the substrate(s) that is transported by the transporter and may play a role in determining substrate specificity. These types of data are critical for understanding transporter function and potentially designing molecular approaches to inhibit transporter function toward development of novel therapeutics for Lyme disease. Development of a phoA-gene fusion reporter system for use in *B. burgdorferi* will provide a new molecular genetics tool for analyzing the topology of transmembrane proteins in *B. burgdorferi*.

The *E. coli* gene *phoA* codes for the enzyme alkaline phosphatase. As a phosphatase, it cleaves phosphate groups, and can be detected when cleavage of a chromogenic substrate results in a color change. The enzyme is called “alkaline” because it is active only in alkaline conditions, or non-reducing environments, such as the periplasm in Gram-negative bacteria. Conversely, the reducing environment of the cytosol inhibits formation of disulphide bonds critical for active conformation of PhoA (4). This feature makes the enzyme useful in determining whether a protein domain is in the periplasm or in the cytosol. The PhoA is fused to a protein being studied, and if the enzyme has activity then the protein is known to localize to the periplasm (Figure 1).

The purpose of this project is to demonstrate proof-of-principle of a phoA-gene fusion reporter system for use in the Lyme disease spirochete *Borrelia burgdorferi*. PhoA is be
appended to a periplasmic protein as a positive control and to a soluble cytosolic protein as a negative control. In conjunction with the recently adapted LacZ reporter system (11) this system can be used in the future to study the topology of *B. burgdorferi* transmembrane proteins. In other bacterial species, the *phoA/lacZ* fusion is used as the primary technique for analyzing the topology of transmembrane proteins (4, 22). Adding this common tool to the *Borrelia* molecular toolkit will enable future studies to answer questions about transmembrane proteins.

![Diagram of PhoA alkaline phosphatase activity](image)

**Figure 1. Representation of PhoA alkaline phosphatase activity in different cellular compartments.**
PhoA exhibits alkaline phosphatase (AP) activity when fused to periplasmic protein domains, but remains inactive when fused to cytoplasmic domains.
Materials and Methods

Bacterial clones and growth conditions. All low-passage-number *B. burgdorferi* clones were derived from non-infectious clone A3-68, which lacks cp9, lp56, and lp25 (16). *B. burgdorferi* was grown in liquid Barbour-Stoenner-Kelly (BSK) II medium supplemented with gelatin and 6% rabbit serum (1) and plated in solid BSK medium as previously described (19, 21). All spirochete cultures were grown at 35°C and supplemented with 2.5% CO₂. Gentamicin was used at 40 µg/ml, when appropriate. All cloning steps were carried out using *Escherichia coli* DH5α. *E. coli* cultures were grown in LB broth or on LB agar plates containing 10 µg/ml gentamicin.

Generation of pFOP Ec. The 0.4 kb DNA fragment encoding the *flaB* promoter was amplified from A3 genomic DNA using Taq polymerase and primers 1 and 2. This *flaB* p fragment and *Borrelia* shuttle vector pBSV2G (6) were each digested overnight with KpnI and BamHI at 37°C, and then ligated together at 16°C overnight. A 1.6 kb DNA fragment encoding the ORF of *oppAIV* without its stop codon was amplified from A3 genomic DNA using Phusion System (New England Biolabs) and primers 3 and 4. Periplasmic *oppAIV* was cloned into the vector pBSV2G using restriction ends BamHI and Sall. The *E. coli* phoA gene (*phoA Ec*) was amplified without the first 63 nucleic acids, which encode the 21 amino acid signal sequence, from *E. coli* strain BL21 cells by colony PCR using primers 7 and 8. The *phoA Ec* gene was cloned in-frame behind *oppAIV* using restriction ends SalI and PstI. Each successive cloning was confirmed via sequencing. This *flaB* p-*oppAIV-phoA Ec* construct shall be referred to as pFOP Ec.

*B. burgdorferi* A3-68 was transformed with 20 µg of pFOP Ec purified from *E. coli* as previously described using electroporation (6, 10, 21). Gentamycin-resistant colonies were
selected in solid BSK medium and confirmed to be true transformants by PCR using primer pairs 7 and 8. Positive pFOP_{Ec} clones were screened with a panel of primers to ensure maintenance of all plasmids of the parent A3-68 clone (18), and a single clone was selected for further experiments.

**phoA optimization.** *E. coli* phoA codon usage in *B. burgdorferi* was analyzed using the Graphical Codon Usage Analyser version 2.0 (www.gcua.schoedl.de). Almost half of the codons in phoA_{Ec} are rare (used less than 20% of the time) in *B. burgdorferi*. The phoA_{Ec} sequence was optimized through the company Genescript in order to be better suited for *B. burgdorferi* G+C content and codon usage. The optimized gene arrived inside of a pUC57 vector backbone, some of which was subcloned into DH5α upon arrival. The optimized gene shall be referred to as phoA_{Bb}.

**Generation of pFOP_{Bb}.** pFOP_{Ec} and phoA_{Bb} were each digested with SalI and PstI. The pFOP_{Ec} digest was run on a 0.6% agarose gel, and a 7.4 kb fragment containing pBSV2G, the flaB promoter, and oppAIV ORF was purified by gel extraction. The phoA_{Bb} gene was digested out of the pUC57 vector using SalI and PstI, and cloned into the 7.4 kb fragment of pFOP_{Ec}, replacing the phoA_{Ec}. This flaB_p-oppAIV-phoA_{Bb} construct shall be referred to as pFOP_{Bb}.

**Generation of pFPP_{Bb}.** The 0.6 kb DNA fragment encoding the ORF of pncA was amplified from A3 genomic DNA using Phusion polymerase and primers 5 and 6. This pncA fragment and pFOP_{Bb} were each digested with BamHI and SalI overnight at 37°C. The pFOP_{Bb} digest was run on a 0.6% agarose gel, and a 7.1 kb fragment containing pBSV2G, the flaB promoter, and phoA_{Bb} was purified by gel extraction. The pncA gene was cloned into this
fragment of pFOP<sub>Ec</sub>, replacing the <i>oppAIV</i>. This <i>flaB<sub>p</sub>-pncA-phoA<sub>Bb</sub></i> construct shall be referred to as pFPP<sub>Bb</sub>.

Both pFOP<sub>Bb</sub> and pFPP<sub>Bb</sub> constructs were transformed into electrocompetent A3-68 <i>B. burgdorferi</i> and DH5α cells.

### Table 1. List of oligonucleotide sequences used in this study.

<table>
<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>Sequence (5' – 3')&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>1</td>
<td>flaBp 5' (KpnI)</td>
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</tr>
<tr>
<td>2</td>
<td>flaBp 3' (BamHI)</td>
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</tr>
<tr>
<td>3</td>
<td>oppAIV 5' (BamHI)</td>
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</tr>
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<td>4</td>
<td>oppAIV 3' (SalI)</td>
<td>ggggtcgaaccttattgctttatttctcttg</td>
</tr>
<tr>
<td>5</td>
<td>pncA 5' (BamHI)</td>
<td>cgcggatccatatataatagaaaaatagttg</td>
</tr>
<tr>
<td>6</td>
<td>pncA 3' (SalI)</td>
<td>acgcgtcgctatatataatagaaaaatagttg</td>
</tr>
<tr>
<td>7</td>
<td>phoA&lt;sub&gt;Ec&lt;/sub&gt; 5' (SalI)</td>
<td>acgagtccagccgacacccagaaatgctttg</td>
</tr>
<tr>
<td>8</td>
<td>phoA&lt;sub&gt;Ec&lt;/sub&gt; 3' (PstI)</td>
<td>ggggctcagttatttccagccgacccagagctttc</td>
</tr>
<tr>
<td>9</td>
<td>pho&lt;sub&gt;Bb&lt;/sub&gt; F498</td>
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<tr>
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<tr>
<td>14</td>
<td>flaBR3</td>
<td>gctgaattgtctgcttctagct</td>
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</tbody>
</table>

*a Sequence portions in bold denote recognition sites for restriction enzymes (which are indicated in parentheses after the oligonucleotide name).*
The E. coli phoA gene was cloned into pBSV2G behind oppAIV from B. burgdorferi, creating pFOP\textsubscript{Ec} (A, top left). A B. burgdorferi codon-optimized phoA gene, phoA\textsubscript{Bb}, was cloned into pFOP\textsubscript{Ec} replacing phoA\textsubscript{Ec} to create pFOP\textsubscript{Bb} (B, top right). The B. burgdorferi pncA gene was cloned into pFOP\textsubscript{Bb}, replacing oppAIV to create pFPP\textsubscript{Bb} (C, bottom). The flaB promoter drives expression of each construct. The restriction sites used for cloning and the sizes of each DNA fragment are indicated.

**Protein gel electrophoresis and western blot.** PhoA fusion proteins in *E. coli* and *B. burgdorferi* were detected by western blotting. Bacterial protein lysates were generated from 1 ml of overnight *E. coli* cultures (~1x10\textsuperscript{9} cells) and 5-10 ml of stationary phase *B. burgdorferi* cultures (~2x10\textsuperscript{9} cells). After being spun down, cells were washed in 1 ml cold HN (50 mM Hepes, 50 mM NaCl, pH 7.5). Cells were resuspended in \(\frac{1}{2}\) volume HN and \(\frac{1}{2}\) volume protein
loading buffer (Laemmli Sample buffer, β-mercaptoethanol) depending on bacterial cell count. Between 1x10^7 and 3x10^8 cells were loaded per gel lane. Protein lysates and protein standards were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked in 5% skim milk overnight. Immunoblot analysis was performed initially probing with an anti-PhoA monoclonal antibody (Sigma-Aldrich) diluted 1:2000 in TBS-T (Tris-buffered saline, pH 7.5, Tween-20) overnight at 4°C. Goat anti-mouse IgM/IgG (Life technologies) diluted 1:10000 in TBS-T was used as a secondary antibody for 1 hour at room temperature and visualized using SuperSignal West Pico Chemiluminescent Substrate (Promega) and x-ray film exposed for indicated amounts of time.

**RNA extraction and cDNA generation.** RNA was isolated from *E. coli* and *B. burgdorferi* cultures. 10^7 cells were spun down, supernatant discarded, and 25µl RNAlater reagent (Life technologies) were added. Cells were homogenized in phase-lock gel tubes with DEPC-treated water and Trizol reagent (Life technologies). Phases were separated with chloroform and RNA was precipitated with isopropanol. Pellet was washed once, then resuspended in 50µl DEPC-treated water with 1µl RNAase inhibitor. RNA was DNAase treated with 1µl Turbo DNAase enzyme for 1 hr at 37°C. cDNA was generated from RNA using random hexamer primers and the Qscript cDNA kit (Quanta). Total cDNA was analyzed by PCR for *phoA* using primers 9 and 10 (Table 1), with *E. coli* recA (primers 11 and 12) and *B. burgdorferi flaB* (primers 13 and 14) used as controls.

**Alkaline phosphatase plate assay.** Both *E. coli* and *B. burgdorferi* were grown on plates containing 5-Bromo-4-chloro-3-indolyl phosphate (XP; Sigma Aldrich) and gentamicin. XP was added to *E. coli* plating media at 40µg/ml, and to *B. burgdorferi* plates at 0.003 g/liter, 0.016
g/liter, 0.04 g/liter, and at 0.16 g/liter, respectively. Alternatively, 240 µl XP (10 mg/ml) was spread on *B. burgdorferi* plates after colony formation.

**Alkaline phosphatase liquid assay.** *B. burgdorferi* and *E. coli* cultures were harvested at stationary phase and spun down for 10 minutes at 4000 rpm at 20°C. The resulting pellet was washed twice in 1 mL cold HN buffer and spun down for 5 minutes at 10000 rpm at 4°C. The pellet was then resuspended in 1 mL HN buffer, and the bacteria lysed with 30µl chloroform and 15µl 0.1% SDS. Of the lysed cells, 450µl were mixed with 550µl of p-nitrophenyl phosphate (PNPP; Sigma Aldritch) and incubated at room temperature until color change was observed. Upon color change, optical density at 405nm was measured using a DU 800 Coulter&Beckmann Spectrophotometer, blanked with lysed cells diluted with HN. Relative quantity of *E. coli* was measured by optical density of diluted, unlysed cells at 600 nm. The number of *B. burgdorferi* added to the reaction was determined by microscopy using a Petroff-Hausser counting chamber.
Results

Cloning of the periplasmic PhoA fusion protein. To determine whether phoA could be used as a reporter gene in B. burgdorferi, we constructed the shuttle vector pFOPEc. This construct carries the E. coli phoA (phoA<sub>Ec</sub>) gene fused to the periplasm-targeting oppAIV gene, with the constitutive B. burgdorferi flaB promoter. As a spirochete, B. burgdorferi constantly produces FlaB, a component of the flagella that run along the length of the bacterium. Thus, the highly active flaB promoter is useful for expressing a protein or fusion construct in high concentrations in either Borrelia or E. coli (13). The oppAIV gene codes for an oligopeptide permease, a periplasmic transporter protein that is responsible for entry of small peptides into B. burgdorferi (2). This protein is especially important to B. burgdorferi given the reduced genome of the organism, which necessitates scavenging of complex nutrients from the external environment. It was hypothesized that constitutive expression of oppAIV would prove lethal in neither E. coli nor B. burgdorferi. Most importantly for this experiment, localization of this protein to the B. burgdorferi periplasm will bring the co-translated PhoA protein to the periplasm as well. The 5’ signal sequence of PhoA that naturally tags the enzyme to the periplasm was removed because we wanted the enzyme to rely on the destination of the protein it was fused to.
Figure 3. The pFOP\textsubscript{Ec} plasmid carries the \textit{flaB\textsubscript{p}}, opp\textit{AIV} and \textit{phoA}. DNA digest of FOPEc plasmid showing expected sizes of cloned elements. The \textit{flaB\textsubscript{p}} was digested out with KpnI and BamHI. opp\textit{AIV} was digested out with BamHI and SalI. \textit{phoA\textsubscript{Ec}} was digested out with SalI and PstI. Plasmids were digested at 37°C for 2 hours. See Figure 2-C for a schematic of the FOP\textsubscript{Ec} plasmid construction. The size standards are shown in base pairs.

As an initial test of the alkaline phosphatase enzyme activity of the OppAIV-PhoA\textsubscript{Ec} fusion protein, we were encouraged to find that \textit{E. coli} transformed with pFOP\textsubscript{Ec} yielded blue colonies on plates containing the chromogenic substrate for alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate (XP) (Figure 4). This was a good initial indicator that this periplasmic fusion in the pBSV2G shuttle vector behind the flaB promoter was producing active protein. Thus emboldened, we transformed pFOP\textsubscript{Ec} into \textit{B. burgdorferi} non-infectious clone A3-68. It was anticipated that A3-68 transformed with pFOP\textsubscript{Ec} would yield purplish colonies on
plated *B. burgdorferi* solid medium containing XP. The blue color of the cleavage of XP would mix with the normal red coloring of the BSK plating media to make purple. However, no color change was observed in the presence of *B. burgdorferi* harboring pFOPₐₚ. It was unknown whether there was a flaw in the assay or if enzyme was not being produced, so the transformants were tested then for PhoA protein production. Protein lysates were made from *E. coli* and *B. burgdorferi* and analyzed by western blot using an antibody specific for the PhoA protein. The OppAIV-PhoAₐₚ protein fusion was detected in *E. coli* at the predicated molecular mass of 100kDa, but no protein fusion was detected in *B. burgdorferi* lysate (Figure 5-A). We were uncertain of whether the problem was in gene expression or in protein translation. Therefore, the expression of the *phoA* gene was analyzed in *B. burgdorferi* harboring pFOPₐₚ and a *phoA* transcript was detected (Figure 5-B). This suggested that the lack of detection of the fusion protein in *B. burgdorferi* may be due to a post-transcriptional defect.
**Figure 4.** *E. coli* harboring pFOP<sub>Ec</sub> form blue colonies in the presence of a chromogenic substrate for alkaline phosphatase.

LB solid medium with gentamycin was used to grow DH5α clones harboring pBSV2G (left) and pFOP<sub>Ec</sub> (right). 5-Bromo-4-chloro-3-indoly1 phosphate (XP) was added to plating media at 40µg/ml, and plates were incubated overnight before photographs were taken.

**Figure 5.** The oppAIV-phoA<sub>Ec</sub> fusion is transcribed in *B. burgdorferi* but is not translated.

(A) Immunoblot analysis of the OppAIV-PhoA<sub>Ec</sub> fusions in *E. coli* and *B. burgdorferi* protein lysates using mouse anti-PhoA antibodies, then goat anti-mouse antibodies. The blot was visualized with pico-chemiluminescent reagent and exposed on x-ray film for 50 minutes. (B) Reverse transcription analysis of the phoA<sub>Ec</sub> and endogenous flaB transcripts in *B. burgdorferi* harboring pFOP<sub>Ec</sub>. Analyses were performed in the presence (RT+) and absence (RT-) of reverse transcriptase enzyme.
The $\text{phoA}_{\text{Ec}}$ gene requires codon optimization for translation in $B. \text{burgdorferi}$. $B. \text{burgdorferi}$ is unable to translate the $\text{phoA}_{\text{Ec}}$ transcript. A possible cause of this is different codon usage between $E. \text{coli}$ and $B. \text{burgdorferi}$. $B. \text{burgdorferi}$ favors codons heavy in adenines and thymines and has much fewer tRNAs to support guanidine- and cytosine-rich codons. The $B. \text{burgdorferi}$ B31 G+C content is only 28.2% while the $\text{Escherichia coli}$ BL21 G+C content is 50.8%. Codon usage analysis of the $\text{phoA}_{\text{Ec}}$ sequence in $B. \text{burgdorferi}$ suggested that 48.7% codons in this gene are rare in $B. \text{burgdorferi}$, meaning that those codons are used less than 20% of the time. The first codon was in particular extremely rare, used less than 5% of the time in $B. \text{burgdorferi}$. By comparison, this gene demonstrated only 19.4% rare codons according to the $E. \text{coli}$ codon usage. To overcome the challenge of producing the PhoA$_{\text{Ec}}$ protein in $B. \text{burgdorferi}$ we ordered a synthetic $\text{phoA}$, $\text{phoA}_{\text{Bb}}$, with reduced G+C content to better reflect the $B. \text{burgdorferi}$ codon usage. This strategy has been used previous to enhance translation of non-$B. \text{burgdorferi}$ genes in $B. \text{burgdorferi}$ (11). The G+C content of the $B. \text{burgdorferi}$ optimized gene is 36.59%, and only 0.6% of its codons are considered rare in $B. \text{burgdorferi}$, and all of them used at least 10% of the time.

**Cloning of the cytoplasmic PhoA fusion protein.** To ensure that activity of our optimized gene was dependent upon the periplasmic location within the cell, PhoA$_{\text{Bb}}$ was also fused to a soluble, cytoplasmic $B. \text{burgdorferi}$ protein. This fusion should not permit PhoA$_{\text{Bb}}$ to become activated, for the protein would be confined to the cytoplasm. Three different $B. \text{burgdorferi}$ cytoplasmic genes were attempted to be used in this cloning project before moving forward with the $\text{pncA}$ gene.
Our initial approach was to generate the PhoA\textsubscript{Bb} fusion using the \textit{B. burgdorferi} cytoplasmic \textit{sodA} gene, which codes for the enzyme superoxide dismutase. This enzyme is used by \textit{B. burgdorferi} to clear toxic oxygen species during times of oxidative stress (7, 23). Although this gene was successfully cloned in front of \textit{phoA} in \textit{E. coli}, generating the plasmid construct pFSP, we were unable to transform this plasmid into \textit{B. burgdorferi}. Since the enzyme coded for naturally is produced in only high stress conditions, we speculated that forced constitutive expression of this enzyme from our plasmid construct may have been lethal to \textit{B. burgdorferi}.

Our second approach was to use the \textit{B. burgdorferi} cytoplasmic \textit{bbk17 (adeC)} gene, which codes for the enzyme adenine deaminase (13). Despite numerous attempts, we were unable to successfully clone the \textit{bbk17} gene under the control of the \textit{flaB} constitutive promoter in \textit{E. coli}. This gene has been previously cloned into \textit{E. coli} under the control of its endogenous promoter but not behind the constitutive \textit{flaB} promoter.

Our final strategy for generating a cytoplasmically localized PhoA fusion was to use the \textit{pncA} gene. The \textit{pncA} gene codes for a nicotinamidase (17). Nicotinamidases are important because they play a role in nicotinate and nicotinamide metabolism, which regulates and maintains cellular pools of NAD\textsuperscript{+}, an important coenzyme. Both \textit{E. coli} and \textit{B. burgdorferi} were found to be permissive to constitutive production of this protein. It was hypothesized that fusion of PhoA to PncA should localize to the \textit{E. coli} and \textit{B. burgdorferi} cytoplasm, where PhoA will not be activated.
Table 2. The *B. burgdorferi* *pncA* gene can be constitutively expressed in both *E. coli* and *B. burgdorferi*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>in <em>E. coli</em></th>
<th>in <em>B. burgdorferi</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>sodA</em></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>bbk17</em></td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td><em>pncA</em></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 6. The pFPP\(_{bb}\) plasmid carries the *flaB*\(_p\), *pncA* and *phoA*.
The *flaB*\(_p\) was digested out with KpnI and BamHI. *pncA* was digested out with BamHI and SalI. *phoA*\(_{bb}\) was digested out with SalI and PstI. Plasmids were digested at 37°C for 2 hours. See Figure 2-C for a schematic of FPP\(_{bb}\) plasmid construction. The sizes of the DNA ladder are shown in base pairs.

Expected DNA insert sizes:
- *flaB*\(_p\): 367 bps
- *pncA*: 606 bps
- *phoA*\(_{bb}\): 1365 bps
**Alkaline phosphatase production.** Both periplasmic and cytoplasmic PhoA<sub>Bb</sub> fusion proteins are detected in *B. burgdorferi* and in *E. coli*, though *B. burgdorferi* produces far less of the fusion protein per cell. In the western immunoblot shown in Figure 7, *B. burgdorferi* lysate was loaded in 5x higher concentration of cells than *E. coli* was. However, the blot shows roughly 5x less protein produced than by *E. coli*. Thus, it is hypothesized that a greater number of spirochetes and a longer reaction time is needed to elicit the same enzyme activity response.

![Western Immunoblot](image)

**Figure 7.** The OppA<sub>IV</sub>-PhoA<sub>Bb</sub> and PncA-PhoA<sub>Bb</sub> fusion proteins are produced in both *E. coli* and *B. burgdorferi*. Immunoblot of OppA<sub>IV</sub>-PhoA<sub>Bb</sub> (FOP<sub>Bb</sub>) and PncA-PhoA<sub>Bb</sub> (FPP<sub>Bb</sub>) fusion proteins in *E. coli* and *B. burgdorferi* (Bb). *E. coli* lysates were loaded at approximately 5x10<sup>7</sup> bacteria/well. *B. burgdorferi* lysates were loaded at approximately 2.5x10<sup>8</sup> spirochetes/well. Primary antibody was mouse anti-PhoA at 1:2000 in TBS-T, and secondary antibody was goat anti-mouse at 1:10000 in TBS-T. Antibodies were visualized with chemiluminescence and exposed on x-ray film for 45 minutes. *E. coli* and *B. burgdorferi* without the fusion protein constructs served as negative controls. The molecular masses of the protein size standards are shown in kilodaltons.

**Alkaline Phosphatase Activity on plates.** Not only is protein produced from both fusion pFOP<sub>Bb</sub> and pFPP<sub>Bb</sub> constructs, but the constructs have strongly differential activity based on protein localization in *E. coli*. This is seen in the plating assay on LB plates containing 5-Bromo-4-chloro-3-indolyl phosphate (XP) a chromogenic substrate for alkaline phosphatase enzyme.
(Figure 8). *E. coli* colonies with pFOP<sub>Bb</sub> grown in the presence of XP overnight turned blue in color. *E. coli* colonies with pFPP<sub>Bb</sub> grown in the presence of XP overnight remained white. However, *B. burgdorferi* colonies grown in BSK plating media with XP added did not show any color change, even when up to 0.16 g/liter XP was added. Neither did *B. burgdorferi* colonies show color change when as much as 240µl XP was spread on top of plates after colonies had already formed.

![Image of E. coli colonies with pFOP<sub>Bb</sub> and pFPP<sub>Bb</sub>](image)

Figure 8. *E. coli* harboring OppAIV-PhoA<sub>Bb</sub> but not PncA-PhoA<sub>Bb</sub> have alkaline phosphatase activity. DH5α *E. coli* transformed with either) (A) pFPP<sub>Bb</sub> (PncA-PhoA<sub>Bb</sub>) or (B) pFOP<sub>Bb</sub> (OppAIV-PhoA<sub>Bb</sub>) were streaked onto LB plates containing 40 µg/ml 5-Bromo-4-chloro-3-indolyl phosphate (XP). Plates were incubated overnight at 37°C before photographs were taken.
Alkaline Phosphatase Activity in bacterial liquid cultures. In order to assess the alkaline phosphatase activity in *B. burgdorferi* and *E. coli* transformants carrying the *phoA*\textsubscript{Bb} constructs, we adapted a liquid assay protocol using the chromogenic substrate for alkaline phosphatase p-nitrophenyl phosphate (pNPP) and normalized the alkaline phosphatase activity to the concentration of lysed bacteria in each sample. In *E. coli*, a chromogenic reaction was observed upon mixing substrate with lysate after only 10 minutes, and was fairly strongly yellow by the time samples were tested at 1 hr. *E. coli* harboring pFOP\textsubscript{Bb} displayed significant activity compared to *E. coli* containing pFPP\textsubscript{Bb} and to WT *E. coli* lacking the *phoA* gene. Comparing the average alkaline phosphatase units (AP units) of *E. coli* + pFOP to *E. coli* + pFPP with a two-tailed P test yielded a statistically significant P value of 0.0020. Thus, *E. coli* + pFOP has significantly greater activity than *E. coli* + pFPP. *E. coli* + pFOP activity compared to WT *E. coli* yields a P value of 0.0007, also indicating significantly greater activity in *E. coli* harboring a periplasmically localized PhoA protein. *E. coli* + pFPP compared to WT yields a P value of 0.1192, meaning that there is no significant difference in alkaline phosphatase activity of these two strains.

The chromogenic reaction for alkaline phosphatase activity was much slower and weaker in *B. burgdorferi* carrying the PhoA fusions than it was in the *E. coli* strains, taking 3 - 7 hours to begin to see a reaction. It was interesting to note a difference in appearance of the *B. burgdorferi* + pFOP\textsubscript{Bb} lysate to the *B. burgdorferi* + pFPP\textsubscript{Bb} lysate. While chloroform/SDS-lysed *B. burgdorferi* +pFOP\textsubscript{Bb} remained homogeneous with the HN buffer solution, chloroform/SDS-lysed *B. burgdorferi* +pFPP\textsubscript{Bb} seemed to precipitate out with a cloudy appearance. Alkaline phosphatase activity was not significantly higher in in *B. burgdorferi* lysates containing pFOP\textsubscript{Bb}.
than in lysates harboring pFPP_{Bb}. The comparison of the mean AP units of \textit{B. burgdorferi} + pFOP compared to \textit{B. burgdorferi} + pFPP resulted in a P value 0.3485. Thus there is no significant difference between the activities. Both PhoA fusion proteins produce detectable yet low alkaline phosphatase activity in \textit{B. burgdorferi}. 

Figure 9. The alkaline phosphatase activities of the PhoA fusions in E. coli and B. burgdorferi lysates.

Liquid assays were performed on E. coli and B. burgdorferi harboring pFOP\textsubscript{Bb} or pFPP\textsubscript{Bb}. Levels represent ± standard errors of the mean. (A) E. coli alkaline phosphatase units are calculated by $1000 \times \text{OD}_{405}/(\text{time} \times \text{OD}_{600})$. $P = 0.0020$. (B) B. burgdorferi alkaline phosphatase units are calculated by $1000 \times \text{OD}_{405}/(\text{time} \times \text{number of spirochetes})$. $P = 0.348$. $P$ values were calculated based on a Student $t$ test comparing FOP to FPP. OD\textsubscript{405} was blanked using lysed cells diluted in HN buffer.
Discussion

In this project, *E. coli phoA* was introduced into *B. burgdorferi* for the purpose of being used as a reporter gene. When fused to a *B. burgdorferi* protein it would follow that protein to its location within the cell, and have activity based on if the protein ended up in the periplasm or not. Periplasm- and cytoplasm- targeted *phoA* fusion constructs were cloned inside of *E. coli* on shuttle plasmids before being transformed into *B. burgdorferi*.

It was found that *E. coli* *phoA* needed to be codon-optimized because of a post-transcriptional codon usage problem in *B. burgdorferi*. Upon optimization, *B. burgdorferi* produces the fusion protein, but production is still low as compared to the robust band seen from *E. coli* in Figure 7. In the liquid culture assay, when compensating for low production by using ten times as much spirochetes as *E. coli* cells, enzyme activity of periplasm-targeted PhoA in *B. burgdorferi* is still slower and weaker than it is in *E. coli*. Furthermore, in *B. burgdorferi* even cytoplasm-targeted PhoA seems to have the same level of activity as the periplasm-targeted PhoA fusion.

The activity of the cytoplasmic fusion construct in *B. burgdorferi* has some possible explanations. It is possible that the high amount of cells overwhelmed the assay. In a 1995 study by Derman and Beckwith (5), it was found that PhoA localized to the cytoplasm slowly acquires enzymatic activity in *E. coli* cells that are not growing, with as much as 30% of the PhoA becoming active over the time course of the experiment. This is a possible explanation for the low levels of activity in *B. burgdorferi*. However, this problem was not seen to this extent in *E. coli* carrying the pFPPBb construct.
Another explanation is that nicotinamidase, the enzyme coded for by \textit{pncA}, may have partial localization to periplasm. And in fact, in a 2011 paper it was seen that although PncA is mostly found in the cytoplasm, there was some PncA in the membrane fraction (12). It may be that PncA is not the ideal cytoplasm-exclusive targeting protein for a negative test fusion. In future studies, a different cytoplasmic \textit{B. burgdorferi} protein could possibly be used.

One possible explanation for the overall low alkaline phosphatase activity in \textit{B. burgdorferi} is that an additional family of enzymes may be required for proper PhoA disulfide bond formation and dimerization to confer activity (15). A 2010 review by Kadokura and Beckwith (14), describes components involved in disulfide-bond formation, known as Dsb proteins, present in \textit{E. coli} and other species of bacteria. Among this family, the one most responsible for speeding up disulfide bond formation is a thiol-disulfide oxidoreductase known as DsbA. A bioinformatics search through the \textit{B. burgdorferi} genome did not find any significant homologues to DsbA, nor were any conserved domains found. In fact, \textit{B. burgdorferi} has one of the smallest sets of thioredox genes among bacteria, with only three thioredox enzymes identified (8). These enzymes might catalyze limited disulphide bond formation in \textit{B. burgdorferi}, but they may not have specificity to foreign proteins such as PhoA. Thus, the alkaline phosphatase activity of the PhoA fusions could potentially be improved by co-expression of \textit{E. coli} DsbA in \textit{B. burgdorferi}. Other Dsb proteins have roles such as maintaining activity of DsbA (DsbB), and as a periplasmic disulfide-bond isomerase (DsbC). As PhoA only has two pairs of disulfide bonds, the isomerase DsbC is probably not necessary for correct bond formation, but the DsbB could be important for keeping DsbA oxidized and active. Thus, expression of DsbB might also be useful for increasing PhoA activity \textit{B. burgdorferi}. 

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Although many studies have cloned and analyzed proteins from many different bacterial species using PhoA fusion in *E. coli*, for *B. burgdorferi* it is advantageous to study protein functions and localization within *B. burgdorferi* itself. Moreover, studying *B. burgdorferi* proteins in *E. coli* may not be feasible in many cases due to differences in expression, production, and activation, as seen with the challenge of cloning the *B. burgdorferi* *bbk17* gene into *E. coli* in this study. Although it may not be easy to adapt PhoA to have strong reporter activity in *B. burgdorferi*, it is certainly easier than adapting every *B. burgdorferi* protein for use in *E. coli*. Even if proteins are capable of being made *E. coli*, their functionality may not reflect their true performance in *B. burgdorferi*. *B. burgdorferi* is so unusual among bacteria in so many aspects, such as codon usage, nutrient usage, and genome structure, that it is important to study its proteins within its own system.
Conclusion

This thesis has explored adaptation of an important molecular genetics tool from one bacterial system to another. The main finding of this work is that *B. burgdorferi* can be granted alkaline phosphatase activity, though sound reporting on localization of native proteins has not been clearly established. Future directions include co-expressing one or more Dsb proteins in *B. burgdorferi* to assist with proper disulfide bond formation of PhoA, potentially increasing PhoA activity. This might allow chromogenic activity to be observed in *B. burgdorferi* BSK plates and lessen the amount of time and concentration of spirochetes required for the liquid culture assay to change color. Once a more robust alkaline phosphatase activity is achieved, *phoA*$_{Bb}$ can be fused to various domains of *B. burgdorferi* transmembrane proteins of interest, and activity can observed by liquid assay to determine periplasmic or cytoplasmic localization of those domains. It is these proteins and their structures that allow *B. burgdorferi* to scavenge what nutrients it requires from its host. Ultimately, expanding the molecular genetics toolkit used in studies of these bacteria, whose molecular mechanisms for pathogenicity are still so little understood, could open up new treatment opportunities for people suffering from Lyme disease.
References


