Bacterial Innate Immunity: Mechanisms of PhoQ Sensing that Promote *Salmonella* Virulence

Kevin George Hicks

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2015

Reading committee:

Samuel I. Miller, Chair

Colin Manoil

Nina R. Salama

Program Authorized to Offer Degree:

Microbiology

University of Washington

Abstract

Bacterial Innate Immunity: Mechanisms of PhoQ Sensing that Promote *Salmonella* Virulence

Kevin George Hicks

Chair of the Supervisory Committee:

Samuel I. Miller

Department of Microbiology, Genome Sciences, and Medicine

Salmonella PhoQ is a histidine kinase with a periplasmic sensor domain that promotes virulence by detecting the hostile environment of the host macrophage phagosome. PhoQ kinase activity is repressed by divalent cations and induced in environments of acidic pH, limited divalent cations, and cationic antimicrobial peptides (CAMP). Upon activation, PhoQ phosphorylates the response regulator PhoP, coordinating regulation of greater than three hundred genes involved in virulence and intracellular survival. Previously, it was unclear which host signals are sensed by salmonellae to promote PhoPQ-mediated virulence. Using NMR and random activating mutagenesis, we defined conformational changes produced in the PhoQ periplasmic domain on exposure to acidic pH that indicate structural flexibility is induced in α-helices 4 and 5, suggesting this region contributes to sensing fluctuations in periplasmic pH. Therefore, we engineered a disulfide bond between the residues W104C and A128C in the PhoQ periplasmic domain that restrains conformational flexibility between the α-helices 4 and 5 and

the α/β-core of the periplasmic domain. PhoQ$^{W104C\text{-}A128C}$ is responsive to CAMP, but is inhibited for activation by acidic pH and divalent cation limitation. *Salmonella enterica* Typhimurium with the *phoQ*$^{W104C\text{-}A128C}$ allele are virulent in mice and macrophages, indicating that acidic pH and divalent cation sensing by PhoQ are dispensable for virulence.

Table of Contents

List of Abbreviations

α/β-core – The PhoQ PD PDC-fold (α2, β3, β5, β6, and β7) adjacent and in contact with α-helices 4 and 5.

AP – Acidic patch

BMMΦ – Bone marrow-derived macrophage

CAMP – Cationic antimicrobial peptide(s)

Hpi – hours post-infection

HSQC – Heteronuclear single quantum coherence

IP – Intraperitoneal

M-cells – Microfold cells

N-mm – N-minimal media

PAS – Per-Arnt-Sim

PD – Periplasmic domain

PDC – PhoQ-DcuS-CitA

PO – Peroral

SPI – *Salmonella* pathogenicity island

T_m^{app} – Apparent melting temperature

TD – Transmembrane domain

T3SS – Type III secretion system

List of Figures

List of Tables

Acknowledgments

I greatly thank those who facilitated my graduate education and progression as a scientist. First and foremost, I gratefully thank Sam for giving me the opportunity to work in his lab, excellent guidance throughout my education, and training me how to be a rigorous and critical scientist.

I thank my outstanding collaborators Rachel, for her expert advice and critical scientific evaluation, Scott, for his excellent suggestions and NMR contributions, and Kornelius and Enea for skillful protein crystallography.

I thank my thesis committee Rachel, Joseph, Nina, and Colin for insightful and rigorous guidance of my thesis. Furthermore, I thank Nina and Colin for participating as my thesis reading committee.

I thank all of my past and present Miller lab colleagues with special recognition to MP, Hemantha, Zach, Susana, and Richard. They provided expert training, thoughtful insight, excellent guidance, and fantastic comic relief during my graduate education.

I thank my amazing classmates and friends Kyle, Camille, Jaki, and Krystal. We survived the gauntlet and had a great time in the process; Salud!

Paramount, I thank my family and friends: my mom, dad, and sister for infinite love and encouragement, good friends Clarke, Leonard, Miles, Adam, and Kyle for ridiculous fun, and lovingly supportive wife Crystal, adorable daughter Genevieve, and dog Raina for endless fun, love, and laughter.

Chapter 1: Introduction

Salmonellae are a significant cause of global mortality and morbidity

Salmonellae are Gram-negative bacterial pathogens of humans and animals which cause a range of enteric diseases, including acute gastroenteritis and severe febrile illnesses (Pegues and Miller, 2014). Most human infections by nontyphoidal, broad host range serovars including *S. enterica* Typhimurium and *S.* Enteritidis, result in self-limited, uncomplicated gastroenteritis; although more severe disease outcomes occur in young, elderly, and immunocompromised individuals (Sperber and Schleupner, 1987). Nontyphoidal salmonellae are a major cause of global diarrheal disease, causing approximately 93.8 million illnesses of gastroenteritis and 155,000 deaths each year (Majowicz et al., 2010). Furthermore, nontyphoidal salmonellae infections are a significant cause of global disease burden as an estimated 4.8 million disability-adjusted life years are lost annually (Murray et al., 2012). In addition to gastroenteritis, invasive nontyphoidal salmonellae illnesses, which typically do not display with diarrhea, have emerged as a prominent cause of life-threatening bacteremia in African adults and children, with case fatality of 20 – 25% (Feasey et al., 2012). It is estimated that invasive nontyphoidal salmonellae cause 3.4 million illnesses annually (Ao et al., 2015). In contrast to nontyphoidal illnesses, enteric fever (including typhoid fever) is a severe systemic febrile illness caused by host-range restricted *S.* Typhi and *S.* Paratyphi bacteria. An estimated 21.7 million illnesses and 217,000 deaths are attributed to enteric fever caused by *S.* Typhi (Crump et al., 2004). In addition, *S.* Paratyphi causes an estimated 5.4 million illnesses of paratyphoid fever annually. With the rise of multi-drug resistant strains, salmonellae remain a major worldwide health concern, especially in developing regions.

Salmonella pathogenesis: intestinal colonization and systemic dissemination.

Following ingestion, salmonellae survive the transit through the hostile, low pH environment of the stomach using adaptive acid tolerance response systems (Alvarez-Ordonez et al., 2011; Foster and Hall, 1990). These acid tolerance systems are important for *Salmonella* pathogenesis as specific genetic disruptions result in acid sensitivity and attenuation in disease models (Garcia-del Portillo et al., 1993; Riesenberg-Wilmes et al., 1996). Upon transition to the intestine, salmonellae encounter a variety of antimicrobial factors including digestive enzymes, bile salts, secretory IgA, antimicrobial peptides and other innate immune defenses that they must evade to gain access to the intestinal epithelium (Antalis et al., 2007; Cremers et al., 2014; Hofmann and Eckmann, 2006; Michetti et al., 1992; Mukherjee and Hooper, 2015; Ouellette, 2011; Prouty et al., 2004; Prouty et al., 2002; Selsted et al., 1992; Woof and Kerr, 2006). Furthermore, salmonellae must create a niche in the competitive environment of the intestinal microbiota. To out compete the intestinal microbiota, salmonellae induce a robust immune response. Animal models have shown that salmonellae induced colitis is primarily due to components of three major virulence factors: flagellar motility and two type three secretion systems (T3SS) encoded on the *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2) (Schmitt et al., 2001; Tsolis et al., 1999). Innate immune detection of *Salmonella* pathogen-associated molecular patterns (LPS, flagellin, curli) and pathogen-induced processes (SPI T3SS effector translocation) by cytosolic and cell membrane pathogen recognition receptors promotes a robust immune response by inducing production of a variety of pro-inflammatory molecules (Thiennimitr et al., 2012; Vance et al., 2009; Winter et al., 2010a). Intestinal inflammation, a hallmark of nontyphoidal salmonellosis, results in generation of a unique terminal electron acceptor, tetrathionate, allowing *Salmonella* to anaerobically respire ethanolamine, a carbon

source not utilized by the microbiota (Thiennimitr et al., 2011; Winter et al., 2010b). Furthermore, intestinal inflammation results in host expression of the antimicrobial protein, lipocalin-2, which binds enterobactin, an essential iron-chelating siderophore of the microbiota (Raffatellu et al., 2009). By sequestering enterobactin, lipocalin-2 elicits bacteriostatic activity on microbes that rely on this siderophore (Flo et al., 2004). In addition to enterobactin, *Salmonella* has a glycosylated derivative, salmochelin (Hantke et al., 2003), which is not sequestered by lipocalin-2; thus, providing resistance to the antimicrobial properties of lipocalin-2 and a growth advantage (Fischbach et al., 2006; Raffatellu et al., 2009). In effect, salmonellae actively promote inflammation in order to shift the intestinal environment in their favor.

A critical feature for salmonellae to colonize the intestine and induce colitis is their ability to invade non-phagocytic enterocytes by stimulating endocytosis using the SPI-1 T3SS (Francis et al., 1992; Galan and Curtiss, 1989a; Hansen-Wester and Hensel, 2001). Following contact with host intestinal epithelial cells, the SPI-1 T3SS injects virulence effector proteins which orchestrate the manipulation of host-cell systems to induce actin reorganization, polymerization, and bundling, promoting bacterial internalization (Hanisch et al., 2011; Hardt et al., 1998; Hayward and Koronakis, 1999; Humphreys et al., 2012; Humphreys et al., 2013; Jolly et al., 2014; Zhou et al., 2001; Zhou et al., 1999). Following bacterial internalization, the SPI-1 effector, SptP, restores host-cell architecture (Fu and Galan, 1999). *Salmonella* invasion into enterocytes, induction of localized intestinal inflammation, and the destruction epithelial tight junctions are important causes of diarrhea during acute salmonellosis (Hapfelmeier et al., 2004; Pegues and Miller, 2014; Zhang et al., 2002). *Salmonella* cross the intestinal epithelia via translocation by $CD18^{+}$ phagocytes and M-cells (Jones et al., 1994; Vazquez-Torres et al., 1999). Interestingly, the route of translocation across the epithelium determines the immune response,

as only salmonellae invasion of M-cells results in abundant IgA secretion in feces (Martinoli et al., 2007). M-cells sample the antigenic environment of the intestinal lumen by pinocytosis and present antigens to lymphoid cells in the underlying Peyer's patches (Kucharzik et al., 2000). Once in the Peyer's patch, salmonellae are phagocytized by intestinal macrophage, migrate to mesenteric lymph nodes, and can disseminate systemically to the spleen and liver (Jones and Falkow, 1996).

Upon phagocytosis by macrophages, salmonellae are contained within a spacious phagosome (Alpuche-Aranda et al., 1995; Alpuche-Aranda et al., 1994), which over a period of minutes to hours, shrinks and acidifies to form a persistent vacuole known as the *Salmonella*-containing vacuole (SCV) (Oh et al., 1996; Rathman et al., 1996). Salmonellae modify the SCV lipid and protein content and promote distinct morphological changes by injecting effectors from the SPI-2 T3SS into the host cytosol which make the SCV a replicative niche (Haraga et al., 2008). SPI-2 is essential for intracellular bacterial survival as genetic lesions in this locus prevent *Salmonella* from successfully infecting macrophage and mice (Hensel et al., 1995; Ochman et al., 1996). The macrophage phagosome is a hostile environment, replete with antimicrobial factors such as oxygen and nitrogen radicals, proteases, metal scavengers and transporters, acidic pH, and antimicrobial peptides (Flannagan et al., 2009). *Salmonella* serotypes associated with systemic disease sense the phagosomal environment and activate various virulence mechanisms in order to survive within the macrophage, allowing dissemination throughout the host organism (Alpuche-Aranda et al., 1994; Alpuche Aranda et al., 1992; Bader et al., 2003; Chen and Groisman, 2013; Dalebroux and Miller, 2014; Guo et al., 1997; Haraga et al., 2008; LaRock et al., 2015; Miller et al., 1989; Ochman et al., 1996; Shea et al., 1996; Vazquez-Torres et al., 2000). The *Salmonella* PhoPQ two-component system is a master regulator responsible for

sensing the macrophage phagosomal environment and promoting the necessary changes within the bacterium to survive within this niche. The importance of PhoPQ for pathogenesis is evident as deletion or constitutive mutations in PhoPQ result in severe attenuation of *Salmonella* infection in macrophage and murine models (Miller et al., 1989; Miller and Mekalanos, 1990).

The PhoPQ regulon promotes intracellular survival of *Salmonella*

PhoP is the cognate response regulator in the PhoPQ two-component system and member of the OmpR/PhoB family of transcription factors (Bachhawat and Stock, 2007; Miller et al., 1989). The PhoPQ system performs important functions in a variety of proteobacteria including pathogens of animals, invertebrates, and plants, as well as nonpathogenic endosymbionts, commensals, and environmental organisms (Cheng et al., 2010; Derzelle et al., 2004; Erickson et al., 2011; Groisman et al., 1992; Johnson et al., 2001; Lee et al., 2008; Llama-Palacios et al., 2003; Macfarlane et al., 1999; Miller et al., 1989; Moss et al., 2000; Pontes et al., 2011; Rebeil et al., 2013). PhoP was first identified in *S. enterica* Typhimurium by isolation of two mutants which lost nonspecific acid phosphatase activity (Kier et al., 1979). Constitutively activating mutations in PhoP revealed it as the regulator of PhoN, a nonspecific acid phosphatase. These findings lead to the misleading gene identifier "Pho", a designation usually used to identify systems which control phosphate assimilation pathways in response to phosphate starvation, such as the *E. coli* PhoRB two-component system. Following the initial characterization, the PhoPQ two-component system was found to be an essential *Salmonella* virulence factor as null mutations in this operon highly attenuate systemic infections in mice and survival within macrophages (Galan and Curtiss, 1989b; Groisman et al., 1989; Miller et al., 1989). Furthermore, it was shown that mutations in *phoP* make *Salmonella* extremely sensitive to acidic pH and host

antimicrobial peptides suggesting a broad regulatory role in virulence (Bader et al., 2003; Bearson et al., 1998; Fields et al., 1989; Miller et al., 1990). Subsequent characterization of the *Salmonella* PhoP regulon reveals that it positively and negatively regulates greater than 300 genes, or approximately 7% of the *Salmonella* genome, by direct PhoP binding to a consensus PhoP-box DNA sequence (G/T)GTTTA(A/T) proximal to gene promoters and indirect, hierarchical regulation of other regulatory systems including sRNAs and other two-component systems (Belden and Miller, 1994; Groisman, 2001; Lejona et al., 2003; McClelland et al., 2001; Monsieurs et al., 2005; Moon and Gottesman, 2009; Prost et al., 2007b; Zwir et al., 2012; Zwir et al., 2005). Analysis of the PhoP regulon reveals that precise spatial and temporal regulation is required for *Salmonella* to properly transform from an invasive, extracellular bacteria to an intracellular pathogen. PhoP-activated genes include those involved in resistance to reactive oxygen and nitrogen species, extensive cell envelope remodeling in order to resist antimicrobial peptides, inhibition of ATP synthesis, acid tolerance response systems, cation, carbohydrate, and amino acid transport and metabolism pathways, phagosome remodeling through regulation of the SPI-2 T3SS, altering host processing and presentation of *Salmonella* antigens, and a variety of essential virulence factors with unknown functions (Belden and Miller, 1994; Bijlsma and Groisman, 2005; Bourret et al., 2009; Dalebroux et al., 2015; Dalebroux et al., 2014; Foster and Hall, 1990; Golubeva and Slauch, 2006; Guo et al., 1997; Guo et al., 1998; Lee et al., 2013; Monsieurs et al., 2005; Navarre et al., 2005; Prost et al., 2007b; Soncini et al., 1996; Wick et al., 1995; Zwir et al., 2005). PhoP-repressed genes primarily include the SPI-1 T3SS and flagella biosynthesis (Adams et al., 2001; Behlau and Miller, 1993). Intracellular salmonellae strongly downregulate SPI-1 and flagellar components as these are recognized by the host innate immune sensor NLCR4 which initiates inflammasome formation and caspase-1 activation (Broz et al.,

2012). The regulatory complexity of the PhoPQ system is evident as it is both positively and negatively autoregulatory by direct transcription induction by PhoP and posttranslational repression by the PhoP-activated gene, MgrB (Lippa and Goulian, 2009; Soncini et al., 1995). Furthermore, the PhoPQ system regulates other regulatory systems including PmrAB, RstAB, SsrB/SpiR, and SlyA (Bijlsma and Groisman, 2005; Kato and Groisman, 2004; Minagawa et al., 2003; Norte et al., 2003). Although the virulence significance of the PhoPQ system is established, a complete understanding of the *Salmonella* PhoP regulon and the changes it promotes is lacking as approximately a third of the regulon is composed of genes of unknown or predicted function (Bader, unpublished microarray data). In order for the PhoP regulon to be regulated in the appropriate context, the PhoQ sensor must signal when in the appropriate intracellular compartment: the macrophage phagosome.

Architecture of the PhoPQ two-component system

Signal transduction from the macrophage phagosome to the PhoP regulon relies on a complex cascade of protein conformational changes, phosphorelays, and direct protein-protein and protein-DNA interactions; beginning at PhoQ, where direct sensation of the phagosomal environment occurs. PhoPQ is a two-component system divided into the PhoQ sensor histidine kinase and the PhoP response regulator. PhoQ forms a dimer and is anchored in the inner membrane by two α-helical transmembrane domains (TD). The N-terminal periplasmic domain (PD) functions as an environmental sensor, capable of responding to acidic pH, CAMP, and divalent cations (Bader et al., 2005; Garcia Vescovi et al., 1996; Prost et al., 2007a). When repressed by divalent cations, PhoQ adopts a conformational state that promotes phosphatase activity and inhibits kinase activity resulting in repression of the PhoP regulon (Castelli et al.,

2000; Montagne et al., 2001). Upon sensation of acidic pH, CAMP, or divalent cation limitation the PhoQ PD undergoes conformational changes from a repressed to an activated state. The PhoQ PD activated state generates a conformational signal that is transduced through the inner membrane by the TD to the modular, cytoplasmic kinase core of PhoQ; which is well conserved among other prototypical sensor histidine kinases. The cytosolic, inner membrane proximal, HAMP domain receives the activation signal from the TD and undergoes conformational changes involving rotation and tilting of the HAMP α-helical bundle (Matamouros et al., 2015). Structural changes in the HAMP domain associated with activation promote a conformational change in the ATP-binding (CA) domain and the dimerization and histidine phosphotransfer (DHp) domain (Gao and Stock, 2009). Conformational activation of the CA and DHp domains results in autophosphorylation within the PhoQ dimer via phosphotransfer from ATP bound in the CA domain to a conserved histidine in the DHp domain. Phosphorylated PhoQ is specifically recognized by its cognate response regulator, PhoP. Direct PhoP interaction with phosphor-PhoQ results in phosphotransfer from the phospho-His residue to a conserved Asp residue on PhoP. The phosphorylated PhoP transcription factor binds a specific DNA motif upstream or within the RNA polymerase binding site of a large number of genes promoting or inhibiting transcription, respectively (Harari et al., 2009).

PhoQ is activated by acidic pH and CAMP and regulated by divalent cations

The PhoQ PD is a member of the PAS-fold and PDC-fold domain families (Cheung et al., 2008; Cheung and Hendrickson, 2010; Cho et al., 2006). Unlike other PDC-sensors, which bind small ligands in a defined binding pocket or PhoQ PD homologs found in environmental bacteria, the PhoQ PD from bacteria that primarily interact with animals has no apparent binding

pocket due to an occluding structural element: α-helices 4 and 5 (Cheung et al., 2008; Cho et al., 2006; Prost et al., 2008; Prost et al., 2007a). Acidic residues on α4 and α5 and β-strands 5 and 6 in the PhoQ PD form a structural scaffold for binding antimicrobial peptides, as well as the divalent cations Mg^{2+}, Mn^{2+}, and Ca^{2+} (Bader et al., 2005; Cho et al., 2006; Prost et al., 2008; Waldburger and Sauer, 1996). PhoQ kinase activity is repressed and phosphatase activity is dominant at millimolar or greater concentrations of divalent cations (Castelli et al., 2000; Garcia Vescovi et al., 1996; Montagne et al., 2001), presumably due to divalent cation salt-bridges formed between the PD acidic patch and inner membrane phospholipids (Cho et al., 2006). Additionally, PhoQ activity is repressed by feedback inhibition involving the small inner membrane protein, MgrB (Lippa and Goulian, 2009). Although the exact mechanism is unclear, MgrB must interact with the PD to repress PhoQ. Conversely, bacterial growth in sub-millimolar divalent cation conditions results in PhoQ activation and increased kinase activity (Garcia Vescovi et al., 1996), presumably due to disruption of salt-bridges between the PhoQ PD and inner membrane. However, the macrophage phagosome has a magnesium concentration of approximately one millimolar and a calcium concentration of approximately five hundred micromolar, suggesting PhoQ is not activated by divalent cation limitation during intracellular infection (Christensen et al., 2002; Martin-Orozco et al., 2006). At one millimolar divalent cation concentration, PhoQ can be activated by exposure to mild acidic pH or sub-inhibitory concentrations of CAMP (Bader et al., 2005; Prost et al., 2007a). PhoQ pH-activation is minimal at neutral (pH 7.5) and strongly acidic (pH 4.5) conditions and maximal at mildly acidic conditions (pH 5.5). CAMP that strongly activate PhoQ include an ancient class of antimicrobial peptides conserved from invertebrates to mammals and are characterized as relatively small, amphipathic, positively charged α-helical peptides which insert and disrupt bacterial lipid

membranes; examples include melittin from honey bees, murine CRAMP, and the human platelet factor C18G and cathelicidin LL-37 (Bader et al., 2003; Bader et al., 2005; Guani-Guerra et al., 2010; Richards et al., 2012; Shprung et al., 2012). Polymyxin B, a cyclic, amphipathic CAMP from *Bacillus polymyxa* activates PhoQ, albeit to a less extent than the aforementioned α-helical CAMPS (Bader et al., 2003). The extent human defensins activate PhoQ remains unclear (Miller et al., 1990); although, the porcine CAMP, protegrin-1, which is similar to human, cysteine-rich, β-sheet defensins moderately activates PhoQ (Bader et al., 2003). Acidic pH and CAMP are relevant host signals as the macrophage phagosome acidifies to approximately pH 5.5 and contains CAMP (Alpuche Aranda et al., 1992; Martin-Orozco et al., 2006; Rathman et al., 1996; Rosenberger et al., 2004). Furthermore, neutralization of acidified macrophage intracellular compartments with chemical inhibitors results in decreased PhoQ-mediated gene expression and attenuates intracellular salmonellae infection (Alpuche Aranda et al., 1992; Martin-Orozco et al., 2006; Rathman et al., 1996). Combined, these findings suggested a model in which acidic pH and CAMP, but not divalent cation limitation, activate PhoQ within the macrophage phagosome; however, the individual contribution of these signals to PhoQ-mediated virulence remained unknown.

Acidic pH and CAMP additively activate PhoQ suggesting that the PD has distinct sensing mechanisms for these stimuli (Prost et al., 2007a). A variety of experimental data indicate that CAMP directly compete with divalent cations for binding sites within the PhoQ PD acidic patch, leading to a model in which CAMP activates PhoQ by disrupting salt-bridges with the inner membrane (Bader et al., 2005). The mechanism by which PhoQ is activated by acidic pH is distinct from CAMP and involves perturbations to a network of residues surrounding H157 within the α/β-core of the PD (Prost et al., 2007a). Additionally, divalent cations and CAMP bind

the PhoQ PD acidic patch at pH 5.5, suggesting that protonation of the acidic patch is not the mechanism by which PhoQ is activated by acidic pH (Prost et al., 2007a). Furthermore, activating and repressing signals that are generated in the PhoQ PD must pass through the T48 D179 K186 (TDK) network, a small triad of residues on the N- and C-terminal α-helices (α1 and α6) of the PD, which is situated at the inner membrane interface between the PD and TD. The conformation of the TDK network is critical for proper PhoQ signal transduction as a variety of point-mutations mutations in this triad result in constitutive activation or repression of the system (Cho et al., 2006; Gunn et al., 1996; Sanowar et al., 2003).

In this study, we defined conformational changes that occur within the PhoQ PD on exposure to acidic pH. Characterization of the conformational changes induced by acidic pH inspired the construction of PhoQ variants which are impaired for acidic pH and divalent cation sensing, but retain their ability to respond to CAMP. Prior to this study, it was unclear which signals were important for PhoQ-mediated virulence. Utilizing these PhoQ variants, we have now established that acidic pH and divalent cation sensing are dispensable signals for PhoQ-mediated systemic virulence of *S. enterica* Typhimurium, suggesting that CAMP or other host molecules facilitate PhoQ-dependent pathogenesis. Our findings highlight the importance of *in vivo* validation of signal specificity for protein sensors, as *in vitro* or culture models of signal transduction do not necessarily reflect the true *in vivo* stimulants. Furthermore, during the course of this study, we developed tools and methodologies that may provide novel insight to the mechanisms by which PhoQ senses acidic pH, CAMP, and divalent cations, signal specificity of PhoQ in other pathogenic and nonpathogenic proteobacteria, and intracellular processes of host cells that are essential for PhoQ activation *in vivo*.

Chapter 2: Materials and Methods

Bacterial Strains and Growth Conditions

Bacterial strains, plasmids and primers used in this study can be found in Tables 1 and 2. *Salmonella enterica* Typhimurium strain 14028s was the wild type strain used in this study and all subsequent strains and mutants were derived from this strain. Unless otherwise stated, all alkaline phosphatase activity assays were performed in the CS1081 background with CS1084 as the wild type control and various alleles of *phoQ* basally expressed from pBAD24. Alkaline phosphatase activity assays were also performed on wild type (KH127) and $phoQ^{W104C\text{-}A128C}$ (KH130) recombined on to the chromosome of CS1081. Bacterial strains where grown in either LB broth or modified N-minimal media (N-mm) as indicated. Activation of the *phoN::TnphoA* reporter was utilized as previously described (Bader et al., 2005; Prost et al., 2007a). Briefly, bacterial strains were grown over night in modified N-mm pH 7.5 containing 1 mM $MgCl_2$. In the morning, cultures were washed once in the appropriate media and diluted 1:100 in to fresh modified N-minimal media containing either 10 μM, 1 mM, or 10 mM $MgCl_2$ and buffered with either 0.1 M Tris or 0.1 M MES to pH 7.5 or pH 5.5, respectively. Unless stated otherwise, the base growth media is N-minimal media pH 7.5 supplemented with 1 mM $MgCl_2$ and 100 $\mu g \cdot mL^{-1}$ ampicillin. Following dilution into fresh media, cultures were grown for 5 hours shaking at 37°C. To study *phoN::TnphoA* reporter activation in the presence of CAMP, overnight cultures were washed once in N-minimal media pH 7.5 containing 1 mM $MgCl_2$ and diluted 1:100 into the same growth media. Cultures were then grown to OD_{600} 0.2, treated with 5 $\mu g \cdot mL^{-1}$ of C18G peptide (Anaspec), and grown shaking at 37°C for 90 minutes. Following incubation, alkaline phosphatase activity was measured. Alkaline phosphatase activity assays were performed

according to standard protocol on cultures grown in duplicate and repeated on at least three independent occasions.

Genetic Techniques

All PhoQ alleles with point-mutations were generated on pBAD24-*phoQ* or pET11a-*phoQ* using the appropriate primers pairs from Table 2 and a standard site-directed mutagenesis protocol or Gibson assembly (Gibson et al., 2009). To generate $phoQ^{W104C\text{-}A128C}$ on the *S. enterica* Typhimurium 14028s chromosome, lambda red allelic exchange methods were utilized (Gerlach et al., 2007). Briefly, to engineer $phoQ^{W104C\text{-}A128C}$ on the *S. enterica* Typhimurium chromosome a tetracycline resistant cassette (*tetRA*) was amplified using primers KH45 and KH46. The resulting phoQ::*tetRA* amplicon was recombined into CS093 generating *phoQ::tetRA* (KH23). Primers KH93 and KH94 were used to amplify $phoQ^{W104C\text{-}A128C}$ from pBAD24-$phoQ^{W104C\text{-}A128C}$ (CS1382). The $phoQ^{W104C\text{-}A128C}$ amplicon was recombined into *phoQ::tetRA* (KH23). Positive clones for $phoQ^{W104C\text{-}A128C}$ recombination were identified via Bochner selection (Bochner et al., 1980). Chromosomal $phoQ^{W104C\text{-}A128C}$ was then transduced into a clean *S. enterica* Typhimurium 14028s background via P22 phage transduction. $phoQ^{W104C\text{-}A128C}$ positive clones were confirmed via DNA sequencing.

To identify residues in the PhoQ PD that when mutated result in increased *phoN::TnphoA* activity, we performed a random mutagenesis screen as previously described (Cho et al., 2006). Briefly, pBAD24-*phoQ* was randomly mutagenized using primers LP135 and LP136 to introduce one mutation per 500 bp in the *phoQ PD* using the GeneMorph II EZClone Domain Mutagenesis Kit (Stratagene). The resulting mutagenized pBAD24-*phoQ* plasmids were transformed into CS1081 and grown overnight on LB plates containing XP substrate (Sigma

104), ampicillin 100 μg·mL^{-1}, and 10 mM MgCl2. In the morning, plates were screened for blue colonies indicative of *phoN::TnphoA* alkaline phosphatase activity and PhoQ activation by divalent cation limitation. Approximately 50,000 colonies were screened. 103 blue colonies were chosen and sequenced, yielding 26 single amino acid substitutions. Mutations identified in the screen were independently engineered in a clean pBAD24-*phoQ* background using the appropriate primers pairs from Table 2 and a standard site-directed mutagenesis protocol. Phenotypes were confirmed by alkaline phosphatase activity assays in duplicate on at least three separate occasions.

Protein Expression and Purification

The PhoQ PD (strain CS1101) was purified as previously described (Bader et al., 2005). PhoQ$^{W104C\text{-}A128C}$ PD (strain KH85) was purified from SHuffle T7 Express *E. coli* (NEB). Purification and storage of PhoQ$^{W104C\text{-}A128C}$ PD was performed according to the same methods as wild type PhoQ PD. Disulfide bond formation in PhoQ$^{W104C\text{-}A128C}$ PD was confirmed by SDS-PAGE. Briefly, strain CS1101 or KH85 were grown in LB media supplemented with 100 mg·L^{-1} ampicillin for all non-labeling experiments. For ^{15}N-labeling and NMR experiments, Strain CS1101 was grown in MOPS minimal medium supplemented with 100 mg·L^{-1} ampicillin and 1 g·L^{-1} ^{15}N-ammonium chloride. Expression strains were grown to mid-log phase and IPTG was added to 0.5 mM. Cultures were induced for 4-6 hours, harvested by centrifugation and lysed using a French Pressure Cell. Inclusion bodies were isolated by centrifugation, washed once in 50 mM sodium phosphate pH 8.0 300 mM NaCl, resuspended in 20 mM sodium phosphate pH 8.0 100 mM NaCl 7 M urea, and incubated on ice for one hour. Samples were then ultracentrifuged at 50,000 rpm for 30 minutes. The supernatant was rapidly diluted into ice cold

20 mM sodium phosphate pH 8.0. Samples were filtered and purified using a 5 mL HisTrap HP nickel column (Amersham) according to standard protocol. Purified protein was then applied to a Superdex-200 gel filtration column (Amersham) equilibrated with 20 mM sodium phosphate pH 6.5 150 mM NaCl 0.1 mM EDTA. PhoQ containing fractions were pooled, concentrated to approximately 0.25 mM and stored at -80°C in 10% glycerol. $PhoQ^{W104C\text{-}A128C}$ PD was expressed by growing strain KH85 at 37°C to mid log phase in LB medium supplemented with 100 $mg \cdot L^{-1}$ ampicillin. IPTG was added to 0.5 mM and protein expression was maintained overnight at 20°C.

NMR Spectroscopy and Chemical Shift Perturbation Analysis

(^{1}H, ^{15}N)-HSQC-NMR spectra of PhoQ PD were collected as a function of pH previously (Prost et al., 2007a). Briefly, uniformly ^{15}N- and ^{13}C-labeled PhoQ PD was prepared to 1.2 mM in 20 mM sodium phosphate buffer pH 6.5, 150 mM NaCl, 20 mM $MgCl_2$, 0.1 mM EDTA, and 10% (v/v) D_2O. The pH was lowered by approximately 0.5 units at a time by addition of microliter aliquots of 500 mM DCl. HSQC spectra from the pH-titration were collected at pH 6.5, 6.0, 5.5, 4.9, 4.1, and 3.5. Standard triple-resonance experiments were collected at pH 3.5 for assignments. Assignments from pH 3.5 were translated to higher pH conditions by tracking chemical shifts through the titration series. NMR experiments were performed at 25°C on a Bruker DMX 500 MHz spectrometer equipped with a triple-resonance, triple-axis gradient probe. Data were processed and analyzed using the programs NMRPipe/NMRDraw (Delaglio et al., 1995) and NMRView (Johnson and Blevins, 1994).

To identify regions of the PhoQ PD affected by pH, (^{1}H,^{15}N)-HSQC-NMR spectra of the PhoQ PD were compared at pH 6.5 and 3.5. Resonances that experienced a chemical shift

perturbation (CSP) greater than 0.08 ppm and/or that broadened beyond detection were considered significantly affected by pH. CSPs were calculated using the formula $((\Delta^{1}H) + (\Delta^{15}N/5))^{1/2}$. Resonances that did not meet these criteria were considered unaffected. 36 residues within the PhoQ PD could not be unambiguously categorized into these groups because of missing/ambiguous assignments and/or crowding in the spectra.

Gene Expression from Bacteria Grown in Culture

Wild type (CS093), *phoQ*$^{W104C\text{-}A128C}$ (KH163), and *ΔphoQ* (CS1350) *S. enterica* Typhimurium were grown overnight in N-mm 1 mM $MgCl_2$ pH 7.5. In the morning, the cultures were normalized to OD_{600} 2.0 and diluted 1:50 into fresh N-mm 1 mM $MgCl_2$ pH 7.5 and grown at 37 °C, 250 rpm. At approximately OD_{600} 0.2, the cultures were normalized to OD_{600} 0.2·mL^{-1}, washed once, and resuspended in 1 mL of either N-mm pH 7.5 1 mM $MgCl_2$, pH 5.5 1 mM $MgCl_2$, pH 7.5 10 μM $MgCl_2$, or pH 7.5 1 mM $MgCl_2$ 5 μg·mL^{-1}. The cultures were grown shaking at 37 °C, 250 rpm. After 1 hour, the cultures were immediately pelleted at 4 °C, the media was aspirated, and placed on ice. RNA was collected using the Trizol Max Bacterial RNA Isolation Kit (ambion) and RNeasy mini kit (Qiagen). cDNA was generated using SuperScript III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen). Quantitative RT-PCR was performed using SYBR GreenER qPCR SuperMix Universal (Invitrogen) and a BioRad CFX96 thermocycler for *S. enterica* Typhimurium *rpoD, pagD, pagO, phoN,* and *phoP* target transcripts using the appropriate qRT primers found in Table 2. Relative gene expression was determined using the $2^{-\Delta\,\Delta C_T}$ method (Livak and Schmittgen, 2001). *rpoD* was used as the calibrator and gene expression was normalized to *ΔphoQ*.

Protein Crystallization, Data Collection and Structure Determination

The *S. enterica* Typhimurium PhoQ$^{W104C-A128C}$ PD structure (PDB 4UEY) was acquired by crystallizing the purified protein using a Mosquito crystallization robot (TTP Labtech) and Nextal Classic Suite, Nextal Classic Suite II, Protein complex Suite (Qiagen) and JBScreen Classic HTS II (Jena Bioscience). The progress of crystallization at 20 °C was monitored using a temperature controlled robot (Rock imager system, Formulatrix). Crystals appeared after two weeks. Optimized crystals of the PhoQ$^{W104C-A128C}$ PD were formed in 0.1 M Bis-Tris pH 6.5 200 mM Magnesium chloride 25% (w/v) PEG3350. Crystals of the PhoQ$^{W104C-A128C}$ PD were mounted in nylon loops (Hampton Research) and directly frozen in liquid nitrogen. Diffraction data of the crystals were collected at ALBA synchrotron (BL13 XALOC, Barcelona, Spain). Crystals were kept at 100 K and 200 diffraction images at one degree were recorded on a Pilatus 6M detector (Dectris, Baden). Diffraction data were processed and scaled using the XDS software package (Kabsch, 2010). Data were truncated at lower resolution according to the recently defined CC* correlation factor (Karplus and Diederichs, 2012). Molecular replacement trials were performed using the program MOLREP and the model of the *S. enterica* Typhimurium PhoQ PD from the PDB databank (PDB 1YAX) (Cho et al., 2006; Vagin and Teplyakov, 2010). The structure was refined using the PHENIX program package (Afonine et al., 2012) after rebuilding the structure in COOT (Emsley et al., 2010). Structure details and PDB entries are given in Table 3. Model quality was assessed using the Molprobity server (http://molprobity.biochem.duke.edu/).

Circular Dichroism Spectroscopy

Prior to CD data collection, purified PhoQ PD and PhoQ$^{W104C-A128C}$ PD were exchanged into 20 mM sodium phosphate buffer pH 5.5 150 mM NaCl 1 mM $MgCl_2$ using a 5 mL HiTrap desalting column (Amersham) and treated with or without an approximate 1000 molar excesses of TCEP hydrochloride (Sigma) pH 5.5 for 4 hours to reduce the disulfide bond formed between W104C and A128C. Following TECP treatment, protein samples were exchanged in to 20 mM sodium phosphate buffer pH 5.5 150 mM NaCl 1 mM $MgCl_2$, with or without 1 mM TCEP and equilibrated overnight at 4°C. Following buffer exchange and equilibration, protein samples were concentrated and prepared to 17 μM for CD analysis. Disulfide bond reduction was monitored by SDS-PAGE prior to performing CD experiments. All CD data collection was performed on an Aviv model 420 spectrometer fitted with a total fluorescence accessory module and thermoelectric cuvette holder using a 1 mm pathlength quartz cuvette. Wavelength scans were performed for each sample prior to thermal denaturation from 260 to 195 nm at 25°C, sampling every 1 nm, with a 3 second averaging time per reading. CD-monitored thermal denaturation data was collected at 212 nm, from 25°C to 95°C, in 1°C increments, with a 3 second averaging time per reading, and 30 second temperature equilibration between readings. Raw thermal denaturation data was normalized to give the fraction unfolded protein assuming a two-state denaturation process (Kamal et al., 2002). All CD experiments were reproduced on at least three separate occasions.

Mouse Infections: Single-strain and Competition

BALB/c or A/J mice were ordered from Jackson Laboratories and virulence phenotypes for strains of *S. enterica* Typhimurium were determined by competition or single-strain

inoculation. Competition experiments were performed similarly to previously described (Freeman et al., 2003). Briefly, cultures of KH111, KH112, KH113, and KH114 were grown overnight in LB media with the appropriate antibiotic and prepared by serial dilution in PBS. The inoculum for intraperitoneal (IP) competition experiments was prepared by equally mixing 2.5 x 10^5 cfu of KH111 (strain A) with 2.5 x 10^5 cfu of KH112, KH113, or KH114 (strain B) in 2 mL PBS. The inoculum for peroral (PO) competition experiments was prepared by equally mixing 5 x 10^7 cfu of KH111 (strain A) with 5 x 10^7 cfu of KH112, KH113, or KH114 (strain B) in 2 mL PBS. 6- to 8-week old female BALB/c mice were administered 0.2 mL of the mixture, for a total inoculation of 1 x 10^5 bacteria for IP infections or 5 x 10^8 bacteria for PO infections. For PO competition experiments, mice were deprived food for 5 hours prior to administering bacteria by oral gavage. The inoculum was confirmed for each experiment by plating dilutions on LB media supplemented with either 50 $\mu g \cdot mL^{-1}$ kanamycin or 100 $\mu g \cdot mL^{-1}$ ampicillin. Mice were euthanized by CO_2 asphyxiation at 48-hpi (IP) or 96-hpi (PO) and spleens were harvested and homogenized in PBS. Homogenized spleens were serial diluted and plated on LB media supplemented with either 50 $\mu g \cdot mL^{-1}$ kanamycin or 100 $\mu g \cdot mL^{-1}$ ampicillin in order to determine the $cfu \cdot mL^{-1}$ bacterial burden for each strain. The competitive index (CI) for each strain was calculated using the following formula: $CI = (\text{strain B } cfu \cdot mL^{-1 \text{ spleen}} / \text{strain A } cfu \cdot mL^{-1 \text{ spleen}}) / (\text{strain B } cfu \cdot mL^{-1 \text{ inoculum}} / \text{strain A } cfu \cdot mL^{-1 \text{ inoculum}})$.

For single-strain experiments, cultures of CS093, KH163, and CS1350 were grown overnight in LB media and prepared by serial dilution in PBS. The inoculum was confirmed for each experiment by plating dilutions on LB media. 6- to 8-week old female BALB/c or A/J mice were infected IP with approximately 1 x 10^3 cfu in 0.2 mL PBS. Mice were euthanized by CO_2 asphyxiation at 48- and 96-hpi and spleens were harvested and homogenized in PBS.

Homogenized spleens were serial diluted and plated on LB media in order to determine the cfu·mL^{-1} bacterial burden for each strain. All mouse experiments were performed with IACUC approval.

In Vitro Bacterial Growth Curve

Wild type (CS093), *phoQ*$^{W104C\text{-}A128C}$ (KH163), and *ΔphoQ* (CS1350) were grown over night in N-mm pH 7.5 1 mM $MgCl_2$. The following morning, the strains were washed in the appropriate N-mm, normalized, and diluted to 0.05 OD_{600} in either N-mm pH 7.5 or pH 5.5 supplemented with 1 mM $MgCl_2$. The strains were grown in a rolling drum at 37°C. At the indicated time-points, the bacterial strains were diluted 1:10 in PBS and their OD_{600} was monitored.

Macrophage Growth Conditions and Bacterial Infections

Bone marrow was isolated from the femurs of BALB/c mice obtained from Jackson Laboratories and differentiated for 7 days in RPMI 1640 media (Gibco #22400-089) supplemented with 10% FBS and L-929 cell supernatant following standard protocols. Following differentiation, bone-marrow derived macrophage were seeded into 24-well plates and incubated overnight. Bone-marrow derived macrophage were infected in triplicate with CS093, KH163, or CS1350 *S. enterica* Typhimurium and bacterial survival determined using a standard gentamicin-protection assay. Briefly, CS093, KH163, and CS1350 were grown overnight in LB media. The following morning, bacterial cultures are washed in PBS and suspended in RPMI 1640 at the appropriate concentration. BALB/c bone-marrow derived macrophage in 24-well plates (2 x 10^5 per well) were washed with PBS and infected in triplicate with CS093, KH163, or CS1350

(M.O.I. of 10) in RPMI 160 supplemented with 10% FBS, synchronized by centrifugation at 1000 rpm for 5 min at RT, and incubated for 30 min. Following incubation, infected macrophage monolayers were washed with PBS, incubated with media supplemented with 100 μg·mL gentamicin^{-1} (Sigma) for 90 minutes and maintained at 15 μg·mL^{-1} gentamicin for the duration of the experiment. Bacterial intracellular survival was determined by lysing infected macrophage with 1% Triton X-100 in PBS at the indicated time-points and plating serial dilutions on LB media for cfu counting.

Gene Expression from Bacteria within Infected Macrophages

BALB/c bone marrow-derived macrophage were seeded into 6-well plates (1 x 10^7 per well) and infected in triplicate with CS093, KH163, or CS1350 *S. enterica* Typhimurium using a standard gentamicin-protection protocol. 30 minutes post-infection, extracellular bacteria were harvested, lysed in Max Bacterial Enhancement Reagent (Ambion) and RNA was stabilized with Trizol (Ambion). Four hours post-infection, media was aspirated, infected macrophage were solubilized in Trizol to stabilize total RNA and triplicates where pooled. Trizol samples were stored at -80°C. RNA was prepared according to the Trizol Reagent protocol, treated with TURBO DNA-free DNase (Ambion), and RNA quality was monitored using a 2200 TapeStation (Agilent Technologies). cDNA was generated using SuperScript III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen). Quantitative RT-PCR was performed using SYBR GreenER qPCR SuperMix Universal (Invitrogen) and a BioRad CFX96 thermocycler for *S. enterica* Typhimurium *rpoD, pagD, pagO, phoN,* and *phoP* target transcripts using the appropriate qRT primers found in Table 2. Relative gene expression was determined using the 2^-

$^{\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). *rpoD* cDNA generated from extracellular bacteria harvested 30 minutes post-infection was used as the calibrator.

Three-Dimensional Structure Analysis

Analysis and modeling of the three-dimensional protein structures was carried out using the PyMOL molecular viewer (Schrodinger, 2010).

Table 1. Bacterial Strains and Plasmids Used in this Study

Strain	Description	Source
CS093	14028s wild type *S. enterica* Typhimurium	ATCC
CS1081	CS093 *phoQ*::*TPOP phoN*::*TnphoA*	Bader et al. 2005
CS1083	CS1081 pBAD24	Bader et al. 2005
CS1084	CS1081 pBAD24-*phoQ*	Bader et al. 2005
CS1399	CS1081 pBAD24-$phoQ^{I88N}$	This work
CS1400	CS1081 pBAD24-$phoQ^{Y89N}$	This work
KH45	CS1081 pBAD24-$phoQ^{I102C}$	This work
KH140	CS1081 pBAD24-$phoQ^{L105D}$	This work
CS1402	CS1081 pBAD24-$phoQ^{T124N}$	This work
CS1403	CS1081 pBAD24-$phoQ^{V126E}$	This work
CS1404	CS1081 pBAD24-$phoQ^{T129I}$	This work
CS1405	CS1081 pBAD24-$phoQ^{T131P}$	This work
CS1406	CS1081 pBAD24-$phoQ^{L132P}$	This work
KH28	CS1081 pBAD24-$phoQ^{L133C}$	This work
CS1407	CS1081 pBAD24-$phoQ^{D150G}$	This work
CS1408	CS1081 pBAD24-$phoQ^{A153P}$	This work
CS1409	CS1081 pBAD24-$phoQ^{M155V}$	This work
CS1410	CS1081 pBAD24-$phoQ^{V178D}$	This work
CS1374	CS1081 pBAD24-$phoQ^{W104C}$	This work
CS1386	CS1081 pBAD24-$phoQ^{A128C}$	This work
CS1382	CS1081 pBAD24-$phoQ^{W104C\text{-}A128C}$	This work
KH48	CS1081 pBAD24-$phoQ^{W104S}$	This work
KH49	CS1081 pBAD24-$phoQ^{A128S}$	This work
KH50	CS1081 pBAD24-$phoQ^{W104S\ A128S}$	This work
CS1101	BL21 pET11a-*phoQ* 45-190-$(His)_6$	Bader et al. 2005
KH85	NEB SHuffle T7 express pET11a-$phoQ^{W104C\text{-}A128C}$ 45-190-$(His)_6$	This work
KH23	*phoQ::tetRA*	This work
KH163	$phoQ^{W104C\text{-}A128C}$	This work

CS1350	*ΔphoQ*	Prost et al. 2008
KH127	*phoQ phoN105::TnphoA*	This work
KH130	$phoQ^{W104C\text{-}A128C}$ *phoN105::TnphoA*	This work
KH111	CS093 $pWSK129^{Kan}$	This work
KH112	CS093 $pWSK29^{Amp}$	This work
KH113	$phoQ^{W104C\text{-}A129C}$ $pWSK29^{Amp}$	This work
KH114	*ΔphoQ* $pWSK29^{Amp}$	This work

Table 2. Primers Used in this Study

tttttPrimer # (name)	Sequence (5' – 3')
LP135 (RM_Fwd)	CTGGTCGGCTATAGCGTAAGTTTTG
LP136 (RM_Rev)	CACGTATACGAACCAGCTCCACAC
LP178 (I88N_Fwd)	CGACCATGACGCTGAATTACGATGAAACGG
LP179 (I88N_Rev)	CCGTTTCATCGTAATTCAGCGTCATGGTCG
LP180 (Y89N_Fwd)	CCATGACGCTGATTAACGATGAAACGGGC
LP181 (Y89N_Rev)	GCCCGTTTCATCGTTAATCAGCGTCATGG
KH81 (I102C_Fwd)	GACGCAGCGCAACTGTCCCTGGCTGATTAAAAG
KH82 (I102C_Rev)	CTTTTAATCAGCCAGGGACAGTTGCGCTGCGTC
LP184 (T124N_Fwd)	CTTCCATGAAATTGAAAACAACGTAGACGCCACC
LP185 (T124N_Rev)	GGTGGCGTCTACGTTGTTTTCAATTTCATGGAAG
LP186 (V126E_Fwd)	GAAATTGAAACCAACGAAGACGCCACCAGCAC
LP187 (V126E_Rev)	GTGCTGGTGGCGTCTTCGTTGGTTTCAATTTC
LP188 (T129I_Fwd)	CAACGTAGACGCCATCAGCACGCTGTTG
LP189 (T129I_Rev)	CAACAGCGTGCTGATGGCGTCTACGTTG
KH192 (L105D_Fwd)	GCGCAACATTCCCTGGGATATTAAAAGCATTCAAC
KH193 (L105D_Rev	GTTGAATGCTTTTAATATCCCAGGGAATGTTGCGC
LP190 (L131P_Fwd)	CAACGTAGACGCCACCAGCCCACTGTTGAGCGAAGAC CATTC
LP191 (L131P_Rev)	GAATGGTCTTCGCTCAACAGTGGGCTGGTGGCGTCTA CGTTG
LP192 (L132P_Fwd)	GACGCCACCAGCACGCCATTGAGCGAAGACCATTC
LP193 (L132P_Rev)	GAATGGTCTTCGCTCAATGGCGTGCTGGTGGCGTC
KH85 (L133C_Fwd)	CACCAGCACGCTGTGTAGCGAAGACCATTC
KH86 (L133C_Rev)	GAATGGTCTTCGCTACACAGCGTGCTGGTG
LP194 (D150G_Fwd)	GTACGTGAAGATGGCGATGATGCCGAG
LP195 (D150G_Rev)	CTCGGCATCATCGCCATCTTCACGTAC
LP196 (A153P_Fwd)	GAAGATGACGATGATCCCGAGATGACCCAC
LP197 (A153_Rev)	GTGGGTCATCTCGGGATCATCGTCATCTTC
LP198 (M155V_Fwd)	GACGATGATGCCGAGGTAACCCACTCGGTAGC

LP199 (M155V_Rev)	GCTACCGAGTGGGTTACCTCGGCATCATCGTC
LP200 (V178D_Fwd)	CCATCGTGGTGGACGATACCATTCCG
LP201 (V178D_Rev)	CGGAATGGTATCGTCCACCACGATGG
LP141 (W104C_Fwd)	GCGCAACATTCCCTGCCTGATTAAAAGCATTC
LP142 (W104C_Rev)	GAATGCTTTTAATCAGGCAGGGAATGTTGCGC
LP145 (A128C_Fwd)	GAAACCAACGTAGACTGCACCAGCACGCTGTTG
LP146 (A128C_Rev)	CAACAGCGTGCTGGTGCAGTCTACGTTGGTTTC
KH61 (W104S_Fwd)	CAGCGCAACATTCCCAGCCTGATTAAAAGCATTC
KH62 (W104S_Rev)	GAATGCTTTTAATCAGGCTGGGAATGTTGCGCTG
KH63 (A128S_Fwd)	GAAACCAACGTAGACAGCACCAGCACGCTGTTG
KH64 (A128S_Rev)	CAACAGCGTGCTGGTGCTGTCTACGTTGGTTTC
LP164 (T48C_Fwd)	GTAAGTTTTGATAAAACCTGCTTTCGTTTGCTGCGCG
LP165 (T48C_Rev)	CGCGCAGCAAACGAAAGCAGGTTTTATCAAAACTTAC
LP168 (K186C_Fwd)	CCATTCCGATAGAACTATGCCGCTCCTATATGGTGTG
LP169 (K186C_Rev)	CACACCATATAGGAGCGGCATAGTTCTATCGGAATGG
KH35 (T48S_Fwd)	GTTTTGATAAAACCAGCTTTCGGCTGCG
KH36 (T48S_Rev)	CGCAGCAAACGAAAGCTGGTTTTATCAAAA
KH39 (K186S_Fwd)	CATTCCGATAGAACTAAGTCGCTCCTATATGGTG
KH40 (K186S_Rev)	CACCATATAGGAGCGACTTAGTTCTATCGGAATG
KH45 (PhoQ_tetRA_knock-in_Fwd)	GAATAAATTTGCTCGCCATTTTCTGCCGCTGTCGCTGC GGTTAAGACCCACTTTCACA
KH46 (PhoQ_tetRA_knock-in_Rev)	CCTCTTTCTGTGTGGGATGCTGTCGGCCAAAAACGACC TCCTAAGCACTTGTCTCCTG
KH93 (ST-PhoQ_N-term_Fwd)	ATGAATAAATTTGCTCGCCATTTTC
KH94 (ST-PhoQ_N-term_Rev)	TTATTCCTCTTTCTGTGTGGG
KH265 (ST-rpoD_Fwd_qRT)	GGGATCAACCAGGTTCAATG

KH266 (ST-rpoD_Rev_qRT)	GGACAAACGAGCCTCTTCAG
KH269 (ST-pagD_Fwd_qRT)	GTTCAGGCCATTGTTCTGGT
KH270 (ST-pagD_Rev_qRT)	TAATCTGCCTGGCTTGCTTT
KH273 (ST-pagO_Fwd_qRT)	CGGGCTTAACTATCGCAATC
KH274 (ST-pagO_Rev_qRT)	CAGCAGAAATAAGCGCAGTG
KH275 (ST-phoP_Fwd_qRT)	TGCCAGGGAAGCTGATTACT
KH276 (ST-phoP_Rev_qRT)	CAGCGGCGTATTAAGGAAAG
KH277 (ST-phoN_Fwd_qRT)	CCGGCTTACCGCTATGATAA
KH278 (ST-phoN_Rev_qRT)	CGCTTACATCTGCATCCTCA

Chapter 3: Conformational Dynamics in the PhoQ PD Associated with Activation and Repression

Activating mutations in the PhoQ PD localize proximal to α-helices 4 and 5

The structural mechanisms by which salmonellae PhoQ senses external stimuli are not well characterized. To define important residues and structural components involved in activation and repression, the *S. enterica* Typhimurium PhoQ PD was targeted for random mutagenesis and a genetic screen for derepressing mutations was performed similar to Cho *et al* utilizing the PhoQ-dependent *phoN::TnphoA* reporter (Cho et al., 2006; Miller et al., 1989). The screen identified eleven mutated residues in the PhoQ PD (I88N, Y89N, T124N, V126E, T129I, T131P, L132P, D150G, A153P, M155V, V178D), in addition to three point-mutations (I102C, L105D, L133C) introduced by site-directed mutagenesis predicted to disrupt interactions between α-helices 4, 5, and the α/β-core, that resulted in elevated levels of PhoQ-dependent *phoN::TnphoA* activity relative to wild type when grown in the presence of repressing concentrations of divalent cations (Figure 1A). The activating mutations identified in this study are located on α-helices 2, 4, and 5 and β-strands 3, 5, 6, and 7 (Figure 1B and C). A majority of the activating mutations are buried in the PD structure, with only four of the mutated residues having side-chains with solvent accessibility (T124, T131, D150, and A153). With the exception of I88 and Y89 (on β-strand 3), all the mutated residues are in proximity to one another (within 5 Å), forming a network of contacts through the core of the domain that connects helices α4 and α5 to the α/β-core (Figure 1D). Overall, the mutations identified in this screen and by site-directed mutagenesis suggest that the structural relationship between α4 and α5 and the rest of the PD plays a critical role in the PhoQ activation state.

Residues in the PhoQ PD are dynamic during pH-titration

PhoQ is activated in acidic conditions *in vitro* and within the acidified environment of the *Salmonella*-containing vacuole (SCV) after phagocytosis (Alpuche Aranda et al., 1992; Martin-Orozco et al., 2006; Prost et al., 2007a). However, the mechanism by which the PhoQ PD senses acidic pH is not well characterized. Previously, we reported that the (^{1}H, ^{15}N)-HSQC-NMR spectrum of the *S. enterica* Typhimurium PhoQ PD is highly sensitive to changes in pH (Prost et al., 2007a). Therefore, to further understand PhoQ dynamics during activation by acidic pH we collected a series of (^{1}H, ^{15}N)-HSQC-NMR spectra of the PhoQ PD as a function of pH (Figure 2A). To extract residue information from the spectra, resonance assignments were determined for the PhoQ PD at pH 3.5, the condition that yielded the greatest number of observable resonances. Of the one hundred and thirty eight residues that can yield HSQC signals, one hundred and twenty resonances could be assigned in the spectrum at pH 3.5 (Figure 2B). The remarkably well dispersed spectrum indicates that the PD remains stably folded, even at pH of 3.5.

In the absence of other pH-dependent processes, resonances that arise from residues that undergo a protonation/deprotonation event will shift in a continuous manner. Such processes will appear in the so-called "fast-exchange" NMR regime due to the rapid on/off rate of protons. Many resonances in the PD spectra exhibited pH-dependent fast-exchange behavior, consistent with ionization of the many histidine and acidic residues. In addition, some resonances broadened and disappeared from the spectrum as a function of pH. This behavior corresponds to intermediate-to-slow exchange and is indicative of a conformational change or the existence of multiple states that interconvert slowly. Thus, pH-dependent changes in the PhoQ PD HSQC spectra reveal regions of the domain that experience changes in functional group ionization and conformational dynamics.

pH-responsive residues in the PhoQ PD localize proximal to the interface between α-helices 4 and 5 and the α/β-core

Spectra collected at pH 3.5 and pH 6.5 were compared to identify regions in the PhoQ PD that are sensitive to changes in pH (Figure 3A). Resonances that experienced significant pH-dependent chemical shift perturbations (CSPs > 0.08 ppm) or broadened beyond detection, localize to regions of the protein that contain ionizable functional groups and/or experience conformational dynamics; thereby defining pH-responsive regions in the domain. Of the one hundred and twenty assigned residues in the PhoQ PD, resonances from forty two residues were affected by transition from pH 6.5 to 3.5 (Figure 3B). Due to resonance overlap and broadening, it is difficult to partition the two spectroscopic effects throughout the comparison. Approximately twenty affected resonances broadened beyond detection at pH 6.5, consistent with pH-dependent conformational dynamics in the PhoQ PD. Furthermore, 66 resonances were relatively unaffected, indicating that the PhoQ PD has pH-insensitive regions.

Assignments for the HSQC spectrum allowed us to identify residues in the PhoQ PD structure that experience pH-dependent changes (Figure 3C). A majority of residues affected by pH localize to α1, α2, α4, and α5 and proximal regions, including β5, β6, and β7. pH-sensitive regions identified in the NMR experiments overlap or are proximal to many of the mutations identified in our screen for activating mutations in the PhoQ PD (Figure 3B, asterisks). Similar to the activating mutations, a majority of the pH-sensitive residues form an interconnected network which spans α4 and α5 and the α/β-core (Figure 3D). These data suggested that PhoQ PD residues and structural features important for activation and repression undergo conformational change during pH titration. A majority of the residues that were unaffected by changes in pH mapped distally to α4 and α5, providing support for the hypothesis that the detection and

response to pH is contained within localized structural elements of the PD. Altogether, these observations are consistent with a model where fluctuations in pH promote local conformational dynamics between α4 and α5 and the α/β-core as part of the pH sensing mechanism.

Figure 1. Residues involved in PhoQ activation and repression form a buried network connecting α4 and α5 to the α/β-core. (A) Mutations identified by random and site-directed mutagenesis confer increased PhoQ-dependent *phoN::TnphoA* alkaline phosphatase activity when grown in N-mm supplemented with 10 mM $MgCl_2$. The data shown is a representative from at least three independent experiments performed in duplicate and represented as the mean ± SD. **(B)** Activating mutations from panel A (magenta) mapped onto the *S. enterica* Typhimurium PhoQ PD primary and secondary structure (residues 45 – 188). **(C)** The locations of activating mutations from panel A (magenta sticks) mapped onto the PhoQ PD crystal structure (PDB 1YAX). Secondary structural features with activating mutations are labeled with yellow circles (NT, N-termini; CT, C-termini). **(D)** Continuous surface representation (1.4 Å probe) of activating mutations from panel C mapped onto the PhoQ PD crystal structure.

Figure 1

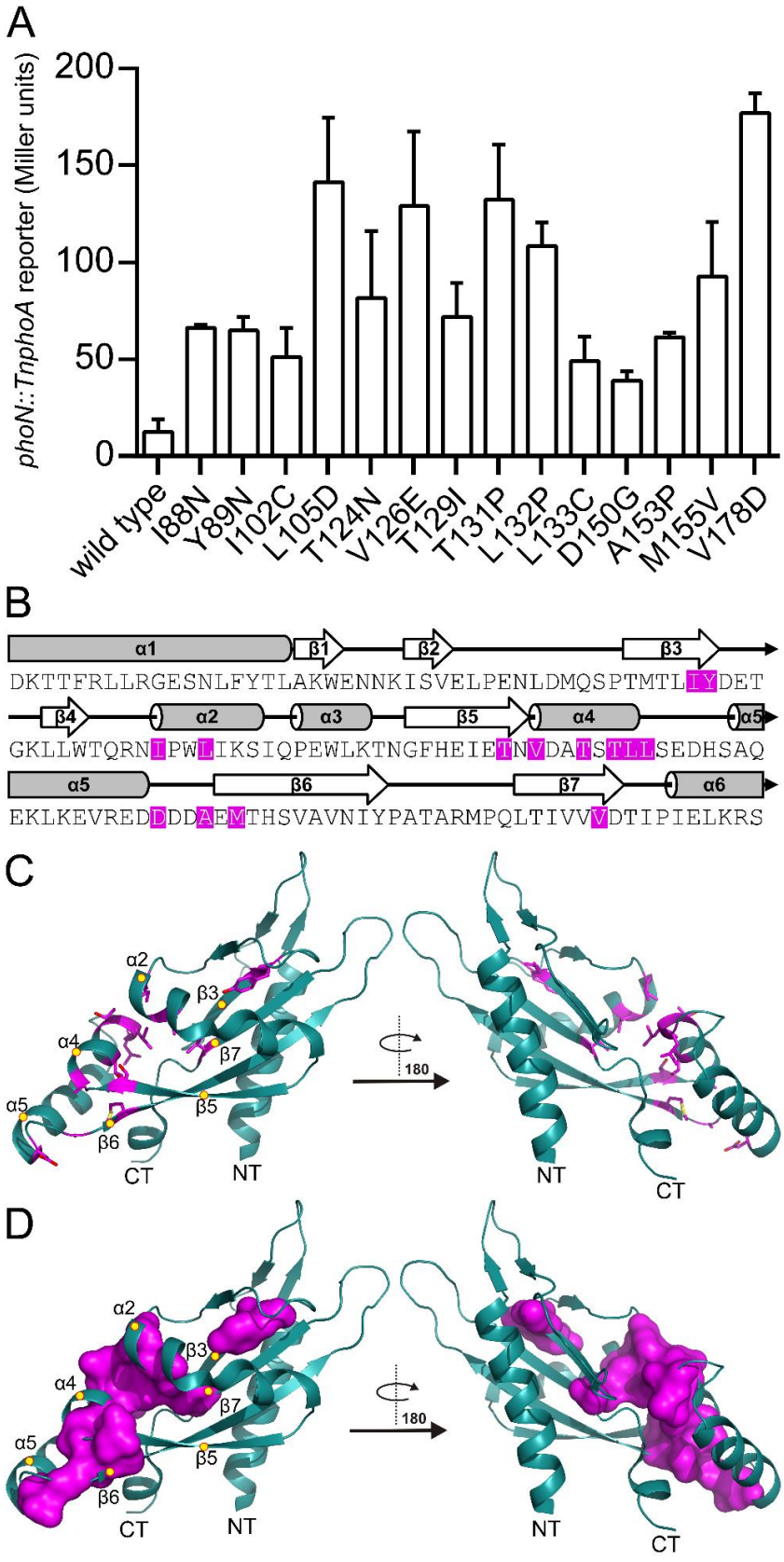

A
phoN::TnphoA reporter (Miller units)
200
150
100
50
0
wild type
I88N
Y89N
I102C
L105D
T124N
V126E
T129I
T131P
L132P
L133C
D150G
A153P
M155V
V178D
B
α1
β1
β2
β3
DKTTFRLLRGESNLFYTLAKWENNKISVELPENLDMQSPTMTLIYDET
β4
α2
α3
β5
α4
α5
GKLLWTQRNIPWLIKSIQPEWLKTNGFHEIETNVDATSTLLSEDHSAQ
α5
β6
β7
α6
EKLKEVREDDDDAEMTHSVAVNIYPATARMPQLTIVVVDTIPIELKRS
C
α2
β3
α4
β7
α5
β5
β6
CT
NT
180
NT
CT
D
α2
β3
α4
β7
α5
β5
β6
CT
NT
180
NT
CT

Figure 2. The annotated PhoQ PD (^{1}H, ^{15}N)-HSQC-NMR spectrum reveals significant peak shifting and broadening during pH titration. (A) (^{1}H, ^{15}N)-HSQC-NMR spectra of neutral to acidic pH-titration of the PhoQ PD. The pH-titration is represented as a magenta (pH 6.5) to black (pH 3.5) color gradient. The pH-titration spectra includes pH 6.5, 6.0, 5.5, 4.9, 4.1, and 3.5. **(B)** The assigned (^{1}H, ^{15}N)-HSQC-NMR spectra of the *S. enterica* Typhimurium PhoQ PD at pH 3.5. Residue numbers are labeled proximal to their corresponding peak.

Figure 2

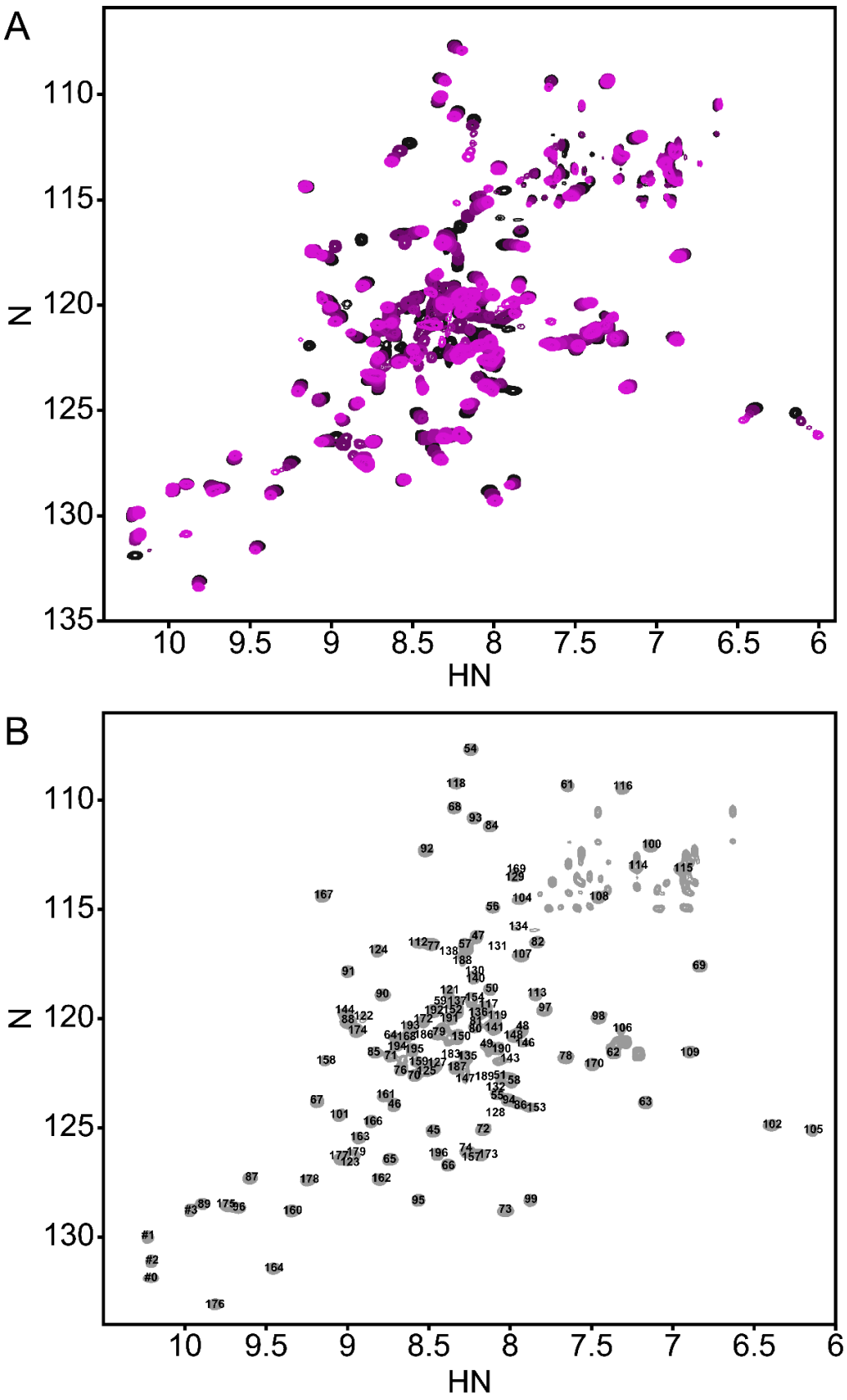
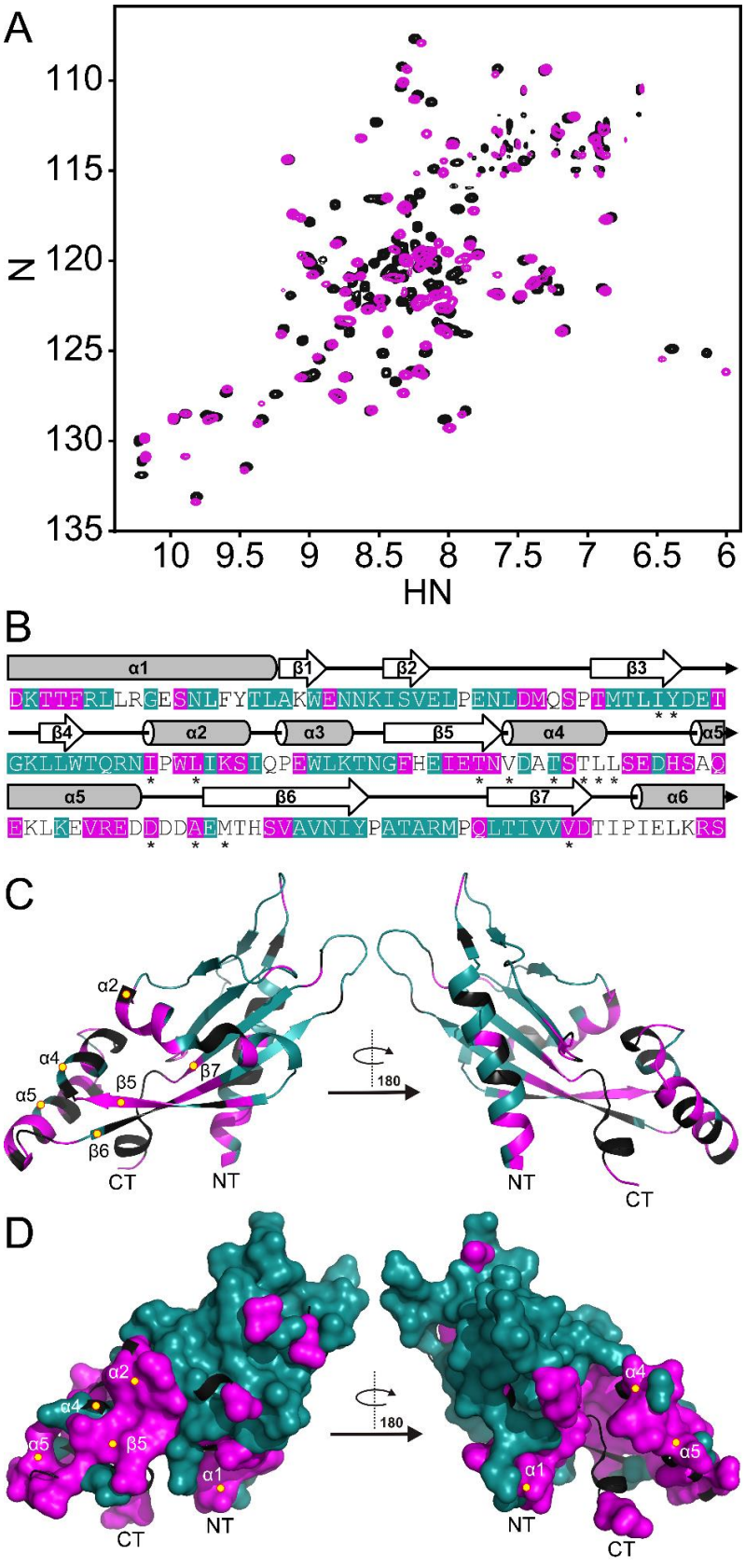


Figure 3. The PhoQ PD experiences significant pH-dependent perturbations which map to α4 and α5 and the α/β-core. (A) Comparison of (^{1}H, ^{15}N)-HSQC-NMR spectra of the PhoQ PD at pH 6.5 (magenta) and pH 3.5 (black). **(B)** Residues that experience CSPs > 0.08 ppm and/or peak broadening determined from the spectral comparison in panel A are mapped onto the *S. enterica* Typhimurium PhoQ PD (residues 45 – 188) primary and secondary structures (pH-sensitive residues, magenta; pH-insensitive residues, teal; ambiguous or non-assigned residues, no color). The locations of activating mutations from Figure 2—figure supplement 1 are indicated with asterisks. **(C)** pH-sensitive residues from panel A mapped onto the PhoQ PD structure (PDB 1YAX); pH-sensitive residues (magenta), pH-insensitive residues (teal), and ambiguous or non-assigned residues (black). pH-sensitive secondary structural features are labeled with yellow circles (NT, N-termini; CT, C-termini). **(D)** Continuous surface representation (1.4 Å probe) of pH-sensitive (magenta) and pH-insensitive (teal) residues from panel C mapped onto the PhoQ PD crystal structure.

Figure 3


A
110
115
120
125
130
135
N
10 9.5 9 8.5 8 7.5 7 6.5 6
HN
B
α1 β1 β2 β3
DKTTFRLLRGESNLFYTLAKWENNKISVELPENLDMQSPTMTLIYDET
β4 α2 α3 β5 α4 α5
GKLLWTQRNIPWLIKSIQPEWLKTNGFHEIETNVDATSTLLSEDHSAQ
α5 β6 β7 α6
EKLKEVREDDDDAEMTHSVAVNIYPATARMPQLTIVVDTIPIELKRS
C
α2
α4
β5
β7
α5
β6
CT
NT
180
NT
CT
D
α2
α4
α5
β5
α1
CT
NT
180
α4
α5
α1
NT
CT

Chapter 4: Construction and Characterization of *phoQ*$^{W104C\text{-}A128C}$

The PhoQ PD W104C-A128C disulfide bond forms in the *Salmonella* periplasm

The NMR and mutagenesis data suggested that α4 and α5 are dynamic and that the relationship between the two helices and the core of the PD likely plays a role in activation. To test this hypothesis, we engineered a PhoQ PD mutant that contains an internal disulfide bond predicted to restrict conformational dynamics between α4 and α5 and the α/β-core. Cysteine residues were introduced at positions W104 (on α2) and A128 (on α4) based on their side-chain surface exposure, relative geometries, and C^{β} distance (~6 Å) observed in the *S. enterica* Typhimurium PhoQ PD structure (PDB 1YAX). Non-reducing SDS-PAGE and western blotting of membranes harvested from *phoQ*$^{W104C\text{-}A128C}$ *S. enterica* Typhimurium revealed a faster migrating PhoQ species relative to wild type, suggesting W104C and A128C form an intramolecular disulfide bond when expressed in bacteria (Figure 4). When treated with sample buffer supplemented with β-mercaptoethanol to reduce the disulfide bond, PhoQ$^{W104C\ A128C}$ migrated similarly to the wild type protein. Membranes harvested from *phoQ*$^{W104C\text{-}A128C}$ *S. enterica* Typhimurium grown in N-minimal media (N-mm) at pH 7.5 or pH 5.5, supplemented with ten micromolar or one millimolar $MgCl_2$, or CAMP showed no observable differences in PhoQ$^{W104C\text{-}A128C}$ disulfide bond formation by SDS-PAGE, suggesting formation of the W104C-A128C disulfide bond is not dependent on growth conditions or PhoQ activation state. These data indicated that the disulfide bond formed between W104C-A128C is stably maintained within the *S. enterica* Typhimurium periplasm.

The W104C-A128C disulfide bond inhibits PhoQ activation by acidic pH and divalent cation limitation, but does not restrict activation by CAMP.

The PhoQ$^{W104C-A128C}$ disulfide mutant was designed to inhibit motion between α2 and α4, allowing us to determine whether the dynamics of α4 and α5 play a critical role in activation. When exposed to acidic pH or low divalent cation growth media, activation of the PhoQ-dependent *phoN::TnphoA* reporter in *S. enterica* Typhimurium was significantly reduced in *phoQ*$^{W104C-A128C}$ relative to wild type (Figure 5A). Additionally, the previously identified T48I activating mutation in the T48 D179 K186 (TDK) network in the PhoQ PD (Cho et al., 2006; Garcia Vescovi et al., 1996; Miller and Mekalanos, 1990; Sanowar et al., 2003) was suppressed by the W104C-A128C disulfide bond, supporting the hypothesis that α4 and α5 and the TDK network are an interconnected signaling element (Figure 5B). Interestingly, the T48I mutation potentiates CAMP activation in the *phoQ*$^{T48I\ W104C-A128C}$ background by an unknown mechanism. Importantly, CAMP still activated the *phoN::TnphoA* reporter in *phoQ*$^{W104C-A128C}$ and *phoQ*$^{T48I\ W104C-A128C}$ *S. enterica* Typhimurium at or above wild type levels, indicating that these mutant proteins are functional (Figure 5A and B). Chromosomal *phoQ*$^{W104C-A128C}$ had a similar phenotype to *phoQ*$^{W104C-A128C}$ expressed from the pBAD24 vector, indicating the phenotype is not an artifact of expression *in trans* (Figure 6A). Furthermore, the *phoQ*$^{W104C-A128C}$ phenotype does not appear to be exclusive to *phoN* as other PhoQ-regulated genes—*pagD*, *pagO* and *phoP*—are significantly reduced for induction by acidic pH and divalent cation limitation, but are induced by exposure to CAMP, similar to wild type bacteria (Figure 7). Serine substitutions at W104 and A128 did not recapitulate the phenotype observed for *phoQ*$^{W104C-A128C}$, but rather resulted in increased *phoN::TnphoA* reporter activity relative to wild type (Figure 6B). Additionally, neither single cysteine nor single serine substitutions at W104 or A128

recapitulated the *phoQ*$^{W104C\text{-}A128C}$ phenotype (Figure 6C). These results confirmed that a disulfide bond is required for the *phoQ*$^{W104C\text{-}A128C}$ phenotype. Similar to residues identified in our screen for activating mutations, the replacement of partially buried, hydrophobic residues at positions W104 and A128 with smaller, polar side-chains promoted activation. Altogether, these data confirmed that restricting conformational flexibility or movement of α4 and α5 inhibits activation by acidic pH, divalent cation limitation, and activating mutations in the TDK network. These data suggest that CAMP activates PhoQ by a mechanism that is distinct and separable from the mechanism by which acidic pH or divalent cation limitation activate PhoQ.

Figure 4. The PhoQ$^{W104C-A128C}$ disulfide bond forms in the *Salmonella* periplasm. Non-reducing SDS-PAGE and Western blotting of wild type and *phoQ$^{W104C-A128C}$* membranes with an anti-PhoQ PD antibody. Bacteria were grown in parallel in basal (pH 7.5) or activating (pH 5.5, 10 μM $MgCl_2$, CAMP) N-mm. Membranes were treated with or without sample buffer containing β-mercaptoethanol to show the effect of disulfide reduction on migration rate.

Figure 4

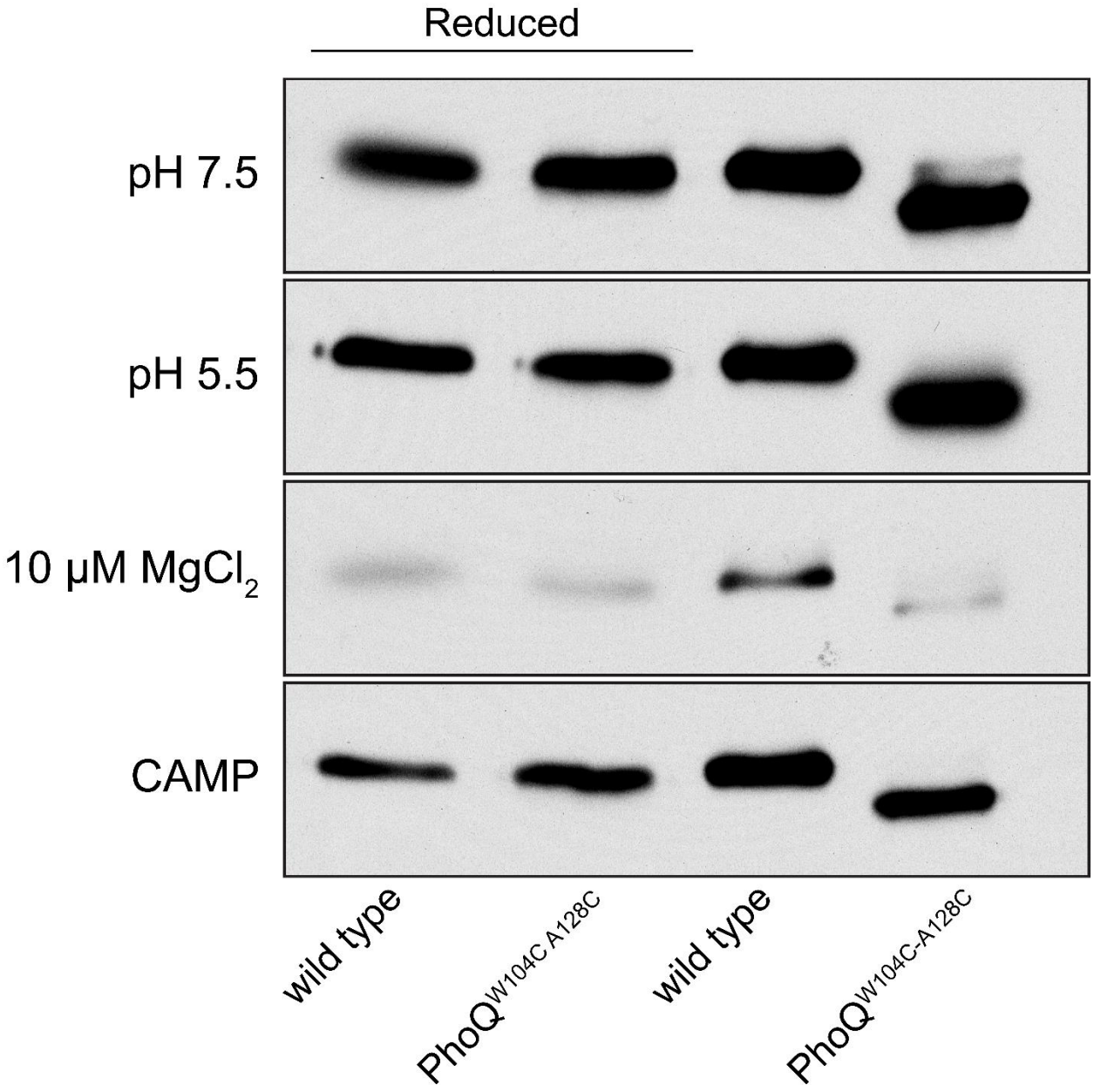

Reduced
pH 7.5
pH 5.5
10 μM MgCl2
CAMP
wild type
PhoQW104C A128C
wild type
PhoQW104C-A128C

Figure 5. A disulfide bond between α-helices 2 and 4 inhibits PhoQ activation by acidic pH and divalent cation limitation, but does not inhibit activation by CAMP. PhoQ-dependent *phoN::TnphoA* alkaline phosphatase activity of **(A)** wild type and $phoQ^{W104C\text{-}A128C}$ or **(B)** $phoQ^{T48I}$ and $phoQ^{T48I\ W104C\text{-}A128C}$ *S. enterica* Typhimurium strains grown in basal (pH 7.5) or activating (pH 5.5, 10 µM $MgCl_2$, or CAMP) N-mm. **(A and B)** The data shown are representatives from at least three independent experiments performed in duplicate and presented as the mean ± SD. Unpaired students T-test were performed between wild type and $phoQ^{W104C\text{-}A128C}$ or $phoQ^{T48I}$ and $phoQ^{T48I\ W104C\text{-}A128C}$ for all conditions; (*) $P \leq 0.05$, (NS) not significantly different.

Figure 5

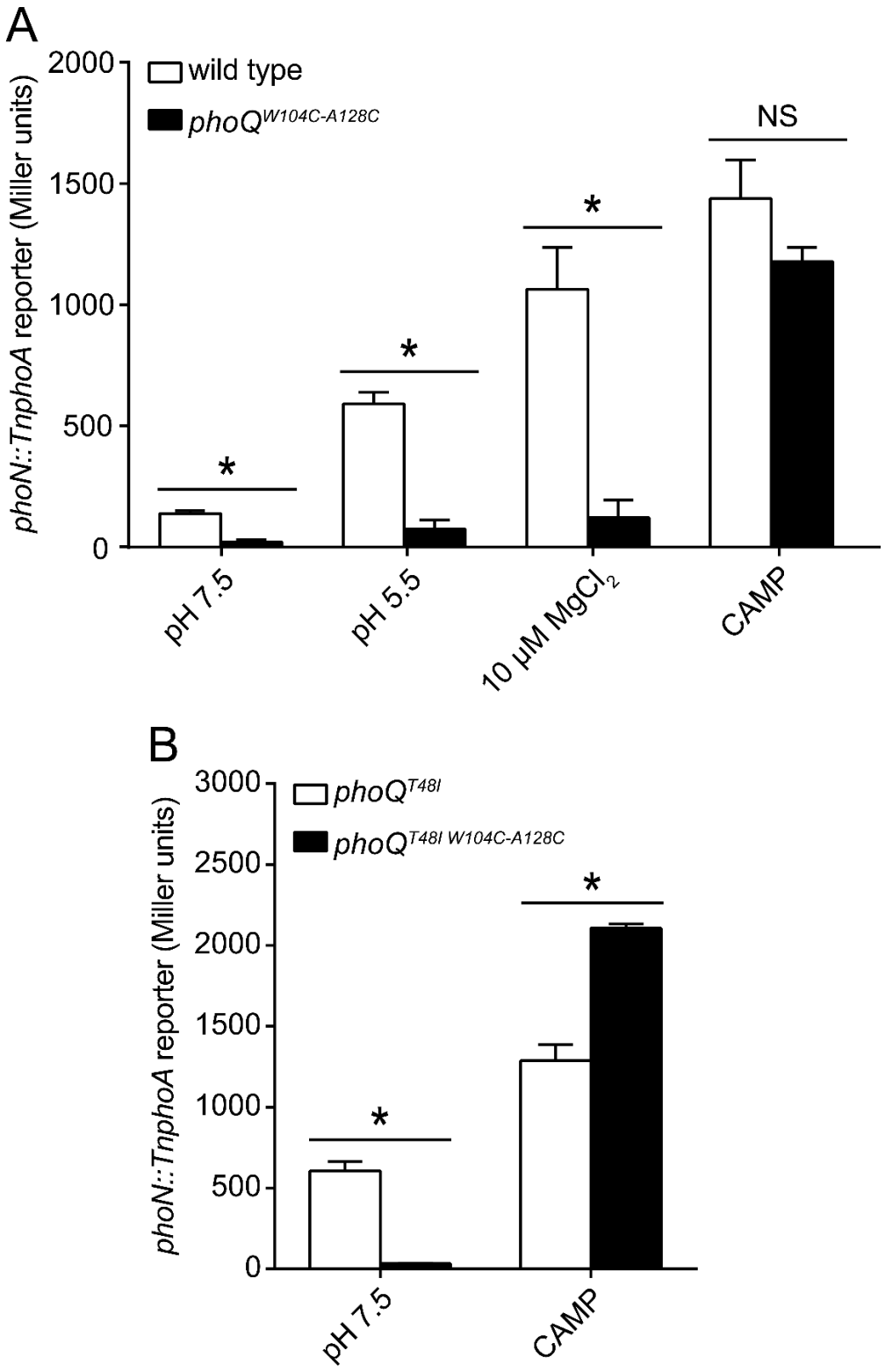

A
2000
1500
1000
500
0
wild type
phoQW104C-A128C
phoN::TnphoA reporter (Miller units)
NS
*
*
*
pH 7.5
pH 5.5
10 μM MgCl2
CAMP
B
3000
2500
2000
1500
1000
500
0
phoQT48I
phoQT48I W104C-A128C
phoN::TnphoA reporter (Miller units)
*
*
pH 7.5
CAMP

Figure 6. Mutations at W104 or A128 do not inhibit activation by acidic pH or divalent cation limitation. (A, B, and C) PhoQ-dependent *phoN::TnphoA* alkaline phosphatase activity of *S. enterica* Typhimurium strains grown in basal (pH 7.5) or activating (pH 5.5, 10 μM $MgCl_2$, CAMP) N-mm. **(A)** Wild type and $phoQ^{W104C\text{-}A128C}$ in single-copy on the *S. enterica* Typhimurium chromosome, under native regulation. **(B)** Wild type and $phoQ^{W104S\ A128S}$ *S. enterica* Typhimurium. **(A and B)** Unpaired students T-test were performed between wild type and $phoQ^{W104C\text{-}A128C}$ or $phoQ^{W104S\ A128S}$ for all conditions; (*) $P \leq 0.05$. **(C)** Single cysteine or serine mutations at position W104 and A128 in PhoQ. **(A, B, and C)** The data shown are representatives from at least three independent experiments performed in duplicate and presented as the mean ± SD.

Figure 6

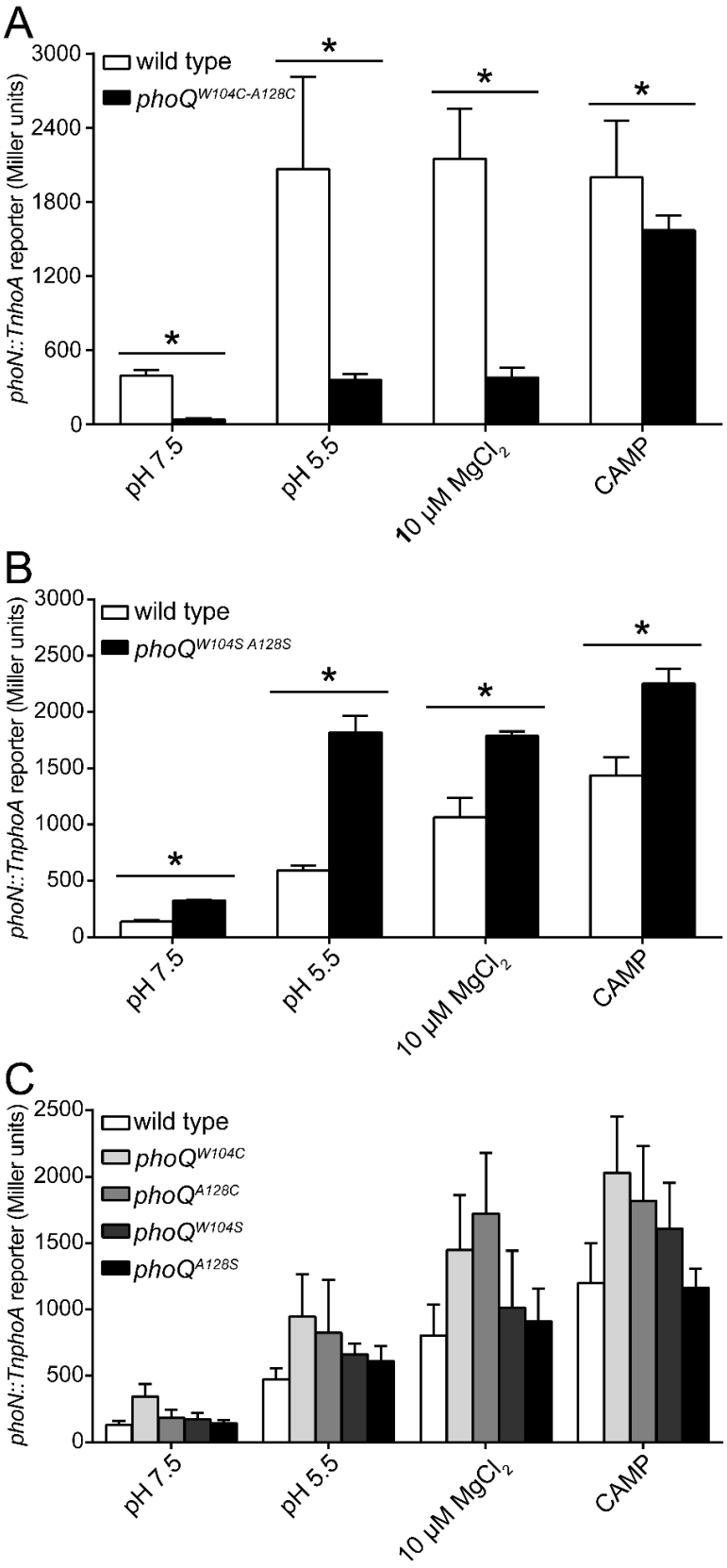
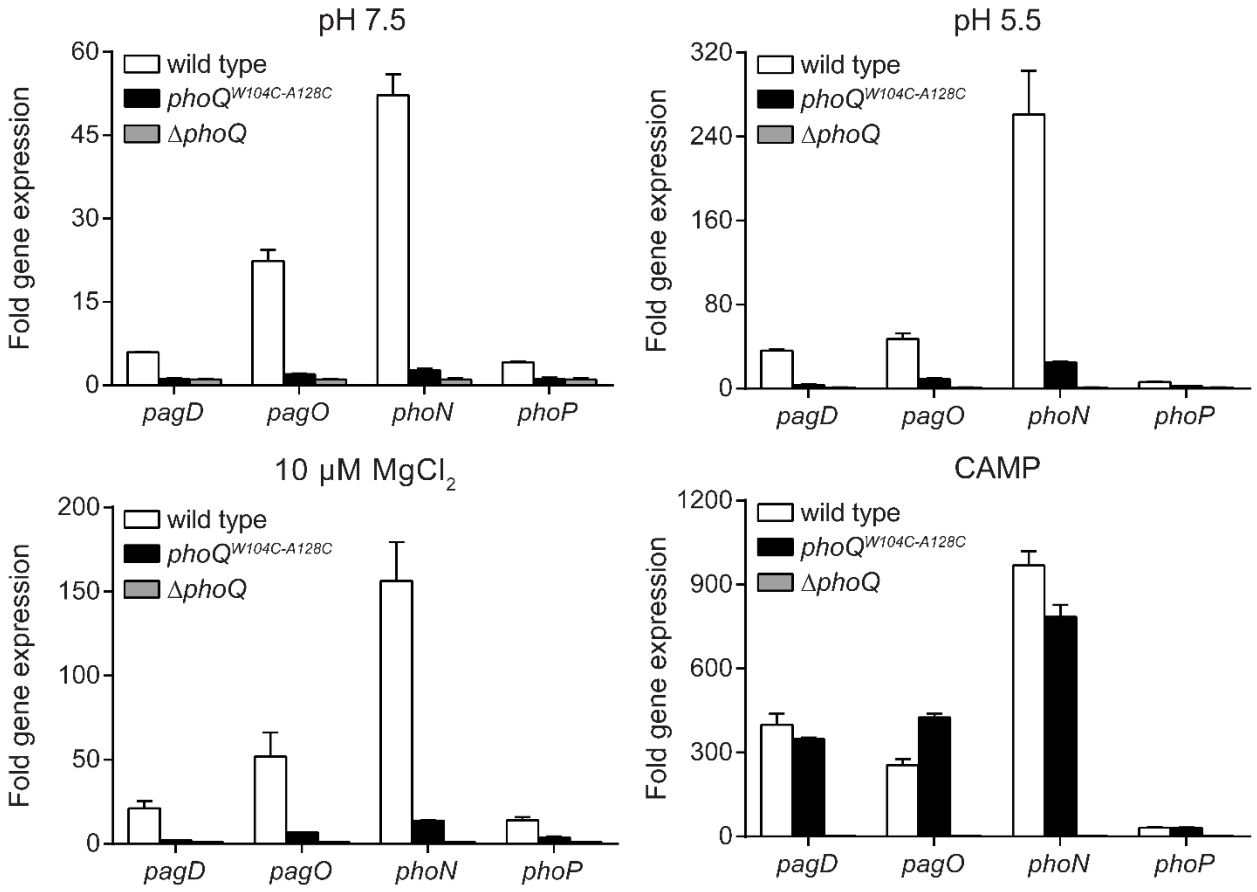

A
wild type
phoQW104C-A128C
phoN::TnphoA reporter (Miller units)
3000
2400
1800
1200
600
0
*
pH 7.5
pH 5.5
10 μM MgCl2
CAMP
B
wild type
phoQW104S A128S
phoN::TnphoA reporter (Miller units)
3000
2500
2000
1500
1000
500
0
*
pH 7.5
pH 5.5
10 μM MgCl2
CAMP
C
wild type
phoQW104C
phoQA128C
phoQW104S
phoQA128S
phoN::TnphoA reporter (Miller units)
2500
2000
1500
1000
500
0
pH 7.5
pH 5.5
10 μM MgCl2
CAMP

Figure 7. Multiple PhoQ-dependent genes in *phoQ*$^{W104C\text{-}A128C}$ salmonellae are induced by CAMP, but not by acidic pH or divalent cation limitation. PhoQ-dependent gene expression from *S. enterica* Typhimurium strains grown in basal (pH 7.5) or activating (pH 5.5, 10 μM $MgCl_2$, or CAMP) N-mm. Gene expression was normalized to *rpoD* and represented as fold-induction relative to *ΔphoQ*. The data shown is a representative from at least three independent experiments performed in duplicate and presented as the mean ± SD.

Figure 7


pH 7.5
pH 5.5
10 μM MgCl2
CAMP
wild type
phoQW104C-A128C
ΔphoQ
Fold gene expression
pagD
pagO
phoN
phoP
0
15
30
45
60
80
160
240
320
50
100
150
200
300
600
900
1200

Chapter 5: Structural and Biochemical Analysis of the PhoQ$^{W104C-A128C}$ PD

The PhoQ$^{W104C-A128C}$ PD has a similar tertiary structure to wild type

A disulfide bond spanning helices α2 and α4 inhibits activation of PhoQ$^{W104C-A128C}$ by acidic pH and divalent cation limitation. Given the remarkable phenotype of this mutant, we sought to ascertain whether the PhoQ$^{W104C-A128C}$ PD maintains a similar structure to the wild type PD. A crystal structure of the *S. enterica* Typhimurium PhoQ$^{W104C-A128C}$ PD (PDB 4UEY) was solved at 1.9 Å resolution (Table 3 and Figure 8). As predicted, the PhoQ$^{W104C-A128C}$ PD formed an intramolecular disulfide bond between W104C and A128C, covalently linking α2 and α4 (Figure 8A inset). The protomers in the PhoQ$^{W104C-A128C}$ PD structure are highly similar to each other, with an average root mean squared deviation (r.m.s.d.) of 0.3. Furthermore, the disulfide mutant structure is similar to previously solved structures of wild type *S. enterica* Typhimurium (PDB 1YAX) and *E. coli* (PDB 3BQ8) PhoQ PD, with an average r.m.s.d. value of 1.07 Å (Figure 8B). These data further demonstrate that the PhoQ$^{W104C-A128C}$ PD forms an intramolecular disulfide bond and a structure similar to the wild type PhoQ PD.

The PhoQ$^{W104C-A128C}$ PD has increased stability relative to wild type

We hypothesize that the W104C-A128C disulfide may stabilize conformational dynamics between α4 and α5 and the α/β-core, preventing acidic pH from promoting a flexible, active state. This hypothesis was tested by performing thermal melts on purified PhoQ PD and PhoQ$^{W104C-A128C}$ PD at pH 5.5 by following the CD signal of each protein as a function of temperature. We first confirmed that purified PhoQ$^{W104C-A128C}$ PD forms a disulfide as visualized as a shift in SDS-PAGE migration rate relative to PhoQ PD and TCEP-reduced PhoQ$^{W104C\ A128C}$

(Figure 9A). The CD spectra revealed that the PhoQ PD and PhoQ$^{W104C-A128C}$ PD with or without TCEP are folded and have relatively similar secondary structure at pH 5.5 and 25°C (Figure 9B). Thermal denaturation of the PhoQ PD at pH 5.5 proved to be irreversible. Therefore, we reported the apparent transition temperatures (T_m^{app}). While the wild type PhoQ PD unfolded with a T_m^{app} of 56°C in the presence and absence of TCEP (Figure 9C), the PhoQ$^{W104C-A128C}$ PD had a significantly increased T_m^{app} of 75°C. When reduced with TCEP, the PhoQ$^{W104C\ A128C}$ PD was slightly destabilized relative to the wild type, with a T_m^{app} 52°C. Therefore, the W104C-A128C disulfide increased the intrinsic stability of the PD at pH 5.5 relative to wild type. Furthermore, the observations that reduced PhoQ$^{W104C\ A128C}$ PD is less stable than wild type and that *phoQ*$^{W104S\ A128S}$ bacteria had increased PhoQ-dependent gene reporter activity relative to wild type (Figure 6B) suggests that substituting small polar side-chains at these positions in the PD results in decreased stability and increased PhoQ activity. Combined, these results suggest that the mechanism by which the W104C-A128C disulfide bond inhibits PhoQ activation by acidic pH and divalent cation limitation involves a loss of conformational flexibility between α4 and α5 and the α/β-core.

The PhoQ$^{W104C-A128C}$ PD experiences pH-dependent conformational dynamics

The W104C-A128C disulfide bond between α-helices 2 and 4 in the PhoQ PD significantly increased the intrinsic stability of the soluble domain, relative to wild type. Thus, the W104C-A128C disulfide bond likely reduces conformational changes experienced between α-helices 4 and 5 and the α/β-core of the PhoQ PD. However, increased stability does not necessarily indicate that the protein is experiencing a global reduction in conformational dynamics. To ascertain the extent of conformational perturbations and protonation-events

experienced by the PhoQ$^{W104C-A128C}$ PD, we monitored the (^{1}H, ^{15}N)-HSQC-NMR spectra of the soluble domain as a function of pH (Figure 10). The experimental setup was similar to the wild type pH-titration series and includes seven spectra from the PhoQ$^{W104C-A128C}$ PD buffered in environments of pH 6.5 down to pH 3.5. Similar to wild type, the PhoQ$^{W104C-A128C}$ PD spectra experienced significant pH-dependent fast-exchange behavior, observed as extensive peak shifting, that is indicative of protonation/deprotonation events. However, unlike wild type, the PhoQ$^{W104C-A128C}$ PD did not experience significant intermediate-to-slow exchange behavior, observed as peak broadening, which is indicative of multiple states or conformational changes. Furthermore, similar to wild type, the PhoQ$^{W104C-A128C}$ PD displayed a significant subset of peaks that were not perturbed by fluctuations in pH, suggesting that pH-dependent changes are localized. These data may indicate that the PhoQ$^{W104C-A128C}$ PD experiences extensive protonation/deprotonation events but relatively small conformational changes.

As an independent approach to monitor pH-dependent conformational dynamics, tryptophan fluorescence spectroscopy was performed on the wild type and PhoQ$^{W104C-A128C}$ PD buffered in environments ranging from pH 7.5 to 3.5 (Figure 11). The wild type and PhoQ$^{W104C-A128C}$ PD has four and three tryptophan residues, respectively, allowing protonation or conformational changes to be observed in the chemical environments surrounding these residues. Similar to wild type, the PhoQ$^{W104C-A128C}$ PD experienced tryptophan quenching as the proton concentration was increased from pH 7.5 to pH 3.5. These observations suggest that the wild type and the PhoQ$^{W104C-A128C}$ PD experience protonation to residues in proximity to these tryptophan residues or that the tryptophan residues are moving into a charged environment. Relative to wild type, the PhoQ$^{W104C-A128C}$ PD had a reduced fluorescent intensity maximum under all conditions. The reduction in fluorescent intensity in the PhoQ$^{W104C-A128C}$ PD was most

likely caused by the loss of one tryptophan due to the W104C mutation. These data support the observations from the NMR pH-titration and suggest that the PhoQ$^{W104C-A128C}$ PD experiences pH-dependent conformational dynamics primarily as protonation-events.

Table 3. Crystallographic data collection and refinement.

	PhoQ$^{W104C-A128C}$ PD
Data collection	
Space group	C2
Cell dimensions	
a, *b*, *c* (Å)	128.04, 45.37, 81.37
α,β,γ (°)	90, 102.53, 90
Resolution (Å)	31.3 – 1.9 (2.01 – 1.90)
R_{sym} or R_{merge}	0.05 (0.51)
I / σI	12.9 (1.6)
Completeness (%)	97.8 (93.8)
Redundancy	3.6 (3.2)
Refinement	
Resolution (Å)	31.3 – 1.90 (1.95 – 1.90)
No. reflections	35633
R_{work} / R_{free}	0.23/0.26 (0.38/0.44)
No. atoms (all)	
Protein	3391
Water	138
Ca^{2+}	-
B-factors	
Protein	44.8
Water	40.6
R.m.s. deviations	
Bond lengths (Å)	0.007
Bond angles (°)	1.2
Ramachandran statistics	
Residues in favored region No (%)	409 (98.3)
Residues in allowed region No (%)	7 (1.7)
Residues in outlier region No (%)	0 (0)
PDB-entry	4UEY
Crystallization conditions	0.1 M Bis-Tris pH 6.5, 200 mM $MgCl_2$, 25% Peg3350

*Values in parentheses are for highest-resolution shell.

Figure 8. The PhoQ$^{W104C-A128C}$ PD is structurally similar to wild type. (A) 1.9 Å crystal structure of the *S. enterica* Typhimurium PhoQ$^{W104C-A128C}$ PD (PDB 4UEY). The W104C-A128C disulfide bond (inset) is located between α2 and α4. Secondary structural features are annotated with yellow circles (NT, N-termini; CT, C-termini). **(B)** Structural comparison of the PhoQ$^{W104C-A128C}$ PD (blue), the wild type *S. enterica* Typhimurium PhoQ PD (teal, PDB 1YAX) and the wild type *E. coli* PhoQ PD (magenta, PDB 3BQ8).

Figure 8

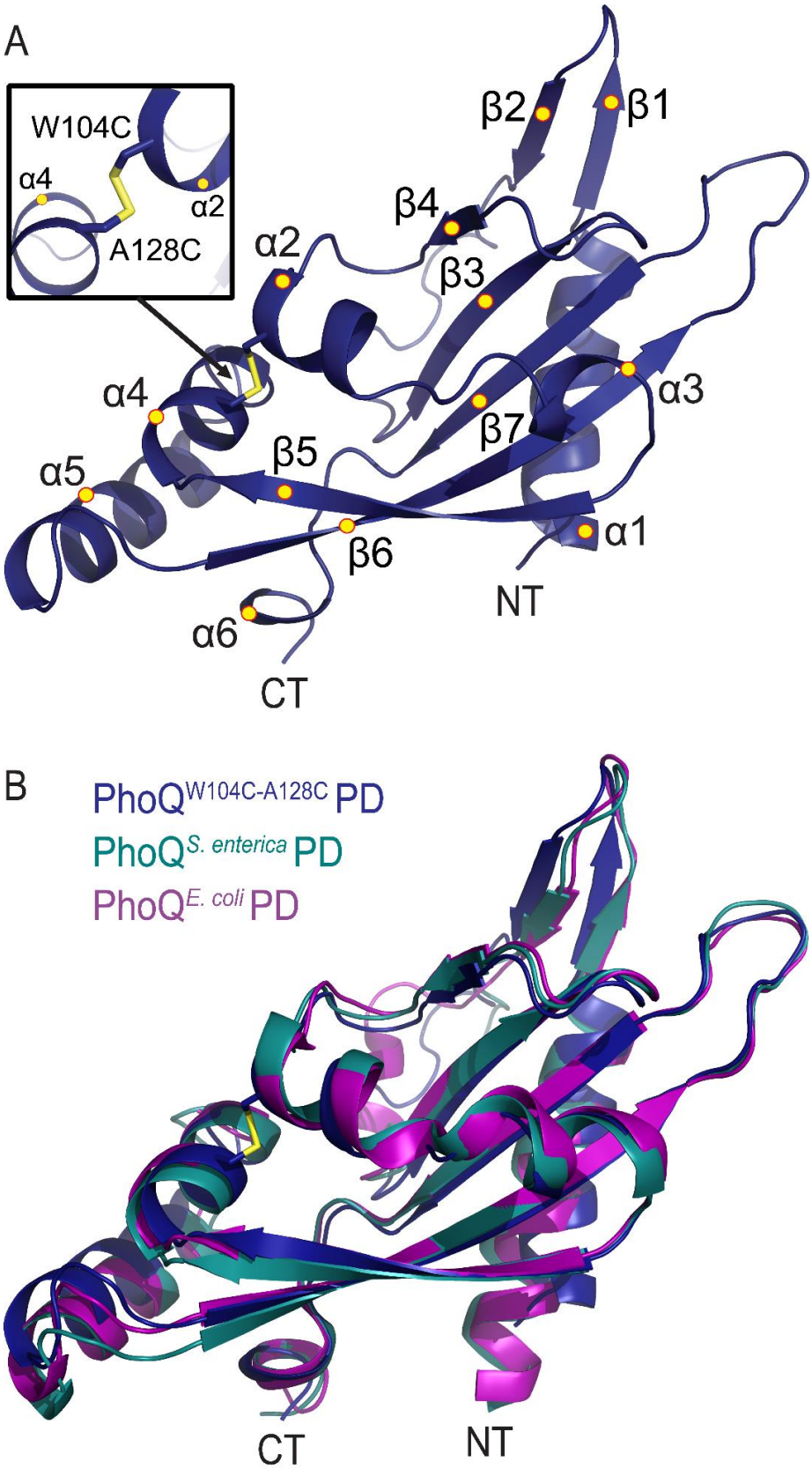

A
W104C
α4
α2
A128C
β2
β1
β4
α2
β3
α4
α3
β7
β5
α5
α1
β6
NT
α6
CT
B
PhoQW104C-A128C PD
PhoQS. enterica PD
PhoQE. coli PD
CT
NT

Figure 9. The W104C-A128C disulfide bond increases stability of the PhoQ PD. (A) Non-reducing SDS-PAGE of purified wild type *S. enterica* Typhimurium PhoQ PD and PhoQ$^{W104C-A128C}$ PD treated with or without TCEP reducing agent. **(B)** CD wavelength scan of the wild type PhoQ PD, PhoQ$^{W104C-A128C}$ PD, and PhoQ$^{W104C\ A128C}$ PD treated with TCEP reducing agent at 25°C buffered to pH 5.5. **(C)** Thermal denaturation of wild type *S. enterica* Typhimurium PhoQ PD and PhoQ$^{W104C-A128C}$ PD treated with or without TCEP monitored by CD spectroscopy at 212 nm. Raw data was normalized to give the fraction unfolded protein assuming a two-state denaturation process. A sigmoidal curve was fit to the processed data. The data shown is a representative from three independent experiments.

Figure 9

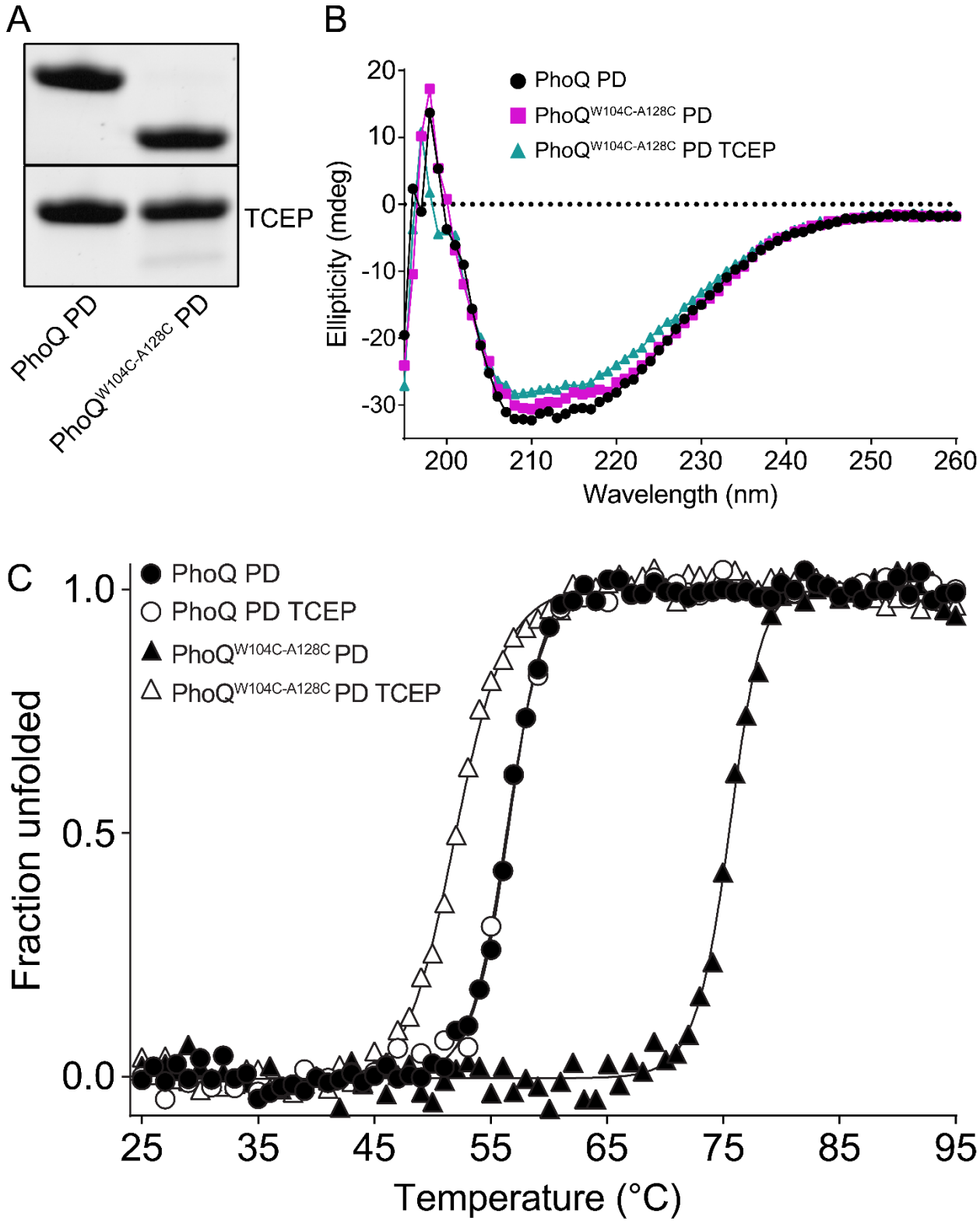

A
TCEP
PhoQ PD
PhoQ^W104C-A128C PD
B
PhoQ PD
PhoQ^W104C-A128C PD
PhoQ^W104C-A128C PD TCEP
Ellipticity (mdeg)
Wavelength (nm)
C
PhoQ PD
PhoQ PD TCEP
PhoQ^W104C-A128C PD
PhoQ^W104C-A128C PD TCEP
Fraction unfolded
Temperature (°C)

Figure 10. The (^{1}H, ^{15}N)-HSQC-NMR spectra of the PhoQ$^{W104C\text{-}A128C}$ PD experience significant pH-dependent peak shifting. (^{1}H, ^{15}N)-HSQC-NMR spectra of neutral to acidic pH-titration of the PhoQ$^{W104C\text{-}A128C}$ PD. The pH-titration is represented as a cyan (pH 6.5) to black (pH 3.5) color gradient. The pH-titration spectra includes pH 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, and 3.5.

Figure 10

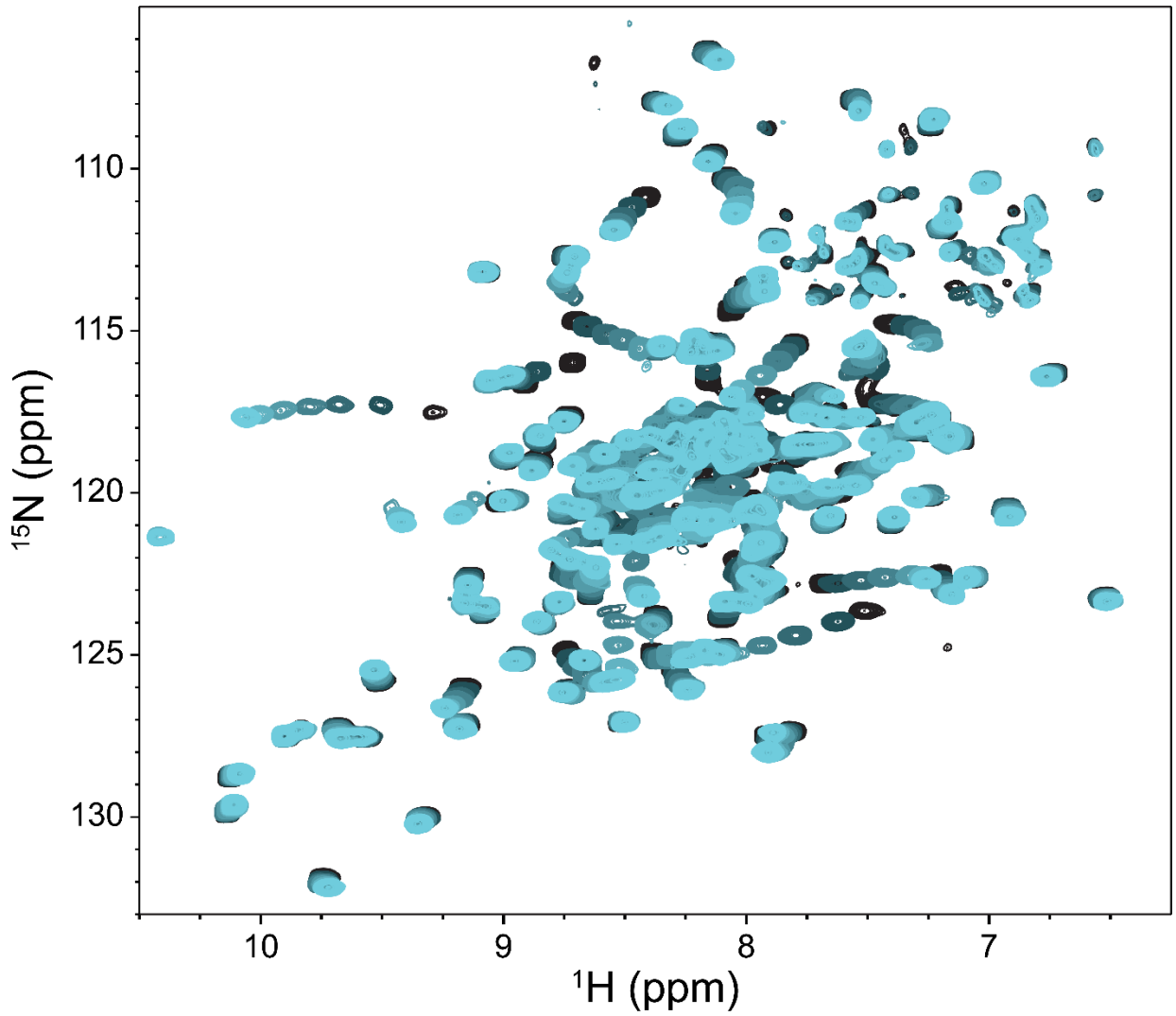
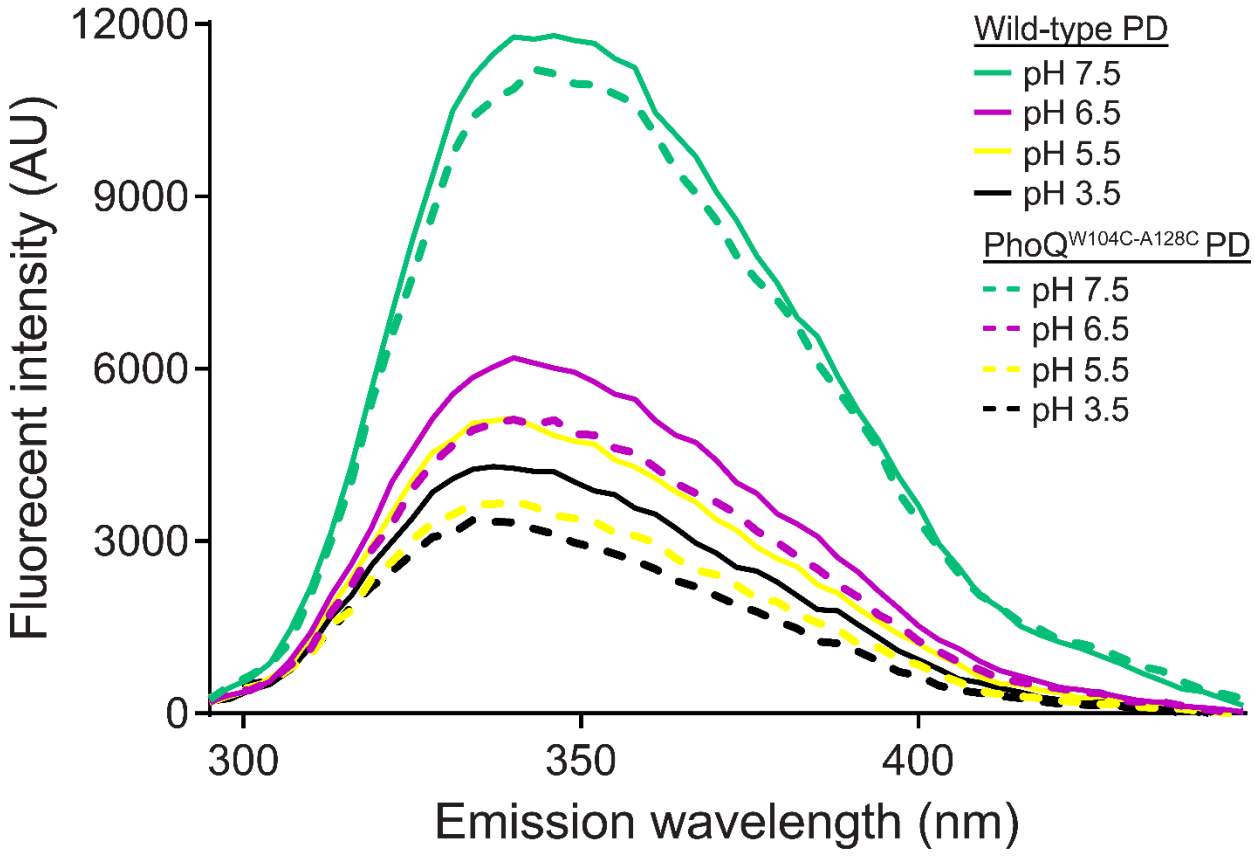


Figure 11. The wild-type and PhoQ$^{W104C-A128C}$ PD experience significant tryptophan fluorescence quenching. Purified wild-type and PhoQ$^{W104C-A128C}$ PD were equilibrated in neutral to acidic sodium phosphate buffers (pH 7.5, 6.5, 5.5, and 3.5) and fluorescent intensity was monitored as a function of wavelength (295 – 448 nm). All samples were normalized to their corresponding buffer controls. The data shown is a representative from three independent experiments performed in triplicate.

Figure 11


12000
9000
6000
3000
0
Fluorecent intensity (AU)
300
350
400
Emission wavelength (nm)
Wild-type PD
pH 7.5
pH 6.5
pH 5.5
pH 3.5
PhoQW104C-A128C PD
pH 7.5
pH 6.5
pH 5.5
pH 3.5

CHAPTER 6: Host Signals Sensed by PhoQ$^{W104C\text{-}A128C}$ Promote *Salmonella* virulence

phoQ$^{W104C\text{-}A128C}$ salmonellae are competent for survival during systemic virulence in mice.

Prior to this study, the contribution of specific stimuli to PhoQ-mediated bacterial virulence was difficult to ascertain as mutants that only respond to individual signals were not available. With the construction of *phoQ*$^{W104C\text{-}A128C}$ *S. enterica* Typhimurium, the significance of acidic pH and divalent cation sensing by PhoQ to virulence can be directly determined independently of CAMP sensing. Thus, BALB/c mice were infected by the intraperitoneal (IP) route with wild type, *phoQ*$^{W104C\text{-}A128C}$, or *phoQ* null (*ΔphoQ*) bacteria and splenic bacterial burden was determined at 48- and 96-hpi (Figure 12A). Similar to infection with wild type bacteria, mice infected with *phoQ*$^{W104C\text{-}A128C}$ bacteria had increased splenic bacterial burden relative to those infected with *ΔphoQ* bacteria. The equivalent experiment was performed in resistant A/J mice to determine if the virulence phenotype observed for mice infected with *phoQ*$^{W104C\text{-}A128C}$ bacteria was due to the susceptible BALB/c mouse genetic background and to determine whether a subtle fitness defect would be exposed on infection of a relatively resistant inbred mouse. Infecting A/J mice revealed the same relative phenotypes for wild type, *phoQ*$^{W104C\text{-}A128C}$, and *ΔphoQ* bacteria, although, as expected, bacterial burden was lower compared to infected BALB/c mice (Figure 12B). These results indicate that PhoQ sensing of acidic pH and divalent cation limitation are dispensable for systemic virulence of *S. enterica* Typhimurium in susceptible and relatively resistant inbred mice.

phoQ$^{W104C\text{-}A128C}$ salmonellae are competent for survival during intraperitoneal and oral competitions in mice

The importance of PhoQ activation by acidic pH and divalent cation limitation for systemic infection was also assessed by competing *phoQ*$^{W104C\text{-}A128C}$ mutant bacteria with wild type *S. enterica* Typhimurium in IP or peroral (PO) infections of BALB/c mice. The splenic bacterial competitive index (CI) for wild type, *phoQ*$^{W104C\text{-}A128C}$, and *ΔphoQ* bacteria was determined for both IP and PO infections at 48-hpi or 96-hpi, respectively (Figure 13 and 14). Furthermore, PO bacterial burden was quantified for the liver and mesenteric lymph nodes. Consistent with the single strain infections, *phoQ*$^{W104C\text{-}A128C}$ demonstrated no reduction in CI and, in contrast, was more competitive than wild type. *ΔphoQ* showed the expected reduction in CI. Altogether, these data indicate that PhoQ activation by acidic pH and divalent cation limitation are dispensable for *S. enterica* Typhimurium to out compete strains with these capabilities during systemic infection of susceptible mice. Furthermore, the observation that IP and PO administered *phoQ*$^{W104C\text{-}A128C}$ *S. enterica* Typhimurium have similar competitive indices suggests that acidic pH and divalent cation sensing by PhoQ are not required for survival or spread from the gastrointestinal tract to deep tissue sites.

phoQ$^{W104C\text{-}A128C}$ and *ΔphoQ* salmonellae have increased growth rates in liquid culture

S. enterica Typhimurium is growth restricted in cultured fibroblasts and nonphagocytic stromal cells in the murine lamina propria via PhoPQ-dependent processes (Cano et al., 2001; Nunez-Hernandez et al., 2013). Thus, it was plausible that the competitive advantage observed for *phoQ*$^{W104C\text{-}A128C}$ relative to wild type was due to an increased growth rate resulting from the loss of acidic pH sensing by PhoQ. When grown in N-mm pH 5.5, wild type growth rate was

decreased relative to *phoQ*$^{W104C\text{-}A128C}$ and *ΔphoQ* (Figure 15). Conversely, wild type, *phoQ*$^{W104C\text{-}A128C}$, and *ΔphoQ* grown in N-mm pH 7.5 had similar growth kinetics. These data provide evidence that acidic pH sensing by PhoQ reduces *S. enterica* Typhimurium growth rate *in vitro* and correlates with the *in vivo* competitive advantage that was observed for *phoQ*$^{W104C\text{-}A128C}$ bacteria within mice spleens.

phoQ$^{W104C\text{-}A128C}$ salmonellae are competent for survival during virulence within cultured macrophage.

The contribution of acidic pH and divalent cation limitation as signals for PhoQ-mediated bacterial intracellular survival within macrophages was evaluated by measuring *S. enterica* Typhimurium survival after infection of bone-marrow derived macrophages (BMMΦ) from BALB/c mice. BMMΦ were infected with wild type, *phoQ*$^{W104C\text{-}A128C}$, or *ΔphoQ S. enterica* Typhimurium strains and bacterial burden was determined at 2-, 4-, 8-, and 24-hpi (Figure 16). No difference in bacterial burden was observed between wild type, *phoQ*$^{W104C\text{-}A128C}$, and *ΔphoQ* at 2- or 4-hpi. At 8- and 24-hpi, bacterial burden for *ΔphoQ* was decreased relative to wild type and *phoQ*$^{W104C\text{-}A128C}$. Importantly, bacteria with the *phoQ*$^{W104C\text{-}A128C}$ allele maintained at or above wild type bacterial levels throughout infection. These data indicate that activation of PhoQ by acidic pH and divalent cation limitation are dispensable for *S. enterica* Typhimurium survival within BMMΦ from inbred mice.

PhoQ-dependent gene expression is induced in *phoQ*$^{W104C-A128C}$ *Salmonella* within cultured macrophages.

The discovery of the *phoQ*$^{W104C-A128C}$ phenotype allows for a unique opportunity to examine the contribution of acidic pH and divalent cation sensing to PhoQ-dependent gene expression during infection of macrophages. Therefore, BMMΦ from BALB/c mice were infected with wild type, *phoQ*$^{W104C-A128C}$, or *ΔphoQ S. enterica* Typhimurium. Following incubation, PhoQ-dependent gene expression was determined for intracellular *S. enterica* Typhimurium (Figure 17). Wild type bacteria experienced 41-, 29-, 36-, and 51-fold increases in gene expression for *pagD*, *pagO*, *phoN*, and *phoP*, whereas *phoQ*$^{W104C-A128C}$ experienced increases of 12-, 9-, 9- and 33-fold, respectively, relative to *ΔphoQ* bacteria. These data may indicate that wild type acidic pH or divalent cation sensing contribute an approximate 3- to 4-fold increase in PhoQ-dependent gene expression relative to *phoQ*$^{W104C-A128C}$; however, a significant amount of gene expression (≥9-fold) appeared to be independent of acidic pH or divalent cation sensing. These findings are consistent with *in vitro* results which show that acidic pH and CAMP are additive signals for PhoQ (Prost et al., 2007a). These data indicate that maximal PhoQ-dependent gene expression in macrophages requires acidic pH or divalent cation sensing. Furthermore, these findings reveal that the *phoQ*$^{W104C-A128C}$ allele promotes significant induction of PhoQ-dependent gene expression, suggesting CAMP or alternative host factors, other than acidic pH and divalent cation limitation, are a major signal for *S. enterica* Typhimurium within BMMΦ vacuoles.

Figure 12. Acidic pH and divalent cation sensing by PhoQ is dispensable for salmonellae survival within susceptible and resistant mice. Individual *S. enterica* Typhimurium strains administered IP to **(A)** BALB/c or **(B)** A/J mice. The inoculum is shown at T=0 hpi. Spleens were harvested and bacterial burden quantified. The data shown are representatives from at least three independent experiments performed in quintuplet and presented as the mean ± SD. Unpaired students T-test were performed between all strains (bar) for each time-point or gene. Symbols for significant difference; (¤) wild type and $phoQ^{W104C\text{-}A128C}$ are not significantly different from each other ($P \geq 0.05$), but are significantly different from *ΔphoQ* ($P \leq 0.05$), (*) all strains are significantly different from each other ($P \leq 0.05$)

Figure 12

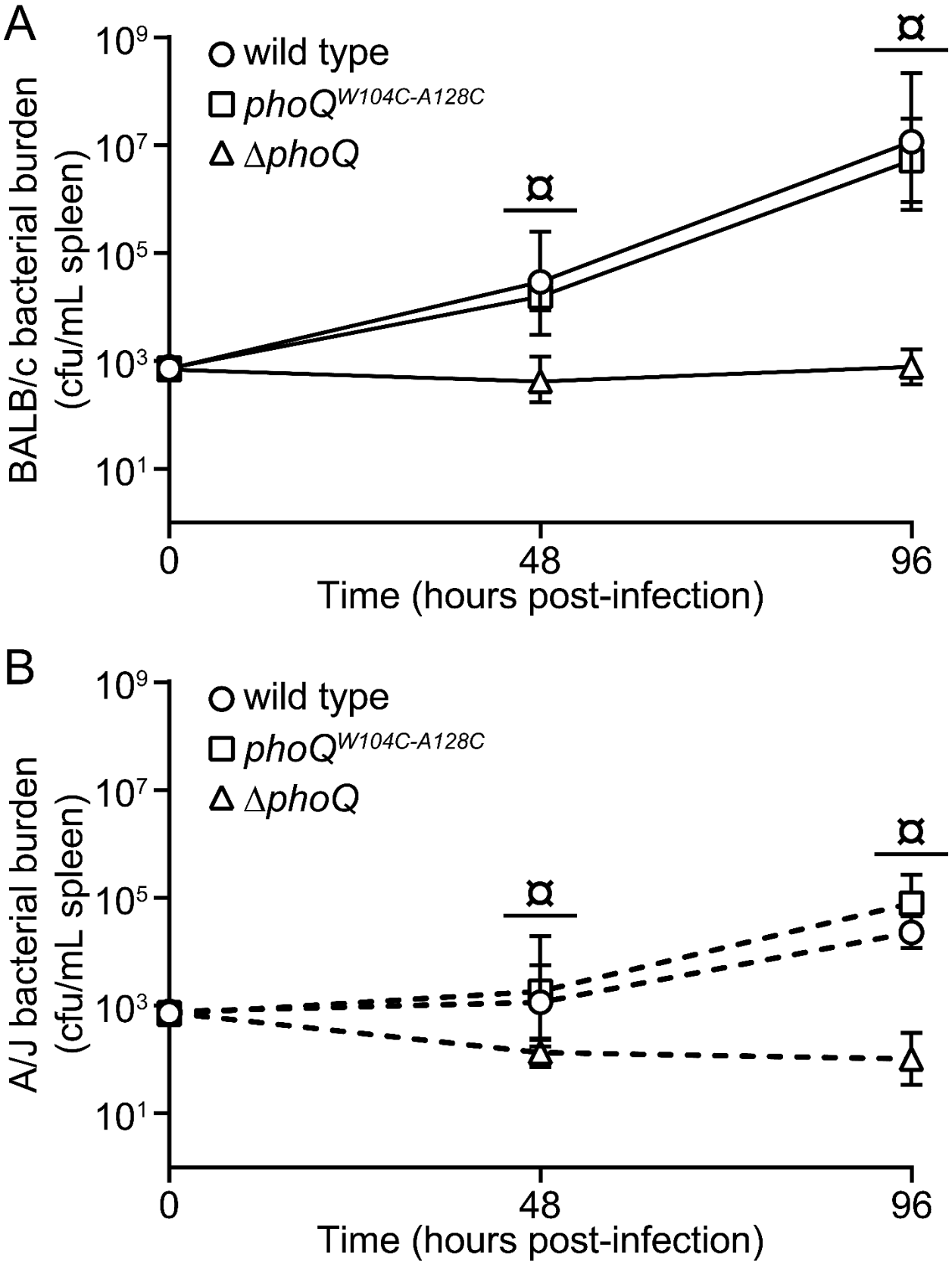

A
BALB/c bacterial burden (cfu/mL spleen)
10^9
10^7
10^5
10^3
10^1
wild type
phoQ$^{W104C-A128C}$
Δ*phoQ*
¤
¤
0
48
96
Time (hours post-infection)
B
A/J bacterial burden (cfu/mL spleen)
10^9
10^7
10^5
10^3
10^1
wild type
phoQ$^{W104C-A128C}$
Δ*phoQ*
¤
¤
0
48
96
Time (hours post-infection)

Figure 13. Acidic pH and divalent cation sensing by PhoQ are dispensable for IP systemic competition of *S. enterica Typhimurium*. Competition between *S. enterica* Typhimurium strains administered IP to BALB/c mice. Spleens were harvested, bacteria quantified 48-hpi, and CI determined. The data shown are representatives from at least three independent experiments performed in quintuplet and presented as the mean ± SD. Unpaired students T-test were performed between all strains (bar); (*) all strains are significantly different from each other ($P \leq 0.05$)

Figure 13

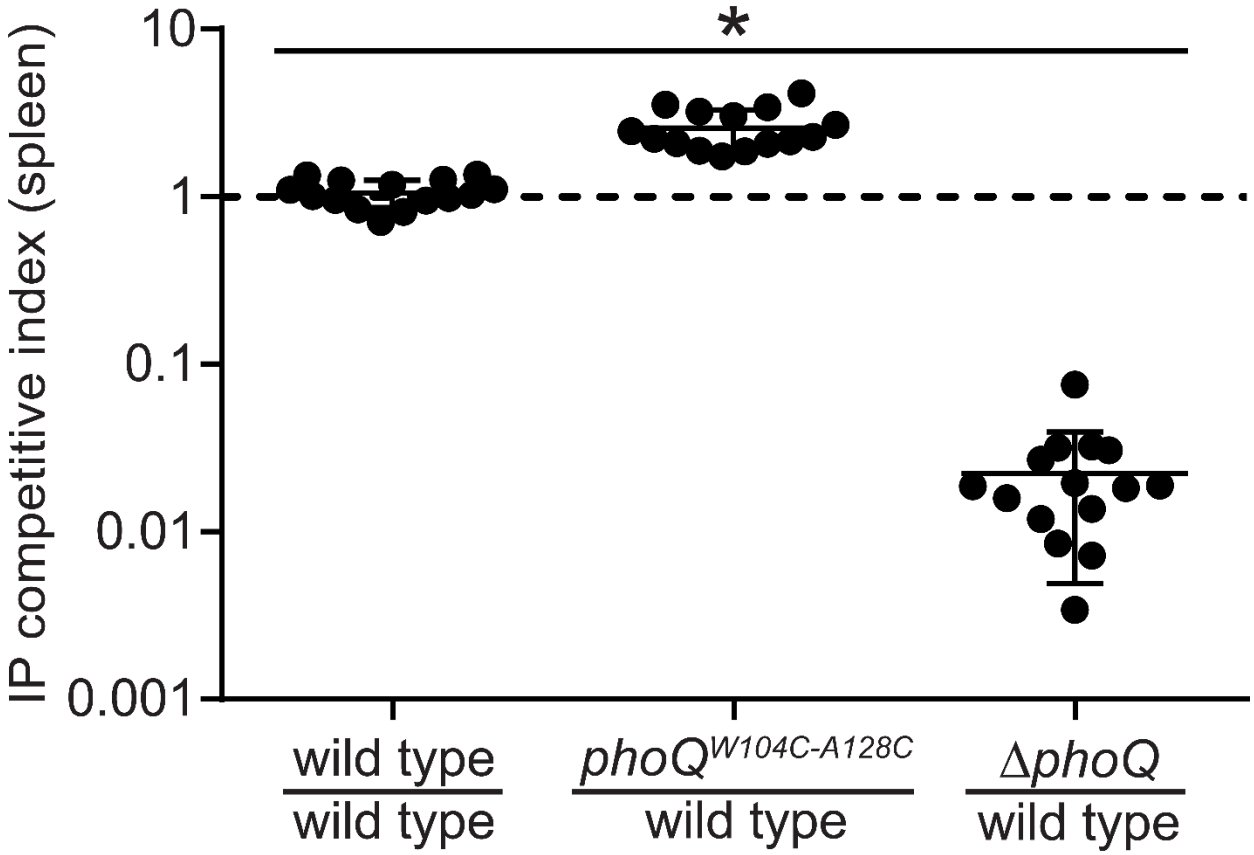

IP competitive index (spleen)
10
1
0.1
0.01
0.001
*
wild type
wild type
phoQW104C-A128C
wild type
ΔphoQ
wild type

Figure 14. Acidic pH and divalent cation sensing by PhoQ are dispensable for PO systemic competition of *S. enterica Typhimurium*. Competition between *S. enterica* Typhimurium strains administered PO to BALB/c mice. **(A)** Spleens, **(B)** livers, and **(C)** MLN were harvested at 96-hpi and bacteria were quantified to determine CI. The data shown are from three independent experiments and presented as the mean ± SD. Data points on the x-axis represent samples with a CI of zero due to the numerator equaling zero in the CI calculation.

Figure 14

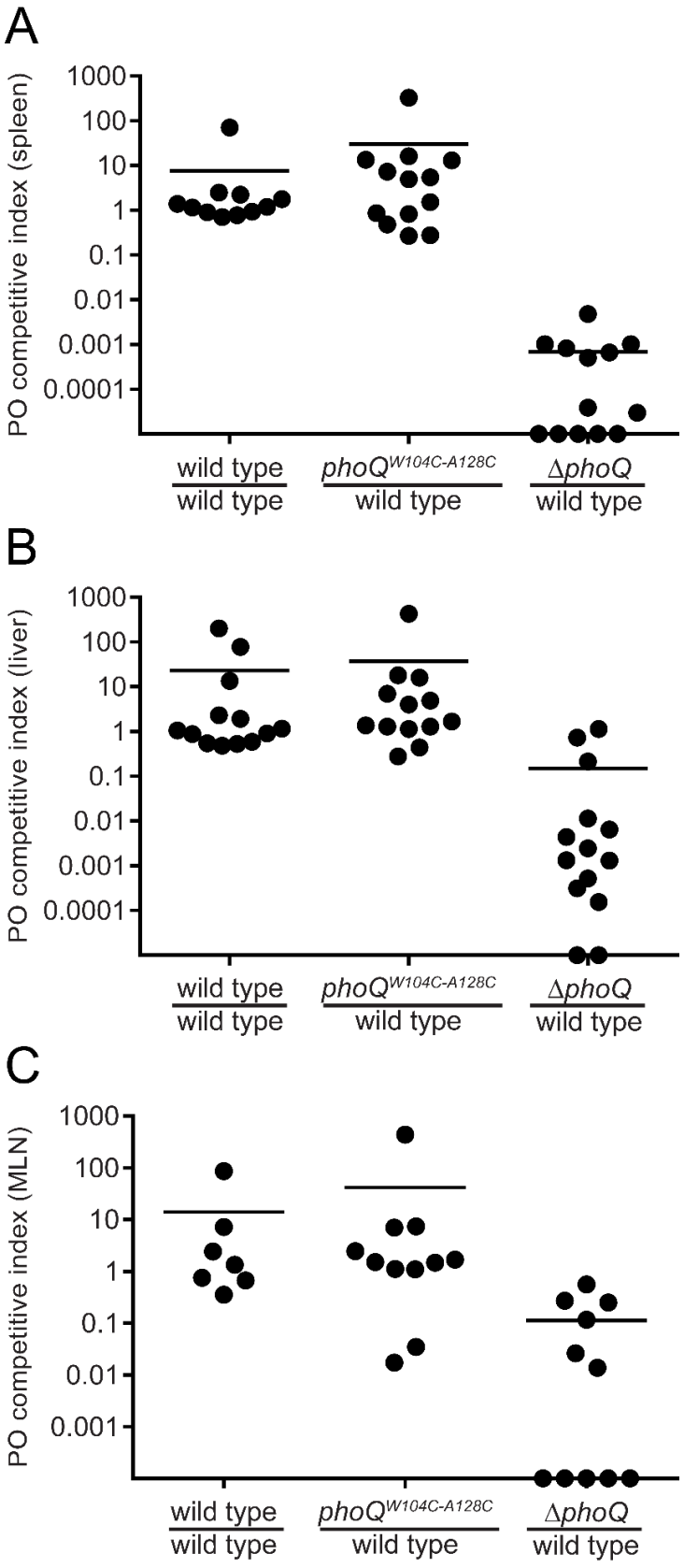

A
PO competitive index (spleen)
1000
100
10
1
0.1
0.01
0.001
0.0001
wild type / wild type
phoQ W104C-A128C / wild type
ΔphoQ / wild type
B
PO competitive index (liver)
1000
100
10
1
0.1
0.01
0.001
0.0001
wild type / wild type
phoQ W104C-A128C / wild type
ΔphoQ / wild type
C
PO competitive index (MLN)
1000
100
10
1
0.1
0.01
0.001
wild type / wild type
phoQ W104C-A128C / wild type
ΔphoQ / wild type

Figure 15. The *in vitro* growth rate of wild type salmonellae is decreased relative to *phoQ*$^{W104C\text{-}A128C}$ and *ΔphoQ* when grown at pH 5.5. *S. enterica* Typhimurium strains were grown in N-mm buffered to pH 7.5 (closed symbols) or pH 5.5 (open symbols) supplemented with 1 mM $MgCl_2$. Bacterial growth was monitored by OD_{600} at the indicated time-points. The data shown is a representatives from at least three independent experiments performed in duplicate and presented as the mean ± SD.

Figure 15

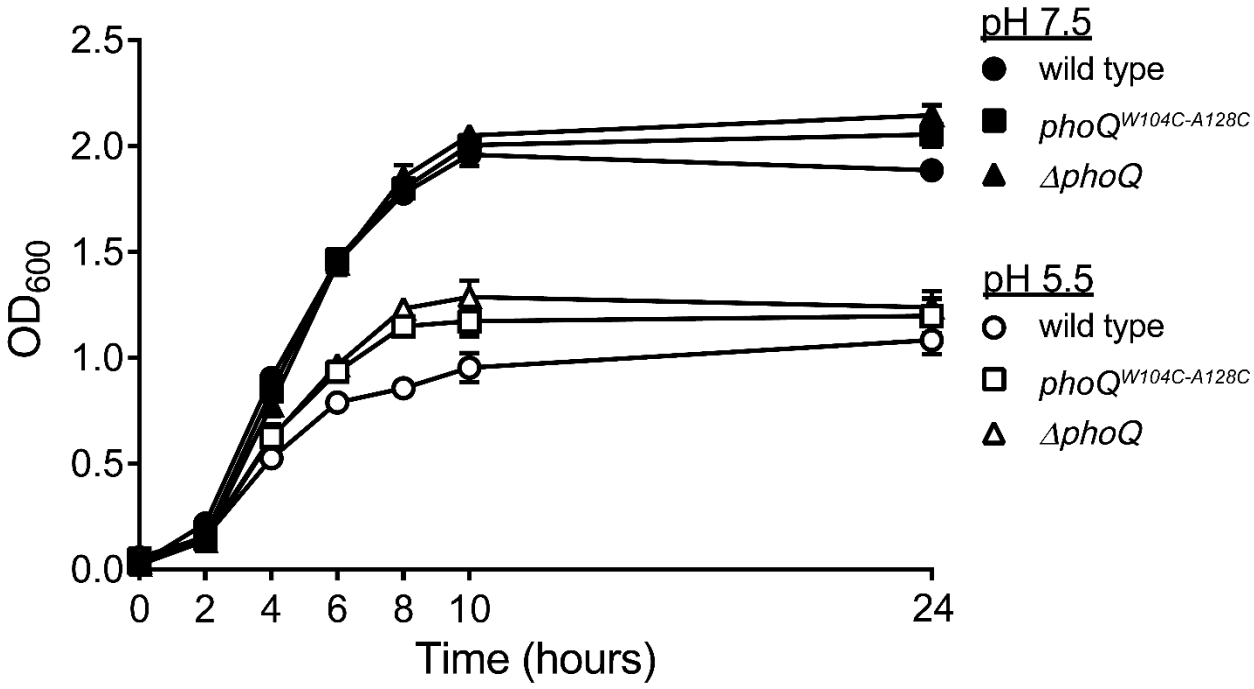

2.5
2.0
1.5
1.0
0.5
0.0
OD600
0
2
4
6
8
10
24
Time (hours)
pH 7.5
wild type
phoQW104C-A128C
ΔphoQ
pH 5.5
wild type
phoQW104C-A128C
ΔphoQ

Figure 16. *phoQ*$^{W104C\text{-}A128C}$ salmonellae survive within cultured macrophage. BALB/c BMMΦ infected with strains of *S. enterica* Typhimurium. Bacteria were harvested and quantified at the indicated time-points. The inoculum is shown at T=0 hpi. The data shown is a representative from at least three independent experiments performed in triplicate and presented as the mean ± SD. Unpaired students T-test were performed between all strains (bar) for each time-point. Symbols for significant difference; (¤) wild type and *phoQ*$^{W104C\text{-}A128C}$ are not significantly different from each other ($P \geq 0.05$), but are significantly different from *ΔphoQ* ($P \leq 0.05$), (*) all strains are significantly different from each other ($P \leq 0.05$)

Figure 16

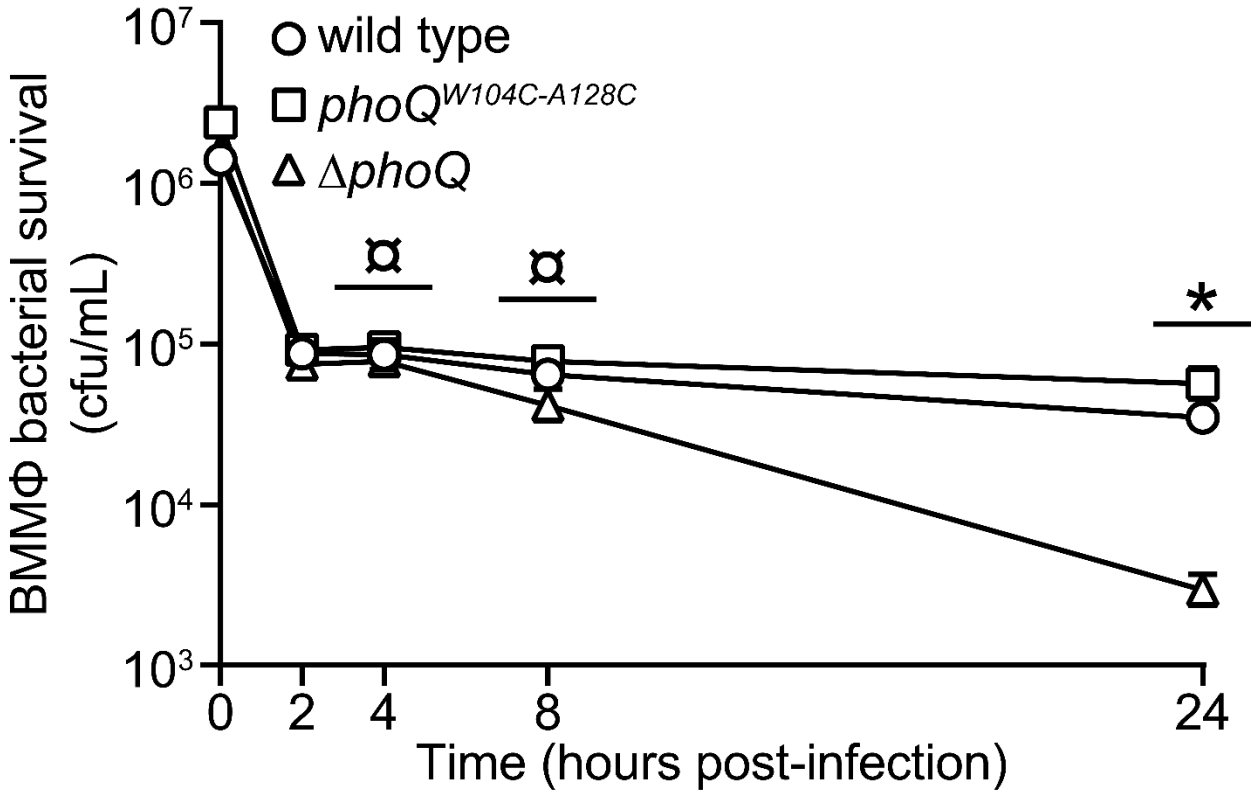
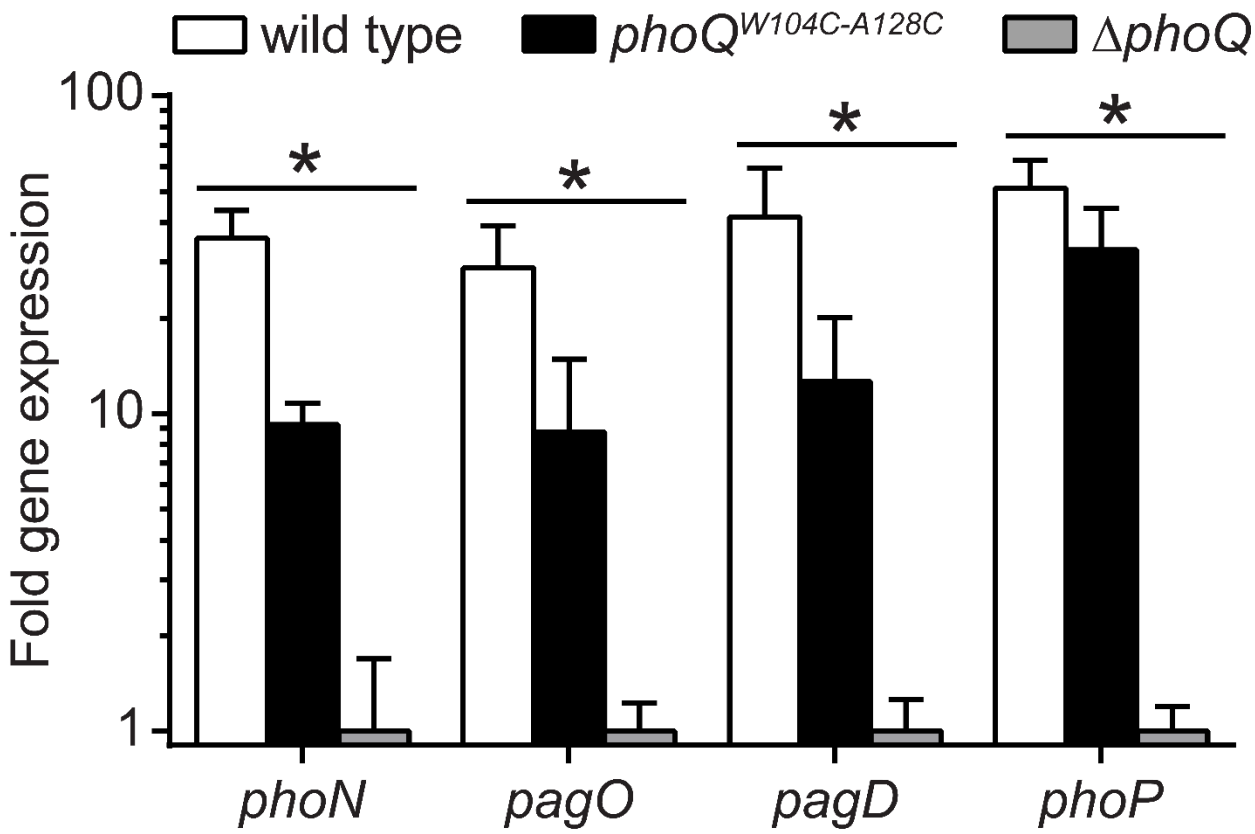

wild type
phoQW104C-A128C
ΔphoQ
BMMΦ bacterial survival (cfu/mL)
10^7
10^6
10^5
10^4
10^3
¤
¤
*
0
2
4
8
24
Time (hours post-infection)

Figure 17. *phoQ*$^{W104C\text{-}A128C}$ salmonellae exhibits PhoQ-dependent gene expression within macrophage. PhoQ-dependent gene expression from *S. enterica* Typhimurium strains within BALB/c BMMΦ 4-hpi. Gene expression was normalized to *rpoD* and presented as fold-induction relative to *ΔphoQ*. The data shown is a representative from at least three independent experiments and presented as the mean ± SD. Unpaired students T-test were performed between all strains (bar) for each gene; (*) all strains are significantly different from each other ($P \leq 0.05$)

Figure 17


wild type
phoQ^W104C-A128C
ΔphoQ
Fold gene expression
100
10
1
*
*
*
*
phoN
pagO
pagD
phoP

Chapter 7: Conclusions

Salmonellae encounter changing environments within the macrophage phagosome and other mammalian host sites during infection. These environments include a variety of antimicrobial factors for which the bacteria must regulate inducible resistance mechanisms in order to survive. These bacterial resistance mechanisms are essential for successful infection, necessitating tight regulation by sensors such as PhoQ. Our study defines α4 and α5 in the PhoQ PD as a pH-responsive structural element that experiences a change in its dynamic behavior upon transition to acidic pH. Furthermore, mutations within the PhoQ PD, predicted to destabilize hydrophobic packing and hydrogen bonding between the α/β-core and α4 and α5, resulted in loss of PhoQ repression. Limiting flexibility between these structural elements by introduction of a disulfide bond inhibited PhoQ activation by acidic pH and divalent cation limitation. We suggest that PhoQ has evolved α4 and α5 as a unique pH-responsive structural element within the PD, effectively replacing the ligand-binding site that is often found in a similar location in other structurally related PDC sensor domains (Cheung and Hendrickson, 2008; Cho et al., 2006).

Model of PhoQ activation and repression

This study provides insights for a refined model of PhoQ activation (Figure 18). At neutral pH and millimolar divalent cation concentration (Figure 18, left) the PhoQ PD is anchored to the inner membrane in a repressed state via cation-bridges, a rigid α/β-core and TDK network, and quiescent α4 and α5. Acidic pH or divalent cation limitation promotes a change in α4 and α5 from a stable to a dynamic state (Figure 18, middle). Acidic pH-induced flexibility in α4 and α5 may destabilize divalent cation salt-bridges between the inner membrane and acidic

patch, thereby promoting a loss of divalent cation-mediated repression. Lack of divalent cation salt-bridges between the PhoQ PD acidic patch and inner membrane due to divalent cation limitation may result in electrostatic repulsion between the acidic patch and inner membrane, releasing α4 and α5, and favoring a more flexible state in this structural element. Changes in the relationship between α4 and α5 and the α/β-core surrounding H157 are transmitted to the dimerization interface and TDK network proximal to the membrane resulting in alterations of the transmembrane domain, cytoplasmic HAMP domain, and ultimately resulting in increased PhoQ kinase activity.

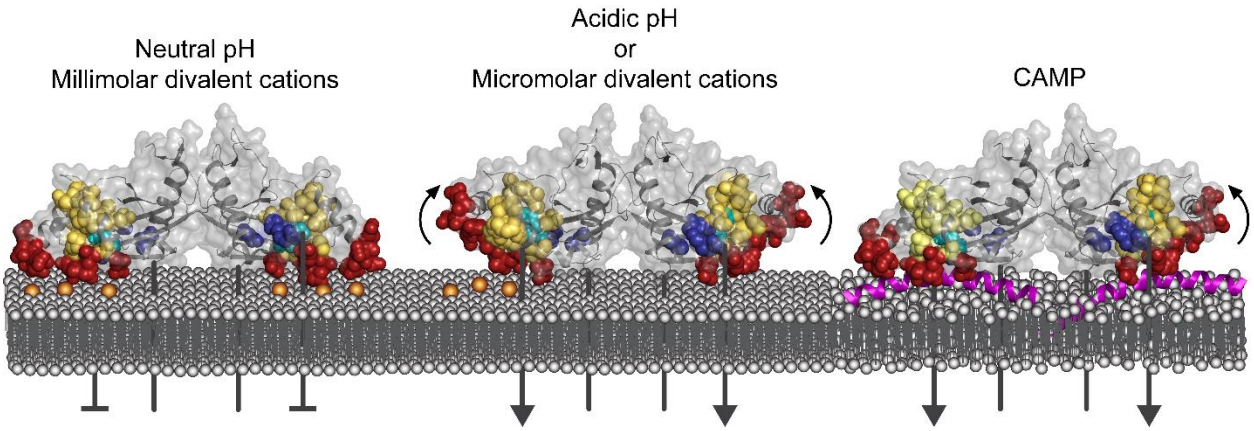
Our previous work suggests that PhoQ activation by acidic pH and CAMP proceed via different mechanisms (Bader et al., 2005; Prost et al., 2007a). In this study, we have shown that activation by acidic pH and divalent cation limitation are separable from CAMP-mediated activation by rigidifying the interaction between α4 and α5 and the α/β-core. Perhaps CAMP circumvents α4 and α5 and activates PhoQ via direct interactions within the transmembrane regions adjacent to the acidic patch or disrupts local phospholipid packing promoting conformational changes in the periplasmic and transmembrane domains (Figure 18, right). Alternatively, it is plausible that CAMP functions as a large steric "wedge". In this scenario, CAMP is recruited to the acidic patch of PhoQ resulting in conformational changes in the PD overcoming any repressive structural constraints between α4 and α5 and the α/β-core.

Our model of PhoQ activation and repression has similarities to a recently proposed two-state computational model in which the PD is predicted to experience broad conformational changes within the periplasmic dimerization interface and acidic patch (Molnar et al., 2014). This model is consistent with predictions previously made in relation to the discovery of the divalent cation bridges between the PhoQ acidic patch and negatively charged membrane

phospholipids (Bader et al., 2005; Cho et al., 2006). Molnar *et al* suggest that the PhoQ PD assumes alternative conformations as the acidic patch moves away from the membrane in the absence of divalent cation. Our findings that restricting movement in α4 and α5 inhibits PhoQ activation by acidic pH and divalent cation limitation supports the model that the acidic patch and α4 and α5 must remain dynamic for proper signaling. Furthermore, our observations that PhoQ activation by acidic pH and divalent cation limitation are separable from CAMP-mediated activation may indicate that distinct conformational states exist for each of the unique PhoQ-activating and -repressing stimuli.

Figure 18. Model of PhoQ activation and repression. (Left) At neutral pH and millimolar divalent cation concentration, the PhoQ PD is maintained in a repressed conformation due to rigidified interactions between the α/β-core (yellow spheres), α4 and α5, and salt-bridges (bronze spheres) formed between the acidic patch (red spheres) and inner membrane. **(Middle)** Transition to a mildly acidic (left protomer) or divalent cation limited (right protomer) environment promotes flexibility in α4 and α5 (bent arrows) and conformational dynamics in the α/β-core surrounding H157 (teal spheres). Movement in α4 and α5 due to acidic pH or divalent cation limitation destabilizes salt-bridges between the acidic patch and inner membrane perturbing the TDK network (blue spheres) resulting in activation. **(Right)** CAMP (magenta helices) intercalates into the inner membrane and promotes PhoQ activation by directly interacting with the PhoQ transmembrane domains and/or by disrupting of local phospholipid packing (left protomer) and/or by overcoming constraints in α4 and α5 (right monomer, bent arrow).

Figure 18


Neutral pH
Millimolar divalent cations
Acidic pH
or
Micromolar divalent cations
CAMP

Mechanisms of pH-sensors

Results presented here indicate that α4 and α5 within the PhoQ PD do not adopt a distinct conformation under activating pH conditions, but rather exchange between a rigid, repressed state and an ensemble of conformations that together constitute the acidic pH-activated state. Unlike other pH-sensors that utilize discrete histidine protonation as a mechanism of activation (Choi et al., 2013; Dawson et al., 2009; Muller et al., 2009; Perier et al., 2007; Williamson et al., 2013), PhoQ activation by acidic pH does not appear to rely strictly on protonation of histidines. Mutagenesis of H157, which is located in the PhoQ PD α/β-core and is observed to form hydrogen bonds with T129 on α4 and the backbone hydroxyl of T180 on the β7-α6 loop, results in a modest increase in activity; however, it does not account for the entire pH-mediated activation state (Prost et al., 2007a). Mutation of the other two histidines in the PD (H137 and H120) did not significantly affect activation or repression of PhoQ (data not shown). An alternate mechanism of PhoQ activation by acidic pH may involve pH-induced conformational changes within the periplasmic dimerization interface. For example, it has been postulated that the CadC pH-sensor senses acidic pH by promoting changes in the dimerization interface as mutations within this regions disrupt pH-sensing (Haneburger et al., 2011). Notably, an intermolecular disulfide at position T61C in the PhoQ PD dimerization interface results in increased PhoQ-dependent reporter activity at pH 5.5, suggesting that conformational changes in the dimerization interface can directly affect signal transduction (data not shown). Furthermore, analytical ultracentrifugation analysis revealed that the PhoQ PD dimer dissociates as the pH decreases (data not shown). Additionally, work by Molnar *et al* support the notion that conformational changes with the dimerization interface are concomitant with activation and repression. Recently, it was shown that the *H. pylori* chemotaxis receptor, TlpB, utilizes a unique

mechanism to sense acidic pH (Goers Sweeney et al., 2012). Interestingly, the TlpB PD may sense pH by adopting a "relaxed" conformation at low pH due to decreased hydrogen bonding to a coordinated urea molecule. It is plausible that similar relaxation may occur in the PhoQ PD between the α/β-core and helices α4 and α5 upon exposure to acidic pH. Therefore, pH-induced conformational changes resulting in structural relaxation or flexibility may be an important mechanism by which pH can be sensed.

The pursuit for host molecules that activate PhoQ

Determining the host signals which activate PhoQ has been difficult; past investigations have relied on alterations to host processes via chemical inhibitors to neutralize acidic compartments or targeted mutagenesis to remove known PhoQ-activating antimicrobial peptides from host animals (Alpuche Aranda et al., 1992; Martin-Orozco et al., 2006; Richards et al., 2012). Although informative, these studies do not account for unintended host-cell changes due to chemical neutralization of acidic vesicles and organelles or uncharacterized host peptides or molecules which may activate the system. For example, distinct pH-gradients are established and required in eukaryotic vesicular trafficking pathways and chemical neutralization of acidic compartments within these pathways results in a variety of cellular disturbances including inhibition of acidic hydrolases and proteases, perturbation of molecular sorting and recycling, endocytosis and exocytosis dysregulation, and disruption of vesicular fusion events (Casey et al., 2010; Dean et al., 1984; Mellman et al., 1986). Furthermore, chemical neutralization of the SCV is detrimental to intracellular *S. enterica* Typhimurium as it results in decreased bacterial survival (Rathman et al., 1996), presumably due to lack of virulence factor expression. Therefore, it is plausible that the use of chemical inhibitors to block or neutralize acidic host-

compartments inhibits processes involved in CAMP maturation or trafficking, thereby preventing activation of PhoQ.

It is plausible that undefined host molecules or conditions, that require defined pH-gradients, activate PhoQ *in vivo*. Our observations that activation by acidic pH and divalent cation limitation are not required for significant increases in PhoQ-dependent gene expression in BALB/c macrophage and that PhoQ-dependent gene expression is induced in CRAMP-deficient macrophage (Richards et al., 2012) indicate that uncharacterized host factors, which are likely to be a variety of different cationic antimicrobial molecules, activate PhoQ. Furthermore, multiple host peptides may activate PhoQ *in vivo* as various host and synthetically derived cationic peptides can activate the system; this is consistent with the large PhoQ PD acidic patch which likely evolved to bind diverse CAMP (Bader et al., 2003; Bader et al., 2005; Shprung et al., 2012). From these observations, it is reasonable to speculate that the acidic patch, located on the α4/α5 structural unit within the PhoQ PD of bacteria that interact with animals, evolved to sense a variety of cationic peptides, as the PhoQ PD from environmental bacteria such as *Pseudomonas aeruginosa* lack these important structural features (Prost et al., 2008). Though it is tempting to speculate that CAMP may be the dominant PhoQ-stimulant during systemic infection, it is important to remember that sensing may be redundant or host-compartment specific. Further experiments will need to be performed to examine the contribution of acidic pH and divalent cation sensing to PhoQ-mediated bacterial survival during transition from the intestinal tract to systemic environments and determine if "bacterial innate immunity" or the recognition of multiple mammalian signals is redundant. Additionally, perhaps acidic pH and divalent cation sensing by PhoQ are functions required for survival in *ex vivo* environments, beyond animal hosts.

Periplasmic disulfide formation in the macrophage phagosome

The work in this study led to the construction of a specific *S. enterica* Typhimurium PhoQ mutant that is inhibited for activation by acidic pH or divalent cation limitation. This mutant has wild type virulence in susceptible and resistant mouse models of systemic infection suggesting that, at least in these models, acidic pH and divalent cation sensing are dispensable for virulence. Although we have shown that the PhoQ$^{W104C-A128C}$ disulfide forms in purified proteins and in bacteria grown in culture, we did not measure disulfide formation for W104C-A128C within host tissues. Host compartments replete with strong oxidizing agents, such as the macrophage phagosome, could potentially disrupt disulfide bond formation. However, multiple salmonellae virulence factors and homeostatic processes that occur in the macrophage phagosome require disulfide bond formation, indicating that *Salmonella enterica* has robust mechanisms to regulate redox potential and resist hyperoxidation of thiols in the periplasm (Ellermeier and Slauch, 2004; Lippa and Goulian, 2012; Miki et al., 2004). Additionally, we have observed altered PhoQ-dependent gene expression from *phoQ*$^{W104C-A128C}$ *S. enterica* Typhimurium within macrophage phagosomes similar to *in vitro* grown bacteria. Therefore, it is highly likely that efficient formation of the W104C-A128C disulfide bond occurs inside host compartments.

In summary, we provide novel detail to the mechanism by which *S. enterica* Typhimurium PhoQ is activated by acidic pH. We have identified residues and secondary structural elements within the PD which contribute to acidic pH sensing and are important for PhoQ signal transduction. Furthermore, structural studies have led to the engineered bifurcation

of PhoQ signaling capabilities; separating acidic pH and divalent cation sensing from CAMP signaling. This discovery has allowed us to determine the contribution of acidic pH and divalent cation sensing to *S. enterica* Typhimurium virulence and will provide valuable insights to the spatial-temporal regulation of PhoQ during pathogenesis.

Chapter 8: Preliminary Data and Future Directions

Characterization of PhoQ sensor variants that do not respond to specific signals

The PhoQ PD directly senses acidic pH, CAMP, and divalent cation concentration (Bader et al., 2005; Garcia Vescovi et al., 1996; Prost et al., 2007a). The observations that acidic pH and CAMP additively activate PhoQ and that titrating divalent cation concentration modulates activation by these stimuli suggests that these chemically distinct signals regulate PhoQ activation and repression by separate structural mechanisms. We have shown that engineering a disulfide bond between α2 and α4 in the PhoQ PD results in inhibition of activation by acidic pH and divalent cation limitation, but CAMP maintains activation potential (Figure 5A). These findings suggest that CAMP activates PhoQ by a different mechanism than acidic pH and divalent cation limitation. Furthermore, the construction of the PhoQ$^{W104C\text{-}A128C}$ protein provides the first evidence that it is possible to internally separate PhoQ activation mechanisms by rational point-mutation, suggesting that construction or identification of other PhoQ variants that only respond to one or more signals may be possible. To identify signal specific PhoQ variants, I focused my search on the PhoQ PD TDK network due to the observations that this network is critically important for proper PhoQ signal transduction.

The PhoQ TDK network is a triad of residues located on the N- and C-termini of the PD at the inner membrane interface between the PD and TD. In the *Salmonella* PhoQ PD crystal structure (PDB 1YAX), the triad is observed as a hydrogen bonded and ionically stabilized, interconnected network between T48, D179, and K186, respectively. Presumably, activating or repressing conformations generated in the PhoQ PD must articulate through the TDK network before passing through the TD to the kinase core. This is exemplified by the observations that the

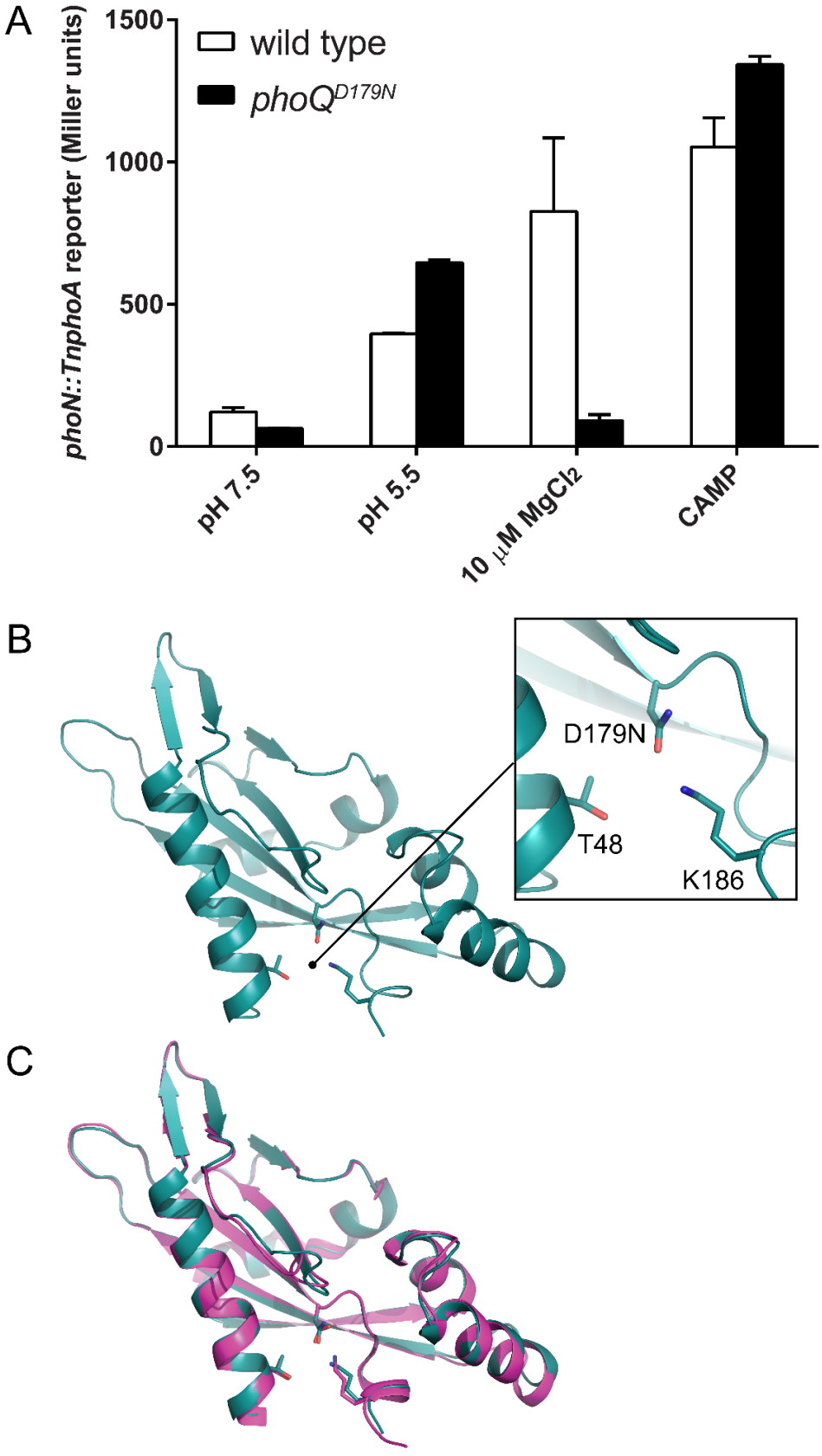
TDK network is a hot-spot for highly activating or repressing mutations relative to the rest of the PhoQ PD (Cho et al., 2006; Sanowar et al., 2003). Furthermore, the T48I activating mutation is suppressed by the W104C-A128C disulfide, suggesting that the TDK network forms an interconnected signaling element with α/β-core and α-helices 4 and 5 (Figure 5B). Although activating and repressing mutations have been identified in the TDK network, the influence of these mutations on PhoQ activation by acidic pH, CAMP, and divalent cation limitation has not been explored. Therefore, I generated a variety of mutations in T48, D179, or K186 to analyze the contribution of sidechain length, charge, polarity, steric effects, and hydrophobicity for PhoQ activation by acidic pH, CAMP, and divalent cation limitation (data not shown). Many of these mutations in the TDK network gave striking and unexpected signaling phenotypes. Of these mutations, I focused on the unique and applicable phenotype of $PhoQ^{D179N}$.

The D179N mutation in the PhoQ TDK network is a conservative mutation as substitution of an aspartic acid for asparagine results in a sidechain with the same length and hydrogen bonding capacity, but without negative charge (Figure 19). Interestingly, $PhoQ^{D179N}$ can be activated by acidic pH and CAMP near wild-type levels, but is not responsive to divalent cation limitation (Figure 19A). These findings suggest that divalent cation sensing by PhoQ proceeds by an unexpected and different mechanism then acidic pH or CAMP. Furthermore, when evaluated with the $PhoQ^{W104C-A128C}$ phenotype, these findings suggest that all known PhoQ stimuli activate by distinct and possibly separable mechanisms. The discovery of $PhoQ^{D179N}$ opens up the possibility to determine the significance of divalent cation sensing by PhoQ for *Salmonella* virulence and intracellular signaling, a longstanding point of contention in the literature.

The mechanism by which the D179N mutation promotes these PhoQ signaling phenotypes is unclear and currently under investigation. To determine whether the D179N mutation promotes structural perturbations, we solved a 2.5 Å resolution the crystal structure of the $PhoQ^{D179N}$ PD in the presence of divalent cations (Figure 19B). Interestingly, the $PhoQ^{D179N}$ PD is similar to the wild-type structure indicating the mutation does not dramatically alter PhoQ structure (Figure 19C). Given that the $PhoQ^{D179N}$ phenotype mimics a divalent cation bound state under divalent cation limiting conditions, it is tempting to speculate that D179 might form a cryptic divalent cation binding site. In this scenario, at low divalent cation concentrations D179 forms an ionic interaction with K186 and PhoQ is activated. When exposed to high concentrations of divalent cations, D179 may form a divalent cation salt-bridge with the inner membrane, similar to the PhoQ PD AP, resulting in PhoQ repression. Divalent cation binding to D179 may disrupt the ionic interaction with K186, promoting a conformational change in K186 associated with PhoQ repression. Thus, the D179N charge state mimics divalent cation bound D179, as it is not negatively charged and cannot form an ionic interaction with K186. We are currently performing experiments to test these hypotheses. Defining the mechanism behind the $PhoQ^{D179N}$ phenotype will provide novel insights to how PhoQ is regulated by divalent cations.

Figure 19. PhoQ^{D179N} is inhibited for divalent cation sensing and has similar structure to wild type. (A) PhoQ-dependent *phoN::TnphoA* alkaline phosphatase activity of wild-type and *phoQ^{D179N} S. enterica* Typhimurium grown in basal (pH 7.5) or activating (pH 5.5, 10 μM $MgCl_2$, or CAMP) N-mm. The data shown are representatives from at least three independent experiments performed in duplicate and presented as the mean ± SD. **(B)** 2.5 Å resolution crystal structure of the PhoQ^{D179N} PD. The D179N mutation is located in the TDK network (inset, sticks). **(C)** Structural comparison of the PhoQ^{D179N} PD (teal) and the wild-type *S. enterica* Typhimurium PhoQ PD (magenta, PDB 1YAX).

Figure 19


A
1500
1000
500
0
wild type
phoQD179N
phoN::TnphoA reporter (Miller units)
pH 7.5
pH 5.5
10 μM MgCl2
CAMP
B
D179N
T48
K186
C

PhoQ sensing mechanisms and signal specificity in diverse bacterial pathogens

Beyond *Salmonella,* the PhoPQ two-component system is an important virulence factor found in a variety of pathogenic proteobacteria that experience distinct environments during infection (e.g. *Klebsiella*, *Shigella*, *Neisseria*, *Yersinia*, etc.). Thus, PhoQ sensing capabilities experience different selection pressures depending on the host-niche of the pathogen: intracellular versus extracellular and phagosomal versus cytoplasmic. For example, comparison of signal specificity between the environmental, opportunistic pathogen *P. aeruginosa* and *S. enterica* Typhimurium revealed that *Salmonella* PhoQ specifically evolved to sense antimicrobial peptides (Prost et al., 2008). Additionally, PhoQ sensing capabilities are significantly diminished in the insect endosymbiont, *Sodalis glossinidius* (Pontes et al., 2011). Relatively little is known about the signal specificities of PhoQ in other pathogenic bacteria. Furthermore, it is often assumed that PhoQ signals in diverse pathogens are the same as *Salmonella*.

An amino acid sequence alignment of the PhoQ PD from a limited set of human proteobacterial pathogens reveals distinct differences in the senor domain (Figure 20A). Critical features in the *Salmonella* PD including the acidic patch, TDK network, α-helices 4 and 5, and pH-sensitive residues are only loosely conserved in other pathogens. Markedly, the *Salmonella* PhoQ PD acidic patch, which binds CAMP and divalent cations, is significantly diminished in *K. pneumoniae* and *Y. pestis*. Furthermore, α-helices 4 and 5, which are important for acidic pH and divalent cation sensing in *Salmonella*, have low sequence conservation relative to other regions in the domain. In distantly related pathogens, such as *N. meningitidis*, the PhoQ PD has very little sequence conservation relative to the other organisms. These sequence alignments may

suggest that the PhoQ sensor experiences different selective pressures in accordance with the signals specific to the host-niche of the pathogen.

To gain insights to the signal specificity of PhoQ in diverse pathogens, sensor domain chimeras with *Salmonella* PhoQ provide a relative robust and cross-comparative system. To begin, the sensing capabilities of *Y. pestis* PhoQ were examined. Similar to Prost *et al*, a PhoQ chimera was constructed (PhoQ$^{Y.\ pestis\ \text{chimera}}$) in which the *S. enterica* Typhimurium PhoQ PD was substituted for the *Y. pestis* PhoQ PD within full-length *S. enterica* Typhimurium PhoQ. *phoQ*$^{Y.\ pestis\ chimera}$ was introduced into *S. enterica* Typhimurium *in trans* and the resulting strain was grown in basal and various activating conditions (Figure 20B). Interestingly, acidic pH and divalent cation sensing is completely absent in PhoQ$^{Y.\ pestis\ \text{chimera}}$ relative to wild-type *Salmonella* PhoQ. However, activation by CAMP is equivalent between wild type and *phoQ*$^{Y.\ pestis\ chimera}$, suggesting the construct is likely functional. These finding may suggest that *Y. pestis* PhoQ does not have the capabilities to sense acidic pH and divalent cation limitation, but can sense CAMP. Furthermore, these findings may suggest that acidic pH and divalent cation sensing by PhoQ is a unique adaptation by certain organisms. Additional characterization is required to confirm that PhoQ$^{Y.\ pestis\ \text{chimera}}$ is functional in *Salmonella* and that *Y. pestis* PhoQ is indeed only a CAMP sensor. If proven dysfunctional, characterizing the mechanism by which PhoQ$^{Y.\ pestis\ \text{chimera}}$ senses CAMP may provide valuable information about the mechanism by which *Salmonella* PhoQ is activated by CAMP. PhoQ chimeras will be constructed to define PhoQ signal specificity in other bacteria *in vitro* and during infection. Such experiments will provide a better understanding of the mechanisms for diverse signal recognition by PhoQ and the host signals that are detected by different pathogenic organisms.

Figure 20. A *Salmonella* PhoQ *Y. pestis* PD chimera does not respond to acidic pH and divalent cation limitation, but is responsive to CAMP. (A) Amino acid multiple sequence alignment of the PhoQ PD from select proteobacterial pathogens. Conservation is shown below the sequence alignment; "*", conserved; ":", conservative substitution; ".", semi-conservative substitution. *N. meningititis* sequence and conservation is set apart to show relative sequence divergence. Select *Salmonella* PhoQ PD features are shown above their respective residues: acidic patch (red bars), TDK network (blue bars), α-helices 4 and 5 (green bars), and pH-sensitive residues (magenta bars). **(B)** PhoQ-dependent *phoN::TnphoA* alkaline phosphatase activity of wild-type and $phoQ^{Y.\ pestis\ chimera}$ *S. enterica* Typhimurium grown in basal (pH 7.5) or activating (pH 5.5, 10 μM $MgCl_2$, or CAMP) N-mm. The data shown are representatives from at least three independent experiments performed in duplicate and presented as the mean ± SD.

Figure 20

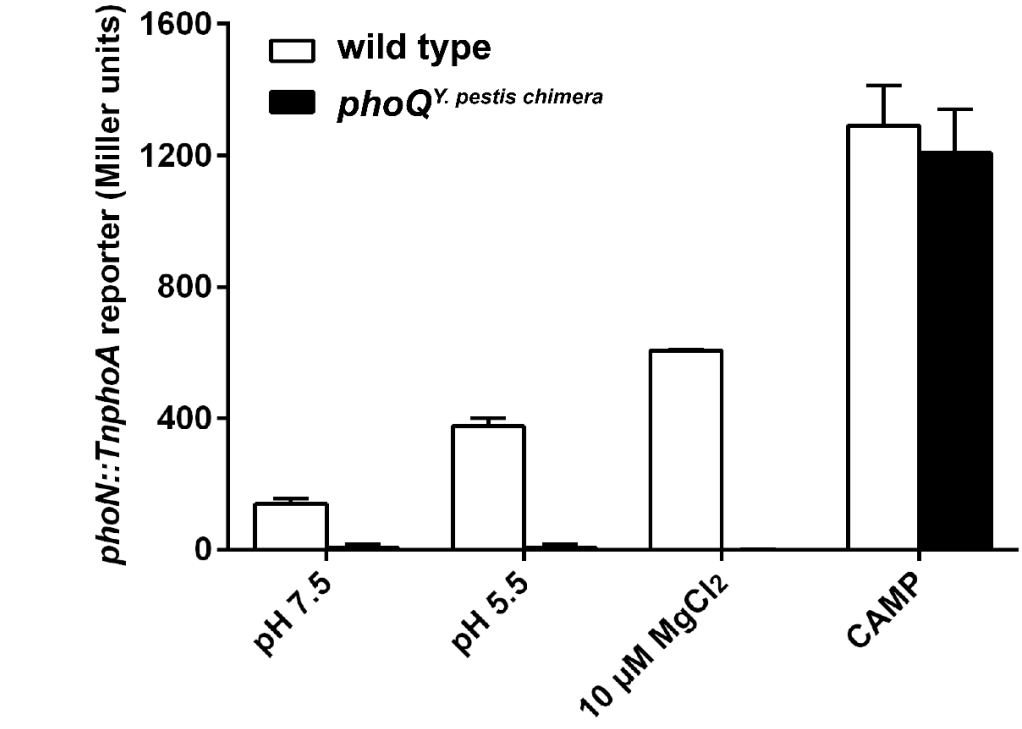
A

```
S. Typhimurium    DKTTFRLLRGESNLFYTLAKWENNKISVELPENLDMQSPTMTLIYDETGK
S. flexneri       DKTTFRLLRGESNLFYTLAQWENNKLHVELPENIDKQSPTMTLIYDENGQ
K. pneumoniae     DKTTFRLLRGESNLFYMLARWENGAIDVDIPENLNMESPTVTLIYDEQGK
Y. pestis         --STFRAHRGESNLFFSLAQWHNNKLSISVPPELELNVPSLVLIYDKDGN
                    :***  *******: **:*.*. : :.:* ::: : *::.****: *:
N. meningitidis   ---ETTLMGSIISAFRARGDAGAREILTEWKDSP-VSSGVYVIQGDEKKD
                           .  . *   .      :  .   .   .    .:  *:  .

S. Typhimurium    LLWTQRNIPWLIKS-IQPEWLKTNGFHEIETNVDATSTLLSEDHSAQEKL
S. flexneri       LLWAQRDVPWLMKM-IQPDWLKSNGFHEIEADVNDTSLLLSGDHSIQQQL
K. pneumoniae     LLWAQRDVPWLAKR-IQPEWLKRNGFHEIEADVDSSSMLLRNNHEIQEQL
Y. pestis         ILWRQRHVPELESH-IEKSWLQKPGFYELDTGTHISRMMMGDNPKAQDQL
                  :** **.:* * .  *: .**:  **:*:::... :  ::  : . *::*.
N. meningitidis   ILHRYIDSYTIERARLFAAGHPHSNLVHIEYDRFGEEYLFFTKDWDKLQA
                  :*    .   :    :        .: .:: .      ::  .   : :

S. Typhimurium    KEVREDDDDAEMTHSVAVNIYPATARMPQLTIVVVDTIPIELKRS---
S. flexneri       QEVREDDDDAEMTHSVAVNVYPATSRMPKLTIVVVDTIPVELKSS---
K. pneumoniae     DAIREQGDDSEMTHSVAINLYPATSKMPQLSIVVVDTIPVELKRS---
Y. pestis         KKY-DDTDDSALTHSVSVNTYAATSRLPQLTIVVVDTIPQELQRTDLV
                      :: **: :*****:* *.**:::*:*:******** **: :
N. meningitidis   RRLPSPLLIPGLPLAPIWHELIILSFIIIVGLLMAYILAGNIAKPIRI
                      .    . :. :   :     : :  : :::.  :. ::  .
```

B



Dissection of intracellular host processes required for PhoQ regulation

The intracellular host signals essential for *Salmonella* PhoQ activation remain elusive. Originally, through the use of intracellular buffering agents, it was postulated that phagosomal acidic pH is the PhoQ activating signal in macrophage (Alpuche Aranda et al., 1992). However, the use of chemical inhibitors of intracellular acidic pH is most likely pleotropic and disrupts unintended crucial intracellular trafficking pathways (Casey et al., 2010). Soon afterwards, *in vitro* studies suggested that PhoQ activation is regulated by divalent cations (Garcia Vescovi et al., 1996). However, during *Salmonella* infection, macrophage phagosomal Ca^{2+} and Mg^{2+} concentrations are maintained at approximately 500 micromolar and 1 millimolar, respectively; suggesting PhoQ is not activated by intracellular divalent cation limitation (Christensen et al., 2002; Martin-Orozco et al., 2006). Following these studies, it was shown *in vitro* that PhoQ is directly activated by acidic pH and CAMP (Bader et al., 2005; Prost et al., 2007a). Furthermore, evidence suggests that CAMP activate PhoQ in *vivo* (Richards et al., 2012; Rosenberger et al., 2004). The findings presented in this study suggest that acidic pH and divalent cation sensing by PhoQ are dispensable for salmonellae virulence, further supporting a role for PhoQ activation by CAMP during infection. Additionally, when integrated with previous findings, our data may suggest that PhoQ activation signals are redundant in the host. It remains to be shown what host molecules and intracellular processes are essential for PhoQ activation in host tissues.

Data presented in this study suggests that PhoQ is activated by CAMP when Salmonellae are within macrophages. To begin to understand the host intracellular processes required for *Salmonella* PhoQ activation, mechanisms of CAMP maturation were targeted. Cathelicidin-related antimicrobial peptides, such as LL-37 in humans and CRAMP in mice, are a family of CAMP found in lysosomes of macrophages and neutrophils and are known to activate PhoQ *in*

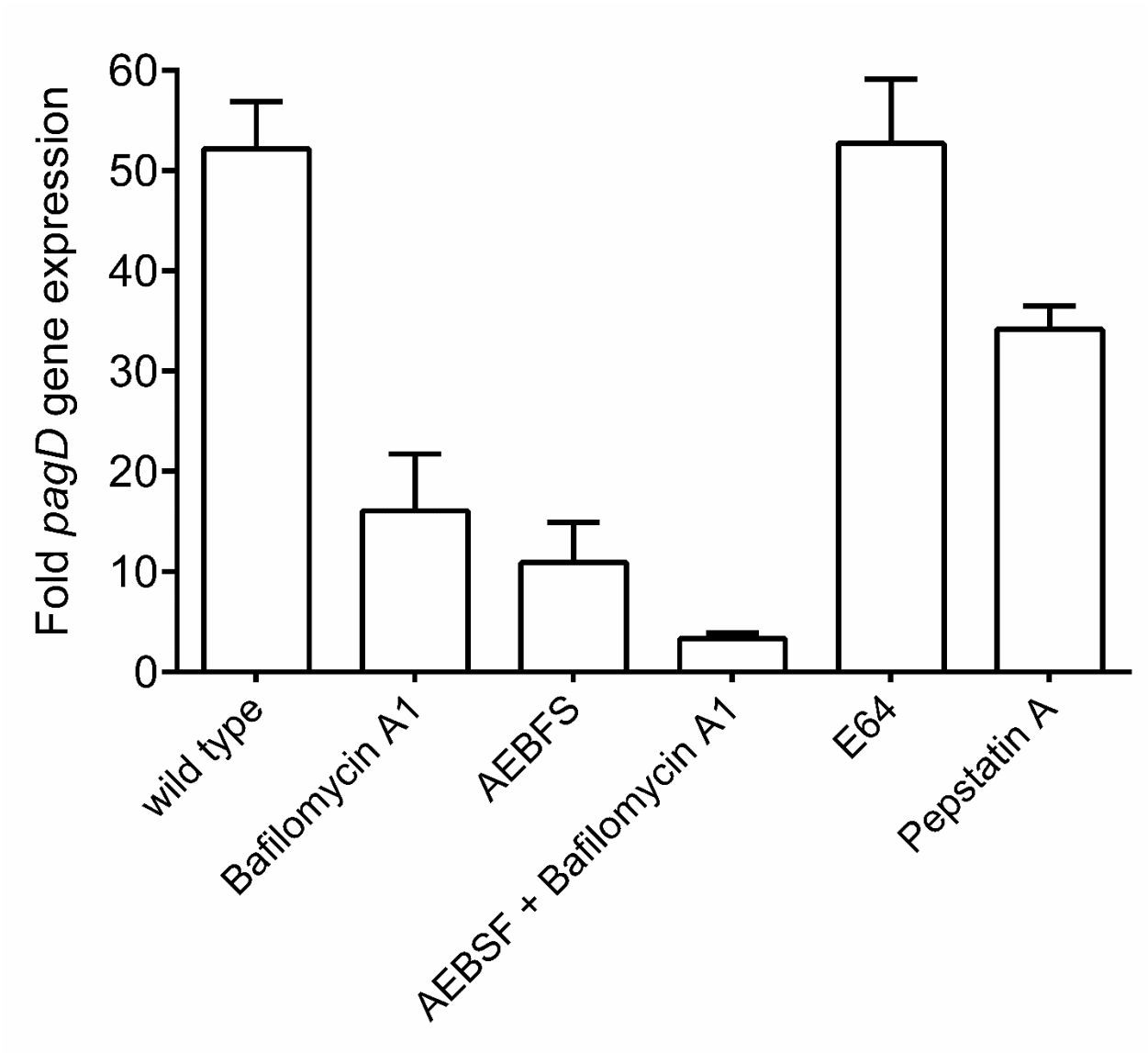
vitro (Bader et al., 2005; Vandamme et al., 2012). Serine protease activity is required to convert cathelicidins from an inactive proprotein to an active, antimicrobial peptide (Scocchi et al., 1992). Previously, it was shown that serine protease inhibitor treatment of macrophages infected with *Salmonella* results in a decrease in filamentous bacteria, which correlates with an inhibition of CAMP activity (Rosenberger et al., 2004). To determine whether intracellular protease activity is required for PhoQ activation, *S. enterica* Typhimurium-infected macrophages were treated with inhibitors of serine (AEBFS), cysteine (E-64), or aspartyl (pepstatin A) proteases and PhoQ-dependent gene expression was monitored (Figure 21). Interestingly, AEBFS resulted in an approximate 3-fold decrease in *Salmonella* PhoQ-dependent *pagD* gene expression within macrophage. Intracellular PhoQ activation appears to be serine protease specific as neither E-64 nor pepstatin A promoted substantial perturbations in *pagD* expression. Furthermore, simultaneously inhibiting macrophage serine proteases and the H+ vacuolar ATPase (V-ATPase) with bafilomycin A1 resulted in an additive decrease in PhoQ-dependent gene expression. This data suggest that serine protease activity is required for PhoQ activation in macrophage. Furthermore, it appears that serine protease inhibition and pH neutralization of macrophage organelles and vacuolar compartments may prevent PhoQ activation by distinct and additive mechanisms.

It is tempting to speculate that serine protease inhibition promotes a decrease in PhoQ activation in macrophages due to specific inhibition of cathelicidin maturation. However, it is possible that other uncharacterized CAMP require serine protease activity in order to activate PhoQ. Furthermore, serine proteases are required for a variety of intracellular processes and there disruption may prevent proper intracellular trafficking of PhoQ stimulating molecules to the SCV (Bond and Butler, 1987). The observations that PhoQ activity is dependent on the pH of

intracellular compartments has been previously described (Alpuche Aranda et al., 1992; Martin-Orozco et al., 2006). Perhaps acidic pH in the SCV is required to activate serine proteases that, in return, cleave and activate CAMP. It has long be established that many proteases found in acidic intracellular compartments require acidic pH to function (Muller et al., 2012). Alternatively, the observations that neutralization of intracellular compartments and serine protease inhibition additively decrease PhoQ activation may suggest that these inhibitors interfere with distinct processes or that their combinatorial treatment results in fully penetrant inhibition of a particular process. Further experiments are required to determine the specific molecules and processes essential for PhoQ activation in host tissues and whether signal specificity is redundant.

Figure 21. Inhibition of macrophage V-ATPase and serine protease activity decreases intracellular salmonellae PhoQ activation. PhoQ-dependent *pagD* gene expression from *S. enterica* Typhimurium within BALB/c BMMΦ 4-hpi treated with AEBFS, E-64, or pepstatin A protease inhibitors and/or bafilomycin A1. Gene expression was normalized to *rpoD* and presented as fold-induction relative to *ΔphoQ*. The data shown is a representative from at least two independent experiments and presented as the mean ± SD.

Figure 21


Fold pagD gene expression
60
50
40
30
20
10
0
wild type
Bafilomycin A1
AEBFS
AEBSF + Bafilomycin A1
E64
Pepstatin A

Bibliography

Adams, P., Fowler, R., Kinsella, N., Howell, G., Farris, M., Coote, P., and O'Connor, C.D. (2001). Proteomic detection of PhoPQ- and acid-mediated repression of Salmonella motility. Proteomics *1*, 597-607.

Afonine, P.V., Grosse-Kunstleve, R.W., Echols, N., Headd, J.J., Moriarty, N.W., Mustyakimov, M., Terwilliger, T.C., Urzhumtsev, A., Zwart, P.H., and Adams, P.D. (2012). Towards automated crystallographic structure refinement with phenix.refine. Acta Crystallogr D Biol Crystallogr *68*, 352-367.

Alpuche-Aranda, C.M., Berthiaume, E.P., Mock, B., Swanson, J.A., and Miller, S.I. (1995). Spacious phagosome formation within mouse macrophages correlates with Salmonella serotype pathogenicity and host susceptibility. Infection and immunity *63*, 4456-4462.

Alpuche-Aranda, C.M., Racoosin, E.L., Swanson, J.A., and Miller, S.I. (1994). Salmonella stimulate macrophage macropinocytosis and persist within spacious phagosomes. J Exp Med *179*, 601-608.

Alpuche Aranda, C.M., Swanson, J.A., Loomis, W.P., and Miller, S.I. (1992). Salmonella typhimurium activates virulence gene transcription within acidified macrophage phagosomes. Proc Natl Acad Sci U S A *89*, 10079-10083.

Alvarez-Ordonez, A., Begley, M., Prieto, M., Messens, W., Lopez, M., Bernardo, A., and Hill, C. (2011). Salmonella spp. survival strategies within the host gastrointestinal tract. Microbiology *157*, 3268-3281.

Antalis, T.M., Shea-Donohue, T., Vogel, S.N., Sears, C., and Fasano, A. (2007). Mechanisms of disease: protease functions in intestinal mucosal pathobiology. Nat Clin Pract Gastroenterol Hepatol *4*, 393-402.

Ao, T.T., Feasey, N.A., Gordon, M.A., Keddy, K.H., Angulo, F.J., and Crump, J.A. (2015). Global burden of invasive nontyphoidal Salmonella disease, 2010(1). Emerg Infect Dis *21*.

Bachhawat, P., and Stock, A.M. (2007). Crystal structures of the receiver domain of the response regulator PhoP from Escherichia coli in the absence and presence of the phosphoryl analog beryllofluoride. Journal of bacteriology *189*, 5987-5995.

Bader, M.W., Navarre, W.W., Shiau, W., Nikaido, H., Frye, J.G., McClelland, M., Fang, F.C., and Miller, S.I. (2003). Regulation of Salmonella typhimurium virulence gene expression by cationic antimicrobial peptides. Molecular microbiology *50*, 219-230.

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

Bearson, B.L., Wilson, L., and Foster, J.W. (1998). A low pH-inducible, PhoPQ-dependent acid tolerance response protects Salmonella typhimurium against inorganic acid stress. Journal of bacteriology *180*, 2409-2417.

Behlau, I., and Miller, S.I. (1993). A PhoP-repressed gene promotes Salmonella typhimurium invasion of epithelial cells. Journal of bacteriology *175*, 4475-4484.

Belden, W.J., and Miller, S.I. (1994). Further characterization of the PhoP regulon: identification of new PhoP-activated virulence loci. Infection and immunity *62*, 5095-5101.

Bijlsma, J.J., and Groisman, E.A. (2005). The PhoP/PhoQ system controls the intramacrophage type three secretion system of Salmonella enterica. Molecular microbiology *57*, 85-96.

Bochner, B.R., Huang, H.C., Schieven, G.L., and Ames, B.N. (1980). Positive selection for loss of tetracycline resistance. Journal of bacteriology *143*, 926-933.

Bond, J.S., and Butler, P.E. (1987). Intracellular proteases. Annu Rev Biochem *56*, 333-364.

Bourret, T.J., Song, M., and Vazquez-Torres, A. (2009). Codependent and independent effects of nitric oxide-mediated suppression of PhoPQ and Salmonella pathogenicity island 2 on intracellular Salmonella enterica serovar typhimurium survival. Infection and immunity *77*, 5107-5115.

Broz, P., Ohlson, M.B., and Monack, D.M. (2012). Innate immune response to Salmonella typhimurium, a model enteric pathogen. Gut Microbes *3*, 62-70.

Cano, D.A., Martinez-Moya, M., Pucciarelli, M.G., Groisman, E.A., Casadesus, J., and Garcia-Del Portillo, F. (2001). Salmonella enterica serovar Typhimurium response involved in attenuation of pathogen intracellular proliferation. Infection and immunity *69*, 6463-6474.

Casey, J.R., Grinstein, S., and Orlowski, J. (2010). Sensors and regulators of intracellular pH. Nat Rev Mol Cell Biol *11*, 50-61.

Castelli, M.E., Garcia Vescovi, E., and Soncini, F.C. (2000). The phosphatase activity is the target for Mg2+ regulation of the sensor protein PhoQ in Salmonella. The Journal of biological chemistry *275*, 22948-22954.

Chen, H.D., and Groisman, E.A. (2013). The biology of the PmrA/PmrB two-component system: the major regulator of lipopolysaccharide modifications. Annual review of microbiology *67*, 83-112.

Cheng, H.Y., Chen, Y.F., and Peng, H.L. (2010). Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in Klebsiella pneumoniae CG43. J Biomed Sci *17*, 60.

Cheung, J., Bingman, C.A., Reyngold, M., Hendrickson, W.A., and Waldburger, C.D. (2008). Crystal structure of a functional dimer of the PhoQ sensor domain. The Journal of biological chemistry *283*, 13762-13770.

Cheung, J., and Hendrickson, W.A. (2008). Crystal structures of C4-dicarboxylate ligand complexes with sensor domains of histidine kinases DcuS and DctB. J Biol Chem *283*, 30256-30265.

Cheung, J., and Hendrickson, W.A. (2010). Sensor domains of two-component regulatory systems. Curr Opin Microbiol *13*, 116-123.

Cho, U.S., Bader, M.W., Amaya, M.F., Daley, M.E., Klevit, R.E., Miller, S.I., and Xu, W. (2006). Metal bridges between the PhoQ sensor domain and the membrane regulate transmembrane signaling. J Mol Biol *356*, 1193-1206.

Choi, C.H., Webb, B.A., Chimenti, M.S., Jacobson, M.P., and Barber, D.L. (2013). pH sensing by FAK-His58 regulates focal adhesion remodeling. J Cell Biol *202*, 849-859.

Christensen, K.A., Myers, J.T., and Swanson, J.A. (2002). pH-dependent regulation of lysosomal calcium in macrophages. J Cell Sci *115*, 599-607.

Cremers, C.M., Knoefler, D., Vitvitsky, V., Banerjee, R., and Jakob, U. (2014). Bile salts act as effective protein-unfolding agents and instigators of disulfide stress in vivo. Proceedings of the National Academy of Sciences of the United States of America *111*, E1610-1619.

Crump, J.A., Luby, S.P., and Mintz, E.D. (2004). The global burden of typhoid fever. Bulletin of the World Health Organization *82*, 346-353.

Dalebroux, Z.D., Edrozo, M.B., Pfuetzner, R.A., Ressl, S., Kulasekara, B.R., Blanc, M.P., and Miller, S.I. (2015). Delivery of cardiolipins to the Salmonella outer membrane is necessary for survival within host tissues and virulence. Cell host & microbe *17*, 441-451.

Dalebroux, Z.D., Matamouros, S., Whittington, D., Bishop, R.E., and Miller, S.I. (2014). PhoPQ regulates acidic glycerophospholipid content of the Salmonella Typhimurium outer membrane. Proceedings of the National Academy of Sciences of the United States of America *111*, 1963-1968.

Dalebroux, Z.D., and Miller, S.I. (2014). Salmonellae PhoPQ regulation of the outer membrane to resist innate immunity. Current opinion in microbiology *17*, 106-113.

Dawson, J.E., Seckute, J., De, S., Schueler, S.A., Oswald, A.B., and Nicholson, L.K. (2009). Elucidation of a pH-folding switch in the Pseudomonas syringae effector protein AvrPto. Proc Natl Acad Sci U S A *106*, 8543-8548.

Dean, R.T., Jessup, W., and Roberts, C.R. (1984). Effects of exogenous amines on mammalian cells, with particular reference to membrane flow. Biochem J *217*, 27-40.

Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J., Bax, A.C.I.N.J.B.N.M.R.J., and Pmid (1995). NMRPipe: a multidimensional spectral processing system based on UNIX pipes. Journal of biomolecular NMR *6*, 277-293.

Derzelle, S., Turlin, E., Duchaud, E., Pages, S., Kunst, F., Givaudan, A., and Danchin, A. (2004). The PhoP-PhoQ two-component regulatory system of Photorhabdus luminescens is essential for virulence in insects. Journal of bacteriology *186*, 1270-1279.

Ellermeier, C.D., and Slauch, J.M. (2004). RtsA coordinately regulates DsbA and the Salmonella pathogenicity island 1 type III secretion system. Journal of bacteriology *186*, 68-79.

Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and development of Coot. Acta Crystallogr D Biol Crystallogr *66*, 486-501.

Erickson, D.L., Russell, C.W., Johnson, K.L., Hileman, T., and Stewart, R.M. (2011). PhoP and OxyR transcriptional regulators contribute to Yersinia pestis virulence and survival within Galleria mellonella. Microb Pathog *51*, 389-395.

Feasey, N.A., Dougan, G., Kingsley, R.A., Heyderman, R.S., and Gordon, M.A. (2012). Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. Lancet *379*, 2489-2499.

Fields, P.I., Groisman, E.A., and Heffron, F. (1989). A Salmonella locus that controls resistance to microbicidal proteins from phagocytic cells. Science *243*, 1059-1062.

Fischbach, M.A., Lin, H., Zhou, L., Yu, Y., Abergel, R.J., Liu, D.R., Raymond, K.N., Wanner, B.L., Strong, R.K., Walsh, C.T., *et al.* (2006). The pathogen-associated iroA gene cluster mediates bacterial evasion of lipocalin 2. Proceedings of the National Academy of Sciences of the United States of America *103*, 16502-16507.

Flannagan, R.S., Cosio, G., and Grinstein, S. (2009). Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nature reviews Microbiology *7*, 355-366.

Flo, T.H., Smith, K.D., Sato, S., Rodriguez, D.J., Holmes, M.A., Strong, R.K., Akira, S., and Aderem, A. (2004). Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature *432*, 917-921.

Foster, J.W., and Hall, H.K. (1990). Adaptive acidification tolerance response of Salmonella typhimurium. Journal of bacteriology *172*, 771-778.

Francis, C.L., Starnbach, M.N., and Falkow, S. (1992). Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with Salmonella typhimurium grown under low-oxygen conditions. Molecular microbiology *6*, 3077-3087.

Freeman, J.A., Ohl, M.E., and Miller, S.I. (2003). The Salmonella enterica serovar typhimurium translocated effectors SseJ and SifB are targeted to the Salmonella-containing vacuole. Infection and immunity *71*, 418-427.

Fu, Y., and Galan, J.E. (1999). A salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. Nature *401*, 293-297.

Galan, J.E., and Curtiss, R., 3rd (1989a). Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. Proceedings of the National Academy of Sciences of the United States of America *86*, 6383-6387.

Galan, J.E., and Curtiss, R., 3rd (1989b). Virulence and vaccine potential of phoP mutants of Salmonella typhimurium. Microb Pathog *6*, 433-443.

Gao, R., and Stock, A.M. (2009). Biological insights from structures of two-component proteins. Annu Rev Microbiol *63*, 133-154.

Garcia-del Portillo, F., Foster, J.W., and Finlay, B.B. (1993). Role of acid tolerance response genes in Salmonella typhimurium virulence. Infection and immunity *61*, 4489-4492.

Garcia Vescovi, E., Soncini, F.C., and Groisman, E.A. (1996). Mg2+ as an extracellular signal: environmental regulation of Salmonella virulence. Cell *84*, 165-174.

Gerlach, R.G., Holzer, S.U., Jackel, D., and Hensel, M. (2007). Rapid engineering of bacterial reporter gene fusions by using Red recombination. Applied and environmental microbiology *73*, 4234-4242.

Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., 3rd, and Smith, H.O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature methods *6*, 343-345.

Goers Sweeney, E., Henderson, J.N., Goers, J., Wreden, C., Hicks, K.G., Foster, J.K., Parthasarathy, R., Remington, S.J., and Guillemin, K. (2012). Structure and Proposed Mechanism for the pH-Sensing Helicobacter pylori Chemoreceptor TlpB. Structure (London, England : 1993).

Golubeva, Y.A., and Slauch, J.M. (2006). Salmonella enterica serovar Typhimurium periplasmic superoxide dismutase SodCI is a member of the PhoPQ regulon and is induced in macrophages. Journal of bacteriology *188*, 7853-7861.

Groisman, E.A. (2001). The pleiotropic two-component regulatory system PhoP-PhoQ. Journal of bacteriology *183*, 1835-1842.

Groisman, E.A., Chiao, E., Lipps, C.J., and Heffron, F. (1989). Salmonella typhimurium phoP virulence gene is a transcriptional regulator. Proceedings of the National Academy of Sciences of the United States of America *86*, 7077-7081.

Groisman, E.A., Heffron, F., and Solomon, F. (1992). Molecular genetic analysis of the Escherichia coli phoP locus. Journal of bacteriology *174*, 486-491.

Guani-Guerra, E., Santos-Mendoza, T., Lugo-Reyes, S.O., and Teran, L.M. (2010). Antimicrobial peptides: general overview and clinical implications in human health and disease. Clin Immunol *135*, 1-11.

Gunn, J.S., Hohmann, E.L., and Miller, S.I. (1996). Transcriptional regulation of Salmonella virulence: a PhoQ periplasmic domain mutation results in increased net phosphotransfer to PhoP. Journal of bacteriology *178*, 6369-6373.

Guo, L., Lim, K.B., Gunn, J.S., Bainbridge, B., Darveau, R.P., Hackett, M., and Miller, S.I. (1997). Regulation of lipid A modifications by Salmonella typhimurium virulence genes phoP-phoQ. Science *276*, 250-253.

Guo, L., Lim, K.B., Poduje, C.M., Daniel, M., Gunn, J.S., Hackett, M., and Miller, S.I. (1998). Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell *95*, 189-198.

Haneburger, I., Eichinger, A., Skerra, A., and Jung, K. (2011). New insights into the signaling mechanism of the pH-responsive, membrane-integrated transcriptional activator CadC of Escherichia coli. The Journal of biological chemistry *286*, 10681-10689.

Hanisch, J., Kolm, R., Wozniczka, M., Bumann, D., Rottner, K., and Stradal, T.E. (2011). Activation of a RhoA/myosin II-dependent but Arp2/3 complex-independent pathway facilitates Salmonella invasion. Cell host & microbe *9*, 273-285.

Hansen-Wester, I., and Hensel, M. (2001). Salmonella pathogenicity islands encoding type III secretion systems. Microbes and infection / Institut Pasteur *3*, 549-559.

Hantke, K., Nicholson, G., Rabsch, W., and Winkelmann, G. (2003). Salmochelins, siderophores of Salmonella enterica and uropathogenic Escherichia coli strains, are recognized by the outer membrane receptor IroN. Proceedings of the National Academy of Sciences of the United States of America *100*, 3677-3682.

Hapfelmeier, S., Ehrbar, K., Stecher, B., Barthel, M., Kremer, M., and Hardt, W.D. (2004). Role of the Salmonella pathogenicity island 1 effector proteins SipA, SopB, SopE, and SopE2 in Salmonella enterica subspecies 1 serovar Typhimurium colitis in streptomycin-pretreated mice. Infection and immunity *72*, 795-809.

Haraga, A., Ohlson, M.B., and Miller, S.I. (2008). Salmonellae interplay with host cells. Nature reviews Microbiology *6*, 53-66.

Harari, O., del Val, C., Romero-Zaliz, R., Shin, D., Huang, H., Groisman, E.A., and Zwir, I. (2009). Identifying promoter features of co-regulated genes with similar network motifs. BMC bioinformatics *10 Suppl 4*, S1.

Hardt, W.D., Chen, L.M., Schuebel, K.E., Bustelo, X.R., and Galan, J.E. (1998). S. typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell *93*, 815-826.

Hayward, R.D., and Koronakis, V. (1999). Direct nucleation and bundling of actin by the SipC protein of invasive Salmonella. Embo J *18*, 4926-4934.

Hensel, M., Shea, J.E., Gleeson, C., Jones, M.D., Dalton, E., and Holden, D.W. (1995). Simultaneous identification of bacterial virulence genes by negative selection. Science *269*, 400-403.

Hofmann, A.F., and Eckmann, L. (2006). How bile acids confer gut mucosal protection against bacteria. Proceedings of the National Academy of Sciences of the United States of America *103*, 4333-4334.

Humphreys, D., Davidson, A., Hume, P.J., and Koronakis, V. (2012). Salmonella virulence effector SopE and Host GEF ARNO cooperate to recruit and activate WAVE to trigger bacterial invasion. Cell host & microbe *11*, 129-139.

Humphreys, D., Davidson, A.C., Hume, P.J., Makin, L.E., and Koronakis, V. (2013). Arf6 coordinates actin assembly through the WAVE complex, a mechanism usurped by Salmonella to invade host cells. Proceedings of the National Academy of Sciences of the United States of America *110*, 16880-16885.

Johnson, B.A., and Blevins, R.A. (1994). NMR View: A computer program for the visualization and analysis of NMR data. Journal of biomolecular NMR *4*, 603-614.

Johnson, C.R., Newcombe, J., Thorne, S., Borde, H.A., Eales-Reynolds, L.J., Gorringe, A.R., Funnell, S.G., and McFadden, J.J. (2001). Generation and characterization of a PhoP homologue mutant of Neisseria meningitidis. Molecular microbiology *39*, 1345-1355.

Jolly, C., Winfree, S., Hansen, B., and Steele-Mortimer, O. (2014). The Annexin A2/p11 complex is required for efficient invasion of Salmonella Typhimurium in epithelial cells. Cell Microbiol *16*, 64-77.

Jones, B.D., and Falkow, S. (1996). Salmonellosis: host immune responses and bacterial virulence determinants. Annu Rev Immunol *14*, 533-561.

Jones, B.D., Ghori, N., Falkow, S.C.I.N.J.E.M.J., and Pmid (1994). Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. The Journal of experimental medicine *180*, 15-23.

Kabsch, W. (2010). Xds. Acta Crystallogr D Biol Crystallogr *66*, 125-132.

Kamal, J.K., Nazeerunnisa, M., and Behere, D.V. (2002). Thermal unfolding of soybean peroxidase. Appropriate high denaturant concentrations induce cooperativity allowing the correct measurement of thermodynamic parameters. The Journal of biological chemistry *277*, 40717-40721.

Karplus, P.A., and Diederichs, K. (2012). Linking crystallographic model and data quality. Science *336*, 1030-1033.

Kato, A., and Groisman, E.A. (2004). Connecting two-component regulatory systems by a protein that protects a response regulator from dephosphorylation by its cognate sensor. Genes Dev *18*, 2302-2313.

Kier, L.D., Weppelman, R.M., and Ames, B.N. (1979). Regulation of nonspecific acid phosphatase in Salmonella: phoN and phoP genes. Journal of bacteriology *138*, 155-161.

Kucharzik, T., Lugering, N., Rautenberg, K., Lugering, A., Schmidt, M.A., Stoll, R., and Domschke, W. (2000). Role of M cells in intestinal barrier function. Ann N Y Acad Sci *915*, 171-183.

LaRock, D.L., Chaudhary, A., and Miller, S.I. (2015). Salmonellae interactions with host processes. Nature reviews Microbiology *13*, 191-205.

Lee, E.J., Pontes, M.H., and Groisman, E.A. (2013). A bacterial virulence protein promotes pathogenicity by inhibiting the bacterium's own F1Fo ATP synthase. Cell *154*, 146-156.

Lee, S.W., Jeong, K.S., Han, S.W., Lee, S.E., Phee, B.K., Hahn, T.R., and Ronald, P. (2008). The Xanthomonas oryzae pv. oryzae PhoPQ two-component system is required for AvrXA21 activity, hrpG expression, and virulence. Journal of bacteriology *190*, 2183-2197.

Lejona, S., Aguirre, A., Cabeza, M.L., Garcia Vescovi, E., and Soncini, F.C. (2003). Molecular characterization of the Mg2+-responsive PhoP-PhoQ regulon in Salmonella enterica. Journal of bacteriology *185*, 6287-6294.

Lippa, A.M., and Goulian, M. (2009). Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. PLoS genetics *5*, e1000788.

Lippa, A.M., and Goulian, M. (2012). Perturbation of the oxidizing environment of the periplasm stimulates the PhoQ/PhoP system in Escherichia coli. Journal of bacteriology *194*, 1457-1463.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Llama-Palacios, A., Lopez-Solanilla, E., Poza-Carrion, C., Garcia-Olmedo, F., and Rodriguez-Palenzuela, P. (2003). The Erwinia chrysanthemi phoP-phoQ operon plays an important role in

growth at low pH, virulence and bacterial survival in plant tissue. Molecular microbiology *49*, 347-357.

Macfarlane, E.L., Kwasnicka, A., Ochs, M.M., and Hancock, R.E. (1999). PhoP-PhoQ homologues in Pseudomonas aeruginosa regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Molecular microbiology *34*, 305-316.

Majowicz, S.E., Musto, J., Scallan, E., Angulo, F.J., Kirk, M., O'Brien, S.J., Jones, T.F., Fazil, A., and Hoekstra, R.M. (2010). The global burden of nontyphoidal Salmonella gastroenteritis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America *50*, 882-889.

Martin-Orozco, N., Touret, N., Zaharik, M.L., Park, E., Kopelman, R., Miller, S., Finlay, B.B., Gros, P., and Grinstein, S. (2006). Visualization of vacuolar acidification-induced transcription of genes of pathogens inside macrophages. Mol Biol Cell *17*, 498-510.

Martinoli, C., Chiavelli, A., and Rescigno, M. (2007). Entry route of Salmonella typhimurium directs the type of induced immune response. Immunity *27*, 975-984.

Matamouros, S., Hager, K.R., and Miller, S.I. (2015). HAMP Domain Rotation and Tilting Movements Associated with Signal Transduction in the PhoQ Sensor Kinase. MBio *6*, e00616-00615.

McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., *et al.* (2001). Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. Nature *413*, 852-856.

Mellman, I., Fuchs, R., and Helenius, A. (1986). Acidification of the endocytic and exocytic pathways. Annu Rev Biochem *55*, 663-700.

Michetti, P., Mahan, M.J., Slauch, J.M., Mekalanos, J.J., and Neutra, M.R. (1992). Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen Salmonella typhimurium. Infection and immunity *60*, 1786-1792.

Miki, T., Okada, N., and Danbara, H. (2004). Two periplasmic disulfide oxidoreductases, DsbA and SrgA, target outer membrane protein SpiA, a component of the Salmonella pathogenicity island 2 type III secretion system. The Journal of biological chemistry *279*, 34631-34642.

Miller, S.I., Kukral, A.M., and Mekalanos, J.J. (1989). A two-component regulatory system (phoP phoQ) controls Salmonella typhimurium virulence. Proc Natl Acad Sci U S A *86*, 5054-5058.

Miller, S.I., and Mekalanos, J.J. (1990). Constitutive expression of the phoP regulon attenuates Salmonella virulence and survival within macrophages. J Bacteriol *172*, 2485-2490.

Miller, S.I., Pulkkinen, W.S., Selsted, M.E., and Mekalanos, J.J. (1990). Characterization of defensin resistance phenotypes associated with mutations in the phoP virulence regulon of Salmonella typhimurium. Infection and immunity *58*, 3706-3710.

Minagawa, S., Ogasawara, H., Kato, A., Yamamoto, K., Eguchi, Y., Oshima, T., Mori, H., Ishihama, A., and Utsumi, R. (2003). Identification and molecular characterization of the Mg2+ stimulon of Escherichia coli. Journal of bacteriology *185*, 3696-3702.

Molnar, K.S., Bonomi, M., Pellarin, R., Clinthorne, G.D., Gonzalez, G., Goldberg, S.D., Goulian, M., Sali, A., and DeGrado, W.F. (2014). Cys-Scanning Disulfide Crosslinking and Bayesian Modeling Probe the Transmembrane Signaling Mechanism of the Histidine Kinase, PhoQ. Structure *22*, 1239-1251.

Monsieurs, P., De Keersmaecker, S., Navarre, W.W., Bader, M.W., De Smet, F., McClelland, M., Fang, F.C., De Moor, B., Vanderleyden, J., and Marchal, K. (2005). Comparison of the PhoPQ regulon in Escherichia coli and Salmonella typhimurium. J Mol Evol *60*, 462-474.

Montagne, M., Martel, A., and Le Moual, H. (2001). Characterization of the catalytic activities of the PhoQ histidine protein kinase of Salmonella enterica serovar Typhimurium. Journal of bacteriology *183*, 1787-1791.

Moon, K., and Gottesman, S. (2009). A PhoQ/P-regulated small RNA regulates sensitivity of Escherichia coli to antimicrobial peptides. Molecular microbiology *74*, 1314-1330.

Moss, J.E., Fisher, P.E., Vick, B., Groisman, E.A., and Zychlinsky, A. (2000). The regulatory protein PhoP controls susceptibility to the host inflammatory response in Shigella flexneri. Cell Microbiol *2*, 443-452.

Mukherjee, S., and Hooper, L.V. (2015). Antimicrobial defense of the intestine. Immunity *42*, 28-39.

Muller, S., Dennemarker, J., and Reinheckel, T. (2012). Specific functions of lysosomal proteases in endocytic and autophagic pathways. Biochim Biophys Acta *1824*, 34-43.

Muller, S., Gotz, M., and Beier, D. (2009). Histidine residue 94 is involved in pH sensing by histidine kinase ArsS of Helicobacter pylori. PloS one *4*, e6930.

Murray, C.J., Vos, T., Lozano, R., Naghavi, M., Flaxman, A.D., Michaud, C., Ezzati, M., Shibuya, K., Salomon, J.A., Abdalla, S., *et al.* (2012). Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet *380*, 2197-2223.

Navarre, W.W., Halsey, T.A., Walthers, D., Frye, J., McClelland, M., Potter, J.L., Kenney, L.J., Gunn, J.S., Fang, F.C., and Libby, S.J. (2005). Co-regulation of Salmonella enterica genes required for virulence and resistance to antimicrobial peptides by SlyA and PhoP/PhoQ. Molecular microbiology *56*, 492-508.

Norte, V.A., Stapleton, M.R., and Green, J. (2003). PhoP-responsive expression of the Salmonella enterica serovar typhimurium slyA gene. Journal of bacteriology *185*, 3508-3514.

Nunez-Hernandez, C., Tierrez, A., Ortega, A.D., Pucciarelli, M.G., Godoy, M., Eisman, B., Casadesus, J., and Garcia-del Portillo, F. (2013). Genome expression analysis of nonproliferating intracellular Salmonella enterica serovar Typhimurium unravels an acid pH-dependent PhoP-PhoQ response essential for dormancy. Infection and immunity *81*, 154-165.

Ochman, H., Soncini, F.C., Solomon, F., and Groisman, E.A. (1996). Identification of a pathogenicity island required for Salmonella survival in host cells. Proceedings of the National Academy of Sciences of the United States of America *93*, 7800-7804.

Oh, Y.K., Alpuche-Aranda, C., Berthiaume, E., Jinks, T., Miller, S.I., and Swanson, J.A. (1996). Rapid and complete fusion of macrophage lysosomes with phagosomes containing Salmonella typhimurium. Infection and immunity *64*, 3877-3883.

Ouellette, A.J. (2011). Paneth cell alpha-defensins in enteric innate immunity. Cell Mol Life Sci *68*, 2215-2229.

Pegues, D.A., and Miller, S.I. (2014). Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, Vol 2 (Elsevier Health Sciences).

Perier, A., Chassaing, A., Raffestin, S., Pichard, S., Masella, M., Menez, A., Forge, V., Chenal, A., and Gillet, D. (2007). Concerted protonation of key histidines triggers membrane interaction of the diphtheria toxin T domain. J Biol Chem *282*, 24239-24245.

Pontes, M.H., Smith, K.L., De Vooght, L., Van Den Abbeele, J., and Dale, C. (2011). Attenuation of the sensing capabilities of PhoQ in transition to obligate insect-bacterial association. PLoS genetics *7*, e1002349.

Prost, L.R., Daley, M.E., Bader, M.W., Klevit, R.E., and Miller, S.I. (2008). The PhoQ histidine kinases of Salmonella and Pseudomonas spp. are structurally and functionally different: evidence that pH and antimicrobial peptide sensing contribute to mammalian pathogenesis. Molecular microbiology *69*, 503-519.

Prost, L.R., Daley, M.E., Le Sage, V., Bader, M.W., Le Moual, H., Klevit, R.E., and Miller, S.I. (2007a). Activation of the bacterial sensor kinase PhoQ by acidic pH. Mol Cell *26*, 165-174.

Prost, L.R., Sanowar, S., and Miller, S.I. (2007b). Salmonella sensing of anti-microbial mechanisms to promote survival within macrophages. Immunol Rev *219*, 55-65.

Prouty, A.M., Brodsky, I.E., Falkow, S., and Gunn, J.S. (2004). Bile-salt-mediated induction of antimicrobial and bile resistance in Salmonella typhimurium. Microbiology (Reading, England) *150*, 775-783.

Prouty, A.M., Van Velkinburgh, J.C., and Gunn, J.S. (2002). Salmonella enterica serovar typhimurium resistance to bile: identification and characterization of the tolQRA cluster. Journal of bacteriology *184*, 1270-1276.

Raffatellu, M., George, M.D., Akiyama, Y., Hornsby, M.J., Nuccio, S.P., Paixao, T.A., Butler, B.P., Chu, H., Santos, R.L., Berger, T., *et al.* (2009). Lipocalin-2 resistance confers an advantage to Salmonella enterica serotype Typhimurium for growth and survival in the inflamed intestine. Cell host & microbe *5*, 476-486.

Rathman, M., Sjaastad, M.D., and Falkow, S. (1996). Acidification of phagosomes containing Salmonella typhimurium in murine macrophages. Infection and immunity *64*, 2765-2773.

Rebeil, R., Jarrett, C.O., Driver, J.D., Ernst, R.K., Oyston, P.C., and Hinnebusch, B.J. (2013). Induction of the Yersinia pestis PhoP-PhoQ regulatory system in the flea and its role in producing a transmissible infection. Journal of bacteriology *195*, 1920-1930.

Richards, S.M., Strandberg, K.L., Conroy, M., and Gunn, J.S. (2012). Cationic antimicrobial peptides serve as activation signals for the Salmonella Typhimurium PhoPQ and PmrAB regulons in vitro and in vivo. Frontiers in cellular and infection microbiology *2*, 102.

Riesenberg-Wilmes, M.R., Bearson, B., Foster, J.W., and Curtis, R., 3rd (1996). Role of the acid tolerance response in virulence of Salmonella typhimurium. Infection and immunity *64*, 1085-1092.

Rosenberger, C.M., Gallo, R.L., and Finlay, B.B. (2004). Interplay between antibacterial effectors: a macrophage antimicrobial peptide impairs intracellular Salmonella replication. Proceedings of the National Academy of Sciences of the United States of America *101*, 2422-2427.

Sanowar, S., Martel, A., and Moual, H.L. (2003). Mutational analysis of the residue at position 48 in the Salmonella enterica Serovar Typhimurium PhoQ sensor kinase. Journal of bacteriology *185*, 1935-1941.

Schmitt, C.K., Ikeda, J.S., Darnell, S.C., Watson, P.R., Bispham, J., Wallis, T.S., Weinstein, D.L., Metcalf, E.S., and O'Brien, A.D. (2001). Absence of all components of the flagellar export and synthesis machinery differentially alters virulence of Salmonella enterica serovar Typhimurium in models of typhoid fever, survival in macrophages, tissue culture invasiveness, and calf enterocolitis. Infection and immunity *69*, 5619-5625.

Schrodinger, LLC (2010). The PyMOL Molecular Graphics System, Version 1.3r1.

Scocchi, M., Skerlavaj, B., Romeo, D., and Gennaro, R. (1992). Proteolytic cleavage by neutrophil elastase converts inactive storage proforms to antibacterial bactenecins. Eur J Biochem *209*, 589-595.

Selsted, M.E., Miller, S.I., Henschen, A.H., and Ouellette, A.J. (1992). Enteric defensins: antibiotic peptide components of intestinal host defense. The Journal of cell biology *118*, 929-936.

Shea, J.E., Hensel, M., Gleeson, C., and Holden, D.W. (1996). Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium. Proceedings of the National Academy of Sciences of the United States of America *93*, 2593-2597.

Shprung, T., Peleg, A., Rosenfeld, Y., Trieu-Cuot, P., and Shai, Y. (2012). Effect of PhoP-PhoQ activation by broad repertoire of antimicrobial peptides on bacterial resistance. The Journal of biological chemistry *287*, 4544-4551.

Soncini, F.C., Garcia Vescovi, E., Solomon, F., and Groisman, E.A. (1996). Molecular basis of the magnesium deprivation response in Salmonella typhimurium: identification of PhoP-regulated genes. Journal of bacteriology *178*, 5092-5099.

Soncini, F.C., Vescovi, E.G., and Groisman, E.A. (1995). Transcriptional autoregulation of the Salmonella typhimurium phoPQ operon. Journal of bacteriology *177*, 4364-4371.

Sperber, S.J., and Schleupner, C.J. (1987). Salmonellosis during infection with human immunodeficiency virus. Rev Infect Dis *9*, 925-934.

Thiennimitr, P., Winter, S.E., and Baumler, A.J. (2012). Salmonella, the host and its microbiota. Current opinion in microbiology *15*, 108-114.

Thiennimitr, P., Winter, S.E., Winter, M.G., Xavier, M.N., Tolstikov, V., Huseby, D.L., Sterzenbach, T., Tsolis, R.M., Roth, J.R., and Baumler, A.J. (2011). Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota. Proceedings of the National Academy of Sciences of the United States of America *108*, 17480-17485.

Tsolis, R.M., Adams, L.G., Ficht, T.A., and Baumler, A.J. (1999). Contribution of Salmonella typhimurium virulence factors to diarrheal disease in calves. Infection and immunity *67*, 4879-4885.

Vagin, A., and Teplyakov, A. (2010). Molecular replacement with MOLREP. Acta Crystallogr D Biol Crystallogr *66*, 22-25.

Vance, R.E., Isberg, R.R., and Portnoy, D.A. (2009). Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. Cell host & microbe *6*, 10-21.

Vandamme, D., Landuyt, B., Luyten, W., and Schoofs, L. (2012). A comprehensive summary of LL-37, the factotum human cathelicidin peptide. Cell Immunol *280*, 22-35.

Vazquez-Torres, A., Jones-Carson, J., Baumler, A.J., Falkow, S., Valdivia, R., Brown, W., Le, M., Berggren, R., Parks, W.T., and Fang, F.C. (1999). Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. Nature *401*, 804-808.

Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D.W., Lucia, S.M., Dinauer, M.C., Mastroeni, P., and Fang, F.C. (2000). Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science (New York, NY) *287*, 1655-1658.

Waldburger, C.D., and Sauer, R.T. (1996). Signal detection by the PhoQ sensor-transmitter. Characterization of the sensor domain and a response-impaired mutant that identifies ligand-binding determinants. The Journal of biological chemistry *271*, 26630-26636.

Wick, M.J., Harding, C.V., Twesten, N.J., Normark, S.J., and Pfeifer, J.D. (1995). The phoP locus influences processing and presentation of Salmonella typhimurium antigens by activated macrophages. Molecular microbiology *16*, 465-476.

Williamson, D.M., Elferich, J., Ramakrishnan, P., Thomas, G., and Shinde, U. (2013). The mechanism by which a propeptide-encoded pH sensor regulates spatiotemporal activation of furin. J Biol Chem *288*, 19154-19165.

Winter, S.E., Keestra, A.M., Tsolis, R.M., and Baumler, A.J. (2010a). The blessings and curses of intestinal inflammation. Cell host & microbe *8*, 36-43.

Winter, S.E., Thiennimitr, P., Winter, M.G., Butler, B.P., Huseby, D.L., Crawford, R.W., Russell, J.M., Bevins, C.L., Adams, L.G., Tsolis, R.M., *et al.* (2010b). Gut inflammation provides a respiratory electron acceptor for Salmonella. Nature *467*, 426-429.

Woof, J.M., and Kerr, M.A. (2006). The function of immunoglobulin A in immunity. J Pathol *208*, 270-282.

Zhang, S., Santos, R.L., Tsolis, R.M., Stender, S., Hardt, W.D., Baumler, A.J., and Adams, L.G. (2002). The Salmonella enterica serotype typhimurium effector proteins SipA, SopA, SopB, SopD, and SopE2 act in concert to induce diarrhea in calves. Infection and immunity *70*, 3843-3855.

Zhou, D., Chen, L.M., Hernandez, L., Shears, S.B., and Galan, J.E. (2001). A Salmonella inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. Molecular microbiology *39*, 248-259.

Zhou, D., Mooseker, M.S., and Galan, J.E. (1999). Role of the S. typhimurium actin-binding protein SipA in bacterial internalization. Science *283*, 2092-2095.

Zwir, I., Latifi, T., Perez, J.C., Huang, H., and Groisman, E.A. (2012). The promoter architectural landscape of the Salmonella PhoP regulon. Molecular microbiology *84*, 463-485.

Zwir, I., Shin, D., Kato, A., Nishino, K., Latifi, T., Solomon, F., Hare, J.M., Huang, H., and Groisman, E.A. (2005). Dissecting the PhoP regulatory network of Escherichia coli and Salmonella enterica. Proceedings of the National Academy of Sciences of the United States of America *102*, 2862-2867.