Ligand Sensing and Signal Transduction by the *Salmonella enterica* Serovar Typhimurium PhoQ Histidine Kinase Sensor

by

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ABSTRACT

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The *Salmonella typhimurium* PhoP/PhoQ two-component system controls the expression of numerous genes involved in virulence. This system is activated *in vivo* within acidified macrophage phagosomes and is repressed *in vitro* in high concentrations of divalent cations such as Mg^{2+}, Ca^{2+}, and Mn^{2+}.

The *pho*-24 allele of *phoQ* harbors a single amino acid substitution (T48I) in the periplasmic domain of the PhoQ histidine kinase sensor. This mutation has been shown to increase net phosphorylation of the PhoP response regulator. The mechanism by which *pho-24* attenuates virulence is defined. The effect on signaling by PhoP/PhoQ of various amino acid substitutions at this position (PhoQ-T48X; X = A, S, V, I and L) were analyzed. Mutations T48V, T48I and T48L were found to affect signaling by PhoP/PhoQ both *in vivo* and *in vitro*. The data are consistent with a model in which the residue at position 48 of PhoQ contributes to a conformational switch between kinase- and phosphatase-dominant states.

The purification and functional reconstitution of $PhoQ_{His}$ is also reported. Reconstituted $PhoQ_{His}$ exhibited all of the catalytic activities described for histidine sensor kinases. This tool allowed us to assess the role of the divalent cations Mg^{2+}, Ca^{2+}, and Mn^{2+} on reconstituted $PhoQ_{His}$ catalytic activities by varying the concentration of divalent cation acting as a ligand for the reconstituted $PhoQ_{His}$, while maintaining a constant concentration of catalytic Mg^{2+}. High concentrations (5 mM) of Mn^{2+}, and to a lesser extent Ca^{2+}, are more potent than Mg^{2+} at repressing the net phosphorylation of PhoP by reconstituted $PhoQ_{His}$, consistent with *in vivo* results.

Antimicrobial peptides were also shown to directly activate the *S. typhimurium* PhoQ kinase sensor. The alpha-helical antimicrobial peptides C18G and LL-37 activation of reconstituted $PhoQ_{His}$ was shown and this activation can be competed with Mg^{2+}. These findings contribute to a model in which antimicrobial peptides and divalent cations both play a role in initiating signal transduction through the PhoQ histidine kinase sensor.

RÉSUMÉ

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Le système bi-protéique PhoP/PhoQ de *Salmonella typhimurium* contrôle l'expression de plusieurs gènes de virulence. Ce système est activé *in vivo* lors de l'acidification des phagosomes des macrophages et réprimé *in vitro* en présence de fortes concentrations en ions divalents tel le Mg^{2+}, Ca^{2+} ou le Mn^{2+}.

L'allèle *pho*-24 du gène *phoQ* possède une substitution simple à la position 48 (T48I) située dans le domaine périplasmique du récepteur histidine kinase PhoQ. Il a été démontré que cette mutation spécifique augmentait la phosphorylation nette de la protéine PhoP de ce système bi-protéique. De plus, le mécanisme par lequel l'allèle *pho*-24 atténu la virulence a été déterminé. Ici, nous avons analysé l'implication du résidu 48 sur la signalisation par PhoP/PhoQ en faisant une série de substitutions (PhoQ-T48X; X= A,S,V,I et L). Les mutations T48V, T48I et T48L ont montré un effet sur la signalisation autant *in vivo* qu'*in vitro*. Ces données prouvent le modèle selon lequel le résidu à la position 48 de PhoQ contribue à un changement conformationel entre les activitées kinases et de phosphatases de cette protéine. Nous avons aussi fait la purification et la reconstitution fonctionnelle de $PhoQ_{His}$. Ce mutant arbore toutes les caractéristiques décrites pour le récepteur histidine kinase PhoQ sauvage. Cet outil nous permet d'établir le rôle des cations divalents Mg^{2+}, Ca^{2+} ou le Mn^{2+} dans la regulation de l'activité catalytique, tout en maintenant une concentration constante de Mg^{2+} pour la fonction enzymatique. Une inhibition de la phosphorylation nette de PhoP par la protéine $PhoQ_{His}$ a été détectée lors de l'utilisation de forte concentration (5 mM) d'ions divalents. Ce phénomène est le plus marquant avec le Mn^{2+}, mais aussi observable avec le Ca^{2+} et le Mg^{2+} ($Mn^{2+}>Ca^{2+}>Mg^{2+}$). Cette répression de l'activité catalytique de $PhoQ_{His}$ est en accord avec les données recueillies *in vivo*.

Il a aussi été démontré que des peptides antimicrobiens ont une activité directe sur le récepteur PhoQ de *Salmonella typhimurium*. Ainsi, les peptides C18G et LL-37, qui ont une conformation en hélice alpha, utilisés dans la reconstitution avec $PhoQ_{His}$ montrent une activation du système bi-protéique qui compétitionne avec

celle du Mg^{2+}. Ces découvertes contribuent au modèle selon lequel autant les peptides antimicrobiens que les ions divalents jouent un rôle dans l'initiation du signal de transduction produit par PhoP/PhoQ.

ACKNOWLEDGEMENTS

It is a privilege to thank those who have made this thesis possible. First and foremost, I acknowledge my supervisor Dr. Hervé Le Moual. I am honoured to call myself the first Le Moual doctoral student. I am simply indebted to your guidance and support that have allowed for my development as a scientist.

Drs. James Coulton and Albert Berghuis served as members of my Ph. D advisory committee and they contributed to this thesis through their suggestions and discussions. I am also indebted to Dr. Coulton for additional support, acting as my advocate on innumerable occasions and generously providing his time to discuss my scientific development.

Past and present members of the Le Moual lab, particularly Phillipe Perron-Savard, Sio Mei Lai, and Valerie Le Sage have acted as technical resources and more importantly, valuable colleagues. Other departmental members, particularly Valérie Hay, have aided with scientific and emotional support. Valérie Hay is also to be acknowledged for translation of the abstract to french.

I am also grateful to past and present members of McGill University Varsity Rowing Heavyweight Women and particular Montreal Rowing Club members. I cannot imagine having survived this Ph. D without their enthusiasm, laughter and fierce friendship.

My deepest love and gratitude go to my family. There is no doubt that they have bestowed on me an abundance of love, humour, perspective, sound advice and patience. This thesis is their accomplishment as much as mine.

Finally, I acknowledge financial support from the Department of Microbiology and Immunology, the Faculty of Medicine, and the Natural Sciences and Engineering Research Council of Canada.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. Identified an additional PhoP constitutively active mutant *of Salmonella typhimurium* PhoQ, PhoQ-T48V, and a PhoP constitutively inactive mutant, PhoQ-T48L.

2. Established that the PhoP constitutively active phenotype (PhoQ-T48I/V mutants) is due to decreased phosphatase activity while the PhoP constitutively inactive phenotype (PhoQ-T48L mutant) is due to both decreased autokinase activity and increased phosphatase activity.

3. Proposed a model in which position 48 of the PhoQ periplasmic sensor domain contributes to a conformational switch between kinase- and phosphatase-dominant states.

4. Established the conditions by which the purified *Salmonella typhimurium* $PhoQ_{His}$ protein can be reconstituted into *Escherichia coli* liposomes, including solubilization, purification, reconstitution, and orientation of $PhoQ_{His}$ in proteoliposomes.

5. Demonstrated that reconstituted $PhoQ_{His}$ exhibits all of the catalytic activities of a histidine kinase sensor. Thus, the development of this tool will allow for the study of divalent cations and other ligands on the regulation of solely the PhoQ protein.

6. Showed that Mn^{2+} is a more potent regulator of reconstituted $PhoQ_{His}$ activity than Ca^{2+} and Mg^{2+}. Thus Mn^{2+} may be as relevant as Ca^{2+} and Mg^{2+} for *Salmonella in vivo*.

7. Demonstrated that reconstituted $PhoQ_{His}$ directly responds to α-helical antimicrobial peptides. Therefore antimicrobial peptides are an additional ligand for the PhoQ sensor kinase.

8. Showed solely reconstituted $PhoQ_{His}$ is sufficient to respond to antimicrobial peptides, strongly arguing that the periplasmic domain is directly involved in peptide signaling.

9. Demonstrated that reconstituted $PhoQ_{His}$ activation by α-helical antimicrobial peptides can be competed with Mg^{2+}, suggesting that metals may compete with antimicrobial peptides for binding to the periplasmic domain.

CONTRIBUTIONS OF AUTHORS

In accordance with the guidelines of the Faculty of Graduate Studies and Research, McGill University, concerning thesis preparation, I have opted to present the experimental portion of this thesis (Chapters 2, 3, 4, and 5) in the form of manuscripts. Therefore, I have included the text of manuscripts which have been published (Chapters 2, 3, and 5) or in preparation (Chapter 4).

Chapters 2, 3 and 5 all contain their own Abstract (Chapters 2 and 3) or Summary (Chapter 5), Introduction, Materials and Methods (Chapter 2) or Experimental (Chapter 3) or Experimental Procedures (Chapter 5), Results, Discussions, Acknowledgements and References. Chapter 4 was submitted as a note and therefore reflects this style with the absence of divisions. A preface serves as a connecting text between manuscripts and is found prior to Chapters 2, 3, 4 and 5. A general introduction and current review of literature are provided in Chapter 1 and general conclusions and opportunities for future research are presented in Chapter 6. References are found at the end of each chapter. Page numbers of this thesis are found at the bottom center of each page.

The manuscripts comprising the experimental portion of this thesis are listed below with the contributions of each author with respect to the experimental work. I am responsible for all experimental work and I composed the text of each manuscript unless indicated otherwise. All of my work was conducted under the sole supervision of Dr. Hervé Le Moual and in his laboratory.

1. **Sanowar, S. , Martel, A., and Le Moual, H.** 2003. Mutational analysis of the residue at position 48 in the *Salmonella enterica* Serovar Typhimurium PhoQ sensor kinase. J. Bacteriol. **185:** 1935-1941 (presented as Chapter 2 of this thesis).

   A . Martel performed the site-directed mutagenesis and cloning of some plasmids.

Dr. H. Le Moual prepared the pPROLAR plasmid with ampicillin resistance cassette. He also wrote and edited this manuscript. I assisted in the writing and editing.

2. **Sanowar, S., and Le Moual, H.** 2005. Functional reconstitution of the *Salmonella typhimurium* PhoQ histidine kinase sensor in proteoliposomes. Biochem J. **390:**769-776 (presented as Chapter 3 of this thesis).

   Dr. H. Le Moual assisted in writing and edited this manuscript.

3. **Sanowar, S., and Le Moual, H.** 2005. Divalent-cation regulation of the *Salmonella enterica* Serovar Typhimurium PhoQ sensor kinase in proteoliposomes. (In preparation; presented as Chapter 4 of this thesis).

   Dr. H. Le Moual edited this manuscript.

4. **Bader M.W., Sanowar, S., Daley, M. E., Schneider, A.R., Cho, U., Xu, W., Klevit, R.E., Le Moual, H., and Miller, S.I.** 2005. Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell. **122:**461-472 (presented as Chapter 5 of this thesis).

   I performed experiments and writing for the PhoQ reconstitution.
   M. E. Daley and **R. E. Klevit** performed the NMR experiments.
   W. Xu and **U. Cho** crystallized the PhoQ periplasmic domain.
   A. R. Schneider made the labeled PhoQ periplasmic domain for the NMR experiments.
   Dr. S. I. Miller edited the manuscript and provided funding.
   Dr. H. Le Moual edited the text and figure pertaining to the reconstitution experiments. Also provided funding for these experiments.
   M. Bader conducted all other experiments, writing and editing.

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CHAPTER 1:
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OVERVIEW OF THE LITERATURE REVIEW

This thesis explores the topic of ligand sensing and signal transduction of the PhoQ sensor kinase in *Salmonella enterica* serovar Typhimurium *(Salmonella typhimurium*). This literature review will provide the background necessary for understanding this topic and is comprised of five main parts. Part one, "The molecular basis of *Salmonella* pathogenesis", will describe the organism and bacterial virulence factors allowing penetration of the intestinal epithelial and replication in the submucosal macrophage. Part two, "Genetic and environmental control of *Salmonella* pathogenesis" will discuss the control of the *Salmonella* virulence program, with emphasis on genetic and environmental regulators. Since several two-component regulatory systems are involved in controlling *Salmonella* pathogenesis, part three, "Two-component signal transduction" will detail the structure and function of these systems while concentrating on the initial steps of ligand recognition and signal transduction through the histidine sensor kinase. This will lead directly to part four, "The PhoP/PhoQ two-component system", where we will focus on a particular two-component system governing many critical *Salmonella* responses during infection. Part five, "Ligands of the *Salmonella* PhoQ kinase sensor" will detail our current understanding of environmental ligands recognized by the PhoQ kinase sensor and their regulation of PhoQ activity. This final section will also review cationic antimicrobial peptides, small innate immune molecules recently proposed to be sensed by the *Salmonella* PhoQ kinase sensor. This review will allow for a thorough understanding of the mechanisms involved in ligand recognition and signal transduction of the *Salmonella typhimurium* PhoQ sensor kinase.

THE MOLECULAR BASIS OF SALMONELLA PATHOGENESIS

Salmonella is a significant threat to worldwide health with ingestion of contaminated food and water causing a range of diseases from localized gastroenteritis to the more systemic typhoid fever. *Salmonella*-induced enterocolitis is the single most common cause of death from food-borne illnesses associated with microbial pathogens in the United States (Mead *et al.*, 1999). This gram-negative organism has also garnered interest for its ability to provide a model system in which to study bacterial virulence factors of an intracellular pathogen. The availability of genetic techniques, experimental models, including knock-out mice, and the genome sequences of several *Salmonella* strains (McClelland *et al.*, 2001; Parkhill *et al.*, 2001), have allowed *Salmonella* to act as a model organism for the study of host-pathogen interactions.

Despite the heterogeneity of hosts infected by *Salmonella* genus, the genetic composition of the genus is quite similar with only two species, *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is further divided into several subspecies and serovars and includes the human restricted *S. enterica* serovar Typhi (hereto referred as *S. typhi*), etiological agent of typhoid fever, and the broader *S. enterica* serovar Typhimurium (hereto referred as *S. typhimurium*) which causes gastroenteritis in humans and a systemic typhoid-like disease in mice. The observations that two different *Salmonella* serovars cause comparable diseases in different hosts and that one serovar causes different diseases depending on the host suggests that *Salmonella* infection provides a unique opportunity to study the contribution of bacterial and host factors to disease. *Salmonella enterica* possess sophisticated determinants to engage the host innate immune system, to sense the host environment and modulate host cellular processes. Studies of this functional interface between pathogen and host provide insights not only into the molecular mechanisms governing bacterial infection but also further our knowledge of basic host cellular processes.

Interactions with the intestinal epithelium

Salmonella infections are initiated when the ingested pathogen survives passage through the acidic environment of the stomach and proceeds to breach the intestinal tract barrier to enter the bloodstream and colonize the liver, spleen and other tissues. Gastric acidity represents a barrier to microbial infection and *Salmonella* has shown adaptive acid-tolerance on exposure to low pH, possibly aiding its survival through the stomach (Garcìa-del-Portillo *et al.*, 1993). Bacteria must also survive antimicrobial peptides and other antimicrobial substances synthesized and secreted by intestinal Paneth cells (reviewed in Ayabe *et al.*, 2004). One of these antimicrobial peptides, human defensin-5 (HD-5) has been shown to be critical for controlling *Salmonella* infections. Genetic transplantation of HD-5 into mice results in dramatic improvement in the resistance of mice to intestinal infections with *Salmonella typhimurium* (Salzman *et al.*, 2003). Various *Salmonella* fimbriae are involved in allowing the bacteria to adhere to the intestinal epithelial cells (Baumler *et al.*, 1996). Although nonphagocytic enterocytes are used for *Salmonella* invasion through bacterial-mediated endocytosis, membranous epithelial (M) cells represent the major point of entry for the bacterium in humans (Jepson and Clark, 2001). M cells are specialized antigen-sampling cells of the mucosal immune system and can be found in the regions overlaying Payer's patches. The role of these cells is to sample the intestinal lumen and transcytose these materials to the underlying lymphoid tissue (Kraehenbuhl and Neutra, 2000). *Salmonella* may also passively cross the intestinal barrier following phagocytosis by migrating CD18-positive phagocytes (Vaquez-Torres *et al.*, 1999).

Much research has been focused on elucidating the molecular mechanisms governing bacterial-mediated endocytosis. Most of the determinants for bacterial-mediated endocytosis are encoded within the *Salmonella* pathogenicity island 1 (SPI-1) located at centisome 63 on the *Salmonella* chromosome (Fig. 1A). SPI-1 encodes a type III secretion system (TTSS-1) and some of its secreted effector proteins are needed for entry into both M cells and enterocytes (reviewed in Galan, 2001). Upon contact with the intestinal epithelial cells, the TTSS-1 is assembled from more than

20 structural proteins to form a supramolecular needle complex that spans the bacterial inner and outer membranes and the host cell membrane. A series of bacterial effector proteins are injected directly into the host cell to engage host components of the actin cytoskeleton and promote bacterial uptake (Fig. 2). Three *Salmonella* effector proteins SopE, SopE2 and SopB activate host Rho GTPases Cdc42 and Rac (Hardt *et al.*, 1988; Stender *et al.*, 2000; Bakshi *et al.*, 2000; Zhou *et al.*, 2001) to trigger a series of signal transduction events that lead to actin cytoskeleton rearrangements and bacterial entry as well as pro-inflammatory cytokines production (Hobbie *et al.*, 1997; Chen *et al.*, 1996). Bacterial entry also necessitates the bacterial effector proteins SipA and SipC, which act as actin-binding proteins (Zhou *et al.*, 1999; Hayward and Koronakis, 1999). *Salmonella* is also actively involved in the recovery from these actin cytoskeleton rearrangements with the bacterial effector protein SptP interacting with Cdc42 and Rac to oppose the activity of SopE, SopE2 and SopB, allowing the rebuilding of the host actin cytoskeleton (Fu and Galán, 1999).

In addition to invasion of the intestinal epithelial barrier, some *Salmonella* serovars also induce a secretory response in the intestinal epithelium and initiate the recruitment and transmigration of neutrophils into the intestinal lumen (Galyov *et al.*, 1997), partially due to the production of cytokines including interleukin-8, a potent neutrophil chemokine, by the intestinal epithelial cells (McCormick *et al.*, 1993). Several Toll-like receptors are expressed in the intestine and these, along with the cytoplasmically expressed nucleotide-binding oligomerization domain (NOD)-containing proteins, regulate inflammatory processes and apoptosis in response to microbial products (reviewed in Eckmann, 2004). The interactions between these microbial sensors and *Salmonella* in the intestine have not been well documented and more detailed studies of the expression and function of sensors will aid in completing our understanding of innate intestinal immunity. Once *Salmonella* crosses the intestinal epithelium, the bacteria encounter submucosal macrophages and disseminate to the reticulo-endothelial system to colonize the liver and spleen (Vasquez-Torres *et al.*, 1999).

Interactions in the intracellular environment

Following invasion, the bacteria survive and replicate within a modified phagosome compartment, the *Salmonella*-containing vacuole (SCV). *Salmonella* intracellular proliferation has been observed in epithelial cells, fibroblast cells and macrophages with differing phenotypes (reviewed in Garcìa-del Portillo, 2001). *In vivo* studies indicate that macrophages are the main host cells permissive for bacterial growth (Richter-Dahlfors *et al.*, 1995; Salcedo *et al.*, 2001). Maintenance of the SCV is accomplished by the actions of bacterial effectors secreted through a second TTSS (TTSS-2) found on pathogenicity island-2 (SPI-2). The effectors SifA and SseJ regulate the stability and lipid acquisition to the membrane of the SCV (Ruiz-Albert *et al.*, 2002, Ohlson *et al.*, 2005). The SPI-2 TTSS also has been shown to block assembly and delivery of the NADPH oxidase, which catalyzes the production of reactive oxygen species within the phagosome (Vasquez-Torres *et al.*, 2000). Whereas the involvement of TTSS-2 in pathogenesis is clear, the precise functions of its effector proteins remain relatively undefined when compared to TTSS-1 effector proteins. Recent studies have focused on identifying novel TTSS-2 effector proteins and elucidating their function *in vivo* will further our understanding of the systemic phase of *Salmonella* infection (Brumell *et al.*, 2003; Knodler *et al.*, 2003; Kujat Choy *et al.*, 2004).

Exposure of mammalian macrophages to lipopolysaccharide (LPS), or specifically lipid A, triggers the expression of cytokines and proteins, including cationic antimicrobial peptides, through TLR-4 receptors (Janeway and Medzhitov, 2002). In mice, murine cathelicidin-related antimicrobial peptide (CRAMP) has been shown to be critical in controlling intracellular *Salmonella*. A peptide-sensitive *Salmonella* mutant showed enhanced survival within macrophages derived from CRAMP-deficient mice (Rosenburger *et al.*, 2004). *S. typhimurium* has been shown to alter its lipid A structure in response to environmental conditions found within the phagolysosome by palmitoylation, deacylation, the formation of a 2-hydroxymyristate (hydroxylation), and the additions of 4-amino-4-deoxy-L-arabinose and phosphoethanolamine (reviewed in Ernst *et al.*, 2001). This remodeling of the LPS, as

well as modifications in the protein composition in the bacterial membrane, promotes resistance to antimicrobial peptides (Guo *et al.*, 1998) and decreases the proinflammatory response (Guo *et al.*, 1997).

Most studies of *S. typhimurium* infection have been carried out with genetically susceptible mice lacking the natural-resistance-associated macrophage protein-1 (Nramp-1). This host protein is implicated in restricting the intracellular replication of several pathogens including *S. typhimuirum*. A bacteriostatic effect against intracellular *Salmonella* is observed when macrophage isolated from a susceptible mice are transfected with a wild-type *Nramp1* gene (Govoni *et al.*, 1999). Nramp-1 localizes to the SCV membrane where it acts to transport the divalent cations Fe^{2+} and Mn^{2+} and is suggested to counteract the ability of *S. typhimurium* to block SCV fusion with late endocytic compartments (Brumell *et al.*, 2002). *Salmonella* also possess an Nramp homologue, MntH, which acts as a Mn^{2+} transporter (Kehres *et al.*, 2000). A second *Salmonella* transport system, SitABCD also transports Mn^{2+} and Fe^{2+} (Kehres *et al.*, 2002). It has recently been shown that both *mntH* and *sitA* genes are upregulated when *Salmonella* is internalized by Nramp-1 expressing macrophages (Zaharik *et al.*, 2004). This study also confirms that in activated macrophages and congenic mice, the transport of divalent cations is essential for *Salmonella* virulence.

The composition of the SCV microenvironment has been subject to much debate. Intracellular *Salmonella* have been observed not to be starved for amino acids or Fe^{2+} and the intracellular intravacuolar environment thought to be low in phosphate and magnesium but high in potassium (Eriksson *et al.*, 2003). Recent studies have attempted to measure elemental concentrations in phagosomes of macrophages infected with wild-type *Mycobacterium tuberculosis* to define the conditions within the pathogen-containing compartment (Wagner *et al.*, 2005). Similar studies conducted with *Salmonella* would be beneficial, measuring elemental concentrations rather then inferring their presence or absence based on upregulation of expression of the genes encoding various ion transporters. Defining the composition of the

intestinal lumen and the phagolysosome is crucial to our ability to identify many of the environmental signals regulating the *Salmonella* virulence program.

GENETIC AND ENVIRONMENTAL CONTROL OF *SALMONELLA* PATHOGENESIS

Control of the complex processes of invasion and intracellular replication involves a number of genetic regulators and environmental stimuli. The regulators allow for the expression of virulence genes not only in the correct anatomical location but also at the correct time and sequence during infection in response to sensing of environmental signals.

Control of *Salmonella* Invasion

The control of invasion involves several transcriptional regulators encoded by the SPI-1 itself, including HilA, HilD, HilC and InvF (reviewed in Altier, 2005; Jones, 2005) or elsewhere on the chromosome, including RtsA and RstB (Ellermier and Slauch, 2003) (Fig. 3). These regulators, in turn, are controlled by both positive and negative regulators outside the SPI-1, including the two-component regulatory systems BarA/SirA, PhoP/PhoQ, OmpR/EnvZ and the Csr post-transcriptional control system. At the center of the regulatory scheme lies HilA. HilA activates the expression of the invasion genes, including those of the TTSS-1, by binding to promoter sequences upstream of the invasion genes or indirectly through a second transcriptional regulator InvF (Lostroh and Lee, 2001; Darwin and Miller 2000). Three regulators, HilC, HilD, and RstA, activate the expression if SPI-1 genes by binding upstream of *hilA* to induce its expression, while in turn, the expression of *hilC* and *rstA* are induced by the SirA/BarA and OmpR/EnvZ two-component systems working through HilD (Ellermeier *et al.*, 2005). HilE has been identified as a negative regulator of *hilA* expression by inhibiting HilD and the two-component systems PhoP/PhoQ and PhoB/PhoR have been shown to act through HilE along with the FimYZ response regulator type proteins (Baxter and Jones, 2005).

Optimal expression of *hilA* occurs in conditions of low oxygen, high osmolality, exponential growth phase, and slightly alkaline pH (Bajaj *et al.*, 1995) and modulation of *hilA* expression appears to be a central mechanism for regulating invasion gene expression to environmental cues. These environmental signals along with bile, Mg^{2+} concentrations, and short chain fatty acids are thought to be sensed by known and unknown two-components systems that are integrated into the genetic control of *hilA* expression (Ellermier *et al.*, 2005). The effects of certain environmental conditions may also be mediated by small DNA binding proteins, such as Hha and Fis, which alter DNA supercoiling and transcription of *hilA* as well as at other levels upstream and downstream of *hilA* (Jones, 2005).

Despite the identification of the environmental cues for invasion, little is known about the signaling mechanisms that allow for the sensing and processing of these cues. It may be possible to abrogate the effects of certain environmental signals on the invasive phenotype if they are mediated in a linear pathway by two-component regulatory systems, such as for the PhoR/PhoB two-component system which increases the expression of *hilE* in response to phosphate (Jones, 2005). It has also been shown that acetate, a short-chain fatty acid, at concentrations found in the ileum induces the invasion and expression of SPI-1 and requires the SirA response regulator protein (Lawhon *et al.*, 2002). High concentrations of two other short-chain fatty acids, propionate and butyrate, which rise in the cecum and the colon, inhibit SPI-1 gene expression (Lawhon *et al.*, 2002). Bile, secreted into the proximal small intestine, also represses SPI-1 gene expression at a level at or above BarA/SirA in the regulatory scheme (Prouty and Gunn, 2000). The sensing of intestinal components assures the repression of the invasion phenotype at times when invasion is not required and its activation at the optimal anatomical location.

Control of *Salmonella* intracellular replication and survival

The SPI-2 TTSS is required for systemic infection and its gene expression is induced inside the host cell. Several studies have examined SPI-2 TTSS expression in different conditions thought to reflect those found in SCVs. SPI-2 TTSS genes were

expressed when bacteria were grown in different minimal media, low osmolality, low concentrations of Mg^{2+}, Ca^{2+}, or phosphate, and acidic pH (Deiwick *et al.*, 1999; Lee *et al.*, 2000; Miao *et al.*, 2002).

The SsrA/SsrB two-component system regulates the expression of components of the SPI-2 TTSS as well as genes encoding certain SPI-2 effectors (Brumell *et al.*, 2003; Cirillo *et al.*, 1998; Worley *et al.*, 2000). The expression of SsrA/SsrB is regulated in turn by the OmpR/EnvZ two-component system (Lee et *al.*, 2000). Single and double mutants were used recently to study the relative contributions of these two regulatory systems in response to low osmolality, acidic pH, or the absence of Ca^{2+}. It was shown that SsrA/SsrB is essential for the induction of SPI-2 gene expression in response to each of these signals (Garmendia *et al.*, 2003). OmpR/EnvZ was found to play a minor role in sensing these signals and required a functional SsrA/SsrB system to mediate the effects of signals on SPI-2 gene expression (Garmendia *et al.*, 2003).

The PhoP/PhoQ two-component regulatory system permits *S. typhimurium* to determine its subcellular location, i.e. inside macrophages and to activate the virulence factors essential for survival. Mutations in the PhoP/PhoQ operon result in attenuated virulence, including the inability to survive within macrophages (Fields *et al.*, 1986; Miller *et al.*, 1989). Despite this essential role of PhoP/PhoQ in intramacrophage survival, the role of this system in controlling the expression of SPI-2 is somewhat controversial. PhoP/PhoQ has been shown to modulate SPI-2 activation using an analysis of secreted SPI-2 proteins (Deiwick *et al.*, 1999). The system has also been observed to play a role in SPI-2 expression under Mg^{2+} limiting conditions but not be required for SPI-2 expression in RAW264.7 macrophage cells (Lee *et al.*, 2000). *In vitro* accumulation of the TTSS-2 translocated proteins SseK1 and SseK2 required functional SsrB/SsrA and PhoP/PhoQ systems (Kujat Choy *et al.*, 2004). The transcription of *spiC*, encoding a TTSS-2 secreted effector protein, was found to be regulated by PhoP/PhoQ through PhoP controlled-expression of the *ssrB* and *ssrA* genes at the transcriptional and post-transcriptional level (Bijlsma and Groisman, 2005). However, other investigators have concluded that the PhoP/PhoQ

system is unlikely to be directly involved in SPI-2 activation. Using a luciferase reporter construct, it was observed that SPI-2 expression was not affected by a constitutively phosphorylated PhoP *in vitro* or by a PhoP mutant in RAW264.7 cells (Miao *et al.*, 2002). The PhoP/PhoQ system was also shown not be required for SPI-2 expression in the presence of phosphorylated OmpR (Kim and Falkow, 2004). The complex interplay of three two-component systems demonstrates that two-component systems are ideally suited to integrate the multiple signals sensed in the vacuolar environment where *Salmonella* resides (Fig. 4).

TWO-COMPONENT SIGNAL TRANSDUCTION

We have seen that as a pathogen moves through its host, it must sense its changing surroundings and respond appropriately in order to adapt and produce a successful infection. In bacteria, two-component signal transduction systems (TCS) fulfill this role by coupling an environmental stimulus with a response (reviewed in Goudreau and Stock, 1998; Stock *et al.*, 2000). Prototypical two-component systems are composed of a histidine kinase sensor (HK) together with its paired response regulator (RR). Genome sequences have revealed that TCS are widespread in bacteria accounting for the majority of signaling pathways but are quite rare in eukaryotes, in which kinase cascades involving Serine/Threonine and Tyrosine phosphorylation predominate (Stock *et al.*, 2000). The number of TCSs present across bacterial species varies greatly with species having smaller genomes and stricter environmental niches having few TCSs, such as the *Helicobacter pylori* genome encoding 4 TCSs. Bacteria with sizeable genomes and which grow in several environmental niches and therefore possess a large repertoire of genes for adaptability can express many different TCSs such as the 30 to 40 TCSs found in *E. coli* (Mizuno, 1997). Yet these conceptually similar systems must process different signals, interact with only their partner, and activate specific genes (Hoch and Varughese, 2001). Because of their involvement in the pathogenesis of many bacterial species and absence from mammals, these systems also provide an attractive target for the development of

novel antimicrobial drugs. In addition to controlling the expression of virulence factors required for infectivity, several TCSs regulate the resistance to antimicrobial products including vancomycin, β-lactams, tetracycline and antimicrobial peptides such as polymyxin (reviewed in Matsushita and Janda, 2002). A small number of inhibitors have been identified based on inhibition of the HK autophosphorylation. Possible targets also include HK extracellular sensor domains, ATP-binding sites and negative regulators (Matsushita and Janda, 2002).

Structure/Function of Histidine Kinases

The HK detects changes in the chemical or physical environment and transduces these extracellular signals into a cytoplasmic adaptive response through a phosphorylation cascade involving both the HK and the RR. The adaptive response is usually mediated through RR transcriptional modulation of specific genes. HKs are mainly integral membrane proteins, an optimal location to respond to external signals. Response regulators are soluble cytoplasmic proteins since the genes they regulate are cytoplasmic and they interact with the cytoplasmic domains of target proteins. Classically, the HK is composed of a periplasmic sensor domain delimited by two transmembrane domains and a cytoplasmic domain composed of a linker domain and the catalytic transmitter domain, which contains both an ATP binding domain and a highly conserved histidine (His) residue (Fig. 5). The RR is a soluble protein composed of an N-terminal receiver domain which contains the invariant aspartate (Asp) residue that accepts the phosphoryl group and a C-terminal effector domain that usually binds to DNA in a phosphorylation-dependent manner (Fig. 5). The HK autophosphorylates in an ATP-dependent manner on the His residue and subsequently transfers the phosphoryl group to the Asp residue in the RR receiver domain. This information transfer from one location to another requires specific recognition between the interacting components to ensure the correct response.

One of the hallmarks of TCS is its modular nature and although all TCS utililize the His-Asp phosphotransfer pathway, some systems integrate modules into more complex multicomponent phosphorelay systems (Appleby *et al.*, 1996). In these

systems, the first domain phosphorylated by the kinase sensor relays its phosphoryl group to a second phosphotransfer domain that serves as the primary phosphoryl group donor to the response regulator. The first three components may be on separate proteins as in the *Bacillus subtilis* sporulation phosphorelay or combined in a multidomain protein, as in the BvgS kinase sensor of *Bortedella pertussis*. A specialized version of a TCS that regulates microbial motility utilizes chemotaxis transducers known as methyl-accepting chemotaxis proteins that detect stimuli and transmit the signal to a non-sensing HK, CheA, which then transmits it to its cognate RR, CheY (Bourret and Stock, 2002). All TCSs, regardless of their domain architecture, rely on phosphoryl transfer for signal propagation.

Several isolated domains of HKs have been structurally characterized (Tanaka *et al.*, 1998; Tomomori *et al.*, 1999; Marina *et al.*, 2001; Bader *et al.*, 2005). The N-terminal dimerization sub-domain of the *Escherichia coli* EnvZ transmitter domain forms a four-helix bundle through the packing of two identical subunits and contains the phosphorylated His residue found in the H box (Fig. 6A; Tomomori *et al.*, 1999). The C-terminal catalytic ATP-binding sub-domain of the *E. coli* EnvZ and PhoQ proteins is an α/β sandwich distinct from the Serine/Threonine/Tyrosine fold but shows similarity to DNA gyrase and Hsp90 (Fig. 6B; Tanaka *et al.*, 1998; Marina *et al.*, 2001). The transmitter domains show many conserved amino motifs and are of similar lengths. Conserved residues are found within the N, G1, F, and G2 boxes that border the ATP-binding pocket of the transmitter domain (reviewed in Dutta *et al.*, 1999). In the EnvZ catalytic domain (Fig. 6A), the AMP-PNP ring is in close proximity to the N-box namesake N347, D373 of the G1 box, I378, L386, and the F-box namesake F387. In addition, the glycine-rich domains of the G1 (D373-G377) and G2 (G403-G405) boxes are essential for kinase acitivity. In contrast, the N-terminal sensing domains of HKs are extremely diverse, heterogeneous in both size and amino acid sequence, reflecting the variety of signals sensed. Most of the ligands for HKs are also unknown. The periplasmic domain of the *S. typhimurium* PhoQ protein has been crystallized and the dimeric domain forms a flat surface that comes in close contact to the membrane and binds phospholipids via divalent-cation bridges

with the bottom portion of the domain containing a highly negatively charged surface (Bader *et al.*, 2005). The crystal structure of the *Klebsiella pneumoniae* CitA periplasmic domain is similar to a PAS domain, a versatile ligand-binding structural domain (Reinelt *et al.*, 2003). The *E. coli* periplasmic sensor domain of EnvZ, an osmosensing HK, has also been investigated by pull-down assays and analytical ultracentrifugation (Khorchid *et al.*, 2004). The EnvZ sensor dimer is composed of an N-terminal α-helical domain with possible regulatory function and a C-terminal core domain which mediates homodimerization. Mutations in the N-terminal α-helical domain have been shown previously to produce a high-osmolality phenotype, indicating that this region of the periplasmic domain may play a role in mediating osmotic signal transduction (Waukau and Forst, 1999). A complete three-dimensional structure of the full-length HK protein has yet to be determined. This would aid in determining the interactions and movements between the respective domains that allow for signal transmission. The mechanisms of ligand recognition and transmembrane signal transduction also remain unclear.

The linker region, which connects the transmembrane domain to the cytoplasmic transmitter domain, is proposed to be crucial for propagation of the signal from the membrane into the cytoplasm (Zhu and Inouye, 2003; 2004). The linker region, referred to as the HAMP domain, is a common structural element found not only in HKs but also various other sensor proteins such as chemoreceptors, nucleotidyl kinases and phosphatases. Although HAMP linkers share little sequence homology, they share a similar helix-turn-helix fold based on secondary structure predictions and cysteine scanning mutagenesis of the Tar chemoreceptor linker (Butler and Falke, 1998). Several functional hybrid HKs have been constructed by fusing the sensor module of chemoreceptors to the transmitter domain of EnvZ using the HAMP domain from either protein. In Taz1, the sensor module of the Tar chemoreceptor is fused to the transmitter module of EnvZ using the Tar linker (Utsumi *et al.*, 1989). In Tez1, the sensor module of Tar is fused to the transmitter module of EnvZ using the EnvZ linker (Zhu and Inouye, 2003). Unlike Taz1, Tez1 could not respond to the Tar ligand, aspartate. In Tez1A1 and TezPQ, where a single

alanine insertion at the transmembrane/linker junction (Tez1A1) and a proline to glutamine mutation at position 185 (TezPQ), the aspartate regulated phenotype was restored (Zhu and Inouye, 2003). These results suggested that the N-terminal junction region modulates the alignment between the two helices in the linker region upon signal input, and that the signal propagation occurs through the resultant conformational change into the downstream catalytic domain of EnvZ to regulate its enzymatic activities (Zhu and Inouye, 2003). *In vivo* complementation experiments were also carried out with Tar-EnvZ hybrid mutants carrying the Tar linker or EnvZ linker in combination with an autophosphorylation site mutant (H243V) or a G2 box mutant (G405A) (Zhu and Inouye, 2004). The effects of mutations at one of the receptor heterodimer or heterodimer linkers on the activities of the EnvZ transmitter domain were investigated. It was concluded that an intrasubunit asymmetric signal transduction occurs through the HAMP linker in a receptor heterodimer. In contrast, for native HKs or receptor proteins, intrasubunit interactions between two linkers normally mediate symmetric signal transduction through both subunits in a dimer.

Catalytic Activities of Histidine Kinases

The chemistry of two-component signal transduction involves three phosphotransfer reactions. Two of these reactions are catalyzed by the HK: autokinase and phosphatase (Stock *et al.*, 2000). Upon detection of a specific stimulus by the HK sensory domain, the γ-phosphoryl group from ATP is transferred to the invariant histidine residue of the HK transmitter domain. Autophosphorylation occurs in *trans* within the receptor dimer with one HK monomer catalyzing the phosphorylation of the conserved His residue in the second monomer (Yang and Inouye, 1991). Subsequently, the phosphoryl group at the His residue is rapidly transferred to an invariant residue in the RR receiver domain in a reaction catalyzed by the RR. Most HKs also possess a phosphatase activity allowing for the dephosphorylation of the RR (Aiba *et al.*, 1989). The conserved His residue is proposed to play a critical role in both the kinase and phosphatase reactions. Replacement of the conserved His in EnvZ, the site of autophosphorylation, with

other amino acid residues almost abolishes the phosphatase activity (Zhu *et al.*, 2000). Therefore the kinase and phosphatase activities are not independent but rather are proposed to share the same active center containing the conserved His of the HK and the conserved Asp of the RR (Zhu *et al.*, 2000).

The balance between the phosphatase and autokinase activities of the sensor kinase controls the net phosphorylation of the response regulator, and, in turn, the adaptive response (Russo and Silhavy, 1991; Yang and Inouye, 1991). It has been proposed that signals transduced across the membrane alter the spatial arrangement between the ATP-binding domain and the central dimerization domain of sensor kinases (Inouye *et al.*, 2003; Jin and Inouye, 1993). By using the Taz1 chimera, it was found that aspartate binding to the Taz1 decreases the phosphatase activity of Taz1 while the kinase activity remains constant (Jin and Inouye, 1993). In contrast, it has been proposed that the kinase activity of EnvZ varies in response to changes in external osmolarity, while the phosphatase activity remains constant (Russo and Silhavy, 1991). This proposal was based on mathematical modeling of porin gene expression and analysis of EnvZ mutants deficient in either kinase or phosphatase activity (Russo and Silhavy, 1991; Hsing and Silhavy, 1997; Hsing *et al*, 1998). The FixL sensor kinase of *Rhizobium meliloti* was found to coordinately modulate kinase and phosphatase activities in response to changes in oxygen levels (Lois *et al.*, 1993). The binding of the PII protein to the nitrogen regulator II (NRII or NtrB) inhibits the kinase activity and activates the phosphatase activity of NRII (Jiang and Ninfa,1999; Jiang *et al.*, 2000). Binding of PII to the C-terminal ATP-binding domain of NRII is thought to result in conformation changes that are transmitted to other domains causing the catalytic domain to assume a phosphatase-dominant conformation (Jiang *et al.*, 2000). In the case of the PhoQ kinase sensor, regulation of the catalytic activities is believed to occur through Mg^{2+} binding to the extracellular PhoQ sensory domain (Garcia-Vescovi *et al.*, 1996). Mg^{2+} recognition elicits a conformational change that alters intracellular enzymatic activities (Garcia-Vescovi *et al.*, 1996). Recent studies, using the PhoQ protein overexpressed in membranes, have addressed which of the PhoQ catalytic activities are affected by the Mg^{2+}-induced

conformational switch. It was proposed that the *S. typhimurium* PhoQ phosphatase activity is the only target of extracellular Mg^{2+} (Castelli *et al.*, 2000). Other reports suggested that Mg^{2+} binding to the PhoQ sensory domain regulates both the phosphatase and autokinase activities (Montagne *et al.*, 2001; Chamnongpol *et al.*, 2003).

Ligand-mediated regulation of the autokinase and phosphatase catalytic activities of HKs remains unclear. The prevailing view is that these opposing activities are coordinately and reciprocally regulated by ligand recognition (Russo and Silhavy, 1993). Two models for how this regulation is achieved have been proposed: the on/off model and the rheostat model. The on/off model suggests that the HK is either in an autokinase-competent or a phosphatase-competent conformation and that ligand binding switches the protein from one mutually exclusive conformation to another, allowing only one activity to occur to modulate the levels of phosphorylated RR. The rheostat model suggests that autokinase-dominant and phosphatase-dominant conformations of the HK exist and that ligand binding turns up one activity while lowering the opposing activity to allow one activity to predominate. Levels of RR are suggested to be controlled in a rheostat-like manner for the OmpR/EnvZ and NRI/NRII systems (Inouye *et al.*, 2003; Jiang *et al.*, 2003).

THE PhoP/PhoQ SYSTEM

The PhoP/PhoQ system was first described in *Salmonella* by the identification of genes encoded in the *pho* locus involved in the transcriptional modulation of the acid phosphatase gene *phoN* (Kier *et al.*, 1979). The PhoP/PhoQ system is prototypical with a transmembrane histidine kinase sensor, PhoQ, and soluble response regulator, PhoP, capable of transcriptional modulation. PhoP/PhoQ homologues are found in many other Gram-negative pathogens, including pathogens that do not have an intracellular lifestyle. PhoP/PhoQ homologues are found in *Escherichia coli* (Kasahara *et al.*, 1992), *Pseudomonas aeruginosa* (Macfarlane *et al.*, 1999), *Providencia stuartii* (Rather *et al.*, 1998) *Yersinia pestis* (Oyston *et al.*, 2000),

Shigella flexneri (Moss *et al.*, 2000), *Neisseria meningitidis* (Johnson *et al.*, 2001), *Erwinia caratova* (Flego *et al.*, 2000), and *Erwinia chrysanthemi* (Llama-Palacios *et al.*, 2005). In *S. enterica* and *P. aeruginosa*, PhoP/PhoQ has been shown to control LPS modifications and resistance to cationic antimicrobial peptides in response to external Mg^{2+} concentrations (Guo *et al.*, 1997; 1998; Macfarlane *et al.*, 1999). In *E. caratovora*, the PhoQ homolog has been shown to respond to external Ca^{2+} but not Mg^{2+} (Flego *et al.*, 2000). Other PhoQ homologs have been shown to respond to changes in environmental pH or unidentified ligands other than divalent cations (Llama-Palacios *et al.*, 2005; Rather *et al.*, 1998). Although the PhoP/PhoQ system recognizes different ligands in different species, inactivation of the *phoP* gene prevents the proliferation within phagocytic cells in *Salmonella*, *Shigella* and *Yersinia*, suggesting a role for the system in survival within the phagosome in several species (Groisman, 2001).

The *Salmonella* PhoP/PhoQ System

Amongst regulators of *Salmonella* pathogenesis, the PhoP/PhoQ two-component system is of unique interest. This system is not only essential for the SPI-1 gene repression, but is also required for intracellular replication and survival. Thus, *Salmonella* PhoP/PhoQ may act as a genetic switch activating traits required for intramacrophage survival while repressing those no longer needed for invasion (Altier, 2005). The system controls the expression of more than 40 genes required for entry into epithelial cells, survival within macrophage, resistance to cationic antimicrobial peptides, pH and bile salts, and LPS modifications (reviewed in Ernst *et al.*, 1999; Groisman, 2001). PhoP regulates the expression of genes directly by binding to the promoter regions of genes having a direct repeat sequence (Lejona *et al.*, 2003). Indirect PhoP regulation also occurs through stimulation of a second TCS, PmrA/PmrB, (Gunn and Miller, 1996; Wosten *et al.*, 2000) and possibly through the SlyA DNA-binding protein (Navarre *et al.*, 2005). Mutations of either the *phoP*, or *phoQ* genes, result in defects for intramacrophage survival and attenuation in mice (Fields *et al.*, 1986; Miller *et al.*, 1989). Likewise, strains harbouring the *pho-24*

allele of PhoQ (mutation T48I in the PhoQ periplasmic domain) are attenuated for virulence in mice (Miller and Mekalanos, 1990). These strains have been shown to constitutively express PhoP-activated genes and repress PhoP-repressed genes (Miller and Mekalanos, 1990).

LIGANDS OF THE SALMONELLA PhoQ KINASE SENSOR

Divalent Cation Ligands of *Salmonella* PhoQ

The PhoQ protein has been shown to sense the divalent cations Mg^{2+}, Ca^{2+}, and Mn^{2+}, whereas the divalent cations Ni^{2+}, Cu^{2+}, Co^{2+} and Ba^{2+} have no effect (Garcia Vescovi *et al.*, 1996). *In vivo* experiments suggest that PhoQ may have distinct non-interacting binding sites for Mg^{2+} and Ca^{2+} (Garcia Vescovi *et al.*, 1997). However, it has been proposed that extracellular Mg^{2+} is the physiological signal controlling PhoP/PhoQ based on the PhoP-dependent upregulation of Mg^{2+} transporters (Groisman, 1998). *In vitro* experiments demonstrated that the purified periplasmic domain of PhoQ binds Mg^{2+} but not Ba^{2+}, a divalent cation unable to modulate the transcription of PhoP-regulated genes (Waldburger and Sauer, 1996; Garcia Vescovi *et al.*, 1997). Sensing of extracellular Mg^{2+} is suggested to allow *Salmonella* to distinguish between its extracellular and intracellular environments. The PhoP/PhoQ system is activated within the SCVs (Alpuche Aranda *et al.*, 1992) and is repressed *in vitro* during bacterial growth in high concentrations of Mg^{2+}, Ca^{2+}, and Mn^{2+} (Garcia Vescovi *et al.*, 1996). Consistent with these observations, several PhoP-dependent phenotypes are regulated by divalent cations in wild-type organisms. Wild-type *Salmonella* grown in Luria-Bertani broth is more than 1 000-fold more resistant to the antimicrobial peptide magainin-2 than to organisms grown in Luria-Bertani broth supplemented with 25 mM Mg^{2+}(Garcia Vescovi *et al.*, 1996). The biological consequence of Mg^{2+} binding to the periplasmic domain is to decrease the levels of phosphorylated PhoP. Consequently, increasing concentrations of Mg^{2+} stimulate the phosphatase activity of PhoQ (Fig. 7, Castelli *et al.*, 2000; Montagne *et al.*, 2001). In the absence of Mg^{2+}, the inhibitory signal of divalent cations is lifted, and PhoQ

autokinase activity dominates allowing phosphorylation of PhoQ and transfer of the phosphoryl group to PhoP (Fig. 7).

Detection of divalent cations is mediated through the PhoQ periplasmic sensory domain although the identity of the cation-binding site remains unclear. Many mutants of the periplasmic domain of PhoQ have been isolated. The *S. typhimurium* strain harbouring the T48I substitution is less sensitive to repression by Ca^{2+} but responds like wild-type to Mg^{2+} (Garcia Vescovi *et al.*, 1996; 1997). The *E. coli* and *S. typhimurium* PhoQ proteins harbour an acidic cluster (EDDDDAE) with a stretch of negatively charged residues in their periplasmic domain (residues 148-154). An *E. coli* strain harbouring a mutant PhoQ protein in the acidic cluster exhibited limited repression by Mg^{2+} (Waldburger and Sauer, 1996). In *S. typhimurium*, the residues within the acidic cluster along with conserved residues G93, W97, H120, and T156 contribute to a normal Mg^{2+} response, and their deletion or mutation severely compromsises PhoQ function (Garcia Vescovi *et al.*, 1996; Chamnongpol *et al.*, 2003). The mutation of D179 in the *E. coli* PhoQ protein results in loss of Mg^{2+} and Ca^{2+} regulation (Minagawa *et al.*, 2005). Cumulatively, these studies suggest that divalent cations bind directly to the PhoQ protein, although the involvement of the acidic cluster remains unclear.

Other Ligands of *Salmonella* PhoQ

The notion that divalent-cation mediated repression may only be part of the PhoQ sensing mechanism has existed for some time. Because mild acid pH promotes the transcription of certain PhoP-activated genes, it has been proposed that *Salmonella* PhoQ senses pH (Alpuche Aranda *et al.*, 1992; Bearson *et al.*, 1998). However, other groups reported that the growth of *Salmonella* in mild acid pH activates only a subset of PhoP-activated genes and this activation occurs in a *phoQ* null mutant or with a mutated PhoP protein (Garcia Vescovi *et al.*, 1996; Soncini and Groisman, 1996; Bearson *et al.*, 1998). *S. typhimurium* can also resist the action of extremely high concentrations of bile and this resistance required an activated PhoP/PhoQ system (Van Velkinburgh and Gunn, 1999). However, the *tolQRA* cluster

required for resistance to bile was not regulated by PhoP/PhoQ and no other PhoP-regulated genes necessary for bile resistance have been identified (Prouty *et al.*, 2002). Therefore, although transcription of PhoP-regulated genes can be regulated by signals other than certain divalent cations, these signals may be sensed by sensors other than PhoQ, activate regulators other than PhoP, and affect only a subset of the genes under PhoP/PhoQ control. Furthermore, although the PhoP/PhoQ system may be required for a resistance phenotype, it does not imply direct sensing or regulation by the system, as seen in the case of bile resistance. Bacterial resistance to oxidative stress and cationic antimicrobial peptides (CAMPs) involves a number of genes under the control of PhoP. PhoQ has also been proposed to sense CAMPs. Recently, the *S. typhimurium* PhoP/PhoQ system has been shown to respond to the presence of sublethal concentrations of the α-helical peptide C18G (Bader *et al.*, 2003). It was unknown whether PhoQ sensed C18G directly or was induced by the alteration of outer membrane permeability sensed by other regulatory systems and relayed to PhoP/PhoQ. CAMP interaction with the PhoQ periplasmic sensor domain would imply a bacterial sensor that senses small molecules of the host innate immune system (Bader *et al.*, 2003).

Antimicrobial Peptides

Antimicrobial Peptide Interactions with Microbial Pathogens

CAMPs represent a difficult challenge for microbes, including *Salmonella*. In the appropriate host location CAMPs mediate killing of *Salmonella,* such as CRAMP-mediated killing of *Salmonella* in macrophages (Rosenburger *et al.*, 2004). HD-5 has been shown to be critical for controlling *Salmonella* infections. Genetic transplantation of HD-5 into mice results in dramatic improvement in the resistance of mice to intestinal infections with *Salmonella typhimurium* (Salzman *et al.*, 2003). The mechanism of action of CAMPs exploits the fundamental difference between bacterial and mammalian membranes. Bacterial cytoplasmic membranes are composed heavily of negatively charged phospholipids while mammalian membranes

are composed mainly of lipids having no net charge on their outer leaflet. This charge differential allows for specific peptide interaction and insertion into bacterial membranes by displacement of lipids, alteration of the membrane structure and in certain cases entry of the peptide to the target cell's interior (Zasloff, 2002). Some of these host CAMPs also appear to have host immunomodulatory functions in addition to their antimicrobial killing capacity including the induction or modulation of chemokine and cytokine production, alteration of gene expression in host cells, and inhibition of proinflammatory responses of host cells to bacterial components such as LPS (reviewed in Bowdish *et al.*, 2005a). These immunomodulatory activities of CAMPs are often mediated at concentrations much lower than those required for antimicrobial killing and this is a key element of their therapeutic potential. The relative significance of the direct antimicrobial activities and immunomodulatory properties of CAMPs in host defense remains unclear. Recently it was demonstrated that antimicrobial activity is not always necessary for protection *in vivo*. Synthetic peptides based on the peptide LL-37 without any antimicrobial activity were constructed and shown to be protective in relevant animal models of *Staphylococcus aureus* and *Salmonella typhimurium* infection (Bowdish *et al.*, 2005b).

Structure and Classification of Antimicrobial Peptides

CAMPs are evolutionary ancient molecules with a widespread distribution across the animal and plant kingdoms (reviewed in Zasloff, 2002; Ganz, 2003). CAMPs are 12-50 amino acids long with a net positive charge of +2 to +9, which is due to an excess of basic arginine and lysine residues, and approximately 50% hydrophobic amino acids (Hancock, 2001). All antimicrobial peptides are derived from larger precursors having both signal sequences and prodomains and undergo post-translational modifications including proteolytic processing (Zasloff, 2002). They fold into amphiphilic structures with hydrophobic and hydrophilic faces but apart from this fundamental structural property they vary considerably in sequence and structure. Structural groups include β-sheets stabilized by disulfide bridges, α-helices, cyclized peptides, and less commonly, extended and loop structures. Two

major families of mammalian CAMPS have been identified, cathelicidins and defensins.

Cathelicidins

Cathelicidins are α-helical peptides having a highly conserved N-terminal domain, termed the cathelin, linked to a C-terminal peptide with antimicrobial activity (Eckmann, 2005). The sole human cathelicidin is LL-37, a homolog to the mouse peptide CRAMP. LL-37 is the proteolytically processed extracellular form of hCAP-18, a peptide that is constitutively produced in the secondary granules of neutrophils and in epithelial and mast cells (Eckmann, 2005). In neutrophils, LL-37 is proteolytically cleaved by proteinase 3 (Sorensen *et al.*, 2001), while additional proteases appear to be important in other cellular locations. Serine proteases in human sweat glands generate additional shorter peptides from LL-37 with increased antimicrobial activity and the serum protease gastricsin processes hCAP-18 into a longer variant of LL-37 in the vagina (Murakami *et al.*, 2004; Sorenson *et al.*, 2003). Therefore, postsecretory processing of LL-37/hCAP-18 can modulate antimicrobial activity and specificity in different tissues. In the gastrointestinal tract, LL-37 is produced constitutively in the colon and duodenum but not in the epithelial cells of the small intestine (Hase *et al.*, 2002). LL-37 is also produced in the stomach and found in gastric juices suggesting that it may be active upon passage into the proximal small intestine (Eckmann, 2005). LL-37 expression is induced upon exposure to proinflammatory mediators or during the course of infection or inflammation (Bowdish *et al.*, 2005). It can be found at unstimulated mucosal surfaces at concentrations of around 2 μg/mL, and at concentrations exceeding 50 μg/mL in inflamed epithelium (Bals *et al.*, 1998).

Defensins

Defensins have three characteristic pairs of intramolecular disulfide bonds and a β-sheet structure. Based on the arrangement and spacing of disulfide bonds, defensins are divided into three groups, α-defensins, β-defensins, and the θ-defensins which are

not found in humans. Whereas α-defensins are contained mainly in granules of neutrophils and in Paneth cells, β-defensins are produced mainly by epithelial surfaces (Ganz, 2003).

Comparative genomics have shown that humans carry 11 α-defensins genes, of which six are expressed while the remaining five are thought to be pseudogenes (Patil *et al.*, 2004). Mice have a larger number of α-defensin genes, termed cryptdins, consisting of 26 genes and at least one pseudogene (Patil *et al.*, 2004). In addition, solely mice have a family of cryptdin-related sequences (CRS) peptide genes that encode peptides that form covalently homo- and heterodimers having potent antimicrobial activity (Hornef *et al.*, 2004).

Expression of α-defensins is limited to very few cell types. Of the six human α-defensins, four are expressed by neutrophils (human neutrophil peptides, HNP1-4) while the remaining two are expressed by Paneth cells in the crypts of the small intestine (human defensins, HD-5 and -6) (Eckmann, 2005). α-defensins are encoded as tripartite prepropeptide sequences, in which a 90-100 amino-acid precursor contains am N-terminal signal sequence (~10 amino acids), an anionic propiece (~45 amino acids), and a C-terminal mature cationic defensin (~30 amino acids) (Ganz, 2003). Neutrophil α-defensins are synthesized in neutrophil precursor cells in the bone marrow termed promyelocytes. The signal sequence is rapidly removed and the proteolytic cleavage to form mature defensins occurs in maturing granules. Having matured in the bone marrow and assembled their granules, neutrophils cease granule and α-defensins synthesis and are released into the blood. During phagocytosis of pathogens, defensin-rich primary granules fuse with phagocytic vacuoles (Ganz, 2003). In the case of mouse Paneth cells α-defensins (cryptidins), the metalloproteinase matrilysin (MMP-7) is required for processing and activation of the peptides. Disruption of MMP-7 leads to increased acute susceptibility to oral *Salmonella* infection due to a lack of bioactive cryptidins in the small intestine (Wilson *et al.*, 1999). In humans, after Paneth cell degranulation is induced by the entry of bacteria into the intestinal lumen, trypsin activates HD-5 by cleavage of its propiece (Ganz, 2003).

The structure of β-defensin precursors is simpler, consisting of a signal sequence, a short or absent propiece, and the mature peptide at the C-teminus (Ganz, 2003). The repertoire of human β-defensins includes at least 10 and as many as 31 genes, several of which are pseudogenes (Eckmann, 2005). These genes are expressed by the epithelium in the lung, skin, kidneys, testis and intestines. In the intestinal tract, human β-defensin-1, hBD-1 is constitutively expressed and not upregulated by proinflammatory stimuli or infection with invasive bacteria (O'Neil *et al.*, 1999). HBD-2 is not expressed constitutively in the intestinal tract, but rather is strongly induced by infection with enteric pathogens or proinflammatory mediators (O'Neil *et al.*, 1999). HBD-3 and hBD-4 have been reported to be expressed constitutively in the intestinal tract with increased levels seen in patients with Crohn disease (Fahlgren *et al.*, 2004). β-defensins, like α-defensins, can kill a wide variety of microbes *in vitro*, but the physiological roles of β-defensins remain poorly defined (Eckmann, 2005). Under optimal conditions of low ionic strength and low divalent cation concentrations, antimicrobial activity is observed at concentrations of defensins as low as 1-10 μg/mL (μM) (Ganz, 2003). Many immunodulatory activities have been attributed to defensins, including the mobilization monocytes, mast cells, immature dendritic cells and lymphocytes, as well as enhance the production of cytokines and chemokines, cause mast cell degranulation and interact with complement components (Lehrer, 2004).

Resistance to Antimicrobial Peptides

The PhoP/PhoQ system mediates resistance to CAMPs including resistance to the α-helical peptides magainin 2 and C18G (Guina *et al.*, 2000; Shi *et al.*, 2004). Magainin 2 is produced by frog skin while C18G is synthetically derived from the C-terminus of platelet factor IV. The C18G peptide analog was shown to have increased antimicrobial activity in human serum against cefepime-altered bacteria (Darveau *et al.*, 1992). Two PhoP-activated outer membrane proteins have been implicated in the resistance to C18G: PagP and PgtE. PagP mediates the palmytoyalation of the lipid A in LPS (Guo *et al.*, 1998) while PgtE, a protease, cleaves C18G (Guina *et al.*, 2000).

Counteracting the activity of CAMPs presents a difficult problem for bacteria since antimicrobial peptides target the fundamental property of the bacterial membrane. In addition, the diversity of structure and absence of consensus sequences make CAMPs difficult to target by protein degredation or neutralization. Despite these limitations bacteria possess strategies for dealing with CAMPs. Oral inoculation of mice with wild-type *Salmonella typhimurium* decreased the expression of cryptdins, the mouse equivalent of human α-defensins CAMPs, while a *Salmonella* mutant defective for the SPI-1 TTSS lacked this inhibitory effect (Salzman *et al.*, 2003b). *Salmonella* secreted effector proteins may also be involved in downregulating CAMP production in Paneth cells of the mouse intestine. *Salmonella* also responds to CAMPs through PhoP/PhoQ-mediated decoration of the outer membrane with positively charged moieties ethanolamine and 4-aminoarabinose. This modified lipid A species showed reduced ability to induce cellular signaling through TLR-4, suggesting a specific function of lipid A modifications in the pathogenesis of salmonellae infection in addition to CAMP resistance (Kawasaki *et al.*, 2005). Therefore, the *Salmonella* PhoP/PhoQ two-component system controls LPS modifications and, in turn resistance to antimicrobial peptides and altered TLR-4 signalling.

RATIONALE AND OBJECTIVES OF THE THESIS

For a system whose importance to *Salmonella* virulence is indisputable, we understand very little of the molecular mechanisms governing PhoQ ligand recognition and transmembrane signaling. Much of the current research is focused on events downstream of PhoQ, such as PhoP-mediated gene regulation. Fundamental questions concerning PhoQ remain. Firstly, what are the ligands of PhoQ and where do they bind? Secondly, how does ligand binding transduce the signal through the membrane? Thirdly, how are PhoQ catalytic activities regulated by ligands? This thesis aims to explore these questions and add to our current knowledge of molecular mechanisms governing ligand recognition and transmembrane signaling.

To study signaling by the PhoP/PhoQ system, we have previously developed *in vitro* assays to measure the net global or individual catalytic activities of PhoQ and PhoP (Montagne *et al.*, 2001). Chapter 2 uses these techniques along with developed *in vivo* assays that assess the global PhoP/PhoQ activity to define the molecular mechanisms by which the mutation T48I (the *pho-24* allele of PhoQ) attenuates virulence in mice. Site-directed mutagenesis was used to generate various amino acid substitutions and PhoQ proteins carrying these various mutations were tested for *in vivo* Mg^{2+} regulation and for *in vitro* global and individual catalytic activities. These strategies establish a role for the residue at position 48 of PhoQ in the conformational switch between kinase- and phosphatase-dominant states.

To date, all of the *in vitro* analyses of PhoQ catalytic activities have been performed using *E. coli* or *S. enterica* membranes overexpressing the PhoQ protein (Castelli *et al.*, 2000; Montagne *et al.*, 2001). These results led to conflicting results regarding the regulation of PhoQ by Mg^{2+}. PhoQ phosphatase activity has been stated to be the only target for Mg^{2+} regulation (Castelli *et al.*, 2000). We have shown that Mg^{2+} reciprocally regulates PhoQ autokinase and phosphates activity in *E. coli* membranes (Montagne *et al.*, 2001). To clarify this question we have developed a reconstituted PhoQ system. Chapter 3 details the successful purification and reconstitution of *S. typhimurium* PhoQ. Purified $PhoQ_{His}$ was inserted into liposomes

and the orientation of the protein and proteoliposome integrity were determined. Reconstituted $PhoQ_{His}$ was also tested for *in vitro* global, autokinase, and phosphate activity. Functional reconstitution of PhoQ provides a tool for investigating ligand-mediated signal transduction.

The PhoP/PhoQ system is repressed *in vitro* during the bacterial growth in high concentrations of Ca^{2+}, Mg^{2+}, and Mn^{2+} (Garcia Vescovi *et al.*, 1996). Chapter 4 describes the effect of these divalent cations on reconstituted $PhoQ_{His}$ catalytic activities. *In vivo* and *in vitro* assays were performed at 5 mM concentrations divalent cation as well as at more physiological concentrations of divalent cation to determine the divalent-cation requirements for maximal repression and activation of the reconstituted $PhoQ_{His}$ protein. Proteoliposome integrity was also assessed in the presence of divalent cations. The contribution of divalent cations to regulation of *in vivo* PhoQ activity in light of the results is discussed.

S. typhimurium PhoP/PhoQ has been shown to respond to the presence of sublethal concentrations of the α-helical peptide C18G (Bader *et al.*, 2003). It was unknown whether PhoQ sensed C18G directly or was induced by the alteration of outer membrane permeability sensed by other regulatory systems and relayed to PhoP/PhoQ. Chapter 5 details the CAMP activation of PhoQ and the dissection of the molecular mechanisms governing this response and its distinction from divalent cation-mediated repression. *In vivo* assays were performed in the presence of CAMPs and Mg^{2+} to determine if PhoQ is regulated by CAMPs. A PhoQ mutant lacking the periplasmic sensor domain and a chimera of PhoQ carrying the sensor domain of *P. aeruginosa* were used to assess the role of the sensor domain in responding to CAMPs. The reconstituted PhoQ protein was used to test PhoQ-mediated peptide signaling *in vitro*. The dimeric PhoQ sensor domain was crystallized with Ca^{2+} to provide details of divalent cation binding. Binding of CAMPs to the PhoQ sensor domain was followed by iron-mediated cleavage, fluorescence with dansylated C18G, and nuclear magnetic resonance (NMR). PhoQ mutants deficient in peptide signaling were constructed and their effects on peptide-mediated were analyzed. A model for

CAMP activation of PhoQ is proposed and the potential implications for PhoQ activation within the macrophage phagosome are discussed.

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FIGURE LEGENDS

Figure 1. TTSS-1 genetic organization. TTSS-1 components encoded on SPI-1 include subunits of the type III secretion apparatus, effectors secreted by the apparatus, factors required for their translocation, and transcriptional regulators (modified from Altier *et al.*, 2005).

Figure 2. Model for *Salmonella*-induced gastroenderitis. Upon interactions with the brush border of the intestinal epithelium, *Salmonella* delivers effectors through the SPI-1 TTSS. SopE, SopE2 and SopB activate the Rho-GTPases Cdc42 and Rac. Actin cytoskeleton rearrangements, further modulated by SipA, result in bacterial uptake. Stimulation of the Erk, Jnk, and p38 MAP kinases results in a nuclear response leading to production of cytokines and infiltration of polymorphonuclear leukocytes. Toll-like receptors (presumably located on the basolateral side) may be stimulated by a variety of bacterial products such as LPS, lipoprotein, and flagellin to further the immune response. SptP restores the integrity of the intestinal cell by reversing the activation of Cdc42 and Rac. Once inside the cell, SPI-2 TTSS is stimulated (Galán, 2001).

Figure 3. A model for SPI-1 regulation. Blue arrows indicate activation of gene expression. Repression is indicated as red line. Solid lines represent direct transcriptional regulation. Short-dashed lines represent regulation that is not known to be direct or indirect. Long-dashed lines represent post-translational effects. Environmental signals are shown in green (modified from Ellermeier *et al.*, 2005).

Figure 4. A model for SPI-2 signaling and regulation. Different colours indicate the different two-component systems. Question marks indicate unknown phosphorylation states of the response regulators. Possible role of PhoP/PhoQ system is discussed in text (Kim and Falkow, 2004).

Figure 5. Schematic representation of a classical two-component system. The histidine kinase sensor is usually a transmembrane protein and the response regulator is a soluble cytoplasmic protein. Domain organization and reactions catalyzed by the system are highlighted.

Figure 6. Three-dimensional structures of histidine kinase sensor isolated domains. a) Structure of EnvZ catalytic domain. The catalytic domain (blue) contains highly conserved sequence motifs (magenta with conversed amino acids shown as ball and stick structures) of the N, G1, F and G2 boxes that form the binding site for ATP (nonhydrolyzable analog shown in green). **b)** Structure of EnvZ catalytic domain. The dimerization domain (gold) contains the conserved His residue (blue) that is the site of phosphorylation (Stock *et al.*, 2000).

Figure 7. The PhoP/PhoQ system is regulated by divalent cations. In high divalent cation concentration (millimolar concentrations), the PhoQ phosphatase activity predominates. On entry into macrophage, divalent cation depletion (micromolar concentrations) is sensed by PhoQ and the system is activated through PhoQ autophosohorylation and phosphotransfer by PhoP.

Figure 1. TTSS-1 genetic organization.

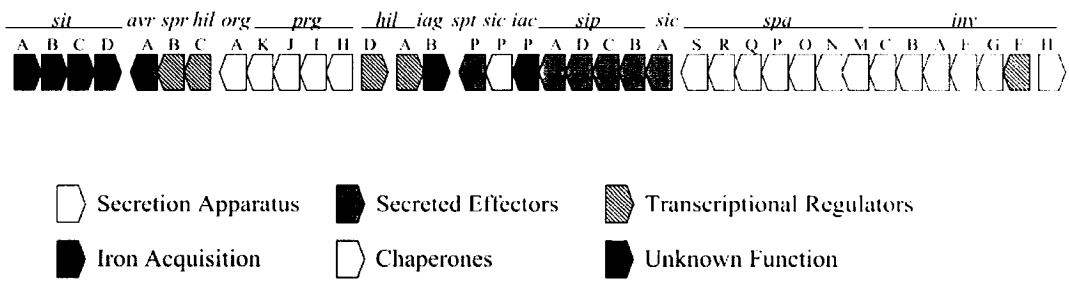


Figure 2. Model for *Salmonella*-induced gastroenderitis.

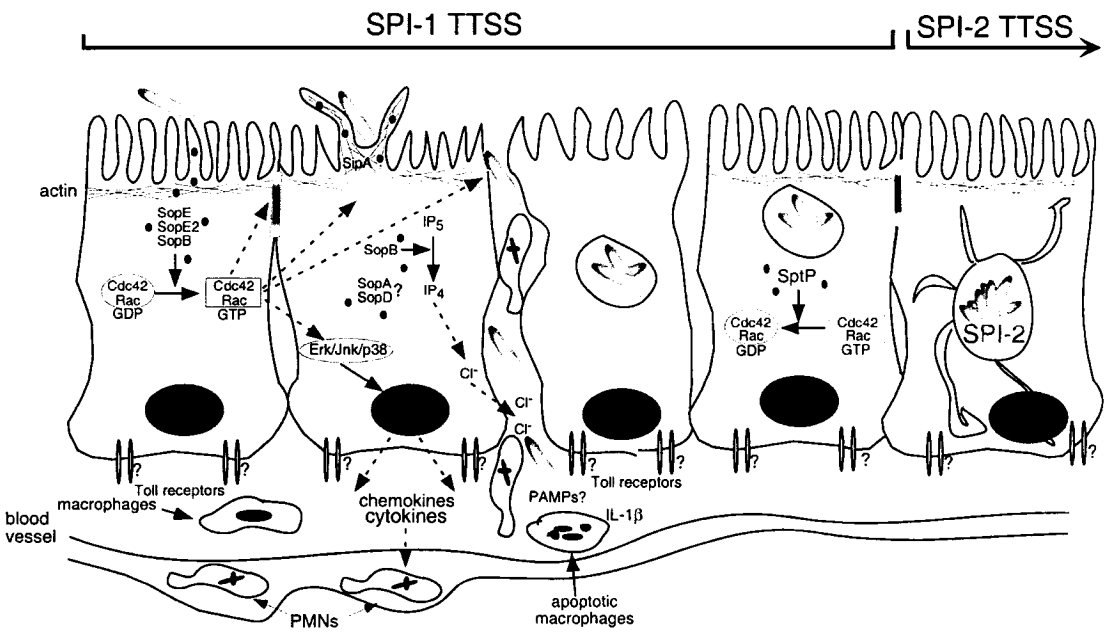


Figure 3. A model for SPI-1 regulation.

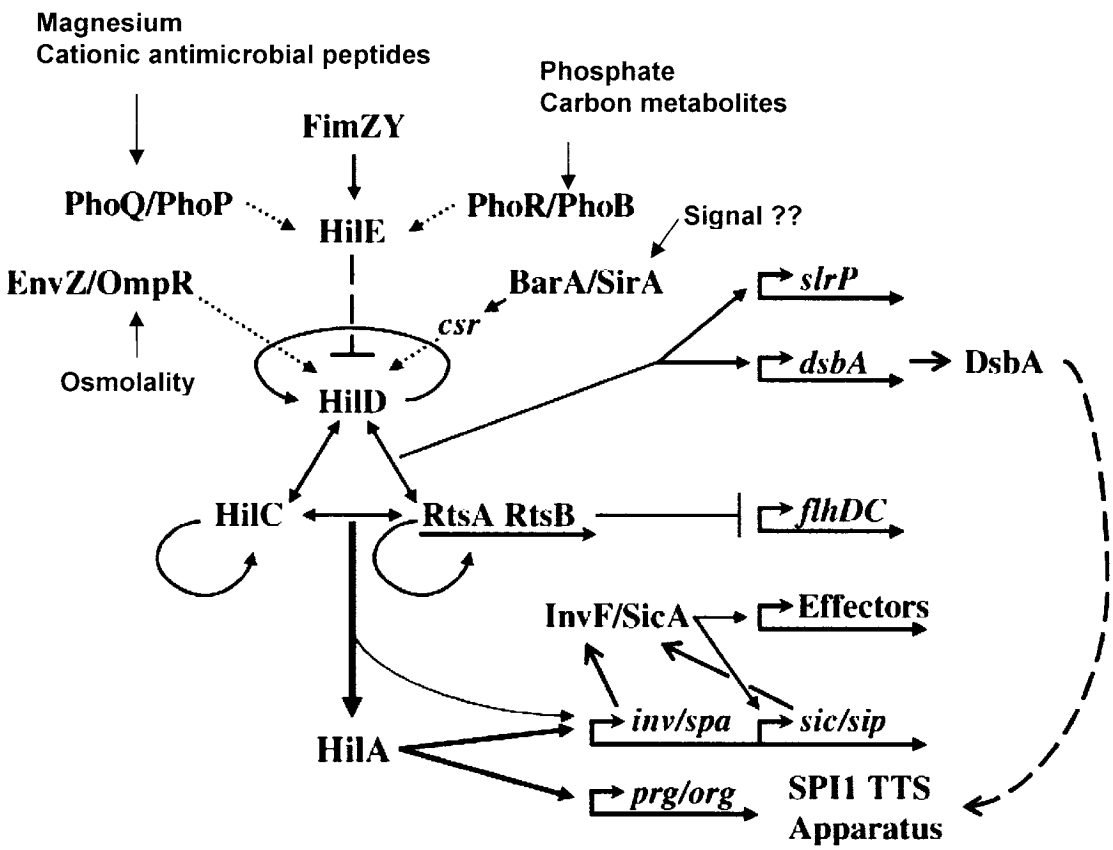


Figure 4. A model for SPI-2 signaling and regulation.

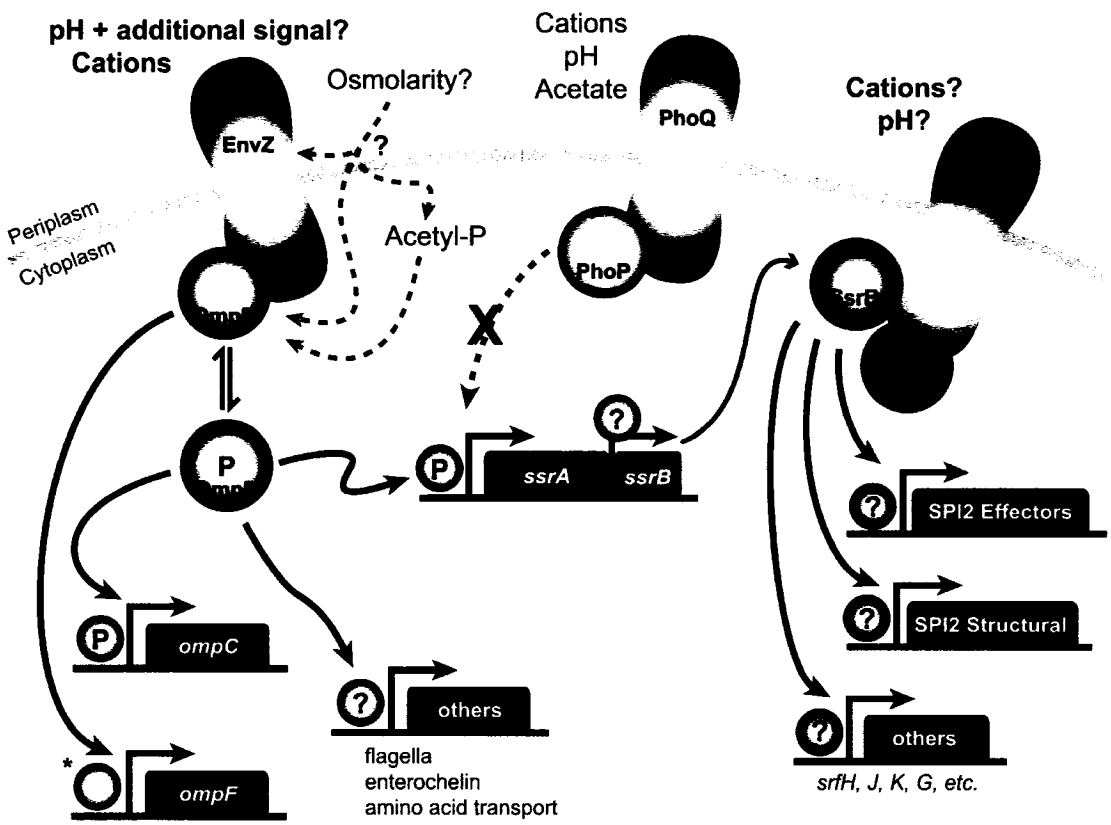


Figure 5. Schematic representation of a classical two-component system.

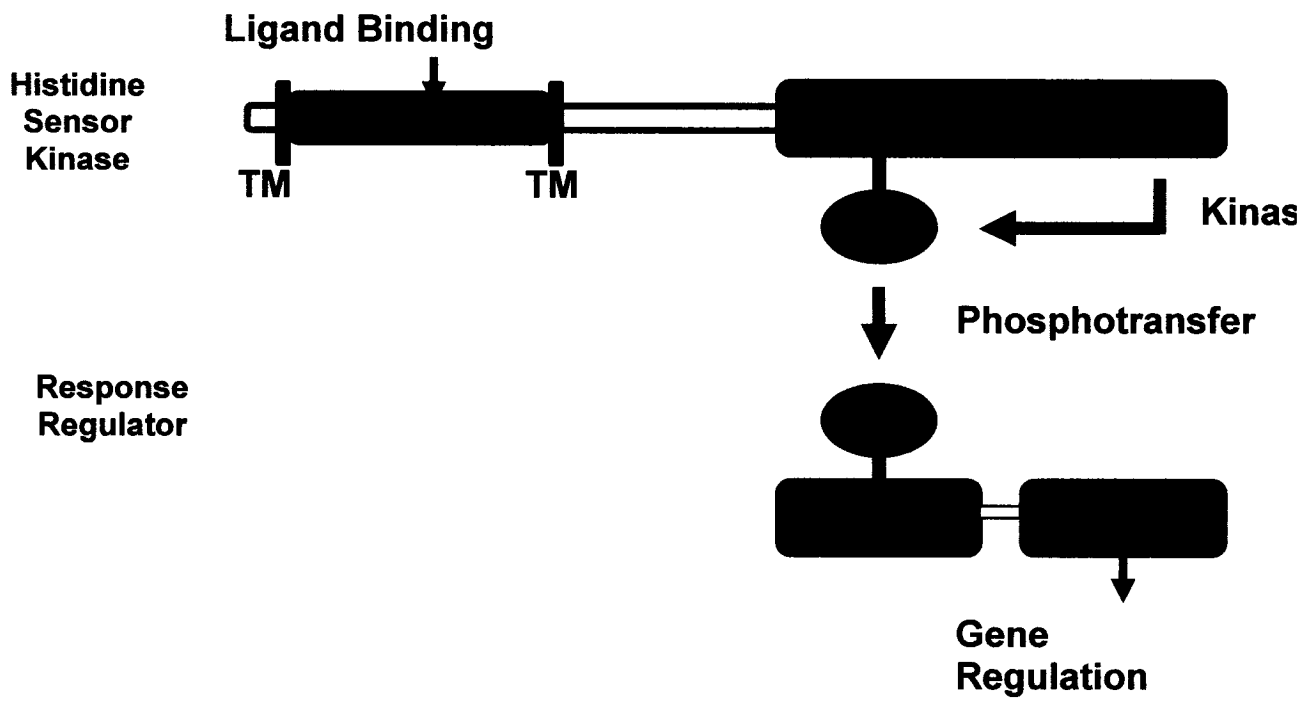


Figure 6. Three-dimensional structures of histidine kinase sensor isolated domains.

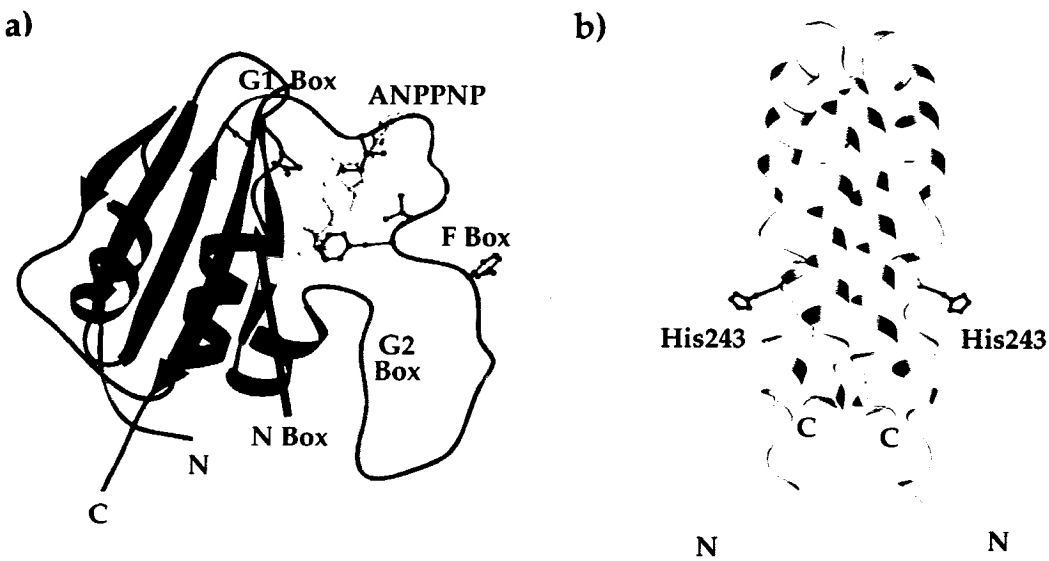
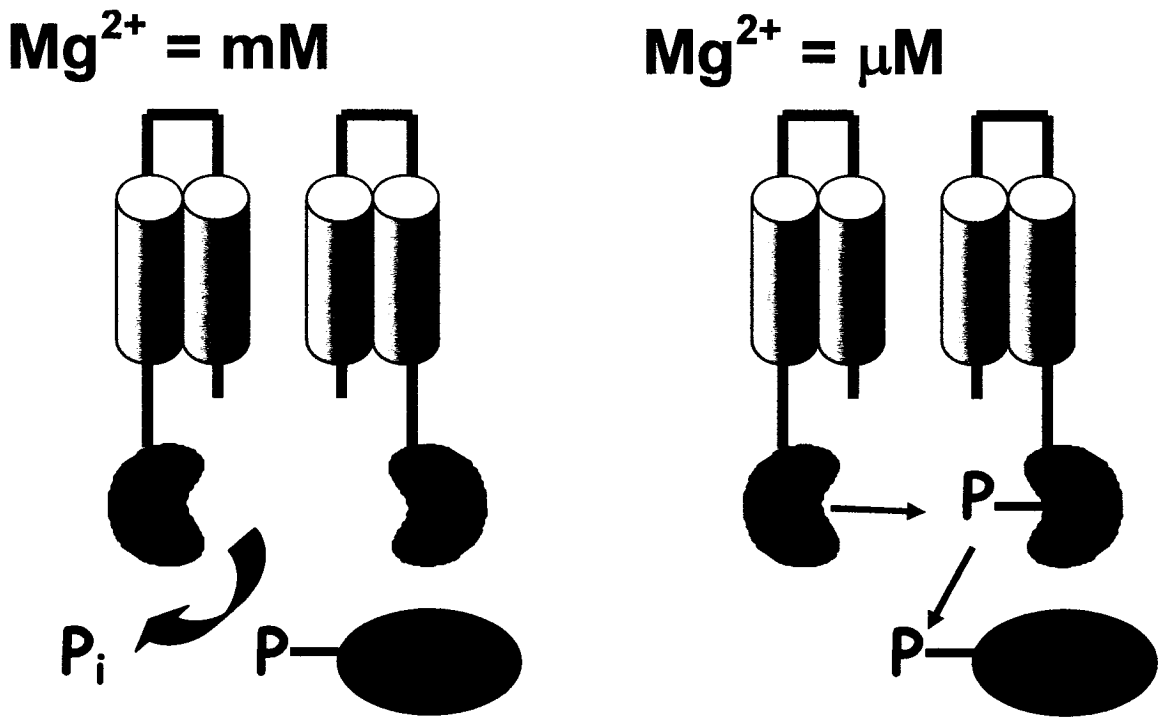


Figure 7. The PhoP/PhoQ system is regulated by divalent cations.



PREFACE TO CHAPTER 2

The experiments conducted in this chapter were designed to investigate the role of the residue at position 48 of the *Salmonella* PhoQ kinase sensor. This study defines the molecular mechanism by which the mutation T48I (*pho-24* allele of PhoQ) attenuates virulence in mice. We used site-directed mutagenesis combined with *in vivo* and *in vitro* assays to show that mutations T48I and T48V conferred a PhoP-constitutively active phenotype. In addition, the mutation T48L conferred a PhoP-constitutively inactive phenotype. The data establish a role for the residue at position 48 of PhoQ periplasmic domain in the conformational switch between kinase- and phosphatase-dominant states of PhoQ.

CHAPTER 2:

Mutational Analysis of the Residue at Position 48 in the *Salmonella enterica* Serovar Typhimurium PhoQ Sensor Kinase

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Running title: MUTATIONAL ANALYSIS OF POSITION 48 OF PhoQ

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ABSTRACT

The PhoP/PhoQ two-component regulatory system of *Salmonella enterica* serovar Typhimurium plays an essential role in controlling virulence by mediating the adaptation to Mg^{2+} depletion. The *pho*-24 allele of *phoQ* harbors a single amino acid substitution (T48I) in the periplasmic domain of the PhoQ histidine kinase sensor. This mutation has been shown to increase net phosphorylation of the PhoP response regulator. We analyzed the effect on signaling by PhoP/PhoQ of various amino acid substitutions at this position (PhoQ-T48X; X = A, S, V, I and L). Mutations T48V, T48I and T48L were found to affect signaling by PhoP/PhoQ both *in vivo* and *in vitro*. Mutations PhoQ-T48V and PhoQ-T48I increased both the expression of the *mgtA*::*lacZ* transcriptional fusion and the net phosphorylation of PhoP, conferring to cells a PhoP-constitutively active phenotype. In contrast, mutation PhoQ-T48L barely responded to changes in the concentration of external Mg^{2+}, *in vivo* and *in vitro*, conferring to cells a PhoP-constitutively inactive phenotype. By analyzing *in vitro* the individual catalytic activities of the PhoQ-T48X sensors, we found that the PhoP-constitutively active phenotype observed for the PhoQ-T48V and PhoQ-T48I proteins is solely due to decreased phosphatase activity. In contrast, the PhoP-constitutively inactive phenotype observed for the PhoQ-T48L mutant resulted from both decreased autokinase activity and increased phosphatase activity. Our data are consistent with a model in which the residue at position 48 of PhoQ contributes to a conformational switch between kinase- and phosphatase-dominant states.

INTRODUCTION

Two-component regulatory systems allow bacteria to adapt to a variety of environmental changes by modulating the expression of specific genes. The *Salmonella enterica* serovar Typhimurium PhoP/PhoQ two-component system plays an essential role in coordinating the expression of various virulence factors (9, 23). By modulating the expression of more than forty different genes, it controls adaptation to Mg^{2+}-limiting environments, protein secretion by the type III secretion system located on *Salmonella* pathogenicity island 1 (SPI1), entry into epithelial cells, survival within macrophages, lipopolysaccharide (LPS) modifications and resistance to antimicrobial peptides (reviewed in references 8 and 13).

The PhoQ protein is a transmembrane histidine kinase sensor that is present in the inner membrane as a homodimer (Fig. 1). Each subunit consists of an N-terminal periplasmic ligand-recognition domain (residues 40 to 194) flanked by two transmembrane segments (TM1 and TM2) and a C-terminal cytoplasmic signaling domain (residues 215-487). The PhoQ protein has been shown to sense some external divalent cations such as Mg^{2+}, Ca^{2+}, and Mn^{2+} (10). It has been proposed that Mg^{2+} is the physiological signal controlling the PhoP/PhoQ system (12). Limiting concentrations of Mg^{2+} in the extracellular environment activate the cytoplasmic phosphorylation cascade by promoting the ATP-dependent autophosphorylation of PhoQ on an invariant His residue (His 277) (3, 26). Subsequently, the phosphoryl group is transferred from PhoQ to a highly conserved Asp residue (Asp 55) of the response regulator PhoP. DNA binding of phospho-PhoP promotes both transcription of PhoP-activated genes and repression of PhoP-repressed genes. In contrast, high concentrations of external Mg^{2+} stimulate the PhoQ phosphatase activity that dephosphorylates phospho-PhoP (3, 26). In turn, this represses transcription of PhoP-activated genes and derepresses PhoP-repressed genes. Thus, environmental Mg^{2+} controls the intracellular level of phospho-PhoP by coordinately regulating the PhoQ autokinase and phosphatase activities (26).

The *pho-24* allele of *phoQ* that was isolated after diethyl sulfate mutagenesis of *S. enterica* serovar Typhimurium LT2 resulted in constitutive expression of *phoN*, a PhoP-activated gene that encodes a periplasmic acid phosphatase (19). Like *Salmonella phoP* or *phoQ* defective strains, those strains harboring the *pho-24* allele are attenuated for virulence in mice (11, 23, 24). The *pho-24* allele confers a PhoP-constitutive phenotype ($PhoP^c$) to cells that is characterized by the constitutive expression and repression of PhoP-activated genes and PhoP-repressed genes, respectively (24). This altered pattern of gene expression results in pleiotropic effects that include decreased invasion of epithelial cells (2, 27), defects in stimulating macropinocytosis and spacious phagosome formation in macrophages (1). It has subsequently been shown that a change of Thr to Ile at position 48 of the PhoQ protein was responsible for the phenoptypes associated with the *pho-24* allele (10, 14). *Salmonella* strains harboring the *pho-24* allele of *phoQ* have been used in various studies to establish the involvement of the PhoP/PhoQ system in lipid A modifications (15), in resistance against host antimicrobial peptides (16, 25) and in resistance to bile (29). In addition, these strains have been used as carriers for heterologous antigens in live oral vaccines (17). Although the *pho-24* mutation has been shown to increase net phosphorylation of PhoP (14), the mechanism by which this mutation affects signaling by the PhoP/PhoQ system remains unknown.

Our study assesses the molecular mechanism by which a mutation at position 48 in the PhoQ periplasmic domain affects catalytic activities in the cytoplasmic signaling domain. We generated mutants of the *phoQ* gene with amino acid substitutions at position 48 and analyzed the catalytic activities of the encoded proteins both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strain XL-1 blue (Stratagene) was used for all DNA manipulations. *E. coli* strain BL21(DE3)pLysE (Novagen) was used for protein expression. *E. coli* strain MG1607 [F⁻ Δ*(argF-lac)U169 araD139 rpsL150 ptsF25 fibB5301 rbsR deoC relA1 mgtA*::λp*lac*Mu55 *phoQ*608::Tn*10*dCam], which was kindly provided by Dr. R. Utsumi, Kinki University, Japan (18), was used for β-galactosidase analyses. Bacterial cultures were grown at 37°C with aeration in Luria-Bertani (LB) broth containing the appropriate antibiotics (100 μg of ampicillin per ml and 30 μg of chloramphenicol per ml).

Plasmids and DNA manipulations. Plasmid pET-Q containing the *S. enterica* serovar Typhimurium *phoQ* gene under the control of the T7 promoter (26), was used to generate the series of mutants at position 48 (pET-Q-T48X). Site-directed mutagenesis was performed according to Deng and Nickoloff (6). The native Thr residue at position 48 was replaced by a Ser residue using primer Q-T48S (5'-AGTTTTGATAAAACCAGCTTTCGTTTGC-3'), an Ala residue using primer Q-T48A (5'-GTTTTGATAAAACCGCCTTTCGTTTGCTG-3'), an Ile residue using primer Q-T48I (5'-AGTTTTGATAAAACCATCTTTCGTTTGC-3'), a Val residue using primer Q-T48V (5'-GTTTTGATAAAACCGTCTTTCGTTTGCTG-3'), and a Leu residue using primer Q-T48L (5'-GTTTTGATAAAACCCTCTTTCGTTTGCTG-3'). Plasmid pET-P_{His} encoded the *S. enterica* serovar Typhimurium PhoP protein fused to a C-terminal His-tag ($PhoP_{His}$; 26).

To carry out β-galactosidase analyses, we constructed the plasmid pPRO-Q_{His}, in which the *S. enterica* serovar Typhimurium *phoQ* gene fused to a C-terminal His-tag is overexpressed under the control of the *tac* promoter. This medium-copy-number plasmid harboring the p15A origin of replication was derived from pPROLar.A122 (Clontech) by inserting the *lacI*^{q} coding sequence and replacing the kanamycin resistance gene by the *ampR* gene. The pPRO-Q_{His}-T48X series of

plasmids was constructed by subcloning the various 732-bp *Kpn*I-*Mlu*I fragments from the pET-Q-T48X plasmids into pPRO-Q_{His} that had been digested with the same enzymes.

β-galactosidase assays. *E. coli* MG1607 cells transformed with the pPRO-Q_{His}-T48X series of plasmids were grown overnight at 37°C in LB supplemented with 0.1% glucose. Cultures were diluted 1:100 in LB supplemented with 0.1% glucose in the presence or absence of 10 mM $MgCl_2$. Cultures were grown to an OD_{600} ≈0.8 before inducing with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. Cell lysates were prepared by using the Reporter Lysis Buffer (Promega). β-galactosidase activity was measured using ONPG as a substrate, as previously described (22). Expression levels of the $PhoQ_{His}$-T48X mutants were monitored by Western blotting. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. His-tagged proteins were detected by using the His•Tag monoclonal antibody (Novagen) and the His•Tag AP LumiBlot Reagents (Novagen), as recommended by the manufacturer.

Preparation of membranes enriched in the various PhoQ-T48X proteins. *E. coli* BL21(DE3)pLysE cells, transformed with the pET-Q-T48X series of plasmids, were grown at 37°C in LB supplemented with appropriate antibiotics. Transcription of the *phoQ* gene was induced at an OD ≈0.8 by adding 0.5 mM IPTG. After 4 h of induction, cells were harvested by centrifugation and membranes were prepared as described previously (20). The total amount of protein in membranes was measured by the Bradford method using the standard procedure of the Bio-Rad protein assay in the presence of 0.1% SDS. Expression of the various PhoQ-T48X proteins was visualized on a 12.5% SDS-PAGE gel. After the Coomassie blue-stained gels were scanned, the percentage corresponding to the overproduced PhoQ-T48X proteins in membranes was estimated.

Expression and purification of $PhoP_{His}$. *E. coli* Bl21(DE3)pLysE cells transformed with the pET-P_{His} plasmid were grown at 22°C in LB supplemented with appropriate antibiotics. Cultures were induced overnight with 1mM IPTG at an OD

≈0.8 and cells were harvested by centrifugation, resuspended in loading buffer consisting of 20 mM Tris-HCl (pH=7.9), 500 mM NaCl, 50 mM imidazole, 1 mM PMSF and sonicated at 4°C. Cell debris was removed by centrifugation, and the supernatant was loaded onto a 1 ml HiTrap chelating column (Amersham Biosciences). The column was extensively washed with loading buffer and eluted using a 0-600 mM imidazole gradient in loading buffer. Fractions containing $PhoP_{His}$ were pooled and dialyzed extensively against 20 mM Tris-HCl (pH=7.5), 50 mM NaCl and 10% glycerol. The concentration of purified $PhoP_{His}$ was measured by using the Bio-Rad protein assay.

***In vitro* reconstitution of the PhoP/PhoQ signaling cascade.** The net phosphorylation of PhoP was measured by incubating membranes containing the PhoQ-T48X proteins (approximately 1.5 μM of PhoQ-T48X) with a 6-fold molar excess of $PhoP_{His}$ in a 15-μl volume of phosphorylation buffer (50 mM Tris-HCl [pH 7.5], 200 mM KCl, 0.1 mM EDTA, 5% glycerol) supplemented with 5 mM $MgCl_2$. Reactions were initiated by the addition of 0.1 mM [$\tilde{\gamma}$ P] ATP (10 Ci/mmol), incubated for 20 min at 22°C to reach an apparent steady state and stopped by addition of 4 x Laemmli SDS sample buffer (250 mM Tris-HCl [pH 6.8], 8% SDS, 40% glycerol, 0.02% bromophenol blue, 4% β-mercaptoethanol). Reaction products were heated at 37°C for 3 min and applied to 10% SDS-PAGE gels. Gels were dried and exposed to a PhosphorImager (Molecular Dynamics). Phosphorylated proteins were quantified by image analysis using the Image Quant software (Molecular Dynamics).

***In vitro* autophosphorylation assays.** Membranes containing the PhoQ-T48X proteins (approximately 1.5 μM of PhoQ-T48X) were autophosphorylated with 0.1 mM [$\tilde{\gamma}$ P] ATP (10 Ci/mmol) in a 15-μl volume of phosphorylation buffer supplemented with 0.1mM $MgCl_2$ (26). The phosphorylation reactions were continued at 22°C for various times before being stopped by addition of 4 x Laemmli SDS sample buffer. Reaction products were analyzed by SDS-PAGE, as described above.

***In vitro* phosphatase assays.** Purified $PhoP_{His}$ was phosphorylated with membranes containing the overexpressed PhoQ-T48V protein in a 75-μL volume of phosphorylation buffer supplemented with 5mM $MgCl_2$ and 0.1 mM [$\tilde{\gamma}$ P] ATP (10 Ci/mmol). After 20 min of incubation at 22°C, the reaction mixture was centrifuged at 16,000 × *g* for 10 min at 4°C to pellet membranes. The supernatant was loaded on a Micro Bio-Spin Chromatography column (Bio-Rad) to remove unincorporated ATP, generated ADP and $MgCl_2$. The flow-through containing phospho-$PhoP_{His}$ was immediately used for the phosphatase assay. Phospho-$PhoP_{His}$ (15 μM) was mixed with membranes containing the PhoQ-T48X proteins (approximately 1.5 μM of PhoQ-T48X) in 15-μl volume of phosphorylation buffer supplemented with 10 mM $MgCl_2$. At various time points, reactions were stopped by the addition of 4 x Laemmli SDS sample buffer. Phosphorylated products were analyzed as described above.

RESULTS

Isolation of the PhoQ-T48X mutations. The residue at position 48 is located at the N-terminus of the periplasmic domain of PhoQ, in close proximity to the first transmembrane segment (TM1; Fig. 1A). Secondary structure predictions of the PhoQ periplasmic domain suggested that the Thr at position 48 is part of an α-helix that is probably continuous with the postulated α-helical TM1 (21). Although the Thr residue at position 48 is conserved in many PhoQ homologues, other amino acid residues (Ser, Ile or Leu) are present at this position in the most distantly related PhoQ homologues (Fig. 1B). To understand the molecular mechanism by which the mutation T48I (the *pho-24* allele of *phoQ*) affects the PhoQ catalytic activities, we replaced the native Thr residue by Ala, Ser, Val, Ile and Leu residues using site-directed mutagenesis (6). The rationale in mutating the Thr at position 48 of PhoQ is based on polarity, size and hydrophobicity of the side chains.

Effect of the PhoQ-T48X mutations on the expression of a PhoP-activated gene. To examine the effects of the PhoQ-T48X mutations on the entire PhoP/PhoQ signaling pathway, *in vivo*, we measured the expression of *mgtA* (a PhoP-activated gene that encodes a Mg^{++} transporter) by measuring the β-galactosidase activity of the *mgtA*::*lacZ* transcriptional fusion (18). *E. coli* MG1607 cells (a *phoQ* defective strain; 18) were transformed with the various pPRO-Q_{His}-T48X plasmids encoding the *S. enterica* serovar Typhimurium PhoQ mutated proteins. To quantify the expression of the PhoQ-T48X proteins by Western blotting, we used plasmids encoding the PhoQ protein fused to a C-terminal tag of 6 histidine residues ($PhoQ_{His}$-T48X). The catalytic activities of the $PhoQ_{His}$ protein were essentially similar to those of the untagged PhoQ protein, as judged by both *in vivo* and *in vitro* assays (data not shown).

As found in previous studies (10, 28), β-galactosidase activity of cells producing wild-type $PhoQ_{His}$ was decreased by about 10-fold when LB was supplemented with 10 mM $MgCl_2$, indicating that expression of *mgtA* was repressed by high concentrations of Mg^{2+} (Fig. 2). Levels of β-galactosidase activity observed

for cells producing either $PhoQ_{His}$-T48S or $PhoQ_{His}$-T48A were essentially similar to those obtained for cells producing wild-type $PhoQ_{His}$, whether or not LB was supplemented with $MgCl_2$ (Fig. 2). In contrast, mutations T48V and T48I in the $PhoQ_{His}$ protein increased the levels of β-galactosidase activity by 5-fold in the absence of $MgCl_2$ and by 14- to 23-fold in the presence of 10 mM $MgCl_2$. Although the $PhoQ_{His}$-T48I or $PhoQ_{His}$-T48V mutants responded to high concentrations of external $MgCl_2$, levels of β-galactosidase activity produced by these cells grown in the presence of 10 mM $MgCl_2$ were higher than the level of β-galactosidase activity obtained with cells producing wild-type $PhoQ_{His}$ grown in the absence of $MgCl_2$. Interestingly, cells that produced the $PhoQ_{His}$-T48L protein showed low levels of β-galactosidase activity whether or not LB was supplemented with 10 mM $MgCl_2$ (Fig. 2). Similar results were obtained by measuring the β-galactosidase activity of the *mgrB*::*lacZ* transcriptional fusion (18; data not shown). These data indicated that mutations T48V, T48I and T48L in the $PhoQ_{His}$ protein affect the PhoP/PhoQ signaling cascade. As observed previously for mutation T48I (24), mutation T48V confers to cells a PhoP-constitutively active phenotype. In contrast, mutation T48L appeared to barely respond to changes in the concentrations of external $MgCl_2$, conferring to cells a PhoP-constitutively inactive phenotype.

***In vitro* global activity of the PhoP/PhoQ-T48X systems.** The level of phospho-PhoP inside the cell depends on both the autokinase and phosphatase activities of PhoQ. To assess *in vitro* the global activity of the PhoP/PhoQ-T48X systems, we reconstituted functional signaling pathways and measured the resulting net phosphorylation of PhoP. The various membranes containing the PhoQ-T48X proteins were incubated with a sixfold molar excess of $PhoP_{His}$ in the presence of 5 mM $MgCl_2$. Figure 3 shows the amounts of [^{32}P]-phospho-$PhoP_{His}$ resulting from the catalytic activities of the various PhoQ-T48X proteins after a 20 min incubation period. For membranes containing the PhoQ-T48A, T48S and T48L mutants, levels of radiolabeled $PhoP_{His}$ were similar to those obtained with wild-type PhoQ (Fig. 3). Sixfold and ninefold increases in radiolabeled $PhoP_{His}$ were observed with membranes containing the PhoQ-T48I and PhoQ-T48V mutants, respectively (Fig. 3).

These data, consistent with our *in vivo* data shown in Fig. 2, confirmed that a Val or Ile residue at position 48 increases the net phosphorylation of PhoP. They also indicated that the Thr to Leu substitution did not affect the net phosphorylation of PhoP under these experimental conditions.

Influence of Mg^{2+} on the *in vitro* global activity of the PhoP/PhoQ-T48L system. With the exception of the PhoQ-T48L protein, all the PhoQ-T48X sensors responded to changes in the concentrations of external $MgCl_2$ (Fig. 2). To further investigate the apparent inability of this mutated protein to respond to $MgCl_2$, we performed *in vitro* global assays at increasing concentrations of $MgCl_2$. As shown in Fig. 4, increasing the concentration of $MgCl_2$ from 1 to 10 mM decreases levels of radiolabeled $PhoP_{His}$ by about 10-fold for wild-type PhoQ. In contrast, the PhoQ-T48L mutant only produced a twofold decrease in the level of radiolabeled $PhoP_{His}$ (Fig. 4). These data indicated that the PhoQ-T48L sensor barely responds to the absence of Mg^{2+}, suggesting that the Thr to Leu substitution may lock the protein in a conformation that confers a PhoP-constitutively inactive phenotype.

***In vitro* autokinase activity of the PhoQ-T48X sensors.** We wished to determine which of the individual PhoQ catalytic activities, autokinase or phosphatase were affected by the mutations at position 48. We measured the initial rates of autophosphorylation of the various PhoQ-T48X sensors present in membranes. Autophosphorylation assays were performed as described in Materials and Methods using similar amounts of the various PhoQ-T48X proteins as judged by SDS-PAGE. The PhoQ-T48I and PhoQ-T48V mutants were autophosphorylated at rates that are almost identical to that obtained for wild-type PhoQ (Fig. 5). In contrast, the Thr to Leu substitution (PhoQ-T48L) decreased the rate of autophosphorylation by about threefold (Fig. 5). Both the PhoQ-T48S and PhoQ-T48A mutants were autophosphorylated at rates that are fivefold slower than that obtained for the wild-type protein (Fig. 5). These data showed that the rates of PhoQ autophosphorylation vary significantly with the nature of the substituting amino acid at position 48.

***In vitro* phosphatase activity of the PhoQ-T48X sensors.** Substitutions at position 48 may not only affect PhoQ autokinase activity, but also affect its phosphatase activity. Therefore, we compared the phosphatase activities of the various PhoQ-T48X sensors *in vitro*. Purified [^{32}P]phospho-PhoP$_{His}$ was incubated with membranes containing the various PhoQ-T48X proteins. Aliquots were removed at different time points and the time course of the reaction was followed as shown in Fig. 6. A control reaction in which [^{32}P]phospho-PhoP$_{His}$ was incubated with control membranes lacking PhoQ indicated that [^{32}P]phospho-PhoP$_{His}$ was stable during the reaction period. The phosphatase activities of PhoQ-T48I, PhoQ-T48V were decreased compared to that of wild-type PhoQ (Fig. 6). In contrast, the PhoQ-T48L protein and to a lesser extent, the PhoQ-T48A protein showed increased phosphatase activities compared to wild-type PhoQ (Fig. 6). The phosphatase activity of PhoQ-T48S was essentially similar to that of wild-type PhoQ (Fig. 6). These data indicated that PhoQ phosphatase activity is also significantly affected by the various substitutions at position 48. Thus, we conclude that both the autokinase and phosphatase activities of PhoQ may be affected by the mutations at position 48.

DISCUSSION

Previous studies have shown that a change of a Thr to Ile at position 48 of the PhoQ histidine kinase sensor (*pho-24* allele of *phoQ*) results in a constitutively active PhoP/PhoQ regulatory system in *S. enterica* serovar Typhimurium (10, 14, 24). In our study, we examined the influence of various amino acid substitutions (Ala, Ser, Val, Ile and Leu) at position 48 of PhoQ on signal transduction by PhoP/PhoQ. We identified three distinct classes of mutations, two of which altered expression of the *mgtA*::*lacZ* transcriptional fusion (Fig. 2).

The first class is represented by the mutations T48A and T48S. These mutations did not significantly alter the Mg^{2+}-regulated expression of the *mgtA*::*lacZ* transcriptional fusion (Fig. 2). *In vitro* reconstitution of the PhoP/PhoQ-T48X systems showed that these mutations did not have an effect on the net autophosphorylation of PhoP (Fig. 3). However, analyzes of the individual PhoQ catalytic activities showed that both mutations affected autokinase activity and to a lesser extent, phosphatase activity (Fig. 5 and 6). Therefore, we conclude that in spite of differences in the individual activities, the balance between autokinase and phosphatase activity remained essentially unchanged for PhoQ-T48A and PhoQ-T48S. Interestingly, the side chains of Ala and Ser are smaller than that of the native Thr. Accordingly, we propose that small side chains or loss of the hydroxyl group at position 48 do not cause major structural alterations leading to important changes in the global activity.

The second class is represented by the mutations T48V and T48I (*pho-24* allele of *phoQ*). Both mutations increased expression of the *mgtA*::*lacZ* transcriptional fusion and conferred a PhoP-constitutively active phenotype (Fig. 2; 24). Both mutated proteins responded to increasing concentrations of Mg^{2+}, albeit to a lesser extent than wild-type PhoQ (Fig. 2; 10). The increased net phosphorylation of PhoP observed for mutant T48V was essentially similar to that observed previously for the mutation T48I (Fig. 3; 14). By analyzing *in vitro* the individual autokinase and phosphatase activities, we found that PhoQ-T48V and PhoQ-T48I have decreased

phosphatase activity, whereas the rates of autophosphorylation were identical to that of wild-type PhoQ (Fig. 5 and 6). Thus, the PhoP-constitutively active phenotype observed for the PhoP/PhoQ-T48V and PhoP/PhoQ-T48I systems is solely due to decreased phosphatase activity of PhoQ. For these mutated PhoQ sensors the balance between autokinase and phosphatase activity shifted toward a kinase-dominant state. It is noteworthy that both Val and Ile are hydrophobic β-branched residues, whereas the native Thr is a polar β-branched residue. Importantly, only hydrophobic residues that are β-branched but not γ-branched conferred a PhoP-constitutively active phenotype. These observations suggest that the hydrophobicity as well as the shape of the residue at position 48 may be critical for PhoQ transmembrane signaling.

The third class is represented by the mutation T48L that confers a PhoP-constitutively inactive phenotype. For this mutation, expression of the *mgtA*::*lacZ* transcriptional fusion was repressed irrespective of Mg^{2+} (Fig. 2). When compared to wild-type PhoQ, limiting concentrations of Mg^{2+} had minimal effect on the net phosphorylation of PhoP measured *in vitro* (Fig. 4). We found that this phenotype resulted from both a decreased autokinase activity and an increased phosphatase activity (Fig. 5 and 6). Thus, the PhoQ-T48L sensor is in a phosphatase-dominant state irrespective of Mg^{2+}. Leu is a hydrophobic residue that is isomeric to Ile but γ-branched. Thus, the longer side chain length of the Leu residue at position 48 may provide a steric hindrance that is responsible for the PhoP-constitutively inactive phenotype observed for the PhoP/PhoQ-T48L system. This substantiates our hypothesis that both the shape and the hydrophobicity of residue 48 are critical for PhoQ transmembrane signaling.

The residue at position 48 of PhoQ may influence signal transduction by directly affecting Mg^{2+}-binding or by interfering with the Mg^{2+}-induced conformational signal that is transmitted through the membrane bilayer. The direct involvement of residue 48 in Mg^{2+}-recognition is most unlikely, because the response to increasing concentrations of external Mg^{2+} is essentially similar for four of the five mutated PhoQ proteins analyzed (Fig. 2). It appears more likely that residue 48 is an important structural determinant for transmitting the Mg^{2+}-induced transmembrane

signal. Our data support our hypothesis that the side-chain of residue 48 is involved in the conformational changes responsible for the switch between the kinase-dominant state and the phosphatase-dominant state.

Residue 48 of PhoQ is located at the N-terminus of the periplasmic domain in close proximity to the cytoplasmic membrane (Fig. 1A). In closely related PhoQ homologues, like the PhoQ proteins of other enterobacteria, the Thr residue at position 48 is strictly conserved (Fig. 1B). In the *P. aeruginosa* and *X. fastidiosa* PhoQ homologues, the residue 48 consists of an Ile and a Leu, respectively (Fig. 1B). Our results show that these mutations affect signaling of the *Salmonella* PhoQ protein. It is possible that in the three-dimensional structure of these proteins compensating mutations counterbalance the presence of an Ile or a Leu at position 48. Alternatively, it is possible that the nature of the residue at position 48 modulates the level of expression of PhoP-regulated genes. It is important to note that the environmental signal sensed by some of these PhoQ homologues may be different than Mg^{2+} (13). In other histidine kinase sensors, the corresponding region has been shown to be important for either transmembrane signaling or ligand recognition. The highly conserved I-box that includes residues 41 to 53 of various EnvZ homologues has been shown to be involved in the signal transduction mechanism (30). The P-box of the NarQ and NarX sensors that corresponds to a stretch of 17 conserved amino acids has been shown to be required for nitrate sensing and/or transmembrane signaling (4, 5, 31). Although the corresponding region in PhoQ homologues is not as highly conserved as the I-box or the P-box (Fig. 1B), the location of position 48 implies that this region is important for transmembrane signaling.

Taken together, our data indicate that substitutions at position 48 of PhoQ affect the balance between autokinase and phosphatase activities. Our findings define the structural requirement at the position 48 of PhoQ and suggest that this residue is critical for the transmission of the ligand-induced conformational signal through the membrane bilayer. Future studies will determine whether the conformational changes that involve residue 48 affect the dimeric interface of the protein or the structure of isolated subunits.

ADDENDUM IN PROOF

After this paper was submitted, Regelmann et al., (A. G. Regelmann, J. A. Lesley, C. Mott, L. Stokes, and C. D. Waldburger, J. Bacteriol. 2002, **184:**5468-5478, 2002) reported a similar study on the *E. coli* PhoQ sensor kinase. Although the PhoQ proteins of *S. enterica* serovar Typhimurium and *E. coli* are 86% identical and exhibit a Thr at position 48, the PhoQ-T48I mutants display opposite phenotypes.

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FIGURE LEGENDS

FIG. 1. Location of position 48 in the periplasmic domain of PhoQ. (A) Schematic representation of the PhoQ sensor kinase. The location of position 48 is shown by an arrow. The cytoplasmic domain representation is based on the NMR structures of the *E. coli* EnvZ sensor kinase (7). It consists of a catalytic ATP-binding domain and a dimerization domain that contains the invariant histidine residue. (B) Partial alignment of the amino acid sequences of the *S. enterica* PhoQ protein to homologues in *E. coli*, *K. pneumoniae*, *Y. pestis*, *P. stuartii*, *E. carotovora*, *P. aeruginosa* and *X. fastidiosa*. The arrow indicates position 48 (*S. enterica* numbering). The bold line under the sequences indicates the N-terminal transmembrane segment (TM1), which has been predicted on the basis of a hydrophobicity plot.

FIG. 2. Regulation of the *mgtA::lacZ* transcriptional fusion by the PhoQ-T48X mutants. (A) *E. coli* cells MG1607 carrying the pPRO-T48X-His plasmids were grown in LB broth or LB broth supplemented with 10 mM $MgCl_2$. β-galactosidase activities are expressed as Miller milliunits. Data are the means of triplicates values with standard deviations. (B) Western blot analysis of the expression level of the various PhoQ-T48X mutants.

FIG. 3. *In vitro* global activity of the PhoP/PhoQ-T48X regulatory systems. Membranes containing the PhoQ-T48X proteins were incubated at 22°C with a sixfold molar excess of $PhoP_{His}$ in the presence of [$\tilde{\gamma}$ P] ATP and 5 mM $MgCl_2$. After 20 min, reactions (15 µl) were stopped by the addition of Laemmli SDS sample buffer. Aliquots were subjected to SDS-PAGE. The amount of ^{32}P associated with the $PhoP_{His}$ protein was quantitated with a PhosphorImager. Data are the means of triplicates values with standard deviations.

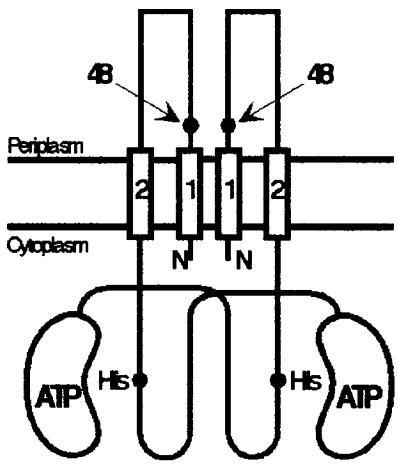
FIG. 4. Influence of Mg^{2+} on the *in vitro* global activity of the PhoP/PhoQ or PhoP/PhoQ-T48L regulatory systems. Membranes containing the PhoQ or PhoQ-T48L proteins were incubated at 22°C with a sixfold molar excess of $PhoP_{His}$ in the presence of [$\tilde{\gamma}$ P] ATP and increasing concentrations of $MgCl_2$. After 20 min, reactions (15 μl) were stopped by the addition of Laemmli SDS sample buffer. Aliquots were subjected to SDS-PAGE. The amount of ^{32}P associated with the $PhoP_{His}$ protein was quantitated with a PhosphorImager.

FIG. 5. Initial rates of autophosphorylation of the PhoQ-T48X proteins. Membranes containing wild-type PhoQ (□), PhoQ-T48A (▲), PhoQ-T48S (○), PhoQ-T48V (Δ), PhoQ-T48I (●) or PhoQ-T48L (◆) were incubated in the presence of [$\tilde{\gamma}$ P] ATP and 0.1 mM $mgCl_2$ for 2 min at 22°C. Reactions (15μl) were stopped by the addition of Laemmli SDS sample buffer. Aliquots were subjected to SDS-PAGE. The amount of ^{32}P associated with the PhoQ protein was quantitated with a PhosphorImager.

FIG. 6. Dephosphorylation of phospho-$PhoP_{His}$ by the PhoQ-T48X proteins. [^{32}P]-phospho-$PhoP_{His}$ was prepared and purified as described under Materials and Methods. Purified [^{32}P]-phospho-PhoP-$_{His}$ was incubated with either control membranes lacking PhoQ (Ctl.) or membranes containing PhoQ-T48X proteins. At indicated time points, aliquots (15 μl) were removed and reactions were stopped by the addition of Laemmli SDS sample buffer. Aliquots were subjected to SDS-PAGE. The amount of ^{32}P associated with the PhoP protein was determined with a PhosphorImager.

FIG. 1. Sanowar *et al.*

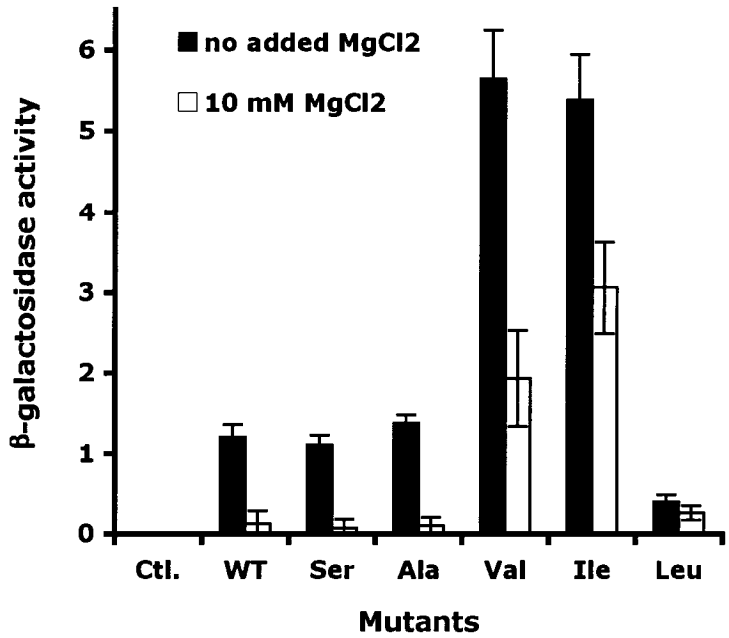
A]



B]

FIG. 2. Sanowar *et al.*

A]



B]

FIG. 3. Sanowar *et al.*

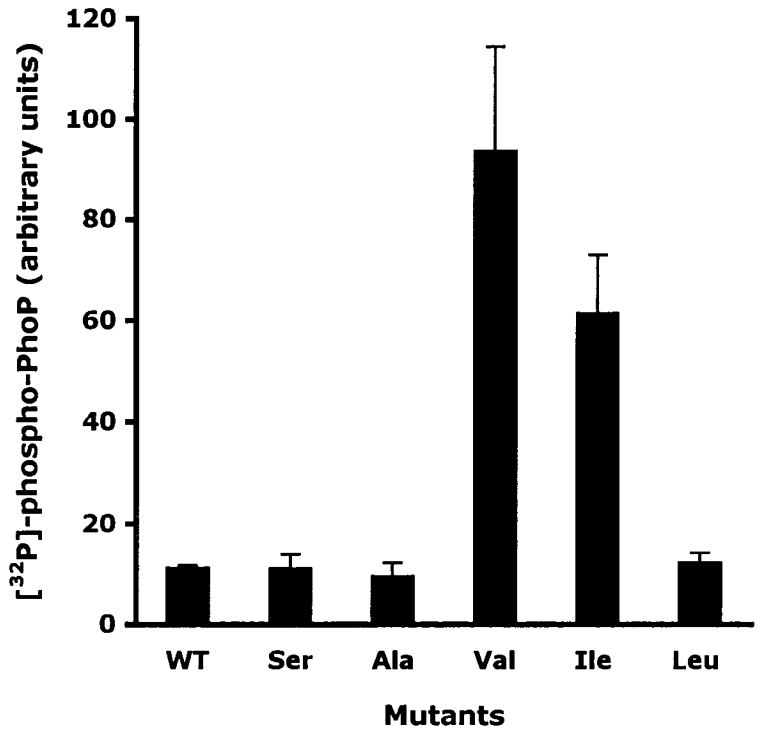


FIG. 4. Sanowar *et al.*

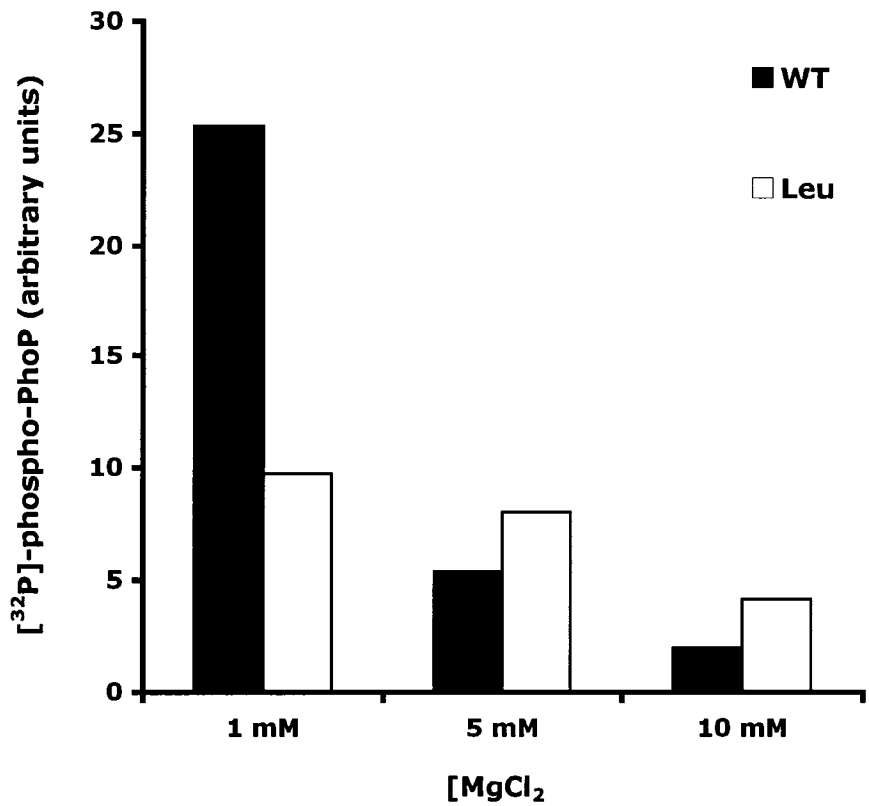


FIG. 5. Sanowar *et al.*

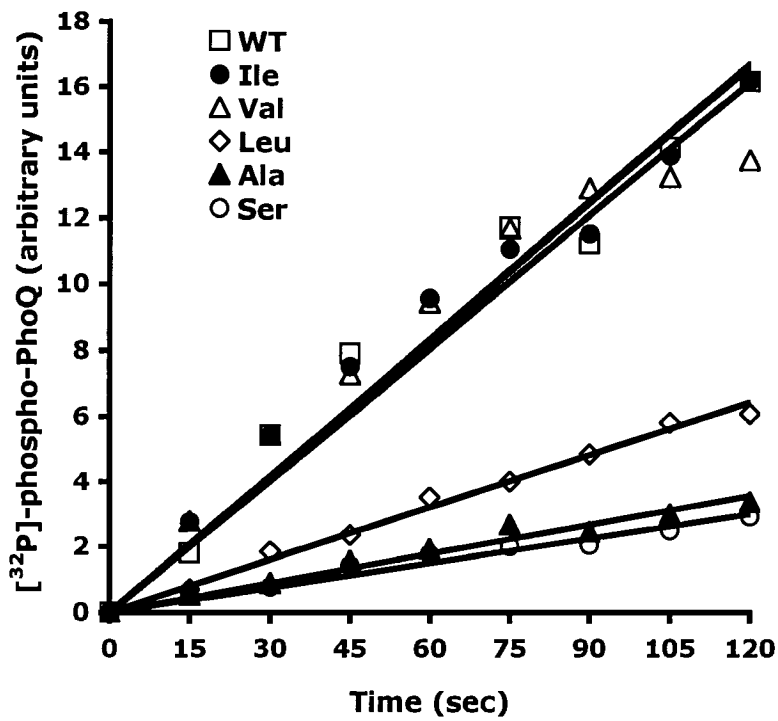
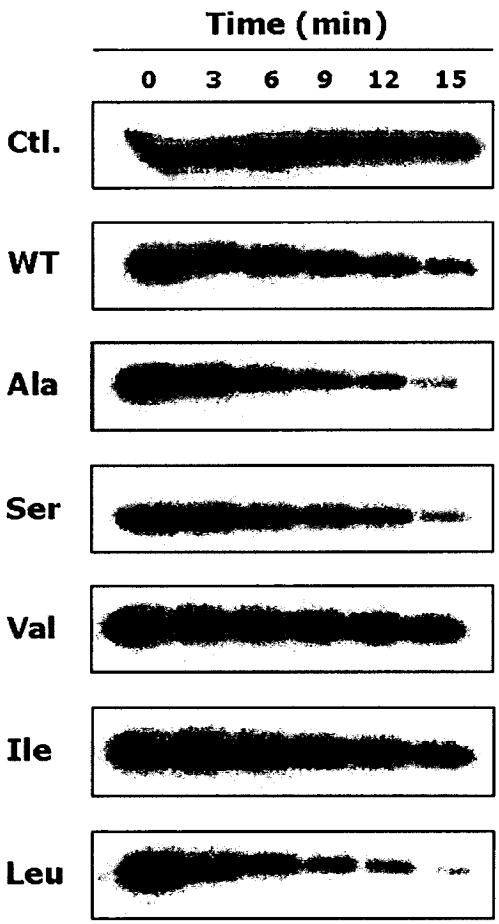


FIG. 6. Sanowar *et al.*



PREFACE TO CHAPTER 3

This study was undertaken in response to conflicting results obtained for the Mg^{2+}-mediated regulation of the PhoQ histidine kinase sensor performed with PhoQ protein overexpressed in membranes. In order to study divalent cation-mediated regulation of PhoQ in the absence of other membrane proteins, we report the purification and reconstitution of *S. typhimurium* $PhoQ_{His}$ into *E. coli* liposomes. Reconstituted $PhoQ_{His}$ inserted into liposomes in a unidirectional orientation. Reconstituted $PhoQ_{His}$ was capable of autokinase and the phosphoryl group was transferred to purified PhoP. Reconstituted $PhoQ_{His}$ was also capable of phosphatase activity and this activity was greatly stimulated in the presence of intraluminal ADP. We demonstrate the successful PhoQ reconstitution and validate use of the reconstituted system for studying ligand-mediated regulation of PhoQ activities.

CHAPTER 3:

Functional reconstitution of the *Salmonella typhimurium* PhoQ histidine kinase sensor in proteoliposomes

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Short title: Functional reconstitution of the PhoQ kinase sensor

Key words: membrane receptor, two-component systems, phosphorylation, bacterial signal transduction.

Abbreviations used: IPTG, isopropyl-β-D-thiogalactopyranoside; ONPG, *o*-nitrophenyl β-D-galactopyranoside; DM, dodecyl-β-D-maltoside; DDM, decyl-β-D-maltopyranoside; LDAO, lauryldimethylamine-*N*-oxide; BCA, bicinchoninic acid; BME, β-mercaptoethanol; 5-IAF, 5-iodoacetamide fluorescein; AmdiS, 4-acetamido-4'-maleidylstilbene-2,2'-disulfonic acid; NEM, *N*-ethylmaleimide; AMP-PNP, adenosine 5'-(*β*, *γ*-imido)triphosphate; GMP-PNP, guanosine 5'-(*β*, *γ*-imido)triphosphate.

ABSTRACT

Two-component signal transduction systems are widespread in bacteria. They are usually composed of a transmembrane histidine kinase sensor and a cytoplasmic response regulator. The PhoP/PhoQ two-component system of *Salmonella typhimurium* contributes to virulence by coordinating the adaptation to low concentrations of environmental Mg^{2+}. Limiting concentrations of extracellular Mg^{2+} activate the PhoP/PhoQ phosphorylation cascade modulating the transcription of PhoP-regulated genes. In contrast, high concentrations of extracellular Mg^{2+} stimulate the dephosphorylation of the response regulator PhoP by the PhoQ kinase sensor. Here, we report the purification and functional reconstitution of $PhoQ_{His}$, a PhoQ variant with a C-terminal His tag, into *Escherichia coli* liposomes . The functionality of $PhoQ_{His}$ was essentially similar to that of PhoQ as shown *in vivo* and *in vitro*. Purified $PhoQ_{His}$ inserted into liposomes in a unidirectional orientation with the sensory domain facing the lumen and the catalytic domain facing the extraluminal environment. Reconstituted $PhoQ_{His}$ exhibited all the catalytic activities that have been described for histidine kinase sensors. Reconstituted $PhoQ_{His}$ was capable of autokinase activity when incubated in the presence of Mg^{2+}-ATP. The phosphoryl group could be transferred from reconstituted $PhoQ_{His}$ to PhoP. Reconstituted $PhoQ_{His}$ catalyzed the dephosphorylation of phospho-PhoP and this activity was stimulated by the addition of extraluminal ADP.

INTRODUCTION

Two-component regulatory systems are used by bacteria to sense and respond to a variety of environmental signals, usually by modulating the expression of specific genes. Prototypical two-component systems consist of a transmembrane histidine kinase sensor that detects specific environmental signals and a cytoplasmic response regulator that elicits the cellular response [1, 2]. Signal transduction across the cytoplasmic membrane modulates an intracellular phosphorylation cascade [3, 4]. The kinase sensor autophosphorylates in an ATP-dependent manner on a highly conserved His residue of its cytoplasmic catalytic domain. Subsequently, the phosphoryl group is transferred from the His residue to an invariant Asp residue of the response regulator receiver domain. In turn, this affects the DNA-binding properties of the response regulator effector domain. Most kinase sensors also possess a phosphatase activity that allows the dephosphorylation of the response regulator. The balance between the autokinase and phosphatase activities of the kinase sensor controls the net phosphorylation of the response regulator [5-7].

In *Salmonella typhimurium*, the PhoP/PhoQ two-component system is composed of the PhoQ histidine kinase sensor and the PhoP response regulator. It controls the expression of more than 40 genes in response to changes in the extracellular concentrations of divalent cations such as Mg^{2+}, Ca^{2+}, and Mn^{2+}[8]. It has been proposed that Mg^{2+} acts as the physiologically relevant signal controlling the PhoP/PhoQ system [9]. Limiting concentrations of extracellular Mg^{2+} (micromolar range) activate the PhoP/PhoQ phosphorylation cascade promoting the transcriptional modulation of PhoP-regulated genes. High concentrations of extracellular Mg^{2+} (millimolar range) stimulate the phosphatase activity of the PhoQ sensor kinase leading to dephosphorylation of the PhoP response regulator. Although much research has addressed the details of the catalytic mechanisms for autokinase and phosphatase activities, many questions remain about how environmental signals regulate these activities [10-12]. In the case of the PhoQ kinase sensor, regulation of

the catalytic activities occurs through Mg^{2+} binding to the extracellular PhoQ sensory domain [8]. Mg^{2+} recognition is believed to elicit a conformational change that controls the PhoQ enzymatic activities [8]. Recent studies, using the PhoQ protein overexpressed in membranes, have addressed whether one or both PhoQ catalytic activities are regulated by the Mg^{2+}-induced conformational change [10, 11].

In this study, we characterized the catalytic activities of the purified $PhoQ_{His}$ protein reconstituted into *E. coli* phospholipids. We showed that reconstituted $PhoQ_{His}$ possesses autokinase activity and the ability to transfer the phosphoryl group to PhoP. In addition, we found that reconstituted $PhoQ_{His}$ exhibits phosphatase activity that is stimulated by the presence of ADP.

EXPERIMENTAL

Plasmid constructs

E. coli strain XL-1 Blue (Stratagene) was used for all DNA manipulations. The molecular cloning techniques used were from Sambrook et al. [13] or as recommended by the manufacturer. Plasmids pET-Q and pET-P_{His} encoding the full-length PhoQ and $PhoP_{His}$ proteins, respectively, have been described previously [10]. For construction of plasmid pET-Q_{His}, the *phoQ* gene of *S. typhimurium* was amplified by PCR from plasmid pET-Q using the forward primer QS-5'-NDE (5'-GGGCCGCCATATGAATAAATTTGCTCGCCATTTTCTG-3'), which contains a *Nde*I restriction site, and the reverse primer QS-3'-XHO (5'-CGGCTCGAGTTCCTCTTTCTGTGTGGGATGCTG-3'), which contains a *Xho*I restriction site. The PCR products were purified using the QIAquick PCR purification kit (Qiagen), digested with *Nde*I and *Xho*I and inserted into the expression vector pET-20b(+) (Novagen) previously digested with the same restriction enzymes. To carry out *in vivo* analyses, we generated plasmid pPRO-Q by inserting the *phoQ* coding sequence into plasmid pPRO [14]. Plasmid pPRO-Q_{His} was described previously [14].

In vivo analysis

E. coli strain MG1607 contains a chromosomal *mgtA::lacZ* transcriptional fusion and a disruption of the *phoQ* gene [15]. β-galactosidase assays were conducted as described previously [14]. Briefly, MG1607 cells were transformed with the pPRO, pPRO-Q or pPRO-Q_{His} plasmids and grown overnight at 37 °C in Luria-Bertani (LB) broth supplemented with 0.2 % (w/v) glucose and 100 μg/mL ampicillin. Cultures were diluted 1:100 in the above medium, in the absence or presence of 10 mM $MgCl_2$, and grown to late-log phase before inducing with 1 mM IPTG for 2 h. Cell lysates were prepared by using the Reporter Lysis Buffer (Promega). β-galactosidase activity was measured using ONPG as a substrate. One unit of β-galactosidase hydrolyzes 1 μmole of ONPG/min at pH 7.5 and 37 °C.

Overexpression of $PhoQ_{His}$ and PhoP

E. coli strain BL21(DE3)pLysE (Novagen) was used for protein expression. Cells were transformed with either the pET-20b(+), pET-Q or pET-Q_{His} plasmids and grown at 37 °C with aeration in LB supplemented with 100 μg/mL ampicillin and 30 μg/mL chloramphenicol. Cells grown to late-log phase were induced with 0.5 mM IPTG for 4h. Cells were harvested by centrifugation (5 000 ***g***, 10 min, 4 °C) and membrane vesicles were prepared as described previously [14]. Overexpression and purification of the PhoP variant harbouring a C-terminal His tag was conducted as described previously [14].

Solubilization and purification of $PhoQ_{His}$

$PhoQ_{His}$-containing membranes were solubilized with 0.1 % (v/v) Triton X-100, 0.1 and 1 % (w/v) DM, 0.1 and 1 % (w/v) DDM, or 0.1 % (v/v) LDAO. Membrane vesicles were mixed with the detergent and incubated on ice for 1hr. Insoluble material was removed by centrifugation (13 000 ***g***, 30 min, 4 °C) and the supernatant was used for SDS/PAGE analysis and for *in vitro* global activity assays as described below. For purification of the $PhoQ_{His}$ protein, $PhoQ_{His}$-containing membranes were solubilized with 1 % (w/v) DM as described above. Following centrifugation, the supernatant was loaded onto a 1 ml HiTrap chelating column (Amersham Biosciences) equilibrated in loading buffer (20 mM Tris/HCl, pH 7.9, 500 mM NaCl, 50 mM imidazole, 0.1 % DM). The column was extensively washed with loading buffer and eluted by applying a 50 mM to 1 M imidazole gradient in loading buffer. Fractions containing $PhoQ_{His}$ were pooled and dialyzed extensively against 20 mM Tris/HCl, pH 7.9, 50 mM NaCl, 10 % (v/v) glycerol, and 0.1 % DM. The concentration of purified $PhoQ_{His}$ was measured using the BCA protein assay (Pierce) with dilutions of BSA as standards.

Reconstitution of $PhoQ_{His}$ into liposomes

E. coli phospholipids (*E. coli* polar lipid extract, acetone/ether washed) were purchased from Avanti Polar Lipids (Alabaster, AL). Liposomes for reconstitution

were prepared as described in Racher et al. [16] using a buffer of 50 mM Tris/HCl, pH 8.0, 10 mM BME and stored at - 70 °C until needed. Triton X-100, DDM, DM and LDAO at various concentrations were initially tested for their suitability to reconstitute $PhoQ_{His}$. $PhoQ_{His}$ reconstitution was performed essentially as described for the *E. coli* ProP and PutP transporters [16, 17]. Liposomes were thawed at room temperature, extruded 19 times through a 400 nm polycarbonate filter and diluted to 5 mg/mL with phosphorylation buffer (50 mM Tris/HCl, pH 7.5, 200 mM KCl, 0.1 mM EDTA, 5 % (v/v) glycerol). Liposomes were destabilized by the addition of 0.2 % (w/v) DDM and the sample was incubated for 30 min at 22 °C with agitation. The purified $PhoQ_{His}$ protein was added at a ratio of 80:1 (lipid: protein) (w/w), and the sample was incubated at 22 °C with agitation for another 30 min. Detergents were removed by adding Bio-Beads SM-2 (Bio-Rad) at a wet weight bead:detergent ratio of 10:1 (w/w). The sample was incubated at 22 °C for 1h with agitation, and a second aliquot of Bio-Beads SM-2 was added. The sample was then incubated with agitation at 4 °C overnight. The proteoliposome solution was removed from the Bio-Beads SM-2 and centrifuged at 470 000 ***g*** for 30 min at 4 °C. The pellet was resuspended in phosphorylation buffer and used immediately. The concentration of $PhoQ_{His}$ in proteoliposomes was determined by SDS/PAGE using known concentrations of the purified protein. Urea extraction was performed as described previously [18], by incubating proteoliposomes with 6.0 M urea at 4 °C for 30 min. Following centrifugation (470 000 ***g***, 30 min, 4 °C), the supernatant and the resuspended pellet were analyzed by SDS/PAGE.

$PhoQ_{His}$ orientation in proteoliposomes

Orientation of $PhoQ_{His}$ was determined through the use of membrane permeable and impermeable thiol-reactive reagents. Fluorescent reagents were purchased from Molecular Probes (Eugene, OR). Proteoliposomes (approximately 1.5 μM of $PhoQ_{His}$) were subjected to one of the following treatments with an incubation of 10 min at 22 °C between reagent additions. For the determination of maximal labelling, proteoliposomes were solubilized with 1 % (w/v) DM and subsequently labelled with

0.33 mM 5-IAF. To determine the percentage of $PhoQ_{His}$ molecules with the catalytic domain extraluminal, proteoliposomes were incubated with 0.33 mM 5-IAF to label externally exposed cysteines. To determine the percentage of $PhoQ_{His}$ molecules with the catalytic domain intraluminal, proteoliposomes were incubated with 0.33 mM AmdiS to block externally exposed cysteines, solubilized with 1 % (w/v) DM and then incubated with 0.33 mM 5-IAF to label solely internally exposed cysteine residues. To control for unspecific labelling and fluorescence, proteoliposomes were incubated with 10 mM NEM, a non-fluorescent membrane permeable thiol-reactive reagent, and then incubated with 0.33 mM 5-IAF. All reactions were stopped by the addition of 4 × Laemmli loading buffer (250 mM Tris/HCl, pH 6.8, 8 % (w/v) SDS, 40 % (v/v) glycerol, 0.02 % bromophenol blue, 4 % (v/v) BME) and analyzed by SDS/PAGE. Fluorescence of proteins labeled with 5-IAF was visualized using an FX Scanner (Bio-Rad) using the internal 532 nm laser for excitation and the 555 nm Longpass filter for emission.

Proteoliposome integrity

Proteoliposome integrity was assessed by determining the extent of leakage of β-galactosidase trapped within liposomes or proteoliposomes. Proteoliposomes were prepared as described above and preloaded with 7.5 units β-galactosidase. Following centrifugation, proteoliposomes were resuspended in phosphorylation buffer or phosphorylation buffer supplemented with 10 mM $MgCl_2$, $CaCl_2$ or $MnCl_2$ and incubated at 22 °C for up to 150 min. Samples were then centrifuged at 470 000 ***g*** for 30 min at 4 °C and the resulting supernatants and resuspended pellets were added to an equal volume of Reporter Lysis Buffer (Promega). β-galactosidase activity was measured using ONPG as the substrate.

***In vitro* global activity assays**

The net phosphorylation of PhoP was measured by incubating intact PhoQ or $PhoQ_{His}$-containing membranes, solubilized $PhoQ_{His}$-containing membranes or

proteoliposomes (all approximately 1.5 μM of PhoQ or $PhoQ_{His}$) with an 8-fold molar excess of PhoP in a 15-μl volume of phosphorylation buffer supplemented with 5 mM $MgCl_2$. Reactions were initiated by the addition of 0.1 mM [γ-^{32}P] ATP (10 Ci/mmol), incubated at 22 °C for various time points and stopped by the addition of 4 × Laemmli loading buffer. Reaction products were heated at 37 °C for 3 min and applied to 10 % SDS/PAGE gels. Gels were dried under vacuum and exposed to a phosphor screen. Phosphorylated proteins were visualized using an FX Scanner and quantified by image analysis using the Quantity One software (Bio-Rad). All arbitrary units of intensity were converted to concentrations of [^{32}P]phosphorylated protein based on a standard curve.

***In vitro* autophosphorylation assays**

Proteoliposomes (approximately 1.5 μM $PhoQ_{His}$) were incubated with 0.1 mM [γ-^{32}P]ATP (10 Ci/mmol) in a 15-μl volume of phosphorylation buffer supplemented with 5 mM $MgCl_2$. The phosphorylation reactions were continued at 22 °C for various time points before being stopped by addition of 4 × Laemmli loading buffer. Phosphorylation products were analyzed by SDS/PAGE as described above.

***In vitro* phosphatase assays**

Purified [^{32}P]phospho-PhoP was prepared as previously described [14]. Radiolabelled phospho-PhoP (12 μM) was mixed with proteoliposomes (approximately 1.5 μM of $PhoQ_{His}$) preloaded with 5 mM of $MgCl_2$ in 15-μl volume of phosphorylation buffer supplemented with 5 mM $MgCl_2$ and various concentrations of ADP, GDP, AMP-PNP or GMP-PNP when appropriate. Stability of [^{32}P]phospho-PhoP in the presence of ADP or AMP-PNP was assessed as described above except that proteoliposomes were omitted from the reaction. After incubating at 22 °C for various times, reactions were stopped by the addition of 4 × Laemmli loading buffer. Phosphorylated products were analyzed by SDS/PAGE as described above.

RESULTS

Influence of the C-terminal His Tag on PhoQ activity

To facilitate purification of PhoQ, the *phoQ* gene was cloned into pET-20b(+) to yield plasmid pET-Q_{His}, which encodes the full-length PhoQ protein with six consecutive histidine residues at the C-terminus ($PhoQ_{His}$). We first examined the effect of the C-terminal His tag on PhoQ activity *in vivo* by measuring the expression of *mgtA* (a PhoP-activated gene that encodes a Mg^{2+} transporter) through the β-galactosidase activity of a *mgtA::lacZ* transcriptional fusion [15]. As shown in Figure 1A, the resulting levels of β-galactosidase activity were essentially similar for cells producing PhoQ and $PhoQ_{His}$ in the absence of $MgCl_2$. In the presence of 10 mM $MgCl_2$, β-galactosidase activity was reduced by 10-fold and 8-fold for cells producing PhoQ and $PhoQ_{His}$, respectively (Figure 1A). Thus, the C-terminal His tag does not grossly affect PhoQ activity and regulation by Mg^{2+}, *in vivo*.

To further compare PhoQ and $PhoQ_{His}$, we assessed the global activity of the PhoP/PhoQ system, *in vitro*, by measuring the net phosphorylation of PhoP resulting from both the autokinase and phosphatase activities of the PhoQ proteins. Membrane fractions containing either the PhoQ or $PhoQ_{His}$ proteins were incubated with an 8-fold molar excess of PhoP in the presence of [γ-^{32}P]ATP and 5 mM $MgCl_2$. Figure 1B shows the amounts of [^{32}P]phospho-PhoP resulting from the activity of PhoQ or $PhoQ_{His}$ after a 20 min incubation period. Levels of radiolabelled phospho-PhoP were increased by 2-fold when incubated with $PhoQ_{His}$ compared to PhoQ. Analysis of the individual activities showed that the phosphatase activity of $PhoQ_{His}$ is slightly reduced compared to that of PhoQ, while the autokinase activities of both PhoQ proteins are similar (results not shown). These *in vitro* data, consistent with the *in vivo* results shown in Figure 1A, show that the activity of $PhoQ_{His}$ is not severely affected by the C-terminal His tag. Thus, $PhoQ_{His}$ was used for further experimentation.

Catalytic activity of the solubilized and purified $PhoQ_{His}$

To purify $PhoQ_{His}$, optimal solubilization conditions were first identified. The detergents Triton X-100, LDAO, DM and DDM were used to solubilize membrane vesicles containing the overexpressed $PhoQ_{His}$ protein. All detergents tested were found to be 60 to 80 % efficient in solubilizing $PhoQ_{His}$ as compared to a non-solubilized control (Figure 2A). When the series of solubilized $PhoQ_{His}$ was assayed for *in vitro* global activity by incubating for 20 min with an 8-fold molar excess of PhoP in the presence of [γ-^{32}P]ATP and 5 mM $MgCl_2$, $PhoQ_{His}$ solubilized in 0.1 % LDAO, DDM or DM retained more than 50 % activity as compared to a non-solubilized control (Figure 2B). Although maximal activity was obtained with 0.1 % DDM (Figure 2B, lane 5), 0.1 % DM was deemed optimal for $PhoQ_{His}$ purification. $PhoQ_{His}$ was purified by Ni^{2+}-NTA chromatography (Figure 2C) and the identity of the purified protein confirmed by Western blotting using a monoclonal antibody directed against the His tag (results not shown). No activity of purified $PhoQ_{His}$ was detected in the *in vitro* global activity assay regardless of the detergent used (results not shown). Approximately 2.5 mg of purified $PhoQ_{His}$ was obtained from 50 mg of total membrane proteins.

Functional reconstitution of $PhoQ_{His}$ in proteoliposomes

Purified $PhoQ_{His}$ was reconstituted into detergent-destabilized liposomes prepared from *E. coli* phospholipids, based on the detergent-mediated method [19]. Purified $PhoQ_{His}$ in 0.1 % DM was added to liposomes destabilized with 0.2 % DDM and detergents were removed by adsorption to Bio-Beads SM-2. The efficiency of $PhoQ_{His}$ reconstitution was assessed by comparing solubilized proteoliposomes to an amount of purified $PhoQ_{His}$ representing 100 % incorporation on SDS/PAGE. The efficiency was determined to be approximately 86 %. Urea extraction was performed to show that the $PhoQ_{His}$ proteins are correctly inserted into liposomes. SDS/PAGE analysis of proteoliposomes treated with urea and subjected to centrifugation showed that about 80 % of the $PhoQ_{His}$ proteins are resistant to urea treatment (results not shown).

Orientation of $PhoQ_{His}$ in proteoliposomes

$PhoQ_{His}$ contains only two cysteine residues at positions 392 and 395 in the cytoplasmic catalytic domain. By exploiting the location of these cysteine residues, we determined the orientation of $PhoQ_{His}$ in proteoliposomes through the differential use of thiol-reactive probes. A control reaction representing 100 % labelling was determined by solubilizing proteoliposomes with 1 % DM and labelling cysteines with 5-IAF (Figure 3A, lane 1). The percentage of $PhoQ_{His}$ molecules with the catalytic domain facing the extraluminal environment was determined by labelling externally exposed cysteines with the membrane impermeable probe 5-IAF (Figure 3A, lane 2). These data suggest that nearly all $PhoQ_{His}$ proteins are oriented with the catalytic domain extraluminal. To confirm this, the percentage of $PhoQ_{His}$ molecules with the catalytic domain facing the intraluminal environment was determined by labelling externally exposed cysteines with the membrane impermeable thiol-reactive probe AMdiS, solubilizing proteoliposomes with 1 % DM and subsequently labelling the remaining (intraluminal) exposed cysteines with 5-IAF (Figure 3A, lane 3). Very little fluorescence attributable to 5-IAF was detected, suggesting that very few $PhoQ_{His}$ proteins are oriented with the catalytic domain intraluminal. Proteoliposomes labelled with the membrane permeable thiol-reactive probe NEM followed by 5-IAF showed little fluorescence, indicating specific labelling of thiol groups (Figure 3A, lane 4). Following the fluorescence experiments, SDS/PAGE gels were stained with Coomassie brilliant blue to confirm that similar amounts of proteins were used in all reactions (Figure 3B). Thus, the orientation of $PhoQ_{His}$ in proteoliposomes was found to be essentially unidirectional, with the catalytic domain extraluminal (Figure 3C). To confirm that the sensory domain of reconstituted $PhoQ_{His}$ is facing the lumen of proteoliposomes, we assessed the ability of $PhoQ_{His}$ to sense the intraluminal environment. Proteoliposomes were preloaded with the various divalent cations known to be sensed by the PhoQ sensory domain [8] and *in vitro* global activity assays were performed. The presence of 5 mM intraluminal $MgCl_2$, $CaCl_2$ or $MnCl_2$ inhibited the phosphorylation of PhoP compared to proteoliposomes preloaded with no divalent cations (S. Sanowar and H. Le Moual, unpublished work). Altogether,

these data provide strong evidences that $PhoQ_{His}$ in proteoliposomes is able to sense the intraluminal environment through its sensory domain and signal to the extraluminal catalytic domain (Figure 3C).

Proteoliposome integrity

To assess proteoliposome leakage, liposomes or proteoliposomes were preloaded with β-galactosidase and incubated for up to 150 min in buffer or buffer supplemented with 10 mM $MgCl_2$, $CaCl_2$ or $MnCl_2$. The amount of β-galactosidase that leaked from the vesicles was measured using the substrate ONPG. There was no detectable leakage of β-galactosidase from liposomes or proteoliposomes exposed to these divalent cations compared to a control exposed to buffer alone (results not shown). The amount of β-galactosidase activity released upon solubilization of the vesicles was similar for all samples. We concluded that $MgCl_2$, $CaCl_2$ or $MnCl_2$ at a concentration of up to 10 mM do not disrupt the membrane permeability barrier.

***In vitro* autokinase activity of reconstituted $PhoQ_{His}$**

In the *in vitro* autokinase assay, we measured the amount of phospho-$PhoQ_{His}$ generated at various time points. To maximize the $PhoQ_{His}$ autokinase activity, proteoliposomes were preloaded with phosphorylation buffer devoid of $MgCl_2$ and incubated in the presence of extraluminal [γ-^{32}P]ATP and 5 mM $MgCl_2$. Over time we observed a slow and sustained net accumulation of [^{32}P]phospho-$PhoQ_{His}$ that reached an apparent steady state level after 120 min (Figure 4). These data demonstrate that reconstituted $PhoQ_{His}$ is capable of autokinase activity.

***In vitro* global activity of reconstituted $PhoQ_{His}$**

To examine the kinetics of net phosphorylation of PhoP by reconstituted $PhoQ_{His}$, proteoliposomes preloaded with phosphorylation buffer devoid of $MgCl_2$ were incubated with an 8-fold excess of PhoP in the presence of [γ-^{32}P]ATP and 5 mM $MgCl_2$. Over time, we observed a slow and sustained net accumulation of [^{32}P]phospho-PhoP that reached an apparent steady state after a 120 min incubation

(Figure 5). Thus, the phosphoryl group can be transferred from reconstituted $PhoQ_{His}$ to PhoP. As expected from the molar excess of PhoP used in the assay, higher levels of [^{32}P]phospho-PhoP were obtained compared to levels of [^{32}P]phospho-$PhoQ_{His}$ (Figures 4 and 5).

In vitro phosphatase activity of reconstituted $PhoQ_{His}$ is stimulated by nucleotides

To assess the phosphatase activity of reconstituted $PhoQ_{His}$, we incubated purified [^{32}P]phospho-PhoP with proteoliposomes preloaded with phosphorylation buffer supplemented with 5mM $MgCl_2$. Aliquots were removed at different time points and the time course of the reaction was followed. A control reaction in which [^{32}P]phospho-PhoP was incubated with liposomes lacking $PhoQ_{His}$ indicated the intrinsic stability of [^{32}P]phospho-PhoP. As shown in Figure 6A, reconstituted $PhoQ_{His}$ dephosphorylated only 20% of [^{32}P]phospho-PhoP after 60 min. Thus, under these experimental conditions reconstituted $PhoQ_{His}$ had little phosphatase activity. ADP and the nonhydrolyzable ATP analogue, AMP-PNP, have been shown to stimulate the phosphatase activity of the EnvZ and NRII kinase sensors [20-22]. To assess the effect of nucleotides on the phosphatase activity of reconstituted $PhoQ_{His}$, we conducted phosphatase assays in the presence of various concentrations of extraluminal ADP, AMP-PNP, GDP or GMP-PNP. As shown in Figure 6B, levels of [^{32}P]phospho-PhoP decreased when assays were performed in the presence of either ADP or AMP-PNP. In contrast, the presence of GDP or GMP-PNP had no effect on the levels of [^{32}P]phospho-PhoP (Figure 6B). To assess whether ADP and AMP-PNP act on reconstituted $PhoQ_{His}$ or directly on phospho-PhoP, we tested the stability of [^{32}P]phospho-PhoP in the presence of 1 mM of these nucleotides. As shown in Figure 6C, AMP-PNP stimulated the dephosphorylation of [^{32}P]phospho-PhoP in a $PhoQ_{His}$-independent manner. In contrast, ADP had no major effect on the stability of [^{32}P]phospho-PhoP (Figure 6C), suggesting that it promotes the dephosphorylation of phospho-PhoP by stimulating the phosphatase activity of reconstituted $PhoQ_{His}$. As shown in Figure 6A, ADP at a concentration of 1 mM increased [^{32}P]phospho-PhoP

dephosphorylation to 70% after 45 min. These data suggest that ADP affects the conformation of the $PhoQ_{His}$ cytoplasmic domain.

DISCUSSION

The PhoP/PhoQ two-component system of *S. typhimurium* controls the expression of many genes, including virulence factors, in response to depletion of environmental Mg^{2+} [8, 23, 24]. To date, the catalytic activities of the PhoQ kinase sensor have been characterized, *in vitro*, using membrane preparations in which PhoQ is overproduced [10, 11, 14]. In this study, we developed a purified and reconstituted system to study transmembrane signalling by PhoQ. $PhoQ_{His}$ was purified and reconstituted into *E. coli* phospholipids. Under our experimental conditions, insertion of $PhoQ_{His}$ into phospholipid vesicles was unidirectional with the sensory domain facing the lumen. We found that reconstituted $PhoQ_{His}$ is functional and catalyzes autophosphorylation, the transfer of the phosphoryl group to PhoP and the dephosphorylation of phospho-PhoP.

To facilitate purification, we used a PhoQ variant with a C-terminal His tag ($PhoQ_{His}$). The possibility that the tag severely affects the PhoQ catalytic activities is most unlikely, since both the tagged and non-tagged proteins show essentially similar activity *in vivo* and *in vitro* (Figure 1) as well as Mg^{2+} regulation, *in vivo* (Figure 1A). To date the few kinase sensors that have been studied in a reconstituted system are KdpD, EnvZ and DcuS of *E. coli* [25-27]. In contrast to the solubilized KdpD and DcuS sensor kinases [25, 27], $PhoQ_{His}$ retained the ability to autophosphorylate and transfer its phosphoryl group to $PhoP_{His}$ upon solubilization (Figure 2B). Similar results to $PhoQ_{His}$ were obtained with solubilized EnvZ [26]. These differences may be due to the detergent used in the solubilization procedures. Following purification using Ni^{2+}-NTA affinity chromatography, $PhoQ_{His}$ was inactive (results not shown), the reason for which is unclear. It is possible that purified $PhoQ_{His}$ lost its dimeric structure that is crucial for *trans*-autophosphorylation, which occurs between two PhoQ monomers [28, 29]. Another possibility is that $PhoQ_{His}$ necessitates lipid components for activity, which would be retained upon solubilization but washed away during purification. Importantly, $PhoQ_{His}$ regained its ability to

autophosphorylate and transfer its phosphoryl group to PhoP, once reconstituted into proteoliposomes (Figures 4 and 5). These results suggest that a lipid environment is necessary to provide the appropriate structural arrangement required for $PhoQ_{His}$ activity.

Although reconstituted $PhoQ_{His}$ exhibited all catalytic activities that are the hallmark of histidine kinase sensors, it showed significant differences compared with PhoQ or $PhoQ_{His}$ overproduced in bacterial membranes [10, 11, 14]. Reconstituted $PhoQ_{His}$ autophosphorylates slowly and the phospholinkage appears to be stable for at least 150 min (Figure 4). In contrast, kinetics of autophosphorylation of PhoQ overproduced in *E. coli* membranes were strongly biphasic, with a rapid phosphorylation phase followed by a slower dephosphorylation phase [10]. This difference in the stability of phospho-PhoQ between the two systems can be explained in two ways. Firstly, interaction of $MgCl_2$ (5 mM) with the periplasmic sensory domain of PhoQ in *E. coli* membranes may impose on PhoQ a conformation that promotes its dephosphorylation. The absence of $MgCl_2$ in the lumen of proteoliposomes would prevent reconstituted $PhoQ_{His}$ from adopting such a conformation, leading to phospholinkage stability. Secondly, dephosphorylation of overproduced PhoQ may be due to a Mg^{2+}-dependent phosphatase present in *E. coli* membranes but absent in the purified and reconstituted system. In cells, response regulator molecules appear to exist in excess over kinase sensor molecules [30]. By using an 8-fold molar excess of PhoP with respect to $PhoQ_{His}$ in proteoliposomes, a 2-fold amplification of the signal was observed as shown by the difference between the levels of [^{32}P]phospho-$PhoQ_{His}$ and [^{32}P]phospho-PhoP obtained at 150 min (Figures 4 and 5). Amplification of the signal to higher extents may be limited by the intrinsic autophosphatase activity of PhoP observed *in vitro* [11]. Indeed, we found that the half-life of [^{32}P]phospho-PhoP is approximately 80 min in the presence of 5 mM $MgCl_2$ (Figure 6A). Kinetics obtained for PhoP phosphorylation (Figure 5) and PhoQ autophosphorylation (Figure 4) are consistent with the fact that transfer of the phosphoryl group to the response regulator is fast compared to the

autophosphorylation of kinase sensors, which constitutes the rate-limiting step of the phosphorylation cascade [3].

We found that reconstituted $PhoQ_{His}$ has little phosphatase activity in the absence of ADP (Figure 6A). In contrast, PhoQ or $PhoQ_{His}$ overproduced in *E. coli* membranes were able to completely dephosphorylate [^{32}P]phospho-PhoP without additional ADP, keeping all other experimental conditions similar [10, 14]. This highlights another major difference between the reconstituted system and membrane preparations in which PhoQ is overexpressed. One possibility is that $PhoQ_{His}$ in proteoliposomes cannot adopt a phosphatase-dominant conformation in the absence of ADP, even though $MgCl_2$ is present at a concentration of 5 mM in the lumen of proteoliposomes. We found that ADP and AMP-PNP, but not by GDP and GMP-PNP, increased the dephosphorylation of phospho-PhoP possibly by stimulating the phosphatase activity of reconstituted $PhoQ_{His}$ (Figure 6B). Strikingly, we found that AMP-PNP directly affects the stability of phospho-PhoP (Figure 6C), the mechanism by which is still unclear. In contrast, ADP did not affect phospho-PhoP stability (Figure 6C). Thus, we conclude that ADP stimulates the phosphatase activity of reconstituted $PhoQ_{His}$ by interacting with the catalytic domain. It has been proposed that signals transduced across the membrane alter the spatial arrangement between the ATP-binding domain and the central dimerization domain of sensor kinases [6, 31]. Our data suggest that in the absence of ADP, these domains cannot adopt proper positioning for maximal phosphatase activity. Binding of ADP to the ATP-binding domain of reconstituted $PhoQ_{His}$ appears to provide a conformation capable of enhanced phosphatase activity (Figure 6A). Altogether, these results indicate that reconstituted $PhoQ_{His}$ is not locked in a kinase-dominant conformation and can switch to a phosphatase-dominant conformation in the presence of ADP.

This *in vitro* reconstituted PhoP/PhoQ system will allow us to systematically vary the concentration of intraluminal divalent cations acting as ligands (Mg^{2+}, Ca^{2+} and Mn^{2+}), while maintaining a constant concentration of extraluminal catalytic Mg^{2+}.

Future studies will clarify whether both the autokinase and phosphatase activities of PhoQ are targets for regulation by divalent cations, as suggested previously for Mg^{2+} [10]. In addition, this system will aid in elucidating the molecular mechanism of signal transduction across the cell membrane.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1 ***In vivo*** **and** ***in vitro*** **activity of the PhoQ and $PhoQ_{His}$ kinase sensors**

(**A**) *In vivo* assays. Transcription of the *mgtA* gene (a PhoP-activated gene) from the *E. coli* MG1607 strain carrying plasmids pPRO (control plasmid), pPRO-Q (PhoQ) or pPRO-Q_{His} ($PhoQ_{His}$). Cells were grown in LB medium or LB medium supplemented with 10 mM $MgCl_2$. β-galactosidase activity from the *mgtA::lacZ* transcriptional fusion was measured as described in the Experimental section. Data are the mean of triplicate values with standard deviations. One unit of β-galactosidase hydrolyzes 1 μmole of ONPG/min at pH 7.5 and 37 °C. (**B**) *In vitro* global activity of the PhoP/PhoQ and PhoP/$PhoQ_{His}$ systems. The net phosphorylation of PhoP by PhoQ or $PhoQ_{His}$ present in membrane fractions was measured. Membrane fractions from *E. coli* cells transformed with the empty plasmid or plasmids encoding either PhoQ or $PhoQ_{His}$ (approximately 1.5 μM) were incubated with an 8-fold molar excess of PhoP in phosphorylation buffer supplemented with 0.1 mM [γ-^{32}P]ATP and 5 mM $MgCl_2$. After 20 min of incubation at 22 °C, reactions were stopped by the addition of 4 × Laemmli loading buffer and analyzed by SDS/PAGE.

Figure 2 **Solubilization and purification of $PhoQ_{His}$**

(**A**) Solubilization of $PhoQ_{His}$. Membrane fractions containing the overexpressed $PhoQ_{His}$ protein were solubilized in 20 mM Tris/HCl, pH 7.5, 10 % (v/v) glycerol and detergent. Lane 1, non-solubilized membranes; lane 2, 0.1 % (v/v) Triton X-100; lane 3, 0.1 % (v/v) LDAO; lane 4, 1 % (w/v) DDM; lane 5, 0.1 % (w/v) DDM; lane 6, 1 % (w/v) DM; lane 7, 0.1 % (w/v) DM. Following a one hour incubation on ice, insoluble material was removed by centrifugation and the supernatants were analyzed by SDS/PAGE. (**B**) Net phosphorylation of PhoP by solubilized $PhoQ_{His}$. *In vitro* global activity assays were conducted as indicated in the Figure 1 legend. Lanes are as in

(A). (**C**) SDS/PAGE analysis of $PhoQ_{His}$ purification. The gel was stained with Coomassie blue. Lane 1, control membrane fractions from cells transformed with the empty plasmid; lane 2, membrane fractions from cells overexpressing $PhoQ_{His}$; lane 3, purified $PhoQ_{His}$ (approximately 4 μg). The molecular weight markers are shown on the left.

Figure 3 Orientation of $PhoQ_{His}$ in proteoliposomes

(**A**) $PhoQ_{His}$-proteoliposomes (1.5 μM $PhoQ_{His}$) were solubilized with 1 % DM and/or incubated with various thiol-reactive probes. Reactions were stopped by the addition of 4 × Laemmli loading buffer and analyzed by SDS/PAGE. Fluorescence of $PhoQ_{His}$ labelled with 5-IAF was visualized as described in the Experimental section. Lane 1, proteoliposomes were solubilized with 1 % DM and labelled with 5-IAF; lane 2, proteoliposomes were labelled with 5-IAF; lane 3, proteoliposomes were treated with AmdiS, solubilized with 1 % DM and labelled with 5-IAF; lane 4, proteoliposomes were treated with NEM and labelled with 5-IAF. (**B**) SDS/PAGE analysis of $PhoQ_{His}$-proteoliposomes used in (A). (**C**) Schematic representation of the topology of $PhoQ_{His}$ in proteoliposomes. The catalytic domain of $PhoQ_{His}$ (Cat) is facing the extraluminal environment. The two cysteines, which are part of the catalytic domain, are indicated as C.

Figure 4 Time course of reconstituted $PhoQ_{His}$ autophosphorylation

$PhoQ_{His}$-proteoliposomes (1.5 μM $PhoQ_{His}$) preloaded with phosphorylation buffer devoid of $MgCl_2$ were incubated at 22 °C in phosphorylation buffer supplemented with 0.1 mM [γ-^{32}P]ATP and 5 mM $MgCl_2$. At the indicated time points, reactions were stopped by the addition of 4 × Laemmli loading buffer and analyzed by SDS/PAGE. The amounts of [^{32}P]phospho-$PhoQ_{His}$ were determined with a phosphorimager.

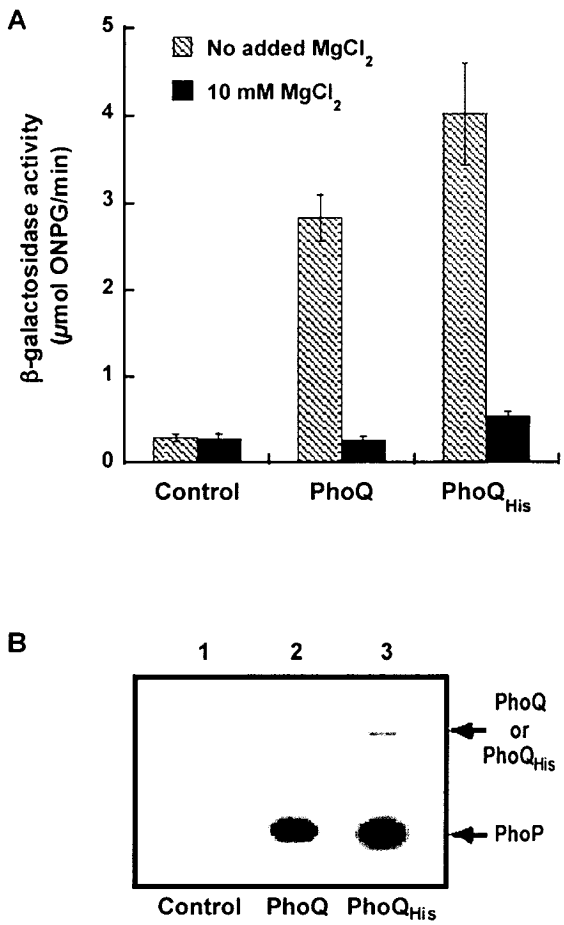
Figure 5 Time course of net phosphorylation of PhoP by reconstituted $PhoQ_{His}$

$PhoQ_{His}$-proteoliposomes (1.5 μM $PhoQ_{His}$) preloaded with phosphorylation buffer devoid of $MgCl_2$ were incubated at 22 °C with an 8-fold molar excess of PhoP in phosphorylation buffer supplemented with 0.1 mM [γ-^{32}P]ATP and 5 mM $MgCl_2$. At time points, reactions were stopped by the addition of 4 × Laemmli loading buffer and analyzed by SDS/PAGE. The amounts of [^{32}P]phospho-PhoP were determined with a phosphorimager.

Figure 6 *In vitro* phosphatase activity of $PhoQ_{His}$ in proteoliposomes

(**A**) Time course of reconstituted $PhoQ_{His}$ phosphatase activity. $PhoQ_{His}$-proteoliposomes were preloaded with phosphorylation buffer supplemented with 5 mM $MgCl_2$. *In vitro* phosphatase assays were performed as described in the Experimental section with phosphorylation buffer supplemented with 5 mM $MgCl_2$ and nucleotides when indicated. Reaction products were analyzed by SDS/PAGE. The amounts of [^{32}P]phospho-PhoP remaining were determined with a phosphorimager. Intrinsic dephosphorylation of [^{32}P]phospho-PhoP (○), dephosphorylation of [^{32}P]phospho-PhoP by reconstituted $PhoQ_{His}$ in the absence (●) or in the presence of 1 mM extraluminal ADP (■). (**B**) Effect of extraluminal nucleotides on the dephosphorylation of [^{32}P]phospho-PhoP. *In vitro* phosphatase assays were performed for 20 min as described in (A) with phosphorylation buffer supplemented with 5 mM $MgCl_2$ and GDP, GMP-PNP, ADP or AMP-PNP at the concentrations indicated. (**C**) Effect of ADP and AMP-PNP on the stability of [^{32}P]phospho-PhoP. Intrinsic dephosphorylation of [^{32}P]phospho-PhoP in the presence of phosphorylation buffer (○),phosphorylation buffer supplemented with 1 mM ADP (■) or phosphorylation buffer supplemented with 1 mM AMP-PNP (●).

Figure 1: Sanowar and Le Moual



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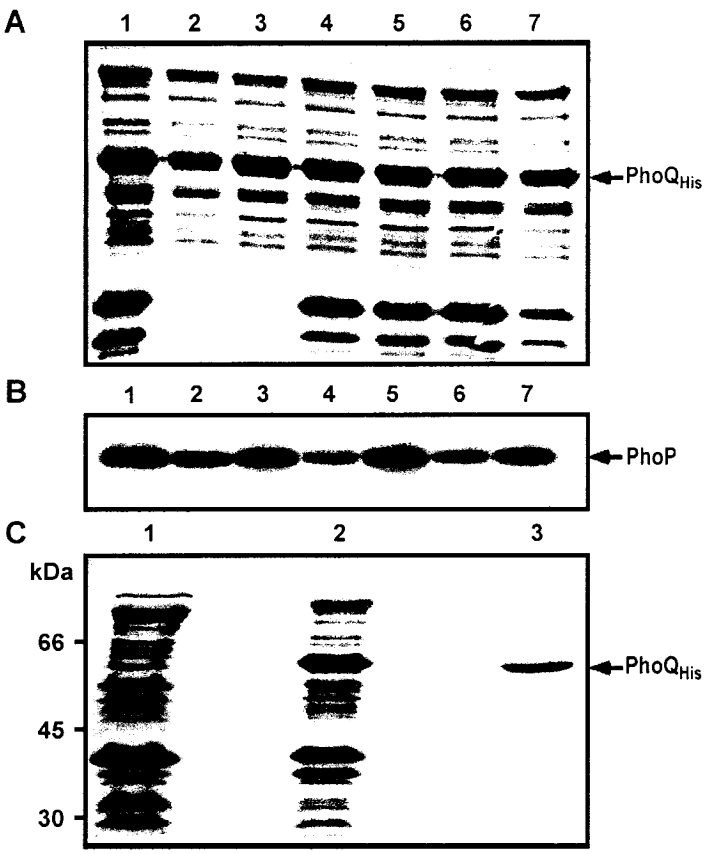

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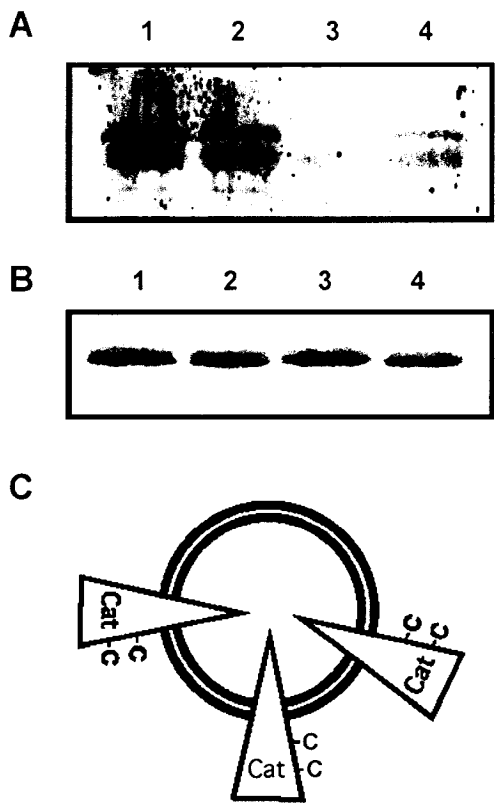

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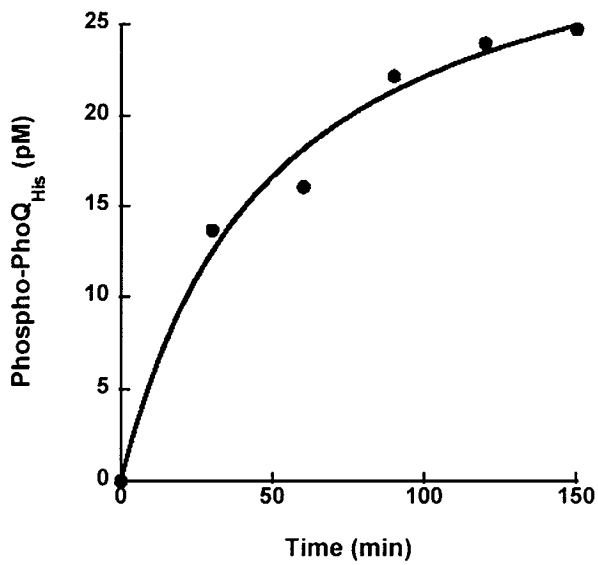

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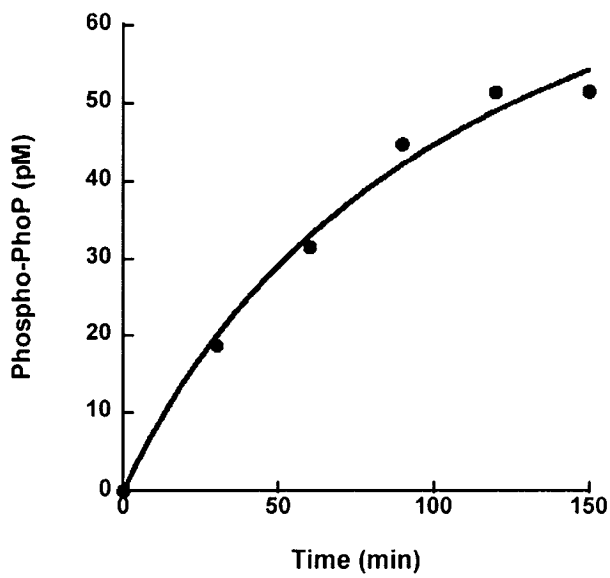
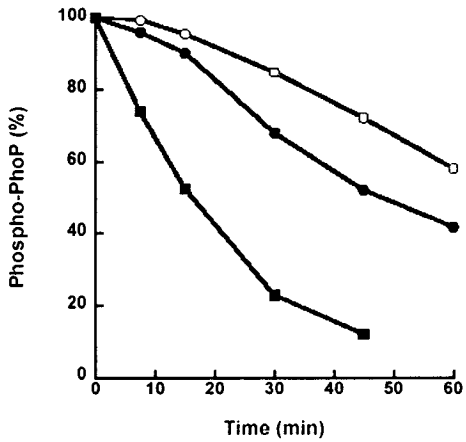


Figure 6: Sanowar and Le Moual

A



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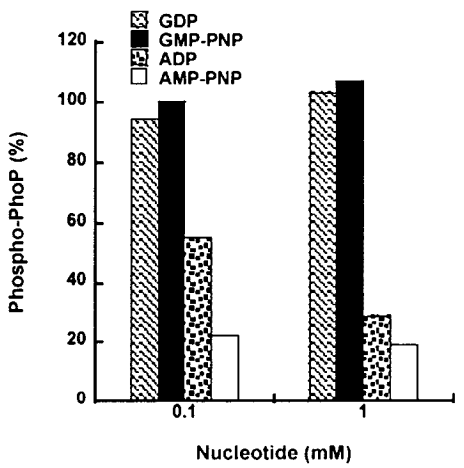

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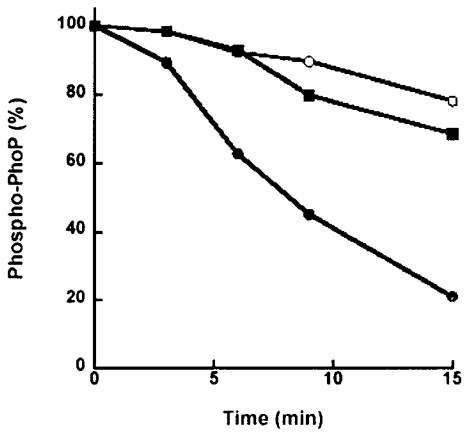

PREFACE TO CHAPTER 4

This study is a direct continuation of Chapter 3 where we reconstituted the PhoQ histidine kinase sensor. Here we used the *in vitro* reconstituted PhoP/PhoQ system to systematically compare the effects of intraluminal divalent cations acting as ligands. We show that high concentrations (5 mM) of Mn^{2+}, and to a lesser extent Ca^{2+}, are more potent than Mg^{2+} at repressing PhoP/PhoQ signal transduction *in vivo* and *in vitro*. Physiological concentrations of these divalent cations are capable of repressing the PhoP/PhoQ system to similar extents. These findings suggest that Mn^{2+} and Ca^{2+} may be as relevant as Mg^{2+} for *Salmonella* survival within macrophages.

CHAPTER 4:

Divalent Cation-mediated Repression of the *Salmonella enterica* Serovar Typhimurium PhoQ Sensor Kinase in Proteoliposomes

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The PhoP/PhoQ two-component regulatory system of *Salmonella typhimurium* plays a central role in controlling virulence by coordinating entry into epithelial cells, survival within macrophages and resistance to antimicrobial peptides. Here, we showed that 5 mM concentrations of Mn^{2+}, and to a lesser extent Ca^{2+}, are more potent than Mg^{2+} at repressing PhoP/PhoQ signal transduction *in vivo* and *in vitro*. More physiological concentrations of these divalent cations are capable of repressing the PhoP/PhoQ system to similar extents. These findings suggest that Mn^{2+} and Ca^{2+} may be as relevant as Mg^{2+} for *Salmonella* survival within macrophages.

Salmonella enterica serovar Typhimurium (*S. typhimurium*) encodes a number of two-component regulatory systems that are critical for intestinal epithelial invasion and intramacrophage survival. Loss of the BarA/SirA two-component system greatly reduces invasion across the intestinal epithelium (Ahmer *et al.*, 1999; Altier *et al.*, 2000). The OmpR/EnvZ system is required for *Salmonella* replication and survival within macrophages, and regulates the expression of the SsrA/SsrB system involved in intracellular bacterial replication (Lee *et al.*, 2000). The PhoP/PhoQ system is required for survival within macrophages and also regulates the PmrA/PmrB two-component system. The PhoP/PhoQ system control the expression numerous genes required for virulence, growth at low Mg^{2+} concentrations, LPS modifications and resistance to antimicrobial peptides (Ernst *et al.*, 2001; Groisman, 2001; Gunn *et al.*, 1998).

The *Salmonella* PhoP/PhoQ is composed of the PhoQ transmembrane histidine kinase sensor that detects the environmental signal and the PhoP cytoplasmic response regulator that elicits the cellular response through transcriptional regulation. This system is activated *in vivo* within acidified macrophage phagosomes and is repressed during bacterial growth in high concentrations of the divalent cations Mg^{2+}, Mn^{2+} and Ca^{2+} (Garcia-Vescovi *et al.*, 1996). Other divalent cations, including Ni^{2+}, Cu^{2+}, and Ba^{2+}, have no effect on PhoQ/PhoP-dependent gene expression *in vitro* (Garcia-Vescovi *et al.*, 1996). It has been proposed that Mg^{2+} acts as the physiologically relevant signal controlling the PhoP/PhoQ system (Groisman, 1998). In addition, the PhoP/PhoQ system has been shown to be activated by cationic antimicrobial peptides that are suggested to compete directly with Mg^{2+} for binding to PhoQ (Bader *et al.*, 2003; 2005) Upon macrophage uptake, *Salmonella* cells reside in modified phagolysosomes termed *Salmonella*-containing vacuoles (SCVs) (Garcìa-del Portillo, 2001). Although concentrations of divalent cations within SCVs are largely unknown, divalent cation transport is believed to be at the interface of host-pathogen interactions. The eukaryotic divalent cation transporter Nramp1 acts at the SCV membrane to export Mn^{2+} with high affinity and Fe^{2+} with a lower affinity from the SCV lumen to the host

cytoplasm (Forbes and Gros, 2001). The *Salmonella* Nramp1 homolog, MntH, imports Mn^{2+} from the SCV lumen to the bacterial cytoplasm (Kehres *et al.*, 2000; Boyer *et al.*, 2002). An additional *Salmonella* transporter, SitABCD, transports Mn^{2+} at slightly alkaline pH (Kehres *et al.*, 2002). Since the PhoP/PhoQ system is active at low cation concentrations *in vitro* and fully induced during bacterial replication in macrophages, it has been proposed that these conditions exist within the bacterial phagosome and function as a signal for PhoQ activation. In this study we explored the effect of the divalent cations Mg^{2+}, Mn^{2+} and Ca^{2+} on PhoP/PhoQ-mediated signaling. We show that physiological concentrations of these divalent cations repress the net phosphorylation of PhoP *in vivo* and *in vitro* and that maximal activation of PhoQ can only be achieved by an environment that is depleted in Mg^{2+}, Mn^{2+} and Ca^{2+}.

Divalent cations repress PhoP/PhoQ-regulated gene expression. The ability of different divalent cations to modulate transcription of PhoP-dependent genes has been previously demonstrated with Mg^{2+} being less effective than Ca^{2+} (Garcia-Vescovi *et al.*, 1996). In addition, it was reported that Mn^{2+} could replace Mg^{2+} however it is unclear at what concentrations (Garcia-Vescovi *et al.*, 1996). We examined divalent cation repression by measuring the expression of *mgtA* (a PhoP-activated gene that encodes a Mg^{2+} transporter) by following the β-galactosidase activity of the *mgtA::lacZ* transcriptional fusion (Kato *et al.*, 1999). *E. coli* MG1607 cells (a *phoQ* defective strain; Kato *et al.*, 1999) were transformed with plasmid pPRO-Q_{His} encoding the *S. typhimurium* PhoQ protein and grown in the presence or absence of various divalent cations and assayed as described previously (Sanowar *et al.*, 2003; Sanowar and Le Moual, 2005). It has previously been shown that a C-terminal His tag does not grossly affect PhoQ activity and regulation by Mg^{2+} *in vivo* and *in vitro* (Sanowar and Le Moual, 2005), therefore this study uses C-terminal His tagged-PhoQ for experimentation. In agreement with previous reports, (Garcia-Vescovi *et al.*, 1996), β-galactosidase activity of cells producing wild-type $PhoQ_{His}$ was decreased in the presence of certain divalent cations, with a 3-fold decrease when LB was supplemented with 5 mM $MgCl_2$, a 5-fold decrease when supplemented with 5 mM

$CaCl_2$, and a 8-fold decrease when supplemented with $MnCl_2$ (Fig. 1A). These results indicate that expression of *mgtA* was repressed to a greater extent by Mn^{2+}, than Ca^{2+} and Mg^{2+} at the same concentration. If one considers more physiological relevant concentrations of these divalent cations, namely 5 mM, 1 mM and 0.1 mM $MgCl_2$, $CaCl_2$ and $MnCl_2$, respectively, essentially similar extents of repression of *mgtA* were observed, with a 2-fold decrease measured for Mg^{2+} and a 1.5-fold decrease measured for both Ca^{2+} and Mn^{2+} (Fig. 1B). These results suggest that all three divalent cations are capable of repressing the PhoP/PhoQ system at physiological concentrations.

Divalent cations decrease PhoQ-mediated signaling *in vitro*. In the PhoP/PhoQ signal transduction cascade, divalent cations regulate the activity of the system by acting as a ligand for the periplasmic domain of the PhoQ sensor kinase. Moreover, Mg^{2+} is also required in the cytoplasmic catalytic domain for activity. We have developed a reconstituted system that allows for independent manipulation of the intraluminal and extraluminal Mg^{2+} concentrations to measure reconstituted $PhoQ_{His}$ activity in response to intraluminal ligands (Sanowar and Le Moual, 2005; Bader *et al.*, 2005). Here, we examined the ability of intraluminal divalent cations to regulate reconstituted $PhoQ_{His}$ activity by preloading vesicles with various concentrations of $MgCl_2$, $CaCl_2$ or $MnCl_2$ and measuring the subsequent phosphorylation of purified PhoP after 20 min while keeping the extraluminal $MgCl_2$ concentration at 5 mM. When $PhoQ_{His}$-containing vesicles were preloaded with 5 mM $MgCl_2$, $CaCl_2$ or $MnCl_2$, the amount of net phosphorylated PhoP decreased 1.4-fold, 2.7-fold and 21.3-fold, respectively, compared to in the absence of intraluminal divalent cation (Fig. 2A). Thus, these data indicate that Mn^{2+}, and to a lesser extent Ca^{2+}, are more potent than Mg^{2+} at repressing the activity of the PhoP/PhoQ system, *in vitro*. At physiological intraluminal divalent cation concentrations (5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM $MnCl_2$), the amount of net phosphorylated PhoP decreased 1.2-fold, 1.7-fold, and 2.8-fold for Mg^{2+}, Ca^{2+} and Mn^{2+}, respectively (Fig. 2B). These results suggest that the PhoQ protein responds to the dynamic range of physiological Mg^{2+}, Ca^{2+}, and Mn^{2+}.

Divalent cations do not disrupt proteoliposome integrity. Proteoliposome integrity was monitored by determining the extent of leakage of β-galactosidase and [γ-^{32}P]ATP. Liposomes or proteoliposomes were preloaded with β-galactosidase and incubated for 180 min in buffer or buffer supplemented with 10 mM $MgCl_2$, $CaCl_2$ or $MnCl_2$ as previously described (Sanowar and Le Moual, 2005). The amount of β-galactosidase that leaked from the vesicles was measured using the substrate ONPG. There was no detectable leakage of β-galactosidase from liposomes or proteoliposomes exposed to these divalent cations compared to a control exposed to buffer alone (Fig. 3A). The amount of β-galactosidase activity released upon solubilization of the vesicles with detergent was similar for all samples. We concluded that $MgCl_2$, $CaCl_2$ or $MnCl_2$ at a concentration of up to 10 mM do not disrupt the membrane permeability barrier. Additional tests were conducted using [γ-^{32}P]ATP. Liposomes were prepared and exposed to 10 mM Mg^{2+}, Ca^{2+}, Mn^{2+} or 0.1 mM Mn^{2+} and a trace amount of [γ-^{32}P]ATP (0.04 Ci/mmol) for 180 min at 22 °C. Samples were then centrifuged at 470 000 ***g*** for 30 min at 4 °C and the radioactivity present in the resulting supernatants and resuspended pellets was followed with a scintillation counter. There was no detectable leakage of [γ-^{32}P]ATP across the liposome membrane exposed to Mg^{2+} and Ca^{2+} compared to a control exposed to buffer alone (Fig. 3B). Partial leakage was observed across the liposome membrane of samples exposed to Mn^{2+} (Fig. 3B). However, no leakage was detected at the physiologically relevant concentration of 0.1 mM Mn^{2+} (Fig. 3B).

Concluding remarks. Identifying which divalent cation is physiologically relevant for *Salmonella* survival within macrophages is important to better understand *Salmonella* pathogenesis. The cytoplasmic concentration of Mg^{2+} (1-10 mM) is at least one order of magnitude higher than those of Ca^{2+} (100 μM) and Mn^{2+} (10-100 μM) (Finney and O'Halloran, 2003). However, the concentrations of these cations in SCVs are largely unknown. Based on the fact that PhoP-activated genes are induced within SCVs, and that some of these genes encode Mg^{2+} transporters (*mgtA* and *mgtB*), it has been proposed that Mg^{2+} is most likely the physiological ligand

(Groisman, 1998). Our data show that high concentrations (5 mM) of Mn^{2+}, and to a lesser extent Ca^{2+}, are more potent than Mg^{2+} at repressing PhoP/PhoQ signal transduction (Fig. 1A, Fig. 2A). It should be noted that although these *in vivo* and *in vitro* experiments are in good agreement for $Mg^{2+}$and Ca^{2+}, the amount of repression measured for Mn^{2+} was greater *in vitro* than that measured *in vivo*. It is possible that the partial leakage of proteoliposome observed with exposure to high concentrations of Mn^{2+} (Fig. 3B) accounts for this difference. Although *E. coli* phospholipids were used to prepare proteoliposomes, it is possible that differences between the *in vivo* and *in vitro* results reflect differences in membrane architecture between cells and proteoliposomes, namely the lack of an outer membrane with its LPS, phospholipids and protein components, and peptidoglycan. Metals are necessary for cell wall integrity with Ca^{2+} and Mg^{2+} as the preferred metals (Beveridge, 1989). LPS phosphoryl groups have been implicated as the primary sites for metal interaction (Beveridge, 1988). It is possible that although the divalent cation concentrations used *in vivo* and *in vitro* were the same, the concentration of divalent cations available to PhoQ differs due to LPS and peptidoglycan binding of metals in whole cells. Regardless, physiological concentrations of these divalent cations are capable of repressing the PhoP/PhoQ system to similar extents (Fig. 1B, Fig. 2B). Thus, it appears that the PhoQ protein evolved to respond to the dynamic range of physiological Mg^{2+}, Ca^{2+} and Mn^{2+}. Furthermore, this suggests that maximal activation of PhoQ can only be achieved by an environment that is depleted in Mg^{2+}, Mn^{2+} and Ca^{2+} and that Mn^{2+} may be as important as Mg^{2+} for *Salmonella* survival inside SCVs. The SCV membrane contains the Nramp1 transporter that exports various cations including Mn^{2+} from the SCV lumen to the cytoplasm of the host cell (Jabado *et al.*, 2003). *Salmonella* also possesses transporters that import Mn^{2+} (MntH, SitABCD) to the bacterial cytoplasm (Kehres *et al.*, 2000; 2002). Thus, it is likely that the Mn^{2+} concentration in the SCV lumen is lower than that of the host cell cytoplasm (10-100 μM). Recent reports suggest that Ca^{2+} concentrations may in fact be higher in SCVs than the host cell cytoplasm with acidified phagosomes having 400-600 μM Ca^{2+} (Christensen *et al.*, 2002). In addition, phagosomes have been

proposed to contain millimolar concentrations of Mg^{2+} at the time of PhoQ-mediated gene transcription (Grinstein S., personal communication). Therefore although we have shown that physiological concentrations of these divalent cations are capable of repressing the PhoP/PhoQ system, the signals for maximal PhoQ activation remain unknown and are unlikely to be depletion of Mg^{2+}, Mn^{2+} and Ca^{2+}.

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FIGURE LEGENDS

FIG. 1. *In vivo* repression of $PhoQ_{His}$ activity by divalent cations. *E. coli* cells MG1607 carrying pPRO (control plasmid) or pPRO-Q_{His} ($PhoQ_{His}$) were grown in LB broth or LB broth supplemented with various concentrations of divalent cations. β-galactosidase activities are expressed as Miller milliunits. Data are the means of triplicates values with standard deviations. One unit of β-galactosidase hydrolyzes 1 μmole of ONPG/min at pH 7.5 and 37 °C. (A) Cells carrying pPRO-Q_{His} were grown in the presence of 5 mM $MgCl_2$, $CaCl_2$, or $MnCl_2$. (B) Cells carrying pPRO-Q_{His} were grown in 5 mM $MgCl_2$, 1 mM $CaCl_2$, or 0.1 mM $MnCl_2$.

FIG. 2. *In vitro* global activity repression of the PhoP/$PhoQ_{His}$ system by divalent cations. The net phosphorylation of PhoP by reconstituted $PhoQ_{His}$ was measured. $PhoQ_{His}$-proteoliposomes (1.5 μM $PhoQ_{His}$) preloaded with phosphorylation buffer in the presence or absence of divalent cations were incubated at 22 °C in phosphorylation buffer supplemented with 0.1 mM [γ-^{32}P]ATP and 5 mM $MgCl_2$. After 20 min, reactions were stopped by the addition of 4 × Laemmli loading buffer and analyzed by SDS/PAGE. The amounts of [^{32}P]phospho-$PhoP_{His}$ were determined with a phosphorimager. Data are the means of triplicates values with standard deviations. (A) $PhoQ_{His}$-proteoliposomes were preloaded with phosphorylation buffer in the presence or absence of 5 mM $MgCl_2$, $CaCl_2$, or $MnCl_2$. (B) $PhoQ_{His}$-proteoliposomes were preloaded with phosphorylation buffer in the presence or absence of 5 mM $MgCl_2$, 1 mM $CaCl_2$, or 0.1 mM $MnCl_2$.

FIG. 3. Proteoliposome integrity. The extent of leakage of β-galactosidase and [γ-^{32}P]ATP across liposome or proteoliposome membrane was measured. Data are the means of triplicates values with standard deviations. (A) Proteoliposomes or liposomes were preloaded with 7.5 units β-galactosidase and exposed to phosphorylation buffer or phosphorylation buffer supplemented with 5 mM $MgCl_2$, $CaCl_2$ or $MnCl_2$ and incubated at 22 °C for 180 min. Samples were then centrifuged and the resulting supernatants and resuspended pellets were assayed for β-

galactosidase activity using ONPG as the substrate. (B) Liposomes were prepared and exposed to to phosphorylation buffer or phosphorylation buffer supplemented with 5 mM $MgCl_2$, $CaCl_2$ or $MnCl_2$ or 0.1 mM $MnCl_2$ and trace amounts of [γ^{32}P]ATP (0.04 Ci/mmol) and incubated at 22 °C for 180 min. After centrifugation, supernatant and resuspended pellets were added to scintillation fluid and radioactivity was measured with a scintillation counter.

FIG. 1. Sanowar and Le Moual

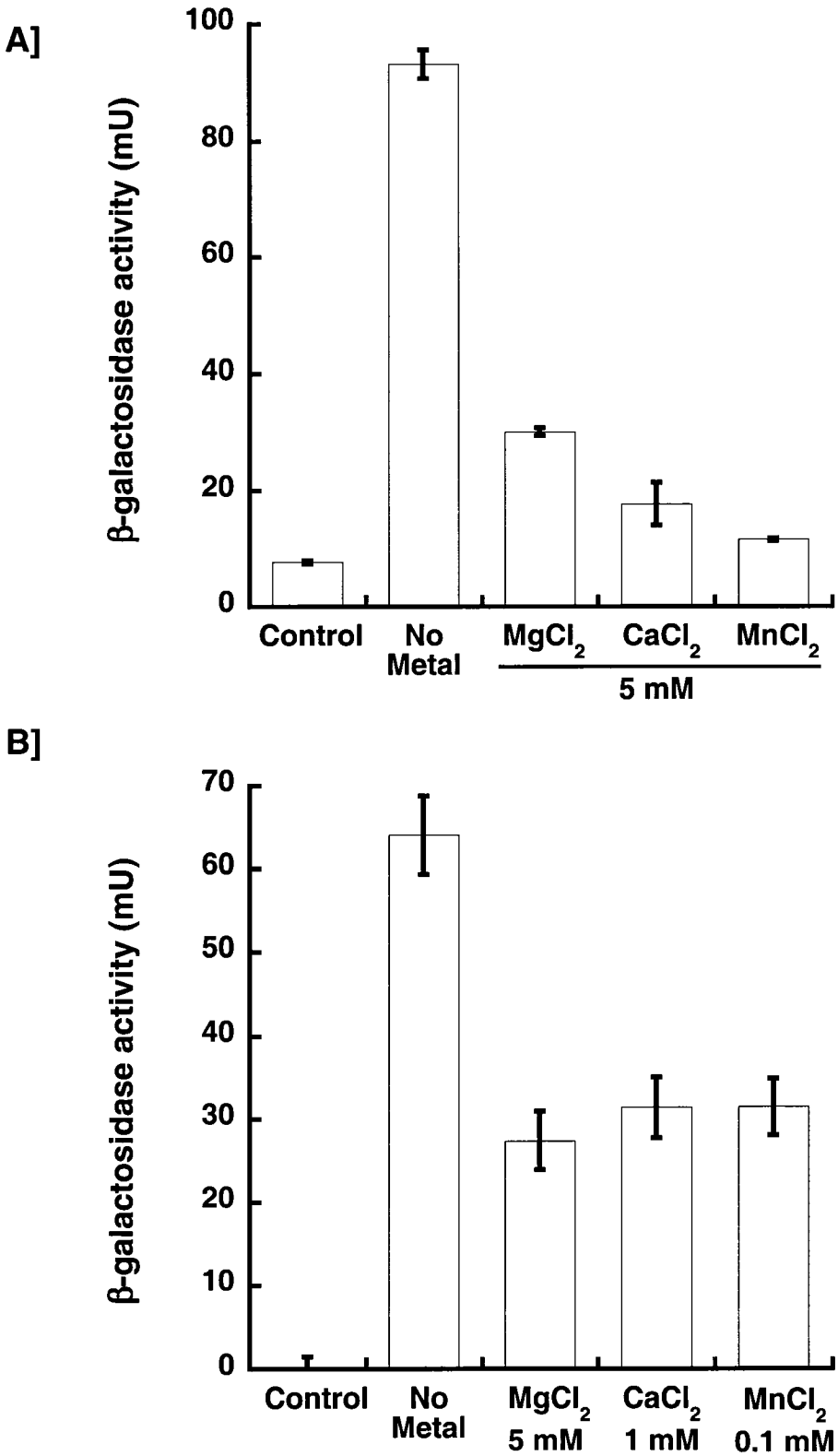


FIG. 2. Sanowar and Le Moual

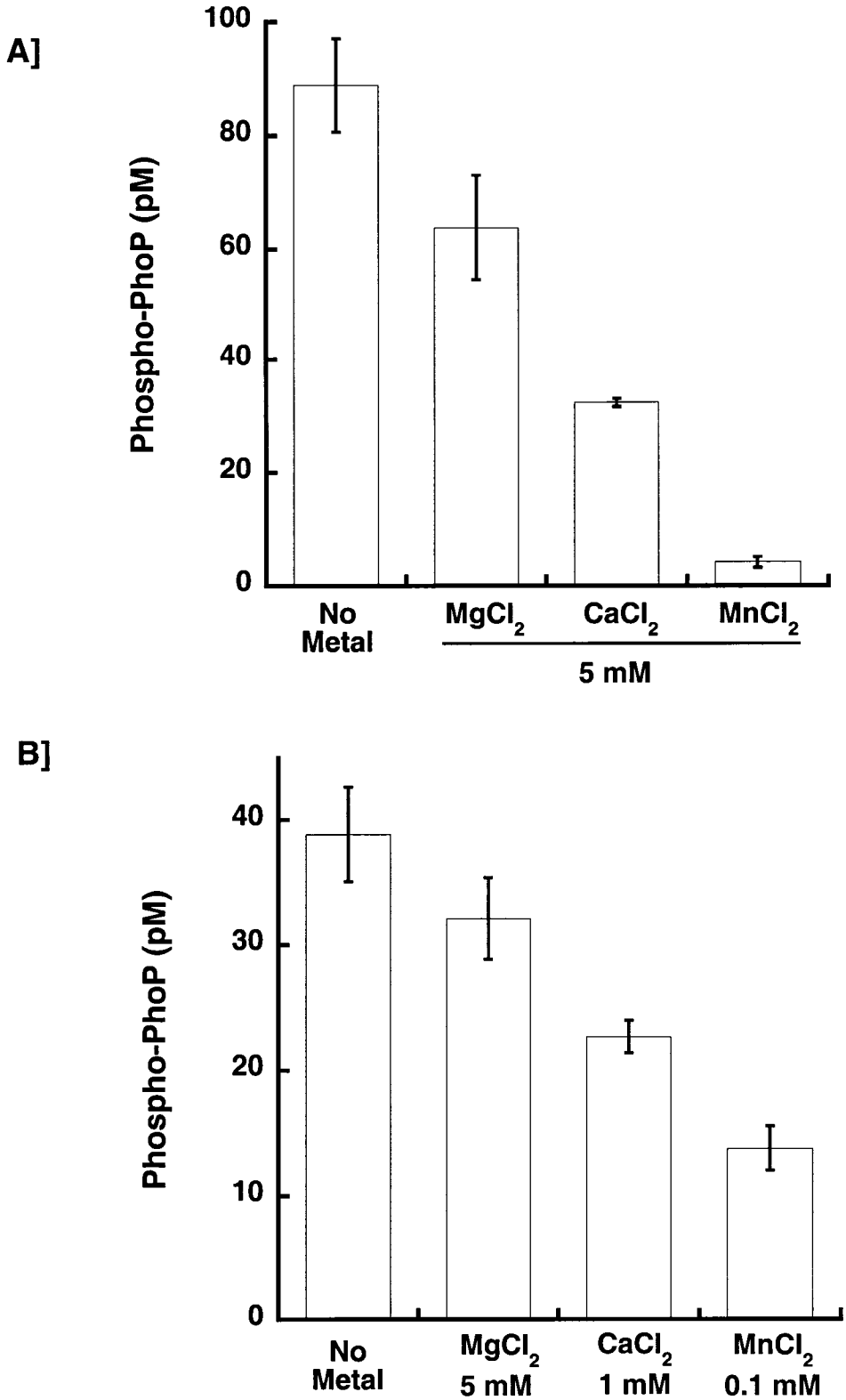
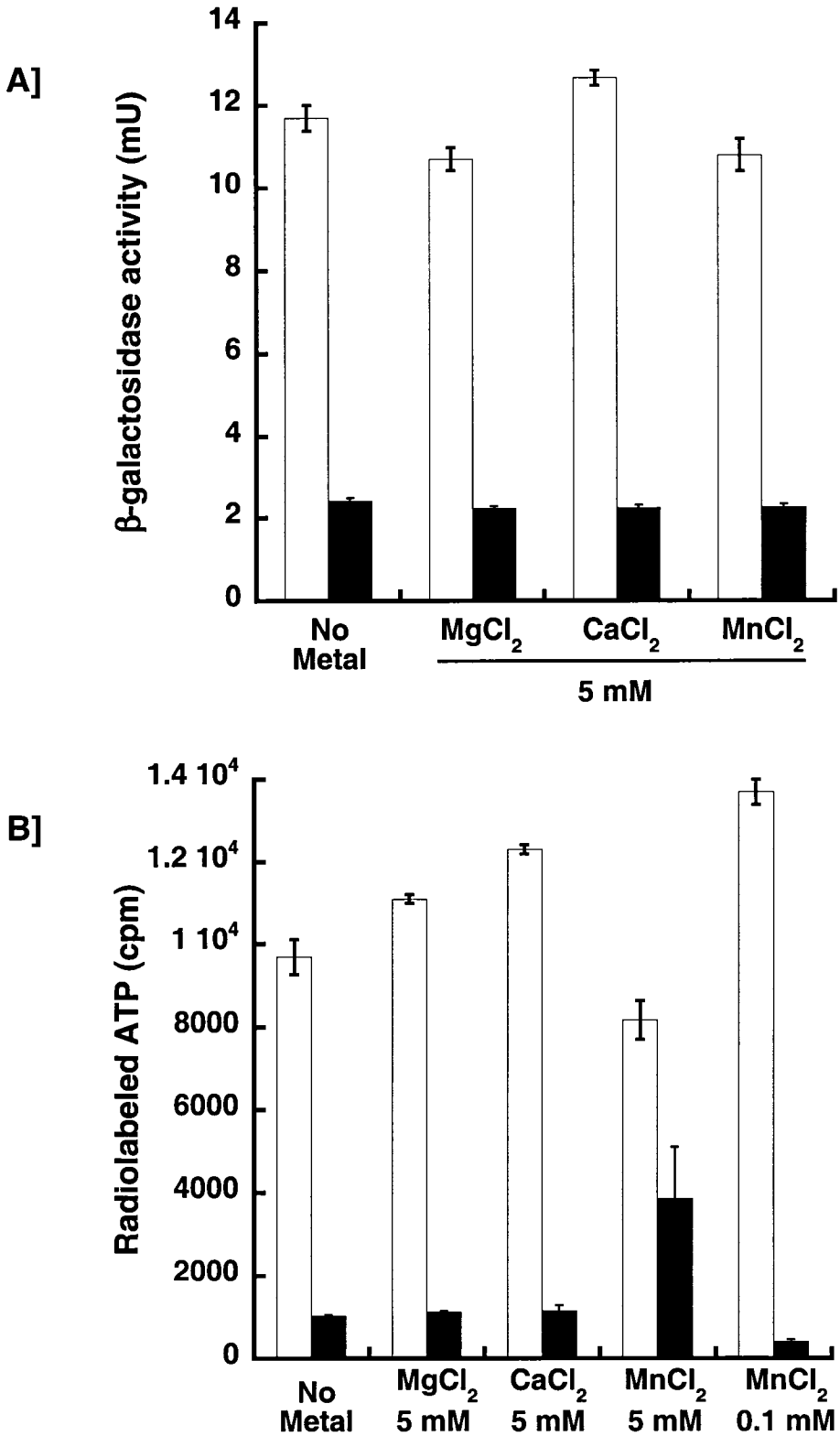


FIG. 3. Sanowar and Le Moual



PREFACE TO CHAPTER 5

In this chapter we examine antimicrobial peptide activation of the PhoQ histidine kinase sensor. We also dissect the molecular mechanisms governing the response to antimicrobial peptides and how this response differs from that of divalent cation-mediated repression. We show that antimicrobial peptides are sensed directly by the PhoQ periplasmic sensor domain. Using the reconstituted PhoQ protein, antimicrobial peptides were shown to increase the net accumulation of phosphorylated PhoP. Addition of Mg^{2+} and antimicrobial peptides to reconstituted PhoQ decreased the net accumulation of phosphorylated PhoP observed with antimicrobial peptides alone, indicating that divalent cations may compete with antimicrobial peptides for binding to the PhoQ periplasmic sensor domain. Divalent cations were shown to bind the PhoQ sensor domain through an acidic surface proximal to the membrane. PhoQ mutants deficient in peptide signalling mapped to this acidic region, demonstrating that residues involved in the response to antimicrobial peptides also participate in the binding of divalent cations. These results produced a model for PhoQ activation and the implications for PhoQ activation within macrophages is discussed.

CHAPTER 5:

Recognition of Antimicrobial Peptides by a Bacterial Sensor Kinase

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Running title: Bacterial Sensing of Antimicrobial Peptides

Summary

PhoQ is a membrane-bound sensor kinase important for the pathogenesis of a number of Gram-negative bacterial species. PhoQ and its cognate response-regulator PhoP constitute a signal transduction cascade that controls inducible resistance to host antimicrobial peptides. We show that enzymatic activity of *Salmonella typhimurium* PhoQ is directly activated by antimicrobial peptides. A highly acidic surface of the PhoQ sensor domain participates in both divalent cation and antimicrobial peptide binding as a first step in signal transduction across the bacterial membrane. Identification of PhoQ signaling mutants, binding studies with the PhoQ sensor domain, and crystallographic analysis of this domain can be incorporated into a model in which antimicrobial peptides displace divalent cations from PhoQ metal binding sites to initiate signal transduction. Our findings reveal a molecular mechanism by which bacteria sense small innate immune molecules to initiate a transcriptional program that promotes bacterial virulence.

Introduction

Multicellular organisms restrict the growth of invading bacteria by both innate and adaptive immunity. One important component of innate immunity are cationic antimicrobial peptides, which are evolutionarily conserved and found in diverse organisms including amoeba, fruit flies, plants, and mammals (Ganz, 2003; Hancock and Diamond, 2000). Antimicrobial peptides have tremendous sequence diversity, but many share common structural features, including a net positive charge and an amphipathic structure, which promotes their ability to interact with negatively charged biological membranes (Dathe and Wieprecht, 1999). The role of antimicrobial peptides in resistance to microbial pathogens is now well established. For example, they make up the majority of effectors of the *Drosophila melanogaster* immune system (Tzou et al., 2002). In mammals, the peptide CRAMP controls the lesion size resulting from Group A Streptococcus skin infections (Nizet et al., 2001), while increased expression of human defensin HD-5 in transgenic mice can alter the host-pathogen relationship (Salzman et al., 2003).

Bacteria have developed mechanisms to resist killing by antimicrobial peptides. These mechanisms have been best characterized for *Salmonella typhimurium*, but are also present in other bacterial pathogens (Miller et al., 2005; Peschel, 2002). Resistance to antimicrobial peptides is typically acquired by modifications of the bacterial cell surface, such as lipopolysaccharide (LPS). LPS is anchored in the gram-negative membrane via its lipid A portion, which becomes heavily modified in antimicrobial peptide resistant strains (Miller et al., 2005). These modifications include addition of aminoarabinose and phosphoethanolamine (Gunn et al., 1998; Lee et al., 2004; Moskowitz et al., 2004), deacylation, hydroxylation, and palmitoylation of lipid A (Guo et al., 1998; Trent et al., 2001). A role for lipid A modifications during virulence is now generally accepted. Lipid A modifications are induced during *Salmonella* invasion of macrophages (Gibbons et al., 2005). Loss of the ability to add aminoarabinose to lipid A reduced the pathogenicity of *Salmonella typhimurium* for highly susceptible inbred mice (Gunn et al., 2000). These

phenotypes are not exclusive to Salmonellae, as mutants of *Bordetella pertussis* and *Legionella pneumonia* that can not add palmitate to lipid A display colonization defects in animal models of infection and survival within human macrophages respectively (Preston et al., 2003; Robey et al., 2001).

The virulence and lipid A modifications of a variety of Gram-negative bacteria are regulated by a two-component system termed PhoPQ (Ernst et al., 1999; Grabenstein et al., 2004; Guo et al., 1997; Moss et al., 2000; Rebeil et al., 2004). The PhoPQ system constitutes a signal transduction cascade composed of the membrane-bound sensor-kinase PhoQ and the cytosolic response regulator PhoP. Activation of the PhoQ kinase leads to autophosphorylation of PhoQ and subsequent phosphorylation of PhoP, which promotes the transcription of a large number of genes including those encoding enzymes involved in lipid A and protein remodeling of the outer membrane. Both *phoP* and *phoQ* are essential for Salmonellae virulence in mice and humans highlighting the importance of these genes during bacterial infections (Fields et al., 1989; Galan and Curtiss, 1989; Hohmann et al., 1996; Miller et al., 1989).

The *Salmonella* PhoP/PhoQ system is activated *in vivo* within acidified macrophage phagosomes. It is repressed *in vitro* during bacterial growth in high concentrations of the divalent cations Ca^{2+}, Mg^{2+}, or Mn^{2+} (Garcia Vescovi et al., 1996). Repression by these metals is dependent on the PhoQ periplasmic sensor domain which must transduce signals across the membrane to activate PhoQ phosphatase activity (Castelli et al., 2000; Montagne et al., 2001). The PhoPQ system is active at low cation concentrations *in vitro* and fully induced during bacterial replication in macrophages. It has therefore been proposed that these conditions exist within the bacterial phagosome and function as a signal for PhoQ activation. Since divalent cation-mediated repression may only be one part of the PhoQ sensing mechanism, we recently began a search for other signals that activate PhoQ. Since PhoPQ are essential to protect bacteria from antimicrobial peptides, we reasoned that such peptides could serve as signals for PhoQ activation (Bader et al., 2003). Previous work demonstrated that the PhoPQ system responds to sublethal

concentrations of antimicrobial peptides to promote resistance to peptide-mediated killing. However, as antimicrobial peptides permeabilize the outer membrane, it was unclear whether the mechanism of PhoPQ activation involved an indirect effect of membrane damage or a direct effect of peptide binding to PhoQ.

The studies reported here were initiated to dissect the molecular mechanism governing the response of PhoPQ to antimicrobial peptides and to distinguish it from divalent cation-mediated repression. We found that antimicrobial peptides constitute signals that are directly sensed by the PhoQ sensor-kinase resulting in increased phospho-transfer from PhoQ to PhoP. This study provides evidence that antimicrobial peptides and divalent cations compete for binding to a highly acidic region of PhoQ suggesting that antimicrobial peptides initiate activation of PhoQ by displacing bound divalent cations. Our results reveal a novel example of a sensor-kinase from an animal pathogen that is activated by host-derived molecules.

Results

Antimicrobial peptides activate PhoPQ-regulated gene expression at intermediate Mg^{2+} concentrations.

Antimicrobial peptide activation of PhoPQ-regulated gene expression was previously demonstrated in growth medium that was undefined for divalent cations. As medium containing high concentrations of divalent cations represses PhoQ mediated expression, the activation of PhoQ was compared in medium containing both antimicrobial peptides and Mg^{2+}. A strain expressing a PhoQ-regulated protein fusion to Salmonellae acid phosphatase (PhoN) was chosen for analysis. The activity of this fusion protein was measured in growth medium containing the α-helical antimicrobial peptides LL-37 or C18G, and varying concentrations of Mg^{2+}. LL-37 and C18G were used because Salmonellae likely encounter this class of peptides within macrophages (Rosenberger et al., 2004). Figure 1A shows that sublethal concentrations of antimicrobial peptides significantly induced PhoPQ-dependent gene expression. Stimulation of PhoP activity by LL-37 and C18G occurred at physiologic Mg^{2+} concentrations (1 mM), but was impaired above 5 mM. This indicates that the effects of LL-37 can be competed by Mg^{2+} either binding to the bacterial cell surface or to PhoQ directly. Fusion protein activities in the presence of C18G and LL-37 were comparable to those observed in the same medium containing low concentrations of Mg^{2+} (10 μM), suggesting that PhoP was fully induced by these peptides.

Activation did not occur in a PhoQ null mutant, but was restored when PhoQ was introduced on a plasmid, indicating that the PhoQ sensor-kinase is required for signaling (Figure 1B). The induction levels in the complemented strain were similar to a strain expressing a constitutive PhoQ variant further confirming full induction of PhoQ by antimicrobial peptides.

The periplasmic domain of PhoQ is required for the transcriptional response to antimicrobial peptides.

The effect of antimicrobial peptides could be an indirect consequence of membrane permeabilization or a direct effect on the PhoQ protein. As illustrated in Figure 2A, PhoQ is comprised of a periplasmic sensor domain, two transmembrane domains, and a cytosolic catalytic domain that undergoes autophosphorylation upon activation. The PhoQ sensor domain consists of 145 amino acids and plays an important role in cation-mediated repression of the PhoPQ system (Garcia Vescovi et al., 1996; Waldburger and Sauer, 1996). Therefore, a strain containing a deleted *phoQ* that expresses a mutant PhoQ(Δ51-181) lacking its periplasmic sensor domain was constructed. The resulting strain failed to induce PhoP-dependent gene activation in the presence of C18G (Figure 2B) or other antimicrobial peptides (n ot shown), indicating that the PhoQ periplasmic domain is required for the response to antimicrobial peptides.

We wished to obtain further genetic evidence for a role of the PhoQ sensor domain during growth in the presence of C18G. It has been reported that the periplasmic domain of *Salmonella typhimurium* and other enteric bacterial PhoQ is strikingly different on the primary sequence level from the sensing domain of *Pseudomonas aeruginosa* PhoQ (Lesley and Waldburger, 2001). Despite this difference, both proteins respond equally to growth in Mg^{2+} limited medium suggesting that they share a common signaling mechanism. A strain expressing a chimeric protein consisting of *Salmonella typhimurium* PhoQ with the *Pseudomonas aeruginosa* sensor domain was constructed and tested for response to divalent cations and peptides. This strain responded robustly to limitation of cations, consistent with a previous report for *Escherichia coli* PhoQ (Figure 2B) (Lesley and Waldburger, 2001). Interestingly, no increase of PhoN fusion activity was observed in the presence of C18G. Taken together, the results provide strong genetic support for an involvement of the *Salmonella typhimurium* periplasmic domain in antimicrobial peptide signaling. This indicates that the response is not conserved in PhoQ from the environmental and opportunistic pathogen *Pseudomonas aeruginosa*, although this bacterium may have other mechanisms to respond to antimicrobial peptides (McPhee et al., 2003).

PhoQ kinase activity increases on exposure of purified protein to antimicrobial peptides.

Though the periplasmic domain is clearly required for PhoQ mediated signaling on exposure to antimicrobial peptides, it was plausible that the signal was mediated indirectly as a result of outer membrane permeability or through another protein. In order to distinguish between these possibilities, full-length PhoQ was purified from membranes and then reconstituted into phospholipid vesicles (Figure 3A). Using fluorescently labeled thiol-reactive probes directed against the two cysteine residues at positions 392 and 395 of the PhoQ cytoplasmic domain, we determined that PhoQ exhibits an inside-out orientation (>95%) with the periplasmic domain facing the vesicle inside and the histidine-kinase domain exposed to the assay buffer (data not shown). PhoQ activation by antimicrobial peptides was then measured by the subsequent phosphorylation of purified PhoP (Figure 3B). When PhoQ containing vesicles were preloaded with 1 μg/ml of C18G, both increased autophosphorylation of PhoQ and phospho-transfer to purified PhoP were observed (Figure 3B). PhoQ activation was concentration dependent and increased to nearly 10-fold in the presence of 5 μg/ml C18G (Figure 3C). The concentration of C18G that exhibited the highest level of PhoQ activity is similar to the concentration that gave robust induction in the *in vivo* experiments (Figure 1A). Thus, our *in vivo* data are in good agreement with the *in vitro* reconstituted system. To assess whether PhoQ activity was due to a non-specific effect of C18G on the conformation or permeability of phospholipid vesicles generated in this system, C18G was added to the assay buffer at 1 μg/ml, but omitted from the vesicle lumen. As shown in Figure 3D, no activation of PhoQ was observed under these conditions. This result strongly argues that the periplasmic domain, which is present on the vesicle inside, is involved in peptide signaling and the observed activation is not due to non-specific effects of C18G, such as membrane permeabilization. To further substantiate this finding, vesicles were preloaded with both C18G (1 μg/ml) and 5 mM $MgCl_2$, which is known to bind to the sensor domain of PhoQ. Figure 3D shows that the extent of PhoQ catalyzed phosphate transfer from ATP to PhoP decreased significantly in the presence of 5

mM $MgCl_2$. The competition at very high Mg^{2+} indicates that metal may compete with C18G for binding to the periplasmic domain in the vesicle lumen, further supporting a role for this domain in signaling. To measure the kinetics of PhoQ activation initial rates of PhoP phosphorylation were measured in the presence of peptides C18G and LL-37. Figure 3E shows that both peptides lead to an increase in the initial rate of PhoQ-dependent phosphorylation of PhoP. The initial rate obtained with α-helical peptide magainin-2 (10 μg/ml) was similar to the one observed for LL-37 (not shown). Therefore, PhoQ directly responds to the presence of α-helical peptides since no other component is necessary for PhoQ activation in the *in vitro* system. Our results further indicate that antimicrobial peptides directly activate PhoQ through interactions with its periplasmic sensor domain.

The acidic surface of the PhoQ periplasmic domain binds divalent cations.

We have recently solved the crystal structure of the PhoQ periplasmic sensor domain (see accompanying manuscript). A central finding of this work was the identification of a negatively charged surface of PhoQ (Figure 4A), which is predicted to contact the negatively charged membrane through a series of three Ca^{2+} bridges. The Ca^{2+} cations serve to alleviate charge repulsion between the acidic surface and the membrane through ionic bridges between membrane phospholipids and membrane proximal acidic residues of PhoQ. We sought independent evidence for divalent cation binding in solution by the PhoQ sensor domain. using Fe^{2+}-catalyzed oxidative cleavage (Figure 4B). Fe^{2+} ions have been shown to interact with Mg^{2+} binding sites in many proteins and this property can be exploited to obtain information about the position of metal binding sites in proteins. When incubated in the cleavage reaction mixture, the PhoQ sensor domain yielded one major cleavage product as visualized by SDS-PAGE (Figure 4B, inset). No cleavage occurred in the presence of 20 mM $MgCl_2$ consistent with Fe^{2+} binding at one or more PhoQ metal binding sites. The N-terminus of the cleavage product was identical with the original construct (i.e., MDKT) and the average mass (determined by MALDI mass spectrometry) was 12,854 Da. Taken together, the results identify the major cleavage product as residues

1-153 with its C-terminal sequence DDDDA[153] (predicted mass 12,871 Da). Cleavage at this sequence is consistent with one of the three Ca^{2+}-binding sites in the crystal structure, specifically site III, in which D150 and D151 coordinate the cation (see accompanying manuscript). The lack of Fe^{2+}-catalyzed cleavage at either of the other two cation binding sites observed in the crystal is notable, as these two sites are formed at the dimer-dimer interface in the crystal. The result in solution indicates that isolated dimers of PhoQ in solution may bind divalent cations at these two sites with lower affinity.

Antimicrobial peptides and divalent cations bind to an acidic surface of PhoQ.

Our data suggest that 1) the periplasmic domain is required for peptide sensing and 2) peptides and divalent cations compete for binding to the periplasmic domain of PhoQ. We utilized three *in vitro* assays as independent tests for binding of antimicrobial peptides to PhoQ. We determined the extent of iron-mediated cleavage of the PhoQ sensor domain in the presence and absence of C18G peptide. As shown in Figure 4B, hydroxyl radicals generated in the reaction mixture specifically cleave the PhoQ sensor domain. As shown in Figure 5A, when incubated with 10 μg/ml C18G, PhoQ was strongly protected from cleavage consistent with the proposal that C18G masks Mg^{2+} binding sites making them inaccessible for iron-mediated cleavage. To gain further evidence for binding of C18G to PhoQ, a fluorescent derivative of the peptide was synthesized (Figure 5B). The dansylated peptide exhibited low fluorescence yield with λ_{max}=525 nm when diluted into assay buffer. When incubated with the purified sensor domain however, the fluorescence yield increased dramatically with the maximum fluorescence shifting to a lower wavelength (λ_{max}=514 nm). Such changes are indicative of a more hydrophobic environment of the dansyl group, which can be best explained by binding of the peptide to PhoQ. The presence of 5 mM $MgCl_2$ significantly inhibited the increase in fluorescence intensity of dansyl-C18G due to PhoQ (Figure 5B). C18G binding showed saturation behavior at low micromolar peptide concentrations (Figure 5C) with an apparent K_d=2.8 μM. Such concentrations are sufficient to activate PhoQ enzymatic activity *in vivo* and *in vitro*

and compete effectively with 1 mM $MgCl_2$ for PhoQ (Fig. 1A). Binding of C18G to PhoQ is specific, as the purified sensor domain of the *Klebsiella pneumonia* histidine-kinase CitA, which is similar to PhoQ in size, net charge, and three-dimensional structure (PAS fold), did not affect the fluorescence spectra over a range of peptide concentrations tested (Fig. 5C). Unlabelled C18G effectively outcompeted dansylated C18G showing that binding was specific and not due to some non-specific feature of the dansyl group (Figure 5C). Taken together, our results show that antimicrobial peptides compete effectively with metal to interact directly with the sensor domain of PhoQ at a site that overlaps with the metal binding site cleaved by Fe^{2+}, and therefore involves the PhoQ acidic surface.

NMR spectra of the periplasmic domain of PhoQ in the presence of antimicrobial peptides could provide an additional highly sensitive assay for the similarity of conformational changes on binding peptides and divalent cations. Unfortunately, when incubated with C18G or LL-37, PhoQ and antimicrobial peptide formed an insoluble precipitate (at the high concentrations required for NMR), impeding analysis. We therefore turned to polymyxin nonapeptide, a derivative of the antimicrobial peptide polymyxin B that robustly activates PhoQ-mediated signaling *in vivo* (3.5-fold in the presence of 20 μg/ml). Polymyxin nonapeptide lacks a fatty acid and is therefore less potent than polymyxin, probably as a result of its decreased hydrophobicity and decreased ability to penetrate the outer membrane. Polymyxin nonapeptide has a net positive charge and amphipathicity characteristic of this class of peptides and it was suitable for NMR analysis. Spectra of PhoQ in the presence and absence of polymyxin nonapeptide are shown in Figure 5C. Remarkably, many of the peaks that appear in the presence of Mg^{2+} (green spectra) also appear when peptide is added (Figure 5C). The peaks that appear in the spectrum of PhoQ in the absence of peptide and divalent cation (black spectrum Figure 5C) are not perturbed by the addition of the peptide. This indicates that the stable structured core of the PhoQ dimer does not undergo a peptide-induced conformational change. The spectrum of PhoQ in the presence of peptide contains new peaks that are dispersed throughout the spectrum, indicating that peptide binding induces an additional PhoQ

stable structure (red spectra). Remarkably, the peptide-induced peaks are coincident with a subset of the peaks that appear when either Mg^{2+} or Ca^{2+} is added to PhoQ (compare red to green spectra; see accompanying manuscript). At the highest peptide concentration used, 10-12 peaks of the ca. twenty that appear with divalent cation are still undetectable or very weak in the PhoQ-peptide spectrum. Addition of 20 mM Mg^{2+} to a sample containing PhoQ and peptide restored the remaining peaks and produced a spectrum indistinguishable from that obtained for PhoQ plus Mg^{2+} in the absence of peptide (data not shown). The NMR spectra provide strong evidence that peptide and divalent cations bind at overlapping sites and that the binding events in solution elicit similar conformational effects that stabilize a region of structure that is intrinsically flexible in the absence of a ligand.

PhoQ periplasmic domain mutants defective in the response to antimicrobial peptides are in residues that participate in the binding of divalent cations.

The acidic surface seems well suited to interact with cationic antimicrobial peptides, which have a net positive charge and a tendency to partition into lipid bilayers. We predicted that amino acid changes in the acidic surface would impair the ability of PhoQ to respond to antimicrobial peptides. Most single mutations in the acidic surface that we constructed exhibited only slight effects on peptide mediated signaling, probably due to the remaining negative charge (not shown). However, we were able to identify two amino acid substitutions, E184K and T156K, which lie within the acidic surface and exhibit a defect in C18G-induced activation as measured by the PhoN reporter fusion *in vivo* (Figure 6). One of these mutants was identified in a genetic screen for a lack of repression of PhoQ in high Mg^{2+} medium (see accompanying manuscript). The double T156K/E184K mutant exhibited a further decrease in peptide-mediated signaling suggesting that these amino acid changes act additively. PhoQ T156K/E184K was also impaired in its response to LL-37 (2.4-fold vs. 8.2-fold activation with 5 μg/ml LL-37). Remarkably, both T156 and E184 participate in the binding of Ca^{2+} ion II in the PhoQ crystal, and as a consequence amino acid substitutions in these residues display higher activities at 10 mM

concentrations of calcium or magnesium (Cho et al.). However, it is important to point out that amino acid substitutions in these residues do not change the basal levels of activity at intermediate magnesium concentrations. Thus, the activation defect seen with peptide is not due to decreased synthesis of these proteins, which was also confirmed by Western blot analysis (Figure 6, inset). Taken together, our data indicate that antimicrobial peptides and metals may compete for overlapping binding sites on PhoQ. Peptide binding may initiate signaling through the PhoQ transmembrane domains leading to increased phosphorylation of PhoP.

Discussion

PhoQ is an antimicrobial peptide sensor.

Histidine sensor-kinases are signaling proteins that play an essential role during many aspects of bacterial physiology including bacterial infections. Although a number of small molecules that initiate specific responses through the activity of histidine-kinases have been identified, the identity of most signals recognized by this class of receptors remains unknown, even though many bacterial genomes contain up to 60 such proteins. These identified small molecule signals are often metabolic components such as phosphorus, nitrogen, citrate, and quinones, but also include the antibiotic vancomycin, plant phenolics, and autoinducer molecules (homoserinelactones) involved in quorum or bacterial density sensing (Arthur et al., 1992; Georgellis et al., 2001; Gilles-Gonzalez et al., 1991; Kaspar et al., 1999; Lee et al., 1995; Miller and Bassler, 2001). In addition, a specific bacterial peptide pheromone ComX is sensed by the sensor kinase ComP to promote competency in Bacillus spp. (Magnuson et al., 1994). In this study, we identify antimicrobial peptides as a direct signal for the activation of the PhoQ histidine-kinase. PhoQ plays a prominent role in Gram-negative bacterial virulence suggesting that antimicrobial peptides could be a signature of host environments for bacterial pathogens. Therefore PhoQ sensing of antimicrobial peptides, an essential and highly conserved component of innate immunity, activates virulence programs within bacterial pathogens.

We had previously shown that the PhoPQ activation is a prominent part of the response to sublethal concentrations of antimicrobial peptides, but it remained uncertain whether PhoQ sensed peptides directly or responded to a secondary consequence of peptide exposure such as membrane damage (Bader et al., 2003). The work reported herein provides a variety of genetic, biochemical, and structural evidence that the periplasmic domain of PhoQ directly binds antimicrobial peptides to transduce a signal that promotes kinase activity in its cytoplasmic domain. Deletion of the periplasmic domain abolished PhoQ mediated signaling, and amino changes in this domain were defined that lack a response to antimicrobial peptides. Most

convincingly, the reconstitution of the PhoPQ system with purified components into membrane vesicles clearly demonstrated that PhoQ and its native lipid environment are the only components required for sensing antimicrobial peptides (Figure 3). In this *in vitro* system, the net phosphorylation of PhoP by PhoQ was greatly enhanced by various antimicrobial peptides. Since the sensor domain of PhoQ faces the vesicle lumen in this system, we were able to show that α-helical peptides such as C18G and LL-37only stimulate catalytic activity of PhoQ when exposed to the periplasmic and not the cytosolic domain. Since phosphotransfer begins with phosphorylation of another molecule of PhoQ, binding of peptide may facilitate the interaction of the histidine-kinase domains within PhoQ dimers. Therefore it is reasonable to conclude that PhoQ functions as a direct sensor with binding of antimicrobial pepide resulting in a conformational change, which has an effect through the membrane on the cytoplasmic histidine-kinase domain.

The PhoQ acidic surface binds divalent cations to repress and antimicrobial peptides to activate kinase activity.

The crystal structure of the PhoQ sensor domain has recently been solved (accompanying manuscript). A prominent feature of the structure is that it displays an extensively negatively charged surface that is predicted to face and lay flat on the membrane. Many amino acid substitutions associated with impaired Mg^{2+} repression of PhoQ map to this surface, supporting a role for this structural feature in signaling. A central finding of our study is that signaling by antimicrobial peptides is tightly linked to divalent cation mediated repression of PhoQ. The PhoPQ system is fully inducible by peptides LL-37 and C18G in the range of physiological Mg^{2+} concentrations (1-2 mM), but induction is severely impaired at higher Mg^{2+} concentrations. A similar effect was observed when 5 mM Mg^{2+} was included in the vesicle lumen in the *in vitro* reconstituted system indicating that Mg^{2+} and antimicrobial peptides exert their effects at similar binding sites on PhoQ. Based on these observations and our finding that the PhoQ acidic surface is involved in metal binding we hypothesized that antimicrobial peptides may compete with metals for the

same binding sites. Two amino acid changes, E184K and T156K, identified in a screen for decreased responses to peptides C18G and LL-37, map directly to the PhoQ Ca^{2+}-binding site II observed in the crystal structure. E184 is at the very C-terminus of the sensor domain, which connects to the second transmembrane domain. Thus, our mutational analysis indicates that peptide binding at this site may trigger subsequent transmembrane signaling. Taken together, our results provide a mechanism for antimicrobial peptide signaling through PhoQ and lead us to conclude that metal and peptides act at similar sites. Therefore it is plausible that antimicrobial peptides function to displace metal directly from the anionic surface and that their amphipathic structure enables an interaction with the membrane that promotes a conformational change in PhoQ, leading to its activation. In its role as an antimicrobial peptide sensor, PhoQ peptide binding and enzymatic activation occurs in a micromolar range even in the range of low millimolar magnesium concentrations. Therefore PhoQ can sense antimicrobial peptides even under most physiologic magnesium concentrations.

Three independent approaches (NMR, iron cleavage, and fluorescence spectroscopy) provide strong evidence for direct binding of the antimicrobial peptides C18G and polymyxin-nonapeptide to the PhoQ sensor domain. Each of the three assays allowed us to conclude that antimicrobial peptides interact with the acidic surface of PhoQ (Figure 6). Binding to the acidic domain was concentration dependent and displayed saturation at micromolar peptide concentrations. The binding constant for peptides of full-length PhoQ may be lower due to the membrane proximity of the acidic surface and the higher local concentration of antimicrobial peptides near the membrane. The molecular mechanism by which the PhoQ sensor domain accommodates a large variety of antimicrobial peptides is currently unknown. It is nevertheless tempting to speculate that the negatively charged surface of the PhoQ sensor domain provides enough plasticity to accommodate a large variety of positively charged peptides despite their diversity on the primary sequence level. Consistent with this model, most single amino acid substitutions that introduce

positive charges on this surface (with the exception of T156K and E184K) do not significantly affect antimicrobial peptide dependent activation (data not shown).

A model for PhoQ activation

The above data in conjunction with the PhoQ crystal structure allow us to propose a mechanism by which PhoQ is activated by antimicrobial peptides and repressed by divalent metals (Figure 7). We propose that the PhoQ sensor domain undergoes a conformational change upon metal depletion due to repulsion between its negatively charged surface and the membrane. In support of this, structural changes of the PhoQ sensor domain have been inferred as a result of proteolytic digestion patterns when cells are depleted of metals (Garcia Vescovi et al., 1996), and the accompanying manuscript indicates that part of this mechanism involves charge repulsion as a result of loss of metal bridges between the membrane and PhoQ. Because of its proximity to the membrane, the negatively charged surface of PhoQ is also perfectly suited to sense the presence of membrane-active peptides. It has been shown that many antimicrobial peptides interact electrostatically with negatively charged phosphate groups of outer membrane lipid A as a first step before inserting into the membrane (Piers and Hancock, 1994; Sawyer et al., 1988) . We speculate that, similar to the interaction with lipid A, which causes the displacement of magnesium bound to lipid A phosphate groups, antimicrobial peptides displace divalent cations from PhoQ as part of their activation mechanism. Binding to PhoQ may promote a conformational change in the PhoQ dimer that is then propagated through the membrane (Figure 7). Such a model is supported by our peptide binding studies, which show that cation and peptide binding sites overlap (Figure 5). Our NMR data also demonstrate that both peptide and divalent cation binding to the PhoQ sensor domain lead to the stabilization of a flexible region of the PhoQ sensor domain. However, the binding of peptides may lead to significant structural distortions in the full-length membrane-bound protein by disrupting the interaction of divalent cations bound to the acidic surface. This is supported by our mutational and structural analysis that demonstrates that structural integrity at the membrane proximal side of the PhoQ sensor domain is

required to promote a fully repressed conformation (see accompanying manuscript). As antimicrobial peptides, due to their membrane-active nature, will come into close proximity to this side of the sensor domain and binding to the acidic domain may initiate transmembrane signaling, thus activating PhoQ. Therefore our current working model includes that peptide binding to the acidic surface displaces at least some metal bridges between PhoQ and the membrane. Peptide binding may function as a lever to lift the acidic surface off the membrane with a resultant change in conformation that leads to signal transduction.

What activates PhoQ within macrophage phagosomes?

A general principle of bacterial virulence is that microbes sense their presence in host-tissues to coordinately express virulence determinants in the correct microenvironment (Mekalanos, 1992). The nature of these microenvironments has long been a topic of debate among those studying bacterial pathogens. Since studies by Pappenheimer in 1936 demonstrated that Diphtheria toxin production was increased in growth medium of low iron, it has been known that bacterial pathogenic factors can be regulated *in vitro* by specific ionic concentrations of growth medium (Pappenheimer, 1993). It has been debated whether similar conditions, usually low iron, Ca^{2+} or Mg^{2+}, temperature, pH, or osmolarity reflect mammalian environments. Evidence from plant pathogenic bacteria has shown that virulence programs are induced by specific plant compounds (Lee et al., 1995) raising the possibility that animal pathogens recognize specific mammalian molecules as signals rather than ionic states of host tissues. Our results suggest one such signal is antimicrobial peptides.

The activity of PhoQ is required for a very central aspect of *Salmonella* pathogenesis: their survival and replication in macrophages (Galan and Curtiss, 1989; Groisman et al., 1989; Miller et al., 1989). Activation of PhoQ and subsequent phosphorylation of PhoP occurs upon bacterial phagocytosis by macrophages and leads to PhoP-dependent gene expression (Alpuche Aranda et al., 1992). As PhoQ seems to be rather non-specific in binding divalent cations (see accompanying

manuscript), it is difficult to imagine how the absence of one cation, such as Mg^{2+}, activates PhoQ under these conditions. Although the idea that phagosomes represent a low Ca^{2+}/Mg^{2+} environment has been promoted for many years (Garcia-del Portillo et al., 1992; Pollack et al., 1986), this proposal would require that immediately upon phagocytosis macrophages are depleted of all divalent cations repressing PhoQ, including Ca^{2+}, Mg^{2+}, and Mn^{2+}. Such a scenario is rather unlikely given that the Ca^{2+} concentration was shown to increase upon acidification of phagosomes and acidification is required for PhoQ mediated gene expression within macrophages (Alpuche Aranda et al., 1992; Christensen et al., 2002). In addition, recent work suggests that Mg^{2+} may indeed play a much less important role during macrophage infection than has previously been proposed, as phagosomes contain millimolar concentrations of Mg^{2+} at the time of PhoQ-mediated gene transcription (Grinstein S., personal communication). Such concentrations would be sufficient to almost completely repress PhoP dependent gene expression if magnesium was the relevant physiologic signal. Since PhoP is also activated at low pH and activation of PhoQ within macrophage phagosomes requires acidification (Alpuche Aranda et al., 1992; Bearson et al., 1998) it is plausible that phagosome acidification promotes the sensing of antimicrobial peptides by PhoQ or that PhoQ also directly senses varying proton concentrations This work provides direct evidence and support for the idea that antimicrobial peptides are a direct signal for PhoQ activation *in vivo* and is consistent with recent work by Finlay's group provided evidence that *Salmonella* resistance to the α-helical macrophage peptide CRAMP is an important function of PhoPQ virulence promotion (Rosenberger et al., 2004). Since LL-37, the human homologue of CRAMP, activates PhoQ, these macrophage peptides may be the actual signal *in vivo* for induction of the PhoQ virulence program. We therefore propose that antimicrobial peptides and low pH, rather than low Mg^{2+} concentrations, may be the major signals sensed by PhoQ *in vivo* to activate gene expression within host tissues.

In summary, we have revealed a molecular mechanism by which bacteria sense the presence of antimicrobial peptides. This study provides data indicating the molecular mechanism by which the PhoQ histidine-kinase, which is central for the

virulence of many Gram-negative bacteria, including animal, plant and insect pathogens, responds to molecules that are increasingly recognized for their role in innate immunity. Antimicrobial peptides are ubiquitous and ancient host defense molecules, and bacterial species have evolved mechanisms to respond directly to the presence of these peptides in a fashion analogous to the recognition of bacterial ligands by Toll-like receptors.

Experimental Procedures

Growth of bacterial strains. Strains were grown in LB or N-minimal media. To study antimicrobial peptide mediated induction of the PhoP regulon, the reporter *phoN*::Tn*PhoA* was chosen (Miller et al., 1989). Strains were grown overnight and diluted 1:100 into fresh medium. When N-minimal media was used, bacteria were washed twice in the same media before inoculation. Strains were then grown to an optical density of 0.2 and antimicrobial peptides were added at the indicated concentrations. After addition of peptide, strains were grown for an additional 60-90 minutes. Alkaline Phosphatase assays were performed using a standard protocol and cultures grown in duplicate at three different occasions. Error bars represent standard deviations of the mean.

***In vitro* reconstitution of the PhoP/PhoQ Signaling Cascade.** Proteoliposomes were preloaded with phosphorylation buffer (50 mM NaPi, pH 7.5, 200 mM KCl, 0.1 mM EDTA, 5% glycerol) supplemented with various concentrations of antimicrobial peptides C18G and LL37, or 5 mM $MgCl_2$ as indicated. The net phosphorylation of PhoP was measured by incubating proteoliposomes containing 1.5 μM PhoQ with an 8-fold molar excess of PhoP in a 15-μl volume of phosphorylation buffer supplemented with 5 mM $MgCl_2$. Reactions were initiated by the addition of 0.1 mM [γ-^{32}P]ATP (10 Ci/mmol), incubated at 22 °C for 20 min and stopped by the addition of Laemmli loading buffer. Reaction products were heated to 37 °C for 3 min and applied to 10% SDS-PAGE gels. Phosphorylated protein was visualized using an FX Scanner (Bio-Rad) and quantified by image analysis using the Quantity One software (Bio-Rad). Intensity units were converted to pM of [^{32}P]-protein calculated from a standard curve.

Peptide binding studies. C18G, dansylated-C18G and LL-37 were synthesized by the UBC peptide synthesis facility (Vancouver) using an Applied Biosystems automated synthesizer. Peptides were HPLC purified after synthesis. Fluorescence

spectra of dansylated-C18G (dC18G) were recorded at room temperature from 400-650 nm at an excitation wavelength of 340 nm. Peptide dC18G was incubated at increasing concentrations in 20 mM Tris-HCl pH 7.4 with or without PhoQ sensor domain (1 μM). Samples were incubated for 20 minutes at room temperature prior to measurements. The fluorescence of unbound peptide was subtracted from the measured fluorescence. Data were fit to a simple equilibrium model $F=F_{max}*P/(K_d+[P])$, where F is the observed fluorescence change of the sample with protein minus the peptide only control, F_{max} is the maximal fluorescence change, [P] is the peptide concentration and K_d is the dissociation constant for the complex. As a control, binding of dC18G was also tested with purified CitA sensor domain. CitA was purified as described previously (Kaspar et al., 1999).

NMR Spectroscopy. The NMR sample of the apo form of the PhoQ periplasmic domain contained 0.8 mM uniformly ^{15}N-labeled PhoQ in 20 mM imidazole buffer at pH 6.5, with 150 mM NaCl, 0.1 mM EDTA and 10% (v/v) D_2O. Additional NMR samples were identical, but also contained either 20 mM $MgCl_2$ or 3.5 mM polymyxin nonapeptide. NMR experiments were performed at 25 °C on a Bruker DMX 500 MHz spectrometer equipped with a triple-resonance, triple-axis gradient probe. Data were processed and analyzed using the programs NMRPipe/NMRDraw (Delaglio et al., 1995) and NMRView (Johnson and Blevins, 1994).

Iron-cleavage assay and MALDI analysis. PhoQ sensor domain (6 μM) was incubated in 20 mM Tris-HCl pH 7.4, 20 mM DTT with either 20 mM $MgCl_2$ or 10 μg/ml C18G or alone for 10 minutes. $FeSO_4$ was then added to a final concentration of 0.05 mM. Samples were taken at indicated time points, mixed with Laemmli buffer, boiled and separated by SDS-PAGE. For MALDI analysis, samples were mixed with 0.1% TFA and 5% acetonitrile, desalted by ZipTip (Millipore, C18). 1 μl of the eluate was mixed with matrix solution and analyzed in a Bruker Biflex mass spectrometer in linear mode with 28% laser power. Approximately 500 shots were recorded per sample. N-terminal sequencing was performed by Proteome Factory

(Berlin).

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Figure legends

Figure 1. Induction of PhoPQ-dependent gene expression by antimicrobial peptides.

A protein fusion between PhoP-dependent acid phosphatase (PhoN) and *E. coli* PhoA served as a reporter. (A) Activation by peptides C18G (5 μg/ml) and LL-37 (5 μg/ml) was measured in N-minimal medium (1 mM or 5 mM $MgCl_2$) and compared to cells grown in 0.01 mM $MgCl_2$. (B) Activities were measured in LB medium. Strain MB101, which carries a *phoQ::tet* allele, did not exhibit peptide mediated induction, but could be complemented with a plasmid carrying full-length *phoQ* under the control of the arabinose promoter (pBAD24-phoQ). Fusion protein levels were compared to induction levels in a PhoQ constitutive background ($PhoQ^c$). Error bars represent standard deviations of the mean of at least three independent experiments.

Figure 2. The PhoQ sensor domain is involved in responding to antimicrobial peptides. A) PhoQ consists of two transmembrane domains, a periplasmic sensor domain, and a cytosolic domain that contains the catalytic ATP binding domain and the phospho-transfer domain. B) The *Salmonella* PhoQ sensor domain is required for peptide-mediated activation. PhoQ lacking the sensor domain, PhoQ(Δ51-181), fails to respond to peptide C18G. A chimeric protein carrying the PhoQ sensor domain from *Pseudomonas aeruginosa* (phoQ-chimera) responds to varying concentrations of Mg^{2+} (10 mM versus 10 μM in N-minimal medium), but fails to mediate C18G dependent activation of the PhoPQ system. Activities were measured in the presence (grey bars) or absence (white bars) of 5 μg/ml C18G in LB medium.

Figure 3. Reconstitution of PhoQ-mediated peptide signaling *in vitro*.

A) An *in vitro* system to study PhoQ activity. PhoQ-dependent phosphorylation of

purified PhoP can be measured with [γ-^{32}P]-ATP. B) Antimicrobial peptide C18G stimulates PhoQ autophosphorylation and PhoP phosphorylation. Vesicles were preformed in the presence of 1 μg/ml C18G, washed and incubated in assay buffer containing [γ-^{32}P]-ATP. Purified PhoP was present at 8-molar excess to PhoQ (1.5 μM). Autoradiograph of SDS-PAGE is shown. C) Activation of PhoQ depends on C18G concentration. Vesicles were preloaded in the presence of increasing concentrations of C18G (0-5 μg/ml) and phospho-transfer to PhoP was determined as under B). D) Activation of PhoQ is initiated by exposure of the sensor domain to peptides. No activation of PhoQ occurs when C18G was omitted from the vesicle lumen and added externally (1 μg/ml). When vesicles were preloaded with 5 mM $MgCl_2$ and 1 μg/ml C18G, activation was significantly reduced. Experiments in C) and D) were carried out in triplicate and repeated at least twice. Typical results are shown. E) Kinetics of PhoQ catalyzed phospho-transfer. C18G and LL-37 enhance the initial rates of PhoP phosphorylation. Vesicles were preloaded with no antimicrobial peptide (▲), LL-37 (10 μg/ml, ●) or C18G (1μg/ml, ■). PhoQ-mediated phosphorylation of PhoP was measured at various time points as under B).

Figure 4. Binding of divalent cations to the PhoQ sensor domain. A) The crystal structure of the dimeric PhoQ sensor domain (*upper panel*) forms a flat surface that comes in close contact to the membrane and binds to phospholipids via divalent cation bridges. The bottom part of this domain contains a highly negatively charged surface as visualized by GRASP surface representation (*lower panel*, view from the membrane) and participates in metal binding. Red represents negatively charged residues; NT: N-terminus; CT: C-terminus. B) The acidic surface is involved in metal binding. Fe^{2+}-catalyzed cleavage of the purified periplasmic domain (6 μM) in the presence of $MgCl_2$ (20 mM, *upper panel*) and absence of $MgCl_2$ (*lower panel*) was assessed by MALDI mass spectrometry. The mass of the cleavage product observed in the absence of $MgCl_2$ in combination with N-terminal sequencing suggests that cleavage occurred at a specific site in the acidic surface. Fe^{2+}-catalyzed

cleavage was also visualized by SDS-PAGE (*inset*). Lane 1: - $MgCl_2$; lane 2: + 20 mM $MgCl_2$.

Figure 5. Binding of antimicrobial peptides to the PhoQ sensor domain.

A) Peptide C18G impairs iron-mediated cleavage of the PhoQ sensor domain. The purified sensor domain was incubated in buffer containing DTT/Fe^{2+} to mediate iron-dependent protein cleavage. Samples were removed at indicated time points and separated by SDS-PAGE. Reactions were also carried out in the presence of 20 mM $MgCl_2$ (*middle panel*) or 10 μg/ml C18G (*right panel*). Cleavage is inhibited by both Mg^{2+} and C18G suggesting the presence of common binding sites. B) Dansylated-C18G binds to PhoQ. 1 μM dansyl-C18G (*d*) was incubated in 20 mM Tris pH 7.4 and fluorescence spectra were recorded at an excitation wavelength of 340 nm. PhoQ (2 μM) was then added to the same cuvette (*a*). The presence of $MgCl_2$ at 1 mM (*b*) or 5 mM (*c*) impairs peptide binding. Protein only control (*e*). C) Titration of 1μM PhoQ (▲) and 1 μM CitA (O) with danslyated C18G (dC18G). dC18G fluorescence in the absence of protein was subtracted from the data. The data were fit to a simple equilibrium model. As a control, phoQ (1 μM) was incubated with 4 μM dC18G and titrated with unlabelled peptide (V). D) Superimposed two-dimensional ^{1}H-^{15}N TROSY-HSQC spectra of the PhoQ sensor domain in its apo-form (black) and in the presence of 3.5 mM polymyxin nonapeptide (PMNP, red) or 20 mM $MgCl_2$ (green). All spectra were collected at a protein concentration of 0.8 mM. The peptide-bound form reveals a subset of those peaks that appear in the presence of Mg^{2+}, indicating overlapping binding sites for peptide and divalent cations.

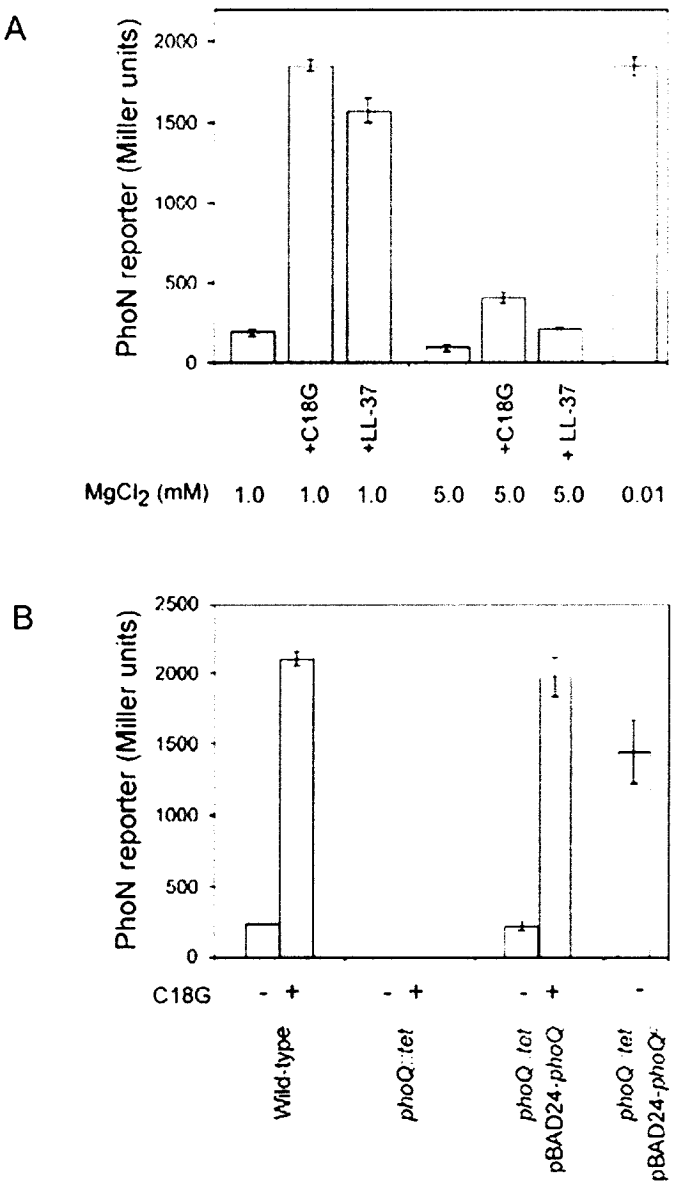
Figure 6. PhoQ mutants deficient in peptide signaling map to the acidic region.

Identification of mutants impaired in peptide-mediated repression. Mutants in the acidic region were screened for a defect in C18G mediated signaling. Strains were grown to OD_{600}=0.2 in LB medium and treated with 2.5 μg/ml C18G for 90 minutes. PhoQ mutants T156K and E184K and a double mutant in these two residues displayed defective C18G-mediated induction of phoN (+). Basal levels of activities

were approximately the same (-) for all mutants and wild-type. *Inset.* Expression levels of mutant proteins determined by Western blot analysis with an antibody against the PhoQ sensor domain. *From left to right.* Wild-type, *phoQ*, E184K, T156K, T156K E184K. All three mutants are expressed at similar levels of wild-type. Cells were grown in the presence of 0.2% arabinose to facilitate detection of PhoQ and variants.

Figure 7. Model of PhoQ activation by antimicrobial peptides. Divalent cations, such as Ca^{2+} or Mg^{2+} (shown as green balls), bind to the acidic surface (red) of the PhoQ sensor domain and form bridges to membrane phospholipids. Metal binding to the acidic surface represses PhoQ activity by locking the PhoQ sensor domain in an inactive conformation (1). Cationic antimicrobial peptides interact with membrane phospholipids and thus come in close contact with Ca^{2+}- and Mg^{2+}-binding sites of PhoQ. Antimicrobial peptides compete with divalent cations for binding to PhoQ and displace divalent cations from PhoQ (2). This results in a conformational change in the PhoQ sensor domain and/or the dimerization interface, and subsequent autophosphorylation of PhoQ (red balls) (3).

Figure 1.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PhoN reporter (Miller units)
2000
1500
1000
500
0
+C18G
+LL-37
+C18G
+ LL-37
MgCl2 (mM) 1.0 1.0 1.0 5.0 5.0 5.0 0.01
B
PhoN reporter (Miller units)
2500
2000
1500
1000
500
0
C18G - + - + - + -
Wild-type
phoQ::tet
phoQ::tet pBAD24-phoQ
phoQ::tet pBAD24-phoQ

Figure 2.

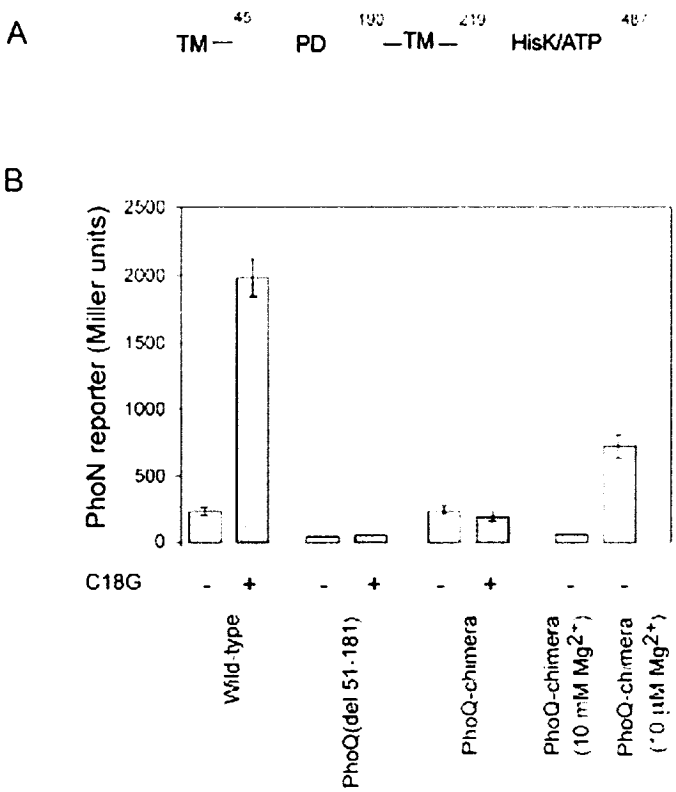
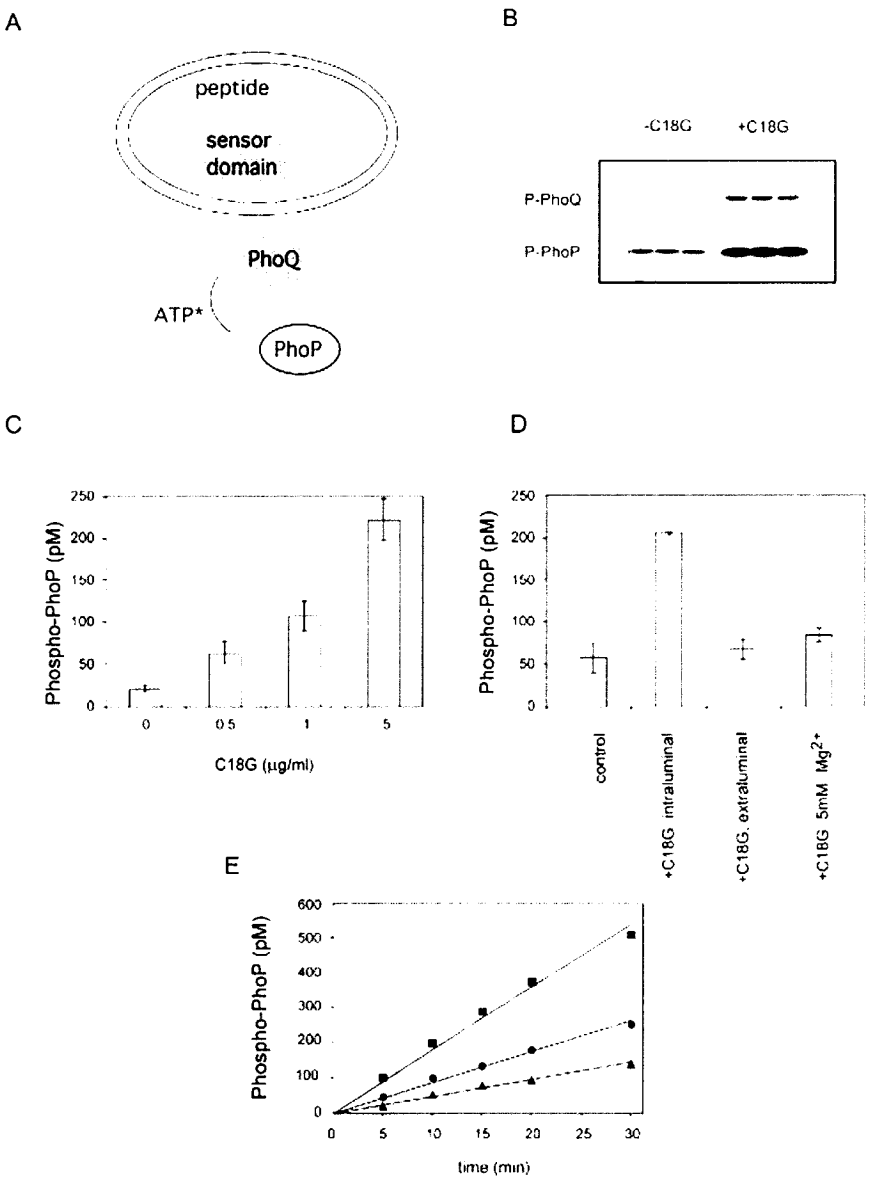

A
TM
PD
TM
HisK/ATP
B
PhoN reporter (Miller units)
2500
2000
1500
1000
500
0
C18G
- + - + - + - -
Wild-type
PhoQ(del 51-181)
PhoQ-chimera
PhoQ-chimera (10 mM Mg2+)
PhoQ-chimera (10 µM Mg2+)

Figure 3.


A
peptide
sensor
domain
PhoQ
ATP*
PhoP
B
-C18G
+C18G
P-PhoQ
P-PhoP
C
Phospho-PhoP (pM)
250
200
150
100
50
0
0
0.5
1
5
C18G (µg/ml)
D
Phospho-PhoP (pM)
250
200
150
100
50
0
control
+C18G intraluminal
+C18G extraluminal
+C18G 5mM Mg2+
E
Phospho-PhoP (pM)
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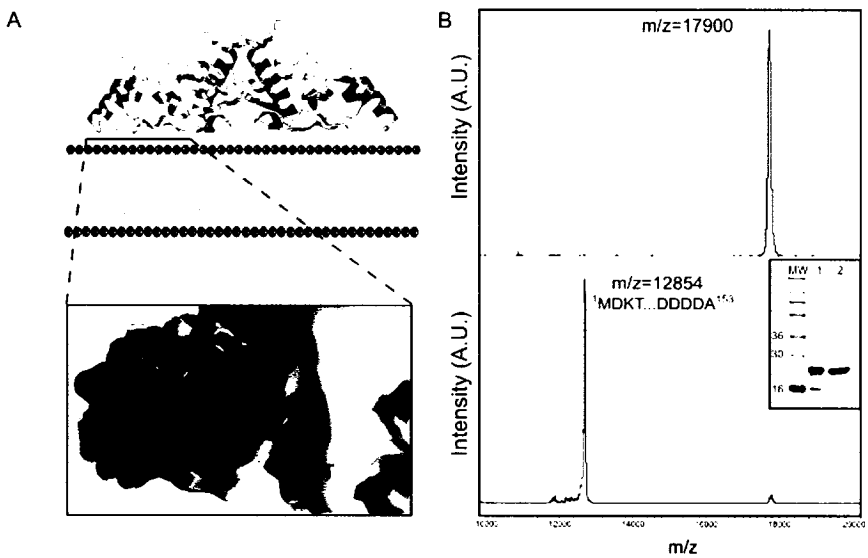
500
400
300
200
100
0
0
5
10
15
20
25
30
time (min)

Figure 4.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B
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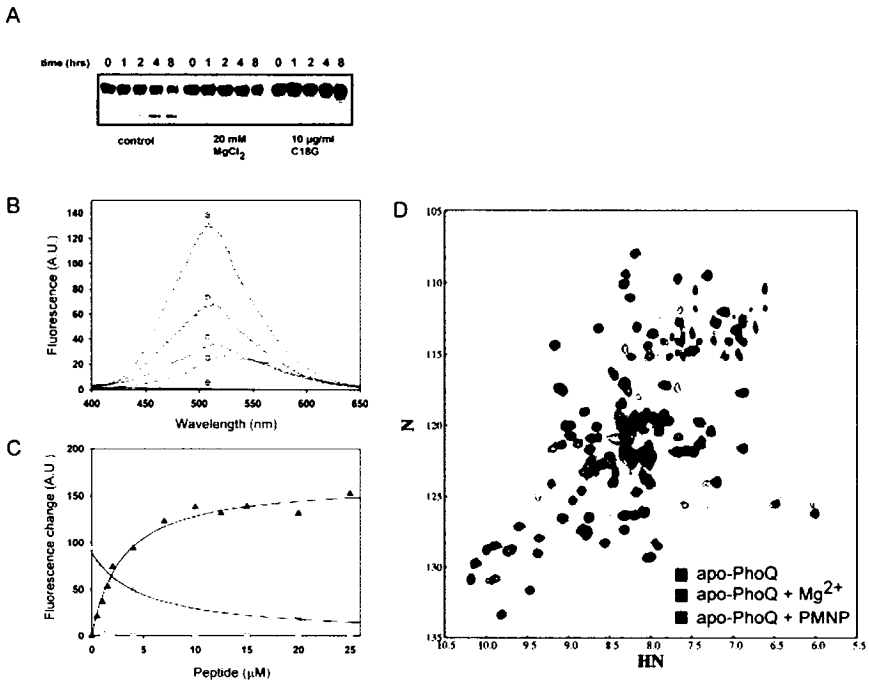
36
30
16
Intensity (A.U.)
m/z

Figure 5.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time (hrs) 0 1 2 4 8 0 1 2 4 8 0 1 2 4 8
control
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10 μg/ml C18G
B
Fluorescence (A.U.)
Wavelength (nm)
C
Fluorescence change (A.U.)
Peptide (μM)
D
apo-PhoQ
apo-PhoQ + Mg2+
apo-PhoQ + PMNP
HN

Figure 6.

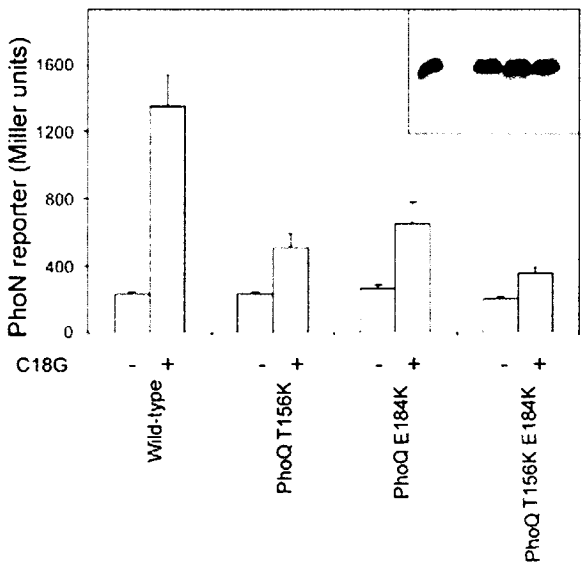
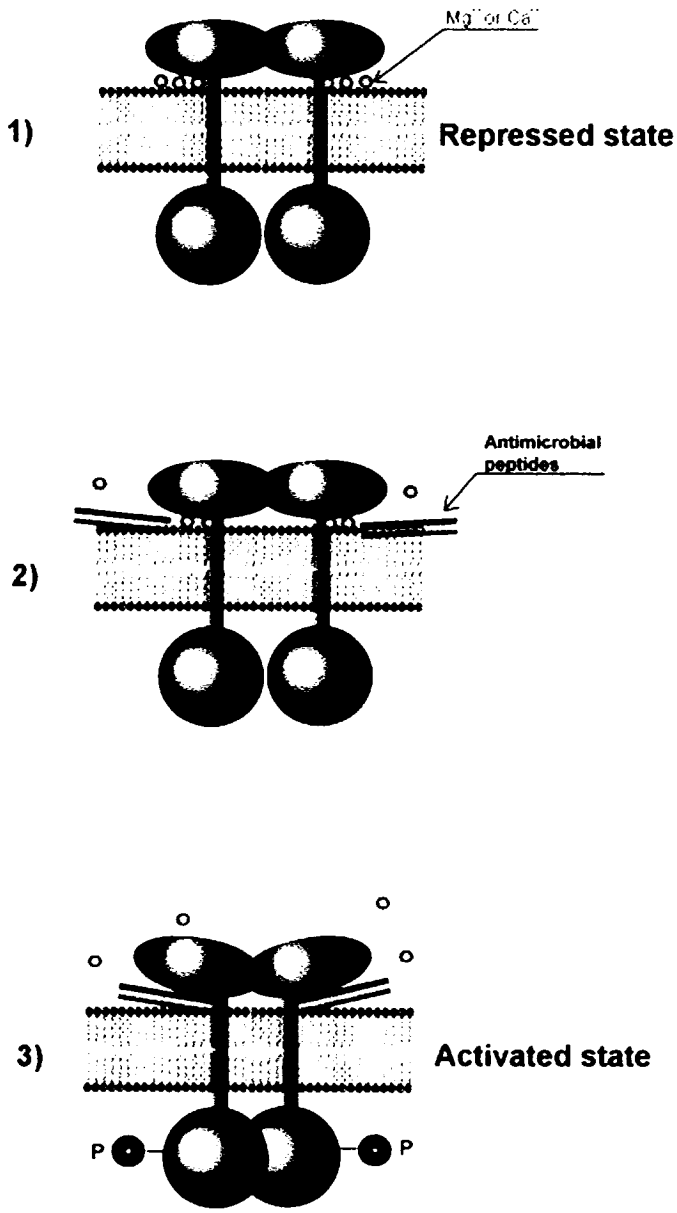

PhoN reporter (Miller units)
1600
1200
800
400
0
C18G
- +
- +
- +
- +
Wild-type
PhoQ T156K
PhoQ E184K
PhoQ T156K E184K

Figure 7.


1)
Repressed state
2)
Antimicrobial peptides
3)
Activated state
P
P

CHAPTER 6:
General Conclusions and Opportunities for Research

This thesis was designed to further our understanding of ligand recognition and transmembrane signal transduction by the PhoQ histidine kinase sensor. Site-directed mutagenesis combined with *in vivo* and *in vitro* activity assays were used to define the molecular mechanism by which the mutation T48I (*pho-24* allele of *Salmonella typhimurium* PhoQ) attenuates virulence in mice (Sanowar *et al.*, 2003). This study also showed that residue 48 of PhoQ, located in the periplasmic domain proximal to membrane, is involved in the conformational changes responsible for the switch between the kinase-dominant and the phosphatase-dominant state of the protein.

Although this study demonstrated the involvement of the residue at position 48 in conformational changes, it does not define the mechanism. Completion of this study would require targeted-disulfide cross-linking and solvent exposure experiments. By introducing a cysteine at residue 48 in a Cys-less PhoQ variant (PhoQ has two native cysteines at positions 392 and 395 located in the cytoplasmic portion of the protein), the orientation of residue 48 can be determined. High efficiency of disulfide formation would indicate that the residues at position 48 of each dimer subunit faces the dimer interface and may be involved in inter-dimer contact. Low efficiency of disulfide formation would indicate that the side chains of position 48 face the protein core or the solvent. Mg^{2+} regulation of cross-linked PhoQ-T48C would also be determined. Solvent exposure studies measures the ability of a given cysteine to react with fluorescent thiol-specific probes. The reactivity of large and charged probes unable to access the protein core will give an indication of residue 48 solvent accessibility. These studies would determine if the residue at position 48 is involved in inter-subunit interactions and impacts the dimeric interface.

Functional reconstitution of the *S. typhimurium* PhoQ allows for the discrimination of divalent cations acting as ligand versus fulfilling catalytic requirements. This tool also allows the study of PhoQ Mg^{2+}-regulation in the absence of other membrane proteins. This thesis reports the functional reconsitution of *S. typhimurium* PhoQ into *Escherichia coli* liposomes (Sanowar and Le Moual, 2005).

Reconstituted PhoQ exhibited all catalytic acitivities associated with histidine kinase sensors, including autokinase, phosphotransfer and phosphatase activities.

It remains unclear how ligand binding to the kinase sensor, often through extracellular domains, controls the phosphorylation of the conserved cytosolic histidine residue. Mg^{2+} sensing by PhoQ was been shown to reciprocally regulate the autokinase and phosphatase activities, with high concentrations of Mg^{2+} decreasing autokinase activity and increasing phosphatase activity (Montagne *et al.*, 2001; Chamnongpol *et al.*, 2003). In contrast, other investigators have found that high concentrations of Mg^{2+} have no effect on PhoQ autokinase activiy (Castelli *et al.*, 2000). Experiments detailing the influence of intraluminal Mg^{2+} on the global and individual activities of reconstituted PhoQ will clarify Mg^{2+} regulation of PhoQ. Initial results indicate that increasing the intraluminal Mg^{2+} concentration decreases the net phosphorylation of extraluminal PhoP by reconstituted PhoQ. This result suggests that levels of phosphorylated PhoP are controlled in a rheostat-like manner. Increasing intraluminal Mg^{2+} concentrations also inhibits the PhoQ autokinase activity, providing strong evidence for the Mg^{2+} regulation of the autokinase activity. The phosphatase activity of PhoQ with increasing concentrations of Mg^{2+} has yet to be measured and these experiments would need to be completed to fully address Mg^{2+} regulation of PhoQ. The role of antimicrobial peptides, such as C18G, on Mg^{2+}-regulation of PhoQ should also be addressed by completing the above experiments in the presence of peptide.

This thesis also reports the use of the *in vitro* reconstituted PhoP/PhoQ system to systematically compare the effects of intraluminal divalent cations acting as ligands. High concentrations of Mn^{2+}, and to a lesser extent Ca^{2+}, are more potent than Mg^{2+} at repressing PhoP/PhoQ signal transduction *in vivo* and *in vitro*. Physiological concentrations of these divalent cations are capable of repressing the PhoP/PhoQ system to similar extents. These data are the first reports of Mn^{2+} mediated-regulation of PhoQ activity. These data also demonstrate divalent-cation mediated repression of reconstituted PhoQ activity, indicating that reconstituted PhoQ

is the sole component needed. These findings suggest that Mn^{2+} and Ca^{2+} may be as relevant as Mg^{2+} for controlling *Salmonella* survival within macrophages.

The role of divalent-cation mediated repression of PhoQ *in vivo* is unclear, in part due to the lack of accurate measurements of divalent cation concentrations within the SCVs. Macrophage lysosomal concentrations of free Ca^{2+}, using both ratiometric and time-resolved fluorescence microscopy, has been reported to be 400-600 μM (Christensen *et al.*, 2002). Measuring the concentrations of Mg^{2+} in macrophage phagosomes is hindered by the lack of a Mg^{2+}-specific fluorescent probe. Others have used hard X-ray microprobe to compare the elemental concentrations in phagosomes infected with wild-type *Mycobacterium tuberculosis* and an Mramp (*Mycobacteria* Nramp homolog) knockout mutant (Wagner *et al.*, 2005). The use of this technique to measure elemental concentrations, such as Mg^{2+} and Mn^{2+}, in phagosomes and SCVs at different time points would be benifical to discussions of physiological PhoQ ligands.

The molecular mechanisms by which binding of divalent cations represses the PhoQ cytosolic domain is unkown. The recent solving of the crystal structure of the *S. typhimurium* PhoQ periplasmic sensor domain in both the metal-free and Ca^{2+}-bound states (Bader *et al.*, 2005; unpublished data) combined with the identification of mutants that are not repressed by divalent cations will aid in the elucidation of mechanisms by which extracellular domains trigger transmembrane signaling. The metal-bound crystal structure confirmed the metal binding surface on the PhoQ periplasmic domain to bridge the acidic surface and the membrane (Bader *et al.*, 2005; unpublished data). By comparing the metal-bound and the free PhoQ periplasmic domain structures, the role of the acidic surface of the PhoQ sensor domain in the initiation of signal propagation can be studied. In the absence of metals, negatively-charged residues of the sensor domain are repulsed from the membrane surface and the periplasmic dimer orientation is proposed to allow the formation of an appropriate dimer structure of the cytosolic domain that promotes trans-autophosphorylation. In the presence of metals, most of the negatively charged residues are bridged via metal ions with the membrane surface, triggering changes in

the dimer interface between the PhoQ sensor domains, and locking PhoQ in a phosphatase-dominant conformation.

Although the crystal structure of the PhoQ sensor domain confirms the site of metal binding, it infers the mechanism of signal propagation based on the assumption that the location of the sensor dimers N- and C-terminal residues correspond to the positions of the first and second PhoQ transmembrane domain, TM1 and TM2, as well as the cytosolic catalytic domains. The crystallization of the full length PhoQ protein, in both the metal bound and metal free states, would provide the complete picture of PhoQ domain positioning. The three-dimensional backbone stucture of a membrane protein with two transmembrane helices in micelles has been reported (Howell *et al.*, 2005). The structural determination of membrane proteins is progressing since the first high resolution structure of a membrane protein in 1985 (Diesenhofer *et al.*, 1985) but the structure of a full-length histidine kinase sensor has yet to be reported. Studies examining the role of conserved polar residues present in the PhoQ TM segments would also further our understanding of the mechanisms governing signal propagation. PhoQ TM sequence alignments show that they are highly conserved amongst *Enterobacteria* and contain a highly conserved proline residue in TM2 (Pro208) along with other conserved polar residues in both TMs (TM1: Thr21 and Ser29; TM2: Tyr 197 and Asn 202). The presence of conserved polar residues in the TMs may indicate that they are involved in the propagation of conformational changes during transmembrane signaling or PhoQ dimerization. Polar residues in transmembrane α-helices may alter the structural details of the hydrophobic sequences and control intermolecular contacts. Site-directed mutagenesis studies of these TM residues will identify key residues involved in the propagation of the signal through conformational changes. Targeted disulfide cross-linking on adjacent residues will allow for the determination of residue orientation, ie. facing the dimer core or facing the membrane. Little is known about the mechanisms for the flow of information from the extracellular domains through the membrane to the cytosolic kinase domains and identification of these mechanisms may yield a

common mechanism used by two-component systems and other signal transduction systems.

This thesis also reports a novel example of a bacterial kinase sensor activated by small molecules of host innate immunity. Antimicrobial peptides are shown to be ligands sensed directly by *S. typhimurium* PhoQ (Bader *et al.*, 2005). Previous work demonstrated that the PhoP/PhoQ system responds to sublethal concentrations of antimicrobial peptides (Bader *et al.*, 2003). However, it was unclear whether the mechanism of activation involved an indirect effect of membrane damage or a direct effect of peptide binding to PhoQ. The studies conducted in this thesis demonstrate that antimicrobial peptides constitute signals sensed by PhoQ, resulting in increased phosphotransfer from PhoQ to PhoP using the *in vitro* reconstituted PhoP/PhoQ system (Bader *et al.*, 2005). Evidence is also presented that antimicrobial peptides and divalent cations compete for binding to the highly acidic region of the PhoQ periplasmic sensor domain seen in the crystal structure.

The molecular mechanisms by which binding of antimicrobial peptides activates the PhoQ cytosolic domain, like that of divalent-cation mediated repression, is also unkown. Antimicrobial activation of PhoQ by antimicrobial peptides is proposed to occur by peptides competing and displacing the divalent-cations bound to the acidic surface of PhoQ to result in conformational changes in the PhoQ sensor domain leading to signal transduction and subsequent autophosphorylation of PhoQ (Bader *et al.*, 2005). The crystallization of the PhoQ sensor domain in the presence of antimicrobial peptides combined with the already available structures of metal-bound and free PhoQ sensor domains, would allow for confirmation of this model.

The antimicrobial peptide activation of PhoQ has been demonstrated with members of the α-helical structural class (Bader *et al.*, 2005). It would be important to identify whether antimicrobial peptide activation of PhoQ is limited to one structural class or family of peptides. Defensins, particularly human defensin-5 (HD-5) given its role in controlling *Salmonella* infection (Salzman *et al.*, 2003), would be interesting pepides to investigate. Several α-defensins and β-defensins, including HD-5, are commercially available. Conducting *in vivo* assays and *in vitro* assays with

reconsitituted PhoQ would identify which antimicrobial peptides are able to activate, or perhaps repress, PhoQ. This will also address the issue of where PhoQ peptide activation is occurring (i.e. which host microenvironment). Competition assays between various antimicrobial peptides and divalent cations, including Mn^{2+}, may reveal that the balance is shifted to divalent-cation repression with certain peptides. It would be expected that the antimicrobial peptides found in the intestinal lumen, such as HD-5, would be unable to activate PhoQ since the PhoP/PhoQ system is believed to be inactive until the intracellular phase of *Salmonella* infection. These experiments would refine our current view of antimicrobial peptide PhoQ activation.

A fundamental question in bacterial pathogenesis is the nature of signals sensed by bacteria. PhoQ is activated when bacteria are phagocytosed by macrophages and reside within acidified modified phagolysosomes (Alpuche-Aranda *et al.*, 1992; Ernst *et al.*, 2001). *In vitro*, PhoQ is active in growth medium depleted of divalent cations such as Ca^{2+} and Mg^{2+}, while PhoQ activity is tighly repressed in medium with millimollar concentrations of such divalent cations (Garcia Vescovi *et al.*, 1996). *In vitro*, antimicrobial peptides activate PhoQ activity (Bader *et al.*, 2005). The nature of the macrophage phagosome microenvironment has been the subject of much debate. Defining the components and concentrations of the phagosome is essential to understanding PhoQ activation *in vivo*. Accurate measurements of divalent cation concentrations combined with the knowledge of which specific antimicrobial peptides are present in the phagosome and at what concentrations, would clarify whether PhoQ activation is the result of antimicrobial peptide activation or the lifting of divalent-cation repression due to low concentrations of divalent cations. The antimicrobial peptide repertoire of the macrophage could be defined using mRNA in situ hybridization or RNA isolation followed by RT-PCR as has been done for several antimicrobial peptides (Schnapp *et al.*, 1998; Oullette *et al.*, 1999). The role of pH in PhoQ activation has also yet to be clarified. PhoQ activation within macrophage phagosomes requires acidification (Alpuche Aranda *et al.*, 1992; Bearson *et al.*, 1998). Repeating the antimicrobial peptide activation experiments at various pHs, *in vivo* and *in vitro*, may give interesting results.

The study of the *S. typhimurum* PhoP/PhoQ two-component system is at heart the study of the complex cross-talk between a microorganism and its host. The intricacies of the relationship merit investigation and will significantly enhance our understanding of the basic mechanisms of ligand-recognition and signal transduction that allow for the coupling of virulence programmes to environmental signals.

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APPENDIX: Research Compliance Certificates