Epigenetic Control ofVirulence Gene Expression in 
*Pseudomonas aeruginosa* by a LysR-Type Transcription Regulator

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**Abstract**

Phenotypic variation within an isogenic bacterial population is thought to ensure the survival of a subset of cells in adverse conditions. The opportunistic pathogen *Pseudomonas aeruginosa* variably expresses several phenotypes, including antibiotic resistance, biofilm formation, and the production of CupA fimbriae. Here we describe a previously unidentified bistable switch in *P. aeruginosa*. This switch controls the expression of a diverse set of genes, including *aprA*, which encodes the secreted virulence factor alkaline protease. We present evidence that bistable expression of *PA2432*, herein named *bexR* (bistable expression regulator), which encodes a LysR-type transcription regulator, controls this switch. In particular, using DNA microarrays, quantitative RT–PCR analysis, chromatin immunoprecipitation, and reporter gene fusions, we identify genes directly under the control of BexR and show that these genes are bistably expressed. Furthermore, we show that *bexR* is itself bistably expressed and positively autoregulated. Finally, using single-cell analyses of a GFP reporter fusion, we present evidence that positive autoregulation of *bexR* is necessary for bistable expression of the BexR regulon. Our findings suggest that a positive feedback loop involving a LysR-type transcription regulator serves as the basis for an epigenetic switch that controls virulence gene expression in *P. aeruginosa*.

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**Introduction**

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen of humans. It can cause infection in a wide variety of tissues in the immunocompromised host, and is the leading cause of morbidity and mortality in cystic fibrosis (CF) patients [1]. This breadth of infectious capacity is thought to result from differential gene expression, as genomic variability between clinical and environmental isolates is low and the genome of *P. aeruginosa* encodes a high proportion of transcription regulators [2,3]. Studying the mechanisms and outcomes of transcription regulation in *P. aeruginosa* may offer some insight into how cohorts of virulence factors are coordinately expressed to influence pathogenesis in a range of pseudomonal infections.

Bacteria are traditionally thought to use transcription regulation to adapt to changing environmental conditions, such as the presence of a new carbon or energy source, a change in temperature or pH, or introduction to a host environment. However, in harsh environmental conditions that exert a sudden selective pressure on a population of cells, the time needed to respond using a genetic regulatory network may prove fatal. The ability of isogenic populations of bacteria to exhibit phenotypic variation allows them to cope with such situations by pre-adapting a subset of the population to the sudden introduction of harsh conditions. Several examples of phenotypic variation in *P. aeruginosa* have been identified, such as the phase-variable expression of the *cupA* fimbrial gene cluster under anaerobic conditions and the transient formation of antibiotic resistant, hyperadherent rough small-colony variants under antibiotic exposure [4–7]. These phenotypes may contribute to the ability of infecting bacteria to withstand chemical or mechanical insults encountered during colonization of the CF lung. Examples such as these suggest that phenotypic variation by *P. aeruginosa* allows the organism to thrive in a complex environment. However, the mechanisms by which these phenotypes are variably expressed are unknown.

Phenotypic variation in bacteria can arise from a variety of mechanisms, both genetic and epigenetic in nature. Classical phase-variation is thought to be genetically mediated, such as the variable expression of the flagellum in *Salmonella enterica* serovar Typhimurium, which is mediated by specifically catalyzed changes in promoter DNA orientation [8]. Phase-variation can also be mediated by epigenetic mechanisms, such as the one involving DNA methylation that controls the phase-variable expression of pyleonphritis-associated pili genes in uropathogenic *Escherichia coli* [9,10]. Phenotypic heterogeneity can arise in the absence of DNA sequence variation or DNA modification in bistable systems (i.e., systems that can exist in one of two alternative expression states, and reversibly switch between them), such as in the case of the lysogenic switch of bacteriophage λ [11,12]. Bistability can arise
Author Summary

Bistable switches allow the expression of a gene, or set of genes, to switch from one stable expression state to another and can generate cells with different phenotypes in an isogenic population. In this work we uncover a previously unidentified bistable switch that controls virulence gene expression in the opportunistic pathogen P. aeruginosa. This switch is controlled by a LysR-type transcription regulator that we call BexR. As well as identifying specific genes that are regulated by BexR, we show that bexR is itself bistably expressed and positively autoregulated. Furthermore, we present evidence that positive autoregulation of bexR is necessary for bistable expression of the BexR regulon. Our findings support a model for BexR-mediated bistability in which positive feedback amplifies bexR expression in a stochastically determined subset of cells, giving rise to heterogeneous expression of BexR target genes within a cell population. By generating diversity in an isogenic population of P. aeruginosa this bistable switch may ensure the survival of a subset of cells in adverse conditions, such as those encountered in the host. Our study defines an epigenetic mechanism for phenotypic variation in P. aeruginosa.

when there exists a mechanism for amplifying differences in protein levels between individual cells and stably propagating these differences to daughter cells (reviewed in [13]). The bistable expression of genes can be achieved using a positive regulatory feedback loop, as is the case in the development of competence under nutrient limitation in Bacillus subtilis; positive feedback of ComK, the master regulator of competence, is required for bistable development of competence in B. subtilis [14,15]. Thus, the architecture of a particular gene regulatory circuit can enable stochastic, reversible differentiation of subsets of bacterial populations into distinct cell types.

Here we uncover a previously unidentified bistable switch in P. aeruginosa controlled by BexR, a LysR-type transcription regulator. We demonstrate that bexR is itself bistably expressed in a BexR-dependent manner and that BexR positively regulates the expression of its own gene. Using DNA microarrays and quantitative real-time RT-PCR (qRT-PCR), we define the bistable regulon of BexR, which contains a diverse set of genes and includes aprA, which encodes the virulence factor alkaline protease. We show further that BexR acts directly at the promoters of many of its regulatory targets, including that of its own gene. Finally, we describe a series of single-cell population analyses that suggest that this bistable switch requires bexR autoregulation. We propose a model for the BexR switch in which positive feedback amplifies bexR expression in a stochastically determined subset of cells, giving rise to bistable expression of BexR target genes in an isogenic population.

Results

BexR Is a Positive Regulator of PA1202 Bistability

In the course of unrelated microarray experiments, we observed a small set of genes that exhibited variable expression between replicates of wild-type cultures of P. aeruginosa strain PAO1 (data not shown). This set includes PA1202, which encodes a hypothetical protein with homology to a predicted hydrolase of Escherichia coli, and PA2432 (herein named bexR for bistable expression regulator), which is predicted to encode a member of the LysR family of transcription regulators. To confirm that PA1202 is expressed in a variable manner, we constructed a strain of PAO1 in which lacZ was placed downstream of the PA1202 gene (Figure 1A). This strain exhibits reversible bistable expression of the lacZ reporter. Specifically, wild-type cells of this reporter strain give rise to both blue (“ON”) and white (“OFF”) colonies on LB agar plates containing X-Gal (Figure 1B). When re-streaked on LB agar with X-Gal, ON colonies give rise to both ON and OFF colonies, and OFF colonies give rise to both OFF and ON colonies. Because our initial microarray analyses suggested that bexR, which encodes a putative transcription activator, co-varied with PA1202, we hypothesized that BexR may positively regulate expression of PA1202 and that bistable expression of bexR may be responsible for the observed bistability in PA1202 expression. To begin to test this hypothesis, we constructed an unmarked in-frame deletion of bexR in PAO1 PA1202 lacZ. Compared to the wild-type reporter strain, the ΔbexR mutant exhibits constitutively low-level expression of PA1202 (Figure 1B). Ectopic expression of bexR in the ΔbexR mutant resulted in increased PA1202 expression (Figure 1C), suggesting that BexR positively regulates expression of PA1202. However, bistable expression of PA1202 is lost when bexR is expressed ectopically; PAO1 ΔbexR PA1202 lacZ grows only as ON colonies on LB agar with X-Gal when carrying a plasmid containing bexR (data not shown), suggesting that native regulation of bexR is necessary for bistable PA1202 expression. Quantification of the frequency at which this switch in expression state occurs reveals a relatively infrequent switch with a bias in favor of the OFF to ON transition (Table 1).

The bexR Gene Is Bistably Expressed and Positively Autoregulated

To determine whether bexR, like PA1202, is expressed in a bistable manner, we constructed a reporter strain in which the putative bexR promoter region was placed upstream of a GFP-lacZ reporter in single copy at the FCTX attachment site in the PAO1 chromosome (Figure 1D) [16,17]. Individual cells of wild-type PAO1 carrying this PbexR-GFP-lacZ reporter either express the GFP reporter, or do not, leading to heterogeneity in the cell population (Figure 1E). Interestingly, cells lacking BexR exhibit constitutively low-level expression of the reporter, suggesting that bistable expression from the bexR promoter also depends on BexR. Bistable expression from the bexR promoter was also observed at the colony level, suggesting long-term maintenance of the BexR expression state (Figure S1). The frequency of switching between expression states is similar for bexR and PA1202, further supporting the hypothesis that bistable expression of bexR is upstream of PA1202 bistability (Table 1 and Table S1). Truncation of the bexR upstream sequence indicated that a 195 bp fragment of upstream DNA is still sufficient to drive expression of a lacZ reporter (integrated in single copy in the chromosome) when bexR is expressed from a plasmid, whereas an 80 bp fragment is not (Figure 1F). Thus, the 195 bp region of DNA immediately upstream of bexR presumably contains the bexR promoter and BexR binding site(s). Thus, BexR positively regulates expression of PA1202 and of its own gene, and bexR is itself bistably expressed, suggesting that other BexR target genes may also be expressed in a bistable manner.

BexR Regulates Expression of a Diverse Set of Genes, Including That Encoding the Virulence Factor AprA

To determine the full extent of the BexR regulon in PAO1, we compared the mRNA content of PAO1 ΔbexR cells containing either a bexR expression vector or an empty vector in both mid-logarithmic and stationary phases of growth using DNA micro-
Figure 1. BexR is a positively autoregulated, bistably expressed regulator of PA1202. (A) Schematic of PA1202 lacZ reporter strains. (B) Phenotypes of wild-type and ΔbexR PA1202 lacZ reporter strains when plated on LB agar containing X-Gal. (C) Quantification of PA1202 lacZ expression in cultures of the wild-type reporter strain in both ON and OFF states, and the ΔbexR reporter strain with empty vector and bexR expression vector. (D) Schematic of attB::PbexR-GFP-lacZ reporter strain. (E) Micrographs of wild-type and ΔbexR attB::PbexR-GFP-lacZ fluorescent reporter cells stained with the membrane dye TMA-DPH. (F) Quantification of PbexR-lacZ expression in PAO1 ΔbexR using varying lengths of bexR promoter DNA in cells with empty vector and bexR expression vector. Error bars in (C,F) represent 1 SD from the mean β-galactosidase activity.

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Table 1. Switching frequencies.

| Strain Genotype          | Transition | Switching Frequency (per cell generation, x10^-4) |
|--------------------------|------------|-----------------------------------------------|
| PA1202 lacZ              | OFF → ON   | 47.8 ± 3.0                                    |
|                          | ON → OFF   | 0.8 ± 0.2                                     |
| ΔbexR PA1202 lacZ        | OFF → ON   | <0.4 ± 0.1                                    |

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Because bexR is itself bistably expressed we would predict that the expression of BexR target genes in wild-type cells should co-vary with the bexR expression state. To test this prediction, we isolated mRNA from cultures of wild-type attB::PbexR-lacZ OFF, attB::PbexR-lacZ ON and ΔbexR attB::PbexR-lacZ reporter strains at both mid-logarithmic and stationary growth phases and profiled relative transcript abundance by qRT-PCR. We observed an approximately 10-fold difference in abundance of bexR transcripts between OFF and ON cultures in mid-logarithmic phase, and an approximately 6-fold difference between OFF and ON cultures in stationary phase (Figure 2A). Consistent with the idea that BexR target genes are expressed in a bistable manner in wild-type cells, expression of members of the putative PA1202 operon, from PA1202 to PA1205, all co-varied with bexR expression (Figure 2B), as did PA0572 and aprA (though for aprA the difference in transcript abundance between ON and OFF cultures was only 2-fold) (Figure 2C). We were unable to observe significant bistable expression of the other apr genes, possibly due to the relatively modest effect of BexR on their expression. The abundance of the arrays. A total of 71 genes exhibited between a 2- and 70-fold change in expression, with most genes upregulated by ectopic expression of bexR (Figure S2). PA1202 was upregulated 70-fold upon ectopic expression of bexR in mid-logarithmic phase. Several genes downstream of PA1202 were also strongly upregulated by ectopic expression of bexR, suggesting that these comprise a BexR-regulated operon. This putative operon includes PA1203, which is predicted to encode a redox protein, PA1204, which is predicted to encode a NADPH-dependent FMN reductase, and PA1205, which is predicted to encode a homolog of pirin, a widely conserved protein with oxygenase activity [18]. PA2698, which is also predicted to encode a hydrolase, was upregulated 7-fold by ectopic expression of bexR, suggesting that a cohort of several enzymes are coordinately regulated by BexR. Several multidrug efflux pumps appeared to be regulatory targets of BexR, as downregulation of mexEF-opmN by 6- to 10-fold and upregulation of mexGH-opmD by 7- to 10-fold was observed during ectopic expression of bexR. Several quorum-sensing-regulated genes encoding secreted proteins were also positively regulated by ectopic bexR expression, such as PA0572, which encodes a LasR-regulated Xcp secretion substrate with a predicted Zn-metallo-protease motif [19–21]. Finally, the LasR-regulated genes aprX, aprE, aprF and aprA, which encode components of the alkaline protease production and secretion machinery, were positively regulated by BexR. aprA, which encodes the alkaline protease precursor protein, plays a role in virulence in a Drosophila melanogaster orogastric model of pseudomonal infection, where it is thought to protect P. entomophila from antimicrobial peptides [22]. These results suggest that BexR controls the expression of a diverse set of genes, including some that encode predicted enzymes and others that encode quorum-regulated secreted proteases.

The BexR Regulon

Because bexR is itself bistably expressed we would predict that the expression of BexR target genes in wild-type cells should co-vary with the bexR expression state. To test this prediction, we isolated mRNA from cultures of wild-type attB::PbexR-lacZ OFF, attB::PbexR-lacZ ON and ΔbexR attB::PbexR-lacZ reporter strains at both mid-logarithmic and stationary growth phases and profiled relative transcript abundance by qRT-PCR. We observed an approximately 10-fold difference in abundance of bexR transcripts between OFF and ON cultures in mid-logarithmic phase, and an approximately 6-fold difference between OFF and ON cultures in stationary phase (Figure 2A). Consistent with the idea that BexR target genes are expressed in a bistable manner in wild-type cells, expression of members of the putative PA1202 operon, from PA1202 to PA1205, all co-varied with bexR expression (Figure 2B), as did PA0572 and aprA (though for aprA the difference in transcript abundance between ON and OFF cultures was only 2-fold) (Figure 2C). We were unable to observe significant bistable expression of the other apr genes, possibly due to the relatively modest effect of BexR on their expression. The abundance of the
The transcript was not significantly different between $D_{bexR}$ and wild-type cultures, suggesting that the observed bistability of aprA and PA0572 (which, like lasA, are LasR-regulated [19]) is not due to differences in LasR function between ON and OFF cultures (Figure 2C). Microarray analysis of cells ectopically expressing $bexR$ suggests that two operons encoding multidrug efflux pumps are reciprocally regulated by BexR (Figure S2). However, this was not observed in wild-type cells in the OFF and ON states (data not shown). Taken together, our data indicate that BexR is responsible for coordinate bistable expression of a variety of genes in wild-type $P.\ aeruginosa$, including two that encode quorum sensing-regulated secreted proteases (PA0572 and aprA).

BexR Acts Directly at Target Promoters

To address whether BexR directly regulates transcription of its target genes, we used chromatin immunoprecipitation (ChIP). We constructed a strain in which the native chromosomal copy of the $bexR$ gene has been modified to encode a version of BexR containing a vesicular stomatitis virus glycoprotein (VSV-G) epitope tag at its C-terminus (BexR-V). This strain retained the ability to bistably express PA1202 lacZ on LB agar containing X-Gal, suggesting that the VSV-G epitope tag does not interfere with BexR activity (data not shown). We immunoprecipitated BexR-V-associated DNA from wild-type ON cultures grown to both mid-logarithmic and stationary phase and quantified occupancy of BexR-V at candidate target promoters relative to a control region not expected to bind BexR-V. BexR-V strongly occupies its own promoter, as well as those of PA1202 and PA0572 (Figure 3). Furthermore, BexR-V occupied the aprX and aprA promoters, but not the intervening DNA upstream of aprD. This suggests that BexR-V has at least two distinct binding sites within the apr locus. All occupancies were significantly higher than those observed in both wild-type OFF cultures and in a non-epitope tagged control strain (Figure S3). These results suggest that BexR regulates many of its target genes directly.

Positive Feedback of $bexR$ Is Required for Bistability

The evidence presented thus far suggests that $bexR$ encodes a bistably expressed transcription regulator that positively regulates its own expression. This is reminiscent of the competence switch in $B.\ subtilis$. In this system, ComK, the master regulator of competence, positively regulates transcription of its own gene, thereby enabling a non-linear response to increasing concentrations of ComK, which leads to bistability in the development of competence. Using single-cell fluorescent reporter analysis, it has been shown that the ComK positive feedback loop is required for bistable expression of competence [14,15]. We hypothesized that, in a similar manner, the positive feedback loop controlling $bexR$ expression is required for bistable expression of the BexR regulon (i.e. positive feedback of $bexR$ creates a condition of hypersensitivity to variation in levels of BexR protein). If this hypothesis is correct a gradual increase in basal $bexR$ expression should increase the proportion of ON relative to OFF cells specifically in a strain with an intact positive feedback loop. In a strain that lacks this positive feedback loop, a graded increase in $bexR$ expression should lead to a corresponding increase in expression of $bexR$-regulated genes with no detectable bistability.

Wild-type $P.\ aeruginosa$ cells containing a $P_{bexR}$-GFP-lacZ reporter construct integrated in single copy into the chromosome can be
seen to exhibit BexR-dependent bistable expression of this reporter by fluorescence microscopy (Figure 1E). Consistent with this observation, quantification of the fluorescence of individual cells within a culture derived from either an ON colony or an OFF colony reveals that cells in the ON and OFF expression states can be distinguished from one another, and that each culture contains both ON and OFF cells (Figure 4). To analyze the effect of positive feedback on bexR bistability, we constructed a pair of strains containing the P\textsubscript{bexR}\textsuperscript{-D-thiogalactoside (IPTG)-inducible copy of bexR (also provided in single copy from the chromosome from a different locus). One of these strains contained an unmarked, in-frame deletion of bexR (the minus feedback strain, Figure 5A); whereas the other contained wild-type bexR at its native locus (the plus feedback strain, Figure 5B). In the absence of IPTG, only cells of the reporter strain with the intact positive feedback loop displayed bistability, and contained two populations of cells corresponding to those in the ON and OFF expression states (manifest in Figure 5B [and Figure 6B] as a population of cells with an essentially bimodal distribution of fluorescence intensities). Furthermore, a gradual increase in expression of ectopically expressed bexR occurred in an increase in the proportion of ON relative to OFF cells only in the plus feedback strain (Figure 5B); in the strain lacking the positive feedback loop, cells responded relatively uniformly to increasing synthesis of ectopically expressed bexR (manifest in Figure 5A as a population of cells with a normal distribution of fluorescence intensities, whose average fluorescence intensity increases with IPTG concentration).

Importantly, for IPTG concentrations at which the average cell fluorescence intensity was similar between cells with and without feedback, two distinct cell populations (ON and OFF) were observed only in cells with an intact positive feedback loop (Figure 5). In particular, cells of the plus feedback strain at 0.5 mM IPTG had a mean fluorescence intensity of 1014 arbitrary units, which is similar to the mean fluorescence intensity of 1720 arbitrary units exhibited by the minus feedback strain at 4 mM IPTG. Whereas the mean reporter gene expression of these two cell populations, and thus the average abundance of BexR protein per cell, was quite similar under these two conditions, the existence of two subpopulations of cells occurred only in the presence of bexR autoregulation (Figure 5). These results suggest that positive feedback of bexR is necessary for bistability.

Feedback-mediated bistable systems often exhibit a capacity for history-dependent behavior, or hysteresis [reviewed in 23]. Systems exhibiting hysteric behavior may have different responses under identical conditions, depending on the conditions previously experienced. For example, in bistable expression of the lac operon of E. coli at low concentrations of a non-metabolizable lactose analog, the concentration of inducer at which initially uninduced cells turn on is higher than that at which initially induced cells turn off [24,25]. The behavior of this system at concentrations of inducer between these thresholds therefore depends on conditions previously encountered. Thus, systems with positive feedback can exhibit memory of previous expression states. To investigate the possibility that positive feedback of bexR can impart a memory of previous expression states on the system, we utilized the plus and minus feedback strains described above (Figure 5) and observed their response over time to a pulse of ectopically expressed bexR, induced by a 2 hour exposure to 20 mM IPTG. In cells without an intact positive feedback loop, the IPTG pulse was sufficient to raise the mean fluorescence intensity to that seen in wild-type ON cells (Figure 6A and Figure 4). However, this degree of expression from the P\textsubscript{bexR}\textsuperscript{-D-thiogalactoside (IPTG)-lacZ reporter was quickly lost upon removal of IPTG and subculturing of cells into fresh media. In contrast, cells of the plus feedback strain maintained their induced state for many generations after the removal of IPTG, suggesting that a brief period in which cells experience a high intracellular concentration of BexR is sufficient to induce a long-lasting ON state (Figure 6B). Indeed, a pulse with IPTG for only 30 minutes is sufficient to induce a transition to a sustained ON state in the plus feedback strain (Figure S4). Only after 31 generations following removal of IPTG, do a portion of the cells begin to transition to the OFF state (Figure 6B). Taken together, the results of our single-cell population analyses suggest a mechanism in which variation in basal expression of bexR in OFF cells is amplified by a positive feedback loop in a stochastically determined subset of cells that then transitions to the ON state and is maintained in that state by continued autoactivation of BexR (Figure 7).

**Discussion**

The results above characterize a heretofore undescribed bistable switch in *P. aeruginosa* that controls virulence gene expression. We have shown that bexR, which encodes a LysR-type transcription regulator, is bistably expressed, and that this bistability results in altered expression of several downstream genes, including those in the uncharacterized PA1202 operon and aprA, which encodes the virulence factor alkaline protease. Furthermore, reporter assays show that BexR can positively regulate its own expression. ChIP analysis indicates that BexR acts directly at the sites of many target promoters, including that of its own gene. Finally, single-cell analyses of the response of a cell population to a graded source of BexR, or a pulse of BexR, suggests that positive autoregulation is
Figure 5. Positive feedback of bexR is required for bistability of the regulon. Cells of both the minus feedback strain (PAO1 ΔbexR attB::PbexR-GFP-lacZ attTn7::TOPLAC-bexR, see diagram in (A), and the plus feedback strain (PAO1 attB::PbexR-GFP-lacZ attTn7::TOPLAC-bexR, see diagram in (B) were grown in LB media containing IPTG at the indicated concentrations, and examined and measured for fluorescence upon reaching mid-logarithmic phase by fluorescence microscopy. Black dots on scatter plots correspond to the automatically measured fluorescence intensity of individual cells (in arbitrary units, AU) in a sample size of 250 cells. The red bar represents the mean fluorescence intensity of cells in a sample. Representative micrographs of selected samples are shown, with green fluorescence displayed in pseudocolor on the top panels and the corresponding DIC image on the bottom panels. Scale bar, 3 μm.
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Figure 6. The feedback-mediated BexR switch exhibits hysteresis. Cells of both the minus feedback strain (PAO1 ΔbexR attB:Ptet::GFP-lacZ attTn7::TOPLAC-bexR, see diagram, Figure 5A), and the plus feedback strain (PAO1 attB:Ptet::GFP-lacZ attTn7::TOPLAC-bexR, see diagram, Figure 5B) were grown to early logarithmic phase and were exposed to a pulse of IPTG (20 mM) for 2 hours (2 h IPTG pulse) to induce ectopic expression of bexR, or not exposed to IPTG (no IPTG pulse). Cells were then washed (to remove IPTG) and grown in fresh media for 31 generations. Cells were examined and measured for fluorescence after 0, 2, 6, 11, 19, and 31 generations post treatment with or without IPTG by fluorescence microscopy. For scatter plots, the number of generations of growth in media without IPTG after the pulse is given on the horizontal axes. Black dots correspond to the automatically measured fluorescence intensity of individual cells (in arbitrary units, AU) in a sample size of 250 cells. The red bar represents the mean fluorescence intensity of cells in a sample. Representative micrographs of selected samples are shown, with green fluorescence displayed in pseudocolor on the top panels and the corresponding DIC image on the bottom panels. Scale bar, 3 μm.

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phenotypic heterogeneity within an isogenic population, thereby creating a subset of cells capable of surviving the onset of an otherwise lethal situation. For example, some bacteria have the ability to survive antibiotic treatment without evolving "bona fide" resistance by stochastically entering a dormant "persister" state during vegetative growth [26]. A recent study suggests that a bexR transposon mutant has 2-fold increased sensitivity to the fluoroquinolone antibiotic ciprofloxacin, which is used in treatment of P. aeruginosa infections in CF patients, though the potential mechanism for this increased sensitivity was not addressed [27,28]. Although our findings raised the possibility that bistable bexR expression might lead to heterogeneity in ciprofloxacin resistance, we found no evidence that bexR contributed to the resistance of P. aeruginosa to ciprofloxacin, at least in strain PAO1 [data not shown].

Bistable expression of virulence factors has been previously reported in P. aeruginosa. For instance, the Type III secretion system is only expressed in a subset of cells grown in inducing conditions [29]. Additionally, the cnpA fimbral gene cluster is bistably expressed by P. aeruginosa when grown in anaerobic conditions [5]. Bistable expression of several virulence factors independently of one another may create several subtypes of cells with differing virulence potential within an isogenic population of infecting bacteria. Thus, bistable expression of virulence factors may represent a strategy employed by P. aeruginosa to generate cell types specialized to survive within different niches in the host.

In P. entomophila, AprA has a significant role in virulence in a D. melanogaster oral model of infection, where it is thought to protect the bacterium from the effects of host-produced antimicrobial peptides [22]. Although oral models of D. melanogaster infection with P. aeruginosa have been used to successfully characterize bacterial virulence, these models have not been used to test the role of AprA in P. aeruginosa virulence [30,31]. If alkaline protease does play a role in defense against antimicrobial peptides in P. aeruginosa, upregulating apec ~2-fold in a subset of cells through BexR-mediated bistability may preemptively adapt a portion of the cell population to the sudden introduction to a particular host environment. P. aeruginosa alkaline protease has been shown to degrade a variety of human proteins and tissues and inhibit immune cell function, presumably by acting at the cell surface to modify phagocytic and chemotactic receptors (reviewed in [32]). Alkaline protease has also been suggested to play a role in corneal keratitis [33], although this role for AprA has been disputed more recently by the comparison of isogenic mutant strains [34]. However, our observation that wild-type strains of P. aeruginosa bistably express aprA may complicate the interpretation of earlier work. Interestingly, the rhizobacterium P. brassicacearum exhibits phenotypic variation in expression of an alkaline protease homolog, though whether this is mediated by bistability of a BexR homolog is unknown [35]. It has been suggested that heterogeneous production of extracellular proteases by an isogenic population of bacteria is an example of cooperative or altruistic behavior, as these proteases diffuse freely through the growth medium and can equally benefit all members of the population [36]. Thus, bistable production of alkaline protease or PA0572, a predicted protease, may serve to benefit both ON and OFF cells in a population. Whether bistable expression of aprA or other members of the BexR regulon, has a role in mammalian virulence remains to be seen.

In contrast with aprA, many other regulatory targets of BexR are poorly characterized hypothetical genes. BexR-mediated bistability does not appear to be limited to P. aeruginosa PAO1, as the homolog of PA1202 in P. aeruginosa PA14, a more virulent clinical strain, is also bistably expressed in a BexR-dependent manner (Figure S5). This conservation across diverse strains of P. aeruginosa suggests an important biological role for BexR-mediated bistability. In this regard, a particularly interesting target of BexR is the PA1202 operon, which is strongly positively regulated by BexR. Several genes in this operon, such as PA1202 and PA1205, are predicted to encode enzymes with catabolic activity directed against small molecules. This may point to a role for the BexR regulon in the ability of P. aeruginosa to metabolize and thereby detoxify certain small molecules. Co-regulation of a diverse set of genes by BexR may indicate that it is involved in manifestation of more than one phenotype. That these genes are expressed in a bistable manner suggest that their expression or repression may be detrimental to growth under certain conditions.

Feedback-Mediated Bistability and the BexR Switch

We propose that positive feedback of bexR provides a mechanism for amplification and propagation of cell-to-cell variability in BexR levels. This regulatory circuit is similar to the one governing competence development in B. subtilis. Experiments
in this system have suggested that noisy expression of comK results in ComK levels in a subpopulation of cells crossing a threshold level for comK autoactivation, causing differentiation into the competent state [14,15,37]. Noise in bexR expression may also provide the basis for generating cell-to-cell variability in BexR levels. The frequency of the BexR switch differs from that of the ComK switch. Whereas P. aeruginosa has been directly observed to enter a competent state in approximately 3.6% of cell division events [38], P. aeruginosa enters into the BexR-ON state approximately 10-fold less frequently, and the BexR-OFF state even less so (Table 1 and Table S1). These low frequencies are often in parallel with classical phase-variation systems, but in the case of BexR, the expression state stability appears to be epigenetically mediated. This low switching frequency may be a function of the high degree of hysteresis observed in the BexR switch. Biological systems capable of hysteretic behavior can retain a memory of previous exposure to inducing conditions, and this has been observed in both naturally occurring and synthetic systems [25,39]. Strictly speaking, hysteresis is not a necessary characteristic of bistable systems, as a synthetic feedback-mediated bistable system was observed to exhibit clear bistability but display no history-dependent response [40]. Nevertheless, hysteresis is often associated with bistable systems, and that it is observed in the BexR switch may suggest that retaining memory of previous conditions is beneficial to the cell.

In P. aeruginosa, regulation of ComK levels is achieved by degradation of ComK by the MecA/ClpCP complex and the inhibition thereof by ComS [41,42]. Our single-cell population analyses indicate that directly modulating BexR levels by induction of ectopic synthesis can affect the frequency at which cells differentiate into the ON state (Figure 5). Modulation of BexR levels or activity in wild-type cells may provide a mechanism for fine-tuning the dynamics of this bistability. There may be accessory factors, perhaps themselves BexR-regulated, that affect BexR levels or activity. A mechanism for modulating BexR autoactivation dynamics may allow P. aeruginosa to regulate switching frequency in response to external conditions. As LysR-type transcription activators often bind to small molecules to alter their DNA-binding and regulatory properties, it is possible that the dynamics of the BexR switch may be tunable by a coinducer molecule [43]. However, no such molecule has yet been identified.

The results presented here outline a model for differentiation into the BexR-ON state, but do not address the mechanism by which a BexR-ON cell can revert to the BexR-OFF state. Previous studies suggest that escape from a positive feedback loop is often mediated by an accessory process. For example, escape from competence in P. aeruginosa occurs when reduction in ComS levels promotes ComK proteolysis by the MecA/ClpCP complex, relieving ComK autoactivation [38]. The switch from BexR-ON to BexR-OFF may also involve some antagonistic process. Unlike several other feedback-mediated bistable switches, the switch from ON to OFF in the case of BexR appears to occur only in a stochastically determined subset of cells. For example, escape from competence in P. aeruginosa occurs because comS transcription is repressed by ComK in the competent state and ComS protein gradually depletes in all cells [38]. In contrast, the BexR-ON state is relatively stable and heritable, and is lost only in a subpopulation of cells. The existence of a stochastic process mediating the switch to BexR-OFF that is distinct from the one mediating the switch to BexR-ON, is further supported by the ~60-fold directional bias in switching frequencies (Table 1). This process may take the form of transcription regulation of bexR or post-translational modulation of BexR levels or activity, and we are currently investigating these possibilities.

**Materials and Methods**

**Bacterial Strains and Plasmids**

P. aeruginosa strains PAO1 and PA14 were provided by Arne Rietsch (Case Western Reserve University). E. coli DH5α FTQ (Invitrogen) was used as the recipient strain for all plasmid constructions, whereas E. coli strain SM10 (Apri) was used to make plasmids into P. aeruginosa.

The PA1202 lacZ reporter strain PAO1 PA1202 lacZ contains the lacZ gene integrated immediately downstream of the PA1202 gene on the PAO1 chromosome and was made by allelic exchange. PCR products 486 bp and 513 bp in length flanking the 3’ end of PA1202 were amplified and spliced together to add KpnI, NotI and SpI sites two bases after the PA1202 stop codon. This PCR product was cloned as a SacI/PoI fragment into pEXG2 [44]. The lacZ gene was subsequently cloned into this construct as a KpnI/SphI fragment derived from pf18-lacZ [Arne Rietsch, unpublished work], generating plasmid pEXF1202-lacZ. This plasmid was then used to create reporter strains PAO1 PA1202 lacZ and PA14 PA1202 lacZ by allelic exchange.

The deletion construct for the bexR gene (PA2432) was generated by amplifying regions 398 bp and 360 bp in length that flank bexR in the PAO1 genome by the PCR and then splicing the flanking regions together by overlap extension PCR; deletions were in-frame and contained the 9-bp linker sequence 5’-GCGGCCGCCC-3’. The resulting PCR product was cloned on an EcoRI/BamHI fragment into plasmid pEX18Gm [45], yielding plasmid pEXM2432. This plasmid was then used to create strains PAO1 ΔbexR, PAO1 PA1202 lacZ ΔbexR and PA14 PA1202 lacZ ΔbexR by allelic exchange [45]. Deletions were confirmed by the PCR.

The attB:PbexR-lacZ reporter strains contain fragments of the bexR promoter fused to the lacZ gene and integrated in single copy into the attB locus in the PAO1 chromosome and were made by site-specific integration followed by backbone excision through transient synthesis of FLP recombinase from plasmid pFLP2 [17,45]. PCR products spanning from 91, 198 or 297 bp to 3 bp upstream of the bexR start codon were amplified and cloned as mini-CTX-lacZ [17], which contains a consensus Shine-Dalgarno sequence downstream of lacZ, yielding plasmids mini-CTX-PP2432-lacZ1, mini-CTX-PP2432-lacZ2 and mini-CTX-PP2432-lacZ3, respectively. These plasmids were then used to create reporter strains PAO1 attB:PbexR-lacZ PAO1 ΔbexR attB:PbexR-lacZ PAO1 attB:PbexR-lacZ PAO1 ΔbexR attB:PbexR-lacZ PAO1 ΔbexR attB:PbexR-lacZ An EcoRI/XhoI fragment of mini-CTX-PP2432-lacZ3 was subcloned into mini-CTX-GFP-lacZ [16], yielding plasmid mini-CTX-PP2432-GFP-lacZ3. This plasmid was then used to create the fluorescent reporter strains PAO1 attB:PbexR-GFP-lacZ and PAO1 ΔbexR attB:PbexR-GFP-lacZ.

The BexR-VSV-G integration vector was generated by first cloning a PCR-amplified DNA fragment containing ~300 bp of sequence from the 3’ portion of the bexR gene on a HindIII/Vial fragment into plasmid pP30A-YTAP [4], generating plasmid pP30A-BexR-TAP. This HindIII/Vial fragment was then subcloned into pP30AFT-MvaT-V [46], generating plasmid pP30AFT-BexR-V. This plasmid was used to make strain PAO1 PA1202 lacZ BexR-V by homologous recombination at the bexR locus followed by backbone excision through transient synthesis of FLP recombinase from plasmid pFLP2 [45]. Production of the BexR-V protein was confirmed by Western blotting with an anti-VSV-G antibody (Sigma).

Plasmid pBexR is a derivative of pPSV35 [44] and directs the synthesis of the BexR protein under control of the IPTG-inducible.
lacUV5 promoter. The plasmid was made by subcloning an EcoRI/ HindIII DNA fragment containing a consensus Shine-Dalgarno sequence and the belR gene into pPSV35.

The attTn7::TOPLAC-belR strains contain a construct which directs the synthesis of the BelR protein under control of the IPTG-inducible TOPLAC promoter stably integrated into the genome in single copy at the attTn7 locus. The TOPLAC promoter in this construct is a derivative of the lac promoter that contains two lac operator sequences centered at positions +63.5 and +11. The sequence of this promoter is 5'-CACACTGTCGCTGAGGCTAAATATGCACCTCAATTATTGTGAAATGAGTCGAAGGCTCTTTTACACGCTTCTGTCCGGCGGTGATATGTGTGG-3', where the -35 and -10 sequences are underlined and the lac operator sequences are shown in bold. The belR gene is inserted downstream of the TOPLAC promoter with a consensus Shine-Dalgarano sequence. This construct was inserted between the ends of the Tn7 transposon on pUC18-mini-Tn7T-LAC [47], generating plasmid pUC18-mini-Tn7T-TOPLAC-belR. This plasmid was used to make strains PAO1 attb:B: belR-3-GFP-lacZ attTn7::TOPLAC-belR and PAO1 ΔbelR attb:B: belR-3-GFP-lacZ attTn7::TOPLAC-belR by site-specific recombination [47].

β-Galactosidase Assays

Cells were grown with aeration at 37°C to mid-logarithmic phase in LB supplemented as needed with gentamicin (25 μg/ml) and IPTG (0.1 mM). Cells were permeabilized with sodium dodecyl sulfate and CHCl3 and assayed for β-galactosidase activity as described previously [48]. Assays were performed at least twice in triplicate on separate occasions. Representative data sets are shown.

RNA Isolation

Cultures of PAO1 ΔbelR attb:B: belR-3-lacZ and PAO1 attb:B: belR-3-lacZ in the OFF and ON states were inoculated in quadruplicate at starting OD600 of ~0.01 and grown with aeration to an OD600 of ~0.5 (representing mid-logarithmic phase) and to an OD600 of ~2.4 (representing stationary phase) at 37°C in LB. Cells were then harvested by centrifugation and RNA prepared essentially as described [49]. Transcripts were quantified by quantitative real-time RT-PCR as described [50].

Switching-Frequency Calculations

Switching-frequency calculations were performed essentially as described [51], except that cells were plated on LB agar plates containing 50 μg/ml X-Gal and grown at 37°C. Error values represent 1 standard deviation (SD) from the mean switching frequency.

Microarray Experiments

Cultures of PAO1 ΔbelR containing plasmid pPSV35 [44] or pBexR were grown with aeration at 37°C in LB containing gentamicin (25 μg/ml). Triplicate cultures of each strain were inoculated at a starting OD600 of ~0.01 and grown to an OD600 of ~0.5 (representing mid-logarithmic phase) and to an OD600 of ~2.3 (representing stationary phase). RNA isolation, cDNA synthesis, and cDNA fragmentation and labeling were performed essentially as described previously [49]. Labeled samples were hybridized to Affymetrix GeneChip P. aeruginosa genome arrays (Affymetrix). Data were analyzed for statistically significant (p<0.05, fold change >2) changes in gene expression using GeneSpring GX.

Chromatin Immunoprecipitation (ChIP)

Cultures of PAO1 PA1202 lacZ BexR-V in either the ON or OFF state were inoculated in quadruplicate at a starting OD600 of ~0.01 and grown with aeration to an OD600 of ~0.5 (representing mid-logarithmic phase) and to an OD600 of ~2.0 (representing stationary phase) at 37°C in LB. ChIP was then performed with 3 ml of culture and fold enrichment values were measured by quantitative PCR relative to the PA2155 promoter essentially as described [46].

Quantitative Fluorescence Microscopy

For fluorescence micrograph analysis, cultures were fixed with formaldehyde and glutaraldehyde at 2.4% and 0.04%, respectively, and cells were allowed to fix for 30 minutes at room temperature. Cells were washed three times with PBS and imaged on a Nikon TE2000 inverted microscope outfitted with a Nikon Intensiluminator, a CoolSnap HQ2 charge-coupled device camera from Photometrics and a Nikon CFI Plan Apo VC x100 objective lens (1.4 NA) for differential interference contrast (DIC) imaging. For GFP images the ET-GFP filter set (Chroma 49002) was used. Images were captured using Nikon Elements software, which was also used for quantification of fluorescence in individual cells. This was done by automatically defining cell boundaries using the DIC image, excluding cells that were poorly focused, narrower than 0.3 μm, longer than 4.0 μm or shorter than 0.5 μm, and using these regions to quantify the GFP image. Values given are subtracted for background fluorescence. At least 400 cells were imaged for each timepoint, and the fluorescence intensities of a random subset of 250 cells are displayed in scatter plots. Images were exported to Adobe Photoshop CS4 for preparation.

For the hypersensitivity experiment (Figure 5), cells were grown with aeration at 37°C to mid-logarithmic phase in LB supplemented as needed with IPTG and prepared for microscopy as described above. The experiment was performed at least twice in duplicate on separate occasions. A representative data set from a single replicate is shown.

The hysteresis experiment (Figure 6) was performed by growing cells with aeration at 37°C in LB and either treating them with 20 mM IPTG for 2 hours or 30 minutes immediately before reaching mid-logarithmic phase, or not treating them with IPTG. A sample was then taken and prepared for microscopy (corresponding to the 0 generation time point) as described above while the remaining cells were washed with LB to remove the IPTG, and inoculated into fresh media at a 1:4 dilution. Cells were then grown continuously for 2 generations to mid-logarithmic phase, a sample was taken and prepared for microscopy (corresponding to the 2 generation time point) and a fresh culture was inoculated at a 1:16 dilution with the remaining cells. Cells were then grown continuously for 4 generations to mid-logarithmic phase, a sample was taken and prepared for microscopy (corresponding to the 6 generation time point) and a fresh culture was inoculated at a 1:16 dilution with the remaining cells. Cells were then grown continuously for 5 generations to late-logarithmic phase, a sample was taken and prepared for microscopy (corresponding to the 11 generation time point) and a fresh culture was inoculated at a 1:32 dilution with the remaining cells. Cells were then grown continuously for 3 generations to late-logarithmic phase and a fresh culture was inoculated at a 1:16 dilution. Cells were then grown continuously for 3 generations to mid-logarithmic phase, a sample was taken and prepared for microscopy (corresponding to the 19 generation time point), remaining cells were allowed to grow for 1.5 generations to early stationary phase and used to inoculate a fresh culture at a 1:100 dilution.
dilution. Cells were then grown continuously for 7 generations (overnight) and used to inoculate a fresh culture at a 1:100 dilution. Cells were then grown continuously for 3.5 generations to mid-logarithmic phase and a sample was taken and prepared for microscopy (corresponding to the 31 generation time point). The experiment was performed at least three times in duplicate on separate occasions. A representative data set from a single replicate is shown.

Supporting Information

Figure S1 bexR is bistably expressed on solid media in a bexR-dependent manner. The bexR promoter was fused to lacZ and stably integrated in single copy into the PAO1 chromosome. The phenotypes of wild-type and ΔbexR strains of this reporter plated on LB agar containing X-Gal are shown. Found at: doi:10.1371/journal.pgen.1000779.s001 (1.71 MB TIF)

Figure S2 BexR regulates expression of a diverse set of genes. Cells of PAO1 ΔbexR with either empty vector or bexR-overexpression vector were grown to mid-logarithmic (ML) and stationary (ST) phase, and mRNA content was profiled by microarray. Found at: doi:10.1371/journal.pgen.1000779.s002 (0.09 MB PDF)

Figure S3 BexR occupies the promoters of target genes. Cells of PA14 1P1A202 lacZ bexR-V in both ON and OFF states were grown to mid-logarithmic and stationary phase, and DNA associated with BexR-V was analyzed by ChIP. A mock IP control was performed with cells of ON-state PAO1 PA14 1P1A202 lacZ which do not synthesize VSV-G-tagged BexR. Error bars represent one standard deviation from the mean fold enrichment. Found at: doi:10.1371/journal.pgen.1000779.s003 (8.17 MB TIF)

Figure S4 A 30 minute pulse of IPTG is sufficient to induce hysteresis. Cells of the plus feedback strain (PAO1 attB::PbexR-GFP-lacZ attTn7::TOPLAC-bexR, see diagram, Figure 5B) were grown to early logarithmic phase and were exposed to a pulse of IPTG (20 mM) for 30 minutes (30 min IPTG pulse) to induce ectopic expression of bexR, or not exposed to IPTG (see Figure 6B, no IPTG pulse). Cells were then washed (to remove IPTG) and grown in fresh media for 31 generations. Cells were examined and measured for fluorescence after 0, 2, 6, 11, 19, and 31 generations post treatment with or without IPTG by fluorescence microscopy. For scatter plots, the number of generations of growth in media without IPTG after the pulse is given on the horizontal axes. Black dots correspond to the automatically measured fluorescence intensity of individual cells (in arbitrary units, AU) in a sample size of 230 cells. The red bar represents the mean fluorescence intensity of cells in a sample. Representative micrographs of selected samples are shown, with green fluorescence displayed in pseudocolor on the top panels and the corresponding DIC image on the bottom panels. Scale bar, 3 μm. Found at: doi:10.1371/journal.pgen.1000779.s004 (6.44 MB TIF)

Figure S5 The PA1202-orthologous PA14 48760 operon also exhibits bexR-dependent bistability in P. aeruginosa strain PA14. (A) Schematic of PA14 48760 lacZ reporter strains. (B) Phenotypes of wild-type and ΔbexR PA14 48760 lacZ reporter strains when plated on M63 minimal agar containing X-Gal. (C) Quantification of PA14 48760 lacZ expression in cultures of the wild-type and ΔbexR reporter strains. Error bars represent one standard deviation from the mean β-galactosidase activity. Found at: doi:10.1371/journal.pgen.1000779.s005 (3.13 MB TIF)

Table S1 Switching frequencies. Found at: doi:10.1371/journal.pgen.1000779.s006 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: KHT IVG SLD. Performed the experiments: KHT IVG. Analyzed the data: KHT IVG SLD. Contributed reagents/materials/analysis tools: KHT IVG SLD. Wrote the paper: KHT SLD.

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