SI Text

Strain Construction. Strains and plasmids are summarized in SI Table 1. $P1_{kc}$ transductions were performed using standard procedures (1). To construct a constitutive CFP reporter, a promoterless *cfp* gene was cloned downstream of the *tetA* promoter (2) and integrated in single copy into the chromosome at the phage attachment site HK_{att}. In the absence of the TetR repressor, the *tetA* promoter is constitutive. The *cfp* gene was isolated from pMG34, which was digested with SpeI, polished with T4 DNA Polymerase, and then digested with XbaI. The fragment that contained *cfp* was cloned into a SalI-XbaI fragment of pAH70 (3). The *tetA* promoter (2) was constructed using the primers 5'-CTAGAGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCc-3' and 5'-ccgggGAGTGGTAAAATAACTCTATCAATGATAGAGTGTCAACT-3', where the uppercase portion is the *tetA* promoter, the underlined portion corresponds to an XbaI-digested end, and the lowercase portion corresponds to an XmaI-digested end. These oligos were annealed and ligated to an XbaI-XmaI fragment of pTM14. The resulting plasmid, pTM27 was integrated via the helper plasmid pAH69 (3) into the chromosome of MG1655 at the phage attachment site HK_{att}. This resulted in the constitutive CFP reporter strain TIM10. The residual *kan* gene was subsequently disrupted by electroporating into a TIM10 strain carrying pKD46 (4) the PCR product created by amplifying the chloramphenicol cassette within pKD3 with the primers 5'-GCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCGC TGGAGCTGCTTCGAA-3' and 5'-CGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATAA TGAATATCCTCCTTAG-3'. The *cat* gene, which lies between FRT sites, was removed using the helper plasmid pCP20 (4). The resultant strain, designated TIM64, constitutively expresses CFP and does not contain an antibiotic resistance marker.

To construct YFP reporters, promoter regions were cloned upstream of a promoterless *yfp* into pCAH63 (3). The resultant plasmids were then integrated in single copy into the chromosome at λ_{att}. The *yfp* gene was isolated from pMG32, which was digested with HindIII, polished with T4 DNA Polymerase, and then digested with XbaI. The fragment that contained *yfp* was then cloned into the vector pCAH63, which had been digested with BamHI, polished with T4 DNA polymerase, and subsequently digested with XbaI. The resultant plasmid, pTM74, contains a multicloning site upstream of the promoterless *yfp* gene. Promoter regions of *mgtA* (4464888-4464168), *mgrB* (1907100-1906801), *hemL* (175031-174898) and *phoPQ* (1189838-1189703) were amplified by PCR and cloned into pTM74 using EcoRI and BamHI sites to yield pTM78, pTM79, pTM82, and pTM83, respectively. These plasmids were integrated in single-copy into the phage λ_{att} site of MG1655 using the helper plasmid pInt-TS (3). The residual *cat* gene linked to the *mgtA* and *mgrB* promoters was replaced with *kan* by electroporating a PCR product, created from the template pKD4 with the primers 5'-ATATCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCGCT GGAGCTGCTTCGAA-3' and 5'-ATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAATATG AATATCCTCCTTAG-3', into the appropriate strains carrying pKD46. TIM91, TIM92, TIM146 and TIM148, which are the two-color fluorescent reporters of *mgtA*, *mgrB*, *hemL*, and *phoPQ*, respectively, were created by moving these markers into TIM64 by P1

transduction and removing the *kan* marker of the *mgtA* and *mgrB* reporter strains using pCP20. The reporter construct from TIM91 was used for the *mgtA* transcription data in Figs. 1, 3, and 4. In Fig. 3*c*, this construct is designated UTR1. A second *mgtA* reporter, designated UTR2 in Fig. 3*c*, was constructed using the *mgtA* promoter region (4465101-4465400). The sequence was cloned into pCAH63 as above to give pTM158 and then integrated at the λ_{att} site in TIM64 and TIM80 to give TIM257 and TIM260, respectively.

The deletions Δ*lacZYA*, Δ*phoP*, and Δ*phoQ* were constructed by lambda-Red based recombination following the protocols in ref. 4. These deletions resulted in replacement of the sequence intervals (365490-360520), (1189571-1189086), and (1188998-1187539) with the scar GTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTAAGGAGGATATTCAT.

Construction of Two-Color Fluorescent Promoter Reporters with Mutated PhoP-Boxes. Primers used for constructing the various mutations are listed in SI Table 2. To mutate the *mgrB* promoter, overlap extension PCR was performed using the template pTM79, which contains the wild-type *mgrB* promoter. First, a fragment (base pairs 1907100-1906858) was created by PCR amplification using the primers *mgrB*-promoter-upper and either *mgrB*-promoter-lower (for pTM92, pTM95, and pTM99) or pTM122-lower primer (for pTM122). A second fragment containing the desired mutation was amplified by PCR using the primers *mgrB*-promoter-lower and either pTM92-, pTM95-, pTM99 or pTM122-upper. The first and second fragments described above were purified and combined, and another round of PCR amplification was performed using the *mgrB*-promoter-upper and -lower primers. The final PCR product was cloned into pTM74 using EcoRI and BamHI sites, and the resultant plasmid was sequenced to confirm the sequence. The plasmids were integrated into the chromosome and transduced into TIM64 as described above. The mutated *mgtA* promoter (hybrid1) was created in an analogous manner using the primers indicated in SI Table 2 below.

Isolation of $PhoP_{ca}$**.** To isolate a *phoP* allele that produces a constitutively active PhoP ($PhoP_{ca}$), the *phoP* gene was first mutated by site-directed mutagenesis so that it encodes a PhoP protein with a D51A substitution. This allele was then further mutated by error-prone PCR and cloned into the plasmid pDSW204 (5). The resultant library was electroporated into the strain TIM233, which lacks the wild-type copy of *phoP* and contains a copy of *yfp* under control of the *mgrB* promoter. Several colonies that showed high YFP fluorescence on LB/ampicillin plates were selected and checked in liquid culture with several different PhoP-reporters. The *phoP* allele that was selected (designated $phoP_{ca}$) contains several mutations, which result in the following amino acid substitutions in PhoP: D51A, Q202R, T211I, L222M.

Fluorescence Measurements. Measurement and analysis of cellular fluorescence followed the protocols described in ref. 6. Fluorescence measurements in Fig. 1 were obtained using a Zeiss Standard research microscope with a 2FL fluorescence adaptor, a 100-W mercury lamp and a Nikon ×60 PlanApo NA 1.4 objective lens. Filter sets, D436/20 excitation, 455dclp dichroic, and D480/40 emission for CFP fluorescence and HQ500/20 excitation, Q5151p dichroic, and HQ535/30 emission for YFP fluorescence

were from Chroma (Brattleboro, VT). Images were acquired with a Hamamatsu (Bridgewater, NJ) C4742-95 cooled charge-coupled device camera.

Fluorescence measurements in the remaining figures were obtained using an Olympus IX81 microscope with a 100 W mercury lamp and ×100 UPlanApo NA 1.35 objective lens. The specifications of the filter sets were the same as those on the Zeiss microscope described above. Images were acquired with a SensiCam QE cooled charge-coupled device camera (Cooke Corporation, Romulus, MI) and IPLab v3.7 software (Scanalytics, Fairfax, VA).

Cellular fluorescence was measured in images as described in ref. 6, using the CFP fluorescence to construct masks. In all cases, the standard error of the mean of the cellular fluorescence for a single culture was negligible compared with the range of mean values obtained from separate cultures.

PhoP-6xHis Purification and Gel Shift Assays. To construct the PhoP-6xHis expression plasmid, *phoP* was amplified by PCR from MG1655 genomic DNA using primers 5'- tacatATGCGCGTACTGGTTGTTGA-3' and 5'- tgctcgagGCGCAATTCGAACAGATAGC-3', where the lowercase letters contain NdeI and XhoI restriction sites, respectively. The PCR product was digested with NdeI and XhoI and ligated into an NdeI-XhoI fragment of pET22b to give pTM50.

To purify PhoP, a 100-ml LB/ampicillin (50 μg/ml) culture of *E. coli* strain BL21(DE3) harboring pTM50 was grown at 37ºC to OD_{600} 0.5. IPTG was added to a final concentration of 1 mM, and the culture was grown for an additional 4 h. At that time, cells were harvested, lysed by sonication, and spun at 4ºC for 15 min at 30,000 × g. The soluble fraction (approximately 5 ml) was incubated with 1.25 ml of 50% Ni-NTA agarose (Qiagen, Valencia, CA) at 4ºC for 1 h. The agarose bed was washed twice and eluted into four 0.5-ml fractions. The purity of the fractions was determined by electrophoresis with 12% SDS-polyacrylamide gels and nonspecific staining of protein with Coomassie. Protein levels were determined by BCA Protein Assay Kit (Pierce, Rockford, IL). The fraction containing the highest level of PhoP-H6 was dialyzed exhaustively into 25 mM Tris•HCl, 50 mM KCl (pH 8.0) and stored at 4ºC.

DNA sequences consisting of the following 300 bp of each PhoP-regulated promoter were amplified by PCR: *mgtA* (4465101-4465400), *phoPQ* (1189691-1189990), *mgrB* (1906801-1907100), and *hemL* (174903-175202). PCR products were purified by agarose gel electrophoresis.

DNA binding reactions were performed on ice in a 20-μl volume. The DNA concentration in each reaction was approximately 10 nM. PhoP was added to various final concentrations to start the reactions, which were then kept on ice for 10 min. At this point, 3 μl of DNA loading buffer (40% sucrose/0.25% bromophenol blue/0.25% xylene cyanol) was added to each reaction. Approximately 5.75 μl of each sample was loaded onto 5% polyacrylamide gel (0.5× TBE) and run at 250 V. DNA was stained with SYBR Green (Pierce, Rockford, IL) and visualized with a Typhoon imager (GE, Piscataway, NJ). Image J (NIH, Bethesda, MD) was used to quantify DNA levels.

Determination of $[Mg^{2+}]_{50\%}$ and Normalization of Stimulus-Response Curves. Each YFP/CFP versus Mg^{2+} curve $C_i(x)$ in Fig. 1*b* (where i denotes a particular promoter), was

fitted to a curve of the form: $A_i + \frac{B_i}{1+\frac{[Mg^{++}]}{D_i}}$ using a routine in the G programming language (National Instruments, Austin, TX) based on the Levenberg-Marquardt algorithm. The constant D_i, which parameterizes the concentration of Mg^{2+} that gives half-maximal activity after subtracting the baseline value A_i, is denoted by $[Mg^{2+}]_{50\%}$ in Fig. 1*c*. We defined normalized curves (Fig. 1*d*) by subtracting A_i and rescaling by B_i: normalized $C_i(x) = (C_i(x)-A_i)/B_i$. Note that this normalization effectively forces the curves to converge to 1 and 0 at low and high magnesium, respectively. Our choice of the above fitting function was simply based on the fact that it gave good fits for all of the curves. Any other procedure for choosing the constants A_i and B_i so that the curves converge at low and high magnesium will give similar results as in Fig. 1*d*.

Analysis of Hypothetical *in Situ* PhoP-P-Binding Model Described in Fig. 2. As shown in Fig. 2, we assume that the level of PhoP-P bound to a particular promoter is determined by a model of simple binding, i.e., $\%\ \text{bound}\left([\text{PhoP}-\text{P}]\right)_i = \frac{100}{1+\frac{K_{di}}{[\text{PhoP}-\text{P}]}}$, where i = A, B, or C denote particular promoters and K_{di} denotes the dissociation constant of promoter i for PhoP-P. Furthermore, we assume the rate of transcription for promoter i, which we denote by T_i, is proportional to the fractional binding (% bound) of PhoP-P: T_i ([PhoP-P]) = a_i + b_i (% bound ([PhoP-P])$_i$), where a_i and b_i are constants.

To compare the transcription profiles of the promoters over a range of stimuli (see Fig. 2*b*), we define the normalized transcriptional response of promoter i to [PhoP-P] to be: $\frac{T_i([\text{PhoP}-\text{P}])-T_i(P_1)}{T_i(P_2)-T_i(P_1)} = \frac{\%\,\text{bound}([\text{PhoP}-\text{P}])_i - \%\,\text{bound}(P_1)_i}{\%\,\text{bound}(P_2)_i - \%\,\text{bound}(P_1)_i}$, where P_1 and P_2 are the PhoP-P levels corresponding to low and high stimuli (e.g., high and low $[Mg^{2+}]$), respectively.

The fold-increase in transcription of a particular promoter corresponding to [PhoP-P] changing from P1 to P2 is given by $\frac{T_i(P_2)-T_i(0)}{T_i(P_1)-T_i(0)} = \frac{\%\,\text{bound}(P_2)}{\%\,\text{bound}(P_1)}$, where $T_i(0)$ denotes the transcription rate in the absence of PhoP-P. If $K_{di} >> P_2$, then $\%\ \text{bound}\left([\text{PhoP}-\text{P}]\right)_i \approx \frac{[\text{PhoP}-\text{P}]}{K_{di}}$ and $T_i([\text{PhoP}-\text{P}]) \approx a_i + b_i \frac{[\text{PhoP}-\text{P}]}{K_{di}}$. The fold-increase is then $\approx \frac{P_2}{P_1}$. Thus, as illustrated in Fig. 2*c*, two promoters with different PhoP-P dissociations constants that are both much greater than P_2 will show the same fold-increase.

Determination of $[\text{PhoP-P}]_{\text{effective}}$ and Transcription as a Function of $[\text{PhoP-P}]_{\text{effective}}$. We model transcription of the i^{th} promoter by: $\left(\frac{\text{YFP}}{\text{CFP}}\right)_i = a_i + \frac{b_i\,[\text{PhoP}-\text{P}]}{[\text{PhoP}-\text{P}]+K_{d,i}}$. The constant a_i is set equal to the fluorescence ratio $(\text{YFP/CFP})_i$ for a Δ*phoP* strain. The

constant b_i parameterizes the maximal level of transcriptional activation and $K_{d,i}$ is the dissociation constant for PhoP-P. We can choose one promoter to map transcription levels onto effective levels of [PhoP-P]. Since this mapping is prone to error for transcription curves that approach saturation, we chose the *phoPQ* promoter, which has the highest dissociation constant based on the results in Fig. 3*d*. In addition, we chose *phoPQ* over *hemL* because the low fluorescence levels associated with transcription from the *hemL* promoter result in large errors. For the *phoPQ* promoter, we found that we obtained excellent fits (shown in Fig. 4*b* and described below) if we assumed that the effective levels of [PhoP-P] corresponding to the various levels of $PhoP_{ca}$ expression in Fig. 4*a* remain well below the dissociation constant of the *phoPQ* promoter ($K_{d,phoPQ}$). Therefore we approximate transcription of *phoPQ* by $\left(\frac{\mathrm{YFP}}{\mathrm{CFP}}\right)_{phoPQ} = a_{phoPQ} + c_{phoPQ}\,[\mathrm{PhoP-P}]$, where $c_{phoPQ} = b_{phoPQ} / K_{d,phoPQ}$. This relation is more restrictive than the general expression above since the two unknown parameters b_{phoPQ} and $K_{d,phoPQ}$ have been replaced with the single parameter, c_{phoPQ}. We denote the values of YFP/CFP for the *phoPQ* promoter corresponding to the six levels of $PhoP_{ca}$ expression in Fig. 4*a* (0, 10, 30, 60, 100, 175 μM IPTG) by $(\mathrm{YFP/CFP})_{phoPQ,i}$ (i = 1, ...6). We then convert these transcription levels into effective [PhoP-P] levels by inverting the above linear relation: $[\mathrm{PhoP-P}]_i = \dfrac{\left(\frac{\mathrm{YFP}}{\mathrm{CFP}}\right)_{phoPQ,i} - a_{phoPQ}}{c_{phoPQ}}$.

As above, we model transcription of the *mgtA* promoter by: $\left(\frac{\mathrm{YFP}}{\mathrm{CFP}}\right)_{mgtA} = a_{mgtA} + \dfrac{b_{mgtA}\,[\mathrm{PhoP-P}]}{[\mathrm{PhoP-P}]+1}$, where a_{mgtA} is the $(\mathrm{YFP/CFP})_{mgtA}$ fluorescence ratio for a Δ*phoP* strain. We have set $K_{d,mgtA} = 1$ to express all concentrations in units of $K_{d,mgtA}$. We then fit this curve to the six points of $(\mathrm{YFP/CFP})_{mgtA,,i}$ associated with $PhoP_{ca}$ induction and with the six values of $[\mathrm{PhoP-P}]_i$ determined above, using a routine based on the Levenberg-Marquardt algorithm. The two fitting parameters are b_{mgtA}, and c_{phoPQ}. Thus, the fit determines the curve for *mgtA* transcription and the constant c_{phoPQ} in terms of the values of $[\mathrm{PhoP-P}]_i$. We similarly model transcription of the *mgrB* promoter by: $\left(\frac{\mathrm{YFP}}{\mathrm{CFP}}\right)_{mgrB} = a_{mgrB} + \dfrac{b_{mgrB}\,[\mathrm{PhoP-P}]}{[\mathrm{PhoP-P}] + K_{d,mgrB}}$, where a_{mgrB} is the $(\mathrm{YFP/CFP})_{mgrB}$ fluorescence ratio for a Δ*phoP* strain. We then fitted this curve to the six points of $(\mathrm{YFP/CFP})_{mgrB,,i}$ corresponding to $PhoP_{ca}$ expression and with the $[\mathrm{PhoP-P}]_i$ values determined above. The two fitting parameters are b_{mgrB} and $K_{d,mgrB}$. For the *hemL* promoter, the levels of transcription for the highest levels of $PhoP_{ca}$ expression were apparently well below saturation, as was the case for *phoPQ*. As a result, we could not separately determine b_{hemL} and $K_{d,hemL}$ by fitting to a similar functional form to that used for *mgrB* above. Therefore, we instead fitted the data to the line: $\left(\frac{\mathrm{YFP}}{\mathrm{CFP}}\right)_{hemL} = a_{hemL} + c_{hemL}\,[\mathrm{PhoP-P}]$, where a_{hemL} is the $(\mathrm{YFP/CFP})_{hemL}$ fluorescence ratio for a Δ*phoP* strain and c_{hemL} is the fitting parameter. The results of the fits for *mgtA*, *mgrB*, and *hemL* are the dotted curves shown in Fig. 4*b*.

To determine $[\text{PhoP-P}]_{\text{effective}}$ for stimulation with LL-37 (Fig. 4*b*), we used the formula $[\text{PhoP}-\text{P}]=\dfrac{\left(\dfrac{\text{YFP}}{\text{CFP}}\right)_{phoPQ}-\text{a}_{phoPQ}}{\text{c}_{phoPQ}}$, where c_{phoPQ} and a_{phoPQ} were determined as described above. This value of $[\text{PhoP-P}]_{\text{effective}}$ was used to plot *mgtA*, *mgrB*, and *hemL* transcription levels of cells stimulated by LL-37 in Fig. 4*b*.

For determining $[\text{PhoP-P}]_{\text{effective}}$ as a function of $[Mg^{2+}]$ (Fig. 4*c*), we found that the YFP/CFP fluorescence levels of the *phoPQ* reporter strain grown in various concentrations of magnesium (Fig. 1*b*) were too low to give accurate measures of $[\text{PhoP-P}]_{\text{effective}}$. Therefore, we used two independent methods, based on transcription of *mgtA* and transcription of *mgrB*, by inverting the above expressions for YFP/CFP in terms of [PhoP-P]:

$$[\text{PhoP}-\text{P}]=\frac{\left(\dfrac{\text{YFP}}{\text{CFP}}\right)_{mgtA}-\text{a}_{mgtA}}{\text{b}_{mgtA}+\text{a}_{mgtA}-\left(\dfrac{\text{YFP}}{\text{CFP}}\right)_{mgtA}},\ \text{and}\ [\text{PhoP}-\text{P}]=\frac{\left[\left(\dfrac{\text{YFP}}{\text{CFP}}\right)_{mgrB}-\text{a}_{mgrB}\right]\text{K}_{\text{d},mgrB}}{\text{b}_{mgrB}+\text{a}_{mgrB}-\left(\dfrac{\text{YFP}}{\text{CFP}}\right)_{mgrB}}.$$

The error bars in Fig. 4*c* come from the errors in b_{mgtA}, b_{mgrB}, and $\text{K}_{\text{d},mgrB}$, which were determined from the nonlinear fits described above. The experimental error in (YFP/CFP) values was negligible compared with these errors.

To construct the in situ binding curves in Fig. 4*d*, the $[\text{PhoP-P}]_{\text{effective}}$ values from Fig. 4*c* were averaged for each Mg^{2+} level. The % $[\text{PhoP-P}]_{\text{effective}}$ bound to *mgtA* and *mgrB* promoter was calculated by normalizing the transcription level with the saturation level of each promoter as determined in Fig. 4*b*. These points are plotted in Fig. 4*d*. The dotted curves correspond to the expected values of bound [PhoP-P] based on the fits for *mgtA* and *mgrB* obtained in Fig. 4*b*.

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