



Published in final edited form as:

Mol Microbiol. 2011 October ; 82(1): 145–163. doi:10.1111/j.1365-2958.2011.07804.x.

The broadly conserved regulator PhoP links pathogen virulence and membrane potential in *Escherichia coli*

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Summary

PhoP is considered a virulence regulator despite being conserved in both pathogenic and non-pathogenic *Enterobacteriaceae*. While *Escherichia coli* strains represent non-pathogenic commensal isolates and numerous virulent pathotypes, the PhoP virulence regulator has only been studied in commensal *E. coli*. To better understand how conserved transcription factors contribute to virulence, we characterized PhoP in pathogenic *E. coli*. Deletion of *phoP* significantly attenuated *E. coli* during extraintestinal infection. This was not surprising since we demonstrated that PhoP differentially regulated the transcription of >600 genes. In addition to survival at acidic pH and resistance to polymyxin, PhoP was required for repression of motility and oxygen-independent changes in the expression of primary dehydrogenase and terminal reductase respiratory chain components. All phenotypes have in common a reliance on an energized membrane. Thus, we hypothesized that PhoP mediates these effects by regulating genes encoding proteins that generate proton motive force. Indeed, bacteria lacking PhoP exhibited a hyper-polarized membrane and dissipation of the transmembrane electrochemical gradient increased susceptibility of the *phoP* mutant to acidic pH, while inhibiting respiratory generation of the proton gradient restored resistance to antimicrobial peptides independent of lipopolysaccharide modification. These findings demonstrate a connection between PhoP, virulence, and the energized state of the membrane.

Keywords

uropathogenic; proton motive force; prophage; UTI; antimicrobial peptide

Introduction

Signal transduction in bacteria mediates changes in gene expression in response to specific external cues. Bacterial pathogens must respond to cues within the host environment to survive immune defense mechanisms and produce virulence factors. The response regulator PhoP has been studied primarily in *Salmonella enterica* serovar Typhimurium (herein referred to as *Salmonella*) where it regulates virulence in response to signals encountered by the bacterium within the mammalian host (Soncini *et al.*, 1996, Prost *et al.*, 2007, Bader *et al.*, 2005). In contrast, PhoP has been proposed to regulate genes that promote survival in non-host environments for commensal *E. coli* (Zwir *et al.*, 2005). Because PhoP and its cognate sensor kinase PhoQ are broadly conserved among many pathogenic and non-pathogenic bacterial species, it has been generally accepted that this two-component

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regulatory system has evolved to control virulence in pathogens while retaining core functions in non-pathogens (Winfield & Groisman, 2004).

PhoP was first identified and suggested to perform a regulatory function in *Salmonella* because a strain with a mutation in *phoP* demonstrated aberrant non-specific acid phosphatase activity (Kier *et al.*, 1979). The relationship between PhoP and virulence was later established by the identification of *phoP* in a genetic screen that selected for mutants in *Salmonella* that had impaired survival in macrophages (Fields *et al.*, 1986) and the finding that *phoP* mutants are attenuated *in vivo* (Fields *et al.*, 1989, Miller *et al.*, 1989, Galan & Curtiss, 1989). Attenuation of virulence is related to the *phoP* mutant's exquisite susceptibility to extracts prepared from neutrophil granules and purified defensins (Fields *et al.*, 1989, Miller *et al.*, 1990). The mechanism responsible for the connection between PhoP and *Salmonella* virulence is the ability to activate lipopolysaccharide (LPS) modification (Bader *et al.*, 2005, Guo *et al.*, 1997, Bader *et al.*, 2003) and acid resistance genes in direct response to cationic antimicrobial peptides and acidic pH produced by neutrophils and macrophages (Alpuche Aranda *et al.*, 1992, Prost *et al.*, 2007, Bearson *et al.*, 1998). PhoP can also be phosphorylated and control its large regulatory network in response to low concentrations of Ca^{2+} and Mg^{2+} at the periplasmic face of the bacterial cytoplasmic membrane (Soncini *et al.*, 1996).

Synergism between antimicrobial peptides, acidic pH, and the oxidative burst is critical for bacterial killing by phagocytes (Babior, 1978, Vazquez-Torres *et al.*, 2000). Unlike antimicrobial peptides that either permeabilize or traverse the Gram negative bacterial outer membrane and facilitate acidification of the periplasm to directly activate PhoPQ (Bader *et al.*, 2003, Bader *et al.*, 2005), reactive oxygen species produced by phagocytes can freely access the bacterial periplasm (Borregaard *et al.*, 1984, Korshunov & Imlay, 2002). In *Salmonella*, PhoP controls production of the phage-encoded superoxide dismutase SodCI (Golubeva & Slauch, 2006) that is required for host colonization because it protects the cell from killing by the phagocyte oxidase (Figueroa-Bossi & Bossi, 1999, De Groote *et al.*, 1997). Collectively, periplasmic SodCI, enzymes that modify the outer leaflet LPS on the outer membrane, and acid resistance functions are well known PhoP-dependent responses that prevent pathogen killing by host neutrophils and macrophages.

Pathogens, whether intracellular or extracellular, must possess mechanisms to avoid being cleared by host neutrophils or macrophages. Although PhoP has been characterized extensively in the intracellular pathogen *Salmonella*, and in non-pathogenic strains of *E. coli* (Minagawa *et al.*, 2003, Monsieurs *et al.*, 2005), its role in controlling virulence gene expression in pathogenic *E. coli* is largely unknown. We reasoned that *phoP* would be critical for pathogenic *E. coli* virulence because of the central role played by the PhoP regulatory system in pathogen survival in a mammalian host (Miller *et al.*, 1989, Oyston *et al.*, 2000). The integration of pathogenicity islands and lysogenic bacteriophages into the *E. coli* genome has created distinct *E. coli* intestinal and extraintestinal pathotypes that cause a spectrum of diseases in humans (Welch *et al.*, 2002). We chose to study PhoP in extraintestinal pathogenic *E. coli* because this pathotype is the only pathogenic *E. coli* exclusively capable of causing disseminated infections in humans.

Our findings demonstrate that loss of *phoP* attenuates uropathogenic *E. coli* virulence during extraintestinal infection. Indeed, among the hundreds of genes that were differentially expressed between wild-type bacteria and the *phoP* mutant are those required for motility, acid survival, and lipopolysaccharide modification. These findings paralleled *phoP* mutant phenotypes of hyper-motility, decreased survival at pH 2.5, and increased susceptibility to antimicrobial peptides, respectively. Alterations in the expression of genes encoding respiratory chain components also occurred in the *phoP* mutant, suggesting that the

cytoplasmic membrane is hyper-polarized in the absence of PhoP. All of the described phenotypes have in common a reliance on an energized membrane. This led us to hypothesize that PhoP control of the energized bacterial membrane represents a conserved function that connects pathogen virulence and normal physiology. In this study, we provide evidence for this assertion.

Results

PhoP is required for *E. coli* virulence during acute urinary tract infection

Although PhoP has not been studied in pathogenic *E. coli*, results from studies in *Salmonella* led to the assumption that PhoP is likely a regulator of virulence in *E. coli*. To address this, we generated a *phoP* deletion mutant in *E. coli* CFT073, a strain isolated from the blood and urine of a hospitalized patient with acute pyelonephritis (Welch et al., 2002, Mobley et al., 1990). Using a well-established murine model of ascending infection, we found that deletion of *phoP* resulted in nearly complete attenuation of strain CFT073 during acute cystitis, where 80% of mice lacked detectable bacteria in the bladder 48 h after transurethral independent challenge ($P=0.0257$) (Fig. 1A). The *phoP* mutant, complemented with *phoPQ* on a plasmid, had >100-fold increase in median CFU/g of bladder when compared to Δ *phoP* containing plasmid vector alone ($P=0.0068$). As for the wild-type strain, the complemented mutant had detectable bacteria in 100% of mice at 48 h post-inoculation (Fig. 1B).

Identification of the pathogenic *E. coli* PhoP regulon

To seek an explanation for attenuation of the *phoP* mutant and to define the pathogenic *E. coli* PhoP regulon, we identified genes with significant changes in expression between the parental CFT073 strain and the *phoP* mutant using microarrays that contained probes representing all 5,379 CFT073 ORFs. The transcription profiles between the parental CFT073 strain and the *phoP* mutant were compared from aerated, mid-exponential phase cultures in LB medium ($OD_{600}=0.6$). We reasoned this would identify genes regulated directly or indirectly by PhoP because (i) LB medium is sufficiently Mg^{2+} -limited to activate *phoPQ*-dependent gene expression (Bishop et al., 2004, Jia et al., 2004, Garcia Vescovi et al., 1996), and (ii) each *phoP*-dependent phenotype was tested using bacteria cultured under these growth conditions. The microarray analysis showed that deletion of *phoP* resulted in the differential expression of 620 genes ($P<0.05$). Of these, 306 and 314 were directly or indirectly activated or repressed by PhoP, respectively (Fig. 2).

The transcription profile of the *phoP* mutant (Fig. 2, outer ring) demonstrated activation (green bars) of the colibactin polyketide genotoxin locus (Nougayrede et al., 2006) encoded on PAI-CFT073-*asnW*, activation of acid fitness island (AFI) genes, repression (red bars) of 36 flagellar and chemotaxis genes (Table S1), and repression of 139 genes encoded within prophage islands are PhoP-dependent. The CFT073 genome contains 14 pathogenicity islands including 5 prophage regions that are absent in the commensal *E. coli* K-12 strain MG1655 (Fig 2, central blue ring). The gene expression data are consistent with previous studies that described the activation and repression of a similarly large number of genes controlled by PhoP in commensal *E. coli* (Minagawa et al., 2003, Monsieurs et al., 2005) and *Salmonella* (Bader et al., 2003, Zwir et al., 2005).

Assessment of established PhoP phenotypes

As predicted by the established role of PhoP in regulating polymyxin and acid resistance genes, deletion of *phoP* in pathogenic *E. coli* resulted in significantly decreased transcription of *arnBCADTEF*, which encode enzymes that catalyze LPS modification by the addition of UDP-4-amino-4-deoxy-L-arabinose to lipid A (Gunn et al., 1998), and genes that encode structural components of the glutamate-, arginine-, and lysine-dependent acid resistance

(AR) systems (Foster, 2004) (Table 1). Indeed, the LPS and AR phenotypes were clearly affected by mutation of *phoP*. *E. coli* strains produce all of the enzymes necessary for the aminoarabinose modification of LPS; this promotes resistance to host antimicrobial peptides (Trent *et al.*, 2001). This modification to LPS is induced by PhoP in *Salmonella*, however, a role for PhoP in L-Ara4N modification of lipid A has been presumed to be absent in *E. coli* because low $[Mg^{2+}]$ failed to promote resistance to an antimicrobial peptide in a commensal strain, and *E. coli* lacks a functional PmrD that links PhoP to the PmrA regulon (Winfield & Groisman, 2004). Although low $[Mg^{2+}]$ can induce palmitoylation of lipid A (Guo *et al.*, 1998) and antimicrobial peptides can induce expression of the PhoP regulon independent of $[Mg^{2+}]$ (Bader *et al.*, 2005), a *phoP* mutant in *E. coli* has not been directly tested for resistance to antimicrobial peptides. To determine if PhoP affects this virulence property, we assessed the survival of the *phoP* mutant when exposed to bactericidal concentrations of the antimicrobial peptide polymyxin B. Following exposure to polymyxin B, we found that deletion of *phoP* in pathogenic *E. coli* resulted in a 100-fold reduction in CFU (Fig. 1C), where the *phoP* mutant displayed only 1% the survival rate of the wild-type strain or complemented mutant (Fig. 1D). The increased polymyxin sensitivity of the *phoP* mutant relative to wild-type was independent of $[Fe^{3+}]$ (Fig. S1), a signal known to induce expression of LPS modification genes through activation of the iron-responsive *basR* (*pmrA*) transcription factor (Winfield & Groisman, 2004).

A key role for PhoP in *Salmonella* is to activate LPS modification and other virulence genes during exposure to acidic pH generated by neutrophils and macrophages in host tissues (Alpuche Aranda *et al.*, 1992). A common link between PhoP in pathogens and non-pathogens may feature the response to acidic pH because commensal *E. coli* lacking *phoP* has impaired survival at pH 2.5 (Zwir *et al.*, 2005). Consistent with PhoP regulating acid resistance genes, the pathogenic *E. coli phoP* mutant displayed decreased viability when shifted from neutral conditions to pH 2.5 (Fig. 1E); the increased susceptibility to extreme acid could be reversed by complementing the *phoP* mutation with *phoPQ* (Fig. 1F).

Deletion of *phoP* results in a hyper-motile phenotype

It was also possible to phenotypically validate the gene-chip data by examining motility in soft agar and assessing flagellin production by western blot. In these studies, as expected by the increased flagellar gene transcription in the absence of PhoP, the *phoP* mutant displayed a hyper-motile phenotype and increased production of FliC (Fig. 3). The increased expression of class II (*fliA*) and class III (*fliC*) flagellar genes was confirmed by qPCR and could be independent of class I transcription because expression of *flhDC* appeared unaffected in the *phoP* mutant (Fig. S2). Despite significant increases in the expression of 36/50 flagellar genes in the absence of *phoP* (Table S1), the precise PhoP-mediated control of motility also specifically excluded 5 of 6 type III protein export apparatus components (*flhA*, *flhB*, *fliP*, *fliQ*, *fliR*, but not *fliO*) (Fig. S3). Hyper-motility and increased FliC production in the *phoP* mutant, was restored to normal levels observed in the wild-type strain by complementation with the wild-type *phoPQ* allele from either the parental CFT073 or commensal K12 strain MG1655 (Fig. 3).

Loss of PhoP creates a hyper-polarized membrane

Decreased survival at acidic pH, greater susceptibility to polymyxin B, and increased motility, all directly resulted from the loss of PhoP. Each phenotype potentially shares a common dependence on the energized membrane or would be affected by perturbations in membrane potential. For example, hyper-motility, as observed in the absence of PhoP, could be due to an increase in membrane potential that would generate a large proton motive force and lead to increased proton translocation through the stator complex that drives rotation of the flagellum to propel bacteria. Consistent with the hypothesis that the PhoP regulon

controls membrane potential, genes encoding proteins involved in electron transfer, respiration, and ion transport were also shown to be regulated by PhoP in the microarray experiments. Specifically, the altered expression of genes encoding alternative respiratory chain components in *phoP* mutant bacteria indicated a connection between PhoP and membrane energetics. For example, loss of PhoP resulted in significantly decreased expression of cytochrome bd-II oxidase subunits *appB* and *appC*, the electron transfer NADH:quinone oxidoreductase *wrbA*, and the NADPH:quinone oxidoreductase *qor* ($P < 0.05$) (Table 2). In bacteria with a functional PhoP response, PhoP-dependent activation of genes encoding terminal oxidase cytochrome bd-II App and quinone oxidoreductases WrbA and Qor would greatly reduce the extrusion of protons during respiration because these non-electrogenic respiratory components (OH^+/e) do not pump protons (Bekker *et al.*, 2009, Natalello *et al.*, 2007). Despite aerobic logarithmic growth conditions in the absence of exogenous nitrate, microarray data also indicated that the respiratory nitrate reductase (NRZ) transcripts *narU*, *narW*, and *narV* were decreased 6.0- to 15.0-fold in the *phoP* mutant ($P < 0.01$), while the expression of periplasmic nitrate reductase cytochrome c (Nap) was increased 2.0- to 4.0-fold ($P < 0.03$) (Table 2). The anaerobic hydrogenase 1 b-type cytochrome *hyaABCD* that is normally induced under anaerobiosis and repressed by nitrate demonstrated a 6.0- to 12.0-fold decrease ($P < 0.01$) in transcription in the absence of PhoP (Table 2).

PhoP-dependent modulation of primary dehydrogenase and terminal reductase respiratory component gene expression would configure electron transfer systems that limit generation of the proton motive force (μH^+). μH^+ is composed of two components, ΔpH and $\Delta\psi$, and reflects the electrochemical gradient of protons and charge, respectively, across the cytoplasmic membrane. Consistent with our assertion that PhoP controls the expression of genes that encode proteins that affect inner transmembrane potential, a number of ion transport genes [*yhiD* (*mgtC*) Mg^{2+} ; *ybaR* Cu^{2+} ; *yadQ* Cl^- ; and *trkA* K^+] displayed significantly decreased transcription ($P < 0.05$) in the *phoP* mutant (Table 2).

To determine if PhoP controls membrane potential in *E. coli*, we used the carbocyanine dye 3'3'-diethyloxacarbocyanine iodide (DiOC_2) to measure relative changes in membrane potential between wild-type CFT073 and the *phoP* mutant. DiOC_2 exhibits green fluorescence in all bacterial cells and its emission shifts to red fluorescence when the dye self-associates at high intracellular concentrations (Sims *et al.*, 1974). Accumulation of intracellular DiOC_2 is directly dependent on membrane potential (positive outside, negative inside). The red:green ratio of DiOC_2 was over 2-fold greater for logarithmic phase ΔphoP cells than the ratio observed for wild-type CFT073 ($P < 0.05$) (Fig. 4A). This difference between wild-type and *phoP* mutant bacteria was notable because CFT073 lacking fumarase activity ($\Delta\text{fumA}\Delta\text{fumB}\Delta\text{fumC}$), which is defective in generating μH^+ due to the disruption of aerobic and anaerobic fumarate conversions, exhibited a 2-fold reduction in red:green emission as compared to wild-type bacteria (Fig. 4A). Thus, the increased DiOC_2 uptake in *phoP* mutant bacteria results from a hyper-polarized membrane caused by the absence of PhoP. CFT073 mutant bacteria lacking non-electrogenic respiratory components of the TCA cycle ($\Delta\text{sdhB}\Delta\text{frdB}$), which do not extrude protons during electron transfer, do not exhibit any difference in the ratio of red:green fluorescence (Fig. 4A). Depolarizing the electrochemical gradient across the inner membrane using the protonophore *m*-chlorophenyl carbonyl cyanide hydrozone (CCCP) collapsed the proton gradient and resulted in the expected loss of red fluorescence for all strains (Fig. 4A). These data demonstrated that controlling membrane potential or limiting generation of μH^+ is a PhoP-dependent function that could also contribute to PhoP-mediated activities such as survival at acidic pH or resistance to cationic antimicrobial peptides.

PhoP-dependent LPS modification is necessary but not sufficient for resistance to polymyxin B

The most widely accepted strategy to resist killing by cationic bactericidal compounds is to reduce the negative charge of the outer membrane and decrease the affinity of the charged peptides for the outer membrane by modification of the outer leaflet LPS. Aminoarabinose modification of the LPS structure requires *arnBCADTEF* and has been shown previously to be induced by low pH in *E. coli* (Trent et al., 2001). Because the operon that encodes the enzymes required to synthesize L-Ara4N was induced by PhoP (Table 1), we generated an *arnT* deletion mutant in CFT073 to determine if bacteria lacking lipid A modification with L-Ara4N would have a polymyxin B susceptibility phenotype similar to bacteria lacking PhoP. We chose to inactivate *arnT* because 4-amino-4-deoxy-L-arabinose transferase activity is required for the attachment of L-Ara4N to lipid A and is critical for resistance to polymyxin B (Breazeale et al., 2005). Experiments were performed in the presence of 10 μM Fe^{3+} to induce expression of *pmrA* (*basR*) because PmrA-dependent lipid A modification can occur independently from PhoP due to a non-functional *pmrD* in *E. coli* (Wosten et al., 2000, Winfield & Groisman, 2004). We found that deletion of *arnT* in CFT073 resulted in an intermediate level of polymyxin B sensitivity between wild-type and the *phoP* mutant (Fig. 4B). We reasoned that an additional modification of lipid A or the loss of the PhoP-dependent alteration of potential across the inner membrane could be responsible for the difference in antimicrobial peptide sensitivity between $\Delta\textit{phoP}$ and $\Delta\textit{arnT}$ bacteria.

Respiratory generation of proton motive force is required for bactericidal activity of polymyxin B

We tested the relationship between μH^+ and PhoP by disrupting the electrochemical gradient and determining resistance to polymyxin B. By uncoupling oxidative phosphorylation from μH^+ generation using sodium azide (NaN_3) or depolarizing the membrane using CCCP, we observed an increase in the *phoP* mutant strain's resistance to antimicrobial peptides to wild-type levels (Fig 5A). In addition, finding that NaN_3 or CCCP also increased wild-type resistance to polymyxin B supported the notion that, in general, reducing the proton gradient across the bacterial inner membrane is an effective means to abrogate the bactericidal effects of cationic antimicrobial compounds (Matsuzaki et al., 1995). Dissipating the electrochemical gradient with CCCP or NaN_3 and washing the cells to remove uncoupler prior to exposing either CFT073 or *phoP* mutant bacteria to polymyxin B was not sufficient to protect the cells (Fig 5B). This suggested that the protective effect of reducing μH^+ is not a pre-set state of the membrane.

Excess Mg^{2+} most likely protects cells from antimicrobial peptides through competitive inhibition at negatively charged sites on the LPS. We found that excess Mg^{2+} , which would increase the net surface charge of outer membrane LPS (Vaara, 1992) and could contribute to reversal of inner transmembrane $\Delta\psi$, protected both wild-type and $\Delta\textit{phoP}$ bacteria from killing by polymyxin B, similar to the protection afforded by the addition of CCCP and NaN_3 (Fig. 5C). This Mg^{2+} -protection against polymyxin B would be predicted to be independent of *phoP*, which would be repressed under this condition. Because PhoP is involved in the maintenance of a robust permeability barrier (Murata et al., 2007), we also tested polymyxin resistance in combination with EDTA, a compound that chelates divalent cations and renders the bacterial LPS and outer membrane more permeable for antimicrobial peptides. We found that EDTA increased sensitivity to polymyxin B for both wild-type CFT073 and the *phoP* mutant and that CFT073 maintained greater resistance to polymyxin than the *phoP* mutant strain (Fig. 5C).

Treatment of *E. coli* or *Salmonella* with millimolar concentrations of EDTA is known to induce lipid A palmitoylation mediated by PagP (Bishop *et al.*, 2000). We constructed CFT073 strains lacking *pagP* (*crcA* in *E. coli*) and a double mutant lacking *pagP* and *arnT* to further differentiate the contribution of LPS modifications or permeability defects at the outer membrane from changes in the transmembrane potential across the inner membrane. Susceptibility to polymyxin B was determined in the presence of 10 μ M Fe³⁺ to activate PmrA and to avoid potential cross-regulatory effects between PhoP and PmrA. In the absence of EDTA, the *arnT* mutant bacteria displayed a 5-fold decrease in polymyxin B resistance relative to wild-type, while the *arnTpagP* double mutant demonstrated susceptibility similar to the *phoP* mutant despite the *pagP* mutant being nearly indistinguishable from wild-type (Fig. 5D). All the bacteria tested, when pre-cultured in 0.5 mM EDTA and exposed to polymyxin B and EDTA demonstrated a marked increase in susceptibility to polymyxin B with 0.5 mM EDTA, (Fig. 5D). Interestingly, while CCCP abolished killing by polymyxin B for all strains and constructs tested, only the LPS modification mutants lacked the same magnitude increase in resistance to killing by polymyxin B as the *phoP* mutant bacteria when respiratory cytochromes were uniformly inhibited by NaN₃ (Fig. 5D). These changes in both outer membrane LPS charge and inner membrane potential appear to reflect an active response because pre-treatment or culturing CFT073 and Δ *phoP* with EDTA, NaN₃, CCCP, or excess [Mg²⁺] alone did not significantly affect bacterial viability, nor did pre-treatment alter the polymyxin resistance phenotype (Fig. S4). Together, these findings provide evidence that controlling μ H⁺ represents a mechanism for PhoP-mediated antimicrobial peptide resistance, independent from LPS modification and maintaining a robust permeability barrier in *E. coli*.

Acid resistance requires PhoP-dependent changes in μ H⁺

Because resistance to acidic pH has been shown to involve enzymatic consumption of cytoplasmic protons and a reversal in membrane potential (Foster, 2004, Richard & Foster, 2004), we determined if the survival defect of the *phoP* mutant at acidic pH is due to the inability to alter respiratory components, reverse membrane potential, or consume intracellular protons in response to acid induction of PhoP. CCCP specifically decreased the ability of the *phoP* mutant but not that of wild-type bacteria to survive when shifted from neutral pH to pH 5.0 (Fig. 6). These findings demonstrated that the role for PhoP-activated genes encoding glutamate-, lysine-, and arginine-decarboxylases that consume cytoplasmic protons is distinct from PhoP regulation of genes encoding proteins that control membrane potential because wild-type CFT073 was unaffected by CCCP in these experiments (Fig. 6). The increased sensitivity to acid for *phoP* mutant bacteria was independent of respiratory generation of μ H⁺ because NaN₃ did not alter susceptibility to acid for either wild-type or the mutant bacteria (Fig. 6). The lack of resistance to moderate acid for the *phoP* mutant during membrane depolarization is notable because *phoP* mutant bacteria did not have a survival defect at pH 5.0 in the absence of CCCP nor did CCCP treatment at neutral pH cause a reduction in viability (Fig. 6).

PhoP represses the induction of a fully functional lysogenic bacteriophage

Synergism between acidification, antimicrobial peptides, and reactive oxygen species allows neutrophils and macrophages to eradicate bacterial pathogens from host tissues (Babior, 1978, Vazquez-Torres *et al.*, 2000). Consistent with a role for PhoP regulating genes that encode proteins for bacterial defense against oxidative attack, expression of superoxide dismutase (*sodC*) and catalase (*katG*) genes was significantly decreased >4.0-fold ($P < 0.05$) in the absence of PhoP (Table 2). PhoP was also required for induction of genes that code for the Suf system that is involved in Fe-S cluster repair during oxidative damage (Table 2). When reactive oxygen species breach the inner membrane, superoxides damage Fe-S-containing proteins (Jang & Imlay, 2007) and promote the release of free iron, the latter

rapidly induces DNA damage by driving Fenton reactions (Park & Imlay, 2003). Oxidative DNA damage can cause prophage induction (Little, 2005), which could occur during exposure to reactive oxygen species generated by the phagocyte NADPH oxidase. In keeping with the hypothesis that PhoP controls a large number of genes that encode a diverse set of proteins involved in comprehensive protection against bactericidal activities of phagocytes (acidification, antimicrobial peptides, and the oxidative burst), the microarray results also indicated that more than 100 genes in prophage islands are repressed by PhoP (Fig. 2). To test phage induction in the absence of PhoP-mediated repression of prophage genes, we assayed culture supernatants from both the *phoP* mutant and parental CFT073 for the ability to form plaques on a restriction/modification (r^-/m^-)-deficient *E. coli* strain (Bertani & Weigle, 1953). Supernatant prepared from logarithmic phase CFT073 produced few (<10) plaques (Fig. 7A), while supernatant from *phoP* mutant bacteria consistently produced significantly more plaques (>100) (Fig. 7B). That CFT073 carries an active phage was not previously appreciated. By quantifying burst size (no. plaques/CFU), we found that mitomycin C (MMC), a potent inducer of DNA damage and phage lambda in *E. coli* (Little, 2005), caused maximal prophage induction for both CFT073 and *phoP* mutant bacteria. However, the burst size in the absence of MMC induction was over 10-fold greater for the *phoP* mutant than for wild-type bacteria (Fig. 7E). Finding that bacteria lacking PhoP have increased phage induction suggests that PhoP-mediated repression of prophage genes would increase bacterial survival by preventing lytic phage replication during oxidative damage caused by the phagocyte NADPH oxidase. To exclude the possibility that the plaques originated from the indicator strain, PCR amplification of phage lysate produced in the (r^-/m^-) strain using probes specific for each predicted cI repressor gene from the CFT073 genome (Fig. 7F) indicated that the active prophage (Φ_{icdA}) originated from the CFT073 chromosome.

Discussion

Our results suggest that PhoP targets, represented by seemingly unrelated outputs of PhoP activity (sensitivity to acid, antimicrobial peptide resistance, and motility) act through a common mechanism that is dependent on the energized state of the membrane (Glagolev & Skulachev, 1978, Foster, 2004, Grundling *et al.*, 2001). Coordinating gene expression with changes in membrane potential or regulating genes that encode proteins that control the state of the energized membrane is relevant for both pathogens avoiding host defenses and for non-pathogens surviving in the environment. PhoP regulation of genes encoding products that control membrane potential is supported by PhoP-dependent activation of transcription of genes encoding non-electrogenic respiratory chain components that do not pump protons, ion importers that actively transport cations against an electrochemical gradient, and cytoplasmic enzymes that mediate acid resistance (Fig. 8). The loss of PhoP-mediated control of the energized membrane is manifested by decreased survival at acidic pH, increased susceptibility to polymyxin B, and hyper-motility in *phoP* mutant bacteria.

By decreasing flagellar gene transcription, reducing motility, and activating acid resistance responses, proteins encoded by genes regulated by PhoP would affect ΔpH by limiting an increase in internal $[H^+]$ and preventing acidification of the cytoplasm. The results of experiments where maintenance of cytoplasmic pH was abolished by membrane depolarization with CCCP (Matin *et al.*, 1982, Krulwich *et al.*, 1978) suggest that the loss of PhoP-activation of genes encoding the cytoplasmic enzymes glutamate-, lysine-, and arginine-decarboxylases and antiporters that consume protons and extrude decarboxylated products, respectively, could account for the specificity of the protonophore's effect on acid survival for bacteria lacking *phoP*. In addition to consuming intracellular protons, AR responses in *E. coli* and other bacteria have been previously shown to promote survival in extreme acid by reversing membrane potential $\Delta\psi$ to reduce proton influx (Richard &

Foster, 2004, Konings *et al.*, 2002, Michels & Bakker, 1985). Because the weak acid CCCP freely diffuses through phospholipid bilayers, discharges ΔpH by release of the ionizable H^+ inside the cytoplasm, and leaves the cell as an anion, destroying $\Delta\psi$, the specific mechanism controlled by PhoP to promote survival during exposure to acidic pH, appears dependent on both ΔpH and $\Delta\psi$. The ΔpH -dependence of the PhoP acid response is demonstrated by reduced survival of the ΔphoP mutant at pH 5.0 only when the ΔpH is discharged with CCCP. This defect is caused by the inability to activate genes that encode cytoplasmic enzymes that consume protons, while the inability to regulate genes that encode functions required for $\Delta\psi$ reversal results in reduced survival at pH 2.5, in the absence of PhoP. Consistent with this hypothesis, using the membrane potential-dependent uptake of DiOC_2 we demonstrated that loss of PhoP creates a hyper-polarized membrane (more negative-inside); PhoP-dependent activation of the Acid Fitness Island (AFI) genes, and genes encoding P-type ATPases that import cations: YhiD (MgtC), YbaR, TrkA, and the Cl^- anion efflux channel YadQ would prevent hyperpolarization during acid stress (positive-inside) and would contribute to $\Delta\psi$ reversal (Iyer *et al.*, 2002). It is possible that bacteria lacking *phoP* could have increased DiOC_2 uptake due to an altered permeability barrier (Murata *et al.*, 2007). However, hyper-motility, acid susceptibility, decreased expression of genes encoding non-electrogenic respiratory components, regulation of genes encoding ion transporters, and PhoP-dependent manipulation of polymyxin resistance by poisoning respiration, taken together, add up to compelling evidence that PhoP control of membrane potential represents the unifying mechanism to resist host antibacterial defenses.

PhoP-mediated control of membrane potential is further supported by the well-known PhoP activation of *mgtA* and genes encoding additional magnesium transporters during growth in the absence of divalent cations (Soncini *et al.*, 1996). These P-type ATPases mediate active import of ions against an electrochemical gradient. Upon first consideration, this may seem like an unusual property since bacterial cells are normally inside-negative allowing polyvalent cations like magnesium to freely diffuse into the cell through the constitutively active CorA channel (Maguire, 2006). However, a PhoP-activated cell, in our model, would be positive-inside and thus would require active transport to import these positively charged ions (Fig. 8). Indeed, active transport of ions against an electrochemical gradient is particularly intriguing given that activated phagocytes, which generate acidification and antimicrobial peptides to kill microbes, require counter-ion conductance and voltage-gated channels that prevent host cell membrane depolarization to sustain NADPH oxidase function and effectively control bacterial infections (DeCoursey *et al.*, 2003, Di *et al.*, 2006, Forbes & Gros, 2001). Specifically, phagocyte killing of bacteria is known to require the cation/ H^+ transporter Nramp1 (Gruenheid *et al.*, 1997, Forbes & Gros, 2003) and the CFTR Cl^- channel (Di *et al.*, 2006). Bacteria, in turn, would benefit by reversing membrane potential or reducing their inside-negative potential in order to resist killing by these host defenses. Furthermore, ion transporters in both *Salmonella* and *E. coli* have been shown to be involved in the response to reactive oxygen (Kehres *et al.*, 2000). The importance of energy transformations by the bacterial membrane having a key role during survival against host defenses is also supported by a recent study by Karlinsey *et al.* that demonstrated maintenance of proton motive force, μH^+ , is required for *Salmonella* virulence in Nramp1-expressing mice (Karlinsey *et al.*, 2010).

Self-promoted uptake of host-derived antimicrobial peptides by bacteria has been proposed to be μH^+ -dependent (Falla *et al.*, 1996, Matsuzaki *et al.*, 1995). Consistent with this, we found that dissipating μH^+ with CCCP abrogated the bactericidal effects of polymyxin B. The contribution of charge modifications to the LPS in the outer membrane to resistance to antimicrobial peptides is well documented; in this study, in contrast to *phoP* mutant bacteria, the susceptibilities of the ΔarnT and ΔpagP mutant bacteria or the $\Delta\text{arnT}\Delta\text{pagP}$ double mutant to polymyxin B were unaffected by uniformly inhibiting respiratory cytochromes

with NaN_3 . To distinguish PhoP-dependent sensitivity to polymyxin from PmrA-dependent effects, we maintained high $[\text{Fe}^{3+}]$ throughout our polymyxin experiments to activate PmrA. Because *E. coli* lacks a functional PmrD (Winfield & Groisman, 2004), PmrA-dependent L-Ara4N modification of lipid A can occur independent of PhoP in both the wild-type and *phoP* mutant bacteria that were both protected by poisoning respiration with NaN_3 . Interestingly, the increase in free iron following phagocyte-induced oxidative damage to bacterial iron-sulfur clusters would also activate the iron-responsive PmrA and PmrA-dependent polymyxin resistance genes independent from PhoP. Similarly, PagP-mediated palmitate addition to lipid A, which confers resistance to antimicrobial peptides other than polymyxin B (Murray *et al.*, 2007), can be induced by NaCl, EDTA, or ammonium metavanadate independent of PhoP (Jia *et al.*, 2004, Zhou *et al.*, 1999). Collectively, these findings suggest that the mechanism responsible for susceptibility of the *phoP* mutant to polymyxin B is distinct from a defect in LPS modification alone. Thus, resistance to polymyxin B involves both charge modifications that occur at the outer leaflet LPS of the outer membrane and modulation of the respiratory generation of the transmembrane electrochemical gradient across the inner membrane.

Synergism between acidification, antimicrobial peptides, and reactive oxygen species allows neutrophils and macrophages to eradicate bacterial pathogens from host tissues (Babior, 1978, Vazquez-Torres *et al.*, 2000). This assault on bacteria leads to the penetration of superoxide into the bacterial periplasm and cytoplasm. The PhoP-activated *sodC* gene product can detoxify superoxide in the periplasm (Kim *et al.*, 2010). When reactive oxygen species breach the inner membrane, superoxide and hydrogen peroxide damage iron-sulfur clusters (Jang & Imlay, 2007) and promote the release of free iron, which rapidly induces DNA damage and bacterial cell death by driving Fenton reactions (Park & Imlay, 2003). Consistent with a defense against superoxide, in the absence of PhoP, we found decreased expression of *sodC* and the *suf* genes; the latter is required for repair and activation of Fe-S-containing enzymes (Imlay, 2008). In addition, PhoP was required for uniform repression of prophage genes in uropathogenic *E. coli*; in the absence of PhoP, we observed an increase in the frequency of spontaneous prophage induction and following induction with the potent DNA-damaging agent MMC. It is reasonable to speculate that a complete PhoP-mediated defense against killing by phagocytes would involve repression of prophage in addition to resistance to antimicrobial peptides and survival at acidic pH because oxidative DNA damage is known to cause prophage induction (Little, 2005).

These results provide compelling evidence linking PhoP, generation of μH^+ , and resistance to host defenses such as acidification, antimicrobial peptides, and reactive oxygen species. In our view, this is clearly reflected by the fact that *phoP* was originally identified in genetic screens that selected for *Salmonella* mutants with increased sensitivity to phagocyte granule extracts and reduced intracellular survival in macrophages (Fields *et al.*, 1989, Fields *et al.*, 1986). For *E. coli*, it has been demonstrated that the antimicrobial peptide LL-37 cathelicidin contributes to protection of the host during invasive urinary tract infection (Chromek *et al.*, 2006) and extraintestinal *E. coli* infections in the urinary tract elicit a massive inflammatory response characterized by neutrophil influx (Godaly *et al.*, 2001, Bergsten *et al.*, 2004). Additionally, bacterial survival during bacteremia is required for extraintestinal virulence, because these *E. coli* strains are confronted with antimicrobial effectors during meningitis or urosepsis (Barondess & Beckwith, 1990, Smith *et al.*, 2010, Wooster *et al.*, 2006). We speculate that the conserved processes controlled by PhoP for *E. coli* to evade host defenses within the bladder are responsible for the attenuation of *phoP* mutant bacteria during extraintestinal infection because PhoP controls a plethora of fitness traits centered on surviving bactericidal activities of phagocytic cells in a large number of enteric pathogens.

Our findings demonstrate that a basic physiological process, modulating the electrochemical gradient across the inner membrane, can be an integral component of bacterial pathogenesis. Specifically, the PhoP regulon activates layers of protective counter-measures against mammalian host defenses including i) modification of lipid A at the outer membrane, ii) superoxide dismutase and ion transport in the periplasm, iii) energy transformations by the cytoplasmic membrane, and iv) cytoplasmic responses to oxidative damage. Basic principles of physiology, shared by nearly all living cells, are beginning to be appreciated as playing a key role in processes that are essential for pathogenesis. For example, bacterial respiration coordinates the timing of type three secretion system (TTSS) assembly with movement of *Shigella* from the intestinal lumen to the mucosa (Marteyn *et al.*, 2010). *Salmonella* pathogenicity island (SPI-2) TTSS stability and effector translocation are determined by changes in the external pH inside *Salmonella*-containing host cell vacuoles (Yu *et al.*, 2010). These recent reports in conjunction with our findings reveal a relationship between fundamental bioenergetics and pathogenesis. The connection between PhoP and μH^+ matches the large PhoPQ regulatory circuit with physiological changes that are most likely to occur through stress encountered during host-pathogen interactions.

Experimental Procedures

Bacterial strains and culture conditions

E. coli strain CFT073 was isolated from the blood and urine of a patient with acute pyelonephritis (Mobley *et al.*, 1990). Unless otherwise noted, bacteria were cultured in Lysogeny broth (LB) medium containing appropriate antibiotics (100 $\mu\text{g ml}^{-1}$ ampicillin and/or 25 $\mu\text{g ml}^{-1}$ kanamycin) at 37°C with aeration.

Construction of mutants and complementation

Deletion mutants were generated using lambda red recombinase (Datsenko & Wanner, 2000). Primers homologous to sequences within the 5' and 3' end of *phoP*, *fumC*, *crcA* (*pagP*), or *arnT*, respectively, were designed and used to replace the target genes with a nonpolar kanamycin resistance cassette (Datsenko & Wanner, 2000). To determine whether the kanamycin resistance cassette recombined within the target gene site, primers that flank the target gene sequence were designed and used for PCR. After amplification, each PCR product was compared to wild-type PCR product when visualized on a 0.8% agarose gel stained with ethidium bromide. For $\Delta\textit{sdhB}\Delta\textit{frdB}$, homologous sequences flanking the *frdA* gene were used to remove the target gene and replace it with a nonpolar chloramphenicol resistance cassette in a kanamycin resistant $\Delta\textit{sdhB}$ strain of CFT073 (Alteri *et al.*, 2009). For the fumarase triple mutant, the kanamycin cassette that replaced *fumC* was unmarked using the FRT sites within the cassette (Datsenko & Wanner, 2000), and *fumB* and *fumA* were replaced using chloramphenicol and kanamycin cassettes, respectively. For *phoP* complementation, the *phoPQ* genes were amplified from CFT073 genomic DNA using Easy-A high-fidelity polymerase (Stratagene) and cloned into pGEN-MCS (Lane *et al.*, 2007) using appropriate restriction enzymes as previously described (Alteri *et al.*, 2009). The sequence of pGEN-*phoPQ* was verified by DNA sequence analysis prior to electroporation into the CFT073 $\Delta\textit{phoP}$ mutant strain.

Murine infection model

Six- to 8-week old female CBA/J mice (20 to 22 g; Jackson Laboratories) were transurethrally inoculated as previously described (Hagberg *et al.*, 1983). Mice were anesthetized with 100 mg ketamine, 10 mg xylazine kg^{-1} body weight. Prior to inoculation, overnight cultures of strain CFT073 were pelleted and resuspended in PBS. Animals were inoculated transurethrally over a 30 sec period with a 50 μl bacterial suspension per mouse using a sterile polyethylene catheter (I.D. 0.28 mm x O.D. 0.61 mm) connected to an

infusion pump (Harvard Apparatus), with total inoculum equaling 1×10^8 CFU/mouse. For determination of CFUs, bladders and kidneys were harvested from euthanized animals at 48 h post-inoculation, weighed, and homogenized in PBS with a GLH homogenizer (Omni International). Tissue homogenates were analyzed for colonization by plating on LB agar using an Autoplate 4000 spiral plater (Spiral Biotech). Colonies were enumerated using a QCount automated plate counter (Spiral Biotech) and *P*-values were determined using the non-parametric Mann Whitney significance test. All procedures were conducted according to protocols approved by University Committee on the Care and Use of Animals at the University of Michigan.

Polymyxin B resistance and acid survival assays

CFU ml^{-1} and percent wild-type survival were determined for CFT073, ΔphoP , and $\Delta\text{phoP} + \text{phoPQ}$ by incubating 10^7 CFU logarithmic phase cells in LB medium containing $10 \mu\text{M}$ FeCl_3 and $2 \mu\text{g ml}^{-1}$ polymyxin B (PB) for 45 min at 37°C or in LB medium buffered to pH 7.0, 5.0, or 2.5 with 2-(*N*-morpholino) ethanesulfonic acid for 1 h at 37°C . For some experiments, 10 mM MgCl_2 , 0.5 mM EDTA, $50 \mu\text{M}$ *m*-chlorophenyl carbonyl cyanide hydrozone (CCCP) or 0.1% sodium azide (NaN_3) were added to cells simultaneously with the addition of polymyxin B or a shift in pH. Pre-treatment of cells prior to exposure to polymyxin B or acidic pH was performed by incubating a 1:10 dilution of cells cultured to $\text{OD}_{600} = 0.8$ in LB medium containing 10 mM MgCl_2 , 0.5 mM EDTA, $50 \mu\text{M}$ CCCP or 0.1% NaN_3 for 1 h. Following pre-treatment, cells were washed with LB medium and incubated in fresh LB containing $2 \mu\text{g ml}^{-1}$ PB, buffered LB pH 7.0, or pH 5.0 as described above. Viable counts were determined from triplicate experiments by plating serial dilutions on LB agar and significance was assessed using Welch's *t* test.

Microarrays and quantitative real-time PCR

For the extraction of total RNA, 1 ml of bacteria culture was added to 0.125 ml of ice cold phenol-ethanol stop solution (5% phenol in ethanol), and the bacteria were collected by centrifugation. Cells were lysed and RNA was extracted using the RNeasy kit (Qiagen) following the manufacturer's recommended protocol. Following elution, nucleic acid concentrations were determined by spectrophotometry (NanoDrop) and residual DNA contamination was removed by incubating the samples with 4U of TURBO DNase (Ambion). After DNase inactivation, the RNA was recovered, quantified, and used as a template for PCR to confirm inactivation of contaminating DNA. For microarrays, RNA purity and integrity were verified by using a BioAnalyzer 2100 (Agilent). RNA was labeled, hybridized, and scanned by using Affymetrix *E. coli* Genome 2.0 GeneChips according to the manufacturer's recommendations. Expression values for each gene were assessed using a robust multi-array average (RMA) (Irizarry *et al.*, 2003). A principal components analysis (PCA) was fit onto the expression values to determine that the replicated samples grouped together and a linear model designed specifically for microarray analysis was fit to the data to compute the contrast of interest (Smyth, 2004) and filter out probesets with a variance < 0.05 . Probesets were adjusted for multiple comparisons using a false discovery rate (FDR) of 0.05 (Klipper-Aurbach *et al.*, 1995). Expression levels with a fold-change > 2.0 and an adjusted *P*-value < 0.05 from three independent replicates of each strain were compared by using the Affymetrix and Limma packages of Bioconductor implemented in the R statistical environment. For qPCR, transcripts were quantified on an MX3000P real-time PCR machine (Stratagene) using Brilliant SYBR green QPCR mix (Stratagene). Transcript levels were normalized to the level of *gapA* (glyceraldehyde 3-phosphate dehydrogenase A) and changes were determined using an experiment-specific calibrator using MXPro v 3.00 software package (Stratagene).

Bacteriophage induction

Supernatants prepared from cells cultured in LB medium or LB medium containing 5 $\mu\text{g ml}^{-1}$ mitomycin C (MMC) were mixed in molten top agar containing $>10^9$ CFU *E. coli* strain C (Bertani & Weigle, 1953) and poured onto TCMG (1% tryptone, 0.5% NaCl, 0.1M MgSO_4 , 0.85% Difco Agar) to visualize plaques following overnight incubation at 37°C. Burst size was calculated as the ratio of plaque forming units (PFU) to CFU. Phage lysate was prepared by culturing *E. coli* C in LB medium with a re-isolated plaque selected on a lawn of *E. coli* C.

Soft agar motility and detection of FliC

For the assessment of swimming motility, 50 μl of overnight cultures of wild-type CFT073, ΔphoP , ΔphoP containing pGEN+*phoP*_{CFT073}, pGEN+*phoP*_{K12}, or pGEN were reinoculated into 2 ml of sterile LB medium and incubated at 37°C with aeration (200 rpm) to an OD₆₀₀ of 1.0. Cultures were stabbed into 0.25% tryptone agar plates (1% tryptone, 0.5% NaCl, 0.25% Difco Agar) using a sterile inoculating needle and incubated at 30°C for 18 hr. For preparation of flagella, 1 ml aliquots of bacteria cultured to OD₆₀₀ = 1.0 were vortexed for 5 min, centrifuged to remove bacterial cells, and supernatants were quantified and standardized using the BCA total protein kit (Thermo Scientific). Detection of FliC by Western blotting was performed using anti-H1 antisera.

Analysis of membrane potential

To determine relative membrane potential between CFT073 wild-type, ΔphoP , $\Delta\text{fumA}\Delta\text{fumB}\Delta\text{fumC}$, and $\Delta\text{sdhB}\Delta\text{frdA}$, 10^7 CFU of logarithmic phase bacteria were diluted into 1 ml sterile PBS containing 10 μl of 3 mM 3,3'-diethyloxycarbocyanine iodide (DiOC₂) (Sims et al., 1974) (Sigma) in DMSO. 10 μl of 10 mM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma) was added to depolarized control samples. Samples were incubated at 37°C for 15 min, dispensed into opaque black microtiter plates, and fluorescence was measured using 488 nm excitation with fluorescein and Texas Red emission filters on a Synergy HT plate-reader operating KC4 software (Bio-Tek). Measurements were normalized using the emission from DiOC₂ blank wells and the ratio of red:green fluorescence for each sample was determined using arbitrary units calculated by dividing the mean fluorescence by CFU for each channel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to David Friedman for many helpful discussions and guidance in performing the phage experiments. We also thank Guy Plunkett III for generously sharing genomics expertise, and Erika Flannery for thoughtful review of the manuscript. This work was supported by public health service grants AI43363 and AI59722 from the National Institutes of Health to H.L.T.M

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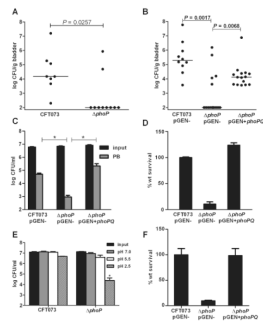


Fig. 1. PhoP is required for virulence, resistance to antimicrobial peptides, and survival at acidic pH in *E. coli*. A. Bladder colonization levels at 48 h post-transurethral inoculation following independent challenge with wild-type uropathogenic *E. coli* CFT073 and $\Delta phoP$ strains. B. *In vivo* complementation of $\Delta phoP$ with *phoPQ* (pGEN+*phoPQ*) restores colonization of female CBA/J mice at 48 h following independent challenge. C. CFU ml⁻¹ and D. percent wild-type (wt) survival for CFT073, $\Delta phoP$, and $\Delta phoP+phoPQ$ following 45 min incubation of 10⁷ CFU logarithmic phase cells in fresh LB medium containing 10 μ M FeCl₃ and 2 μ g/ml polymyxin B (PB). E. CFU ml⁻¹ and F. percent wild-type (wt) survival for CFT073, $\Delta phoP$, and $\Delta phoP+phoPQ$ at 1 h post-inoculation of buffered LB medium pH 2.5 with 10⁷ CFU of bacteria. In A. and B. black dots represent the log CFU g⁻¹ from individual mice and horizontal bars represent the median CFU g⁻¹. *P*-values were determined using the non-parametric Mann Whitney significance test. In C.-F. Bars represent mean values and significant differences (*P* < 0.01) determined by Welch's *t* test are indicated with an asterisk.

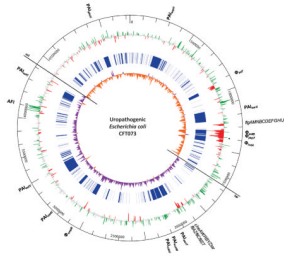


Fig. 2.

Whole genome view of PhoP-dependent gene expression and identification of the large PhoP regulon in pathogenic *E. coli*. Log₂-fold changes in gene expression between exponential phase uropathogenic *E. coli* CFT073 wild-type and $\Delta phoP$ were determined using microarrays and plotted as rays from the outer ring. Individual genes that have significantly decreased transcription in the absence of *phoP* (*) are considered directly or indirectly activated by PhoP (green) while significant increases in gene expression represent directly or indirectly PhoP-repressed genes (red). Gene expression levels that were not considered significant are colored grey. Lines in the center ring (blue) mark uropathogenic *E. coli* CFT073 genes absent in commensal *E. coli* K12 strain MG1655 that were identified using Mauve whole genome sequence alignments. The inner-most ring represents positive (purple) and negative (orange) values for GC skew $(C-G)/(C+G)$ calculated using a 10 kb sliding-window. Local changes in base composition bias indicate recent integration sites of foreign DNA or recombination events. The values switch polarity at the origin (*ori*) and terminus (*ter*). Coordinates from the CFT073 chromosome, the Acid Fitness Island (AFI), flagellar gene operons, pathogenicity (PAI), and prophage (Φ) islands are labeled on the periphery.

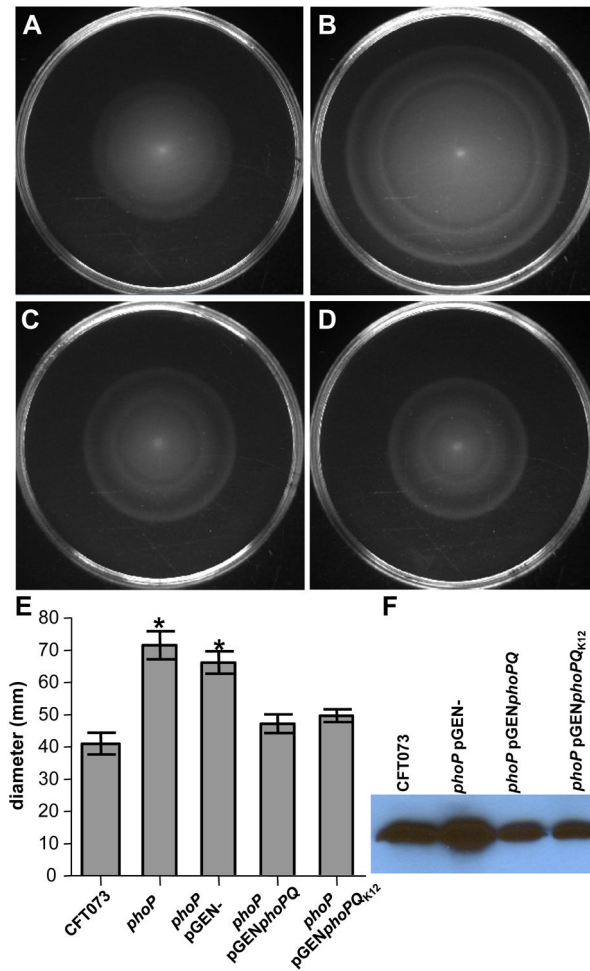
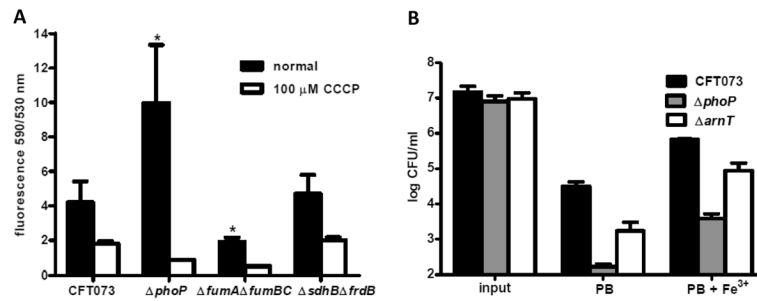


Fig. 3. PhoP represses motility in pathogenic *E. coli*. Microarray analysis showed increased expression of 37/44 flagellar genes in the absence of *phoP*. A.-D. Soft agar motility plates of A. CFT073, B. Δ *phoP*, C. Δ *phoP* complemented with *phoPQ* or with D. *phoPQ* from *E. coli* K12 MG1655. E. Diameter of motility from triplicate experiments (* P < 0.05). F. Western blot with anti-H1 FliC antisera.

**Fig. 4.**

Prevention of membrane hyper-polarization and lipid A modification with L-Ara-4N are necessary for PhoP-dependent antimicrobial peptide resistance phenotype. A. Ratio of red:green fluorescence for 10^7 CFU of logarithmic phase CFT073, $\Delta phoP$, $\Delta fumA\Delta fumB\Delta fumC$, and $\Delta sdhB\Delta frdA$ incubated in $30 \mu\text{M}$ 3',3'-diethyloxacarbocyanine iodide (DiOC_2) (black bars). Depolarized control cells were incubated in $30 \mu\text{M}$ DiOC_2 and $100 \mu\text{M}$ carbonyl cyanide 3-chlorophenylhydrazine (CCCP) (white bars). The green fluorescent carbocyanine dye (DiOC_2) shifts to red fluorescence when the dye self-associates at high intracellular concentrations. Bars represent mean values and significant differences determined by Welch's *t* test are indicated with an asterisk. B. CFU/ml for CFT073, $\Delta phoP$, and $\Delta arnT$ following 45 min incubation of 10^7 CFU logarithmic phase cells in fresh LB medium containing $10 \mu\text{M}$ FeCl_3 and $2 \mu\text{g ml}^{-1}$ polymyxin B (PB) or LB medium containing only $2 \mu\text{g ml}^{-1}$ PB. Viable counts were determined from triplicate experiments by plating serial dilutions on LB agar.

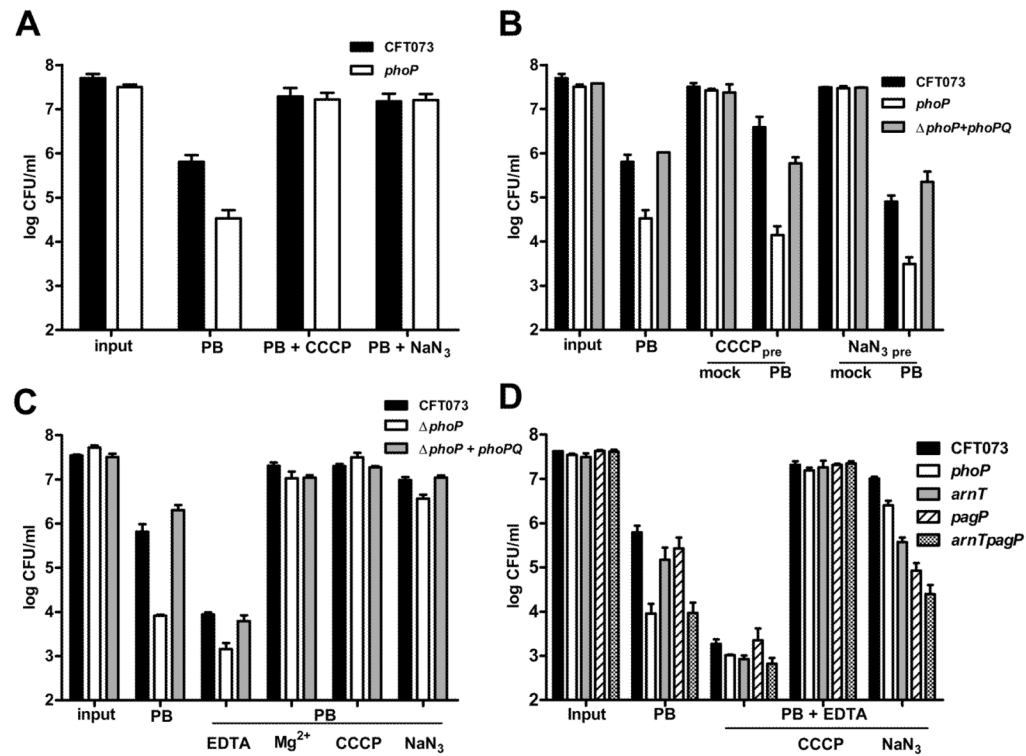


Fig. 5. Influence of the transmembrane proton motive force on *E. coli* susceptibility to antimicrobial peptides. A. Viable counts of CFT073 (black bars) and $\Delta phoP$ (white bars) following 45 min incubation in 2 $\mu\text{g ml}^{-1}$ PB with and without 50 μM *m*-chlorophenyl carbonyl cyanide hydrozone (CCCP) or 0.1% sodium azide (NaN₃). B. CFT073, $\Delta phoP$, and $\Delta phoP+phoPQ$ cells grown to OD₆₀₀ = 0.8 were diluted 1:10 (input) and pre-treated with CCCP or NaN₃ for 1 h. Following pre-treatment, cells were washed with LB medium and incubated in fresh LB medium (mock) or LB containing 2 $\mu\text{g/ml}$ PB for 45 min. C. CFT073, $\Delta phoP$, and $\Delta phoP+phoPQ$ were grown and incubated in PB alone or PB containing CCCP or NaN₃ as described in (A) or in PB containing 0.5 mM EDTA or 10 mM MgCl₂. D. CFT073, $\Delta phoP$, $\Delta arnT$, $\Delta pagP$, and $\Delta arnT\Delta pagP$ were incubated in LB medium containing 10 μM FeCl₃ and 2 $\mu\text{g ml}^{-1}$ PB or LB medium containing 0.5 mM EDTA, 2 $\mu\text{g ml}^{-1}$ PB and CCCP or NaN₃. Bacteria treated with EDTA were pre-cultured in LB medium with 0.5 mM EDTA. Viable counts in A.–D. were determined from triplicate experiments by plating serial dilutions on LB agar.

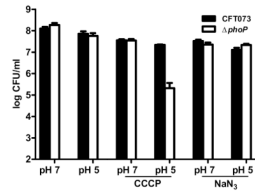


Fig. 6.

PhoP-mediated control of membrane potential is required for *E. coli* resistance to acidic pH. CFT073 (black bars) and $\Delta phoP$ (white bars) were cultured to $OD_{600} = 0.8$ were diluted and incubated in buffered LB medium at pH 7 and pH 5 with and without CCCP and NaN_3 for 1 h. Viable counts were determined from triplicate experiments by plating serial dilutions on LB agar.

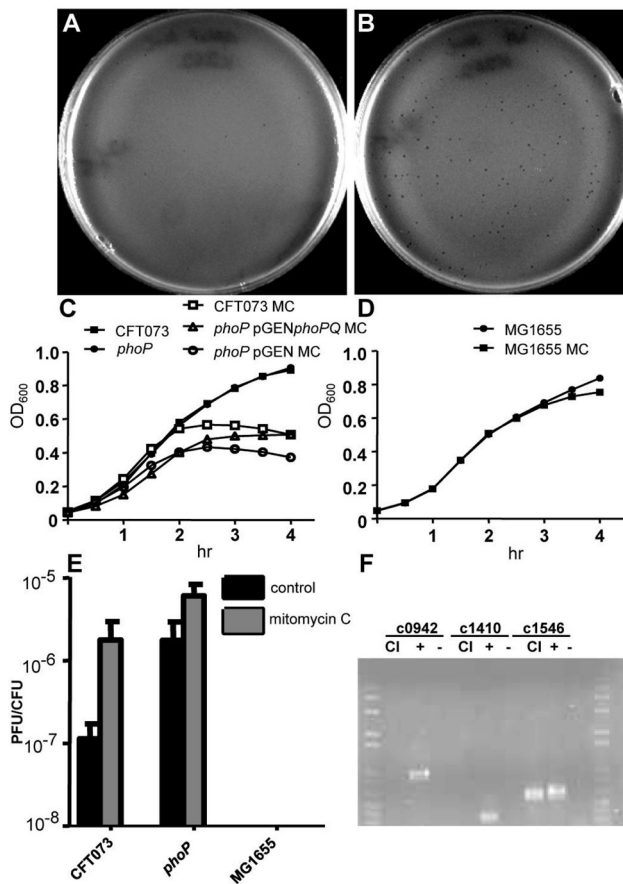


Fig. 7. PhoP represses prophage induction. A. CFT073 and B. ΔphoP supernatants prepared from cells cultured in LB medium to $\text{OD}_{600} = 1.0$ were mixed in molten top agar containing $>10^9$ CFU *E. coli* strain C and poured onto TCMG agar plates to visualize plaques. C. Growth of CFT073 (□), ΔphoP (○), and complemented mutant (Δ) in LB medium containing $5 \mu\text{g ml}^{-1}$ mitomycin C (MMC). CFT073 (■) and ΔphoP (●) growth in LB medium alone is shown for comparison. D. Growth of MG1655, which lacks a functional prophage, in the presence (■) and absence of MMC (●). E. Burst size (no. plaques CFU^{-1}) for CFT073, ΔphoP , and MG1655 cultured in LB or LB medium containing $5 \mu\text{g ml}^{-1}$ MMC. F. CFT073 genes homologous to lambda *cI* were amplified by PCR from *E. coli* C lysate (CI) following incubation with plaques isolated from MC induced CFT073 supernatant. Genomic DNA purified from CFT073 (+) and *E. coli* C (-) were included as controls.

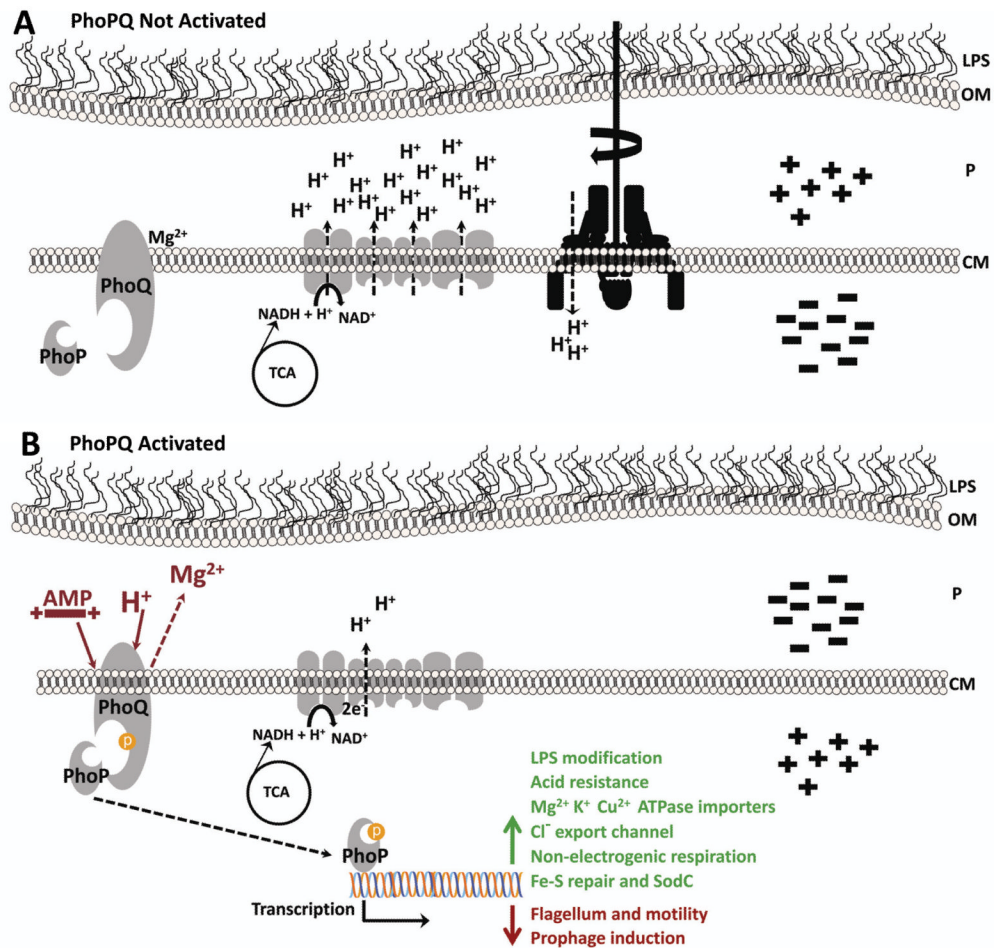


Fig. 8. The PhoP regulon controls energy transformations and membrane potential as one protective counter-measure against mammalian host defenses. A. PhoPQ is not activated. Proton motive force (μH^+), consisting of a gradient of protons (ΔpH) and charge ($\Delta\psi$), across the cytoplasmic membrane (CM), is generated by respiration using electron transfer systems. Protons are pumped into the periplasm (P) by electrogenic respiratory chain components concomitant with the re-oxidation of NAD^+ from NADH produced by the tricarboxylic acid (TCA) cycle. The transmembrane potential (inside-negative) allows protons to diffuse into the cytoplasm, with the $[\text{H}^+]$ gradient, to drive processes including ATP synthesis and rotation of the flagellum and motility. B. In response to antimicrobial activities of host phagocytes, PhoPQ is activated by acidification and cationic antimicrobial peptides (AMP) that have traversed the bacterial outer membrane (OM). PhoP-mediated induction (green arrow and text) of acid resistance responses, non-electrogenic respiratory chain components that do not pump protons, active import of cations, and efflux of Cl^- create a reversed membrane potential (inside positive) and reduce the bactericidal effects of AMPs and inorganic acid by reducing their ability to disrupt the CM or acidify the cytoplasm. Activated PhoP represses (red arrow and text) the transcription of flagellar and prophage genes, expression of the latter can be induced by oxidative DNA damage caused by Fenton chemistry during assault by the phagocyte NADPH oxidase.

Table 1

Microarray result for lipopolysaccharide modification and acid resistance gene transcription in the absence of *phoP*.

Gene	Function	log 2 fold-change	P-value
<i>gadA</i>	glutamate decarboxylase isozyme	-4.9026	0.0206
<i>gadY</i>	ncRNA	-4.3170	0.0065
<i>gadE</i>	DNA-binding transcriptional activator	-4.1352	0.0209
<i>gadB</i>	glutamate decarboxylase isozyme	-4.1239	0.0240
<i>hdeD</i>	acid-resistance membrane protein	-4.1196	0.0170
<i>gadC</i>	acid sensitivity protein	-4.0879	0.0158
<i>hdeB</i>	acid-resistance protein	-3.3836	0.0344
<i>hdeA</i>	acid-resistance protein	-3.2784	0.0240
<i>gadW</i>	putative transcriptional regulator GadW	-3.0689	0.0214
<i>gadX</i>	DNA-binding transcriptional regulator GadX	-2.5727	0.0226
<i>arnB</i>	UDP-4-amino-4-deoxy-L-arabinose--aminotransferase	-1.5925	0.0375
<i>arnE</i>	UDP-4-amino-4-deoxy-L-arabinose--flippase	-1.5471	0.0090
<i>arnD</i>	UDP-4-amino-4-deoxy-L-arabinose--deformylase	-1.3614	0.0307
<i>arnA</i>	UDP-4-amino-4-deoxy-L-arabinose--formyltransferase	-1.2314	0.0455
<i>arnC</i>	UDP-4-amino-4-deoxy-L-arabinose--transferase	-1.3350	0.0786
<i>arnT</i>	4-amino-4-deoxy-L-arabinose transferase	-0.9219	0.0786

Table 2

Microarray result for primary dehydrogenase, terminal reductase, and oxidation-related transcription in the absence of *phoP*.

Gene	Function	Log 2 fold-change	P-value
<i>yhiD</i>	Mg(2+) transport ATPase inner membrane protein	-3.9398	0.0002
<i>narU</i>	Nitrite extrusion protein 2	-3.8395	0.0093
<i>hyaB</i>	hydrogenase 1, large subunit	-3.6945	0.0076
<i>hyaA</i>	hydrogenase 1, small subunit	-3.5637	0.0049
<i>narW</i>	Respiratory nitrate reductase 2 delta chain	-3.4979	0.0042
<i>hyaD</i>	Hydrogenase 1 maturation protease	-2.9709	0.0049
<i>sufB</i>	component of SufBCD complex	-2.4839	0.0069
<i>narV</i>	Respiratory nitrate reductase 2 gamma chain	-2.4373	0.0060
<i>hyaC</i>	Ni/Fe-hydrogenase 1 B-type cytochrome subunit	-2.4161	0.0029
<i>sufA</i>	iron-sulfur cluster assembly scaffold protein	-2.4119	0.0174
<i>sufE</i>	cysteine desulfuration protein SufE	-2.3753	0.0076
<i>sufD</i>	component of SufBCD complex	-2.3616	0.0149
<i>appC</i>	Cytochrome BD-II oxidase subunit I	-2.3207	0.0215
<i>katE</i>	hydroperoxidase HPiI(III) (catalase)	-2.3165	0.0396
<i>appB</i>	Cytochrome BD-II oxidase subunit II	-2.2607	0.0515
<i>sodC</i>	Superoxide dismutase [Cu-Zn] precursor	-2.0652	0.0132
<i>hyaF</i>	protein involved in nickel incorporation into hydrogenase-1 proteins	-1.9418	0.0130
<i>sufC</i>	cysteine desulfurase ATPase component	-1.7518	0.0051
<i>appA</i>	phosphoanhydride phosphorylase	-1.6539	0.0429
<i>wrbA</i>	electron transport component WrbA	-1.5563	0.0300
<i>ybaR</i>	Copper-transporting P-type ATPase	-1.3787	0.0349
<i>yohF</i>	oxidoreductase with NAD(P)-binding Rossmann-fold domain	-1.3696	0.0368
<i>fhlA</i>	Formate hydrogenlyase transcriptional activator	-1.3173	0.0184
<i>hyaE</i>	protein involved in processing of HyaA and HyaB proteins	-1.2780	0.0126
<i>nhaA</i>	Na(+)/H(+) antiporter 1	-1.2602	0.0213
<i>gcd</i>	Glucose dehydrogenase	-1.1960	0.0375
<i>qor</i>	quinone oxidoreductase, NADPH-dependent	-1.0977	0.0320
<i>ugd</i>	UDP-glucose 6-dehydrogenase	-0.9449	0.0277
<i>yadQ</i>	chloride channel protein	-0.9051	0.0396
<i>hemH</i>	ferrochelataase	-0.8957	0.0269
<i>trkA</i>	potassium transporter peripheral membrane component	-0.7195	0.0220
<i>frdC</i>	fumarate reductase subunit C	0.6357	0.0383
<i>frdB</i>	fumarate reductase (anaerobic), Fe-S subunit	0.6576	0.0442
<i>ccmA</i>	Heme exporter protein A	0.7094	0.0308
<i>napG</i>	quinol dehydrogenase periplasmic component	0.8784	0.0174
<i>napH</i>	quinol dehydrogenase membrane component	0.9033	0.0113
<i>pdhR</i>	transcriptional regulator of pyruvate dehydrogenase complex	1.0407	0.0348
<i>napC</i>	nitrate reductase, cytochrome c-type, periplasmic	1.1595	0.0259

Gene	Function	Log 2 fold-change	P-value
<i>ykgF</i>	electron transport amino acid dehydrogenase	1.6450	0.0054
<i>ykgE</i>	oxidoreductase	1.6883	0.0033
<i>napB</i>	nitrate reductase, small, cytochrome C550 subunit, periplasmic	1.9532	0.0143