

Dissecting the PhoP regulatory network of *Escherichia coli* and *Salmonella enterica*

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Genetic and genomic approaches have been successfully used to assign genes to distinct regulatory networks. However, the present challenge of distinguishing differentially regulated genes within a network is particularly hard because members of a given network tend to have similar regulatory features. We have addressed this challenge by developing a method, termed Gene Promoter Scan, that discriminates coregulated promoters by simultaneously considering both multiple *cis* promoter features and gene expression. Here, we apply this method to probe the regulatory networks governed by the PhoP/PhoQ two-component system in the enteric bacteria *Escherichia coli* and *Salmonella enterica*. Our analysis uncovered members of the PhoP regulon and interactions with other regulatory systems that were not discovered in previous approaches. The predictions made by Gene Promoter Scan were experimentally validated to establish that the PhoP protein uses multiple mechanisms to control gene transcription, regulates acid resistance determinants, and is a central element in a highly connected network.

promoter | machine learning | gene transcription | acid pH

A critical challenge of the postgenomic era is to understand how genes are differentially regulated. Because the prevalent mechanisms controlling gene expression operate at the level of transcription initiation, computational techniques have been developed that identify *cis* regulatory features (1, 2) and map such features into expression patterns (3, 4) to classify genes into distinct networks. However, these methods are not focused on the more challenging problem of distinguishing between differentially regulated genes within a network. Here, we use a unsupervised machine learning method (5–7), Gene Promoter Scan (GPS), to investigate the regulatory interactions governed by the PhoP/PhoQ two-component system, a central element in one of the most interconnected bacterial genetic networks thus far described.

The PhoQ protein is a sensor for extracytoplasmic Mg^{2+} that modifies the phosphorylated state of the DNA-binding protein PhoP (8–10). The PhoP protein controls the expression of a large number of genes that mediate adaptation to low Mg^{2+} environments and/or virulence in several bacterial species including *Salmonella enterica*, *Shigella flexneri*, *Yersinia pestis*, *Erwinia carotovora*, *Neisseria meningitidis*, *Photobacterium luminescens* and *Escherichia coli* (see ref. 11 for a review). It has been proposed that the PhoP protein recognizes the direct hexanucleotide repeat (T/G)GTTTA, separated by five nucleotides, which has been termed the PhoP box (12). Indeed, experiments carried out with the PhoP-activated *mgtA* promoter of *Escherichia coli* demonstrated the critical role that certain PhoP box nucleotides play in *mgtA* expression and that the purified PhoP protein and RNA polymerase were sufficient to promote *mgtA* transcription *in vitro* (13). However, there is uncertainty about what constitutes a PhoP binding site because many PhoP-regulated promoters do not conform to the *mgtA* promoter model (14, 15). Moreover, the identification of PhoP-regulated targets is confounded by the fact that many genes are indirectly regulated by

PhoP, which controls other two-component regulatory systems at the transcriptional (e.g., RstA/RstB) (14), posttranscriptional (e.g., SsrB/SpiR) (J. Bijlsma and E.A.G., unpublished results), and posttranslational (e.g., PmrA/PmrB) (16) levels. In this article, we use GPS to uncover previously uncharacterized members of the PhoP regulatory network and demonstrate that PhoP controls transcription from different classes of promoters and has an unsuspected role in the regulation of acid resistance genes.

Materials and Methods

Bacterial Strains, Growth Conditions, and Enzymatic Assays. Strains used in this study are listed in Table 1. The *Escherichia coli* K-12 *phoP* strain EG12976 was constructed from the wild-type MG1655 strain by P1 transduction with a lysate prepared by using the *phoP* strain FS1002 (17) as a donor and selected on a LB plate containing 50 μ g/ml kanamycin. Construction of other strains is described in *Supporting Text*, which is published as supporting information on the PNAS web site. For the GeneChip (Affymetrix, Santa Clara, CA) experiment, strains MG1655 and EG12976 were grown overnight in N-minimal medium (pH 7.7) and 10 mM $MgCl_2$ and washed three times with N-minimal medium without Mg^{2+} . The OD_{600} was adjusted to 0.5 and 1/100 volume of cells was added to 30 ml of N-minimal medium (pH 7.7) and 10 μ M $MgCl_2$ and was shaken at 250 rpm (Innova Incubator Shaker Model 4230, New Brunswick Scientific) until OD_{600} equals 0.25. Cells were chilled on ice and immediately harvested to prepare total RNA. β -gal activity was carried out after growth of bacteria in N-minimal medium (pH 7.7) and 10 mM Mg^{2+} , 10 μ M Mg^{2+} , or 10 μ M Mg^{2+} plus 100 μ M Fe^{3+} as described in ref. 18.

RNA Isolation, Processing, and Analysis. Total *Escherichia coli* RNA was isolated by using 600 μ l of the Tissue and Cell Lysis Solution of the Masterpure RNA Purification Kit (Epicentre Technology, Madison, WI). Enrichment of mRNA was done according to the manufacturer's instructions (Affymetrix). Alternatively, total RNA was isolated as described above with the following modification: after DNaseI treatment, total RNA was purified by using the Megaclear Purification Kit (Ambion, Austin, TX). mRNA enrichment was performed for this total RNA (50 μ g) by using the MICROBExpress Bacterial mRNA Purification Kit (Ambion). Biotin labeling, hybridization, and scanning were done as described in the manufacturer's instructions (Affymetrix). We used the Affymetrix GeneChip to examine four experiments by using strains MG1655 and EG12976 grown in low

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Abbreviations: cfu, colony-forming units; GPS, Gene Promoter Scan.

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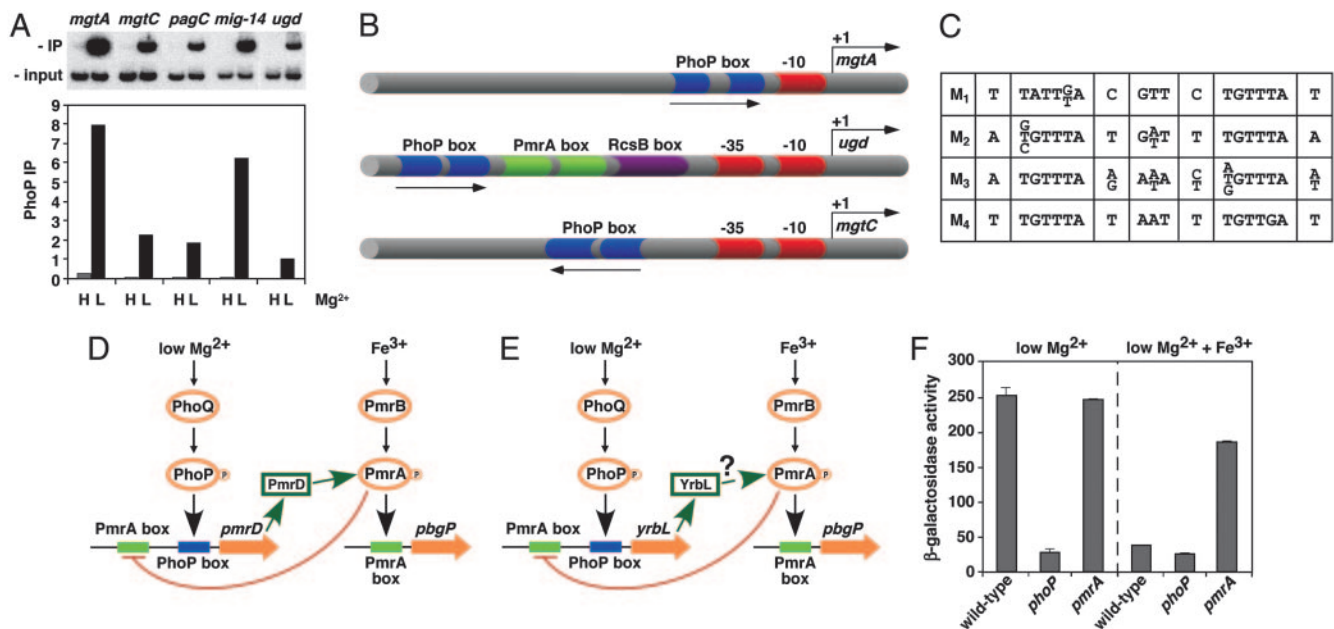


Fig. 1. Experimental validation of GPS predictions and schematic representation of PhoP-regulated promoters. (A) Chromatin immunoprecipitation (ChIP) of PhoP-regulated promoters predicted by GPS demonstrates binding of the PhoP-HA protein to the PhoP-activated *mgtA*, *mgtC*, *pagC*, *mig-14*, and *ugd* promoters, which are found in different profiles. Binding is detected in organisms experiencing low (L) (i.e., 10 μ M) but not high (H) (i.e., 10 mM) Mg²⁺, which correspond to conditions that activate and repress, respectively, the PhoP/PhoQ system. PhoP immunoprecipitation (IP) values correspond to the DNA bound by the PhoP protein (IP) divided by the total DNA before precipitation (input). (B) Representation of PhoP-regulated promoters identified by GPS and experimentally validated. Arrows, transcription start sites; red symbols, RNA polymerase sites; blue symbols, PhoP binding sites; green symbols, PmrA binding sites; purple symbols, RcsB binding sites. Drawing is not to scale. (C) The PhoP binding site submotifs. We built a model for the PhoP binding site by applying an extension of the CONSENSUS/PATSER program (25) by using promoters exhibiting coherent expression patterns (Fig. 3A). This initial model was used for identifying promoter motifs, which were clustered into four subsets based on their degree of sequence similarity to one another (M₁–M₄). All four submotifs have an hexameric direct repeat separated by 5 bp and preserved those conserved positions critical for PhoP-promoted transcription of the *mgtA* promoter (13). (D) Model for a multicomponent feedback loop in *Salmonella* involving the regulatory protein PmrA and its posttranslational activator PmrD. Transcription of the *pmrD* gene is induced in low Mg²⁺ in a PhoP-dependent fashion and repressed in the presence of Fe³⁺ in a PmrA-dependent manner. The PhoP and PmrA proteins exert their regulatory effects directly on the *pmrD* promoter, which has binding sites for these two proteins. (E) Model for regulatory interactions at the *Escherichia coli yrbL* promoter, which harbors PhoP- and PmrA-binding sites arranged in a fashion similar to those present in the *Salmonella pmrD* promoter. (F) Transcription of the *yrbL* gene is induced in low Mg²⁺ in a PhoP-dependent fashion and repressed by Fe³⁺ in a PmrA-dependent manner. β -gal activity (Miller units) of *Escherichia coli* strains harboring a *lac*-transcriptional fusion immediately downstream of the *yrbL* gene was determined in wild type, *phoP*, and *pmrA* backgrounds by using organisms grown in N-minimal medium (pH 7.7) and 10 μ M Mg²⁺ with or without 100 μ M Fe³⁺.

Classification of PhoP-Regulated Promoters. The use of relaxed thresholds resulted in the expected increased recovery of false positive candidates. Thus, we incorporated additional promoter features into the analysis in an effort to retrieve and classify bona fide PhoP-regulated promoters. Some of these features are as follows: the discrimination of PhoP box submotifs (M₁–M₄) (Fig. 3B), the orientation (O₁–O₂) (Fig. 3C) and distance of the PhoP box relative to the RNA polymerase site (P₁–P₃) (Fig. 3D), the class of σ 70 promoter (because σ 70 is responsible for transcription of PhoP-regulated genes; ref. 13) (P₁–P₃) (Fig. 3D), the presence of potential binding sites for 60-plus transcription factors (27) (I₀–I₄) (Fig. 3E), and whether the position of the PhoP box suggests a promoter is activated or repressed (A₁–A₃) (Fig. 3F).

We built models for each of these features by using the 43 promoters described above and 12 promoters identified in the initial analysis and known to be regulated by PhoP (Table 3, which is published as supporting information on the PNAS web site). Then, we generated profiles consisting of overlapping groups of promoters with common sets of features (Fig. 3G and H). For example, one of the profiles encompassed promoters that belong to the same expression (E₁), PhoP box submotif (M₂), and promoter class (P₁) (Fig. 1H) and harbored not only the prototypical PhoP-regulated *phoP* and *mgtA* promoters (13, 14) but also the *yhiW* promoter, which was not known to be under PhoP control. Yet, another profile comprised promoters that

share the promoter class (P₂), PhoP box orientation (O₁), and regulatory interactions (I₃) and included the *ompT*, *pipD*, *ugtL*, and *ybjX* promoters. We used the generated promoter profiles to search the intergenic regions of the *Escherichia coli* and *S. enterica* genomes and recovered additional putative promoters belonging to the PhoP regulon. Finally, we revised the original profiles by incorporating the latter promoters, which increased the robustness of the profiles (for examples of identified profiles, see Fig. 4, which is published as supporting information on the PNAS web site).

Our search successfully retrieved 92% of a group of 26 PhoP-regulated promoters that had not been used in the development of the models ($P < 10^{-5}$). Analysis of 487 additional promoters known to be regulated by transcription factors other than PhoP (27) indicated that our method recovered PhoP-regulated promoters with a specificity of 94.6% and a sensitivity of 92.3% (correlation coefficient, 93.9%).

Experimental Validation of Regulatory Interactions Mediated by PhoP. Transcriptional control of atypical promoters. The PhoP-activated *mig-14*, *mgtC*, *virK*, and *pagC* promoters of *Salmonella* do not harbor a typical PhoP box in place of the –35 region, as found in archetypal PhoP-regulated promoters such as the *mgtA* promoter (13). This finding has led to the proposal that PhoP regulates these promoters indirectly through another regulatory protein(s) (15). However, we determined that these promoters

do harbor a PhoP box but in the opposite orientation and located further upstream of the RNA polymerase site than the archetypal PhoP-regulated promoters (15, 28). Moreover, the statistical inclusion of these promoters within a profile supported the notion that they are regulated directly by PhoP despite their unusually oriented PhoP box.

We verified the predictions described above by using chromatin immunoprecipitation to probe promoter occupancy by the PhoP protein *in vivo*. We established that when *Salmonella* experiences low Mg^{2+} , the PhoP protein binds to both the archetypal *mgtA* promoter as well as the *mig-14*, *mgtC*, and *pagC* promoters (Fig. 1A). In contrast, there was no binding to these promoters when *Salmonella* experienced high Mg^{2+} , a condition that represses transcription of PhoP-activated genes. Furthermore, we conducted site-directed mutagenesis of the hexameric repeat sequences that makes up the PhoP box in the *mgtC* promoter (see *Supporting Text*) and demonstrated that, despite its orientation, the PhoP box is essential for the low- Mg^{2+} PhoP-dependent expression of the *mgtC* gene (data not shown). These results demonstrate that the PhoP protein can regulate transcription directly from promoters harboring PhoP binding sites in either orientation and at various distances from the RNA polymerase binding site (Fig. 1B).

A possible multicomponent loop involving the PhoP-activated *yrbL* gene. We have previously identified a multicomponent loop in *Salmonella* where the PhoP-dependent PmrD protein activates the regulatory protein PmrA, and activated PmrA represses transcription from the *pmrD* promoter, which harbors binding sites for both the PhoP and PmrA proteins (18) (Fig. 1D). We hypothesized that the PhoP-activated *yrbL* gene of *Escherichia coli* might participate in an analogous loop with the PmrA protein because both the *Salmonella pmrD* and *Escherichia coli yrbL* promoters can be found in the same promoter profile and harbor similarly arranged PhoP and PmrA boxes (Fig. 1D and E). In agreement with this notion, transcription of the *Escherichia coli yrbL* gene was induced in low Mg^{2+} in a PhoP-dependent fashion and repressed by PmrA in response to Fe^{3+} (Fig. 1F), which is the specific signal that activates the PmrA protein (11).

The PhoP/PhoQ system controls acid resistance genes in *Escherichia coli*. We classified the PhoP-activated genes identified in the genome-wide transcription experiment into two groups: E_1 and E_2 , corresponding to genes that were up-regulated strongly and mildly, respectively. Interestingly, most of the genes in expression group E_2 (Fig. 3A) encode proteins implicated in acid resistance in *Escherichia coli* (29–31), suggesting a role of PhoP in the control of acid resistance. Although these acid resistance genes displayed similar expression, they can be grouped into three profiles based on the presence of a PhoP box, the PhoP box submotif (M_1 – M_4), the presence of binding sites for other regulatory proteins (I_0 – I_4), and the distance from the PhoP box to the RNA polymerase site (P_1 – P_3) (Fig. 5, which is published as supporting information on the PNAS web site). One profile contained acid resistance structural genes lacking a recognizable PhoP box, such as *dps* and *gadA*. A second profile comprised a different set of acid resistance structural genes, including *hdeD* and *hdeAB*, which are predicted to have a class II promoter with a PhoP box close to the RNA polymerase site (P_1). The third profile, which harbors the PhoP box-containing promoters of the acid resistance regulatory genes *yhiE* and *yhiW* (also termed *gadE* and *gadW*, respectively), shared the promoter class and distance of the PhoP box to the RNA polymerase site (P_3).

We verified that PhoP plays a critical role in the control of acid resistance in a series of experiments demonstrating that: (i) expression of the *hdeA* (Fig. 2A) and *yhiW* (Fig. 2B) genes is regulated by Mg^{2+} in a PhoP-dependent fashion; (ii) the low Mg^{2+} activation of *hdeA* requires the regulatory gene *yhiW* (Fig. 2A), which encodes a protein that can bind to the *gadA* promoter

(32); (iii) the PhoP protein footprints the *hdeA* and *yhiW* promoters (Fig. 2C), protecting regions that correspond to the PhoP-binding sites predicted by GPS (Fig. 2D), indicating that PhoP regulates these targets directly; and (iv) the *phoP* mutant was hypersensitive to acid pH after logarithmic growth, like the *yhiW* mutant (Fig. 2E).

Discussion

The use of the GPS methodology to scan, analyze and group regulatory regions allowed us to unveil previously uncharacterized PhoP-regulated promoters, thereby uncovering an unsuspected complexity in the regulatory targets that are under direct and indirect transcriptional control of the PhoP protein. Our analysis revealed several classes of PhoP-regulated promoters that differ in the location and orientation of the PhoP box relative to the RNA polymerase binding site (Fig. 1B) and the presence of a -35 region and/or binding sites for other transcription factors (Fig. 3E). In addition, by distinguishing classes of PhoP box submotifs (Fig. 1C) supported by many promoters (Fig. 3B) and clarifying the exceptions to the original consensus (12), we could increase the specificity of the PhoP binding-site model without affecting its sensitivity to weak sites. This approach allowed us to discover promoters with a weak degree of matching to the consensus sequence. The experimental verification that the PhoP protein binds to different classes of promoters *in vivo* (Fig. 1A) and *in vitro* (Fig. 2C), together with the demonstration that the PhoP box is necessary for *mgtC* transcription despite its unusual orientation (data not shown), argues against proposals that atypical PhoP-dependent promoters are regulated by the PhoP protein only indirectly (15). Moreover, as transcription from certain PhoP-dependent promoters requires additional regulatory proteins, such as RcsB (33), SlyA (34), and YhiW (Fig. 2A), our results indicate that the PhoP protein uses multiple mechanisms to promote transcription. The control of different classes of promoters may allow the PhoP protein to coordinate the expression of a variety of products that may be required in different amounts and/or for different extents of time.

We established that the PhoP/PhoQ system controls expression of acid resistance genes in *Escherichia coli*. These results were unexpected because acid resistance genes were not identified as PhoP-regulated genes in two previous microarray experiments that compared expression of wild-type and *phoP* *Escherichia coli* strains (14, 35) nor had mutations in *phoP* been uncovered in screenings for acid regulatory genes in *Escherichia coli*. The difference with these reports might be due to the fact that we used defined media instead of complex media to grow the bacteria. Alternatively or in addition, it may reflect the ability of the GPS method to recover promoters, even if they are not strongly up-regulated by a transcription factor. The PhoP-dependent induction of acid resistance genes occurred at the mildly alkaline pH 7.4, suggesting that these genes may also help alleviate low Mg^{2+} stress.

The GPS method considers gene expression as one feature among many. This attribute of GPS allowed us to distinguish between acid resistance genes (Fig. 5), which otherwise would have stayed undifferentiated within the same expression group if analyzed by methods that back correlate gene expression with *cis* regulatory features (3, 4). Moreover, it predicted that PhoP uses at least two modes of regulation to control transcription of acid resistance genes: a feed-forward loop and classical transcriptional cascade (Fig. 2F). This prediction was based on: first, the identification of a PhoP box in the promoter of only a subset of acid resistance genes; second, considering different PhoP submotifs (as opposed to determining regulon membership by using a strict statistical cut off to examine the degree of matching to a consensus sequence); and third, by incorporating multiple promoter features into the analysis to recover profiles compris-

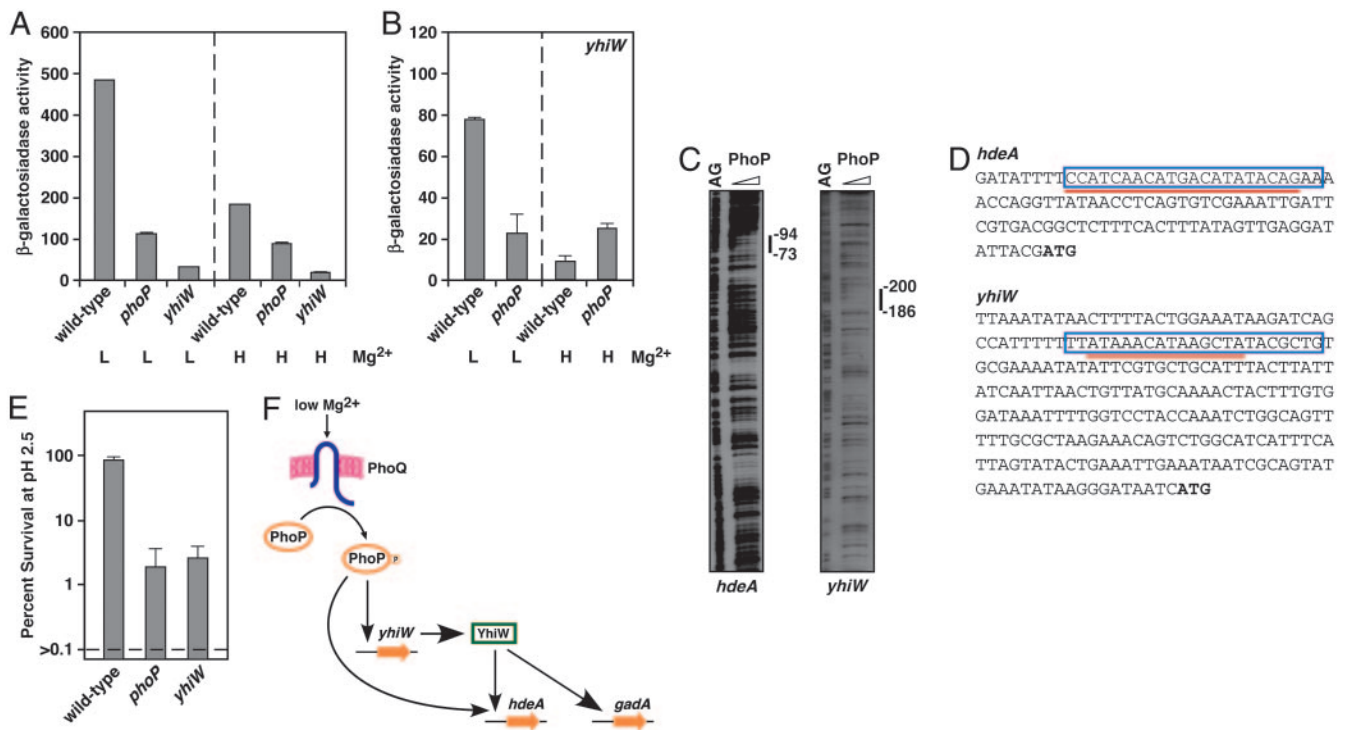


Fig. 2. The PhoP protein controls expression of acid resistance genes in *Escherichia coli*. (A) Transcription of the acid resistance *hdeA* gene is induced in low Mg²⁺ in a PhoP- and YhiW-dependent fashion. β -gal activity (Miller units) of strains harboring a *lac*-gene transcriptional fusion to the *hdeA* gene was determined in wild-type, *phoP*, and *yhiW* backgrounds by using organisms grown in N-minimal media (pH 7.4) and 10 μ M low (L) or 10 mM high (H) Mg²⁺. (B) Transcription of the acid resistance regulatory gene *yhiW* is modulated by Mg²⁺ in a PhoP-dependent manner. β -gal activity (Miller units) of strains harboring *lac*-transcriptional fusions to the *yhiW* gene was determined in wild-type and *phoP* backgrounds by using organisms grown in N-minimal media (pH 7.4) and 10 μ M (L) or 10 mM (H) Mg²⁺. (C) The PhoP protein footprints the *hdeA* and *yhiW* promoters. We used 0, 25, 50, and 100 pmol of *Salmonella* PhoP-His-6 (from left to right). AG corresponds to a sequencing ladder showing the A and G nucleotides. (D) Nucleotide sequence of the promoter regions of the *hdeA* and *yhiW* genes. Boxed sequences correspond to PhoP binding sites predicted by GPS. The red underline corresponds to DNA sequences protected by the purified PhoP-H6 protein as shown in C. Bold ATG sequences correspond to the predicted start codons. (E) The *phoP* and *yhiW* mutants are hypersensitive to acid pH. Logarithmically growing wild-type, *phoP*, and *yhiW* strains were exposed to pH 2.5 in LB broth adjusted with HCl for 1 hour at 37°C and then diluted and plated to determine the number of cfu. The percent survival was calculated as described in *Materials and Methods*. Data correspond to three repeats of the experiment. Percent survival values were converted to logarithmic values ($\log_{10} x$, where x equals the percent survival) for calculation of geometric means and standard errors. The survival levels of the wild-type strain were normalized to 100% and those of the mutant strains are displayed relative to those of the wild-type strain. The actual survival of the wild-type strain was 5.6%. (F) Model for the control of acid resistance genes by the PhoP protein in *Escherichia coli*. The *hdeA* gene is regulated in a feed-forward loop by the PhoP and YhiW proteins, whereas the *gadA* gene is regulated in a classical transcriptional cascade where the PhoP protein promotes transcription of the regulatory gene *yhiW* and the YhiW protein binds to the *gadA* promoter.

ing small numbers of promoters that would otherwise be subsumed by “typical” profiles encompassing large numbers of promoters. And it was also based on a model for the sequences recognized by the YhiW protein that we generated from analyzing promoters that are coordinately regulated by the YhiW protein (36). We provided experimental evidence for our model by demonstrating that a functional *phoP* gene is necessary for transcription of acid resistance genes after growth in low Mg²⁺ (Fig. 2A and B), the PhoP protein footprints the promoters of both structural and regulatory acid resistance genes (Fig. 2C), and inactivation of the *phoP* gene rendered *Escherichia coli* hypersusceptible to acid pH, as susceptible as mutants in the well characterized acid resistance regulatory gene *yhiW* (Fig. 2E).

A distinguishing characteristic of our approach is that promoters for orthologous genes are considered individually. This tenet is in contrast to some phylogenetic footprinting methods (1) that often ignore regulatory differences among closely related organisms due to their strict reliance on the conservation of regulatory motifs across bacterial species. Thus, we could uncover cases of phenotypic differences between closely related species resulting from the differential regulation of homologous genes. For example, the *ugd* promoter of *Salmonella* (Fig. 1B), but not of *Escherichia coli*, harbors a PhoP box that is located

much further upstream from an RNA polymerase binding site than in other PhoP-regulated promoters (Table 3). Despite the lack of conservation in this atypically located regulatory site, we demonstrated that the PhoP protein binds to the *Salmonella ugd* promoter *in vivo* (Fig. 1A) and *in vitro* (33), inactivation of the PhoP box in the *ugd* promoter abolishes PhoP-dependent *ugd* transcription (33), and *Escherichia coli* is unable to promote *ugd* transcription under the PhoP-dependent conditions that activate *ugd* expression in *Salmonella* (C. Mouslim and E.A.G., unpublished results).

In addition to controlling transcription directly at a variety of different promoters, the PhoP protein regulates several dozen genes indirectly through transcriptional, posttranscriptional, and posttranslational cascades promoting expression and/or activity of other two-component systems and regulatory proteins. This conclusion suggests that the PhoP/PhoQ system is a central element in a highly connected network and places PhoP in the same group of transcriptional regulators as Crp, Fnr, and ArcA, which also govern expression of large number of targets in *Escherichia coli*, often by working with additional regulatory proteins (37). The high connectivity of the PhoP/PhoQ system may be due to its role in maintaining Mg²⁺ homeostasis, and the fact that the essential role that Mg²⁺ plays in all cells demands

the adjustment of numerous cellular activities when an organism experiences Mg^{2+} -limiting environments. Alternatively or in addition, the high connectivity could reflect the apparent use of Mg^{2+} as a signal to denote extracellular and intracellular environments during infection of a mammalian host (38).

In summation, we have described previously uncharacterized regulatory targets of and interactions by the bacterial PhoP protein. The analysis of individual networks in eukaryotes is likely to be even more complex because a single nucleotide difference in the binding site for a transcription

factor can dictate the requirement for coactivator proteins (39). Thus, such analysis will require approaches like the one presented here that can uncover subtle differences between regulatory targets.

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