ANALYSIS OF A BACTERIAL SERINE/THREONINE KINASE

by

Adwoa Manu-Boateng

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ABSTRACT

RdoA is a bacterial protein kinase from *Salmonella enterica* serovar Typhimurium first noted for its regulation of *dsbA* expression in this organism. The crystal structure of RdoA’s homologue, YihE from *Escherichia coli*, revealed a basic bi-lobal kinase domain that is a hallmark of the eukaryotic Ser/Thr, Tyr protein kinase superfamily. YihE however, bears the greatest structural similarity to choline kinase and aminoglycoside 3’-phosphotransferase [APH(3’)]-IIIa which are both atypical kinases. RdoA and YihE have demonstrated the capacity for autophosphorylation in vitro and the ability to phosphorylate myelin basic protein, however, the native kinase target protein has not been identified. Based on structural alignment with APH(3’)-IIIa, predictions were made of key residues involved in ATP binding and catalysis and five YihE mutants were generated. Both the wildtype and YihE mutants were cloned for expression as N-terminal histidine-tagged proteins. In the work presented here, these proteins have been overexpressed and purified for further study. Mutational analyses revealed that four of the five mutants had decreased kinase activity in comparison to the wildtype protein, thereby establishing the mutated residues as important for enzymatic activity. Several attempts were made to elucidate the substrate of RdoA/YihE, however, it remains unknown. Further investigation is necessary to identify its substrate(s) and to pinpoint its physiological significance. RdoA is a member of the Cpx regulon and its absence stimulates Cpx activation. Since the Cpx system is involved in regulating expression of cell surface appendages and is one of three envelope stress response systems, it is hypothesized that RdoA serves to relay Cpx activation signals. This is supported by
studies on the effect of pH on Cpx activity in wildtype and rdoA− cells presented here. RdoA homologues are present in at least 85 different genera. This level of conservation is indicative of an important biological role for this previously uncharacterized bacterial protein kinase.
ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>AI</td>
<td>autoinducer</td>
</tr>
<tr>
<td>AMPPNP</td>
<td>adenyl-5'-yl imidodiphosphate</td>
</tr>
<tr>
<td>APH(3’)-IIIa</td>
<td>aminoglycoside 3’-phosphotransferase IIIa</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>β-GP</td>
<td>β-glycerophosphate</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming units</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetracetic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose binding protein</td>
</tr>
<tr>
<td>MyBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>NTA</td>
<td>nitrilotriacetic acid</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>optical density at 600 nanometers</td>
</tr>
<tr>
<td>O/N</td>
<td>overnight</td>
</tr>
<tr>
<td>ONPG</td>
<td>o-nitrophenyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PTS</td>
<td>phosphoenolpyruvate carbohydrate phosphotransferase system</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TCS</td>
<td>two-component system</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TEV</td>
<td>tobacco etch virus</td>
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CHAPTER 1

INTRODUCTION

With the vast number and range of substrates, alongside the potential for complex regulatory networks, posttranslational modification of proteins by phosphorylation is a major regulatory mechanism for both eukaryotic and prokaryotic organisms (Johnson and Lewis, 2001; Kennelly, 2003). Many cellular processes such as transcription, metabolism and cell movement are controlled by phosphorylated proteins (Raggiaschi et al., 2005). For many years however, protein phosphorylation was considered limited to complex organisms, those composed of numerous differentiated cells (Johnson and Lewis, 2001; Kennelly and Potts, 1996). The existence of bacterial kinases was not accepted until the late 1970s. A decade later, the discovery of the two-component system proved that protein kinases were ubiquitous amongst prokaryotes. Even so, kinases targeting hydroxyl amino acids like serine, threonine and tyrosine were considered unique to eukaryotic organisms while prokaryotes favoured phosphorylation of histidine or carboxyl amino acids like aspartate (Johnson and Lewis, 2001; Kennelly and Potts, 1996). As the cartoon below illustrates (Figure 1), researchers have been challenged to prove that when it comes to protein phosphorylation, what eukaryotic organisms do, the prokaryotes are capable of as well. Advances in science, particularly in the fields of genomics and proteomics have led to the discovery of serine, threonine and even tyrosine phosphorylation in bacteria raising doubts over the compartmentalization of eukaryotic and prokaryotic protein kinases.
Figure 1. A comical depiction of the search for phosphotyrosine in bacteria (Grangeasse et al., 2007).
RdoA is a serine/threonine kinase from *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) and a member of the Cpx regulon. The crystal structure of YihE, the *Escherichia coli* (*E. coli*) homologue, has been recently solved (Zheng et al., 2007) demonstrating the protein’s structural similarity to eukaryotic protein kinases. RdoA/YihE is widespread in bacteria with over 100 homologous proteins in 85 different genera (Martin, 2007). Although its substrate is not known, *rdoA/yihE* phenotypes indicate the target may be a global regulatory protein or possibly RdoA has several targets.

The goal of this work was to further characterize RdoA including studies of its mechanism of action and elucidation of its substrate(s). The following chapters detail the steps undertaken to achieve this goal. Chapter 2 lays the foundation necessary to grasp the information in subsequent chapters. The materials and methods utilized in the study of RdoA/YihE are described in Chapter 3 followed by a presentation of results in Chapter 4. The final chapter is a discussion of these results with suggestions for future directions.
CHAPTER 2
LITERATURE REVIEW

2.1 Phosphorylation in *Salmonella*

Protein kinase activity was first detected in *Salmonella* by Wang and Koshland Jr. (1978) in the late 1970s. Since that time several protein kinases have been characterized in this organism particularly those which are part of the canonical two-component system. Phosphorylation of protein and non-protein substrates plays an important role in *Salmonella* physiology governing chemotaxis, the expression of virulence traits and synthesis of cell structures such as lipopolysaccharide (LPS).

Resistance to several antibiotics and host defense factors is due in part to the LPS found on the surface of Gram negative pathogens. The LPS molecule is divided into three regions; a membrane anchor known as lipid A, short branched sugar chains or the core oligosaccharide and the O-antigen, which is a structurally diverse polysaccharide. The addition of phosphate groups to the core oligosaccharide is essential for forming a stable outer membrane in these bacteria. Yethon *et al.* (1998) have shown that WaaP and WaaY are kinases responsible for phosphorylation of the LPS core. *S. typhimurium waaP* mutants have increased sensitivity to polymyxin and a loss of virulence in mouse models of infection (Yethon *et al.*, 2000).

Another *Salmonella* kinase, whose target is not proteinacious, is LsrK (*luxS* regulated kinase) from the quorum sensing pathway. Quorum sensing, the regulation of specific bacterial activities according to cell density, is achieved through the release and detection of signaling molecules known as autoinducers (AI) (Bassler, 1999).
These are synthesized by individual cells and released into the environment. Upon reaching a critical extracellular concentration, AI molecules diffuse back into the cell where they impart their regulatory behaviour. In Gram negative bacteria, quorum sensing is typically controlled by the LuxI/LuxR (or equivalent) system. LuxI synthesizes the AI while LuxR is able to detect and bind the AI, altering transcription of downstream target genes. The AI-2 quorum sensing molecule is thought to function in interspecies cell to cell communication and is synthesized by the LuxS synthase. In *S. typhimurium*, LsrK is a kinase that phosphorylates AI-2. This phosphorylation event serves two purposes: a) the phosphorylated form of AI-2 is thought to be responsible for transcription of the *lsr* operon via inactivation of the inhibitory LsrR protein and b) similar to carbohydrate phosphorylation in the phosphoenolpyruvate carbohydrate phosphotransferase system (reviewed in the next section), phosphorylation of AI-2 sequesters the signaling molecule in the cytoplasm following import (Taga et al, 2003).

Protein kinases associated with bacterial organisms include enzymes of the phosphoenolpyruvate (PEP) carbohydrate phosphotransferase and two-component systems. Transport of various carbohydrates across the cytoplasmic membrane and retention of these carbohydrates through the addition of a phosphate group is accomplished by the PEP carbohydrate phosphotransferase system or PTS (Postma *et al.*, 1993). The two component system utilizes a histidine-aspartate phosphorelay to modify gene expression in response to environmental stimuli (Stock *et al.*, 2000; West and Stock, 2001). In addition to regular cell physiology both these systems play a role in *Salmonella* virulence as described below.
PTSs couple the translocation of a carbohydrate to its phosphorylation. Overall the process involves phosphotransfer from PEP, energetically equivalent to ATP, to substrate and results in release of the phosphocarbohydrate into the cytoplasm (Lengeler et al., 1994; Postma et al., 1993). Carbohydrates accumulated by other means require more than one ATP equivalent per monosaccharide unit for their transport and phosphorylation (Postma et al., 1993). The PTS is therefore an efficient means of actively accumulating carbohydrates especially for anaerobic bacteria, which must make judicious use of ATP. Components of the mannitol, glucose and mannose PTSs are illustrated in Figure 2. Enzyme I (E1) and the histidine protein (HPr) are soluble cytoplasmic proteins common to all PTSs. At room temperature, E1 monomers dimerize and autophosphorylate in the presence of magnesium and PEP (Han et al., 1990). Phosphorylation occurs on a histidine residue in the N-terminal domain. The C-terminal domain appears to be involved in dimerization and interaction with PEP (LiCalsi et al., 1991). Autophosphorylation is followed by the dissociation of the dimer and phosphorylation of HPr. Variations in PTSs arise from differences in the many enzyme II (E2) proteins; unlike the EI and HPr proteins, EIIs are carbohydrate specific. They may be a single membrane protein with three autonomous domains (A, B and C) or two to four proteins where at least one is membrane bound (refer to Figure 2). The C and sometimes D domain(s) form a channel in the membrane to transport the carbohydrate while the A and B domains relay the HPr phosphoryl group to the carbohydrate upon its entry into the cytoplasm (Postma et al., 1993).

E1 is invariantly present in all PTSs and although it is not required for growth in a rich medium, Kok et al. (2003) postulated that E1 may be required for infection. The
Figure 2. The mannitol, glucose and mannose PTSs. E1 and HPr are common to all PTSs while the E2 enzymes are carbohydrate specific. E1 monomers dimerize then autophosphorylate in the presence of PEP and magnesium. Following autophosphorylation, E1 dimers dissociate and phosphorylate HPr which in turn phosphorylates the carbohydrate after its membrane translocation facilitated by E2 (Postma et al., 1993).
role of E1 in the virulence of *Haemophilus influenza*, *Staphylococcus aureus* and *S. typhimurium* was investigated by deletion of *pst1* which encodes E1. The ability of *pst1* mutants to cause infection in mice was compared in the three bacteria. The strongest attenuation was seen in *S. typhimurium* which has the most complex PTS. For wildtype cells as few as 7 colony forming units (cfu) were required to produce an LD$_{50}$ in BALB/c mice, while $3.8 \times 10^3$ were required for an LD$_{50}$ in the *pst1* mutant. Additionally, the intracellular growth rate (cfu 24hr post infection/cfu 3hr post infection) of wildtype *S. typhimurium* in a macrophage-like mouse cell line was two times higher than in the mutant. *S. typhimurium pst1* mutants are unable to ferment glucose, mannose or maltose; this defect may be responsible for the observed virulence effects (Kok *et al.*, 2003).

These findings highlight the requirement for protein phosphorylation in *Salmonella* under certain conditions and its impact on virulence.

Protein kinases play an indispensable role in information transfer within the cell. Serine (Ser), threonine (Thr), tyrosine (Tyr), histidine (His) and aspartate (Asp) are the commonly phosphorylated amino acids (Johnson and Lewis, 2001). Phosphorylation of the latter two is usually associated with two-component systems which are described in more detail in later sections. Briefly, they include a sensor histidine kinase and its cognate response regulator. Together these proteins link external stimuli to a reactionary cellular response. Two-component systems (TCSs) are involved in bacterial motility, as well as metabolism and stress responses. Although these functions alone are not cause for pathogenesis, they are important in establishing an infection. The expression of virulence factors are in some cases controlled by TCSs. In *Salmonella*, the PhoP-PhoQ TCS is a master regulator of many virulence traits (Beier and Gross, 2006). PhoQ, the
sensor kinase, responds to the periplasmic concentration of divalent cations such as magnesium and calcium. Millimolar amounts of magnesium repress the PhoPQ system while micromolar concentrations activate the system. It is thought that magnesium binds directly to PhoQ’s periplasmic domain so that the lack of binding initiates signal transduction. Expression of at least forty proteins is thought to be under control of the PhoPQ TCS (Véscoví et al., 1994). This is based on two dimensional gel analyses. The PhoPQ system was first recognized for its role in virulence in 1989. Miller et al. discovered that mutations in the phoP locus resulted in a marked decrease in the virulence of S. typhimurium as assessed by the LD50 (Miller et al., 1989). Prior to this finding, it was noted that S. typhimurium mutants defective in macrophage survival had mutations that mapped to the phoP locus. Bijlsma and Groisman (2005) linked the PhoPQ system to intramacrophage survival by showing that expression of the SsrBA TCS, which regulates genes encoding a type III secretion system and effector proteins, is regulated by PhoPQ. This is one example of the complex regulatory network of TCSs in Salmonella that control the expression of genes necessary for growth and metabolism, stress management and virulence. At the heart of this regulation is the phosphorylation of signaling components.

Increasingly, Ser/Thr phosphorylation has been reported in Salmonella. PutA which autophosphorylates on Ser, Thr and Tyr is involved in proline utilization. Phosphorylation and the intracellular proline concentration prompt PutA to act as a transcriptional repressor of putA and putP (encodes a proline permease) or to oxidize proline to glutamate (Ostrovsky and Maloy, 1995). Another example is SteC, a Salmonella effector protein delivered into host cells by its type three secretion system
(TTSS) (Poh et al., 2007). It is one of only four TTSS effectors known to display kinase activity. Amino acids 232-280 of SteC bear residues that are highly conserved amongst the Ser/Thr and Tyr protein kinases. It is able to autophosphorylate in vitro and phosphorylates myelin basic protein (used experimentally to detect Ser/Thr kinase activity). SteC is required for the formation of the F-actin meshwork that surrounds Salmonella-containing vacuoles (Poh et al., 2007). RdoA, a Ser/Thr recently identified in S. typhimurium is the focus of this research. Its structure and impact on cell physiology are discussed in detail in following sections. Together with the kinases just mentioned, RdoA demonstrates that Salmonella harbours proteins belonging to the Ser/Thr and Tyr protein kinase superfamily described below.

2.2 The Serine/Threonine, Tyrosine Protein Kinase Superfamily

The Ser/Thr and Tyr protein kinases share a common catalytic domain that is approximately 280 amino acids in length and contains twelve conserved subdomains numbered I-V, VIa, VIb and VII-XI (Shi et al., 1998). Absolute conservation is relatively low with only twelve residues amongst the eukaryotic protein kinases conserved with near 100% frequency. A nucleotide binding domain spans subdomains I-IV. Its key features are the middle glycine (G52) and a highly conserved aliphatic hydrophobic residue at the end of the sequence GXGXXGXXV in subdomain I, an absolutely conserved lysine residue (K72) in subdomain II and another absolutely conserved residue, glutamate (E91) in subdomain III. Among the residues thought to participate directly in catalysis are an aspartate and asparagine, D166 and N171 respectively, from subdomain VIb and a conserved aspartate (D184) in subdomain VII.
Other features include the highly conserved glutamate (E208) in subdomain VIII, aspartate (D220) from subdomain IX and the arginine (R280) of subdomain XI (Shi et al., 1998). Each subdomain contains up to 20 amino acid residues and together they fold into a characteristic bilobal structure. The smaller amino (N)-terminal lobe is composed of mostly β-strands while the carboxyl (C)-terminal is highly α-helical. A cleft separating the lobes is the site of catalysis (Hanks and Hunter, 1995). Cyclic AMP (cAMP)-dependent protein kinase, or protein kinase A (PKA), is one of the simplest and best understood members of the Ser/Thr, Tyr protein kinase family (Taylor et al., 1999). Its basic bilobal catalytic domain structure is shown in Figure 3. The N-terminal lobe is a five-stranded β-sheet with a conserved α-helix, the C helix, which links the third and fourth β strands. This lobe encompasses subdomains I-IV and along with a short linker contains most of the necessary features for ATP binding. Subdomain I residues fold into β-strands 1 and 2, its glycine rich loop is essential for positioning of ATP or the nucleotide that is acting as phosphate donor. K72 from subdomain II (forms β-strand 3) is required for maximum enzyme activity. The C helix mentioned above represents subdomain III. A nearly invariant glutamate residue, E91, is found within this helix and serves to stabilize the interaction between K72 and the α- and β-phosphates of ATP. Subdomain IV corresponds to β-strand 4 and contains no remarkable features. Linking the N- and C-terminal lobes are β-strand 5 and helix D consisting of residues from subdomain V. The C-terminal lobe is large and with the exception of four β-strands is α-helical. It includes subdomains VIa-XI. Docking of the substrate to be phosphorylated and phosphate transfer occur here. Subdomain VIb folds into β-strands 6 and 7 with an intervening loop. The catalytic loop, as it is called, contains D166 that serves as a base
Figure 3. PKA catalytic domain structure. The structure consists of two lobes or domains; a small N-terminal domain made predominantly of β-strands and larger α-helical C-terminal domain. A cleft between the two domains is the site of catalysis. Depicted on the left-hand side is PKA in standard view, the right-hand side depicts the same image in an open book format. This format is generated by rotating the standard view 90° about the vertical axis, dividing the two lobes at their linker region, then oppositely rotating each lobe 90° about the horizontal axis. β-strands are represented by arrows while α-helices are shown as cylinders. Conserved structural elements are coloured yellow and highly conserved amino acid residues are illustrated as green ball-and-stick models. ATP and magnesium ions (grey spheres) are shown interacting with both lobes (Scheeff and Bourne, 2005).
catalyzing the phosphotransfer reaction from kinase to substrate (Hanks and Hunter, 1995; Taylor et al., 1999). D166 removes the hydroxyl proton from the phosphate-receiving serine, threonine or tyrosine residue in the substrate. The resulting nucleophile then attacks the ATP molecule, acquiring its γ-phosphate (Al-Obeidi et al., 1998). N171 helps stabilize the catalytic loop by hydrogen bonding with D166. D184 from subdomain VII chelates magnesium ions that bridge the β- and γ-phosphates of ATP. Subdomain VIII, which houses E208, folds into a chain facing the cleft. It appears to play a major role in substrate recognition. Also, residues in this subdomain, such as T197 in PKA, are important for kinases that are activated by means of autophosphorylation. Helix F contains subdomain IX residues, namely D220 which stabilizes the catalytic loop through hydrogen bonding. Subdomain X is poorly conserved, altogether missing in some kinases. When present, it corresponds to helix G. Finally subdomain XI at the C-terminal of the kinase domain covers helices H and I. R280 which lies between the helices forms an ion pair with E208 from subdomain VIII that helps stabilize the large lobe (Hanks and Hunter, 1995).

2.3 RdoA – a Ser/Thr Kinase from Salmonella

RdoA or regulator of disulfide oxidoreductase A is a bacterial Ser/Thr kinase discovered recently in S. typhimurium and is the focus of this work. Depicted below (Figure 4) is the crystal structure of YihE, RdoA’s E. coli homologue (Zheng et al., 2007). The two proteins share 96% amino acid sequence similarity (Suntharalingam et al., 2003). Overall YihE has the typical protein kinase structure with an N-terminal lobe made predominately of β-sheets and a larger, mainly α-helical C-terminal lobe. A hinge region
Figure 4. YihE structure. This bacterial kinase has an N-terminal lobe made predominantly of β-strands, a cleft, then a large α-helical C-terminal lobe (shown in red) which is divided into two smaller lobes separated by a second cleft. Conserved kinase structural motifs are shown in yellow (Zheng et al., 2007).
of approximately 10 amino acid residues connects the two lobes forming an open cleft, the putative site of phosphotransfer. In YihE, there is a second larger cleft in the C-terminal region that is thought to be involved in substrate binding (Zheng et al., 2007). Autophosphorylation of RdoA/YihE on an unknown number of serine and threonine residues has been demonstrated in vitro, however to date its physiological substrate has not been identified.

Based on structural homology searches, YihE may be classified as an atypical kinase; these are phosphotransferases that share homology with the typical protein kinases, however they are lacking some of the usual kinase motifs (Scheeff and Bourne, 2005). For example, the sequence GXGXXG from subdomain I, conserved in typical protein kinases, is absent in YihE. YihE bears significant structural similarity to two members of the atypical protein kinase class – choline kinase and APH(3')-IIIa (Zheng et al., 2007); see Figure 5. This eukaryotic choline kinase is involved in the biosynthesis of phosphatidylcholine, an important component of eukaryotic cell membranes and the precursor of various second messengers (Exton, 1994). The prokaryotic aminoglycoside phosphotransferases confer resistance to a wide range of aminoglycoside antibiotics by phosphorylating their 3’ hydroxyl group thereby rendering them inactive (Smith and Baker, 2002; Wright and Thompson, 1999). They share catalytically essential subdomains II-VII and IX and utilize ATP in a fashion similar to the protein kinases despite lacking several of the protein kinase subdomains. The current model of YihE is without bound substrate, however structural similarity between it and APH(3’)-IIIa allow predictions on the location of key residues involved in ATP binding and phosphotransfer. A superimposed image of the proposed ATP binding site in YihE and APH(3’)-IIIa is
Figure 5. Structural alignment of YihE with APH(3′)-IIIa and choline kinase – two atypical kinases. (A) YihE in green aligned with APH(3′)-IIIa in cyan. (B) YihE again in green aligned with choline kinase in violet (Zheng et al., 2007).
depicted in Figure 6. In APH(3′)-IIIa, H188 forms hydrogen bonds with D190 and I207, these correspond to H199 and V216 in YihE, the two interactions facilitate orientation of the side chains of the catalytically important residues D190 and 208 (D201 and D217 in YihE). APH(3′)-IIIa D208 coordinates two magnesium ions for catalysis while D190, like D166 of the protein kinase superfamily, acts as the catalytic base. S27 is also an important residue in APH(3′)-IIIa that interacts with ATP’s β-phosphate. Its counterpart in YihE is S36.

In spite of a lack of amino acid sequence homology, less than 10% similarity, the APH small molecule kinases share many structural features of the Ser/Thr and Tyr protein kinases. In fact, APH(3′)-IIIa is able to phosphorylate MARCKS (myristolated alanine-rich C-kinase substrate) peptide, protamine and myelin basic protein which are used to test for Ser/Thr protein kinase activity in vitro (Daigle et al., 1999). Whereas APH(3′)-IIIa and choline kinase are both small molecule kinases, the large cleft in YihE’s C-terminal lobe suggests it has the ability to bind larger substrates (Zheng et al., 2007).

2.3.2 RdoA Phenotypic Characterization

The rdoA phenotype raises several questions while providing some clues as to the identity of its substrate. The protein itself is localized in the cytoplasm and more highly translated during late log and stationary phase growth in S. typhimurium (He, 2005). It is also more highly expressed when the cells are grown in alkaline conditions (pH 8.5 compared to pH 7) and is required for growth as the pH exceeds 8 (He, 2005). The cell’s
Figure 6. Superimposition of the proposed active site of YhiE (green) with the active site of APH(3′)-IIIa (cyan). APH(3′)-IIIa is shown complexed with AMPPNP. The side chains of six highly conserved residues are illustrated as stick models. Bracketed amino acid codes correspond to YihE (Zheng et al., 2007).
long term survival is diminished in the absence of RdoA by a few orders of magnitude.

Flagellar phase variation is also influenced by RdoA. This is a process whereby *S. typhimurium* is able to express one of two flagellin proteins; FljB or FliC. The expression of one or the other in a cell population is reportedly random, governed by a DNA inversion event (Andrewes, 1922; Zieg *et al*., 1977), however experiments have shown there is a clear bias towards FliC in wildtype cells that shifts towards FljB expression in *rdoA* mutants (Richards, 2006). The switch from FliC to FljB is also seen in wildtype cells under alkaline conditions (Richards, 2006). In terms of an *rdoA*− phenotype, another piece of the puzzle is evidence of its involvement in curli expression. Curli fibers are surface appendages that are important for biofilm formation and the establishment of infection (Barnhart and Chapman, 2006). Under normal conditions, curli subunits or curlin expression is repressed, in part due to the action of CpxR at the curlin promoter. Expression is de-repressed however in the absence of RdoA or CpxR (Zheng *et al*., 2007). In *Shigella flexneri*, the effect of *yihE* on global gene expression was examined by microarray analysis (Li *et al*., 2001). Transcription of approximately 100 genes was altered in the absence of *yihE*. Several of the genes encoded hypothetical proteins with unknown function however many of the changes were decreases in the expression of genes involved in metabolism. For example *sucAB*, *sucCD sdhCDAB* and *gltA* which encode enzymes from the tricarboxylic acid (TCA) cycle were all down-regulated. Also affected were amino acid biosynthesis, intracellular transport and expression of cell surface structures (Li *et al*., 2001). In a subsequent study by Edwards-Jones *et al*. (2004), LPS synthesis was examined in a *yihE* mutant prompted by the observation that the galactose utilization operon, *galETK*, was depressed in this mutant.
(Li et al., 2001). Edwards-Jones et al. (2004) found that the yihE mutant was impaired in LPS synthesis and linked this to inefficient production of UDP-glucose and UDP-galactose resulting from depression of the galETK operon. This effect on LPS synthesis is not seen in *S. typhimurium* (Richards, 2006).

The range of phenotypes observed in an rdoA/yihE mutant suggests it has several targets or may act on a global regulatory protein. It is also evident that the activity of RdoA is associated with that of the Cpx system. This is consistent with the presence of a CpxR binding site within *rdoA’s* promoter, meaning that *rdoA* gene expression is regulated at least in part by Cpx signal transduction (Suntharalingam et al., 2003). It has been shown that RdoA is more highly expressed upon activation of the Cpx system and that the absence of RdoA leads to Cpx activation (He, 2005). The role of RdoA may be to propagate the Cpx activation signal. In order to better understand the relationship between RdoA and the Cpx system, a discussion of the Cpx TCS follows.

### 2.4 Two-Component Systems General Overview

With representatives in life’s three major kingdoms (Wolanin et al., 2002), the two-component signal transduction system is one means by which external stimuli are linked to specific adaptive cellular responses (West and Stock, 2001). Despite the name, many two-component systems have more than just two components. What defines the system, however, is its histidine kinase sensor and response regulator (West and Stock, 2001). The histidine kinase is a transmembrane sensor embedded in the cell’s inner membrane with domains in both the periplasm and cytoplasm. Stimuli are perceived by the kinase causing dimerization and then autophosphorylation at a conserved histidine residue. A
phosphotransfer reaction catalyzed by its cognate response regulator causes phosphate transfer from the kinase to a conserved aspartate in the response regulator (Stock et al., 2000). Most response regulators have two domains, a regulatory or receiver domain and an effector domain. The effector domains of response regulators vary in structure due to their diverse functions however most act as transcription factors and contain helix-turn-helix DNA binding motifs. Phosphorylation of the regulatory domain causes a conformational change in the protein that enables the response regulator to carry out its specific function (West and Stock, 2001).

2.4.1 The Cpx Envelope Stress Response System

*S. typhimurium*’s conjugative plasmid expression or Cpx system is one of several two-component systems harboured by this bacterium. The system’s histidine kinase is called CpxA and its response regulator is CpxR (Ruiz and Silhavy, 2005). The Cpx system has a third component, a periplasmic inhibitor protein known as CpxP. In the absence of stimulating signals, CpxP is thought to interact with CpxA in a manner that prevents activation of the system. Upon arrival of activating stimuli, CpxP is titrated away from CpxA allowing autophosphorylation and signal propagation (Danese and Silhavy, 1998 and Isaac et al., 2005). The Cpx system is well established as an envelope stress response system that monitors the cell’s outer compartment. In this capacity, the system responds to changes in the environment or within the cell that could compromise the integrity of the extra-cytoplasmic compartment. In *E. coli*, several signals lead to activation of the Cpx system. These include alkaline pH (Danese and Silhavy, 1998), changes in membrane composition (Mileykovskaya and Dowhan, 1997), overexpression
of the outer membrane protein NlpE and the inner membrane protein YafY (Snyder et al., 1995). Off-pathway pilins, those that fail to associate with their cognate chaperone (Jones et al., 1997) and spheroplast formation (Raivio et al., 2000) also induce Cpx activation. Many of these signals result in the accumulation of misfolded proteins in the periplasm. In response, activation of the Cpx system leads to the transcription of a periplasmic protease (DegP) and various folding factors (DsbA and PpiA) along with the cpxA, cpxR and cpxP genes themselves (Connolly et al., 1997; DeWulf et al., 1999; DiGiuseppe and Silhavy, 2003). A model for this system is shown in Figure 7.

2.4.2 The Cpx System Beyond Envelope Stress

A much broader role for the Cpx system is slowly emerging that goes beyond responding to envelope stress. The system is implicated in the expression of various outer membrane structures such as the curli fibers mentioned previously. CpxR acts with OmpR, the response regulator of the Env/OmpR two component system, to regulate curli gene expression in response to osmolarity (Jubelin et al., 2005). CpxR inhibits curli expression by binding upstream of the csgD promoter whose gene product positively regulates curli production. The Cpx signaling pathway also affects Pap and bundle forming pili expression. Pap pili are hair-like appendages extending from the surface of uropathogenic E. coli (UPEC). In the absence of CpxR, UPEC express fewer and shorter pili (Hung et al., 2001) however, Cpx activation has also been shown to repress pap transcription and pili expression (Hernday et al., 2004). Additionally, expression of bundle forming pili from enteropathogenic E. coli (EPEC) is diminished in strains lacking cpxR (Nevesinjac and Raivio, 2005). This is a small sampling of the outcome of
Figure 7. The Cpx envelope stress response. A stimulus such as misfolded pilin subunits triggers activation of the Cpx stress response. CpxP binds the misfolding proteins allowing CpxA to autophosphorylate then phosphorylate CpxR. CpxR-P then induces transcription of genes whose products help alleviate the stress (adapted from Isaac et al., 2005).
Cpx activation, its diverse regulon includes genes involved in chemotaxis, and drug resistance in addition to those already mentioned. It has been noted that the range of cellular functions affected by Cpx activation correlates with those of cells grown in biofilms (Dorel et al., 2006), yet the biogenesis of adherence structures such as curli and pap pili is repressed upon Cpx activation. Dorel et al. hypothesize that to join a biofilm, each cell must first express the correct adherence structure, then turn off its expression to conserve energy. The Cpx system may act as an “off” switch for the expression of these surface appendages once cell-cell contact has been made. In fact, surface sensing by the Cpx system has been demonstrated in *E. coli* supporting this hypothesis. Otto and Silhavy (2002) found that attachment to an abiotic surface, glass beads, activated the Cpx system. When compared to planktonic cells, attached cells exhibited an approximate three fold increase in the transcription both *cpxR* and *cpxP* (Otto and Silhavy, 2002). With an affect on so many cell phenotypes, a complete picture of the role Cpx plays in cell physiology is yet to be revealed. Furthermore, the question of how RdoA, a Cpx regulon member, fits into this picture remains unanswered.

### 2.4.3 Two Component Signaling and Ser/Thr Kinases

A recent report examined the interplay between two-component signaling and a Ser/Thr kinase cascade. This example comes from *Myxococcus xanthus* (*M. xanthus*) where the first bacterial Ser/Thr kinase was characterized (Nariya and Inouye, 2005). Two Ser/Thr kinases from *M. xanthus*, Pkn8 and Pkn14, form a signaling cascade where Pkn8 phosphorylates Pkn14. Phosphorylated Pkn14 in turn phosphorylates a transcriptional activator MrpC (*Myxococcus* regulatory protein C) whose expression is controlled by the
MrpAB two-component system. The *mrp* locus is autoregulated and required for formation of *M. xanthus* fruiting bodies. Expression of MrpC is dependent on MrpB and the two regulate *mrpAB* expression. Pnk8/Pkn14 phosphorylation has a negative effect on the expression of *mrpC* and in the absence of either kinase, MrpC levels remain high causing *M. xanthus* to undergo accelerated development (Lux and Shi, 2005; Nariya and Inouye, 2005). Nariya and Inouye are the first to publish data showing a relationship between His/Asp and Ser/Thr phosphorylation in signaling, however, the affect of CpxR-P on *rdoA* transcription (He, 2005; Suntharalingam *et al*., 2003), and also RdoA on *cpxP* transcription as this work demonstrates, reveal that *M. xanthus* is not alone in combining these two modes of phosphorylation to propagate signals. The coordinated action of Ser/Thr and His kinases adds another dimension to the study of phosphorylation as a regulatory mechanism, as well as to the elucidation of RdoA’s native substrate and its physiological role within the cell.

2.5 Project Objectives and Hypothesis

In order to identify a protein as the physiological substrate of a kinase, one criterion is the demonstration of phosphorylation using purified proteins *in vitro* (Kobe *et al*., 2005). Traditionally, protein phosphorylation has been studied through the use of inorganic phosphate isotopes, such as $^{32}$P or $^{33}$P (Hunter, 2000; Raggiaschi *et al*., 2005). A kinase and potential substrate(s) can be incubated with $\gamma$-$^{32}$P labeled ATP. The transfer of phosphate from ATP to substrate by the kinase can be monitored by one or two dimensional gel electrophoresis followed by autoradiography to visualize phosphorylated proteins. The goal of this study was to clone and purify RdoA, using the purified protein
to search for a substrate amongst the myriad of *Salmonella* proteins. The hypothesis is that RdoA has either multiple substrates or its substrate is a global gene regulator. This hypothesis stems from the observed phenotypes of *rdoA* mutants which include heightened pH sensitivity, decreased long term survival (He, 2005) and a switch in flagellin phase variation (Richards, 2006). Beyond identification of RdoA’s substrate, an understanding of its mechanism of action in relation to other kinases is sought. The final aspect of this project is further examination of the *rdoA* phenotype.
CHAPTER 3
MATERIALS AND METHODS

3.1 Bacterial Strains, Plasmids and Culture Conditions

A description of the *E. coli* and *S. typhimurium* bacterial strains and plasmids used for this work is provided in Tables 1, 2 and 3 respectively. Luria Bertani (LB) broth [1% (wt/vol) tryptone, 0.5% (wt/vol) NaCl and 0.5% (wt/vol) yeast extract] or solid plates [1.5% (wt/vol) agar added to LB broth] were used for bacterial growth. Cells were grown at room temperature (approximately 22°C), 30°C or 37°C with aeration and supplemented with antibiotics, *Isopropyl β-D-1-thiogalactopyranoside* (IPTG) or arabinose where appropriate. Cell growth was monitored by optical density measurements at a wavelength of 600nm (OD$_{600}$).

3.2 Plasmid Isolation

Plasmids were isolated using the Sigma GenElute Plasmid Miniprep Kit (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) following the manufacturer’s instructions.

3.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as described by Laemmli (1970). Protein samples were mixed 5:1 with 6X SDS loading buffer [0.35M Tris-HCl pH 6.8, 30% (vol/vol) glycerol, 10% (wt/vol) SDS, 0.6M DTT and 0.012% (wt/vol) bromophenol blue], boiled for 5min then centrifuged at 13,000 X g for 30sec in order to remove insoluble material. Samples
**Table 1. E. coli Strains**

<table>
<thead>
<tr>
<th>Lab Strain Designation</th>
<th>Description</th>
<th>Plasmid</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLM142</td>
<td>araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB3501 deoC1 ptsF25 rbsR, mot&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Lab strain collection</td>
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<td>NLM411</td>
<td>See NLM142</td>
<td>pAMB1</td>
<td>This work</td>
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<tr>
<td>NLM264</td>
<td>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA Δ(lacIZYA-argF) U169 deoR [80 Δ(lacZ) m15]</td>
<td>(Hanahan, 1983)</td>
<td></td>
</tr>
<tr>
<td>NLM402</td>
<td>See NLM264</td>
<td>pTL61T</td>
<td>This work</td>
</tr>
<tr>
<td>NLM364</td>
<td>Host for T7-based expression vector fhuA2 [lon] ompT lacZ::T7 gene1 gal sulAll Δ(mcrC-mrr) 114::IS10 R(mcr-73::miniTn10 TetS) 2 R(zgb-210::Tn10 TetS) endA1 [dcm]</td>
<td>New England Biolabs</td>
<td></td>
</tr>
<tr>
<td>NLM418</td>
<td>See NLM364</td>
<td>pJW861</td>
<td>This work</td>
</tr>
<tr>
<td>NLM419</td>
<td>See NLM364</td>
<td>pJW862</td>
<td>This work</td>
</tr>
<tr>
<td>NLM420</td>
<td>See NLM364</td>
<td>pJW863</td>
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<td>See NLM364</td>
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<td>NLM387</td>
<td>Host for Ptac-based expression vector F-araΔ(lac-proAB) [φ80dlacΔ(lacZ) m15] rpsL(StrR) thi hsdR</td>
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<td>pCH3</td>
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<td>NLM392</td>
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<td>NLM393</td>
<td>F- ara-14 leuB6 secA lacY1 proc14 tsx-67 Δ(ompT-fePC)266 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi l</td>
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<td>See NLM393</td>
<td>pCH3</td>
<td>This Work</td>
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<td>NLM394</td>
<td>F- ara-14 leuB6 fhuA2 Δ(argF-lac) U169 lacY1 lon::miniTn10(Tet&lt;sup&gt;+&lt;/sup&gt;) glnV44 galK2 rpsL20(Str&lt;sup&gt;+&lt;/sup&gt;) xyl-5 mtl-5 Δ(malB) zjc::Tn5(Kan&lt;sup&gt;+&lt;/sup&gt;) Δ(mcrM-mrr)</td>
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<td>NLM395</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; lac&lt;sup&gt;+&lt;/sup&gt; lac&lt;sup&gt;+&lt;/sup&gt; pro /ara Δ(lac-pro) Δ(tsp)=Δ(prc)::Kan&lt;sup&gt;+&lt;/sup&gt; eda51::Tn10(Tet&lt;sup&gt;+&lt;/sup&gt;) gyrA rpoB thi-1 argl(am)</td>
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<tr>
<td>NLM409</td>
<td>miniF lac&lt;sup&gt;+&lt;/sup&gt;( Cam&lt;sup&gt;+&lt;/sup&gt;) fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10--Tet&lt;sup&gt;+&lt;/sup&gt;)2 [dcm] R(zgb-210::Tn10--Tet&lt;sup&gt;+&lt;/sup&gt;) endA1 Δ(mcrC-mrr)114::IS10</td>
<td>(Zheng et al., 2007)</td>
<td>pPI1224</td>
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Table 2. *S. typhimurium* Strains

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<td>NLM2217</td>
<td>SL1344 his strep&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>NLM2239</td>
<td>NLM 2217 rdoA null</td>
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<td>(Suntharalingam <em>et al</em>., 2003)</td>
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<tr>
<td>NLM2326</td>
<td>NLM2217, cpxAR null</td>
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<td>(Richards, 2006)</td>
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<td>See NLM2217</td>
<td>pAMB1</td>
<td>This work</td>
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<td>See NLM2239</td>
<td>pAMB1</td>
<td>This work</td>
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<tr>
<td>NLM2352</td>
<td>See NLM2239</td>
<td>pND18</td>
<td>This work</td>
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<tr>
<td>NLM2353</td>
<td>See NLM2326</td>
<td>pAMB1</td>
<td>This work</td>
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<tr>
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<tr>
<td>----------</td>
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<tr>
<td>pBAD18</td>
<td>4.6kb derivative of pBR322 with a multiple cloning site downstream of the P&lt;sub&gt;BAD&lt;/sub&gt; promoter</td>
<td>(Guzman &lt;i&gt;et al.&lt;/i&gt;, 1995)</td>
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<td>pND18</td>
<td>pBAD18 with &lt;i&gt;nlpE&lt;/i&gt; cloned into the SalI and HindIII sites</td>
<td>(Snyder &lt;i&gt;et al.&lt;/i&gt;, 1995)</td>
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<tr>
<td>pTL61T</td>
<td>8.6kb cloning vector for lacZ reporter constructs</td>
<td>(Linn and St. Pierre, 1990)</td>
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<td>pAMB1</td>
<td>pTL61T with &lt;i&gt;cpxP&lt;/i&gt; promoter region cloned into the HindIII and XbaI sites upstream of lacZ</td>
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<td>pMAL-c2x</td>
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<td>New England Biolabs</td>
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<td>pCH3</td>
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<td>pET15b</td>
<td>5.7kb cloning vector for constructing N-terminal His-tagged proteins</td>
<td>Novagen</td>
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<td>pPI1224</td>
<td>pET15b vector modified to include a 7-amino acid spacer followed by the TEV protease recognition sequence cloned into the NcoI and BamHI sites, &lt;i&gt;yihE&lt;/i&gt; was PCR amplified with unique BglII and EcoRI sites then cloned into the BamHI and EcoRI sites of the vector</td>
<td>(Zheng &lt;i&gt;et al.&lt;/i&gt;, 2007)</td>
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<td>pJW861</td>
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<td>pJW865</td>
<td>pET15b with &lt;i&gt;yihED217A&lt;/i&gt; cloned into the BamHI and EcoRI sites</td>
<td>(Zheng &lt;i&gt;et al.&lt;/i&gt;, 2007)</td>
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</table>
were then loaded onto polyacrylamide gels in a miniature gel electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA, USA) and electrophoresed at 50V through a 5% stacking gel [850μL 30:0.8% (wt/vol) acrylamide:bis-acrylamide, 1.25mL 0.5M Tris-HCl buffer pH 6.8 with 0.4% (wt/vol) SDS, 2.92mL dH2O, 55μL 10% (wt/vol) ammonium persulfate (APS) and 6μL TEMED] and 150V through a 12% resolving gel [3mL 30:0.8% (wt/vol) acrylamide:bis-acrylamide, 1.5M Tris-HCl pH 8.8 with 0.04% (wt/vol) SDS, 2.6mL dH2O, 25μL 10% (wt/vol) APS and 10μL TEMED] in SDS running buffer [25mM Tris-HCl, 192mM glycine and 0.1% (wt/vol) SDS, pH 8.3]. Fermentas PageRuler Unstained Protein Marker (Fermentas Canada Inc., Burlington, ON, Canada), Prestained Protein Marker Broad Range and Protein Marker Broad Range (New England Biolabs Inc., Beverly, MA, USA) markers were used for reference. Proteins were visualized by placing gels in Colloidal Coomassie stain [1.6% (vol/vol) ortho-phosphoric acid, 8% (wt/vol) ammonium sulfate and 0.08% (wt/vol) Brilliant Blue G250] overnight followed by destaining with water.

### 3.4 Protein Assays

Protein concentrations were determined spectrophotometrically using the BCA Protein Assay Kit from Pierce (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. Bovine serum albumin (BSA) was used as a standard.
3.5 Expression and Purification of RdoA

3.5.1 Overexpression of MBP-RdoA

RdoA can be expressed from pCH3 as a maltose binding protein (MBP) fusion. This pMAL-c2X (New England Biolabs Inc.) based vector allows for cloning downstream of malE (encodes MBP) and a Factor Xa recognition sequence. The resulting cytoplasmic fusion protein can be overexpressed, purified by amylose affinity chromatography then cleaved to free the protein of interest from MBP. Expression of MBP-RdoA was investigated under various conditions. Eighty millilitres LB + glucose supplemented with 100\(\mu\)g/mL ampicillin were inoculated with 0.8mL NLM387 overnight (O/N) culture. Cells were grown at 37°C until the OD_{600} reached approximately 0.5. A 1mL sample was taken to serve as the uninduced cell population. MBP-RdoA expression was then induced with IPTG at a final concentration of 0.3mM. Incubation continued for various lengths of time at 22, 30 or 37°C. After induction at 37°C for 2 hr, another 1mL sample was drawn for the induced cell population. Samples were centrifuged at 13,000 X g for 2min then resuspended in 50\(\mu\)L 1X SDS loading buffer (uninduced cells) or 100\(\mu\)L 1X SDS loading buffer (induced cells). The cells in the remainder of each culture were harvested by centrifugation (4000 X g for 15min) then resuspended in 5mL column buffer (50mM Tris-HCl pH 7.4, 200mM NaCl and 1mM EDTA) and frozen O/N at -20°C. Cells were thawed in cold water, sonicated (9 X 15sec, cooled on ice in between bursts) then centrifuged once more to separate the soluble and insoluble proteins (9,000 X g for 20min). The supernatant was collected as the soluble extract and the pellet was resuspended in 5mL column buffer. Samples were mixed 1:1 with 2X SDS loading buffer and subjected to SDS-PAGE.
3.5.2 MBP-RdoA Expression in Protease Deficient Strains

To limit RdoA degradation during purification, pCH3 was moved to four *E. coli* protease deficient strains. NLM strains 392, 393, 394 and 395 were transformed with the plasmid. MBP-RdoA was then expressed from these strains as described above except induction was carried out only at 22°C for 5hrs. In order to assess protein expression in these strains, samples were collected as described above and analyzed by SDS-PAGE.

3.5.3 Batch Purification of MBP-RdoA

Small scale purification of MBP-RdoA was conducted in 1.5mL microfuge tubes. One hundred millilitres LB + glucose was inoculated with 2mL NLM 411 O/N culture and grown to an OD$_{600}$ of 0.5. MBP-RdoA expression was induced with 0.3mM IPTG for 5 hrs at 22°C. Cells were harvested by centrifugation, resuspended in 5mL column buffer, lysed by sonication then centrifuged to remove the insoluble debris as described above. Meanwhile 500μL amylose beads (New England Biolabs Inc.) were washed with column buffer. Washing was done by adding 500μL column buffer to the beads and mixing briefly by inverting the microfuge tube or pipetting. The bead suspension was centrifuged at 13,000 X g for 20sec and the buffer pipetted off. This was repeated 7 times. Five hundred microlitres of the clarified extract were added to the equilibrated beads and incubated at 4°C for 30min with mixing. The suspension was again centrifuged and the supernatant (“flow through”) removed. The beads were washed 10 times with 500μL column buffer and the fusion protein was eluted by washing 8 times with 100μL column buffer + 10mM maltose. Samples underwent SDS-PAGE analysis.
3.5.4 MBP-RdoA Purification by Column Chromatography

Following attempts at batch purification, the fusion protein was purified over a column. MBP-RdoA was overexpressed under conditions outlined in the preceding section. Five millilitres of the amylose beads were loaded onto a 20mL column (Bio-Rad Laboratories) and equilibrated with 20mL column buffer. Five millilitres of the clarified cell extract was diluted with 10mL column buffer then applied to the column. The flow through was collected and the column washed with 50mL column buffer. MBP-RdoA was eluted with 10mL column buffer + 10mM maltose in 10 fractions. Samples were analyzed by SDS-PAGE.

3.6 Expression and Purification of YihE

3.6.1 YihE Overexpression

YihE was previously cloned into a modified pET15b vector. From this vector, YihE is expressed as an N-terminal His-tagged protein with an intervening Tobacco Etch Virus (TEV) protease recognition sequence. Expression is under control of a T7/lacO promoter and can be induced by the addition of IPTG. To determine conditions which would yield high levels of YihE, NLM409 O/N culture was used to inoculate 50mL LB with 200μg/mL ampicillin. Cultures were grown at 37°C until OD$_{600}$ reached 0.8 at which point IPTG was added to a final concentration of 0.3mM and incubation continued at 22, 30 or 37°C. A 1mL sample was taken every hour until five hours of induction. Cells were pelleted, lysed with BugBuster reagent (Novagen Inc. Mississauga, ON, Canada) then analyzed by SDS-PAGE. Overexpression of YihE was carried out at 22°C as recommended by J. Zheng despite evidence that higher temperatures yield higher levels
of expression. For large scale purification, 1L of LB with 200μg/mL ampicillin was inoculated with 10mL NLM409 O/N culture. Cells were grown at 22°C until their OD$_{600}$ reached 0.8. YihE expression was induced with 0.3mM IPTG and growth continued at 22°C for an additional 5hr. Cells were harvested by centrifugation at 4,000 X g for 15min and stored O/N at -20°C.

3.6.2 His-YihE Purification by Column Chromatography

Frozen cell pellets were thawed in cold water and resuspended in 50mL buffer [50mM NaH$_2$PO$_4$, 300mM NaCl, 10mM imidazole, 0.1% (vol/vol) Triton X-100, 1mM PMSF] then sonicated 9 X 15sec with cooling on ice in between bursts. The insoluble cell debris was removed by centrifugation at 4°C for 20 minutes at 13,000 X g. Clarified cell extract was added to 5mL equilibrated Ni-NTA agarose (QIAGEN Inc., Mississauga, ON, Canada) and mixed at 4°C for 30 minutes then poured into a 20mL column allowing the flow through liquid to pass. The column was washed with 40mL followed by 10mL of column buffer containing 20mM and 50mM imidazole respectively. His-YihE was eluted with 15mL column buffer containing 250mM imidazole in approximately 1mL fractions and stored at 4°C for future work. Samples were subjected to SDS-PAGE analysis.

3.6.3 Cleavage of His-YihE

Cleavage of the 8X His-tag was accomplished using the AcTEV protease (Invitrogen Canada Inc., Burlington, ON, Canada). This recombinant protease from TEV recognizes the sequence E-N-L-Y-F-Q-G cleaving between Q and G. To determine optimal
conditions for cleavage 200 units of the protease (one unit of enzyme reportedly cleaves ≥ 85% of 3μg of protein in one hour at 30°C) were incubated with 1mg of protein then divided into three aliquots for incubation at 22, 16 or 4°C for 2, 4, 8 and 24 hours. Samples were collected at the times indicated and underwent SDS-PAGE analysis.

3.6.4 Protease Removal

Because the AcTEV protease is itself His-tagged, it can be separated from the cleaved protein by nickel affinity chromatography. Cleaved samples were diluted to reduce the imidazole concentration to 25mM. Samples were applied to the column and the flow through containing pure YihE was collected and stored at 4°C for future work.

3.7 Kinase Assays

3.7.1 Detection of YihE Kinase Activity

Myelin basic protein (MyBP) is commonly used as a substrate for serine/threonine kinases. Phosphorylation of MyBP as well as autophosphorylation (or phosphorylation of its histidine tag) was used as an indicator of wildtype and mutant YihE kinase activity. Purified kinase was incubated with an equivalent amount of MBP; 5μg of each was typically used. These were incubated with 5μCi [γ-33P]ATP at 37°C for 1 hour in a kinase reaction buffer consisting of 20mM HEPES pH 7.4, 25mM β-glycerophosphate, 1mM NaF, 1mM DTT and 20mM MgCl₂. The reaction was terminated with the addition of 6X SDS loading buffer then heated to 98°C for 5min. Samples were subjected to SDS-PAGE (without staining), soaked for 5 minutes in a solution of 30% (vol/vol) methanol and 5% (vol/vol) glycerol then dried between cellophane. Incorporation of the
radiolabel was detected by exposing the dried gel to a phosphor-imaging screen (Kodak, Rochester, NY, USA) O/N. The phosphorescence signal recorded on this screen was captured digitally using the Personal Molecular Imager FX software (Bio-Rad Laboratories).

3.7.2 Labeling of Cell Lysates

Labeling experiments involving cell lysate were performed as described above except cell lysates rather than MyBP were used as substrate. Cell lysates were prepared as follows: 2-3mL of an O/N culture was used to inoculate 100mL LB. In the preparation of NLM2352 lysates, the media was supplemented with 100μg/mL ampicillin. For the NLM2239 strain, cells were grown into stationary phase (OD$_{600}$ = 1.2-1.3). For NLM2352, cells were grown until an OD$_{600}$ of 0.8 was reached at which point arabinose was added to the culture to a final concentration of 0.4% (wt/vol) to induce expression of NlpE. Cells were grown for an additional 2 hours then harvested by centrifugation at 4,000 X g for 15 minutes. The cell pellet was resuspended in 1-2mL of kinase reaction buffer then sonicated (9 X 15sec bursts, cooling on ice in between) to lyse the cells. The cell extract was clarified by centrifugation at 13,000 X g for 5 min. Total protein concentration was determined by BCA protein assay kit (Pierce Biotechnology Inc.) according to the manufacturer’s instructions.

3.7.3 Pull-Down Assay

This experiment was done in an attempt to capture YihE’s substrate using a histidine-tagged YihE mutant immobilized on a nickel column. The regular overexpression and
chromatography procedures were followed up until washing of the column with column buffer containing 50mM imidazole. At this point NLM2352 lysate, prepared as described above was added to the column. After passage of the flow through, the column was washed with 60mL column buffer containing 20mM imidazole. The YihE mutant and potentially bound substrate were eluted from the column with 10mL buffer containing 250mM imidazole. Samples from this experiment were used in a labeling assay with wildtype YihE.

3.8 Construction of the cpxP::lacZ Reporter

A cpxP::lacZ reporter was constructed to monitor the activation status of the Cpx system. The cpxR-cpxP intergenic region was amplified from S. typhimurium genomic DNA using primers NM143 (ATC GCC AAG CTT CAG CTC TCG GTC ATC ATC AA: 5’ cpxP promoter primer) and NM144 (TAG CCT CTA GAG CGG TAA CTT TGC GCA TC: 3’ cpxP promoter primer) in the following PCR program: 95°C for 2min (pause to add Vent DNA polymerase). Three cycles of 95°C for 1min, 48°C for 1min and 72°C for 30sec and 30 cycles of 95°C for 1min, 59°C for 1min and 72°C for 30sec then held at 4°C. The products were electrophoresed on a 1.5% agarose gel at 75V for 1.5hr and observed under UV illumination. The band of interest was excised and purified using a QIAGEN Gel Extraction Kit (QIAGEN Inc.) then digested with HindIII and XbaI restriction enzymes (New England Biolabs Inc.). The vector, pTL61T, was minipreped using the Sigma GenElute Miniprep Kit (Sigma-Aldrich Canada Ltd.) and also digested with HindIII and XbaI. Vector and insert were ligated using the T4 ligase (New England Biolabs Inc.) and products of the ligation reaction were used to transform NLM264.
Potential clones were selected on ampicillin (100μg/mL) then screened on X-GAL/ampicillin plates and finally tested for the new plasmid using restriction analysis.

3.9 β-galactosidase Assays

The cpxP::lacZ reporter was used to assess the effect of pH on Cpx activity. Samples for β-galactosidase analysis were prepared by using 1mL NLM411, 2346, 2347 or 2353 O/N culture to inoculate 25mL of LB pH 7 supplemented with 100μg/mL ampicillin and buffered with 0.1M sodium phosphate. Cells were grown at 37°C until they reached an OD₆₀₀ of 0.7-0.8 which corresponds to late log phase. The cells were then harvested by centrifugation and resuspended in 1mL fresh LB. Two hundred and fifty microlitres of this suspension was added to flasks containing 25mL of LB at pH 5, 6, 7 or 8 buffered with 0.1M sodium phosphate. Cells were grown for an additional hour then a 1mL sample was taken. These cells were washed once with 1mL LB then 200μL were taken for subsequent β-galactosidase analysis. The sample’s OD₆₀₀ was measured using the remaining 800μL. Tubes containing 0.9mL of Z-buffer [60mM Na₂HPO₄, 40mM NaH₂PO₄H₂O, 10mM KCl and 1mM MgSO₄7H₂O, adjusted to pH 7, adding 0.27% (vol/vol) β-mercaptoethanol before use] were prepared in advance and stored at 4°C. To these tubes, 100μL of each sample (in duplicate) were added. Using Pasteur pipets, one drop of 1% (wt/vol) SDS and two drops of chloroform were added to permeabilize the cells. Tubes were then vortexed for 20 seconds and placed in a water bath at 30°C. Prior to this, a 4mg/mL solution of o-nitrophenyl-β-D-galactopyranoside (ONPG) was made by dissolving ONPG in Z-buffer. Two hundred microlitres of this solution were added to each tube giving a final concentration of 2mM. ONPG is a chromogenic substrate for the
β-galactosidase enzyme that when cleaved produces galactose and o-nitrophenol, the latter is yellow in solution. The reaction was terminated by increasing the pH of the solution with the addition of Na₂CO₃ (500μL of a 1M solution were added giving a final concentration of 0.3M) and the time taken for each tube to turn yellow recorded. Tubes were centrifuged briefly to reduce cell debris then a 1mL sample taken for spectrophotometric measurements. The amount of o-nitrophenol produced is proportional to the activity of the enzyme and can be quantified by measuring the absorbance at 420nm. Since cells are present in the solution a portion of the absorbance at 420nm is due to light scattering from the cells. Measuring the absorbance at 550nm is used to account for this. β-galactosidase activity was then calculated using the Miller equation below:

\[
\text{Activity} = \frac{\text{OD}_{420} - (1.75 \times \text{OD}_{550})}{\text{OD}_{600} \times \text{time (min)} \times \text{vol. of cells added (mL)}} \times 1000
\]
4.1 Protein Purification

In order to obtain purified kinase for biochemical and functional analyses, two affinity based purification schemes were employed. The first involved expression of RdoA as a fusion protein from NEB’s pMAL-c2x vector. This vector allows expression of maltose binding protein (MBP) fusions. RdoA was cloned downstream of \textit{malE} (encodes MBP) and a Factor Xa recognition sequence forming the plasmid pCH3 (cloning was performed by He, 2005). The resulting cytoplasmic fusion protein can be overexpressed, purified by affinity chromatography, and then cleaved to free RdoA from MBP. YihE was cloned into a modified pET15b vector for expression as an N-terminal His-tagged protein with an intervening protease recognition sequence (Zheng \textit{et al.} 2007). Following overexpression and purification by Ni affinity chromatography, the tag can be cleaved by Invitrogen’s TEV protease.

4.1.1 Overexpression and Purification of RdoA

The expression of MBP-RdoA from plasmid pCH3 was investigated under various conditions to determine which would yield the highest level of soluble fusion protein. The steps taken to overexpress MBP-RdoA and separate the cell soluble and insoluble fractions are detailed in the Materials and Methods section. From Figure 8A it is clear that overexpression at a lower temperature (in this case room temperature) for longer
Figure 8. Expression and purification of RdoA. (A) Overexpression of MBP-RdoA in NLM387. Cells were grown at 37°C with aeration to an OD$_{600}$ of 0.5 then induced with 0.3mM IPTG under the conditions indicated. Cells were harvested by centrifugation and lysed by sonication. The soluble and insoluble cells fraction were mixed with SDS sample buffer then electrophoresed on a 12% polyacrylamide gel and visualized by Colloidal Coomassie staining. (B) Overexpression of MBP-RdoA in Protease Deficient Strains NLM 392, 393, 394 and 395. Cells were grown at 37°C with aeration to an OD$_{600}$ of 0.5 then induced with 0.3mM IPTG at 22°C for 5hr. Cells were then harvested by centrifugation and lysed by sonication. Uninduced and induced whole cells along with the induced soluble cell fractions were mixed with SDS sample buffer and subjected to SDS-PAGE on a 12% gel followed by Colloidal Coomassie staining. WC = whole cells, S = soluble fraction. (C) Purification of MBP-RdoA by Column Chromatography. MBP-RdoA was purified from crude cell extracts over an amylose column as described in the Materials and Methods. Lane 1, uninduced cells; lane 2 cells following overexpression of MBP-RdoA; lane 3, partially purified MBP-RdoA eluted from column.
periods of time results in the greatest yield of soluble fusion protein. It appears the
growth of the cells and levels of protein expression from the vector at higher
temperatures overwhelms the cell’s capacity to produce properly folded proteins.
Misfolded proteins can interact with the cells membrane(s) (Davis et al., 1986), this
likely accounts for the increased amount of insoluble fusion protein produced in cells
grown at 37°C. Although the overall expression level is high in these cells, much of the
protein is of no use in an insoluble form. Strong detergents or denaturants could
solubilize the protein however, these might compromise the overall structure and/or
enzymatic activity leading to problems in the long run. It was therefore determined that
to overexpress MBP-RdoA, induction at 22°C for 5hrs was most favorable.

To limit degradation during the purification process, protease inhibitors such as
PMSF are routinely included in cell lysis buffers; for this work a different approach was
taken. Protease deficient strains NLM 392-395 were transformed with pCH3 by
electroporation. NLM392 is deficient in the DnaJ chaperone and many unstable proteins
are reportedly more stable in this mutant (Straus et al., 1988). NLM393 is an ompT
mutant. In addition to periplasmic proteins, this periplasmic protease can degrade
cytoplasmically expressed proteins following cell lysis (Grodberg and Dunn, 1988). The
cell’s major ATP-dependent protease has been knocked out in NLM394, a lon mutant
(Grossman et al., 1983). And finally NLM395 lacks the Prc protease that is responsible
for degrading cytoplasmic and periplasmic proteins after cell lysis (Silber et al., 1992).
The expression of MBP-RdoA from these strains was analyzed by SDS-PAGE. NLM393
yielded considerably more soluble fusion protein (Figure 8B) and was chosen as the
background strain for any further expression and purification. The degree to which
degradation is inhibited in this strain was never determined since purification of RdoA with the pMAL system was not carried out to completion in the work reported here.

After overexpression of MBP-RdoA, column chromatography was used to separate MBP-RdoA from other proteins in cell extracts. The amylose column retained several proteins in addition to the one of interest, see Figure 8C. Despite washing of the column to the point where proteins were no longer detected in the wash buffer, many proteins were eluted from the column along with the fusion protein. From 100mL of culture, 4mg of protein were recovered from the column but only a portion of the total yield was MBP-RdoA. Improving the purification to obtain higher levels of pure fusion protein would require additional procedures such as size exclusion chromatography. This was not pursued once it became known that a His-tagging method being used to purify YihE yielded a more pure product. This method for purifying RdoA was initially ruled out because of concerns over the effect a His-tag would have on protein kinase activity (N. Martin personal communication). However, the vector used for cloning His-tagged YihE allows for cleavage of the tag after purification. Several unsuccessful attempts were made to clone RdoA as a His-tagged protein. Under the assumption that cloning problems would eventually be resolved, work was initiated in purifying YihE for use in the interim. Because of their sequence similarity and failure to clone RdoA, YihE was substituted for RdoA in kinase activity studies and substrate search.

4.1.2 Overexpression and Purification of YihE

His-YihE expression under various temperatures and for varying lengths of time was examined. Following IPTG induction, cells were incubated for 1-5 hours at 22, 30 or
37°C. The soluble cell fractions from these cells are depicted in Figure 9A. His-YihE expression increased with increasing temperature and time, however for purification, 22°C for 5 hours was chosen for induction. It was recommended induction be carried out at room temperature or lower (J. Zheng, personal communication) to ensure proper protein folding while still yielding sufficient levels of protein for purification. Given the outcome of similar expression trials with the MBP-RdoA fusion protein, it was expected that the same conditions would yield similar levels of His-YihE. However 1 or 2 hours induction at 37°C produced more His-YihE than 5 hours induction at 22°C. The difference in size may account for the differences in expression. MBP-RdoA is approximately double the size of His-YihE and possibly requires more time for folding or a lower temperature to slow expression allowing for proper folding.

Before beginning large scale purification of His-YihE by Ni affinity chromatography, small trials were conducted to establish optimal conditions, particularly how to retain the highest level of YihE while washing away non-specific proteins. Crude extract was prepared from 100mL culture induced to overexpress His-YihE then applied to the Ni column. The protein was eluted from the column using buffers with increasing imidazole concentration. Twenty millimolar imidazole washed away many proteins from the column without loss of the tagged protein (data not shown). At 50mM imidazole, His-YihE is eluted to a small degree from the column along with non-specific proteins associated with the column (data not shown). After 50mM imidazole a substantial amount of His-YihE is eluted from the column, therefore, to wash the column buffers containing 20mM followed by 50mM imidazole were chosen. This removes the majority but not all non-specific proteins (Figure 9B). The yield from this purification strategy is
Figure 9. Expression and purification of YihE. (A) Overexpression of His-YihE. Conditions leading to optimal expression of His-YihE were investigated. 50mL of LB were inoculated with 1mL O/N culture of NLM409 and grown at 37°C with aeration to an OD₆₀₀ of 0.8. Cells were induced with 0.3mM IPTG then further incubated under conditions indicated above. Cells were harvested then lysed to collect the soluble protein fraction and 10μL of each sample was electrophoresed on a 12% gel followed by Colloidal Coomassie staining. (B) Purification of His-YihE by Nickel Affinity Chromatography. His-YihE was purified from NLM409 cells induced to overexpress the tagged protein. Standard column chromatography procedures were followed, see Materials and Methods for details. Lane 1, uninduced cells; lane 2, crude extract following overexpression; 3, purified protein after elution from column; 4, purified protein following cleavage of His-tag. (C) Cleavage of His-YihE. Cleavage of the 8X His-tag was accomplished using the AcTEV protease. 1mg of protein was incubated with 200 units of the protease and divided into three equal parts to be incubated at 4, 16 or 22°C for 2, 4, 8 and 24hr. 10μL samples were collected at each time point and analyzed by SDS-PAGE followed by Colloidal Coomassie staining.
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approximately 15mg per litre of culture compared to approximately 7mg per litre
obtained from MBP-RdoA purification. The end product was also higher in purity with
this method. Perhaps washing the amylose column with a low concentration maltose
buffer before elution of the fusion protein would have improved MBP-RdoA purification.
This is something to consider in future work with affinity tagged proteins.

The final steps in His-YihE purification are cleavage of its His-tag and removal of
the protease used to cleave the tag. The AcTEV protease from Invitrogen recognizes the
sequence E-N-L-Y-F-Q-G cleaving between Q and G. Since the protease is itself His-
tagged, it was removed after cleavage by Ni affinity chromatography. The manufacturers
of AcTEV suggest cleavage be carried out at 30°C but recommend determining optimal
cleavage conditions for each protein. Therefore a few reactions were set up as described
in the Materials and Methods section and the result of these trials are illustrated in Figure
9C. The cleavage was successful at temperatures much lower than 30°C which is
favorable for protein stability. Even at 4°C the tag was removed within 4hrs of
incubation and after 24hrs the protein remained intact. It was concluded that cleavage
could be carried out O/N in the fridge without concerns over degradation.

4.2 Analysis of YihE Kinase Activity

Structural alignment with APH(3′)-IIIa and choline kinase places RdoA/YihE in the
category of atypical protein kinases which share some but not all features of the Ser/Thr,
Tyr kinase super family (Scheeff and Bourne, 2005; Zheng et al., 2007). The ability of
wildtype YihE to autophosphorylate and phosphorylate MyBP was investigated. After
confirming wildtype kinase activity, YihE mutants (provided by Dr. Z. Jia) were
examined to gain insight into YihE’s mechanism of action as an atypical kinase. Single amino acid substitutions were made in residues predicted to influence ATP binding and phosphate transfer. Five mutations were studied and suggest YihE may, in fact, function in a manner similar to that of the eukaryotic Ser/Thr protein kinases.

4.2.1 Optimization of Time and Temperature for Kinase Reactions

A kinase like any enzyme has a set of conditions under which it functions optimally. These include, but are not limited to, a range of temperature and pH. Additionally the concentration of substrate, time allowed for phosphotransfer and the presence of cofactors has an impact on a kinase reaction. For this work, kinase reactions were performed in a buffer used to identify RdoA as a protein Ser/Thr kinase (He, 2005). A review of the literature revealed that others studying kinase activity performed reactions in similar buffers at temperature ranging from room temperature to 37°C for as little as 10min up to 1hr (Motley and Lory, 1999; Neu et al., 2000; Peck, 2006). Phosphorylation of His-YihE and MyBP was investigated at 22, 30 and 37°C for 10, 20, 30 and 60min. This was by no means an exhaustive study to optimize reaction conditions but provided some starting parameters based on previously published experiments. Incorporation of the label into His-YihE and MyBP increased with increasing time and temperature (Figure 10). Of the conditions tested, 60min at 37°C resulted in the greatest incorporation of $\gamma^{33}P$ and was chosen for future work. It cannot be definitively concluded, however, that YihE kinase activity is at its optimum under these conditions since a longer period of time or higher temperature were not tested. It should also be noted that neither the $\gamma^{33}P$-ATP nor MyBP concentrations are limiting.
Figure 10. Optimization of time and temperature for kinase reactions. 10μg of purified YihE (uncleaved) and 10μg MyBP were incubated with 10μCi [γ-33P]ATP at 22, 30 or 37°C and samples taken at 10, 20, 30 and 60min adding SDS sample buffer to stop the kinase reaction. Samples then underwent SDS-PAGE followed by autoradiography to determine conditions best suited for the kinase reaction. (A) Autoradiogram showing phosphorylation of His-YihE under various conditions. (B) Autoradiogram showing phosphorylation of MyBP under various conditions.
4.2.2 Autophosphorylation of YihE

Cleavage of His-YihE’s tag and removal of the protease unfortunately greatly reduces the concentration of pure protein since the sample must be diluted before each of these procedures. Using a Centricon device (Millipore Inc., Nepean, ON, Canada) to concentrate the sample to previous levels was marginally successful at best. Therefore, His-YihE eluted from the column was used in kinase reactions acknowledging the possibility that the tag might influence YihE autophosphorylation. After equalizing the concentration of cleaved and tagged YihE, their ability to autophosphorylate was compared to determine the effect, if any, of the tag on phosphorylation. The results were somewhat unexpected. As seen in Figure 11, after cleavage YihE autophosphorylation is hardly detectable suggesting signals perceived beforehand were due largely to the tag. This experiment validated initial concerns over using a His-tagging method to purify RdoA/YihE and underscores the importance of assessing the effect a tag might have on the protein of interest and raises questions as to why a kinase would also phosphorylate residues within its affinity tag. In the end it was decided to proceed with the tagged YihE since a) conditions under which the tag was removed may contribute to the decrease in autophosphorylation, b) using His-YihE is one means of determining whether or not the sample still possesses kinase activity by checking for “autophosphorylation” and c) both the uncleaved and cleaved protein (data not shown) were able to phosphorylate MyBP.

4.2.3 Mutational Analysis

Five YihE mutants, each with a single amino acid substitution in a predicted key residue, were examined for diminished kinase activity. Mutant S36P harbours a serine to
Figure 11. Autophosphorylation of YihE. The ability of YihE to autophosphorylate *in vitro* was examined. (A) Protein loading control; 2μg of His-YihE or YihE (after cleavage of His-tag) were electrophoresed on a 12% polyacrylamide gel then stained with Colloidal Coomassie stain. Lane 1, uncleaved protein; 2, cleaved protein. (B) Two micrograms of His-YihE or YihE were incubated with 2.5μCi γ<sup>33</sup>P-ATP at 37°C for 1hr. Reaction products were electrophoresed on a 12% polyacrylamide gel then visualized by autoradiography. Lane 1, uncleaved protein; 2, cleaved protein.
proline switch at position 36. YihE S36 aligns with S27 of APH(3′)-IIIa which interacts with the β-phosphate of ATP (Thompson et al., 2002). The aspartate at position 201 has been replaced with alanine in the D201A mutant. This aspartate is the predicted catalytic base for phosphotransfer therefore the expectation is that this mutant will not possess catalytic activity. Nearby, D217 which is predicted to coordinate two magnesium ions for catalysis has also been changed to alanine which should impede ATP binding in mutant D217A. Another important aspartate residue in the protein kinase family is D220 which stabilizes the catalytic loop through hydrogen bonding (Hanks and Hunter, 1995). In YihE, D220 has been substituted for alanine. Finally the N206A mutation maps to a highly conserved APH(3′)-IIIa asparagine residue, N195. N195 interacts with one of two magnesium ions at the active site (Smith and Baker, 2002).

The outcome of YihE mutational analysis is shown in Figure 12. Two of the five mutants, N206A and D220A, showed marked decreases while mutations D201A and D217A essentially abolished kinase activity. If predictions are correct, these two mutations affect ATP binding and the reaction that removes ATP’s γ-phosphate. This experiment demonstrates that although YihE may fall under the category of atypical kinases, its mechanism of action mirrors that of a typical Ser/Thr kinase.

4.3 Substrate Search

Phosphoprotein identification is challenging since at any given time or under a given set of conditions, only a subset of all cellular proteins are expressed and only a fraction of these are phosphorylated. A low level of expression and the action of phosphatases can further impede their identification (Raggiaschi et al., 2005). The characterization of
Figure 12. Kinase Activity of YihE mutants. The ability of five YihE mutants to autophosphorylate and phosphorylate MyBP was investigated. 5μg of each mutant and 5μg MyBP were incubated with 5μCi [γ-33P]ATP for 1hr at 37°C. The reaction was terminated by adding SDS sample buffer to the reaction then samples were subjected to SDS-PAGE and autoradiography. (A) Protein loading control for YihE, Colloidal Coomassie stained SDS-PAGE gel. (B) Autoradiogram.
RdoA/YihE thus far has focused on its biochemical activity, however, a complete picture of RdoA/YihE activity should include identification of its target protein. In order to present a protein as a kinase substrate, one must demonstrate it becomes phosphorylated by the kinase \textit{in vitro} and/or \textit{in vivo}. To this end, a few approaches have been employed that involve tracking phosphorylation in cell lysates and also using YihE mutants to identify its substrate.

### 4.3.1 Search Amongst NLM2239 Lysate

Cell lysate from NLM2239 (an \textit{rdoA}− \textit{S. typhimurium} strain) was first used as a source of substrate. Cells from 100mL culture were grown to stationary phase and soluble proteins were extracted as described in the Methods and Materials section. The soluble protein prep was then incubated with YihE and [\(\gamma^{33}\text{P}\)]-ATP. The expectation was that a number of proteins would become phosphorylated in the presence of ATP due to kinases in the lysate. If adding YihE to the reaction altered the phosphoprotein profile, the difference could be attributed to YihE phosphorylating its substrate(s). Surprisingly, only one band appeared after incubation of the lysate with ATP (Figure 13), its phosphorylation did not depend on YihE. However the intensity of the band and therefore the degree of \(^{33}\text{P}\) incorporation did depend on the amount of lysate added. As this amount increased, the intensity decreased most likely due to the action of phosphatases in the lysate.

### 4.3.2 Phosphatase Inhibitor Assay

The kinase reaction buffer in use contains 1mM NaF and 25mM \(\beta\)-GP. Based on results from labeling assays it was decided that at these concentrations, the inhibitors were not
Figure 13. Labeling of NLM2239 cell lysate. Cell lysate was prepared from a *Salmonella rdoA* strain as described in the Materials and Methods. The cell lysate was then used as a substrate for YihE in a kinase assay. Decreasing amounts of the cell lysate were incubated with 5μg of the kinase and 5μCi \([\gamma^{33}\text{P}]\text{ATP}\) for 1hr at 37°C. The reaction was stopped by adding SDS sample buffer then underwent SDS-PAGE and autoradiography.
sufficient to stop de-phosphorylation. The concentration of each was changed to 5, 25 or 50mM in the kinase reaction buffer and NLM2352 lysate (the reason for using this strain is explained in the following section) was labeled with $^{33}$P. Increasing the phosphatase concentration did not have as pronounced an effect on phosphorylation as decreasing/increasing the amount of cell lysate, although inhibitor does offer protection against phosphatase activity, compare Figure 14 lanes 4-12 to lanes 1-3. In subsequent assays involving labeling of cell lysates, 25mM NaF and $\beta$-GP were used since this allows a higher level of phosphorylation than no or 5mM inhibitor without the smearing observed with 50mM. It still appears the amount of lysate has the greatest impact on phosphorylation (Figure 14). With increasing cell lysate, there is a disappearance of the approximately 30kDa band accompanied by the appearance of bands near 60 and 100kDa that cannot be rationalized as due to the action of phosphatases. Possibly the increased amount of cell lysate increases the abundance of the higher molecular weight proteins so that their phosphorylation becomes visible by autoradiography. Protease activity may account for the disappearance of the lower molecular weight (MW) band.

With phosphatase inhibitor, particularly at the 25 and 50mM concentrations, individual bands cannot be discerned in the ~10-30kDa range, there is however a strong signal emitted from this region. Whether this is due to the presence of several phosphoproteins of similar size is not certain. Another possibility is that the phosphatase inhibitors increase ATP-dependent protease activity by inhibiting de- phosphorylation of these proteases. The smear observed could be the resulting degradation products of one or more phosphoproteins.
**Figure 14.** Phosphatase Inhibitor Assay. NaF and β-GP phosphatases were used in an attempt to increase the level of phosphorylated proteins in cell lysate labeling experiments. Zero, 5, 25 or 50mM of each inhibitor were incubated with increasing amounts of cell lysate in the presence of 5μCi [γ-33P]ATP for 1hr at 37°C. Kinase activity was terminated with the addition of SDS sample buffer and samples were subjected to SDS-PAGE and autoradiography. Lanes 1-3, [0mM] inhibitor with 1.2, 2.4 and 12μg protein respectively; 4-6, [5mM] inhibitor with 1.2, 2.4 and 12μg protein respectively; 7-9, [25mM] inhibitor with 1.2, 2.4 and 12μg protein respectively; 10-12, [50mM] inhibitor with 1.2, 2.4 and 12μg protein respectively.
4.3.3 Pull-down Experiments

Uncovering a substrate through labeling of cell lysates in the presence of YihE was not producing the desired results; a more targeted approach was needed. RdoA is more highly expressed during the later stages of cell growth and when the Cpx system is activated (He, 2005). Since the Cpx system is a stress response system and \textit{rdoA} transcription increases under stress conditions sensed by Cpx such as alkaline pH, this increased transcription may coincide with an increase in the abundance of RdoA’s substrate. It was hypothesized that cell lysates prepared from stationary phase cells expressing NlpE, an activator the Cpx stress response, would contain higher levels of RdoA’s substrate, which would facilitate its identification. NLM2239 cells were transformed with pND18 to create strain NLM2352. Cell lysates were prepared from NLM2352 cells induced to express NlpE and grown into stationary phase. In trying to identify kinase substrates, other researchers have employed pull-down experiments where a kinase is immobilized on a column and used to capture its substrate (Powell \textit{et al.,} 2002). For this method to work the substrate must interact strongly and long enough with the kinase so that it will remain bound until the two are eluted from the column. This type of approach was attempted without success using wildtype YihE, after which it was repeated with the YihE mutants. The rationale being that since the mutants are less able to phosphorylate a substrate like MyBP (with the exception of one), they may bind their substrate and remain bound to it for a longer period of time due to their reduced ability for phosphotransfer. The YihE mutants were overexpressed in NLM364 as previously described followed by affinity chromatography, however, the protein was not eluted from the column, and rather cell lysate from NLM2352 was added followed by washing then
elution of each YihE mutant. The elute was used in a kinase assay. Since four out of five mutants have reduced kinase activity, wildtype YihE was added to each elute to phosphorylate any potential substrate trapped and released by the mutants. As seen in Figure 15, two phosphorylated bands appear in addition to YihE when ATP is added to the products of each mutant’s pull-down. Neither of these bands appears when ATP is added to the lysate alone. It seems the pull-down experiment has enriched at least two proteins which correspond to MW of approximately 70 and 30kDa. It is unlikely that the proteins are YihE substrates however, since they are phosphorylated in the absence of fully functional YihE. The 70kDa band may be a YihE dimer that has trapped ATP or another cellular kinase that has been captured by the column and undergoes autophosphorylation upon the addition of ATP. Comparing Figures 14 and 15 reveals that in the phosphatase inhibitor assay (Figure 14), an approximately 30kDa protein is phosphorylated in the absence of YihE. Additionally, labeling of NLM2239 lysates resulted in phosphorylation of an approximately 30kDa protein also in the absence of YihE. Interestingly, the 30 and 70kDa bands are less intensely or not at all phosphorylated in the presence of wildtype YihE (Figure 15).

Three of the mutations, S36P, D201 and N206A, appear to act as dominant negative mutations. When wildtype YihE is incubated with each of these mutants and ATP, there is a decrease in wildtype YihE autophosphorylation and in the case of YihE S36P a decrease in autophosphorylation of both the wildtype and mutant. An inhibitory interaction may take place between the wildtype and mutant proteins or there may be competition for ATP. There is 6-7 times more mutant than wildtype protein in lanes 5, 7 and 9. The mutants may trap ATP, preventing the wildtype YihE from becoming
Figure 15. Pull-down and subsequent labeling assay with YihE mutants. Five Histagged YihE mutants were immobilized on a Ni column to which NLM2352 cell lysates from were added in an attempt to trap a substrate. Each odd numbered lane contains 2.5µg of wildtype YihE. Lane 1 contains 5µg MyBP and serves as a positive control for YihE activity. Lanes 2 and 3 contain 15µg of protein from the cell lysate and lanes 4-13 contain the elute from the indicated mutant’s pull-down. The amounts of eluted protein added to the labeling assays were approximately 18, 18, 14, 12, and 8µg for S36P, D201A, N206A, D217A and D220A respectively.
autophosphorylated, however they are unable to hydrolyze ATP and become autophosphorylated themselves.

4.4 RdoA and Cpx Signaling

Placing cells in an alkaline pH environment is one way in which Cpx activity is induced in the laboratory. In *E. coli* and *Shigella sonnei* the effect of alkaline pH on Cpx activity has been documented (Danese and Silhavy, 1998; Nakayama and Wantanbe, 1995), however, this has not been studied directly in *Salmonella*. With the use of a *cpxP:*lacZ reporter, the effect of alkaline pH on *cpxP* transcription was investigated. β-galactosidase assays were performed on wildtype, *rdoA*− and *cpxAR*− strains harbouring this reporter.

4.4.1 The Effect of pH on Cpx Activity

In wildtype *S. typhimurium*, Cpx dependent transcription of *cpxP* occurs even at a pH of 5 (Figure 16B). There is no change in *cpxP* transcription from pH 5 to 6 followed by a substantial increase from pH 6 to 7 and no change from pH 7 to 8 (Figure 16B). A similar trend was observed when the reporter was moved to a wildtype *E. coli* background (Figure 16A). Danese and Silhavy (1998) studied the effect of pH on *cpxP* transcription in *E. coli*. They found the system was off below pH 5.3 and saw an almost fifty fold increase in transcription from pH 5.3 to 8.4. *cpxP* transcription beyond pH 8 was not examined in the current work so possibly there is an increase at pHs higher than 8 as seen in the Daness and Silhavy study or it may be that pH regulated Cpx activity differs between the two organisms.
Figure 16. β-galactosidase Assays; the effect of pH on cpxP transcription in S. typhimurium. cpxP transcription under different pH conditions was measured in various strains using a cpxP::lacZ reporter. 25mL of LB buffered with 100mM sodium phosphate were inoculated 1:25 with O/N cultures. Cultures were grown at 37°C with aeration until an OD₆₀₀ between 0.7-0.8 was reached. The cells were then sub-cultured into fresh media at pH 5, 6, 7 or 8 buffered with 100mM sodium phosphate and grown for an additional hour after which the β-galactosidase activity of each culture was determined as described in the Materials and Methods section. (A) NLM411, n=5. (B) NLM2346, n=3. (C) NLM2347, n=4. (D) NLM2353, n=2. Asterisks indicate a statistically significant difference from one pH to the next higher pH.
Because the levels of β-galactosidase activity seen in a cpxAR null background strain are similar to those measured from the vector alone (data not shown), cpxP transcription depends solely on CpxA and/or CpxR. The role of RdoA in Cpx’s pH response was also investigated using an rdoA- S. typhimurium strain. The absence of rdoA activates the Cpx system particularly at an alkaline pH (compare Figure 16B and C) when rdoA is more highly expressed (He, 2005). However, as with the other strains investigated, in an rdoA null background there was no statistically significant difference in cpxP transcription from pH 7 to 8.
CHAPTER 5

DISCUSSION

The discovery of Pkn1, a Ser/Thr kinase from *M. xanthus* (Munoz-Dorado *et al.*, 1991), fractured the dividing wall between prokaryotic and eukaryotic Ser/Thr protein kinases. This work led the way to characterizing other bacterial Ser/Thr kinases such as RdoA and YihE which are the topic of this thesis. RdoA is a Ser/Thr kinase discovered in *S. typhimurium* with homologues in several bacterial species (He, 2005; Martin, 2007). Both the *E. coli* and *S. typhimurium* proteins undergo autophosphorylation and are able to phosphorylate MyBP *in vitro* (Zheng *et al.*, 2007; this work). Recent publication of YihE’s crystal structure revealed its kinase domain bears structural similarity to members of the Ser/Thr, Tyr protein kinase superfamily as well as having its own unique features. YihE structurally resembles choline kinase and APH(3’)-IIIa, two atypical kinases, although there is little direct amino acid sequence similarity. The goal of this project was to further characterize RdoA, including elucidation of its substrate, and by doing so gain insights into its role as a member of the Cpx regulon.

5.1 Expression and Purification of RdoA and YihE

Two methods were employed for overexpression and purification of RdoA and YihE. With the pMAL system, RdoA was cloned as an MBP fusion protein. High levels of MBP-RdoA were obtained from overexpression, however, a one-step purification scheme does not result in a sample of high purity (Figure 8C). On the contrary, YihE was expressed with an N-terminal His-tag and purified with greater success (Figure 9B).
drawback to this protocol is the impact of the tag on kinase activity. This can be overcome, however, by removal of the tag.

5.2 RdoA/YihE Kinase Activity

The capacity for RdoA to autophosphorylate has been demonstrated in vitro (He, 2005). The same studies were carried out in this work with YihE revealing that it also undergoes autophosphorylation. Interestingly, the tag associated with YihE to facilitate its purification contributes residues that are phosphorylated in vitro (Figure 11). When expressed from pPI224, the amino acid sequence upstream of YihE reads MGSSHHHHHHHHDDYDIPTTENLYFQGS. By comparing lanes 1 and 2 of Figure 11 it is clear that at least one of these residues becomes phosphorylated in the presence of $\gamma^{33}\text{P}]\text{ATP}$. Young et al. (2003) and Boitel et al. (2003) had similar findings while characterizing PknB, a kinase from Mycobacterium tuberculosis. Up to two phosphoserines were detected on a peptide corresponding to the His-tag fragment in the former study (Young et al., 2003) while the latter identified a monophosphorylated His-tag peptide (Boitel et al., 2003). YihE’s tag contains several residues with the potential to become phosphorylated. It is likely that phosphorylation occurs on one or more of the available serine or threonine residues. The intense signal generated from His-YihE phosphorylation compared to that of YihE suggests the tagged protein has multiple phosphorylation sites while the native protein may only have one or two. Interestingly, there are 23 serine and threonine residues in the YihE protein, yet it favours phosphorylation of residues, potentially serine and/or threonine, within the affinity tag.
Perhaps due to their arrangement at the protein’s N-terminus they are more accessible or surrounding residues may create a more favourable environment for autophosphorylation.

Phosphorylation of residues in a region termed the activation segment is an important aspect of regulation in many protein kinases, converting the enzyme from an inactive to active state. The phospho-modification may promote correct domain orientation, proper alignment of ATP binding residues or relieve autoinhibition by the activation segment (Johnson et al., 1996). Autophosphorylation as a means of activation occurs in kinases such as PKA (Steinberg et al., 1993), PknB (Boitel et al., 2003; Young et al., 2003) and phosphorylase kinase (Wang et al., 1976). PknB is autophosphorylated on Thr171 and Thr173, and phosphorylation of both residues is required for full enzymatic activity. PknB T171A and T173A mutants were 15 and 20 times less active than the wildtype, respectively, in a MyBP phosphorylation assay. Activity was reduced 300-fold in the T171A/T173A double mutant and an N-terminal His-tag also showed a lower degree of phosphorylation in the three mutants compared to wildtype (Boitel et al., 2003). For most Ser/Thr kinases and all Tyr kinases, a catalytic aspartate is directly preceded by an arginine residue giving these kinases the name RD kinases. Ionic interactions are required between this arginine and a phosphate or carboxylate group (Johnson et al., 1996). An additional role for phosphorylation of residues in the activation segment is the neutralization of charge at the catalytic site. As Figure 17 illustrates, there is a clustering of positively charged residues in this region in PKA. Without the phosphate group, charge repulsion would not allow these catalytically important residues to be brought into their correct alignment (Johnson et al., 1996). RdoA and YihE are not RD kinases and they do not contain an activation loop, however,
Figure 17. Clustering of residues at PKA’s catalytic site. Thr197-P makes contact with Lys189 and Arg195 as well as His87 and Thr195 (not shown in this diagram). The phosphate’s dianionic group is necessary to overcome repulsion from the close arrangement of several positive charges so that the ATP molecule and catalytically important residue may be correctly aligned. (Johnson et al., 1996).
they undergo autophosphorylation. Neither their phosphorylated residue(s) nor the significance of these phosphorylation events is known.

Five YihE mutants were examined for a loss of *in vitro* kinase activity. Single amino acid substitutions were made in conserved residues clustered in the proposed ATP binding pocket based on alignment with APH(3’)-IIIa. A loss of kinase activity, to varying degrees, was observed in four of the five mutants (Figure 12). The difference in activity observed amongst the mutants and in comparison to wildtype YihE is cause for further discussion. Beginning with the S36P mutant, this mutation does not interfere with kinase activity as measured in the current study. Compared to wildtype YihE, YihE S36P was able to autophosphorylate and phosphorylate MyPB to a degree equal to or greater than the wildtype enzyme. S27 is highly conserved amongst the aminoglycoside phosphotransferases. Although these enzymes lack the GXGXXG ATP binding motif found in subdomain II of the protein kinases, a GXS motif plays the same role in APH(3’)-IIIa (Smith and Baker, 2002). The serine residue hydrogen bonds with ATP’s β-phosphate. The effect a mutation in this residue would have on kinase activity has been studied and S27 has been shown to be important for phosphoryl transfer (Thompson *et al.*, 2002). This work reveals that a similar mutation in YihE does not result in a loss of activity. A nearby lysine residue, conserved in the aminoglycoside phosphotransferases and the protein kinases (Hanks and Hunter, 1995; Wright and Thompson, 1999; Smith and Baker, 2002), also anchors the ATP molecule through hydrogen bonding and may compensate for the loss of S36. The D201A mutation, as expected, virtually abolishes kinase activity. D201 corresponds to APH(3’)-IIIa D190, which has been identified by mutagenesis as the catalytic base (Hon *et al.*, 1997). Surrounding this highly conserved
and catalytically important residue are N195 and D208 (APH(3’)-IIIa numbering). Both are involved in coordinating magnesium ions at the active site. Two magnesium ions are found associated with AMPPNP, the bound nucleotide, in APH(3’)-IIIa’s crystal structure. One forms a bridge between the α- and β- phosphate groups, the other bridges the β- and γ-phosphates of AMPPNP. The side chains of N195 and D208 interact with the former magnesium ion while D208 interacts with the latter (Smith and Baker, 2002). D208N and D208E mutations in APH(3’)-IIIa result in enzymes lacking detectable kinase activity while an N195A mutant has a decreased affinity for ATP (Boehr et al., 2001). This work demonstrates that the equivalent residues in YihE also positively influence enzymatic activity since the corresponding mutations in YihE lead to a marked decrease in γ-33P labeling (Figure 12). The YihE D220A mutant is also impaired in its ATP binding and/or phosphotransfer capability, although to a lesser extent than the D201A, D217A and N206A mutants. YihE D220 has not been aligned with a key residue in APH(3’)-IIIa however it lies in the ATP binding pocket (J. Zheng personal communication) and is highly conserved amongst YihE homologues (He, 2005). For members of the Ser/Thr, Tyr protein kinase superfamily, D220 stabilizes the catalytic loop through hydrogen bonding (Hanks and Hunter, 1995). YihE D220 may play a similar role.

Mutagenesis studies to date have identified several residues important for YihE kinase activity while providing evidence that the mechanism of YihE catalysis and various residues implicated in ATP positioning, are consistent with members of the protein kinase superfamily. Remarkably, YihE lacks the GXGXXG loop important for ATP positioning in this superfamily (Zheng et al., 2007). The crystal structure of YihE
in complex with an ATP analogue would contribute valuable information towards the characterization of this bacterial kinase. Additionally, for members of the kinase superfamily an invariant lysine residue in subdomain II is important for phosphotransfer (Carrera et al., 1993). Key amino acid residues that could be examined in future studies include this lysine.

5.3 RdoA/YihE Substrate Search

One basic method of identifying the substrate of a kinase involves incubating cell extracts with magnesium, a radio-nucleotide and the kinase of interest to see which proteins in the extract become labeled. Labeling of *S. typhimurium* cell lysates in the presence of YihE has not lead to the identification of a substrate (Figure 13). An attempt to co-purify a substrate using YihE mutants was also unsuccessful (Figure 15). Drawbacks to this approach can be that the presence of other kinases in the cell extract can lead to background phosphorylation and subsequent protein purification to identify a potential substrate may be difficult (Peck, 2006). One way of circumventing the first drawback is using high concentrations of the added kinase however, since many protein kinases can phosphorylate proteins *in vitro* that are not their physiological substrates, caution must be taken when interpreting the results of such an experiment (Cohen and Knebel, 2006). Ion exchange chromatography is another way of reducing background phosphorylation since this procedure separates most kinases from their substrates while concentrating substrates thereby increasing the likelihood they will be detected by their cognate kinase (Knebel et al., 2001). Another interesting technique involves introducing a functionally silent mutation into the ATP binding site of the kinase under investigation. The mutation
replaces a bulky amino acid with a small one like glycine or alanine creating space in the ATP binding pocket for a bulky ATP analogue. In a reaction mixture, only the mutated kinase will be able to utilize the bulky ATP analogue reducing background (Shah and Shokat, 2003). The elucidation of RdoA’s substrate may require using one or more of the modifications described above. Phosphoprotein enrichment from the NLM2352 strain and fractionation of the cell lysate based on molecular weight are also worth exploring. To date, only the cell’s soluble protein fraction has been examined for an RdoA/YihE substrate, the rationale being that since RdoA is a cytoplasmic protein (He, 2005), its substrate should also reside in the cytoplasm. It is feasible however, that the substrate is a membrane protein with a cytoplasmic domain. Devising a system to solubilize the membrane fraction without interfering with enzymatic activity would expand the search to insoluble proteins that have domains exposed to the cytoplasm.

The current reaction buffer is well suited for kinase reactions involving purified, soluble protein (Figure 10) but may require adjustments for the cell lysate labeling experiments employed here. A pH optimum or ideal magnesium concentration has not been determined for RdoA/YihE. RdoA/YihE may, in fact, utilize another divalent cation such as manganese. PknB, a kinase from Mycobacterium tuberculosis, for example, functions better in the presence of MnCl₂ rather than MgCl₂ (Boitel et al., 2003). Another concern is the adequate inhibition of protein phosphatases. NaF and β-GP have been used as phosphatase inhibitors with varying success. At high concentrations (50mM) they result in high background and smearing on autoradiograms (Figure 14). It is possible that β-GP inhibits kinase activity. Phosphorylase kinase, for instance, is activated either by autophosphorylation or via phosphorylation by PKA. The
former method of activation is inhibited by the presence of \( \beta \)-GP, while PKA catalyzed activation is not (Wang et al., 1976). In order to proceed with the labeling of cell lysates, the effect of \( \beta \)-GP on RdoA/YihE kinase activity must be determined and if need be, a more suitable phosphatase inhibitor found.

Richards (2006) has suggested integration host factor (IHF) as a putative substrate of RdoA. IHF is a regulatory protein able to bind DNA causing a bend of up to \( 180^\circ \) (Rice, 1997). Notably, like RdoA, IHF expression is growth phase dependent (Aviv et al., 1994) and increases with the onset of stationary phase (Ditto et al., 1994). IHF is composed of \( \alpha \)- and \( \beta \)-subunits encoded by the \( \text{ihfA} \) and \( \text{ihfB} \) genes respectively and can exist as a homo- or heterodimer. \( \text{ihfAB} \) and \( \text{ihfA} \) mutants express only FljB flagellins while in the absence of \( \text{ihfB} \) only FliC is detected (Mangan et al., 2006). This change in flagellin expression could be linked to that observed in an \( rdoA^- \) mutant. IHF regulation is complex due to its ability to form homo- and heterodimers. If RdoA acts on IHF, phosphorylation might mediate the change between its various forms and or affect its DNA binding ability causing changes in gene expression (Richards, 2006).

5.4 RdoA and the Cpx pH Response

Subjecting cells to growth at alkaline pH is one way in which Cpx activity is induced in the laboratory, however the pH threshold of this response in Salmonella had not been investigated. An area of interest in the current work is RdoA’s function within the Cpx stress response. After construction of a \( \text{cpxP}::\text{lacZ} \) reporter, the effect of alkaline pH on \( \text{cpxP} \) transcription was investigated. \( \beta \)-galactosidase assays were performed on wt, \( rdoA^- \) and \( cpxAR^- \) strains harbouring this reporter (Figure 16). The absence of \( rdoA \) activates
the Cpx system particularly at an alkaline pH (compare Figure 16B and C). This is consistent with the observation that \textit{rdoA} is more highly expressed under alkaline conditions (He, 2005) and supports the hypothesis that RdoA is involved in propagating the Cpx response signal. As the pH approaches and exceeds 7, a stress signal appears to be generated and the Cpx system is activated to respond to the alkaline condition. Part of this response involves up-regulation of RdoA whose activity potentially plays a role in stress alleviation. In the absence of RdoA, it appears the signaling pathway that would normally lead to recovery from the stressful condition is broken. The Cpx system is further activated and maintained in an active state.

5.5 Summary and Future Directions

The work presented here describes the purification and characterization of a bacterial kinase and attempts to identify its substrate. This work demonstrates that YihE, and by extension RdoA, functions in a manner likened to that of other Ser/Thr kinases. Mutagenesis of an invariant lysine residue and determination of the affect of this mutation will further add to the characterization. It has been shown that YihE undergoes autophosphorylation, however, which residues are phosphorylated remains unknown. Mass spectrometry analysis will be required to map out the phosphorylated residue(s). Studies on the effect of pH on Cpx activity support a role for RdoA in relaying the Cpx activation signal. IHF has been hypothesized as a substrate of RdoA/YihE (Richards, 2006), cloning of \textit{ihf} is being pursued in order to test this hypothesis.
REFERENCES


