

Stochastic activation of the response regulator PhoB by noncognate histidine kinases

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Abstract

Two-component systems (TCS) are the most prevalent gene regulatory mechanism in bacteria. A typical TCS is comprised of a histidine kinase (HK) and a partner response regulator (RR). Specific environment signals lead to autophosphorylation of different HKs, which in turn act as phosphoryl donors for autophosphorylation of their partner RRs. Nonpartner HKs and RRs also interact, giving rise to cross regulation among TCSs in response to diverse signals.

PhoR (HK) and PhoB (RR) constitute the TCS for detection of environmental (extracellular) inorganic phosphate (P_i). The PhoR/PhoB TCS controls the expression of a large number of genes for acquisition of alternative phosphorus sources, including *phoA*, which encodes the non-specific phosphohydrolase bacterial alkaline phosphatase (Bap). Cross activation of PhoB by the nonpartner HK CreC is now a classic example of cross regulation among TCSs. A systematic search for other cross talking HKs revealed five additional HKs that activate (phosphorylate) PhoB (J. M. B. and B. L. W., unpublished data).

Examination of cross activation of PhoB by these non-partner HKs by flow cytometry at the single-cell level revealed a bimodal, “all-or-none,” distribution pattern for expression of a *phoAp-gfp* (green fluorescent protein) reporter fusion. Although the basis of the observed stochastic behavior is unclear, it seems to reflect an inherent property of TCSs. We propose that cells exploit the stochastic character of TCSs to achieve nongenetic (epigenetic) diversity within genetically homogeneous cell populations in order to facilitate adaptation to environmental changes.

Keywords: ArcB, BaeS, cell populations, CreC, cross regulation, cross talk, EnvZ, *Escherichia coli*, flow cytometry, gene expression, *gfp*, KdpD, *phoA*, PhoR, promoter reporter fusion, protein phosphorylation, QseC, signal transduction, single-cell, two-component regulatory system

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1 Introduction

Stochastic changes in gene expression have been observed in diverse biological systems from bacteria to multicellular organisms [Elowitz *et al.*, 2002; Ferrell, Jr. and Machleder, 1998; McAdams and Arkin, 1997; Ozbudak *et al.*, 2002; Thattai and van Oudenaarden, 2001]. Many have proposed that cells exploit stochastic, “all-or-none,” effects as a means to achieve genetic and nongenetic diversity within cell populations in order to survive in different environments. Stochastic mechanisms are also thought to be a driving force in the determination of cell fate and development in complex organisms. Thus, a better quantitative understanding of the stochastic behavior of gene expression will have broad implications in cell biology.

Two-component systems (TCSs) are the most prevalent mechanism of transmembrane signal transduction in bacteria. TCSs control gene expression for many key processes in cell physiology, cell-cell communication, bacterial development and, in the case of pathogens, bacterial virulence. TCSs are important for cell survival and adaptation in a changing environment, in particular, for the dynamic changes that occur in bacterial-host interactions during infection. A typical TCS is comprised of a histidine kinase (HK) and partner response regulator (RR). Detection of an environmental signal by the HK leads to its autophosphorylation and subsequent transfer of the phosphoryl group to an RR. Free and phospho-RRs in turn activate or inhibit gene expression in response to various environmental (or intracellular) cues.

The PhoR (HK)/PhoB (RR) TCS controls the expression of many genes in response to environmental (extracellular) inorganic phosphate (P_i). During growth with P_i in excess, genes for use of alternative phosphorus (P) sources (e. g., *phoA*, encoding the non-specific alkaline phosphatase (Bap) for use of a variety of organic phosphates) are normally turned off. When growth is restricted due to limitation of extracellular P_i , PhoR is autophosphorylated and acts as a phosphoryl donor for activation (autophosphorylation) of PhoB, resulting in activation (or inhibition) of a number of downstream target genes that comprise the phosphate (Pho) regulon.

Regulatory connections have been observed between different TCSs and for TCSs with other global regulators (e. g., [Hengge-Aronis, 2002; Suzuki *et al.*, 2002]). One of the first examples of physiologically useful signaling of this sort (also called cross regulation [Wanner, 1992]) is the activation of the RR PhoB by the nonpartner HK CreC [Parkinson, 1988; Wanner and Latterell, 1980]. Activation of PhoB by CreC and PhoR is highly regulated by the carbon source and environmental P_i , respectively, presumably as a means to coordinate carbon, energy, and P_i metabolism [Wanner, 1993]. Although CreC-dependent activation of PhoB is strongly affected by various catabolites, the precise signal for CreC is unknown [Wanner *et al.*, 1996]. Another example of cross regulation is the activation of the RR OmpR by the non-partner HK ArcB. In this case, activation of OmpR by its partner HK EnvZ is controlled by the medium osmolarity (the signal for EnvZ), whereas the activation of OmpR by ArcB responds to anaerobiosis (the signal for ArcB) [Matsubara *et al.*, 2000]. Observations such as these suggest that many TCSs may respond to signals from different HKs as a way to facilitate adaptation to changing environments.

With the goal of understanding the biological basis of cross regulation, we previously conducted a systematic search for non-partner HKs that activate (phosphorylate) PhoB. To do this, we expressed genes for different HKs in single copy on the chromosome from the rhamnose-regulated *rhaBp* [Haldimann *et al.*, 1998]. Among fourteen non-partner HKs tested, four HKs (ArcB, CreC, KdpD, and QseC) showed strong activation, two HKs (BaeS

and EnvZ) showed weak activation, and eight HKs (BarA, BasS, CpxA, CusS, PhoQ, RstB, TorS, and YedV) showed no activation of PhoB [Blackman *et al.*, 2005]. Careful examination of PhoB activation by these non-partner HKs revealed substantial variability in the degree of activation, and often as much as 10 to 15% differences among replicas of the same cell cultures. The observed variability was reminiscent of the Bap clonal variation phenotype, a metastable phenotype that was previously shown to be associated with the activation of PhoB by CreC [Wanner, 1986a; Wanner, 1986b; Wanner *et al.*, 1988].

The Bap clonal variation phenotype probably reflects heterogeneity of the cell population with respect to *phoAp* transcription [Wanner, 1987]. Accordingly, we tested for whether the variability among replicas for cross activation of PhoB by non-partner HKs is attributable to heterogeneity of the respective cell populations. We used flow cytometry to do single-cell profiling of PhoB activation by these HKs in cells carrying a *phoAp-gfp* (green fluorescent protein) reporter fusion. Cells expressing different non-partner HKs displayed bimodal behavior with respect to expression of the *phoAp-gfp* transcriptional fusion. Accordingly, cells appear to display the stochastic character of gene expression in a cross-regulation network of TCSs, which would allow for generation of nongenetic diversity within a genetically homogeneous cell population that would facilitate adaptation to different environments.

2 Methods

Strains. All bacteria are isogenic with *E. coli* K-12 BW26910 (*lacIp4000 (lacI^q) rrnB3 ΔlacZ4787 ΔphoR574 ΔcreABCD154 hsdR514 DE(araBAD)567 DE(rhaBAD)568 rph-1*) and carry a chromosomal *rhaBp*-HK fusion, with or without the gene for its partner RR and the genes for acetyl phosphate synthesis. All fusions were constructed by λ Red-mediated recombination essentially as described elsewhere [Datsenko and Wanner, 2000], except by using PCR fragments generated with a specially designed template plasmid having an outwardly directed *rhaBp* [Datsenko and Wanner, 2005]. The DE(*rhaBAD*)568 mutation [Haldimann *et al.*, 1998] permits use of L-rhamnose as a gratuitous inducer by eliminating genes for rhamnose catabolism. The DE(*ackA pta*)160 mutation [Metcalf *et al.*, 1996] was introduced to eliminate acetyl phosphate synthesis.

Reporter fusions. Transcriptional *phoAp*- and *rhaBp-gfp* fusions were made using CRIM plasmids [Zhou *et al.*, 2004], which were integrated into the chromosome or a specially engineered low copy number F plasmid [Firth *et al.*, 1996; Zhou *et al.*, 2005], as described previously [Haldimann and Wanner, 2001].

Cell growth and sample preparation. Cells were routinely grown in MOPS minimal medium with excess P_i (2 mM K₂HPO₄ [Wanner, 1994]) in 16 by 150-mm tubes with 2-ml medium containing 0.1% glycerol or in 250-ml deep-baffled flasks with 35-ml medium containing 1% glycerol. 1 mM L-Rhamnose was used for induction of HK synthesis. For time course studies, “starter” inocula were prepared by inoculating tube cultures with freshly-grown single colonies from an agar medium of similar composition and incubating about 12 hours in a roller at 37°C. Unless indicated otherwise, 10- to 40- μ l portions from cultures that were grown without rhamnose were diluted ca. 1,000 to a low cell density (about 10⁴ cells per ml) into flasks containing medium with L-rhamnose. These subcultures were incubated about ten hours till reaching a visibly turbid cell density when sampling commenced. For cell culture density measurements, 100- μ l portions were added to 150- μ l “fix” (0.5-ml formaldehyde per 100 ml water) in wells of a 96-well microplate, mixed by pipetting, and measured in a Molecular Devices 340PC plate reader at A₄₁₀. For Bap assays, 100- μ l portions were added to 200- μ l 1M Tris·HCl buffer (pH 8.0) and 100 μ g/ml chloramphenicol in microfuge tubes (in

an ice-water bath), vortexed briefly and kept on ice until assayed. For flow cytometry, 100- μ l portions were added to empty microfuge tubes and kept on ice until assayed.

Bap assay. Bap activities were quantitatively determined by a microplate colorimetric assay, essentially as described for measuring β -galactosidase activities [Zhou *et al.*, 2003], except for use of *p*-nitrophenol phosphate (*p*NPP) as substrate and 1 M Tris·HCl as buffer. 10- to 30- μ l portions of the Bap assay samples were added to 200- μ l Tris·HCl assay buffer containing 100 μ g/ml chloramphenicol and 15 mM OTG (1-S-octyl- β -D-thioglucoiside) in microplate wells. The microplate was then incubated with shaking at 37°C for 10 minutes. Assays were started by addition of 20- μ l 1.0% *p*NPP (prewarmed) to the microplate wells with a multichannel pipettor. Readings were taken at regular intervals (5, 10, and 20 min and later times) until all samples reached an A_{410} of 0.2 to 0.6 or 16 h, whichever occurred first. A no cell (buffer plus *p*NPP) blank was run in each microplate. Enzyme units were calculated in terms of nanomoles per min per ml culture using the molar extinction coefficient ϵ_{410} of 1.62×10^4 . Specific activities are enzyme units per cell culture A_{410} . After subtraction of the blank, values were calculated using the equations:

Activity (nmoles/ml/min) = $3.0 \text{ (CDF)} \cdot [A_{410}(t_e) - A_{410}(t_0)] \cdot 8.33 \text{ (SDF)} \cdot 61.73 \text{ (CF)} / (t_e - t_0)$, where CDF is the Culture Dilution Factor (which is 3.0 if 100 μ l sample was added 200 μ l buffer), SDF is the sample dilution factor (which is 8.33 if 30 μ l was added to give a (final) 250 μ l reaction), and CF is the conversion factor (61.73, i. e., $10^6/16200$, for conversion of absorbance to nanomoles per ml). $A_{410}(t_e)$ and $A_{410}(t_0)$ are the absorbances at the end (t_e) and start (t_0), respectively. Specific activity = Activity / [$2.5 \text{ (FDF)} \cdot A_{410} \text{ (cells)}$], where FDF is the Fix Dilution Factor and $A_{410} \text{ (cells)}$ is the cell density.

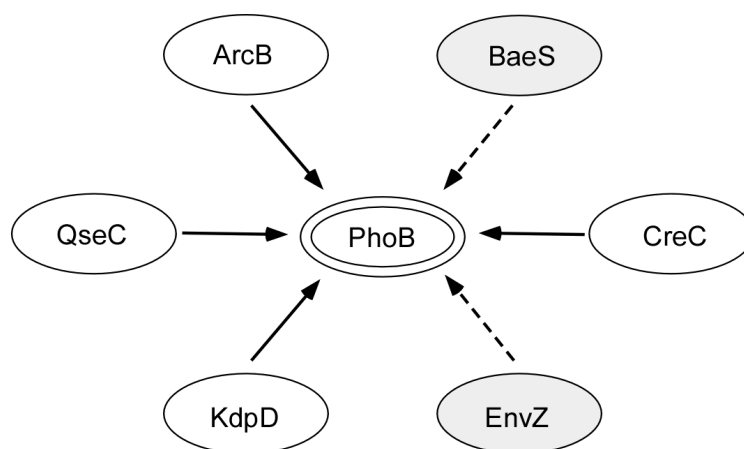


Fig. 1. Activation of PhoB by noncognate HKs. Hollow ovals with thick arrows show HKs that strongly activate PhoB; filled ovals with dashed arrows show HKs that weakly activate PhoB. See text.

Flow cytometry and data analysis. For collecting *gfp* expression data, samples were held on ice until assayed in a CYTOMICS FC500 (Beckman-Coulter, Hialeah, FL, USA) flow cytometer. The CYTOMICS FC500 is equipped with 2 air-cooled lasers, a blue Argon laser (excitation wavelength at 488 nm) and a red Helium-Neon laser (excitation wavelength at 633 nm). Up to seven optical parameters can be collected simultaneously: Forward Scatter (FS), related to the size of the particles; Right Angle Light Scatter (RALS), related to the shape and the internal structure (granularity) of the particles; up to 5 fluorescent signals centered approximately at 525, 575, 610, 675 and 755 nm. GFP fluorescence data were obtained by excitation with the 488-nm Argon laser and collecting the emission centered around 525 nm. SYTO 17 (Molecular Probes, Inc., Eugene, OR, USA), a red fluorescent DNA stain excitable with the Helium-Neon laser, was used to distinguish cells from the background signal (noise,

detritus). The signal from SYTO 17 was collected at 675 nm. After measuring GFP fluorescence, cell samples were incubated with 5 nM SYTO 17 for 5 min on ice. At least 10,000 cells were collected for data in results. Data analysis was performed on WinMDI, a freeware package developed by J. Trotter (<http://facs.scripps.edu/software.html>).

3 Results

Activation of PhoB by noncognate HKs. Six noncognate HKs activate PhoB in the absence of its partner PhoR (Fig. 1). These HKs were tested for activation of PhoB in both the presence and absence of their partner RRs [Blackman *et al.*, 2005]. ArcB, CreC, KdpD, and QseC were found to activate PhoB both in the presence and absence of its partner RR ArcA, CreB, KdpE, and QseB, respectively. In contrast, BaeS and EnvZ were found to activate PhoB only in the absence of its partner RR BaeR and OmpR, respectively. Interaction of HKs with their partner RRs is expected to affect how strongly the HK interacts with (activates) nonpartner RRs such as PhoB. Accordingly, it is reasonable to suppose that an HK that activates PhoB both in the presence and absence of its partner RRs shows stronger interaction with PhoB than an HK that activates PhoB only in the absence of its partner RRs.

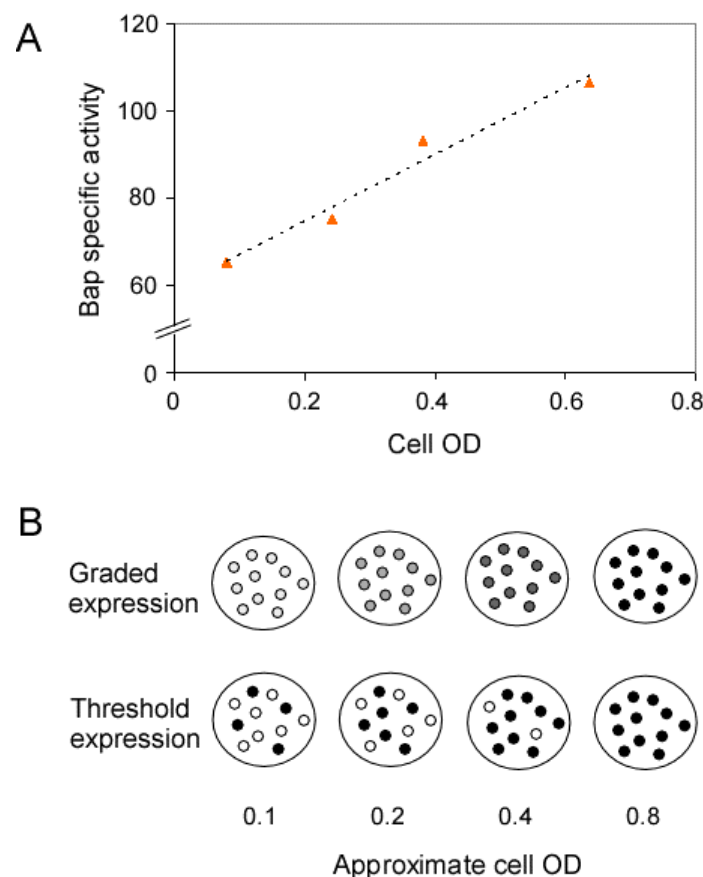


Fig. 2. Time course for PhoB activation by ArcB. (A) *E. coli* BW31820 (like BW26910, except *rhaBp-arcB ΔarcA Δ(ackA pta)*) was grown in 1 % glycerol MOPS medium with 1 mM L-rhamnose for 10 hours from a low cell density. The culture was periodically sampled for cell density and Bap activity measurements once the density reached an A_{410} of about 0.1, as described in Materials. Bap specific activities (nanomoles product made by minute per cell OD) and cell optical densities (A_{410}) are shown. (B) Graphic representation of graded and threshold expression. See text.

Time course for activation of PhoB by ArcB. We previously found substantial variation in the amount of activation of PhoB by non-partner HKs when comparing replica cultures

[Blackman *et al.*, 2005]. We therefore examined the time course for activation of PhoB by ArcB by measuring Bap activity (Fig. 2A). Unexpectedly, our data showed that the Bap specific activity increased with increased cell density. Because replica cultures were occasionally sampled at different cell densities in earlier studies, the large variations seem to result from cell density effects on activation of PhoB.

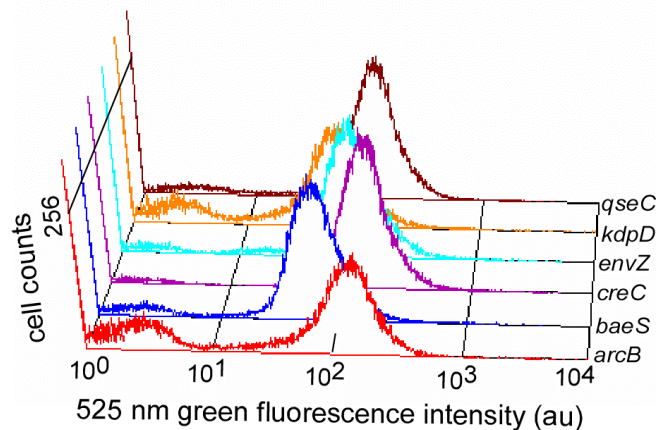


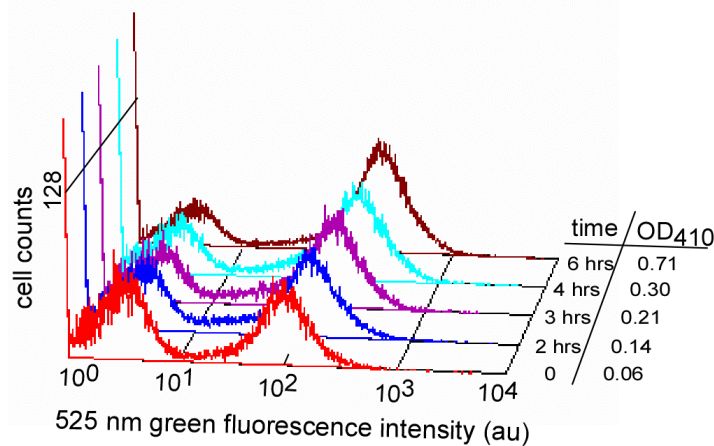
Fig. 3. Single-cell profiling of PhoB activation by noncognate HKs. Single colonies for *E. coli* strains carrying *phoAp-gfp* and *rhaBp*-HK fusions were inoculated into 2-ml 0.1% glycerol MOPS medium with 1 mM L-rhamnose and grown for ten hours in 16-mm tubes, as described in Materials. Green fluorescence was measured by flow cytometry as described in Materials. Results are shown for BW31820 (like BW26910, except *rhaBp-arcB ΔarcA Δ(ackA pta)*), BW31817 (like BW26910, except *rhaBp-baeS ΔbaeR Δ(ackA pta)*), BW31828 (like BW26910, except *rhaBp-creCD ΔcreAB Δ(ackA pta)*), BW31827 (like BW26910, except *rhaBp-envZ ΔompR Δ(ackA pta)*), BW31824 (like BW26910, except *rhaBp-kdpD ΔkdpE Δ(ackA pta)*), and BW31819 (like BW26910, except *rhaBp-qseC ΔqseB Δ(ackA pta)*).

Two mechanisms would account for cell density-dependent increased specific activities (Fig. 2B). The entire cell population could show graded expression with respect to ArcB/PhoB-dependent *phoA* expression. For example, a subtle change in anaerobiosis at higher cell densities could lead to greater stimulation of ArcB and increased activation of PhoB with increased cell density. Alternatively, the cell population could show threshold expression. That is, the proportion of cells showing ArcB/PhoB-dependent *phoA* expression could increase with increased cell density. This could result from a cell density effect on stimulation of ArcB in individual cells. Single-cell profiling was carried out in order to distinguish if the cell density effect on the activation of PhoB results from a graded or threshold response.

Single-cell profiling of PhoB activation. To test for graded versus threshold effects on PhoB activation, reporter strains carrying a *phoAp-gfp* fusion on a low copy F plasmid were made for all six *rhaBp*-HK fusion strains. Examination of these strains following growth overnight (12 hours) in carbon (glycerol)-limited cultures revealed a bimodal distribution of fluorescence intensities for all six *rhaBp*-HK fusion strains (Fig. 3). The lower and higher fluorescence intensity peaks for these strains coincide with intensity values of non-activated and activated subpopulations, as determined by comparison with cultures grown without and with L-rhamnose for induction of HK synthesis (data not shown). As shown in Fig. 3, non-activated and activated subpopulations were seen for all six HK reporter strains, however the bimodal distribution patterns were more evident for ArcB and KdpD than for the other HKs. These data show that activation of PhoB by non-partner HKs shows a threshold response.

The variability observed for activation PhoB by these HKs probably resulted from heterogeneity of the cell population with respect to *phoAp* transcription.

A



B

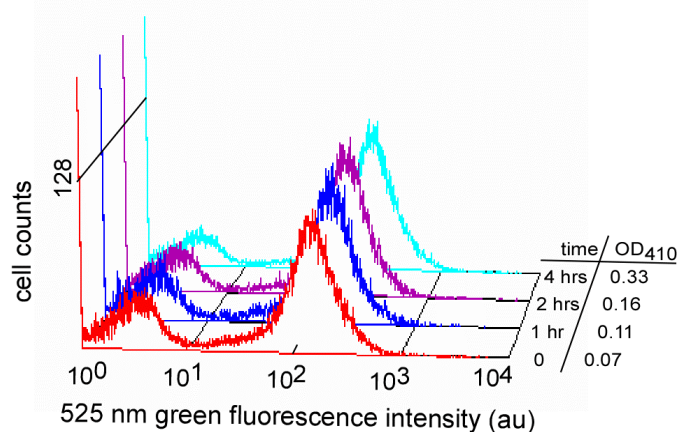


Fig. 4. Time course for single-cell profiling of PhoB activation by ArcB. Single colonies for *E. coli* strains carrying a *phoAp-gfp* fusion were grown in 1 % glycerol MOPS medium with 1 mM L-rhamnose for 10 hours from a low cell density. Cultures were periodically sampled for cell density and green fluorescence, by flow cytometry, as described in Materials. Cultures of *E. coli* BW31815 (like BW26910, except *rhaBp-arcB ΔarcA*) were inoculated from starter cultures that had been grown without (A) or with L-rhamnose (B). Analogous experiments were carried out with BW31820 (like BW26910, except *rhaBp-arcB ΔarcA Δ(ackA pta)*).

Further studies were carried out for activation of PhoB by ArcB. We also examined the effect of the incubation time with the inducer (L-rhamnose). As described in Materials, we routinely inoculate shake flask cultures at a low cell density and then incubate these cultures for about 10 hours until they become visibly turbid when we begin sampling them. To minimize heterogeneity, we routinely use starter inocula that are prepared by inoculation of small, carbon-limited cultures with single colonies from an agar medium of similar composition. Starter cultures are grown for about 12 hours and are usually prepared with medium lacking an inducer. Accordingly, we tested whether addition of L-rhamnose to the starter cultures has an effect. Similar bimodal distribution patterns for *phoAp-gfp* expression were seen with cultures that had been inoculated from uninduced (normal) or induced starter cultures, as shown in Fig. 4A and 4B, respectively. In other words, both non-activated and activated subpopulations were observed regardless of whether cells had been grown with L-rhamnose (for induction of *arcB* expression) for ten or more than twenty hours. With increased cell density the non-activated subpopulation (without green fluorescence) decreased

from about 50% to 30% of the total population, while the activated subpopulation (with green fluorescence) steadily increased from approximately 50% to 70% during this period (Fig. 4A). Concomitant increases in Bap specific activities were also seen (data not shown). As expected, the proportion of non-activated cells was significantly lower in cultures that had been grown for a longer period in the presence of L-rhamnose (Fig. 4B). One possible effector of the cell density-dependent increased PhoB activation by ArcB could be acetyl phosphate. We therefore also examined the effect of a $\Delta(ackA\ pta)$ mutation. Similar results were found for activation of PhoB by ArcB in single-cell profiling of $\Delta(ackA\ pta)$ mutant (data not shown).

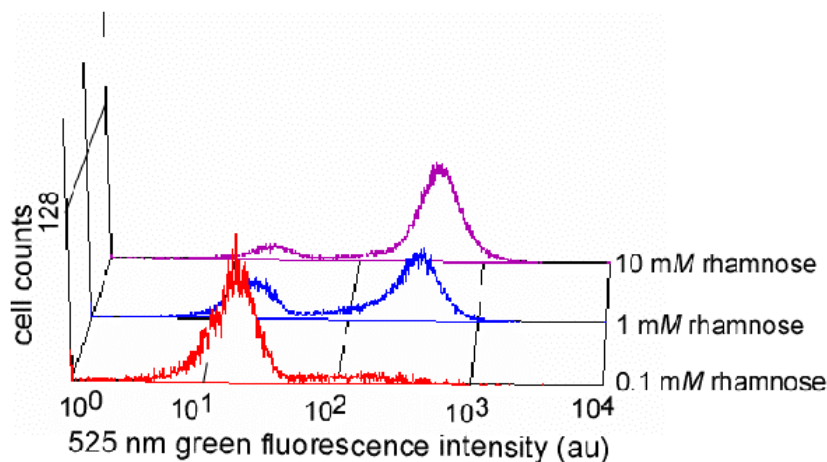


Fig. 5. Single-cell profiling of PhoB activation by ArcB with differing L-rhamnose concentrations. *E. coli* BW32405 (like BW26910, except *phoAp-gfp rhaBp-arcB Δ arcA Δ (ackA pta)*) was grown in 1 % glycerol MOPS medium with 0.1, 1, and 10 mM L-rhamnose for 10 hours from a low cell density. Cultures were sampled for green fluorescence by flow cytometry at cell density A_{410} about 0.2.

Effect of L-rhamnose concentration on activation. The classic all-or-none phenomenon resulted from use of a suboptimal inducer concentration [Novick and Weiner, 1957]. We therefore examined the effect of varying L-rhamnose concentration from 0.1 to 10 mM. To rule out the possibility that the heterogeneity resulted from use of a low copy plasmid (e. g., due to an effect on F plasmid replication), these experiments were done with a strain carrying a chromosomal *phoAp-gfp* reporter fusion. Single-cell profiling of the chromosomal *phoAp-gfp* reporter fusion also revealed bimodal distribution of fluorescence intensity (Fig. 5). While GFP fluorescent data showed a definite effect of inducer concentration on the binary response of *phoAp-gfp* expression (Fig. 5), a bimodal distribution of non-activated and activated cells was clearly evident even at the highest inducer concentration. While we do not understand the basis for the effect of the L-rhamnose concentration, 0.1 mM is more than ten-fold above the K_t for rhamnose uptake [Muiry *et al.*, 1993]. Thus, it is unlikely that uptake is limiting because we routinely use 1 mM, which is more than 100-fold greater than the K_t . Further, a *rhaBp-gfp* fusion showed homogenous induction of GFP fluorescence under these conditions (data not shown).

4 Discussion

Four signaling pathways were previously shown to activate (phosphorylate) the RR PhoB, including its partner HK PhoR, the nonpartner HK CreC, the small molecule acetyl phosphate

(an intermediate of the acetate kinase (AckA)-phosphotransacetylase (Pta) pathway), and the nonpartner HK EnvZ. Also, activation by EnvZ was seen only in the absence of OmpR and required acetyl phosphate synthesis [Kim *et al.*, 1996]. We recently conducted a systematic search for additional nonpartner HKs that activate PhoB. We did this in a $\Delta creC \Delta phoR$ mutant (BW26910) to prevent interference by CreC and PhoR. Because HKs are stimulated by different signals, many of which are unknown, we used a strategy that did not require an exogenous signal. We sought HKs that activate PhoB upon (modest) overexpression from chromosomal *rhaBp*-HK transcriptional fusions. We previously found that overexpression of CreC or PhoR result in activation of PhoB in the absence of a signal (unpublished data).

Fourteen RRs belong to the OmpR/PhoB family (also simply called the PhoB family). We tested all thirteen HKs that are partners of other PhoB family RRs for cross activation of PhoB. We also tested the HK BarA (the partner of the NarL family RR UvrY [Pernestig *et al.*, 2001]) because BarA has been shown elsewhere to activate the PhoB family RR OmpR [Nagasawa *et al.*, 1992]. Our search revealed four HKs (ArcB, CreC, KdpD, and QseC) that strongly activate PhoB, two HKs (BaeS and EnvZ) that weakly activate PhoB, and eight other HKs that failed to activate PhoB [Blackman *et al.*, 2005]. These data provide additional support for cross activation of PhoB by CreC and EnvZ. They also show that there is a high degree of specificity not only between partner HKs and RRs, but also between non-cognate HKs and RRs of the same family.

Cross activation of PhoB by CreC displays a metastable, clonal variation phenotype, resulting from an all-or-none effect on transcription of *phoA* and other Pho regulon promoters in single cells [Wanner *et al.*, 1988; Wanner, 1996]. This unusual switching behavior is no doubt partly a consequence of the autoamplification of *phoBR* operon expression by PhoB [Guan *et al.*, 1983]. We have also seen a similar Bap clonal variation phenotype in mutants that affect signaling by other HKs, including ArcB (unpublished data). We therefore examined activation of PhoB by nonpartner HKs by single-cell profiling of strains carrying a *phoAp-gfp* reporter fusion.

Our GFP expression data showed that all six non-partner HKs lead to bimodal (all-or-none) distribution of fluorescence intensities, even following several generations of growth for induction of HK synthesis. Accordingly, the stochastic activation of PhoB by noncognate HKs appears to be a fundamental property of PhoB signaling system. In preliminary studies, we have also now observed bimodal behavior with respect to expression of the *phoAp-gfp* upon activation of PhoB by PhoR in response to P_i limitation (L. Z. and B. L. W., unpublished data).

Several features of the PhoB TCS may account for its bimodal behavior upon activation of different HKs, especially the observed cell density effect on activation of PhoB by ArcB. First, PhoB synthesis itself is controlled by an autoamplification loop. Second, activation of PhoB by ArcB (and probably other HKs) probably results a system operating near a threshold. It is unclear what property of the system is near a threshold. It could be the amount of ArcB or active (autophosphorylated) ArcB. It could be the amount of PhoB or active (phosphorylated) PhoB. Or, it could be the amount of signaling molecule or some other factor. Many of these factors are difficult to assess in large cell populations, and surely most difficult in single cells. Modeling of these and other factors [Kierzek *et al.*, 2001] should provide insight into what further experiments are needed to understand more fully the operation of the PhoR/PhoB TCS.

E. coli encodes thirty-one simple or complex TCSs, which include thirty-four RRs, twenty-three orthodox HKs, five hybrid (composite) HKs (ArcB, BarA, EvgS, RcsC, TorS), and two unorthodox HKs (CheA, YojN). Therefore, more than forty distinct His \rightarrow Asp signaling pathways operate in *E. coli* in response to different stimuli [Mizuno *et al.*, 2002]. Additional

His→Asp phosphotransfer reactions have been shown to occur between nonpartner HKs and RRs, at least *in vitro* [Yamamoto *et al.*, 2005]. We tested only fourteen non-partner HKs for activation of PhoB, including two hybrid HKs (ArcB and BarA). Whether other HKs cross activate PhoB has not been tested. Cross regulation may also occur among many other TCSs.

Even in the absence of full understanding of the underlying mechanism, our results have implications with regards to cross regulation among TCSs. For example, because signaling by CreC has only a small effect in the presence of PhoR, it has been difficult to establish its physiological significance [Wanner *et al.*, 1996]. The finding that activation of PhoB by noncognate HKs results in heterogeneous response at the single-cell level suggests that only a subpopulation is activated under these conditions. Accordingly, the small effects seen earlier may result from high activation of a subpopulation. Non-cognate interactions of this sort may be critically important in the coordination of cell growth and metabolism, as suggested earlier [Wanner, 1992].

Stochastic changes in gene expression result in phenotypic variation within genetically homogenous cell populations. Classic example of this behavior is the all-or-none switch resulting in the spontaneous release of phage λ from a population of lysogenic cells [Witkin and Wermundsen, 1977]. Phenotypic variation has also been seen among subpopulations during formation of bacterial colonies arising from the growth of single cells [Shapiro, 1992]. Analogous stochastic changes in gene expression may also contribute to bottleneck effects during infection by bacterial pathogens.

In summary, the bimodal behavior of *phoAp-gfp* expression resulting from activation of PhoB by nonpartner HKs observed in this work shows that cross-regulation leads to heterogeneity of the cell population. In nature such heterogeneity could play an important role in adaptation to a changing environment. By turning on gene expression in a subpopulation, a small number of cells while in the process of adapting to one stimulus could always be ready, “primed,” for diverse changes to achieve rapid and successful adaptations to fluctuating environments.

5 Acknowledgement

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6 References

Blackman, J. M., Zhou, L., Datsenko, K. A., and Wanner, B. L. unpublished data. 2005. Ref Type: Unpublished Work

Datsenko, K.A. and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA 97, 6640-6645.

Datsenko, K. A. and Wanner, B. L. unpublished data. 2005. Ref Type: Unpublished Work

Elowitz, M.B., Levine, A.J., Siggia, E.D., and Swain, P.S. (2002). Stochastic gene expression in a single cell. Science 297, 1183-1186.

Ferrell, J.E., Jr. and Machleder, E.M. (1998). The biochemical basis of an all-or-none cell fate switch in *Xenopus* oocytes. Science 280, 895-898.

- Firth,N., Ippen-Ihler,K., and Skurray,R.A. (1996). Structure and function of the F factor and mechanism of conjugation. In *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, F.C.Neidhardt, R.Curtiss, III, J.L.Ingraham, E.C.C.Lin, K.B.Low, B.Magasanik, W.S.Reznikoff, M.Riley, M.Schaechter, and H.E.Umberger, eds. (Washington, DC: ASM Press), pp. 2377-2401.
- Guan,C.-D., Wanner,B.L., and Inouye,H. (1983). Analysis of regulation of *phoB* expression using a *phoB-cat* fusion. *J. Bacteriol.* *156*, 710-717.
- Haldimann,A., Daniels,L.L., and Wanner,B.L. (1998). Use of new methods for construction of tightly regulated arabinose and rhamnose promoter fusions in studies of the *Escherichia coli* phosphate regulon. *J. Bacteriol.* *180*, 1277-1286.
- Haldimann,A. and Wanner,B.L. (2001). Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies in bacteria. *J. Bacteriol.* *183*, 6384-6393.
- Hengge-Aronis,R. (2002). Signal Transduction and Regulatory Mechanisms Involved in Control of the sigma(S) (RpoS) Subunit of RNA Polymerase. *Microbiol. Mol. Biol. Rev.* *66*, 373-395.
- Kierzek,A.M., Zaim,J., and Zielenkiewicz,P. (2001). The effect of transcription and translation initiation frequencies on the stochastic fluctuations in prokaryotic gene expression. *J. Biol. Chem.* *276*, 8165-8172.
- Kim,S.-K., Wilmes-Riesenberg,M.R., and Wanner,B.L. (1996). Involvement of the sensor kinase EnvZ in the *in vivo* activation of the response regulator PhoB by acetyl phosphate. *Molec. Microbiol.* *22*, 135-147.
- Matsubara,M., Kitaoka,S.I., Takeda,S.I., and Mizuno,T. (2000). Tuning of the porin expression under anaerobic growth conditions by his- to-Asp cross-phosphorelay through both the EnvZ-osmosensor and ArcB- anaerosensor in *Escherichia coli*. *Genes Cells* *5*, 555-569.
- McAdams,H.H. and Arkin,A. (1997). Stochastic mechanisms in gene expression. *Proc. Natl. Acad. Sci. USA* *94*, 814-819.
- Metcalf,W.W., Jiang,W., Daniels,L.L., Kim,S.-K., Haldimann,A., and Wanner,B.L. (1996). Conditionally replicative and conjugative plasmids carrying *lacZ α* for cloning, mutagenesis, and allele replacement in bacteria. *Plasmid* *35*, 1-13.
- Mizuno,T., Aiba,H., Oshima,T., Mori,H., and Wanner,B.L. (2002). Genome-wide analysis of *E. coli* histidine kinases. In *Histidine Kinases in Signal Transduction*, M.Inouye and R.Dutta, eds. (New York: Elsevier Sciences), pp. 191-202.
- Muiry,J.A., Gunn,T.C., McDonald,T.P., Bradley,S.A., Tate,C.G., and Henderson,P.J. (1993). Proton-linked L-rhamnose transport, and its comparison with L-fucose transport in *Enterobacteriaceae*. *Biochem. J.* *290 (Pt 3)*, 833-842.
- Nagasawa,S., Tokishita,S., Aiba,H., and Mizuno,T. (1992). A novel sensor-regulator protein that belongs to the homologous family of signal-transduction proteins involved in adaptive responses in *Escherichia coli*. *Molec. Microbiol.* *6*, 799-807.
- Novick,A. and Weiner,M. (1957). Enzyme induction as an all-or-none phenomenon. *Proc. Natl. Acad. Sci. USA* *43*, 553-556.
- Ozbudak,E.M., Thattai,M., Kurtser,I., Grossman,A.D., and van Oudenaarden,A. (2002). Regulation of noise in the expression of a single gene. *Nat. Genet.* *31*, 69-73.
- Parkinson,J.S. (1988). Protein phosphorylation in bacterial chemotaxis. *Cell* *53*, 1-2.

- Pernestig,A.K., Melefors,O., and Georgellis,D. (2001). Identification of UvrY as the cognate response regulator for the BarA sensor kinase in *Escherichia coli*. *J. Biol. Chem.* *276*, 225-231.
- Shapiro,J.A. (1992). Differential action and differential expression of DNA polymerase I during *Escherichia coli* colony development. *J. Bacteriol.* *174*, 7262-7272.
- Suzuki,K., Wang,X., Weilbacher,T., Pernestig,A.K., Melefors,O., Georgellis,D., Babitzke,P., and Romeo,T. (2002). Regulatory circuitry of the CsrA/CsrB and BarA/UvrY systems of *Escherichia coli*. *J. Bacteriol.* *184*, 5130-5140.
- Thattai,M. and van Oudenaarden,A. (2001). Intrinsic noise in gene regulatory networks. *Proc. Natl. Acad. Sci. USA* *98*, 8614-8619.
- Wanner,B.L. (1996). Phosphorus assimilation and control of the phosphate regulon. *In: Escherichia coli and Salmonella Cellular and Molecular Biology*, Neidhardt, F.C., Curtiss, R., III, Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M., and Umberger, H.E. (eds.), ASM Press, Washington, DC, , pp. 1357-1381.
- Wanner,B.L. (1993). Gene regulation by phosphate in enteric bacteria. *J. Cell. Biochem.* *51*, 47-54.
- Wanner,B.L. (1992). Minireview. Is cross regulation by phosphorylation of two-component response regulator proteins important in bacteria? *J. Bacteriol.* *174*, 2053-2058.
- Wanner,B.L. (1986a). Bacterial alkaline phosphatase clonal variation in some *Escherichia coli* K-12 *phoR* mutant strains. *J. Bacteriol.* *168*, 1366-1371.
- Wanner,B.L. (1986b). Novel regulatory mutants of the phosphate regulon in *Escherichia coli* K-12. *J. Mol. Biol.* *191*, 39-58.
- Wanner,B.L. (1987). Control of *phoR*-dependent bacterial alkaline phosphatase clonal variation by the *phoM* region. *J. Bacteriol.* *169*, 900-903.
- Wanner,B.L. (1994). Gene expression in bacteria using *TnphoA* and *TnphoA'* elements to make and switch *phoA* gene, *lacZ* (op), and *lacZ* (pr) fusions. *In: Methods in Molecular Genetics*, vol. 3, Adolph, K.W. (ed.), Academic Press, Orlando, pp. 291-310.
- Wanner,B.L., Jiang,W., Kim,S.-K., Yamagata,S., Haldimann,A., and Daniels,L.L. (1996). Are the multiple signal transduction pathways of the Pho regulon due to cross talk or cross regulation? *In: Regulation of gene expression in Escherichia coli*, Lin, E.C.C., and Lynch, A.S. (eds), R. G. Landes Company, Austin, pp. 297-315.
- Wanner,B.L. and Latterell,P. (1980). Mutants affected in alkaline phosphatase expression: evidence for multiple positive regulators of the phosphate regulon in *Escherichia coli*. *Genetics* *96*, 242-266.
- Wanner,B.L., Wilmes,M.R., and Young,D.C. (1988). Control of bacterial alkaline phosphatase synthesis and variation in an *Escherichia coli* K-12 *phoR* mutant by adenyl cyclase, the cyclic AMP receptor protein, and the *phoM* operon. *J. Bacteriol.* *170*, 1092-1102.
- Witkin,E.M. and Wermundsen,I.E. (1977). Induction of lambda prophage and of mutations to streptomycin resistance in separate small fractions of a lysogenic derivative of *Escherichia coli* B/r by very low doses of ultraviolet light. *Mol. Gen. Genet.* *156*, 35-39.
- Yamamoto,K., Hirao,K., Oshima,T., Aiba,H., Utsumi,R., and Ishihama,A. (2005). Functional characterization *in vitro* of all two-component signal transduction systems from *Escherichia coli*. *J. Biol. Chem.* *280*, 1448-1456.

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Zhou,L., Zhang,K., and Wanner,B.L. (2004). Chromosomal expression of foreign and native genes from regulatable promoters in *E. coli*. *Methods in Molecular Biology* 267, 123-134.