Ligand Sensing and Signal Transduction by the Two-Component System PhoP/PhoQ

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For Toby

ABSTRACT

The two-component system (TCS) PhoP/PhoQ promotes *Salmonella typhimurium* adaptation to the changing environments encountered during infection. It is crucial for the regulation of virulence genes that are activated within acidified macrophage phagosomes. PhoQ is activated by antimicrobial peptides (AMP) and repressed by high concentrations of divalent cations such as Mg^{2+}, Ca^{2+} and Mn^{2+}.

Acidification of the environment was shown to directly activate the *S. typhimurium* PhoQ, with maximal activity at pH 5.5. We demonstrated that reconstituting PhoQ in proteoliposomes in the presence of the AMPs at acidic pH has an additive effect on the activation of PhoQ. Since AMPs and acidic pH are encountered during infection they are likely physiological ligands that activate the PhoPQ in the intracellular environment. NMR spectroscopy indicates that at low pH the PhoQ periplasmic domain ($PhoQ_{Peri}$) adopts a specific conformation. We investigated the effect of pH acidification on the intrinsic tryptophan fluorescence of $PhoQ_{Peri}$ and determined that the fluorescent spectrum undergoes fluorescence quenching and blue-shifting. These characteristics were attributed to a π–stacking interaction between residues H120-W113. Mutational analysis indicates that the PhoQ kinase activity is optimal when aromatic residues are present at positions 113 and 120. All the data taken together argue for a pH-dependent dynamic π–stacking interaction between W113 and H120.

The *Citrobacter rodentium* genome sequence contains a *phoPQ* operon homologous (~79% identity) to that of *S. typhimurium*. We report that *C. rodentium* PhoQ senses fluctuations in Mg^{2+} concentrations and acidic pH. Surprisingly, PhoQ was not activated by the presence of AMPs. However, activation by AMPs is observed when *C. rodentium* PhoP/PhoQ was expressed in a *S. typhimurium* background. We identified an outer membrane protease of the omptin family that was responsible for inhibiting PhoQ activation by AMPs. In stark contrast to *S. typhimurium*, which relies on LPS modifications to resist AMPs, our results suggest that *C. rodentium* promotes resistance through a PhoP/PhoQ-dependent OM protease to inhibit disruption of the outer membrane by AMPs.

RÉSUMÉ

Le système à deux composantes PhoP/PhoQ est utilisé par la bactérie *Salmonella typhimurium* pour répondre aux changements environnementaux qui ont lieu durant une infection. Ce système orchestre la virulence et permet la survie de la bactérie à l'intérieur des macrophages de l'hôte. La protéine senseur PhoQ est activée par des signaux spécifiques tels que les peptides antimicrobiens (PAM) et réprimée par des fortes concentrations en Mg^{2+}, Ca^{2+} and Mn^{2+}.

PhoQ est directement activé par une acidification du milieu avec une activité maximale à pH 5.5. Nous avons démontré par reconstitution de PhoQ dans des liposomes que la présence de PAMs a un effet additif sur l'activation de PhoQ par un pH acide. Au cours d'une infection, *S. typhimurium* est en contact avec les PAMs et un pH acide qui sont probablement les ligands physiologiques de PhoQ. La spectroscopie par RMN a montré que le domaine periplasmique de PhoQ ($PhoQ_{Peri}$) adopte une conformation spécifique en présence d'un pH acide. Nous avons examiné l'effet du pH acide sur la fluorescence intrinsèque des résidus tryptophane de la protéine $PhoQ_{Peri}$ et déterminé que le spectre de fluorescence subit un «quenching» et un «bleu-shifting». Ces caractéristiques ont été attribuées à une interaction dite «π-stacking» des résidus H120 et W113. Une analyse par mutagenèse de ces résidus indique qu'une activité optimale de la kinase PhoQ est obtenue lorsque les résidus 113 et 120 présentent un cycle aromatique. Ces résultats semblent indiquer que l'interaction dynamique «π-stacking» entre les résidus 113 et 120 dépend du pH environnant.

La séquence du génome de *Citrobacter rodentium* présente un opéron *phoPQ* (~79% identité) homologue à celui de *S. typhimurium*. Nous avons déterminé que PhoQ de *C. rodentium* perçoit les variations de pH et en Mg^{2+} du milieu environnant. De manière surprenante, les PAMs ne causent aucune augmentation d'activité de PhoQ. Néeanmoins, lorsque le système PhoP/PhoQ de *C. rodentium* est exprimé chez *S. typhimurium* les PAMs activent PhoQ. Nous avons identifié une protéine de la membrane externe appartenant à la famille des omptin qui est responsable de l'inactivité de PhoQ en présence des PAMs. Ces résultats suggèrent que le mécanisme de résistance aux PAMs de *C. rodentium* serait régulé par le système PhoP/PhoQ et

une protéase qui empêcherait la destruction de la membrane externe par les PAMs. Ce mécanisme de défense est différent de celui du système PhoP/PhoQ de *S. typhimurium* qui repose essentiellement sur des modification du LPS.

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First and foremost I would like to thank my supervisor Dr. Hervé Le Moual for advancing my scientific pursuits. His enthusiasm and patience has provided me with confidence, while his guidance and support has allowed me to think critically and to expand my scientific ability

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. Provided evidence that *Salmonella typhimurium* PhoQ directly responds to acidic pH and that in the presence of AMPs the two signals have an additive effect on the phosphorylation of PhoP.

2. Demonstrated a pH-dependent conformational change of the *S. typhimurium* PhoQ periplasmic domain using fluorescence spectroscopy.

3. Identified an evolutionarily conserved His-Trp π-cation interaction within the PhoQ periplasmic domain of Gram-negative pathogens.

4. Set up a method of creating chromosomal reporter fusions in *C. rodentium* by conjugation.

5. Identified Mg^{2+} and acidic pH as *in vitro* ligands of *C. rodentium* PhoQ.

6. Identified a PhoP/PhoQ-dependent outer membrane protease of the omptin family in *C. rodentium*, named CroP.

7. Illustrated that *in vitro C. rodentium* PhoQ is not activated by AMPs. This lack of activation was attributed to the presence of CroP, which inhibited AMPs from breaching the outer membrane and binding to the periplasmic domain of PhoQ.

8. Proposed a novel mechanism of antimicrobial peptide resistance for *Citrobacter rodentium*.

CONTRIBUTION OF AUTHORS

In accordance with guidelines of the Faculty of Graduate Studies and Research, McGill University, concerning thesis preparation, the experimental portion of this thesis (Chapters 2, 3 and 4) is prepared in the form of manuscripts.

The manuscripts are listed below and a description of the contributions of each co-author with respect to the experimental work for each manuscript. I, Valerie Le Sage, am responsible for the rest of the work presented in Chapters 2-4. Contributions not from the authors listed are presented in the acknowledgement section of each Chapter. All the work presented in this thesis was reviewed, edited and conducted under the supervision of Dr. Hervé Le Moual.

CHAPTER 2

Activation of the bacterial sensor kinase PhoQ by acidic pH

Lynne R. Prost, Margaret E. Daley, Valerie Le Sage, Martin W. Bader, Hervé Le Moual, Rachel E. Klevit and Samuel I. Miller

Lynne R. Prost, **Martin W. Bader** and **Samuel I. Miller** performed the *in vivo* transcription assays.

Margaret E. Daley and **Rachel E. Klevit** provided the NMR spectroscopy data.

Lynne R. Prost and **Margaret E. Daley** were responsible for drafting the manuscript.

CHAPTER 3

A tryptophan residue involved in a π-cation interaction with a protonated histidine residue probes pH-dependent conformational changes in the periplasmic domain of the *Salmonella* PhoQ sensor kinase

Valerie Le Sage, Aaron Bilek, Samuel I. Miller and Hervé Le Moual

Aaron Bilek assisted with the site-directed mutagenesis experiments.

Samuel I. Miller provided us with bacterial strains and plasmids.

CHAPTER 4

An outer membrane protease of the omptin family prevents activation of the PhoPQ two-component system by antimicrobial peptides in *Citrobacter rodentium*

Valerie Le Sage, Lei Zhu, Andrea Portt, Samantha Gruenheid and Hervé Le Moual

Lei Zhu and **Samantha Gruenheid** provided the *phoPQ* mutant *C. rodentium* strain.

Andrea Portt assisted with the creation of the *croP* mutant *C. rodentium* strain.

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LIST OF ABBREVIATION

ABC	ATP-binding cassette
A/E	attaching and effacing
AMP	antimicrobial peptide
Asp	aspartate
ATR	acid tolerance response
CA	catalytic and ATP-binding
cfu	colony forming units
CAMP	cationic antimicrobial peptide
CCCP	carbonyl cyanide meta-chlorophenylhydrazone
CR	*Citrobacter rodentium*
CRAMP	cathelin-related antimicrobial peptide
Cys	cysteine
dC18G	dansylated C18G
DHp	dimerization and histidine phosphotransfer
DM	dodecyl-b-D-maltoside
EHEC	enterohaemorrhagic *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
EtBr	ethidium bromide
GAP	GTPase-activating protein
GEF	guanidine nucleotide exchange factor
GHL	GyraseB, Hsp90 and MutL
GI	gastrointestinal
HAMP	histidine kinases, adenylylcyclases, methyl-accepting chemotaxis proteins and phosphatases
hBD-1	human β-defensin-1
HD-5	human α-defensin
His	histidine
HSQC	heteronuclear single quantum coherence
iNOS	inducible nitric oxide synthase
IPTG	isopropyl-1-thio-β-Dgalactopyranoside

K_{SV}	Stern-Volmer collisional quenching constant
L-Ara4N	aminoarabinose
LB	luria broth
LC MS/MS	Liquid chromatography electrospray ionization tandem mass spectrometry
LEE	locus of enterocyte effacement
Ler	LEE-encoded regulator
LOV2	light-oxygen-voltage
LPS	lipopolysaccharide
NMR	nuclear magnetic resonance
NOS2	nitric oxide synthase
NPN	1-N-phenyl-napthylamine
Nramp1	natural resistance-associated macrophage protein 1
ORF	open reading frame
OM	outer membrane
PAGE	polyacrylamide gel electrophoresis
PAI	pathogenicity island
PAS	Per-Ant-Sim
PBS	phosphate-buffered saline
PDE	phosphodiesterase
PEtN	phosphoethanolamine
$PhoQ_{Peri}$	PhoQ periplasmic domain
phox	phagocyte oxidase
PMN	polymorphonuclear leukocyte
PMNP	polymyxin nonapeptide
RIVET	resolvase-based *in vivo* expression technology
RR	response regulator
Sif	*Salmonella*-induced filament
SCV	*Salmonella*-containing vacuole
SK	sensor kinase
ST	*Salmonella typhimurium*
T3SS	type III secretion system

Tir	transmembrane intimin receptor
TCS	two-component system
TM	transmembrane
wHTH	wing helix-turn-helix

PREFACE TO CHAPTER 1

This thesis explores the PhoP/PhoQ two-component system in two pathogenic bacteria: *Salmonella enterica* serovar Typhimurium and *Citrobacter rodentium*. This literature review provides the necessary background for understanding this topic and is comprised of 7 main sections. Part 1 - "Two-component signal transduction" describes the structure and function of these systems, concentrating on the novel crystallographic data that have produced a model for sensor kinase signal transduction. This leads directly to Part 2 - "The PhoP/PhoQ two-component system", which details the ligands of the PhoQ sensor kinase and the regulon that is controlled by the PhoP response regulator. Part 3 - "The molecular basis of *Salmonella typhimurium* virulence" describes the organism and the virulence factors that determine intestinal epithelium invasion and survival within submucosal macrophages. Part 4 - "Regulation of *Salmonella* pathogenesis" expands on the previous section to discuss the regulation of *Salmonella* virulence, with emphasis on genetic and environmental regulators. The balance that determines health or disease of an individual is described in Part 5 - "Host innate defense and *Salmonella* resistance", which details host mechanisms to defend against the invading organism and elaborates on *Salmonella* evasion countermeasures. Part 6 - "*Citrobacter rodentium* pathogenesis" reviews the organism, the environmental niche and virulence factors of this pathogen. The final setion, Part 7 - "Introduction to the thesis" describes the rationale and objectives of the research.

CHAPTER 1

Literature review, rationale and objectives of the thesis

PART 1 - TWO-COMPONENT SIGNAL TRANSDUCTION

Bacteria live in unpredictable environments where nutrient levels, temperature, acidity, osmolarity and many other conditions are ever changing. Bacterial survival requires continuous monitoring of external conditions so as to adapt themselves accordingly. Consequently, bacteria have developed sophisticated signaling systems to rapidly adjust their structure, physiology or behavior in response to environmental changes (Stock *et al.*, 2000; Goudreau & Stock, 1998). These systems are comprised of signaling proteins, which are built from input or output domains and transmitter or receiver modules that can be integrated into a wide variety of cellular signaling circuits. The simplest circuit, called the two-component system (TCS) (Figure 1), consists of two proteins: a membrane-bound sensor kinase (SK) and a cytoplasmic response regulator (RR). Typically, the genes that code for cognate pairs of SKs and RRs are found together in a single operon, indicating a functional relationship between the two partners. The SK monitors a given environmental parameter through its N-terminal input domain. Once the stimulus is detected, the C-terminal transmitter module communicates the signal to the N-terminal receiver module of the RR. The RR is then activated and triggers the C-terminal output domain to down- or up-regulate the transcription of genes involved in chemotaxis, quorum sensing, nutrient uptake, nitrogen fixation, osmoregulation, sporulation and pathogenic host invasion.

TCS communication requires specific recognition between the cognate SK and RR to transfer information through phosphorylation and dephosphorylation reactions. TCS phosphotransfer pathways utilize histidine (His) and aspartic acid (Asp) residues as opposed to eukaryotic organisms that take advantage of serine, threonine and tyrosine phosphoester bonds. The modular nature of the TCS has the advantage of expanding a basic phosphotransfer pathway to a more complex phosphorelay pathway (Appleby *et al.*, 1996). A phosphorelay pathway has multiple His- and Asp-containing domains where the first domain is phosphorylated by a hybrid SK and relays its phosphoryl group to a second phosphotransfer domain that serves as the primary phosphoryl group donor for the RR (Figure 2). The arrangement of these modules may differ as the first three components may be on separate proteins, for example the

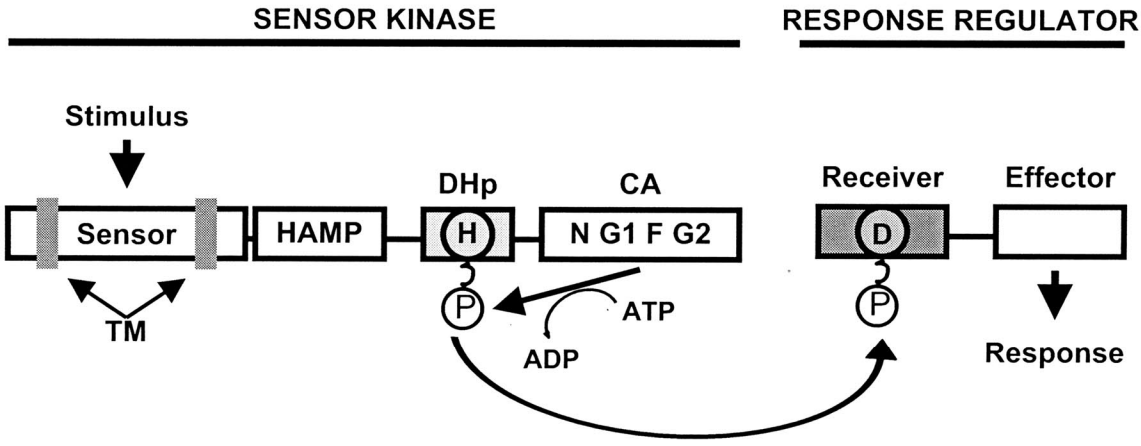


Figure 1. Schematic diagram of the classical two-component system.

The sensor kinase is typically a dimeric transmembrane (TM) protein with a conserved phosphodonor histidine (H) in the DHp (dimerization and histidine phosphotransfer) domain. Receipt of a stimulus activates autophosphorylation of the SK by the catalytic and ATP-binding (CA) domain. The phosphyl group is donated to the response regulator receiver domain, which carries the phosphoacceptor aspartate (D). Once active the response regulator effector domain binds DNA to modulate gene transcription.

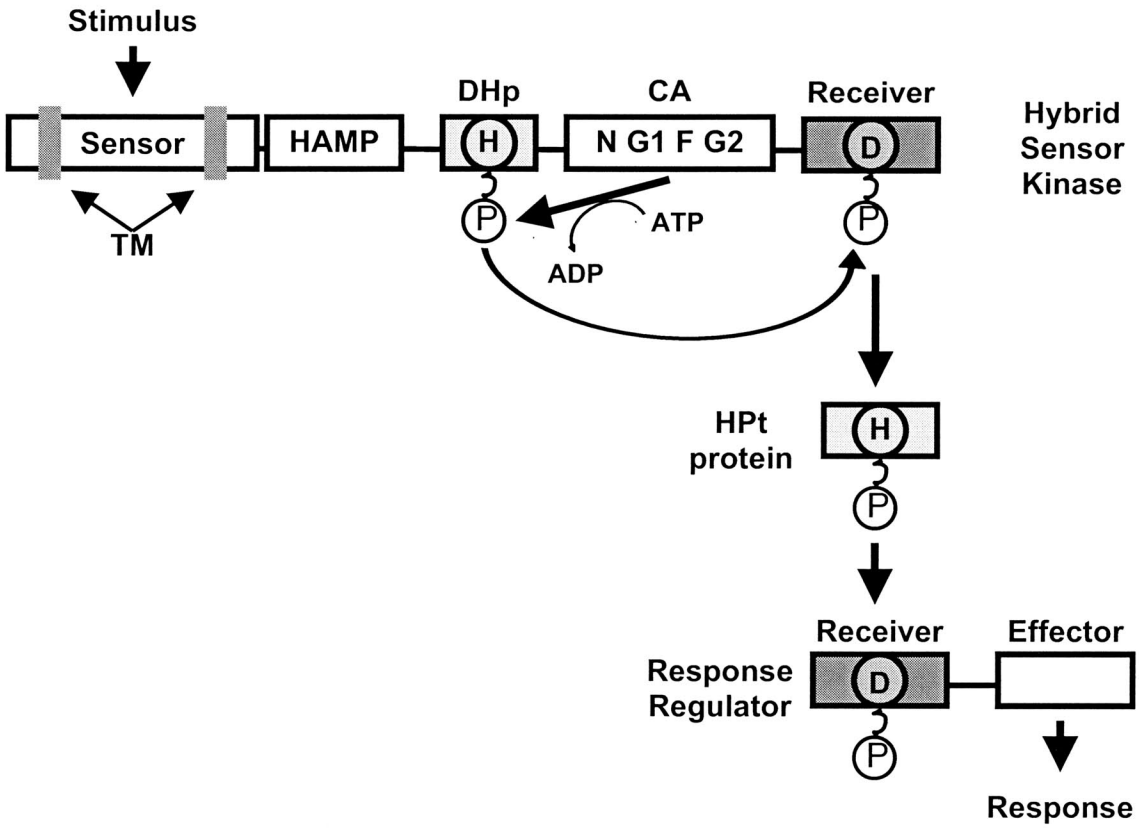


Figure 2. Schematic representation of a multi-component phosphorelay.

A hybrid sensor kinase autophosphorylates upon recognition of a specific stimulus. The phosphoryl group is transferred intramolecularly to a C-terminal receiver domain. A histidine phosphotransfer (HPt) protein will shuttle the phosphoryl group from the sensor kinase to the cognate response regulator that mediates the cellular response. Adapted from West & Stock, 2001.

sporulation control system of *Bacillus subtilis* (Hoch, 1993) or combined as a multi-domain protein, as in the *Bordetella pertussis* SK, BvgS (Uhl & Miller, 1994).

Genome sequence analysis indicates that TCSs are rare in eukaryotes but abound in bacteria, where they constitute the majority of signaling pathways (Stock *et al.*, 2000). The number of TCSs varies widely across bacterial species depending on the size of the genome and the complexity of its environmental niche (Galperin, 2005; Ulrich & Zhulin, 2007). Although, some bacteria such as *Mycoplasma* have no TCSs, most contain several dozen (*Escherichia coli* and *Bacillus anthracis*) while relatively few contain over 100 TCSs, these include *Myxococcus xanthus* and *Cyanobacteria* (Gao *et al.*, 2007).

The modular design of bacterial TCSs allows for versatility and adaptability but their absence from animals and involvement in essential bacterial processes makes them an ideal therapeutic target. To date, a small number of inhibitors have been designed to interfere with the autophosphorylation of SKs (Matsushita & Janda, 2002). Structure-based drug design has been used to screen for potential inhibitors of YycG, a SK from *Staphylococcus epidermidis* (Qin *et al.*, 2006). One inhibitor displays significant *S. epidermidis* killing but more importantly has a bactericidal effect on the bacteria within mature biofilms (Qin *et al.*, 2007). A screen of small organic molecules identified an inhibitor (LED209) of the enterohemorrhagic *Escherichia coli* SK QseC (Rasko *et al.*, 2008). QseC activates virulence genes upon recognition of host-derived adrenergic signals and bacterial autoinducer-3 (Hughes & Sperandio, 2008; Sperandio *et al.*, 2003). LED209 inhibits binding of specific signals to QseC, thus halting virulence gene activation without affecting bacterial growth (Rasko *et al.*, 2008). Targeting drugs to inhibit virulence instead of growth is a novel strategy that provides less selective pressure for the generation of microbial resistance (Hung *et al.*, 2005; Cegelski *et al.*, 2008; Hughes & Sperandio, 2008). Additionally, homologues of QseC are present in at least 25 other plant and human pathogens including *Salmonella enterica* (Bearson & Bearson, 2008) and *Francisella tularensis* (Weiss *et al.*, 2007). Administering LED209 to mice infected with either *S. enterica* or *F. tularensis* increased the longevity of these mice as compared to the untreated controls (Rasko *et al.*, 2008). As an alternative to inhibitors that block SKs, it is hoped

that a targeted approach to specific RRs will also prove to be a new avenue of drug research (Gao *et al.*, 2007).

SENSOR KINASES

The SK superfamily is characterized by a highly conserved and uniquely folded kinase core that is necessary for ATP binding and catalyzes the phosphorylation of a His side chain. Preceding this sequence is a highly divergent sensing domain that provides the SK with a specific regulatory function. Typically SKs are membrane-bound homodimers that regulate the two-component pathway through strict control of the RR's phosphorylated state.

Activity of the SK is dependent on detection of an external signal through the sensor domain. Recognition of the specific ligand leads to a conformational change that is propagated downward through the transmembrane (TM) helices and linker domain to align the catalytic domain with the conserved His residues. Autophosphorylation of the SK dimer occurs in *trans* with one SK monomer catalyzing the addition of a phosphoryl group from ATP to the His residue in the second monomer (Yang & Inouye, 1991). The second reaction in the pathway transfers the phosphoryl group from the SK-His residue to a conserved Asp residue in the RR. Lastly, the phosphatase activity of the SK towards its cognate RR hydrolyzes the phosphoester bond of the phosphoryl group (Aiba *et al.*, 1989). The conserved His residue plays a critical role in both kinase and phosphatase activities for example, mutation of this amino acid in the osmosensing SK, EnvZ, abrogates both catalytic activities (Zhu *et al.*, 2000). It is clear that the kinase and phosphatase activities are not independent of each other but share the same active site containing the conserved His of the SK (Zhu *et al.*, 2000).

Net phosphorylation of the RR is the result of a balance between the kinase and phosphatase activities of the SK (Russo & Silhavy, 1991; Yang & Inouye, 1991). The phosphorylated form of the RR dominates when the SK kinase activity is "on" whereas an active SK phosphatase activity results in the dephosphorylated form of the RR to dominate the cell. The two SK activities are antagonistic and tightly regulated by ligand recognition (Russo & Silhavy, 1993). For example, binding of the signal

transduction protein PII to the *E. coli* nitrogen regulator NRII (or NtrB) inhibits the kinase activity while increasing the phosphatase activity of the SK (Jiang & Ninfa, 1999; Jiang *et al.*, 2000). Stimulation of the phosphatase activity is not simply the result of dampening the kinase activity but results from PII binding causing a conformational change that is transmitted to the catalytic domain that assumes a phosphatase dominant conformation (Jiang *et al.*, 2000).

A mechanistic understanding of how a membrane-embedded SK converts recognition of specific signal(s) to regulation of catalytic activities is currently limited. The crystallization of individual domains has provided much structural information; however, without a full-length three-dimensional SK structure the mechanism of signal propagation remains elusive.

The extracellular sensor domain

SKs can detect a variety of chemical and physical stimuli that is reflected by the extensive structural diversity of their sensing domains. Concurrently, the periplasmic domain structures of *Klebsiella pneumoniae* CitA and *E. coli* DcuS were solved (Reinelt *et al.*, 2003; Pappalardo *et al.*, 2003). Both of these SKs were shown to have a Per-Ant-Sim (PAS) domain fold that before then had only been described in cytoplasmic proteins, such as the oxygen sensor FixL (Gong *et al.*, 1998). Thus far, more than 1100 proteins have been found to have a PAS domain, which transforms an input signal into a conformational change that ultimately results in a global subunit-subunit interaction (Taylor & Zhulin, 1999). The PAS family has a very low sequence identity but members share a conserved central β-sheet structure and a length of ~130 residues (Gilles-Gonzalez & Gonzalez, 2004). The mechanism of signal input and transmission by PAS domains is of great interest but remains poorly defined.

Structural studies of the *E. coli* phosphodiesterase DOS and the LOV2 (light-oxygen-voltage) domain from a light-activated plant kinase (phototropin) provide insight into mechanisms of PAS domain mediated signal propagation. Comparing the crystal structures of the inactive (Fe^{3+}) and active (Fe^{2+}) DOS homodimer indicates a change in the relative orientation of the two subunits (Kurokawa *et al.*, 2004). In the inactive state, the heme iron is ligated to a His residue, its subsequent reduction causes

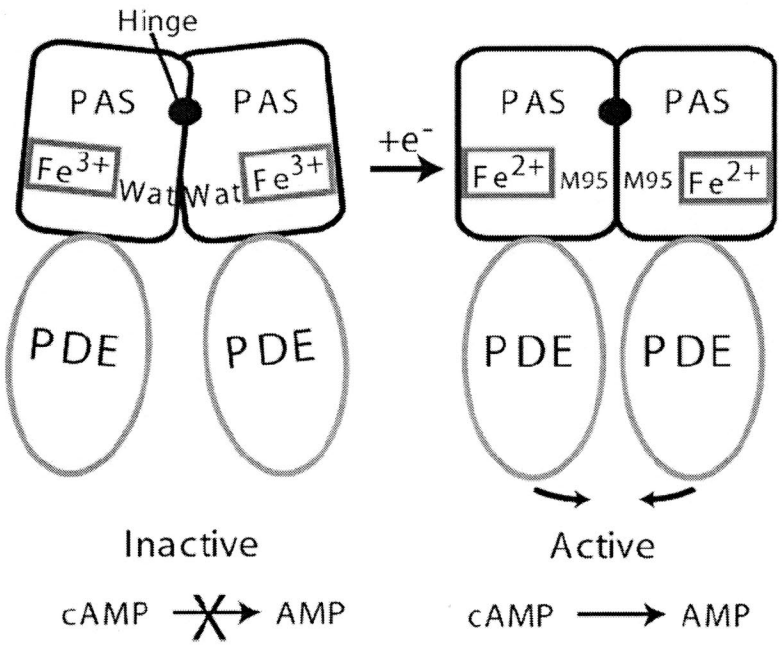


Figure 3. Proposed model for the regulation of the phosphodiesterase (PDE) activity of the *Esherichia coli* DOS.

Redox-induced scissor-type motion of the *E. coli* DOS homodimer alters the relative position of the PDE domain to switch the catalytic activity on and off.

Figure from Kurokawa *et al.*, 2004.

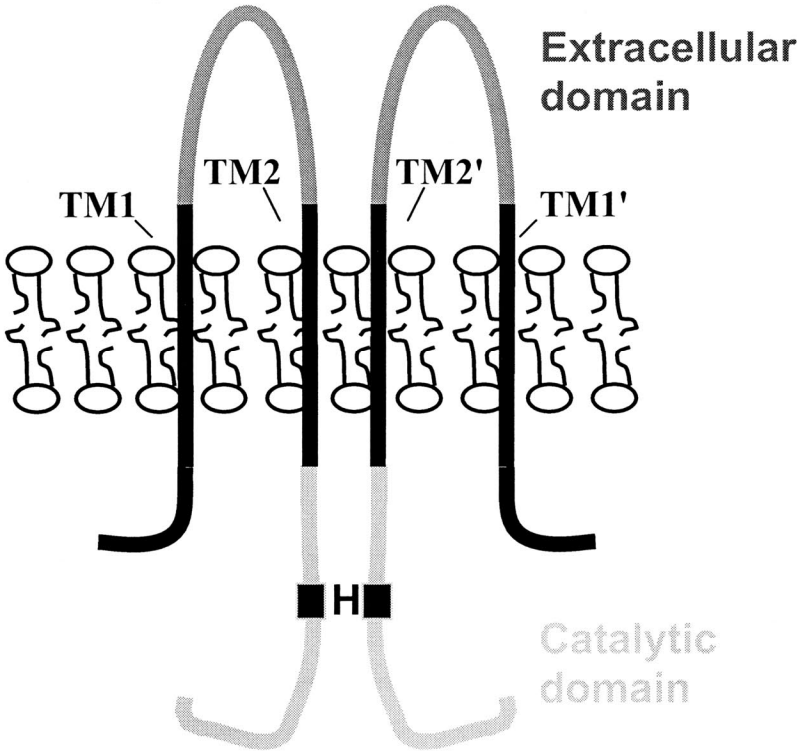


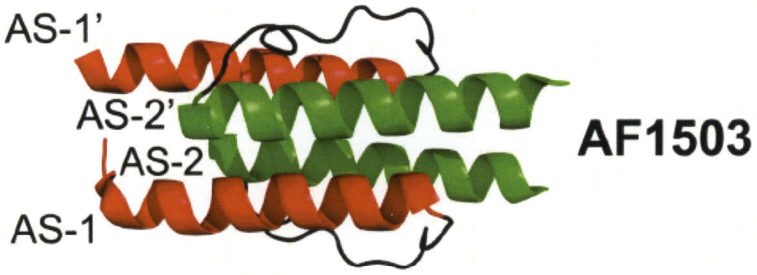
Figure 4. Schematic representation of a membrane-embedded sensor kinase homodimer. The transmembrane helices form a 4-helix bundle and play an important role in transduction of signal from the extracellular domain to the catalytic domain.

the heme to be transferred to a methionine residue in the FG loop. The flexible FG loop becomes rigid and the subunits rotate relative to each other in a "scissor-type" motion that controls downstream signaling and activation of the phosphodiesterase activity (Figure 3). In contrast, the LOV2 domain undergoes light-induced structural changes whereby two strands (Hß and Iß) within the PAS core will displace the Jα helix (Harper *et al.*, 2003). In the dark (inactive) the Jα helix associates with the core but upon light emission the helix is destabilized activating the kinase activity. These activation mechanisms probably extend into other PAS domains despite the diversity of known cofactors and signals (Khorchid & Ikura, 2006).

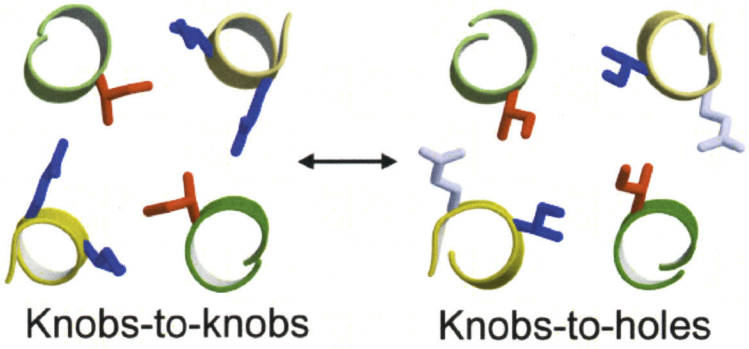
The transmembrane domain

Soluble extracellular ligands are perceived by the extracellular domain that is frequently flanked on either side by two TM segments (TM1 and TM2) (Hoch, 2000). In the SK dimer, each monomer contributes two TMs (TM1, TM2, TM1′ and TM2′) (Figure 4). Current information on the mechanism of TM signaling has been provided by studies of the aspartate receptor (Tar) and the ribose and galactose receptor (Trg). This has been accomplished through the use of site-directed cysteine (Cys) replacement and sulfhydryl chemistry. *In vitro* studies of Tar reveal that signaling is blocked when TM1-TM2 (Chervitz & Falke, 1995) but not TM1-TM1′ (Chervitz *et al.*, 1995) are immobilized by engineered disulfide bridges. The induction of disulfide linkages formation between the TM helices of Trg in the presence and absence of ligand results in the formation of the same 19 TM Cys pairs spanning the neighboring helices (Hughson & Hazelbauer, 1996). Identical cross-linking in the absence and presence of ligand indicates a very small movement between the TM helices. Interestingly, the rate of disulfide bond formation clearly demonstrates that ligand occupancy alters the rate of cross-linking solely between the TM1-TM2 pair (Hughson & Hazelbauer, 1996). Together these results show that a subtle conformational change of TM2 occurs relative to TM1 while a stable interaction is maintained across the interface between the homologous helices in the TM domain (TM1-TM1′) (Khorchid & Ikura, 2006).

A.



B.



C.

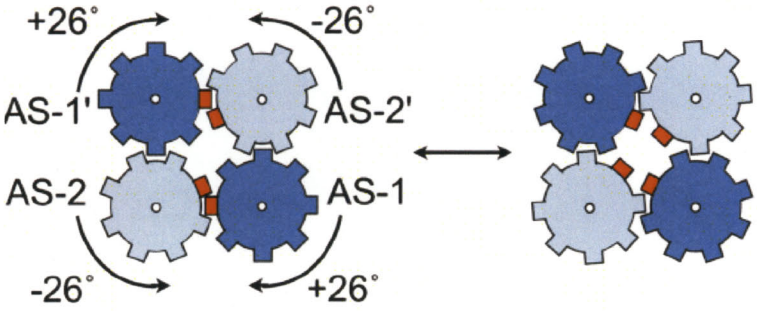
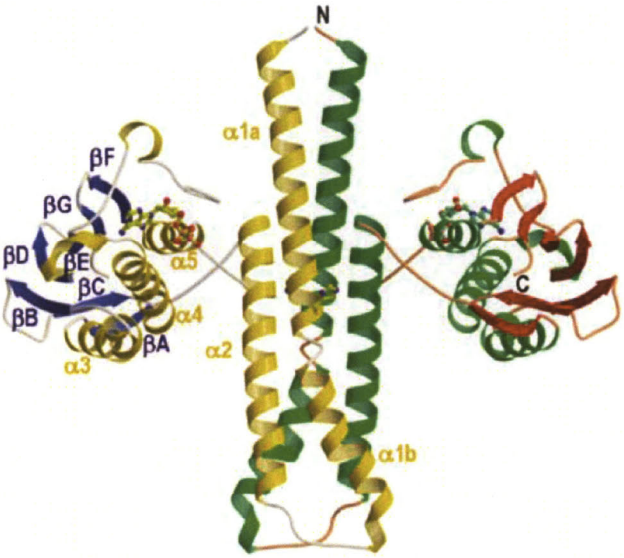


Figure 5. The Af1503 HAMP domain as a model for SK HAMP domains.

A. Crystal structure of the *Archaeoglobus fulgidus* Af1503 HAMP domain. **B.** The knobs-to-knobs packing state of the Af1503 HAMP domain is converted to a knobs-to-holes by a 26° rotation of the helices. **C.** Proposed direction of rotation of interlocking helices in the Af1503 HAMP domain

Figure from Hulko *et al.*, 2006.

A.



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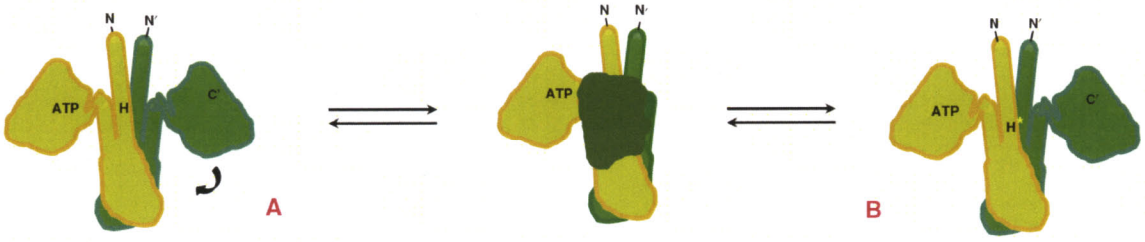

Figure 6. The structure of the sensor kinase catalytic core.

A. Crystal structure of the intact catalytic core from the *Thermotoga maritima* sensor kinase TM0853. **B.** Schematic representation of the proposed mechanism of SK autophosphorylation (A→B). The N- and C-termini, ATP and the conserved His residue are indicated on the SK dimer (orange and green). The phosphoryl group is indicated by a yellow asterisk.

Figures from Marina *et al.*, 2005.

Several mechanisms have been proposed for signal transmission by the TM helices. The planarity of the membrane limits the motion to include rotation, piston-like displacement or pivot movement (Moukhametzianov *et al.*, 2006; Scott & Stoddard, 1994). Regardless of the specific motion, the movement is presumed to be subtle with a 1-2 Å piston displacement (Hughson & Hazelbauer, 1996; Bass & Falke, 1999) or a ~ 4° rotation of the TM2 signaling helix (Cochran & Kim, 1996; Kwon *et al.*, 2003).

The HAMP domain

The SK linker domain connects TM2 and the cytoplasmic domain and is known to play an important role in signal transduction. However, the linker domain is capable of responding to environmental cues, such as osmotic stress. A classic example is the *E. coli* osmosensing SK EnvZ, which is membrane-bound and contains a periplasmic domain. Removal of the EnvZ periplasmic domain results in a SK that maintains its response to osmotic stress (Leonardo & Forst, 1996). Osmosensing is abrogated by mutation of the EnvZ linker domain and when the protein is not integrated into a membrane, which serves to fix the linker in place (Park & Inouye, 1997). Secondary structural analysis of different signal-transducing proteins reveals a highly conserved helix-turn-helix fold although the amino acid sequence has low homology. The motif is called HAMP for the proteins that carry the domain: histidine kinases, adenylylcyclases, methyl-accepting chemotaxis proteins and phosphatases (Aravind & Ponting, 1999).

Although the Smart database identifies HAMP domains in 30% of all SKs, direct structural analysis remains elusive. It has been proposed that lack of a stable crystal structure indicates frailty of the HAMP domain, which might be important for signal transduction (Kishii *et al.*, 2007). Exchanging HAMP domains between receptors, for example, between the SKs CpxA and NarX and between the SK EnvZ and the chemoreceptor Tar (Appleman & Stewart, 2003; Utsumi *et al.*, 1989) maintains the same activity as the native form. This indicates that HAMP domains have a similar mode of action, even though they have divergent amino acid sequences.

Hulko *et al.*, published the first structure of a HAMP domain from the hyperthermophilic archeaon, *Achaeoglobus fulgidus* protein Af1503, a putative TM receptor. Although not a SK, this structure provides information on the conformational change that leads to signal transduction. The HAMP domain is a dimer, where each monomer contributes two helices to form a parallel four-helix coiled-coil, a structure that is traditionally characterized by knobs-into-holes packing (Figure 5A). Interestingly, Af1503 exhibited a knobs-to-knobs packing state (Figure 5B). The proposed mechanism of signal transduction occurs through a 26° rotation of the individual helices that changes the packing state from a knobs-to-knobs into a knobs-to-holes (Figure 5C). Structural and functional studies show that equilibrium between these two packing states is dependent on the side chain of residue 291. Mutation A291V will bias the conformation towards a knobs-to-holes state (Hulko *et al.*, 2006). A chimera created from the *E. coli* Tar receptor and the Af1503 HAMP domain results in active Tar-mediated signaling, whereas replacement with the Af1503 A291V mutant results in an inactive Tar receptor. Corroborating evidence from true SKs, EnvZ and NarX indicate that mutation of the residue corresponding to A291V favor the kinase activity (Appleman & Stewart, 2003; Tokishita *et al.*, 1992).

The HAMP domain is responsible for converting an upstream motion of the TM helices into a downstream rotation. Considering the vast number of HAMP domains, it is likely that they are adapted to process a variety of different signals.

The catalytic core

The cytoplasmic portion of the SK is highly conserved in sequence and structure among family members. The catalytic core contains two functionally distinct domains being the N-terminal dimerization and histidine phosphotransfer (DHp) domain and the catalytic and ATP-binding (CA) domain at the C-terminus. Autophosphorylation occurs in the H box of the dimeric DHp domain and is mediated by the CA domain in the presence of ATP (Tomomori *et al.*, 1999). The CA domain is monomeric and encompasses the highly conserved sequence motifs N, G1, F and G2 (Parkinson & Kofoid, 1992). X-ray crystallography of the CA domains from *Thermotoga maritima* CheA, and *E. coli* PhoQ and NtrB (Bilwes *et al.*, 1999; Marina

et al., 2001; Song *et al.*, 2004) indicate a mixed α/β sandwich fold that is structurally related to the ATP-binding domains of GyraseB, Hsp90 and MutL (GHL) ATPase family (Dutta & Inouye, 2000).

In 2005, the first intact SK cytoplasmic domain was crystallized (Figure 6A) and provided the first picture of the interdomain interactions between the DHp and CA domains (Marina *et al.*, 2005). According to the structure the His residue and the ATP nucleotide are at a distance of 25 Å and at an angle that makes the phosphotransfer reaction impossible (Marina *et al.*, 2005). Figure 6 illustrates the proposed model of the SK autokinase activity. In the unphosphorylated state, the CA domain is loaded with ATP, awaiting sensor domain activation, at which point the CA domain swings into the DHp domain via the α2 helix, which acts as a flexible hinge. This conformational change brings the ATP and His residue into close proximity and enables the phosphostransfer. Once phosphorylated, the conformation of the kinase core relaxes and the CA domain vacates its position to allow the RR access to the phosphorylated His residue (Figure 6B).

RESPONSE REGULATORS

A search of approximately 400 bacterial and archaeal genome sequences provides a list of over 9000 RRs (Ulrich & Zhulin, 2007; Galperin, 2006). Most RRs have two domains: the N-terminal receiver domain and the C-terminal effector domain. RRs are classified based on the sequence similarity of their effector domain. Sixty percent of all RRs fall into the DNA-binding subfamily while the other RR subfamilies are characterized as having enzymatic, protein-protein interaction or RNA-binding domains (Galperin, 2006). Crystallization of a full-length RR has been hampered by the flexible linker and consequently there is only one structure available (Buckler *et al.*, 2002). RR activation occurs when the cognate SK phosphorylates the receiver domain. However, the mechanism by which phosphorylation activates the RR remains unclear and appears to vary between individual RRs. The phosphorylation-induced conformational change appears to result in the release of the inhibitory closed-conformation and/or dimerization of the RR. Full-length *E. coli* PhoP in

solution appears as a monomer in the inactive state and as a dimer in the active state (Bachhawat & Stock, 2007).

The receiver domain is structurally conserved and consists of a five stranded parallel β–sheet surrounded by five amphipathic helices. The highly conserved residues cluster in and around the active-site cleft (Robinson *et al.*, 2000). The chemotaxis protein, CheY, serves as a representative model for RR receiver domains (Stock *et al.*, 1990; Volz, 1993). The phosphoaccepting Asp57 residue is surrounded by Asp 12 and Asp13 which participate in the phosphotransfer reaction by binding the necessary Mg^{2+} ion. The receiver domain actively participates in the phosphotransfer reaction as demonstrated *in vitro* by the fact that the small molecule, acetyl phosphate, can act as a phosphodonor for CheY (Lukat *et al.*, 1992). Even though small molecules are capable of phosphorylating RRs the reaction occurs more rapidly in the presence of the cognate SK (Zapf *et al.*, 1996; Mayover *et al.*, 1999). In addition, RRs possess an autophosphatase activity that limits the lifetime of the phosphorylated RR from seconds to hours, depending on the RR's physiological function (West & Stock, 2001).

The variable DNA-binding effector domains are further subdivided into three major families based on their structural homology: the OmpR/PhoB winged-helix domains, the NarL/FixJ four-helix domains and the NtrC ATPase-coupled transcription factors (Stock *et al.*, 1989). Members of the OmpR/PhoB subfamily are the most abundant RRs and their DNA-binding domains consist of a winged helix-turn-helix (wHTH) domain (Kenney, 2002). The wHTH domain interacts with the major groove of DNA through the recognition helix that is flanked by two loops or "wings", which interact with the minor grooves. Regardless of the structural similarity between this subfamily different mechanisms of action will alter gene expression. For example, OmpR initiates transcriptional activation by interacting with the α-subunit of the RNA polymerase (Garrett & Silhavy, 1987; Matsuyama & Mizushima, 1987) while PhoB interacts with σ^{70} (Makino *et al.*, 1993; Kumar *et al.*, 1994).

PART 2 - THE PHOP/PHOQ TCS

Pathogenic bacteria necessitate the use of TCSs to coordinate the expression of virulence factors in a precise and timely fashion. The PhoP/PhoQ TCS was first identified through a screen for mutants with elevated nonspecific phosphatase activity (Kier *et al.*, 1979). PhoP/PhoQ is a prototypical TCS composed of a transmembrane SK, PhoQ and a soluble RR, PhoP that modulates gene transcription. PhoP/PhoQ TCSs have been identified in many pathogenic *Enterobacteria* including mammalian pathogens such as *Salmonella enterica* (Miller *et al.*, 1989), *Escherichia coli* (Kasahara *et al.*, 1992), *Yersina pestis* (Oyston *et al.*, 2000), *Yersinia pseudotuberculosis* (Flamez *et al.*, 2007), *Pseudomonas aeruginosa* (Ernst *et al.*, 1999), *Providencia stuartii* (Rather *et al.*, 1998), and *Shigella flexneri* (Moss *et al.*, 2000), the insect pathogen *Photorhabdus luminescens* (Derzelle *et al.*, 2004), and the plant pathogens *Erwinia caratova* (Flego *et al.*, 2000) and *Erwinia chrysanthemi* (Llama-Palacios *et al.*, 2003). PhoP/PhoQ is a signal transduction pathway that is central to the virulence of most of these pathogens, since deletion of the *phoP* and/or *phoQ* genes usually results in attenuation of virulence in their respective hosts.

The most extensively studied PhoP/PhoQ system is in *Salmonella enterica* serovar Typhimurium (hence referred to as *S. typhimurium*) where it has been directly associated with the virulence of this bacterium (Groisman *et al.*, 1992). *S. typhimurium* PhoP/PhoQ actively regulates gene transcription within host macrophage phagosomes (Alpuche Aranda *et al.*, 1992), the mouse intestinal lumen and tissue (Merighi *et al.*, 2005), indicating that PhoQ recognizes signal(s) within these environments.

The sensor kinase PhoQ

S. typhimurium PhoQ is 487 amino acids in length and contains a periplasmic domain of 146 residues that senses environmental changes (Miller *et al.*, 1989). The structure of the periplasmic domain contains an unusual PAS fold that is characterized by two novel α-helices (α4/α5) instead of a small molecule binding cleft (Cho *et al.*, 2006). The helices contribute negatively charged residues to an acidic cluster of amino acids (EDDDDAE), which are involved in ligand binding (Figure 7). The periplasmic

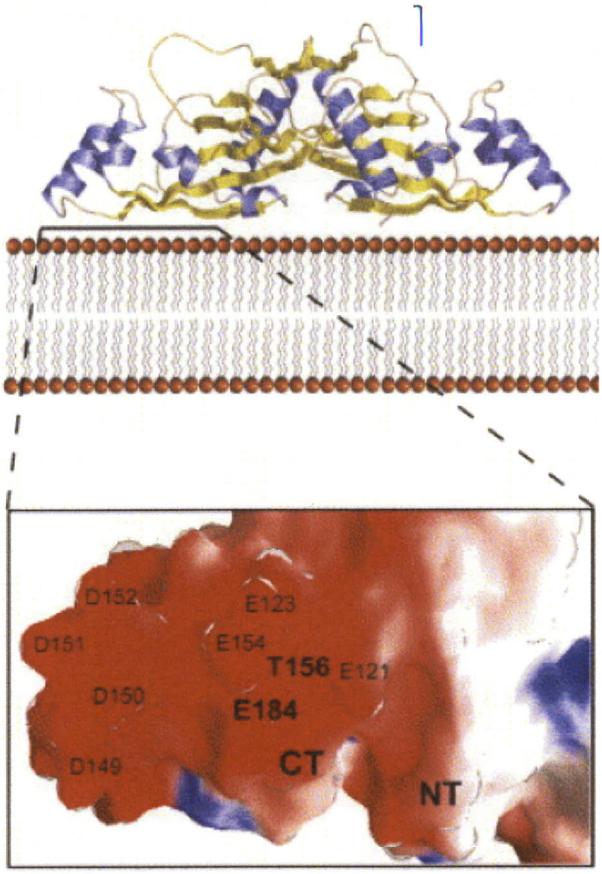

Figure 7. Crystal structure of the dimeric PhoQ periplasmic domain.

The PhoQ dimer forms a flat surface that comes into close contact with the membrane. The inset shows a highly negatively charged cluster of amino acids that participate in the biding of divalent cations. Antimicrobial peptides compete with divalent cations for binding to PhoQ. The location of the acidic cluster suggests that PhoQ contacts the membrane through a series of divalent-cation bridges.

Figure form Bader *et al.*, 2005.

domain is essential for signal recognition since truncated mutants are unable to respond to PhoQ ligands, Mg^{2+} and antimicrobial peptides (AMP) (Castelli *et al.*, 2000; Montagne *et al.*, 2001; Bader *et al.*, 2005).

PhoQ ligand – Divalent cations

PhoQ specifically senses Mg^{2+}, Ca^{2+} and Mn^{2+} but not other divalent cations, such as Ni^{2+}, Cu^{2+}, Co^{2+} and Ba^{2+} (Garcia Vescovi *et al.*, 1996). Divalent cation-depleted media results in an upregulation of PhoP-activated gene expression (Garcia Vescovi *et al.*, 1996). In contrast, the presence of millimolar concentrations of Mg^{2+}, Ca^{2+} or Mn^{2+} represses the expression of PhoP-activated genes (Garcia Vescovi *et al.*, 1996). *In vitro*, Mg^{2+} has been shown to bind the PhoQ periplasmic domain (Waldburger & Sauer, 1996; Vescovi *et al.*, 1997). PhoQ binding Mg^{2+} causes elevated levels of unphosphorylated PhoP, which is the direct result of an increase in PhoQ phosphatase activity (Castelli *et al.*, 2000; Montagne *et al.*, 2001). *In vivo* experiments suggest that Ca^{2+} binds differently to the PhoQ periplasmic domain as Ca^{2+} does not effectively repress PhoP-activated genes as well as Mg^{2+} (Vescovi *et al.*, 1997).

The PhoQ periplasmic domain was crystallized in the presence of Ca^{2+} ions and provides information on the repressed state structure of the SK (Cho *et al.*, 2006). Based on the positioning of the N- and C-termini the acidic cluster of amino acids is predicted to face the negatively charge phospholipid membrane (Figure 7). Consequently, the charge repulsion pushes the PhoQ periplasmic domain away from the membrane. In the presence of Ca^{2+}, the charge repulsion is alleviated because the ions form bridges between the PhoQ acidic cluster and the phospholipid membrane. NMR analysis in the absence of divalent cations produces a spectrum with a degree of flexibility whereas the presence of Mg^{2+} or Ca^{2+} stabilizes the conformation (Cho *et al.*, 2006). The wealth of structural and biochemical data has produced a model for Mg^{2+} repression of PhoQ (Prost & Miller, 2008). Helices $\alpha4/\alpha5$ are repulsed away from the membrane in the absence of divalent cations. This action leads to a structural flexibility of the PhoQ periplasmic domain that is translated downwards to the cytoplasmic domain to induce autophosphorylation.

Originally, Mg^{2+} was believed to be the primary signal that mediates PhoP/PhoQ activation within the macrophage phagosome because *S. typhimurium phoPQ* mutants are unable to grow under Mg^{2+}-depleted conditions and PhoP upregulates the expression of inducible Mg^{2+} transporters (Soncini *et al.*, 1996). Additionally, the concentration of Mg^{2+} in phagosomes was assumed to be in the micromolar range. In 2006, it was demonstrated that within 30 minutes of *S. typhimurium* being engulfed within the phagosome the concentration of Mg^{2+} stabilizes at 1 mM (Martin-Orozco *et al.*, 2006). This divalent cation concentration represses the activity of PhoQ so it seems unlikely that Mg^{2+} is the activating signal within the phagosome. Ca^{2+} can also be eliminated as a physiological ligand because the concentration increases as the phagosome acidifies and finally settles between 0.5-1 mM (Christensen *et al.*, 2002).

PhoQ ligand – Antimicrobial peptides

To this end, more physiologically relevant ligands present during a *S. typhimurium* infection have been examined as possible activators of PhoQ. Macrophage phagosomes contain AMPs that play an important role in controlling *S. typhimurium* replication *in vivo* (Rosenberger *et al.*, 2004). In the presence of low millimolar concentrations of Mg^{2+}, subinhibitory concentrations of cationic AMPs activate PhoQ (Bader *et al.*, 2003; Bader *et al.*, 2005). AMPs cause bacterial membrane damage, which activates gene transcription by RpoS whereas, PhoQ activation is the direct result of AMP binding to the periplasmic domain. Reconstituting the PhoP/PhoQ system in liposomes in the presence of AMPs results in an increase in PhoP phosphorylation by PhoQ, even in the presence of 1 mM Mg^{2+} (Bader *et al.*, 2005). Cationic AMPs are positively charged and attracted to the acidic amino acid cluster of PhoQ. Interestingly, high millimolar concentrations of Mg^{2+} are able to compete with AMPs for binding to the PhoQ periplasmic domain suggesting that they both share the same binding site. This data is consistent with the proposed model of PhoQ activation that AMPs will force apart PhoQ and the membrane similar to the charge repulsion by virtue of it binding to the acidic face and larger than divalent cations (Prost & Miller, 2008).

It is unclear whether or not PhoQ from other pathogenic species sense the same signal(s) since direct ligand activation has not been studied. It has been suggested that other PhoPQ systems are able to respond to the presence of AMPs (Prost & Miller, 2008) as *E. chrysanthemi*, *S. flexneri* and *P. luminsecens phoPQ* mutants are more susceptible to the presence of cationic AMPs, as compared to wild-type (Llama-Palacios *et al.*, 2003; Derzelle *et al.*, 2004; Moss *et al.*, 2000). The PhoQ periplasmic domain amino acid sequences of these three pathogens align with *S. typhimurium* and include the α4/α5 helices.

In contrast to these eukaryotic-associated bacteria the opportunistic pathogen *P. aeruginosa* typically inhabits soil and water. Alignment of the *S. typhimurium* and *P. aeruginosa* PhoQ amino acid sequences reveal a high degree of divergence in the periplasmic domain, which suggests different mechanisms of signal detection (Lesley & Waldburger, 2001). *P. aeruginosa* PhoQ lacks the α4/α5 helices and even though PhoQ is repressed by divalent cations, it is not activated by AMPs (Prost *et al.*, 2008; Bader *et al.*, 2005). Although soil dwelling organisms encounter AMPs (Polymyxin B) produced by other microorganisms (*Bacillus polymyxa*) the diversity is less extensive than in plant and animal hosts. Evolution of the PhoQ acidic face coincided with those bacteria having to sense and respond to multiple signals within a complex eukaryotic environment (Prost & Miller, 2008). The competition between divalent cations and AMPs may serve to determine the subcellular environment of the invading microbe during the infection cycle (Zwir *et al.*, 2005).

The response regulator PhoP

PhoP is categorized as a member of the OmpR/PhoB subfamily (Volz, 1993) with 35% identity and 76% similarity to PhoB. PhoP is a 26.2 kDa protein containing an N-terminal regulatory domain and C-terminal DNA-binding domain tethered together by a flexible linker. Transcriptional regulation of many PhoP genes is complex and involves several regulatory circuits (Navarre *et al.*, 2005; Barchiesi *et al.*, 2008; Kong *et al.*, 2008) Many PhoP-regulated genes harbor the so-called PhoP box (Soncini *et al.*, 1995). Two direct repeats, T/GGTTTA, separated by a 5-nucleotide spacer make up the PhoP box (Kato *et al.*, 1999) to which the PhoP dimer binds. The

PhoP box is usually found within the -35 region of a promoter, for example the *mgtA* promoter. However, PhoP boxes have also been identified in the opposite orientation and at various distances from the RNA polymerase binding site (Zwir *et al.*, 2005).

The PhoP/PhoQ regulon

In 1990, two-dimensional SDS-PAGE analysis determined the PhoP/PhoQ regulon to comprise 40 genes (Miller & Mekalanos, 1990), presently with the advance of microarray analysis the estimate is over 200 genes (Monsieurs *et al.*, 2005). The PhoP/PhoQ is a master regulator of virulence that governs a regulon of genes that includes those necessary for: magnesium transport, LPS modification and survival within macrophages (Fields *et al.*, 1989; Behlau & Miller, 1993; Soncini *et al.*, 1996; Guo *et al.*, 1997; Bearson *et al.*, 1998; Adams *et al.*, 2001).

Mg^{2+} transport

PhoPQ controls the expression of genes required for the growth in Mg^{2+}-deficient environments (Soncini *et al.*, 1996). *S. typhimurium* encodes three Mg^{2+} transporters, *corA*, *mgtA* and *mgtB* (Smith & Maguire, 1998), the latter two are PhoP-regulated. MgtA and MgtB are P-type ATPases that independently take up Mg^{2+} (Tao *et al.*, 1995). *Salmonella* requires both MgtA and MgtB for growth in low Mg^{2+} liquid media (Soncini *et al.*, 1996).

LPS Modification

PhoP/PhoQ governs the transcription of genes that modify LPS, specifically the lipid A component, these include *pagP*, *lpxO* and *pagL* (Figure 8). The outer membrane protein, PagP adds palmitate to lipid A. Interestingly, PagP homologues have been identified in *Yersinia* and *Bordetella* species, and *E. coli* and although *Pseudomonas* can add palmitate it lacks a PagP homologue (Bishop *et al.*, 2000). LpxO catalyzes the incorporation of a hydroxyl group into lipid A (Gibbons *et al.*, 2000). Finally, it has been shown that lipid A deacylase activity is the result of PagL (Trent *et al.*, 2001).

A subset of LPS modification genes are regulated by another TCS, PmrA/PmrB (Roland *et al.*, 1993). PmrA is the RR and PmrB is the SK that responds to extracellular ferric iron (Fe^{3+}) and acidic pH (Wosten *et al.*, 2000; Perez & Groisman, 2007). PmrA/PmrB mediates the addition of ethanolamine and aminoarabinose (L-Ara4N) to lipid A through the upregulation of *pmrC*, *pbgP* and *ugd* (Gunn *et al.*, 1998; Guo et al., 1997). The first step of the L-Ara4N modification requires the *ugd* gene product, a UDP-glucose dehydrogenase to synthesize the L-Ara4N precursor. The addition of L-Ara4N to lipid A is performed by the enzymes, which are encoded within the *pbgP* operon (*pmrHFIJKLM*) (Figure 8 and 9). These modifications of the lipid A part of LPS are indirectly regulated by the PhoPQ TCS (Figure 9). PhoP/PhoQ regulates the expression of the connector protein, PmrD that binds and stabilizes phosphorylated PmrA so the RR will continue to transcribe PmrA-regulated in the presence of PhoP/PhoQ-activating signals (Kato & Groisman, 2004; Kox *et al.*, 2000).

Survival within macrophages

LPS modifications contribute to *Salmonella* survival within the macrophage phagosome by increasing resistance to AMPs (Fields *et al.*, 1989). *In vitro* and *in vivo* intramacrophage survival also requires the PhoP-regulated gene *mgtC* (Blanc-Potard & Groisman, 1997). An *mgtC* mutant is unable to grow in a Mg^{2+}-depleted environment so is predicted to function in Mg^{2+} acquisition (Blanc-Potard & Groisman, 1997).

Interestingly, the intacellular pathogen *Mycobacterium tuberculosis* has a MgtC homologue that is required for growth in low Mg^{2+} environments and within the macrophage (Buchmeier *et al.*, 2000). However, the phylogenetically closely related *E. coli* lacks the *mgtC* gene and is unable to grow in low Mg^{2+} concentrations (Blanc-Potard & Groisman, 1997). This suggests that the acquisition of *mgtC* aided in promoting the development of *Salmonella* as an intracellular pathogen (Groisman, 2001).

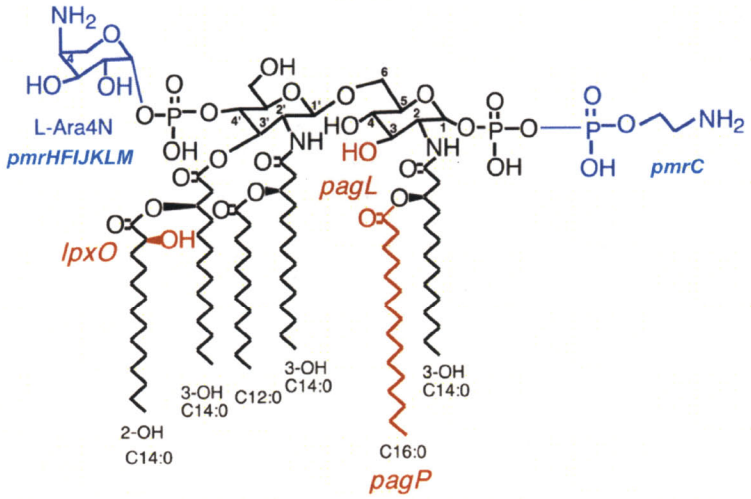

Figure 8. PhoPQ-mediated modifications of LPS in *Salmonella typhimurium*.

The PhoPQ-mediated (red) and the PmrAB-mediated (blue) structural modifications of the lipid A component of LPS are shown. Structural changes include the addition of aminoarabinose (L-Ara4N) by the proteins encoded by the *pmrHFIJKLM* operon and phosphoethanolamine (PEtN) by the *pmrC* gene product. The *pagP* gene mediates the addition of palmitate (C16). Deacylation of the 3 position 3-hydroxymyristate (3-OH C14) is carried out by the *pagL* gene product. Finally, the *lpxO* gene product hydroxylates the fatty acid at position 3' of lipid A.

Figure adapted from Prost *et al.*, 2007.

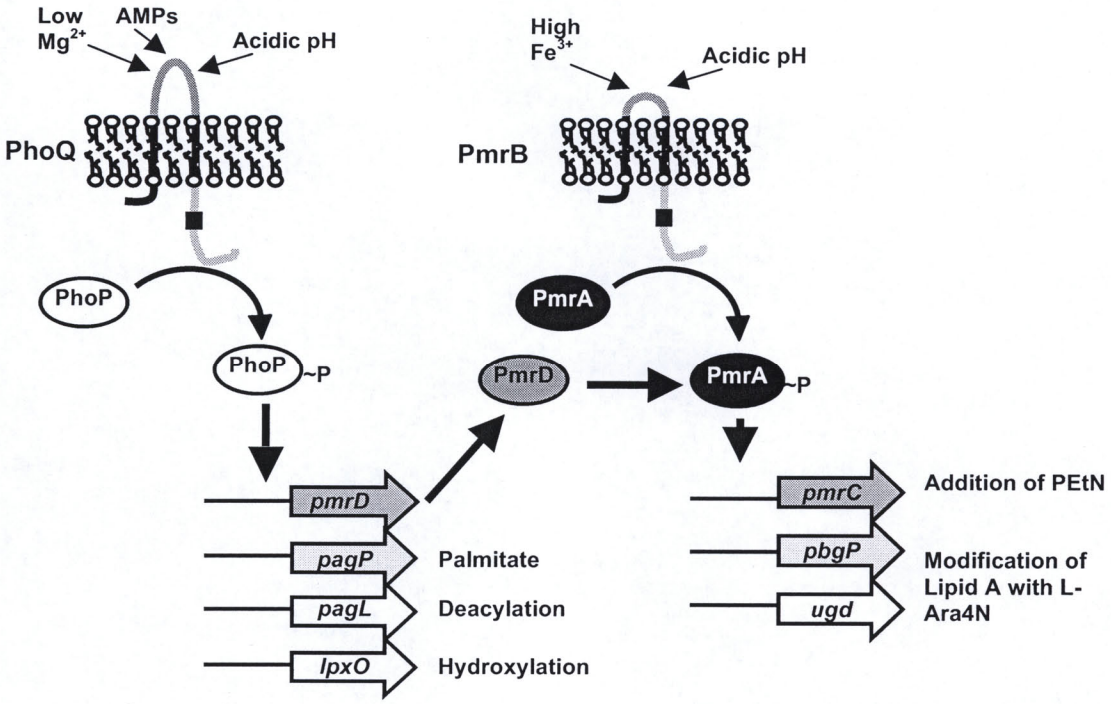

Figure 9. Diagram of the PhoP/PhoQ regulatory cascade in *Salmonella typhimurium*. Low Mg^{2+}, AMPs and acidic pH activate the PhoQ/PhoP system, which promotes the expression of p*mrD* and LPS modification genes (*pagP*, *lpxO* and *pagL*). Fe^{3+} and acidic pH can directly activate the PmrA/PmrB system to upregulate the transcription of genes that add phosphoethanolamine (PEtN) and aminoarabinose (L-Ara4N) to lipid A. PmrA/PmrB can be indirectly activated by PhoP/PhoQ through the connector protein PmrD that binds and stabilizes phosphorylated PmrA. The LPS modifications (Figure 7) mediated by both two-component systems promote survival
Adapted from Groisman, 2001.

PART 3 - THE MOLECULAR BASIS OF *SALMONELLA TYPHIMURIUM* VIRULENCE

Salmonellae are Gram-negative microorganisms that are capable of infecting a wide variety of animals (Pegues *et al.*, 2005). In humans, they cause a range of conditions known as salmonellosis, which includes gastroenteritis, enteric fever and bacteremia (Ohl & Miller, 2001). The *Salmonella* genus is comprised of only two species: *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is further divided into subspecies and serovars, including the etiological agent of typhoid fever *S. enterica* serovar Typhi (hereto referred to as *S. typhi*) and *S. typhmurium* that afflicts humans with gastroenteritis and mice with a systemic typhoid-like disease (Groisman & Saier, 1990). The bacteria are transmitted through contaminated food and water. In humans, a *S. typhimurium* infection is characterized by gastroenteritis, associated with intestinal inflammation and diarrhea. This disease is usually self-limiting and confined to the intestine, however immunologically compromised patients can fall victim to a systemic infection. In industrialized countries, non-typhoidal salmonellosis is the most common food-borne bacterial disease (Schlundt *et al.*, 2004). In the United States, there are nearly 1.4 million food-borne infections annually (Mead *et al.*, 1999), although the illness is self-limiting this converts into an estimated cost of $1.4 billion/year in lost wages, recall expenses and medical bills (Roberts *et al.*, 1989).

As a species *Salmonellae* can be exquisitely host-adapted or have a broad host range producing a variety of diseases in different hosts. The genome sequences from several *Salmonella* strains (McClelland *et al.*, 2001; Parkhill *et al.*, 2001) along with a growing number of available knock-out mice make *Salmonella* a model organism for the study of host-pathogen interactions.

Salmonella typhimurium *virulence determinants*

Pathogenic bacteria have evolved through the acquisition of virulence determinants via lateral gene transfer (Ochman *et al.*, 2000; Schmidt & Hensel, 2004). Pathogenicity islands (PAI) are chromosomal regions, which contain virulence genes that are absent from non-pathogenic strains. Although PAIs have divergent functions they are all characterized by a G+C content that differs from the rest of the genome

and frequently encode remnants of mobile elements (Schmidt & Hensel, 2004; Gal-Mor & Finlay, 2006). PAIs are heavily regulated to avoid the genes that they encode compromising bacterial fitness upon acquisition. The histone-like protein H-NS prevents the uncontrolled expression of PAI genes by binding to A+T-rich genes and repressing transcription. If the PAI is beneficial, a counteracting regulatory mechanism evolves to regulate its temporal expression (Lucchini *et al.*, 2006; Navarre *et al.*, 2006; Oshima *et al.*, 2006).

S. typhimurium encode an arsenal of virulence genes involved in adhesion, biofilm formation, iron acquisition and acid resistance as well as a number of large PAIs, referred to as SPI (*Salmonella* pathogenicity island) where, SPI-1 and SPI-2, have been characterized extensively. SPI-1 and SPI-2 each encode a Type III secretion system (T3SS) that injects effector proteins into eukaryotic cells to promote bacterial colonization and survival. T3SS are structurally akin to a molecular syringe and evolutionarily related to flagella. T3SS are 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. The SPI-1 T3SS mediates invasion into host cells and translocates bacterial proteins across the plasma membrane while the SPI-2 T3SS is important for intracellular survival by transporting effectors across the macrophage phagosomal membrane. Temporal and spatial regulation of these virulence factors through networks of regulators is key to a successful infection.

Salmonella invasion

Adherence to host cells is a precursor of invasion and initial contact with epithelial cells is mediated by fimbriae (Baumler *et al.*, 1996). *Salmonella* species primarily invade enterocytes by directing their own uptake through the induction of membrane ruffles on the surface of host cells (Francis *et al.*, 1993). Efficient invasion is dependent on the SPI-1 T3SS (T3SS-1) as mutants deficient in secretion are 10- to 100- fold more attenuated in the mouse model of systemic infection (Galan & Curtiss, 1989; Jones *et al.*, 1994). Contact with the intestinal epithelium triggers T3SS-1 assembly and subsequent injection of at least five effector proteins that orchestrate efficient *Salmonella* invasion (Figure 10). The three bacterial effectors, SopE, SopE2

and SopB mediate actin cytoskeletal reorganization, membrane ruffling and bacterial internalization by macropinocytosis. SopE and SopE2 are highly related guanine nucleotide exchange factors (GEFs) that stimulate the host Rho family GTPases: RhoG, Rac1 and Cdc42 (Hardt *et al.*, 1998; Bakshi *et al.*, 2000; Stender *et al.*, 2000; Friebel *et al.*, 2001). The phosphoinositide phosphatase SopB stimulates an endogenous exchange factor to indirectly activate RhoG (Patel & Galan, 2006). Activation of Rho GTPases ultimately results in stimulation of actin polymerization at the site of membrane ruffling through a cascade of N-WASP and Arp2/3 recruitment (Criss & Casanova, 2003; Unsworth *et al.*, 2004; Shi *et al.*, 2005). The bacterial effectors SipA and SipC bind actin directly to modulate the spacial localization of actin beneath the invading bacteria thus promoting efficient uptake (Hayward & Koronakis, 1999; Zhou *et al.*, 1999). After bacterial internalization, the process is reversed by SptP, a GTPase-activating protein (GAP) for member of the Rho family, which rebuilds the host actin cytoskeleton to its basal state (Fu & Galan, 1999).

In addition to invasion, T3SS-1 has been implicated in the induction of gastroenteritis and intestinal disease. Several MAPK pathways are triggered by the stimulation of Cdc42 by the *Salmonella* effectors, SopE, SopE2 and SopB (Hobbie *et al.*, 1997; Chen *et al.*, 1996). This leads to the activation of transcription factors (AP-1 and NF-κB) that increase the production of pro-inflammatory cytokines, such as IL-8 to cause intestinal inflammation and polymorphonuclear leukocytes (PMN) migration. SopB promotes intestinal disease by increasing a compound (D-myo-inositol 1,4,5,6-tetrakisphosphate) that increases chloride secretion and fluid flux (Zhou *et al.*, 2001; Norris *et al.*, 1998).

Salmonella survival

Intracellular proliferation of *S. typhimurium* occurs primarily within macrophages but has been observed in non-phagocytic cells, such as enterocytes (Salcedo *et al.*, 2001; Richter-Dahlfors *et al.*, 1997). *Salmonella* strains deficient in their ability to survive and replicate within macrophages are avirulent in murine models of infection (Fields *et al.*, 1986). Once intracellular, *Salmonella* resides within

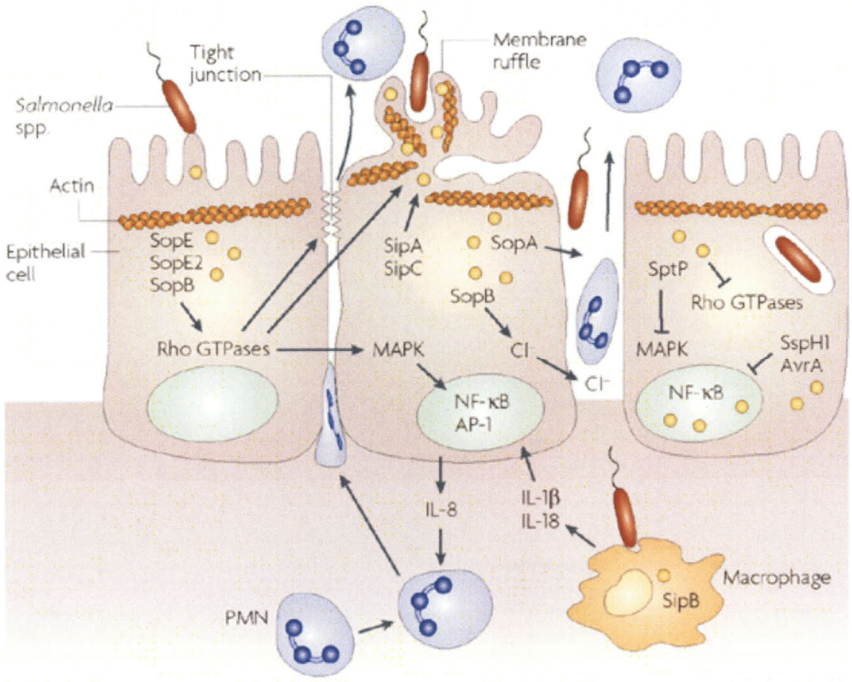

Figure 10. Invasion of host cells by *Salmonella*.

Interaction with the intestinal epithelium causes the SPI-1 T3SS to assemble and translocate the effector proteins (yellow circles) into the host cell. SopE, SopE2 and SopB activate Rho GTPases, which results in actin cytoskeleton rearrangements and destabilization of tight junctions. In addition, MAPK signaling is activated and results in a nuclear response leading to production of cytokines and migration of polymorphonuclear leukocytes (PMN). Membrane ruffles are further promoted by SipA and SipC to complete bacterial uptake. SopA further promotes the transmigration of PMN, while SopB stimulates Cl^- secretion. SptP restores the integrity of the intestinal cell by reversing the action of Rho GTPases.

Figure from Haraga *et al.*, 2008.

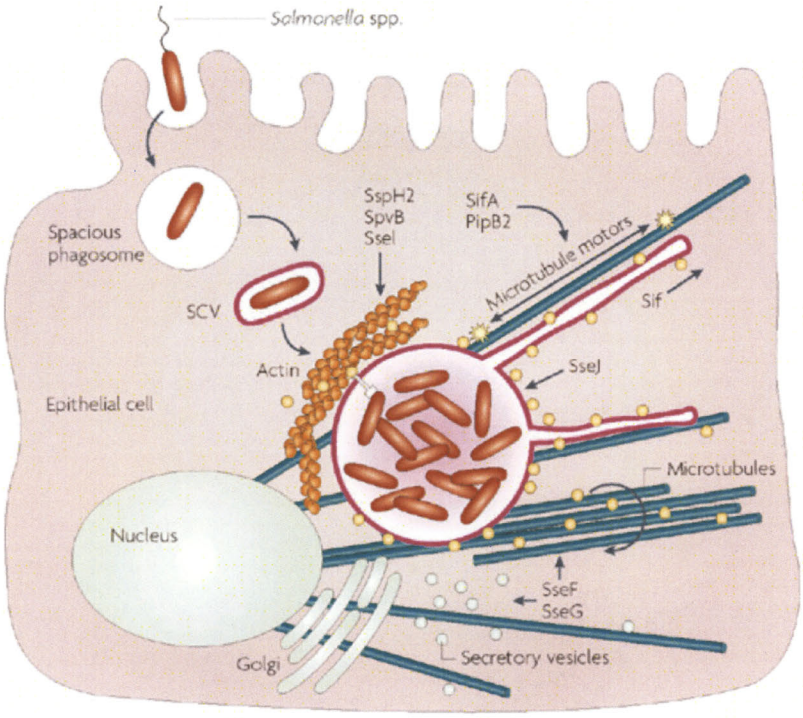

Figure 11. Model for *Salmonella*-containing vacuole formation.

Once inside the macrophage, *Salmonella* is housed in a spacious phagosome, which is modified into a *Salmonella*-containing vacuole (SCV). SCVs are characterized by an acidic pH and the presence of the late endosomal marker LAMP-1 (purple). The SPI-2 T3SS is stimulated and translocates effector proteins (yellow circles) across the phagosomal membrane. SifA and PipB2 cause the formation of *Salmonella*-induced filaments (Sif) along microtubules (green) and regulate the accumulation of microtubule-motor proteins (yellow stars). SseF and SseG recruit Golgi-derived vesicle to the SCV by redirecting their traffic and also cause microtubule bundling. The effectors SspH2, SpvB and SseI are believed to have an important role in the accumulation of actin around the SCV, while the deacylase SseJ helps to maintain the integrity of the SCV membrane.

Figure from Haraga *et al.*, 2008.

a modified phagosome known as the *Salmonella*-containing vacuole (SCV) (Figure 11). The bacterium regulates SCV biogenesis by limiting the interactions with the endocytic system. Early endosome markers such as EEA1, Rab5 and transferrin receptor characterize the initial SCV membrane. These first markers are rapidly replaced within an hour with late endosome markers, LAMPs and vacuolar ATPase (Steele-Mortimer *et al.*, 1999; Smith *et al.*, 2005). Finally, *Salmonella* prevents the SCV-lysosome fusion however some studies indicate that lysosomes ultimately fuse with the SCV over many hours (Oh *et al.*, 1996; Drecktrah *et al.*, 2007). Although it has been suggested that this delay in lysosome recruitment is a mechanism of resistance against the phagolysosomal environment (Hashim *et al.*, 2000; Hang *et al.*, 2006). The sequential membrane exchange is accompanied by a decrease in SCV pH to 5.5 (Alpuche Aranda *et al.*, 1992; Rathman *et al.*, 1996).

In vivo, SPI-2 null mutants are severely attenuated for virulence (Hensel *et al.*, 1995; Shea *et al.*, 1996). T3SS-2 effectors are involved in various aspects of endocytic trafficking, including delaying lysosome fusion to SCVs (Uchiya *et al.*, 1999), blocking NADPH oxidase-dependent killing by macrophages (Vazquez-Torres *et al.* 2000; Gallois *et al.*, 2001) and interfering with the localization of inducible nitric oxide synthase (iNOS) to the SCV (Chakravortty *et al.*, 2002). Thus far, 20 T3SS-2 effector proteins have been identified although the majority has not been associated with a specific role in promoting replication or modifying vesicular movement (Haraga *et al.*, 2008). This can be attributed to the redundancy in function of each individual effector so that single deletion mutants usually have no phenotype either *in vivo* or *in vitro* (Steele-Mortimer, 2008). Mutation in genes coding for SifA, SseJ, SseF, SseG, SopD2 and PipB2 cause a virulence defect in mice (Beuzon *et al.*, 2000; Jiang *et al.*, 2004; Ruiz-Albert *et al.*, 2002; Freeman *et al.*, 2003; Knodler *et al.*, 2003; Deiwick *et al.*, 2006). Five of these effectors are involved, to different extents, in the formation of long filamentous membrane structures called *Salmonella*-induced filaments (Sifs). While SseJ has a negative regulatory effect since an *sseJ* deletion mutant has increased Sif formation (Birmingham *et al.*, 2005). Sifs may function to increase the size of the SCV to accommodate bacterial replication and/or redirect nutrient-rich organelles to the SCV (Haraga *et al.*, 2008).

Once *Salmonella* is internalized the T3SS-1 is downregulated and the upregulation of SPI-2 genes promotes intracellular survival. This picture of *Salmonella* interacting with host cells has become more complex as it is now apparent that T3SS-1 effectors are translocated across the vacuolar membrane for some time after invasion (Giacomodonato *et al.*, 2007). Four T3SS-1 effectors, SopA, SipA, SopB and SopD are directly involved in SCV biogenesis. After invasion, the translocation of SopA across the SCV is associated with an increase in *Salmonella* escape from the SCV (Zhang *et al.*, 2005). Hours after invasion, SipA and SopB play a role in the redistribution of late endosomes and aid in the accumulation of phosphatidylinositol-3-phosphate (PI3P), respectively (Brawn *et al.*, 2007; Dukes *et al.*, 2006). SopD is expressed following invasion but its role in SCV biogenesis remains unclear (Brumell *et al.*, 2003).

PART 4 - REGULATION OF *SALMONELLA* PATHOGENESIS

Many genetic regulators and environmental stimuli control the complex process of invasion and intracellular replication. The regulators allow for the expression of virulence genes in the correct anatomical location and at the appropriate time during infection in response to environmental signals.

Regulation of SPI-1

SPI-1 controls invasion through transcriptional regulators encoded within the PAI, including HilA, HilD, HilC and InvF (Altier, 2005; Jones, 2005) and outside it, including RtsA and RtsB (Ellermeier & Slauch, 2003). Systems outside of SPI-1 positively and negatively modulate the expression of these regulators, including the TCSs BarA/SirA, PhoP/PhoQ, OmpR/EnvZ and the csr post-transcriptional control system.

The levels of HilA are maximal under conditions of low oxygen, high osmolarity, exponential growth phase and slightly alkaline pH (Bajaj *et al.*, 1995). HilA is the central regulator of SPI-1 and is known to bind directly to promoters and activate genes necessary for the T3SS-1 (Lee *et al.*, 1992). A *hilA* deletion mutant is phenotypically equivalent to a deletion of the entire SPI-1 locus in the BALB/c mice

infection model (Ellermeier *et al.*, 2005). HilC, HilD and RtsA are AraC-like transcriptional activators that bind to the *hilA* promoter to control its gene expression (Schechter & Lee, 2001; Olekhnovich & Kadner, 2002; Ellermeier & Slauch, 2003). Many environmental signals feed into the SPI-1 system through EnvZ/OmpR, BarA/SirA and Fur. These three regulatory systems require the presence of HilD to impact on the expression of the *hilA* gene product (Ellermeier *et al.*, 2005). HilE is the major negative regulator of SPI-1 that binds and inhibits HilD (Baxter *et al.*, 2003). Interestingly, PhoP/PhoQ, PhoR/PhoB and the regulators, FimZ and FimY are negative regulators of SPI-1 and affect the regulation of *hilE* (Ellermeier & Slauch, 2007).

Regulation of SPI-2

The complex regulatory cascade that modulates the expression of SPI-2 genes is not completely understood. The SsrB/SsrA TCS is encoded within the SPI-2 PAI and is considered to be the master regulator of SPI-2 gene induction (Worley *et al.*, 2000). The RR SsrB binds DNA to upregulate the expression of SPI-2 genes but the specific signals that activate the SK SsrA are unknown. The OmpR/EnvZ TCS regulates *ssrBA* expression by binding to its promoter (Lee *et al.*, 2000). SPI-2 genes are known to be actively expressed intracellularly by *S. typhimurium* (Valdivia & Falkow, 1997; Cirillo *et al.*, 1998; Deiwick *et al.*, 1999). Independently exposing *S. typhimurium* to acidic pH or inorganic phosphate limitation leads to activation of SPI-2 gene expression with different kinetics (Lober *et al.*, 2006).

Contradictory evidence has been presented for the involvement of PhoP/PhoQ in modulating the expression of *ssrAB*. PhoP/PhoQ regulates the expression of three SsrB-dependent effectors encoded outside the SPI-2 PAI (Worley *et al.*, 2000). In addition to activating effectors, the T3SS-2 is upregulated under Mg^{2+} or Ca^{2+}-deprivation conditions (Deiwick et al., 1999; Garmendia *et al.*, 2003), which are known PhoP/PhoQ-activating conditions (Garcia Vescovi *et al.*, 1996). SseK1 and SseK2 are T3SS-2 effectors that require SsrB/SsrA and PhoP/PhoQ in order to accumulate *in vitro* (Kujat Choy *et al.*, 2004). PhoP/PhoQ is necessary for transcription of *spiC*, a T3SS-2 secreted effector, through the direct binding to the

promoter of *ssrB* (Bijlsma & Groisman, 2005). However, other investigators have concluded that PhoP/PhoQ is not directly involved in SPI-2 activation (Miao *et al.*, 2002; Kim & Falkow, 2004).

The virulence-associated transcriptional regulator SlyA contributes to the regulation of SPI-2 genes, in a partially overlapping manner with OmpR and dependent on SsrA (Linehan *et al.*, 2005). A *slyA* mutant is similar to an *ompR* mutant as both mutants lack Sif formation and exhibit reduced expression of the SsrA-dependent SPI-2 gene, *sifA* (Linehan et al., 2005; Garmendia et al., 2003). The *ssrA* promoter in *S. typhimurium* contains a SlyA box analogous to the one found in the SlyA-dependent *ugtL* gene (Shi *et al.*, 2004). It seems that the SsrB/SsrA TCS requires input from a number of different regulatory systems over time to convey specific signals to modulate the expression of the T3SS and its effectors (Bijlsma & Groisman, 2005).

Recent work has suggested that the distinct roles of SPI-1 and SPI-2 are not as independent and location restricted as first thought. SPI-2 genes are not solely expressed after the bacterium is internalized into host cells. Recent studies have shown that SPI-2 genes can be expressed in the intestinal lumen (Merighi et al., 2005; Brown *et al.*, 2005). Cross-talk between SPI-1 and SPI-2 occurs through the SPI-1 encoded regulator HilD in a growth phase-dependent manner in Luria-Bertani (LB) broth (Bustamante *et al.*, 2008). The SPI-1-encoded regulator HilD has been shown to counteract H-NS repression and bind directly to the promoter of *ssrBA* (Bustamante *et al.*, 2008). The sophisticated level of regulation evident in *Salmonella* is an example of a complex regulatory network that integrates ancestral and recently acquired regulatory elements to efficiently coordinate virulence factors during infection.

PART 5 – HOST INNATE DEFENSE AND *SALMONELLA* RESISTANCE

Prior to establishing a successful infection, *S. typhimurium* must first overcome several obstacles. Firstly, the pathogen needs to survive passage through the acidic environment of the stomach before reaching the gastrointestinal (GI) tract. Once there, the physical barrier of the epithelial cells, the presence of AMPs (defensins and

cathelicidins) and innate immune components of the blood such as macrophages protect the host GI tract from infection.

Antimicrobial peptides - Defensins

Of the two major families of AMPs in mammalian epithelia defensins predominate in the small intestine (Dann & Eckmann, 2007). Defensins are small cationic AMPs and characteristically contain three disulfide bonds and a β-sheet structure. Defensins are divided into three groups α-, β- and θ-defensins based on the arrangement of their disulfide bonds (Figure 12).

Paneth cells and neutrophils produce α-defensins that kill a broad range of microbes *in vitro* (Eckmann, 2005). α-defensins are encoded as tripartite prepropeptide sequences, in which a 90-100 amino acid precursor contains an 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). Human have six known α-defensins while mice express over 20 α-defensins, termed cryptdins. Of these human HD-5 and HD-6, and the cryptdins are stored as inactive propeptides (Cunliffe, 2003). Consequently, maturation requires posttranslational proteolysis of the human defensins by trypsin (Ghosh *et al.*, 2002), while the cryptdins are activated by the metalloproteinase matrilysin (Weeks *et al.*, 2006). Mice unable to process α-defensins have a greater sensitivity to oral infection by *S. typhimurium* (Wilson *et al.*, 1999), while transgenic mice carrying an additional human α-defensin (HD-5) are highly resistant to *Salmonella* infections (Salzman *et al.*, 2003).

β-defensins have a simpler structure that contains a signal sequence, a short or absent propiece and the mature peptide at the C-terminus (Ganz, 2003). Humans have at least 10 and as many as 40 β-defensin genes. These are secreted mainly by epithelial surfaces of the lungs, skin, kidney, testis and intestine (Pazgier *et al.*, 2006). Produced by epithelial cells, human β-defensin-1 (hBD-1) is constitutively expressed but can be upregulated further by the presence of albumin and amino acids (Sherman *et al.*, 2006). In contrast, the expression of hBD-2 is strongly inducible by infection with enteric pathogens or proinflammatory mediators (O'Neil *et al.*, 1999). Decreased mucosal hBD-2 expression has recently been associated with a higher risk of

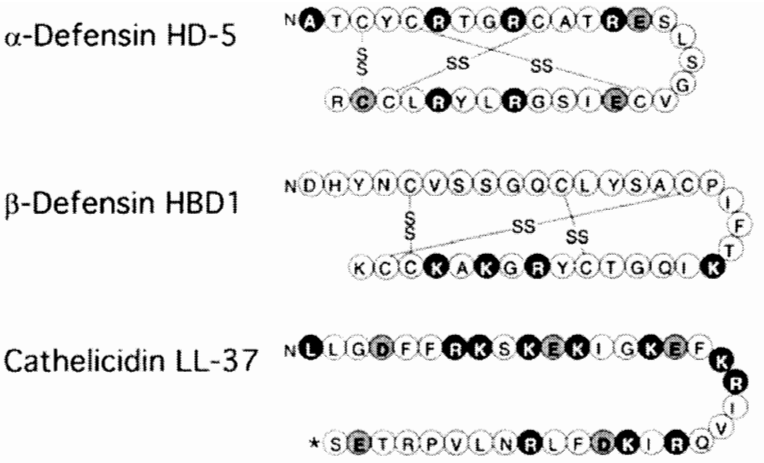

Figure 12. Structure of human antimicrobial peptides.

α- and β-defensins differ in length and the pairing pattern of their disulfide bonds. The positively (black) and negatively (gray) amino acids are indicated.

Figure from Muller *et al.*, 2005.

developing Crohn's disease (Fellermann *et al.*, 2006). Furthermore, the inducible β-defensins hBD-3 and hBD-4 have a markedly decreased or absent expression in epithelial cells of Crohn's disease patients (Fahlgren *et al.*, 2004). The physiological role of β-defensins is poorly defined but they are known to kill a wide variety of microbes *in vitro* (Dann & Eckmann, 2007). Under optimal conditions of low ionic strength and low divalent cation concentrations, β-defensins are active at concentrations as low as 1-10 μM (Ganz, 2003). Defensins also have immunomodulatory activities, such as mobilizing monocytes, mast cells, immature dendritic cells and lymphocytes. In addition they enhance production of cytokines and chemokines, cause mast cells degranulation and interact with complement components (Lehrer, 2004).

Antimicrobial peptides – Cathelicidins

The second major family of AMPs consists of a group of α-helical cationic peptides called cathelicidins. Cathelicidins are characterized by the presence of a conserved pro-peptide sequence, termed cathelin, which is linked to a C-terminal cationic peptide (Eckmann, 2005). These AMPs have a broad spectrum of action against Gram-positive and Gram-negative bacteria, fungi, parasites and enveloped viruses and have been identified in several mammalian species (Bals & Wilson, 2003).

A single cathelicidin has been identified in both humans and mice, being LL-37 (Figure 12) and the cathelin-related antimicrobial peptide (CRAMP), respectively (Larrick *et al.*, 1995; Gallo *et al.*, 1997). Neutrophils, epithelial and mast cells constitutively expressed hCAP-18, which undergoes proteolytic processing to become the mature form, LL-37 (Eckmann, 2005). The postsecretory maturation of LL-37/hCAP-18 modulates antimicrobial activity and tissue specificity. Further induction of LL-37 expression occurs in the colon under conditions of inflammation associated ulcerative colitis but not Crohn's disease (Wehkamp *et al.*, 2007; Schauber *et al.*, 2006). LL-37 can be found on unstimulated mucosal surfaces at concentrations of ~2 μg/mL and at concentrations exceeding 50 μg/mL in inflamed epithelium (Bals *et al.*, 1998). The mechanism of killing by LL-37 is poorly defined. The current model suggests that LL-37 covers the bacterial membrane to form a pore, which causes

leakage of cytoplasmic contents (Henzler Wildman *et al.*, 2003). The optimal killing capacity of LL-37 is achieved in conjunction with other antimicrobial components such as β-defensin 2 (Ong *et al.*, 2002), lysozyme and lactoferrin (Bals *et al.*, 1998).

Cathelicidins have a α-helical structure, including LL-37 and CRAMP. Other examples of α-helical peptides include magainin 2 and C18G, which are derived from frog skin and platelet factor IV, respectively. The last 13 amino acids of the C-terminus of platelet Factor IV, peptide C13, were found to be antibacterial in human serum against bacteria exposed to the cephalosporin antibiotibic, cefepime (Darveau *et al.*, 1992). Peptide analogs were optimized for a more potent antimicrobial activity. C18G was shown to have a 80-fold increase in antimicrobial activity as compared to C13. C18G is often used as the prototypical α-helical peptide because it is easier to synthesize.

Macrophages

Macrophages are phagocytic cells that engulf and digest microorganisms. They are found in many areas, principally the lymph nodes and are a key component of the innate immune system. Macrophage deficiencies in phagocyte oxidase (phox) and inducible nitric oxide synthase (NOS2) result in *Salmonella* survival (Shiloh *et al.*, 1999). The production of inflammatory cytokines, such as IFNγ by macrophages plays an important role in the host response to *Salmonella* infection (Hess *et al.*, 1996). The divalent cation transporter Nramp1 (natural resistance-associated macrophage protein) is exclusively expressed by immune cells and localizes to the phagosomal membrane after phagocytosis (Cellier *et al.*, 1995). Recruitment of Nramp1 to the SCV removes Fe^{2+} and Mn^{2+} to create an environment that impairs bacterial replication and abrogates the ability of *S. typhimurium* to block late endocytic compartments fusion with the SCV (Cuellar-Mata *et al.*, 2002).

Mammalian macrophages are activated by lipid A of lipopolysaccharide (LPS), which is recognized by the TLR-4 receptor and triggers the expression of cytokines and proteins including AMPs (Janeway & Medzhitov, 2002). Bacterial membranes are negatively charged while mammalian membranes are composed of lipids having no net charge. Cationic AMPs are preferentially attracted to the bacterial phospholipids as

a consequence of the charge interaction. Although, the microbicidal activity of cathelicidins is principally directed towards the membrane to cause damage by forming ion channels or aqueous pores, some AMPs have as yet unidentified intracellular targets (Hale & Hancock, 2007). In murine macrophages, the expression of CRAMP increases to impair *S. typhimrium* growth (Rosenberger *et al.*, 2004). CRAMP is critical in controlling intracellular *Salmonella* since peptide-sensitive mutants have enhanced survival in macrophages derived from CRAMP-deficient mice.

Salmonella resistance to host defenses

Studies have shown that *Salmonella* is able to resist innate immune factors, namely acidic pH, Nramp1, AMPs, nitric oxide (Shiloh & Nathan, 2000) and oxygen radicals (Fang *et al.*, 1999).

Most *Enterobacteria* are able to mount a resistance mechanism to low acidity known as the acid tolerance response (ATR), *S. typhimurium* is no exception (Garcia-del Portillo *et al.*, 1993). Expression of more than 50 acid shock proteins are upregulated upon ATR triggering, through specific signaling pathways, including PhoPQ, which coordinates resistance to inorganic acid stress (Bearson *et al.*, 1998). To counteract the effects of Nramp1 *Salmonella* has two divalent cation transporters, MntH to import Mn^{2+} and SitABCD for Fe^{2+} and Mn^{2+} (Kehres *et al.*, 2000; Kehres *et al.*, 2002). Both *mntH* and *sitA* are essential for *Salmonella* virulence and shown to be upregulated upon bacterial internalization by Nramp1 producing macrophages (Zaharik *et al.*, 2004).

S. typhimurium promotes remodeling of its outer membrane to increase resistance to AMPs and dampen host recognition of LPS. The expression levels of E-selectin from human endothelial cells and TNF-α from human monocyte-derived macrophages are higher for LPS from a *phoP* null mutant as compared to the LPS from a constitutive PhoP mutant, which has all the LPS modifications (Guo *et al.*, 1997).

Export of AMPs from the bacterium through energy-dependent transporters is a mechanism of AMP resistance used by *S. typhimurium*. ATP-binding cassette (ABC)

transporters, such as the *yejABEF* and *sapABCDF* operons, have been implicated in AMP resistance (Groisman *et al.*, 1992; Eswarappa *et al.*, 2008).

Proteolytic enzymes that digest or inactivate AMPs are another mechanism of bacterial resistance to innate host defense. An outer membrane protease, PgtE, was recently identified in *S. typhimurium* and promotes resistance to α-helical AMPs (Guina *et al.*, 2000). PgtE is a member of the omptin family of proteases, which are present in many Gram-negative pathogenic *Enterobacteria,* including *Yersinia pestis* (Pla), *Shigella flexneri* (SopA) and *E. coli* (OmpT and OmpP) (Kukkonen & Korhonen, 2004; Hritonenko & Stathopoulos, 2007). S. *typhimurium* PgtE is post-transcriptionally regulated by PhoP/PhoQ and cleaves AMPs such as C18G *in vitro*, but its *in vivo* contribution to AMP resistance appears marginal when expressed from a single chromosomal copy (Guina *et al.*, 2000). *In vivo*, *Y. pestis* Pla activity is essential for tissue invasion (Sodeinde *et al.*, 1992; Lathem *et al.*, 2007). Pla promotes degradation of fibrin clots by activating plasminogen into plasmin through inactivation of the α_2-antiplasmin inhibitor (Kukkonen *et al.*, 2001). A recent study reveals that Pla is also able to inactivate AMPs like LL-37 and CRAMP (Galvan *et al.*, 2008). Furthermore, *E. coli* OmpT efficiently degrades the AMP protamine *in vitro* (Stumpe *et al.*, 1998). It is clear that omptins play a role in AMP resistance *in vitro.* However, *in vivo* the relationship between omptins and bacterial pathogenesis is tenuous.

PART 6 - *CITROBACTER RODENTIUM* PATHOGENESIS

In the 1960s and 1970s, *Citrobacter rodentium* was described in scientific literature as an atypical *C. freundii* strain responsible for enterocolitis in mice. Up until 1993, only *C. freundii*, *C. koseri* and *C. amalonaticus* were officially recognized species of *Citrobacter*. Once DNA-DNA hybridization was used to reclassify the taxon (Brenner *et al.*, 1993) all the atypical *C. freundii* strains were renamed *C. rodentium*.

C. rodentium is a non-invasive pathogen and the etiological agent of transmissible murine colonic hyperplasia (Schauer *et al.*, 1995). The disease is characterized by epithelial cell hyperproliferation in the descending colon and is host-adapted to infect mice. The hyperplastic state can be a precursor for colon

tumorigenesis (Barthold *et al.*, 1977) and is thought to be akin to humans having an increased risk of colorectal cancer when suffering from ulcerative colitis (Lipkin, 1975). *C. rodentium* adheres to colonic epithelial cells and subverts the host cytoskeleton to produce attaching and effacing (A/E) lesions beneath the adherent bacteria (Knutton *et al.*, 1987). This histopathology is associated with local destruction of the brush border microvilli. These changes in ultrastructure are identical to those produced by the human pathogens enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) (Rothbaum *et al.*, 1983; Donnenberg *et al.*, 1993). In the developing world, EPEC is a major cause of infantile diarrhoea while EHEC causes food-borne bloody diarrhoea in developed countries. EPEC and EHEC have a strict host specificity for humans so *C. rodentium* is a convenient *in vivo* model to study these human pathogens. The genome has recently been sequenced and is approximately 5,346,659 bp in length. *C. rodentium* contains three cryptic plasmids: pCRP1 (65 kb), pCRP2 (60 kb) and pCRP3 (3 kb) (Deng *et al.*, 2001).

Citrobacter *niche*

Environmental conditions within the intestinal lumen are characterized by high osmolarity, a neutral pH (except in the stomach and duodenum) and the presence of bicarbonate and AMPs. The concentration of free divalent cations has not been precisely measured. The concentration of total Mg^{2+} was approximated at over 20 mM, although the concentration of free Mg^{2+} is probably closer to 0.8 mM, which is the same as plasma (Jittakhot *et al.*, 2004; Laires *et al.*, 2004).

In the murine intestine, CRAMP mRNA is constitutively expressed and immunostaining for CRAMP indicates that it is largely restricted to the surface colon epithelial cells (Iimura *et al.*, 2005). This colonic distribution mirrors that of the human cathelicidin LL-37 (Hase *et al.*, 2002). CRAMP is a functionally important non-redundant component of the innate immune system in the colon. Synthetic CRAMP and CRAMP present in colonic epithelial cells extracts exert an antimicrobial activity against *C. rodentium*. However, CRAMP is only important during the first week of *C. rodentium* colonization as CRAMP-deficient mice are more susceptible to

infection and have significantly higher bacterial counts as compared to wild-type mice (Iimura *et al.*, 2005).

Citrobacter *pathogenesis*

EPEC, EHEC and *C. rodentium* possess a PAI called locus of enterocyte effacement (LEE) (Figure 13) that causes the A/E pathology. The LEE location of integration suggests that although the three pathogens acquired the PAI by horizontal gene transfer *C. rodentium* did so much later than EPEC and EHEC (Deng *et al.*, 2001). Transplanting the LEE from a strain of EPEC that causes diarrhea in rabbits into the innocuous *E. coli* K12 strain confers the ability to generate A/E lesions in this model (Karaolis *et al.*, 1997; McDaniel & Kaper, 1997). Five operons encoding 41 genes reside within the LEE of each A/E pathogen, and can be classified into three groups based on function. The first region encodes a T3SS. Several of these genes have homology to other pathogenic bacteria including *Yersinia*, *Shigella* and *Salmonella*. The middle region contains the *eae* and *tir* genes, which encode the adhesin intimin and its transmembrane intimin receptor (Tir), respectively. The last region comprises effector proteins secreted by the T3SS (Garmendia *et al.*, 2005).

C. rodentium is transmitted through the fecal-oral route and requires a high oral infectious dose of 10^9 bacteria. It is interesting to note that the *C. rodentium* shed from the stool of infected mice has an infectious dose that is 1000-fold lower and suggests a hyper-infectious host-adapted state (Wiles *et al.*, 2005; Bishop *et al.*, 2007). In the colon, A/E pathogens typically use adhesins to mediate primary attachment to the host epithelial cells (Johnson & Barthold, 1979). The T3SS assembles in a timely fashion with EspA units polymerizing to form the shaft of the needle and together EspB/D constitute the translocation pore in the host cell plasma membrane. Tir is secreted and localizes to the host cell plasma membrane (Deibel *et al.*, 1998; Kenny *et al.*, 1997). Intimin is an integral bacterial outer membrane protein that binds Tir to form a very tight interaction (Frankel *et al.*, 2001; Liu *et al.*, 1999). A/E pedestals form underneath adherent bacteria as a consequence of other effectors triggering actin polymerization. The function of these pedestals is unclear but may prevent the bacteria from being washed away with the flow of diarrhea.

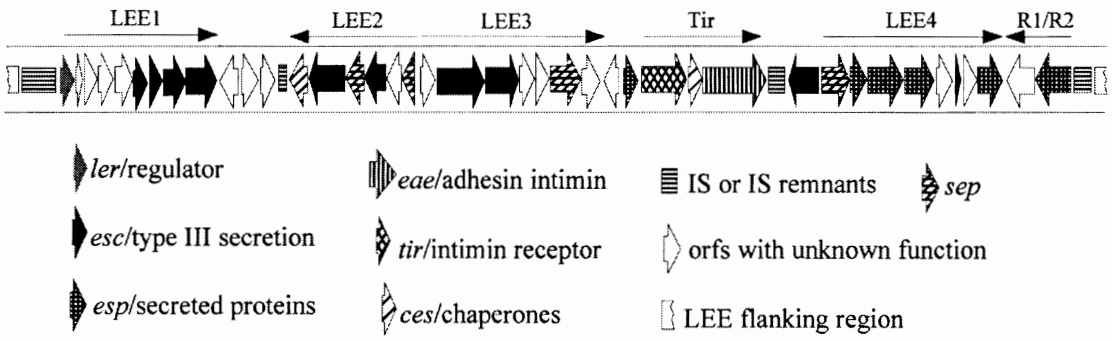

Figure 13. *C. rodentium* LEE genetic organization.

The direction of each arrow indicates the orientation of the gene. The major operons encoded by the LEE (LEE1, -2, -3 and -4, Tir, R1/R2) are shown along with their transcriptional directions.

Adapted from Deng *et al.*, 2001

Regulation of the LEE operons is a complex process that is dependent on environmental factors, quorum sensing and several regulators. Most LEE genes are positively regulated by Ler (LEE-encoded regulator), which is encoded within the LEE. Ler activates transcription by alleviating the repression enforced by the DNA-binding protein H-NS (Bustamante *et al.*, 2001; Umanski *et al.*, 2002).

Two-component systems of Citrobacter rodentium

Integration of virulence genes into a regulatory network is necessary for pathogenesis. To date, no TCS has been characterized in *C. rodentium*. However, genomic analysis of the phylogenetically related *E. coli* K12 revealed the presence of 29 SKs, 32 RRs and a single HPt (Mizuno, 1997). *E. coli* possesses a PhoP/PhoQ TCS that is homologous to *S. typhimurium* but part of a different signal transduction network (Eguchi *et al.*, 2004). The *E. coli* EvgS/EvgA TCS modulates the expression of PhoP-regulated genes through a small inner membrane protein, B1500 (Eguchi *et al.*, 2007). Expresssion of the *b1500* gene is upregulated by EvgS/EvgA so that the gene product can interact with the PhoQ SK to activate the PhoP/PhoQ system (Eguchi *et al.*, 2007).

PART 7 – INTRODUCTION TO THE THESIS

Our laboratory's focus is on the PhoP/PhoQ two-component system, which senses and responds to specific environmental cues. PhoP/PhoQ is essential for virulence in a number of Gram-negative plant and animal pathogens. This thesis focuses on the TCS PhoP/PhoQ in the extensively characterized *S. typhimurium* and *C. rodentium*, which is a model system for the study of EHEC and EPEC.

It has been proposed that Mg^{2+} is the physiological signal controlling PhoP/PhoQ and that detection of extracellular Mg^{2+} should allow *Salmonella* to distinguish between extracellular and intracellular environments (Garcia Vescovi *et al.*, 1996). It is clear that PhoQ is activated by the presence of AMPs found within the macrophage phagosome (Bader *et al.*, 2005). One of our research goals is to demonstrate that acidification of the macrophage phagosome is directly sensed by the *S. typhimurium* PhoQ SK, which has long been proposed as an activating ligand of

PhoQ. The second objective of this research was to decipher the mechanism of signal transduction in response to acidic pH. More specifically, to identify the amino acid(s) involved in the pH-dependent activation of PhoQ and to characterize the associated conformational change using fluorescence spectroscopy.

PhoP/PhoQ plays a pivotal role in the survival and prolifereation of the intracellular pathogen *S. typhimurium* by sensing and adapting to the AMPs and acidic pH within the macrophage phagosome. Since AMPs are secreted in the lumen of the small intestine and colon of mammals, the PhoP/PhoQ systems of other enteric extracellular pathogens such as EPEC, EHEC and *C. rodentium* should sense AMPs in order to promote resistance. The availability of the *C. rodentium* genome sequence enabled the identification of a *phoPQ* operon, which is highly homologous to the TCS in *S. typhimurium.* The final objective was to characterize the ligands of *C. rodentium* PhoQ and to determine the role in virulence of the PhoP/PhoQ TCS in *C. rodentium*.

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PREFACE TO CHAPTER 2

The notion that divalent-cation depletion within the macrophage phagosome is the activating signal of *Salmonella typhimurium* PhoQ has existed for some time. However, recent studies have measured the concentration of Mg^{2+} within the phagosome and determined it to be in the low millimolar range. These concentrations are known to repress the activation of PhoQ. Because mild acid pH promotes the transcription of certain PhoP-activated genes, it has long been speculated that *Salmonella* PhoQ senses pH.

Chapter 2 is a published manuscript that describes the characterization of acidic pH as a physiologically relevant activating signal of PhoQ in the macrophage phagosome. *In vivo* transcription assays and reconstitution of PhoQ in proteoliposomes demonstrate that acidic pH directly activates PhoQ in the presence of millimolar concentrations of Mg^{2+}. This Chapter also describes NMR analysis that illustrates that the PhoQ periplasmic domain undergoes conformational changes in the presence of acidic conditions. Taken together, these data have allowed us to form a model for PhoQ-mediated activation by acidic pH.

CHAPTER 2

Activation of the bacterial sensor kinase PhoQ by acidic pH

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ABSTRACT

The *Salmonellae* PhoQ sensor kinase senses the mammalian phagosome environment to activate a transcriptional program essential for virulence. The PhoQ periplasmic domain binds divalent cations, forming bridges with inner membrane phospholipids to maintain PhoQ repression. PhoQ also binds and is activated by cationic antimicrobial peptides. In this work, PhoQ is directly activated by exposure of the sensor domain to pH 5.5. NMR spectroscopy indicates that at acidic pH, the PhoQ periplasmic domain adopts a conformation different from that in the presence of divalent cations or antimicrobial peptides. The conformation is partially simulated by mutation of histidine 157, which is part of an interaction network that distinguishes the repressed conformation. The effects of antimicrobial peptides and pH on PhoQ activity are additive. We propose a model of activation by antimicrobial peptides via disruption of the cation bridges and/or by acidification of the periplasm through destabilization of the interaction network.

INTRODUCTION

Salmonella species infect a range of hosts and are causative agents of the human diseases typhoid fever and gastroenteritis (Pegues *et al.*, 2005). During systemic infection, *S. typhimurium* survives phagocytosis by host macrophages to promote disease (Fields *et al.*, 1986; Leung & Finlay, 1991). Within the phagosome the bacteria are exposed to a variety of antimicrobial factors including toxic oxygen and nitrogen species, antimicrobial peptides, proteases, and acidic pH. *Salmonella* sense the phagosome environment through specific sensing mechanisms and avoid killing by using these sensors to change gene expression. One such sensing system important for *Salmonella* pathogenesis is the two-component system PhoPQ, which controls various potential virulence mechanisms including resistance to antimicrobial peptides (Fields *et al.*, 1989; Miller *et al.*, 1990; Gunn & Miller, 1996), small molecule transporters, and survival in acidic pH environments (Foster & Hall, 1990). PhoQ is a membrane-bound sensor kinase that is activated by the intracellular environment (Miller *et al.*, 1989). Activation leads to autophosphorylation of PhoQ, followed by phosphorylation of the response regulator protein PhoP, which controls transcription of a large group of genes (Miller *et al.*, 1989; Belden & Miller, 1994; Soncini *et al.*, 1996; Gunn & Miller, 1996).

PhoQ is an inner membrane protein with two transmembrane regions, a periplasmic sensor domain, and a cytosolic kinase domain (see Figure 3A) which autophosphorylates through an intramolecular interaction to promote phosphorylation of the transcriptional activator PhoP. PhoQ is repressed in growth medium with divalent cation (Mg^{2+}, Mn^{2+}, or Ca^{2+}) concentrations of 5 mM or greater (Garcia Vescovi *et al.*, 1996). Bacterial cells grown in low micromolar concentrations of these three divalent cations, an environment that results in significant growth inhibition, have increased PhoQ-mediated gene expression. However, because the concentration of Mg^{2+} inside *Salmonella*-containing vacuoles has been estimated at 1 mM (Martin-Orozco *et al.*, 2006), it is unclear whether the results using medium limited for divalent cations, which destabilizes the bacterial outer membrane, are directly relevant to intracellular activation. At 1 mM Mg^{2+} or Ca^{2+}, PhoQ can be activated by host antimicrobial peptides (Bader *et al.*, 2005). In the crystal structure of PhoQ in its

repressed state, Ca^{2+} ions bind aspartic and glutamic acid residues in an acidic patch of PhoQ that putatively faces the membrane. These Ca^{2+} ions are hypothesized to form bridges between the protein surface and the inner membrane (Cho *et al.*, 2006). NMR experiments demonstrate that Mg^{2+} and Ca^{2+} bind to identical sites in the periplasmic domain and that 20 mM concentrations of either divalent cation produces the same structural (repressed) state (Cho *et al.*, 2006). A variety of experimental data indicate that PhoQ can be activated by displacement of divalent cations from this acidic region by antimicrobial peptides (Bader *et al.*, 2005).

PhoQ-mediated gene expression can also be activated in medium of low pH (Alpuche Aranda *et al.*, 1992; Bearson *et al.*, 1998; Martin-Orozco *et al.*, 2006). It would be reasonable for PhoQ to sense this change, as it encounters acidic pH (~5.5) in the macrophage phagosome. It is plausible that such a change could destabilize the divalent cation bridges that function to maintain repression of the system. In further support of this idea, PhoP-mediated gene expression is part of the adaptive acid tolerance response (Foster & Hall, 1990; Bearson *et al.*, 1998). Furthermore, PhoQ-dependent gene expression *in vivo* after phagocytosis by macrophages requires acidification of the *Salmonellae*-containing phagosomes (Alpuche Aranda *et al.*, 1992; Martin-Orozco *et al.*, 2006). Because many of these effects could be a result of indirect effects on the bacteria or the phagosome, we examined whether acidic pH is a direct activating signal for PhoQ.

RESULTS

PhoQ Is Activated in Acidic Growth Medium with Millimolar Concentrations of Divalent Cations

Though previous studies have suggested that PhoP-dependent genes can be activated in growth medium at acidic pH, the use of growth medium with defined divalent cation and hydrogen ion concentrations has not been reported. A bacterial strain expressing a reporter protein fusion to the PhoP-controlled gene encoding acid phosphatase (PhoN) was grown in culture medium buffered so that pH was subject to only minor fluctuations over the course of an experiment. These media allow activation at incremental pH changes to be measured reliably. Figure 1A shows that PhoPQ-dependent gene expression increases with decreasing pH independent of divalent cation concentration. pH-dependent activation was observed at 1 mM $MgCl_2$, where PhoN expression increased 3-fold by lowering the pH from 7.5 to 5.5. Interestingly, activation was maximal at pH 5.5 and decreased at pH 5.0–4.5. Notably, at pH 5.5, activation reached the same level as with divalent cation limitation at pH 7.5. Culture growth was severely inhibited in medium containing 10 mM Mg^{2+} at acidic pH. Despite this growth limitation, we did not observe increased reporter gene expression at low divalent cation concentration with acidification (data not shown). The Mg^{2+} concentration inside *Salmonella*-containing phagosomes is estimated to be 1 mM (Martin-Orozco *et al.*, 2006), suggesting that the observed pH response may be relevant to infection. Activation was observed even in the presence of 10 mM $MgCl_2$, a concentration that strongly represses PhoQ at neutral pH. Despite this repression at pH 7.5 in the presence of a high concentration of $MgCl_2$, we observed a 5.5-fold increase in PhoN expression by lowering the pH from 7.5 to 5.5. Activation by acidic pH in the presence of divalent cations was not observed in a PhoQ null mutant and was restored when the mutation was complemented with PhoQ expressed from a plasmid (Figure 1B), confirming that PhoQ is required for the observed pH response.

PhoQ Is Directly Activated by Exposure of the Periplasmic Domain to Acidic pH

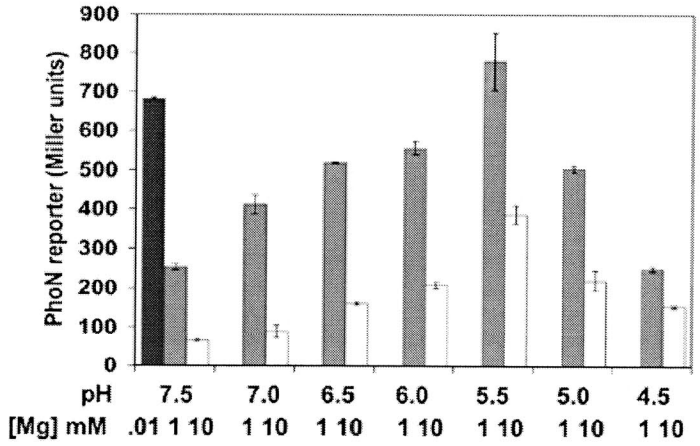
To demonstrate direct activation of PhoP by PhoQ upon exposure to acidic pH, we used a reconstituted *in vitro* system described previously (Bader *et al.*, 2005;

Sanowar & Le Moual, 2005). Full-length PhoQ was purified from membranes and reconstituted into vesicles. PhoQ inserts in a unidirectional orientation with the periplasmic domain facing the lumen of the vesicles. This allowed us to lower the pH inside the vesicles, while keeping a constant pH of 7.5 outside the vesicles for optimal PhoQ kinase activity (measured as the amount of phosphorylated PhoP generated). As shown in Figure 1C, increased phospho-PhoP was observed at acidic pH in the absence of $MgCl_2$. Similar results were obtained when $MgCl_2$ at a concentration of 1 mM was present inside the vesicles (data not shown). Lowering intralumenal pH from 7.5 to 5.5 resulted in a 3.5-fold increase of PhoP phosphorylation. As observed in the *in vivo* experiments, activation was maximal at pH 5.5 and decreased at pH 5.0–4.5. Together, these results suggest that PhoQ is capable of activating PhoP directly upon exposure to acidic pH in millimolar concentrations of divalent cation.

Antimicrobial Peptides and Acidic pH Activate PhoQ Additively

Two signals have now been shown to directly activate PhoQ in the presence of millimolar concentrations of divalent cations, subinhibitory (micromolar) concentrations of antimicrobial peptides and acidic pH. To determine whether these signals could have additive effects, we grew the PhoN reporter strain in media at pH 7.5, 6.5, or 5.5; with or without antimicrobial peptide; and with 1 mM (physiological concentration) or 10 mM (repressing concentration) Mg^{2+}. The synthetic antimicrobial peptide C18G was used as a prototypical α-helical, cationic peptide to mimic one that might be encountered *in vivo*. This peptide has similar activation of the PhoQ system to the natural mouse and human antimicrobial peptides CRAMP and LL-37 but is cheaper and easier to synthesize. As seen in Figure 2A, the two signals act additively to activate PhoP-dependent gene expression. With 1mM Mg^{2+} in the absence of peptide, activation increases with decreasing pH, as observed above. When peptide is present under these conditions, the activation at each pH was even greater. The same trend is observed in 10 mM Mg^{2+}; namely, addition of peptide increases the pH activation. However, the activation at 10 mM Mg^{2+} is not as strong, indicating that while pH and peptide can overcome strong divalent cation repression, divalent cations

A


PhoN reporter (Miller units)
900
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700
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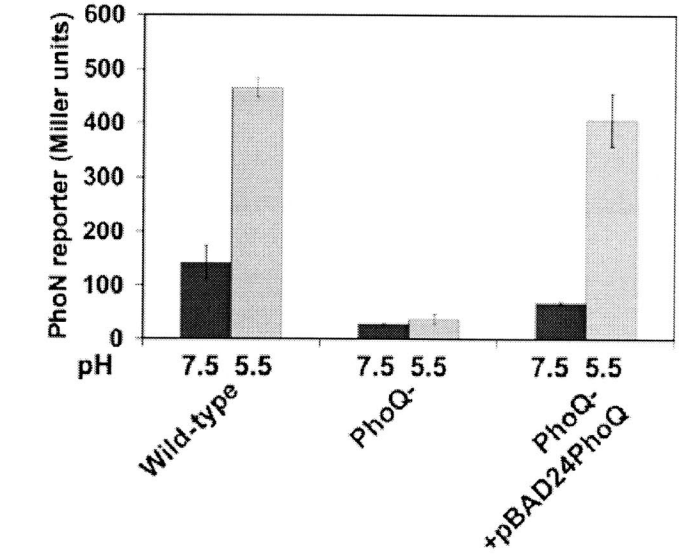
B


PhoN reporter (Miller units)
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pH 7.5 5.5 7.5 5.5 7.5 5.5
Wild-type
PhoQ-
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+pBAD24PhoQ

C

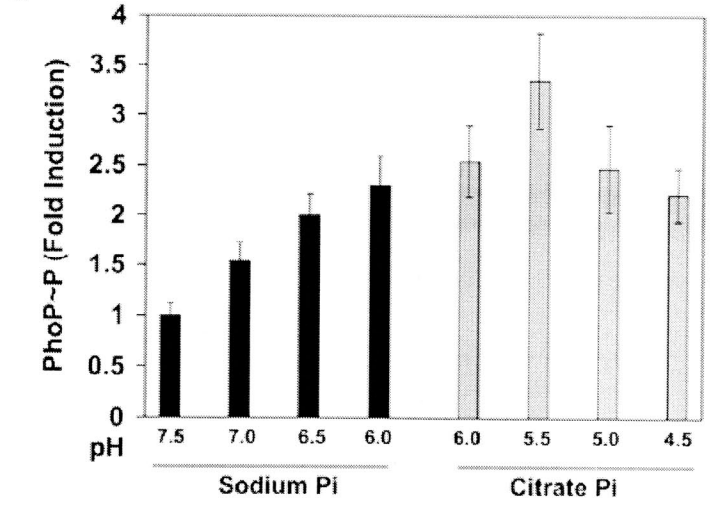
PhoP~P (Fold Induction)
4
3.5
3
2.5
2
1.5
1
0.5
0
pH 7.5 7.0 6.5 6.0 6.0 5.5 5.0 4.5
Sodium Pi
Citrate Pi

Figure 1. PhoP-Dependent Gene Activation by Acidic pH Is Mediated Directly by PhoQ.

(A) PhoP-dependent gene activation increases in acidified media. A reporter fusion between PhoP-dependent acid phosphatase (PhoN) and *E. coli* PhoA was used to measure activation. Cultures were grown in N-minimal medium buffered with MES and containing 1 or 10 mM $MgCl_2$, as indicated.

(B) The PhoP-dependent pH response requires PhoQ. Activities were measured in the PhoQ wild-type strain CS120 and MB101, which carries a *phoQ::tet* allele. The response could be complemented in MB106, which has a plasmid carrying PhoQ under the control of the arabinose promoter (pBAD24-phoQ). Cultures were grown in N-minimal medium buffered with MES at pH 7.5 (black bars) and 5.5 (gray bars), and containing 10 mM $MgCl_2$.

(C) PhoQ-mediated phosphorylation of PhoP depends on the acidification of intralumenal pH. The vesicles were formed in the presence of sodium phosphate (black bars) or citrate phosphate (gray bars) adjusted to the appropriate pH. Vesicles were incubated in the presence of [γ-^{32}P]ATP and an 8-fold molar excess of PhoP for 20 min at 22°C. The amounts of [^{32}P]phospho-PhoP were determined with a phosphoimager. All graphed values are mean ± standard deviation.

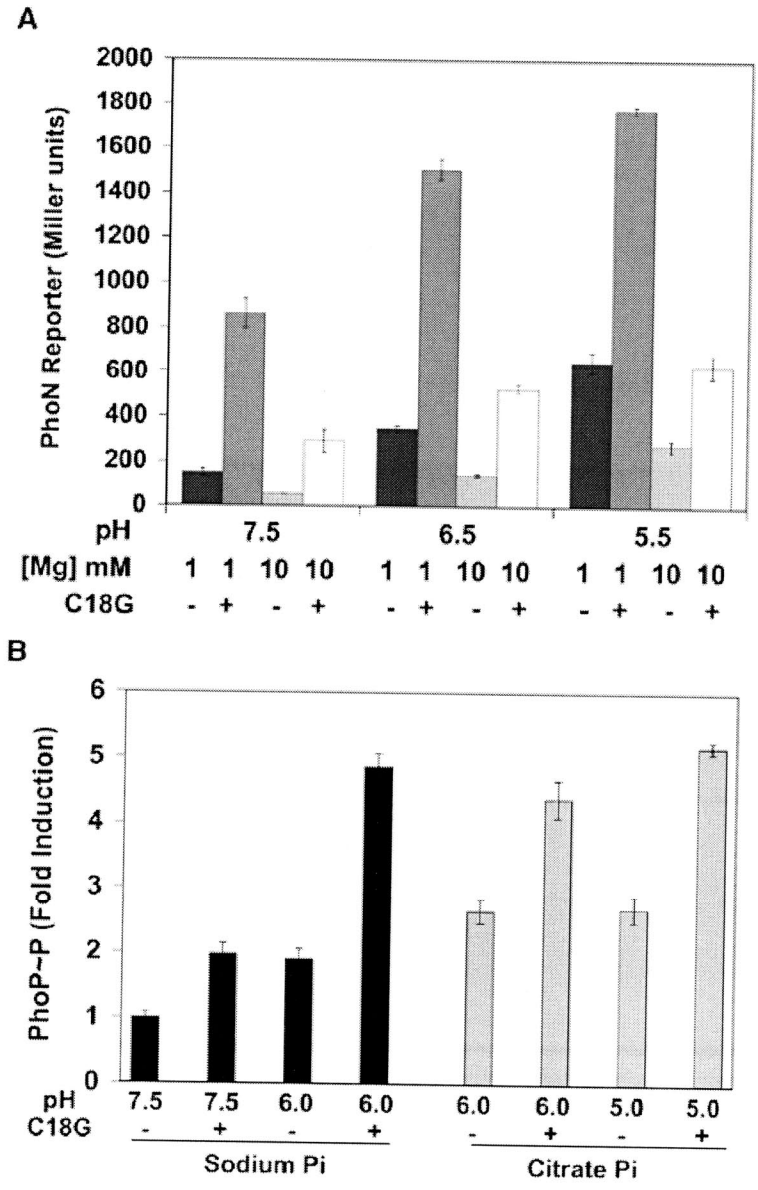

Figure 2. Peptides and pH Have an Additive Effect on PhoPQ Activation.

(A) PhoP-dependent gene activation relies on Mg^{2+}, peptide, and pH. Cultures were grown in N-minimal medium buffered with MES and containing 1 mM or 10 mM $MgCl_2$. C18G was added to 5 mg/ml where indicated. **(B)** The presence of peptide further increases PhoP phosphorylation at all pH values. Vesicles were formed in the presence of sodium phosphate (black bars) or citrate phosphate buffer (gray bars) supplemented or not with 1 mg/ml C18G. Phosphorylation of PhoP was determined as described in Figure 1C. All graphed values are mean ± standard deviation.

still exert some repressing effect at all pH values. This may be in part because the divalent cations will compete with peptide for binding to lipopolysaccharide (LPS) as well as PhoQ and penetration through the outer membrane is required for activation of inner membrane PhoQ.

To determine whether the additive nature of peptide and pH on PhoPQ activation is a direct result of sensing the two signals by PhoQ, we examined the combined effect of pH and peptide on phosphorylation of PhoP using the *in vitro* reconstituted system. As shown in Figure 2B, PhoP phosphorylation was enhanced 2- to 3-fold in the presence of peptide at each pH tested. This demonstrates that the additive effect of pH and peptide on PhoP activation is mediated by PhoQ and suggests that PhoQ is able to sense both signals directly.

Conformation of the PhoQ Sensor Domain Is Sensitive to pH Changes

Based on the above data with purified PhoQ reconstituted into membrane vesicles, we hypothesized that a change in external pH could alter the conformation of the PhoQ periplasmic sensor domain as part of environmental sensing. Therefore, we used NMR spectroscopy to monitor conformational changes in the purified PhoQ periplasmic domain (Figure 3A). Chemical shift perturbations of backbone amide NMR resonances, as observed in ^{1}H-^{15}N HSQC spectra, are sensitive indicators of alterations in chemical environment. ^{1}H-^{15}N HSQC spectra of a ^{15}N-labeled protein can be acquired under varying conditions, allowing changes that occur in the protein to be monitored at the amino acid residue level. Such NMR experiments on the isolated periplasmic sensor domain of PhoQ have previously demonstrated changes in peak intensity associated with the binding of divalent cations. These results are consistent with stabilization of a region of PhoQ that is intrinsically flexible in the absence of ligand (Cho *et al.*, 2006).

The TROSY-HSQC spectrum of Mg^{2+}-bound PhoQ sensor domain at pH 6.5 contains ~120 peaks (Figure 3B, black spectrum). Acidification of the protein sample by ~0.5 pH unit at a time leads to significant shifts in the positions of a subset of resonances throughout the spectrum. Several such shifts are highlighted in the inset boxes of Figure 3B. As the protein reaches pH 5.5–5.0 in the titration (the point of

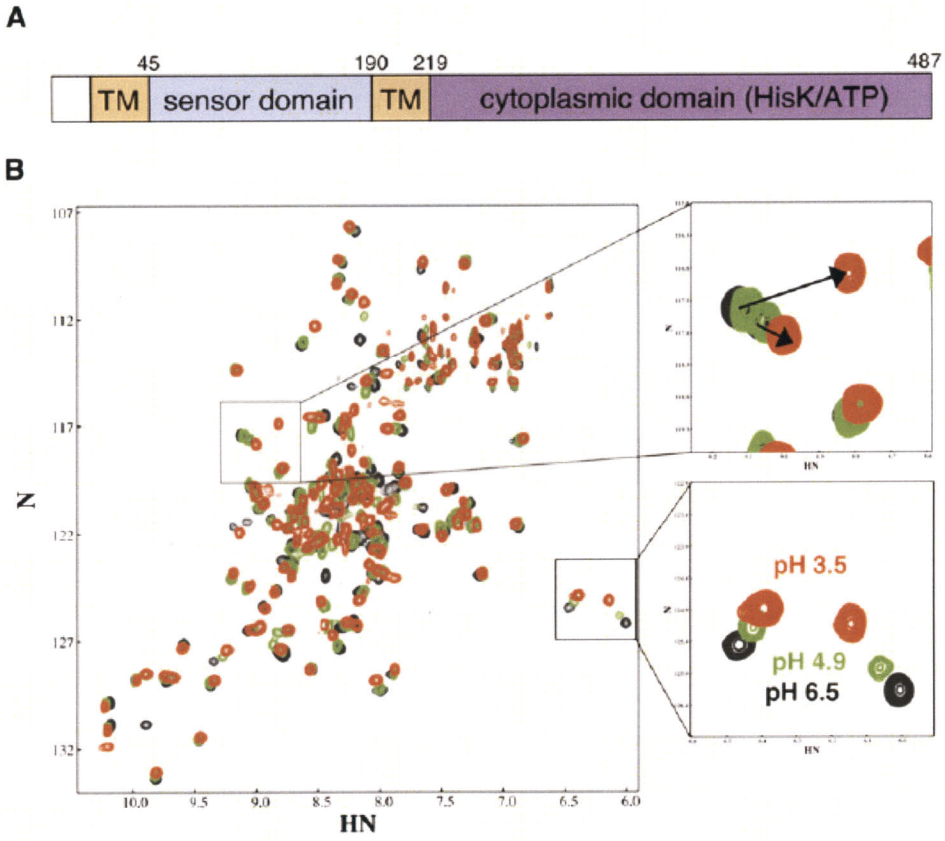

Figure 3. The PhoQ Sensor Domain Is Sensitive to Acidification.

(A) A schematic of the domain organization of PhoQ shows it consists of two transmembrane regions, a periplasmic sensor domain, and a cytosolic domain that contains the catalytic ATP-binding domain and the phosphotransfer domain.

(B) Selected points of the titration of the PhoQ sensor domain in the presence of 20 mM Mg^{2+}. Superimposed two-dimensional HSQC spectra are shown at pH 6.5 (black), 4.9 (green), and 3.5 (red). The insets highlight two regions of the spectrum that are subject to large shifts upon acidification.

maximal pH activation), the spectrum undergoes a general loss of peak intensity and some peaks broaden beyond detection (Figure 3B, green spectrum). Peak broadening of this type arises when the chemical groups involved exist in multiple states and exchange among them. Peak intensity is recovered as the pH decreases below 5.0, indicating stabilization of a ''low pH'' conformation (Figure 3B, red spectrum). Remarkably, the PhoQ sensor domain is extremely stable and remains folded at pH 3.5, where many proteins would be expected to unfold.

NMR was also used to monitor a pH titration performed in the absence of divalent cations (data not shown). As previously reported (Cho *et al.*, 2006), the starting spectrum at pH 6.5 contained about 20 fewer peaks than observed in the presence of 20 mM Mg^{2+}; however, below pH 4.9 the missing peaks grew in intensity and appeared at the same shifted position as in the Mg^{2+}-containing titration. Although pH 3.5 is not a physiologically relevant state, we note that the PhoQ sensor domain spectrum at this pH is identical in the presence and absence of Mg^{2+}, likely indicating that the protein adopts a stably folded structure that does not bind Mg^{2+}. The pH 5.5 spectrum is different from that of the sensor domain in the absence of divalent cations at neutral pH, perhaps indicating that activation can be achieved via different conformational states.

The PhoQ Sensor Domain Still Binds Mg^{2+} at pH 5.5

Aspartic acid and glutamic acid side chains serve as the ligands for cation binding to PhoQ (Cho *et al.*, 2006). As pH decreases, the carboxylate groups may become protonated, thereby dissociating the divalent cations. To examine the possibility that acidic pH activation of PhoPQ is simply due to the loss of Mg^{2+} binding to the periplasmic sensor domain, we collected NMR spectra at pH 5.5, the point of maximal PhoQ kinase activity and PhoQ-dependent gene expression. As mentioned above, at pH 5.5 a subset of peaks in the spectrum experience significant line broadening due to exchange among multiple states, suggesting increased flexibility. This is particularly apparent in the spectrum of apo-PhoQ (Figure 4, black spectrum), in which many peaks are broadened beyond detection. Many of the missing peaks regain intensity upon addition of 20 mM Mg^{2+} (Figure 4, red spectrum),

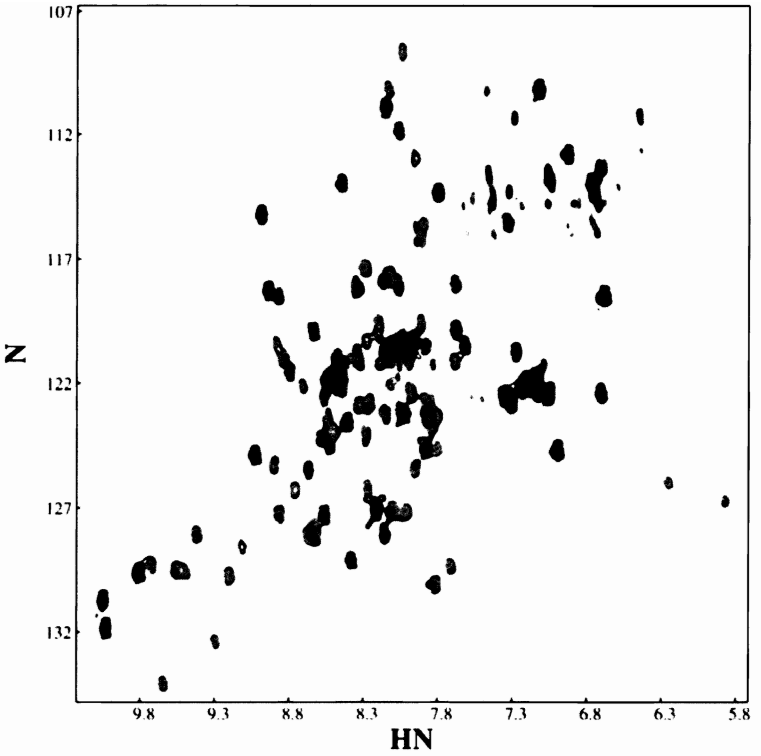

Figure 4. PhoQ Still Binds to Mg^{2+} and PMNP at pH 5.5.

Superimposed HSQC spectra of the PhoQ sensor domain at pH 5.5 in the absence (black) and presence of 4 mM PMNP (green) or 20 mM Mg^{2+} (red).

indicating that the sensor domain still binds Mg^{2+} at pH 5.5. Therefore hydrogen ions are not directly competing for binding to divalent cation-binding sites even in the absence of the increased stability of the inner membrane phospholipids. However, peaks grow in at shifted positions relative to the pH 6.5 spectrum, indicating a pH-specific conformational change as described above which is different from that related to the presence of divalent cations. The peaks already present in the apo-PhoQ spectrum are not perturbed by the addition of Mg^{2+}, suggesting that the core of the protein is stably structured and does not undergo a global conformational change. The new peaks that appear upon addition of Mg^{2+} are dispersed throughout the spectrum, indicating that binding stabilizes additional folded structure in regions that were previously flexible. These NMR data provide strong evidence that the specific PhoQ periplasmic domain conformation observed at pH 5.5 is not caused solely by the loss of Mg^{2+}-binding sites in PhoQ.

We have previously shown that polymyxin has the ability to activate PhoQ-mediated signaling in vivo and that polymyxin nonapeptide (PMNP) is a suitable antimicrobial peptide for NMR analysis, binding the sensor domain directly at pH 6.5 (Bader *et al.,* 2005). The spectrum of PhoQ in the presence of peptide at pH 5.5 is shown in Figure 4 (green spectrum). Many of the peaks that appear in the presence of peptide are coincident with a subset of those peaks that appear when Mg^{2+} is added to PhoQ (compare green and red spectra). Binding of either divalent cations or antimicrobial peptides causes changes in the intensity of the peaks. However, as shown in Figure 4, at pH 5.5 all of the peaks are overlapped and are in a shifted position relative to the pH 6.5 spectrum (refer to Figure 3B, black spectrum). Taken together, these data strongly support the likelihood that loss of divalent cation binding is not responsible for PhoQ-mediated gene expression at pH 5.5 and that in the presence of divalent cations or antimicrobial peptide the sensor domain still responds to environmental pH.

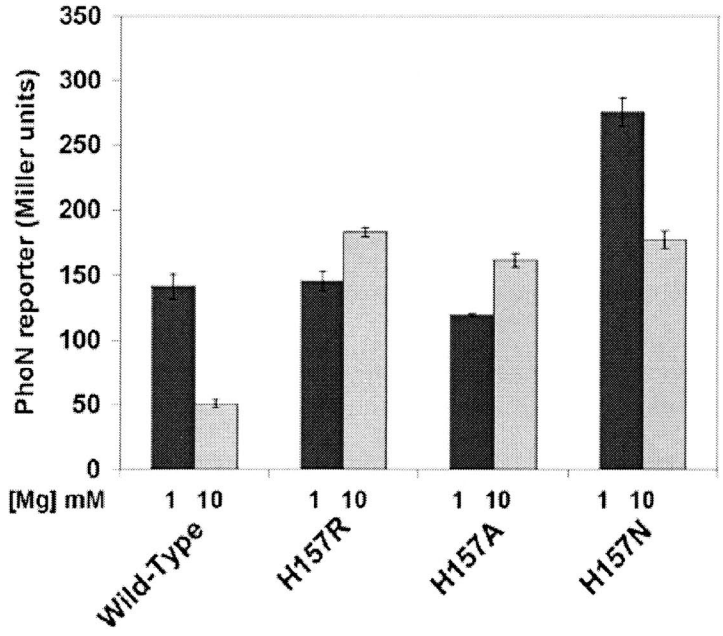

Figure 5. Mutations in H157 Result in Divalent Cation Derepression.

Strain MB106, which carries a PhoQ null allele and the plasmid pBAD24-PhoQ, was used as the wild-type strain. The indicated mutations were made in pBAD24-PhoQ using QuikChange mutagenesis. Cultures were grown in N-minimal media buffered with MES and supplemented with 1 or 10mM $MgCl_2$, as indicated. All graphed values are mean ± standard deviation.

PhoQ Histidine 157 Is Important for Repression of PhoQ-Mediated Gene Expression

PhoQ activation by pH is maximal at around pH 5.5, close to the pH value at which histidine residues can become protonated. These observations led us to the hypothesis that protonation of a histidine in a critical region of the protein might cause the conformational switch associated with activation. In a previous genetic screen, we identified a histidine to arginine (R) mutation at amino acid 157 (H157) which resulted in derepression in the presence of 10 mM divalent cation (Cho *et al.*, 2006). In the repressed state conformation, the H157 side chain is buried in the structural core of the sensor domain and participates in an interaction network that is in part comprised of hydrogen bonds (Cho *et al.*, 2006). Introduction of a charged side chain, either by mutation or by protonation, into the buried network might destabilize it and thereby destabilize the repressed conformation. To investigate the possibility that protonation of H157 is responsible for pH-mediated gene expression, we also mutated this residue to alanine (A) and asparagine (N) and tested all three mutant proteins for PhoP-dependent reporter activity. As shown in Figure 5, all H157 mutations tested are derepressed at pH 7.5 compared to wild-type in the presence of 10 mM Mg^{2+}. Therefore, it is not the introduction of a positive charge that destabilizes the repressed state, but specifically the loss of the imidazole side chain at position 157. In addition, the mutants are still pH responsive and are also derepressed compared to wild-type at pH 6.5 and 5.5 (data not shown). Thus, the presence of a histidine at position 157 is required to maintain wild-type repression of PhoQ, but protonation of this histidine is not a specific requirement for activation. Only the H157N mutant is derepressed compared to wild-type in the presence of 1 mM Mg^{2+}; the H157R and H157A mutants only show derepression at 10 mM Mg^{2+}. The asparagine side-chain mutant, but not arginine or alanine, retains hydrogen-bonding properties similar to the wild-type histidine, suggesting that though this function is important to maintain repression in the presence of divalent cations, additional properties of the histidine side chain are essential and specific for maintaining repression at 1mM divalent cation concentration. Taken together these data indicate that activation by acidic pH is not simply a result of protonation of histidine 157.

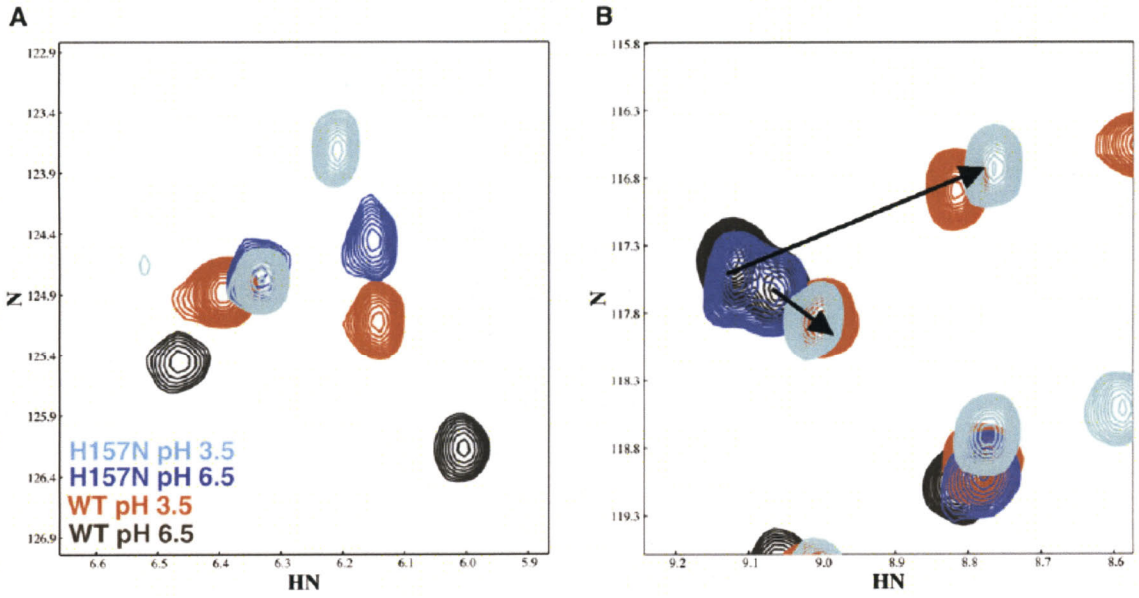

Figure 6. The H157N Mutation in the Sensor Domain Partly Mimics the Low pH Conformation.

The wild-type pH 6.5 (black) and pH 3.5 (red) spectra are compared to the spectra of H157N in the presence of 20 mM Mg^{2+} at pH 6.5 (dark blue) and pH 3.5 (light blue). The regions shown are the same as the insets shown in Figure 3B. The region in **(A)** shows peaks that appear in the low pH conformation in the H157N mutant at neutral pH, indicating the region around H157 as one pH-sensitive region. In **(B)** it is clear that there are other regions of the protein that are unaffected by the mutation at H157 yet are still titrated by low pH.

Mutation of Histidine 157 Promotes the Low pH Activated Conformation of the Sensor Domain at Neutral pH

Our *in vivo* experiments indicate that substitution of H157 with arginine, alanine, or asparagine favors the derepressed state. To obtain structural insight into this state, we used NMR spectroscopy to examine conformational changes associated with mutation of H157. We hypothesized that destabilization of the interaction network involving H157 could be important for pH activation because it was unlikely to be explained by divalent cation displacement from PhoQ-membrane bridges. To investigate the role of H157 in PhoQ's response to changes in Mg^{2+} concentration, the residue was substituted with either an arginine or an asparagine in the context of the sensor domain for NMR studies. Each of these mutations results in a well-folded protein with a similar conformation (see Figure S1 in the Supplemental Data). At pH 6.5 the spectrum of the H157R PhoQ sensor domain is nearly identical in the absence and presence of Mg^{2+}, consistent with the original mutant's phenotype of not responding to changes in Mg^{2+} concentration and with the lack of effect of Mg^{2+} concentration observed in the reporter assay (Figure 5). In contrast, the spectrum of H157N is sensitive to Mg^{2+} at pH 6.5, again recapitulating the Mg^{2+} sensitivity exhibited by full-length H157N PhoQ in the reporter assay (Figure 5). A subset of peaks grow in intensity in the presence of 20 mM Mg^{2+}, similar to the behavior previously reported for the wild-type sensor domain (Cho *et al.*, 2006). The remarkable correspondence between behaviors observed in the PhoN reporter strain and those observed for purified PhoQ sensor domain by NMR indicate that the conformational information provided by the NMR experiments is directly relevant to the mechanism of activation.

Numerous peaks in the H157N spectrum at pH 6.5 have shifted to new positions compared to the wild-type spectrum (Figure S2). Some peak shifting is expected as mutation of a residue affects the environment of residues surrounding it and thus their corresponding peaks in the spectrum. In this case, the derepressing H157N mutation gives rise to a considerably different spectrum in which a subset of the shifted peak positions closely correspond to those of the acidic pH conformation of wild-type PhoQ (Figure 6A, compare red and blue peaks). Of the ~30 peaks in the

wild-type spectrum whose shifts can be followed through the pH titration (Figure 3B), about one-third have the peak position of the H157N mutant spectrum at pH 6.5 close to the wild-type pH 3.5 position. Some of these resonances can be titrated even further in the H157N spectra upon lowering the pH to 3.5 (i.e., Figure 6A, compare blue to cyan peaks). However, the mutation does not completely reflect the pH-sensitive conformational change of the PhoQ periplasmic domain, because there is a second set of resonances in the wild-type spectra (roughly 20 peaks by the above count), which are insensitive to the mutation of H157 but are still undergoing chemical shifts induced by acidic pH (Figure 6B). Therefore these data indicate that destabilization of the hydrogen bonding interaction network in part simulates a conformational change induced by pH and identifies this network of interactions involving H157 as part of the mechanism of pH sensing.

DISCUSSION

PhoQ Is a Direct Sensor for Acidic pH

In the present study, we demonstrate that the *Salmonella* bacterial sensor kinase PhoQ can be directly activated by acidic pH. As *Salmonellae* encounter a pH of ~5.5 within the host cell phagosome, this is likely a physiologically relevant signal for activation of PhoQ. Incremental decreases in pH led to increases in PhoP-dependent gene activation as well as phosphorylation of PhoP. Furthermore, the sensor domain changes conformation upon acidification. NMR experiments at pH 5.5 in the presence or absence of Mg^{2+} demonstrate PhoQ's divalent cation-binding ability and rule out the trivial solution that activation at acidic pH is due to loss of Mg^{2+} binding. Taken together, our data provide strong evidence that the periplasmic domain of PhoQ adopts a pH-specific conformation as part of environmental sensing, ultimately leading to events catalyzed by its cytoplasmic domain, including intermolecular phosphorylation of another PhoQ in its dimer, phosphorylation of PhoP, and activation of PhoP-dependent gene transcription.

The Mechanism of PhoQ Acidic pH-Mediated Activation

A central finding of our study is that the direct response of PhoQ to acidification is mediated by a conformational change of the periplasmic domain, as demonstrated by NMR. In particular, the NMR spectrum of the derepressed mutant H157N appears to identify a pH-sensitive region of the protein, as this mutation partially mimics the activated acidic pH-induced conformation of wild-type PhoQ. *In vivo* PhoP-dependent gene expression data indicate that H157 mutants are unable to be fully repressed by divalent cations, consistent with results from the initial screen from which H157R was identified (Cho *et al.*, 2006). Although the original mutant substitutes a large and positively charged arginine for the histidine, removal of any functionality at residue 157 by replacement with alanine yields a protein with indistinguishable behavior to H157R. Both mutants are repressed to about the level of the wild-type protein at 1 mM Mg^{2+}, and both mutants have lost their ability to be repressed by higher concentrations of Mg^{2+}. These results also show unequivocally that the imidazole of H157 is required for cation-dependent repression of PhoQ. We

designed the H157N mutation to maintain the hydrogen-bonding capability of the wild-type residue. Remarkably, this mutant does retain the ability to respond to changes in Mg^{2+} concentration, consistent with the idea that the interaction network that includes H157 is required for the ability of the sensor domain to take on a repressed conformation in the presence of a high concentration of Mg^{2+}.

H157 is located on the central b sheet of the periplasmic PAS domain of PhoQ. The side chain takes part in a network of interactions that provide links to both the C-terminal end of the domain and the α4/α5 helix-turn-helix motif (see Figure 7). The two helices (shown in red in Figure 7) contain the acidic patch where cation binding is observed in the crystal structure (Cho *et al.*, 2006). As the crystals were grown in the presence of high divalent cation concentration, and cations are observed in the crystal structure, it is likely that this structure represents the repressed conformation. Our data demonstrate that the network of interaction that includes H157 is essential for divalent cation-mediated repression and is also associated with pH-mediated activation.

An important conclusion from the correlation of the NMR and *in vivo* data is that the divalent cation, antimicrobial peptide, and pH responses are separable, though partially overlapping, phenomena. Gene expression data from mutants H157R and H157A clearly show loss of divalent cation repression while they maintain the ability to respond to pH, indicating that the two functions can be separated. In addition, NMR data indicate that two different types of conformational change can occur in the PhoQ sensor domain—with changing divalent cation or antimicrobial peptide concentration peaks only change in intensity, whereas with changing pH, peaks shift and change intensity. Furthermore, the H157N mutation partially mimics the acidic pH-activated state. This suggests separate but overlapping mechanisms for activation of PhoQ, depending on the environmental signal being sensed.

Promotion of a more flexible state of the periplasmic domain may be responsible for a subtle conformational change that is transmitted through the membrane to the kinase domain, resulting in signal transduction. NMR peak broadening indicative of a system sampling multiple conformational states is observed in response both to loss of divalent cations or at pH 5.5, suggesting that induced flexibility of the interaction network or another region of the protein may be the

overlapping component of conformational activation. A model for stimulus-induced destabilization of N- or C-terminal helices has been proposed as a molecular mechanism of activation in photoactivatable PAS domains (Lee *et al.*, 2001; Harper *et al.*, 2003). Our NMR data suggest that PhoQ sensor domain dynamics are maximal at pH 5.5: the pH where PhoQ appears to be most activated. The results may indicate that a signal induced destabilization mechanism may extend to the PhoQ PAS domain sensing the very different stimuli of pH and antimicrobial peptides.

We suggest a model for PhoQ sensor domain activation as shown schematically in Figure 7. Many NMR peaks are not affected under any condition, indicating a stable core of the domain that does not undergo dramatic change, represented in blue on the structure. Helices α4 and α5, shown in red, are involved in divalent cation binding and are hypothesized to form bridges with the membrane. The H157 interaction network, shown in green, is required for maintaining divalent cation repression, presumably via structural rigidity that holds the a helices down against the membrane, because despite the divalent cation bridges, the two acidic surfaces of PhoQ and the membrane likely experience electrostatic repulsion. When this rigidity is disrupted by either binding of antimicrobial peptide to the acidic region or by acidification of the periplasm, the interaction network is destabilized and electrostatic repulsion causes the α4/α5 helices to move away from the membrane. This interaction network provides links among the core of the protein, the acidic cluster, and the C terminus of the domain, where the signal is transduced through the membrane to the cytoplasmic domain.

Activation of PhoPQ in Host Macrophages

A functional PhoPQ system is required for *Salmonella* virulence for mice and survival within macrophages. For PhoPQ to upregulate transcription of the genes involved in intracellular survival, PhoQ must receive an activating signal from within the macrophage phagosome. The identity of this signal has been a topic of much debate. Based on the data presented in this article, we propose that acidic pH and antimicrobial peptides work together as activating signals of PhoQ in vivo. It has been shown previously that PhoQ is also a direct sensor for antimicrobial peptides (Bader *et*

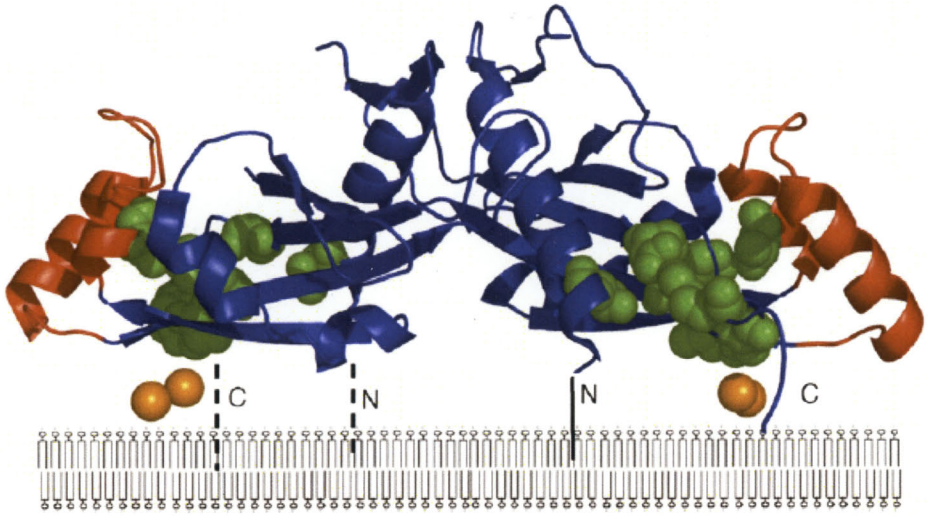

Figure 7. The Interaction Network Involving H157 in the *Salmonella* PhoQ Sensor Domain Dimer.

The PhoQ sensor domain (PDB code 1YAX) is colored to illustrate the interaction network surrounding H157. The core of the protein is colored blue and the α4/α5 helices are red. The residues participating in the interaction network comprised of hydrogen bonds and electrostatic contacts are colored green with their side chains displayed in space-filling format. The proposed membrane-facing surface is the bottom of the molecule, with the N and C termini labeled where they would enter the membrane. The gold spheres represent the two Ca^{2+} ions observed in the crystal structure; a third Ca^{2+} ion not observed in the crystal structure was found biochemically (Bader *et al.*, 2005) and is not shown. The acidic patch involved in divalent cation binding extends along the bottom of the protein, starting at the end of the α5 helix and including several glutamic acid residues that are also part of the interaction network.

al., 2005), another component of the host antimicrobial response. Together, acidic pH and antimicrobial peptides may represent the phagosome environment to PhoQ, resulting in phosphorylation of PhoP and virulence gene expression to promote bacterial survival within phagocytes. In support of this idea, we demonstrate that the two potential physiologic signals, antimicrobial peptides and acidic pH, can activate PhoPQ additively. Both PhoP-dependent gene activation and PhoP phosphorylation are greater in the presence of C18G and acidic pH than acidic pH alone.

The hypothesis that acidic pH is involved in PhoQ activation is strongly supported by many studies beginning with the work from Alpuche Aranda *et al.* in 1992 (Rathman *et al.*, 1996; Martin-Orozco *et al.*, 2006). However, the work presented here provides biochemical data to demonstrate that the pH effect is a direct effect on PhoQ. We have shown a direct phosphorylation of PhoP by PhoQ upon exposure to acidic pH and have also shown that the activation is likely mediated by a conformational/dynamic change in the PhoQ periplasmic sensor domain. In addition, it has been unclear whether acidic pH is a relevant signal for PhoQ because PhoP-dependent gene activation does not reach the levels seen in growth medium with low divalent cations. However, our results demonstrate that in defined medium, activation by acidic pH can reach the levels observed in low divalent cation medium. With the recent evidence that antimicrobial peptides directly activate PhoQ, it is possible that these two signals act together to fully activate the PhoPQ regulon in vivo within macrophage phagosomes. Indeed, we found that the two signals together activate PhoPQ much more strongly than acidic pH alone or low divalent cations. In fact, both signals may be required within phagosomes because antimicrobial peptides may be required to permeabilize the outer membrane and lower periplasmic pH to that of the phagosome.

In summary, we provide evidence that the sensor kinase PhoQ of *Salmonella* is able to directly sense acidic pH. The ability of this molecule to sense antimicrobial peptides has been previously established, and we show that these signals can work together to further activate PhoPQ. Both acidic pH and antimicrobial peptides are likely encountered during infection, and PhoPQ mediates responses to both. Thus,

they are likely signals to allow PhoPQ to recognize and respond to the intracellular environment.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Genetic Methods

For a detailed list of strains and primers used in this study, see Table S1. Strain CS120 was used as the wild-type reporter strain, except where indicated otherwise. For generation of mutants, plasmid pMB106 was used as a template for QuikChange mutagenesis (Stratagene) using the forward primers listed in Table S1 and corresponding reverse primers.

Bacterial Growth Conditions

Strains were grown in a modified N-minimal medium containing 100 mM MES instead of 100 mM Tris, pH adjusted as indicated. To study activation of the PhoP regulon, a strain containing *phoN::*Tn*phoA* was used (Miller *et al.*, 1989). For *in vivo* assays, strains were grown overnight, washed twice with N-minimal media, and then diluted 1:100 into fresh medium. Where peptide was used, strains were then grown to an OD_{600} of 0.2–0.3, and C18G was added at the indicated concentrations. After addition of peptide, strains were grown for another 60–90 min. Where peptide was not used, strains were grown for ~4.5 hr. Alkaline phosphatase activity assays were performed according to a standard protocol on cultures grown in duplicate on at least three independent trials.

Purification of Proteins

For NMR analysis, proteins were purified as previously described (Bader *et al.*, 2005). Briefly, strain MB141 was grown in MOPS-minimal medium supplemented with 1 g/l ^{15}N-ammonium chloride to mid-log phase, when IPTG was added to 0.5 mM. Cultures were grown an additional 4–6 hr, harvested by centrifugation, and lysed using a French Pressure Cell. Inclusion bodies were isolated by centrifugation at 3,500 x g, then washed once in 50 mM sodium phosphate (pH 8.0) and 300 mM NaCl; resuspended in 20 mM sodium phosphate (pH 8.0), 100mM NaCl, and 7M urea; and incubated on ice for 1 hr. Samples were then ultracentrifuged at 50,000 rpm for 45 min. The supernatant was rapidly diluted into ice-cold 20 mM sodium phosphate (pH 8.0). Samples were filtered and purified using a 5 mM HiTrap

nickel column (Amersham) according to a standard protocol. Purified protein was then applied to a Superdex-75 gel filtration column (Amersham) equilibrated with 20 mM sodium phosphate (pH 6.5), 150 mM NaCl, and 0.1 mM EDTA. PhoQ-containing fractions were pooled and concentrated to ~1 mM.

Purification of full-length PhoQ for reconstitution into vesicles was performed as previously described (Sanowar & Le Moual, 2005). Briefly, bacterial cultures were grown in LB for 4 hr after induction with 0.5 mM IPTG. Membrane fractions were prepared as described previously (Sanowar *et al.*, 2003). Membrane proteins were solubilized with 1% dodecyl-b-D-maltoside (DM). PhoQ was purified by nickel-NTA affinity chromatography and reconstituted into liposomes prepared from purified *E. coli* phospholipids (Avanti Polar Lipids).

In Vitro *Reconstitution of PhoPQ*

Proteoliposomes were loaded with phosphorylation buffer containing 200 mM KCl, 0.1 mM EDTA, 5% glycerol, and 40 mM sodium phosphate or citrate phosphate buffer adjusted to the appropriate pH. C18G at a concentration of 1 mg/ml was also included where indicated. The activity of PhoQ was assessed by the net phosphorylation of PhoP measured by incubating proteoliposomes containing 1.5 mM PhoQ with an 8-fold molar excess of PhoP in phosphorylation buffer at pH 7.5 (Bader *et al.*, 2005). Reactions were initiated by the addition of 0.1 mM [γ-32P]ATP and then incubated at 22°C for 20 min. Phosphorylated protein was visualized using an FX Scanner (Bio-Rad) and quantified by analysis with Quantity One software (Bio-Rad).

NMR Spectroscopy

The NMR sample of the PhoQ periplasmic domain (45-190) used in the pH titration contained 1.2 mM uniformly ^{15}N-labeled PhoQ in 20 Mm sodium phosphate buffer at pH 6.5, with 150 mM NaCl, 20 mM $MgCl_2$, 0.1 mM EDTA, and 10% (v/v) D_2O. The pH was lowered ~0.5 units at a time with the addition of microliter aliquots of 500 mM DCl. The other NMR samples of wild-type PhoQ sensor domain were prepared in the same manner using 0.8 mM uniformly ^{15}N-labeled PhoQ adjusted to the stated pH and contained 20 mM $MgCl_2$ or 4 mM PMNP where required. The

sample of H157N PhoQ contained 0.5 mM uniformly ^{15}N-labeled protein either apo or in the presence of 20 mM $MgCl_2$ with the pH adjusted as stated. All 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 & Blevins, 1994).

SUPPLEMENTARY DATA

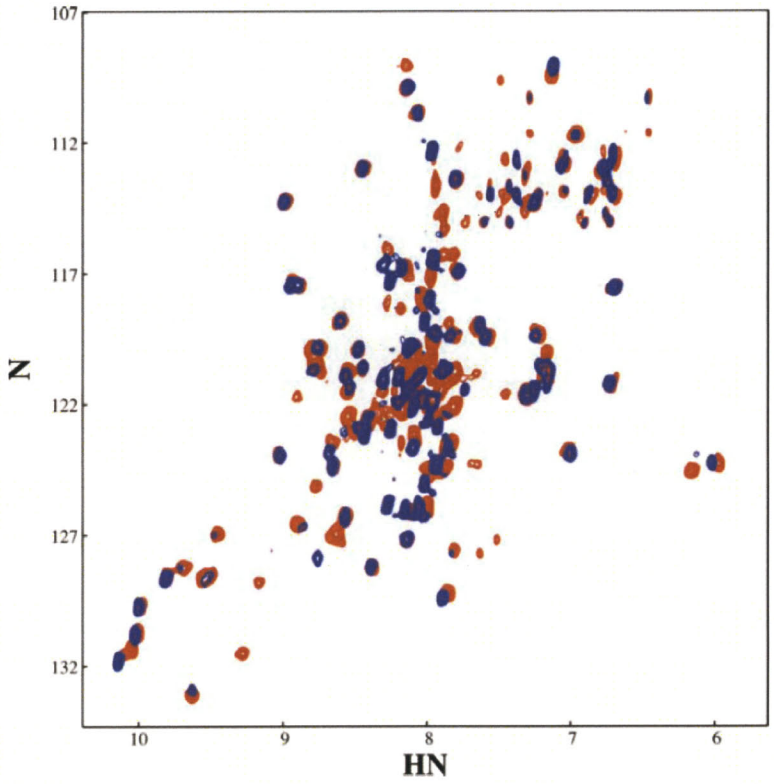

Figure S1. HSQC spectra of 0.67 mM H157R PhoQ-SD (blue) and 0.48 mM H157N PhoQ-SD (red) with 20 mM $MgCl_2$ at pH 6.5. Mutation of H157 to either R (blue spectrum) or N (red spectrum) results in a well-folded protein with a similar structural state. However, as described in the text, H157R is no longer Mg-responsive, while H157N is, as demonstrated by the increased number of peaks.

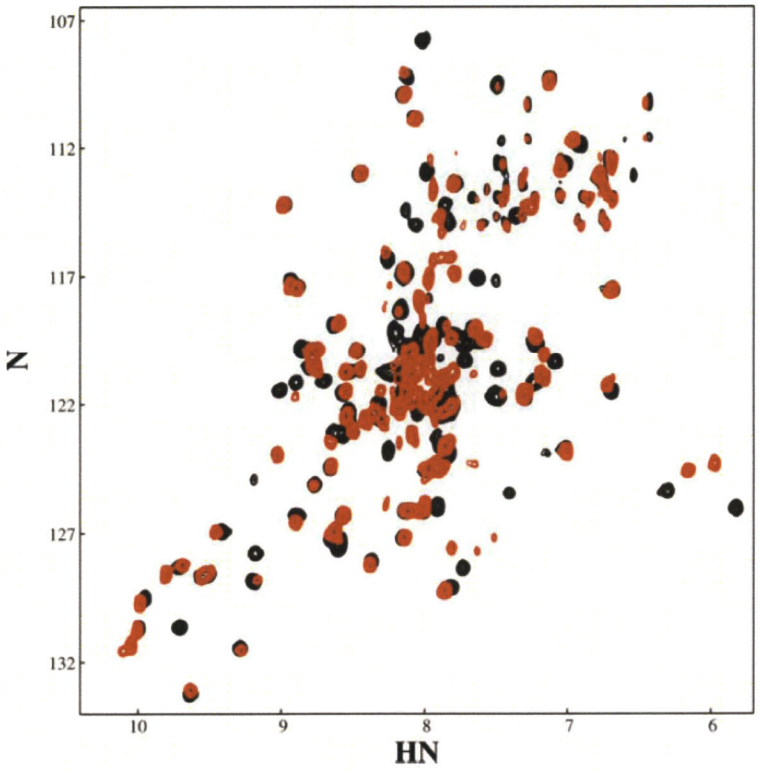

Figure S2. HSQC spectra of 0.8 mM WT PhoQ-SD (black) and 0.48 mM H157N PhoQ-SD (red) with 20 mM $MgCl_2$ at pH 6.5. Mutation of H157N results in a stable and well-folded protein as indicated by the red spectrum. This de-repressing point mutation causes the spectrum to be considerably different than the WT "repressed state" spectrum.

Table S1. Strains and Primers Used in This Study

Strains
CS120 14028s *phoN105::*Tn*phoA*
MB101 CS120 *phoQ::*Tn10d(T-POP)
MB106 MB101 pBAD24-*phoQ*
MB141 BL21(DE3) pET11a-*phoQ* 45-190-(His)6
MB187 MB101 pBAD24-*phoQ* H157R
MB211 BL21(DE3) pET11a-*phoQ* H157R 45-190-(His)6
LP36 MB101 pBAD24-*phoQ* H157A
LP42 BL21(DE3) pET11a-*phoQ* H157N 45-190-(His)6
LP43 MB101 pBAD24-*phoQ* H157N

Primers
LP68 (H157A) GCGGAGATGACCGCCTCGGTAGCGG
LP70 (H157N) GCGGAGATGACCAACTCGGTAGCGG
MB169 (H157R) CCGAGATGACCCGCTCGGTAGCGGT

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PREFACE TO CHAPTER 3

A major theme discussed in Part 1 of Chapter 1 is the availability of crystal structures for all the domains of the two-component system and how this structural information is used to propose models of signal transduction through the membrane. Chapter 2 uses NMR to examine the conformational change that the PhoQ periplasmic domain undergoes as a consequence of acidification.

Having demonstrated that PhoQ is directly responsible for sensing pH (Chapter 2) we wanted to identify the pH-sensing residue(s). We used fluorescence spectroscopy in order to probe conformational changes of the PhoQ periplasmic domain in response to acidification. Although promising, the interaction between H120 and W113 was not responsible for pH–sensing since mutation of these residues in the context of the full-length PhoQ SK did not abolish pH responsiveness.

CHAPTER 3

A tryptophan residue involved in a π-cation interaction with a protonated histidine residue probes pH-dependent conformational changes in the periplasmic domain of the *Salmonella* PhoQ sensor kinase

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ABSTRACT

The PhoP/Q two-component signal transduction system controls *Salmonella* virulence by regulating gene expression in response to various environmental cues. The PhoQ sensor kinase is activated by limiting concentrations of divalent cations, cationic antimicrobial peptides and acidic pH. PhoQ autophosphorylates and transfers its phosphoryl group to the transcriptional regulator PhoP that controls expression of a large number of genes relevant to *Salmonella* survival within host cells. The effect of pH acidification on the intrinsic tryptophan fluorescence of the PhoQ periplasmic domain ($PhoQ_{Peri}$) was studied to detect conformational changes. pH titration experiments revealed that at least one of the four tryptophan residues of $PhoQ_{Peri}$ is quenched with a pK_a of 7.5, suggesting involvement of a histidine residue. Tryptophan fluorescence spectra of single mutants of $PhoQ_{Peri}$ showed that the tryptophan at position 113 (W113) is quenched by the histidine at position 120 (H120) in a pH-dependent manner. The three-dimensional structure of $PhoQ_{Peri}$ indicates ring stacking between W113 and H120. Acrylamide fluorescence quenching experiments showed that W113 is less accessible to the quencher at pH 5.5 than at pH 8.0. Mutagenesis analysis indicated that the regulation of the PhoQ kinase activity is optimal when aromatic residues are present at positions 113 and 120. This π–stacking interaction is conserved among PhoQ homologues of Gram-negative pathogens. These results argue for a pH-dependent dynamic π–stacking interaction between W113 and H120. They also identify W113 as a good fluorescent indicator of conformational change induced by pH acidification, a condition encountered by *Salmonella* upon entry into host cells.

INTRODUCTION

Two-component systems (TCSs) are signal transduction pathways utilized by bacteria to sense changing environmental conditions and respond accordingly by modulating the transcription of specific genes (Stock *et al.*, 2000; Szurmant *et al.*, 2007). The simplest form of TCS consists of a histidine kinase sensor and a response regulator. The sensor kinase is usually an integral membrane protein with an extracellular sensory domain and a cytosolic signaling domain that autophosphorylates on a conserved histidine residue in response to specific environmental cues. The response regulator is a cytoplasmic protein that acts as a transcriptional regulator. Transfer of the phosphoryl group from the sensor kinase to an invariant aspartate residue of the response regulator promotes the transcriptional regulation of target genes.

The PhoP/Q TCS is composed of the sensor kinase PhoQ and the response regulator PhoP. PhoP/Q appears to be central to the virulence of a number of Gram-negative bacteria including insect, plant and mammalian pathogens (Moss *et al.*, 2000; Oyston *et al.*, 2000; Llama-Palacios *et al.*, 2003; Derzelle *et al.*, 2004). In the facultative intracellular pathogen, *Salmonella enterica*, the PhoP/Q TCS is critical for survival within host macrophages and successful infection (Groisman, 2001; Prost et al., 2007b). Deletion of the *phoP* or *phoQ* genes, or expression of a constitutively active PhoQ protein result in attenuation of virulence (Miller *et al.*, 1989; Miller & Mekalanos, 1990). Together with other TCSs, PhoP/Q is part of a regulatory network that controls the expression of more than 200 genes, including genes associated with magnesium uptake, modifications of the lipopolysaccharide, resistance to cationic antimicrobial peptides (CAMPs) and other virulence traits (Groisman, 2001; Monsieurs *et al.*, 2005). Following uptake by host macrophages, *Salmonella* resides within vacuoles, known as *Salmonella*-containing vacuoles (SCVs), in which bacterial cells survive and replicate (Miller *et al.*, 1989; Prost *et al.*, 2007b). The vacuolar environment of the SCV is characterized by defenses of innate immunity such as CAMPs, acidic pH in the range of 5.0-6.5 and a constant concentration of Mg^{2+} around 1 mM (Rosenberger *et al.*, 2004; Alpuche Aranda *et al.*, 1992; Martin-Orozco *et al.*, 2006). The PhoQ sensor kinase has been proposed to sense the intra-vacuolar

environment of SCVs (Prost *et al.*, 2007b). Several lines of experimental evidence have shown that the periplasmic PhoQ sensory domain directly recognizes CAMPs and acidic pH leading to increased kinase activity and PhoP phosphorylation (Bader *et al.*, 2005; Prost *et al.*, 2007a). In contrast, high concentrations of divalent cations such as Mg^{2+}, Ca^{2+} and Mn^{2+} have been shown to repress PhoP/Q by promoting the PhoQ-mediated dephosphorylation of PhoP (Garcia Vescovi *et al.*, 1996; Castelli *et al.*, 2000; Montagne *et al.*, 2001). CAMPs appear to compete with divalent cations for binding to the PhoQ acidic patch that faces the phospholipid membrane (Bader *et al.*, 2005). Although it has been shown that acidic pH is perceived directly by the PhoQ periplasmic domain, the mechanism of pH sensing and subsequent conformational changes that lead to signal transduction are still unclear (Prost & Miller, 2008). NMR experiments have shown that the conformation of the isolated PhoQ sensory domain is sensitive to pH changes both in the presence and absence of divalent cations or CAMPs (Prost *et al.*, 2007a). Fluorescence spectroscopy is a technique often used to study protein conformational changes. Tryptophan residues are intrinsic fluorescent probes that are highly sensitive to changes in their local environment (Lakowicz, 1999). The PhoQ periplasmic domain contains four tryptophan residues that are all partially exposed (Cho *et al.*, 2006; Cheung *et al.*, 2008).

In this study, we analyzed the tryptophan fluorescence of the PhoQ sensory domain to detect pH-dependent changes of structure. We identified a pH-dependent dynamic π–stacking interaction between W113 and H120. A sequence alignment of PhoQ sensory domains from different Gram-negative species in combination with activity assays of PhoQ mutants at positions 113 and 120 highlighted the importance of this evolutionarily conserved stacked ring arrangement for optimal PhoQ response to environmental signals.

RESULTS

Tryptophan fluorescence of wild-type PhoQ sensory domain

Fluorescence experiments were performed to investigate conformational changes that occur in the PhoQ periplasmic sensory domain ($PhoQ_{Peri}$) upon pH acidification. $PhoQ_{Peri}$ contains four tryptophan residues at positions 65, 97, 104 and 113 that could act as fluorescent reporters of structural changes. We monitored the intrinsic tryptophan fluorescence emission of the purified $PhoQ_{Peri}$ protein at various pHs by exciting the protein at 295 nm, a wavelength that avoids excitation of tyrosine (Lakowicz, 1999). The pH was incrementally lowered from 11 to 3.5, resulting in a dramatic decrease of 6-fold in the fluorescence intensity of $PhoQ_{Peri}$ (Figure 1A). This decrease in fluorescence intensity was not due to changes in the oligomeric state of the protein, since $PhoQ_{Peri}$ at a concentration of 30-100 μM was mostly monomeric at both pH 8.0 and 5.5, as determined by sedimentation velocity experiments (results not shown). The emission maximum of $PhoQ_{Peri}$ at pH $\geq$ 7.5 was 347 nm and became blue-shifted by 6 nm at pH $\leq$ 6.5 (Figure 1A and Table 1). Figure 1(B) shows the titration curve of fluorescence intensity of $PhoQ_{Peri}$ as a function of pH. The fluorescence of tryptophans was greatly affected by changes in pH between 6.0 and 9.0 with approximately 80% of the fluorescence being quenched over this pH range. This indicates that at least one tryptophan is quenched by the protonation of a neighboring ionizable residue. A pK_a of 7.5 was obtained by fitting the data to a sigmoidal equation, suggesting that the ionizable residue may be a histidine. These findings indicate that pH acidification results in an important change in the microenvironment of at least one of the four tryptophan residues of $PhoQ_{Peri}$.

W113 is quenched in a pH-dependent manner

To determine the contribution of each tryptophan in the pH-dependent fluorescence spectrum of $PhoQ_{Peri}$, we individually substituted tryptophan residues by phenylalanines. Similar to wild-type, emission spectra of the mutants W65F, W97F and W104F were quenched in a pH-dependent manner and fluorescence spectra were blue-shifted by approximately 6 nm at pH $\leq$ 6.5 (Table 1). In contrast, the W113F mutant showed a fluorescence intensity that was essentially independent of pH (Figure

Table 1. Wavelength emission maxima and relative intensities at these wavelengths for wild-type and mutant $PhoQ_{Peri}$ proteins

Protein	λmax (nm) pH 8.0	Relative Intensity	λmax (nm) pH 5.5	Relative Intensity	Ratio of intensities pH 8.0/pH 5.5
Wild-type	347±0.9	720	341±0.7	190	3.78
W65F	347±0.3	612	340±1.0	160	3.82
W97F	347±0.5	603	343±0.6	249	2.42
W104F	347±0.9	640	340±1.3	240	2.66
W113F	340±0.6	290	340±0.6	250	1.16
H120N	346±0.8	690	346±0.5	630	1.09
H120V	345±0.3	629	345±0.5	622	1.01
H137N	347±0.3	790	341±0.3	210	3.76
H157N	347±0.7	710	340±0.7	210	3.38

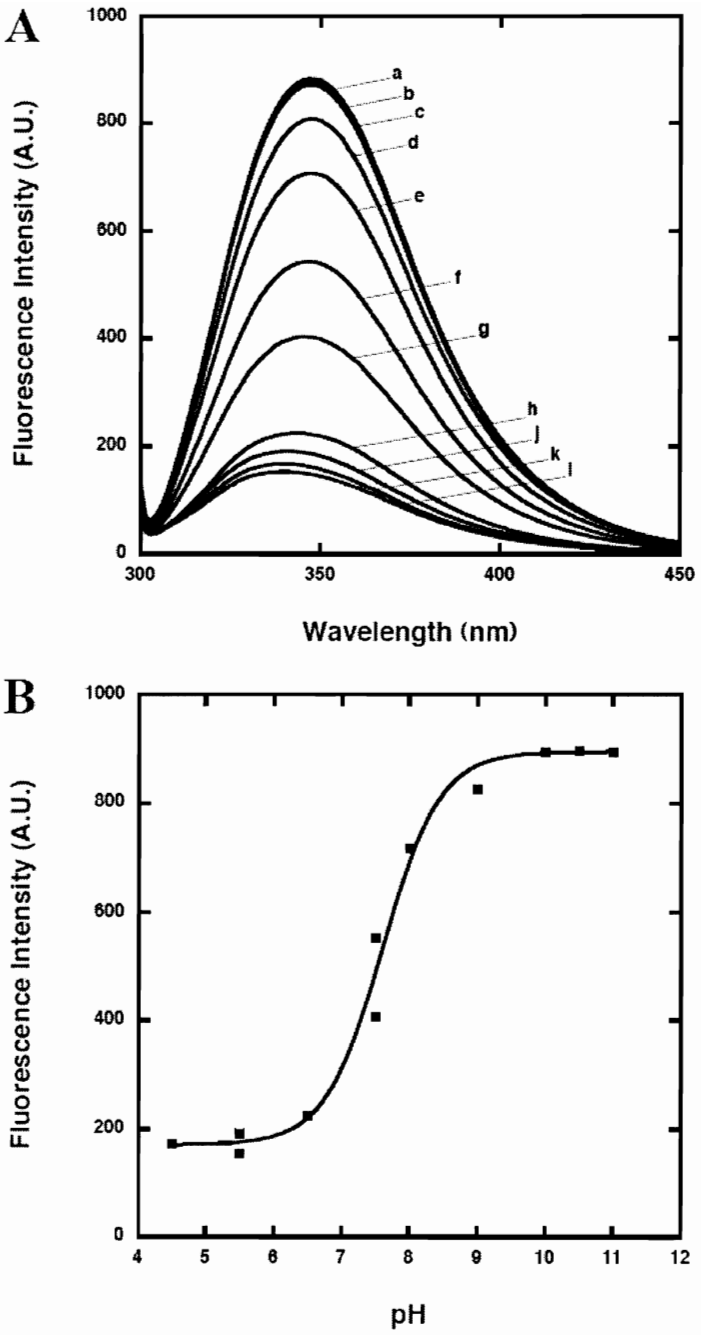

A
Fluorescence Intensity (A.U.)
Wavelength (nm)
a
b
c
d
e
f
g
h
j
k
l
B
Fluorescence Intensity (A.U.)
pH

Figure 1. Effect of pH on the fluorescence emission spectra of $PhoQ_{Peri}$.

A, Fluorescence emission spectra of $PhoQ_{Peri}$ (6.75 μM) were recorded at 20 °C in 20 mM of the specified buffers supplemented with 150 mM NaCl. Excitation wavelength was set at 295 nm. Each spectrum was corrected for buffer background. Buffers used are CAPS pH 11(a), pH 10.5 (b), pH 10.0 (c), Tris-HCl pH 9.0 (d), pH 8.0 (e), pH 7.5 (f), NaPi pH 7.5 (g), MES pH 6.5 (h), pH 5.5 (i), CitPi pH 5.5 (j) and pH 3.5 (k). **B**, pH titration of $PhoQ_{Peri}$. The fluorescence intensity of the emission maxima was plotted as a function of pH. The solid line represents the best fit to a sigmoidal curve.

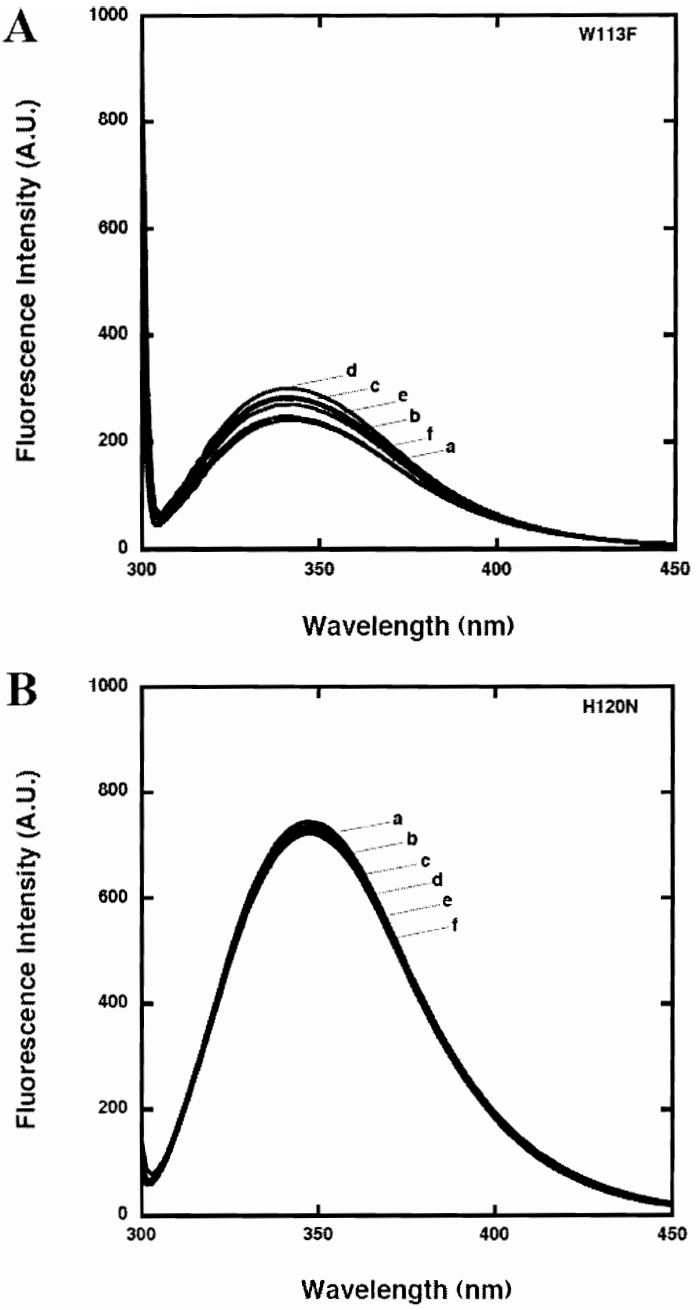


Figure 2. Fluorescence emission spectra of the W113F **(A)** and H120N **(B)** $PhoQ_{Peri}$ proteins.

Mutant $PhoQ_{Peri}$ (6.75 μM) were dialyzed against 20 mM CAPS pH 11.0 (a), Tris-HCl pH 9.0 (b), pH 8.0 (c), NaPi pH 7.5 (d), CitPi pH 5.5 (e) and pH 3.5 (f), supplemented with 150 mM NaCl. The tryptophan residues were excited at 295 nm and the emission spectra recorded at 20 °C.

2A). No blue shifting was observed for mutant W113F, the maximum emission was close to that observed for wild-type $PhoQ_{Peri}$ at pH $\leq$ 6.5, regardless of pH (Figure 2A and Table 1). These data show that W113 is the tryptophan residue that is quenched in a pH-dependent manner.

Protonation of His 120 quenches W113

$PhoQ_{Peri}$ contains three histidine residues that were individually mutated to asparagines (H120N, H137N and H157N) to identify the quencher of W113. The emission spectra of mutants H137N and H157N were quenched in a pH-dependent manner as wild-type $PhoQ_{Peri}$ (Table 1). In contrast, no significant change in fluorescence intensity was observed for the mutant H120N by lowering the pH (Figure 2B). In addition, no blue shift at pH $\leq$ 6.5 was observed for this mutant (Figure 2B and Table 1). Similar conclusions were reached for the mutant H120V (Table 1). These data clearly implicate the protonation of H120 as the cause of the pH-dependent fluorescence quenching of W113. This finding is in good agreement with the crystal structures of $PhoQ_{Peri}$, in which W113 is 3.5 Å away from H120 (Cho *et al.*, 2006; Cheung *et al.*, 2008). The fact that the indole ring of W113 lies parallel to the imidazole ring of H120 suggests that these two residues may be involved in a π–stacking interaction (Figure 3).

Acrylamide quenching of $PhoQ_{Peri}$

Lowering the pH below 6.5 induced a consistent blue shift of approximately 6 nm in wild-type and mutant $PhoQ_{Peri}$ proteins, with the exception of W113F and H120N (Table 1). This result suggests that pH acidification affects the conformation of W113, which becomes more buried as a result of its interaction with protonated H120. To confirm these results, acrylamide quenching experiments were performed to evaluate the solvent exposure of W113 at different pHs (Eftink & Ghiron, 1976; Eftink & Ghiron, 1981). Since a $PhoQ_{Peri}$ protein containing a single tryptophan residue at position 113 was poorly expressed and highly unstable, quenching experiments were performed using wild-type $PhoQ_{Peri}$ and mutants W113F and H120N. Intrinsic fluorescence was measured in the presence of increasing

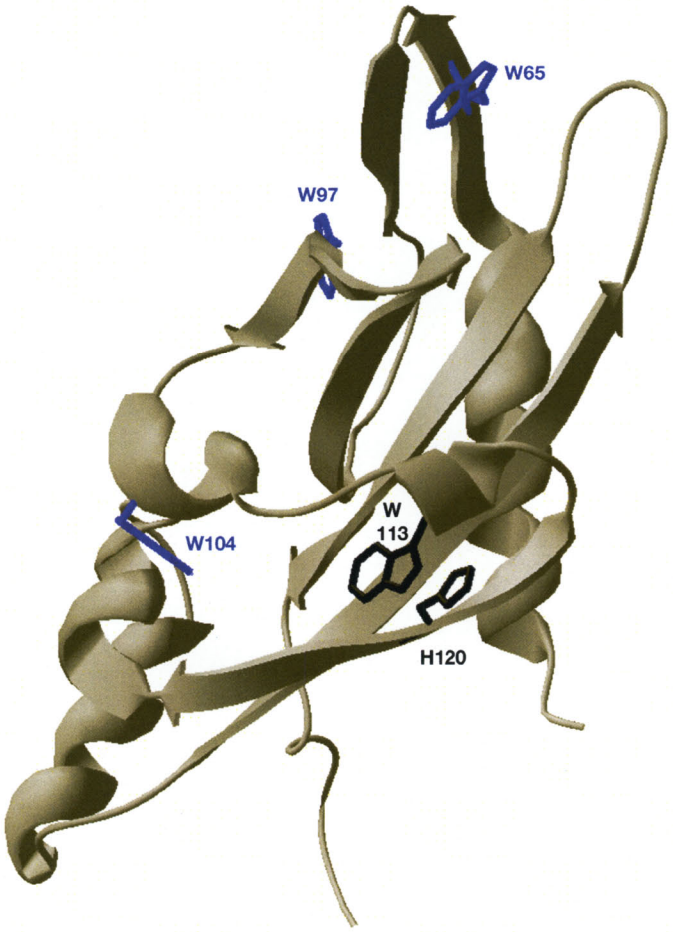


Figure 3. Ribbon representation of the α3-helix and the β5-sheet of PhoQ periplasmic domain.

Protein Data Bank entry 1YAX. Side chains are shown as stick representations.

concentrations of the neutral collisional quencher, acrylamide. Quenching experiments were performed under conditions at which the side chain of H120 is expected to be protonated (pH 5.5) and non-protonated (pH 8.0) (Figure 1B). The Stern-Volmer plots shown in Figure 4 illustrate that wild-type and mutant $PhoQ_{Peri}$ proteins have a linear relationship at acrylamide concentrations in the range of 0-0.2 M, which is consistent with the fact that all tryptophans are partially accessible to the solvent and form a single class of fluorophores (Cho *et al.*, 2006; Cheung *et al.*, 2008). Data were fitted to the Stern-Volmer equation to obtain the collisional quenching constants (K_{SV}). The Stern-Volmer plots obtained for wild-type $PhoQ_{Peri}$ show that lowering pH to 5.5 attenuates acrylamide quenching (Figure 4A). The K_{SV} value obtained at pH 5.5 is 1.8-fold lower that than that obtained at pH 8.0 (Table 2), indicating that at least one tryptophan of $PhoQ_{Peri}$ is more accessible to the quencher at pH 8.0 than pH 5.5. To determine whether W113 is the tryptophan quenched by acrylamide in a pH-dependent manner, we analyzed the W113F mutant. As shown in Figure 4(B) and Table 2, the Stern-Volmer plots and K_{SV} values obtained for this mutant at pH 8.0 and 5.5 were similar. In good agreement, Stern-Volmer plots and K_{SV} values obtained for the H120N mutant were also essentially similar at pH 8.0 and 5.5 (Figure 4C and Table 2). These acrylamide quenching data are consistent with the 6 nm blue shift observed for $PhoQ_{Peri}$ at acidic pH. Together, these data indicate that W113 is less accessible to the solvent at acidic pH and that H120 is responsible for this pH-dependent conformational alteration.

In vivo *activity assays*

To examine the possibility that H120 is involved in pH sensing, various mutations were introduced at positions 113 and 120 of full-length PhoQ. The activity of the PhoQ mutants was measured in response to acidic pH, in the context of a PhoQ-null mutant *Salmonella* reporter strain transformed with the wild-type or mutant pBAD-Q plasmids (Bader *et al.*, 2005; Prost *et al.*, 2007a). Bacterial cells were grown in N-minimal medium buffered at pH 7.5 or 5.5 and the alkaline phosphatase activity of the PhoP-regulated *phoN::*Tn*phoA* reporter was measured (Bader *et al.*, 2005; Prost *et al.*, 2007a). For wild-type PhoQ, lowering the pH from 7.5 to 5.5 increased PhoN

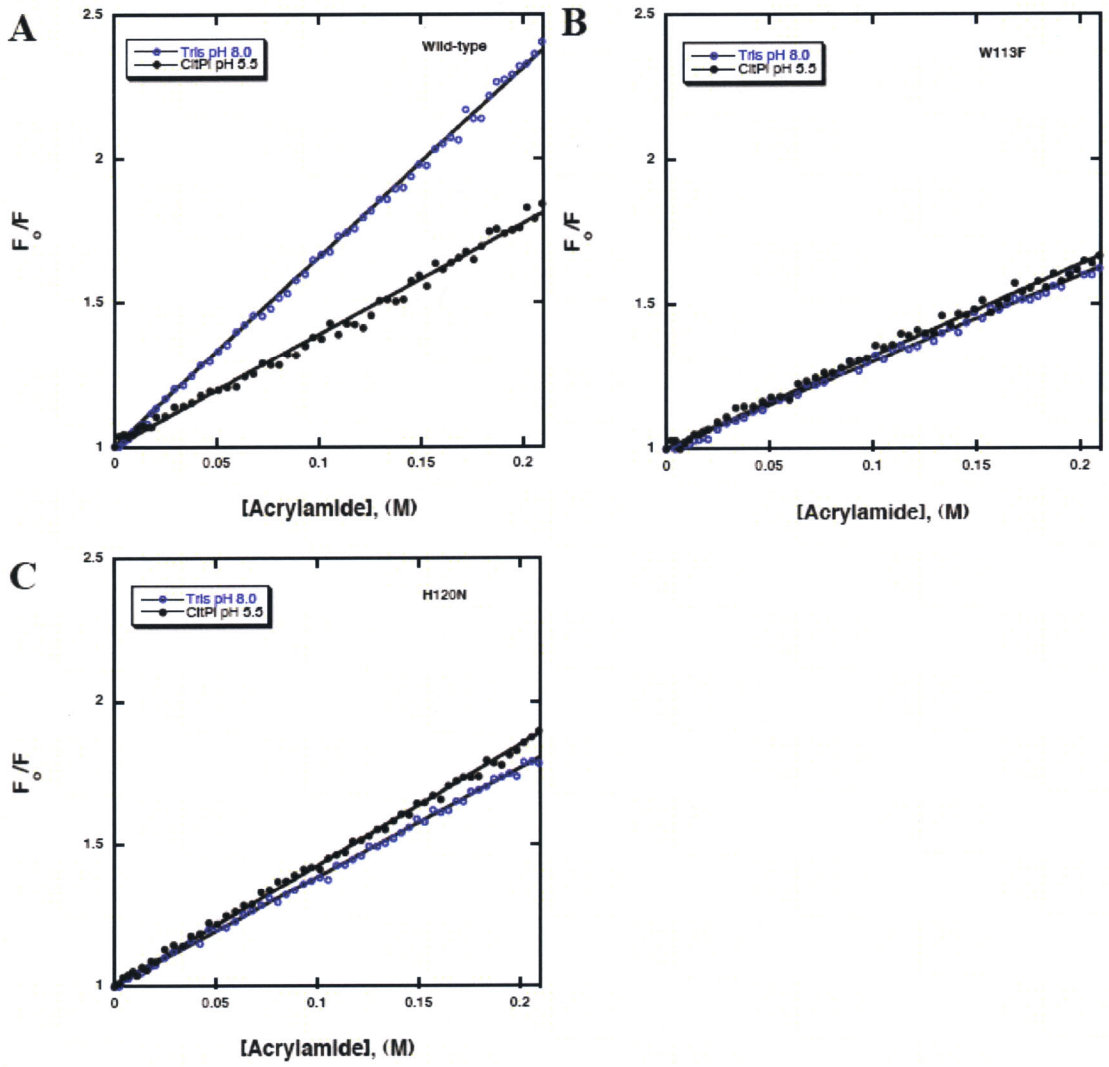
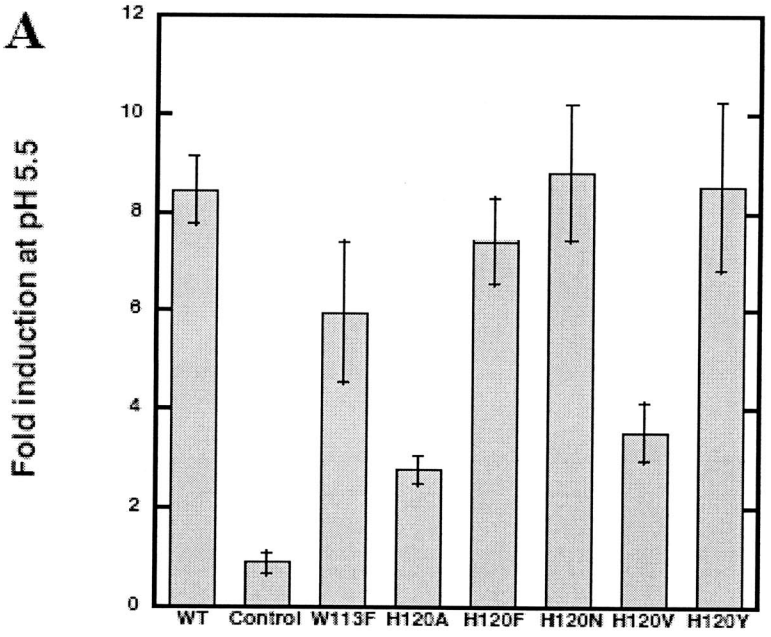


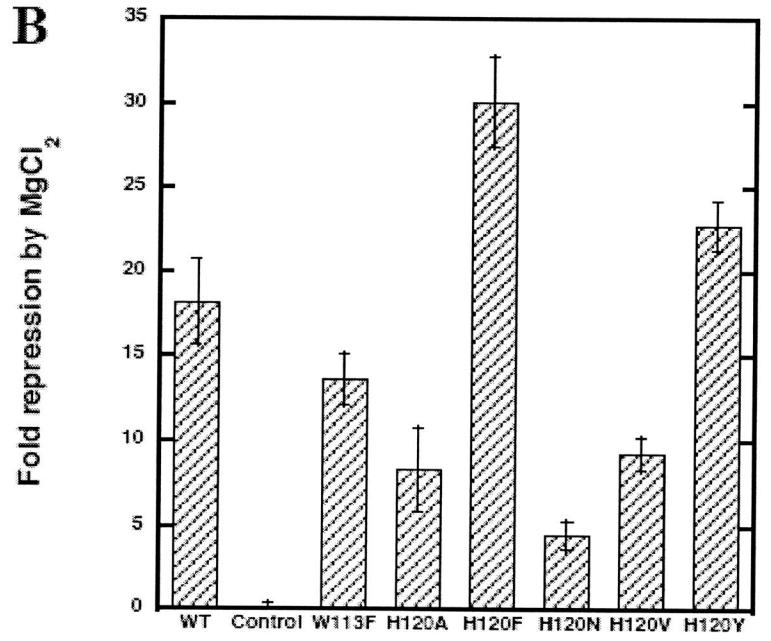
Figure 4. Stern-Volmer plots for acrylamide quenching of wild-type **(A)**, W113F **(B)** and H120N **(C)** of $PhoQ_{Peri}$.

Quenching experiments were performed with proteins in Tris-HCl pH 8.0 (blue) or citrate phosphate pH 5.5 (black). Emission intensities were collected at the maximal wavelengths (Table. 1). The plots presented herein are averages from three scans.

Table 2. Stern-Volmer quenching constants K_{SV} obtained for $PhoQ_{Peri}$ wild-type and mutants W113F and H120N at pH 8.0 and pH 5.5

$PhoQ_{Peri}$	K_{SV}	
	pH 8.0	pH 5.5
Wild-type	6.59±0.31	3.49±0.65
W113F	3.22±0.36	3.38±0.29
H120N	4.1±0.07	3.91±0.16


A
Fold induction at pH 5.5
12
10
8
6
4
2
0
WT
Control
W113F
H120A
H120F
H120N
H120V
H120Y


B
Fold repression by MgCl2
35
30
25
20
15
10
5
0
WT
Control
W113F
H120A
H120F
H120N
H120V
H120Y

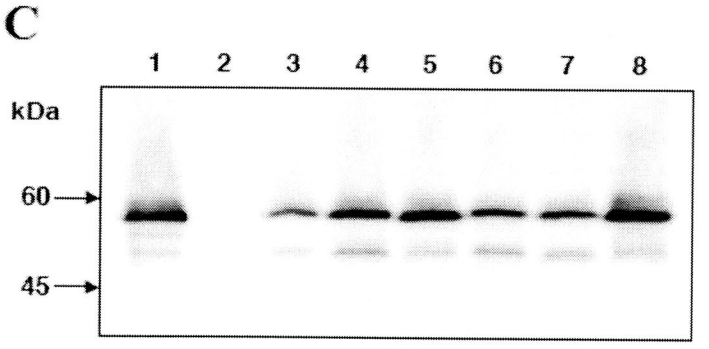

C
1
2
3
4
5
6
7
8
kDa
60
45

Figure 5. *In vivo* activity of wild-type and mutant PhoQ proteins.

A. PhoPQ-dependent activation of *phoN* by pH acidification. Strain MB101 was transformed with plasmids pBAD24-Q (wild-type), pBAD24 (negative control) or pBAD24-derived plasmids expressing different PhoQ mutants. Cultures were grown in N-minimal medium buffered with Bis-Tris at pH 7.5 or 5.5. Fold induction values correspond to the ratios of the alkaline phosphatase activity obtained for cells grown at pH 5.5 over the alkaline phosphatase activity obtained for cells grown at pH 7.5. Values are mean ± standard deviation of three independent experiments. **B**. PhoPQ-dependent repression of *phoN* by $MgCl_2$. Strain MB101 transformed with plasmids pBAD24-Q and pBAD24 were used as the wild-type and control strains, respectively. Cultures were grown in N-minimal medium buffered with Bis-Tris at pH 7.5 and containing 8 μM or 10 mM $MgCl_2$. Fold repression values correspond to the ratios of the alkaline phosphatase activity obtained for cells grown in the presence of 8 μM $MgCl_2$ over the alkaline phosphatase activity obtained for cells grown in the presence of 10 mM $MgCl_2$. Values are mean ± standard deviation of three independent experiments **C**. Expression levels of wild-type and mutant full-length PhoQ proteins were determined by Western blot analysis using an antibody against the PhoQ periplasmic domain. From the left to the right: wild-type, negative control, W113F, H120A, H120F, H120N, H120V and H120Y.

expression by 8-fold (Figure 5A). The mutants felt into two categories: W113F, H120F, H120N and H120Y resulted in induction levels of PhoN expression between 6- and 8-fold, indicating that they retained the ability to be activated by acidic pH (Figure 5A). In contrast, the aliphatic substitutions, H120A and H120V, responded poorly to acidic pH and resulted in a 3- and 3.5-fold induction, respectively (Figure 5A). All these mutants are still pH responsive, indicating that the presence of an ionizable imidazole side chain at position 120 of PhoQ is not required for pH sensing. These data also highlight the importance of an aromatic or imidazole ring at position 120, although an asparagine at this position functions as well as the native histidine.

Millimolar concentrations of Mg^{2+} are known to repress the transcription of PhoP-activated genes (Garcia Vescovi *et al.*, 1996). Thus, the importance of residue H120 in the PhoQ response to Mg^{2+} was examined by determining the fold repression ratio of cells grown in the presence of either 8 μM or 10 mM $MgCl_2$. As shown in Figure 5(B), a similar pattern of PhoQ responsiveness was observed, with the exception of mutant H120N. The introduction of an aromatic ring at position 120 increased PhoQ responsiveness to Mg^{2+} depletion. Mutants H120F and, to a lesser extent, H120Y showed higher fold-induction (30- and 22.5-fold) than wild-type PhoQ (18-fold). In contrast, H120A (8-fold) H120N (5-fold) and H120V (9-fold) showed reduced responsiveness to Mg^{2+} depletion, compared to wild-type (Figure 5B). Mutant W113F responded to Mg^{2+} depletion slightly less well than wild-type (Figure 5B). Western blot analysis of the mutant PhoQ expression levels indicates that the different mutant phenotypes are not due to various levels of the PhoQ protein (Figure 5C). Altogether, these results clearly show that H120 is not involved in pH sensing. They also highlight the importance of the stacking interaction between positions 113 and 120 and indicate that responses to variations in pH and Mg^{2+} concentrations are grossly superimposable, although an asparagine at position 120 responds to pH acidification but not to Mg^{2+}.

Evolutionary conserved π-stacking arrangement of the PhoQ protein

To investigate whether the π-stacking interaction between W113 and H120 was conserved among PhoQ homologues, we aligned the region that encompasses

residues 113 and 120 from various Gram-negative pathogens. These included closely related species of the family *Enterobacteriaceae* and the more distantly related *Pseudomonas* species. As shown in Figure 6, all PhoQ homologues of the *Enterobacteriaceae* family contain a tryptophan at position 113. While many of them have a histidine residue at position 120, three out of nine contain a tyrosine at this position. Although the imidazole side chain of histidine can be readily protonated, it appears unlikely that the tyrosine side chain (pK_a = 11.1) undergoes ionization within a physiological pH range. Interestingly, a π-stacking interaction involving a tyrosine (position 113) and a phenylalanine (position 120) is also conserved in *P. aeruginosa* PhoQ, which is known to have different sensing capabilities (Bader *et al.*, 2005). Thus, across all species, the side chains of amino acids 113 and 120 consist of aromatic or imidazole rings that potentially form cation-π or π-stacking interactions. This interaction, which has been conserved across evolution, may be necessary for protein stability and/or function.

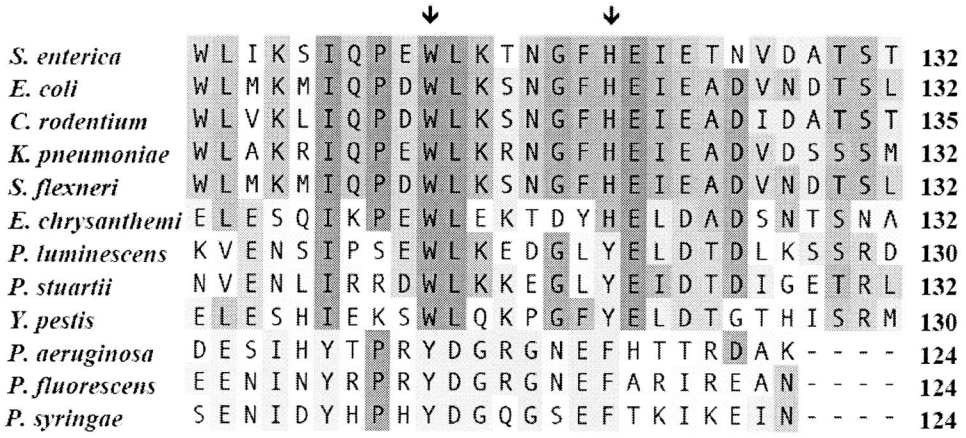


Figure 6. Partial sequence alignment of the PhoQ region encompassing W113 and H120. The arrows indicate positions 113 and 120.

DISCUSSION

The *Salmonella* PhoQ sensor kinase has been shown to sense several environmental cues that are encountered during the intracellular stage of infection (Prost *et al.*, 2007b; Prost & Miller, 2008). PhoP/Q is activated by CAMPs and acidic pH, and repressed by divalent cations such as Mg^{2+} and Ca^{2+}. The molecular mechanism by which PhoQ senses divalent cations has been unraveled recently. Crystallization of the PhoQ sensory domain ($PhoQ_{Peri}$) in the presence of Ca^{2+} ions revealed that divalent cations form metal bridges between the bacterial membrane and a negatively charged surface of PhoQ, which is known as the acidic cluster (Cho *et al.*, 2006). CAMPs were found to compete with divalent cations for binding to the PhoQ acidic cluster (Bader *et al.*, 2005). Acidic pH is another environmental signal sensed directly by the PhoQ sensory domain (Prost *et al.*, 2007a). The underlying mechanism of pH sensing and subsequent structural changes that modulate the PhoQ kinase activity remain to be elucidated. Using intrinsic tryptophan fluorescence, we found that pH acidification greatly affects the fluorescence properties of $PhoQ_{Peri}$. This work identifies a pH-dependent π-stacking interaction between the imidazolium form of H120 and W113 that is responsible for fluorescence quenching of W113. At acidic pH, W113 becomes less accessible to the solvent than at neutral pH. This may be due to a subtle conformational change and/or to its interaction with the imidazolium ring of H120.

The pH dependence of $PhoQ_{Peri}$ fluorescence intensity is associated with W113, and not with any of the other three tryptophan residues (Table 1 and Figure 2A). Based on a pK_a value of 7.5 and mutagenesis of the three histidine residues of $PhoQ_{Peri}$, H120 was identified as being the residue responsible for the pH-dependent fluorescence quenching of W113 (Figure 2B). These results show that protonation of H120 at pH $\leq$ 7.5 causes a decrease in the fluorescence intensity of W113. This result is consistent with previous studies showing that the imidazolium form of histidine is a more efficient quencher of tryptophan in solution than the imidazole form (Steiner & Kirby, 1969; Willaert & Engelborghs, 1991). In addition, fluorescence quenching of W113 by H120 appears to be due to a direct interaction between these two residues, since mutating either W113 or H120 abolished fluorescence quenching (Figure 2). The

3D structure of $PhoQ_{Peri}$ showed that the imidazole ring of H120 at a distance of 3.5 Å from the indole ring of W113, corroborating a direct interaction between these two residues (Figure 3). The imidazole side chain of H120 could interact with the indole ring of W113 in two different ways. First, the NH of the indole ring of W113 could form a hydrogen bond with one of the two nitrogens of the imidazole ring of H120, as proposed for the T4 lysozyme Q105H mutant (Van Gilst & Hudson, 1996). This possibility appears unlikely, because the side chains of W113 and H120 are not oriented in a way that allows linear hydrogen bonding geometry between the donor and acceptor (Stickle *et al.*, 1992). In addition, protonation of H120 at acidic pH would result in the breakage of this hydrogen bond and one would expect an increase in the fluorescence intensity of W113 rather than a decrease, as exemplified by fluorescence analysis of the T4 lysozyme Q105H mutant (Van Gilst & Hudson, 1996). The second possibility is that H120 and W113 interact through a π-stacking (or cation-π) interaction, as observed for barnase (Loewenthal *et al.*, 1991; Loewenthal *et al.*, 1992). This possibility is favored by the fact that the imidazole and indole rings are essentially parallel in the 3D structure of $PhoQ_{Peri}$ (Figure 3). It is also consistent with our observation that protonation of H120 upon pH acidification induces quenching of the W113 fluorescence, as shown for barnase (Loewenthal *et al.*, 1991). Other pH-dependent π-stacking interactions between histidine and tryptophan residues have been identified in proteins such as the *Achromobacter* protease I, the M2 proton channel from influenza A virus and the microsomal apocytochrome b5 (Okada *et al.*, 2001; Shiraki *et al.*, 2002; Wang *et al.*, 2006).

Emission spectra of wild-type $PhoQ_{Peri}$ recorded at acidic pH consistently exhibited a 6-nm blue shift (Table 1). In contrast, both W113F and H120N mutants produced consistently blue-shifted emission spectra regardless of pH. These data suggest that W113 is shielded from the solvent as a consequence of pH acidification and that shielding of W113 depends on its interaction with H120. This pH-dependent change in the environment of W113 is strongly supported by acrylamide quenching experiments. The Stern-Volmer quenching constant of wild-type $PhoQ_{Peri}$ at pH 8.0 is significantly higher than that obtained at pH 5.5 (Table 2). In contrast, mutants W113F and H120N showed quenching constants that are essentially similar at both pHs.

Altogether, these data are consistent with a model suggesting that acidic pH changes the conformation of W113 that shifts to a more solvent-inaccessible environment in order to interact with protonated H120. This cation-π interaction involves the electrostatic attraction between the negative electrostatic potential associated with the indole ring of W113 and protonated H120. Although unprotonated H120 could potentially interact with W113 at pH ≥ 7.5, the interaction is expected to be much weaker (Ma & Dougherty, 1997). In good agreement, fluorescence intensities observed for mutants H120N and H120V at all pHs are very close to that of the wild-type protein at pH 8.0 (Table 1), indicating the absence of quenching of W113 by the unprotonated H120. Further work involving high-resolution methods such as NMR spectroscopy will be necessary to confirm this model. The pK_a value of H120 was determined to be 7.5, which is significantly higher than the value of 6.6 obtained for free histidine in solution or histidine residues in unfolded proteins (Tanokura *et al.*, 1976; Anderson *et al.*, 1990). An elevated pK_a value for H120 is consistent with previous work showing that the imidazolium form of histidine is stabilized by the negative electrostatic potential of the indole ring (Loewenthal *et al.*, 1992; Ma & Dougherty, 1997).

Histidine residues involved in pH-sensing have been documented in a number of proteins, such as K^+ channels, the ovarian cancer G-protein-coupled receptor 1 (OGR1) and the M2 proton channel from influenza virus (Chanchevalap *et al.*, 2000; Ludwig *et al.*, 2003; Hu *et al.*, 2006). In the latter case, the protonated form of the pH-sensing histidine (H37) is involved in a cation-π interaction with W41 (Okada *et al.*, 2001). To investigate the possibility that protonation of H120 is responsible for the PhoQ activation by acidic pH, several mutants at position 120 were generated and assayed *in vivo* for their responsiveness to acidic pH. Our data clearly showed that pH sensing by PhoQ is not attributable to H120, since replacement of H120 with non-ionizable residues like phenylalanine or asparagine preserves pH-mediated gene expression (Figure 5A). Other studies have shown that pH sensing may involve negatively charged residues such as aspartate and glutamate (Schuerch *et al.*, 2005; Jasti *et al.*, 2007; Kim *et al.*, 2008). Furthermore, these studies have demonstrated that pH sensitivity can be distributed over multiple residues involved in an interaction

network (Jasti *et al.*, 2007). The PhoQ sensory domain contains a negatively charged surface that is in close proximity to the membrane and was shown to be involved in the binding of divalent cations (Cho *et al.*, 2006). It is possible that several residues of the PhoQ negatively charged surface sense pH and initiate the conformational rearrangement leading to kinase activation. It should be noted that H120 is adjacent to E121 and E123, two glutamates that are part of the negatively charged surface.

Previous studies have shown that interactions between histidine and aromatic residues either stabilize protein structure or influence protein function. For example, mutational studies performed on barnase have shown that the cation-π interaction between the H18 and W94 stabilizes the protein by 1 kcal/mol, highlighting its contribution to protein stability (Loewenthal *et al.*, 1992). In the *Achromobacter* protease I, a chymotrypsin-type serine protease, a cation-π interaction between W169 and H210 regulates the proteolytic activity in a pH-dependent manner by interfering with the hydrogen bond network of the catalytic triad (Shiraki *et al.*, 2002). NMR analyses of the PhoQ periplasmic domain have shown that acidification of pH to 5.5 increases structural flexibility of a subset of residues (Prost *et al.*, 2007a). Strikingly, the PhoQ periplasmic domain was found to be extremely stable and remained folded at pH 3.5 (Prost *et al.*, 2007a). It is thus suggested that the cation-π interaction between W113 and H120 is important for maintaining stability in the protein at acidic pH. This interaction would compensate for the induced flexibility associated with the conformational change that the PhoQ periplasmic domain undergoes at acidic pH and maintain a properly folded protein. Although this cation-π interaction is not strictly conserved in all PhoQ homologues, it is striking that residues harboring aromatic or imidazole side chains are invariably present at positions 113 and 120 (Figure 6). Remarkably, the W-Y and Y-F amino acid pairs found in *Yersinia pestis* and *Pseudomonas aeruginosa*, respectively, have the potential to form a π-stacking arrangement and influence protein stability, although in a pH-independent manner.

The results of this study identified a cation-π interaction between W113 and H120 that is formed in the PhoQ periplasmic domain upon pH acidification. This interaction is most likely part of a more global remodeling of a network of interactions associated with pH acidification. This pH-dependent interaction makes W113 an

excellent fluorescent indicator of the conformational change induced by low pH. Sensing of acidic pH by PhoQ leads to the activation of downstream virulence genes and promotes *Salmonella* survival in host macrophages.

EXPERIMENTAL PROCEDURES

Generation of mutant $PhoQ_{Peri}$ constructs

Plasmid pET-Q_{Peri} encoding the wild-type PhoQ periplasmic sensory domain (residues 45-190) fused to a C-terminal His tag ($PhoQ_{Peri}$) (Bader *et al.*, 2005) was used as a template for all mutagenesis reactions. Mutants of $PhoQ_{Peri}$ were generated using the QuikChange® site-directed mutagenesis kit (Startagene) following the manufacturer's protocol. Mutants W65F, W97F, W104F, W113F, H120N, H120V, H137N and H157N were generated using the primers listed in Table S1. A triple mutant W65F-W97F-W104F was also generated using the QuikChange® multi site-directed mutagenesis kit (Stratagene), plasmid $PhoQ_{Peri}$-W104F as a template and primers W65F and W97F. Mutations were confirmed by DNA sequencing using a 3730xl DNA analyzer system (Applied Biosystems) at the McGill University and Génome Québec Innovation Centre.

Generation of mutant $PhoQ_{Fl}$ constructs

To express full-length PhoQ ($PhoQ_{Fl}$) in *S. enterica* strain MB101, the PhoQ coding sequence was cleaved with enzymes *Kpn*I and *Pst*I from plasmid pPRO-Q (Sanowar & Le Moual, 2005) and ligated into plasmid pBAD24 (Guzman *et al.*, 1995) cut with the same enzymes. The resulting plasmid pBAD-Q was used as a template for site-directed mutagenesis to generate mutants $PhoQ_{Fl}$ W113F, H120N and H120V (primers listed in Table S1). Additional H120 mutants were constructed using primer H120X, in which nucleotides coding for H120 were randomized (Table S1).

Expression and purification of wild-type and mutant $PhoQ_{Peri}$ proteins

The wild-type and mutant $PhoQ_{Peri}$ proteins were expressed in *E. coli* ArcticExpress® BL21 (Stratagene). Overnight cultures were grown in LB (Luria-Bertani) broth supplemented with the appropriate antibiotics. A 1:100 dilution of the bacterial culture without antibiotics was grown at 30 °C to late-logarithmic phase, induced with 0.5 mM IPTG (isopropyl-1-thio-β-Dgalactopyranoside) and incubated for an additional 20 hours at 10 °C. Cells were harvested by centrifugation and resuspended in loading buffer (20 mM sodium phosphate pH 7.5, 500 mM NaCl, 5

mM imidazole and 1mM PMSF). Cells were disrupted by sonication and lysates were centrifuged at 200,000 *g* for 30 min. The cytoplasmic fraction (supernatant) was loaded onto a 1-ml Ni^{2+}-NTA column (Amersham Biosciences). The column was washed with loading buffer and eluted using a 5 to 600 mM imidazole gradient in loading buffer. Fractions containing $PhoQ_{Peri}$ were dialyzed against 20 mM sodium phosphate pH 7.5, 150 mM NaCl and stored at 4 °C. The concentration of purified protein was determined using the BCA protein assay (Pierce).

Tryptophan fluorescence

Fluorescence measurements were done on a Cary Eclipse spectrofluorometer (Varian Inc.) with the cell compartment thermoregulated at 20 °C. Aliquots of purified $PhoQ_{Peri}$ (6.75 μM) were dialyzed against 20 mM of the following buffers supplemented with 150 mM NaCl: citrate phosphate (pH 3.5-5.5), sodium phosphate (pH 6.0-8.0), Tris/HCl (pH 7.5-9.0) and Caps (pH 10- 11). The excitation wavelength was set at 295 nm to minimize the contribution of tyrosine residues. Tryptophan emission spectra were recorded from 300 to 450 nm, with excitation and emission slit widths set at 5 nm. Each spectrum was the average of 10 consecutive scans. All spectra were corrected by subtracting the blank spectrum corresponding to buffer alone.

Fluorescence quenching

Collisional fluorescence quenching was performed at 20 °C with 6.75 μM of $PhoQ_{Peri}$ in 20 mM of either Tris/HCl (pH 8.0) or citrate phosphate (pH 5.5) supplemented with 150 mM NaCl. Acrylamide was dissolved in the same buffers and aliquots were progressively added and allowed to equilibrate for 15 sec. Quenching experiments were performed with excitation at 295 nm and the emission intensity recorded at the maximum emission wavelength. Values were corrected for the buffer blank and dilution was taken into account. The corrected data were plotted according to the Stern-Volmer equation that relates the drop in fluorescence to the concentration of quencher as shown in Equation 1: $F_0/F=1+K_{SV}[Q]$ where F_0 and F are fluorescence intensities in the absence and presence of quencher, respectively, K_{SV} is the Stern-

Volmer quenching constant and Q is the molar concentration of the quencher (Eftink & Ghiron, 1976). To determine K_{SV}, data were fitted to the above equation using the KaleidaGraph 4.03 software.

In vivo *activity assays*

The activity of PhoP/Q was examined using MB101, a strain expressing the PhoP-regulated reporter *phoN*::Tn*phoA* and carrying a *phoQ* null allele (Table S1). MB101 transformed with plasmids pBAD-Q and pBAD24 were used as the wild-type and negative control strains, respectively. Bacterial cultures were grown overnight in LB, washed twice with PBS, diluted 1:100 into N-minimal medium (Nelson & Kennedy, 1971) and grown for 3.5 hours at 37 °C. To measure the effect of pH acidification on the PhoP/Q activity, N-minimal medium was buffered with either 100 mM Bis-Tris (pH 7.5 and 5.5) or 100 mM MES (pH 7.5 and 5.5) (Prost *et al.*, 2007a). With both buffer systems, pH was stable over the course of the experiment. To measure the effect of divalent cations on the PhoP/Q activity, cells were grown in N-minimal medium in the presence of 8 μM or 10 mM $MgCl_2$. Transcription of the *phoN* gene was determined by measuring the alkaline phosphatase activity, according to a standard protocol on cultures grown in duplicate on three independent trials. PhoQ expression from the pBAD24-derived plasmids was analyzed by Western blotting. Blots were incubated with a polyclonal antibody directed against the PhoQ periplasmic domain (1:10,000) and a 1:10,000 dilution of anti-rabbit secondary antibody conjugated to horseradish peroxidase (Sigma). Blots were developed using the Immobilon Western horseradish peroxidase substrate (Millipore) according to the manufacturer's instructions.

SUPPLEMENTARY DATA

Table S1. Bacterial strains, plasmids and primers used in this study

	Relevant characteristics	Reference or source
S. enterica strains		
MB101		Bader *et al.*, 2005
E. coli strains		
XL1 Blue		Stratagene
BL21 (DE3) pLysE		Novagen
BL21 ArticExpress		Stratagene
Plasmids		
pET-Q_{Peri}	*phoQ* 45-190-$(His)_6$ into pET11a	Bader *et al.*, 2005
pBAD24		Guzman *et al.*, 1995
pBAD-Q	*phoQ* coding sequence into pBAD24	This study
Primers		
W65F	TATACCCTCGCCAAATTCGAAAATAATAAAATCAGCG	
W97F	ACGGGCAAATTATTATTTACGCAGCGCAACATTCCC	
W104F	CGCAGCGCAACATTCCCTTTCTGATTAAAAGCATTC	
W113F	AGCATTCAACCGGAATTCTTAAAAACGAACGGC	
H120N	AAAACGAACGGCTTCAATGAAATTGAAACCAACG	
H120V	AAAACGAACGGCTTCGTTGAAATTGAAACCAACG	
H137N	CTGTTGAGCGAAGACAATTCCGCGCAGGAAAAAC	
H157N	GATGCCGAGATGACCAACTCGGTAGCGGTAAAT	
H120X		
GGTTAAAAACGAACGGCTTCXXXGAAATTGAAACCAACGTAGAC		

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PREFACE TO CHAPTER 4

In 2005, a study determined that the antimicrobial peptide CRAMP secreted by intestinal murine epithelial cells was necessary for controlling *Citrobacter rodentium* infection during the first 7 days. We proposed that *C. rodentium* must have a mechanism of AMP resistance since the pathogen resides in an environment where CRAMP is present. Results from a BLAST genome search of the *Citrobacter rodentium* genome showed that there was a close homologue of the *Salmonella typhimurium phoPQ* operon.

Chapter 4 is a manuscript in preparation for submission to Molecular Microbiology. The section investigates the physiological signals that could activate the PhoP/PhoQ system within the mouse colon. *S. typhimurium* PhoP/PhoQ is used to compare and contrast the activity of the system in *C. rodentium*. This Chapter also describes the role that PhoP/PhoQ plays in *C. rodentium* virulence. Based on experimental results, Chapter 4 presents evidence that each is part of a different regulatory system, which promotes resistance to AMPs.

CHAPTER 4

An outer membrane protease of the omptin family prevents activation of the PhoPQ two-component system by antimicrobial peptides in *Citrobacter rodentium*

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To be submitted to Molecular Microbiology

ABSTRACT

The PhoPQ two-component system is present in a number Gram-negative bacterial species, including the facultative intracellular pathogen, *Salmonella enterica*. PhoPQ of *S. enterica* plays a crucial role in resistance to antimicrobial peptides (AMPs) by controlling covalent modifications of lipid A upon recognition of α-helical AMPs, such as human LL-37 or mouse CRAMP. A homologue of the *phoPQ* operon was found in the genome of the murine enteric extracellular pathogen, *Citrobacter rodentium*. Here we report that, although a *C. rodentium phoPQ* deletion mutant was defective in AMP resistance, PhoQ was unable to mediate gene activation in the presence of α-helical AMPs. The unresponsiveness of PhoQ was dependent on a specific feature of this pathogen as AMPs induced transcriptional activation of the PhoP-regulated gene, *mgtA*, when the *C. rodentium phoPQ* operon was expressed in a *S. enterica phoPQ* mutant. Analysis of the outer membrane (OM) fractions of the *C. rodentium* wild-type and *phoPQ* strains led to the identification of a protease of the omptin family, that we named CroP. Deletion of *croP* in *C. rodentium* resulted in a strain that was highly susceptible to α- helical AMPs, similarly to the *phoPQ* mutant, indicating a direct role of CroP in AMP resistance. In addition, CroP was found to greatly contribute to the protection of the OM from AMP damage, by actively degrading α-helical AMPs before reaching the periplasmic space. Accordingly, transcriptional activation of *C. rodentium* PhoPQ by α-helical AMPs was restored in the *croP* mutant. This study indicates that the extracellular *C. rodentium* and the intracellular *S. enterica* pathogens use different mechanisms to resist α-helical AMPs. This finding may be applicable to other extracellular enteric pathogens such as EPEC and EHEC.

INTRODUCTION

Antimicrobial peptides (AMPs) are important components of the innate immune system that are involved in the host defense against microorganisms (Hancock and Lehrer, 1998; Zasloff, 2002; Hancock and Sahl, 2006). Although diverse in sequence and structure, AMPs are characterized by their small size, amphipathic properties and cationic net charges. There are two major classes of mammalian AMPs, the cathelicidins, such as human LL-37 and mouse CRAMP that form α-helices and the α- and β-defensins that adopt a β-sheet structure (Ganz, 2003; Zanetti, 2004). Various cell types including phagocytic cells, goblet cells and epithelial cells, produce AMPs in a constitutive or inducible manner. In Gram-negative bacteria, AMPs interact first with the lipid A moiety of lipopolysaccharide (LPS) to disrupt the outer membrane (OM) and gain access to the periplasmic space. Most AMPs appear to exert their bactericidal function by disrupting the cytoplasmic membrane (Brogden, 2005; Melo *et al.*, 2009). Bacterial pathogens have developed several mechanisms to resist killing by AMPs. These resistance mechanisms include the protease-mediated inactivation of AMPs, the export of AMPs by efflux pumps and the covalent modifications of bacterial membrane components that result in a decrease of the overall negative charge and, in turn, minimize membrane interaction with cationic AMPs (Miller *et al.*, 2005; Peschel and Sahl, 2006).

The PhoPQ two-component system (TCS) is composed of the sensor kinase PhoQ and the cognate response regulator PhoP. PhoPQ is found in a variety of Gram-negative pathogens, including the facultative intracellular *S. enterica*, which is by far the best-characterized PhoPQ system. *S. enterica* PhoPQ is a master regulator of virulence that is critical for bacterial survival within macrophage phagosomes (Groisman *et al.*, 1989; Miller *et al.*, 1989; Groisman, 2001; Prost and Miller, 2008). PhoQ, like most sensor kinases, possesses both kinase and phosphatase activities that play opposite roles in controlling the phosphorylation level of PhoP. These activities are regulated by several environmental cues that are sensed by the PhoQ periplasmic sensor domain. Limiting concentrations of the divalent cations Mg^{2+} and Ca^{2+} were shown to promote PhoQ autophosphorylation and, in turn, PhoP phosphorylation (Garcia Vescovi *et al.*, 1996; Castelli *et al.*, 2000; Montagne *et al.*, 2001; Sanowar and

Le Moual, 2005). Recent studies revealed that PhoQ also responds to α-helical AMPs and acidic pH, leading to the activation of the PhoPQ signaling pathway (Bader *et al.*, 2005; Prost *et al.*, 2007). Acidic pH and α-helical AMPs are likely the physiologically relevant ligands of *S. enterica* PhoQ, since the vacuolar environment of phagosomes is characterized by the presence of AMPs, a pH in the range of 5.0-6.5 and a constant concentration of Mg^{2+} around 1 mM (Alpuche Aranda *et al.*, 1992; Martin-Orozco *et al.*, 2006). In agreement, PhoPQ has been shown to be critical for *S. enterica* to resist the antimicrobial activity of CRAMP in murine macrophages (Rosenberger *et al.*, 2004). PhoQ-activating signals, such as α-helical AMPs, promote structural modifications of the lipid A moiety of LPS (Miller *et al.*, 2005). PhoP directly regulates the expression of the lipid A-modifying enzymes *lpxO*, *pagL* and *pagP*. The PmrAB TCS regulates expression of other lipid A-modifying enzymes like the *pmrC* gene and the *pmrHFIJKLM* operon. In *S. enterica*, PhoPQ promotes activation of PmrA-regulated genes by transcriptionally activating the expression of the PmrD protein that binds the response regulator PmrA and prevents its dephosphorylation (Kox *et al.*, 2000; Kato and Groisman, 2004).

The involvement of PhoPQ in resistance to various AMPs has also been reported for other pathogens of the family *Enterobacteriaceae*. These include the mammalian pathogens *Yersinia pseudotuberculosis* and *Yersinia pestis* (Marceau *et al.*, 2004; Winfield *et al.*, 2005), *Shigella flexneri* (Moss *et al.*, 2000), as well as the insect pathogen *Photorhabdus luminescens* (Derzelle *et al.*, 2004) and the plant pathogen *Erwinia chrysanthemi* (Llama-Palacios *et al.*, 2003). In contrast, *Pseudomonas aeruginosa* PhoQ is not activated by AMPs but responds to both divalent cations and acidic pH (Prost *et al.*, 2008). This discrepancy was attributed to the absence of a cluster of acidic amino acids from the *P. aeruginosa* PhoQ periplasmic domain. Thus, a PhoQ response to AMPs can be directly correlated with the presence of this acidic cluster in the PhoQ periplasmic domain, which interacts with the membrane through divalent cation bridges (Bader *et al.*, 2005; Cho *et al.*, 2006; Prost and Miller, 2008).

The role of OM proteases in bacterial resistance to AMPs is less well established than that of lipid A modifications. OM proteases of the omptin family

were found in various Gram-negative pathogens including *S. enterica* (PgtE), *Y. pestis* (Pla), *S. flexneri* (SopA) and *E. coli* (OmpT and OmpP) (Kukkonen and Korhonen, 2004; Hritonenko and Stathopoulos, 2007). Although *S. enterica* PgtE cleaves AMPs such as C18G *in vitro*, its contribution to AMP resistance appears to be marginal when expressed from a single chromosomal copy (Guina *et al.*, 2000). *In vivo*, *Y. pestis* Pla activity is essential to tissue invasion (Sodeinde *et al.*, 1992; Lathem *et al.*, 2007). Pla promotes degradation of fibrin clots by activating plasminogen into plasmin and inactivating the α_2-antiplasmin inhibitor (Kukkonen *et al.*, 2001). A recent study revealed that Pla was also able to inactivate AMPs such as LL-37 and CRAMP, *in vitro* (Galvan *et al.*, 2008). Furthermore, *E. coli* OmpT was reported to efficiently degrade the AMP protamine (Stumpe *et al.*, 1998).

Citrobacter rodentium is an extracellular enteric pathogen that causes transmissible murine colonic hyperplasia (Schauer and Falkow, 1993). Similar virulence factors make *C. rodentium* an excellent model organism for the study of the human pathogens enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC). Colonization of the intestine by these foodborne pathogens produces attaching and effacing (A/E) lesions (Mundy *et al.*, 2005), which are characterized by intimate bacterial attachment to intestinal epithelial cells, formation of actin-rich pedestals underneath adherent bacteria and destruction of brush border microvilli. Host defenses against these pathogens include the secretion of AMPs such as ß-defensins and cathelicidins (O'Neil *et al.*, 1999; Hase *et al.*, 2002; Schauber *et al.*, 2003). During infection, *C. rodentium* resides in close proximity to colonic epithelial cells expressing CRAMP, which has antimicrobial activity against this pathogen (Iimura *et al.*, 2005).

Having identified a homologue of the *phoPQ* operon in the *C. rodentium* genome sequence, we examined whether *C. rodentium* PhoQ recognizes α-helical AMPs and responds accordingly by promoting resistance. We present evidence that *C. rodentium* PhoQ is not activated by α-helical AMPs because they are degraded by CroP, an outer membrane protease of the omptin family, before they reach the periplasmic space. This study shows that *C. rodentium* and *S. enterica* use different strategies to resist the deleterious effect of α-helical AMPs.

RESULTS

C. rodentium PhoPQ is involved in the adaptation to AMPs and Mg^{2+}-depleted environments

The genome of the mouse enteric pathogen *C. rodentium* has been sequenced and is being annotated (http://www.sanger.ac.uk/Projects/C_rodentium). Open reading frames (ORFs) with high identities to the *S. enterica* amino acid sequences of PhoP (82%) and PhoQ (78%) were identified from the *C. rodentium* genome sequence. As in the *S. enterica phoPQ* operon, the two ORFs overlapped by one nucleotide, suggesting that they are part of the same transcriptional unit. A consensus PhoP box that consists of the two half-sites GGTTTA and TGTTTA separated by 5 nucleotides was identified 58 nucleotides upstream of the ATG start codon, suggesting that *C. rodentium* PhoP binds to and autoregulates its own promoter (Soncini *et al.*, 1995)

To investigate the role of PhoPQ in *C. rodentium*, we generated a *phoPQ* deletion mutant and investigated its sensitivity to various environmental stresses. In agreement with previous studies, the viability of the *phoPQ* mutant was highly compromised by the presence of 5 mM EDTA (Fig. 1A). Resistance to EDTA was rescued by complementation with the *phoPQ* operon present on the low-copy number plasmid pCR*phoPQ* (Fig. 1A). In contrast, no difference in survival was observed when the wild-type and *phoPQ* strains were challenged with either mild detergent (0.05% SDS) or high osmotic pressure (2.4 M NaCl) (data not shown). Western blot analysis of whole cell lysates from wild-type and *phoPQ* mutant *C. rodentium* grown in media containing either 8 μM or 10 mM $MgCl_2$ is shown in Fig. 1B. In wild-type *C. rodentium*, the PhoPQinducing condition (8 μM $MgCl_2$) produced elevated levels of PhoP (lane 1), while the expression of PhoP under PhoPQ-repressing condition (10 mM $MgCl_2$) was drastically repressed (lane 2). As expected, no expression was observed in the *phoPQ* mutant under both growth conditions (lanes 3 and 4). These data indicate that *C. rodentium* PhoPQ responds to external Mg^{2+}, as described previously for *S. enterica* and *E. coli* (Garcia Vescovi *et al.*, 1996; Kato *et al.*, 1999).

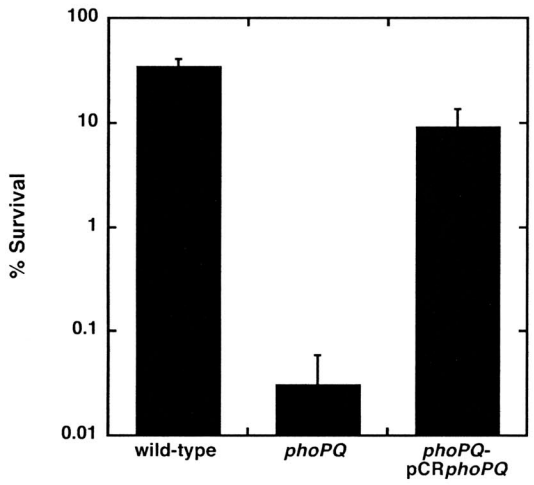
Since *S. enterica* PhoPQ is important for resistance to α-helical AMPs (Bader et al., 2005), we tested the involvement of *C. rodentium* PhoPQ in resistance to the mouse cathelicidin CRAMP, L-C18G and polymyxin B (PMB). Disk diffusion assays

were performed in the presence of increasing concentrations of these AMPs. As shown in Fig. 1C, wild-type *C. rodentium* was resistant to lethal concentrations of CRAMP and L-C18G. In contrast, we observed that challenge with PMB readily killed *C. rodentium* (Fig. 1C). The *C. rodentium phoPQ* mutant was more susceptible than wild-type to increasing concentrations of L-C18G and complementation of the *phoPQ* mutant with pCR*phoPQ* restored resistance to L-C18G (Fig. 1D). Similar results were obtained using CRAMP (data not shown). Altogether, these results indicate that *C. rodentium* PhoPQ is involved in the adaptive response to AMPs and Mg^{2+}-depleted environments. They also suggest that *C. rodentium* PhoQ senses both Mg^{2+} and AMPs as its *Salmonella* homologue (Garcia Vescovi *et al.*, 1996; Bader *et al.*, 2005).

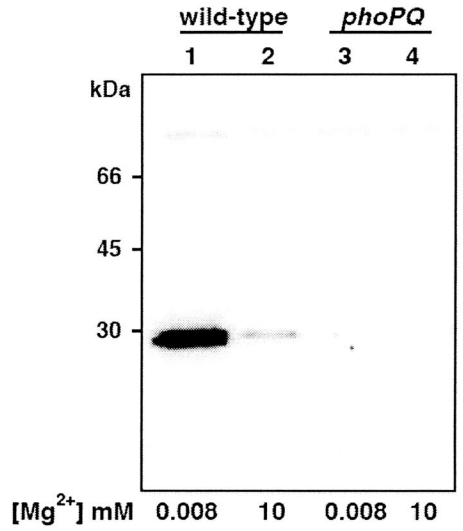
C. rodentium *PhoQ responds to Mg2+ and pH acidification but not to AMPs*

To further investigate the environmental cues sensed by *C. rodentium* PhoQ, we generated a β-galactosidase fusion with the *C. rodentium mgtA* gene, which codes for a Mg^{2+} transporter. As in *S. enterica* and *E. coli*, this gene harbors a PhoP box (TGTATAxxxxxCGTTTA) that contains all the essential nucleotides (underlined) for PhoP recognition (Kato *et al.*, 1999; Yamamoto *et al.*, 2002; Lejona *et al.*, 2003). Wild-type and *phoPQ* reporter strains were grown under PhoQ-activating and PhoQ-repressing conditions. When comparing the activity of *mgtA::lacZ* in 8 μM and 10 mM Mg^{2+}, a 22-fold repression was observed (Fig. 2A). Regulation of *mgtA* expression by Mg^{2+} was PhoPQ-dependent since the *phoPQ* mutant was unable to modulate *mgtA*::*lacZ* gene transcription (Fig. 2A). Acidic pH was tested as a PhoQ-activating signal, since *S. enterica* PhoQ was shown to respond to pH acidification (Prost et al., 2007). A 3.7-fold increase in *mgtA::lacZ* gene transcription was observed when the bacteria were transferred from a medium of neutral pH to one of acidic pH (Fig. 2B). The *phoPQ* mutant was unresponsive to pH acidification (Fig. 2B). Strikingly, sublethal concentrations of L-C18G (2 μM) or CRAMP (10 μM) were unable to increase expression of the *mgtA*::*lacZ* fusion (Fig. 2C). This is in sharp contrast to what has been observed for *S. enterica* PhoQ (Bader et al., 2005) and suggests that *C. rodentium* PhoQ might be unable to recognize AMPs.

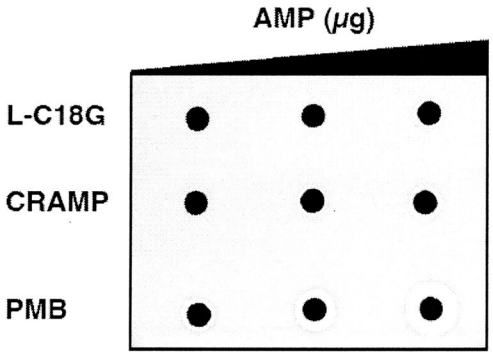
A.


100
10
1
0.1
0.01
% Survival
wild-type
phoPQ
phoPQ-
pCRphoPQ

B.


wild-type
phoPQ
1
2
3
4
kDa
66
45
30
[Mg2+] mM
0.008
10
0.008
10

C.


AMP (μg)
L-C18G
CRAMP
PMB

D.

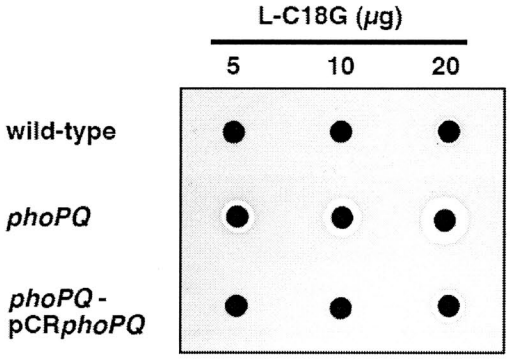

L-C18G (μg)
5
10
20
wild-type
phoPQ
phoPQ -
pCRphoPQ

Figure 1. *C. rodentium* PhoPQ is necessary for resistance to AMP and growth in Mg^{2+}-depleted environments.

A. Wild-type and *phoPQ* mutant *C. rodentium* strains were grown in LB in the presence or absence of 5 mM EDTA for 1 hour. Survival was defined by the colony forming units (cfu) of challenged cells over the cfu of unchallenged cells, multiplied by 100. Values correspond to the mean ± standard deviation of three independent experiments performed in triplicate.

B. Anti-PhoP immunoblot of crude bacterial extracts isolated from wild-type and *phoPQ* mutant cells grown in N-minimal media with 8 μM or 10 mM $MgCl_2$, as described under *Experimental procedures*.

C. Disk diffusion assay of wild-type *C. rodentium* in the presence of disks containing increasing concentrations of L-C18G (5, 10, 20 μg), CRAMP (50, 100, 250 μg) and PMB (0.5, 1, 2.5 μg).

D. Disk diffusion assay with L-C18G. Disks were impregnated with the indicated AMP amount and positioned on the agarose containing either the wild-type, *phoPQ* mutant or *phoPQ* mutant complemented with pCR*phoPQ C. rodentium* strains.

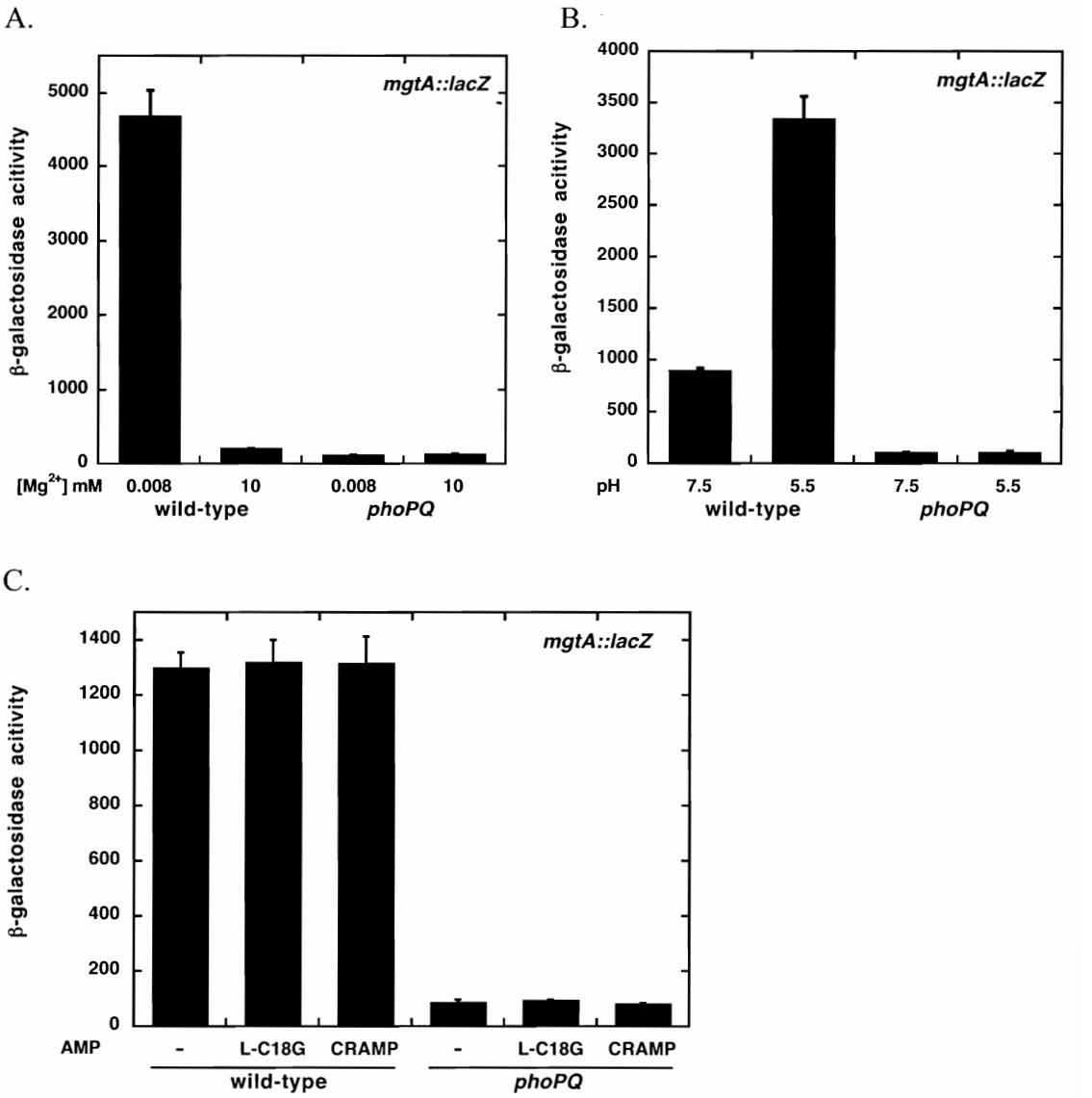


Figure 2. PhoPQ is regulated by Mg^{2+}-deprivation and acidic pH but not AMPs. β-Galactosidase activity from *mgtA::lacZ* transcriptional fusion in wild-type or *phoPQ* mutant *C. rodentium*. Cultures were grown in N-minimal media supplemented with: **A.** 8 μM or 10 mM $MgCl_2$. **B.** 1 mM $MgCl_2$ at pH 7.5 or 5.5. **C.** 1 mM $MgCl_2$ in the absence or presence of 2 μM L-C18G and 10 μM CRAMP. Values are mean ± standard deviation of cultures grown in triplicate and are representative of at least three independent trials.

A.

B.

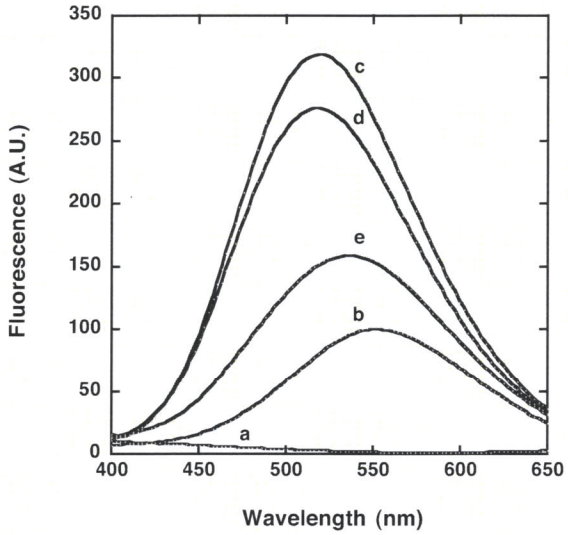


Figure 3. Dansylated C18G interacts with the periplasmic domain of *C. rodentium* PhoQ.

A. Alignment of the periplasmic domain of PhoQ from *C. rodentium* (CR) and *S. enterica* (ST). Residues indicated by the asterix have been shown to be important for AMP recognition in *S. enterica*.

B. 1 μM dansylated C18G (b) was incubated in 20 mM Tris-HCl pH 7.5, excited at 340 nm and the spectra were recorded. $PhoQ_{Peri}$ (1 μM) was added without (c) or with $MgCl_2$ at a concentration of 1 mM (d) or 10 mM (e). (a) represents protein only control.

The C. rodentium *PhoQ periplasmic domain binds AMPs*

The sequence of the *S. enterica* PhoQ periplasmic domain ($PhoQ_{Peri}$) contains a cluster of acidic amino acids (EDDDDAE) that has been proposed to be involved in the recognition of both divalent cations and AMPs (Bader *et al.*, 2005; Cho *et al.*, 2006). Alignment of the amino acid residues of the *C. rodentium* and *S. enterica* PhoQ periplasmic domains showed that this acidic cluster is strictly conserved (Fig. 3A). A construct of *C. rodentium* $PhoQ_{Peri}$, corresponding to residues 45-190 of the full-length protein, was expressed in *E. coli* and purified to homogeneity. $PhoQ_{Peri}$ was used to measure the binding of the dansylated derivative of C18G (dC18G) by monitoring fluorescence emission as described previously (Bader *et al.*, 2005). As shown in Figure 3B, dC18G fluoresces with a λmax of 550 nm (line b). The fluorescence intensity of dC18G increased by approximately 3-fold upon addition of equimolar amounts of *C. rodentium* $PhoQ_{Peri}$ (line c). In addition the λmax was blue-shifted from 550 to 520 nm (lines b and c). These changes in fluorescence reflect movement of the dansyl group to a more hydrophobic environment and illustrate that dC18G binds to *C. rodentium* $PhoQ_{Peri}$. The addition of 10 mM $MgCl_2$ caused a reduction in the fluorescence intensity and a red-shift of the λmax to 537 nm (line e), confirming that Mg^{2+} effectively competes with dC18G for binding to $PhoQ_{Peri}$. These results are very similar to those obtained previously using the *S. enterica* $PhoQ_{Peri}$ protein (Bader *et al.*, 2005). The ability of *C. rodentium* $PhoQ_{Peri}$ to bind dC18G in combination with its sequence identity to *S. enterica* strongly suggests that *C. rodentium* PhoQ can be activated by the presence of AMPs.

C. rodentium *PhoPQ responds to AMPs when expressed in* S. enterica

To further demonstrate that *C. rodentium* PhoQ has the potential to recognize AMPs, we complemented the *phoPQ* mutant strains of *S. enterica* and *C. rodentium* with pCR*phoPQ* and pST*phoPQ*, respectively. The activation of PhoPQ was measured by monitoring the β-galactosidase activity of the *mgtA::lacZ* fusion. Interestingly, in the context of the *S. enterica phoPQ* strain, complementation with *C. rodentium phoPQ* mediated a 2.4- and 1.8-fold increase in β-galactosidase activity in the presence of L-C18G and CRAMP, respectively (Fig. 4A). This level of activation was

comparable to that obtained by complementation of the *S. enterica phoPQ* strain with its native *phoPQ* operon (Fig. 4A). Conversely, *S. enterica phoPQ* introduced in a *C. rodentium* background was unable to activate transcription of the *mgtA::lacZ* fusion in response to either L-C18G or CRAMP (Fig. 4B). These data clearly show that the differential PhoPQ-dependent regulation of *mgtA*::*lacZ* in response to AMPs is due to the bacterial species expressing the operon and not a feature of PhoPQ itself.

Permeability of the C. rodentium *and* S. enterica *OMs*

In *S. enterica*, PhoPQ-mediated lipid A modifications contribute to the strengthening of the OM and resistance to AMPs (Guo *et al.*, 1998; Murata *et al.*, 2007). The OM permeability of *C. rodentium* and *S. enterica* strains grown under PhoPQ-inducing conditions was examined by measuring the influx of ethidium bromide (EtBr), as described previously (Murata *et al.*, 2007). The permeability of the *C. rodentium* OM to EtBr was greater than that of *S. enterica* as shown by the 4-fold difference between the influx rates of the two wild-type strains (Fig. 5A and B). The *C. rodentium phoPQ* mutant was more permeable than the wild-type strain with a rate of EtBr influx that was 1.7-fold higher (Fig. 5A). When the same experiment was performed with *S. enterica*, a 5.2-fold increase in EtBr influx was observed for the *phoPQ* mutant as compared to wild-type (Fig. 5B). These data suggest that PhoPQ-mediated lipid A modifications contribute less to the strengthening of the OM in *C. rodentium* than in *S. enterica*. Thus, it appears unlikely that PhoPQ-mediated lipid A modifications are responsible for the inability of AMPs to penetrate the *C. rodentium* OM and, in turn, activate PhoQ.

Differential disruption of the C. rodentium *and* S. enterica *OMs by AMPs*

Initially, AMPs interact with LPS and then penetrate the OM by self-promoted uptake to access the cytoplasmic membrane (Hancock and Lehrer, 1998). NPN (1-phenylnapthylamine), which fluoresces in the hydrophobic environment of damaged membranes, was used as a probe to measure OM disruption induced by sublethal concentrations of L-C18G, CRAMP or PMB (Loh *et al.*, 1984). Addition of PMB to wild-type *C. rodentium* caused a rapid increase in fluorescence intensity that reached a

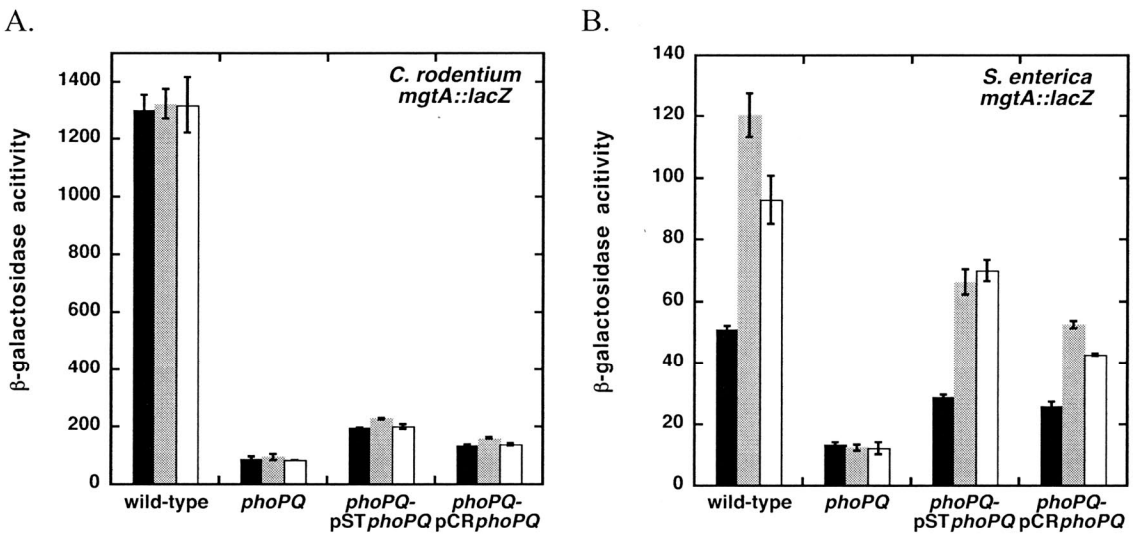


Figure 4. *C. rodentium* PhoQ is activated by AMPs when expressed in *S. enterica.* β-Galactosidase activity expressed by *C. rodentium* (**A**) and *S. enterica* (**B**) *phoPQ* mutant strains grown in N-minimal media supplemented with 1 mM $MgCl_2$ harboring the *mgtA::lacZ* fusion. The transcriptional activity was investigated in the absence of AMP (black bars), and the presence of either 2 μM L-C18G (grey bars) or 10 μM CRAMP (white bars). The values of ß-galactosidase activity are the average of three independent assays.

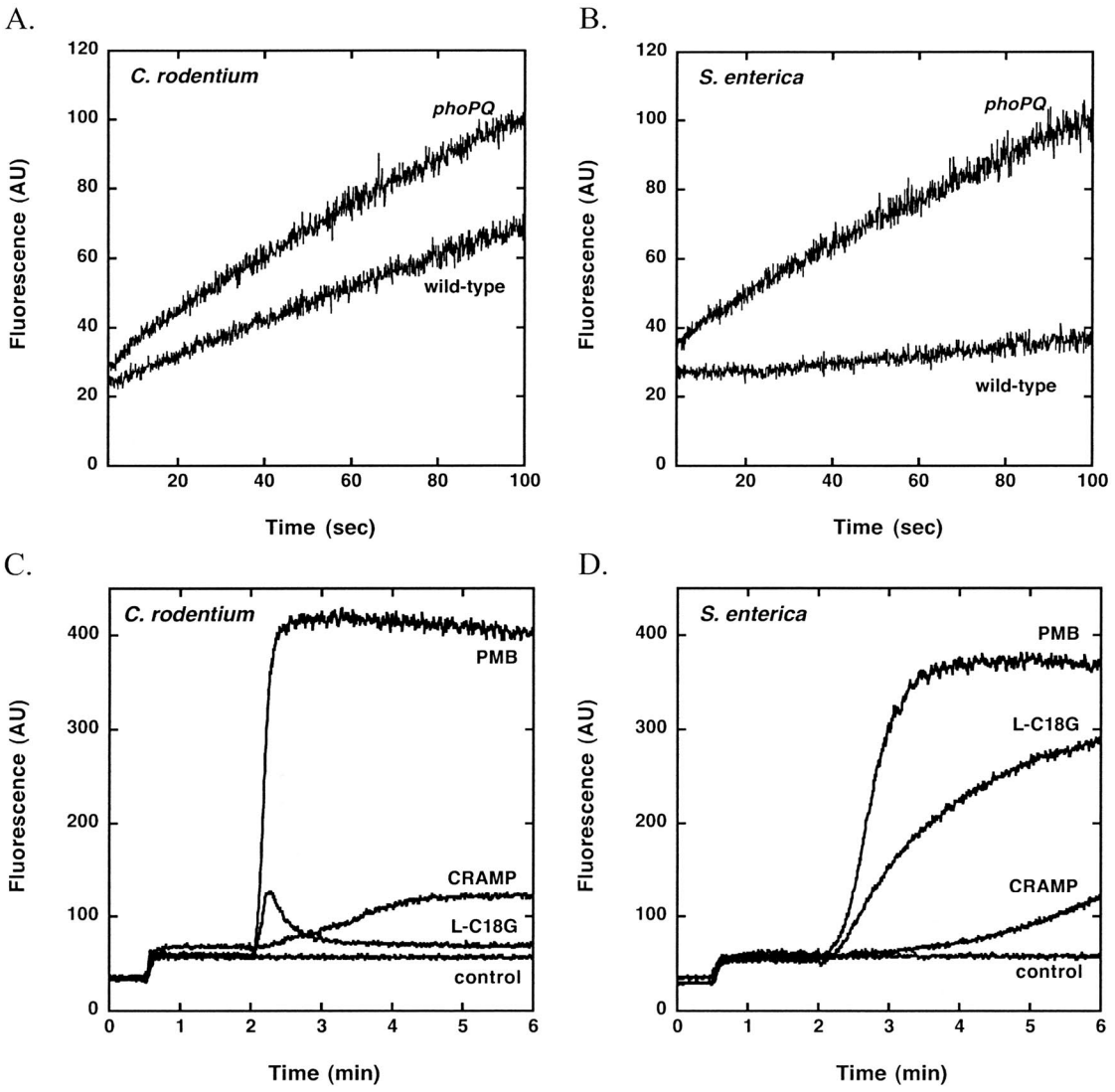
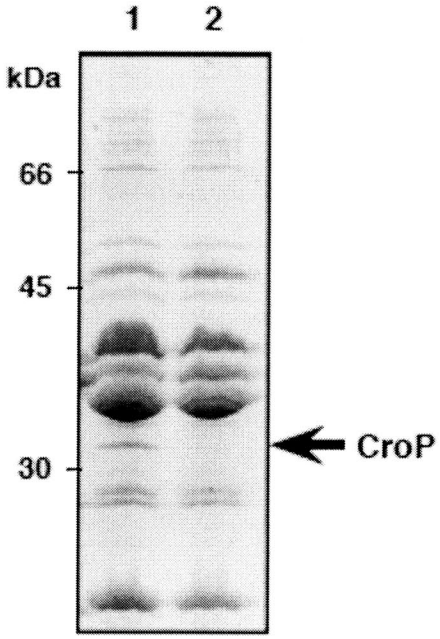


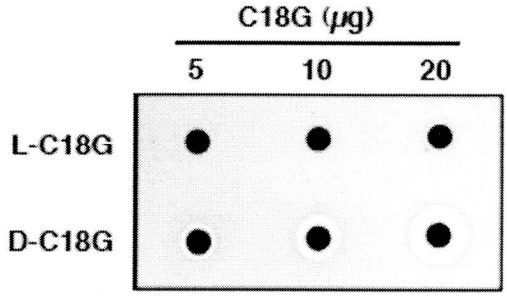
Figure 5. The OM of *C. rodentium* differs from that of *S. enterica.*

Ethidium influx into wild-type and *phoPQ* mutant cells of *C. rodentium* (**A**) and *S. enterica* (**B**). Cultures were grown in N-minimal media supplemented with 8 μM $MgCl_2$. Cells were washed and resuspended in 50 mM sodium phosphate buffer (pH 7.5) and the ethidium influx was performed in the presence of 5 μM CCCP. Uptake of NPN across the wild-type *C. rodentium* (**C**) and *S. enterica* (**D**) OM in the absence of AMP (control) and the presence of 2 μM L-C18G, 10 μM CRAMP or 1 μg/mL PMB. The y-axis values represent NPN uptake recorded as the fluorescence intensity observed as NPN entered the damaged membrane. Each experiment is representative of at least three independent trials. AU, arbitrary units.

A.


1
2
kDa
66
45
30
CroP

B.


C18G (μg)
5
10
20
L-C18G
D-C18G

C.

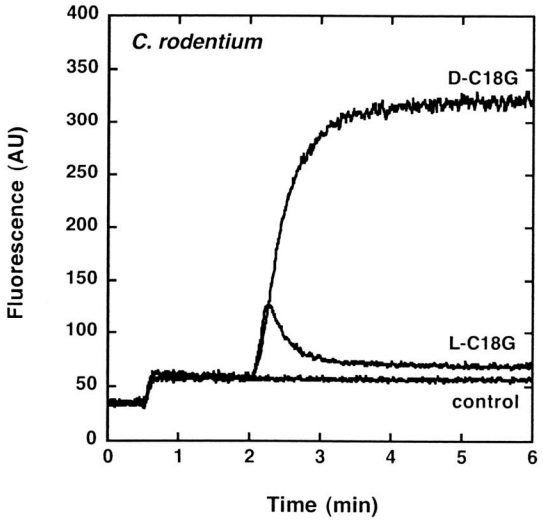

C. rodentium
D-C18G
L-C18G
control
Fluorescence (AU)
Time (min)
0
50
100
150
200
250
300
350
400
0
1
2
3
4
5
6

Figure 6. Evidence of a stereospecific protease involved in AMP resistance.

A. SDS-PAGE of the OM protein fraction from wild-type (lane 1) and *phoPQ* mutant (lane 2) cultures grown in LB for 6 hours. The arrow indicates the band for which mass spectrometry analyses were performed identifying an OM protease of the omptin family (CroP).

B. Disk diffusion assay of wild-type *C. rodentium* to increasing amounts of L-C18G and D-C18G.

C. NPN uptake by wild-type *C. rodentium* OM in the absence of AMP (control) and the presence of either 2 μM L-C18G or 2 μM D-C18G. Each experiment is representative of at least three independent trials.

plateau after 30 seconds (Fig. 5C). In contrast, both L-C18G and CRAMP had a moderate effect on the *C. rodentium* OM integrity. L-C18G caused a biphasic disruption of the *C. rodentium* OM characterized by a slight burst followed by a sustained decrease in fluorescence intensity (Fig. 5C). In contrast, the OM of *S. enterica* was steadily disrupted over the course of the experiment by the addition of each AMP (Fig. 5D). The OM of the *C. rodentium phoPQ* mutant was extensively disrupted, similarly to that of *S. enterica*, by the addition of L-C18G or CRAMP as evidenced by the steady increase in fluorescence over time (data not shown), which correlates with the susceptibility of the *phoPQ* mutant to AMPs (Fig. 1C). These results indicate that α-helical AMPs barely disrupt the *C. rodentium* OM and suggest that these AMPs may not be able to reach the periplasmic space and activate PhoQ.

Identification of a* C. rodentium *OM protease involved in AMP resistance

The OM protein fractions of the *C. rodentium* wild-type and *phoPQ* strains grown in LB were analyzed by SDS-PAGE. As shown in Fig. 6A, the protein profiles were essentially similar with the exception of one band in the wild-type strain (lane 1) having an approximate molecular weight of 33 kDa that was absent from the *phoPQ* mutant (lane 2). Coomassie-stained bands, indicated by the arrow, were cut from lanes 1 and 2, trypsin-digested and submitted to liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS). Mass fingerprinting identified the protein migrating at 33 kDa in the wild-type lane as a homologue of the *E. coli* OM protease OmpP. A BLAST search of the *C. rodentium* genome identified an ORF coding for an OM protease of the omptin family, hereafter named CroP (*C. rodentium* outer membrane protease). The protein sequence deduced from the *croP* gene shares 73 and 74% amino acid sequence identity with *E. coli* OmpP and OmpT, respectively, and 39% sequence identity with *S. enterica* PgtE. The CroP sequence contains the conserved residue pairs (Asp83-Asp85 and Asp210-His212) that are part of the omptin active site (Hritonenko & Stathopoulos, 2007).

To test the involvement of the OM protease CroP in resistance to α-helical AMPs, the enantiomer of L-C18G (D-C18G) was synthesized from all D-amino acids. Wild-type *C. rodentium* was unable to resist increasing concentrations of D-C18G, as

evidenced by the larger zones of inhibition surrounding the disks containing D-C18G (Fig. 6B). In addition, D-C18G caused a rapid increase in NPN fluorescence, indicating extensive membrane damage to wildtype *C. rodentium* (Fig. 6C). These results are indicative of a stereospecific mechanism of AMP resistance that is consistent with the enzymatic action of the CroP OM protease.

CroP contributes to the protection of the OM from AMP damage

To better define the role of CroP in resistance to α-helical AMPs, a *croP* deletion mutant was generated. Disk inhibition assays were performed to evaluate the antimicrobial activity of L-C18G on the *C. rodentium croP* mutant. This mutant was found to be susceptible to L-C18G (Fig. 7A) and CRAMP (data not shown). Complementation of the *croP* mutant with plasmid pCR*croP* restored resistance to L-C18G to a greater extent than wild-type (Fig. 7A and 1D). A *phoPQ croP* double mutant exhibited the same susceptibility as either the *croP* or *phoPQ* single mutants (Fig. 7A and 1D). In agreement with these results, L-C18G was able to disrupt the OM of the *croP* mutant, as illustrated by the rapid increase in NPN fluorescence following the addition of L-C18G (Fig. 7B). Interestingly, the addition of L-C18G to the *croP* mutant complemented with pCR*croP* produced the same baseline signal as the control without AMP, suggesting complete proteolytic inactivation of L-C18G (Fig. 7C). As shown in Fig. 7B and C, the addition of PMB to both the *croP* and the pCR*croP*-complemented strains caused the same rapid increase in fluorescence intensity as the wild-type strain (Fig. 5C), indicating that PMB is not a CroP substrate. Taken together, these results show that CroP plays a crucial role in resistance to α-helical AMPs, likely through its proteolytic activity.

CroP degrades AMPs

To verify that CroP was directly responsible for the proteolytic inactivation of AMPs, cleavage of L-C18G was assayed using the wild-type, *phoPQ* and *croP* mutant strains. As shown in Fig. 8, L-C18G was completely degraded when incubated for 30 min with wild-type *C. rodentium* cells (lane 2). In sharp contrast, no degradation was observed when L-C18G was incubated with either *phoPQ* or *croP* mutant cells (lanes

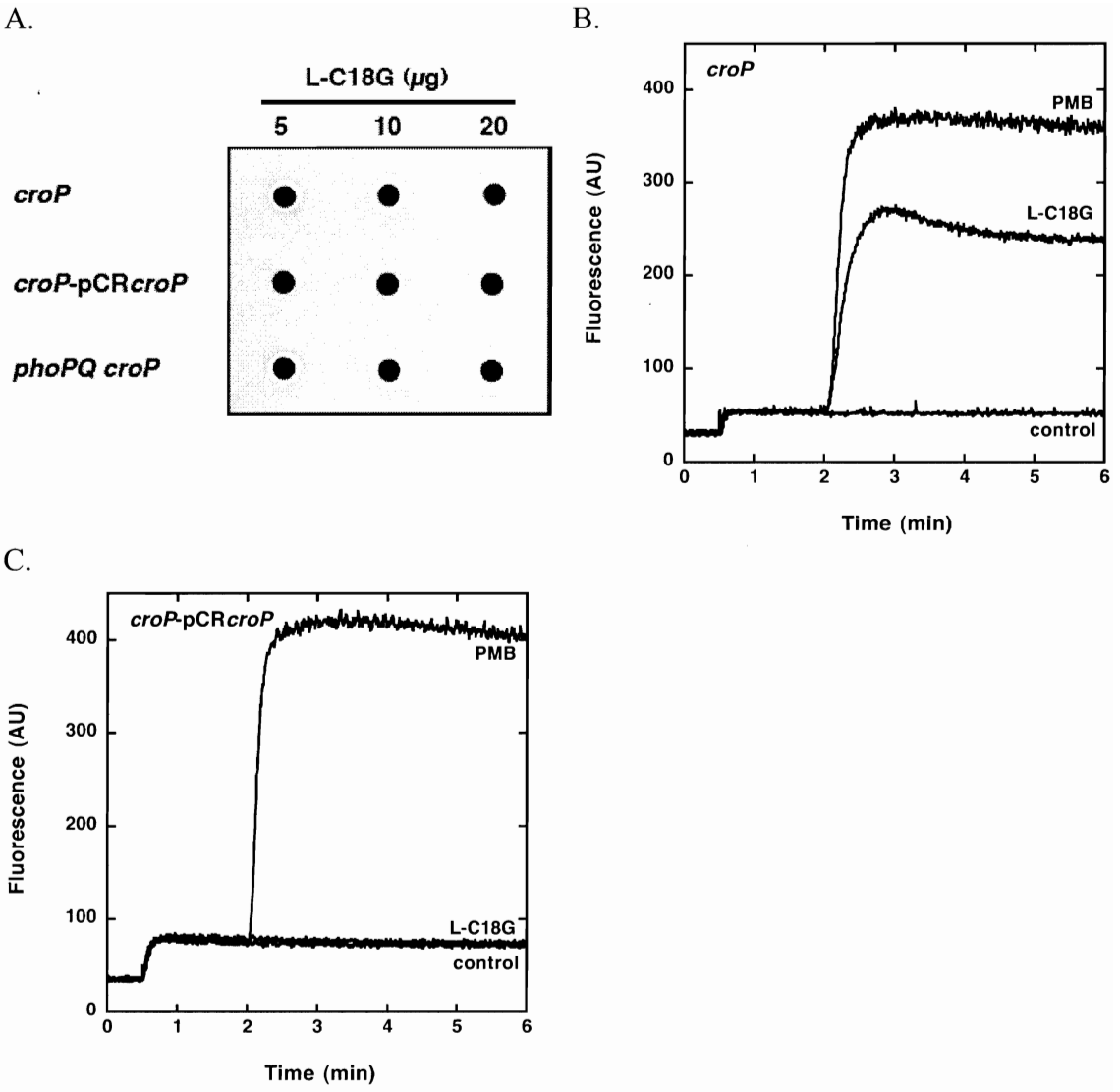


Figure 7. Identification of an OM protease, CroP involved in AMP resistance.

A. Disk diffusion assay of indicated mutants to increasing amounts of L-C18G.

NPN uptake by the OMs of the *croP* mutant (**B**) and the *croP* mutant complemented with pCR*croP* (**C**) in the absence of AMP (control) the presence of either 2 μM L-C18G or 1 μg/mL PMB. Each experiment is representative of at least three independent trials.

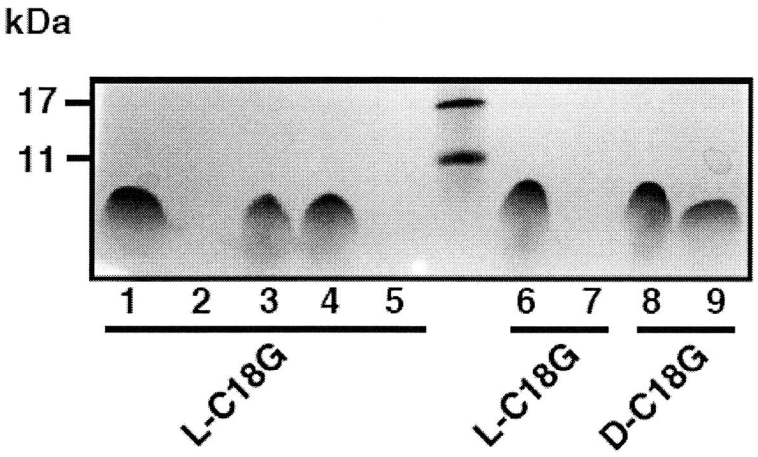


Figure 8. CroP degrades L-C18G but not D-C18G.

Degradation of AMP by different *C. rodentium* mutant strains was assayed by SDS-PAGE. L-C18G or D-C18G (200 μM) was incubated alone (lanes 1, 6 and 8), and in the presence of wild-type (lanes 2 and 9), *phoPQ* mutant (lane 3), *croP* mutant (lane 4) or *croP* mutant complemented with p*croP* (lane 5) for 30 minutes.

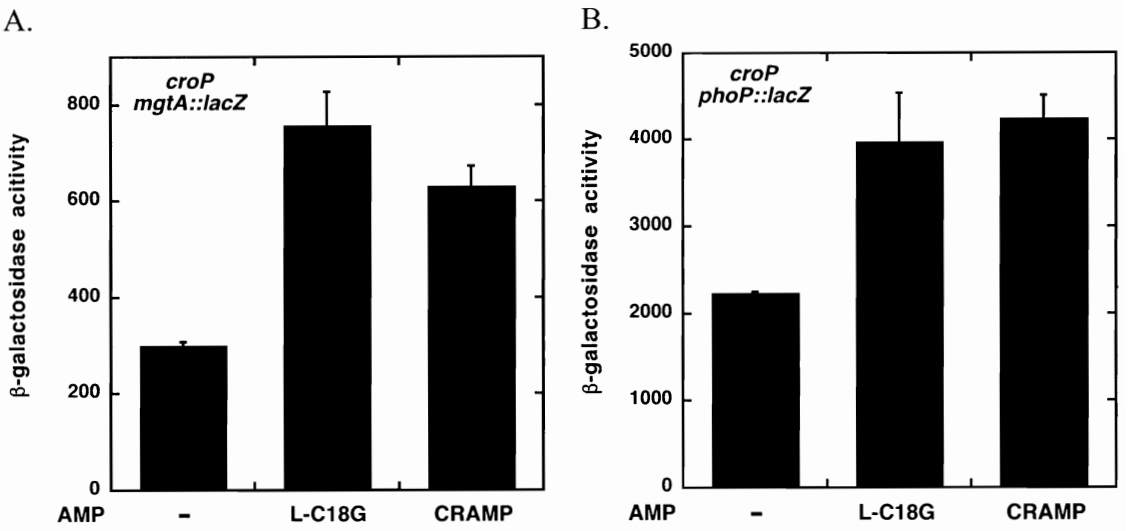


Figure 9. AMP activation of PhoQ is inhibited by the presence of CroP.

ß-Galactosidase assay was performed using *croP* mutant strains which carry the *mgtA::lacZ* (**A**) and *phoP::lacZ* (**B**). Strains were grown in N-minimal media supplemented with 1 mM $MgCl_2$ in the absence or presence of 2 μM L-C18G or 10 μM CRAMP. The values of ß-galactosidase activity are the average of three independent assays.

3 and 4). Similar results were obtained using CRAMP as a CroP substrate (data not shown). In good agreement with our previous results, D-C18G was not degraded when incubated with *C. rodentium* wild-type cells (Fig. 8, lane 9). These data clearly show that the proteolytic activity of CroP, present at the *C. rodentium* OM, is responsible for the degradation of α-helical AMPs. In turn, inactivation of AMPs prevents them from reaching the *C. rodentium* periplasmic space and activating PhoQ.

CroP inhibits PhoQ recognition of AMPs

To ascertain whether AMPs can activate PhoPQ in the context of the *C. rodentium croP* mutant, we assayed the expression of *mgtA::lacZ* and *phoP::lacZ* in the presence of sublethal concentrations of L-C-18G and CRAMP. As shown in Fig. 9, addition of L-C18G or CRAMP induced a 2.5- and 2.1-fold increase in *mgtA::lacZ* activity and a 1.8- and 1.9-fold increase in *phoP::lacZ* activity, respectively. Therefore, these data demonstrate that the presence of CroP at the *C. rodentium* OM is responsible for the unresponsiveness of PhoQ to α–helical AMPs, which are degraded before reaching the periplasmic space.

DISCUSSION

AMPs are an important means of defense against bacterial pathogens. The mouse colonic environment inhabited by *C. rodentium* contains CRAMP, an α-helical AMP that is known to have antimicrobial activity against this extracellular pathogen (Iimura *et al.*, 2005). To determine whether PhoPQ plays a role in resistance to CRAMP, we characterized the *C. rodentium* PhoPQ TCS and compared it to its homologue in *S. enterica*. The *C. rodentium phoPQ* mutant shares many characteristics of the *S. enterica phoPQ* mutant, namely an inability to grow in Mg^{2+}-depleted environments and an increased sensitivity to AMPs. The activity of *C. rodentium* PhoQ was repressed by millimolar concentrations of Mg^{2+} and activated by acidic pH. A striking difference, revealed by this study, is the apparent unresponsiveness of *C. rodentium* PhoPQ to α-helical AMPs such as CRAMP and L-C18G. This finding is in sharp contrast to what has been observed for *S. enterica* (Bader *et al.*, 2003; Bader *et al.*, 2005).

CroP prevents AMPs from reaching the periplasm

PhoQ is an important sensor of the host environment, but it remains unclear whether all PhoQ homologues respond to the same stimuli. We found that α-helical AMPs do not activate *C. rodentium* PhoPQ. However, our study provides several lines of evidence showing that *C. rodentium* PhoQ has the potential to recognize and be activated by AMPs. First, *C. rodentium* PhoQ possesses the acidic cluster that participates in the binding of both divalent cations and AMPs in *S. enterica* PhoQ (Fig. 3A) (Cho *et al.*, 2006). The isolated PhoQ periplasmic sensor domain bound dC18G, as its *S. enterica* homologue (Fig. 3B) (Bader *et al.*, 2005). AMPs activated PhoPQ when the *C. rodentium phoPQ* operon was expressed in *S. enterica* (Fig. 4A). Finally, AMPs activated PhoPQ in the *C. rodentium croP* mutant (Fig. 9). Clearly, the difference in recognition of α-helical AMPs by the *C. rodentium* and *S. enterica* PhoQ proteins is attributable to the presence of the CroP OM protease in *C. rodentium*. CroP protects the *C. rodentium* OM from damage by degrading AMPs before they reach the periplasmic space and interact with PhoQ. Although, *S. enterica* possesses the PgtE OM protease that shares 40% amino acid sequence identity with CroP, PgtE was

shown to have a minor effect on AMP degradation when expressed from a single chromosomal copy. Only when *pgtE* was overexpressed on a high-copy number plasmid was resistance of *S. enterica* to C18G increased (Guina *et al.*, 2000). Proteases of the omptin family share the same catalytic mechanism and exhibit sequence identities ranging from 40 to 80% (Hritonenko & Stathopoulos, 2007). However, their physiological substrates are poorly defined. The *Y. pestis* Pla OM protease has been shown to cleave protein substrates that are relevant to pathogenesis (Sodeinde *et al.*, 1992; Lathem *et al.*, 2007). In contrast, *E. coli* OmpT and OmpP might be more specific for small peptides (Stumpe *et al.*, 1998). The fact that CroP is only 40% identical to PgtE is consistent with the possibility that CroP degrades primarily biologically active AMPs, whereas PgtE cleaves larger proteins such as plasminogen and the C3b, C4b and C5 components of complement (Kukkonen *et al.*, 2004; Ramu *et al.*, 2007).

Is PhoPQ activated in the intestinal lumen?

For the intracellular pathogen *S. enterica*, several lines of evidence have shown that PhoPQ is activated within macrophage phagosomes by AMPs and acidic pH (Alpuche Aranda *et al.*, 1992; Rosenberger *et al.*, 2004; Martin-Orozco *et al.*, 2006). For pathogens like *C. rodentium*, which reside extracellularly in the intestinal lumen throughout the infection process, it is unclear whether PhoP-activated genes are expressed during infection. Environmental conditions in the intestinal lumen are characterized by high osmolarity, neutral pH, and the presence of AMPs. In the gut lumen, the concentration of total Mg^{2+} has been estimated at up to 20 mM, however, the concentration of free Mg^{2+} has not been precisely measured and is likely lower, in the millimolar range (Laires *et al.*, 2004). AMPs and Mg^{2+} were found to compete for the same PhoQ-binding site. Consequently, AMPs were unable to activate PhoPQ when the Mg^{2+} concentration was in the range of 5-10 mM (Bader *et al.*, 2005). Assuming that the Mg^{2+} concentration in the intestinal lumen is high enough to competitively inhibit activation of PhoQ by AMPs, PhoPQ-dependent LPS modifications would be absent. Thus, it seems reasonable to propose that *C. rodentium* adopted an alternative mechanism to resist the effect of AMPs. A mechanism of AMP

resistance that relies on an OM protease may be beneficial to *C. rodentium* in a high Mg^{2+} environment.

C. rodentium *may have a limited ability to modify LPS*

Many genes that encode LPS-modifying enzymes in *S. enterica* appear to be absent from the *C. rodentium* genome. Of the PhoP-regulated genes *lpxO*, *pagL* and *pagP,* only *pagP* is present in the *C. rodentium* genome. The PhoP-regulated *pmrD* gene, whose gene product connects the PhoPQ and PmrAB signaling pathways, is absent from the *C. rodentium* genome. This suggests that PhoPQ does not indirectly control PmrA-regulated genes involved in lipid A modifications, as described previously for *Y. pestis* (Winfield & Groisman, 2004). Consistent with the fact that *C. rodentium* was highly susceptible to PMB (Fig. 1C), the PmrA-regulated *pmrHFIJKLM* operon (also known as *pbgP* operon) is absent from the *C. rodentium* genome. This operon is responsible for the addition of 4-aminoarabinose to lipid A, a modification that is essential to PMB resistance (Gunn *et al.*, 1998). Although it is possible that LPS-modifying genes are harbored by *C. rodentium* plasmids, as described for *E. coli* O157:H7 (Kaniuk *et al.*, 2004), it appears that *C. rodentium* possesses only the *pagP* and *pmrC* genes that are responsible for the transfer of palmitate and the addition of phosphoethanolamine to lipid A, respectively. This limited number of lipid A modifications may not allow *C. rodentium* to strengthen the permeability barrier of its OM to an extent compatible with AMP resistance. This hypothesis is strongly supported by our EtBr permeability experiments (Fig. 5A). It further highlights the major contribution of the CroP OM protease in resistance to AMPs.

Different strategies for AMP resistance

In *S. enterica*, resistance to AMPs is primarily mediated by LPS modifications. The *S. enterica* PgtE OM protease appears to play a marginal role. Indeed, the proteolytic activity of PgtE does not prevent AMPs from reaching the periplasmic space and activating PhoPQ (Fig. 5D). In contrast, the *C. rodentium* CroP protease appears to be much more potent than its *Salmonella* counterpart at degrading AMPs,

since neither L-C18G nor CRAMP reach the periplasmic space (Fig. 5C). Because *Citrobacter* and *Escherichia* are closely related genera that occupy the same niche inside their respective hosts and share a striking number of virulence factors, it seems likely that the OmpT OM proteases expressed by EHEC and EPEC play a similar role during infection by actively degrading AMPs. A recent study showed that LL-37 and CRAMP are secreted in the human and murine urinary tracts, respectively (Chromek *et al.*, 2006). Since OmpT is an important virulence factor of uropathogenic *E. coli* (UPEC) (Foxman *et al.*, 1995; Kanamaru *et al.*, 2003), it is possible that UPEC OmpT is also involved in the degradation of AMPs.

In summary, this work provides an alternative mechanism by which extracellular enteric pathogens, such as *C. rodentium*, resist AMPs. An OM protease like CroP is potentially active, not only against α-helical peptides of the cathelicidin family, but also against β-defensins that are produced by colonic epithelial cells.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37°C in Luria-Bertani (LB) broth or N-minimal medium supplemented with 0.2% glucose (Nelson and Kennedy, 1971). When appropriate, media were supplemented with the following antibiotics: ampicillin (100 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}), kanamycin (50 μg ml^{-1}), streptomycin (30 μg ml^{-1}) and gentamicin (20 μg ml^{-1}).

Construction of C. rodentium *deletion mutants*

Plasmid purification, cloning and transformation were performed according to standard procedures (Sambrook *et al.*, 1989). All oligonucleotide primers are listed in Table 2. The *C. rodentium phoPQ* and *croP* deletion mutants were generated by *sacB* gene-based allelic exchange (Donnenberg & Kaper, 1991). The upstream and downstream sequences of the *phoPQ* operon were PCR-amplified from *C. rodentium* genomic DNA using primers CR400, CR401, CR402 and CR403. A three-way ligation was performed with the two digested fragments (XbaI-NdeI and NdeI-SacI) and plasmid pRE118 previously cleaved with XbaI and SacI, generating plasmid pCR001. Once the flanking regions of *phoPQ* were confirmed by sequencing, pCR001 was transformed by electroporation into *C. rodentium* DBS100. Transformants were grown on LB agar with kanamycin to select for plasmid insertion into the chromosome of *C. rodentium*. The 5' and 3' flanking regions of *croP* were amplified using the primers CR636, CR637, CR638 and CR639. A two-step ligation was used to insert the digested fragments (XbaI-EcoRV and EcoRV-SacI) into the XbaI and SacI sites of pRE112. The resulting plasmid, pCR002, was transformed into *E. coli* X7213 and introduced into the wild-type and *phoPQ* mutant *C. rodentium* strains through conjugation. Plasmid insertion was selected for using LB agar supplemented with chloramphenicol. Clones of each deletion mutants were grown on peptone agar containing 2% sucrose to select for sucrose-resistant colonies that were either Kan^S or Cm^S, indicating that allelic exchange resulted in the loss of the wild-type copy of *phoPQ* or *croP* along with the plasmid vehicle, respectively. Insertions were

confirmed by PCR and sequencing.

Plasmid construction

Plasmid pCR*phoPQ* was constructed by amplifying the *phoPQ* operon and its promoter from *C. rodentium* genomic DNA using *Pfx* DNA polymerase (Invitrogen) and primers CR541 and CR430. The resulting PCR product was cloned into the XbaI and BamHI restriction sites of plasmid pWSK129. Similarly, pST*phoPQ* was obtained by cloning the PCR-amplified *S. enterica phoPQ* operon and its promoter (primers ST565 and ST564) into pWSK129 previously digested with XbaI and BamHI. The *C. rodentium croP* gene and its promoter was PCR-amplified using primers CR634 and CR639 and cloned into the XbaI and SacI sites of pWSK129, generating plasmid pCR*croP*.

Construction of chromosomal* lacZ *transcriptional fusions

Chromosomal transcriptional fusions between the *mgtA* or *phoP* promoters and the *lacZ* reporter gene were generated in *C. rodentium* and *S. enterica* using the suicide vector pFUSE (Baümler *et al.*, 1996). The *mgtA::lacZ* fusion was constructed by PCR amplifying the *mgtA* promoter using *C. rodentium* genomic DNA and primers CR542 and CR543. The PCR product was digested with XbaI and SmaI and cloned into the corresponding sites of pFUSE. The resulting construct was transferred into the wild-type, *phoPQ* and *croP* mutant *C. rodentium* strains by conjugation and integrated by homologous recombination, as previously described (Daigle *et al.*, 2001). A similar strategy was used to construct *C. rodentium phoP::lacZ* fusions (primers CR540 and CR541). The *S. enterica mgtA*::*lacZ* (primers ST459 and ST458) fusion was integrated into the chromosome of both *S. enterica* wild-type and Δ*phoPQ*.

β-*Galactosidase activity assay*

Bacteria containing *lacZ* fusions were grown overnight at 37°C in LB containing the appropriate antibiotic. To examine regulation of PhoQ by Mg^{2+}, cultures were grown in N-minimal medium (pH 7.5) containing 10 mM $MgCl_2$ at 37°C. At an OD_{600} of 0.4, cells were washed twice in N-minimal medium,

resuspended in fresh media supplemented with either 8 μM or 10 mM $MgCl_2$ and incubated at 37°C for an additional 90 min. To test PhoQ activation by acidic pH, cells were grown to an OD_{600} of 0.4 in N-minimal medium (pH 7.5) supplemented with 1 mM $MgCl_2$. Once washed, cells were resuspended in fresh media containing 1 mM $MgCl_2$ and buffered at pH 7.5 or pH 5.5 and grown for 90 minutes at 37°C. Activation of PhoQ by AMPs was performed in N-minimal medium (pH 7.5) supplemented with 1 mM $MgCl_2$. AMPs were added at an OD_{600} of 0.4 and cultures were incubated at 37°C for 60 min. β-galactosidase activity assays were performed in triplicate, as previously described (Miller, 1972).

Stress survival assay

Aliquots (60 μl) of overnight cultures were inoculated into 2 mL of LB supplemented with either 0.05% SDS, 2.4 M NaCl or 5 mM EDTA and grown for 1 hour at 37°C with aeration. Cultures were serially diluted in phosphate-buffered saline (PBS) and plated on LB agar. Percent survival was determined by dividing colony-forming units (cfu) of stressed cells by cfu of unstressed bacteria, and multiplying by 100.

Disk diffusion assay

Peptides CRAMP GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ and L-C18G ALYKKLLKKLLKSAKKLG were synthesized with a purity of > 85% (BioChemia Inc.). D-C18G, the all D-amino acid enantiomer of L-C18G was synthesized at the Sheldon Biotechnology Centre, McGill University. Polymyxin B was purchased from Sigma. Aliquots (80 μl) of overnight cultures were in inoculated into 1% agarose (20 ml) and poured into 15 cm Petri dishes containing LB agar. Disks containing the indicated amount of AMP were layered on top of the agarose and incubated overnight at 37°C.

Cloning, expression and purification of the* C. rodentium *PhoQ periplasmic domain

The *C. rodentium* PhoQ periplasmic domain ($PhoQ_{Peri}$) was amplified by PCR using primers CR562 and CR563 from genomic DNA. The PCR fragment was

digested with BamHI and XbaI and ligated into plasmid pET11a digested with the same enzymes to generate plasmid pCRQ$_{Peri}$. The *C. rodentium* PhoQ$_{Peri}$ protein was expressed in *E. coli* RIL ArcticExpress® (Stratagene) according to the manufacturer protocol. Cells were harvested and resuspended in 20 mM sodium phosphate buffer (pH 7.5) containing 500 mM NaCl, 20 mM imidazole and PMSF (17 μg.ml-1). Cells were disrupted by sonication, centrifuged at 216 000 *g* for 30 min and the supernatant was applied to a Ni^{2+}-NTA affinity chromatography column according to manufacturer's instructions (GE Healthcare). Purified protein was dialyzed against 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. Protein concentration was determined using the BCA protein assay (Pierce).

Fluorescence spectroscopy

Peptide binding assays were performed as previously described (Bader *et al.*, 2005). PhoQ$_{Peri}$ at a final concentration of 1μM was incubated with 1 μM dansylated C18G (dC18G) in the absence or presence of $MgCl_2$ for 20 min at room temperature. Excitation of dC18G was at 340nm and the fluorescence emission spectra were recorded from 400 to 650 nm using 10 nm slit widths. Each spectrum was the mean of 10 consecutive scans.

Ethidium bromide influx assay

The OM permeability was assessed using ethidium bromide as a fluorescent probe as previously described (Murata *et al.*, 2007). *C. rodentium* and *S. enterica* wild-type and *phoPQ* mutant strains were grown in N-minimal medium containing 10 mM $MgCl_2$ to an OD_{600} of 0.4 before being washed and resuspended in fresh medium containing 8 μM $MgCl_2$. At an OD_{600} of 1.0, cultures were diluted 10-fold in 50 mM sodium phosphate buffer (pH 7.5) and transferred into a quartz cuvette equipped with a stir bar. Cells were incubated at 20°C for 10 min in the presence of 6 μM carbonyl cyanide meta-chlorophenylhydrazone (CCCP) to inactivate efflux pumps. Fluorescence of EtBr-DNA complexes was measured over time at 20°C with excitation and emission wavelengths of 545 and 600 nm, respectively. Excitation and emission slit widths set at 5 and 10 nm, respectively. The experiment was repeated

three times with similar results.

Outer membrane disruption assay

Bacterial cells were grown to an OD_{600} of 0.5-0.6 in N-minimal medium with 1 mM $MgCl_2$. Cells were diluted to an OD_{600} of 0.37 with 5 mM HEPES and transferred into a quartz cuvette equipped with a stir bar. Samples were excited at 350 nm and emitted fluorescence was recorded at 420 nm over time using 5 nm slit widths. Thirty seconds after the start of the experiment, 1-N-phenyl-naphthylamine (NPN, Sigma) was added at a final concentration of 5 µM and AMPs were added 90 sec later.

Outer membrane protein extraction

Outer membranes were isolated as previously described (Hernandez-Alles *et al.*, 1999). Briefly, cells were collected by centrifugation, resuspended in 10 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$ and disrupted by sonication. Unbroken cells were removed by centrifugation at 3 300 *g* for 10 min. Cell membranes were collected by high-speed centrifugation at 100 000 *g* for 1h. The pellet was resuspended in10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ containing 2% sodium lauryl sarcosinate and incubated for 30 min at 25°C. OM proteins were collected by centrifugation at 100 000 *g* for 1 h. After a second treatment with sodium lauryl sarcosinate, OM proteins were resuspended in loading buffer before being resolved on a 12.5 % SDS-PAGE, subsequently stained with Coomassie blue. In-gel trypsin digestion, mass spectrometry and analysis by MASCOT software were carried out at the McGill University and Génome Québec Innovation Centre, Montreal, Canada.

Proteolytic cleavage of AMPs by CroP

Bacterial cells were grown to an OD_{600} of 0.5-0.6 in N-minimal medium with 1 mM $MgCl_2$. Culture aliquots (20 µl) were incubated with 200 µM AMP for 30 min at 37°C. Reactions were stopped by the addition of 25 µl Tricine sample buffer (2x). Reaction aliquots (10 µl) were analyzed by Tris-Tricine SDS-PAGE (10-20 % acrylamide, Bio-Rad) and Coomassie staining.

Western blotting

Wild-type and *phoPQ* mutant strains were grown at 37°C to an OD_{600} of 0.8 in N-minimal medium (pH 7.5) containing either 8 μM or 10 mM $MgCl_2$. Cells were harvested and resuspended in 100 mM sodium phosphate buffer (pH 7.5), 5 mM EDTA and 10 % glycerol. Cells were lysed by sonication and cell debris was removed by centrifugation at 200 000 *g*. Equal amounts of whole cell lysates were run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Blots were developed with an antiserum against *S. enterica* PhoP (1:1000), an anti-rabbit IgG horseradish peroxidase-linked antibody (1:5000) and the Immobilon Western reagent (Millipore).

Table 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or Source
C. rodentium		
DBS100	wild-type *C. rodentium*	Schauer & Falkow, 1993
phoPQ	DBS100 Δ*phoPQ*	This work
croP	DBS100 Δ*croP*	This work
CR003	DBS100 Δ*phoPQ*Δ*croP*	This work
CR101	DBS100 *mgtA::lacZ*, Cm^R	This work
CR102	*phoPQ mgtA::lacZ*, Cm^R	This work
CR103	*croP mgtA::lacZ*, Cm^R	This work
CR106	*croP phoP::lacZ*, Cm^R	This work
S. enterica		
14028s	wild-type *S. enterica*	SGSC
phoPQ	14028s Δ*phoPQ*	SGSC
ST101	14028s *mgtA::lacZ*, Cm^R	This work
ST102	*phoPQ mgtA::lacZ*, Cm^R	This work
Plasmids		
pET11a	T7 expression vector, Amp^R	Stratagene
pFUSE	Suicide vector, *lacZYA*, Cm^R, mob^+ (RP4), ori R6K	Baumler *et al.*, 1996
pRE112	Sucrose sensitive (*sacB*1) suicide vector, Cm^R	Edwards *et al.*, 1998
pRE118	Sucrose sensitive (*sacB*1) suicide vector, Kan^R	Edwards *et al.*, 1998
pWSK129	Low-copy number cloning vector, Kan^R	Wang & Kushner
$pCRQ_{Peri}$	pET11a containing residues 45-190 of the *C. rodentium* PhoQ protein	This work
pCR001	pRE118 containing Δ*phoPQ*	This work
pCR002	pRE112 containing Δ*croP*	This work
pCR*phoPQ*	pWSK129 containing the *C. rodentium phoPQ* operon and its promoter	This work
pST*phoPQ*	pWSK129 containing the *S. enterica phoPQ* operon and its promoter	This work
pCR*croP*	pWSK129 containing the *C. rodentium croP* gene and its promoter	This work

Table 2. Primers used in this study.

Primer #	Name	Sequence
CR400	CR*phoPQ* 5′F	ACGTACTCTAGAGTTAAGCCTTATCTGAAGGGC
CR401	CR*phoPQ* 5′R	GATCAACATATGCGCATTATCCTCAACAACCAG
CR402	CR*phoPQ* 3′F	CGATCACATATGGGGCAGAAGGACGGCTAAG
CR403	CR*phoPQ* 3′R	ATATATGAGCTCCATCGCCTTGCTTCAGGGCA
CR636	CR*croP* 5′F	AGCTAGGGTACCTTGGCCTGCTGATTGAACGCG
CR637	CR*croP* 5′R	AGCTACGATATCCAAACCGGATTCGCCAGACGC
CR638	CR*croP* 3′F	AGCTAGGATATCCTTGAAGGCGTATGGAGTCG CG
CR639	CR*croP* 3′R	AGCTACGAGCTCGCAATACAGGGGATTGAAG GG
CR540	CR*phoP::lacZ* 5′F	AGCTAGTCTAGAGTATCGCGTGGGCCGTGCTG AG
CR541	CR*phoP::lacZ* 3′R	AGCTACCCCGGGGCATCCTGAAGTTGCACC
CR430	CR*phoQ* 3′R	CAGTGGATCCTTAGCCGTCCTTCTGCCCCG
CR542	CR*mgtA::lacZ* 5′F	CCCCCCTCTAGACCGTGCTGATTCATCACCGC TTC
CR543	CR*mgtA::lacZ* 3′R	AGCTACCCCGGGTAACGGATCGCGGTGTACC AGG
CR562	CR $PhoQ_{Peri}$ 5′F	GGAATTCCATATGGATAAGACCACGTTTCGCC TGCTGCG
CR563	CR $PeriQ_{Peri}$ 3′R	CGGGATCCTTAGTGATGGTGATGGTGATGCAT ATAAGAGCGTTTAAGCTCAACGG
ST565	ST*phoP::lacZ* 5′F	AGCTAGTCTAGAGACTCAACCGTCCTGCGTAA CCTGGGT
ST564	ST*phoQ* 3′R	CAGTGGATCCTTATTCCTCTTTCTGTGTGGATG CTGTCG
ST459	ST*mgtA::lacZ* 5′F	CCCCCCTCTAGACCGTGTTGATTCATCACCGC TTC
ST458	ST*mgtA::lacZ* 3′F	CCCCCCCGGGAACCAGACGATAAGGCAAATG ACG

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CHAPTER 5

Conclusions and Perspectives

The *Salmonella* PhoP/PhoQ TCS is a master regulator that controls a large regulon necessary for pathogenesis. Given the fact that the PhoP/PhoQ system is activated inside the phagosome and has a crucial role in resistance to macrophage defenses it is likely that PhoQ is able to perceive intracellular signals. The first objective of this thesis stems from the long-standing controversy regarding the PhoQ-activating ligand(s) within the SCV. We set out to clearly demonstrate that H^+ ions activate the *S. typhimurium* PhoQ SK. The second objective was to describe the mechanism of acidic pH recognition. The third objective was made possible by the recent availability of the *Citrobacter rodentium* genome sequence. Analysis revealed that it contains a homologue of the *phoPQ* operon, which we then proceeded to characterize in order to gain an understanding of its role in this extracellular pathogen.

Acidic pH as a physiological ligand of *Salmonella typhimurium* PhoQ

Acidic pH was first proposed in 1992 as being the physiological ligand that activates *S. typhimurium* PhoQ inside the macrophage. The pH inside the phagosome stabilizes between pH 5.0 and 6.5 (Alpuche Aranda *et al.*, 1992; Martin-Orozco *et al.*, 2006). Acidification of the SCV coincides with the induction of a subset of PhoP-activated genes, which is abolished by pH neutralization of the phagosome (Alpuche Aranda *et al.*, 1992). A long-standing misconception that the concentration of Mg^{2+} and Ca^{2+} inside the SCV was depleted fueled the hypothesis that divalent cations were acting as the physiological ligands of PhoQ (Groisman, 1998).

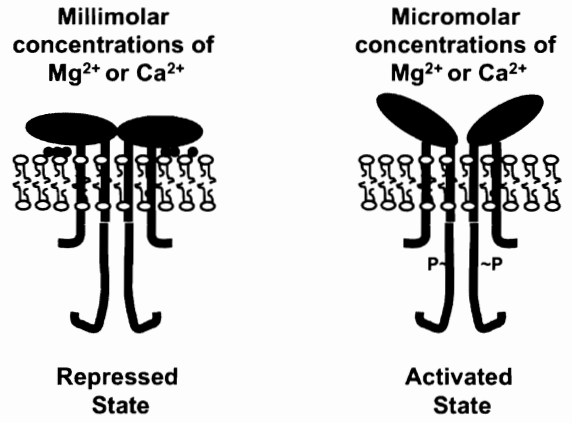
Chapter 2 of this thesis demonstrates for the first time that the SK PhoQ directly recognizes increasing concentrations of H^+ ions. PhoQ reconstituted membrane vesicles loaded with a buffer of acidic pH (5.5) and the physiological concentration of Mg^{2+} (1 mM), produces an increase in the phosphorylation of PhoP (Prost *et al.*, 2007). The mechanism of acidic pH-mediated PhoQ activation occurs through an increase in its autokinase activity while the phosphatase activity remains constant (V. Le Sage and H. Le Moual, unpublished results). Under acidic conditions, a sublethal concentration of AMPs had an additive effect to increase PhoP phosphorylation by PhoQ (Prost *et al.*, 2007). As PhoQ is a sensor of the intracellular environment and both acidic pH and AMPs are present within the macrophage

phagosome it is likely that together these signals maximally activate *S. typhimurium* PhoQ.

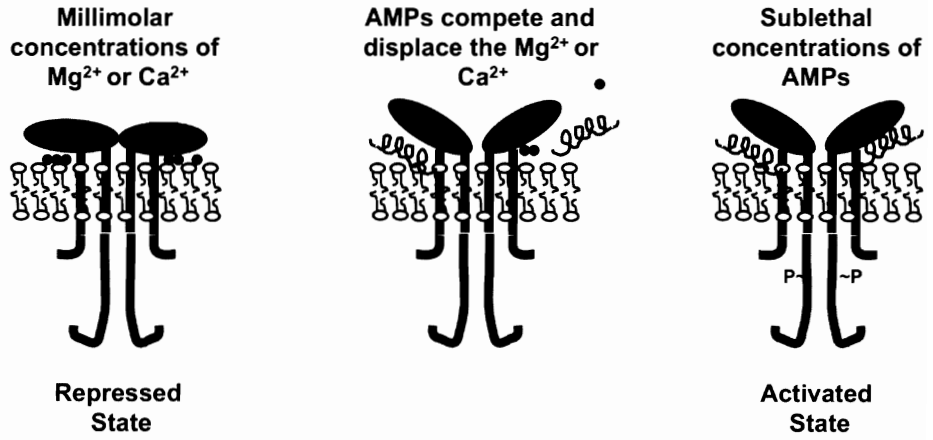
A model for *Salmonella* PhoQ signal integration from three different ligands found inside the SCV has been proposed based on NMR spectroscopy data (Prost & Miller, 2008). Millimolar concentrations of divalent cations bind the acidic patch of the periplasmic domain and lock PhoQ in a repressed state by tethering it to the bacterial membrane (Figure 1A). The presence of Mg^{2+} imposes structural rigidity on the PhoQ periplasmic domain that is alleviated by the absence of metal bridging. The subsequent charge repulsion forces the α4/α5 helices away from the negatively charged membrane to produce a conformational change that results in increased PhoQ autophosphorylation (Figure 1A). Cationic AMPs bind to the periplasmic domain at the same location as divalent cations, and because of their size, push the α4/α5 helices away from the membrane (Figure 1B). Acidic conditions surrounding the PhoQ periplasmic domain influence the core β-sheet hydrogen bonds inducing a flexible structure that allows the α4/α5 helices to distance themselves from the membrane and releasing the metal bridges (Figure 1C). Maximal activation is achieved by a combination of acidic pH and AMPs to, at once, lose structural rigidity and force the helices away from the membrane. Whether this model of signal recognition holds true for other PhoQ SKs remains an open question.

Typically, bacteria favor growth at neutral pH but some are able to adapt to acidic environments. This implies the evolution of a system that senses pH alterations and subsequent implementation of a successful strategy to survive the adverse pH condition. The *phoPQ* operon is present in the genome of a number of Gram-negative bacteria but direct activation of PhoQ under acidic conditions has not been extensively studied. PhoQ activation by H^+ ions is suggested by the observation that *phoP* and/or *phoQ* mutants of *Erwinia chrysanthemi* (Llama-Palacios et al., 2003) and *Yersinia pestis* (Oyston *et al.*, 2000) have a diminished capacity to survive in acidic pH. In contrast, a *phoP* mutant of *Shigella flexneri* survives as well as wild-type under extreme acid (pH 2.5) (Moss *et al.*, 2000). These three PhoP/PhoQ systems have high amino acid sequence homology however the pH susceptibility of each *phoPQ* mutant may suggest that their sensing capabilities are different.

A.


Millimolar concentrations of Mg^{2+} or Ca^{2+}
Micromolar concentrations of Mg^{2+} or Ca^{2+}
P~
~P
Repressed State
Activated State

B.


Millimolar concentrations of Mg^{2+} or Ca^{2+}
AMPs compete and displace the Mg^{2+} or Ca^{2+}
Sublethal concentrations of AMPs
P~
~P
Repressed State
Activated State

C.

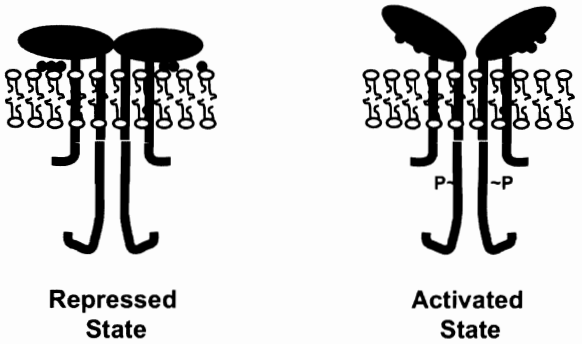

Neutral pH
Acidic pH
P~
~P
Repressed State
Activated State

Figure 1. Model for PhoQ ligand sensing.

Millimolar concentrations of divalent cations, such as Mg^{2+} and Ca^{2+} (green balls) bind to the PhoQ periplasmic domain and tether it to the membrane thus locking PhoQ in a repressed state (**A**). Antimicrobial peptides also bind to the PhoQ periplasmic domain to compete and displace the divalent cations (**B**). Ultimately, this results in a conformational change in PhoQ that causes autophosphorylation. Acidic pH causes activation of PhoQ by inducing flexibility (purple) in the periplasmic domain causing them to distance themselves from the membrane and releasing the metal bridges (**C**).

Recently, the ligands and sensing mechanisms of PhoQ have been described for the opportunistic pathogen *Pseudomonas aeruginosa* (Prost *et al.*, 2008). *P. aeruginosa* PhoQ binds divalent cations using a specific ligand-binding pocket as its periplasmic domain lacks the α4/α5 helices. NMR analysis and fluorescence spectroscopy of the *P. aeruginosa* PhoQ periplasmic domain indicate that the protein undergoes a specific conformational change in response to acidification (Prost *et al.*, 2008). Interestingly, as compared to *S. typhimurium*, *P. aeruginosa* PhoQ is only partially activated by acidic conditions implying a different mechanism of H^+ ion sensing. Because *P. aeruginosa* PhoQ is structurally divergent from *S. typhimurium* PhoQ and senses Mg^{2+} but not AMPs a differing mechanism of H^+ ions perception is reasonable.

It is clear that PhoQ is activated by acidic pH but to what extend the conformational change of the periplasmic domain contributes to this activation has yet to be determined. An examination of the pH responsiveness of different internal PhoQ deletions may indicate the involvement of the bacterial membrane or the SK linker domain instead of or in addition to the periplasmic domain.

Mechanism of pH sensing

NMR spectroscopy of the purified PhoQ periplasmic domain indicates that the protein undergoes structural changes upon pH acidification (Prost *et al.*, 2007). Chapter 3 set out to determine the amino acid residue(s) responsible for PhoQ sensing H^+ ions.

Fluorescence spectroscopy of the PhoQ periplasmic domain demonstrates that incrementally lowering the pH causes quenching of the fluorescence intensity and a 6 nm blue-shift in the maxima. His residues are plausible candidates for sensing pH because they titrate in the physiological pH range. Mutation of each of the three titratable His residues (120, 137 and 157) to nontitratable Asn residues abrogated the pH-dependent quenching and blue-shifting of the fluorescent spectrum in only the H120N mutant. The pH response of the full-length SK differs based on the amino acid substitution at position 120. PhoQ mutants with Asn, Phe or Tyr residue replacements maintain pH-responsiveness, while aliphatic substitutions (Ala and Val) could not

activate the PhoP-dependent *phoN* gene expression. We can conclude that residue H120 is not the pH sensor for the PhoQ SK.

Mutagenesis of the Trp residue at position 113 results in a protein whose fluorescent spectrum is insensitive to changes in pH. The available PhoQ crystal structure (Cho *et al.*, 2006) confirms that H120 is proximal to W113. Structural modeling of the periplasmic domain reveals that W113 and H120 are in an apparent π-stacking arrangement. We propose that above pH 7.5, H120 is not protonated and unable to quench the fluorescence of W113 however below pH 7.5 the His residue becomes protonated and is capable of efficiently quenching the fluorescence of W113 (Van Gilst & Hudson, 1996). The blue-shift in the wild-type spectrum indicates that either the π-stacking is causing W113 to be displaced to a more buried position or that the interaction itself is making the residue less accessible. NMR assignment of the low-pH state will provide insight into the activated conformation of PhoQ and which regions have increased flexibility.

The pK_a of the PhoQ periplasmic domain is ~7.5. This value is often associated with a His residue because its imidazole moiety is the only side chain that ionizes in solution within a physiological pH range. However, recent studies suggest that under certain conditions charged amino acids, including Asp, Glu and Lys can act as pH sensors (Tsuboi *et al.*, 2003; Rapedius *et al.*, 2006; Kim *et al.*, 2008). The pK_a of an isolated Glu or Asp carboxylate group is typically 3-4 whereas juxtaposition of multiple carboxylates can raise the pK_a to 6-8 (Davoodi *et al.*, 1995; Morrill & MacKinnon, 1999). The pH-dependent hemolytic activity of lysteriolysin O from *Listeria monocytogenes* is based on a triad of charged resides (Glu247-Asp320-Glu208) whose carboxylates repel each other at neutral pH causing a partial denaturation that renders the protein inactive (Schuerch *et al.*, 2005). The substitution of Asp320 alone has no effect on the activity of lysteriolysin O and requires an additional mutation of Glu247 to halt the denaturation process making the protein pH-insensitive (Schuerch *et al.*, 2005). The acid-sensing ion channel 1 (ASIC) from chicken epithelial cells is an example of a proton-activated receptor that senses changes in pH over multiple amino acids within its acidic pocket (Jasti *et al.*, 2007).

The negatively charged surface of the PhoQ periplasmic domain contains a cluster of charged amino acids (D149-E154) and several other residues, including D45, E112, E121, E123 and E184 that are highly conserved among other PhoQ SKs. It is possible that any number of these charged amino acids is responsible for the pH-dependent conformational change of the periplasmic domain. Mutant strains should be constructed to assess whether any individual or combination of these residues impacts upon the pH sensing capabilities of PhoQ. Thus, it seems that the molecular mechanism of pH sensing by PhoQ is a complex process involving several residues that may form a network of interaction.

The *Citrobacter rodentium* PhoP/PhoQ two-component system

Diverging from a common ancestor 100-150 million years ago, *Escherichia coli* and *S. typhimurium* are two closely related *Enterobacteria* that have both adapted themselves to cause human pathology (Doolittle *et al.*, 1996). In nonpathogenic *E. coli*, PhoP/PhoQ plays a role in its physiological response to Mg^{2+} starvation and acid resistance but understandably not virulence (Garcia Vescovi *et al.*, 1996). As humans are the only reservoir for enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* efforts at studying host-pathogen interactions are limited to *in vitro* experiments. The genomes of both EHEC and EPEC contain the LEE (locus of enterocyte effacement) pathogenicity island, which encodes the virulence factors responsible for intestinal attaching and effacing (A/E) lesions. The host-adapted murine pathogen *Citrobacter rodentium* has a homologous LEE and provides an excellent small animal model for the pathogenesis of A/E bacteria.

It is obvious that *Salmonella* PhoP/PhoQ is critical for intracellular survival and promotes resistance to host innate defenses as a master regulatory system that controls most virulence factors. Chapter 4 draws similarities and differences between the PhoP/PhoQ TCSs of *C. rodentium* and *S. typhimurium*. The sequences of the *phoPQ* operons are ~79% identical, additionally the *C. rodentium phoPQ* mutant shares many characteristics with the *S. typhimurium phoPQ* mutant, including an inability to grow in Mg^{2+}-depleted conditions and a heightened sensitivity to AMPs. The activity of *C. rodentium* PhoQ is repressed by millimolar concentrations of Mg^{2+}

and activated by acidic pH. In contrast to *S. typhimurium*, *C. rodentium* PhoQ does not respond to the presence of AMPs. However, when the *C. rodentium phoPQ* operon is expressed in a *S. typhimurium phoPQ* mutant strain, AMP activation is restored. Thus, we concluded that sublethal concentrations of AMPs can elicit a response from PhoQ however a component of the *C. rodentium* bacteria is inhibiting recognition of this ligand.

Outer membrane proteins are important for maintaining membrane integrity and selective permeability but can also play a role in bacterial pathogenesis by improving a pathogen's adaptability to various environments. Chapter 4 provides convincing evidence that the OM protease CroP not only inhibits activation of PhoQ by AMPs but is also an important AMP resistance factor. CroP is a member of the omptin family consisting of enterobacterial surface proteases that share a conserved β-barrel fold. Omptins have high sequence identity yet exhibit different functions. Cleavage of the protein/peptide substrate occurs preferentially between two successive basic amino acid residues. The substrate specificity of each individual omptin is determined by the minor sequence variations in the five extracellular loops (L1-L5) near the catalytic residues. PgtE of *S. tyhimurium* and OmpT of *E. coli* degrade cationic AMPs. The amino acid composition of the extracellular loops of CroP differs from OmpT of *E. coli* suggesting that CroP may bind different substrates. It is evident that the presence of CroP in the OM reduces the amount of damage caused by AMPs, through the proteolytic cleavage of α-helical AMP.

Pla of *Y. pestis* inactivates AMPs such as LL-37 and CRAMP (Galvan *et al.*, 2008) but also promotes the degradation of fibrin clots by proteolytic cleavage of plasminogen into plasmin and inactivating the α_2-antiplasmin inhibitor (Kukkonen *et al.*, 2001). Interestingly, analysis of 2D gel electrophoresis in combination with mass spectroscopy identified two other proteins (Dps and a cleavage product of OmpA) whose expression strictly coincides with the presence of CroP in the membrane (A. Portt, V. Le Sage and H. Le Moual, unpublished results). This suggests a possible interaction between CroP and one or both of these OM proteins. Dps is a nonspecific DNA-binding protein from starved cells that protects from oxidative stress (exponential phase) and organizes the chromosome into a stable nucleoprotein

complex during stationary phase. Extensively studied in *E. coli,* OmpA has been attributed many functions, including maintaining membrane integrity (Sonntag *et al.*, 1978), enhancing macrophage uptake of LPS (Korn *et al.*, 1995), promoting the invasion of the central nervous system (Prasadarao *et al.*, 1996) and may form pores (Sugawara & Nikaido, 1992). There is no immediately apparent reason as to why Dps or OmpA would interact with a protease or be involved with AMP resistance. We could speculate that the absence of CroP may interfere with Dps, OmpA or another as yet unidentified system that is involved in AMP resistance. A recent study indicates that OmpA from *Klebsiella pneumoniae* is involved in AMP resistance as an *ompA* mutant is more susceptible to AMPs than wild-type (Llobet *et al.*, 2009). To understand the interaction between Dps and/or OmpA and the protease we intend to create an active site mutant of CroP to verify whether or not proteolytic cleavage is necessary for localization to the OM in the case of Dps. Further work along these lines may confirm a specific interaction between CroP and OmpA using the *C. rodentium croP* mutant to express *E. coli* OmpT, OmpP and *S. typhimurium* PgtE and systematically comparing their proteolytic specificities for OmpA. As the biological roles of these two proteins are still unknown in *Citrobacter* we will examine these in future studies.

Because AMPs act on bacterial membranes, Gram-negative bacteria modify their cell surface in order to decrease OM permeability or to prevent binding of AMPs to the OM. Modification of the lipid A domain of LPS is known to contribute to the virulence and pathogenesis of various species. The natural variation in the structure and function of lipid A reflect the diversity of bacterial survival strategies. In *S. typhimurium*, PhoP-mediated LPS modifications are the primary mechanism of AMP resistance. The Sanger genome sequence project of *C. rodentium* strain ICC168 (http://www.sanger.ac.uk/ Projects/C_rodentium/) provides a searchable database for the identification of *S. typhimurium* LPS modifying enzyme homologues. While *C. rodentium* possesses enzymes for the addition of palmitate (PagP) to lipid A, corresponding enzymes for deacylation (PagL), hydroxylation (LpxO) do not appear to be present. PagP is an OM enzyme of LPS biosynthesis that attenuates the ability of LPS to trigger host defenses by TLR-4 (Miller *et al.*, 2005). Homologues of PagP are

encoded by a number of Gram-negative bacteria but apart from *S. typhimurium* its involvement in AMP resistance is unknown (Lin *et al.*, 2002). *E. coli* K12 and O157:H7 each express a functional PagP homologue that is either expressed at low levels or is present in the OM in a latent state, respectively (Bishop, 2005; Smith *et al.*, 2008). Preliminary evidence indicates that activation of at least one PhoP-regulated gene decreases the permeability of the *Citrobacter* OM to ethidium bromide. We would like to determine the contribution of PagP to the membrane integrity of *Citrobacter* using a *pagP* mutant to analyze its OM permeability and lipid A structure.

Resistance to cationic AMPs in Gram-negative bacteria is also promoted through the upregulation of PhoP-independent LPS modifications. The TCS PmrA/PmrB of *S. typhimurium* responds to Fe^{3+} and acidic pH and regulates 20 confirmed genes and as many as 100 genes according to microarray analysis (Marchal *et al.*, 2004). PmrA activates transcription of genes that modify LPS being *pmrC* (PEtN) and the *pmrHFIJKLM* operon (L-Ara4N). Indirect activation of PmrA/PmrB occurs upon upregulation of the PhoP-activated gene *pmrD* (Kox *et al.*, 2000). In *Salmonella*, PmrD binds and stabilizes the phosphorylated form of PmrA to maintain activation in the presence of PhoP/PhoQ activating signals. In contrast, *E. coli* PmrD is structurally different and is unable to connect PhoP/PhoQ to PmrA/PmrB (Fu *et al.*, 2007). The *C. rodentium* genome lacks the *pmrD* gene as well as the *pmrHFIJKLM* operon. Interestingly, *Citrobacter* PhoP/PhoQ does not directly regulate the expression of *pmrC*::*lacZ* however gene expression was induced under Mg^{2+}-depletion and repressed in the presence of high Mg^{2+} concentrations (V. Le Sage and H. Le Moual, unpublished results). PmrB is not known to respond to changes in Mg^{2+} concentrations suggesting that *pmrC* may be regulated by an additional TCS. For example, in *Salmonella* the PreA/PreB TCS (homologous to QseB/QseC in EHEC) activates transcription of *pmrC* in a PmrA- and PhoP-independent manner (Merighi *et al.*, 2006). By inserting the *pmrC*::*lacZ* reporter into the *pmrAB* mutant we would be able to discriminate between PmrB responding to Mg^{2+} and regulation of *pmrC* by another source. A better understanding of the LPS modifications in *C. rodentium* would come from the structural analysis of the lipid A component from a *pmrAB* deletion mutant.

C3H/HeJ mice are susceptible to *C. rodentium* infections, which result in 100% mortality between days 6 and 10 post-infection (Vallance *et al.*, 2003). To determine the role of PhoP/PhoQ during infection, C3H/HeJ mice were inoculated with a wild-type *C. rodentium* strain and its isogenic *phoPQ* mutant. No significant differences were observed between the mortality of mice infected with the wild-type or *phoPQ* mutant bacterial strains, although there appears to be a trend of delayed death in the *phoPQ* mutant infected mice (L. Zhu and S. Gruenheid, personal communication). These data suggest that *phoPQ* may not be crucial for virulence in the C3H/HeJ mouse model. It is clear that in C57BL/6, CRAMP has antimicrobial activity against *C. rodentium* and as the sole murine cathelicidin is important for controlling the bacterial load during the first 7 days of infection (Iimura *et al.*, 2005). We established that the *C. rodentium phoPQ* operon is necessary for resistance to AMPs but dispensable for virulence. Our results, taken together with the study of Iimura *et al.*, suggest a role for the PhoP/PhoQ TCS in the early stages of *Citrobacter* infection. To this end, a competitive index can be performed with the C57BL/6 mouse strain to determine whether or not there is a difference in the fitness between the wild-type and the *phoPQ* mutant strains early in infection. These experiments would help delineate if the AMP resistance that PhoP/PhoQ confers to *C. rodentium* translates to the mouse infection model.

Why this difference exists between *S. typhimurium* and *C. rodentium* perhaps derives from the environmental conditions that each organism lives in and the evolutionary pressures they have had to overcome. The intracellular *S. typhimurium* resides within macrophage phagosomes, while *C. rodentium* is an extracellular organism that is conditioned to survive and replicate in the colon attached to the epithelium layer. Environmental conditions within the gut lumen are characterized by high millimolar concentrations of Mg^{2+}, a neutral pH and the presence of AMPs. It is clear that *C. rodentium* PhoQ is not activated by AMPs, which begs the question as to whether or not PhoP/PhoQ is active within the gut. The classic model states that *S. typhimurium* PhoQ is activated when the bacterium is intracellular and poorly expressed extracellularly. However, recombination-based *in vivo*-expression technology (RIVET) experiments clearly demonstrate that PhoP-regulated promoters

are active within the intestinal lumen (Merighi *et al.*, 2005). Future work includes the use of *in vivo* bioluminescence imaging to follow the expression of the *phoP* gene within the intestine of the *C. rodentium* infected mice. Similar to the effect of LPS modifications, negative charges in LPS are bridged by Mg^{2+} ions to create tight lateral packing interactions, which largely prevent permeation. So an AMP resistance mechanism that utilizes an OM protease instead of LPS modifications may be a bacterial evolutionary response to the high concentrations of Mg^{2+} in the mouse colon.

The identification of genes activated or repressed by PhoP/PhoQ in *C. rodentium*, as well as the detailed mechanism of its regulation merit further investigation since at least a subset of genes in the regulon is involved in AMP resistance, such as the OM protease CroP. Although, PhoP does not regulate the transcription of *croP* directly it seemed likely that the export to the membrane is modulated in a PhoP-dependent manner. In addition, PhoP may be regulating other, as yet unidentified, genes that modify LPS, as suggested by the EtBr permeability assay. In contrast to *S. typhimurium* PhoP/PhoQ, PhoP/PhoQ in *E. coli* is part of a regulatory network that is controlled by the EvgS/EvgA TCS through B1500, a small inner membrane protein (Eguchi *et al.*, 2007). A BLAST search indicates that no homologues of the EvgS/EvgA TCS or B1500 are present in the *C. rodentium* genome. However, this does not rule out the possibility of another TCS that senses a relevant signal within the intestinal lumen regulating the activity of PhoP/PhoQ.

C. rodentium is used as a model to study EPEC and EHEC. Disruption of the EPEC and EHEC OM by AMPs mirrors the same pattern as *C. rodentium*, which is distinct from that of *S. typhimurium* (V. Le Sage and H. Le Moual, unpublished results). Each pathogenic *E. coli* strain encodes at least one member of the omptin family (OmpT and/or OmpP), suggesting that these OM proteases play an important role in AMP resistance. Future work will determine the contribution of these omptins to pathogenesis.

This work shows that although PhoP/PhoQ in *C. rodentium* and *S. typhimurium* are homologous to each other, they are part of dramatically different regulatory networks, which is undoubtedly a reflection of their extracellular and intracellular habitats.

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APPENDICES

I. Copyright waivers

II. Certificates