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MINIREVIEW

The Pleiotropic Two-Component Regulatory System PhoP-PhoQ†

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PhoP-PhoQ is a two-component system that governs virulence, mediates the adaptation to Mg^{2+}-limiting environments, and regulates numerous cellular activities in several gram-negative species. It consists of the inner membrane sensor PhoQ and the cytoplasmic regulator PhoP. The PhoP-PhoQ system is encoded by the *phoP* locus, which was first identified in *Salmonella enterica* serovar Typhimurium as controlling the expression of a nonspecific acid phosphatase (51). This is the reason for the *pho* in *phoP*, a designation typically denoting loci involved in phosphate metabolism. However, the PhoP-PhoQ system responds to the levels of Mg^{2+} and Ca^{2+} (29) and should not be confused with PhoB-PhoR or PhoP-PhoR, two-component systems governing the adaptation to phosphate-limiting conditions in *Escherichia coli* (83) and *Bacillus subtilis* (49), respectively. The realization that PhoP-PhoQ controls virulence in *Salmonella* (31, 58) promoted new interest in the system in the late 1980s and has rendered the PhoP regulon one of the best characterized regulons in enteric bacteria.

Here, I first discuss how the PhoP-PhoQ two-component system responds to environmental cues and interacts with other regulatory systems to integrate multiple signals into a coordinated cellular response and then I describe the PhoP-regulated genes mediating the various PhoP-controlled functions, including virulence. The *Salmonella*-centric tone of this review reflects the fact that most of the work on PhoP-PhoQ has been carried out with this enteric pathogen. However, many of the findings discussed about *Salmonella* PhoP-PhoQ apply to PhoP-PhoQ homologues in other gram-negative species. A model of the PhoP-PhoQ system is presented in Fig. 1, and the genes and cellular activities regulated by Phop-PhoQ in *Salmonella* are listed in Table 1.

PhoP-PhoQ RESPONDS TO EXTRACYTOPLASMIC LEVELS OF Mg^{2+} AND Ca^{2+}

PhoP-PhoQ constitutes the first example of a regulatory system that uses extracellular Mg^{2+} as a primary signal. Growth in micromolar concentrations of Mg^{2+} promotes transcription of PhoP-activated genes in a PhoP-and PhoQ-dependent manner, whereas growth in millimolar concentrations of Mg^{2+} represses expression of PhoP-activated genes to the levels displayed by *phoP* or *phoQ* null mutants (29, 50, 78). In addition to Mg^{2+}, Ca^{2+} and Mn^{2+} can repress transcription of PhoP-activated genes, whereas Ni^{2+}, Cu^{2+}, Co^{2+}, and Ba^{2+} have no effect (29).

Consistent with Mg^{2+} and Ca^{2+} being the physiological signals controlling the PhoP-PhoQ system, several PhoP-dependent phenotypes are regulated by these divalent cations in wild-type microorganisms. For example, wild-type *Salmonella* grown in Luria-Bertani broth is >1,000-fold more resistant to the antimicrobial peptide magainin 2 than organisms grown in Luria-Bertani broth supplemented with 25 mM Mg^{2+} (29). Likewise, the ability to modify the lipid A moiety of the lipopolysaccharide (LPS) and to exhibit resistance to the antibiotic polymyxin B is regulated by Mg^{2+} in both *Salmonella* (33, 41) and *Pseudomonas aeruginosa* (18, 55). Moreover, the PhoP-PhoQ homologue PehR-PehS governs transcription of the Ca^{2+}-regulated virulence protein PehA in the plant pathogen *Erwinia carotovora* subsp. carotovora (23, 24). On the other hand, the PhoQ protein of *Providencia stuartii* does not appear to respond to Mg^{2+} but to a yet undefined signal (68).

HOW DOES Mg^{2+} CONTROL THE PhoP-PhoQ SYSTEM?

The *Salmonella* PhoQ protein features two transmembrane regions that define a long cytoplasmic C-terminal tail harboring the histidine residue predicted to be the site of autophosphorylation and a periplasmic domain harboring several acidic residues that could be involved in sensing divalent cations. In vivo experiments indicate that the PhoQ protein has distinct noninteracting binding sites for Mg^{2+} and Ca^{2+} (28) and that the PhoP-PhoQ system responds to the periplasmic (as opposed to cytoplasmic) levels of these divalent cations (29). Moreover, in vitro experiments demonstrated that the purified 146-amino-acid periplasmic domain of PhoQ binds Mg^{2+} but not Ba^{2+}, a divalent cation unable to repress transcription of PhoP-regulated genes (28, 82). A cluster of acidic residues in the periplasmic region of the PhoQ protein has been implicated in Mg^{2+} sensing by *E. coli* (82); however, its role is presently unclear because the acidic cluster is not conserved in the PhoQ protein of some gram-negative species that respond to Mg^{2+}.

What are the biochemical consequences resulting from Mg^{2+} binding to the periplasmic domain of the PhoQ protein? High Mg^{2+} promotes the dephosphorylation of phospho-PhoP by the PhoQ protein (as opposed to regulating the autophos-

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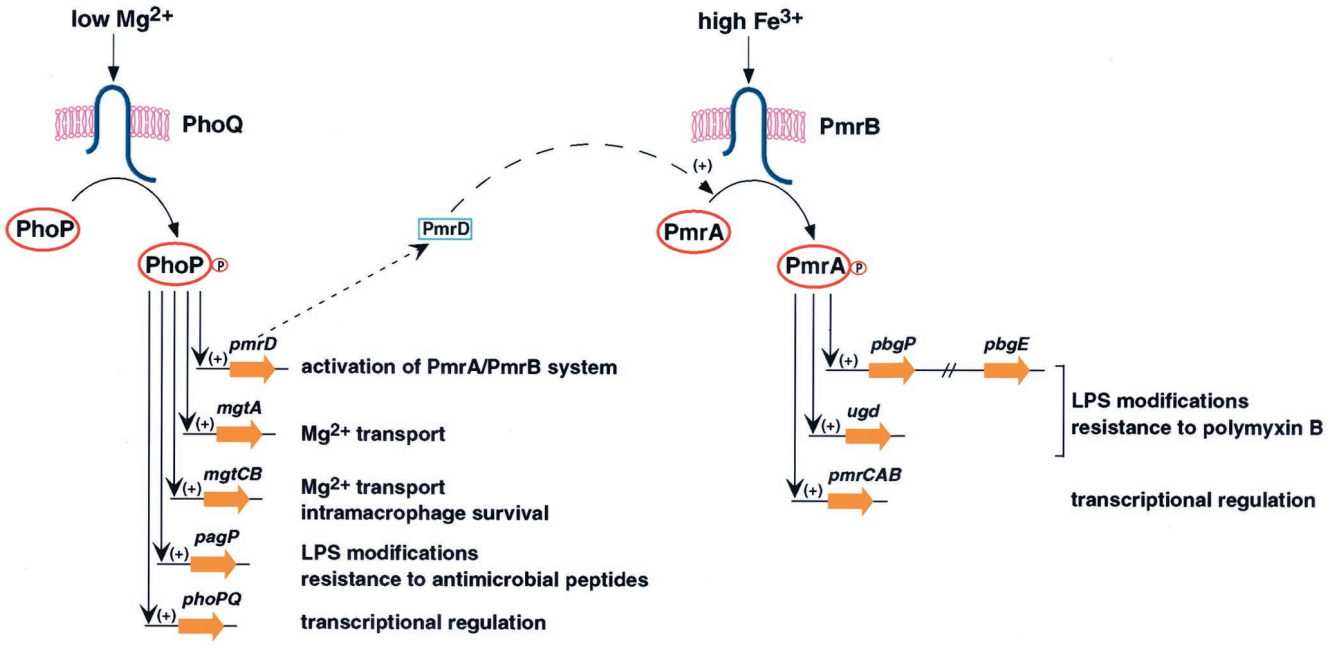

FIG. 1. Model describing the signals controlling expression of PhoP-PhoQ-regulated determinants and the interaction between the PhoP-PhoQ and PmrA-PmrB two-component systems, as well as some of the genes and phenotypes governed by the PhoP-PhoQ system.

phorylation of PhoQ or the phosphorylation of the PhoP protein from phospho-PhoQ.) When grown in the presence of Mg^{2+}, PhoQ abolishes transcription of PhoP-activated genes in a strain harboring a PhoP variant that autophosphorylates from acetyl phosphate (15). And in vitro, Mg^{2+} stimulates dephosphorylation of phospho-PhoP by membranes enriched for the PhoQ protein (14). It has been proposed that dephosphorylation of phospho-PhoP involves the reversal of phosphate transfer from aspartate 57 in PhoP back to histidine 243 in PhoQ because a mutant PhoQ protein harboring a valine residue at position 243 exhibits no phosphatase activity (14). However, additional PhoQ mutants will need to be tested to validate this model because when similar experiments were carried out with the related sensor EnvZ of *E. coli*, only certain amino acid substitutions in the histidine site of phosphorylation resulted in EnvZ proteins lacking phosphatase activity towards phospho-OmpR (48).

pH REGULATES TRANSCRIPTION OF A SUBSET OF PhoP-ACTIVATED GENES

Because mild acid pH promotes transcription of certain PhoP-activated genes, it has been proposed that the *Salmonella* PhoQ protein senses acid pH (4) or responds to both pH and Mg^{2+} (7). However, PhoQ is unlikely to be a pH sensor because growth in mild acid pH promotes transcription only in a subset of PhoP-activated genes and this activation still takes place in a *phoQ* null mutant (7, 29, 79). Likewise, the mild-acid

TABLE 1. Genes and cellular activities regulated by PhoP-PhoQ in *Salmonella*

Gene	Function and /or properties of gene product	Present in *E. coli* K-12?
hilA	Transcriptional regulator of invasion genes	No
mgtA	P-type ATPase Mg^{2+} transporter	Yes
mgtB	P-type ATPase Mg^{2+} transporter	No
mgtC	Mg^{2+} acquisition; intramacrophage survival	No
pagC	Outer membrane protein with sequence similarity to *Enterobacter* OmpX, *Yersinia* Ail, and phage lambda Lom	No
pagP	Outer membrane enzyme mediating transfer of palmitate to lipid A; resistance to peptide C18G	Yes
pbgPE operon	Synthesis and/or incorporation of 4-aminoarabinose into lipid A; resistance to polymyxin	Yes
pcgL	Periplasmic D-Ala-D-Ala dipeptidase	No[a]
pgtE	Outer membrane protease; resistance to peptide C18G	No[a]
phoN	Periplasmic nonspecific acid phosphatase	No
phoPQ	Mg^{2+} -responding two-component system	Yes
pmrAB	Fe^{3+} -responding two-component system	Yes
pmrD	Mediator of transcriptional activation of *pmrA*-regulated genes during growth in low Mg^{2+}	Yes
prgHIJK	Components of Inv-Spa type III secretion system	No
spvB	Mono(ADP-ribosyl)transferase encoded in *Salmonella* virulence plasmid	No
ugd	UDP-D-glucose dehydrogenase	Yes
ugtL	Putative membrane protein	No

[a] *E. coli* K-12 harbors two genes, *ecovanX* and *ompT*, encoding products that are only 40 and 46% identical to the *Salmonella pcgL* and *pgtE* gene products, respectively. However, these genes are not true homologs and, like *Salmonella pcgL* and *pgtE*, the *E. coli* genes appear to have been acquired horizontally.

induction of these genes is not affected by mutation of the *phoP* gene, arguing against the possibility of the PhoP protein itself sensing pH or being activated by a pH sensor. Therefore, although transcription of PhoP-regulated genes can be regulated by signals other than Mg^{2+} and Ca^{2+}, these signals are sensed typically by sensors other than PhoQ, activate regulators other than PhoP, and affect only a subset of the genes belonging to the PhoP-PhoQ regulon.

AUTOGENOUS REGULATION OF THE PhoP-PhoQ SYSTEM

The *phoPQ* operon is autogenously controlled in a positive fashion by the PhoP and PhoQ proteins both in *Salmonella* (77) and *E. coli* K-12 (50). The *phoPQ* operon is transcribed from two promoters, one active only during growth in low Mg^{2+} and dependent on the PhoP and PhoQ proteins and another that is constitutive (i.e., active regardless of the Mg^{2+} concentration or presence of PhoP and PhoQ proteins) (29). However, the regulation of the *phoPQ* operon differs in *Salmonella* and *E. coli* K-12 in that PhoP-PhoQ regulates the promoter distal to the *phoP* initiating codon in *Salmonella* (77) but controls the promoter that is proximal to *phoP* in *E. coli* (50). Thus, while the relative position of the constitutive and regulated promoters is different in *Salmonella* and *E. coli*, both species rely on a positive regulatory loop to control *phoPQ* gene expression, perhaps as a means to amplify the signals sensed by the PhoQ protein.

In *P. aeruginosa*, the *phoPQ* genes are preceded by *oprH* (a gene encoding an outer membrane protein of unknown function) and transcribed from two promoters. There is an Mg^{2+}- and PhoP-regulated polycistronic transcript that expresses the *oprH, phoP*, and *phoQ* genes, whereas a second promoter located within or downstream of *oprH* allows low levels of *phoPQ* transcription (54). This organization is reminiscent of that present in the *Salmonella* locus encoding the two-component system PmrA-PmrB: the *pmrAB* genes are preceded by the *pmrC* gene (which also encodes a putative outer membrane protein) and transcribed by two promoters, one upstream of *pmrC* and one within *pmrC* (39).

THE PhoP-PhoQ TWO-COMPONENT SYSTEM ACTIVATES THE PmrA-PmrB TWO-COMPONENT SYSTEM DURING GROWTH IN LOW Mg^{2+} CONCENTRATIONS

A subset of PhoP-activated genes is regulated via another two-component system (39, 43, 79), PmrA-PmrB (70), where PmrA is the response regulator and PmrB is a sensor kinase that responds to extracytoplasmic ferric iron (86). Thus, either low Mg^{2+} or high Fe^{3+} concentrations promote transcription of PhoP-activated PmrA-dependent genes. In contrast, transcription of those PhoP-activated genes which are PmrA independent is promoted in low Mg^{2+} concentrations. but does not respond to Fe^{3+}.

We are beginning to understand how the Mg^{2+} and Fe^{3+} signals are sensed and transduced into an appropriate cellular response that activates only the correct set of genes. The low Mg^{2+} activation of PmrA-regulated genes, which requires the *phoQ, phoP, pmrD, pmrA*, and *pmrB* genes, occurs by the PhoQ protein serving as an Mg^{2+} sensor that modulates the ability of the PhoP protein to promote transcription of the *pmrD* gene. The *pmrD* gene product then activates PmrA-PmrB at a post-transcriptional level by a yet undefined mechanism (52). On the other hand, Fe^{3+}-promoted activation of PmrA-regulated genes occurs by Fe^{3+} binding to the periplasmic domain of the PmrB protein (86), which presumably promotes PmrB autophosphorylation and the ensuing phosphorylation of the PmrA protein. This would result in transcription of PmrA-activated genes because the PmrA protein binds to the promoters it regulates with higher affinity in its phosphorylated form (85). The Fe^{3+}-promoted pathway is independent of the *phoP, phoQ* (86), and *pmrD* (52) genes, allowing transcription of PmrA-activated genes to take place independently of the PhoP-PhoQ system. In addition, transcription of PmrA-dependent genes can be induced by mild acid pH in strains lacking functional *phoQ, phoP* (79), or *pmrB* (F. C. Soncini and E. A. Groisman, unpublished results) genes by an unknown mechanism.

Why does the PmrA regulon respond to multiple signals? We hypothesize that this is because the PmrA-regulated gene products are useful under the various growth conditions that promote their expression. For example, when bacteria experience low Mg^{2+} environments the PhoP-PhoQ system is activated, resulting in expression of several proteins required for growth in low Mg^{2+} concentrations such as the Mg^{2+} transporters MgtA and MgtB (78). By also promoting *pmrD* transcription, PhoP-PhoQ activates the PmrA-PmrB system, which controls transcription of genes necessary for growth in low Mg^{2+} solid media (78). Perhaps transcription of PhoP-regulated genes is induced at dissimilar Mg^{2+} thresholds depending on whether they are regulated directly or indirectly (e.g., via *pmrD*) by the PhoP protein. On the other hand, the possibility to activate the PmrA-PmrB system independently of PhoP-PhoQ allows the synthesis of proteins conferring protection from the toxic effects of Fe^{3+} in environments with repressing levels of Mg^{2+} (86).

The PhoP-PhoQ and PmrA-PmrB systems are widely distributed among enteric bacteria and have been identified also in gram-negative organisms such as *P. aeruginosa* (31, 55; F. Solomon and E. A. Groisman, unpublished results). In contrast, *pmrD* homologues have not been detected in the genomes of species harboring *phoP-phoQ* and *pmrA-pmrB* genes (M. Winfield and E. A. Groisman, unpublished results). This indicates that PhoP-PhoQ and PmrA-PmrB can exist as "free-standing" two-component systems, each responding to their specific signals. Moreover, it suggests that during evolution the PhoP-PhoQ and PmrA-PmrB systems became connected via the "*pmrD* shunt" in species such as *Salmonella*. This enabled expression of PmrA-activated genes not only when Fe^{3+} concentrations are high but also when Mg^{2+} concentrations are low, thereby providing *pmrD*-containing organisms with the opportunity to expand their niches.

PhoP-REGULATED PROMOTERS

A direct repeat, (T/G)GTTTA, has been identified 25 bp upstream of the transcription start site of three Mg^{2+}-regulated PhoP-activated genes in *E. coli* K-12: *phoPQ, mgtA*, and *mgrB* (50). This repeat is also present in the promoter region of the *Salmonella phoPQ* (77) and *phoN* (35) genes and has been

detected in the putative regulatory region of four additional open reading frames in *E. coli* and three in *Salmonella* (50). It is tempting to speculate that the identified motif represents a bona fide PhoP-binding site. However, this awaits DNAse footprinting analysis of PhoP-regulated promoters because the identified motif is also present in the promoter region of the PhoP-activated PmrA-dependent *ugd* gene (38, 79), which has been shown to bind the PmrA protein (1).

ECLECTIC GENES AND PHENOTYPES CONTROLLED BY PhoP-PhoQ

The PhoP-PhoQ system governs expression of at least 40 proteins in *Salmonella* (59), which constitutes approximately 1% of the open reading frames encoded in the *Salmonella* genome. Some two dozen PhoP-regulated determinants have been identified by classical genetic methods (9, 66, 78) and MALDI-TOF analysis (36). And while additional PhoP-regulated genes are likely to be uncovered with the use of high-density DNA arrays, some general principles have emerged from the analysis of the PhoP-regulated genes already identified. First, the PhoP-PhoQ regulon mediates the adaptation to Mg^{2+}-limiting environments. Second, PhoP-PhoQ governs virulence in several gram-negative species. Third, many of the genes identified as PhoP regulated are species specific and confer unique properties upon the microorganism. And fourth, PhoP-PhoQ governs the modification of many components in the bacterial cell envelope.

Mg^{2+} TRANSPORTERS, LPS MODIFICATIONS, AND ADAPTATION TO Mg^{2+}-LIMITING ENVIRONMENTS

The PhoP-PhoQ system controls the expression of several genes that are necessary for growth in low Mg^{2+} concentrations (78). These include the *mgtA* and *mgtB* genes, which encode two of the three Mg^{2+} transporters of *Salmonella* (75). Like a *phoP* null mutant, *mgtA* and *mgtCB* null mutants exhibit wild-type logarithmic growth but reach a plateau earlier than wild-type *Salmonella* in low Mg^{2+} liquid media, forming elongated cells and losing the ability to form bacterial colonies (78). That lack of either MgtA or MgtB results in a growth defect suggests that both transporters are necessary to maintain physiological levels of Mg^{2+} in the *Salmonella* cytoplasm. Alternatively, the growth defect may be due to the inability of these transporters to take up or export a ligand(s) that is cotransported along with Mg^{2+}. The MgtA and MgtB proteins are P-type ATPases that transport Mg^{2+} down an electrochemical gradient, supporting the notion that a ligand is likely cotransported along with Mg^{2+} (75). While the *mgtA* and *mgtB* genes encode proteins that are 50% identical, they differ in phylogenetic distribution and expression profile: *mgtB* has been detected in only 3 of 10 gram-negative species harboring *mgtA*-hybridizing sequences (11), and acid pH abolishes *mgtA* transcription but has minimal effect on *mgtB* expression (74).

A different set of PhoP-regulated genes is required for growth in low Mg^{2+} solid media (but dispensable for growth in low Mg^{2+} liquid media): the *ugd* gene and the seven-gene operon *pbgPE* (78) (the *pbgPE* operon is designated *pmrF* in reference 38), which are regulated via the PmrA-PmrB system. The *ugd*-and *pbgPE*-encoded proteins mediate the synthesis and/or incorporation of 4-aminoarabinose in the lipid A portion of the LPS (38). This reduces the negative charge of the LPS and renders *Salmonella* resistant to the cationic peptide antibiotic polymyxin B (33, 38), presumably by decreasing binding of the positively charged polymyxin to the surface of the bacterial cell (20, 73).

What is the physiological role of the low Mg^{2+}-promoted PmrA-regulated LPS modifications? We have hypothesized that the LPS is an Mg^{2+} reservoir and constitutes the initial source of Mg^{2+} for the MgtA and MgtB transporters when bacteria face Mg^{2+}-limiting environments (33). According to this hypothesis, the PmrA-controlled modifications of the LPS serve to neutralize negative charges normally neutralized by Mg^{2+} and Ca^{2+} ions. Thus, the failure to modify the surface charge in a *pmrA* mutant would result in electrostatic repulsion between negatively charged LPS molecules, thereby preventing the intimate side-by-side alignments required for the formation of bacterial colonies, which could be important in biofilm formation.

PhoP-REGULATED RESISTANCE TO ANTIMICROBIAL PEPTIDES

Salmonella phoP mutants are highly susceptible to a variety of antimicrobial peptides, including the mammalian defensins, the frog-derived magainin 2, the insect-derived melittin and mastoparan, and the peptide antibiotic polymyxin B, produced by the soil bacterium *Paenibacillus polymyxa* (21, 33, 34, 39, 60). Despite similarities among many of these antimicrobial peptides—such as being small, cationic, and amphipathic (5, 64)—different PhoP-regulated determinants often mediate resistance to different antimicrobial peptides. Some of these determinants modify the LPS, whereas others encode extracytoplasmic proteases with the capacity to cleave antimicrobial peptides.

PagP is a PhoP-activated outer membrane protein responsible for the incorporation of palmitate into the lipid A moiety of the LPS (10), but dispensable for other PhoP-regulated modifications, such as addition of 2-hydroxymyristate or 4-aminoarabinose into lipid A (42). A *Salmonella pagP* mutant exhibits hypersensitivity to the synthetic α-helical peptide C18G but displays wild-type resistance towards polymyxin and protegrin (42). In addition to PagP, the outer membrane protease PgtE contributes to resistance against C18G, at least when the *pgtE* gene is overexpressed to high levels, since a *pgtE* mutant exhibits wild-type resistance to C18G (36). Resistance is associated with C18G cleavage by strains expressing PgtE, whose expression is reportedly controlled by PhoP-PhoQ at a post-transcriptional level (36).

Polymyxin resistance requires both PmrA-dependent and -independent PhoP-activated loci. The PmrA-dependent loci include *ugd* and the seven-gene *pbgPE* operon (also designated *pmrF*) (33, 38), both of which mediate the synthesis and/or incorporation of 4-aminoarabinose into the lipid A moiety of the LPS (38, 40). A PhoP-activated PmrA-independent gene(s) also appears to participate in polymyxin resistance because when *Salmonella* is grown in low Mg^{2+} concentrations (to promote transcription of the whole PhoP regulon) a *phoP* mutant is >20 times more susceptible to polymyxin than a *pmrA* mutant (86). Moreover, when bacteria are grown in high

Fe^{3+} concentrations (to promote expression of PmrA-activated genes independently of PhoP) the *phoP* mutant remains >20 times more susceptible to polymyxin than wild-type *Salmonella* (86).

It was originally suggested that increased susceptibility to antimicrobial peptides is in part responsible for virulence attenuation in *phoP* and *phoQ* mutants (21). However, it is presently unclear what contribution PhoP-regulated peptide resistance makes to *Salmonella* virulence because *pagP* mutants retain wild-type virulence (9) and *pmrA* mutants are only slightly attenuated (40).

PhoP and Mg^{2+} also control resistance to antimicrobial peptides in other gram-negative species. For example, a *phoP E. coli* strain is hypersensitive to killing by magainin 2 and mastoparan (32), and Mg^{2+} modulates resistance of *Shigella flexneri* to magainin 2 in a PhoP-dependent manner (62). In *P. aeruginosa,* wild-type organisms grown in low Mg^{2+} concentrations are more resistant to killing by C18G (18) or polymyxin B (18, 55) than organisms grown in high Mg^{2+} concentrations. On the other hand, the role of PhoP in polymyxin resistance remains controversial in *Pseudomonas*. One group found the *phoP* null mutant more susceptible to polymyxin (18), whereas another group reported the *phoP* mutant to be as resistant as wild-type *Pseudomonas* (55), although *phoP* expression from a heterologous promoter conferred polymyxin resistance even in cells grown in high Mg^{2+} (55).

ROLE OF PhoP-PhoQ IN *SALMONELLA* PATHOGENESIS

Salmonellae are facultative intracellular pathogens that infect a wide variety of animals causing different disease conditions (see reference 72 for a review on *Salmonella* pathogenesis). *Salmonella* strains harboring null alleles of the *phoP* or *phoQ* gene are highly attenuated for virulence: their median lethal doses in intraperitoneally innoculated BALB/c mice are 5 orders of magnitude higher than that of wild-type *Salmonella* (21, 26, 58). This attenuation could be due to one or more of the various in vitro virulence defects displayed by *phoP* and *phoQ* null mutants. These include the inability to survive within macrophages (22, 58) and an increased susceptibility to killing by antimicrobial peptides (21, 34, 36, 42, 60), bile salts (81), and acid pH (7, 25). While many of these PhoP-regulated functions are mediated by genes present also in *E. coli*, PhoP-PhoQ appears to control virulence primarily by regulating transcription of *Salmonella*-specific virulence genes.

The PhoP-activated *mgtC* gene is required for intramacrophage survival in vitro and mouse virulence in vivo (11). Encoded within a *Salmonella*-specific gene cluster designated SPI-3 pathogenicity island, MgtC appears to function in Mg^{2+} acquisition, because an *mgtC* mutant is defective for growth in a low Mg^{2+} medium (11) and the MgtC protein is predicted to localize to the inner membrane (76). MgtC may mediate Mg^{2+} uptake by working in conjunction with another protein(s) because, by itself, it could not mediate transport of radioactive Ni^{2+} or Co^{2+} when used as a surrogate for Mg^{2+} (61). The MgtC protein appears to confer intramacrophage survival by insuring that proper levels of Mg^{2+} are available to the microorganism proliferating within the phagosome because intramacrophage survival was partially restored to both *mgtC* and *phoP* mutants upon addition of Mg^{2+} to the tissue culture media (11).

It is interesting that the phylogenetically distant pathogen *Mycobacterium tuberculosis* harbors an MgtC homolog that is also required for growth in low Mg^{2+} concentrations, proliferation within macrophages, and virulence in mice (13). In contrast, the closely related *E. coli* is missing the *mgtC* gene and cannot grow in Mg^{2+} concentrations as low as *Salmonella* (11). This suggests that acquisition of the *mgtC* gene, and the ensuing ability to grow in low Mg^{2+} concentrations, were important steps in the development of *Salmonella* as an intracellular pathogen.

Because *mgtC* mutants are not as attenuated as *phoP* null mutants, additional PhoP-regulated loci likely mediate *Salmonella* virulence in mice. Two such candidate loci, which have been implicated in intramacrophage survival, are the *spv* genes in the *Salmonella* virulence plasmid (53) and the *ssa* and *sse* genes in the SPI-2 pathogenicity island (16, 45, 63). However, the role of PhoP in transcription of the *spv* (19, 43, 44, 57) and SPI-2 (17, 80) genes remains controversial, suggesting the existence of other, yet unidentified, PhoP-activated virulence genes.

The attenuated phenotype and immunogenic potential of *phoP* mutants of serovar Typhimurium in mice (26, 58) have prompted the development of a vaccine strain of serovar Typhi that appears to be safe and immunogenic in humans (47). Moreover, the PhoP-PhoQ system influences the processing and presentation of antigens by *Salmonella* because activated macrophages process a *phoP* null mutant more efficiently than wild-type *Salmonella*, which in turn, is processed more efficiently than a *pho-24* mutant [84]; see below).

POTENTIAL ROLE FOR PhoP-PhoQ IN PEPTIDOGLYCAN REMODELING

PhoP governs transcription of two adjacent *Salmonella*-specific genes that could be involved in peptidoglycan remodeling: *pcgL*, encoding a D-Ala-D-Ala dipeptidase, and *ugtL*, encoding a protein with sequence similarity to a chitin synthetase from *Schizosaccharomyces pombe* (46). While D-Ala-D-Ala dipeptidases have been traditionally associated with vancomycin-resistant enterococci (69), the PcgL protein is unlikely to be involved in resistance to this glycopeptide antibiotic because gram-negative bacteria are naturally resistant to vancomycin. The *Salmonella* and enterococcal D-Ala-D-Ala dipeptidases exhibit virtually identical substrate specificity in spite of the fact that they are only 40% identical at the amino acid level. On the other hand, these enzymes differ in their subcellular location: periplasmic for PcgL and cytoplasmic for the enterococcal enzyme. While the physiological role of the PcgL and UgtL proteins remains undetermined, these proteins could function in some aspect of peptidoglycan metabolism because D-Ala-D-Ala is produced only for its incorporation into the peptidoglycan and chitin is the yeast equivalent of bacterial peptidoglycan. For example, these proteins could participate in the peptidoglycan remodeling experienced by *Salmonella* during growth in host epithelial cells (67), an environment that promotes expression of PhoP-activated genes (4, 27, 44, 80).

VIRULENCE ATTENUATION ASSOCIATED WITH HYPERACTIVATION OF THE PhoP-PhoQ SYSTEM

One of the paradoxical virulence phenotypes associated with PhoP-PhoQ is that displayed by a strain harboring the *pho-24* allele of *phoQ*, which overexpresses PhoP-activated proteins and further represses PhoP-repressed proteins and is as attenuated for mouse virulence as a *phoP* null mutant (59). Often referred to as PhoP^c (for *PhoP* constitutive [59]), the *pho-24* mutation is neither in the *phoP* gene nor constitutive. The *pho-24* mutant harbors a single amino acid substitution in the periplasmic domain of PhoQ (28) and responds to Mg^{2+} virtually like wild-type *Salmonella*. On the other hand, its PhoQ protein has an altered set point for Ca^{2+}, requiring eight times higher concentrations of this divalent cation to repress transcription to the levels achieved by wild-type PhoQ (28).

The *pho-24* mutant is unable to invade nonphagocytic cells due to repression of transcription of the invasion gene regulator *hilA* (6), which controls expression of several genes within the SPI-1 pathogenicity island (8, 66). However, the *pho-24* mutant is highly attenuated even when mice are inoculated intraperitoneally (i.e., when the invasion genes are not required), unable to replicate within macrophages in vitro, and defective in inducing spacious phagosome formation in macrophages (3).

The virulence attenuation exhibited by the *pho-24* mutant has been attributed to disregulation of PhoP-controlled targets (59), which is consistent with increased phosphorylation of the PhoP protein promoted by membranes from a *pho-24* mutant relative to that promoted by membranes prepared from wild-type *Salmonella* (37). While the mechanism by which hyperactivation of the PhoP-PhoQ system reduces virulence remains unknown, it appears to require the *spv* plasmid virulence genes because constitutive expression of the *phoP* gene from a heterologous promoter results in virulence attenuation only in strains harboring the *spv* genes (57).

EXPRESSION OF PhoP-REGULATED GENES DURING INFECTION: Mg^{2+} LEVELS AS A MEANS OF TELLING INTRACELLULAR FROM EXTRACELLULAR ENVIRONMENTS

The majority of *Salmonella* promoters preferentially induced during infection of host cells are under transcriptional control of the PhoP-PhoQ system: 8 of 14 promoters recovered from macrophages by differential fluorescence induction [80] and 7 of 8 promoters isolated from the spleen and/or cultured macrophages by in vivo expression technology (44) were dependent on *phoP* for intracellular expression. The seven PhoP-dependent promoters recovered by in vivo expression technology are induced in vitro both by low pH and by low Mg^{2+} concentrations and are transcriptionally active in macrophages and epithelial cells (44). This indicates that the signals promoting expression of PhoP-activated genes are common to both cell types and argues against early proposals that PhoP-activated genes are expressed in macrophages but not in epithelial cells (4).

All PhoP-regulated genes mediating *Salmonella* virulence appear to have been acquired by horizontal gene transfer. We hypothesize that these genes have been put under PhoP-PhoQ control to insure that *Salmonella* expresses its virulence genes at the right time and in the right places (e.g., *mgtC* inside the macrophage phagosome). According to this hypothesis, *Salmonella* determines its subcellular location (at least in part) by examining the Mg^{2+} levels in its surroundings via the PhoQ protein: a low Mg^{2+} concentration is an indication of an intracellular environment (i.e., the phagosome), whereas a high Mg^{2+} concentration denotes an extracellular environment (30). (Ca^{2+} and Mn^{2+} also regulate the PhoP-PhoQ system in vitro, but the concentrations of these divalent cations are too low in host tissues to control PhoP-PhoQ in vivo.) This hypothesis is consistent with the expression pattern of PhoP-activated genes, which are transcriptionally induced when the Mg^{2+} concentration is low in vitro (28, 78) and inside host cells in vivo (4, 27, 43, 44, 80). According to this model, PhoP-repressed genes, such as the invasion determinant *prgH*, should be transcribed outside host cells, an environment where the concentrations of Mg^{2+} and Ca^{2+} are high, thereby eliciting entry into host cells.

VIRULENCE ROLE OF PhoP-PhoQ IN OTHER GRAM-NEGATIVE SPECIES

The PhoP-PhoQ system is required for virulence in several species that have very different lifestyles from that of *Salmonella*. For example, *S. flexneri*—the agent of bacillary dysentery—requires a functional *phoP* gene for full virulence (62) despite causing a different disease and normally residing in a different subcellular compartment than *Salmonella* (see reference 71 for a review on *Shigella* pathogenesis). A *Shigella phoP* mutant is hypersensitive to killing by neutrophils (62), the only cell type in which *Shigella*, like *Salmonella*, remains within a membrane bound vacuole (56) and does not escape to the cytosol by lysing the phagosomal membrane.

PhoP also controls virulence in *Yersinia pestis*, the etiologic agent of bubonic plague (see reference 12) for a review on *Yersinia* pathogenesis): a *Yersinia phoP* mutant has a mean lethal dose that is 75-fold higher than that of the isogenic wild-type strain (65). Even though *Yersinia* is normally present extracellularly, initially in the infection it undergoes an intracellular phase within phagocytic cells, where PhoP may be important because a *Yersinia phoP* mutant is defective for survival within macrophages and exhibits increased sensitivity to low pH, oxidative killing, and high osmolarity (65).

Thus, while *Salmonella, Shigella*, and *Yersinia* cause different disease conditions, inactivation of the *phoP* gene prevents proliferation within phagocytic cells of all three species, suggesting a role for these PhoP-PhoQ systems in survival within the phagosome. Surprisingly, PhoP-PhoQ also controls virulence functions in *Erwinia carotovora* supsb. carotovora, a plant pathogen that resides in the intercellular fluid and is not known to enter plant cells (see reference 2 for a review on *Erwinia* pathogenesis). *Erwinia* secretes a large number of cell wall-degrading enzymes that damage different plant tissues, and one of them is an endopolygalacturonase (designated PehA) that is transcriptionally regulated by the PhoP-PhoQ homologue PehR-PehS (23). Ca^{2+} controls the levels of the PehA protein in *Erwinia* and transcription of the *pehA* gene in *E. coli*, in both cases requiring a functional *phoP* gene. In addition, mutants of *Erwinia* defective in either *pehR* or *pehS* are attenuated for virulence on tobacco seedlings. This indicates that

PhoP-PhoQ can control virulence even in pathogens that do not have an intracellular lifestyle.

CONCLUSIONS

The PhoP-PhoQ two-component system governs the adaptation to low Mg^{2+} environments and the response to other stress conditions by regulating expression of as much as 1% of the genes in certain gram-negative species. PhoP-PhoQ mediates its effects often indirectly: via activation of other regulatory systems such as the PmrA-PmrB two-component system. By controlling expression of many horizontally acquired virulence determinants, the PhoP-PhoQ system has become a major regulator of virulence in *Salmonella*.

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