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Bistability in *Pseudomonas aeruginosa*

Abstract

The opportunistic pathogen *P. aeruginosa* is a leading cause of hospital-acquired infections, and is also the primary cause of morbidity and mortality in patients with cystic fibrosis (CF). In this thesis, I describe the identification and characterization of a novel LysR-type transcription regulator (LTTR) of *P. aeruginosa* named BexR. I show that BexR exhibits reversible ON/OFF bistable expression, which leads to the bistable expression of several genes including one encoding a virulence factor. I present results suggesting that this bistable expression depends on positive feedback of BexR. This work illuminates the simplicity with which a transcription regulatory network can exhibit a complex behavior and generate phenotypic diversity in a clonal population.

In the CF lung, *P. aeruginosa* is thought to persist in a biofilm, a growth state that depends on the coordinate activity of many bacterial adherence factors such as the CupA fimbriae. The *cupA* fimbrial genes are repressed by the H-NS (“histone-like nucleoid structuring”) family members MvaT and MvaU, and in the absence of MvaT, are expressed in a bistable or phase-variable manner. Here, I describe the adaptation of a genetic system for depleting both MvaT and MvaU from the cell and present evidence suggesting that the presence of at least one of these two proteins is essential for cell viability.

Regulation of the *cupA* fimbrial genes is complex, and is influenced by several positive regulators. In this thesis I describe the identification and characterization of PrrA, a small regulatory RNA (sRNA) that positively regulates the *cupA* genes. The gene encoding PrrA is a member of the *mexEFoprN* multidrug efflux pump operon, which is positively regulated by the LTTR MexT. I describe the results of a genome-wide survey of the effect of ectopic *prpA*

expression, and discuss how these results suggest that PrrA is a negative regulator of the H-NS family member MvaU. This thesis thus describes the identification and characterization of several novel regulators of virulence in *P. aeruginosa*, reports a synthetic lethal relationship between the H-NS family members MvaT and MvaU and provides a basis for understanding the mechanism behind bistable gene expression in many bacteria.

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For my grandparents

Chapter 1

Introduction

Pseudomonas aeruginosa

During the pre-antiseptic era of surgery, many medical practitioners frequently observed a blue-green pigmentation on the dressings used to bind their patients. The origins of this pigment remained a mystery until 1860, when Fordos reported the isolation of a chloroform-soluble blue-green chemical that turns red upon treatment with hydrochloric acid, which he named “pyocyanine” (pyocyanin) [1]. Twenty-two years later, Gessard described the identification of the bacterium responsible for this pigment, which is known today as *Pseudomonas aeruginosa* [2]. Throughout the early 20th century, it became clear that *P. aeruginosa* could be responsible for a wide range of infections, including endocarditis, corneal keratitis, meningitis and systemic infections of children [3-6]. From these studies, several themes in *P. aeruginosa* infection began to emerge. It became clear that the organism was primarily associated with infection of the very young or very old, or of previously debilitated adults. Secondly, *P. aeruginosa* infections could be grouped into two types: the acute, which came on suddenly and resulted in violent systemic symptoms and death within days, and the chronic, which often lasted much longer [7]. Interestingly, *P. aeruginosa* was also reported as early as 1934 to be a potential hospital-acquired, or nosocomial, pathogen, when a case of meningitis in an adult woman was suggested to be linked to a spinal injection of contaminated anaesthetic [5]. Today, *P. aeruginosa* is responsible for 8% of nosocomial infections, and nosocomial pathogens cost the American healthcare system between \$28.4 and \$45 billion and cause an estimated 1.7 million infections and 99,000 deaths each year [8-10].

In addition to its role as a nosocomial pathogen, *P. aeruginosa* is known today as the primary cause of morbidity and mortality in patients with cystic fibrosis (CF) [11]. CF is a recessive genetic disorder caused by a mutation in the gene encoding the CFTR chloride ion channel [12]. It is the most common autosomal recessive genetic disorder among Caucasians,

with a frequency of about 1 in 2,500 live births [13]. Patients with defective CFTR function exhibit impaired ion secretion across epithelial layers in the lung, pancreas, liver and intestine, resulting in thick, dehydrated secretions such as mucous. It is thought that this altered mucous layer in the lung impairs the “mucociliary escalator”, in which microorganisms that enter the lower respiratory tract of healthy individuals are expelled by mucous flow over the ciliated epithelium. In patients with CF, these microorganisms, including *P. aeruginosa*, remain and are able to cause chronic, persistent infection. During the course of the patient’s life with this chronic infection, recurrent acute exacerbations of lung distress are thought to lead to a progressive loss of lung function that eventually results in death. It is not precisely known what causes these acute episodes, but anti-pseudomonal treatments are thought to be among the most effective interventions in alleviating the symptoms of an exacerbation, suggesting a central role for *P. aeruginosa* in the progression of CF [14]. Thus, managing chronic *P. aeruginosa* infection in CF patients is central to treatment of this disease.

Chronic infection

Chronic infections of *P. aeruginosa* are among the most significant diseases to which this organism contributes. These infections are especially difficult to treat due to the ability of *P. aeruginosa* to form robust surface-associated communities, or biofilms. In the CF lung, *P. aeruginosa* is thought to grow as a biofilm, a growth state which could contribute to the high degree of antibiotic resistance observed in the clinical setting [15,16]. In addition to infection of the CF lung, *P. aeruginosa* is also commonly isolated in cases of medical surface-associated infections such as ventilator-associated pneumonia, catheter-associated urinary tract infection and central line-associated bloodstream infection [8]. In chronic infections, the ability of the organism to associate with surfaces is thought to contribute greatly to the persistent nature of

these infections, and the antibiotic resistance engendered by growth in these biofilm communities makes these infections expensive and difficult, if not impossible, to clear.

Biofilm formation in the laboratory is a well-characterized developmental process, with several sequential steps leading to the formation of a highly structured community (reviewed in [17]). The process begins when motile planktonic cells form a transient association with a surface, which is typically mediated by cell surface-associated adherence factors such as pili or fimbriae [18,19]. Secretion of extracellular polysaccharide (EPS) begins the process of encasing these cells in a polymeric matrix and strengthening the attachment to the surface. Consistent with the importance of EPS production in the early stages of biofilm formation, it has been shown that genes encoding the synthetic machinery for the well-characterized EPS alginate, a main component of biofilms of mucoid *P. aeruginosa*, are induced as early as 15 minutes after initial association with a surface [20]. After the induction of EPS production, the biofilm begins to undergo a highly complex maturation process, forming three-dimensional structures, replete with water channels and pores, that rise well above the surface to which they are attached [21]. This maturation process is associated with massive changes in the proteome and transcriptome of the organism [22,23]. Most notably, cells appear to communicate with one another chemically through quorum sensing (QS) when grown in biofilms [24]. QS is a cell-cell signaling mechanism used by bacteria that was originally thought to simply be a mechanism for sensing cell density, though understanding of its role has advanced through studies of single-species biofilms and multispecies interactions (reviewed in [25]). *P. aeruginosa* has at least three known QS signals, the C4- and 3OC12-acyl homoserine lactones (HSLs) and the *Pseudomonas* quinolone signal (PQS) [26-28]. These signals are involved in a complex signaling cascade that coordinates gene expression between cells grown in a community, and many genes important for biofilm formation and maturation are regulated by QS [29]. When examined on a smaller scale, the differentiation of cells in the biofilm becomes strikingly complex and structured

(reviewed in [17]). Cells occupying different niches or physical locations within the biofilm can exhibit markedly different patterns of gene expression or metabolism, which creates a highly differentiated multicellular “superorganism”. Thus, the mature biofilm is a complex, highly structured community of phenotypically differentiated cells, and is thought to be the result of a sophisticated multistage developmental process.

In contrast to the well-characterized nature of laboratory-grown *P. aeruginosa* biofilms, the role of biofilms in the human host is much less clear. This is due largely to the difficulty of addressing the formation, structure and physiology of biofilms in detail in the infectious setting. Indeed, the evidence that *P. aeruginosa* grows as a biofilm in the CF lung is highly suggestive but not conclusive [30]. Sections of lung biopsy from CF patients show growth of *P. aeruginosa* in clusters that appear similar to the microcolonies observed in early biofilms [31]. The QS signals C4- and 3OC12-acyl HSL have also been identified in sputum isolated from CF patients in ratios similar to those observed in laboratory-grown biofilms [15]. While this evidence is suggestive that *P. aeruginosa* grows in a biofilm-like state in the CF lung, the exact nature of how this chronic pathogen lives out its long-term infection in the CF patient is not well understood.

Several studies have suggested that portions of the infected CF lung contain anaerobic or microaerophilic regions in which bacteria can grow, and that *P. aeruginosa* responds to these signals to upregulate important factors for virulence and persistence. Increased oxygen consumption by the damaged lung epithelium and the increased viscosity and decreased flow of airway mucous can create steep hypoxic gradients in the CF lung in which microcolonies of *P. aeruginosa* can be found [32]. It is also known that nitrate, a potential terminal electron acceptor for *P. aeruginosa* when grown in the absence of oxygen, can be found in relatively high concentrations in the secretions of the CF lung [33,34]. These studies, taken together with a

report that *P. aeruginosa* can form robust biofilms *in vitro* when grown anaerobically in the presence of nitrate, suggest that *P. aeruginosa* can potentially grow as an anaerobic biofilm in the CF lung [35]. *P. aeruginosa* upregulates a number of genes, including those involved in the metabolism and tolerance to nitrate reduction products when grown anaerobically with nitrate [36,37]. Interestingly, these same studies also found that the *cupA* genes, which encode components of a chaperone-usher fimbria required for biofilm formation on abiotic surfaces in cells lacking a type IV pilus, were strongly upregulated during growth in these conditions [18,36,38]. Thus, even with the limitations inherent in studying *P. aeruginosa* growth in the CF lung, several lines of evidence suggest that oxygen-limiting, nitrate-replete conditions in the CF lung can lead to upregulation of the expression of genes encoding an adherence factor that may contribute to growth as a biofilm in this chronic infection.

Chaperone-usher fimbriae

The chaperone-usher fimbriae represent a family of surface appendages present in a wide range of bacteria, united by their common evolutionary heritage and assembly mechanism [39]. The best-characterized of these systems are the P (*pap*) and type I (*fim*) fimbriae of uropathogenic *Escherichia coli*, both of which can contribute to colonization and inflammation in urinary tract infections [40,41]. These and other chaperone-usher fimbriae are encoded by several genes, usually located in an operon, that encode at least three proteins: a major fimbrial subunit, a chaperone and an usher protein. All of these proteins are secreted to the periplasm by the general secretory pathway [42]. Here, the chaperone interacts with the fimbrial subunit to ensure proper folding, and this complex interacts with the usher, an integral outer membrane protein which mediates the secretion and extracellular assembly of the fimbrial subunit into a polymeric fimbria [43]. Interestingly, even though this assembly mechanism is conserved among chaperone-usher fimbriae, a study investigating the promiscuity and interplay between the type I

and F1C fimbriae of *E. coli* suggests that the chaperone proteins cannot function with the homolog of their cognate usher protein (and vice versa), indicating a degree of pairwise specificity based on genetic linkage and coevolution between these two fimbrial assembly components [44].

Like uropathogenic strains of *E. coli*, the genome of *P. aeruginosa* also contains multiple chaperone-usher fimbrial gene clusters (up to five depending on the strain) [18,45,46]. The first of these to be identified was the *cupA* gene cluster, which is required for biofilm formation on abiotic surfaces in *P. aeruginosa* strains lacking type IV fimbriae [18]. This gene cluster, along with the homologous *cupB* and *cupC* gene clusters present in *P. aeruginosa* strain PAO1, is not expressed under normal laboratory conditions, but is expressed in strains lacking the global regulator MvaT [47]. As described above, the *cupA* genes are also induced in response to low oxygen concentrations, or when cells are grown anaerobically with nitrate [36]. This is thought to be due to positive regulation of the *cupA* genes by the regulator of anaerobic nitrate respiration Anr [48]. When considered in light of the mounting evidence that *P. aeruginosa* likely encounters oxygen-limiting conditions when grown in the CF lung (see above), this result raises the intriguing possibility that the CupA fimbriae are adherence factors that contribute to biofilm formation in this chronic infection. The *cupA* fimbrial genes are also positively regulated by CgrABC, proteins which are encoded by genes immediately upstream of the *cupA* genes themselves and are required for their expression [48]. This putative *cgrABC* operon is also repressed by MvaT, a member of the H-NS (histone-like nucleoid-structuring) family of nucleoid-associated proteins. A plasmid carrying the putative *cgrABC* operon can induce *cupA* gene expression in *P. aeruginosa* cells containing MvaT. The CgrABC proteins do not represent conventional positive regulators of transcription, as CgrA is homologous to phosphoadenosine phosphosulfate reductases, CgrB is predicted to be a member of the GNAT (GCN5-related acetyltransferase) family of acetyltransferases and CgrC bears homology to ParB-like DNA-

binding proteins. While it is known that an interaction between CgrA and CgrC is required for *cupA* gene expression, it is unclear how these unusual regulators function to positively regulate the *cupA* genes [49]. It is also known that CgrB can interact with the CgrA-CgrC complex, but whether this interaction is important for *cupA* gene expression is currently not known (H.R. McManus and S.L. Dove, unpublished). Finally, in all growth conditions or genetic backgrounds in which expression of the *cupA* genes is induced, they appear to be expressed in a phase-variable or bistable manner, a phenomenon which is discussed in greater detail below (Figure 1.1) [50]. Regulation of *cupA* gene expression is therefore complex, and involves the convergence of local and global transcription regulators.

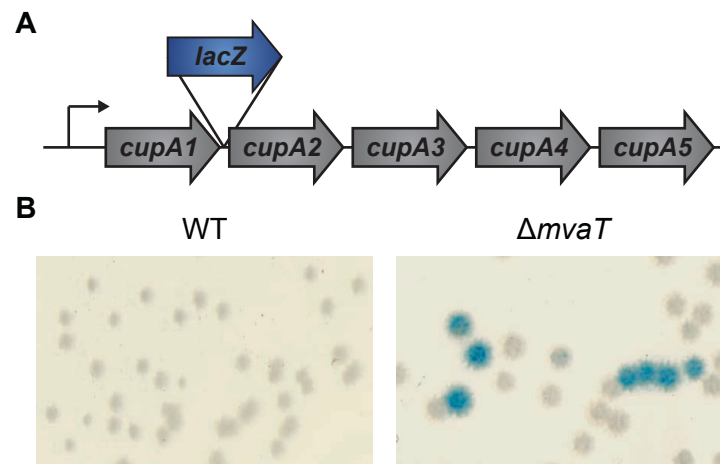


Figure 1.1. MvaT represses phase-variable expression of the *cupA* fimbrial operon. (A) Schematic of the *cupA* operon in *cupA lacZ* reporter strains. (B) Phenotypes of wild-type (WT) and $\Delta mvaT$ *cupA lacZ* reporter strains when plated on LB agar containing X-Gal. Adapted from [50].

The H-NS family members MvaT and MvaU

The global regulator MvaT, which represses *cupA* fimbrial gene expression, is one of the two H-NS family members in *P. aeruginosa* [47]. H-NS family members are present in many Gram-negative bacteria, and are named for their homology or functional similarity to H-NS of *E. coli* [51]. These proteins typically bind to a specific AT-rich target DNA sequence and subsequently oligomerize along adjacent DNA, coating extended lengths of the chromosome and creating a nucleoprotein filament [52]. Where bound, these proteins can alter the superstructure of DNA, modulating supercoiling and bridging the DNA [53,54]. Thus, these proteins are often referred to as “histone-like”, due to their functional similarity to the histones of eukaryotes. H-NS-like proteins can affect gene expression through a variety of mechanisms, including occlusion of RNA polymerase and influence on DNA topology (reviewed in [55]). It has been proposed that H-NS family members, with their propensity for binding to and spreading across AT-rich regions of the genome, act to repress the expression of horizontally-acquired DNA which is often AT-rich relative to the rest of the genome [56,57].

MvaT and its homolog MvaU represent the H-NS family in *P. aeruginosa*, where they repress several genes involved in virulence [47]. Higher-order oligomerization of MvaT is required to repress *cupA* fimbrial gene expression, suggesting that MvaT may function much like other H-NS family members to influence transcription [58]. Both MvaT and MvaU occupy relatively large regions widely spread throughout the *P. aeruginosa* genome in a manner reminiscent of other H-NS family members [59]. There appears to be a degree of functional redundancy between MvaT and MvaU as MvaU can act to repress several MvaT target genes in the absence of MvaT. Depletion of either MvaT or MvaU in the absence of the partner regulator results in a growth defect, suggesting that the presence of either one of these two proteins is essential (see Chapter 3). This is likely due to their function in repressing the expression of

genes of a prophage (S. Castang and S.L. Dove, unpublished). Interestingly, MvaT and MvaU can form heteromeric complexes with one another, further underscoring the interrelatedness in their function [50].

Regulation by small RNAs in P. aeruginosa

Small noncoding RNAs, referred to as sRNAs in bacteria, have rapidly gained attention in the past decade as an additional class of regulators of gene expression. sRNAs are typically between 100 and 300 nucleotides in length, and are encoded by genes located in both monocistronic and polycistronic operons (from which they are often processed), or located on the antisense strand from other, often protein-coding genes [60,61]. Though they were initially characterized in model organisms such as *E. coli*, appreciation for the importance of these sRNAs in controlling gene expression has spurred interest in searching for them in a wide range of bacteria. Advances in computational methods for predicting sRNA genes and, more recently, high-throughput RNA sequencing endeavors have contributed to a large increase in the number of bacterial species known or predicted to encode sRNAs [62]. Trans-acting sRNAs tend to act in one of two non-mutually exclusive mechanisms to alter gene expression, either interacting with target mRNAs through basepairing interactions or by interacting with regulatory proteins (reviewed in [63] and [64]). sRNAs that interact directly with target mRNAs often require the presence of the RNA chaperone protein Hfq to stabilize an otherwise weak basepairing interaction involving between 10 and 25 imperfectly matching bases [60,65]. This direct sRNA-mRNA interaction can affect mRNA fate to reduce production of the specified protein (or proteins) in several distinct ways. The sRNA can bind at or near the ribosome-binding sequence of the target mRNA, inhibiting translation and destabilizing the mRNA [66]. Binding of an sRNA can also promote endonucleolytic mRNA cleavage and subsequent mRNA destabilization [67]. Alternatively, sRNAs can basepair with target mRNAs to alleviate autoinhibitory secondary

structures present in the mRNA that block translation, thus positively regulating protein synthesis [68]. Some sRNAs, especially the cis-antisense sRNAs, which are encoded at the same locus as their target gene on the antisense strand, are thought to target only a single transcript [69,70]. Many trans-encoded sRNAs, such as DsrA of *E. coli*, act directly on several mRNA targets to coordinately regulate production of many proteins, thus acting as global regulators [71]. Similarly, some mRNAs, such as *csgD* of *E. coli*, can respond to several distinct sRNAs to integrate signals from disparate regulatory networks (reviewed in [72]). Thus, several sRNAs can regulate the same targets, and regulatory targets for a particular sRNA can be difficult to identify experimentally without overexpressing the sRNA [73].

sRNAs can act at the post-translational level as well, interacting with and sequestering proteins that are themselves regulators of gene expression. The RNA-binding protein CsrA of *E. coli* can bind to a conserved motif in many different mRNAs, inhibit their translation and promote their degradation [74]. However, the sRNA CsrB can sequester CsrA and thereby prevent translation inhibition and transcript destabilization [75]. Additionally, the 6S RNA can sequester σ^{70} -containing RNA polymerase holoenzyme, preventing transcription from some σ^{70} -dependent promoters [76]. DsrA has been suggested to interact with several proteins *in vivo*, though it is as yet unknown whether these interactions have any physiological outcome [77]. Interestingly, some sRNAs that act as RNAs to affect gene expression can also contain an expressed open reading frame, and both the sRNA and the polypeptide specified therein may play roles in gene regulation [78]. Thus, sRNAs represent a broadly conserved class of regulators in bacteria that affect gene expression in many ways.

P. aeruginosa has been shown to encode several sRNAs that regulate a wide variety of processes. A recent review summarizes 42 sRNAs of *P. aeruginosa* whose production has been experimentally verified [79]. Many of these sRNAs, including PrrF1 and PrrF2, which repress the

translation of several mRNAs that encode iron-containing proteins, and PhrS, which regulates the mRNA encoding PqsR, a regulator of PQS synthesis, likely act by basepairing to their mRNA targets [80,81]. PhrS is also thought to encode a translated short polypeptide, though the function of this peptide is unclear. The sRNAs RsmY and RsmZ are thought to act in a manner analogous to CsrB of *E. coli*: that is, by sequestering the RNA-binding protein RsmA from several mRNA targets, thus affecting their translation [82-84]. *P. aeruginosa* also encodes the 6S RNA, though it is unknown whether it sequesters σ^{70} -containing RNA polymerase like its homolog in *E. coli* [85]. Trans-acting sRNAs of *P. aeruginosa* thus represent an important class of regulators of gene expression that can function through several different mechanisms.

Antibiotic resistance and RND-type efflux pumps

One factor that makes chronic infections with *P. aeruginosa* particularly difficult to treat is the high degree of antibiotic resistance exhibited by the organism in the infectious setting. A recent study found that approximately 55% of all clinical *P. aeruginosa* isolates are resistant to at least one antibiotic, and approximately 15% of isolates are resistant to three or more antibiotics [86]. Growth of the organism in biofilms, which is thought to occur in several chronic infections, has been observed to result in an increase in resistance to a number of antibiotics, including tobramycin, an aminoglycoside antibiotic widely used in treatment of CF patients [87]. This may be due to the contribution of several factors [16]. Firstly, biofilms are heterogeneous with respect to cellular metabolism and growth, and cells in the biofilm that are growing more slowly than others may respond poorly to certain antibiotics that act on growing cells [88,89]. Secondly, the extracellular matrix of a biofilm can provide a diffusion barrier to certain antibiotics such as piperacillin [90]. Lastly, growth in a biofilm can specifically induce expression of antibiotic resistance systems, including (but not limited to) resistance nodulation division (RND)-type efflux pump systems [87,91]. These systems confer resistance to a variety of antibiotics by

proton-motive force-dependent efflux of the antibiotic, and are typically composed of three proteins: an integral membrane protein, a periplasmic membrane-fusion protein and an outer membrane protein [92]. The genome of *P. aeruginosa* strain PAO1 contains genes encoding or predicted to encode at least 10 of these systems, and these systems are implicated in increased resistance to a number of antibiotics, including aminoglycosides, beta-lactams, fluoroquinolones, sulfonamides and many others [93,94].

The role of RND-type efflux pumps in *P. aeruginosa* extends beyond efflux of antibiotics [95]. It has been suggested that the MexEF-OprN pump can reduce synthesis of the QS signal PQS by exporting synthetic precursors from the cytoplasm [96,97]. The MexAB-OprM pump has been similarly implicated in export of 3OC12-HSL, another QS signal [98,99]. RND-type efflux pumps have also been implicated in export of several toxic organic solvents from the cytoplasm [100]. It has also been suggested that the MexAB-OprM pump is required for virulence in a leukopenic mouse model of septicemia [101]. The authors of this study propose that the MexAB-OprM pump is required to export critical virulence determinants from the cell, however, a role for the pump in export of host-derived antimicrobial chemicals from the cytoplasm of bloodborne *P. aeruginosa* is equally likely. Thus, RND-type efflux systems can contribute to resistance to antimicrobial compounds, including several clinically relevant antibiotics, and can affect gene expression and pathogenesis in *P. aeruginosa*.

The LysR-type transcription regulator MexT

Expression of the *mexEFoprN* RND-type efflux pump genes is regulated by several factors, but foremost among them is the LysR-type transcription regulator (LTTR) MexT [102-104]. This protein is encoded by a gene located immediately upstream of and on the same strand as the *mexEFoprN* operon. MexT likely binds to the *mexE* promoter at an imperfectly

palindromic sequence upstream of the *mexE* gene, and can positively regulate transcription of the *mexEFoprN* genes into a polycistronic mRNA ([105] and K.H. Turner and S.L. Dove, unpublished). Strains of *P. aeruginosa* PAO1 lacking MexT display an approximately 500-fold reduction in *mexEFoprN* mRNA abundance, suggesting that expression of this operon is highly positively regulated by MexT (K.H. Turner and S.L. Dove, unpublished). MexT can also regulate the expression of a number of other genes and affect the expression of several virulence factors, including the type III secretion system, pyocyanin and the QS signal PQS [106,107]. A recent study reported that ectopic expression of *mexT* from a plasmid can both positively and negatively regulate the expression of 112 genes, including many encoding virulence factors [108]. The authors further classified these genes into those that depended on the presence of the *mexEF* genes for MexT-mediated regulation and those that did not. Interestingly, of the 40 genes most significantly affected by MexT, none of those whose expression increased upon ectopic *mexT* expression were affected by deletion of the *mexEF* genes, suggesting that MexT positively regulates its MexEF-OprN-independent targets. The predicted promoter regions of many of these genes contained an imperfectly palindromic sequence similar to that found upstream of the *mexEFoprN* genes, and DNA fragments containing this sequence from these target genes exhibited retarded mobility when incubated with purified MexT in electrophoretic mobility shift assays. Thus, the authors suggest that MexT is a global regulator affecting virulence, QS and antibiotic resistance gene expression that functions by binding to these imperfectly palindromic sequences upstream of target genes and positively regulating their expression.

Studies of MexT and its regulation of the *mexEFoprN* operon are often complicated by high mutability in the *mexT* gene and in other genes, both known and unknown, that affect expression of the *mexEFoprN* operon. A strain of *P. aeruginosa* that exhibited an altered antibiotic resistance profile, including increased resistance to chloramphenicol and the

fluoroquinolone norfloxacin, was spontaneously generated in the laboratory and termed an *nfxC* mutant [109]. Subsequent studies made clear that mutations in several genes could yield this characteristically altered antibiotic resistance profile, and that these mutant strains all exhibited increased expression of the *mexEFoprN* operon [110]. For example, mutations in *mexS*, which is predicted to encode an oxidoreductase, have been shown to recapitulate the *nfxC*-type mutant phenotype [111]. Interestingly, an *mvaT* mutant strain also partially exhibits the *nfxC*-type phenotype, though to a significantly lesser degree than spontaneously generated *nfxC*-type mutants [103]. However, many *nfxC*-type mutants that have been isolated or spontaneously generated carry uncharacterized mutations [104]. Spontaneous *nfxC*-type mutants are also found among clinical isolates, suggesting that the MexEF-OprN pump may play a role in some *P. aeruginosa* infections [112-114]. Interestingly, an 8-bp sequence in the *mexT* gene is present in two copies in many *P. aeruginosa* strains, including the sequenced PAO1-UW strain [115]. In strains carrying two copies of this 8-bp sequence, the *mexT* gene carries a frameshift mutation and no longer produces a functional MexT protein. However, many strains, including some spontaneously generated *nfxC*-type mutants, lack one of these repeats, thus producing functional MexT and subsequent high-level expression of the *mexEFoprN* genes [116]. Thus, studies describing potential regulation of MexT target genes must be carefully interpreted in light of these differences in *mexEFoprN* expression.

MexT is a member of the LTTR family of transcription regulators, a family of regulators present in a wide range of bacteria. Many bacteria contain multiple members of this family. Indeed, LTTRs represent the most highly represented family in the Cluster of Orthologous Groups database entry for *P. aeruginosa* PAO1, with 121 predicted members, and may represent the most prevalent family of genes in the PAO1 genome [115]. LTTRs tend to be composed of an N-terminal DNA-binding domain and a C-terminal multimerization and small molecule-binding domain (reviewed in [117]). In many cases, the C-terminal domains of LTTRs

bind small molecules, which induces a conformational shift in the protein and an alteration in DNA-binding, multimerization or transcription activation properties. For example, the well-characterized LTTR TrpI in *P. aeruginosa* activates transcription of the *trpBA* genes, which encode enzymes that catalyze the final steps in tryptophan biosynthesis, in response to the tryptophan precursor indoleglycerol phosphate (InGP) [118,119]. TrpI binds to InGP, which promotes the interaction of two TrpI tetramers bound to two distinct sites upstream of *trpBA* [120,121]. Formation of this multimeric complex is thought to alter DNA topology in such a way as to allow transcription from the *trpBA* promoter and expression of the genes encoding the tryptophan synthetic enzymes [122,123]. However, not all LTTRs are thought to bind small molecules [124]. MexT has been shown to be required for upregulation of the *mexEFoprN* operon in response to chloramphenicol and chemically-induced nitrosative stress, however, it is unknown whether this depends on small molecule binding by MexT [125].

Phenotypic variation

Phenotypic variation, such as that exhibited by the *cupA* fimbrial genes of *P. aeruginosa*, is a common phenomenon in bacteria. The initial description of a phenotypically variable system in bacteria was that of the flagellum of *Salmonella* spp. by Andrewes in 1922 [126]. An isogenic population of *Salmonella* contains subpopulations of cells that express one of two flagellar antigens. A single cell in either expression state tends to give rise to daughter cells of the same expression state upon division, yet can occasionally give rise to a daughter cell of the alternate expression state at some fixed frequency (with a switch occurring in approximately 0.03% of cell divisions) [127]. These flagellar antigens are highly immunogenic in the human host, but antibodies raised against one antigen do not cross-react with the other antigen. It is thought that by stochastically designating a subset of cells in a population to express a different antigen, a population of *Salmonella* can “hedge” against acquired immunity against the flagellum in a human host. Thus, phenotypic variation can protect a population of bacteria against the sudden onset of conditions in which a particular phenotype might be detrimental to bacterial fitness. Moreover, the reversible nature of this switch in expression state ensures that after a selective sweep, population heterogeneity can be restored.

Phase-variation

Since the initial description of phenotypic variation in the *Salmonella* flagellar antigens, it has become clear that the expression of many genes in bacteria exhibits a reversible switch between two expression states (typically ON and OFF). One family of mechanisms by which this variation is achieved is referred to as phase-variation. In phase-variation systems, reversible changes in DNA sequence or structure alter gene expression in a heritable manner (reviewed in [128]). For example, the promoter of the gene encoding one of the two flagellar antigens of *Salmonella* is flanked by inverted repeats [129]. When a recombination event occurs between

these two repeat sequences, the promoter region is inverted, and the promoter can no longer drive expression of an operon containing the flagellar antigen gene and a gene encoding a repressor of the alternative flagellar antigen gene [130,131]. This leads to derepression of the gene encoding the alternative antigen and a switch in expression state. Interestingly, the *fim* locus of uropathogenic *E. coli* strains, which encodes a chaperone-usher fimbria as discussed above, is also subject to phase-variation due to inversion of a promoter flanked by inverted repeat sequences [132]. Many phase-variable systems are controlled by slipped-strand mispairing during DNA replication, in which several short repeats arranged contiguously can induce skipping or duplication of these repeats by DNA polymerase. This occurs in genes encoding enzymes involved in lipooligosaccharide modification in the human pathogen *Haemophilus influenzae*, which leads to nonsense mutations in these genes arising in a phase-variable manner and subsequent variation of the lipooligosaccharide structure [133,134].

Another particularly interesting mechanism of phase-variation is that controlling variable type IV pilin expression in *Neisseria gonorrhoeae*. The gene encoding this pilin, *pilE*, is subject to a complex process known as combinatorial variation, in which six silent, variable *pilS* loci around the genome recombine randomly and at high frequency with the expressed *pilE* locus, with which they have some homology [135]. Recombination between these semi-homologous loci results in the production of a chimeric pilin protein whose composition can change depending on the changing *pilE* sequence. Therefore, this imperfect homologous recombination system can also generate phase-variation in gene expression. Finally, phase-variation as mediated by changes in DNA sequence can also occur due to the action of transposases, as is thought to be the case with the extracellular polysaccharide biosynthesis machinery in certain isolates of *Pseudoalteromonas atlantica* [136]. Thus, high mutability at specific loci in bacterial genomes can serve as a basis for phase-variation of a wide range of phenotypes.

Phase-variation can also be controlled by epigenetic modifications to DNA. In epigenetically mediated phase-variation, alternative methylation states of a target DNA sequence affect the binding of a transcription regulator. This was first described as the basis for phase-variation of the Pap pilus of uropathogenic *E. coli* (a chaperone-usher fimbria) [137]. The promoter region of the *pap* operon contains two 5'-GATC-3' Dam methylase target sequences in close proximity to one another. Methylation of the Dam target sequence proximal to the *pap* promoter ("GATC¹¹³⁰") interferes with binding to that sequence by the DNA-binding protein Lrp [138]. Lrp will instead bind to the promoter distal Dam target sequence ("GATC¹⁰²⁸"), where it acts as a positive regulator of *pap* operon expression [139]. In the alternative state, in which GATC¹⁰²⁸ is methylated and GATC¹¹³⁰ is nonmethylated, Lrp will instead bind to GATC¹¹³⁰ and repress expression of the *pap* operon. It is thought that a switch in expression state can occur when the previously methylated 5'-GATC-3' sequence becomes hemimethylated after DNA replication, allowing Lrp to bind and prevent Dam from methylating the unmethylated strand [140]. Interestingly, the *pap* operon can also be activated by cyclic AMP-CAP, which acts as a global regulator of metabolism in *E. coli*, suggesting that the metabolic state of the cell can also feed into regulation of phase-variation [141]. This system highlights the complex nature of DNA-mediated phase-variation systems and provides a well-characterized example of how phenotypic variation may be achieved without changes to DNA sequence.

Bistability

The mechanisms that generate phenotypic variation in bacteria are typically grouped into one of two categories: phase-variation (described above) and bistability. The precise distinction between these two mechanistic families is often unclear. Some authors classify systems in which phenotypic variation arises from modifications to DNA sequence or structure as phase-variable, and consider all other phenotypic variation systems to be bistable [142,143]. In this work I consider phase-variation as a special case of bistability. The concept of bistability has its roots in physics, where it simply describes any system that can exist in one of two stable states. Examples of these systems include toggle (or “latch”) switches in electronics, which can exist in either the “on” or “off” state, or lasers, which either emit or do not emit photons. Indeed, the word “bistability” itself suggests nothing more than the existence of two stable states. Based on this simple criterion, many biological systems, including phase-variation systems, can be considered bistable. Bistable switches are found in the single cell, including the switch in competence gene expression in *Bacillus subtilis* and the signaling cascades that control the eukaryotic cell cycle [144,145]. Bistable systems can exist at the multicellular or multiorganismal level as well; both the propagation of prions throughout an infected organism and variation between high and low population densities in single-species populations subjected to asymmetric competition are considered bistable processes [146-148]. Therefore, I will use the term “bistability” to describe phenotypic variation only in a general, phenomenological sense, and consider phase-variation to be one mechanism by which bistability can be achieved.

Bistable systems that are not controlled by phase-variation have been characterized in many bacteria, and control variation in a wide range of phenotypes (reviewed in [142]). Bistability can confer a switch-like behavior to the expression of a particular phenotype, and can ensure that the phenotype is stably inherited by daughter cells. For this reason, they often

underlie the behavior of developmental transitions in bacteria, in which a cell transitions sharply into a distinct lifestyle. For example, the well-characterized transition of *B. subtilis* from vegetative growth to sporulation involves the action of numerous bistable switches [149]. Key among these is that mediated by the transcription factor Spo0A. When phosphorylated, Spo0A positively regulates transcription of a number of genes involved in the transition to sporulation [150]. Importantly, phosphorylated Spo0A can also positively regulate transcription of its own gene through positive regulation of the alternative sigma factor σ^H [151]. A bistable switch, mediated by the transcription regulator ComK, also controls development of competence in *B. subtilis* [152]. As a population of *B. subtilis* cells enters stationary phase, a subpopulation of only 10-20% of the cells becomes competent, whereas the remainder of the population remains noncompetent [153,154]. Positive feedback of ComK is required for bistability of this switch [144]. When positive feedback of ComK is intact, the expression level of competence genes can only stably occupy a high or a low state, whereas without positive feedback, a population of cells can occupy a range of intermediate expression states with respect to the competence genes. The switch to competence also highlights a second key feature of bistable systems: it has been shown that a high degree of noise in production of ComK is required for some cells to enter the competent state [155,156]. Studies on the development of competence in *B. subtilis* thus highlight the nature of bistable systems as containing an unstable intermediate “threshold” expression state separating a high or a low stable expression state (Figure 1.2). Furthermore, these studies illustrate that bistable systems can amplify small variations in gene expression at the single cell level to create distinct, stable differences in cellular fate.

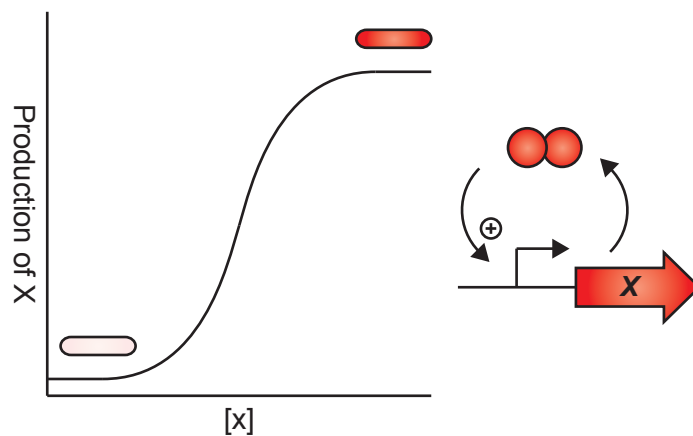


Figure 1.2. A single positive feedback loop can give rise to bistability. In this example, the gene X encodes a transcription activator that binds to the promoter of its own gene as a dimer to activate transcription (right). The graph depicts the production rate of X as a function of its concentration (left). This system has two stable expression states: low concentration and low production of X (light ellipse), and high concentration and high production of X (dark ellipse). These two stable states are separated by an intermediate concentration of X at which its production rate is highly sensitive to variation in abundance of X . This intermediate value is determined by the dissociation constants of the X dimer and of the dimer-DNA complex (for further discussion, see Appendix I).

Positive feedback is a key component of many bistable systems, and can arise from a number of mechanisms. In the case of the competence switch of *B. subtilis*, positive feedback is direct, mediated by binding of ComK to the promoter region of its own gene to positive regulate its own expression. Positive feedback can be indirect as well, as in the case of bistability of the *lac* operon of *E. coli* when cells are grown in low concentrations of the non-metabolizable inducer thio-methylgalactoside (TMG) [157]. In this system, TMG can bind to the transcription repressor LacI and alleviate its repression of the *lac* operon. *lacY*, which encodes a transporter of lactose and similar compounds such as TMG, is one of the genes in the *lac* operon. When the *lac* operon is expressed, cells produce more of the LacY transporter, thus allowing for increased transport of TMG and continued derepression of the *lac* operon. Interestingly, the expression state of this system at low concentrations of TMG depends on previous growth conditions: when cells of *E. coli* are initially grown in the absence of TMG and are subsequently grown in a low concentration of TMG, the *lac* operon is not expressed [158]. However, when cells transition from a high to a low concentration of TMG, the *lac* operon is expressed. Thus, two isogenic populations of cells grown in identical conditions can occupy two distinct expression states depending on the conditions in which they were previously grown, and maintain that expression state for hundreds of generations. This effect is known as hysteresis, and is a key feature of many bistable systems.

Many potential regulatory network configurations can give rise to bistability (Figure 1.3). Experimental and theoretical studies suggest that two criteria are absolutely necessary for bistability in a regulatory switch: positive feedback (discussed above) and hypersensitivity [159-161]. Hypersensitivity is crucial to development of a bistable switch because it creates the potential for an unstable “threshold” expression state between two stable expression states (Figure 1.2). In the competence switch of *B. subtilis*, hypersensitivity arises from cooperative binding of DNA by multimers of the key transcription regulator ComK [162]. However,

hypersensitivity in bistable switches need not depend on cooperative binding. In the bistable galactose uptake system of the budding yeast *Saccharomyces cerevisiae*, hypersensitivity is achieved by molecular titration: galactose binds to the cytoplasmic Gal3p protein, and this complex can bind to the transcription repressor Gal80p and inhibit transport of Gal80p into the nucleus [163]. When Gal80p is sequestered to the cytoplasm, the *GAL3* gene is derepressed, leading to higher production of the Gal3p protein and further cytoplasmic sequestration of Gal80p [164]. It has been shown that at a given concentration of galactose, expression of Gal3p can be bistable [165]. Much like titrating a strong acid into a buffered basic solution, a threshold concentration of Gal3p exists at which a sharp change in the relative concentrations of free to sequestered Gal80p occurs. The value of this threshold concentration of Gal3p and the sharpness of the transition that it enables both depend on the intracellular concentration of Gal80p. Indeed, at a given concentration of galactose, the system is bistable only at an intermediate intracellular concentration of Gal80p [165]. This illustrates an important point about bistable systems more generally: while feedback and hypersensitivity are necessary for the development of bistability, they are not necessarily sufficient to create bistability. The same is true of the *lac* operon bistability in *E. coli*: when the concentration of TMG is significantly lower or higher than the critical intermediate value, expression of the *lac* operon is constitutively off or on, respectively [158]. The biochemical parameters of a regulatory system must therefore fall within a range that enables bistability (see Appendix I for a detailed description of this requirement). Thus, bistability is a condition-dependent property of certain regulatory networks.

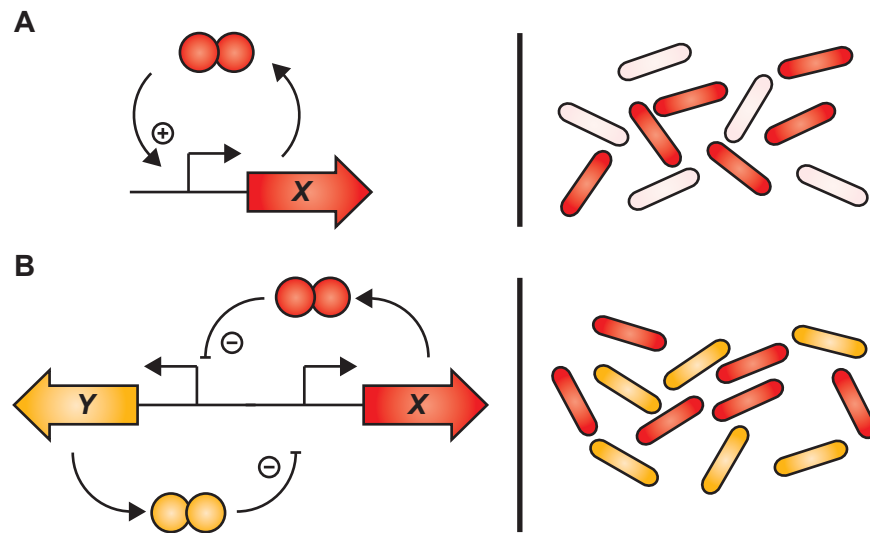


Figure 1.3. Multiple transcription regulatory network configurations can give rise to bistability. (A) A single positive feedback loop (left) can give rise to two expression states in a clonal population of bacteria (right). (B) A double negative feedback loop (left) can also give rise to two expression states in a clonal population of bacteria (right).

Dissertation overview

The opportunistic pathogen *P. aeruginosa* can cause a wide range of medically and economically significant infections in immunocompromised or hospitalized patients. Many of the most clinically challenging of these infections are chronic in nature, and the ability of the organism to persist as a biofilm is thought to contribute greatly to the persistent nature of these infections. Additionally, growth as a biofilm enhances an already potent capacity of *P. aeruginosa* to resist antibiotics, further complicating treatment of these infections. The CupA fimbriae are expressed in microaerophilic or anaerobic conditions (such as those thought to be present in the lungs of *P. aeruginosa*-infected CF patients), where they can contribute to biofilm formation. The genes encoding these fimbriae belong to a class of genes that are repressed by the coordinate function of the H-NS family members MvaT and MvaU. Interestingly, the *cupA* fimbrial genes can exhibit bistable, or potentially phase-variable, expression.

The range of general classes of mechanism by which the common phenotype of phenotypic variation is achieved suggests that phenotypic variation in bacteria has evolved multiple times independently in response to common selective pressures. There are several hypotheses as to what these selective pressures may represent. Originally, it was thought that phenotypic variation evolved to allow invading bacteria to evade the host immune system by ensuring that a subset of a population would express a different particular antigen, such as the flagellar antigens of *Salmonella*. However, the subsequent discovery that many proteins not thought to serve as antigens, such as DNA restriction-modification systems in *Mycoplasma pulmonis*, exhibit reversible switching between stably inherited expression states has led to a broader understanding of the selective pressures that may give rise to this phenotype [166]. In principle, attack by the host immune system is no different to a bacterium than attack by lytic bacteriophage from the standpoint of its effect on fitness. Phenotypic variation, then, can

potentially be considered a “kin selection” strategy, ensuring that the relatives of a given bacterium are prepared to survive in the face of selective pressure.

This thesis reports my and my colleagues’ work on bistable gene expression in *P. aeruginosa*. Specifically, I begin by describing the discovery and characterization of a novel feedback-mediated bistable switch in the BexR transcription regulator in Chapter 2. In Chapter 3 I describe a genetic system used to demonstrate that the loss of both of the H-NS family members MvaT and MvaU cannot be tolerated by the cell. Chapter 4 describes the discovery of the novel small regulatory RNA PrrA, which serves as a regulatory link between the MexEF-OprN RND-type efflux pump and bistable expression of the CupA fimbriae, potentially by negatively regulating MvaU. Finally, I synthesize the experimental work presented in this thesis and present potential future directions for that work in Chapter 5. Our work introduces and characterizes several novel regulators of virulence in *P. aeruginosa*, clarifies the redundant function of the H-NS family members MvaT and MvaU and provides a basis for understanding mechanisms generating bistable gene expression in a wide variety of bacteria.

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References

1. Fordos M-J (1860) Recherches sur la matière colorante des suppurations bleues : pyocyanine. C R Hebd Seances Acad Sci 51: 215–217.
2. Gessard C (1882) Sur les colorations bleue et verle des linges à pansements. C R Hebd Seances Acad Sci 94: 536–538.
3. Fish GW, Hand MM, Keim WF (1937) Acute bacterial endocarditis due to *Pseudomonas aeruginosa* (*B. pyocyaneus*): report of a case. Am J Pathol 13: 121–127.
4. Callan PA (1906) Ulceration and destruction of cornea by *Bacillus pyocyaneus*. Trans Am Ophthalmol Soc 11: 201–204.
5. Shrewsbury JF (1934) *B. pyocyaneus* meningitis with recovery. Br Med J 1: 280–281.
6. Williams EP, Cameron K (1894) Infection by the *Bacillus pyocyaneus* a cause of infantile mortality. Public Health Pap Rep 20: 355–360.
7. Freeman L (1916) Chronic general infection with the *Bacillus pyocyaneus*. Ann Surg 64: 195–202.
8. Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, et al. (2008) Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infect Control Hosp Epidemiol 29: 996–1011.
9. Klevens RM, Edwards JR, Richards CL, Horan TC, Gaynes RP, et al. (2007) Estimating health care-associated infections and deaths in U.S. hospitals, 2002. Public Health Rep 122: 160–166.
10. Scott RD (2009) The direct medical costs of healthcare-associated infections in U.S. hospitals and the benefits of prevention. Centers for Disease Control. Available: http://www.cdc.gov/HAI/pdfs/hai/Scott_CostPaper.pdf. Accessed 19 February 2012.
11. Govan JR, Deretic V (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol Rev 60: 539–574.
12. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, et al. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245: 1066–1073.
13. Ratjen F, Döring G (2003) Cystic fibrosis. Lancet 361: 681–689.
14. Hyatt AC, Chipps BE, Kumor KM, Mellits ED, Lietman PS, et al. (1981) A double-blind controlled trial of anti-*Pseudomonas* chemotherapy of acute respiratory exacerbations in patients with cystic fibrosis. J Pediatr 99: 307–314.
15. Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, et al. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407: 762–764.

16. Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284: 1318–1322.
17. Stoodley P, Sauer K, Davies DG, Costerton JW (2002) Biofilms as complex differentiated communities. *Annu Rev Microbiol* 56: 187–209.
18. Vallet I, Olson JW, Lory S, Lazdunski A, Filloux A (2001) The chaperone/usher pathways of *Pseudomonas aeruginosa*: identification of fimbrial gene clusters (*cup*) and their involvement in biofilm formation. *Proc Natl Acad Sci USA* 98: 6911–6916.
19. O'Toole GA, Kolter R (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* 30: 295–304.
20. Davies DG, Geesey GG (1995) Regulation of the alginate biosynthesis gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture. *Appl Environ Microbiol* 61: 860–867.
21. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, et al. (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280: 295–298.
22. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* 184: 1140–1154.
23. Whiteley M, Banger MG, Bumgarner RE, Parsek MR, Teitzel GM, et al. (2001) Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 413: 860–864.
24. Schuster M, Lostroh CP, Ogi T, Greenberg EP (2003) Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* 185: 2066–2079.
25. Keller L, Surette MG (2006) Communication in bacteria: an ecological and evolutionary perspective. *Nat Rev Microbiol* 4: 249–258.
26. Pearson JP, Gray KM, Passador L, Tucker KD, Eberhard A, et al. (1994) Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci USA* 91: 197–201.
27. Pearson JP, Passador L, Iglewski BH, Greenberg EP (1995) A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 92: 1490–1494.
28. Pesci EC, Milbank JB, Pearson JP, McKnight S, Kende AS, et al. (1999) Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 96: 11229–11234.
29. Whiteley M, Lee KM, Greenberg EP (1999) Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 96: 13904–13909.
30. Moreau-Marquis S, Stanton BA, O'Toole GA (2008) *Pseudomonas aeruginosa* biofilm formation in the cystic fibrosis airway. *Pulm Pharmacol Ther* 21: 595–599.

31. Lam J, Chan R, Lam K, Costerton JW (1980) Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infect Immun* 28: 546–556.
32. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, et al. (2002) Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest* 109: 317–325.
33. Hassett DJ (1996) Anaerobic production of alginate by *Pseudomonas aeruginosa*: alginate restricts diffusion of oxygen. *J Bacteriol* 178: 7322–7325.
34. Jones KL, Hegab AH, Hillman BC, Simpson KL, Jenkins PA, et al. (2000) Elevation of nitrotyrosine and nitrate concentrations in cystic fibrosis sputum. *Pediatr Pulmonol* 30: 79–85.
35. Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, et al. (2002) *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell* 3: 593–603.
36. Alvarez-Ortega C, Harwood CS (2007) Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. *Mol Microbiol* 65: 153–165.
37. Filiatrault MJ, Wagner VE, Bushnell D, Haidaris CG, Iglewski BH, et al. (2005) Effect of anaerobiosis and nitrate on gene expression in *Pseudomonas aeruginosa*. *Infect Immun* 73: 3764–3772.
38. Meissner A, Wild V, Simm R, Rohde M, Erck C, et al. (2007) *Pseudomonas aeruginosa* *cupA*-encoded fimbriae expression is regulated by a GGDEF and EAL domain-dependent modulation of the intracellular level of cyclic diguanylate. *Environ Microbiol* 9: 2475–2485.
39. Nuccio S-P, Bäumler AJ (2007) Evolution of the chaperone/usher assembly pathway: fimbrial classification goes Greek. *Microbiol Mol Biol Rev* 71: 551–575.
40. Roberts JA, Marklund BI, Ilver D, Haslam D, Kaack MB, et al. (1994) The Gal(α 1-4)Gal-specific tip adhesin of *Escherichia coli* P-fimbriae is needed for pyelonephritis to occur in the normal urinary tract. *Proc Natl Acad Sci USA* 91: 11889–11893.
41. Connell I, Agace W, Klemm P, Schembri M, Märdil S, et al. (1996) Type 1 fimbrial expression enhances *Escherichia coli* virulence for the urinary tract. *Proc Natl Acad Sci USA* 93: 9827–9832.
42. Thanassi DG, Saulino ET, Hultgren SJ (1998) The chaperone/usher pathway: a major terminal branch of the general secretory pathway. *Curr Opin Microbiol* 1: 223–231.
43. Jones CH, Jacob-Dubuisson F, Dodson K, Kuehn M, Slonim L, et al. (1992) Adhesin presentation in bacteria requires molecular chaperones and ushers. *Infect Immun* 60: 4445–4451.
44. Klemm P, Jørgensen BJ, Kreft B, Christiansen G (1995) The export systems of type 1 and F1C fimbriae are interchangeable but work in parental pairs. *J Bacteriol* 177: 621–627.
45. He J, Baldini RL, Déziel E, Saucier M, Zhang Q, et al. (2004) The broad host range

pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc Natl Acad Sci USA* 101: 2530–2535.

46. Giraud C, Bernard CS, Calderon V, Yang L, Filloux A, et al. (2011) The PprA-PprB two-component system activates CupE, the first non-archetypal *Pseudomonas aeruginosa* chaperone-usher pathway system assembling fimbriae. *Environ Microbiol* 13: 666–683.

47. Vallet I, Diggle SP, Stacey RE, Cámara M, Ventre I, et al. (2004) Biofilm formation in *Pseudomonas aeruginosa*: fimbrial *cup* gene clusters are controlled by the transcriptional regulator MvaT. *J Bacteriol* 186: 2880–2890.

48. Vallet-Gely I, Sharp JS, Dove SL (2007) Local and global regulators linking anaerobiosis to *cupA* fimbrial gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* 189: 8667–8676.

49. McManus HR, Dove SL (2011) The CgrA and CgrC proteins form a complex that positively regulates *cupA* fimbrial gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* 193: 6152–6161.

50. Vallet-Gely I, Donovan KE, Fang R, Joung JK, Dove SL (2005) Repression of phase-variable *cup* gene expression by H-NS-like proteins in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 102: 11082–11087.

51. Dillon SC, Dorman CJ (2010) Bacterial nucleoid-associated proteins, nucleoid structure and gene expression. *Nat Rev Microbiol* 8: 185–195.

52. Bouffartigues E, Buckle M, Badaut C, Travers A, Rimsky S (2007) H-NS cooperative binding to high-affinity sites in a regulatory element results in transcriptional silencing. *Nat Struct Mol Biol* 14: 441–448.

53. Hinton JC, Santos DS, Seirafi A, Hulton CS, Pavitt GD, et al. (1992) Expression and mutational analysis of the nucleoid-associated protein H-NS of *Salmonella typhimurium*. *Mol Microbiol* 6: 2327–2337.

54. Dame RT, Noom MC, Wuite GJL (2006) Bacterial chromatin organization by H-NS protein unravelled using dual DNA manipulation. *Nature* 444: 387–390.

55. Dorman CJ (2004) H-NS: a universal regulator for a dynamic genome. *Nat Rev Microbiol* 2: 391–400.

56. Lucchini S, Rowley G, Goldberg MD, Hurd D, Harrison M, et al. (2006) H-NS mediates the silencing of laterally acquired genes in bacteria. *PLoS Pathog* 2: e81.

57. Navarre WW, Porwollik S, Wang Y, McClelland M, Rosen H, et al. (2006) Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*. *Science* 313: 236–238.

58. Castang S, Dove SL (2010) High-order oligomerization is required for the function of the H-NS family member MvaT in *Pseudomonas aeruginosa*. *Mol Microbiol* 78: 916–931.

59. Castang S, McManus HR, Turner KH, Dove SL (2008) H-NS family members function coordinately in an opportunistic pathogen. *Proc Natl Acad Sci USA* 105: 18947–18952.

60. Papenfort K, Vogel J (2010) Regulatory RNA in bacterial pathogens. *Cell Host Microbe* 8: 116–127.
61. Vogel J, Bartels V, Tang TH, Churakov G, Slagter-Jäger JG, et al. (2003) RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria. *Nucleic Acids Res* 31: 6435–6443.
62. Livny J, Waldor MK (2007) Identification of small RNAs in diverse bacterial species. *Curr Opin Microbiol* 10: 96–101.
63. Majdalani N, Vanderpool CK, Gottesman S (2005) Bacterial Small RNA Regulators. *Crit Rev Biochem Mol Biol* 40: 93–113.
64. Waters LS, Storz G (2009) Regulatory RNAs in bacteria. *Cell* 136: 615–628.
65. Vogel J, Luisi BF (2011) Hfq and its constellation of RNA. *Nat Rev Microbiol* 9: 578–589.
66. Morita T, Mochizuki Y, Aiba H (2006) Translational repression is sufficient for gene silencing by bacterial small noncoding RNAs in the absence of mRNA destruction. *Proc Natl Acad Sci USA* 103: 4858–4863.
67. Morita T, Maki K, Aiba H (2005) RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev* 19: 2176–2186.
68. Hammer BK, Bassler BL (2007) Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae*. *Proc Natl Acad Sci USA* 104: 11145–11149.
69. Gerdes K, Wagner EGH (2007) RNA antitoxins. *Curr Opin Microbiol* 10: 117–124.
70. Krinke L, Wulff DL (1990) RNase III-dependent hydrolysis of λ cll-O gene mRNA mediated by λ OOP antisense RNA. *Genes Dev* 4: 2223–2233.
71. Lease RA, Cusick ME, Belfort M (1998) Riboregulation in *Escherichia coli*: DsrA RNA acts by RNA:RNA interactions at multiple loci. *Proc Natl Acad Sci USA* 95: 12456–12461.
72. Boehm A, Vogel J (2012) The *csgD* mRNA as a hub for signal integration via multiple small RNAs. *Mol Microbiol* 84: 1–5.
73. Vogel J, Wagner EGH (2007) Target identification of small noncoding RNAs in bacteria. *Curr Opin Microbiol* 10: 262–270.
74. Liu MY, Romeo T (1997) The global regulator CsrA of *Escherichia coli* is a specific mRNA-binding protein. *J Bacteriol* 179: 4639–4642.
75. Liu MY, Gui G, Wei B, Preston JF, Oakford L, et al. (1997) The RNA molecule CsrB binds to the global regulatory protein CsrA and antagonizes its activity in *Escherichia coli*. *J Biol Chem* 272: 17502–17510.
76. Wassarman KM (2007) 6S RNA: a regulator of transcription. *Mol Microbiol* 65: 1425–1431.

77. Brescia CC, Kaw MK, Sledjeski DD (2004) The DNA binding protein H-NS binds to and alters the stability of RNA *in vitro* and *in vivo*. J Mol Biol 339: 505–514.
78. Wadler CS, Vanderpool CK (2007) A dual function for a bacterial small RNA: SgrS performs base pairing-dependent regulation and encodes a functional polypeptide. Proc Natl Acad Sci USA 104: 20454–20459.
79. Sonnleitner E, Haas D (2011) Small RNAs as regulators of primary and secondary metabolism in *Pseudomonas* species. Appl Microbiol Biotechnol 91: 63–79.
80. Sonnleitner E, Gonzalez N, Sorger-Domenigg T, Heeb S, Richter AS, et al. (2011) The small RNA PhrS stimulates synthesis of the *Pseudomonas aeruginosa* quinolone signal. Mol Microbiol 80: 868–885.
81. Wilderman PJ, Sowa NA, FitzGerald DJ, FitzGerald PC, Gottesman S, et al. (2004) Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. Proc Natl Acad Sci USA 101: 9792–9797.
82. Kay E, Humair B, Dénervaud V, Riedel K, Spahr S, et al. (2006) Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. J Bacteriol 188: 6026–6033.
83. Heurlier K, Williams F, Heeb S, Dormond C, Pessi G, et al. (2004) Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. J Bacteriol 186: 2936–2945.
84. Brencic A, Lory S (2009) Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. Mol Microbiol 72: 612–632.
85. Vogel DW, Hartmann RK, Struck JC, Ulbrich N, Erdmann VA (1987) The sequence of the 6S RNA gene of *Pseudomonas aeruginosa*. Nucleic Acids Res 15: 4583–4591.
86. Master RN, Clark RB, Karlowsky JA, Ramirez J, Bordon JM (2011) Analysis of resistance, cross-resistance and antimicrobial combinations for *Pseudomonas aeruginosa* isolates from 1997 to 2009. Int J Antimicrob Agents 38: 291–295.
87. Mah T-F, Pitts B, Pellock B, Walker GC, Stewart PS, et al. (2003) A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature 426: 306–310.
88. Brown MR, Allison DG, Gilbert P (1988) Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? J Antimicrob Chemother 22: 777–780.
89. Kinniment SL, Wimpenny JW (1992) Measurements of the distribution of adenylate concentrations and adenylate energy charge across *Pseudomonas aeruginosa* biofilms. Appl Environ Microbiol 58: 1629–1635.
90. Hoyle BD, Alcantara J, Costerton JW (1992) *Pseudomonas aeruginosa* biofilm as a diffusion barrier to piperacillin. Antimicrob Agents Chemother 36: 2054–2056.
91. Zhang L, Mah T-F (2008) Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. J Bacteriol 190: 4447–4452.

92. Eswaran J, Koronakis E, Higgins MK, Hughes C, Koronakis V (2004) Three's company: component structures bring a closer view of tripartite drug efflux pumps. *Curr Opin Struct Biol* 14: 741–747.
93. Poole K, Srikumar R (2001) Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. *Curr Top Med Chem* 1: 59–71.
94. Poole K (2001) Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *J Mol Microbiol Biotechnol* 3: 255–264.
95. Piddock LJV (2006) Multidrug-resistance efflux pumps - not just for resistance. *Nat Rev Microbiol* 4: 629–636.
96. Köhler T, van Delden C, Curty LK, Hamzehpour MM, Pechère JC (2001) Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa*. *J Bacteriol* 183: 5213–5222.
97. Lamarche MG, Déziel E (2011) MexEF-OprN efflux pump exports the *Pseudomonas* quinolone signal (PQS) precursor HHQ (4-hydroxy-2-heptylquinoline). *PLoS ONE* 6: e24310.
98. Evans K, Passador L, Srikumar R, Tsang E, Nezezon J, et al. (1998) Influence of the MexAB-OprM multidrug efflux system on quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 180: 5443–5447.
99. Pearson JP, van Delden C, Iglewski BH (1999) Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J Bacteriol* 181: 1203–1210.
100. Li XZ, Zhang L, Poole K (1998) Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent tolerance. *J Bacteriol* 180: 2987–2991.
101. Hirakata Y, Srikumar R, Poole K, Gotoh N, Suematsu T, et al. (2002) Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. *J Exp Med* 196: 109–118.
102. Köhler T, Epp SF, Curty LK, Pechère JC (1999) Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. *J Bacteriol* 181: 6300–6305.
103. Westfall LW, Carty NL, Layland N, Kuan P, Colmer-Hamood JA, et al. (2006) *mvaT* mutation modifies the expression of the *Pseudomonas aeruginosa* multidrug efflux operon *mexEF-oprN*. *FEMS Microbiol Lett* 255: 247–254.
104. Kumar A, Schweizer HP (2011) Evidence of MexT-independent overexpression of MexEF-OprN multidrug efflux pump of *Pseudomonas aeruginosa* in presence of metabolic stress. *PLoS ONE* 6: e26520.
105. Maseda H, Uwate M, Nakae T (2010) Transcriptional regulation of the *mexEF-oprN* multidrug efflux pump operon by MexT and an unidentified repressor in *nfxC*-type mutant of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 311: 36–43.
106. Tian Z-X, Mac AogAin M, O'Connor HF, Fargier E, Mooij MJ, et al. (2009) MexT modulates

virulence determinants in *Pseudomonas aeruginosa* independent of the MexEF-OprN efflux pump. Microb Pathogenesis 47: 237–241.

107. Linares JF, López JA, Camafeita E, Albar JP, Rojo F, et al. (2005) Overexpression of the multidrug efflux pumps MexCD-OprJ and MexEF-OprN is associated with a reduction of type III secretion in *Pseudomonas aeruginosa*. J Bacteriol 187: 1384–1391.

108. Tian Z-X, Fargier E, Mac AogAin M, Adams C, Wang Y-P, et al. (2009) Transcriptome profiling defines a novel regulon modulated by the LysR-type transcriptional regulator MexT in *Pseudomonas aeruginosa*. Nucleic Acids Res 37: 7546–7559.

109. Fukuda H, Hosaka M, Hirai K, Iyobe S (1990) New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. Antimicrob Agents Chemother 34: 1757–1761.

110. Masuda N, Sakagawa E, Ohya S (1995) Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 39: 645–649.

111. Sobel ML, Neshat S, Poole K (2005) Mutations in PA2491 (*mexS*) promote MexT-dependent *mexEF-oprN* expression and multidrug resistance in a clinical strain of *Pseudomonas aeruginosa*. J Bacteriol 187: 1246–1253.

112. Fukuda H, Hosaka M, Iyobe S, Gotoh N, Nishino T, et al. (1995) *nfxC*-type quinolone resistance in a clinical isolate of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 39: 790–792.

113. Wolter DJ, Black JA, Lister PD, Hanson ND (2009) Multiple genotypic changes in hypersusceptible strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients do not always correlate with the phenotype. J Antimicrob Chemother 64: 294–300.

114. Llanes C, Köhler T, Patry I, Dehecq B, van Delden C, et al. (2011) Role of the MexEF-OprN efflux system in low-level resistance of *Pseudomonas aeruginosa* to ciprofloxacin. Antimicrob Agents Chemother 55: 5676–5684.

115. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrenner P, et al. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406: 959–964.

116. Maseda H, Saito K, Nakajima A, Nakae T (2000) Variation of the *mexT* gene, a regulator of the MexEF-OprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa*. FEMS Microbiol Lett 192: 107–112.

117. Schell MA (1993) Molecular biology of the LysR family of transcriptional regulators. Annu Rev Microbiol 47: 597–626.

118. Crawford IP, Gunsalus IC (1966) Inducibility of tryptophan synthetase in *Pseudomonas putida*. Proc Natl Acad Sci USA 56: 717–724.

119. Manch JN, Crawford IP (1982) Genetic evidence for a positive-acting regulatory factor mediating induction in the tryptophan pathway of *Pseudomonas aeruginosa*. J Mol Biol 156: 67–77.

120. Chang M, Crawford IP (1990) The roles of indoleglycerol phosphate and the TrpI protein in the expression of *trpBA* from *Pseudomonas aeruginosa*. *Nucleic Acids Res* 18: 979–988.
121. Chang M, Crawford IP (1991) In vitro determination of the effect of indoleglycerol phosphate on the interaction of purified TrpI protein with its DNA-binding sites. *J Bacteriol* 173: 1590–1597.
122. Gao J, Gussin GN (1991) Mutations in TrpI binding site II that differentially affect activation of the *trpBA* promoter of *Pseudomonas aeruginosa*. *EMBO J* 10: 4137–4144.
123. Piñeiro S, Olekhovich I, Gussin GN (1997) DNA bending by the TrpI protein of *Pseudomonas aeruginosa*. *J Bacteriol* 179: 5407–5413.
124. Bender RA (1991) The role of the NAC protein in the nitrogen regulation of *Klebsiella aerogenes*. *Mol Microbiol* 5: 2575–2580.
125. Fetar H, Gilmour C, Klinoski R, Daigle DM, Dean CR, et al. (2011) *mexEF-oprN* multidrug efflux operon of *Pseudomonas aeruginosa*: regulation by the MexT activator in response to nitrosative stress and chloramphenicol. *Antimicrob Agents Chemother* 55: 508–514.
126. Andrewes FW (1922) Studies in group-agglutination I. The Salmonella group and its antigenic structure. *J Pathol* 25: 505–521.
127. Stocker BA (1949) Measurements of rate of mutation of flagellar antigenic phase in *Salmonella typhi-murium*. *J Hygiene* 47: 398–413.
128. van der Woude MW, Bäumlér AJ (2004) Phase and antigenic variation in bacteria. *Clin Microbiol Rev* 17: 581–611.
129. Simon M, Zieg J, Silverman M, Mandel G, Doolittle R (1980) Phase variation: evolution of a controlling element. *Science* 209: 1370–1374.
130. Zieg J, Silverman M, Hilmen M, Simon M (1977) Recombinational switch for gene expression. *Science* 196: 170–172.
131. Johnson RC, Simon MI (1985) Hin-mediated site-specific recombination requires two 26 bp recombination sites and a 60 bp recombinational enhancer. *Cell* 41: 781–791.
132. Abraham JM, Freitag CS, Clements JR, Eisenstein BI (1985) An invertible element of DNA controls phase variation of type 1 fimbriae of *Escherichia coli*. *Proc Natl Acad Sci USA* 82: 5724–5727.
133. Weiser JN, Love JM, Moxon ER (1989) The molecular mechanism of phase variation of *H. influenzae* lipopolysaccharide. *Cell* 59: 657–665.
134. Weiser JN, Maskell DJ, Butler PD, Lindberg AA, Moxon ER (1990) Characterization of repetitive sequences controlling phase variation of *Haemophilus influenzae* lipopolysaccharide. *J Bacteriol* 172: 3304–3309.
135. Segal E, Hagblom P, Seifert HS, So M (1986) Antigenic variation of gonococcal pilus involves assembly of separated silent gene segments. *Proc Natl Acad Sci USA* 83: 2177–2181.

136. Bartlett DH, Wright ME, Silverman M (1988) Variable expression of extracellular polysaccharide in the marine bacterium *Pseudomonas atlantica* is controlled by genome rearrangement. *Proc Natl Acad Sci USA* 85: 3923–3927.
137. Blyn LB, Braaten BA, White-Ziegler CA, Rolfson DH, Low DA (1989) Phase-variation of pyelonephritis-associated pili in *Escherichia coli*: evidence for transcriptional regulation. *EMBO J* 8: 613–620.
138. Nou X, Skinner B, Braaten B, Blyn L, Hirsch D, et al. (1993) Regulation of pyelonephritis-associated pili phase-variation in *Escherichia coli*: binding of the PapI and the Lrp regulatory proteins is controlled by DNA methylation. *Mol Microbiol* 7: 545–553.
139. Braaten BA, Nou X, Kaltenbach LS, Low DA (1994) Methylation patterns in *pap* regulatory DNA control pyelonephritis-associated pili phase variation in *E. coli*. *Cell* 76: 577–588.
140. Hernday AD, Braaten BA, Low DA (2003) The mechanism by which DNA adenine methylase and PapI activate the Pap epigenetic switch. *Mol Cell* 12: 947–957.
141. Weyand NJ, Braaten BA, van der Woude M, Tucker J, Low DA (2001) The essential role of the promoter-proximal subunit of CAP in *pap* phase variation: Lrp- and helical phase-dependent activation of *papBA* transcription by CAP from -215. *Mol Microbiol* 39: 1504–1522.
142. Smits WK, Kuipers OP, Veening J-W (2006) Phenotypic variation in bacteria: the role of feedback regulation. *Nat Rev Microbiol* 4: 259–271.
143. Dubnau D, Losick R (2006) Bistability in bacteria. *Mol Microbiol* 61: 564–572.
144. Maamar H, Dubnau D (2005) Bistability in the *Bacillus subtilis* K-state (competence) system requires a positive feedback loop. *Mol Microbiol* 56: 615–624.
145. Ferrell JE (2008) Feedback regulation of opposing enzymes generates robust, all-or-none bistable responses. *Curr Biol* 18: R244–R245.
146. Laurent M (1996) Prion diseases and the “protein only” hypothesis: a theoretical dynamic study. *Biochem J* 318: 35–39.
147. Kacser H, Small JR (1996) How many phenotypes from one genotype? The case of Prion diseases. *J Theor Biol* 182: 209–218.
148. Dercole F, Ferrière R, Rinaldi S (2002) Ecological bistability and evolutionary reversals under asymmetrical competition. *Evolution* 56: 1081–1090.
149. Chung JD, Stephanopoulos G, Ireton K, Grossman AD (1994) Gene expression in single cells of *Bacillus subtilis*: evidence that a threshold mechanism controls the initiation of sporulation. *J Bacteriol* 176: 1977–1984.
150. Molle V, Fujita M, Jensen ST, Eichenberger P, González-Pastor JE, et al. (2003) The Spo0A regulon of *Bacillus subtilis*. *Mol Microbiol* 50: 1683–1701.
151. Predich M, Nair G, Smith I (1992) *Bacillus subtilis* early sporulation genes *kinA*, *spo0F*, and *spo0A* are transcribed by the RNA polymerase containing σ^H . *J Bacteriol* 174: 2771–2778.

152. Hahn J, Kong L, Dubnau D (1994) The regulation of competence transcription factor synthesis constitutes a critical control point in the regulation of competence in *Bacillus subtilis*. *J Bacteriol* 176: 5753–5761.
153. Nester EW, Stocker B (1963) Biosynthetic latency in early stages of deoxyribonucleic acid transformation in *Bacillus subtilis*. *J Bacteriol* 86: 785–796.
154. Hadden C, Nester EW (1968) Purification of competent cells in the *Bacillus subtilis* transformation system. *J Bacteriol* 95: 876–885.
155. Maamar H, Raj A, Dubnau D (2007) Noise in gene expression determines cell fate in *Bacillus subtilis*. *Science* 317: 526–529.
156. Süel GM, Kulkarni RP, Dworkin J, Garcia-Ojalvo J, Elowitz MB (2007) Tunability and noise dependence in differentiation dynamics. *Science* 315: 1716–1719.
157. Novick A, Weiner M (1957) Enzyme induction as an all-or-none phenomenon. *Proc Natl Acad Sci USA* 43: 553–566.
158. Ozbudak EM, Thattai M, Lim HN, Shraiman BI, van Oudenaarden A (2004) Multistability in the lactose utilization network of *Escherichia coli*. *Nature* 427: 737–740.
159. Thomas R (1998) Laws for the dynamics of regulatory networks. *Int J Dev Biol* 42: 479–485.
160. Gardner TS, Cantor CR, Collins JJ (2000) Construction of a genetic toggle switch in *Escherichia coli*. *Nature* 403: 339–342.
161. Ferrell JE (2002) Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. *Curr Opin Cell Biol* 14: 140–148.
162. Hamoen LW, Van Werkhoven AF, Bijlsma JJ, Dubnau D, Venema G (1998) The competence transcription factor of *Bacillus subtilis* recognizes short A/T-rich sequences arranged in a unique, flexible pattern along the DNA helix. *Genes Dev* 12: 1539–1550.
163. Yano K, Fukasawa T (1997) Galactose-dependent reversible interaction of Gal3p with Gal80p in the induction pathway of Gal4p-activated genes of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 94: 1721–1726.
164. Peng G, Hopper JE (2002) Gene activation by interaction of an inhibitor with a cytoplasmic signaling protein. *Proc Natl Acad Sci USA* 99: 8548–8553.
165. Acar M, Becskei A, van Oudenaarden A (2005) Enhancement of cellular memory by reducing stochastic transitions. *Nature* 435: 228–232.
166. Dybvig K, Yu H (1994) Regulation of a restriction and modification system via DNA inversion in *Mycoplasma pulmonis*. *Mol Microbiol* 12: 547–560.

Chapter 2

**Epigenetic control of virulence gene expression in *Pseudomonas aeruginosa* by a
LysR-type transcription regulator**

Attributions

This chapter is a reprint of a paper published in the December 2009 issue of PLoS Genetics (Turner KH, Vallet-Gely I, Dove SL (2009) Epigenetic control of virulence gene expression in *Pseudomonas aeruginosa* by a LysR-type transcription regulator. PLoS Genet 5(12): e1000779). The “unrelated microarray experiments” referred to on page 48, as well as the construction of PAO1 *PA1202 lacZ*, PAO1 Δ *bexR* and PAO1 Δ *bexR PA1202 lacZ*, were performed by Isabelle Vallet-Gely. I performed all other work described in this chapter. Simon L. Dove and I wrote the paper. This work is reprinted with permission of the authors under the Creative Commons Attribution 3.0 Unported License (CC BY 3.0, details available at <http://creativecommons.org/licenses/by/3.0/>).

**Epigenetic control of virulence 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*.

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*.

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 pyelonephritis-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 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 2.1A). 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 2.1B). 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 $\Delta bexR$ mutant exhibits constitutively low-level expression of PA1202 (Figure 2.1B). Ectopic expression of *bexR* in the $\Delta bexR$ mutant resulted in increased PA1202 expression (Figure 2.1C), suggesting that BexR positively regulates expression of PA1202. However, bistable expression of PA1202 is lost when *bexR* is expressed ectopically; PAO1 $\Delta bexR$ *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 2.1).

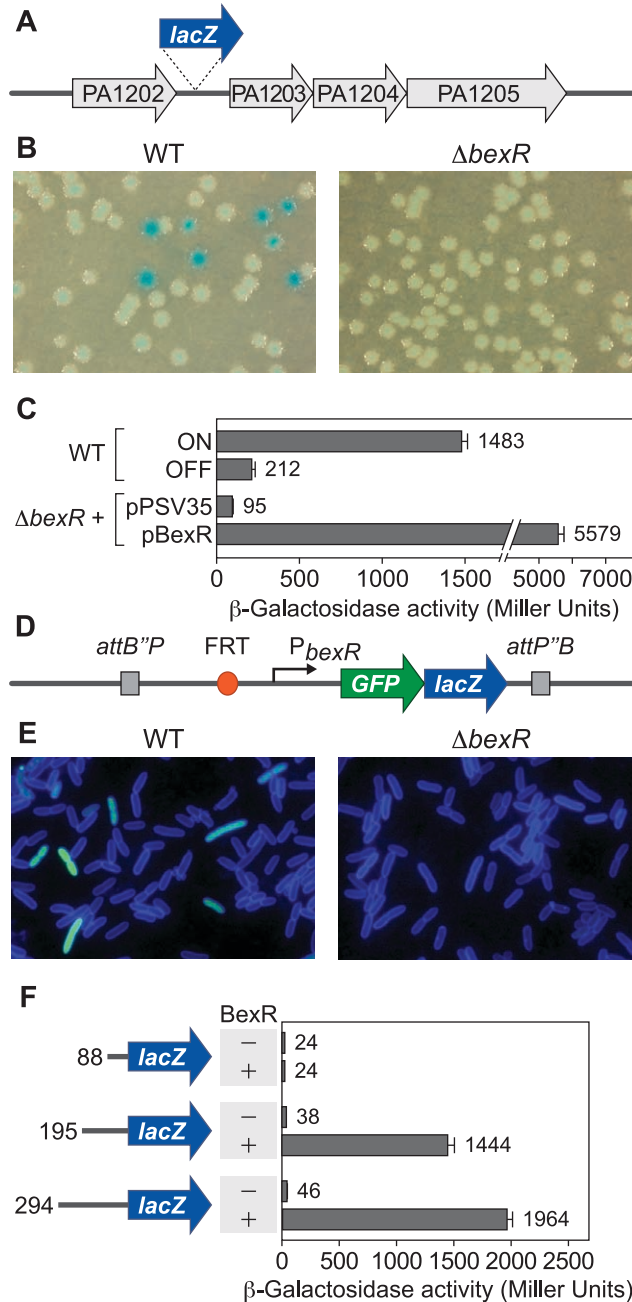


Figure 2.1. BexR is a positively autoregulated, bistably expressed regulator of PA1202. (A) Schematic of *PA1202 lacZ* reporter strains. (B) Phenotypes of wild-type and $\Delta bexR$ *PA1202 lacZ* reporter strains when plated on LB agar containing X-Gal. (C) Quantification of *PA1202 lacZ* expression in cultures of the wild-type reporter strain in both ON and OFF states, and the $\Delta bexR$ reporter strain with empty vector and *bexR* expression vector. (D) Schematic of *attB::P_{bexR}-GFP-lacZ* reporter strain. (E) Micrographs of wild-type and $\Delta bexR$ *attB::P_{bexR}-GFP-lacZ* fluorescent reporter cells stained with the membrane dye TMA-DPH. (F) Quantification of *P_{bexR}-lacZ* expression in PAO1 $\Delta bexR$ 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.

Table 2.1. Switching frequencies.

Strain genotype	Transition	Switching frequency (per cell generation, $\times 10^{-4}$)
<i>PA1202 lacZ</i>	OFF $\rightarrow$ ON	47.8 ± 3.0
	ON $\rightarrow$ OFF	0.8 ± 0.2
$\Delta bexR$ <i>PA1202 lacZ</i>	OFF $\rightarrow$ ON	$< 0.4 \pm 0.1$

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 Φ CTX attachment site in the PAO1 chromosome (Figure 2.1D) [16,17]. Individual cells of wild-type PAO1 carrying this P_{bexR} -*GFP-lacZ* reporter either express the GFP reporter, or do not, leading to heterogeneity in the cell population (Figure 2.1E). 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 2.2). 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 2.1 and Table 2.2). 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 88 bp fragment is not (Figure 2.1F). 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.

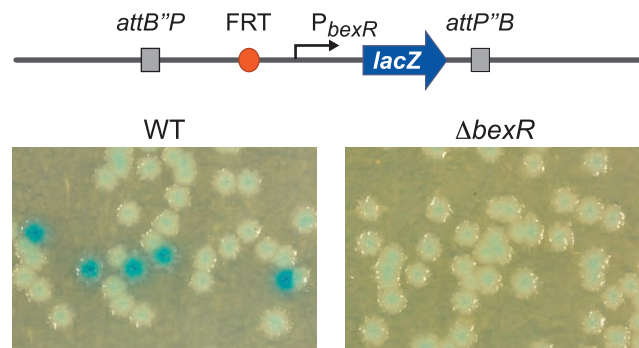


Figure 2.2. *bexR* is bistably expressed on solid media in a *bexR*-dependent manner. The *bexR* promoter was fused to *lacZ* and stably integrated in single copy into the PAO1 chromosome. The phenotypes of wild-type and $\Delta bexR$ strains of this reporter plated on LB agar containing X-Gal are shown.

Table 2.2. Switching frequencies.

Strain genotype	Transition	Switching frequency (per cell generation, $\times 10^{-4}$)
<i>attB::P_{bexR}-lacZ</i>	OFF $\rightarrow$ ON	47.4 ± 6.6
	ON $\rightarrow$ OFF	0.6 ± 0.4
$\Delta bexR$ <i>attB::P_{bexR}-lacZ</i>	OFF $\rightarrow$ ON	$< 0.4 \pm 0.1$

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 $\Delta bexR$ cells containing either a *bexR* expression vector or an empty vector in both mid-logarithmic and stationary phases of growth using DNA microarrays. 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 2.3). 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-oprN* by 6- to 10-fold and upregulation of *mexGHI-opmD* by 7- to 13-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-metalloprotease 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.

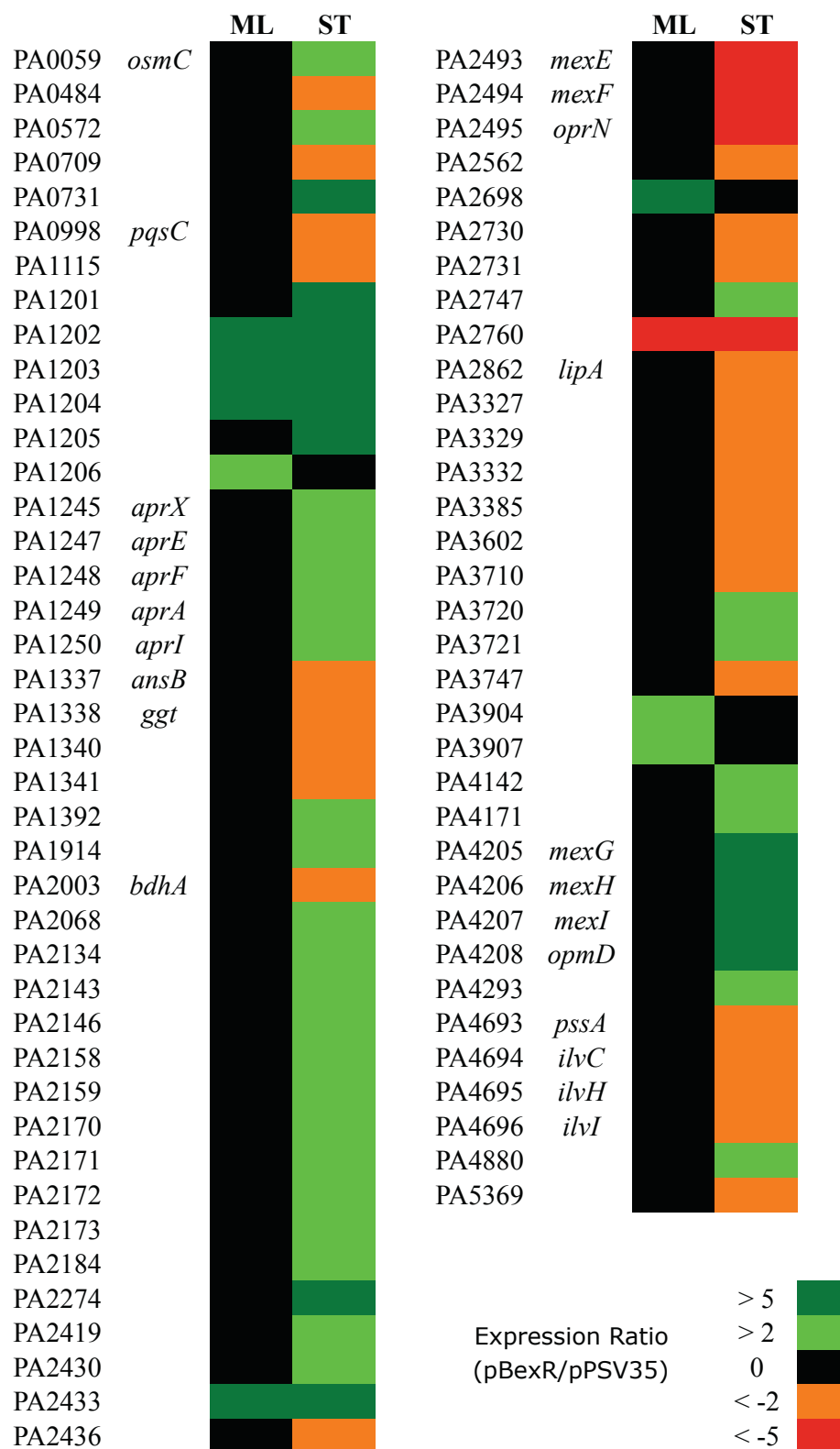


Figure 2.3. BexR regulates expression of a diverse set of genes. Cells of PAO1 $\Delta bexR$ with either empty vector or *bexR*-overexpression vector were grown to mid-logarithmic (ML) and stationary (ST) phase, and mRNA content was profiled by microarray.

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::P_{bexR}-lacZ* OFF, *attB::P_{bexR}-lacZ* ON and $\Delta bexR$ *attB::P_{bexR}-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 2.4A). 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 2.4B), as did PA0572 and *aprA* (though for *aprA* the difference in transcript abundance between ON and OFF cultures was only 2-fold) (Figure 2.4C). 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 *lasA* transcript was not significantly different between $\Delta bexR$ 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 2.4C). Microarray analysis of cells ectopically expressing *bexR* suggests that two operons encoding multidrug efflux pumps are reciprocally regulated by BexR (Figure 2.3). 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*).

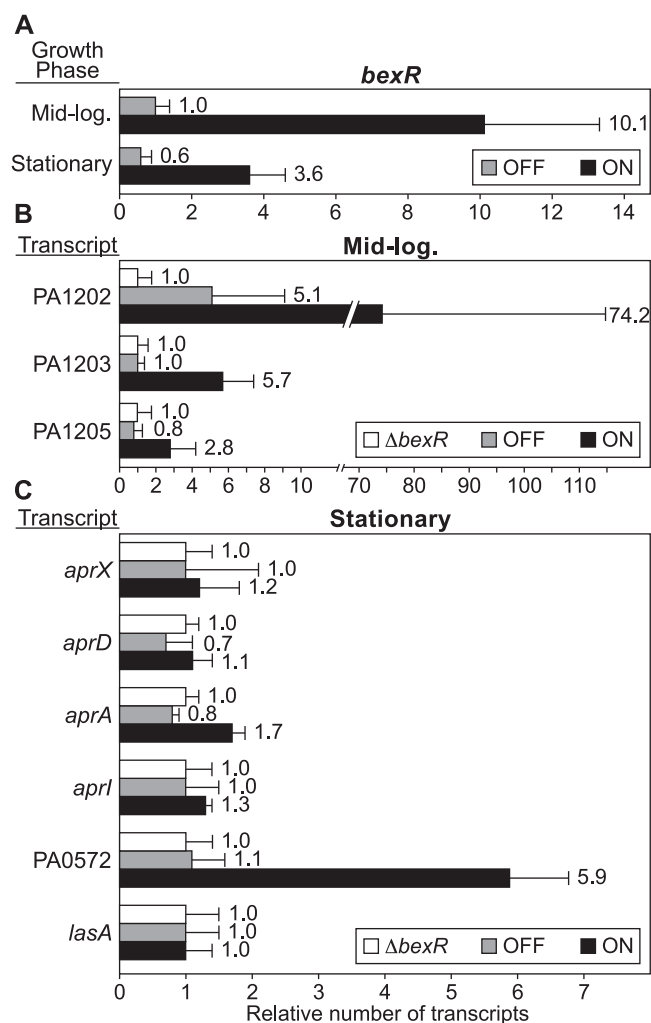


Figure 2.4. BexR-regulated transcripts vary between the OFF and ON states. (A) Relative quantity of *bexR* mRNA in both mid-logarithmic and stationary phase. (B) Relative quantities of PA1202 operon mRNAs in mid-logarithmic phase. (C) Relative quantities of quorum sensing-regulated mRNAs in stationary phase. Error bars represent relative expression values calculated from ± 1 SD from the mean $\Delta\Delta C_t$.

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 2.5). 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 2.6). These results suggest that BexR regulates many of its target genes directly.

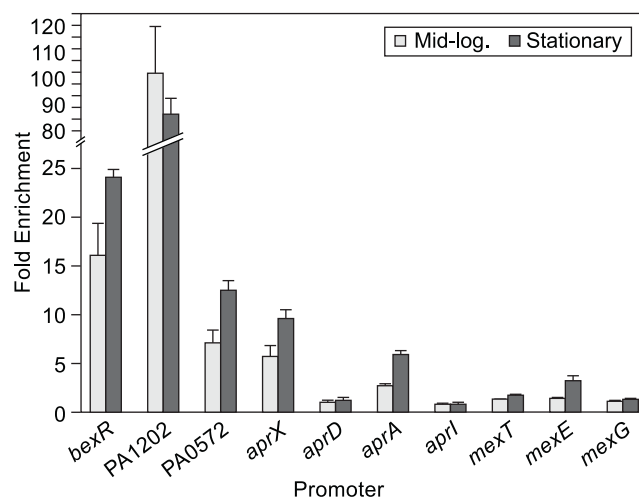


Figure 2.5. BexR occupies the promoters of target genes. ChIP of an epitope-tagged version of BexR reveals preferential binding of the promoters of target genes over a non-binding control region *in vivo* at both mid-logarithmic and stationary phase. Error bars represent 1 SD from the mean fold enrichment.

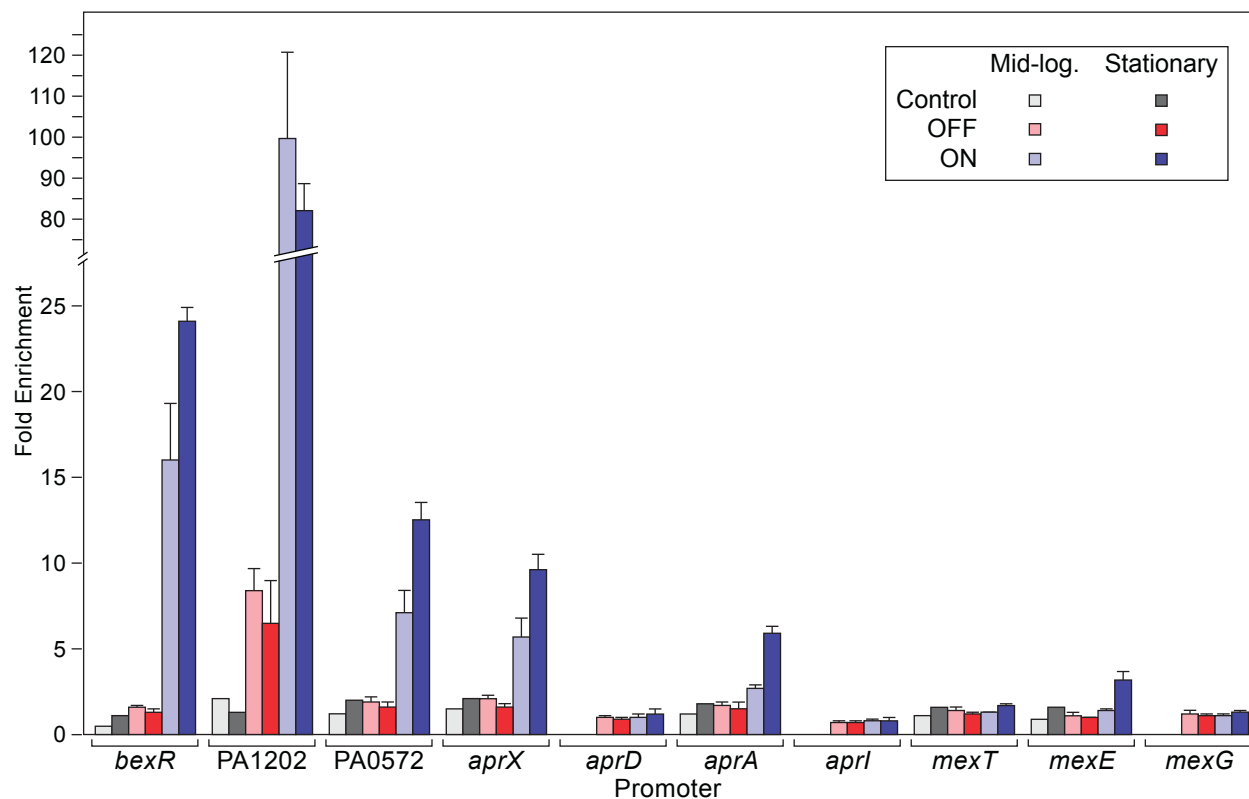


Figure 2.6. BexR occupies the promoters of target genes. Cells of PAO1 *PA1202 lacZ* BexR-V in both ON and OFF states were grown to mid-logarithmic and stationary phase, and DNA associated with BexR-V was analyzed by ChIP. A mock IP control was performed with cells of ON-state PAO1 *PA1202 lacZ* which do not synthesize VSV-G-tagged BexR. Error bars represent one standard deviation from the mean fold enrichment.

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 2.1E). 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 2.7). To analyze the effect of positive feedback on *bexR* bistability, we constructed a pair of strains containing the P_{bexR} -*GFP-lacZ* reporter construct and an isopropyl- β -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 2.8A), whereas the other contained wild-type *bexR* at its native locus (the plus feedback strain, Figure 2.8B). 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 2.8B [and Figure 2.9B] as a population of cells with an essentially bimodal distribution of fluorescence intensities). Furthermore, a gradual increase in ectopically expressed *bexR* resulted in an increase in the proportion of ON relative to OFF cells only in the plus feedback strain (Figure 2.8B); in the strain lacking the positive feedback loop, cells responded relatively uniformly to increasing synthesis of ectopically expressed *bexR* (manifest in Figure 2.8A 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 2.8). In particular, cells of the plus feedback strain at 0.5 mM IPTG had a mean fluorescence intensity of 1814 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 2.8). These results suggest that positive feedback of *bexR* is necessary for bistability.

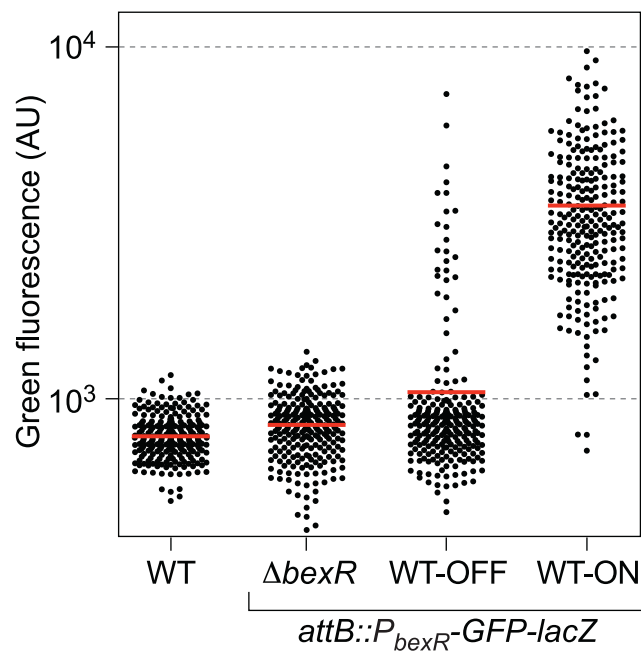


Figure 2.7. Automated fluorescence intensity measurement of single cells reveals bistable expression from the *bexR* promoter. Cultures of wild-type PAO1 (WT), the wild-type $attB::P_{bexR}-GFP-lacZ$ fluorescent reporter strain (originating from ON and OFF colonies) and the $\Delta bexR attB::P_{bexR}-GFP-lacZ$ fluorescent reporter strain (see Figure 2.1D), were grown and examined and measured for fluorescence upon reaching mid-logarithmic phase by fluorescence microscopy. 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.

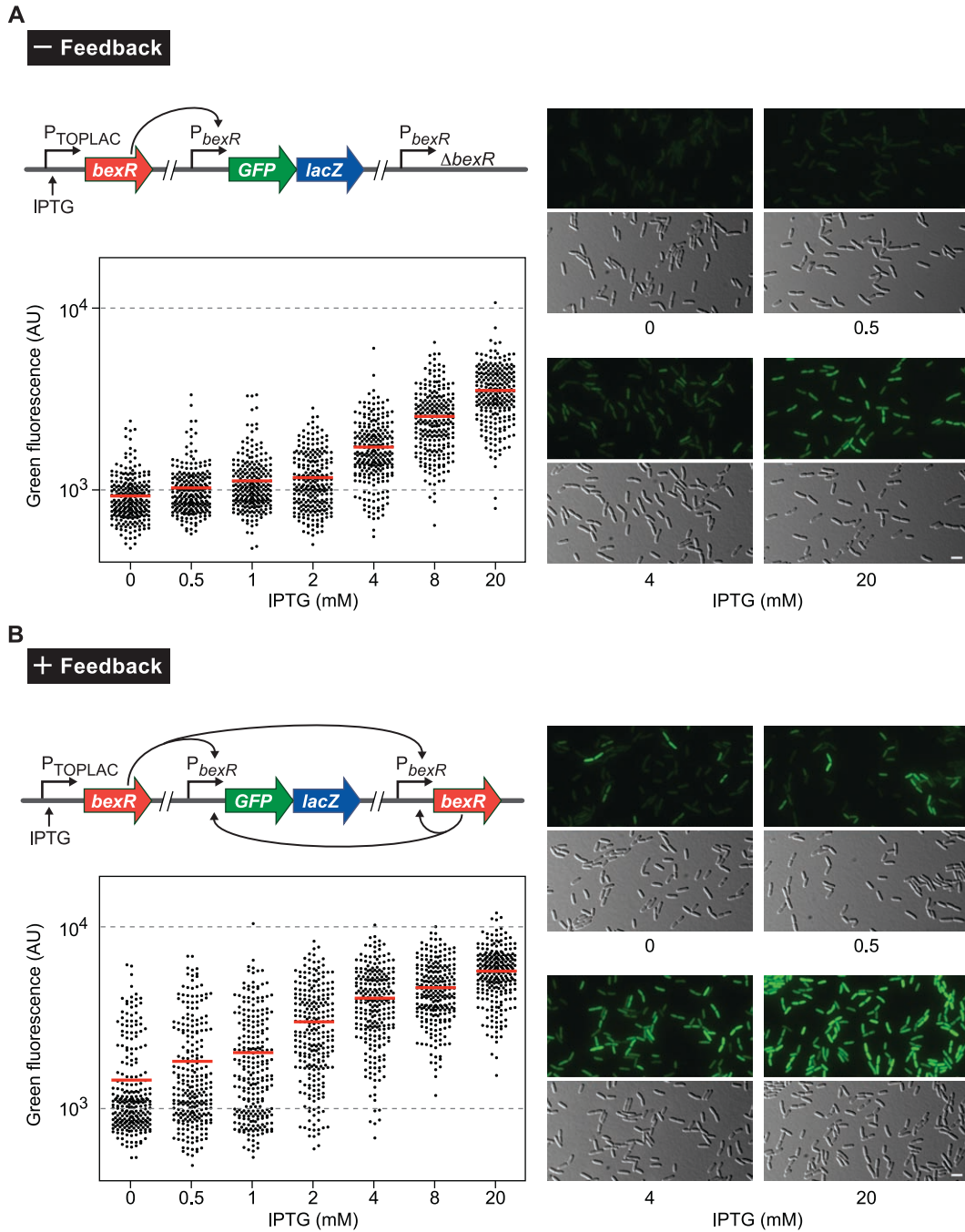


Figure 2.8. Positive feedback of *bexR* is required for bistability of the regulon. Cells of both the minus feedback strain (PAO1 $\Delta bexR$ *attB::P_{bexR}-GFP-lacZ attTn7::TOPLAC-bexR*, see diagram in (A), and the plus feedback strain (PAO1 *attB::P_{bexR}-GFP-lacZ attTn7::TOPLAC-bexR*, see diagram 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 μ m.

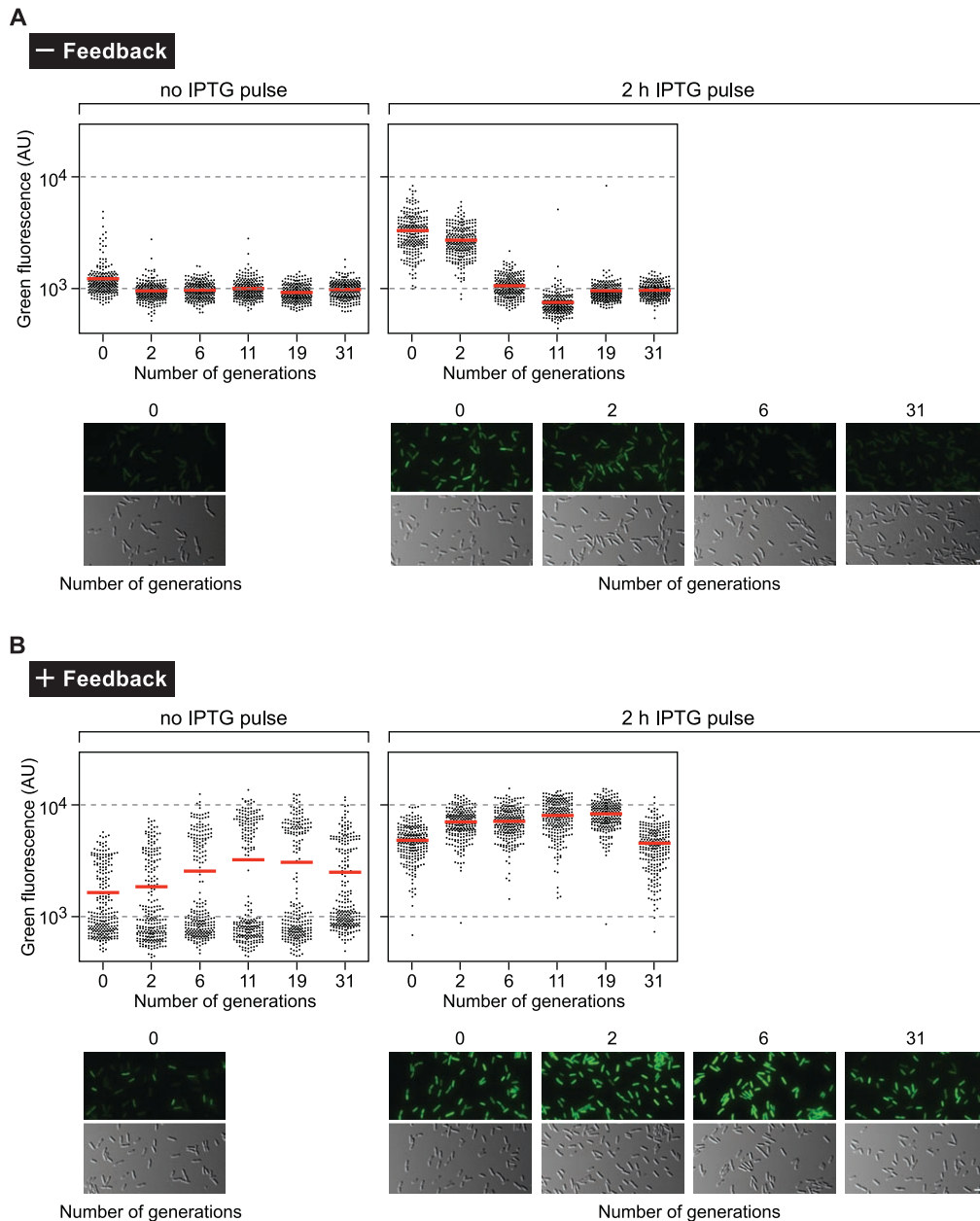


Figure 2.9. The feedback-mediated BexR switch exhibits hysteresis. Cells of both the minus feedback strain (PAO1 $\Delta bexR$ $attB::P_{bexR}$ -GFP-lacZ $attTn7::TOPLAC-bexR$, see diagram, Figure 2.8A), and the plus feedback strain (PAO1 $attB::P_{bexR}$ -GFP-lacZ $attTn7::TOPLAC-bexR$, see diagram, Figure 2.8B) 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 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 μ m.

Feedback-mediated bistable systems often exhibit a capacity for history-dependent behavior, or hysteresis [reviewed in 23]. Systems exhibiting hysteretic 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 2.8) 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 the level seen in wild-type ON cells (Figure 2.9A and Figure 2.7). However, this degree of expression from the P_{bexR} -*GFP-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 2.9B). 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 2.10). Only after 31 generations following removal of IPTG, do a portion of the cells begin to transition to the OFF state (Figure 2.9B). 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 2.11).

+ Feedback

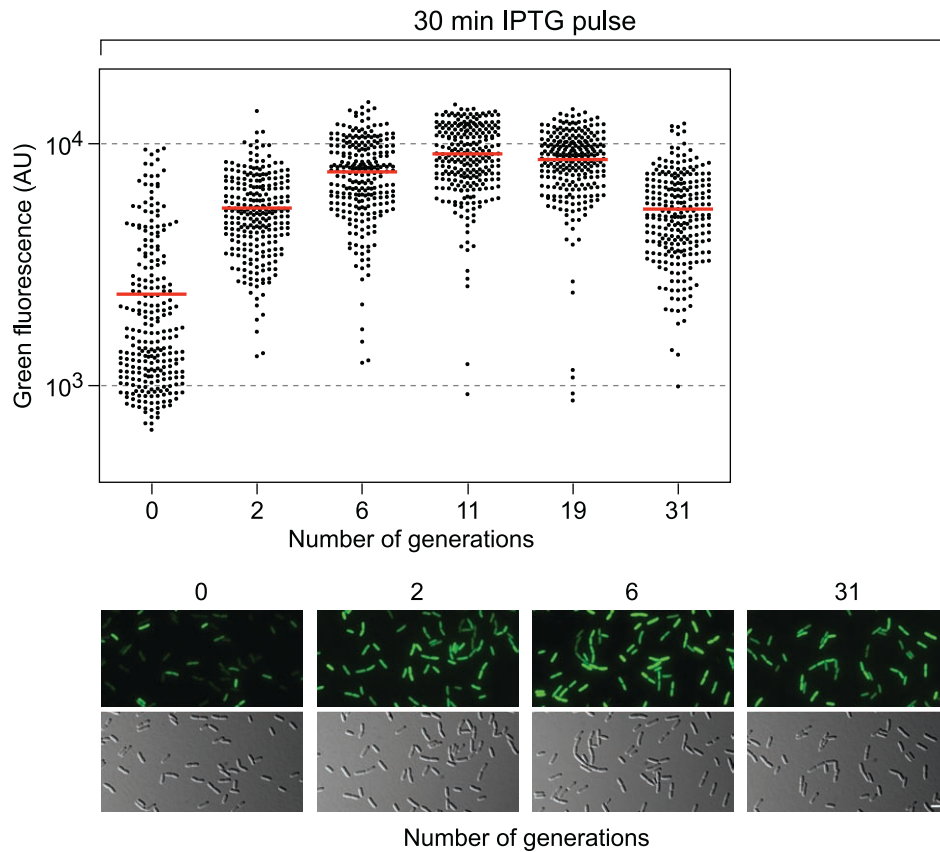


Figure 2.10. A 30 minute pulse of IPTG is sufficient to induce hysteresis. Cells of the plus feedback strain (PAO1 *attB::P_{bexR}-GFP-lacZ attTn7::TOPLAC-bexR*, see diagram, Figure 2.8B) were grown to early logarithmic phase and were exposed to a pulse of IPTG (20 mM) for 30 minutes (30 min IPTG pulse) to induce ectopic expression of *bexR*, or not exposed to IPTG (see Figure 2.9B, 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 μ m.

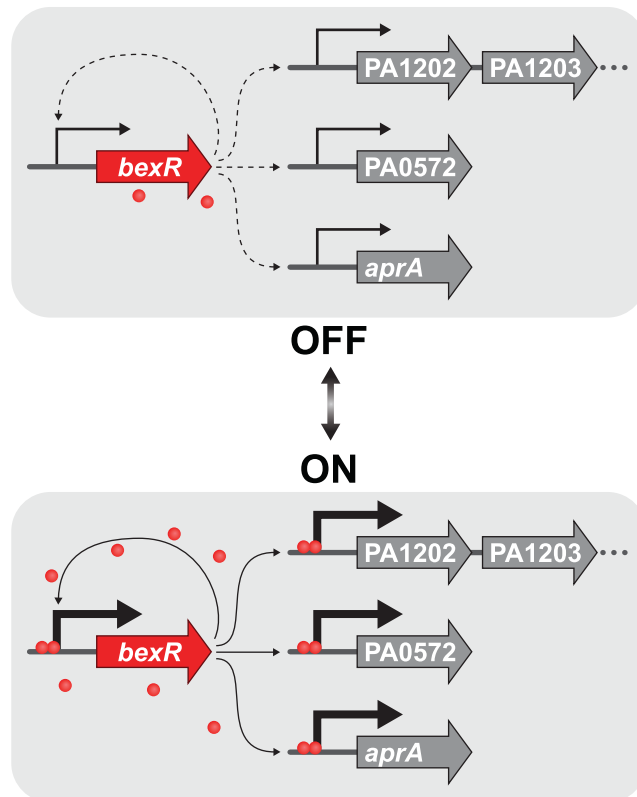


Figure 2.11. A model for the switch to the ON state. Wild-type *P. aeruginosa* is hypersensitized to BexR levels by virtue of positive feedback at the *bexR* locus. Cell-to-cell variability in basal *bexR* expression results in a stochastically determined subset of cells activating the positive autoregulatory loop by BexR binding to its own promoter and activating transcription. At this point, the ON state is maintained by direct positive feedback. Transcription of downstream genes such as *aprA*, PA0572, and the PA1202 operon is upregulated in the ON state by BexR binding to their promoters and activating transcription.

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 necessary for the observed bistability. Taken together, these results outline a novel feedback-mediated bistable switch in an opportunistic pathogen.

Phenotypic outcomes of BexR bistability

Bistability is a mechanism by which bacteria can introduce 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 *cupA* fimbrial 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 *aprA* ~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 2.12). 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 suggests that their expression or repression may be detrimental to growth under certain conditions.

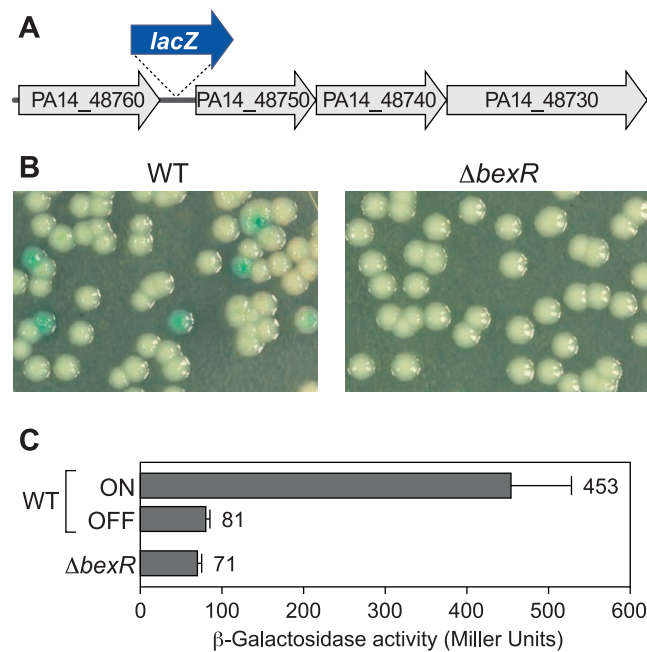


Figure 2.12. The PA1202-orthologous PA14_48760 operon also exhibits *bexR*-dependent bistability in *P. aeruginosa* strain PA14. (A) Schematic of PA14_48760 *lacZ* reporter strains. (B) Phenotypes of wild-type and $\Delta bexR$ PA14_48760 *lacZ* reporter strains when plated on M63 minimal agar containing X-Gal. (C) Quantification of PA14_48760 *lacZ* expression in cultures of the wild-type and $\Delta bexR$ reporter strains. Error bars represent one standard deviation from the mean β -galactosidase activity.

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 *B. subtilis* 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 2.1 and Table 2.2). These low frequencies are on par 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 *B. subtilis*, 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 2.8). 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 *B. subtilis* 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 *B. subtilis* 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 2.1). 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* DH5 α F'IQ (Invitrogen) was used as the recipient strain for all plasmid constructions, whereas *E. coli* strain SM10 (λ pir) was used to mate 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*, *NcoI* and *SphI* sites two bases after the PA1202 stop codon. This PCR product was cloned as a *SacI*/*PacI* fragment into pEXG2 [44]. The *lacZ* gene was subsequently cloned into this construct as a *KpnI*/*SphI* fragment derived from pP18-*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'-GCGGCCGCC-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 Δ *bexR*, PAO1 *PA1202 lacZ* Δ *bexR* and PA14 *PA1202 lacZ* Δ *bexR* by allelic exchange [45]. Deletions were confirmed by the PCR.

The *attB::P_{bexR}-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 *EcoRI/XhoI* fragments into mini-CTX-*lacZ* [17], which contains a consensus Shine-Dalgarno sequence upstream of *lacZ*, yielding plasmids mini-CTX-*PF2432-lacZ.1*, mini-CTX-*PF2432-lacZ.2* and mini-CTX-*PF2432-lacZ.3*, respectively. These plasmids were then used to create reporter strains PAO1 *attB::P_{bexR.1}-lacZ*, PAO1 Δ *bexR attB::P_{bexR.1}-lacZ*, PAO1 *attB::P_{bexR.2}-lacZ*, PAO1 Δ *bexR attB::P_{bexR.2}-lacZ*, PAO1 *attB::P_{bexR.3}-lacZ* and PAO1 Δ *bexR attB::P_{bexR.3}-lacZ*. An *EcoRI/XhoI* fragment of mini-CTX-*PF2432-lacZ.3* was subcloned into mini-CTX-GFP-*lacZ* [16], yielding plasmid mini-CTX-*PF2432-GFP-lacZ.3*. This plasmid was then used to create the fluorescent reporter strains PAO1 *attB::P_{bexR.3}-GFP-lacZ* and PAO1 Δ *bexR attB::P_{bexR.3}-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/NotI* fragment into plasmid pP30 Δ -YTAP [4], generating plasmid pP30 Δ -BexR-TAP. This *HindIII/NotI* fragment was then subcloned into pP30 Δ FRT-MvaT-V [46], generating plasmid pP30 Δ FRT-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 *bexR* gene into pPSV35.

The *attTn7::TOPLAC-bexR* strains contain a construct which directs the synthesis of the BexR 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'-
CACTACGTGCTCGAGGGT**AAATGTGAGCACTCACA**TTTATTCTGAAATGAGCTCTTTACA
CGTCCTGCTGCCGGCTCGTATGTTGTGTGGA**AATTGTGAGCGGATAACA**ATTAAGCTTAGT
CGACAGCTAGCCGGATCC-3', where the -35 and -10 sequences are underlined and the *lac* operator sequences are shown in bold. The *bexR* gene is inserted downstream of the *TOPLAC* promoter with a consensus Shine-Dalgarno sequence. This construct was inserted between the ends of the Tn7 transposon on pUC18-mini-Tn7T-LAC [47], generating plasmid pUC18-mini-Tn7T-TOPLAC-*bexR*. This plasmid was used to make strains PAO1 *attB::P_{bexR}.3-GFP-lacZ attTn7::TOPLAC-bexR* and PAO1 $\Delta bexR$ *attB::P_{bexR}.3-GFP-lacZ attTn7::TOPLAC-bexR* 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 μ g/ml) and IPTG (0.1 mM). Cells were permeabilized with sodium dodecyl sulfate and CHCl_3 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 $\Delta bexR$ *attB::P_{bexR}.3-lacZ* and PAO1 *attB::P_{bexR}.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 ≈ 0.55 (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 $\Delta bexR$ 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 Intensilight illuminator, a Coolsnap HQ2 charge-coupled device camera from Photometrics and a Nikon CFI Plan Apo VC $\times 100$ 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 0.5 μm , longer than 4.0 μm or shorter than 0.5 μm , and using those 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 2.8), 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 2.9) 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 5 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. 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.

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References

1. Govan JR, Deretic V (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol Rev 60: 539–574.
2. Wolfgang MC, Kulasekara BT, Liang X, Boyd D, Wu K, et al. (2003) Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 100: 8484–8489.
3. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrenner P, et al. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406: 959–964.
4. Vallet-Gely I, Donovan KE, Fang R, Joung JK, Dove SL (2005) Repression of phase-variable *cup* gene expression by H-NS-like proteins in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 102: 11082–11087.
5. Vallet-Gely I, Sharp JS, Dove SL (2007) Local and global regulators linking anaerobiosis to *cupA* fimbrial gene expression in *Pseudomonas aeruginosa*. J Bacteriol 189: 8667–8676.
6. Déziel E, Comeau Y, Villemur R (2001) Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. J Bacteriol 183: 1195–1204.
7. Drenkard E, Ausubel FM (2002) *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature 416: 740–743.
8. Simon M, Zieg J, Silverman M, Mandel G, Doolittle R (1980) Phase variation: evolution of a controlling element. Science 209: 1370–1374.
9. Casadesus J, Low D (2006) Epigenetic gene regulation in the bacterial world. Microbiol Mol Biol Rev 70: 830–856.
10. Low DA, Casadesus J (2008) Clocks and switches: bacterial gene regulation by DNA adenine methylation. Curr Opin Microbiol 11: 106–112.
11. Eisen H, Brachet P, Pereira da Silva L, Jacob F (1970) Regulation of repressor expression in λ . Proc Natl Acad Sci USA 66: 855–862.
12. Ptashne M (2004) A genetic switch, third edition: phage lambda revisited. Cold Spring Harbor: Cold Spring Harbor Laboratory Press. 164 p.
13. Dubnau D, Losick R (2006) Bistability in bacteria. Mol Microbiol 61: 564–572.
14. Smits WK, Eschevins CC, Susanna KA, Bron S, Kuipers OP, et al. (2005) Stripping *Bacillus*: ComK auto-stimulation is responsible for the bistable response in competence development. Mol Microbiol 56: 604–614.
15. Maamar H, Dubnau D (2005) Bistability in the *Bacillus subtilis* K-state (competence) system requires a positive feedback loop. Mol Microbiol 56: 615–624.

16. Rietsch A, Wolfgang MC, Mekalanos JJ (2004) Effect of metabolic imbalance on expression of type III secretion genes in *Pseudomonas aeruginosa*. *Infect Immun* 72: 1383–1390.
17. Hoang TT, Kutchma AJ, Becher A, Schweizer HP (2000) Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. *Plasmid* 43: 59–72.
18. Adams M, Jia Z (2005) Structural and biochemical analysis reveal pirins to possess quercetinase activity. *J Biol Chem* 280: 28675–28682.
19. Schuster M, Lostroh CP, Ogi T, Greenberg EP (2003) Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* 185: 2066–2079.
20. Nouwens AS, Beatson SA, Whitchurch CB, Walsh BJ, Schweizer HP, et al. (2003) Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. *Microbiology* 149: 1311–1322.
21. Seo J, Brencic A, Darwin AJ (2009) Analysis of secretin-induced stress in *Pseudomonas aeruginosa* suggests prevention rather than response and identifies a novel protein involved in secretin function. *J Bacteriol* 191: 898–908.
22. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B (2006) Prevalence of local immune response against oral infection in a *Drosophila/Pseudomonas* infection model. *PLoS Pathog* 2: e56.
23. Ninfa AJ, Mayo AE (2004) Hysteresis vs. graded responses: the connections make all the difference. *Sci STKE* 232: pe20.
24. Novick A, Weiner M (1957) Enzyme induction as an all-or-none phenomenon. *Proc Natl Acad Sci USA* 43: 553–566.
25. Ozbudak EM, Thattai M, Lim HN, Shraiman BI, van Oudenaarden A (2004) Multistability in the lactose utilization network of *Escherichia coli*. *Nature* 427: 737–740.
26. Lewis K (2007) Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* 5: 48–56.
27. Breidenstein E, Khaira B, Wiegand I, Overhage J, Hancock R (2008) Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. *Antimicrob Agents Chemother* 52: 4486–4491.
28. Gibson RL, Burns JL, Ramsey BW (2003) Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am J Respir Crit Care Med* 168: 918–951.
29. Rietsch A, Mekalanos JJ (2006) Metabolic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*. *Mol Microbiol* 59: 807–820.
30. Erickson DL, Lines JL, Pesci EC, Venturi V, Storey DG (2004) *Pseudomonas aeruginosa* *relA* contributes to virulence in *Drosophila melanogaster*. *Infect Immun* 72: 5638–5645.
31. Vodovar N, Acosta C, Lemaitre B, Boccard F (2004) *Drosophila*: a polyvalent model to decipher host-pathogen interactions. *Trends Microbiol* 12: 235–242.

32. Matsumoto K (2004) Role of bacterial proteases in pseudomonal and serratial keratitis. *Biol Chem* 385: 1007–1016.
33. Howe TR, Iglewski BH (1984) Isolation and characterization of alkaline protease-deficient mutants of *Pseudomonas aeruginosa* *in vitro* and in a mouse eye model. *Infect Immun* 43: 1058–1063.
34. Pillar CM, Hazlett LD, Hobden JA (2000) Alkaline protease-deficient mutants of *Pseudomonas aeruginosa* are virulent in the eye. *Curr Eye Res* 21: 730–739.
35. Chabeaud P, de Groot A, Bitter W, Tommassen J, Heulin T, et al. (2001) Phase-variable expression of an operon encoding extracellular alkaline protease, a serine protease homolog, and lipase in *Pseudomonas brassicacearum*. *J Bacteriol* 183: 2117–2120.
36. Veening J-W, Igoshin OA, Eijlander RT, Nijland R, Hamoen LW, et al. (2008) Transient heterogeneity in extracellular protease production by *Bacillus subtilis*. *Mol Syst Biol* 4: 184.
37. Maamar H, Raj A, Dubnau D (2007) Noise in gene expression determines cell fate in *Bacillus subtilis*. *Science* 317: 526–529.
38. Süel GM, Garcia-Ojalvo J, Liberman LM, Elowitz M (2006) An excitable gene regulatory circuit induces transient cellular differentiation. *Nature* 440: 545–550.
39. Ajo-Franklin CM, Drubin DA, Eskin JA, Gee EP, Landgraf D, et al. (2007) Rational design of memory in eukaryotic cells. *Genes Dev* 21: 2271–2276.
40. Isaacs FJ, Hasty J, Cantor CR, Collins JJ (2003) Prediction and measurement of an autoregulatory genetic module. *Proc Natl Acad Sci USA* 100: 7714–7719.
41. Turgay K, Hahn J, Burghoorn J, Dubnau D (1998) Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. *EMBO J* 17: 6730–6738.
42. Ogura M, Liu L, Lacelle M, Nakano MM, Zuber P (1999) Mutational analysis of ComS: evidence for the interaction of ComS and MecA in the regulation of competence development in *Bacillus subtilis*. *Mol Microbiol* 32: 799–812.
43. Schell MA (1993) Molecular biology of the LysR family of transcriptional regulators. *Annu Rev Microbiol* 47: 597–626.
44. Rietsch A, Vallet-Gely I, Dove SL, Mekalanos JJ (2005) ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 102: 8006–8011.
45. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP (1998) A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212: 77–86.
46. Castang S, McManus HR, Turner KH, Dove SL (2008) H-NS family members function coordinately in an opportunistic pathogen. *Proc Natl Acad Sci USA* 105: 18947–18952.
47. Choi KH, Gaynor JB, White KG, Lopez C, Bosio, CM, et al. (2005) A Tn7-based broad-range bacterial cloning and expression system. *Nat Methods* 2: 443–448.

48. Dove SL, Hochschild A (2004) A bacterial two-hybrid system based on transcription activation. *Methods Mol Biol* 261: 231–246.
49. Wolfgang MC, Lee VT, Gilmore ME, Lory S (2003) Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. *Dev Cell* 4: 253–263.
50. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402–408.
51. Blyn LB, Braaten BA, White-Ziegler CA, Rolfson DH, Low DA (1989) Phase-variation of pyelonephritis-associated pili in *Escherichia coli*: evidence for transcriptional regulation. *EMBO J* 8: 613–620.

Chapter 3

H-NS family members function coordinately in an opportunistic pathogen

Attributions

This chapter is a reprint of a paper published in the December 2, 2008 issue of Proceedings of the National Academy of Sciences (Castang S, McManus HR, Turner KH, Dove SL (2008) H-NS family members function coordinately in an opportunistic pathogen. Proc Natl Acad Sci 105: 18947-18952). Sandra Castang and Heather R. McManus performed the majority of the experimental work. My contribution to the work involved adapting the inducible protein degradation system that was used to deplete both MvaT and MvaU from cells in Figures 3.7 through 3.9, and I performed the experiments shown in Figure 3.7. Additionally, I wrote several Perl and Java scripts that were used to analyze and process the ChIP-on-Chip data reported in the paper. Finally, I constructed the vector used to introduce a VSV-G tag to the C terminus of MvaU at its native locus in the chromosome. Dr. Castang, Dr. McManus, Simon L. Dove and I wrote the paper. This work is reprinted with permission of the National Academy of Sciences of the USA under their policy on the rights and permissions of authors (<http://www.pnas.org/site/misc/rightperm.shtml>).

H-NS family members function coordinately in an opportunistic pathogen

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Abstract

The histone-like nucleoid structuring protein, H-NS, is a prominent global regulator of gene expression. Many Gram-negative bacteria contain multiple members of the H-NS family of proteins. Thus, a key question is whether H-NS family members have overlapping or distinct functions. To address this question we performed genome-wide location analyses with MvaT and MvaU, the two H-NS family members present in *Pseudomonas aeruginosa*. We show that MvaT and MvaU bind the same chromosomal regions, coregulating the expression of ~350 target genes. We show further that like H-NS in enteric bacteria, which functions as a transcriptional silencer of foreign DNA by binding to AT-rich elements, MvaT and MvaU bind preferentially to AT-rich regions of the chromosome. Our findings establish that H-NS paralogs can function coordinately to regulate expression of the same set of target genes, and suggest that MvaT and MvaU are involved in silencing foreign DNA elements in *P. aeruginosa*.

Introduction

The histone-like nucleoid structuring protein H-NS is a prominent global regulator of gene expression found in a number of different Gram-negative bacteria. Although H-NS silences the expression of hundreds of genes, it also plays a role in DNA compaction [1]. Furthermore, several genome-wide association studies in enteric bacteria have revealed that H-NS displays a marked preference for binding AT-rich regions of DNA [2-5]. In so doing H-NS provides a mechanism for selectively silencing so called xenogeneic DNA (DNA acquired from a foreign source), such as that acquired through horizontal transfer, and whose AT-content is typically higher than that of the cognate genome [3,5-7]. Although traditionally thought of as a nonsequence-specific DNA-binding protein, recent evidence suggests that H-NS binds specific sites on the DNA with high affinity [8]. Once bound to these sites, which are themselves AT-rich, H-NS is thought to oligomerize across adjacent AT-rich regions of DNA, forming an extensive nucleoprotein complex that facilitates gene silencing [8].

Many bacteria contain more than one H-NS family member [9]. Of the two in *Escherichia coli* K-12 (H-NS and its paralog StpA), only H-NS appears to play a significant role in controlling gene expression. StpA is less abundant than H-NS, and although it can associate with H-NS, and can functionally substitute for H-NS, it is not thought to influence the expression of any H-NS-regulated gene [1]. However, in uropathogenic *E. coli*, H-NS and StpA are thought to have both overlapping and distinct functions, with one regulon controlled exclusively by H-NS, and a second smaller regulon under the control of both H-NS and StpA [10]. Nevertheless, in most bacteria it is not known whether H-NS family members have overlapping or distinct functions.

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen that is best known through being the principal cause of morbidity and mortality in cystic fibrosis

(CF) patients [11]. The organism uses an impressive battery of virulence factors to intoxicate the human host, and persists in the chronically infected CF lung as a biofilm (a community of cells encased in a polymeric matrix), a lifestyle that engenders increased resistance to antibiotics, and exacerbates the problem of eradicating the organism from the lung. Foremost among the regulators of virulence gene expression and biofilm formation in *P. aeruginosa* is MvaT, one of the two H-NS family members present in this organism [12-14]. That MvaT belongs to the H-NS family, despite sharing limited sequence similarity with H-NS, is suggested by multiple functional similarities and several structural predictions [15-19].

MvaT was initially identified in *P. aeruginosa* as a global regulator of virulence gene expression [12]. Subsequent microarray analyses revealed that MvaT controls the expression of at least 150 genes in *P. aeruginosa* [13,14], including genes encoding virulence factors, house-keeping genes, and a preponderance of genes encoding proteins associated with the cell surface, some of which play important roles in biofilm formation [13,14]. However, it is not known which of these genes are regulated directly by MvaT. In addition, very little is known about which genes are regulated by the MvaT paralog MvaU. Although MvaU can interact with MvaT [18], and plays a role in regulating the expression of at least one MvaT-regulated gene, deletion of *mvaU* had no effect on the expression of several others [13,18]. Thus, the extent to which the MvaT and MvaU regulons overlap was unknown.

Here we present evidence that the H-NS family members MvaT and MvaU regulate expression of the same set of target genes in *P. aeruginosa*. In particular, using chromatin immunoprecipitation (ChIP) coupled with fully tiled high density DNA microarrays (ChIP-on-chip) we show that MvaT and MvaU occupy the same regions of the chromosome, suggesting that MvaT and MvaU function coordinately. In addition to identifying those genes that are regulated directly by MvaT and MvaU, and extending the MvaT (/MvaU) regulon, these analyses reveal

that, like H-NS from enteric bacteria, MvaT and MvaU bind preferentially to AT-rich regions of the DNA. Furthermore, using a ClpXP protease-based protein degradation system we show that the loss of both MvaT and MvaU from the cell cannot be tolerated. Depletion of MvaT in cells of an *mvaU* mutant strain results in overexpression of target genes, suggesting that the synthetic lethality that ensues is due to misregulated expression of certain target genes. These findings have implications for xenogeneic silencing in *P. aeruginosa*, and the function of H-NS and its paralogs in other bacteria.

Results

ChIP-on-Chip reveals extensive overlap between the MvaT and MvaU regulons

To identify those genes that are controlled directly by MvaT and MvaU, and determine the extent of the overlap between the MvaT and MvaU regulons we used ChIP-on-chip. To facilitate the immunoprecipitation of MvaT and MvaU we constructed two strains of *P. aeruginosa* PAO1 that synthesized epitope-tagged forms of MvaT and MvaU at native levels. In one of these strains (PAO1 MvaT-V) the native copy of the *mvaT* gene specified MvaT with a vesicular stomatitis virus glycoprotein (VSV-G) epitope tag fused to its C terminus (MvaT-V). The function of MvaT was not impaired by the presence of the VSV-G tag as MvaT-V fully repressed expression of the MvaT-regulated *cupA* gene (data not shown). In the other strain (PAO1 MvaU-V) the native copy of the *mvaU* gene specified MvaU with a VSV-G epitope tag fused to its C terminus (MvaU-V).

ChIP-on-chip with cells of the PAO1 MvaT-V strain revealed a total of 111 distinct genomic regions that were significantly enriched for MvaT (Figure 3.1, Figure 3.2 and Table 3.1¹). Peaks corresponding to genomic regions associated with MvaT were located throughout the chromosome and encompassed both intergenic and coding regions (Figure 3.1). Peaks ranged in size from ~ 0.2 to 22 kilobases, with an average width of ~ 2.5 kb (Table 3.1), consistent with the idea that MvaT is a member of the H-NS family of proteins that typically oligomerize across long stretches of target DNA [1,6].

¹ Table 3.1 is available online from the Proceedings of the National Academy of Sciences: http://www.pnas.org/content/suppl/2008/11/21/0808215105.DCSupplemental/ST1_PDF.pdf

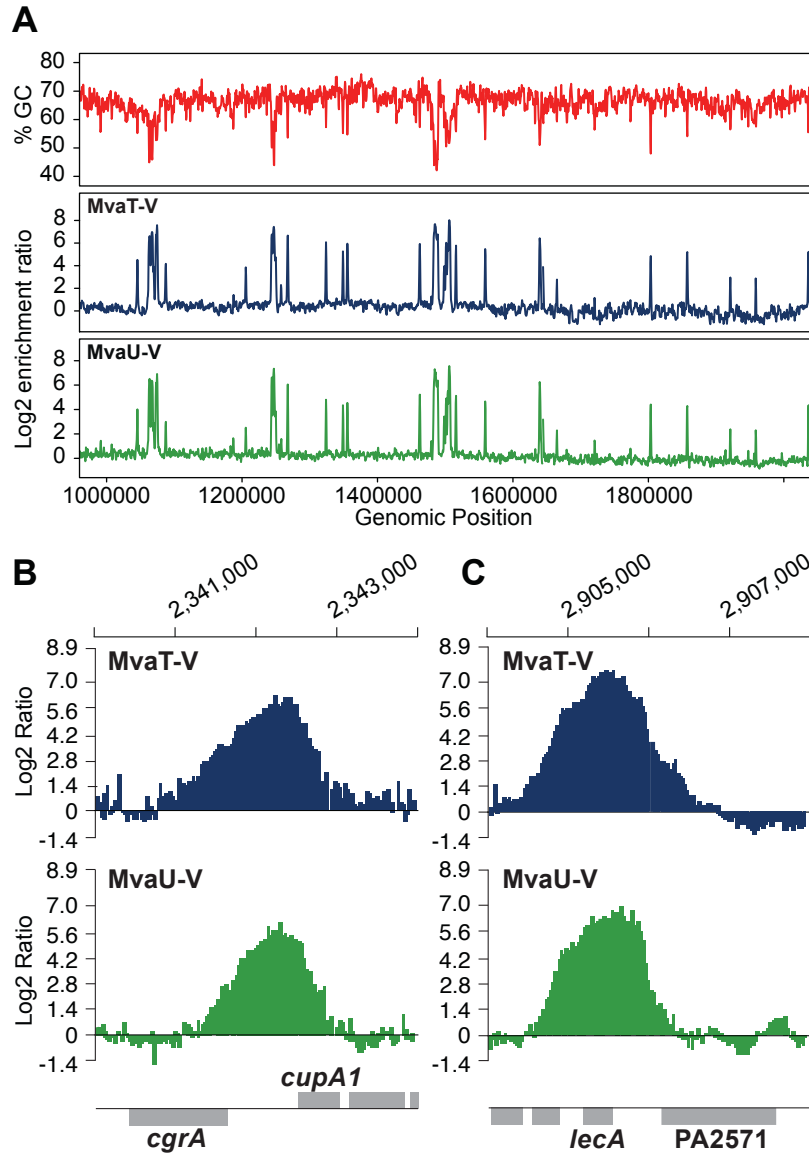


Figure 3.1. MvaT and MvaU occupy the same AT-rich regions of the chromosome. (A) The percent GC content for 1 Mb of the PAO1 chromosome (in windows of 1000 bp across the genome with a window step size of 500 bp) is plotted (in red) against the locations of MvaT-V (plotted as log2 ratios in blue) and MvaU-V (plotted as log2 ratios in green) as determined by ChIP-on-chip. Association of MvaT-V and MvaU-V with (B) the *cgrA*-*cupA1* intergenic region, and with (C) the *lecA* promoter and coding region. Log2 ratio values were normalized and averaged across three replicate arrays.

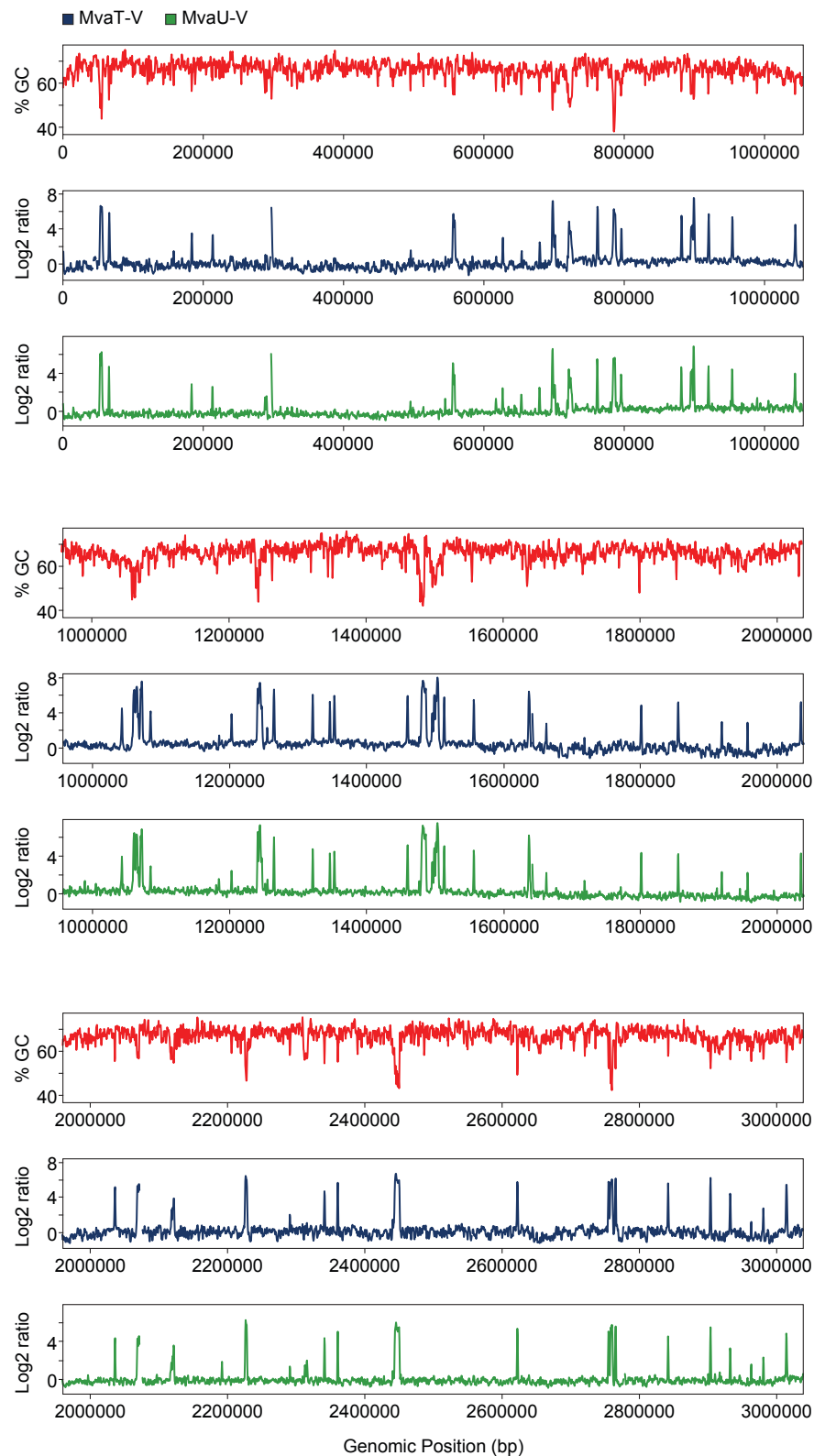


Figure 3.2. MvaT and MvaU occupy the same AT-rich regions of the chromosome. The percent GC content for the PAO1 chromosome (in windows of 1000 bp across the genome with a window step size of 500 bp) is plotted (in red) against the locations of MvaT-V (plotted as log2 ratios in blue) and MvaU-V (plotted as log2 ratios in green) as determined by ChIP-on-chip (Figure continued on next page).

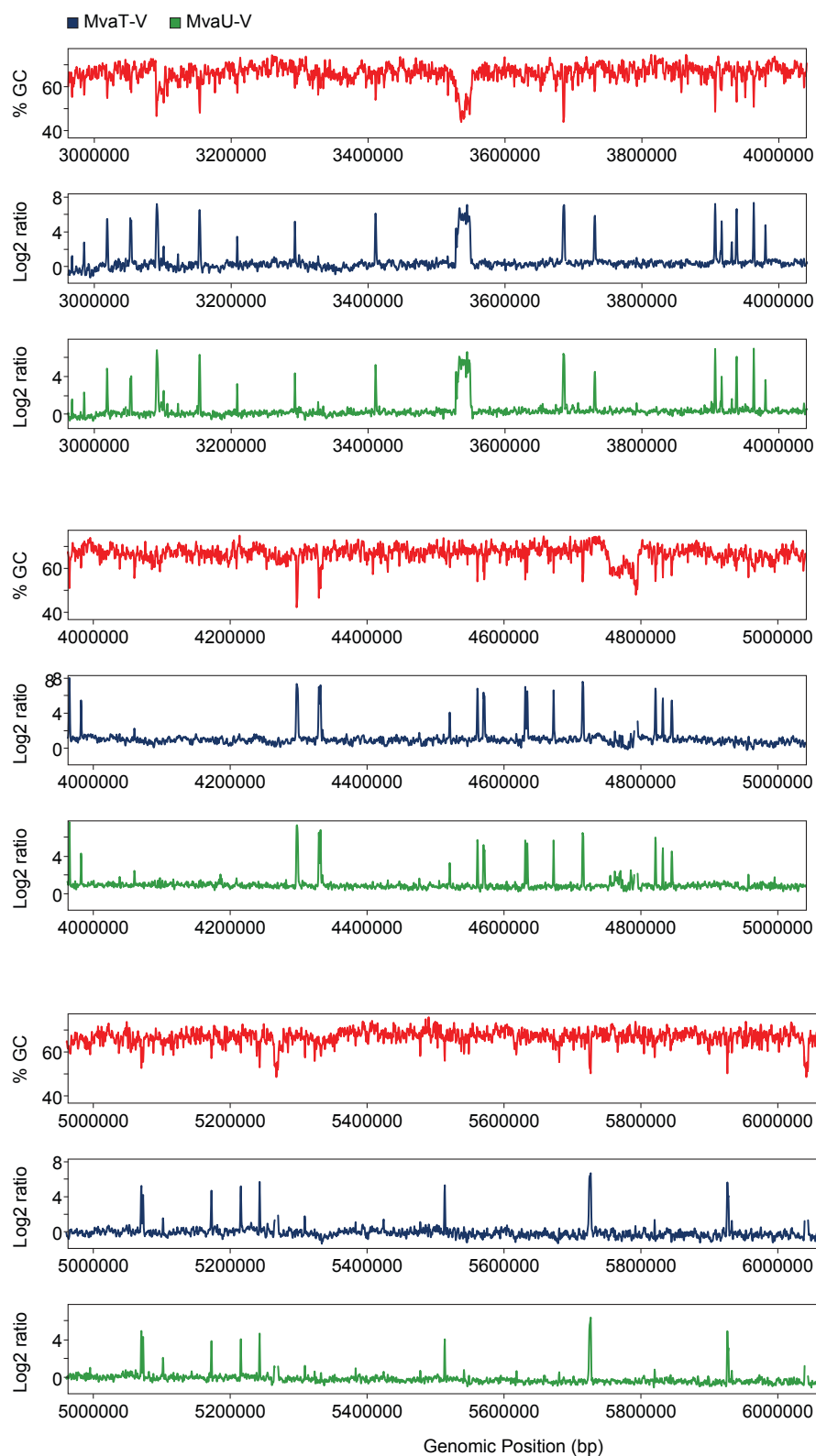


Figure 3.2 (continued). MvaT and MvaU occupy the same AT-rich regions of the chromosome.
(See figure legend previous page).

To validate the MvaT ChIP-on-chip results, independent ChIP experiments were conducted and analyzed by using quantitative real-time PCR (qPCR). Of the 21 MvaT peaks tested, no false positives were detected and none of the 9 negative control regions showed significant enrichment (Figure 3.3A and data not shown). Furthermore, trends in the magnitude of fold enrichment of a specific target region were similar between ChIP-on-chip and ChIP datasets (Figure 3.3A). ChIP-on-chip and ChIP control experiments conducted using a strain that synthesized MvaT with a C-terminal tandem affinity purification (TAP) tag (PAO1 MvaT-TAP) [18], yielded results similar to those obtained with the PAO1 MvaT-V strain (Figure 3.3B and data not shown), illustrating the robust nature of these results. An additional control performed with a strain synthesizing a TAP-tagged form of the putative σ factor PA2896 confirmed the specificity of the association of MvaT with target regions (Figure 3.3B). Finally, qPCR analyses of mock ChIP samples revealed no appreciable enrichment of any of the DNA regions that were tested (Figure 3.3B and data not shown).

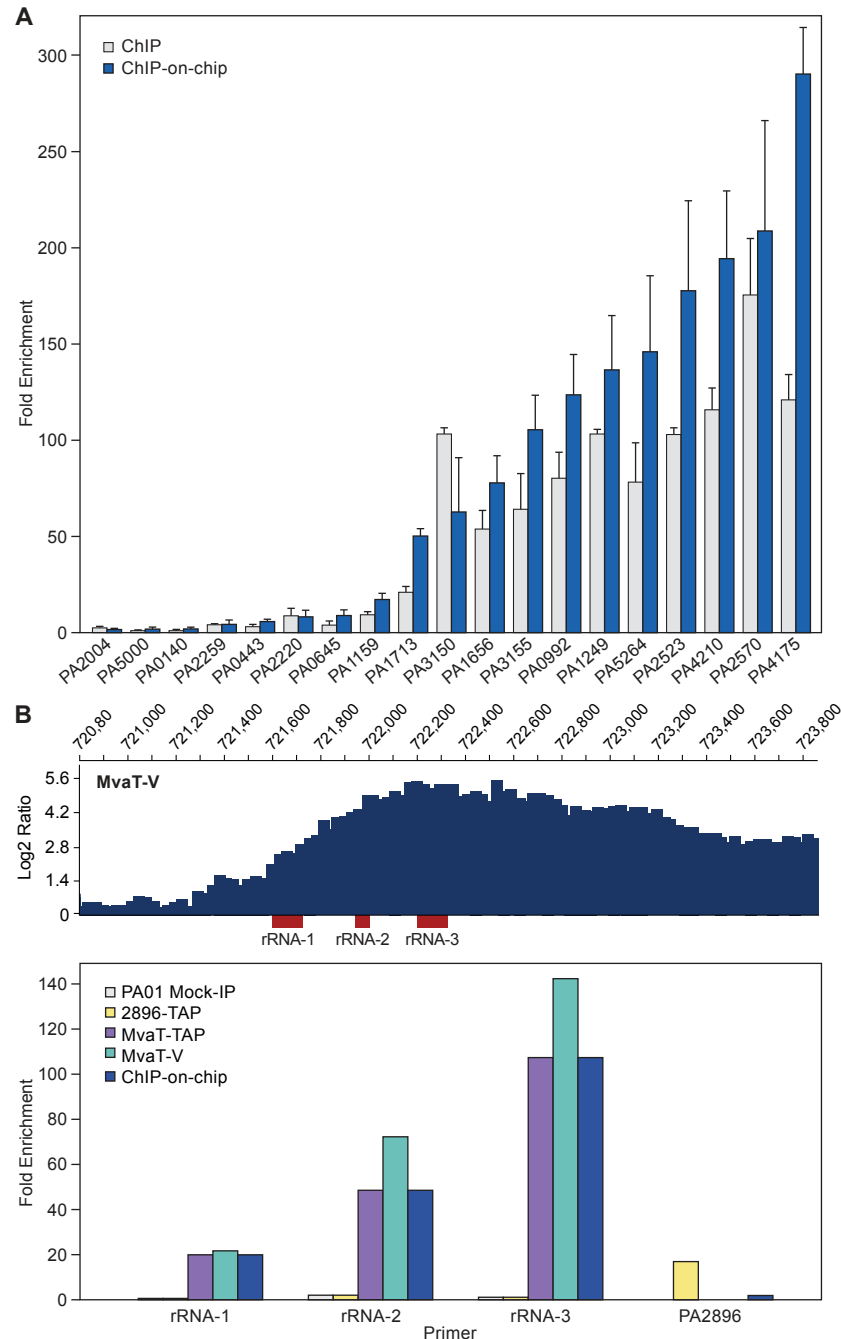


Figure 3.3. Validation of MvaT-V ChIP-on-chip peaks. (A) Independent MvaT-V ChIP experiments were performed and qPCR was used to quantify the fold enrichment (ChIP DNA/input DNA) of regions immediately upstream of several genes of interest. All ChIP fold enrichment scores are calculated relative to an amplicon internal to PA2155 (a poorly expressed gene that shows no MvaT-V or MvaU-V enrichment in ChIP-on-chip datasets) and were averaged across three biological replicates. Corresponding ChIP-on-chip values were obtained by isolating the largest log2 ratio within the genomic regions surrounding each qPCR amplicon, which were then converted to fold enrichment values (ChIP DNA/input DNA). Amplicons are labeled according to the number of the gene immediately downstream of the primer pair. (B) ChIP experiments were conducted on the following strains: PAO1, PAO1 PA2896-TAP, PAO1 MvaT-TAP, and PAO1 MvaT-V. The PAO1 mock-IP was conducted using anti-VSV-G agarose beads. The log2 ratio values represented in the MvaT-V peak (top) were averaged across three replicate arrays. Fold enrichment values (bottom) are from single experiments.

ChIP-on-chip analyses of MvaU-V revealed a total of 102 distinct genomic regions that were significantly enriched for MvaU binding (Figure 3.1, Figure 3.2, and Table 3.2²). Similar to our findings with MvaT, peaks corresponding to those regions of the chromosome associated with MvaU were found throughout the genome with each region spanning anywhere from approximately 0.1 to 21 kb, with an average width of approximately 2 kb. Independent ChIP experiments followed by qPCR were conducted to validate the MvaU-V results, with no false positives evident among the 14 peaks tested, and no appreciable enrichment of MvaU-V at any of the 5 negative control regions that were analyzed (Figure 3.4).

Comparison of the MvaT and MvaU-associated regions revealed a striking correspondence between the two (Figure 3.1 and Figure 3.2). In particular, 99 of the 102 regions associated with MvaU were also associated with MvaT. Furthermore, minimally relaxing the stringency of the criteria we used to identify peaks resulted in MvaU being enriched in all 111 of the regions associated with MvaT (data not shown). The extent of the overlap in the genomic regions associated with MvaT and MvaU strongly suggests that these two proteins control the expression of essentially the same set of genes.

² Table 3.2 is available online from the Proceedings of the National Academy of Sciences: http://www.pnas.org/content/suppl/2008/11/21/0808215105.DCSupplemental/ST2_PDF.pdf

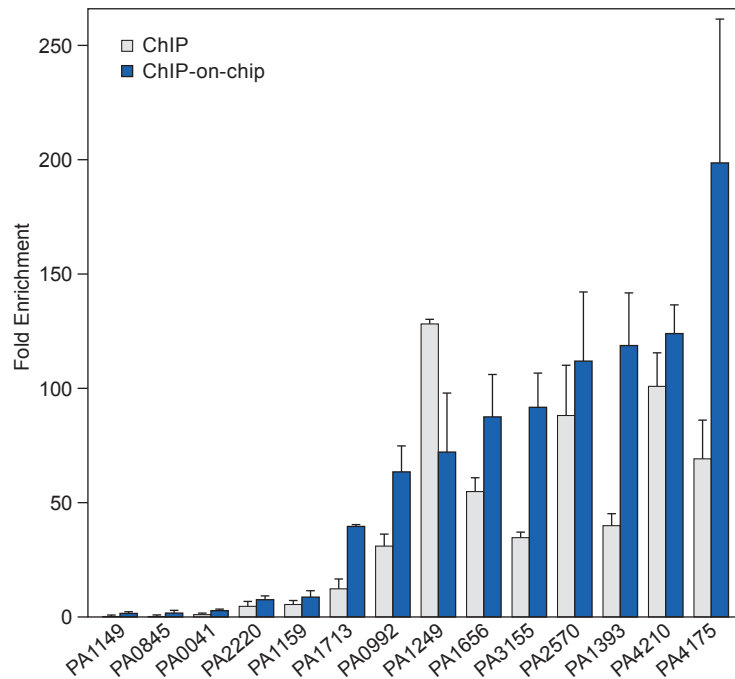


Figure 3.4. Validation of MvaU-V ChIP-on-chip peaks. Independent MvaT-V ChIP experiments were performed and qPCR was used to quantify the fold enrichment (ChIP DNA/input DNA) of regions immediately upstream of several genes of interest. All ChIP fold enrichment scores are calculated relative to an amplicon internal to PA2155 and were averaged across three biological replicates. Corresponding ChIP-on-chip values were obtained by isolating the largest log₂ ratio within the genomic regions surrounding each qPCR amplicon, which were then converted to fold enrichment values (ChIP DNA/input DNA). Amplicons are labeled with the number of the gene immediately downstream of the primer pair.

MvaT and MvaU preferentially associate with AT-rich regions of the chromosome

In *Salmonella enterica* and *E. coli* [2-5], H-NS has been shown to preferentially associate with AT-rich regions of the genome. To determine whether MvaT and MvaU show a similar binding preference, the percentage GC content within 1,000 bp windows across the PAO1 genome was calculated and compared with the average fold enrichment of MvaT-V and MvaU-V (expressed as a log2 ratio) within each window. A strong correlation between MvaT or MvaU enrichment and genomic AT content was found (Figure 3.1, Figure 3.2, and Figure 3.5), suggesting that like H-NS, MvaT and MvaU display a distinct preference for binding AT-rich regions of the DNA and may be involved in xenogeneic silencing in *P. aeruginosa*. Consistent with this idea we found that MvaT and MvaU were associated with approximately two thirds of the so-called regions of genomic plasticity (PGRs) in *P. aeruginosa* PAO1 that represent strain-specific segments of DNA that may contribute to the ability of *P. aeruginosa* to adapt to particular environments [20]. Many PGRs are thought to have been acquired by horizontal gene transfer and contain regions where the AT content is higher than that of the rest of the genome.

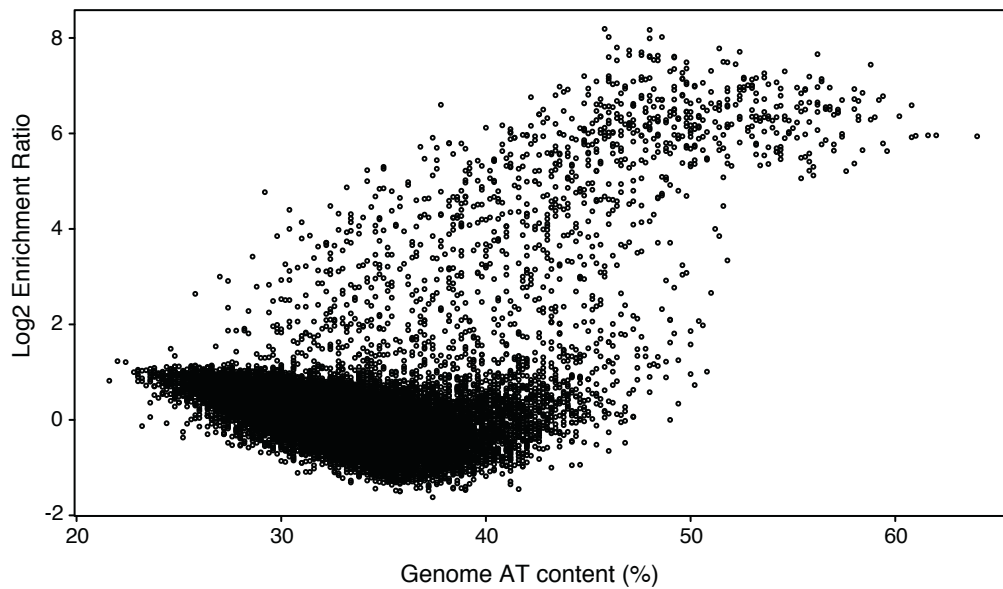


Figure 3.5. MvaT associates with AT-rich regions of the chromosome. Each circle represents a single 500 bp (250 bp step) window of the PAO1 chromosome and illustrates the average AT content (x-axis) and the average ChIP-on-chip log₂ ratio for MvaT-V (y-axis) in each window. The majority of the windows are not enriched for MvaT-V and fall within the log₂ ratio range of -2 to 2. Windows that show enrichment for MvaT-V have a wide range of AT content; however, windows that have AT contents above 53% have log₂ ratios that do not fall below 5, suggesting that MvaT-V associates with most, if not all, of the AT-rich regions of the PAO1 chromosome.

Direct targets of MvaT and MvaU

Our ChIP-on-chip analyses revealed a total of 394 and 311 genes associated directly with MvaT-V and MvaU-V peaks, respectively. Although these numbers are in good agreement with one another, we suggest that the difference between them likely reflects differences in the threshold values used to define peaks. Note that these tallies likely underestimate the total number of genes regulated directly by MvaT and MvaU as they do not take into account genes that are not bound directly by MvaT/MvaU but lie within operons whose regulatory regions are.

To determine whether our results are in accordance with known regulatory roles of MvaT, we compared genes associated with MvaT-bound regions with known MvaT-regulated genes. Of the 104 genes whose expression was shown to be repressed by MvaT [13], 84 (81%) were found to have peaks associated with them or with their corresponding regulatory regions. Conversely, only 7 (14%) of the 49 genes whose expression was activated by MvaT were located in the vicinity of MvaT-associated regions, and 6 of these genes were in the same operon. Taken together, these findings suggest that MvaT functions directly as a repressor, and likely mediates many of its positive effects on gene expression indirectly.

Extension of the MvaT regulon

Our analyses identified an additional 311 MvaT target genes that were not known to belong to the MvaT regulon (Table 3.1) [13,14]. Among these are several of the *phz* genes that are required for synthesis of the toxic secondary metabolite pyocyanin [21], and genes encoding critical regulators of quorum sensing and virulence such as *mvfR*, *lasR*, *rsaL*, *lasI*, *pprB*, *rpoS*, and *rsmZ* [22]. These findings likely explain several of the phenotypes observed for an *mvaT* mutant, including overproduction of pyocyanin and increased synthesis of the C4-HSL and 3O-C12-HSL quorum-sensing signals [12]. Other newly identified MvaT target genes include many

known and putative virulence genes such as *pldA* encoding phospholipase D [23], the *algA* and *algD* genes that are involved in the synthesis of alginate [11], the *exsE* and *exsD* genes encoding regulators of the type III secretion system [24], the *mvaT* gene itself, and the PA1656, *clpV3*, PA2372, and *vgrG3* genes present on the recently identified HSI-II and HSI-III type VI secretion islands, together with the type VI secretion related genes *hcp2* (*hcpA*) and *vgrG2* [25]. Therefore, our findings both confirm identified members of the MvaT regulon and identify previously unknown members of the regulon.

Mechanistic implications

Defining the specific locations of MvaT and MvaU along the *P. aeruginosa* chromosome by ChIP-on-chip suggests specific mechanistic hypotheses for how MvaT and MvaU control the expression of individual target genes. For example, the results depicted in Figure 3.1B suggest that MvaT mediates its effects on expression of the *cupA* fimbrial genes by (i) directly controlling the expression of the *cgr* regulatory genes that positively regulate *cupA* gene expression [26], and by (ii) directly binding the *cupA* promoter and *cupA1* coding region. In regard to the latter it is interesting to note that H-NS can inhibit transcription initiation by occupying regions both upstream and downstream of a promoter [27]. As a second example, the results depicted in Figure 3.1C show that MvaT and MvaU bind both the promoter and entire coding region of the *lecA* gene, raising the possibility that MvaT and MvaU might exert a portion of their repressive effects on *lecA* expression by interfering with transcription elongation [28]. Because many regulators mediate their effects on gene expression by relieving the silencing effects of H-NS proteins [1,7], our findings concerning the locations of MvaT and MvaU provide a resource for those interested in understanding whether particular regulators in *P. aeruginosa* exert their effects by relieving transcriptional silencing mediated by MvaT and MvaU.

MvaT has a marked effect on the ability of MvaU to associate with a subset of genomic regions

We have shown that MvaT and MvaU associate with one another [18], raising the possibility that they bind the same genomic regions because they function exclusively as MvaT-MvaU heteromers. To determine whether MvaT is required in order for MvaU to associate with any genomic region we performed ChIP-on-chip with MvaU-V in a $\Delta mvaT$ mutant strain (PAO1 $\Delta mvaT$ MvaU-V) and compared it with our MvaU ChIP-on-chip data obtained in the presence of MvaT (i.e., from PAO1 MvaU-V). We found that all genomic regions that showed MvaU-V enrichment in the presence of MvaT were still enriched for MvaU-V when MvaT was no longer present (Table 3.2), suggesting that MvaU-V does not require MvaT to interact with genomic DNA. However, in the absence of MvaT, the log₂ enrichment ratios for all MvaU-V peaks were slightly lower, with the majority of peaks (51%) showing a ~3-fold reduction in enrichment in the absence of MvaT (Table 3.2). Interestingly, the log₂ ratio of some peaks decreased substantially in the $\Delta mvaT$ MvaU-V dataset, with ~16% of the peaks showing between a 5- and 10-fold reduction (Figure 3.6 and Table 3.2). For example, in Figure 3.6A, the degree of enrichment of MvaU within the *cgrA-cupA1* region is substantially less in the absence of MvaT than in the presence of MvaT (10-fold versus 64-fold enrichment, respectively, at the maximum peak height) whereas in Figure 3.6B, the degree of association of MvaU with the *lecA* region is similar in the absence or presence of MvaT (74-fold enrichment versus 135-fold, respectively). Taken together, our findings suggest that MvaT and MvaU need not necessarily function as heteromers, and that MvaT can influence the association of MvaU with specific genomic regions.

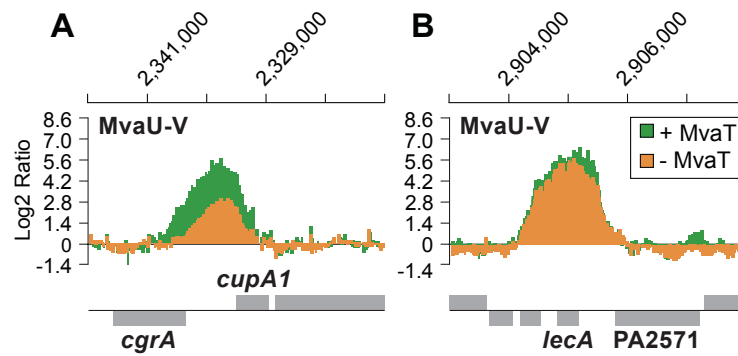


Figure 3.6. MvaT can influence the association of MvaU with target sites. Association of MvaU-V with the *cgrA*-*cupA1* intergenic region (A) and with the *lecA* promoter and coding region (B), in the presence (green) or absence (orange) of MvaT. Log2 ratio values were normalized and averaged across three replicate arrays.

MvaT and MvaU are essential in the absence of the partner regulator

Because MvaT and MvaU regulate a common set of genes, we reasoned that removal of both MvaT and MvaU might result in particularly pronounced effects on the expression of MvaT/MvaU target genes. Repeated attempts at constructing a $\Delta mvaT \Delta mvaU$ mutant failed, suggesting that loss of both MvaT and MvaU resulted in lethality. Therefore, to test our idea we made derivatives of our $\Delta mvaT$ and $\Delta mvaU$ mutant strains [18] in which the respective partner regulator could be depleted. Our strategy for depleting either MvaU or MvaT is indicated in Figure 3.7A, and involved adapting a ClpXP protease-based controllable protein degradation system [29] for use in *P. aeruginosa*. This technique utilizes a small epitope tag that can be fused to the C terminus of a particular protein to mark it for degradation in a controllable fashion. The degradation tag (referred to here as DAS4) contains a low-affinity binding site for the ClpXP protease complex and a high-affinity binding site for SspB - an adaptor protein that feeds the tagged protein to the ClpXP machinery. Degradation of a tagged protein depends on ClpXP and SspB, with the rate of degradation being dependent on the intracellular concentration of SspB [29].

To begin to address the question of whether the combined activities of MvaT and MvaU are essential, we made a version of our PAO1 *cupA lacZ* reporter strain that carried an in-frame deletion of the *sspB* gene (PAO1 $\Delta sspB$ *cupA lacZ*). We then tagged the native copy of the *mvaT* gene in this strain such that it specified MvaT with a VSV-G and a DAS4 tag fused to its C terminus (MvaT-V-DAS4) (see Figure 3.7A). The resulting MvaT depletion strain (PAO1 $\Delta sspB$ *cupA lacZ* MvaT-V-DAS4) therefore made MvaT-V-DAS4 at native levels, and allowed us to determine that the MvaT-V-DAS4 fusion was functional; the MvaT-V-DAS4 fusion fully repressed expression of the *cupA lacZ* reporter (data not shown).

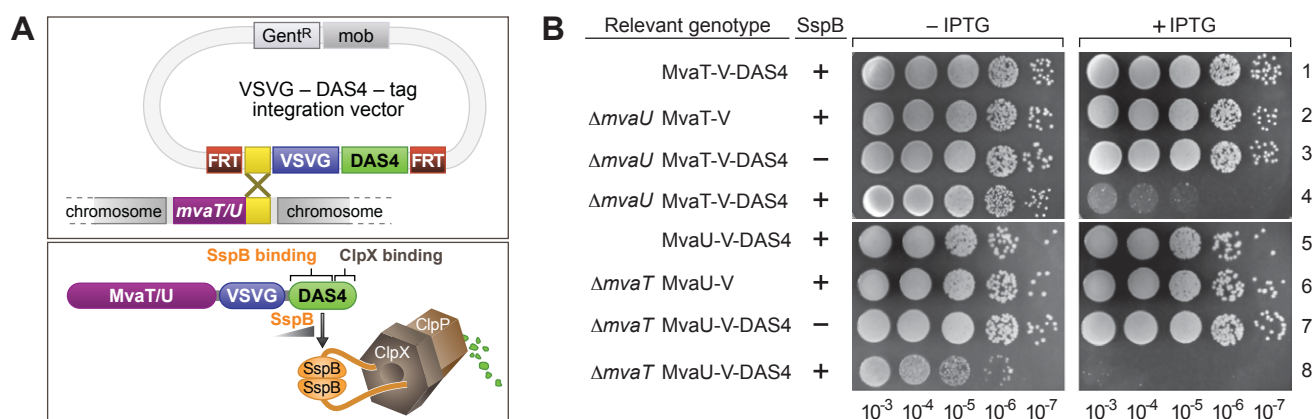


Figure 3.7. Combined loss of MvaT and MvaU results in lethality. (A) ClpXP-based controllable protein degradation system. Representation of the VSVG-DAS4 tag integration vector and its use in making strains synthesizing MvaT-V-DAS4 and MvaU-V-DAS4. The VSV-G epitope tag (blue) and the DAS4 tag (green) are shown together with the gentamicin resistance determinant (Gent^R), the mobilization region (mob) and FRT sites. (B) Depletion of MvaT-V-DAS4 in a $\Delta mvaU$ mutant strain and depletion of MvaU-V-DAS4 in a $\Delta mvaT$ mutant strain results in loss of viability. Plasmids pV-SspB and pPSPK-SspB express *V-sspB* and *sspB* respectively in an IPTG-inducible fashion. Plasmids pPSV35 and pPSPK are empty vector controls. Colonies of plasmid-containing cells were resuspended in LB to the same OD600. Cells were serially diluted to the indicated OD600 and spotted onto LB plates that either did (+) or did not (-) contain 2 mM IPTG. Row 1, PAO1 $\Delta sspB$ *cupA lacZ* MvaT-V-DAS4 carrying pV-SspB. Row 2, PAO1 $\Delta sspB$ $\Delta mvaU$ *cupA lacZ* MvaT-V carrying pV-SspB. Row 3, PAO1 $\Delta sspB$ $\Delta mvaU$ *cupA lacZ* MvaT-V-DAS4 carrying pPSV35. Row 4, PAO1 $\Delta sspB$ $\Delta mvaU$ *cupA lacZ* MvaT-V-DAS4 carrying pV-SspB. Row 5, PAO1 $\Delta sspB$ MvaU-V-DAS4 carrying pPSPK-SspB. Row 6, PAO1 $\Delta sspB$ $\Delta mvaT$ MvaU-V carrying pPSPK-SspB. Row 7, PAO1 $\Delta sspB$ $\Delta mvaT$ MvaU-V-DAS4 carrying pPSPK. Row 8, PAO1 $\Delta sspB$ $\Delta mvaT$ MvaU-V-DAS4 carrying pPSPK-SspB.

To determine whether MvaT was essential in the absence of MvaU, we next made a version of the MvaT depletion strain in which the *mvaU* gene was deleted (PAO1 Δ *sspB* Δ *mvaU* *cupA lacZ* MvaT-V-DAS4). A vector directing the synthesis of SspB in an IPTG-inducible fashion was then introduced into each of the MvaT depletion strains, and cells from single colonies of each transformed strain were serially diluted and spotted onto LB agar plates, or LB agar plates containing IPTG to induce expression of *sspB* and deplete the DAS4-tagged MvaT. Depletion of MvaT resulted in at least a 10⁴-fold decrease in colony forming units only in the absence of MvaU (Figure 3.7B, rows 1 and 4). Additional controls revealed that the loss of viability that results upon depletion of MvaT in the Δ *mvaU* mutant strain depended on the presence of SspB and the presence of the DAS4 tag on MvaT-V-DAS4 (Figure 3.7B). These findings suggest that MvaT is essential when MvaU is absent but not when MvaU is present.

Using an analogous strategy we tested the prediction that MvaU is essential in the absence of MvaT. Depletion of MvaU-V-DAS4 resulted in at least a 10⁴-fold decrease in colony forming units only in the Δ *mvaT* mutant background (Figure 3.7B, rows 5 and 8). Taken together, our findings suggest that MvaT and MvaU have shared functions that are essential in *P. aeruginosa*. They further suggest that a degree of functional redundancy exists between MvaT and MvaU, because cells can tolerate loss of either protein, but not the loss of both.

Transcriptional effects of the combined loss of MvaT and MvaU

We next asked whether we could use our depletion system to determine the transcriptional effects of removing both MvaT and MvaU. For these experiments we used the *mvaU*⁺ and $\Delta mvaU$ pair of MvaT depletion strains described earlier (PAO1 $\Delta sspB$ *cupA lacZ* MvaT-V-DAS4, and PAO1 $\Delta sspB$ $\Delta mvaU$ *cupA lacZ* MvaT-V-DAS4). Cells of each of these MvaT depletion strains were transformed with a vector directing the IPTG-dependent synthesis of a VSV-G tagged version of SspB (V-SspB), and an empty vector control, and grown to mid-log. (Use of V-SspB and MvaT-V-DAS4 allowed the simultaneous analysis of both proteins by western blotting.) Cultures were then divided into two and grown for a further 30 min either in the absence or presence of IPTG. Cells were harvested for western blot analyses and for RNA isolation. As additional controls, cells of a wild-type strain (PAO1 *cupA lacZ*), cells of a $\Delta mvaT$ mutant strain (PAO1 $\Delta mvaT$ *cupA lacZ*), and cells of a $\Delta mvaU$ mutant strain (PAO1 $\Delta mvaU$ *cupA lacZ*) were grown in the same way as the cells used for the depletion studies and were harvested for RNA isolation. The results depicted in Figure 3.8A show that the addition of IPTG to cells of each MvaT depletion strain containing the *sspB* expression vector resulted in both induction of V-SspB synthesis and concomitant depletion of MvaT-V-DAS4 to the extent that MvaT-V-DAS4 could no longer be detected. Thus, induction of V-SspB is rapid and causes depletion of MvaT-V-DAS4. An additional control revealed that the ability of V-SspB to promote depletion of MvaT-V-DAS4 depended on the presence of the DAS4 tag as MvaT-V accumulated in both the presence and absence of SspB (Figure 3.9).

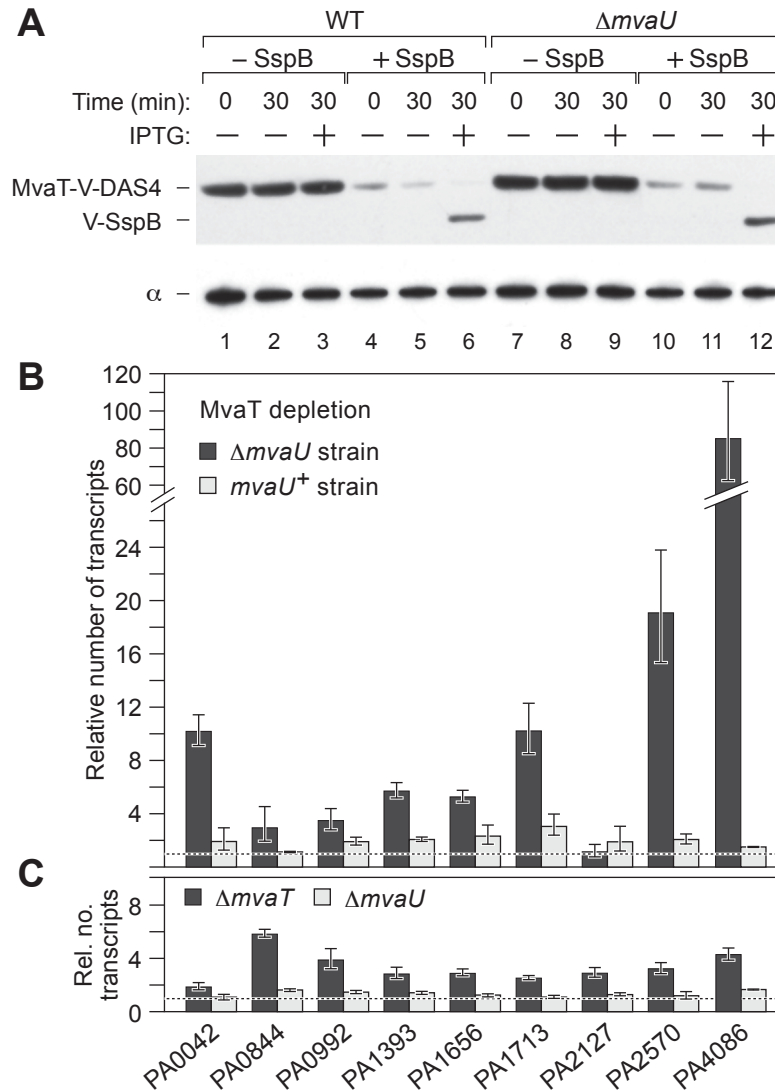


Figure 3.8. Combined loss of MvaT and MvaU has a larger effect on the expression of target genes than loss of MvaT or MvaU alone. Cells of the MvaT depletion strains containing plasmid pV-SspB (expressing V-SspB in an IPTG-inducible manner) or the empty control vector pPSV35, were first grown to mid-log (time 0), then grown for a further 30 minutes either in the absence (-) or presence of IPTG (+). Cells were harvested at the indicated time points and analyzed for protein and RNA. (A) Depletion of MvaT-V-DAS4 and induction of V-SspB analyzed by Western blot. Upper panel, Western blot probed with antibody against VSV-G tag. Lower panel, Western blot probed with antibody against the α subunit of RNA polymerase serves as a control for sample loading. PAO1 $\Delta sspB$ *cupA lacZ* MvaT-V-DAS4 carrying either pPSV35 (lanes 1-3), or pV-SspB (lanes 4-6). PAO1 $\Delta sspB$ $\Delta mvaU$ *cupA lacZ* MvaT-V-DAS4 carrying either pPSV35 (lanes 7-9), or pV-SspB (lanes 10-12). (B) Effect on target gene expression of depleting MvaT in the absence and presence of MvaU. Abundance of transcripts in cells PAO1 $\Delta sspB$ $\Delta mvaU$ *cupA lacZ* MvaT-V-DAS4 ($\Delta mvaU$ strain) carrying pV-SspB relative to those carrying pPSV35 (black bars). Abundance of transcripts in cells PAO1 $\Delta sspB$ *cupA lacZ* MvaT-V-DAS4 ($mvaU^+$ strain) carrying pV-SspB relative to those carrying pPSV35 (grey bars). The indicated transcripts were quantified by qRT-PCR. The dotted line at the bottom of the graph represents transcript abundance in cells of the strains carrying the pPSV35 control vector. (C) Effect of $\Delta mvaT$ and $\Delta mvaU$ mutations on target gene expression. Abundance of transcripts in cells of PAO1 $\Delta mvaT$ *cupA lacZ* (black bars) and cells of PAO1 $\Delta mvaU$ *cupA lacZ* (grey bars) relative to those in cells of the wild-type control PAO1 *cupA lacZ*. The dotted line represents the transcript levels of PAO1 *cupA lacZ*. Error bars in (B) and (C) represent relative expression values calculated from plus or minus one standard deviation from the mean $\Delta\Delta Ct$.

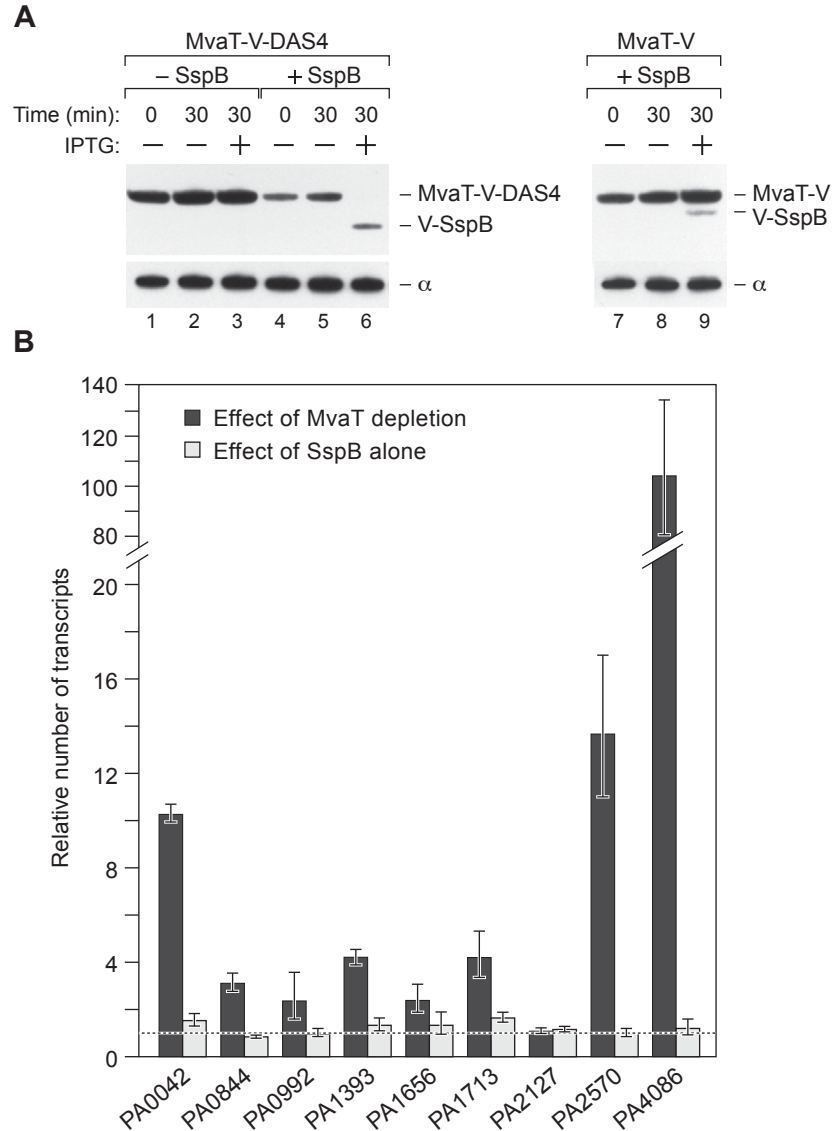


Figure 3.9. Expression of SspB without depletion of MvaT has little effect on expression of MvaT/MvaU target genes. Cells of the MvaT-V-DAS4 depletion strain or cells of the MvaT-V control strain containing plasmid pV-SspB (expressing V-SspB in an IPTG-inducible manner) or the empty control vector pPSV35, were first grown to mid-log (time 0), then grown for a further 30 minutes either in the absence (-) or presence of IPTG (+). Cells were harvested at the indicated time points and analyzed for protein and RNA. (A) MvaT-V-DAS4 but not MvaT-V becomes depleted following induction of V-SspB. Upper panel, Western blot probed with antibody against VSV-G tag detects MvaT-V-DAS4, MvaT-V, and V-SspB. Lower panel, Western blot probed with antibody against the α subunit of RNA polymerase serves as a control for sample loading. Lanes 1-3, lysates from PAO1 Δ sspB Δ mvaU cupA lacZ MvaT-V-DAS4 carrying pPSV35. Lanes 4-6, lysates from PAO1 Δ mvaU Δ sspB cupA lacZ MvaT-V-DAS4 carrying pV-SspB. Lanes 7-9, lysates from PAO1 Δ sspB Δ mvaU cupA lacZ MvaT-V carrying pV-SspB. (B) Effect on target gene expression of depleting MvaT or expressing SspB. Abundance of transcripts in cells PAO1 Δ sspB Δ mvaU cupA lacZ MvaT-V-DAS4 carrying pV-SspB relative to those carrying the empty control vector pPSV35 (black bars). Abundance of transcripts in cells PAO1 Δ sspB Δ mvaU cupA lacZ MvaT-V carrying pV-SspB relative to the depletion strain carrying the empty control vector pPSV35 (grey bars). The indicated transcripts were quantitated by qRT-PCR. The dotted line at the bottom of the graph represents transcript abundance in cells of the depletion strain carrying the pPSV35 control vector. Error bars represent the relative expression values calculated from plus or minus one standard deviation from the mean $\Delta\Delta$ Ct.

We next determined what effects the combined loss of MvaT and MvaU had on the expression of target genes, and whether the transcriptional effects of depleting MvaT were any different in the presence or absence of MvaU. To do this we quantified the transcripts of a subset of MvaT/MvaU target genes after MvaT depletion in the *mvaU*⁺ and $\Delta mvaU$ mutant cells by quantitative real time RT-PCR (qRT-PCR). The same transcripts were also quantified in control cells containing the empty vector in which MvaT was not depleted. The results depicted in Figure 3.8B show that the combined loss of MvaT and MvaU resulted in changes in target gene expression that ranged anywhere from an approximate 3-fold to 90-fold increase. These differences in magnitude presumably reflect, at least in part, differences in the affinity of MvaT/MvaU for a particular region, and/or differences in the mechanism by which the binding of MvaT/MvaU to a particular regulatory region or gene facilitates repression. Furthermore, in most cases, depletion of MvaT had a greater effect on the expression of MvaT/MvaU target genes in the absence of MvaU than in the presence of MvaU (Figure 3.8), confirming that the effects observed upon depletion of MvaT in the $\Delta mvaU$ mutant strain are dependent on the absence of MvaU. Note that the effects on gene expression observed upon depletion of MvaT in the $\Delta mvaU$ mutant cells were not simply because of an effect on growth rate as the *mvaU*⁺ cells and the $\Delta mvaU$ mutant cells grew at similar rates over the course of the experiment. An additional control revealed that the induction of V-SspB alone had little effect on the expression of the MvaT/MvaU target genes (Figure 3.9B). Comparison of the effects of removing both MvaT and MvaU (Figure 3.8B) with the effects of deleting either *mvaT* or *mvaU* (Figure 3.8C) revealed that in general the combined loss of MvaT and MvaU had a much greater effect on the expression of MvaT/ MvaU target genes than the loss of either MvaT or MvaU alone. In fact, deletion of *mvaU* had little effect on the expression of any of the MvaT/MvaU target genes tested (Figure 3.8C). Furthermore, the results depicted in Figure 3.8 B and C suggest that depletion of MvaT in the presence of MvaU tended to have less of an effect on the expression of target genes than did deletion of *mvaT* alone, suggesting that we may not

be depleting all of the available MvaT-V-DAS4, or that we may not be depleting it quickly enough to fully recapitulate the effects of deleting *mvaT*.

Discussion

We have obtained evidence that the two H-NS family members in *P. aeruginosa*, MvaU and MvaT, regulate expression of the identical set of target genes. Specifically, using ChIP-on-chip, we have found that MvaT and MvaU associate with the same regions of the chromosome. These findings provide a demonstration that, on a genome-wide level, a pair of H-NS family members functions coordinately. In addition, our analysis indicates that MvaT and MvaU preferentially associate with AT rich regions suggesting that MvaT and MvaU are involved in silencing foreign DNA elements in *P. aeruginosa*.

Coordinate activity of MvaT and MvaU in P. aeruginosa

We propose that the coordinate activity of MvaT and MvaU may allow for the integration of multiple environmental signals. If the various species of MvaT and MvaU (MvaT homomers, MvaT-MvaU heteromers, and MvaU homomers) [18] recognized certain sites with different affinities, and if the expression of *mvaT* and *mvaU* responded to different environmental signals, this would allow these signals to fine-tune the expression of the MvaT/MvaU regulon by changing the intracellular concentration and thus activity of each protein. Our findings with MvaT and MvaU suggest that H-NS and its paralogs might also function coordinately in other bacteria, a possibility that is consistent with several previous observations [10,19,30].

Using a ClpXP protease-based protein depletion system [29] that we adapted for use in *P. aeruginosa*, we found that the loss of both MvaT and MvaU from the cell resulted in loss of viability. Because neither MvaT nor MvaU on its own is essential, these findings suggest that MvaT and MvaU are, at least by one measure, functionally redundant. We propose, however, that the roles of MvaT and MvaU may not simply be to provide a backup system in the event that the gene encoding one or the other regulator is lost or in the event that the activity of either

regulator is inhibited [7]. Although the functional redundancy between MvaT and MvaU would be of obvious benefit under these circumstances, both MvaT and MvaU are evidently involved in controlling the same set of genes in WT cells, which may be their primary role.

Although we do not yet know why loss of both MvaT and MvaU results in cell death in *P. aeruginosa*, we speculate that the uncontrolled expression of a subset of MvaT/MvaU target genes is responsible. Indeed, in *Salmonella* H-NS appears to be essential; *hns* cannot be deleted in WT cells but can in cells carrying mutations in other global regulatory genes including *rpoS* and *phoP*, suggesting that the essential activity of H-NS involves repression of a gene or set of genes that are positively regulated by *rpoS* and *phoP* [3,5].

The reciprocity between MvaT and MvaU, i.e., the fact that deletion of the gene encoding one of these regulators leads to an increase in production of the other [18], likely contributes to the ability of cells to tolerate loss of MvaT or MvaU, and likely masks the individual contributions of MvaT and MvaU to the regulation of target genes. Our ChIP-on-chip analyses revealed that MvaT and MvaU were associated with both the *mvaT* and *mvaU* genes (Table 3.1 and Table 3.2), suggesting that the reciprocal effects of deleting *mvaT* and *mvaU* result from a direct repressive effect of MvaT and MvaU on the expression of their respective genes.

Xenogeneic silencing in P. aeruginosa

In enteric bacteria H-NS displays a distinct preference for AT-rich regions of DNA, and in so doing provides a mechanism for silencing xenogeneic DNA [7]; this presumably explains why H-NS plays such a prominent role in controlling the expression of virulence genes in enteric pathogens, many of which are located on pathogenicity islands or other AT-rich foreign DNA

elements [1,6,7]. Xenogeneic silencing is thought to minimize any potential fitness cost of inheriting DNA acquired from a foreign source, and at the same time facilitate integration of any inherited genes into the existing regulatory networks of the cell. Despite MvaT and MvaU being somewhat distant homologs of H-NS, we found that MvaT and MvaU display a distinct preference for binding AT-rich regions of the *P. aeruginosa* chromosome. Based on these findings, and the evidence that MvaT and MvaU function as repressors through their DNA-binding (and possible DNA-bridging activities) [17], we propose that MvaT and MvaU function coordinately as xenogeneic silencers in *P. aeruginosa*. H-NS can evidently recognize specific sites on the DNA with high affinity, and once bound to these sites oligomerize across adjacent AT-rich stretches of DNA [8]. We suggest, therefore, that if MvaT homomers, MvaT-MvaU heteromers, and MvaU homomers recognized different specific sites with high affinity, but could still form higher order oligomers with one another, this would expand the number of potential sites that could nucleate the silencing of foreign DNA. Our findings with MvaT and MvaU not only imply that MvaT paralogs are likely involved in xenogeneic silencing in other pseudomonads, they also suggest that xenogeneic silencing is mediated by H-NS family members in bacteria other than those of the enterobacteriaceae.

Materials and methods

Bacterial strains and plasmids

P. aeruginosa strains PAO1, PAO1 *cupA lacZ*, PAO1 $\Delta mvaT$ *cupA lacZ*, and PAO1 $\Delta mvaU$ *cupA lacZ* have been described previously [18]. *Escherichia coli* DH5 α F'IQ (Invitrogen) was used as the recipient strain for all plasmid constructions, whereas *E. coli* strain SM10 (λ pir) was used to mate plasmids into *P. aeruginosa*.

The deletion construct for the *sspB* gene (PA4427) was generated by amplifying regions flanking *sspB* by the PCR and then splicing the flanking regions together by overlap extension PCR. The deletion was in-frame and contained the linker sequence 5'-ATGGCGGCCGCTTAA-3'. The resulting PCR product was cloned on a *Xba*I/*Eco*RI fragment into plasmid pEXG2 [32] yielding plasmid pEXG2- Δ *sspB*. This plasmid together with recipient strains PAO1, PAO1 *cupA lacZ* and PAO1 $\Delta mvaU$ *cupA lacZ* respectively was then used to create the following strains containing in-frame deletions of *sspB* by allelic exchange: PAO1 Δ *sspB*, PAO1 Δ *sspB cupA lacZ*, and PAO1 Δ *sspB mvaU cupA lacZ*. Plasmid pEX- $\Delta mvaT$ [18] was used to create strain PAO1 Δ *sspB mvaT* containing an in-frame deletion of *mvaT* by allelic exchange. Deletions were confirmed by the PCR.

The VSVG-DAS4-tag (V-DAS4-tag) integration vector pVDIV was generated by cloning a DNA fragment containing the linker sequence 5'-AAGCTTGAATTCAGTAGTGAGCTCATATATGCGGCCGCATATACAGATATTGAAATGAATAGATTAGGAAAAGGCGCCGCCAACGACGAGAACTACAGCGAGAACTACGCCGACGCCAGCGAGGATCC-3' into pP30 Δ FRT [18] cut with *Hind*III and *Bam*HI. This linker specifies (in order) *Hind*III, *Eco*RI, *Spe*I, *Sac*I, and *Not*I restriction sites, and specifies the peptide AAAYTDIEMNRLGKGAANDENYSENADAS followed by a stop codon. The peptide consists of

a linker of three alanine residues followed by a VSV-G epitope tag, a single glycine residue and a DAS4 SspB-dependent degradation tag [29]. Plasmid pVDIV therefore carries the mobilization region from RP4 (*mob*), the ColE1 origin of replication, the *aacC1* gene (conferring resistance to gentamicin), FRT sites for efficient Flp recombinase-mediated excision, and DNA specifying the V-DAS4 tag (see Figure 3.7A). Plasmids pMvaT-VDIV and pMvaU-VDIV were made by subcloning ~300bp *HindIII/NotI* DNA fragments from pP30Δ-MvaT-TAP and pP30Δ-MvaU-TAP [18], respectively, corresponding to the 3' portion of the corresponding gene into pVDIV cut with *HindIII* and *NotI*; the portions of the *mvaT* and *mvaU* genes were cloned such that they were in-frame with the DNA specifying the V-DAS4 tag. These plasmids together with recipient strains PAO1 Δ*sspB*, PAO1 Δ*sspB* Δ*mvaT*, PAO1 Δ*sspB* *cupA lacZ*, and PAO1 Δ*sspB* Δ*mvaU* *cupA lacZ* were then used to create strains PAO1 Δ*sspB* MvaU-V-DAS4, PAO1 Δ*sspB* Δ*mvaT* MvaU-V-DAS4, PAO1 Δ*sspB* *cupA lacZ* MvaT-V-DAS4, and PAO1 Δ*sspB* Δ*mvaU* *cupA lacZ* MvaT-V-DAS4 by integration of the plasmid into the chromosome [18] followed by backbone excision by transient synthesis of FLP recombinase from plasmid pFLP2 [34].

The MvaT-V integration vector pP30ΔFRT-MvaT-V was generated by cloning a PCR-amplified DNA fragment containing ~300 bp of sequence from the 3' portion of the *mvaT* gene followed by a *NotI* site, and sequence 5'-ATATACAGATATTGAAATGAATAGATTAGGAAAA-3' encoding a VSV-G epitope tag and a stop codon into pP30ΔFRT [18] cut with *HindIII* and *BamHI*. Plasmid pP30ΔFRT-MvaU-V was made by subcloning the ~300 bp fragment corresponding to the 3' portion of the *mvaU* gene from pMvaU-VDIV into pP30ΔFRT-MvaT-V cut with *HindIII* and *NotI*. Plasmids pP30ΔFRT-MvaT-V and pP30ΔFRT-MvaU-V together with recipient strains PAO1 Δ*sspB* Δ*mvaU* *cupA lacZ* and PAO1 Δ*sspB* Δ*mvaT* were used to make strains PAO1 Δ*sspB* Δ*mvaU* *cupA lacZ* MvaT-V and PAO1 Δ*sspB* Δ*mvaT* MvaU-V, by integration of the appropriate plasmid into the chromosome [18] followed by backbone excision through transient synthesis of FLP recombinase from plasmid pFLP2 [34]. Production of the

MvaT-V and MvaU-V proteins was confirmed by Western blotting with an anti-VSV-G antibody (Sigma-Aldrich).

To make expression plasmid pPSPK-SspB, a DNA fragment containing the *P. aeruginosa sspB* coding sequence (PA4427) flanked 5' by a *Pst*I site and a consensus Shine-Dalgarno sequence and flanked 3' by a *Hind*III site was generated by the PCR, digested with *Hind*III and *Pst*I and cloned into pPSV37 (Arne Rietsch, Case Western Reserve University) cut with *Hind*III and *Pst*I to generate plasmid pPSV37-SspB. Then, the P_{spank} promoter region from plasmid pdr110 (David Rudner, Harvard Medical School, Boston) was amplified by the PCR so as to contain a *Dra*III site 5' of the *lacO2* sequence and a synthetic polylinker containing (in order) *Hind*III, *Sall*, *Nhe*I, *Bam*HI, *Xma*I, *Kpn*I, and *Sph*I sites 3' of the transcription start site and *lacO1* sequence. This DNA fragment was cloned into pPSV37-SspB cut with *Dra*III and *Bam*HI to generate plasmid pPSPK-SspB, which directs the isopropyl-b-D-thiogalactopyranoside (IPTG)-inducible synthesis of the *P. aeruginosa* SspB protein from the P_{spank} promoter and confers resistance to gentamicin. The pV-SspB plasmid was made by cloning a PCR-amplified DNA fragment specifying a consensus Shine-Dalgarno sequence and *P. aeruginosa* SspB with a VSV-G tag fused to its N-terminus (V-SspB) into pPSV35 [32] cut with *Pst*I-*Hind*III. Plasmid pV-SspB therefore directs the IPTG-inducible synthesis of V-SspB and confers resistance to gentamicin.

Plasmids pP30 Δ FRT-MvaT-V and pP30 Δ FRT-MvaU-V together with recipient strains PAO1 and PAO1 Δ *mvaT* were used to make strains PAO1 MvaT-V, PAO1 MvaU-V, PAO1 Δ *mvaT* MvaU-V, by integration of the appropriate plasmid into the chromosome [18] followed by backbone excision through transient synthesis of FLP recombinase from plasmid pFLP2 [34]. Production of the MvaT-V and MvaU-V proteins was confirmed by Western blotting with an anti-VSV-G antibody (Sigma). Strain PAO1 MvaT-TAP synthesizing a TAP-tagged form of MvaT has

been described previously [18]. Plasmid pP30Δ-PA2896-TAP-FRT was generated by cloning an ~300-bp fragment of DNA corresponding to a 3' portion of the PA2896 gene into *HindIII-NotI* cut pP30ΔFRT-TAP; the portion of the PA2896 gene was cloned such that it was in-frame with the DNA specifying the TAP-tag. The control strain PAO1 PA2896-TAP synthesizing a TAP-tagged form of the putative s factor PA2896 was made by integration of the PA2896-TAP-FRT plasmid into the chromosome [18]. Synthesis of the PA2896-TAP fusion was confirmed by Western blotting with PAP [18].

Chromatin Immunoprecipitation (ChIP)

Cultures were inoculated at a starting OD₆₀₀ of ~0.05 and grown with aeration to an OD₆₀₀ of ~0.6 at 37 °C in LB. Formaldehyde was added to 20 ml of culture to a final concentration of 1% and samples were incubated at room temperature for 30 minutes. To quench the cross-linking reaction, glycine was added to a final concentration of 250 mM and followed by a 15 minute incubation at room temperature. Cell pellets were washed three times with 1X PBS and were then resuspended in lysis buffer (20 mM KHEPES, pH 7.9; 50 mM KCl; 0.5 mM DTT; 10% glycerol; Roche Protease Inhibitor Cocktail) and chromosomal DNA was sheared by sonication to an average size of 0.5-1 Kb. After the removal of cell debris by centrifugation, 50 ml of each sample was removed to serve as an input control. The immunoprecipitation (IP) reaction was initiated by the addition of α-VSV-G agarose beads (BETHYL laboratories, Inc.) for VSV-G-tagged proteins, or IgG Sepharose beads (GE Healthcare) for TAP-tagged proteins, to each of the remaining samples. Following incubation at 4°C overnight, beads were pelleted and washed five times with buffer IPP50 (10 mM Tris-HCl, pH 8; 150 mM NaCl; 0.1% NP40) and washed twice with TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8). Beads were resuspended in elution buffer (50 mM Tris-HCl, pH 8; 10 mM EDTA, pH 8; 1% SDS), and immunoprecipitated complexes were removed from the beads by

incubation at 65°C for 15 minutes. The recovered supernatants were placed at 65°C overnight to reverse the cross-links. The input samples were also incubated at 65°C overnight following the addition of 200 ml of TE with 1% SDS.

Quantitative PCR

qPCR was performed by using iTaq SYBR green with ROX (BioRad) and an Applied Biosystems StepOnePlus detection system. ChIP fold enrichment values were calculated as described [31] and represent the relative abundance of a sequence of interest compared with a negative control region. All ChIP fold enrichment values represent the average of three biological replicates, except for those reported in Figure 3.3B which are the values obtained from a single experiment.

ChIP-on-chip

For ChIP-on-chip experiments, high-density oligonucleotide arrays corresponding to the *P. aeruginosa* PAO1 genome were designed and manufactured by Roche NimbleGen, Inc (<http://www.nimblegen.com>). Each array contains 385,382 probes (50 mers) tiled ~20 bp apart. These probes span the entire genome on both the plus and minus strands of the DNA and provided almost complete sequence coverage of the PAO1 genome. Prior to array hybridization, ChIP DNA was amplified using the Invitrogen BioPrime Array CGH kit. Resulting sample DNA was labeled and hybridized by Roche NimbleGen, Inc. Input and experimental DNA samples were labeled with Cy3 and Cy5 respectively and were co-hybridized to arrays, which were subsequently scanned at 5 µm resolution using the GenePix 4000B scanner (Axon Instruments). Raw fluorescence intensity data were obtained from scanned images using NIMBLESCAN™ 2.0 extraction software (Roche NimbleGen). The log₂-ratio of the Cy5-labeled experimental sample versus the Cy3-labeled input sample was calculated for each spot on the

array. The log₂-ratio values for each array were centered around zero by subtracting the bi-weight mean log₂-ratio from each individual value. All ChIP-on-chip experiments were done in triplicate.

Array Data Analysis

To control for any non-specific enrichment, the log₂ ratio values from the mock VSV-G ChIP-on-chip experiments were averaged across the three replicate arrays, resulting in an average background log₂ for each probe. These values were then subtracted from experimental arrays prior to further data analysis. A custom R-language program written by Flavell *et al.*, [35] was used to isolate “peaks” of enrichment of the experimental sample relative to the input sample. This program utilized a fixed percentile cut-off method, which requires at least four consecutive probes to be enriched above a given threshold in order for a region to be considered a peak. The threshold level that yielded the largest number of peaks, while still minimizing the number of peaks not present in all three replicate arrays was used in the final analysis. The MvaT-V arrays were analyzed using a 95% threshold, and the MvaU-V threshold was set at 97%. Following this analysis, peaks that were not present in all three replicate arrays were discarded. A java program was used to obtain annotated PAO1 genes that fell within or near (150 bp on either side) isolated peaks. The max log₂ value for each peak in each array was determined using a Perl script. All the max log₂ values we report are averaged across three replicate arrays. To compare PAO1 genome GC content to ChIP-on-chip log₂ values, a Perl script (derived from a script from Navarre *et al.*, [5]) was used. This script calculates both the average GC content and the average log₂ value for 1000 bp windows, with a 500 bp step size, across the PAO1 genome.

Depletion Experiments on LB-agar plates

To demonstrate synthetic lethality of MvaT and MvaU by depletion, the indicated plasmids were introduced into the indicated strains (Figure 3.7B) by electroporation. Colonies of plasmid-containing cells were selected on LB agar containing gentamicin (30 mg/ml) and resuspended in liquid LB media to an OD₆₀₀ of 0.01 (i.e. 10^{-2}). Ten-fold serial dilutions of cells (20 ml of each) were spotted onto LB agar plates containing gentamicin (30 mg/ml) with or without IPTG (2 mM). Plates were incubated overnight at 37°C prior to being photographed.

RNA isolation and expression analysis

For all experiments duplicate cultures were grown from separate single colonies, and all experiments were repeated at least three times on separate occasions. RNA isolation and cDNA synthesis were essentially as described previously for *P. aeruginosa* [36]. Transcript quantities were determined relative to the amount of *clpX* transcript by qRT-PCR with the iTaq SYBR Green kit (Bio-Rad) and an Applied Biosystems StepOnePlus detection system. The specificity of the PCR primers was verified by melting curve analyses. Relative expression values were calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) as described previously [37] and are the average of two biological replicates. Error bars represent the relative expression values calculated from plus or minus one standard deviation from the mean $\Delta\Delta Ct$. Representative data sets are shown.

Western Blots

Equal numbers of cells were lysed and separated by SDS-PAGE on 12% Bis-Tris NuPAGE gels (Invitrogen) in MES running buffer and transferred to nitrocellulose using the iBlot dry blotting system (Invitrogen). Membranes were blocked with 25 ml of SuperBlock Blocking Buffer (Pierce) in TBS with 0.1% Surfact-Amps 20 (Pierce). Membranes were then probed with

monoclonal antibodies against the VSV-G-tag (Sigma-Aldrich) or the α subunit of RNA polymerase (NeoClone). Proteins were detected using either goat polyclonal anti-rabbit IgG conjugated with horseradish peroxidase or goat polyclonal anti-mouse IgG conjugated with horseradish peroxidase (Pierce), and visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

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References

1. Dorman CJ (2004) H-NS: a universal regulator for a dynamic genome. *Nat Rev Microbiol* 2: 391–400.
2. Grainger DC, Hurd D, Goldberg MD, Busby SJW (2006) Association of nucleoid proteins with coding and non-coding segments of the *Escherichia coli* genome. *Nucleic Acids Res* 34: 4642–4652.
3. Lucchini S, Rowley G, Goldberg MD, Hurd D, Harrison M, et al. (2006) H-NS mediates the silencing of laterally acquired genes in bacteria. *PLoS Pathog* 2: e81.
4. Oshima T, Ishikawa S, Kurokawa K, Aiba H, Ogasawara N (2006) *Escherichia coli* histone-like protein H-NS preferentially binds to horizontally acquired DNA in association with RNA polymerase. *DNA Res* 13: 141–153.
5. Navarre WW, Porwollik S, Wang Y, McClelland M, Rosen H, et al. (2006) Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*. *Science* 313: 236–238.
6. Dorman CJ (2007) H-NS, the genome sentinel. *Nat Rev Microbiol* 5: 157–161.
7. Navarre WW, McClelland M, Libby SJ, Fang FC (2007) Silencing of xenogeneic DNA by H-NS—facilitation of lateral gene transfer in bacteria by a defense system that recognizes foreign DNA. *Genes Dev* 21: 1456–1471.
8. Bouffartigues E, Buckle M, Badaut C, Travers A, Rimsky S (2007) H-NS cooperative binding to high-affinity sites in a regulatory element results in transcriptional silencing. *Nat Struct Mol Biol* 14: 441–448.
9. Tendeng C, Bertin PN (2003) H-NS in Gram-negative bacteria: a family of multifaceted proteins. *Trends Microbiol* 11: 511–518.
10. Müller CM, Dobrindt U, Nagy G, Emödy L, Uhlin BE, et al. (2006) Role of histone-like proteins H-NS and StpA in expression of virulence determinants of uropathogenic *Escherichia coli*. *J Bacteriol* 188: 5428–5438.
11. Govan JRW, Deretic V (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* 60: 539–574.
12. Diggle SP, Winzer K, Lazdunski A, Williams P, Cámara M (2002) Advancing the quorum in *Pseudomonas aeruginosa*: MvaT and the regulation of N-acylhomoserine lactone production and virulence gene expression. *J Bacteriol* 184: 2576–2586.
13. Vallet I, Diggle SP, Stacey RE, Cámara M, Ventre I, et al. (2004) Biofilm formation in *Pseudomonas aeruginosa*: fimbrial *cup* gene clusters are controlled by the transcriptional regulator MvaT. *J Bacteriol* 186: 2880–2890.
14. Westfall LW, Carty NL, Layland N, Kuan P, Colmer-Hamood JA, et al. (2006) *mvaT* mutation modifies the expression of the *Pseudomonas aeruginosa* multidrug efflux operon *mexEF-oprN*. *FEMS Microbiol Lett* 255: 247–254.

15. Tendeng C, Soutourina OA, Danchin A, Bertin PN (2003) MvaT proteins in *Pseudomonas* spp.: a novel class of H-NS-like proteins. *Microbiology* 149: 3047–3050.
16. Rescalli E, Saini S, Bartocci C, Rychlewski L, De Lorenzo V, et al. (2004) Novel physiological modulation of the *Pu* promoter of TOL plasmid. *J Biol Chem* 279: 7777–7784.
17. Dame RT, Luijsterburg MS, Krin E, Bertin PN, Wagner R, et al. (2005) DNA bridging: a property shared among H-NS-like proteins. *J Bacteriol* 187: 1845–1848.
18. Vallet-Gely I, Donovan KE, Fang R, Joung JK, Dove SL (2005) Repression of phase-variable *cup* gene expression by H-NS-like proteins in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 102: 11082–11087.
19. Baehler E, de Werra P, Wick LY, Péchy-Tarr M, Mathys S, et al. (2006) Two novel MvaT-like global regulators control exoproduct formation and biocontrol activity in root-associated *Pseudomonas fluorescens* CHAO. *Mol Plant Microbe Interact* 19: 313–329.
20. Mathee K, Narasimhan G, Valdes C, Qiu X, Matewish JM, et al. (2008) Dynamics of *Pseudomonas aeruginosa* genome evolution. *Proc Natl Acad Sci USA* 105: 3100–3105.
21. Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G, et al. (2001) Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 183: 6454–6465.
22. Schuster M, Greenberg EP (2006) A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. *Int J Med Microbiol* 296: 73–81.
23. Wilderman PJ, Vasil AI, Johnson Z, Vasil ML (2001) Genetic and biochemical analyses of a eukaryotic-like phospholipase D of *Pseudomonas aeruginosa* suggest horizontal acquisition and a role for persistence in a chronic pulmonary infection model. *Mol Microbiol* 39: 291–303.
24. Yahr T, Wolfgang MC (2006) Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. *Mol Microbiol* 62: 631–640.
25. Mougous JD, Cuff ME, Raunser S, Shen A, Zhou M, et al. (2006) A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* 312: 1526–1530.
26. Vallet-Gely I, Sharp JS, Dove SL (2007) Local and global regulators linking anaerobiosis to *cupA* fimbrial gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* 189: 8667–8676.
27. Nagarajavel V, Madhusudan S, Dole S, Rahmouni AR, Schnetz K (2007) Repression by binding of H-NS within the transcription unit. *J Biol Chem* 282: 23622–23630.
28. Dole S, Nagarajaval V, Schnetz K (2004) The histone-like nucleoid structuring protein H-NS represses the *Escherichia coli* *bgl* operon downstream of the promoter. *Mol Microbiol* 52: 589–600.
29. McGinness KE, Baker TA, Sauer RT (2006) Engineering controllable protein degradation. *Mol Cell* 22: 701–707.
30. Beloin C, Deighan P, Doyle M, Dorman CJ (2003) *Shigella flexneri* 2a strain 2457T expresses three members of the H-NS-like protein family: characterization of the Sfh protein. *Mol Genet Genomics* 270: 66–77.

31. Aparicio O, Geisberg JV, Struhl K (2004) in *Current Protocols in Molecular Biology*, eds Ausubel FA, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (Wiley, New York), pp 21.3.1–21.3.17.
32. Rietsch A, Vallet-Gely I, Dove SL, Mekalanos JJ (2005) ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 102: 8006–8011.
34. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP (1998) A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212: 77–86.
35. Flavell SW, Kim TK, Gray JM, Harmin DA, Hemberg M, et al. (2008) Genome-wide analysis of MEF2 transcriptional program reveals synaptic target genes and neuronal activity-dependent polyadenylation site selection. *Neuron* 60: 1022–1038
36. Wolfgang MC, Lee VT, Gilmore ME, Lory S (2003) Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. *Dev Cell* 4: 253–263.
37. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25: 402–408.

Chapter 4

**Identification of a small regulatory RNA that controls *cupA* fimbrial gene
expression in *Pseudomonas aeruginosa***

Attributions

This chapter describes a project that began with a transposon mutant screen that was conducted before my arrival in the lab of Simon L. Dove by Isabelle Vallet-Gely, a former postdoctoral fellow. Beyond the initial implication of *mexT* and *mexF* in regulation of the *cupA* genes (see below), all work reported herein was performed by myself with extensive guidance from Dr. Dove. I also wrote the chapter itself, again with extensive guidance and editing from Dr. Dove. The results reported here have not been published in any peer-reviewed journal, yet they have been presented as posters at departmental conferences and as seminars to research groups I visited during the summer of 2011.

Abstract

The *cupA* genes of the opportunistic pathogen *Pseudomonas aeruginosa* encode structural components and assembly factors of a chaperone-ushe fimbrial structure that allows the organism to adhere to abiotic surfaces. While normally repressed under standard laboratory growth conditions, these genes are expressed in a phase-variable manner in cells grown in oxygen-limiting conditions or in cells lacking the H-NS family member MvaT. MvaT is known to function coordinately with MvaU, another H-NS family member in *P. aeruginosa*, to repress the expression of several hundred target genes. In this work we identify a small regulatory RNA (sRNA) that is required for expression of the *cupA* genes. We report that this sRNA, which we have named PrrA (*Pseudomonas* RNA regulator of *cupA*), is encoded by a previously unannotated gene located directly downstream of the *mexEFoprN* multidrug efflux pump genes. Using genetic analysis, we show that the *mexE* promoter, whose activity is highly positively regulated by MexT, is required for expression of *prpA*, and that MexT-driven expression of *prpA* is required for phase-variable expression of the *cupA* genes. Finally, we report that ectopic expression of *prpA* in cells lacking MvaT results in the derepression of many genes, including *cupA*, that are normally repressed by MvaU, suggesting that PrrA is a negative regulator of this nucleoid-associated protein. This is the first report of sRNA-mediated regulation of fimbrial gene expression in *P. aeruginosa*, and possibly the first description of an sRNA that relieves silencing mediated by a H-NS family member in this opportunistic pathogen.

Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen of humans and is the main cause of morbidity and mortality in patients with cystic fibrosis (CF) [1]. Infections of *P. aeruginosa* in the CF lung are notoriously difficult to treat due to its high intrinsic level of antibiotic resistance, and the organism is thought to contribute to the decline of lung function that eventually leads to death. In the CF lung, *P. aeruginosa* is thought to exist in a biofilm, enclosed in a polymeric matrix consisting of polysaccharides, proteins and extracellular DNA [2]. Central to this lifestyle is the ability of the organism to adhere to surfaces including the lung epithelium, which is achieved largely through adherence factors such as fimbriae. The genome of *P. aeruginosa* contains up to five chaperone-usher fimbrial gene clusters [3-5]. One of these, the *cupA* gene cluster, encodes structural and assembly factors of a fimbrial structure that allows the bacterium to form biofilms on abiotic surfaces in the absence of the type IV pilus [3,6]. Interestingly, expression of the *cupA* fimbrial genes is induced in oxygen-limiting conditions such as those thought to occur in regions of the CF lung, which may contribute to the increase in biofilm growth observed in these conditions [7-9].

Expression of the *cupA* fimbrial gene cluster is tightly regulated and is not observed in wild-type cells grown under standard laboratory conditions. The H-NS family members MvaT and MvaU are thought to repress *cupA* expression both directly and indirectly; MvaT and MvaU occupy the *cupA* promoter region and MvaT has been shown to repress expression of the *cgrABC* genes, which are located immediately upstream of the *cupA* genes and encode regulators that are required for *cupA* expression [7,10-12]. In particular, the *cgrA* and *cgrC* genes encode proteins that form a complex that, along with the *cgrB* gene product, positively regulates expression of the *cupA* genes [7,13]. The CgrABC proteins do not resemble any classical positive regulators of transcription, and their mechanism of action, outside of the fact

that an interaction between the CgrA and CgrC proteins is required for *cupA* gene expression, remains unknown. It has also been shown that Anr, a global regulator of anaerobic nitrate respiration, positively regulates the *cupA* genes, likely also through its effects on the *cgrABC* genes [7]. Interestingly, when expression of the *cupA* fimbrial genes is induced either during growth in oxygen-limiting conditions or in cells lacking MvaT, they are expressed in a bistable or phase-variable manner (i.e. expressed in a reversible ON/OFF fashion) [7,12]. Thus, regulation of *cupA* fimbrial gene expression involves several global and local regulators that converge to effect a complex pattern of expression.

In addition to their role as repressors of the *cupA* genes, the H-NS family members MvaT and MvaU are thought to be involved in the repression of approximately 350 genes across the genome of *P. aeruginosa*, including several virulence-associated genes such as *exsA*, which encodes a regulator of type III secretion genes, and *aprE*, which encodes a component of the alkaline protease secretion machinery [10,11]. Like H-NS family members of other bacteria, most notably the enterobacteriaceae, MvaT and MvaU are found preferentially associated with AT-rich regions of the DNA [14-16]. It is possible that, like H-NS, MvaT and MvaU bind to a specific DNA sequence and oligomerize with high affinity across adjacent regions of the DNA [10,17-20]. Consistent with this idea, the ability of MvaT to form oligomers of a complexity higher than that of a dimer is required in order for this protein to function as a repressor [17]. We have previously reported that MvaT and MvaU occupy essentially the same regions of the chromosome, and that the ability of these proteins to repress expression of genes encompassing or surrounding many of these regions is partially or completely redundant (i.e. in the absence of one of the proteins, the partner protein can function as a repressor in its stead) [10]. When both MvaT and MvaU are absent, the genes with which they normally associate, including the *cupA* gene cluster, are massively derepressed, and cell growth or survival is

impaired. Thus, the overlapping functions of the partner H-NS family members MvaT and MvaU in *P. aeruginosa* appear to be essential for viability of the organism.

In this work, we identify MexT, a positive regulator of the *mexEFoprN* multidrug efflux pump genes, as a positive regulator of the *cupA* fimbrial genes. We show that MexT exerts its effect on the *cupA* genes by positively regulating the expression of a novel small regulatory RNA (sRNA) that is in turn required for *cupA* gene expression. We have named this sRNA PrrA (*Pseudomonas* RNA regulator of *cupA*) and show that the *prpA* gene is located directly downstream of the *mexEFoprN* multidrug efflux pump genes as part of the *mexEFoprN* operon. Finally, we present evidence suggesting that PrrA regulates the *cupA* genes by functioning as a negative regulator of the H-NS family member MvaU. Our findings suggest that transcription activators can exert positive effects on gene expression by activating the expression of sRNAs that relieve repression exerted by H-NS family members. Furthermore, this work outlines a novel link between a multidrug efflux system and an adherence factor in an opportunistic pathogen known for a high degree of resistance to antibiotics and robust biofilm formation.

Results

MexT is a positive regulator of cupA gene expression

In an attempt to identify factors that positively regulate the *cupA* genes in the absence of the H-NS family member MvaT, we mutagenized strain PAO1 $\Delta mvaT$ *cupA lacZ* by random transposon mutagenesis and screened for mutants that exhibited reduced expression of the *cupA lacZ* reporter. To conduct this screen, phase-OFF (i.e. white) colonies grown from a transposon insertion library were restreaked as patches on LB agar supplemented with X-Gal. This patching step allowed us to distinguish between colonies from cells that carried a transposon insertion in a positive regulator of the *cupA* genes and colonies from phase-OFF cells which carried a transposon insertion in a region that does not influence *cupA* gene expression; cells in which the transposon had interrupted a positive regulator of the *cupA* genes grew as white patches, whereas parental-type cells grew as pale blue patches. Using this approach, we isolated two mutants that exhibit reduced expression of the *cupA* genes, and found by sequencing transposon-adjacent DNA that these mutants carry transposon insertions in the *mexT* and *mexF* genes (data not shown). The *mexT* gene encodes a LysR-type transcription regulator that binds to a specific DNA sequence found upstream of the promoters of several target genes [21]. One regulatory target of MexT is the *mexEFoprN* operon, which is positively regulated by MexT and located immediately downstream of the *mexT* gene and encodes an RND-type efflux pump. The MexEF-OprN efflux pump is known to confer a high degree of resistance to several antibiotics, including chloramphenicol, trimethoprim and ciprofloxacin, likely through its activity as a small molecule efflux pump [22].

To determine whether the phenotype observed in the *mexT* insertion mutant was due to loss of the *mexT* gene product itself, we constructed an unmarked, in-frame deletion of the *mexT* gene in strain PAO1 $\Delta mvaT$ *cupA lacZ*. Compared to the parental strain, this $\Delta mexT$

strain exhibits constitutively low-level expression of the *cupA lacZ* reporter when carrying an empty expression vector, pPSV38 (Figure 4.1B). When $\Delta mexT$ cells are transformed with a plasmid, pMexT, that directs the expression of *mexT* from a heterologous promoter, phase-variable expression of the *cupA lacZ* reporter is restored, suggesting that MexT is indeed a positive regulator of *cupA* gene expression. Cultures derived from these $\Delta mexT$ mutant colonies exhibit similar phenotypes when analyzed by β -galactosidase assay, suggesting that this phenotype is exhibited in liquid culture as well (Figure 4.1C). To test whether this phenotype is also observed at the single cell level, a construct in which the *cupA* promoter region drives expression of a *GFP-lacZ* reporter was introduced into strain PAO1 $\Delta mvaT$ at the Φ CTX attachment site. Cells carrying this reporter construct only exhibit bistable expression of the reporter in the presence of MexT (Figure 4.1D). Finally, to test whether MexT was a positive regulator of the *cupA* genes in wild-type cells (i.e. in *mvaT*⁺ cells), we grew strains either with or without the *mexT* deletion carrying the *cupA lacZ* reporter in oxygen-limiting conditions, which normally induces expression of the *cupA* genes [7]. Cells lacking MexT do not express the *cupA lacZ* reporter to wild-type levels in these conditions, and this defect can be complemented by expressing the *mexT* gene in trans (Figure 4.1E). Taken together, these results suggest that MexT is a positive regulator of the *cupA* genes in both cells lacking MvaT and in wild-type cells grown in oxygen-limiting conditions.

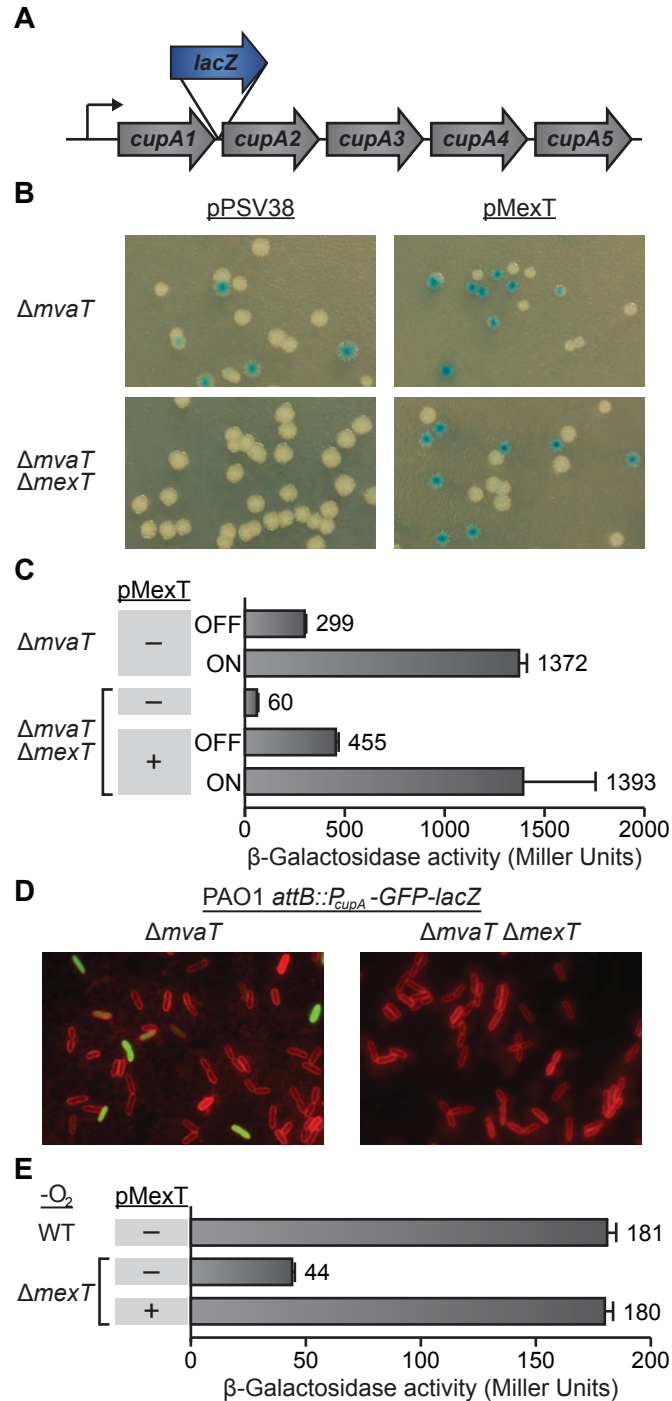


Figure 4.1. MexT is a positive regulator of the *cupA* fimbrial genes. (A) Schematic of the *cupA* operon in all *cupA lacZ* reporter strains. (B) Phenotypes of PAO1 $\Delta mvaT$ *cupA lacZ* and PAO1 $\Delta mvaT \Delta mexT$ *cupA lacZ* reporter strains carrying the indicated plasmids when plated on LB agar containing X-Gal. (C) Quantification of *cupA lacZ* expression in cultures of the reporter strains in B, carrying either pPSV38 (-) or pMexT (+). Cultures were inoculated with either phase-ON (blue) or phase-OFF (white) colonies when applicable. (D) Fluorescence micrographs of cells of the PAO1 $\Delta mvaT$ *attB::P_{cupA}-GFP-lacZ* and PAO1 $\Delta mvaT \Delta mexT$ *attB::P_{cupA}-GFP-lacZ* reporter strains stained with the membrane dye FM4-64FX. (E) Quantification of *cupA lacZ* expression in anaerobic cultures of the PAO1 *cupA lacZ* and the PAO1 $\Delta mexT$ *cupA lacZ* reporter strains, carrying either pPSV38 (-) or pMexT (+).

The mexE promoter is required for cupA expression

MexT is a positive regulator of the *mexEFoprN* operon, which encodes a tripartite multidrug efflux pump (Figure 4.2A) [22,23]. Both the *mexT* and *mexF* genes were identified in our transposon mutagenesis screen for positive regulators of *cupA* gene expression. We therefore tested the possibility that the MexEF-OprN pump itself was required for *cupA* gene expression. To do this, we constructed an in-frame, unmarked, deletion of the *mexEFoprN* operon in PAO1 $\Delta mvaT$ *cupA lacZ* and tested whether cells of the resulting strain expressed the *cupA lacZ* reporter. Cells of the $\Delta mexEFoprN$ strain express the *cupA lacZ* reporter in liquid culture to a similar degree as in the parental strain (Figure 4.2B). When plated on LB agar with X-Gal, cells of the $\Delta mexEFoprN$ strain give rise to both phase-ON and phase-OFF colonies much like cells of the parental strain (data not shown). These results suggest that the *mexEFoprN* genes do not influence expression of the *cupA* genes, and that MexT exerts its effect on *cupA* expression through another mechanism.

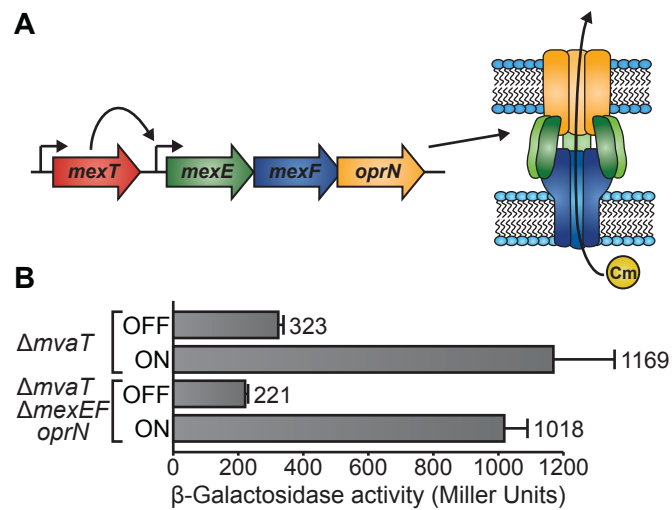


Figure 4.2. Deletion of the genes encoding the MexEF-OprN multidrug efflux pump has no effect on *cupA* fimbrial gene expression. (A) Schematic showing positive regulation of the *mexEFoprN* multidrug efflux pump genes by MexT. (B) Quantification of *cupA lacZ* expression in cultures of the PAO1 $\Delta mvaT cupA lacZ$ and the PAO1 $\Delta mvaT \Delta mexEFoprN cupA lacZ$ reporter strains. Cultures were inoculated with either phase-ON or phase-OFF colonies.

In the course of investigating the potential role of the MexEF-OprN pump in *cupA* gene regulation we introduced an in-frame, unmarked, deletion of the entire *mexTEFoprN* locus to strain PAO1 $\Delta mvaT$ *cupA lacZ*, creating strain PAO1 $\Delta mvaT \Delta mexTEFoprN$ *cupA lacZ* (Figure 4.3A). As expected, cells of this strain exhibit reduced expression of the *cupA* genes, presumably due to the absence of MexT. However, unlike cells of the PAO1 $\Delta mvaT \Delta mexT$ *cupA lacZ* reporter strain in which *mexT* was deleted, phase-variable expression of the *cupA* genes could not be restored to cells of the PAO1 $\Delta mvaT \Delta mexTEFoprN$ *cupA lacZ* reporter strain by a plasmid that expresses *mexT* from a heterologous promoter (Figure 4.3B). Taken together with our finding that the *mexEFoprN* genes themselves are not required for *cupA* gene expression, this result suggests a role for the MexT-regulated *mexE* promoter in positively regulating the *cupA* genes. To test this hypothesis, we constructed a deletion of 40 basepairs (bps) of sequence spanning the MexT-binding site to immediately upstream of the *mexEFoprN* transcription start site [21,24]. This deletion, which we termed ΔP_{mexE} , was then introduced into strain PAO1 $\Delta mvaT \Delta mexEFoprN$ *cupA lacZ* in order to test whether the *mexE* promoter had a role in regulating the *cupA* genes independent of its known role in regulating the *mexEFoprN* genes. Cells of this strain exhibit reduced expression of the *cupA* genes, suggesting that the *mexE* promoter, but not the *mexEFoprN* genes themselves, is required for *cupA* fimbrial gene expression (Figure 4.3C).

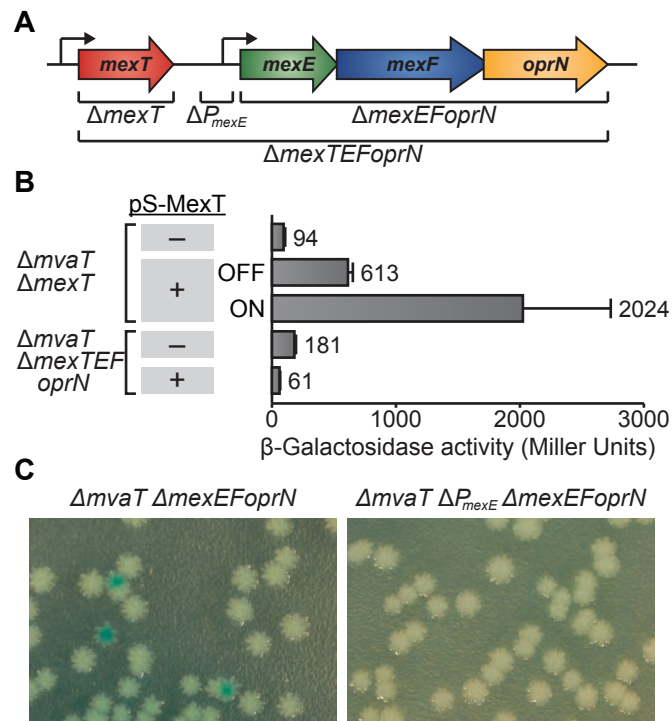


Figure 4.3. The *mexE* promoter is required for *cupA* gene expression. (A) Schematic showing the deletion mutations engineered and tested. (B) Quantification of *cupA lacZ* expression in cultures of the PAO1 $\Delta mvaT \Delta mexT$ *cupA lacZ* and the PAO1 $\Delta mvaT \Delta mexTEFoprN$ *cupA lacZ* reporter strains, carrying either pPSPK (-) or pS-MexT (+). (C) Phenotypes of colonies of the PAO1 $\Delta mvaT \Delta mexEFoprN$ *cupA lacZ* and the PAO1 $\Delta mvaT \Delta P_{mexE} \Delta mexEFoprN$ *cupA lacZ* reporter strains when plated on LB agar containing X-Gal.

A polar mutation immediately after oprN reduces cupA gene expression

The *mexEFoprN* genes are arranged in an operon immediately downstream of the *mexE* promoter. Because the *mexE* promoter is required for *cupA* gene expression in a *mexEFoprN*-independent manner, we hypothesized that there is an unannotated element present downstream of *oprN* produced from the same transcript that is a positive regulator of the *cupA* genes. To begin to test this hypothesis, we constructed a plasmid, pT1000, based on the allelic exchange vector pEXG2 that is designed to reversibly integrate two strong transcription terminators at any locus in the genome [25]. Sequence homologous to the region of the genome after which transcription termination is desired is cloned into pT1000 immediately upstream of the two terminators, and serves to “target” integration of the vector to the desired locus. Upon introduction of the plasmid by conjugation and selection on gentamicin, a primary integrant is generated that carries transcription terminators immediately downstream of the inserted sequence (Figure 4.4A). Importantly, this “targeting” sequence is duplicated downstream of the vector backbone sequence after homologous recombination, thus preserving any potential cis-regulatory sequences required for the function of downstream elements. The vector can subsequently be excised by growing cells on LB agar supplemented with sucrose to ensure any observed phenotype is linked to vector integration. We used this plasmid to test whether termination of transcription immediately after *oprN* can affect *cupA* gene expression.

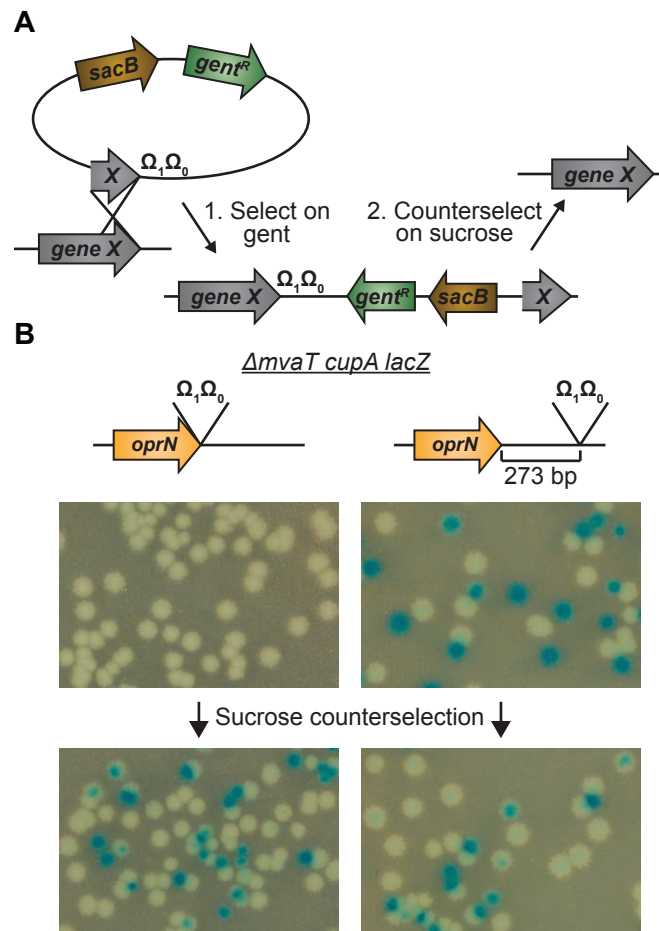


Figure 4.4. Transcription immediately downstream of *oprN* is required for *cupA* gene expression. (A) Schematic of pT1000-mediated reversible insertion of transcription terminators. (B) Phenotypes of PAO1 $\Delta mvaT cupA lacZ$ reporter strains with pT1000 inserted immediately or 273 bps downstream of *oprN* and derivatives of these strains with pT1000 removed by sucrose counterselection when plated on LB agar containing X-Gal.

To test whether transcription of the region immediately downstream of *oprN* is required for *cupA* gene expression, we constructed two versions of pT1000 that insert terminators either immediately downstream of the 3' end of *oprN* or 273 bps downstream of the 3' end of *oprN*. These two plasmids were introduced into strain PAO1 $\Delta mvaT$ *cupA lacZ*. When the terminator array is introduced immediately downstream of *oprN*, phase-variable *cupA* gene expression is not observed. However, when the terminator array is introduced 273 bps further downstream of *oprN*, phase-variable expression of *cupA* remains unaffected (Figure 4.4B). Subsequent growth of these strains on sucrose to counterselect against the terminator vector restores the parental phenotype, indicating that the loss in observable *cupA* gene expression is linked to insertion of the transcription terminators immediately downstream of *oprN*. This result supports the hypothesis that a cotranscribed element immediately downstream of the *mexEFoprN* genes is a positive regulator of the *cupA* genes.

The sRNA PrrA is encoded immediately downstream of oprN

The results outlined above suggest that an unannotated element is encoded within 273 bps beyond the 3' end of *oprN*. Computational analysis of this region identifies sequence encoding a potential Rho-independent transcription terminator spanning from 39 to 75 bps downstream of the *oprN* stop codon (data not shown). Thus, we hypothesized that any RNA species produced from the *mexE* promoter would likely extend no further than 75 bases beyond the *oprN* stop codon. Examination of the sequence between the 3' end of *oprN* and this predicted terminator suggests that it is unlikely to encode a protein, and mutations designed to introduce frameshift mutations to all three reading frames in this region have no effect on *cupA* gene expression (data not shown). This led us to further hypothesize that this region encodes an sRNA that regulates the *cupA* genes. To attempt to identify this putative sRNA, we performed intramolecular RNA circularization followed by cDNA synthesis and amplification

across the ligation junction (Figure 4.5A). This procedure was performed on RNA preparations with and without tobacco acid pyrophosphatase (TAP) treatment to determine whether any species identified originated from nucleotide triphosphate-initiated transcription or were the result of RNA endonuclease cleavage. Amplified products were cloned and sequenced to determine the 5' and 3' termini of any RNA species. We detected several PCR products whose termini suggested that they originated from RNA species of between 57 and 70 bases in length. No product detected from either the untreated or TAP-treated sample had sequence corresponding to a 5' terminus upstream of the cytosine 6 bps downstream of the end of *oprN* (Figure 4.5B). We also detected several possible 3' termini of this RNA species corresponding to between 62 and 75 bps downstream of the *orpN* stop codon. This variability in detected 3' termini may reflect the identification of RNA degradation intermediates, as we often detected long tracts of adenine residues after the 3' terminus, which is a hallmark of degradosome-mediated RNA degradation [26]. These variable 3' termini may also reflect differences in where transcription terminates at the Rho-independent terminator. Thus, we propose that sequence from 6 to between 62 and 75 bps downstream of the *oprN* stop codon encodes an sRNA that we have named PrrA (*Pseudomonas* RNA regulator of *cupA*), and that full-length PrrA is 70 bases in length (Figure 4.5C).

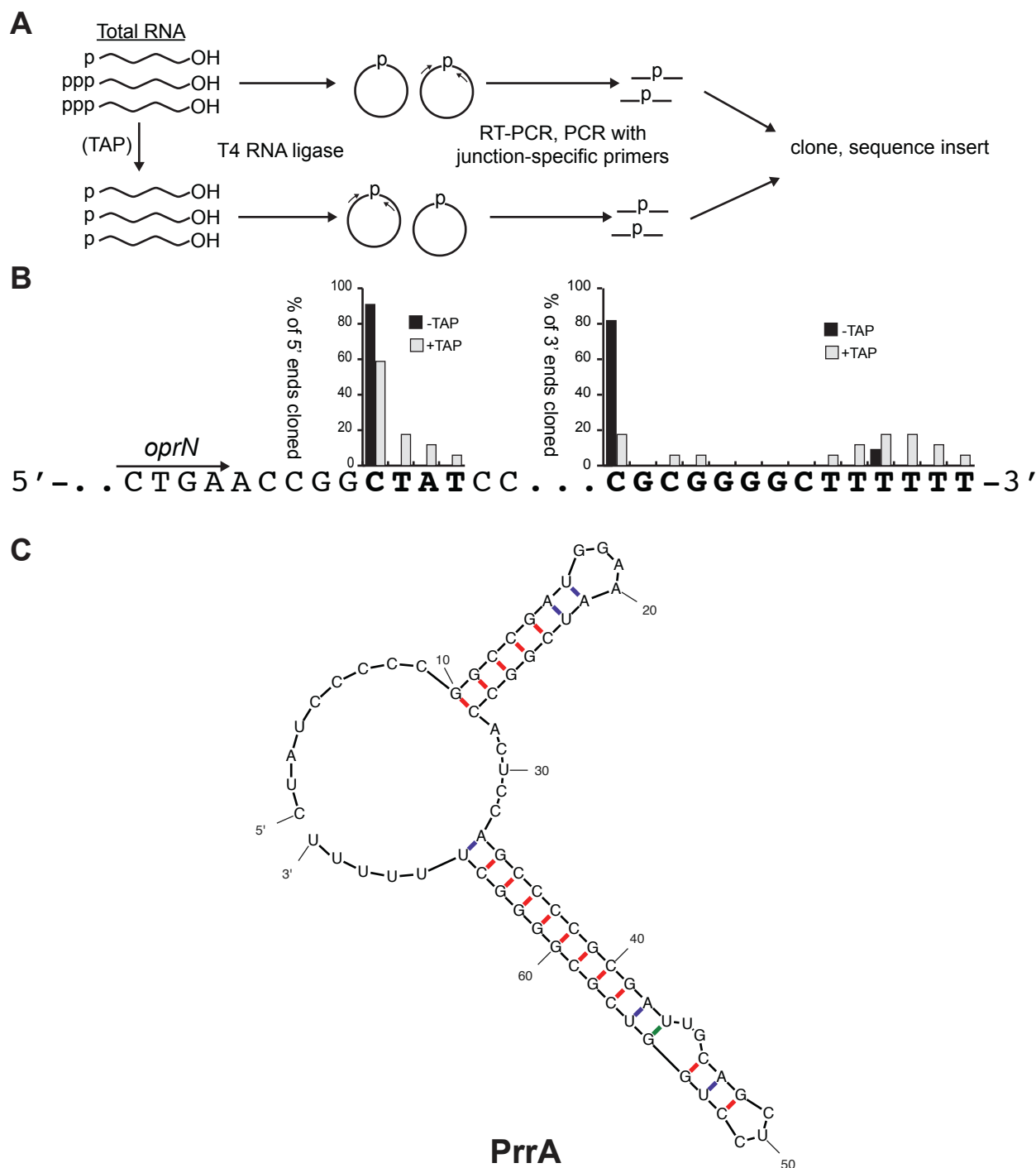


Figure 4.5. Intramolecular circularization of RNA reveals PrrA as a 70 base sRNA. (A) Schematic of protocol for T4 RNA ligase-mediated RNA circularization and transjunctional RT-PCR. (B) Distribution of bases at which 5' and 3' termini of PrrA were detected as determined by DNA sequencing. Sequence beyond the 3' end of *oprN* is shown with the relative frequency at which each particular base was identified as the 5' or 3' terminus from both TAP-treated and TAP-untreated RNA preparations. (C) Predicted structure of PrrA as determined by MFold (<http://mfold.rna.albany.edu/?q=mfold>).

PrrA is a positive regulator of the cupA genes

To determine whether reduced expression of *prrA* is the basis for reduced *cupA* expression in strains lacking MexT, in strains lacking the *mexE* promoter or in strains containing a polar mutation downstream of *oprN*, we took a complementation-based approach. Specifically, we began by testing whether providing the DNA sequence downstream of *oprN* could restore *cupA* expression to cells that contain a deletion of the *mexE* promoter. To test this, we constructed a series of plasmids containing the *mexE* promoter upstream of varying lengths of sequence immediately downstream of *oprN* (Figure 4.6A). These plasmids were introduced to strain PAO1 $\Delta mvaT \Delta P_{mexE} \Delta mexE oprN cupA lacZ$, which does not exhibit phase-variable *cupA* gene expression (Figure 4.3C). When this strain is transformed with a plasmid carrying the *mexE* promoter region plus as few as 272 bps downstream of *oprN* (insert number 3), expression of the *cupA* genes is dramatically increased (Figure 4.6B). However, a strain carrying a plasmid with the *mexE* promoter alone (insert number 4) does not exhibit increased expression of the *cupA* genes. This suggests that PrrA, when expressed in trans under the control of the *mexE* promoter, can complement the loss of the *mexE* promoter at its native locus. Furthermore, since the activity of the *mexE* promoter is highly dependent upon MexT, this result implies that MexT is required for *cupA* gene expression because of its role in positively regulating *prrA* expression [23].

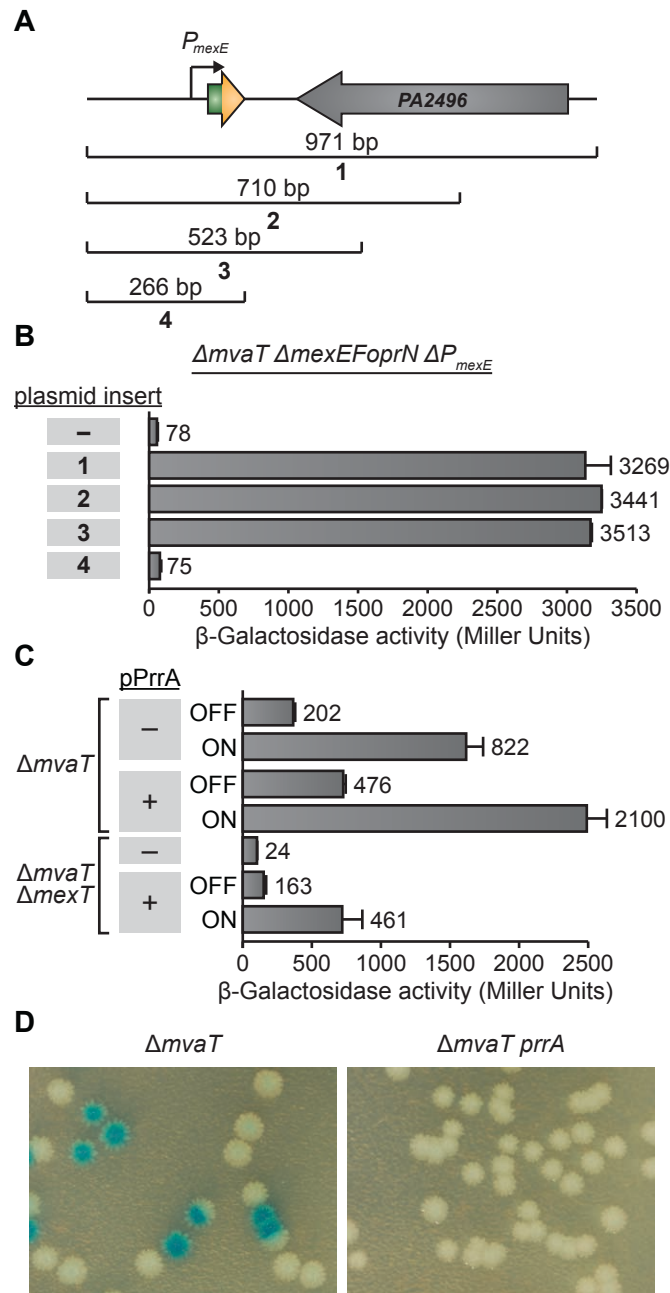


Figure 4.6. PrrA is a positive regulator of the *cupA* genes. (A) Schematic of plasmid inserts tested. These inserts were derived from a $\Delta mexEFoprN$ strain, and the composite green and orange arrow represents the linker sequence left after the $\Delta mexEFoprN$ deletion (see text). (B) Quantification of *cupA lacZ* expression in cultures of the PAO1 $\Delta mvaT \Delta P_{mexE} \Delta mexEFoprN cupA lacZ$ reporter strain, carrying either pPSV38 (-) or pPSV38 containing one of the inserts in A (1-4). (C) Quantification of *cupA lacZ* expression in cultures of the PAO1 $\Delta mvaT cupA lacZ$ and the PAO1 $\Delta mvaT \Delta mexT cupA lacZ$ reporter strains, carrying either pPSV38 (-) or pPrrA (+) and grown in the presence of 10 mM IPTG. (D) Phenotypes of the PAO1 $\Delta mvaT cupA lacZ$ and the PAO1 $\Delta mvaT prrA cupA lacZ$ reporter strains when plated on LB agar containing X-Gal.

To test whether ectopic expression of *prpA* is sufficient to complement the loss of MexT, we provided a PAO1 $\Delta mvaT$ *cupA lacZ* strain lacking MexT with a plasmid, pPrpA, that directs the synthesis of PrpA from an IPTG-inducible promoter. When the parental (i.e. *mexT*⁺) strain is transformed with pPrpA, expression of the *cupA* genes is further increased in cultures derived from both phase-OFF and phase-ON colonies (Figure 4.6C). Providing a derivative of this strain lacking MexT with pPrpA partially restores phase-variable *cupA* gene expression. This suggests that ectopic expression of *prpA* is sufficient to complement the loss of MexT. Finally, to determine whether PrpA is necessary for *cupA* gene expression in PAO1 $\Delta mvaT$ *cupA lacZ*, we constructed a deletion spanning bps 2 through 37 downstream of *oprN*, thus presumably leaving the predicted terminator described above intact but partially removing the *prpA* sequence. The resulting strain, PAO1 $\Delta mvaT$ *prpA cupA lacZ*, does not exhibit phase-variable *cupA* gene expression (Figure 4.6D). Taken together with previously described results, this finding suggests that PrpA is an sRNA of *P. aeruginosa* whose MexT-regulated expression from the *mexE* promoter is required for phase-variable *cupA* fimbrial gene expression. PrpA represents the first sRNA regulator of fimbrial gene expression in *P. aeruginosa*, and provides a novel link between multidrug resistance and expression of an adherence factor in an opportunistic pathogen notorious for high degrees of both resistance to antibiotics and formation of surface-attached communities.

PrpA does not regulate cupA mRNA translation or stability

Many bacterial sRNAs exert their regulatory effects by base-pairing with target mRNAs (reviewed in [27]). These interactions can result in alterations of mRNA secondary structure and often lead to alterations in RNA stability or translation [28]. To investigate whether PrpA is a direct post-transcriptional regulator of the *cupA* mRNA, we designed a construct in which the arabinose-inducible *P_{BAD}* promoter drives expression of a transcript that begins at the *cupA*

transcription start site (as determined by high-throughput sequencing [24]) and contains the *cupA1* and *lacZ* genes in the same manner as the chromosomal *cupA lacZ* fusion (Figure 4.7A) [29]. This construct was introduced to strains PAO1 $\Delta mvaT$ and PAO1 $\Delta mvaT \Delta mexT$, and *lacZ* expression was measured in cells grown in LB media with varying concentrations of arabinose. We found that deletion of *mexT* had no effect on expression of this reporter at any concentration of arabinose that was tested (Figure 4.7B). A similar result was obtained with strains PAO1 and PAO1 $\Delta mexT$ carrying a similar construct (i.e. with *mvaT*⁺ strains) (data not shown). This suggests that PrrA does not directly regulate the *cupA* transcript post-transcriptionally, and, when taken together with the results in Figure 4.1D suggesting that the *cupA* promoter region is regulated by MexT, indicates that the target of PrrA lies upstream of *cupA* transcription.

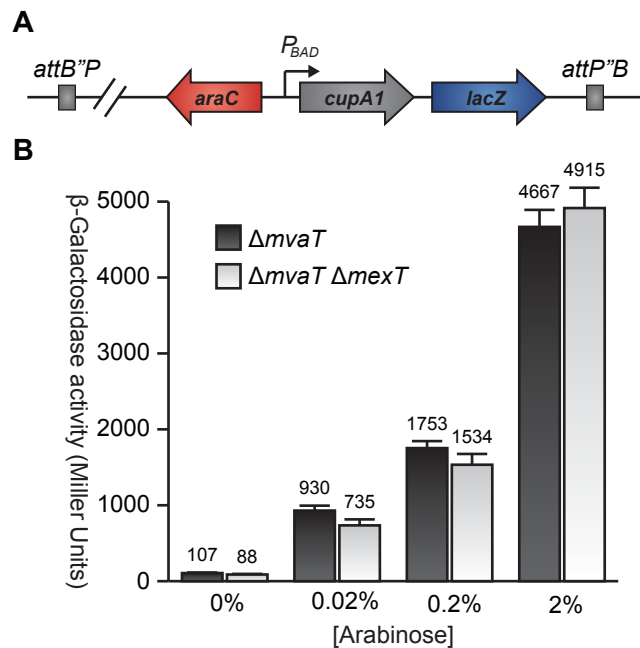


Figure 4.7. MexT does not directly post-transcriptionally regulate the *cupA* genes. (A) Schematic of the construct for generating the arabinose-inducible promoter-driven *cupA lacZ* transcript. (B) Quantification of arabinose-inducible promoter-driven *cupA lacZ* expression in cultures of the PAO1 $\Delta mvaT$ *attB::araC-P_{BAD}-cupA-lacZ* and the PAO1 $\Delta mvaT \Delta mexT$ *attB::araC-P_{BAD}-cupA-lacZ* reporter strains when grown in the presence of the indicated concentrations of arabinose.

Microarray analysis suggests PrrA negatively regulates the H-NS family member MvaU

The results outlined above support the hypothesis that the sRNA PrrA is a positive regulator of the *cupA* fimbrial genes, but do not implicate a direct target for PrrA. To attempt to identify this target, we analyzed the effect of ectopic *prpA* expression on the transcriptome using DNA microarrays. For this experiment, we chose to utilize strain PAO1 $\Delta mvaT \Delta P_{mexE} \Delta mexEFoprN cupA lacZ$, which exhibits highly increased *cupA* gene expression upon introduction of a plasmid carrying the *prpA* gene under the control of the *mexE*-promoter (Figure 4.6B, insert number 3). We therefore compared the transcriptome of this strain carrying this *prpA*-expression plasmid to that of the same strain carrying an empty vector control. A total of 102 genes and intergenic regions represented on the Affymetrix *P. aeruginosa* GeneChip change expression more than 3-fold, all of which are upregulated upon ectopic *prpA* expression (Table 4.1). Strikingly, 75 of these genes and intergenic regions, or the region encompassing their predicted promoters, are located within previously described peaks of occupancy for the H-NS family member MvaU (the other H-NS family member in *P. aeruginosa*, MvaT, can also occupy many of these loci, but the strain profiled by microarray here is a $\Delta mvaT$ mutant) (Figure 4.8) [10]. In strains lacking MvaT, MvaU presumably represses genes associated with these regions of occupancy, and when both proteins are depleted from the cell, expression of some of these genes increases dramatically. Therefore, this high degree of agreement between our previously published profile of MvaU occupancy across the genome and genes whose expression increases upon ectopic *prpA* expression suggests that PrrA is a negative regulator of MvaU. Taken together with the genetic evidence described above, the results of our microarray analysis suggest that the sRNA PrrA represents a link between the LysR-type transcription regulator MexT and global gene regulation through the H-NS family member MvaU (Figure 4.9).

Table 4.1. Ectopic expression of *prpA* in PAO1 $\Delta mvaT \Delta P_{mexE} \Delta mexEFoprN cupA lacZ$ results in increased expression of MvaU target genes.

Locus ID	Gene name	Fold change (PrrA+ vs. PrrA-)	MvaU occupancy in PAO1 $\Delta mvaT$ (Log2) ¹	Peak number ²
PA0051	<i>phzH</i>	4.2	5.70	2
PA0498		4.6	4.86	6
PA0499		3.6	4.86	6
PA0696		3.3	4.78	12
PA0825		11.4	6.21	16
PA0826		5.5	6.21	16
PA0981-2 ig		4.0	6.72	20
PA0996	<i>pqsA</i>	3.4		
PA0997	<i>pqsB</i>	4.0		
PA1000	<i>pqsE</i>	4.0		
PA1001	<i>phnA</i>	4.1		
PA1152-3 ig		3.5	7.30	25
PA1168		7.7	5.55	26
PA1169		4.8	5.55	26
PA1183	<i>dctA</i>	21.2		
PA1216		3.3		
PA1249	<i>aprA</i>	4.6	5.87	29
PA1383		13.9	6.73	32
PA1386		9.4	6.73	32
PA1388		3.3	6.73	32
PA1390		5.0		
PA1391		3.2		
PA1633	<i>kdpA</i>	3.1		
PA1634	<i>kdpB</i>	3.6		
PA1635	<i>kdpC</i>	7.1		
PA1636	<i>kdpD</i>	3.7		
PA1659		4.1	4.83	39
PA1660		3.2		
PA1661		3.4		
PA1663		3.1		
PA1665		5.5		
PA1667		4.1		
PA1669		3.1		
PA1701		3.0		
PA1709	<i>popD</i>	3.2		
PA1720	<i>pscG</i>	3.3		

Table 4.1 (Continued).

Locus ID	Gene name	Fold change (PrrA+ vs. PrrA-)	MvaU occupancy in PAO1 $\Delta mvaT$ (Log2)	Peak number
PA1874		3.3	5.89	43
PA1893		8.5	4.86	44
PA1894		11.8	4.86	44
PA1895		7.7	4.86	44
PA1896		7.7	4.86	44
PA1897		10.1	4.86	44
PA2037		3.4	6.15	47
PA2109		3.3		
PA2128	<i>cupA1</i>	28.9	3.26	50
PA2129	<i>cupA2</i>	28.4	3.26	50
PA2130	<i>cupA3</i>	16.8	3.26	50
PA2131	<i>cupA4</i>	10.4	3.26	50
PA2132	<i>cupA5</i>	21.1	3.26	50
PA2134		5.3	3.26	50
PA2145		5.8	6.02	51
PA2146		3.0	6.02	51
PA2223		4.4	6.23	52
PA2224		18.1	6.23	52
PA2373		4.1	4.33	53
PA2506		7.3		
PA2520	<i>czcA</i>	5.9	4.86	56
PA2521	<i>czcB</i>	9.3	4.86	56
PA2522	<i>czcC</i>	22.2	4.86	56
PA2523		10.4	4.86	56
PA2524		6.4	4.86	56
PA2697		10.7	5.20	61
PA2698		11.9	5.20	61
PA2699		7.0	5.20	61
PA2794		3.7	6.00	65
PA2936		4.0	4.06	67
PA3044	<i>rocS2</i>	4.1	5.33	68
PA3045	<i>rocA2</i>	4.4	5.33	68
PA3326		3.2	4.52	71
PA3328		3.8	4.52	71
PA3331		4.5	4.52	71
PA3520		5.3	5.90	75
PA3550	<i>algF</i>	4.5	4.29	77
PA4080		7.2	5.83	82

Table 4.1 (Continued).

Locus ID	Gene name	Fold change (PrrA+ vs. PrrA-)	MvaU occupancy in PAO1 $\Delta mvaT$ (Log2)	Peak number
PA4080-1 ig		4.7	5.83	82
PA4081	<i>cupB6</i>	5.2	5.83	82
PA4082	<i>cupB5</i>	6.2	5.72	83
PA4084	<i>cupB3</i>	5.4	5.72	83
PA4086	<i>cupB1</i>	12.1	5.72	83
PA4139		9.7	6.00	84
PA4140		7.0	6.00	84
PA4141		5.2	4.92	85
PA4142		6.1	4.92	85
PA4143		4.2	4.92	85
PA4175	<i>prpL</i>	5.3	6.50	86
PA4209	<i>phzM</i>	5.1	6.10	87
PA4211	<i>phzB1</i> ³	9.0	6.10	87
PA4212	<i>phzC1</i>	4.8	6.10	87
PA4213	<i>phzD1</i>	3.4	6.10	87
PA4214	<i>phzE1</i>	3.5	6.10	87
PA4215	<i>phzF1</i>	5.2	6.10	87
PA4216	<i>phzG1</i>	5.3	6.10	87
PA4217	<i>phzS</i>	6.5		
PA4294		3.0	5.77	90
PA4296		6.1	5.77	90
PA4306		5.1	4.30	91
PA4648		3.8	5.07	97
PA5087		3.3	6.18	100
PA5168		3.6		
PA5169		3.3		
PA5264		4.4	4.98	101
PA5530		4.7		

Genes predicted to be in operons as listed on the *Pseudomonas* genome database (<http://www.pseudomonas.com>) are grouped in blocks by similar background shading. ig = intergenic region.

¹This gene, or its predicted promoter region, was found to be associated with or within 500 bp of a peak of occupancy of MvaU as profiled in strain PAO1 $\Delta mvaT$ [10]. The log2 enrichment value of that peak is reported here.

²Peak number as reported in [10], Supplemental Table 2.

³The GeneChip array does not distinguish between the *phzB1-G1* and *phzB2-G2* genes, however, the report of altered *phzM* and *phzS* expression leads us to infer that the *phz1* operon increases expression upon ectopic *prpA* expression.

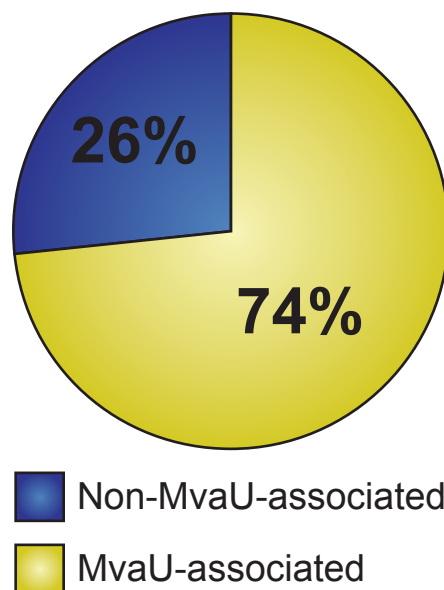


Figure 4.8. A majority of PrrA-regulated genes are associated with MvaU in PAO1 $\Delta mvaT$. Shown is a summary of the data in Table 4.1, describing the number of PrrA-regulated genes and intergenic regions (or their predicted promoter regions) that were found to be associated with MvaU in a previous report [10].

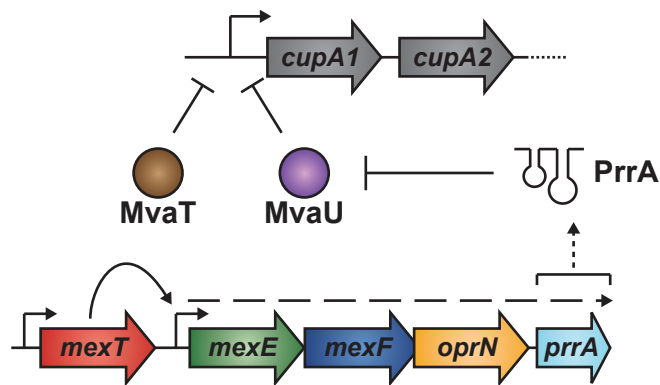


Figure 4.9. A model for PrrA function. In this model, MexT binds to the *mexE* promoter and drives production of a polycistronic transcript that contains the *mexEFoprN* genes, which encode a multidrug efflux pump, and the *prrA* gene, which encodes an sRNA. The PrrA sRNA then inhibits the activity, stability or expression of MvaU, which, along with MvaT, normally represses expression of the *cupA* genes.

Discussion

The results reported in this work describe the identification of a novel sRNA of *P. aeruginosa* encoded in a multidrug efflux operon and its role in regulating the *cupA* fimbrial genes. We have shown that MexT, a LysR-type transcription regulator that positively regulates expression of the *mexEFoprN* multidrug efflux pump genes, is a positive regulator of the *cupA* genes. More specifically, we have shown that the *mexE* promoter, a known MexT target, is required for expression of the *cupA* genes independently of the *mexEFoprN* genes. Using an engineered polar mutation at the end of the *mexEFoprN* operon, we show that expression of an element downstream of these genes is required for expression of the *cupA* genes. We identify this element using intramolecular circularization of RNA templates as a novel sRNA, PrrA. Using deletion and complementation analysis, we obtained evidence that PrrA acts downstream of MexT to regulate the *cupA* genes, and that its expression is also required for *cupA* gene expression. Finally, we report that ectopic expression of *prrA* results in increased expression of many genes that are associated with the H-NS family member MvaU. This work suggests that transcription activators can exert positive effects on gene expression by activating the expression of sRNAs that relieve repression exerted by H-NS family members.

sRNA regulation of a chaperone-usher fimbrial gene cluster

The *cupA* fimbrial genes are one of three chaperone-usher fimbrial gene clusters present in the genome of *P. aeruginosa* PAO1 [3]. The other two fimbrial gene clusters, known as *cupB* and *cupC*, are also repressed by MvaT in wild-type cells, but to a lesser degree than the *cupA* gene cluster [11]. Consistent with this finding, we have previously reported that both MvaT and MvaU occupy the *cupA*, *cupB* and *cupC* gene clusters and likely repress their expression [10]. Thus, the regulation of chaperone-usher fimbrial genes in *P. aeruginosa* is linked by the H-NS family members MvaT and MvaU. To our knowledge, PrrA represents the

first sRNA regulator of fimbrial gene expression in *P. aeruginosa*. sRNAs have previously been shown to regulate adherence factors such as fimbriae in other organisms. The VrrA sRNA of *Vibrio cholerae* negatively regulates the toxin co-regulated pilus, possibly by binding directly to the *tcpA* mRNA [30]. It has also been recently reported that the McaS sRNA of *Escherichia coli* can interact directly with the *csgD* mRNA and inhibit the biogenesis of curli, which are proteinaceous surface appendages important for attachment to surfaces [31]. In addition, the type I fimbriae of uropathogenic *E. coli* are adherence factors that are regulated by an sRNA [32]. Posttranscriptional regulation has also been implicated in expression of the major fimbriae of *Porphyromonas gingivalis*, an oral pathogen, though whether this is sRNA-mediated remains to be seen [33]. In general, sRNA regulation of surface appendages is thought to provide an additional measure of control in the assembly of these complex multi-subunit structures. In the specific case of PrrA, however, the results of our microarray analysis (Table 4.1) suggest that the sRNA may mediate fimbrial gene regulation through modulation of a global regulator, the H-NS family member MvaU.

The mechanistic basis for phase-variable *cupA* gene expression remains unknown, and our characterization of PrrA as a novel positive regulator of the *cupA* genes raises the question of whether or not it is a key component in mediating phase-variable *cupA* gene expression. However, we do not believe PrrA represents a key component in this phase-variable switch, as we have reported evidence suggesting that the *cupA* genes are phase-variably expressed in cells ectopically expressing *prrA* from a heterologous promoter (Figure 4.6C).

Conservation of PrrA

Our description of a novel sRNA gene immediately downstream of *oprN* raises the question of whether or not this gene is conserved among different strains of *P. aeruginosa*, and among other pseudomonads more generally. Alignment of sequences spanning the 3' region of the *oprN* gene through the *prrA* gene in a variety of *P. aeruginosa* strains reveals that sequence beyond the 3' end of *prrA* is markedly more variable than that in the *prrA* gene (Figure 4.10). Furthermore, the *prrA* gene itself is conserved to a degree similar to that observed for *oprN*. Thus, *prrA* appears to be conserved among *P. aeruginosa* isolates. The genus *Pseudomonas* encompasses several species, including the plant pathogens *P. syringae* and *P. brassicacearum*, the insect pathogen *P. entomophila* and the environmental isolate *P. putida*, which is not known to cause disease but can metabolize a wide range of toxic organic solvents. Alignment of *prrA* of *P. aeruginosa* strain PAO1 with sequence downstream of the *oprN* ortholog of a variety of sequenced *Pseudomonas* genomes reveals conservation of certain features (Figure 4.11). All strains appear to encode a similar Rho-independent terminator sequence downstream of *oprN*, as residues predicted to be involved in formation of both a hairpin structure and a poly-U tail are conserved (Figure 4.11, black boxes). However, only some pseudomonads appear to encode the smaller hairpin at the 5' end of PrrA (Figure 4.5C), as *P. syringae* sequences do not contain the residues corresponding to those of *P. aeruginosa* and other pseudomonads (Figure 4.11, red boxes). Intriguingly, in those pseudomonads that do have the two conserved regions predicted to be required for formation of the 5' hairpin, variation in one or two residues in one region is often accompanied by a compensatory change in the other region that would preserve the predicted hairpin structure. This covariation suggests that formation of the 5' hairpin may contribute to PrrA function.



Figure 4.10. PrrA is highly conserved among *P. aeruginosa* strains. Shown is an alignment of the 3' end of *oprN*, the *prrA* gene and downstream sequence from several strains of *P. aeruginosa*.

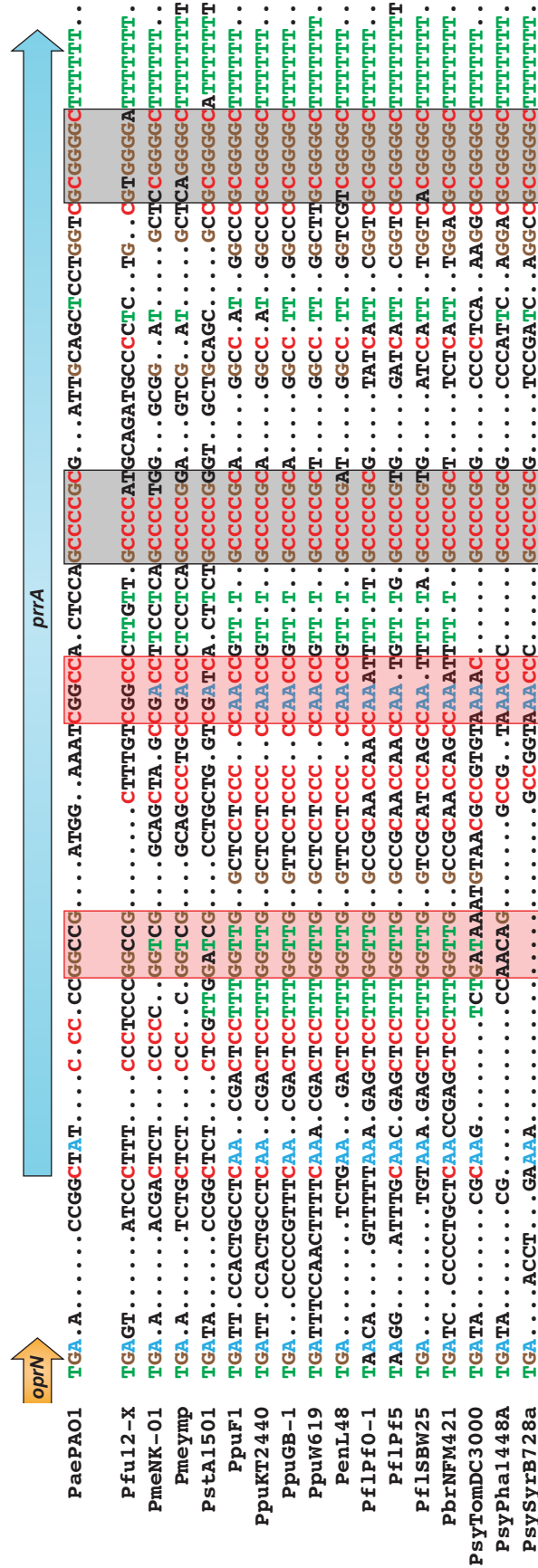


Figure 4.11. Conservation of PrrA among pseudomonads. Shown is an alignment of sequence downstream of orthologs of *oprN* from a variety of *Pseudomonas* spp. Conserved residues are shown in color, red boxes indicate residues predicted to participate in formation of the 5' hairpin and black boxes indicate residues predicted to participate in formation of the Rho-independent transcription terminator hairpin (see text). PaePAO1, *P. aeruginosa* PAO1. Pfu12-X, *P. fulva* 12-X. PmeNK-01, *P. mendocina* NK-01. Pmeymp, *P. mendocina* ymp. PstA1501, *P. stutzeri* A1501. PpuF1, *P. putida* F1. PpuKT2440, *P. putida* KT2440. PpuGB-1, *P. putida* GB-1. PpuW619, *P. putida* W619. PenL48, *P. entomophila* L48. PfIPf0-1, *P. fluorescens* Pf0-1. PfIPf5, *P. fluorescens* Pf5. PfISBW25, *P. fluorescens* SBW25. PbrNFM421, *P. brassicacearum* subsp. *brassicacearum* NFM421. PsyTomDC3000, *P. syringae* pv. *tomato* DC3000. PsyPha1448A, *P. syringae* pv. *phaseolicola* 1448A. PsySyrB728a, *P. syringae* pv. *syringae* B728a.

Does PrrA regulate MvaU?

The global gene regulators MvaT and MvaU belong to the H-NS family of nucleoid-associated proteins (NAPs). Members of this protein family often regulate transcription of many genes at various loci around the genome by virtue of their ability to oligomerize across long regions of DNA [34]. One way in which this regulation can be modulated is by alterations in expression of the NAP itself. It is possible that PrrA functions by negatively regulating the expression of *mvaU*, either directly or indirectly. Indeed, many NAPs in *E. coli* are differentially regulated in response to changing growth phases, providing evidence that intracellular concentrations of NAPs are sensitive to regulatory inputs [34]. The sRNA DsrA negatively regulates expression of H-NS in *E. coli* by binding directly to the *hns* transcript through basepairing interactions [35]. There are six bases of perfect complementarity between the coding region of the *mvaU* transcript and bases 30-35 of PrrA, which is consistent with so-called “seed” regions for some Hfq-dependent sRNA-mRNA interactions (Figure 4.5C and data not shown) [36]. Thus, it is possible that PrrA may basepair directly with the *mvaU* transcript to reduce its translation and/or stability. However, our microarray analysis did not reveal any significant change in *mvaU* transcript abundance upon ectopic *prrA* expression (data not shown).

It is also possible that PrrA may function by binding directly to MvaU, preventing its association with the DNA. Protein-protein interactions have been shown to alter NAP activity. The Hha/YmoA family of proteins has been shown to interact with H-NS family members to impair their ability to oligomerize across DNA and thus regulate transcription of certain genes [37,38]. Additionally, the gp5.5 protein of bacteriophage T7 interacts with the H-NS protein of *E. coli*, where it also interferes with higher-order oligomerization [39]. If the PrrA sRNA functions by directly interacting with and inhibiting MvaU, it would represent the first instance to our

knowledge of direct inhibition of an H-NS family member by an sRNA. NAPs, including StpA, HU and H-NS of *E. coli*, have been reported to interact directly with RNA *in vitro* [40-42]. It has been suggested that the sRNA DsrA can interact with the H-NS protein as well as the *hns* transcript *in vivo*, however, there is no evidence that this RNA-protein interaction inhibits the activity of H-NS [40]. Interaction between StpA and RNA has been observed in a number of assays *in vitro*, and has been suggested to occur *in vivo* as well in StpA-mediated promotion of the trans-splicing of the bacteriophage T4 thymidylate synthase group I intron [42,43]. Thus, it is plausible that PrrA could interact directly with MvaU. Indeed, an interaction between the HU protein and DsrA *in vitro* has been shown to require both the presence of at least one stem-loop structure and an unpaired 5' or 3' overhang in the RNA species, which are elements that are predicted to exist in PrrA (Figure 4.5C) [41]. If PrrA mediates its proposed negative effects on MvaU by a direct sRNA-protein interaction, it would represent the first known RNA inhibitor of a NAP, and we are currently investigating this possibility.

Materials and methods

Bacterial strains, media and chemicals

P. aeruginosa strain PAO1 was provided by Arne Rietsch (Case Western Reserve University). *P. aeruginosa* strains PAO1 $\Delta mvaT$ *cupA lacZ*, PAO1 *cupA lacZ* and PAO1 $\Delta mvaT$ have been described previously [7,12]. *E. coli* DH5 α F'IQ (Invitrogen) was used as the recipient strain for all plasmid constructions, whereas *E. coli* strain SM10 (λ pir) was used to mate plasmids into *P. aeruginosa*. *P. aeruginosa* was grown in LB for all experiments except those whose results are shown in Figure 4.1E; in the latter experiments LB with 2.5 g liter⁻¹ NaCl and 1% KNO₃ (LBN) was used as the growth medium. Anaerobic growth conditions were achieved using an anaerobic growth chamber together with a gas pack (AnaeroPack system; PML Microbiologicals). To confirm that anaerobic conditions were attained, in each experiment a Δanr strain (PAO1 Δanr *cupA lacZ* [7]) was incubated alongside the experimental strains. As reported previously, *anr* mutants do not grow under anaerobic conditions in which nitrate is used as the terminal electron acceptor [44,45].

When growing *E. coli*, antibiotics were used when necessary at the following concentrations: gentamicin (15 μ g ml⁻¹) and tetracycline (10 μ g ml⁻¹). When growing *P. aeruginosa*, antibiotics were used when necessary at the following concentrations: gentamicin (25 μ g ml⁻¹ for liquid cultures, 30 μ g ml⁻¹ for solid LB media and 60 μ g ml⁻¹ for Pseudomonas isolation agar (PIA) media (BD Diagnostic Systems) and tetracycline (35 μ g ml⁻¹ for LB media and 250 μ g ml⁻¹ for PIA media). Phase-ON and phase-OFF colonies of the PAO1 $\Delta mvaT$ *cupA lacZ* reporter strain were visualized following growth on LB agar containing X-gal (75 μ g ml⁻¹).

The PAO1 strain studied herein contains an 8-bp deletion in the *mexT* sequence when compared to the published PAO1-UW sequence [46]. This mutation shifts the reading frame of

mexT and leads to the production of full-length MexT protein, and has been described in several spontaneously generated chloramphenicol-resistant mutants (the so-called *nfxC*-type mutation) [47]. Furthermore, this reading frame shift suggests that the full-length *mexT* coding sequence begins at base 122 in the annotated *mexT* gene (as reported by the Pseudomonas Genome Database, <http://www.pseudomonas.com>). A protein produced from the coding sequence beginning at base 122 and lacking the aforementioned 8 bps has high homology to Cluster of Orthologous Groups COG0583 (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=COG0583>), which represents the LysR-type transcription regulators. The deletion construct for this 915-bp *mexT* gene (PA2492) was generated by amplifying regions 759 bps and 880 bps in length that flank *mexT* 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'-GCGGCCGCC-3'. The resulting PCR product was cloned on an *HindIII/XhoI* fragment into plasmid pEXG2 [48], yielding plasmid pEXG2- Δ *mexT*. This plasmid was then used to create strains PAO1 Δ *mvaT* Δ *mexT* *cupA lacZ*, PAO1 Δ *mexT* *cupA lacZ*, PAO1 Δ *mexT* and PAO1 Δ *mvaT* Δ *mexT* by allelic exchange [49]. Deletions were confirmed by the PCR.

Plasmid pPSV38 is a derivative of pPSV37 [50] in which the promoter region (as defined by a 117-bp *DraIII/EcoRI* fragment) was replaced with that of pUC18-mini-Tn7T-TOPLACUV5 [13]. Briefly, pPSV38 has two *lacO* sequences centered at positions -62.5 and +11 relative to a *lacUV5* promoter. Thus, plasmid pPSV38 drives the IPTG-inducible expression of sequence inserted downstream of this synthetic promoter.

Plasmid pMexT is a derivative of pPSV38 (see above) and directs the synthesis of the MexT protein under the control of the IPTG-inducible *lacUV5* promoter. The plasmid was made

by amplifying the 915-bp *mexT* gene with a consensus Shine-Dalgarno sequence on a *Bam*HI/*Hind*III fragment and cloning it into pPSV38.

The *attB*::*P*_{*cupA*}-GFP-*lacZ* reporter strains contain a fragment of the *cupA* promoter fused to the GFP and *lacZ* genes 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 [49,51]. A PCR product spanning from 794 bps upstream to 172 bps downstream of the *cupA1* start codon was amplified and cloned as a *Hind*III/*Eco*RI fragment into mini-CTX-GFP-*lacZ* [52], which contains a consensus Shine-Dalgarno sequence upstream of GFP-*lacZ*, yielding plasmid mini-CTX-*P*_{*cupA*}-GFP-*lacZ*. This plasmid was then used to create the reporter strain PAO1 Δ *mvaT attB*::*P*_{*cupA*}-GFP-*lacZ*, which was the parental strain for PAO1 Δ *mvaT* Δ *mexT attB*::*P*_{*cupA*}-GFP-*lacZ*.

The deletion construct for the *mexEFoprN* genes (PA2493-5) was generated by amplifying regions 689 bps and 730 bps in length that flank *mexEFoprN* 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'-GCGGCCGCC-3'. The resulting PCR product was cloned on a *Hind*III/*Xho*I fragment into plasmid pEXG2 [48], yielding plasmid pEXG2- Δ *mexEFoprN*. This plasmid was then used to create strain PAO1 Δ *mvaT* Δ *mexEFoprN cupA lacZ* by allelic exchange [49]. The deletion construct for the *mexTEFoprN* genes was then generated by subcloning a 766-bp *Hind*III/*Not*I fragment from pEXG2- Δ *mexT* into plasmid pEXG2- Δ *mexEFoprN* to yield plasmid pEXG2- Δ *mexTEFoprN*. This plasmid was then used to create strain PAO1 Δ *mvaT* Δ *mexTEFoprN cupA lacZ* by allelic exchange [49]. Deletions were confirmed by the PCR.

Plasmid pS-MexT is a derivative of pPSPK [10] (see Chapter 3) and directs the synthesis of the MexT protein under the control of the IPTG-inducible P_{spank} promoter. The plasmid was made by amplifying the 915-bp *mexT* gene with a consensus Shine-Dalgarno sequence on a *Bam*HI/*Hind*III fragment and cloning it into pPSPK.

The deletion construct for the *mexE* (PA2493) promoter was generated by amplifying regions 597 bps and 769 bps in length that flank a 60-bp sequence encompassing the predicted MexT-binding site [21] through the predicted -1 position relative to the *mexEFoprN* transcription start site as determined by high-throughput sequencing [24] from the PAO1 $\Delta mvaT \Delta mexEFoprN cupA lacZ$ genome by the PCR and then splicing the flanking regions together by overlap extension PCR. The resulting PCR product was cloned on a *Hind*III/*Xho*I fragment into plasmid pEXG2 [48], yielding plasmid pEXG2- $\Delta P_{mexE}(\Delta EFN)$. This plasmid was then used to create strain PAO1 $\Delta mvaT \Delta P_{mexE} \Delta mexEFoprN cupA lacZ$ by allelic exchange [49]. Deletions were confirmed by the PCR.

Plasmid pT1000-*oprN* is a derivative of pEXG2 [48] that is designed to allow reversible integration of two strong transcription terminators downstream of the *oprN* gene and was generated by amplifying DNA fragments containing 641 bps of sequence from the 3' portion of the *oprN* gene (without the stop codon) and 378 bps of sequence containing the transcription terminators T_0 and T_1 from bacteriophage λ and the *E. coli* *rrnB* operon, respectively, from pUC18-mini-Tn7T-LAC [53] by the PCR and then splicing these fragments together by overlap extension PCR in such a way as to introduce a *Not*I site and a TGA stop codon in between them. The resulting PCR product was cloned on a *Hind*III/*Kpn*I fragment into plasmid pEXG2 [48], yielding plasmid pT1000-*oprN*. This plasmid was then used to create strain PAO1 $\Delta mvaT oprN-T_0T_1 cupA lacZ$ by homologous recombination. Plasmid pT1000-*oprN*272 was created by amplifying a DNA fragment containing 299 bps of sequence from the 3' portion of the *oprN* gene

and 272 bps further downstream by the PCR. The resulting PCR product was cloned on a *HindIII/NotI* fragment into plasmid pT1000-*oprN*, yielding plasmid pT1000-*oprN*272. This plasmid was then used to create strain PAO1 $\Delta mvaT$ *oprN*272-*T₀T₁* *cupA lacZ* by homologous recombination. Integration of these plasmids was confirmed by the PCR.

The plasmids diagrammed in Figure 4.6A and used in Figure 4.6B carry fragments amplified from a $\Delta mexEFoprN$ strain spanning sequence immediately downstream of *mexT* (containing the *mexE* promoter) and extending to varying distances downstream of *oprN* by the PCR. These PCR products were cloned on *BamHI/HindIII* fragments into pPSV38 to yield plasmids carrying inserts numbered 1-4 as diagrammed in Figure 4.6A. Specifically, insert 1 contains 233 bps of sequence spanning from immediately downstream of *mexT* through the *mexE* start codon, the 9-bp linker sequence 5'-GCGGCCGCC-3' and 729 bps of sequence spanning from the *oprN* stop codon through the entire hypothetical gene PA2496. Inserts 2, 3 and 4 contain truncations of the insert 1 sequence lacking 265bps, 448 bps and 705 bps of sequence from the 3' end, respectively.

Plasmid pPrrA is a derivative of pPSV38 (see above) and directs the synthesis of the PrrA sRNA under the control of the IPTG-inducible *lacUV5* promoter. The plasmid was made by cloning a PCR-amplified DNA fragment containing 76 bps of sequence immediately downstream of the *oprN* gene on a *BamHI/HindIII* fragment into pPSV38.

The deletion construct for the partial *prrA* sequence was generated by amplifying regions 645 bps and 689 bps in length that flank a 36-bp sequence spanning positions 2 through 37 downstream of *oprN* by the PCR and then splicing the flanking regions together by overlap extension PCR. The resulting PCR product was cloned on a *HindIII/XbaI* fragment into plasmid

pEXG2 [48], yielding plasmid pEXG2- $\Delta prrA36$. This plasmid was then used to create strain PAO1 $\Delta mvaT prrA cupA lacZ$ by allelic exchange [49]. The deletion was confirmed by the PCR.

The construct for ectopically producing the *cupA1 lacZ* transcript from a heterologous promoter was generated by amplifying a region 1198 bps in length from pBAD24 containing the *araC* gene and the arabinose-regulated P_{BAD} promoter and a region 1,394 bps in length from the genome of a *cupA lacZ* strain spanning the transcription start site of the *cupA* operon as determined by high-throughput sequencing and a portion of the 5' end of the *lacZ* gene by the PCR and then splicing these regions together by overlap extension PCR [24]. The resulting PCR product, in which the transcription start site of the P_{BAD} promoter is replaced with that of the *cupA* 5' untranslated leader RNA, was cloned on a *KpnI/AatII* fragment into plasmid mini-CTX-*lacZ* [51], yielding plasmid mini-CTX-*araC-P_{BAD}-cupA-lacZ*. This plasmid was then used to create strains PAO1 $\Delta mvaT attB::araC-P_{BAD}-cupA-lacZ$ and PAO1 $\Delta mvaT \Delta mexT attB::araC-P_{BAD}-cupA-lacZ$.

β -galactosidase assays

Cells were either grown with aeration or anaerobically (see above) at 37°C to mid-logarithmic phase in LB supplemented as needed with gentamicin (25 $\mu\text{g ml}^{-1}$) and IPTG (10 mM). Cells were permeabilized with sodium dodecyl sulfate and CHCl_3 and assayed for β -galactosidase activity as described previously [54]. Assays were performed at least twice in triplicate on separate occasions. Representative data sets are shown.

Fluorescence microscopy

For fluorescence micrograph analysis, cultures were stained with the fluorescent membrane dye FM4-64FX (Molecular Probes) at 0.5 $\mu\text{g ml}^{-1}$ for 10 minutes at room

temperature. Cultures were then 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 Intensilight illuminator, a Coolsnap HQ2 charge-coupled device camera from Photometrics and a Nikon CFI Plan Apo VC $\times 100$ 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.

RNA isolation

Cultures of PAO1 were grown with aeration at 37°C to mid-logarithmic phase in LB. Cells were then harvested by centrifugation and total RNA was prepared and treated with DNase I essentially as described [24].

Intramolecular circularization and RT-PCR of RNA templates

Approximately 6.5 μg of a total RNA preparation was treated with 5 U of TAP (Epicentre), in a 50 μl reaction with 1X reaction buffer for 1 hour at 37 °C, and subsequently purified with TRI Reagent (Sigma Life Sciences). TAP-treated and untreated total RNA preparations were treated with 5 U of T4 RNA Ligase (New England Biolabs) in a 20 μl reaction with 1X reaction buffer for 16 hours at 16 °C, and subsequently purified with TRI Reagent. These ligated RNA preparations were incubated with a semi-random oligonucleotide with the sequence 5'-NSNSNSNSNS-3' (NS5) at 25 ng μl^{-1} in a 10 μl reaction for 10 minutes at 70 °C and 10 minutes at 25 °C. These RNA preparations with the hybridized NS5 oligo were then treated with 500 U of SuperScript III (Invitrogen) in a 20 μl reaction with 1X first strand buffer, 10 mM DTT, 1 mM dNTPs and 20 U RNaseIN (Ambion) for 10 minutes at 25 °C, 1 hour at 37 °C, 1 hour at 42 °C, and finally 10 minutes at 70 °C. RNA was removed from these samples by

alkaline hydrolysis and cDNAs were purified by phenol-chloroform extraction. Finally, the predicted intramolecular circularization junction was amplified by the PCR using oligonucleotides with the sequences 5'-GGGCTGGAGTGGCCGATTTC-3' and 5'-CCGCGATTGCAGCTCCTGGT-3'. The amplified products were cloned into pCR2.1-TOPO (Invitrogen) according to the manufacturer's protocol. Individual clones were isolated, and plasmids were prepared and the insert regions sequenced. Nine individual inserts were sequenced from plasmids prepared from TAP-untreated RNA (two of which contained two PrrA sRNAs joined together), and 17 individual inserts were sequenced from plasmids prepared from TAP-treated RNA.

Microarray experiments

Cultures of PAO1 $\Delta mvaT \Delta P_{mexE} \Delta mexEFoprN cupA lacZ$ containing plasmid pPSV38 or a plasmid carrying insert number 3 as outlined in Figure 4.6A were grown in triplicate to an OD₆₀₀ of ≈ 0.5 . RNA isolation, cDNA synthesis and cDNA fragmentation and labeling were performed essentially as described previously [55]. Labeled samples were hybridized to Affymetrix GeneChip *P. aeruginosa* genome arrays (Affymetrix). Data were analyzed for statistically significant ($p < 0.05$, fold change > 3) changes in gene expression using GeneSpring GX.

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References

1. Govan JR, Deretic V (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol Rev 60: 539–574.
2. Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, et al. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407: 762–764.
3. Vallet I, Olson JW, Lory S, Lazdunski A, Filloux A (2001) The chaperone/usher pathways of *Pseudomonas aeruginosa*: identification of fimbrial gene clusters (*cup*) and their involvement in biofilm formation. Proc Natl Acad Sci USA 98: 6911–6916.
4. Mikkelsen H, Ball G, Giraud C, Filloux A (2009) Expression of *Pseudomonas aeruginosa* *CupD* fimbrial genes is antagonistically controlled by RcsB and the EAL-containing PvrR response regulators. PLoS ONE 4: e6018.
5. Giraud C, Bernard CS, Calderon V, Yang L, Filloux A, et al. (2011) The PprA-PprB two-component system activates CupE, the first non-archetypal *Pseudomonas aeruginosa* chaperone-usher pathway system assembling fimbriae. Environ Microbiol 13: 666–683.
6. Meissner A, Wild V, Simm R, Rohde M, Erck C, et al. (2007) *Pseudomonas aeruginosa* *cupA*-encoded fimbriae expression is regulated by a GGDEF and EAL domain-dependent modulation of the intracellular level of cyclic diguanylate. Environ Microbiol 9: 2475–2485.
7. Vallet-Gely I, Sharp JS, Dove SL (2007) Local and global regulators linking anaerobiosis to *cupA* fimbrial gene expression in *Pseudomonas aeruginosa*. J Bacteriol 189: 8667–8676.
8. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, et al. (2002) Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J Clin Invest 109: 317–325.
9. Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, et al. (2002) *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev Cell 3: 593–603.
10. Castang S, McManus HR, Turner KH, Dove SL (2008) H-NS family members function coordinately in an opportunistic pathogen. Proc Natl Acad Sci USA 105: 18947–18952.
11. Vallet I, Diggle SP, Stacey RE, Cámara M, Ventre I, et al. (2004) Biofilm formation in *Pseudomonas aeruginosa*: fimbrial *cup* gene clusters are controlled by the transcriptional regulator MvaT. J Bacteriol 186: 2880–2890.
12. Vallet-Gely I, Donovan KE, Fang R, Joung JK, Dove SL (2005) Repression of phase-variable *cup* gene expression by H-NS-like proteins in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 102: 11082–11087.
13. McManus HR, Dove SL (2011) The CgrA and CgrC proteins form a complex that positively regulates *cupA* fimbrial gene expression in *Pseudomonas aeruginosa*. J Bacteriol 193: 6152–6161.

14. Grainger DC, Hurd D, Goldberg MD, Busby SJW (2006) Association of nucleoid proteins with coding and non-coding segments of the *Escherichia coli* genome. *Nucleic Acids Res* 34: 4642–4652.
15. Lucchini S, Rowley G, Goldberg MD, Hurd D, Harrison M, et al. (2006) H-NS mediates the silencing of laterally acquired genes in bacteria. *PLoS Pathog* 2: e81.
16. Navarre WW, Porwollik S, Wang Y, McClelland M, Rosen H, et al. (2006) Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*. *Science* 313: 236–238.
17. Castang S, Dove SL (2010) High-order oligomerization is required for the function of the H-NS family member MvaT in *Pseudomonas aeruginosa*. *Mol Microbiol* 78: 916–931.
18. Bouffartigues E, Buckle M, Badaut C, Travers A, Rimsky S (2007) H-NS cooperative binding to high-affinity sites in a regulatory element results in transcriptional silencing. *Nat Struct Mol Biol* 14: 441–448.
19. Lang B, Blot N, Bouffartigues E, Buckle M, Geertz M, et al. (2007) High-affinity DNA binding sites for H-NS provide a molecular basis for selective silencing within proteobacterial genomes. *Nucleic Acids Res* 35: 6330–6337.
20. Sette M, Spurio R, Trotta E, Brandizi C, Brandi A, et al. (2009) Sequence-specific recognition of DNA by the C-terminal domain of nucleoid-associated protein H-NS. *J Biol Chem* 284: 30453–30462.
21. Tian Z-X, Fargier E, Mac Aogáin M, Adams C, Wang Y-P, et al. (2009) Transcriptome profiling defines a novel regulon modulated by the LysR-type transcriptional regulator MexT in *Pseudomonas aeruginosa*. *Nucleic Acids Res* 37: 7546–7559.
22. Köhler T, Michéa-Hamzehpour M, Henze U, Gotoh N, Curty LK, et al. (1997) Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. *Mol Microbiol* 23: 345–354.
23. Köhler T, Epp SF, Curty LK, Pechère JC (1999) Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. *J Bacteriol* 181: 6300–6305.
24. Goldman SR, Sharp JS, Vvedenskaya IO, Livny J, Dove SL, et al. (2011) NanoRNAs prime transcription initiation in vivo. *Mol Cell* 42: 817–825.
25. Rietsch A, Mekalanos JJ (2006) Metabolic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*. *Mol Microbiol* 59: 807–820.
26. Blum E, Carpousis AJ, Higgins CF (1999) Polyadenylation promotes degradation of 3'-structured RNA by the *Escherichia coli* mRNA degradosome *in vitro*. *J Biol Chem* 274: 4009–4016.
27. Majdalani N, Vanderpool CK, Gottesman S (2005) Bacterial Small RNA Regulators. *Crit Rev Biochem Mol Biol* 40: 93–113.
28. Gottesman S, Storz G (2011) Bacterial small RNA regulators: versatile roles and rapidly

evolving variations. Cold Spring Harb Perspect Biol 3: 1–16.

29. Hoang HH, Nickerson NN, Lee VT, Kazimirova A, Chami M, et al. (2011) Outer membrane targeting of *Pseudomonas aeruginosa* proteins shows variable dependence on the components of Bam and Lol machineries. mBio 2: e00246-11.

30. Song T, Mika F, Lindmark B, Liu Z, Schild S, et al. (2008) A new *Vibrio cholerae* sRNA modulates colonization and affects release of outer membrane vesicles. Mol Microbiol 70: 100–111.

31. Thomason MK, Fontaine F, De Lay N, Storz G (2012) A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in *Escherichia coli*. Mol Microbiol 84: 17–35.

32. Pichon C, Merle du L, Caliot ME, Trieu-Cuot P, Le Bouguénec C (2011) An *in silico* model for identification of small RNAs in whole bacterial genomes: characterization of antisense RNAs in pathogenic *Escherichia coli* and *Streptococcus agalactiae* strains. Nucleic Acids Res. doi:10.1093/nar/gkr1141.

33. Xie H, Kozlova N, Lamont RJ (2004) *Porphyromonas gingivalis* genes involved in *fimA* regulation. Infect Immun 72: 651–658.

34. Dillon SC, Dorman CJ (2010) Bacterial nucleoid-associated proteins, nucleoid structure and gene expression. Nat Rev Microbiol 8: 185–195.

35. Lease RA, Cusick ME, Belfort M (1998) Riboregulation in *Escherichia coli*: DsrA RNA acts by RNA:RNA interactions at multiple loci. Proc Natl Acad Sci USA 95: 12456–12461.

36. Vogel J, Luisi BF (2011) Hfq and its constellation of RNA. Nat Rev Microbiol 9: 578–589.

37. Nieto JM, Madrid C, Miquelay E, Parra JL, Rodríguez S, et al. (2002) Evidence for direct protein-protein interaction between members of the enterobacterial Hha/YmoA and H-NS families of proteins. J Bacteriol 184: 629–635.

38. Paytubi S, Madrid C, Forns N, Nieto JM, Balsalobre C, et al. (2004) YdgT, the Hha paralogue in *Escherichia coli*, forms heteromeric complexes with H-NS and StpA. Mol Microbiol 54: 251–263.

39. Ali SS, Beckett E, Bae SJ, Navarre WW (2011) The 5.5 protein of phage T7 inhibits H-NS through interactions with the central oligomerization domain. J Bacteriol 193: 4881–4892.

40. Brescia CC, Kaw MK, Sledjeski DD (2004) The DNA binding protein H-NS binds to and alters the stability of RNA *in vitro* and *in vivo*. J Mol Biol 339: 505–514.

41. Balandina A, Kamashev D, Rouviere-Yaniv J (2002) The bacterial histone-like protein HU specifically recognizes similar structures in all nucleic acids. DNA, RNA, and their hybrids. J Biol Chem 277: 27622–27628.

42. Mayer O, Rajkowitsch L, Lorenz C, Konrat R, Schroeder R (2007) RNA chaperone activity and RNA-binding properties of the *E. coli* protein StpA. Nucleic Acids Res 35: 1257–1269.

43. Waldsich C, Grossberger R, Schroeder R (2002) RNA chaperone StpA loosens interactions of the tertiary structure in the *td* group I intron in vivo. *Genes Dev* 16: 2300–2312.
44. Ye RW, Haas D, Ka JO, Krishnapillai V, Zimmermann A, et al. (1995) Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. *J Bacteriol* 177: 3606–3609.
45. Zimmermann A, Reimann C, Galimand M, Haas D (1991) Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on *anr*, a regulatory gene homologous with *fnr* of *Escherichia coli*. *Mol Microbiol* 5: 1483–1490.
46. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrenner P, et al. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406: 959–964.
47. Maseda H, Saito K, Nakajima A, Nakae T (2000) Variation of the *mexT* gene, a regulator of the MexEF-OprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 192: 107–112.
48. Rietsch A, Vallet-Gely I, Dove SL, Mekalanos JJ (2005) ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 102: 8006–8011.
49. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP (1998) A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212: 77–86.
50. Lee P-C, Stopford CM, Svenson AG, Rietsch A (2010) Control of effector export by the *Pseudomonas aeruginosa* type III secretion proteins PcrG and PcrV. *Mol Microbiol* 75: 924–941.
51. Hoang TT, Kutchma AJ, Becher A, Schweizer HP (2000) Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. *Plasmid* 43: 59–72.
52. Rietsch A, Wolfgang MC, Mekalanos JJ (2004) Effect of metabolic imbalance on expression of type III secretion genes in *Pseudomonas aeruginosa*. *Infect Immun* 72: 1383–1390.
53. Choi K-H, Gaynor JB, White KG, Lopez C, Bosio CM, et al. (2005) A Tn7-based broad-range bacterial cloning and expression system. *Nat Methods* 2: 443–448.
54. Dove SL, Hochschild A (2004) A bacterial two-hybrid system based on transcription activation. *Methods Mol Biol* 261: 231–246.
55. Turner KH, Vallet-Gely I, Dove SL (2009) Epigenetic control of virulence gene expression in *Pseudomonas aeruginosa* by a LysR-type transcription regulator. *PLoS Genet* 5: e1000779.

Chapter 5

Discussion

Summary of results

The opportunistic pathogen *Pseudomonas aeruginosa* is a common cause of nosocomial infections and infections of the immunocompromised, and is the primary cause of morbidity and mortality in patients with the recessive genetic disorder cystic fibrosis (CF). This bacterium can cause infections in a wide variety of sites, including the skin, the cornea, the bloodstream and the lungs. *P. aeruginosa* is not thought to be a major component of the human microflora. While it has been found to infect agricultural plants such as lettuce and beans during warm, humid conditions, it is not generally thought to maintain an environmental reservoir as part of the normal flora of other organisms [1]. Outbreaks of *P. aeruginosa* in hospitals are thought to originate from contaminated objects such as water faucets, and the organism may persist on these objects for prolonged periods of time [2,3]. Because of this remarkable ability of *P. aeruginosa* to survive and potentially thrive in a wide variety of conditions, it is considered a “generalist.” This particular aspect of the nature of *P. aeruginosa* came into clearer focus when the sequencing of its genome revealed that it devotes a high proportion of its genetic capabilities to the regulation of transcription (8.4% of *P. aeruginosa* genes are predicted to encode transcription regulators) [4]. *P. aeruginosa*, then, represents a powerful model for the study of transcription regulation in pathogenic bacteria, and studying the regulation of virulence genes in *P. aeruginosa* could reveal potential new targets for therapeutic intervention.

In the preceding chapters, I reported work on the regulation of bistable gene expression in *P. aeruginosa*. Chapter 2 described the identification and characterization of a feedback-mediated bistable switch in gene expression, mediated by the LysR-type transcription regulator (LTTR) BexR. In Chapter 3, I described the adaptation of a targeted, inducible protein depletion system and its use in demonstrating synthetic lethality between the H-NS family members MvaT and MvaU. Finally, Chapter 4 reported the identification of a novel small regulatory RNA

(sRNA), PrrA, that positively regulates the *cupA* fimbrial genes, and described evidence suggesting that it may function through the global regulator MvaU. This final chapter will summarize the findings reported in the previous chapters, suggest future directions for study and synthesize the work presented into themes that relate to the study of bistable gene expression in bacteria more broadly.

BexR comprises a feedback-mediated bistable switch in virulence gene expression

Chapter 2 described a novel bistable switch in expression of a set of genes that occurs in *P. aeruginosa*. This switch is mediated by an LTTR, which we have named BexR (bistable expression regulator), that can bind to the promoter of its target genes and positively regulate their expression. Among these target genes is that encoding the secreted protease AprA, a virulence factor, and those encoding components of its secretion machinery. Interestingly, BexR can bind to the promoter of its own gene as well, and we have shown that it positively regulates its own expression. We presented the results of two experiments examining gene expression at single-cell resolution suggesting that this positive autoregulation forms the basis for bistable expression of BexR and, presumably, its target genes. We described how, without positive autoregulation, expression of *bexR* is no longer bistable and can instead occupy a wide range of expression states. Finally, we presented evidence that positive feedback is necessary for hysteretic, or history-dependent, behavior of the BexR switch, which is a hallmark of feedback-mediated bistable systems. This work represents an illustration of the relative simplicity with which complex patterns in gene expression can be achieved, and can serve as a framework for the characterization of such switches in other bacteria, such as that controlling the type I fimbrial genes in *Streptococcus pneumoniae* (see Appendices II and III).

Synthetic lethality between the H-NS family members MvaT and MvaU

In Chapter 3, I described the adaptation of a targeted protein depletion system and its use in demonstrating that the H-NS family members MvaT and MvaU have a shared, essential function in *P. aeruginosa*. In this system, a modified version of the *ssrA* tag called the DAS4 tag was fused to the C-terminus of either MvaT or MvaU. The DAS4 tag, like the wild-type *ssrA* tag, targets a protein for degradation by the ClpXP protease. However, because of the modifications engineered into the DAS4 tag, it is a poor target for the ClpXP protease in the absence of the SspB adaptor protein [5]. When SspB is ectopically expressed, in this case under the control of an IPTG-inducible promoter, DAS4-tagged MvaT or MvaU are rapidly targeted for degradation. This system was used to show that *P. aeruginosa* cannot tolerate the simultaneous loss of both MvaT and MvaU. This report of my work was placed in the larger context of a study showing that these two H-NS family members function coordinately and associate with over 100 distinct regions of the *P. aeruginosa* chromosome. Finally, the targeted depletion system was used to show that upon loss of both MvaT and MvaU, at least some genes associated with these MvaT and MvaU-associated regions become massively derepressed. This work provides an easily adaptable system that can be used to rapidly deplete *P. aeruginosa* cells of proteins and subsequently examine the effects of their loss. Our group has subsequently used this system to investigate the effects of depletion of the oligoribonuclease Orn in *P. aeruginosa* [6].

The sRNA PrrA may regulate the cupA fimbrial genes through an effect on MvaU

The *cupA* fimbrial genes encode structural components and assembly factors of a fimbrial structure of *P. aeruginosa* that is thought to play a role in biofilm formation [7,8]. These genes are expressed in a bistable or phase-variable manner in either the absence of MvaT or when cells are grown in oxygen-limiting conditions [9,10]. Chapter 4 began by describing the initial identification of the LTTR MexT as a positive regulator of *cupA* fimbrial gene expression.

We then presented evidence that MexT exerted its effects on *cupA* expression through a novel sRNA, which we have named PrrA (*Pseudomonas* RNA regulator of *cupA*). This sRNA is encoded by a previously unannotated gene in the MexT-regulated *mexEFoprN* RND-type efflux pump operon. Finally, by examining the effect of ectopic expression of PrrA on the *P. aeruginosa* transcriptome, we obtained indirect evidence that PrrA may be negatively regulating the H-NS family member MvaU. While further work is required to establish this regulatory connection, these results nonetheless raise exciting possibilities about sRNA regulation of H-NS family members. Furthermore, this work demonstrates how a transcription activator, MexT, can extend its regulatory reach by positively regulating a trans-acting sRNA. If PrrA does indeed negatively regulate MvaU, this work provides a mechanistic link between regulation by an LTTR and global gene regulation by an H-NS family member through an sRNA.

Future directions

Feedback-mediated bistability in bacteria

The work reported in Chapter 2 on the bistable BexR switch raises interesting questions about the prevalence of bistability in nature. This work demonstrates that bistable gene expression does not necessarily require the evolution of sophisticated regulatory systems. A given expression system can exhibit bistability if (1) it responds in a hypersensitive fashion to a change in the abundance of a key component, (2) the key component feeds back positively onto its own production and (3) the biochemical parameters (dissociation constants, rates of production, etc.) of the system are tuned appropriately to generate the possibility for bistability (see Appendix I for a more thorough discussion of the basis for these requirements). These three criteria can be fulfilled in a variety of ways. Hypersensitivity is simply an observed property of any system responding to an input, and any system that responds in a nonlinear fashion to a particular input can be hypersensitive over a given range. In biological terms, this can be

achieved in a number of different ways, such as having a transcription regulator act cooperatively to bind the DNA (see Chapter 1). Similarly, positive feedback can arise from many regulatory network architectures, including direct positive feedback like that seen in the case of the BexR switch. For example, a double negative feedback loop is critical in the bistable switch from lysogeny to lytic growth of bacteriophage λ [11]. Studies have consistently revealed that feedback is a highly conserved property of global transcriptional regulatory networks [12]. Taken together, these observations suggest that bistability, or at least the potential for bistability, is widespread in biology, and the discovery of the bistable BexR switch may presage many such discoveries in the future. Indeed, in the time since the work described in Chapter 2 was performed, we have characterized another such feedback-mediated bistable switch in expression of the type I pilus genes of *S. pneumoniae* (see Appendices II and III).

The dependence of bistability on the values of certain biochemical constants raises interesting questions about the role of the BexR switch in *P. aeruginosa* biology. If bistability can be so sensitive to minor variations in biochemical constants such as dissociation constants or protein synthesis or degradation rates (see Appendix I), then what selective pressures might be exerted on *P. aeruginosa* to ensure that bistable BexR expression is conserved? Unlike phase-variable expression of surface-associated antigens such as the flagellar antigen subtypes of *Salmonella* spp. or lipooligosaccharide sugar moieties of *Neisseria gonorrhoeae*, there are no obvious candidates among the BexR-regulated genes for proteins whose synthesis would be detrimental to the cell in the presence of the host immune system. Perhaps the BexR switch represents a response to the phenomenon of “kin selection” more generally, ensuring that a subset of cells in a clonal population is preadapted for the sudden onset of conditions in which expression of certain factors is beneficial. For example, several secreted proteins that are known or predicted to be proteases are bistably produced in a BexR-dependent fashion, and one of these, AprA, has been suggested to protect *P. entomophila* from antimicrobial peptides

[13]. If these proteases act in the extracellular space to degrade an antimicrobial peptide, they could be considered a “public good”, benefitting all members of the population even though only a subset of the population produces them. This is an interesting potential role for BexR bistability, and future research should focus on (1) how members of the BexR regulon contribute to *P. aeruginosa* physiology and (2) how their bistable expression can benefit the cell. Microarray analysis suggests that BexR can regulate the expression of 71 genes, and that PA1202 and several genes downstream are the most highly positively regulated BexR target genes. However, the function of the proteins encoded by the PA1202 gene cluster is unclear. It is possible that the putative enzymes encoded by this gene cluster represent a pathway for detoxification of an unknown toxic small molecule, yet a screen for small molecules or growth media that conferred a growth advantage to strains constitutively expressing *bexR* over those lacking *bexR* yielded no evidence to this effect (K.H. Turner and S.L. Dove, unpublished).

Interestingly, of the 121 LTTRs predicted to be encoded by *P. aeruginosa*, no two exhibit a higher degree of pairwise homology than BexR and the LTTR that is predicted to be encoded by the PA1201 gene, which is immediately upstream of and on the opposite strand from PA1202 (a common location for LTTRs relative to their target operons). Future studies should thus address whether the putative PA1201 LTTR affects expression of the PA1202 gene cluster or any other BexR target genes. Furthermore, as LTTRs often bind to small molecules through their C-terminal domains to affect their ability to regulate transcription, it is possible that BexR responds to an as-yet unidentified small molecule. If there is a small molecule that can interact with BexR, it could potentially modify the dynamics of BexR bistability in response to environmental conditions. Thus, a potentially promising future direction for studies on the BexR protein will be to investigate how the C-terminal domain of BexR contributes to its ability to regulate bistable expression of the genes under its control.

Regulation of H-NS family members by sRNAs

The effect of ectopic expression of the *prpA* sRNA gene on the transcriptome of *P. aeruginosa*, which was reported in Chapter 4, is highly suggestive of a mechanism of action in which PrrA negatively regulates the H-NS family member MvaU. This hypothesis stems from the observation that a majority of the genes that change expression in response to ectopic *prpA* expression in an *mvaT* mutant strain, including the *cupA* genes, were previously found to be associated with regions of MvaU occupancy when measured in an *mvaT* mutant strain [14]. If PrrA is indeed a negative regulator of *mvaU* expression or MvaU activity, several predictions are raised that can be verified experimentally. For one, it will be interesting to investigate whether depletion of MvaU (perhaps using the DAS4-tagging system described in Chapter 3) in cells of a $\Delta mvaT$ strain can result in the derepression of the genes in the PrrA regulon in a similar fashion. Additionally, in Chapter 4 we reported evidence that MexT is a positive regulator of the *cupA* genes in wild-type (i.e. *mvaT*⁺) cells grown in anaerobic conditions. If MexT exerts its effects on the *cupA* genes through a positive effect on *prpA* in anaerobic conditions, this would suggest that PrrA can positively regulate the *cupA* genes when cells are grown anaerobically, and this prediction should be explicitly tested by measuring *cupA* gene expression in cells with or without *prpA*. If PrrA acts to positively regulate the *cupA* genes in cells grown anaerobically through an effect on MvaU, then MvaU should negatively regulate the *cupA* genes in wild-type (i.e. *mvaT*⁺) cells. Therefore, deletion of *mvaU* should suppress the *mexT* mutant (or potentially the *prpA* mutant) phenotype in these conditions by restoring *cupA* gene expression to wild-type levels in cells grown anaerobically. Furthermore, our hypothesis presumes that the expression of PrrA target genes increases upon ectopic expression of *prpA* because of reduced MvaU occupancy. This presumption can be tested rather simply by chromatin immunoprecipitation-based approaches, which we have previously used to investigate association of MvaU with target regions of DNA [14].

As described in Chapter 1, sRNAs can function at either the post-transcriptional or post-translational level to regulate gene expression. Thus, if further experimentation supports the hypothesis that PrrA may function through MvaU, future studies should address the mechanism by which this occurs. PrrA may negatively regulate production of the MvaU protein, either directly or indirectly. This could be observed by examining the effect of either deletion or ectopic expression of *prpA* on MvaU protein abundance by Western blotting. The possibility that PrrA directly negatively regulates translation or stability of the *mvaU* transcript should be investigated, especially in light of the fact that PrrA contains six bases of perfect complementarity with the coding region of the *mvaU* transcript (see Chapter 4). Known Hfq-dependent sRNA-mRNA interactions typically depend on a “seed” region of as few as six bases that are perfectly complementary between an sRNA and its target that are surrounded by additional bases, some of which are complementary to the target mRNA, as is seen between PrrA and the *mvaU* transcript [15]. If these bases participate in an sRNA-mRNA interaction, point mutations to these bases should abolish the effect of ectopic *prpA* expression on *cupA* gene expression. Furthermore, mutations to the corresponding bases in the *mvaU* transcript designed to restore basepairing should reverse this effect if PrrA functions through basepairing with the *mvaU* transcript (care should be taken to engineer only silent mutations to the *mvaU* coding region to ensure the MvaU protein remains unaltered). It is also possible that, like many sRNAs that function by basepairing, PrrA depends on Hfq for its ability to positively regulate the *cupA* genes. Therefore, an *hfq* mutant strain should exhibit a similar phenotype to a *prpA* mutant strain with respect to expression of the *cupA* genes. However, there are numerous pleiotropic effects associated with mutation of *hfq*, as Hfq is thought to be required for the activity of numerous sRNAs in *P. aeruginosa*. Therefore, even if Hfq were found to be required for phase-variable expression of the *cupA* genes, it would not establish whether Hfq exerted its effects on *cupA* gene expression solely through PrrA.

It is also possible that, like the sRNAs RsmY and RsmZ, PrrA functions as a gene regulator by binding to and sequestering a regulatory protein, in this case MvaU [16-18]. This possibility would be supported by a reduction in the function of MvaU (i.e. reduced association with target DNA) without a corresponding reduction in its abundance (however, if a PrrA-MvaU interaction promoted degradation of MvaU, a reduction in MvaU abundance may still be observed). Several sRNA-protein interactions, including those of RsmY and RsmZ with the protein RsmA, depend on both sequence and secondary structure of the sRNAs. Alignment of sequences homologous to *prrA* from several species of the genus *Pseudomonas* suggests that the 5' hairpin may be a conserved feature of PrrA. Thus, the importance of this region to PrrA function in *P. aeruginosa* should be investigated by mutational analysis. If the 5' hairpin region is required for PrrA function, ectopic expression of a gene encoding a hybrid sRNA containing the 5' hairpin of PrrA and the predicted Rho-independent transcription terminator of a different sRNA (such as RsmZ) should complement a *mexT* deletion for *cupA* gene expression. Biochemical approaches should also be taken to explore a potential interaction between PrrA and MvaU and combined with experiments designed to investigate the importance of this potential interaction *in vivo*.

The work presented in Chapter 4 serves as an example of how a global regulator of gene expression, MexT, can extend its regulatory reach through an sRNA. If PrrA is indeed a negative regulator of MvaU, this reach may extend beyond the control of the *cupA* genes. Notably, however, not all genes associated with MvaU were found to change expression upon ectopic *prrA* expression. The expression of individual MvaU target genes may exist on a spectrum of varying sensitivity to changes in abundance of MvaU. This may be influenced by differences in the affinity of MvaU for regions surrounding these different genes or by the role of other transcription regulators in regulating expression of these target genes. sRNAs play a role in regulating other nucleoid-associated proteins, including H-NS (through basepairing), and it

would be interesting to investigate whether sRNA regulation of these proteins affects expression of their target genes in a similar fashion. The sRNAs MicF and RybB, which are positively regulated by the envelope stress sigma factor σ^E , have been described as playing an important role in extending the cell envelope stress response to negative regulation of translation of outer membrane porins [19]. In this study, the authors emphasize the importance of the rapid response to envelope stress conferred by sRNA-mediated inhibition of translation of proteins that might further exacerbate outer membrane stress. PrrA may similarly represent a rapidly-responding conduit between signals sensed by MexT and derepression of genes under the control of the H-NS family member MvaU. Exploring the connection between PrrA and MvaU may therefore shed further light on the role of sRNAs in regulatory networks, and provide a novel link between an LTTR and an H-NS family member.

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References

1. Green SK, Schroth MN, Cho JJ, Kominos SK, Vitanza-jack VB (1974) Agricultural plants and soil as a reservoir for *Pseudomonas aeruginosa*. Appl Microbiol 28: 987–991.
2. Trautmann M, Lepper PM, Haller M (2005) Ecology of *Pseudomonas aeruginosa* in the intensive care unit and the evolving role of water outlets as a reservoir of the organism. Am J Infect Control 33: S41–S49.
3. Grundmann H, Kropec A, Hartung D, Berner R, Daschner F (1993) *Pseudomonas aeruginosa* in a neonatal intensive care unit: reservoirs and ecology of the nosocomial pathogen. J Infect Dis 168: 943–947.
4. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrenner P, et al. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406: 959–964.
5. McGinness KE, Baker TA, Sauer RT (2006) Engineering controllable protein degradation. Molecular Cell 22: 701–707.
6. Goldman SR, Sharp JS, Vvedenskaya IO, Livny J, Dove SL, et al. (2011) NanoRNAs prime transcription initiation in vivo. Mol Cell 42: 817–825.
7. Meissner A, Wild V, Simm R, Rohde M, Erck C, et al. (2007) *Pseudomonas aeruginosa* *cupA*-encoded fimbriae expression is regulated by a GGDEF and EAL domain-dependent modulation of the intracellular level of cyclic diguanylate. Environmental Microbiology 9: 2475–2485.
8. Vallet I, Olson JW, Lory S, Lazdunski A, Filloux A (2001) The chaperone/ushe pathways of *Pseudomonas aeruginosa*: identification of fimbrial gene clusters (*cup*) and their involvement in biofilm formation. Proc Natl Acad Sci USA 98: 6911–6916.
9. Vallet-Gely I, Donovan KE, Fang R, Joung JK, Dove SL (2005) Repression of phase-variable *cup* gene expression by H-NS-like proteins in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 102: 11082–11087.
10. Vallet-Gely I, Sharp JS, Dove SL (2007) Local and global regulators linking anaerobiosis to *cupA* fimbrial gene expression in *Pseudomonas aeruginosa*. J Bacteriol 189: 8667–8676.
11. Schubert RA, Dodd IB, Egan JB, Shearwin KE (2007) Cro's role in the CI-Cro bistable switch is critical for λ 's transition from lysogeny to lytic development. Genes Dev 21: 2461–2472.
12. Alon U (2007) An introduction to systems biology. Boca Raton, Florida: CRC Press. 301 p.
13. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B (2006) Prevalence of local immune response against oral infection in a *Drosophila/Pseudomonas* infection model. PLoS Pathog 2: e56.
14. Castang S, McManus HR, Turner KH, Dove SL (2008) H-NS family members function coordinately in an opportunistic pathogen. Proc Natl Acad Sci USA 105: 18947–18952.

15. Vogel J, Luisi BF (2011) Hfq and its constellation of RNA. *Nat Rev Microbiol* 9: 578–589.
16. Kay E, Humair B, Dénervaud V, Riedel K, Spahr S, et al. (2006) Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J Bacteriol* 188: 6026–6033.
17. Heurlier K, Williams F, Heeb S, Dormond C, Pessi G, et al. (2004) Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 186: 2936–2945.
18. Brencic A, Lory S (2009) Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Mol Microbiol* 72: 612–632.
19. Gogol EB, Rhodius VA, Papenfort K, Vogel J, Gross CA (2011) Small RNAs endow a transcriptional activator with essential repressor functions for single-tier control of a global stress regulon. *Proc Natl Acad Sci USA* 108: 12875–12880.

Appendix I

Feedback-mediated bistability – a mathematical treatment

Attributions

This appendix comprises a discussion of a hypothetical bistable biological system from a dynamical systems perspective. In it, I hope to make clear the mathematical basis for how a simple transcription regulatory network can exhibit complex behaviors without equally complex control structures such as those controlling the phase-variable systems described in Chapter 1. This appendix describes the mathematical basis for many of the features of bistable systems discussed in Chapter 1. Much of this material arose from the Systems Biology course taught by Alexander van Oudenaarden at MIT, to whom I am grateful for increasing my understanding of the mathematics of systems biology. I wrote this appendix, with critical reading and helpful comments from Addison K. Stark and Simon L. Dove.

Dimerization and promoter binding

Consider a system in which a protein, X , can bind to the promoter of its own gene as a dimer to activate its expression (Figure I.1). The dissociation constant of the dimer into its two monomers is K_1 , and the dissociation constant of the dimer-DNA complex is K_2 :



In this expression, D represents a molecule of target DNA, and X^2 represents a dimer of X .

These two equilibria can be combined, and the ratio of the individual components to the dimer-DNA complex can be expressed as:

$$\frac{[D][X][X]}{[DX^2]} = K_1 K_2 = K_R \quad (I.2)$$

In this equation, $[D]$ is defined as the concentration of unbound DNA, $[DX^2]$ is defined as the concentration of bound DNA, $[X]$ is defined as the concentration of the protein X and K_R is a composite dissociation constant. As these binding and unbinding reactions occur on a much shorter timescale than the synthesis and degradation of X , with time constants on the order of milliseconds, they will be treated as instantaneous for the purposes of this dynamical system. In essence, the relative proportions of the monomer, the unbound dimer and the bound dimer depend solely on the concentrations of X and of the target DNA, and upon any change in either concentration the equilibria are immediately reestablished. This separation of timescales is an important simplification for describing the model, as we shall later see.

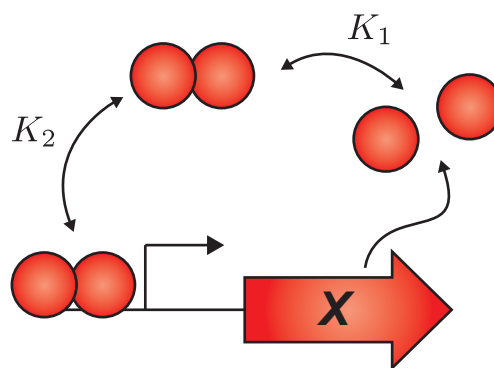


Figure I.1. A model autoregulated system. In this system, the protein X can dimerize with dissociation constant K_1 and this dimer can bind to the promoter of its own gene with dissociation constant K_2 .

A further simplification we will make will be to consider the concentration of target DNA to be constant, assuming that any duplication in the gene content that occurs due to DNA replication during the cell cycle is offset by an increase in the cell volume during growth. In effect, the total concentration of DNA, $[D_{tot}]$, is fixed, and the sum of $[D]$ and $[DX^2]$ is constant. Solving Equation 1.2 for $[D]$ we get the following expression for $[D_{tot}]$:

$$[D_{tot}] = [D] + [DX^2] = \frac{K_R[DX^2]}{[X]^2} + [DX^2] = [DX^2] \frac{K_R + [X]^2}{[X]^2} \quad (1.3)$$

We can now define the fraction of DNA bound by a dimer of X in terms of K_R and $[X]$:

$$f_{\text{bound}} = \frac{[DX^2]}{[D_{tot}]} = \frac{[X]^2}{K_R + [X]^2} \quad (1.4)$$

Similarly, the fraction of unbound DNA is simply:

$$f_{\text{unbound}} = 1 - f_{\text{bound}} = \frac{K_R}{K_R + [X]^2} \quad (1.5)$$

The dissociation constant for a dimer of the CI protein of bacteriophage lambda, a transcription regulator, is approximately 20 nM, and the dissociation constant for a λ CI dimer-DNA complex is approximately 3 nM [1]. Therefore, a value for K_R , which we have defined as the product of our two dissociation constants (Equation 1.2), should be on the order of approximately 60 nM². A plot of the fraction of DNA bound by X as a function of the concentration of X reveals a sigmoidal curve (Figure 1.2). As the concentration of X increases from zero, the fraction of DNA that is bound by a dimer of X increases gradually at first, accelerates rapidly as the concentration nears the square root of K_R , and then increases slowly as all of the DNA becomes bound by X. Thus, a regulator that must dimerize to bind DNA exhibits hypersensitivity in binding to its target.

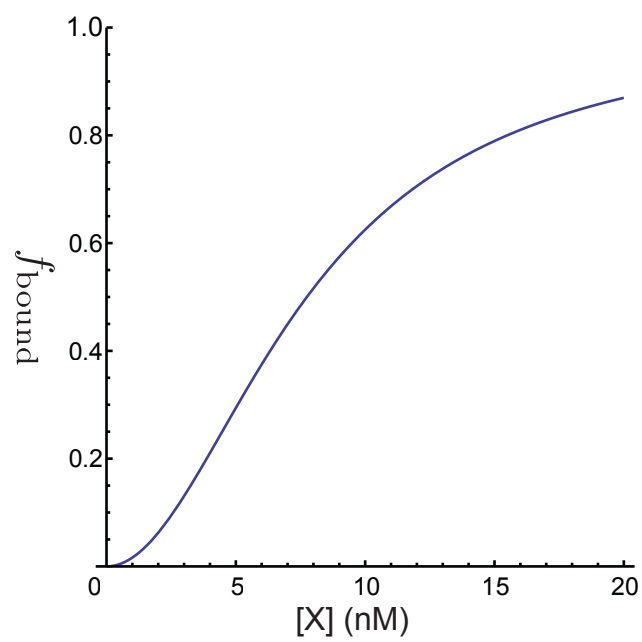


Figure I.2. The fraction of DNA bound by X (f_{bound}) responds to the changing concentration of X with hypersensitivity.

A deterministic, continuous-variable simulation

Now that we have considered the rapid binding reactions in this system, let us consider the dynamics of the slow reactions, namely, the synthesis and degradation of X (Figure I.3). For simplification, we will consolidate all of the processes involved in producing full-length, functional X protein into a single reaction. This production process can occur at one of two rates: k_A , an “activated” synthesis rate at which X is produced when the promoter of its gene is bound by a dimer of X, and k_B , a “basal” synthesis rate at which X is produced when the promoter of its gene is not bound by a dimer of X. We will also assume that degradation of X occurs by normal protein turnover, and is not regulated in any way. This is analogous to exponential decay with time constant γ (the half-life of X is $\ln 2/\gamma$). With these three processes defined, we have completed the description of our model system, and we can begin examining its behavior.

To determine how this system changes from a starting condition over time, we need a differential equation to determine how the concentration of X changes with time. This is simply the sum of the synthesis and degradation rates at a given concentration of X (hereafter referred to as x). Degradation of X is an exponential process, and so the overall degradation rate of X is:

$$g(x) = \gamma x \quad (1.6)$$

However, there are two possible synthesis reactions (Figure I.3), and so we must specify a weight for each term to determine how much the two rate constants, k_A and k_B , contribute to the overall synthesis rate of X, which we will call $f(x)$. We will use our expressions for f_{bound} (Equation I.4) and f_{unbound} (Equation I.5) as these weights:

$$f(x) = k_A f_{\text{bound}} + k_B f_{\text{unbound}} = \frac{k_A x^2 + k_B K_R}{K_R + x^2} \quad (1.7)$$

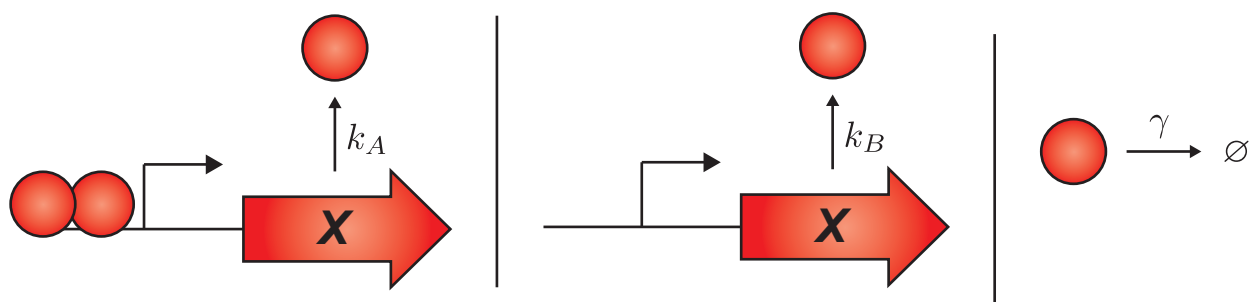


Figure I.3. A diagram of the slow reactions in the model autoregulated system. Shown are the three reactions to be considered as terms in the time-evolution of X . Left, a single molecule of X is synthesized from the gene encoding it when the promoter is bound by a dimer of X with rate constant k_A . Center, a single molecule of X is synthesized from the gene encoding it when the promoter is unbound with rate constant k_B . Right, a single molecule of X is spontaneously degraded in an exponential decay process with rate constant γ .

With the synthesis and degradation rates defined as a function of x (Equations I.6 and I.7), we can specify our differential equation describing the time evolution of x :

$$\frac{dx}{dt} = f(x) - g(x) = \frac{k_A x^2 + k_B K_R}{K_R + x^2} - \gamma x \quad (I.8)$$

This equation completely describes how the concentration of X will change with time, and depends solely on the current concentration of X and the biochemical constants k_A , k_B , K_R and γ . We can now use this equation to simulate the behavior of the system.

Before we begin simulating the system, however, we can examine the properties of the equation describing the time evolution of x to make some qualitative observations about how it may behave. We will assign the following values to the biochemical constants k_A , k_B and γ :

$$\left\{ \begin{array}{ll} k_A = 1.15 & \text{nM min}^{-1} \\ k_B = 0.03 & \text{nM min}^{-1} \\ \gamma = 0.069 & \text{min}^{-1} \end{array} \right\} \quad (I.9)$$

These values are of similar magnitude to those observed in biological systems (see [2]). We will vary the composite dissociation constant K_R and observe how the behavior of the system changes by observing a plot of $f(x)$, the synthesis rate (Equation I.7) and $g(x)$, the degradation rate (Equation I.6) as functions of x . When K_R is high (240 nM^2), $f(x)$ is equivalent to $g(x)$ for only one value of x (Figure I.4, left). This value (marked with a filled circle) is known as a steady state, because dx/dt , which is equivalent to $f(x)-g(x)$ (Equation I.8), evaluates to zero at this value. The concentration of X will not change once this steady state value is reached. Moreover, when the concentration of X is lower than this value, $f(x)$ is greater than $g(x)$, and the concentration will increase. Similarly, when the concentration of X is higher than this value, $f(x)$ is less than $g(x)$, and the concentration will decrease. Therefore, this intersection point reflects a stable steady state, and our model system will always tend towards it when the interaction between X and the promoter of its gene is weak. In biological terms, this means that the production of X is constitutively low. When K_R is low (15 nM^2), $f(x)$ is equivalent to $g(x)$ for only one value of x

(Figure I.4, right). This steady state is also stable, and our model system will always tend towards it when the interaction between X and the promoter of its gene is strong. In biological terms, this means that the production of X is constitutively high.

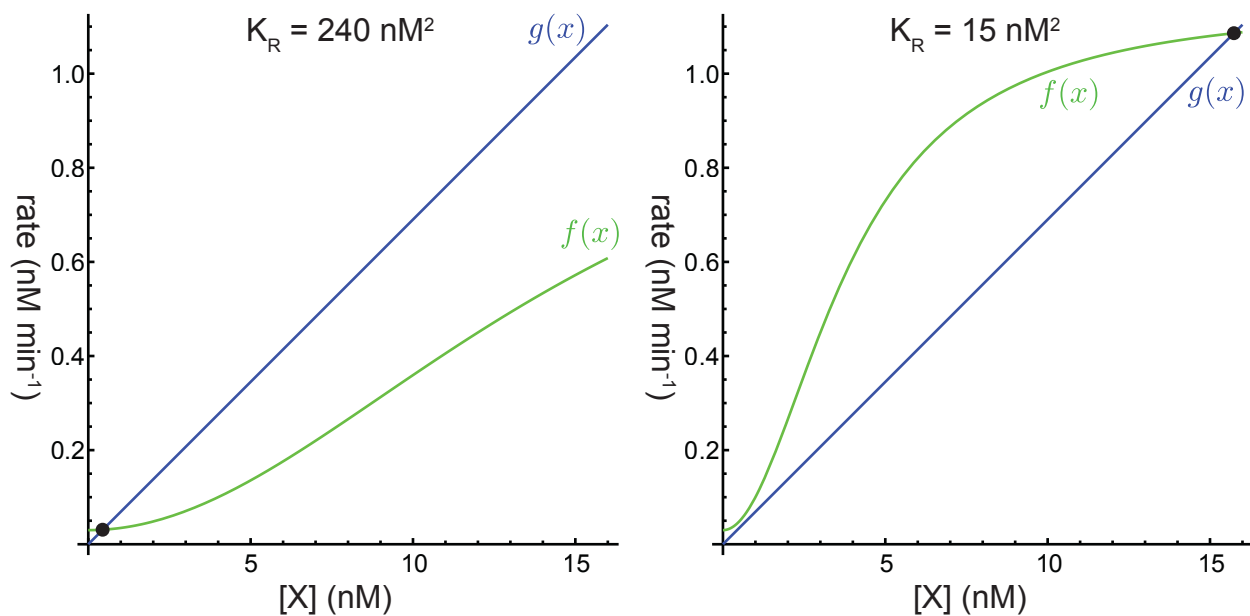


Figure I.4. A comparison of the synthesis and degradation rates as functions of the concentration of X with high and low dissociation constants. Shown are the synthesis ($f(x)$) and degradation ($g(x)$) rates of X, plotted as functions of the concentration of X and superimposed upon one another with high (left) and low (right) K_R . The blue line represents $g(x)$, the green line represents $f(x)$ and the filled circles represent stable steady states (i.e. intersection points where $f(x)$ is greater than $g(x)$ to the left and less than $g(x)$ to the right).

Our model system, then, should not exhibit any peculiar behavior when the interaction between X and the promoter of its gene is very weak or very strong. However, when K_R , our composite dissociation constant, has an intermediate value (60 nM^2), some important features emerge. A plot of $f(x)$ and $g(x)$ at this intermediate value of K_R reveals three points at which the values of these two functions are equivalent (Figure 1.5). Therefore, at this value of K_R the system has three steady states. Interestingly, however, only the lowest and highest steady states (marked with filled circles) are stable steady states, because, as before, $f(x)$ is greater than $g(x)$ to their left and less than $g(x)$ to their right. This means, again, that the concentration of X will tend towards these states when it is close to them. In contrast, the intermediate steady state (marked with an open circle) is unstable, because $f(x)$ is less than $g(x)$ to its left and greater than $g(x)$ to its right. As we shall see, this means that even though a concentration of X of exactly that value would not change over time, when the concentration of X is near that value it will tend to move away from it. Thus, with the values we have chosen for these biological constants, our model system is bistable when K_R is 60 nM^2 .

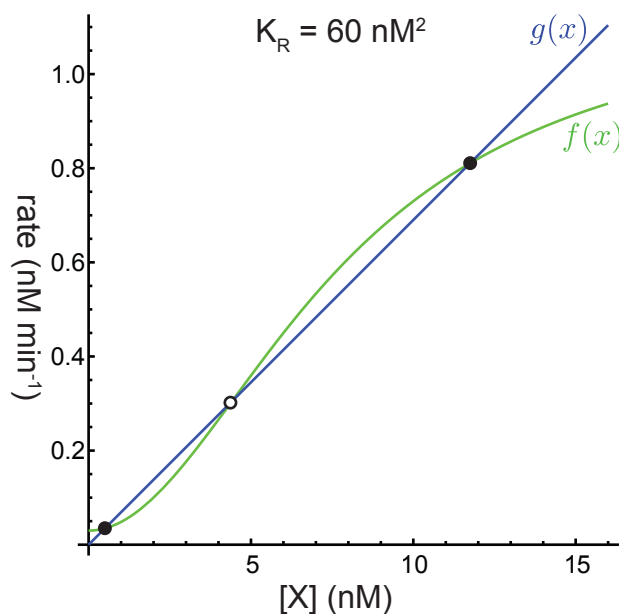


Figure I.5. A comparison of the synthesis and degradation rates as functions of the concentration of X with an intermediate dissociation constant. Shown are the synthesis ($f(x)$) and degradation ($g(x)$) rates of X, plotted as functions of the concentration of X and superimposed upon one another with an intermediate K_R . The blue line represents $g(x)$, the green line represents $f(x)$, the filled circles represent stable steady states (i.e. intersection points where $f(x)$ is greater than $g(x)$ to the left and less than $g(x)$ to the right) and the open circles represent unstable steady states (i.e. intersection points where $f(x)$ is less than $g(x)$ to the left and greater than $g(x)$ to the right).

Now that we have derived a differential equation that can describe how the concentration of X changes over time (Equation I.8) and defined values for the biological constants that should yield interesting behavior, we can simulate the progression of the system over time for a variety of starting conditions. This is done using numerical analysis software such as MATLAB. Essentially, this program iteratively calculates a series of values for x based on an initial condition, which we will choose, and our differential equation (Equation I.8), which describes how x should change over time. Using a variety of starting conditions, this simulation shows that the system will always tend towards the two stable steady states described earlier (Figure I.6). This suggests that a comparable system operating in bacteria can occupy either a constitutively high or constitutively low state, depending on the initial starting concentration of the regulator protein.

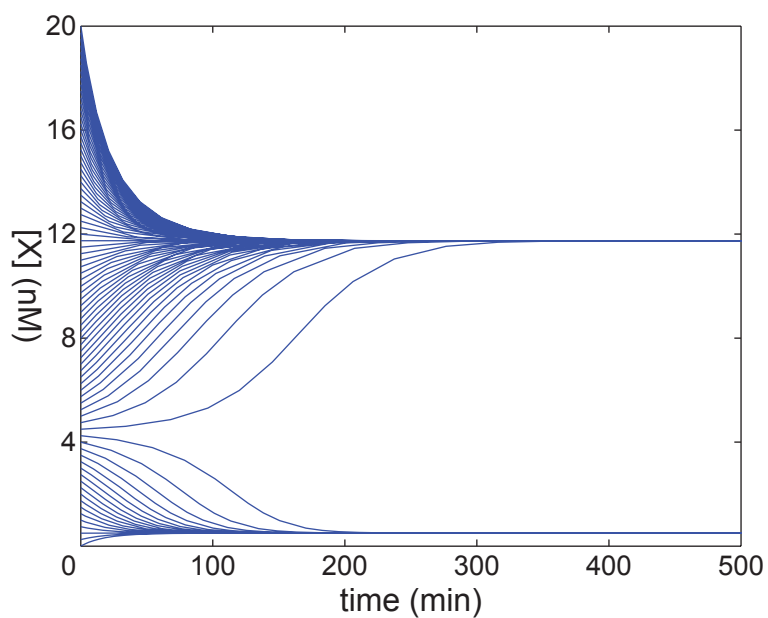


Figure I.6. A continuous-variable, deterministic simulation of the model system. Shown is a plot of the concentration of X as a function of time for several independent continuous-variable, deterministic simulations of the model system. A range of starting concentrations of X were chosen (represented by the left ends of the series of lines on the plot), and the concentration of X was calculated over time using a MATLAB program based on Equation I.8. All possible starting concentrations of X converge to the two stable states.

Randomness and noise – a discrete, stochastic simulation

While the use of this simulation to model the behavior of the system over time can replicate the existence of two steady states, it does not take into account the stochastic, noisy nature of gene expression at the single-cell level (reviewed in [3]). This is because the model utilized above treats the amount of X in the cell as a continuous variable (i.e. one that can take on any non-integer value). This is a valid approximation when the number of molecules of X that we are considering is sufficiently large, as the concentration can change in extremely small increments. However, the number of molecules of a protein in a bacterial cell can often be quite small. For example, there are approximately 40 monomers of the AraC transcription regulator per *E. coli* cell [4]. Thus, to make our model more biologically relevant, we need a model that can count individual molecules of X (i.e. treat x as a discrete variable). Furthermore, the model is entirely deterministic, and the random nature of biochemistry at the single-cell level is not accurately represented. We will therefore also include an element of randomness to our treatment of this system using a Monte Carlo simulation method.

The Gillespie method, a widely used Monte Carlo simulation, allows for the simple translation of systems such as ours from a deterministic, continuous system to a stochastic, discrete system [5]. It relies on the assumption that protein production and degradation are Poisson processes, that is, that individual events do not influence the likelihood of a subsequent event occurring. In a Poisson process with rate r , the waiting times between events are exponentially distributed with parameter r . The Gillespie method relies on this property of Poisson processes to simulate the random occurrence of either the production or degradation of a single molecule. In short, Gillespie states that a single waiting time from one event to the next, Θ , can be calculated as:

$$\theta = -\frac{1}{r} \ln\left(\frac{1}{u}\right) \quad (I.10)$$

In this equation, u represents a number randomly chosen from a uniform distribution between 0 and 1 (i.e. any value between 0 and 1, all with equal probability of being chosen). To simulate our system using this method, we will randomly generate a waiting time until the next production event and until the next degradation event based on the current value of x and compare them. If the waiting time until the production event is shorter, we increment the value of x by one and recalculate the rates, and if the degradation event is calculated to occur first, we decrement the value of x by one and recalculate the rates. We have already defined the rates of production (Equation I.7) and degradation (Equation I.6) as functions of the concentration of X , and we will correct the parameters that determine these rates (k_A , k_B and K_R) using the volume of the cell we are considering to express these parameters in terms of numbers of molecules of X . These corrected parameters will be used to calculate these random waiting times as in Equation I.10.

We will first simulate the system assuming a cell volume of 3 fL, which is only slightly higher than the volume of an *E. coli* cell growing exponentially in nutrient-rich broth (~1.7 fL) [6]. Stochastic simulation of the system in this 3 fL cell using a starting number of molecules of X near the unstable steady state yields more random behavior than that exhibited by the continuous-variable simulation (Figure I.7). First, which stable steady state the system initially occupies is somewhat random, as several independent trials with the same initial condition yield different behaviors over time. Second, it is clear that, in this system, a cell can randomly switch from both the low expression state to the high expression state (note the simulation depicted by the blue line in Figure I.7) and from the high expression state to the low expression state (note the simulation depicted by the red line in Figure I.7). This switch can occur when the number of molecules of X approaches the threshold value determined by the unstable steady state and happens to continue changing until it moves across that value.

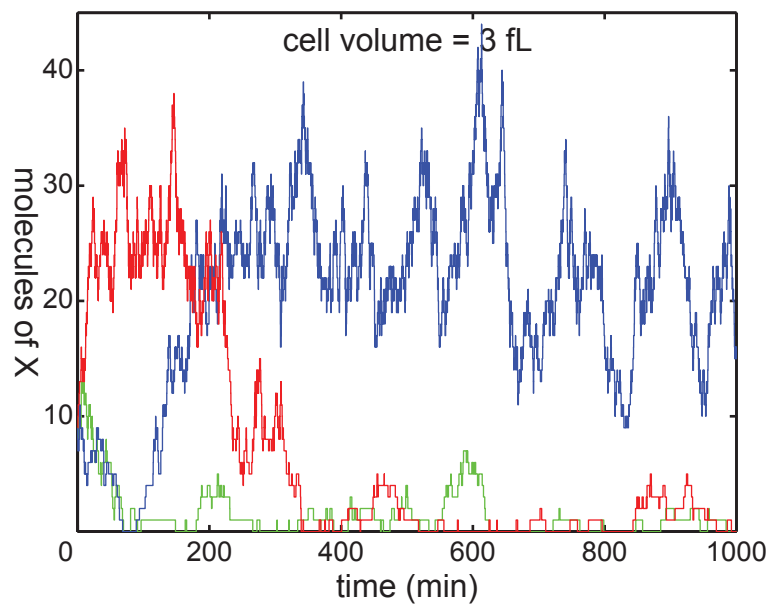


Figure I.7. A discrete-variable, stochastic simulation of the model system in a 3 fL cell. Shown is a plot of the number of molecules of X as a function of time for three independent discrete-variable, stochastic simulations of the model system, assuming a cell volume of 3 fL. A distinctly colored line represents each independent trial. All trials began with the same starting value for the number of molecules of X.

To emphasize the importance of noise in this stochastic simulation, we will simulate the system using a cell volume of 30 fL, which is considerably larger than the average *E. coli* cell. This simulation behaves more reproducibly than that assuming a cell volume of 3 fL (Figure I.8). Its behavior much more closely approximates what occurs in the continuous-variable case (Figure I.6). This is because when the number of molecules of X is higher, it more closely resembles a continuous variable. These simulations show that a model system containing a protein that acts cooperatively to regulate its own expression can exhibit bistable, switching expression when modeled in a sufficiently small single cell.

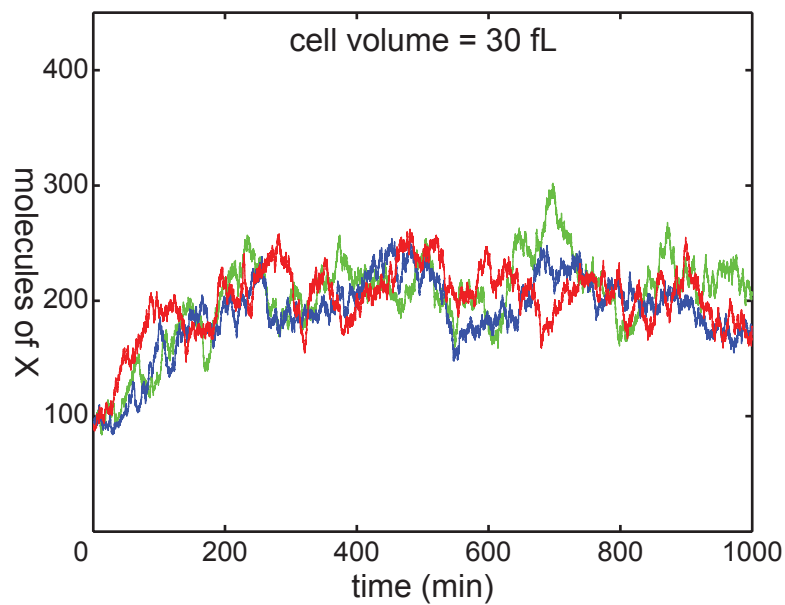


Figure I.8. A discrete-variable, stochastic simulation of the model system in a 30 fL cell. Shown is a plot of the number of molecules of X as a function of time for three independent discrete-variable, stochastic simulations of the model system, assuming a cell volume of 30 fL. A distinctly colored line represents each independent trial. All trials began with the same starting value for the number of molecules of X.

Relevance to biological systems

The model described and simulated above is an idealized version of a biological system that contains many simplifications. Key among them is the assumption that the total concentration of target DNA in the cell is not changing. This is certainly not the case in growing cells, as a given segment of the genome of *E. coli* can duplicate as quickly as every 27 minutes when cells are growing in exponential phase in rich media [7]. The increase in cytoplasmic volume as cells grow in size will constantly lower the DNA concentration in between replications, causing the intracellular target DNA concentration to oscillate sharply. Furthermore, the intracellular concentration of X and its dissociation constants K_1 and K_2 may not be as simple *in vivo* as they appear in the model. For one, site-specific DNA binding proteins also interact non-specifically with DNA, albeit much more weakly than with their specific target sequence [8]. This would suggest that in truth, the equilibrium described in Equation 1.2 is much more complicated. There may also be other proteins, small molecules or DNA sequences that interact with X in ways that would affect the interaction with X and the promoter of its own gene. Also, many other factors could affect the expression of the gene encoding X, such as concentration of RNA polymerase holoenzyme, accessibility of the promoter due to nucleoid-associated proteins, limitation on synthetic substrates such as amino acids or nucleotides, etc. Additionally, it is highly unlikely that cells grow as rapidly or as consistently in nature as they do in mid-exponential phase in the laboratory. Despite the severe limitations that accompany the numerous simplifications in modeling transcription networks, these approaches have been used multiple times to accurately represent behaviors of naturally occurring systems, including the induction of competence and sporulation in *B. subtilis* and bistability in the *lac* operon of *E. coli* [9,10]. Additionally, modeling approaches have been used to design synthetic systems *in silico* that behave in predicted ways when constructed *in vivo* [2,11].

This modeling exercise demonstrates several key points about feedback-mediated bistable systems. First, without hypersensitivity, a system cannot exhibit the sharp response to subtle variation in input conditions required to establish distinct stable states. Secondly, whether these states are possible depends both on the general shape of response functions but also on the biochemistry behind those functions. A system such as that modeled here with too high or too low a response threshold will only contain one stable steady state (Figure I.4). And finally, the impact of the random nature of biological processes such as protein production and degradation is increased at the single-cell level, where the loss or gain of a single molecule has greater weight (Figures I.7 and I.8). Bistable switches exist in all organisms and behave generally like the model system described above: hypersensitivity amplifies noise inherent in processes at the single-cell level, and positive feedback perpetuates the differences created into stable expression states.

MATLAB scripts

MATLAB script I.1: BistableContFunc.m

```
function dydt = f(t,y,flag,kB,KR,kA,gamma)
% [x] = y(1)
dydt = [(kA*y(1)^2+kB*KR)/(KR+y(1)^2)-gamma*y(1)];
```

MATLAB script I.2: BistableContSolver.m

```
% activated synthesis rate
kA=1.15;
% basal synthesis rate
kB=0.03;
% degradation constant
gamma=0.069;
% composite Kd
KR=60;
options=[];
hold off;
for (i=0:.25:20)
    [t1 y1]=ode23('BistableContFunc',[0 500],
        [i],options,kB,KR,kA,gamma);
    plot(t1,y1(:,1),'-');
```


(MATLAB script I.2 continued)

```
    hold on;
end
axis([0 500 0 20]);
```

MATLAB script I.3: BistableDiscreteSim.m

```
% cell volume in fL (Figure I.7 = 3, Figure I.8 = 30)
vol=3;
% correction factor for molecules per cell (1 nM in a 1 fL cell =
% 0.6 molecules)
c=0.6*vol;
% activated synthesis rate (corrected)
kA=1.15*c;
% basal synthesis rate (corrected)
kB=0.03*c;
% degradation constant
gamma=0.069;
% composite Kd (corrected)
KR=60*c^2;
% simulation parameters
initialn=floor(5*c);
running_time=1000;
colors=['r'; 'g'; 'b'];
hold off;
for (i=1:length(colors))
    % index
    k=1;
    clear t;
    clear state;
    n=initialn;
    % time at index k
    t(k)=0;
    % n at index k
    state(k)=n;
    while t(k) < running_time
        k = k+1;
        % evaluate f(x)
        rcreate_n = (kA*n^2+kB*KR)/(KR+n^2);
        % evaluate g(x)
        rdestroy_n = gamma*n;
        % Gillespie f(x) and g(x) times
        time_create = -log(rand(1,1))./rcreate_n;
        time_destroy = -log(rand(1,1))./rdestroy_n;
        % when did the first event happen?
        time = min(time_create, time_destroy);
        % which event happened first?
        if (time_create < time_destroy)
            n = n+1;
        elseif (time_create > time_destroy)
```

(MATLAB script I.3 continued)

```
        n = n-1;
    end
% increment time and state arrays
    t(k) = t(k-1)+time;
    state(k) = n;
end
stairs(t,state,colors(i));
hold on;
end
axis([0 running_time 0 25*c]);
```

References

1. Rusinova E, Ross JB, Laue TM, Sowers LC, Senear DF (1997) Linkage between operator binding and dimer to octamer self-assembly of bacteriophage λ cI repressor. *Biochem* 36: 12994–13003.
2. Elowitz MB, Leibler S (2000) A synthetic oscillatory network of transcriptional regulators. *Nature* 403: 335–338.
3. Raser JM, O'Shea EK (2005) Noise in gene expression: origins, consequences, and control. *Science* 309: 2010–2013.
4. Kolodrubetz D, Schleif R (1981) Identification of *araC* protein and two-dimensional gels, its *in vivo* instability and normal level. *J Mol Biol* 149: 133–139.
5. Gillespie DT (1976) A general method for numerically simulating the stochastic time evolution of coupled chemical reactions. *J Comput Phys* 22: 403–434.
6. Kubitschek HE, Friske JA (1986) Determination of bacterial cell volume with the Coulter Counter. *J Bacteriol* 168: 1466–1467.
7. Skarstad K, Steen HB, Boye E (1985) *Escherichia coli* DNA distributions measured by flow cytometry and compared with theoretical computer simulations. *J Bacteriol* 163: 661–668.
8. Dahirel V, Paillusson F, Jardat M, Barbi M, Victor J-M (2009) Nonspecific DNA-protein interaction: why proteins can diffuse along DNA. *Phys Rev Lett* 102: 228101.
9. Süel GM, Garcia-Ojalvo J, Liberman LM, Elowitz MB (2006) An excitable gene regulatory circuit induces transient cellular differentiation. *Nature* 440: 545–550.
10. Ozbudak EM, Thattai M, Lim HN, Shraiman BI, van Oudenaarden A (2004) Multistability in the lactose utilization network of *Escherichia coli*. *Nature* 427: 737–740.
11. Ajo-Franklin CM, Drubin DA, Eskin JA, Gee EPS, Landgraf D, et al. (2007) Rational design of memory in eukaryotic cells. *Genes Dev* 21: 2271–2276.

Appendix II

**Expression of the type 1 pneumococcal pilus is bistable and negatively regulated
by the structural component RrgA**

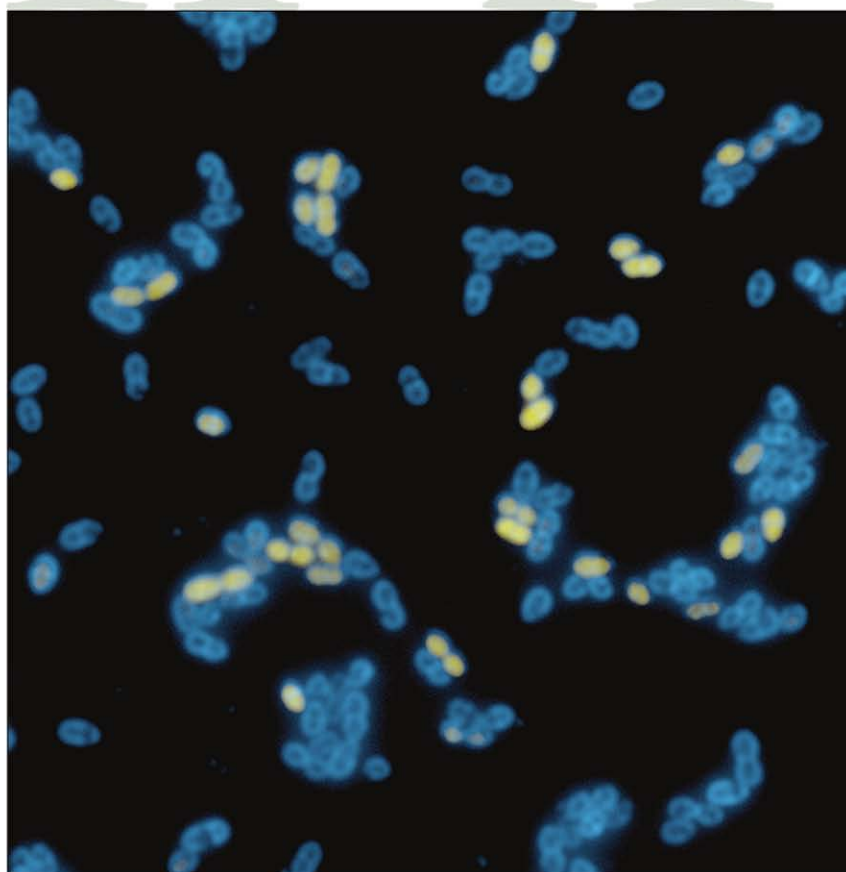
Attributions

This appendix is a reprint of a paper published in the August 2011 issue of *Infection and Immunity* (Basset A, Turner KH, Boush E, Sayeed S, Dove SL, Malley R (2011) Expression of the Type 1 Pneumococcal Pilus Is Bistable and Negatively Regulated by the Structural Component RrgA. *Infect Immun* 79: 2974-2983). Alan Basset and I performed the majority of experimental work in this paper. Elizabeth Boush assisted with the flow cytometric analysis of cell populations and Sabina Sayeed assisted in the genetic manipulation of pneumococcal strains. Dr. Basset, Simon L. Dove, Richard Malley and I wrote the paper. This work is reprinted with permission of the American Society for Microbiology.

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Cover photograph (Copyright © 2011, American Society for Microbiology. All Rights Reserved.):
Bistable expression of the type I pilus genes in *Streptococcus pneumoniae*. Shown are cells of a strain of *S. pneumoniae* carrying the green fluorescent protein (GFP) gene in place of the *rrgC* pilus gene that have been stained with the membrane dye FM 4-64FX (Invitrogen). GFP reporter fluorescence is shown in yellow pseudocolor, and membrane dye fluorescence is shown in cyan pseudocolor.

Expression of the Type 1 Pneumococcal Pilus Is Bistable and Negatively Regulated by the Structural Component RrgA^{▽†}

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Simon L. Dove,[‡] and Richard Malley^{‡*}

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The pneumococcal type 1 pilus, which is present in 25 to 30% of clinical isolates, has been associated with increased adherence and inflammatory responses and is being evaluated as a potential vaccine candidate. Here we show that expression of the pilus is bistable as a result of the molecular interaction between the transcription activator RlrA and a structural component of the pilus called RrgA. Sampling various clinical pneumococcal isolates that harbor the type 1 pilus-encoding islet, we show that distinct populations of cells can be identified with either undetectable or prominent pilus expression. When these two populations are separated and regrown in liquid medium, they are phenotypically different: the nonexpressing population reverts to the previous bimodal distribution, whereas the expressing population retains the same high level of pilus expression. Controlled exogenous expression of the regulatory pilus gene *rlrA* in a strain from which the endogenous version has been deleted increases pilus expression steadily, suggesting that the bistable expression of the pilus observed in wild-type cells is dependent on the native *rlrA* promoter. Finally, we demonstrate that RrgA is a negative regulator of pilus expression and that this repression is likely mediated through direct interaction with RlrA. We conclude that type 1 pilus expression in pneumococcus exhibits a bistable phenotype, which is dependent upon the molecular interplay between the RlrA and RrgA proteins. We suggest that this flexibility in expression may assist adaptation to a range of immune conditions, such as evasion of antipilus antibodies, within potential hosts.

The Gram-positive bacterium *Streptococcus pneumoniae* (pneumococcus), in which the genetic role of DNA was discovered (1), is among the most analyzed organisms. However, aspects of its pathogenicity continue to be elusive. Pneumococcus is one of the major causes of bacterial pneumonia, meningitis, and septicemia, accounting for about 11% of mortality worldwide in children under 5 years of age (31). The bacterium is a frequent colonizer of the nasopharynx in children; most children are colonized by pneumococcus at some point during the first 2 years of life. It remains unclear, however, why some children develop invasive disease, whereas in the majority of cases, colonization remains asymptomatic, and a combination of bacterial virulence and host factors may be responsible.

Currently available pneumococcal vaccines target the main virulence factor, the capsular polysaccharide, and generate systemic immunity via anticapsular antibodies (6, 7). Immunogenicity in infants requires that these polysaccharides be conjugated to carrier proteins, an approach that is practicable for only a minority of the >90 known capsular serotypes. These “conjugate” vaccines, however, have limitations, including selective coverage of capsular types included, only partial protection against mucosal disease, the phenomenon of serotype replacement, and high cost (14, 22). For these reasons, alter-

native vaccine strategies have been sought, including the development of serotype-independent protein-based vaccines (3, 8, 32).

One of the most recent identified antigens proposed as a potential vaccine target is the pneumococcal pilus (13). At least two types of pneumococcal pili exist: type 1, shown to have several properties related to colonization and invasive disease (4) and encoded by the *rlrA* pathogenicity islet present in 25% of strains (5); and a second, type 2, which is rarer (2). Both pili act as adhesins (2, 30); the type 1 pilus was shown to enhance colonization in a mouse model (4) and facilitate the formation of microcolonies and biofilms (28). Type 1-piliated pneumococcal strains were also shown to induce significantly more tumor necrosis factor alpha (TNF- α) in a mouse model of intraperitoneal sepsis than their pilus-negative isogenic controls (4).

The genes encoding the pneumococcal type 1 pilus are found in approximately 25% of clinical isolates (5). Different structural models of the type 1 pilus have been proposed (12, 16, 17, 34), but in general, it is agreed that the RrgB protein forms the backbone, to which are attached two ancillary proteins, RrgA and RrgC, which are thought to be an adhesin and anchor to the cell wall, respectively. Prior to universal immunization with pneumococcal conjugate vaccine Prevnar in the United States, the type 1 pilus was found predominantly in serotypes that were included in the vaccine (5). These serotypes, and thus the prevalence of pilus-positive strains, sharply declined after the introduction of this vaccine, but the percentage of pilus-positive strains returned to prevaccine levels within the subsequent 3 to 5 years (33).

This persistence of the pilus in the pneumococcal population despite the initial impact of immunization suggests that this

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† Supplemental material for this article may be found at <http://iai.asm.org/>.

‡ S. L. Dove and R. Malley contributed equally to this study.

▽ Published ahead of print on 16 May 2011.

TABLE 1. Characteristics of the strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
T4	Spontaneous Sm ^r derivative of TIGR4	www.tigr.org
T4 Δ (pilus)	$\Delta rlrA$ - <i>srtD</i> :: <i>cat</i> Sm ^r Cm ^r	This study
T4 $\Delta rrgA$	$\Delta rrgA$::Janus Km ^r	This study
T4 $\Delta rrgA$ GFP	$\Delta rrgA$::Janus $\Delta rrgC$:: <i>gfp</i> (<i>mut3</i>) Km ^r	This study
T4 $\Delta rrgC$	$\Delta rrgC$::Janus Km ^r	This study
T4 GFP	$\Delta rrgC$:: <i>gfp</i> (<i>mut3</i>) Sm ^r	This study
T4 $\Delta rlrA$ J	$\Delta rlrA$::Janus Km ^r	This study
T4 $\Delta rlrA$	$\Delta rlrA$::Sm ^r	This study
T4 $\Delta rlrA_c$ GFP	$\Delta rlrA$:: <i>cat</i> $\Delta rrgC$:: <i>gfp</i> (<i>mut3</i>) Sm ^r Cm ^r	This study
T4 $\Delta rlrA_c$	$\Delta rlrA$:: <i>cat</i> Sm ^r Cm ^r	This study
T4 <i>rlrA</i> -VSV-G	<i>rlrA</i> -VSV-G Sm ^r	This study
T4 $\Delta rrgA$ <i>rlrA</i> -VSV-G	<i>rlrA</i> -VSV-G $\Delta rrgA$::Janus Km ^r	This study
T4 $\Delta malQ$	$\Delta malQ$::Janus Km ^r	This study
T4 GFP <i>Pmal</i> - <i>rlrA</i> $\Delta rlrA$	$\Delta rrgC$:: <i>gfp</i> (<i>mut3</i>) <i>Pmal</i> $\Delta malQ$:: <i>rlrA</i> $\Delta rlrA$:: <i>cat</i> Sm ^r Cm ^r	This study
T4 GFP <i>Pmal23</i> - <i>rlrA</i> $\Delta rlrA$	$\Delta rrgC$:: <i>gfp</i> (<i>mut3</i>) <i>Pmal23</i> $\Delta malQ$:: <i>rlrA</i> $\Delta rlrA$:: <i>cat</i> Sm ^r Cm ^r	This study
Plasmids		
pAC1000	Derivative of pEV3	15
pQE30- <i>rrgA</i>	Wild-type <i>rrgA</i> gene with His ₆ tag	This study
pQE-30- <i>rrgB</i>	Wild-type <i>rrgB</i> gene with His ₆ tag	This study
pET21b- <i>rlrA</i> -VSV-G	<i>rlrA</i> gene with VSV-G tag	This study

structure provides important selective advantages in colonization; at the same time, the fact that in all populations surveyed, the prevalence does not exceed 30% suggests that there must be a cost to the presence of the pilus, perhaps due to the age-dependent rise in antibodies directed against pilus type 1 proteins (33). This potential fitness cost led us to investigate the mechanism of regulation of pilus gene expression in pneumococcus. Here we show, using various clinical strains grown in different media, that the expression of the type 1 pilus is bistable, with cells within a population either expressing the pilus or not. Moreover, we show that the structural protein RrgA is a negative regulator of this expression via its interaction with the pilus regulator protein RlrA.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains and plasmids are listed in Table 1. Primers used in this study are listed in the table in the supplemental material. Pneumococcal strains were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY), on plates containing tryptic soy agar with 5% sheep's blood (TSA), or in minimal medium (19). Antibiotics (300 μ g/ml kanamycin, 600 μ g/ml streptomycin, and 4 μ g/ml chloramphenicol) were added where indicated. *Escherichia coli* strains were grown in LB medium supplemented with 100 μ g/ml ampicillin when indicated. All pneumococcal mutants were constructed from a spontaneous streptomycin-resistant (Sm^r) derivative of the serotype 4 clinical isolate TIGR4 (strain T4). Bacteria were killed by heating at 58°C for 1 h prior to flow cytometry analysis.

Cloning strategies. Pneumococcal mutants were generated using either a suicide vector, pAC1000, or by use of overlapping PCR strategies, as described previously (15, 18). Where indicated, mutants were made either using the bicistronic Janus cassette (36) or the chloramphenicol resistance gene. Every mutant reported in this study had the corresponding area of interest sequenced and its genotype confirmed.

A strain with full deletion of the pilus islet, T4 Δ (pilus), was constructed by PCR amplification of the upstream and downstream region of the *rlrA* pathogenicity islet with the Dop1/Dop2' and Dop3'/Dop4 primer pairs, respectively. The upstream and downstream regions were combined by overlapping PCR and cloned into vector pGEM-T. The chloramphenicol resistance gene was amplified from plasmid pAC1000 using oligonucleotides (CmOp1/CmOp2) and further cloned in between upstream and downstream regions using the XbaI restriction site, which lead to the creation of the plasmid called pAL1. Finally, the product of the PCR using the Dop1/Dop4 primer pair on the pAL1 plasmid was used to

transform the TIGR4 strain. Colonies that underwent the double-recombination event were selected on TSA plates supplemented with chloramphenicol, and their genotype was confirmed by PCR. Western blot analysis was then performed on cell wall extracts of T4 Δ (pilus) as described previously (20).

Strain T4 $\Delta rrgA$ was generated in the same fashion as strain T4 Δ (pilus) using primer pairs DrrgA1/DrrgA2, DrrgA3/DrrgA43, and DrrgA5/DrrgA6 (36). In order to evaluate the phenotype of a complemented $\Delta rrgA$ strain, the *rrgA* gene was reintroduced in its original locus.

In order to make some of the mutants needed for the study, genes *rrgC*, *rlrA*, and *malQ* were replaced by the Janus cassette independently and *rlrA* was replaced with the *cat* gene by an overlapping PCR strategy using the primers sets described in the table in the supplemental material. Strain T4 GFP was made by replacing the Janus cassette inserted instead of the *rrgC* gene with *gfp*(*mut3*), a *mut3* gene coding for green fluorescent protein (GFP). T4 GFP was then transformed with genomic DNA of T4 $\Delta rlrA_c$ and T4 $\Delta rrgA$; colonies were then selected on plates containing chloramphenicol or kanamycin, respectively, in order to isolate T4 $\Delta rlrA_c$ GFP and T4 $\Delta rrgA$ GFP.

T4 $\Delta rlrA$ was made by removal of the Janus cassette previously inserted into the *rlrA* gene. Strain T4 *rlrA*-VSV-G was then made by inserting a vesicular stomatitis virus glycoprotein (VSV-G) epitope tag at the C terminus of *rlrA*. Strain T4 $\Delta rrgA$ *rlrA*-VSV-G was obtained by transforming strain T4 *rlrA*-VSV-G with genomic DNA of T4 $\Delta rrgA$ and selection using plates supplemented with kanamycin.

An *rlrA* mutant strain with *rlrA* under the influence of a maltose-inducible promoter was constructed as follows. T4 GFP *Pmal*-*rlrA* $\Delta rlrA$ was obtained in a succession of steps, by first transforming the T4 GFP strain with genomic DNA of T4 $\Delta malQ$ and selection on kanamycin. The resulting strain, T4 $\Delta malQ$ GFP, was further transformed with genomic DNA of T4 $\Delta rlrA_c$ and a T4 $\Delta rlrA$ $\Delta malQ$ GFP strain was selected on a blood agar plate supplemented with chloramphenicol. Finally T4 GFP *Pmal*-*rlrA* $\Delta rlrA$ was obtained by replacing the Janus cassette with the *rlrA* gene. Because this strain showed expression of the pilus in THY, even in the absence of added maltose, suggesting leakiness of the promoter, the ribosome binding site (RBS) of the maltose promoter was altered by use of overlapping PCR in order to decrease the activity of the promoter. Several strains containing mutations of the RBS of the *malQ* promoter were evaluated by flow cytometry in the presence of increasing concentrations of maltose in minimal medium. Promoter *Pmal23* was selected as it had negligible pilus expression in the absence of maltose and increasing pilus expression with increasing concentration of maltose. Sequencing of the RBS of *Pmal23* showed that it contained TACGGA instead of ACGAGG present in the RBS of the wild-type T4 strain.

Protein purification and expression. Genes encoding His₆-tagged RrgA and RrgB were cloned into pQE30, whereas the gene encoding His₆-tagged RlrA-VSV-G was cloned into pET21b vector for expression. Plasmids were transformed into *E. coli* strain BL21(DE3) for expression. Bacteria were grown to an

optical density at 600 nm (OD_{600}) of 0.6 and then induced with 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 4 h at 16°C. Bacteria were harvested and then suspended in lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM imidazole, 1× antiprotease [Roche], 0.1% lysozyme, 10 mM $MgCl_2$, silicone, and DNase) before being sonicated six times for 30 s each. Samples were centrifuged for 30 min at 14,000 rpm in the cold, and supernatants were collected. Five hundred microliters of Ni-nitrilotriacetic acid (NTA) was added to samples, and the mixture was incubated for 1 h at 4°C. Samples were loaded onto a column, washed twice with buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 30 mM imidazole) at pH 8.0, and then eluted in buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 250 mM imidazole) at pH 8.0. Protein purity was analyzed on NuPage 4 to 12% Bis-Tris gel (Invitrogen), and protein concentrations were quantified using the bicinchoninic acid (BCA) protein assay (Pierce).

Generation and testing of polyclonal antibodies. Purified recombinant His₆-tagged RrgA and RrgB were used to immunize guinea pigs and rabbits, respectively (Cocalico Biologicals, Inc.). Final antiserum bleeds were tested at various dilutions on pure protein to determine the optimal dilution for flow cytometry or Western blotting.

Flow cytometry analysis. Analysis was performed on a Beckman Coulter MoFlo Legacy flow cytometer. GFP and Alexa 488 were excited by a Coherent Sapphire solid-state laser emitting at 488 nm, whereas Alexa 660 was excited by a Dako red diode laser emitting at 635 nm. Prior to each experiment, the flow cytometer was aligned with Beckman Coulter flow-check Fluorospheres (part no. 6605359) and SPHEROUltra Rainbow calibration particles (catalog no. URCP-38-2K) and then configured to trigger off the side scatter (SSC) parameter. To confirm that the flow cytometer was capturing all of the bacteria via the SSC trigger, three sizes of Sphero polystyrene particles were run through the machine prior to each acquisition: 1.00 to 1.49 μ m (catalog no. PP-10-10), 0.4 to 0.6 μ m (catalog no. PP-01-10), and 0.2 to 0.3 μ m (catalog no. PP-025-10). The flow cytometer settings remained the same for all experiments. Postacquisitional analysis was performed using Beckman Coulter Summit 4.3 software and Tree-star FlowJo 8.7.3. Heat-killed bacteria were blocked for 1 h in buffer 0 (1× phosphate-buffered saline [PBS] with 1% bovine serum albumin [BSA]), treated for 1 h with primary antibody in buffer 1 (1× PBS, 0.05% Tween 20, 1% BSA), washed once with buffer 1, and then treated for 1 h with secondary antibody in buffer 1. Bacteria were washed three times in buffer 1 and analyzed by flow cytometry using MoFlo. The experiments were performed at room temperature. Alexa Fluor 488 or 660 secondary antibodies from Invitrogen were used at a 1/50 dilution.

Micrographs. Cells of *S. pneumoniae* T4 GFP, T4 Δ rhlA GFP, and T4 Δ rgA GFP were suspended from TSA plates into 1 ml of PBS and fixed and imaged essentially as described previously (37).

Separation of bacteria by magnetic beads. To separate bacteria on the basis of pilus expression with magnetic beads, pneumococcal strains were grown to an optical density of 0.5. The equivalent of 5×10^7 CFU was blocked in buffer 0, treated for 1 h with an anti-RrgB antibody at a 1/200,000 dilution in buffer 1, and then washed twice in buffer 2 (buffer 1 with 2 mM EDTA). Bacterial pellets were suspended and incubated for 15 min at 4°C into 160 μ l of buffer 2 and 40 μ l of anti-rabbit IgG microbeads (Milenyi Biotec). Bacteria were washed three times in buffer 2 and passed through MACS separation columns (Milenyi). The flowthrough fraction was collected, and the absence of pilus expression was confirmed by flow cytometry or Western blotting. Eluted bacteria were harvested by centrifugation and examined by flow cytometry and Western blotting.

RNA isolation and real-time RT-PCR. Cells from 2 ml of bacteria at an OD_{600} of 0.5 were harvested and suspended in 100 μ l of Tris-EDTA (TE) (Ambion). Cells were lysed on the amalgamator three times for 16 s after addition of glass beads (Sigma). Immediately afterwards, 350 μ l of RLT buffer with β -mercaptoethanol (10 μ l/ml) was added to samples, mixed, and then centrifuged for 2 min. Supernatants were collected, and 250 μ l of ethanol (100%) was added. Subsequent steps were performed per the instructions included in the RNA minikit from Qiagen. Transcripts were quantified by quantitative real-time reverse transcription (RT)-PCR relative to the *gyrB* transcript essentially as described previously (23).

Coimmunoprecipitation assay using purified proteins or live bacteria. Various amounts of RlrA-VSV-G and RrgA purified proteins were incubated together overnight at 4°C. The following day, 80 μ l of buffer 3 (50 mM Tris-HCl [pH 7.8], 300 mM NaCl, 1 mM EDTA, 1% BSA, 2% Triton X-100) and 20 μ l of agarose-immobilized goat anti-VSV-G antibody (Bethyl Laboratories) were added to each sample and incubated at room temperature for 1 h. After extensive washing in buffer 3, samples were suspended in SDS gel loading buffer, and Western blotting of these samples was performed for detection of RlrA-VSV-G and RrgA proteins. Protein bands were detected using either a rabbit anti-VSV-G affinity-purified antibody (Bethyl Laboratories, Inc.) or antibody against the RrgA protein.

To detect the association of the two proteins in the context of live bacteria, pneumococcal strains were grown in 30 ml of THY at an OD_{600} of 0.8. Bacterial cells were collected and lysed in buffer 4 (50 mM Tris-HCl [pH 7.8], 300 mM NaCl, 1× antiprotease [Roche], 1 mM EDTA, 2% Triton X-100) with 1% sodium deoxycholate and DNase. Samples were incubated for 30 min at 37°C and centrifuged, and supernatants were collected. Forty microliters of agarose-immobilized goat anti-VSV-G antibody was added to the samples, and the mixture was incubated overnight at 4°C. Samples were extensively washed in buffer 4 and suspended in SDS gel loading buffer. A Western blot analysis was performed to check for the presence of both RlrA-VSV-G and RrgA proteins.

ChIP. Cultures of T4 *rhlA*-VSV-G and T4 Δ rgA *rhlA*-VSV-G were inoculated in triplicate from TSA plates at a starting OD_{600} of 0.05 and grown to an OD_{600} of 0.8 in THY broth. Chromatin immunoprecipitation (ChIP) was then performed using 3 ml of culture, and fold enrichment values were measured by quantitative PCR relative to the *gyrB* coding region essentially as described previously (9).

RESULTS

Expression of pneumococcal pilus type 1 is bimodal. In the course of studies focused on the analysis of the inflammatory response to pneumococci, we generated antibodies to the structural proteins of the pilus RrgA and RrgB and evaluated how these antibodies bound to the pneumococcal strain serotype 4 (T4) strain TIGR4 by flow cytometry. We began our studies by growing a clonal population of T4 in liquid medium, incubating it with RrgB polyclonal antibodies, and analyzing the results by flow cytometry. Surprisingly, and in contrast to findings by Moschioni et al. (27), a dual population was observed, with only roughly 30% of the population showing fluorescence following incubation with RrgB antibodies (Fig. 1A, left panel). We repeated this experiment now using the same protocol as described previously (27) and still detected two populations. The same observation was made when cells of strain T4 were grown in various media, such as tryptic soy medium, Dulbecco's modified Eagle's medium (DMEM), pneumococcal minimal medium, or RPMI (data not shown). For convenience, the low-fluorescence subpopulation will be referred as LPE (for low pilus expression) as opposed to HPE (high pilus expression) for the high-fluorescence subpopulation. When a strain deleted for the pilus genes was used, only cells of the LPE population remained, showing that the binding of the antibody was specific for the pilus protein in the HPE population (Fig. 1A, right panel). These findings were reproduced when antibodies directed against the other main pilus structural protein, RrgA, were used; the levels of expression of RrgB and RrgA correlated strongly with one another, as assessed by flow cytometry (Fig. 1B). We were not able to visualize the RrgC protein by flow cytometry, which may be due to its low abundance and/or location at the base of the pilus, potentially rendering it inaccessible to antibodies (29). Since deletion of the *rgc* gene does not affect RrgB polymerization (11), we inserted the *mut3* gene encoding GFP [*gfp(mut3)*] at the site of the *rgc* locus. We observed two populations of cells—one without and one with GFP expression (Fig. 1C, left panel). Overall, GFP expression correlates well with expression of the RrgB protein (Fig. 1C, right panel). This bimodality is not dependent on growth phase, as it was observed in cells harvested from blood agar plates or grown in liquid medium to the early, middle, or late log phase (data not shown). These data thus confirm that the type 1 pilus is expressed in a bimodal manner. Next we evaluated the pattern of pilus expression in a group of 13 clinical isolates

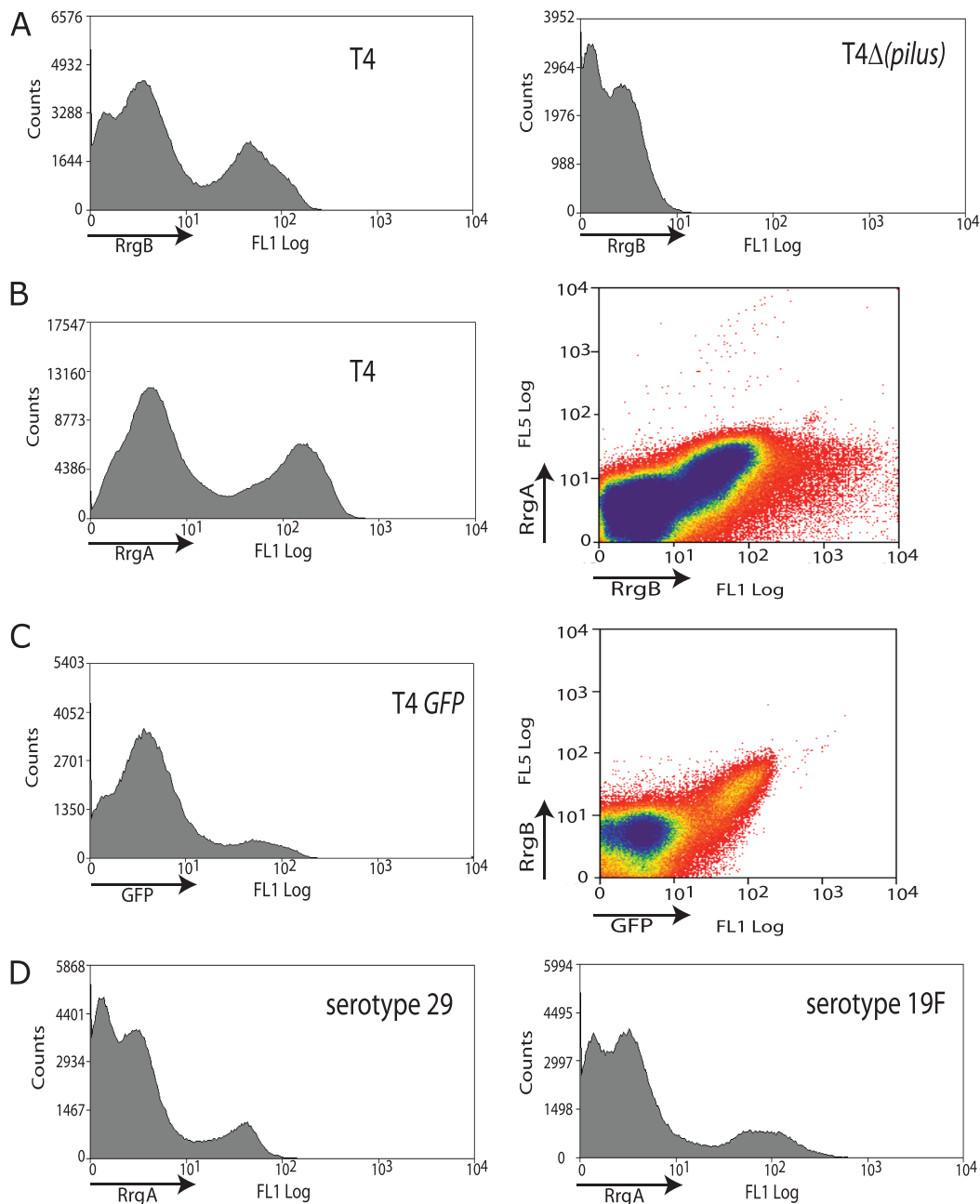


FIG. 1. The type 1 pneumococcal pilus is bimodally expressed. (A) Flow cytometry analysis performed on T4 and T4 Δ (pilus) using anti-RrgB antibody shows a dual population in the T4 strain as opposed to the T4 Δ (pilus) strain. The x axis represents the level of fluorescence, and the y axis represents the number of events counted. (B) Flow cytometry analysis of a T4 strain using anti-RrgA antibody also shows a bimodal population. When antibodies to both proteins were used to stain bacteria with different fluorophores (RrgA with Alexa 488 on FL1 and RrgB with Alexa 660 on FL5 [Invitrogen]), a strong and significant correlation was observed. (C) Flow cytometry of the T4 GFP strain shows that the GFP gene, replacing the *mgC* gene, is also expressed bimodally. GFP expression and RrgB expression also correlate strongly in a T4 GFP strain when analyzed. (D) The pattern of pilus expression in various pneumococcal strains was examined, as detailed in the text; in the majority of cases (see text), bimodal distributions were apparent.

representing serotypes 4, 6B, 7C, 9V, 12F, 13, 14, 15F, 19A, 19F, 29, and 33F (strains derived from the collection described in reference 5) using an antibody to the RrgA protein (which is less variable than RrgB) (13). We observed

the same type of dual population in 10/13 serotypes, most prominently in strains of serotypes 6B, 19F, and 29 (Fig. 1D). These results show that expression of the pilus is bimodal in strains other than T4.

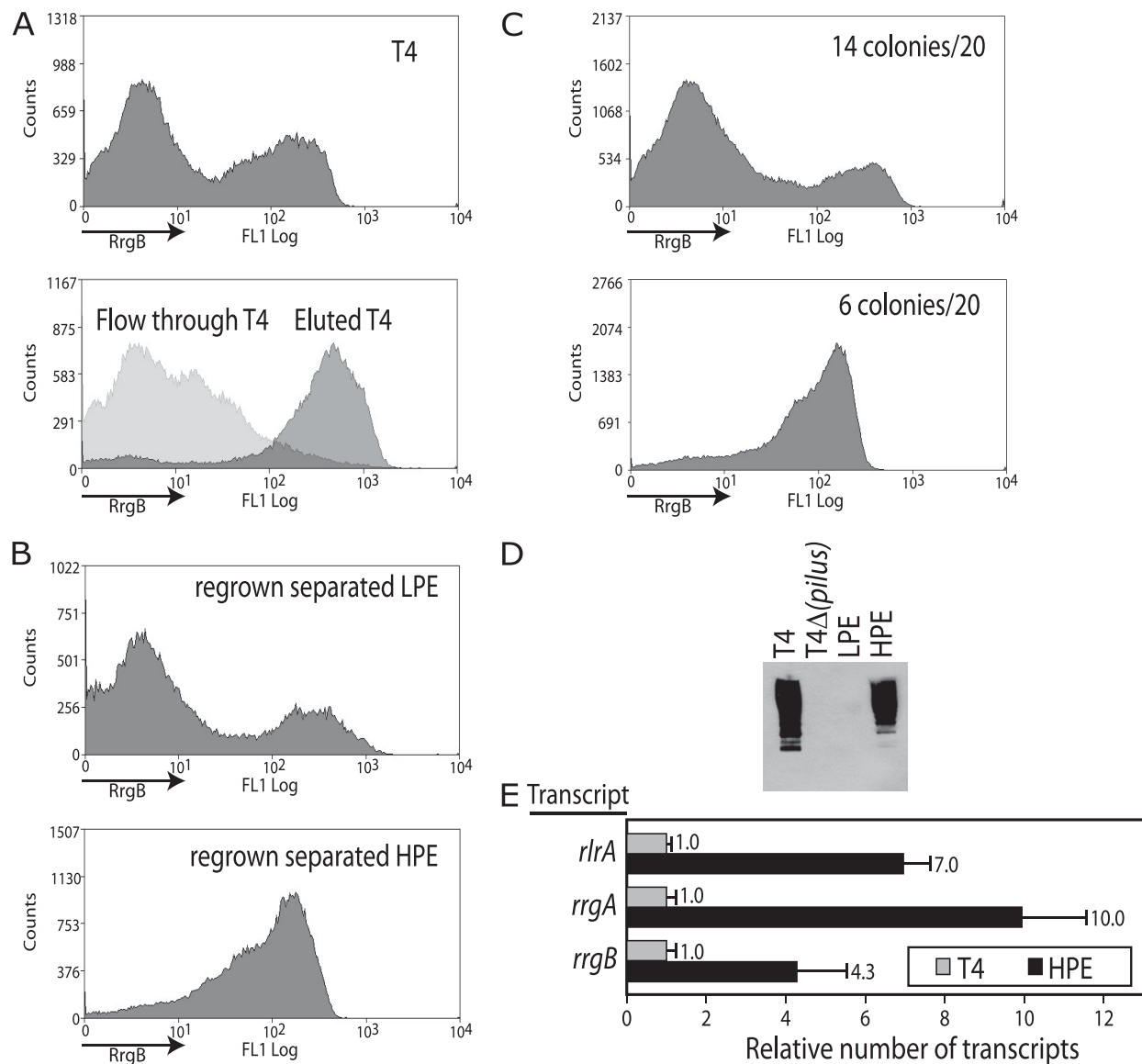


FIG. 2. The type 1 pilus is expressed in a bistable manner. (A) Regrowth of low- and high-pilus-expression (LPE and HPE, respectively) populations. (A) From a population of T4 cells exhibiting both LPE and HPE phenotypes, LPE and HPE cells were separated from one another using antibodies coupled with magnetic beads and immediately analyzed by flow cytometry targeting the RrgB protein. (B) Twenty colonies originating from either LPE or HPE cells were streaked on TSA plates and regrown in THY. The data presented here are representative of the 20 results obtained. LPE reverts to the original bimodal population, whereas the HPE phenotype remains constant. (C) Pattern of pilus expression from 20 isolated colonies from a T4 strain population. (D) Western blot analysis of flowthrough (LPE) and eluted (HPE) cells using anti-RrgB antibody confirms the absence of RrgB protein in LPE populations, in contrast to its presence in HPE cell populations. Note that the concentrations of bacterial cell lysates used in T4, HPE, and LPE lanes were not adjusted, so that relatively more protein from LPE cells was loaded on the gel; despite this, no RrgB band can be observed in the LPE lane. (E) Real-time RT-PCR of T4 and HPE populations performed to evaluate the quantity of *rlrA*, *rrgA*, and *rrgB* transcripts relative to *gyrB* transcript. The abundance of transcripts for the three genes analyzed in cells of a T4 strain was arbitrarily set to 1. The results shown here are representative of the three experiments performed. The expression of the *rlrA*, *rrgA*, and *rrgB* genes is significantly increased in HPE cells compared to cells of a T4 strain.

Type 1 pilus gene expression is bistable. To further evaluate these findings, we separated the LPE and the HPE cell populations using magnetic beads. A clonal population of T4 cells was grown to mid-exponential phase and incubated with antibodies to RrgB, following which, the bacteria were incubated

with anti-rabbit IgG-coupled magnetic beads; after incubation, the antibody-bound and non-antibody-bound bacteria were separated by elution in the presence or the absence of a magnetic field, as described in Materials and Methods. Figure 2A shows the distribution of the original bacterial suspension (top

panel) and the flowthrough (light gray, bottom panel) (LPE) and eluted bacteria (dark gray, bottom panel) (HPE) following this procedure. Interestingly, when cells of the LPE population were plated and 20 of the resultant colonies were subsequently grown in liquid medium once to mid-log phase, the bimodal pattern of pilus expression was restored (reflecting about 70% LPE and 30% HPE cells) (Fig. 2B), demonstrating that the switch to the HPE phenotype can occur very rapidly. In contrast, similar growth of the HPE population did not result in a detectable decrease in pilus expression, with the majority of cells retaining the HPE phenotype. This was also the case when these HPE cells were regrown and plated multiple times; at most, only a small percentage of the population appeared to revert to the LPE state (data not shown). When cells from 20 individual colonies of our TIGR4 reference strain were each grown to mid-log phase in liquid medium, cells in 14 out of 20 (70%) of the cultures exhibited the same bimodal pattern as cells of the original strain, whereas cells in the remaining cultures (30%) were predominantly of the HPE phenotype (Fig. 2C). Thus, while cells in the LPE population gradually revert to a bimodal distribution upon regrowth, the phenotype of the HPE population does not revert at the same rate. Western blotting performed on isolated LPE and HPE populations using anti-RrgB antibodies confirmed that the pilus is not detectable in LPE bacteria, in contrast to HPE bacteria (Fig. 2D), confirming the findings by flow cytometry. The differences in pilus expression can also be noted at the transcriptional level; real-time RT-PCR of regrown HPE bacteria demonstrates strong upregulation of *rlrA* and genes encoding the structural proteins RrgA and RrgB compared to the TIGR4 reference strain (Fig. 2E). These findings demonstrate that cells of the LPE population can upregulate pilus expression such that the total population reaches the previously observed equilibrium, whereas HPE cells tend to be fixed in this phenotypic state. Sequence analysis of the region encompassing *rlrA* and *rrgA* in HPE cells did not show any differences from the wild-type sequence, showing that the HPE phenotype is not the result of genetic mutation.

In summary, our data show that two distinct phenotypes of pilus expression (LPE and HPE) can be detected following growth of a clonal population of pneumococci and, furthermore, that when LPE (but not HPE) cells are isolated, their subsequent growth results in the restoration of a bimodal pilus expression phenotype. Thus, we conclude that expression of the type 1 pilus is bistable.

The bistable phenotype is dependent on the endogenous *rlrA* promoter. Next, we tried to identify potential mechanisms for pilus bistability. Since RlrA has been shown to be the major positive regulator of type 1 pilus expression (15), we evaluated whether bistability was mediated through the *rlrA* promoter. As expected, a strain in which the *rlrA* gene was deleted does not express the pilus (Fig. 3A). These results were confirmed using strain T4 Δ *rlrA* GFP—no fluorescence was observed (Fig. 3B3 and B4). To evaluate whether bistable expression is mediated by the native *rlrA* promoter, we constructed a strain in which the endogenous *rlrA* gene was deleted and an exogenous *rlrA* gene was added under the control of a modified maltose promoter. As shown in Fig. 3C, the increasing concentrations of maltose in minimal media were associated with a gradual and steady increase in pilus expression but without

any evidence of a bimodal distribution. No effect of maltose could be detected on the T4 strain (data not shown). These data support the hypothesis that bistable expression of the pilus is dependent upon having the endogenous *rlrA* promoter driving *rlrA* expression.

RrgA is a negative regulator of pilus expression. In the course of evaluating the role of RrgA in inflammatory responses, we had noted that a deletion of the *rrgA* gene from T4 resulted in a significant increase in the expression of the pilus as determined by Western blotting; furthermore, the level of RrgB expression in the *rrgA* mutant resembled that found in HPE cells (Fig. 4A). Thus, we hypothesized that RrgA may be a negative regulator of pilus expression. When we analyzed the patterns of pilus expression of 20 independent T4 Δ *rrgA* mutants by flow cytometry, we found that every isolate displayed the HPE phenotype (Fig. 4B), as was also noted by microscopy when a strain deleted for the *rrgA* gene and carrying the GFP gene (T4 Δ *rrgA* GFP) was examined (Fig. 3B5 and B6). Complementation of *rrgA* restored the bimodal pattern of expression (Fig. 4C), thus supporting the hypothesis that RrgA negatively regulates bistable expression of the type 1 pilus.

Flow cytometry and Western blotting of T4 *rlrA*-VSV-G and T4 Δ *rrgA* *rlrA*-VSV-G mutants confirmed that the pilus expression was not modified by the presence of the tag. We then performed a chromatin immunoprecipitation (ChIP) experiment comparing cells of wild-type T4 *rlrA*-VSV-G and T4 Δ *rrgA* *rlrA*-VSV-G mutant strains and showed that the deletion of the *rrgA* gene results in a significant increase in amount of RlrA associated with the *rlrA*-*rrgA* intergenic region, whereas there was no evidence of increased association of the RlrA protein with either the *rrgB* or *srtB* promoter regions (Fig. 4D). These results raised the possibility that RrgA may mediate its negative effects on pilus gene expression by interacting directly with RlrA, a positive regulator of pilus gene expression. We therefore tested whether RlrA and RrgA could interact with one another by immunoprecipitation. We mixed the two purified proteins RlrA-VSV-G and RrgA and incubated the mixture with agarose-immobilized goat anti-VSV-G antibody. As shown in Fig. 4E (left panel), Western blotting with an anti-RrgA antibody confirmed that RrgA coprecipitates with RlrA, whereas no RrgA could be detected in the absence of RlrA. A similar finding was observed with lysate obtained from pneumococci expressing wild-type RrgA and a VSV-G-tagged version of RlrA, strongly supporting the idea that the two proteins interact (Fig. 4E, right panel). Thus, our data suggest that the bistable expression of the pilus is a consequence of the molecular interaction between RlrA and the negative regulator RrgA. We suggest that this negative regulation results in less RlrA at the *rlrA* promoter.

DISCUSSION

Gram-positive pili differ from their Gram-negative counterparts by the presence of covalently linked subunits containing a conserved LPXTG motif (or a variant thereof), which is the target of sortase enzymes that catalyze the covalent attachment of the backbone pilins to the cell wall. Published data suggest that the role of pili in Gram-positive bacteria in colonization and disease may be both species and process dependent. In group B streptococcus (GBS) and group A streptococcus

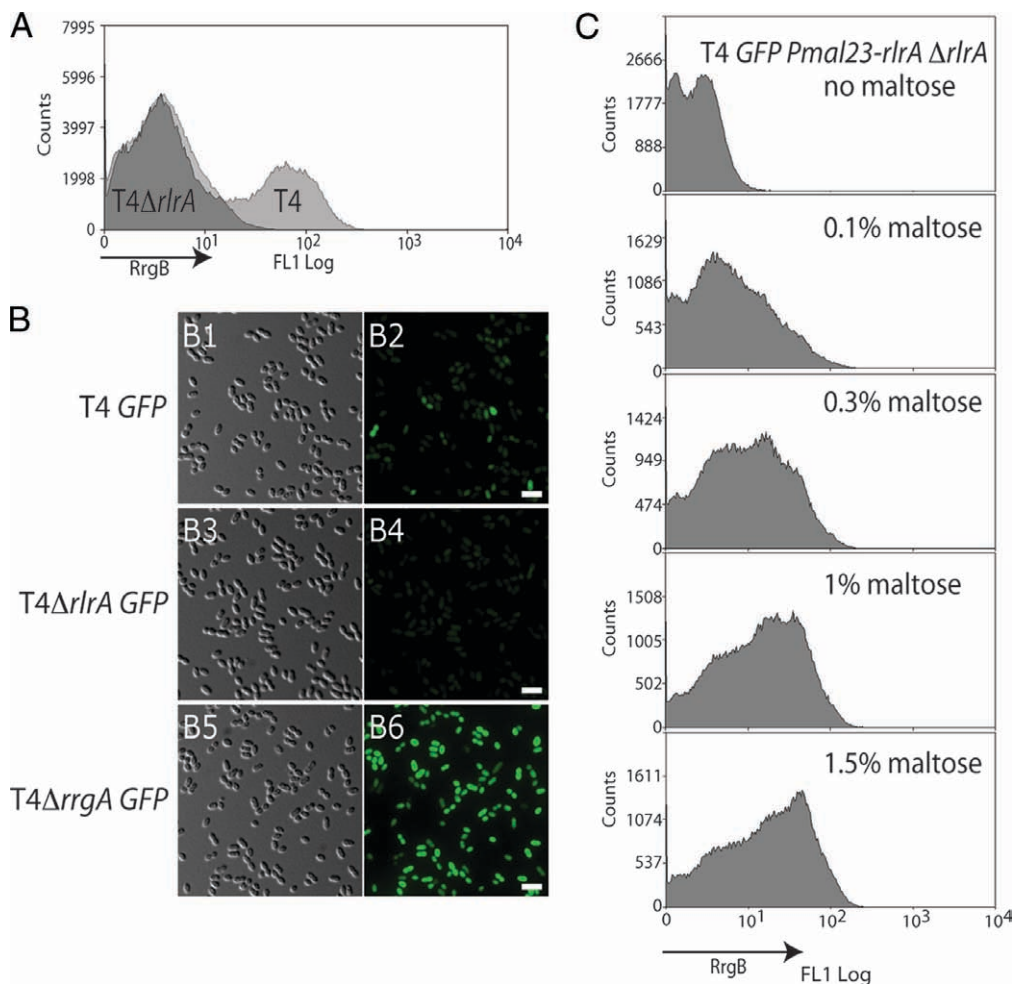


FIG. 3. The native *rlrA* promoter is essential for bistable expression of the pilus. (A) Comparison of pilus expression levels in cells of the T4 $\Delta rlrA$ strain and wild-type T4 strain by flow cytometry using anti-RrgB antibodies. (B) Fluorescent microscopy of T4 GFP, T4 $\Delta rlrA$ GFP, and T4 $\Delta rrgA$ GFP strains. Fluorescence microscopy of the T4 GFP strain (B1 and B2), in which the *rrgC* gene was replaced by the *mut3* gene coding for GFP [*gfp(mut3)*], confirms bistability of pilus expression. In contrast, fluorescence microscopy of T4 $\Delta rlrA$ GFP confirms the absence of pilus expression in the mutant (B3 and B4), whereas GFP expression is noted in all T4 $\Delta rrgA$ GFP cells (B5 and B6). (C) Expression of the pilus in cells of strain T4 GFP *Pmal23-rlrA*, in which the endogenous *rlrA* gene was deleted and an exogenous *rlrA* gene was added under the control of an altered maltose promoter. A gradual but monophasic increase in pilus expression is observed.

(GAS), as well as pneumococcus, pili are shown to be adhesins that contribute to enhanced epithelial cell attachment. However, whereas in GBS and pneumococcus the pilus appears to contribute to virulence in systemic disease (4, 24, 25), the opposite was found in *Streptococcus pyogenes*, where, unexpectedly, the presence of the pilus promotes capture of the organism in extracellular traps, thereby reducing the virulence potential of the organism (10). From an epidemiological perspective, it is also unclear whether the pilus contributes to systemic virulence in pneumococcus, as there is no difference in prevalence of pilus genes in nasopharyngeal and invasive disease isolates (5).

The data presented here add another level of complexity to the issue. Pilus expression in pneumococci appears to be tightly regulated, with only a subset of bacteria expressing the pilus at any one time. This phenotypic variability is critically dependent

on the RrgA protein, one of the structural proteins of the pilus, which physically interacts with the positive regulator RlrA, thus repressing pilus gene expression and contributing to the bistable phenotype that we observed.

Bistability is a mechanism by which bacteria can introduce heterogeneity within an isogenic population of cells, which may provide a selective advantage for subsets of bacteria under adverse conditions and which has been described in many different organisms (39). For example, some bacteria enter a dormant or vegetative state to resist antibiotic treatment, a process that would otherwise require the development or acquisition of antibiotic resistance genes (21). Bistable expression of several virulence factors may be beneficial to an organism as it may create subpopulations of cells within an isogenic population that differ in their adaptation to the environment (38). We believe the bimodal pattern of pilus expression de-

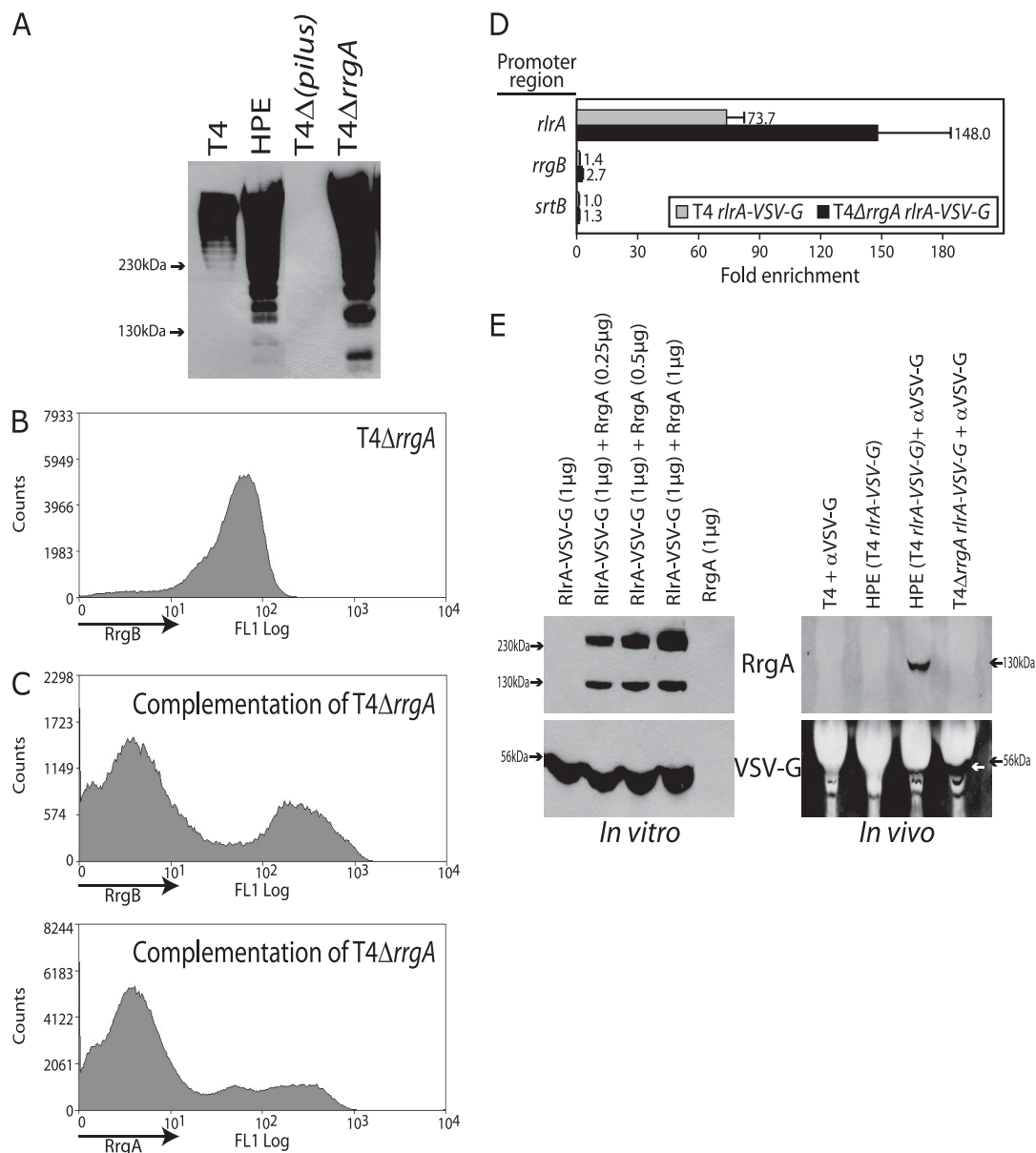


FIG. 4. The RrgA protein is a negative regulator of pilus expression that interacts directly with RlrA. (A) Detection of RrgB by Western blotting in T4, HPE, T4 Δ(*pilus*), and T4 Δ*rrgA* cells. The numbers of bacteria were equivalent in each lane. (B) Cells of the T4 Δ*rrgA* strain display only the HPE phenotype (20 strains evaluated, all strains HPE, and representative results shown). (C) Complementation of the T4 Δ*rrgA* mutant strain restores bistable expression of the pilus. (D) A ChIP experiment was performed measuring the association of RlrA with different promoters of the *rlrA* pathogenicity islet in strains T4 *rlrA*-VSV-G and T4 Δ*rrgA rlrA*-VSV-G. More RlrA was found associated with the *rlrA* promoter in T4 Δ*rrgA rlrA*-VSV-G cells than in T4 *rlrA*-VSV-G cells. RlrA did not detectably associate with the *srtB* promoter. (E) RlrA-VSV-G and RrgA proteins coprecipitate. (Left panel) Various amounts of RlrA-VSV-G and RrgA protein were incubated together, after which RlrA-VSV-G was immunoprecipitated with anti-VSV-G antibodies. There is a dose-dependent increase in detectable RrgA by Western blotting; no RrgA is detected in the absence of RlrA-VSV-G. The two bands corresponding to RrgA are also seen when the purified RrgA protein alone is run on a denaturing SDS polyacrylamide gel. (Right panel) To examine whether the two proteins interact when expressed by pneumococci, lysates of pneumococcal strains with or without a VSV-G-tagged version of RlrA and with or without RrgA were immunoprecipitated using anti-VSV-G (αVSV-G) antibodies. No RrgA protein is immunoprecipitated in the T4 strain in the absence of the VSV-G-tagged RlrA or in a T4 Δ*rrgA rlrA*-VSV-G strain. In contrast, both proteins are readily detected by Western blotting when lysates from a strain that expresses the VSV-G-tagged RlrA and wild-type RrgA are used, confirming that the two proteins interact.

scribed here represents the first example of a bistable system in *S. pneumoniae*.

We show here that type 1 pilus expression in pneumococcus is tightly regulated by a system that results in only a minority (about 30%) of cells within a population expressing the pilus. This bistable expression of the pilus appears to be the consequence of the molecular interaction between RlrA, a positive regulator of pilus gene expression, and RrgA, a negative regulator of pilus gene expression. As we show here, in a strain in which RlrA expression is placed under the control of a heterologous, maltose-inducible promoter, the gradual addition of maltose results in a graded or monophasic increase in pilus expression, implicating the endogenous *rlrA* promoter in the bistable phenotype. Similarly, in the absence of RrgA, virtually all of the cells within a population display an HPE phenotype. We also show here that the two proteins RlrA and RrgA interact in a specific manner. This interaction appears to interfere, either directly or indirectly, with the binding of RlrA to its own promoter. Taken together, these data strongly suggest that the bistable phenotype is the result of the molecular interaction between a positive regulator and negative regulator of the pilus. We imagine that another important determinant of pilus bistability is positive autoregulation of the *rlrA* gene (15). This is consistent with our finding that the native *rlrA* promoter is required for bistable expression of the pilus. Indeed, at least one bistable switch in a pathogenic bacterium has been shown to be mediated by a positive feedback loop involving a transcription regulator (37). However, positive regulation of the *rlrA* gene by RrgA alone evidently does not suffice to mediate bistability, but instead requires input from the negative regulator RrgA.

Pili in pneumococcus have been implicated in a number of biological processes, including increased host cell adherence, biofilm formation, and inflammatory responses. It is plausible that these responses may, under some circumstances, either enhance or diminish the fitness of the organism. In the case of pneumolysin, the cholesterol-dependent cytolysin of pneumococcus, the presence of the toxin is associated with enhanced virulence in animal models, but the interaction of the toxin with Toll-like receptor 4 (TLR4) results in a reduced capacity to colonize the nasopharynx and cause disease in mice (26, 35). A similar situation may be occurring with the pilus, whereby the presence of the adherence-promoting RrgA protein may initially increase the ability of the organism to colonize, but may also interfere with long-term colonization by triggering inflammatory or acquired immune responses in the host.

Given the findings presented here, the role of pilus proteins in pathogenesis and their potential as vaccine candidates must be reexamined. We had previously reported that the presence of the pilus (at the DNA level) in pneumococcal isolates in children was not associated with increased virulence (5). Given the bistable nature of pilus expression, it is now apparent that studies of pilus expression in the context of clinical infection would be necessary to evaluate more fully the role of this structure in virulence. Similarly, while protection against invasive disease was demonstrated when pilus antigens were used as vaccines in mouse models, the bistability of pilus expression could conceivably interfere with such a strategy, since the pilus is not required for virulence in humans (5). Further experiments using the two phenotypic populations among pilated

strains in animal models are planned and may allow for a more complete understanding of the role of the pilus at different stages of pneumococcal pathogenesis and immunity to this respiratory pathogen.

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REFERENCES

1. Avery, O. T., C. M. Macleod, and M. McCarty. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J. Exp. Med.* **79**:137–158.
2. Bagnoli, F., et al. 2008. A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells. *J. Bacteriol.* **190**:5480–5492.
3. Balachandran, P., A. Brooks-Walter, A. Virolainen-Julkunen, S. K. Hollingshead, and D. E. Briles. 2002. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*. *Infect. Immun.* **70**:2526–2534.
4. Barocchi, M. A., et al. 2006. A pneumococcal pilus influences virulence and host inflammatory responses. *Proc. Natl. Acad. Sci. U. S. A.* **103**:2857–2862.
5. Basset, A., et al. 2007. Association of the pneumococcal pilus with certain capsular serotypes but not with increased virulence. *J. Clin. Microbiol.* **45**:1684–1689.
6. Black, S., et al. 2000. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. *Pediatr. Infect. Dis. J.* **19**:187–195.
7. Black, S. B., et al. 2001. Postlicensure evaluation of the effectiveness of seven valent pneumococcal conjugate vaccine. *Pediatr. Infect. Dis. J.* **20**:1105–1107.
8. Briles, D. E., et al. 2000. The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. *Vaccine* **18**:1707–1711.
9. Castang, S., H. R. McManus, K. H. Turner, and S. L. Dove. 2008. H-NS family members function coordinately in an opportunistic pathogen. *Proc. Natl. Acad. Sci. U. S. A.* **105**:18947–18952.
10. Crotty Alexander, L. E., et al. 2010. MIT1 group A streptococcal pili promote epithelial colonization but diminish systemic virulence through neutrophil extracellular entrapment. *J. Mol. Med.* **88**:371–381.
11. Falker, S., et al. 2008. Sortase-mediated assembly and surface topology of adhesive pneumococcal pili. *Mol. Microbiol.* **70**:595–607.
12. Gentile, M. A., et al. 2011. Structural and functional characterization of the *Streptococcus pneumoniae* RrgB pilus backbone D1 domain. *J. Biol. Chem.* **286**:14588–14597.
13. Gianfaldoni, C., et al. 2007. *Streptococcus pneumoniae* pilus subunits protect mice against lethal challenge. *Infect. Immun.* **75**:1059–1062.
14. Hanage, W. P. 2008. Serotype-specific problems associated with pneumococcal conjugate vaccination. *Future Microbiol.* **3**:23–30.
15. Hava, D. L., C. J. Hemsley, and A. Camilli. 2003. Transcriptional regulation in the *Streptococcus pneumoniae* *rlrA* pathogenicity islet by RlrA. *J. Bacteriol.* **185**:413–421.
16. Hillerlingmann, M., et al. 2008. Pneumococcal pili are composed of protofilaments exposing adhesive clusters of Rrg A. *PLoS Pathog.* **4**:e1000026.
17. Hillerlingmann, M., et al. 2009. Molecular architecture of *Streptococcus pneumoniae* TIGR4 pili. *EMBO J.* **28**:3921–3930.
18. Horton, R. M., Z. L. Cai, S. N. Ho, and L. R. Pease. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques* **8**:528–535.
19. Lacks, S. 1966. Integration efficiency and genetic recombination in pneumococcal transformation. *Genetics* **53**:207–235.
20. LeMieux, J., D. L. Hava, A. Basset, and A. Camilli. 2006. RrgA and RrgB are components of a multisubunit pilus encoded by the *Streptococcus pneumoniae* *rlrA* pathogenicity islet. *Infect. Immun.* **74**:2453–2456.
21. Lewis, K. 2007. Persister cells, dormancy and infectious disease. *Nat. Rev.* **5**:48–56.
22. Lipsitch, M. 2001. Interpreting results from trials of pneumococcal conjugate vaccines: a statistical test for detecting vaccine-induced increases in carriage of nonvaccine serotypes. *Am. J. Epidemiol.* **154**:85–92.
23. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods* **25**:402–408.

24. Maisey, H. C., M. Hensler, V. Nizet, and K. S. Doran. 2007. Group B streptococcal pilus proteins contribute to adherence to and invasion of brain microvascular endothelial cells. *J. Bacteriol.* **189**:1464–1467.
25. Maisey, H. C., et al. 2008. A group B streptococcal pilus protein promotes phagocyte resistance and systemic virulence. *FASEB J.* **22**:1715–1724.
26. Malley, R., et al. 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc. Natl. Acad. Sci. U. S. A.* **100**:1966–1971.
27. Moschioni, M., et al. 2010. The two variants of the *Streptococcus pneumoniae* pilus 1 RrgA adhesin retain the same function and elicit cross-protection in vivo. *Infect. Immun.* **78**:5033–5042.
28. Munoz-Elias, E. J., J. Marciano, and A. Camilli. 2008. Isolation of *Streptococcus pneumoniae* biofilm mutants and their characterization during nasopharyngeal colonization. *Infect. Immun.* **76**:5049–5061.
29. Muzzi, A., M. Moschioni, A. Covacci, R. Rappuoli, and C. Donati. 2008. Pilus operon evolution in *Streptococcus pneumoniae* is driven by positive selection and recombination. *PLoS One* **3**:e3660.
30. Nelson, A. L., et al. 2007. RrgA is a pilus-associated adhesin in *Streptococcus pneumoniae*. *Mol. Microbiol.* **66**:329–340.
31. O'Brien, K. L., et al. 2009. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* **374**:893–902.
32. Ogunniyi, A. D., R. L. Folland, D. E. Briles, S. K. Hollingshead, and J. C. Paton. 2000. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect. Immun.* **68**:3028–3033.
33. Regev-Yochay, G., et al. 2010. Re-emergence of the type 1 pilus among *Streptococcus pneumoniae* isolates in Massachusetts, USA. *Vaccine* **28**:4842–4846.
34. Spraggon, G., et al. 2010. Supramolecular organization of the repetitive backbone unit of the *Streptococcus pneumoniae* pilus. *PLoS One* **5**:e10919.
35. Srivastava, A., et al. 2005. The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease. *Infect. Immun.* **73**:6479–6487.
36. Sung, C. K., H. Li, J. P. Claverys, and D. A. Morrison. 2001. An rpsL cassette, Janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. *Appl. Environ. Microbiol.* **67**:5190–5196.
37. Turner, K. H., I. Vallet-Gely, and S. L. Dove. 2009. Epigenetic control of virulence gene expression in *Pseudomonas aeruginosa* by a LysR-type transcription regulator. *PLoS Genet.* **5**:e1000779.
38. Vallet-Gely, I., J. S. Sharp, and S. L. Dove. 2007. Local and global regulators linking anaerobiosis to cupA fimbrial gene expression in *Pseudomonas aeruginosa*. *J. Bacteriol.* **189**:8667–8676.
39. van der Woude, M. W., and A. J. Baumler. 2004. Phase and antigenic variation in bacteria. *Clin. Microbiol. Rev.* **17**:581–611.

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Table S1. Primers used in this study.

Primers	Sequence	Source
	<u>Deletion of the pilus</u>	
Dop1	5-ATGGTCATGATTTTCTTAGTG-3	This study
Dop2'	5-TAAAAGCAACCCTCGCAACTGTCTAGACCAACAGCGTCTCATCTATC-3	This study
Dop3'	5-GATAGATGAGACGCTGTTGGTCTAGACAGTTGCGAGGGTTGCTTTTA-3	This study
Dop4'	5-ATGATAGAGCCGTCGGATGTC-3	This study
CmOp1	5-AGTTTCTAGACGGTATCGATAAGCTTGATG-3	This study
CmOp2	5-TTGATCTAGATGGAGCTGTAATATAAAAAACCTTC-3	This study
	<u>Deletion of <i>rrgA</i> with the <i>Janus</i> cassette</u>	
DrrgA1	5-ACTCTTTGGACTCAGGGAAC-3	This study
DrrgA2	5-ATATTATCATTTCTACACTCTCGAGTTTAGATCTTTAAACAACCTCCATCACAATAG-3	This study
DrrgA3	5-AATTAGATCTTCTATGCCTATTCCAGAGGAAATGGAT-3	This study
DrrgA4	5-AATACTCGAGCTACATTCTCTGTGTTTTTTTA-3	This study
DrrgA5	5-CTATTGTGATGGAAGTTGTTTTAAAGATCTAAACTCGAGAGTGAGAAATGATAATAT-3	This study
DrrgA6	5-CTGGATTGCTTTACCTTTGA-3	This study
	<u>Deletion of <i>rrgC</i> with the <i>Janus</i> cassette</u>	
DrrgC12	5-TGCCGGATCCTAAAAGGGTATTACAGCAGATT-3	This study
DrrgC2n	5-ATCCATTTCTCTGGAATAGGCATAGACATCAATGGCTCCTTTCTCTC-3	This study
DrrgC3n	5-GAGAGAAAGGAGCCATTGATGTCTATGCCTATTCCAGAGGAAATGGAT-3	This study
DrrgC4n	5-ATGATGTACATTGAATATCACTAGGGCCCCCTTCCTTATGCTTTTGGAC-3	This study
DrrgC5n	5-AAAACTCGAGTGGAGCTGTAATATAAAAAACCTTC-3	This study
DrrgC62	5-TGACAAGCTTCAAACCTGTATGTGCCGTAAT-3	This study
	<u>Replacing the <i>Janus</i> cassette into <i>rrgC</i> with <i>gfpmut3</i></u>	
DrrgC-GFP2	5-GAAAAGTTCTTCTCCTTTACTCATCAATGGCTCCTTTCTCTC-3	This study
DrrgC-GFP3	5-GAGAGAAAGGAGCCATTGATGAGTAAAGGAGAAGAACTTTTC-3	This study
DrrgC-GFP4	5-ATGATGTACATTGAATATCATTTGTATAGTTCATCCATGCC-3	This study
DrrgC-GFP5	5-GGCATGGACGAGCTGTACAAGTGATTTCAATGTACATCAT-3	This study
	<u>Deletion of <i>rlrA</i> the <i>Janus</i> cassette</u>	
Dop1	5-ATGGTCATGATTTTCTTAGTG-3	This study
DrlrA2	5-ATCCATTTCTCTGGAATAGGCATAGACATAGTTACCGAATCTTAGTT-3	This study
DrlrA3	5-AACTAAGATTCCGTAAGTATGTCTATGCCTATTCCAGAGGAAATGGAT-3	This study
DrlrA4	5-GTCCAAAAGCATAAGGAAAGGGGCCCTAGTAAGTTCATCGTACTGTCTAC-3	This study
DrlrA5	5-GTAGACAGTACGATGAACCTACTAGGGCCCCCTTCCTTATGCTTTTGGAC-3	This study
RA343	5-CCTCCCCCGACCCTCCGCCACCTGCCTCGGTAGGTACCTTATT-3	This study
	<u>Deletion of the <i>Janus</i> inserted in <i>rlrA</i></u>	
DrlrA1noJ	5-AACTAAGATTCCGTAAGTATGTAGTTCATCGTACTGTCTAC-3	This study
DrlrA2noJ	5-GTAGACAGTACGATGAACCTACATAGTTACCGAATCTTAGTT-3	This study
	<u>Deletion of <i>malQ</i> with <i>Janus</i></u>	
DmalQ1	5-TCATAACATCAGGGACATT-3	This study
malQJ22	5-ATCCATTTCTCTGGAATAGGCATAGAGCATCAACACACCACTTTGACG-3	This study
malQJ32	5-CGTCAAAGTGGTGTGTTGATGCTCTATGCCTATTCCAGAGGAAATGGAT-3	This study
malQJ42	5-GTTGTCAAGTCAAGCAAACCTTCTAGGGCCCCCTTCCTTATGCTTTTGGAC-3	This study
malQJ52	5-GTCCAAAAGCATAAGGAAAGGGGCCCTAGAAGGTTTGCTTGACTTGACAAC-3	This study
DmalQ6	5-GTCAGCAAGGTCATGCAAG-3	This study
	<u>Replacing the <i>Janus</i> cassette in <i>malQ</i> by <i>rlrA</i></u>	
malQ-rlrA2	5-CTTTTTCATGTATTGTTTAGCATAATAGCACCTCGTGTGTTA-3	This study
malQ-rlrA3	5-TAACACACGAGGTGCTATTATGCTAAACAAATACATTGAAAAAG-3	This study
malQ-rlrA4	5-TAGTTGTCTCCTGATTATTGTCTTATAACAAATAGTGAGCCTT-3	This study
malQ-rlrA5	5-AAGGCTCACTATTTGTTATAAGACAATAATCAGGAGACAAC-3	This study

<u>Adding a VSV-G tag into <i>rlrA</i></u>		
VSV-G1	5-CGATTCATTTCAATATCTGTATAGGATCCACCACCTCCTAACAAATAGTGAGCCTTTTAA-3	This study
VSV-G2	5-GTCCAAAAGCATAAGGAAAGGGGCCCTAGTGATATTCATGTACATCAT-3	This study
<u>Deletion of <i>rlrA</i> with <i>cat</i> gene</u>		
rlrA-Cm1	5-TGTTACCTTGGACAGTCG-3	This study
rlrA-Cm2	5-CATCAAGCTTATCGATACCGCATAGTTACCGAATCTTAGTTGC-3	This study
rlrA-Cm3	5-GCAACTAAGATTTCGGTAAGTATGCGGTATCGATAAGCTTGATG-3	This study
rlrA-Cm4	5-GTGTAGACAGTACGATGAAGTATGAGCTGTAATATAAAAACCTTC-3	This study
rlrA-Cm5	5-GAAGGTTTTTATATTACAGCTCCATAAGTTCATCGTACTGTCTACAC-3	This study
rlrA-Cm6	5-CTAGCTTCTTTTCGAGTACTTG-3	This study
<u>Mutation of the Ribosome Binding Site</u>		
RBS1	5-TATTTGTTTAGCATAATAGCANNNNNNGTGTTAAAATAATGGAACGTT-3	This study
RBS2	5-AACGTTCCATTATTTTAAACACNNNNNNNTGCTATTATGCTAAACAAATA-3	This study
<u>Cloning <i>rrgA</i> into pQE-30 plasmid</u>		
pQE30A5	5-ATTAGGATCCAAGATATTTTCAGAAGGCAGTTGCA-3	(15)
pQE30A3	5-TTAAGCATGCTTCTCTCTTTGGAGGAATAGGTTC-3	(15)
<u>Cloning <i>rrgB</i> into pQE-30 plasmid</u>		
pQE30B5	5-ATTAGGATCCCTTGCTGCCTTATTACTGA-3	(15)
pQE30B3	5-TATTGCATGCGATAGTGATTTTTTTGTTGAC-3	(15)
<u>Cloning <i>rlrA</i> into pET21b plasmid</u>		
pET21-rlrA1	5-ATTCGCTAGCCTAAACAAATACATTGAAAAAAG-3	This study
pET21-rlrA2	5-CAGTCTCGAGTTTACCCAAACGATTCAATTC-3	This study
<u>ChIP experiment primers</u>		
KRTgyrBF1	5-TGATGACCGATGCCGATG-3	This study
KRTgyrBF1	5-TTGGGCAATATAAACATAACCAGC-3	This study
KRTlrAF2	5-ATGGGATTGAAAGCGACAAGCCAC-3	This study
KRTlrAR2	5-TACAGCCGATGCTGGTCGATAACT-3	This study
KRTrrgAF2	5-AGATAAGTCTGTGCCGCTGGATGT-3	This study
KRTrrgAR2	5-AGCATAAGTCACAAGCGCTACCCT-3	This study
KRTrrgBF2	5-TCACGGGAATACTCCAAGCCGAA-3	This study
KRTrrgBR2	5-TGCACCTGTAGCATCAACCCATGT-3	This study
KRTPrlrAF2	5-AATCCGTTCACTAACAACGTAGC-3	This study
KRTPrlrAR2	5-CAAGTGTGTCATTGCCAGGTTGAG-3	This study
KRTPrrgBF1	5-ACTGGTGGTATCGGAATGTTGCCA-3	This study
KRTPrrgBR1	5-ACACTTACGGATGTTCCGTGTG-3	This study
KRTPsrtBF1	5-AAGATAGCAGGCTGAAGGGAAGAC-3	This study
KRTPsrtBR1	5-AATCTGCCAGCCTCATATCCGTT-3	This study

Appendix III

**An epigenetic switch mediates bistable expression of the type I pilus genes in
*Streptococcus pneumoniae***

Attributions

This appendix is a reprint of a paper published in the March 2012 issue of the Journal of Bacteriology (Basset A, Turner KH, Boush E, Sayeed S, Dove SL, Malley R (2012) An epigenetic switch mediates bistable expression of the type I pilus genes in *Streptococcus pneumoniae*. J Bacteriol 194: 1088-1091). Alan Basset, Simon L. Dove, Richard Malley and I designed the experiment performed in this paper, which Dr. Basset performed. Elizabeth Boush assisted with the flow cytometric analysis of cell populations and Sabina Sayeed assisted in the genetic manipulation of pneumococcal strains. Dr. Basset, Dr. Dove, Dr. Malley and I wrote the paper. This work is reprinted with permission of the American Society for Microbiology.

An Epigenetic Switch Mediates Bistable Expression of the Type 1 Pilus Genes in *Streptococcus pneumoniae*

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Expression of the pneumococcal type 1 pilus is bistable and positively regulated by the transcription factor RlrA. RlrA is also known to positively control its own expression. Here we present evidence that bistable expression of the type 1 pilus is mediated by the positive-feedback loop controlling *rlrA* expression.

Based on a screen designed to identify pneumococcal virulence genes in a mouse model of infection, the type 1 pilus was identified to be an important virulence factor (6). Further studies in mice have confirmed these findings, as the type 1 pilus was subsequently demonstrated to be a potent inflammatory agonist (1) and an adhesin (11) and to participate in biofilm formation (10). The structural proteins of the pilus have been evaluated in mice as vaccine candidates (5). At the same time, the role of the pneumococcal type 1 pilus in pneumococcal pathogenesis in humans remains uncertain, with epidemiological data conflicting. We have previously demonstrated that the prevalence of pilus genes was similar in strains isolated from blood or the nasopharynx of children; this lack of enrichment of pilated strains in bacteremic isolates strongly argues against a major role of the pilus in invasive disease (2). After the introduction of the pneumococcal conjugate vaccine Prevnar in 2000 in the United States, the prevalence of strains carrying the pilus genes declined dramatically, as a result of the association between the type 1 pilus and the capsular serotypes covered by the vaccine (2). However, several years later, with the emergence of replacement strains not covered by the vaccine, the prevalence of strains carrying the type 1 pilus genes returned to pre-2000 levels, arguing instead that the presence of these genes may confer an advantage to the organism (13).

Complicating the picture further, we and others recently demonstrated that type 1 pilus gene expression is bistable (3, 4). In particular, using flow cytometric analysis with antibodies to structural proteins of the pilus, we identified the existence of two different cell types within a clonal population of *Streptococcus pneumoniae* strain TIGR4 (T4). One of these cell types expressed the pilus (high pilus expression [HPE]), whereas the other did not (low pilus expression [LPE]). The same phenomenon was observed in cell populations of several other clinical pneumococcal strains (3). We were able to show that the bistable phenotype is dependent on the presence of the endogenous *rlrA* promoter and that bistable pilus expression is negatively regulated by RrgA, a structural component of the pilus. Our previous work, however, did not address the mechanistic basis for bistable pilus gene expression. Several mechanisms for the generation of phenotypically distinct subpopulations in a clonal population of bacteria have been described. For instance, phase-variable gene expression can arise from programmed stochastic modifications to DNA sequence or chemistry (reviewed in reference 19). In our case, we were unable to detect any changes to the *rlrA* promoter sequence between HPE and LPE populations (3), suggesting that changes to

DNA sequence are not involved in controlling the observed phenotypic variability in the pneumococcal pilus.

Gene expression systems that involve transcription feedback can also give rise to phenotypic variability. For example, bistability can be mediated by a transcription activator acting cooperatively to positively regulate its own expression; bistability can occur due to the accumulation of the regulator, in a stochastically determined subset of cells, to a concentration above the threshold required to activate expression of the gene encoding the regulator. Such feedback-mediated bistability controls phenotypic variation in a number of bacteria, including the opportunistic pathogen *Pseudomonas aeruginosa* (18). We have previously shown that expression of *rlrA* from its endogenous promoter is required for bistable pilus expression. Furthermore, RlrA is known to regulate expression from its own promoter, creating a potential positive-feedback loop (7). We therefore hypothesized that bistable expression of the type 1 pilus genes is mediated by this positive-feedback loop, with cells in which *rlrA* expression is autoactivated displaying high intracellular concentrations of RlrA with concomitant high-level expression of the pilus genes.

A hallmark of feedback-mediated bistable systems is that they display a capacity for hysteresis (12). That is, the expression state of these systems appears to display a memory of previous expression states. This phenomenon has been observed in bistable expression of the *lac* operon in *Escherichia coli*, cell cycle progression in *Xenopus laevis* oocytes, and numerous synthetic gene expression systems involving positive feedback (reviewed in reference 9). Therefore, if the type 1 pilus genes of *S. pneumoniae* exhibit bistable expression due to a feedback-mediated bistable switch in *rlrA* expression, strains lacking this positive-feedback loop should no longer exhibit a hysteretic response to previous expression states. To test this prediction, we constructed a pair of strains that contain an inducible construct for ectopic expression of *rlrA* and either are wild type for *rlrA* at its native locus (and thus contain an intact positive-feedback loop) or contain a deletion of *rlrA* (and thus lack a positive-feedback loop). If *rlrA* comprises a feedback-

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mediated bistable switch, then only the strain in which the positive-feedback loop is intact should maintain high expression of the RlrA-regulated type 1 pilus after ectopic *rlrA* expression is withdrawn. Using this experimental approach, we show here that positive feedback of *rlrA* is required for persistence of the HPE phenotype, suggesting that bistable expression of the type 1 pilus genes is mediated by the positive-feedback loop that involves RlrA.

In the studies described below, overlapping PCR products were generated and used to transform strains in which the bicistronic Janus cassette (17) replaced the gene of interest. Two different derivatives of TIGR4 (T4) were used in this study, one, called the “minus-feedback” strain (T4 *Pmal23-rlrA* Δ *rlrA*), in which the endogenous *rlrA* gene was deleted and reinserted elsewhere in the genome under the control of a modified maltose promoter (Fig. 1A), and another one, called the “plus-feedback” strain (T4 *Pmal23-rlrA*), which carries both the positively autoregulated endogenous *rlrA* gene and an exogenous copy of the *rlrA* gene under the control of the same modified maltose promoter (Fig. 1C). Both strains were grown for a period of 24 h, harvested for evaluation by flow cytometry, and then reinoculated at a low optical density daily under the indicated conditions over a period of 3 days. The flow cytometry procedure targeting the RrgB proteins of the pilus was performed as described previously (3). These strains were grown in Dulbecco modified Eagle medium (DMEM; Cellgro, Mediatech, Inc.) supplemented with either 1% glucose to repress expression from the maltose promoter (20) or 1% maltose to induce expression from the maltose promoter.

The results obtained for the minus-feedback strain are shown in Fig. 1B. On day 1, the majority of cells in the population exhibit mainly the LPE phenotype, with a minority of cells exhibiting the HPE phenotype. The latter population is likely a consequence of residual expression from the modified maltose promoter. The relative composition of the population of cells obtained after successive growth of cells from day 1 in DMEM supplemented with glucose remains similar over days 2 and 3. The degree to which the pilus is expressed in cells in these populations indicates that in the presence of glucose, exogenous *rlrA* expression is repressed to a level comparable to that of endogenous *rlrA* expression in cells of wild-type *S. pneumoniae* in the LPE state. When cells from day 1 were grown in DMEM supplemented with maltose, a majority of cells in the population shifted from the LPE to HPE states, indicating successful exogenous expression of the *rlrA* gene. Importantly, these cells exhibit a degree of pilus expression similar to that observed in cells of wild-type *S. pneumoniae* in the HPE state, indicating that the *rlrA* gene can be expressed from the maltose promoter under induction to a degree similar to that exhibited by expression from its native locus. When cells from day 2 grown in maltose were regrown on the following day (day 3) in DMEM supplemented with maltose, cells remained in the HPE state, suggesting that pilus expression can be maintained over that time period under ectopic *rlrA* expression. In contrast, when the same cells from day 2 grown in the presence of maltose were regrown in DMEM supplemented with glucose, cells in the HPE state reverted to the LPE state, consistent with repression of ectopic *rlrA* gene expression in the presence of glucose. The results from this strain, which carries a single copy of *rlrA* under the control of the inducible maltose promoter, suggest that pilus gene expression is transient after brief ectopic expression of *rlrA* in the absence of positive feedback at the *rlrA* locus.

We then conducted the same experiment with cells of the plus-feedback strain. The pilus is bistably expressed on day 1 when cells are grown in DMEM supplemented with glucose, and cells exhibit both the LPE and HPE phenotypes (Fig. 1D). The relative composition of the population of cells obtained after successive growth of cells from day 1 in DMEM supplemented with glucose remains similar over days 2 and 3. On day 2, when grown in DMEM supplemented with maltose, the entire population of cells switches to the HPE phenotype in a manner similar to that observed with the minus-feedback strain (Fig. 1D and B). When the cells of the plus-feedback strain are subsequently cultured in DMEM supplemented with glucose on day 3 (i.e., under conditions in which ectopic expression of *rlrA* is repressed), cells remain in the HPE state (Fig. 1D). These results suggest that expression of the type 1 pilus exhibits hysteresis when the positive-feedback loop is intact. This is the first demonstration of hysteresis in bistable expression of the pneumococcal type 1 pilus and supports the hypothesis that positive feedback of *rlrA* mediates bistable pilus gene expression.

These data therefore provide a plausible explanation for the stability of the HPE state: once a certain threshold concentration of RlrA sufficient to activate the positive-feedback loop is reached, autoactivation maintains high-level expression of *rlrA* with concomitant high-level expression of the type 1 pilus genes. It should be noted that our experiments do not specifically address the mechanism by which RlrA positively regulates expression of its own gene. RlrA may function as an activator simply by contacting RNA polymerase; alternatively, it is formally possible that RlrA functions through a more complex mechanism and exerts its effects by influencing the methylation state of the *rlrA* promoter (19).

Given our findings, one could of course wonder why only a fraction of cells expresses the pilus at any one time. However, as we observed (3), there is a demonstrable rate of reversion of HPE cells to the LPE state, and the balance between these rates presumably determines the proportion of cells in each expression state. In relation to this, we have shown previously that RrgA interacts directly with RlrA and negatively regulates pilus gene expression (3). Moreover, others have demonstrated the importance of other regulators in the control of pilus gene expression (8, 15, 16). Thus, the complex interplay between these negative regulators, including RrgA, and the positive regulator RlrA may determine this equilibrium and potentially provide a mechanism for reversion to the LPE phenotype. Interestingly, both RrgA and RlrA are encoded on the *rlrA* pilus pathogenicity island, suggesting that acquisition of this island conferred not only the genes encoding the pilus but also the capacity for its bistable expression. This has important implications for the spread of the type 1 pilus genes among pneumococcal strains in the clinical setting, as well as for horizontal gene transfer more broadly.

It is thus apparent that *S. pneumoniae* maintains very tight control of type 1 pilus gene expression. The type 1 pilus genes are present in only a minority (25%) of strains, and, on the basis of circumstantial epidemiological data, may be more common in immunologically naïve individuals than those with preexisting antibodies (2, 14). Despite near eradication of strains that contain the pilus genes soon after the introduction of the pneumococcal conjugate vaccine, strains that contain the pilus genes have since returned to previous levels, which argues for a relative advantage of these genes under certain conditions. However, the fact that the pilus is bistably expressed, a phenomenon that we propose arises

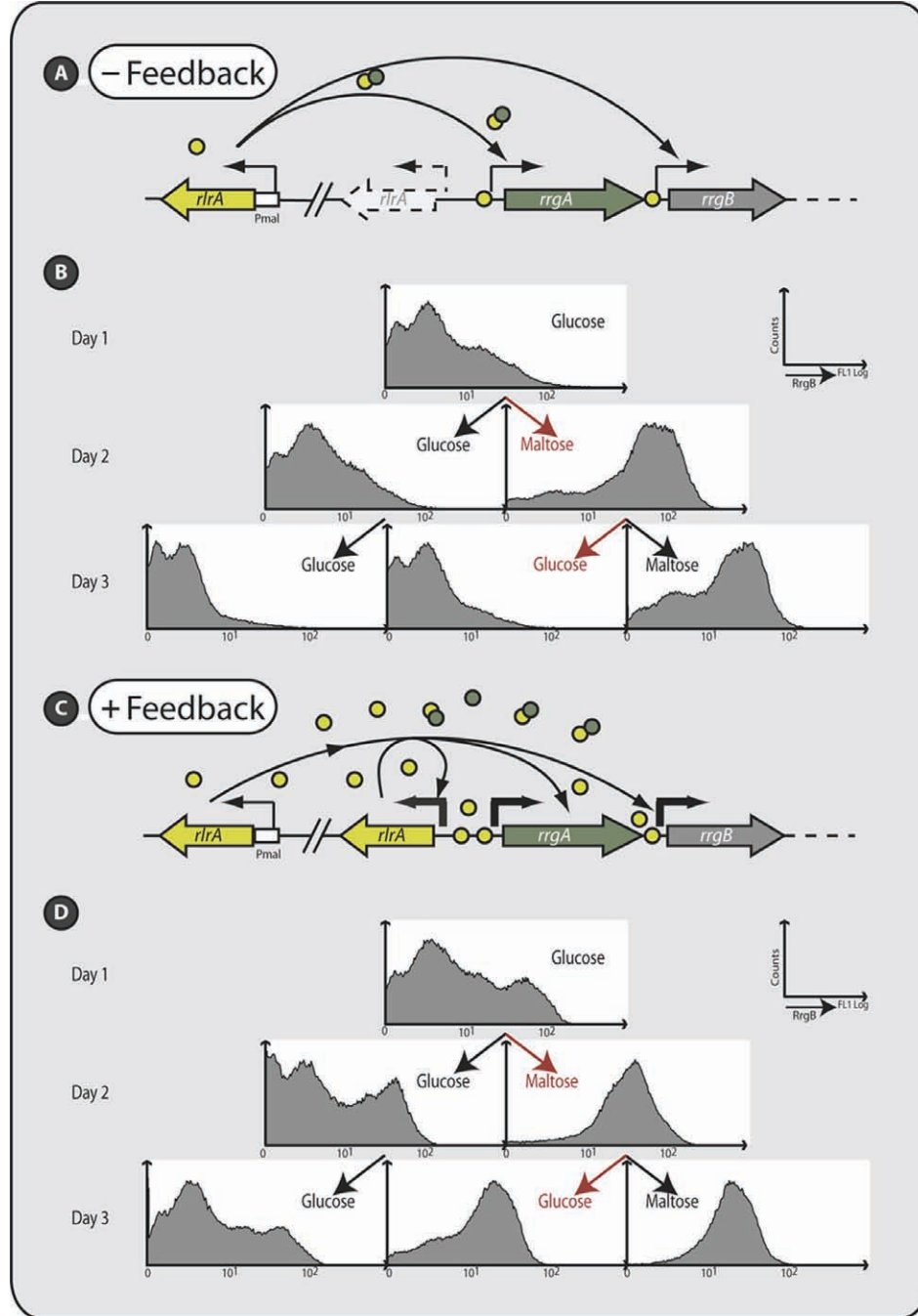


FIG 1 An RlrA-mediated positive-feedback loop mediates bistable expression of the pneumococcal type 1 pilus genes. (A and C) Diagrams of the minus-feedback (A) and plus-feedback (C) strains used in this study. The dashed outline of the *rlrA* gene represents a deletion of this gene. Light circles represent the RlrA protein, and dark circles represent the RrgA protein. (B and D) The minus-feedback (B) and plus-feedback (D) strains were grown for 3 days successively. Each day, cells from the previous day were grown in medium containing either maltose or glucose, for induction or repression, respectively, of ectopic *rlrA* expression from the modified maltose promoter. Each plot is a histogram of fluorescence intensity obtained after analyzing a population of cells stained with antibodies directed against the major pilus subunit protein RrgB. The red arrows indicate a comparison emphasized in the text, namely, growth of the cells before, during, and after a brief pulse of ectopic *rlrA* expression.

due to positive feedback of the positive regulator RlrA, further supports the view that a complex balance of benefits and costs to the expression of pilus genes must exist. These may depend on factors such as age, preexisting immunity, and site of infection or colonization. Overall, our findings underscore the need for thorough investigations of the pattern and kinetics of pilus expression during infection in animal models.

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REFERENCES

1. Barocchi MA, et al. 2006. A pneumococcal pilus influences virulence and host inflammatory responses. *Proc. Natl. Acad. Sci. U. S. A.* 103:2857–2862.
2. Basset A, et al. 2007. Association of the pneumococcal pilus with certain capsular serotypes but not with increased virulence. *J. Clin. Microbiol.* 45:1684–1689.
3. Basset A, et al. 2011. Expression of the type 1 pneumococcal pilus is bistable and negatively regulated by the structural component RrgA. *Infect. Immun.* 79:2974–2983.
4. De Angelis G, et al. 2011. The *Streptococcus pneumoniae* pilus-1 displays a biphasic expression pattern. *PLoS One* 6:e21269.
5. Gianfaldoni C, et al. 2007. *Streptococcus pneumoniae* pilus subunits protect mice against lethal challenge. *Infect. Immun.* 75:1059–1062.
6. Hava DL, Camilli A. 2002. Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. *Mol. Microbiol.* 45:1389–1406.
7. Hava DL, Hemsley CJ, Camilli A. 2003. Transcriptional regulation in the *Streptococcus pneumoniae* *rlrA* pathogenicity islet by RlrA. *J. Bacteriol.* 185:413–421.
8. Hemsley C, Joyce E, Hava DL, Kawale A, Camilli A. 2003. MgrA, an orthologue of Mga, acts as a transcriptional repressor of the genes within the *rlrA* pathogenicity islet in *Streptococcus pneumoniae*. *J. Bacteriol.* 185:6640–6647.
9. Mitrophanov AY, Groisman EA. 2008. Positive feedback in cellular control systems. *Bioessays* 30:542–555.
10. Munoz-Elias EJ, Marciano J, Camilli A. 2008. Isolation of *Streptococcus pneumoniae* biofilm mutants and their characterization during nasopharyngeal colonization. *Infect. Immun.* 76:5049–5061.
11. Nelson AL, et al. 2007. RrgA is a pilus-associated adhesin in *Streptococcus pneumoniae*. *Mol. Microbiol.* 66:329–340.
12. Ninfa AJ, Mayo AE. 2004. Hysteresis vs. graded responses: the connections make all the difference. *Sci. STKE* 2004:pe20.
13. Regev-Yochay G, et al. 2010. Re-emergence of the type 1 pilus among *Streptococcus pneumoniae* isolates in Massachusetts, USA. *Vaccine* 28:4842–4846.
14. Regev-Yochay G, et al. 2009. The pneumococcal pilus predicts the absence of *Staphylococcus aureus* co-colonization in pneumococcal carriers. *Clin. Infect. Dis.* 48:760–763.
15. Rosch JW, Mann B, Thornton J, Sublett J, Tuomanen E. 2008. Convergence of regulatory networks on the pilus locus of *Streptococcus pneumoniae*. *Infect. Immun.* 76:3187–3196.
16. Song XM, Connor W, Hokamp K, Babiuk LA, Potter AA. 2009. The growth phase-dependent regulation of the pilus locus genes by two-component system TCS08 in *Streptococcus pneumoniae*. *Microb. Pathog.* 46:28–35.
17. Sung CK, Li H, Claverys JP, Morrison DA. 2001. An *rpsL* cassette, janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. *Appl. Environ. Microbiol.* 67:5190–5196.
18. Turner KH, Vallet-Gely I, Dove SL. 2009. Epigenetic control of virulence gene expression in *Pseudomonas aeruginosa* by a LysR-type transcription regulator. *PLoS Genet.* 5:e1000779.
19. van der Woude MW, Baumler AJ. 2004. Phase and antigenic variation in bacteria. *Clin. Microbiol. Rev.* 17:581–611.
20. van Wezel GP, White J, Young P, Postma PW, Bibb MJ. 1997. Substrate induction and glucose repression of maltose utilization by *Streptomyces coelicolor* A3(2) is controlled by *malR*, a member of the *lacI-galR* family of regulatory genes. *Mol. Microbiol.* 23:537–549.