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Minireview

MINIREVIEW

Pseudomonas aeruginosa AmpR: an acute–chronic switch regulator

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This is a timely and well-written review summarizing recent findings on the role of the global regulator AmpR on *Pseudomonas aeruginosa* virulence and physiology. The significance of this regulator has broadened from its established role in regulation of beta-lactam resistance to novel, unexpected, multiple regulatory functions including the switch between acute and chronic modes of infection.

ABSTRACT

Pseudomonas aeruginosa is one of the most intractable human pathogens that pose serious clinical challenge due to extensive prevalence of multidrug-resistant clinical isolates. Armed with abundant virulence and antibiotic resistance mechanisms, it is a major etiologic agent in a number of acute and chronic infections. A complex and intricate network of regulators dictates the expression of pathogenicity factors in *P. aeruginosa*. Some proteins within the network play key roles and control multiple pathways. This review discusses the role of one such protein, AmpR, which was initially recognized for its role in antibiotic resistance by regulating AmpC β-lactamase. Recent genomic, proteomic and phenotypic analyses demonstrate that AmpR regulates expression of hundreds of genes that are involved in diverse pathways such as β-lactam and non-β-lactam resistance, quorum sensing and associated virulence phenotypes, protein phosphorylation, and physiological processes. Finally, *ampR* mutations in clinical isolates are reviewed to shed light on important residues required for its function in antibiotic resistance. The prevalence and evolutionary implications of AmpR in pathogenic and nonpathogenic proteobacteria are also discussed. A comprehensive understanding of proteins at nodal positions in the *P. aeruginosa* regulatory network is crucial in understanding, and ultimately targeting, the pathogenic stratagems of this organism.

Key words: *Pseudomonas aeruginosa* virulence; global regulator; antibiotic resistance; quorum sensing; c-di-GMP; ser/thr protein phosphorylation

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium best known for its ability to cause opportunistic human infections. It is the primary cause of fatal lung infections among patients with cystic fibrosis (CF) (Doggett 1969; Lyczak *et al.*, 2002) and the leading cause of secondary infections in immunocompromised patients such as those with AIDS, cancer, and burn wounds (Afessa *et al.*, 1998; Vento *et al.*, 2008; Branski *et al.*, 2009; Hoiby 2011). Unfortunately, *P. aeruginosa* infections are associated with a poor prognosis and have high fatality rates (Aliaga *et al.*, 2002; Hakki *et al.*, 2007; Horino *et al.*, 2012). A wide array of cell-associated and secreted virulence factors ensure the success of *P. aeruginosa* as a pathogen.

Pseudomonas aeruginosa infections are extremely difficult to treat due to its ability to switch from acute to chronic

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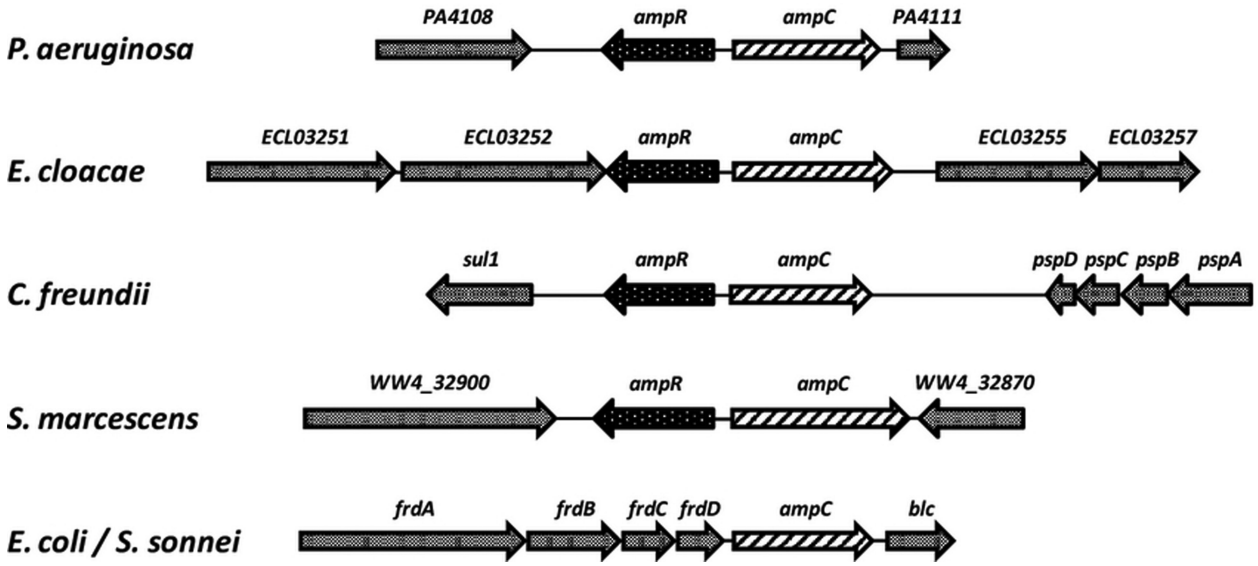

Figure 1. Genetic locus of the *ampR-ampC* module. The open reading frames and operons surrounding *ampR-ampC* in *Pseudomonas aeruginosa* and different enterobacterial species are shown. The presence of a divergently transcribed *ampR* (*P. aeruginosa, Enterobacter cloacae, Citrobacter freundii, and Serratia marcescens*) indicates inducible β-lactamase production, whereas in *Escherichia coli* and *Shigella sonnei*, *ampC* expression is constitutively low.

infection phenotype and develop multidrug resistance (Hogardt and Heesemann 2013). Currently, β-lactams alone or in combination with aminoglycosides form the first line of defense against *P. aeruginosa* (Foundation 2011). However, clinicians worldwide are now faced with *P. aeruginosa* strains that are resistant to most β-lactams, aminoglycosides, and quinolones (Lister *et al.*, 2009). Antibiotic-resistant isolates of *P. aeruginosa* are selectively favored *in vivo* in patients with CF (Chen *et al.*, 1995; Bonfiglio *et al.*, 1998). The development of resistance to almost all clinically relevant antibiotics by *P. aeruginosa* has allowed its classification as an ESKAPE pathogen (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), dreaded in the hospitals as they are capable of confounding any treatment strategy (Rice 2010; Pendleton *et al.*, 2013). Addressing this public health threat will require a better understanding of the molecular mechanisms of antibiotic resistance.

Hyperexpression and presence of β-lactamases in the biofilm matrix, either free or in membrane vesicles, has been linked to the reduced effectiveness of β-lactam therapy (Ciofu *et al.*, 2010; Hengzhuang *et al.*, 2011). A major mechanism of β-lactam resistance in *P. aeruginosa* is the overproduction of the chromosomally encoded, inducible β-lactamase, AmpC (Lodge *et al.*, 1990; Kong *et al.*, 2005b). The LysR-type transcriptional regulator AmpR modulates expression of *ampC* encoding β-lactamase (Lodge *et al.*, 1993; Kong *et al.*, 2005b). Recent studies have shown that, apart from regulating antibiotic resistance, AmpR has an extensive regulon that encompasses several virulence and physiological factors (Balasubramanian *et al.*, 2011, 2012, 2014; Kumari *et al.*, 2014b). Furthermore, AmpR is a key player in the intricate network of regulators that is responsible for mediating virulence and antibiotic resistance in *P. aeruginosa* (Balasubramanian *et al.*, 2013a).

This review summarizes the role of *P. aeruginosa* AmpR in regulating pathogenesis. We also discuss our current understanding of AmpR-mediated regulation of critical virulence and physiological determinants. Specifically, we focus on the role of AmpR in regulating antibiotic resistance and the switch between acute and chronic infection traits. Given that AmpR is also found in many other Gram-negative bacterial pathogens (Seoane *et al.*, 1992; Proenca *et al.*, 1993; Gould *et al.*, 2006), inhibiting its function will likely be a viable option to deal with bacterial infections in the clinical setting.

AMPR IN ENTEROBACTERIA

The regulation of β-lactam resistance is controlled by *ampR–ampC* module (where the gene loci are linked, divergently transcribed, and functionally conserved) in many enterobacterial species (Fig. 1). Chromosomally encoded *ampC* is found in most of the enterobacterial species, albeit with a distinct regulatory pattern. In *Escherichia coli* and *Shigella sonnei*, low-level constitutive expression of *ampC* is directed by a promoter located within the coding sequence of the upstream fumarate reductase operon, *frd* (Grundstrom and Jaurin 1982; Bergstrom *et al.*, 1983; Cole and Nicolas 1986). Resistance to modern β-lactams in these species occurs rather infrequently and is mostly mediated by promoter mutations, novel promoters, weakened attenuators, or multiple, tandem duplications of the *ampC* gene (Normark *et al.*, 1977; Cole and Guest 1979; Edlund *et al.*, 1979; Jaurin *et al.*, 1982; Olsson *et al.*, 1982). In contrast, expression of *ampC* in other organisms such as *Citrobacter freundii*, *Enterobacter cloacae*, and *Serratia marcescens* is induced by β-lactam antibiotics (Lindberg *et al.*, 1985; Cole and Nicolas 1986; Nicolas *et al.*, 1987; Mahlen *et al.*, 2003). Such induction results in therapeutic failure of β-lactam treatment due to stable overproduction of the AmpC. The most significant genetic difference that results in inducible *ampC* is the presence of *ampR*, encoding a transcriptional regulator upstream of, and divergently transcribed from, the *ampC* gene (Fig. 1, Lindberg *et al.*, 1985; Honore *et al.*, 1986). In *C. freundii* and *E. cloacae*, *ampC* expression is repressed and induced by AmpR in the absence and presence of inducers, respectively (Lindberg *et al.*, 1985; Lindberg and Normark 1987).

The expression of *C. freundii* or *E. cloacae ampC-ampR* in *E. coli* results in the synthesis of inducible β-lactamase (Lindberg *et al.*, 1985). Moreover, *ampR* from *E. cloacae* and *C. freundii* can cross-complement each other in *E. coli*, which typically

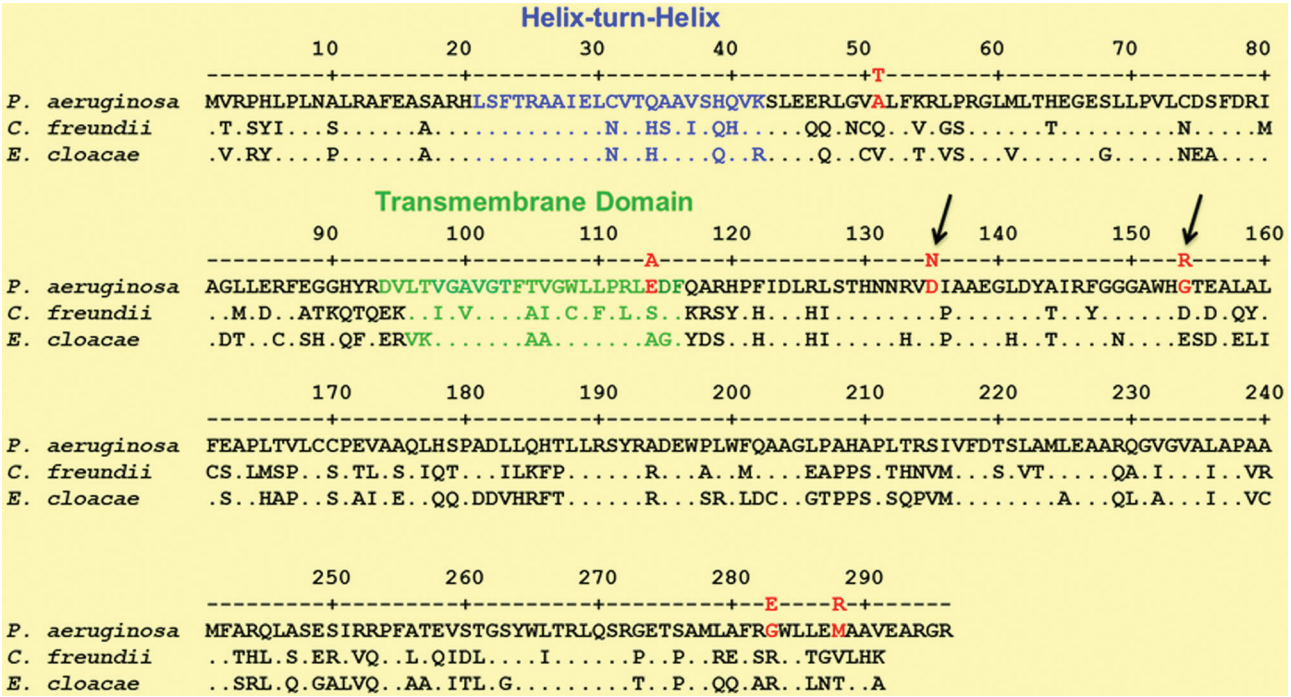

Figure 2. *Pseudomonas aeruginosa* AmpR sequence homology. The *P. aeruginosa* AmpR sequence from the *Pseudomonas* Database (Winsor *et al.*, 2011) was used to determine similarity with its homologs in two other *Enterobacteriaceae* members using CLUSTALW2. Mutations in the *P. aeruginosa* AmpR sequence identified in antibiotic-resistant clinical isolates are shown in red. The arrows indicate the mutations confirmed in the laboratory that contribute to enhanced resistance.

lacks *ampR* (Lindberg and Normark 1987). Together, these findings indicate that all other factors required for *ampC* induction are present in the *E. coli* chromosome. Moreover, the close homology between the 3′-ends of *E. coli* and *E. cloacae frd* operons and the region downstream of the *E. cloacae ampC* promoter suggests that *ampR* may have been deleted from the *ampC* region of the *E. coli* chromosome following the divergence from a common ancestor (Honore *et al.*, 1986).

AMPR-AMPC IN *PSEUDOMONAS AERUGINOSA*

The divergently transcribed *ampR–ampC* gene arrangement in *P. aeruginosa* is similar to that seen in other organisms including *C. freundii* and *E. cloacae* (Fig. 1; Lodge *et al.*, 1993). Sequence analysis revealed that *P. aeruginosa* AmpR bears a high degree of homology to its counterparts in *C. freundii* (58%) and *E. cloacae* (62%) (Lodge *et al.*, 1990). A high degree of homology is also seen in the helix-turn-helix (HTH) and the hydrophobic domains, whereas homology is lower in the effector-binding domains (EBDs) (Fig. 2). Preliminary experiments in our laboratory suggest that AmpR is possibly an inner membrane protein, but similar studies have not been performed on its counterparts in other bacteria. In a previous study, *C. freundii* AmpR was purified from an insoluble cellular fraction in *E. coli*, suggesting its membrane localization (Jacobs *et al.*, 1997), in agreement with the sequence analysis (Fig. 2). It would be interesting to determine whether AmpR is indeed a membrane protein and raises exciting questions on the AmpR-mediated regulatory aspects in bacteria.

Protein modeling shows two C-terminal EBDs and an N-terminal HTH domain, separated by a hydrophobic helix (Fig. 3). The EBD of the *C. freundii* AmpR was recently crystallized and shown to be a dimer (Balcewich *et al.*, 2010). The crystal structure revealed that each EBD has two subdomains that form a pocket between them. The groove on the surface of subdomain I of the EBD, along with the pocket, forms the putative effector-binding site (Balcewich *et al.*, 2010). Recently, *P. aeruginosa* AmpR was also shown to be a dimer (Caille *et al.*, 2014).

In *C. freundii*, AmpR recognizes the 15-bp DNA sequence 5′ TCTGCTGCTAAATTT 3′ in P_{ampC} (Lindquist *et al.*, 1989; Lodge *et al.*, 1990). In *P. aeruginosa*, *in silico* analysis of microarray data revealed an A-T-rich putative AmpR-binding site (5′ TCTGCTCCAAATTT 3′) in the *ampR–ampC* intergenic region (Zeng *et al.*, 2007; Balasubramanian *et al.*, 2012). The binding site was further refined using ChIP Seq data to show that the bases that are critical to AmpR binding are As and Ts at positions 1, 6, 9, 10, and 13 (Balasubramanian *et al.*, 2014).

The HTH motif in *C. freundii* AmpR has been shown to be important in DNA binding (Lindquist *et al.*, 1989). Studies in our laboratory have shown that in *P. aeruginosa*, the third helix of the HTH motif is critical to DNA binding (Caille *et al.*, 2014). Further, amino acid residues in this helix that are important for DNA binding have been identified using mutation analysis (Caille *et al.*, 2014).

AmpR belongs to the LysR family of transcriptional regulators that typically autorepress their own expression (Schell, 1993; Maddocks and Oyston, 2008). Autoregulation has been demonstrated in *C. freundii* (Lindquist *et al.*, 1989) but not in *E. coli* mini cells expressing *C. freundii* AmpR (Lindberg *et al.*, 1985), suggesting that there are exceptions to the autoregulatory process. The *ampR–ampC* intergenic region in *P. aeruginosa* is only 149 bp, and the putative AmpR-binding site overlaps promoters of both *ampR* and *ampC* (Lindquist *et al.*, 1989). This suggests that AmpR binding to this region would allow negative autoregulation, in addition to modulating *ampC* expression (Lindquist *et al.*, 1989). However, in *P. aeruginosa*, autoregulation occurs only under sub-MIC antibiotic exposure in the alginate constitutively producing strain PDO300 and not in the isogenic parent, PAO1 (Kong *et al.*, 2005; Balasubramanian *et al.*, 2011). AmpR autoregulation in

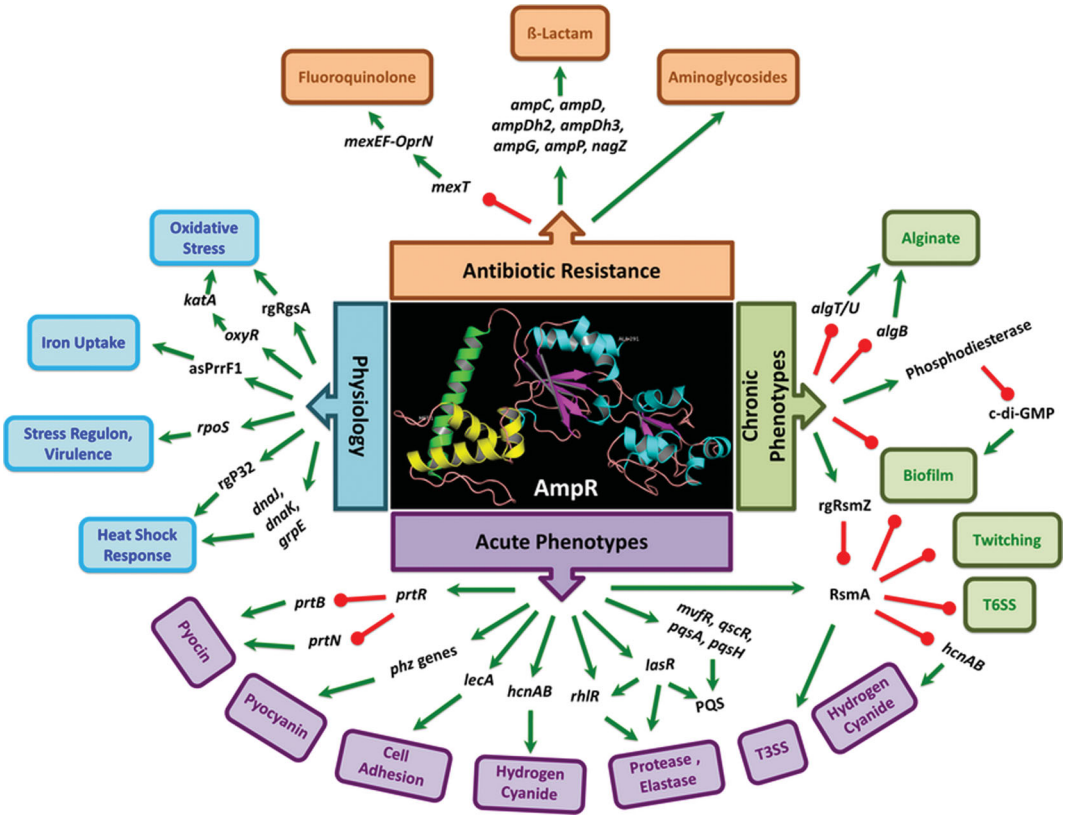

Figure 3. AmpR is a global regulator in *Pseudomonas aeruginosa*. AmpR regulates resistance to different classes of clinically relevant antibiotics, either positively (β-lactams, aminoglycosides) or negatively (quinolones). AmpR plays a key role in determining *P. aeruginosa* virulence and physiology by regulating expression of transcriptional and post-transcriptional regulators that feed into critical networks, such as QS, Gac-Rsm, iron uptake, and stress response pathways. The data in the figure were obtained from gene expression/proteomic/phenotypic assays (Balasubramanian *et al.*, 2012, 2014; Kumari *et al.*, 2014a,b). Only the gene expression confirmed by qPCR and/or phenotypes confirmed by assays are shown here. Arrow colors indicate positive (green) and negative (red) regulation. No distinction has been made between direct and indirect regulations. AmpR model based on the Protein Model Portal (Tacconelli *et al.*, 2014) is shown in the center. The model shows the DNA-binding helix-turn-helix motif (blue), a hydrophobic domain (green), and the C-terminal effector-binding domain (yellow and cyan).

P. aeruginosa thus appears to be dependent on the presence of the alginate master regulator AlgT/U and β-lactam stress (Balasubramanian *et al.*, 2011).

AMPR AS GLOBAL REGULATOR IN *P. AERUGINOSA*

Even though the role of AmpR in β-lactam resistance was known since 1990s, recent studies demonstrated its role in controlling disparate but important pathogenic determinants (Kong *et al.*, 2005b; Balasubramanian *et al.*, 2011, 2012, 2014; Kumari *et al.*, 2014a,b). The major facets of AmpR-mediated virulence regulation that likely impact the clinical success of *P. aeruginosa* are discussed below.

Role of *P. aeruginosa* AmpR in antibiotic resistance

Antibiotic resistance is a major concern when dealing with *P. aeruginosa* infections in the hospitals. The current treatment regimen for *P. aeruginosa* infections is a combination therapy of an antipseudomonal β-lactam in association with either an aminoglycoside or a fluoroquinolone (preferably ciprofloxacin) (Mesaros *et al.*, 2007). Having been identified as a positive regulator of the chromosomal AmpC β-lactamase, the role of AmpR in β-lactam resistance has been established in different bacterial species (Lindberg and Normark 1986; Normark *et al.*, 1986). Not surprisingly, loss of AmpR function in *P. aeruginosa* was found to render the strain sensitive to β-lactam antibiotics (Kong *et al.*, 2005b; Balasubramanian *et al.*, 2012; Kumari *et al.*, 2014a).

In many of the *Enterobacteriaceae* members, the AmpR-AmpC system is tightly linked to other *amp* genes whose purported natural function is in the recycling of cell wall/peptidoglycan. In *P. aeruginosa*, these genes include *ampG* and *ampP* encoding permeases; *ampD, ampDh2,* and *ampDh3* encoding amidases; and *nagZ* encoding a hydrolase (Juan *et al.*, 2006; Asgarali *et al.*, 2009; Kong *et al.*, 2010; Zamorano *et al.*, 2010; Zhang *et al.*, 2010). The cell wall degradation products produced by the action of these genes are presumed to be effectors of AmpR, resulting in activation/repression of *ampC* expression (Jacobs *et al.*, 1994, 1997). In addition to regulating *ampC*, AmpR is required for the expression of all the above genes (Balasubramanian *et al.*, 2012). *Pseudomonas aeruginosa* AmpR thus plays a central role in the cell wall recycling as well as AmpC-mediated β-lactam resistance.

Interestingly, the regulation of β-lactam resistance by *P. aeruginosa* AmpR seems to involve more than one pathway. AmpR positively regulates the expression of MexR, a repressor of the MexAB-OprM efflux pump that is involved in the efflux of β-lactam antibiotics (Balasubramanian *et al.*, 2012). Although it is counterintuitive to AmpR being a positive regulator of β-lactam resistance, it also suggests that we are far from

understanding β-lactam resistance. Moreover, deleting *ampC* in PAO1 (Kumari *et al.*, 2014a) or in PAOΔ*ampR* (D. Zincke, unpublished data) abolishes β-lactam resistance in spite of having a functional MexAB-OprM system. These observations suggest that AmpC is the major resistance determinant and MexAB pump by itself is not enough to confer β-lactam resistance.

Resistance to β-lactams is also regulated by the CreBCD system (Moya *et al.*, 2009; Zamorano *et al.*, 2010). CreBC forms a two-component system that positively regulates expression of an inner membrane protein CreD (Avison *et al.*, 2001); together, they regulate β-lactam resistance (Avison *et al.*, 2004). AmpR regulates CreD in a CreB-independent manner, possibly affecting β-lactam resistance via CreBCD (Balasubramanian *et al.*, 2012). Furthermore, AmpR functions as a negative regulator of a second chromosomal β-lactamase, an oxacillinase termed OxaB (PoxB, PA5514), whose activity spectrum is limited primarily to carbapenems (D. Zincke, unpublished data; Kong *et al.*, 2005). The physiological significance of negative regulation of *oxaB* expression by AmpR is unclear. Electrophoretic mobility shift assays suggest indirect regulation of *oxaAB* operon (*PA5513-5514*) by AmpR under the conditions tested (O. Caille, unpublished data), although it is possible that recognition and binding of P_{oxa} by AmpR requires as yet unidentified signals.

In addition to regulating β-lactam resistance positively, AmpR also regulates quinolone resistance (Balasubramanian *et al.*, 2012). Gene expression and phenotypic assays indicate that AmpR negatively regulates transcription and, ultimately, function of the *mexEF-oprN* efflux system, by modulating expression of *mexT*, encoding the positive regulator (Balasubramanian *et al.*, 2012). Repression of *mexT* expression by AmpR is β-lactam independent (Balasubramanian *et al.*, 2012). It is important to note that AmpR positively and negatively regulates resistance to β-lactams and quinolones, respectively.

In response to β-lactam antibiotic stress, AmpR also negatively regulates the expression of proteins involved in aminoglycoside resistance such as MexXY efflux pump and Aph, an aminoglycoside 3′-phosphotransferase (Kumari *et al.*, 2014b). The regulation of proteins involved in aminoglycosides resistance in response to β-lactam stress is not expected. Furthermore, phenotypic microarray data suggest that AmpR influences resistance to multiple antibiotics (Balasubramanian *et al.*, 2012). Thus, AmpR is among the very few transcriptional regulators in *P. aeruginosa* that modulates resistance to different classes of antibiotics (Fig. 3).

AmpR mediates antibiotic cross-resistance

Currently, combination therapy with two or more antibiotics of different classes is used to tackle *P. aeruginosa* infections (Paul and Leibovici, 2005; Tamma *et al.*, 2012). In recent years, however, the advantages of combination therapy have been questioned in light of data suggesting adverse effects of drugs when combined (Paul *et al.*, 2004, 2006; Boyd and Nailor 2011; Johnson *et al.*, 2011; Tamma *et al.*, 2012). Specifically, while much research is focused on studying bacterial resistance to high antibiotic doses, response to subinhibitory concentrations (SICs) of antibiotics remains largely unexplored. For instance, SIC of carbapenems, specifically imipenem, leads to clinical resistance in *P. aeruginosa* (Livermore, 1987; Kumari *et al.*, 2014a). Often, resistance to multiple classes of antibiotics is mediated by the same mechanism or regulator (MexAB, AmpR), raising the real possibility of antibiotic cross-resistance.

Pre-exposure of *P. aeruginosa* PAO1 cells to SIC of imipenem (as low as 3 ng μL^{-1}) induces transient cross-resistance to other clinically relevant β-lactams, such as piperacillin, ceftazidime, ticarcillin, and aztreonam (Kumari *et al.*, 2014a). This is possibly because imipenem is the most proficient inducer of *ampC* and *ampR* expression compared to other clinically used antibiotics (Kumari *et al.*, 2014a) and residual amounts of imipenem can prime the cells to resist subsequently used β-lactams. Imipenem-mediated cross-resistance is completely dependent on AmpR (Kumari *et al.*, 2014a).

AmpR regulates β-lactam and non-β-lactam resistance in both AmpC-dependent and independent manner. AmpR is a negative regulator of MexXY (Kumari *et al.*, 2014b), suggesting that the *ampR* mutants would exhibit enhanced resistance to aminoglycosides. However, phenotypic analyses show that loss of *ampR* results in enhanced aminoglycoside susceptibility, which can be further enhanced upon pre-exposure to SIC of various β-lactams and non-β-lactams (Kumari *et al.*, 2014a). Thus, although the MexXY proteins are made in higher quantities, the *ampR* mutants are susceptible to aminoglycosides, suggesting the existence of post-translational modification. Moreover, resistance toward aminoglycosides in *P. aeruginosa* clinical isolates is mostly dependent on horizontally acquired enzymes or membrane alterations (Lister *et al.*, 2009), highlighting the multilayered control of antibiotic resistance.

The *ampR* mutant is not only sensitive to many β-lactam antibiotics; it regulates critical virulence factors as discussed in the following sections. Targeting AmpR will thus render the strain less virulent and enhance sensitivity to β-lactams, and possibly aminoglycosides without affecting their efficacy at SIC pre-exposure to antibiotics, making it an attractive drug candidate. The findings by our group and others on development of cross-resistance (Masuda *et al.*, 2000; Kumari *et al.*, 2014a) warrant further studies looking into this very relevant clinical phenomenon.

AmpR regulates QS in *P. aeruginosa*

The pathogenic potential of *P. aeruginosa* is determined by a large number of both cell-associated (flagella, pili, lipopolysaccharide) and secreted virulence determinants such as pyocyanin, exotoxin A, cyanides, proteases, elastases, and rhamnolipids among others. The expression of a vast majority of the secreted virulence factors is under the control of a population-density-dependent gene expression process called quorum sensing (Williams and Camara, 2009). Population density is sensed by small, diffusible molecules, either acyl homoserine lactones (AHLs) or quinolones (PQSs), which are produced by the bacteria (Jimenez *et al.*, 2012). The *las* and *rhl* AHL-dependent systems together affect about 10% of the *P. aeruginosa* transcriptome (Schuster and Greenberg, 2006). The master transcriptional regulator LasR is at the top of the QS hierarchy in *P. aeruginosa* and controls expression of the Las, Rhl, and PQS systems (Latifi *et al.*, 1996; Pesci *et al.*, 1997; McGrath *et al.*, 2004). LasR itself is regulated by many different transcriptional regulators (reviewed in Balasubramanian *et al.*, 2013a).

AmpR regulates expression of the major QS regulators LasR, RhlR, MvfR, and QscR, thereby controlling expression of the entire QS regulatory cascade (Balasubramanian *et al.*, 2014). Recent studies in our laboratory identified a putative AmpR-binding site in P_{lasR} (5′ TTGGTTAATAGTTT 3′) and demonstrated direct AmpR binding to the promoter (Balasubramanian *et al.*, 2014). Consequently, loss of AmpR results in a significant loss in the production of QS-regulated acute virulence factors, such as the proteases LasA and LasB, and pyocyanin

(Balasubramanian *et al.*, 2012). AmpR-mediated QS regulation is also required for full pathogenicity of the bacterium, as demonstrated by reduced virulence of *ampR* mutants in the *Caenorhabditis elegans* acute infection model (Balasubramanian *et al.*, 2012). AmpR thus lies at the heart of the *P. aeruginosa* pathogenesis network by regulating QS.

AmpR regulates physiological processes and metabolism in *P. aeruginosa*

Given the diverse phenotypes regulated by AmpR, it is not surprising that some genes involved in metabolic pathways are included in its regulon (Balasubramanian *et al.*, 2012, 2014). Phenotypic microarray analysis showed that utilization of citrulline, histidine, leucine, serine, shikimic acid, spermidine, and pyridoxal is negatively regulated by AmpR (Balasubramanian *et al.*, 2012). The physiological implications of this negative regulation are as yet unclear. Also, the *ampR* mutant is more sensitive to many agents that affect cell growth (those belonging to the BIOLOG sensitivity panel), suggesting that a functional AmpR is critical to robust survival of *P. aeruginosa* (Balasubramanian *et al.*, 2012).

AmpR is involved in regulating critical physiological processes such as iron acquisition (Balasubramanian *et al.*, 2014). As iron is a limiting factor for growth in the host, bacteria have evolved siderophores to chelate extracellular iron (Meyer *et al.*, 1996; Martin *et al.*, 2011). AmpR positively regulates expression of the major *P. aeruginosa* siderophore genes encoding pyoverdine and pyochelin (Balasubramanian *et al.*, 2014). Loss of *ampR* results in impaired growth under iron-limiting conditions, which can be rescued by making conditions iron replete. This observation and the fact that siderophore genes are downregulated suggest that AmpR affects iron uptake and not utilization in *P. aeruginosa* (Balasubramanian *et al.*, 2014). AmpR also positively regulates expression of the small RNA rgPrrF1, which is involved in iron uptake regulation. However, the master repressor of iron uptake, Fur, is not regulated by AmpR (Balasubramanian *et al.*, 2014), suggesting an AmpR-mediated, Fur-independent regulation mechanism.

AmpR-mediated regulation of the stress response system

Bacterial cells have evolved elaborate mechanisms to counteract various stress conditions. The major stationary phase sigma factor affecting bacterial stress response is RpoS, which also regulates virulence (Suh *et al.*, 1999; Schuster *et al.*, 2004; Potvin *et al.*, 2008). The *P. aeruginosa* RpoS regulon has previously been identified to include 772 genes (Schuster *et al.*, 2004). Gene expression studies in *P. aeruginosa* show an AmpR-dependent positive regulation of RpoS (Balasubramanian *et al.*, 2012). This suggests that AmpR may regulate the stress response via RpoS in *P. aeruginosa*.

A functional AmpR is also required for survival of *P. aeruginosa* upon exposure to heat shock (Balasubramanian *et al.*, 2014). AmpR affects heat tolerance in *P. aeruginosa* by positively regulating genes of the DnaJ-DnaK-GrpE Hsp70 system and the small RNA rgP32, which is part of the *dnaJ-dapB-p32* operon (Stover *et al.*, 2000; Balasubramanian *et al.*, 2014). Due to positive regulation of *rpoS* expression by AmpR, the temperature sensitivity of PAOΔ*ampR* is more enhanced in the stationary phase compared with log phase (Balasubramanian *et al.*, 2014).

RpoS, along with GacA, regulates expression of a small RNA rgRgsA, which contributes to hydrogen peroxide resistance (Gonzalez *et al.*, 2008). AmpR positively regulates expression of the major catalase *katA*, rgRgsA, and 68 other genes involved in *P. aeruginosa* oxidative stress response, including the master regulator OxyR, either directly or indirectly (Balasubramanian *et al.*, 2014). Besides, loss of *ampR* in PAO1 increases its susceptibility to H_2O_2, suggesting a weakened oxidative stress response (Balasubramanian *et al.*, 2014). These findings indicate that AmpR is an integral part of the stress response system in *P. aeruginosa*.

Regulation of secondary-messenger-mediated signaling

The central role of cyclic-di-GMP in several critical bacterial processes such as virulence, stress survival, motility, biofilm formation, and dispersion is well established (Romling *et al.*, 2013; Ryan 2013). Given the importance of this messenger molecule, intracellular levels of c-di-GMP are tightly regulated by diguanylate cyclases and phosphodiesterases, and some proteins have both these domains (Ryan 2013). The *P. aeruginosa* PAO1 genome encodes 39 proteins that contain these domains and are thus capable of modulating intracellular c-di-GMP levels (Stover *et al.*, 2000; Kulasakara *et al.*, 2006). AmpR positively regulates three of these phosphodiesterase-domain-containing proteins, BifA, CdpA, and PA4781 (Kumari *et al.*, 2014). This suggests that AmpR potentially negatively regulates c-di-GMP level in the cells by positively regulating phosphodiesterase gene expression (Kumari *et al.*, 2014) and needs further investigation. Interestingly, the gene upstream of *ampR*, *PA4108*, codes for a phosphodiesterase (Ryan *et al.*, 2009) but was not identified in our transcriptomic or proteomic analyses.

Serine/Threonine/Tyrosine phosphorylation

Ser/Thr/Tyr phosphorylation plays a critical role in determining eukaryotic protein function (Cohen 2000; Hunter 2000). This process has now been demonstrated in prokaryotes also, albeit at much lower levels (Kannan *et al.*, 2007; Macek *et al.*, 2007). Additional studies addressing Ser/Thr/Tyr phosphorylation in bacteria are needed to understand their role in critical regulatory processes.

Previous studies have identified post-translational modifications to play a role in important virulence processes, such as motility and the HCP1-mediated type 6 secretion system in *P. aeruginosa* (Kelly-Wintenberg *et al.*, 1993; Mougous *et al.*, 2007). Phosphoproteome analysis identified AmpR to be a major negative regulator of *P. aeruginosa* protein phosphorylation (Kumari *et al.*, 2014b). The study identified phosphorylation of 45 proteins to be negatively regulated by AmpR, either in the presence or in the absence of β-lactam stress (Kumari *et al.*, 2014b). These include major virulence determinants such as the anaerobic growth regulator Anr, the outer membrane component of the MexAB efflux pump, OprM, a transcriptional activator of the MexEF-OprN efflux system, MexT, and the penicillin-binding proteins MrcB and MurD (Kumari *et al.*, 2014b). Given the important role of these proteins in *P. aeruginosa* physiology and pathogenesis, the effect of phosphorylation on protein function and the role of AmpR in this process need further elucidation.

ROLE OF AMPR IN ACUTE–CHRONIC INFECTION SWITCH

One of the major features of *P. aeruginosa* is its ability to cause both acute and chronic infections. The physiology of the cells is widely different between these two infection phases and is characterized by opposing phenotypes. The infection process is initiated by planktonic cells that express a wide variety of acute virulence factors, including expression of flagella and pili (Vallet *et al.*, 2001; Ma *et al.*, 2009); QS-regulated virulence factors such as proteases, elastases, phenazines, and toxins (Williams and Camara 2009); and type III secretion system (Hauser, 2009). Cells in this stage of infection are typically sensitive to antibiotics (Hogardt and Heesemann 2013), unless initial infection was by an antibiotic-resistant strain. Expression of these acute virulence factors is designed to aid in establishment of infection.

Upon transitioning to chronic infection in patients with CF, chronic obstructive pulmonary disease, emphysema, or otitis media, *P. aeruginosa* forms biofilms that indicate a poor prognosis for patient health (Harmsen *et al.*, 2010). Formation of biofilms is probably the most critical factor that allows *P. aeruginosa* survival in the CF lung, and is associated with acquisition of niche-specific adaptive mutations and diversification (Boles and Singh 2008; Harmsen *et al.*, 2010; Yang *et al.*, 2011; Lopez-Causape *et al.*, 2013). Extensive research over the years has identified some critical determinants that trigger and support the transition from acute to chronic infection. The CF airways are a complex environment that is extensively compartmentalized based on differences in the local inflammatory processes and antibiotic penetration (Bjarnsholt *et al.*, 2009; Hoiby *et al.*, 2010). The host immune response-mediated oxidative stress, inflammation, and antibiotic treatment have been identified as triggers for *P. aeruginosa* diversification in the CF lung biofilms (Mathee *et al.*, 1999; Ciofu *et al.*, 2005; Kohanski *et al.*, 2007; Boles and Singh, 2008; Driffield *et al.*, 2008). In addition to biofilm formation, other major changes associated with the chronic infection process include hypermutability, conversion to mucoidy, and acquisition of antibiotic resistance (Doggett 1969; Oliver *et al.*, 2000). Hypermutability is a determining feature of chronic lung infections. CF lung isolates acquire mutations early on in *lasR* and *mucA*, and later in the antimutator genes *mutS*, *mutT*, *mutL*, *mutY*, *mutM*, and *uvrD*, resulting in many of the phenotypes associated with chronic infections (Ciofu *et al.*, 2010). Even though *mutY* and *mutM* are overexpressed in *ampR* mutants (>2.0-fold), there is no significant difference in mutation frequencies to rifampicin and streptomycin (Balasubramanian *et al.*, 2014). However, given that MutY and MutM are weak antimutators compared with MutS (Ciofu *et al.*, 2010), it is possible that loss of *ampR* in the CF lung potentially alters mutation frequencies, affecting survival in chronic infections. The occurrence and frequency of *ampR* mutations in CF lungs remains to be determined.

Previous studies have demonstrated that the RetS-LadS-GacSA-Rsm regulatory cascade plays a central role in the acute-chronic switch (Lapouge *et al.*, 2008). The hybrid sensor kinases RetS and LadS have opposing effects on the GacS sensor kinase (Laskowski and Kazmierczak 2006; Ventre *et al.*, 2006). RetS forms dimers with GacS inhibiting its function, whereas LadS phosphorylates GacS (Goodman *et al.*, 2009). GacS, through GacA, activates expression of the small regulatory RNAs, rgRsmY and rgRsmZ, which sequester and block activity of the negative regulator RNA-binding protein RsmA (Brencic *et al.*, 2009). RsmA inactivation by rgRsmY/rgRsmZ activates transcription of genes involved in biofilm formation and represses genes involved in acute virulence and motility (Jimenez *et al.*, 2012). RsmA mutants show reduced colonization in the initial infection stages, but ultimately favored chronic infection in a mouse model of acute pneumonia (Mulcahy *et al.*, 2008). AmpR negatively regulates RsmA activity by upregulating the expression of *ladS* and rgRsmZ (Balasubramanian *et al.*, 2014), thus feeding into the acute-chronic regulatory switch.

The loss of *ampR* results in many phenotypes resembling a chronic infection strain. These include loss of QS-dependent (proteases, elastases, pyocyanin) and QS-independent (downregulation of T3SS genes) acute virulence factors, increased fluoroquinolone resistance, and enhanced biofilm formation (Balasubramanian *et al.*, 2012, 2014; Kumari *et al.*, 2014a). Many of these effects of AmpR could be accounted for by the fact that AmpR directly regulates LasR, the QS master regulator (Balasubramanian *et al.*, 2014). Recent proteomic analyses have demonstrated that AmpR positively regulates phosphodiesterases that reduce c-di-GMP levels (Kumari *et al.*, 2014b). High levels of c-di-GMP enhance biofilm formation and promote chronic infection by *P. aeruginosa* (Jimenez *et al.*, 2012). Therefore, modulation of intracellular c-di-GMP levels by regulating phosphodiesterase gene expression is one potential explanation for how AmpR controls biofilm formation. Moreover, AmpR also negatively regulates expression of AlgT/U (Balasubramanian *et al.*, 2011), which controls alginate production, an important component of *P. aeruginosa* biofilms. Thus, AmpR-mediated negative regulation of *algT/U* expression could be an additional biofilm control mechanism. Although alginate itself is not required for biofilm formation (Stapper *et al.*, 2004), copious amounts are typically found in *P. aeruginosa* CF biofilms (Harmsen *et al.*, 2010). As RsmA negatively regulates biofilm formation, one would expect a lower biofilm formation in the *ampR* mutant, due to sequestration of RsmA by rgRsmZ. This is contrary to the negative regulation of biofilm formation by AmpR (Balasubramanian *et al.*, 2012). However, given the complex, multitiered gene regulation in *P. aeruginosa* (Jimenez *et al.*, 2012; Balasubramanian *et al.*, 2013a,b), the relative contributions of the individual regulator signals in determining the final outcome (e.g. a phenotype) remain largely unexplained.

AMPR MUTATIONS IN CLINICAL ISOLATES

The infecting clonal types of *P. aeruginosa* undergo many changes upon infection to adapt and colonize, a process driven by mutations (Folkesson *et al.*, 2012; Wong *et al.*, 2012; Behrends *et al.*, 2013). Several recent studies have identified genes that are mutated in either clinical isolates of *P. aeruginosa* or strains that have been subjected to CF-like growth conditions (Hoffman *et al.*, 2009; Cramer *et al.*, 2010; Feliziani *et al.*, 2010; Chung *et al.*, 2012; Wong *et al.*, 2012; Hogardt and Heesemann, 2013). As part of the adaption process in the CF lung, the isolates lose their ability to produce acute virulence factors and overexpress chronic virulence traits, as discussed earlier. This is facilitated by mutations in *mucA* and *lasR* early on in the infection (Smith *et al.*, 2006; Ciofu *et al.*, 2010), resulting in alginate overproduction (Martin *et al.*, 1993) and downregulation of QS-regulated virulence factors (Venturi 2006).

Being a regulator of several important pathways in *P. aeruginosa* (Balasubramanian *et al.*, 2012, 2014), acquiring mutations in *ampR* to alter antibiotic resistance will likely disturb the balance of the regulatory network in the organism. Thus, the mode of *ampC* de-repression in clinical isolates is often through

mutations in accessory genes that are AmpR-regulated (Balasubramanian *et al.*, 2012), such as the *ampD* alleles encoding amidase and its homologs (Juan *et al.*, 2006; Schmidtke and Hanson, 2008), *nagZ* encoding hydrolase (Zamorano *et al.*, 2010), or genes that are outside of the AmpR regulon, such as *dacB* encoding penicillin-binding protein 4 (Moya *et al.*, 2009). Some strains that have been implicated in outbreaks harbor more than one mutation, resulting in multidrug-resistant (MDR) and extensively drug-resistant (XDR) clones (Deplano *et al.*, 2005; Suarez *et al.*, 2011). The incidence of MDR and XDR clones of *P. aeruginosa* in patients is on the rise and undermines treatment strategies (Mesaros *et al.*, 2007; Pena *et al.*, 2012). Genetic analysis of the molecular mechanisms contributing to enhanced resistance of the XDR clones revealed combinations of resistance to β-lactams (AmpC overproduction and inactivation of OprD), fluoroquinolone resistance (point mutations in GyrA), resistance to gentamycin and tobramycin (*aadB* gene acquired on a class I integron), and upregulation of aminoglycoside resistance (mutation in the *mexZ* repressor of the MexXY-OprM efflux pump) (Cabot *et al.*, 2012).

Some *ampR* mutations in clinical isolates are associated with high levels of β-lactamase production in MDR and XDR high-risk clones of *P. aeruginosa* (Cabot *et al.*, 2012) and are summarized in Fig. 2. Specifically, in a majority of the most prevalent *P. aeruginosa* ST175 high-risk XDR/MDR isolates analyzed, a novel mutation in AmpR (glycine 154-arginine) was the reason for constitutive activation of *ampC* expression (Cabot *et al.*, 2012). In the sporadic XDR/MDR and moderately resistant strains, other *ampR* mutations were detected (E114A, G283E, M288R, A51T; Fig. 2), but these polymorphisms are also found in wild-type strains such as PA14 (Winsor *et al.*, 2011; Cabot *et al.*, 2012). Complementing an *ampR* deletion strain in *trans* with a plasmid harboring *ampR*-G154R enhanced *ampC* expression and resistance to ceftazidime (Cabot *et al.*, 2012). In light of studies that demonstrate AmpR to be a positive regulator of acute virulence factors and antibiotic resistance (Balasubramanian *et al.*, 2012, 2014), it is very possible that locking AmpR in an active conformation contributes to the success of high-risk XDR clones such as ST175. This, however, remains to be examined.

In *C. freundii*, AmpR becomes a constitutive activator of *ampC* expression upon amino acid substitutions R86C, G102E, and D135N (Kuga *et al.*, 2000; Balcewich *et al.*, 2010), of which only the D135N mutation has been found in a clinical isolate (Bagge *et al.*, 2002). Studies in our laboratory have demonstrated that mutating the aspartic acid residue at position 135 to asparagine (D135N) in *P. aeruginosa* AmpR locks it in the constitutively active conformation (Caille *et al.*, 2014). However, the G102E mutation in *P. aeruginosa* AmpR seems to destabilize the protein, leading to the loss of activity (Caille *et al.*, 2014). These studies from our laboratory and elsewhere demonstrate that mutations in *ampR* play an important role in regulating antibiotic resistance in *P. aeruginosa*.

PREVALENCE OF AMPR IN PROTEOBACTERIA

Phylogenetic analysis reveals that AmpR homologs are found in many α-, β-, and γ-proteobacteria (Fig. 4). There appears to be two main branches in the phylogenetic tree (Fig. 4). The first branch contains human pathogens (*P. aeruginosa* and *Enterobacteriaceae* members such as *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae, E. coli, Morganella morganii*, and *Yersinia enterocolitica*) and plant pathogens/symbionts (*Erwinia* sp, *Agrobacterium* sp., *P. fluorescens*, and *Rhizobium* sp). The second branch consists of different *Burkholderia* and *Serratia* species, *Azorhizobium* sp., *Caulobacter* sp., and *Acidovorax* sp., among others. The genus *Burkholderia* forms two different subclades, with the major human pathogens belonging to the *B. cepacia* complex such as *B. cepacia*, *B. cenocepacia*, and *B. multivorans* being part of the same subclade (Fig. 4). It is interesting to note that AmpR in *Serratia,* an *Enterobacteriaceae* member, bears a higher homology to the *Burkholderia* AmpR than to the other *Enterobacteriaceae* members.

In many of these bacteria, as in *P. aeruginosa*, AmpR plays an important role in conferring β-lactam resistance (Seoane *et al.*, 1992; Naas *et al.*, 1995; Trepanier *et al.*, 1997; Weng *et al.*, 2004; Okazaki and Avison, 2008). Considering the global regulatory role of AmpR in *P. aeruginosa*, it would be interesting to see whether it plays a similar role in other pathogenic/nonpathogenic bacteria.

Several members of these genera have previously been identified as emerging human pathogens, especially in the CF lung (Davies and Rubin 2007; Raso *et al.*, 2008), while others are important plant pathogens and are related to *Pseudomonas*. It is therefore not surprising that they all harbor the *ampR* gene, suggesting that they acquired it early on in the evolutionary process. Moreover, given the shared habitat (rhizosphere) for many of these bacteria, it is likely that *ampR* was acquired by horizontal gene transfer.

CONCLUSIONS

Treatment for *P. aeruginosa* infections poses an immense clinical challenge due to its potent virulence arsenal, ability to establish persistent chronic infections, and extensive drug resistance. Research over the years has generated much information about regulation of its pathogenesis, but as the function of nearly half of the genome is unknown (Winsor *et al.*, 2011), we still have a long way to go in understanding the mechanisms involved. The large genome of *P. aeruginosa* allows it to dedicate a huge portion toward the regulation of various virulence determinants. At the same time, there is extensive cross talk between the regulators of various pathways, resulting in a challenging network of systems controlling various aspects of pathogenesis (Balasubramanian *et al.*, 2013a).

AmpR is one of 434 transcriptional regulators identified in the PAO1 genome, many of which remain uncharacterized (Stover *et al.*, 2000; Balasubramanian *et al.*, 2013b). With a large complement of regulatory proteins and accessory metabolic genes, it is no surprise that *P. aeruginosa* is able to adapt and thrive in a wide range of habitats. Analyses of potential and empirically demonstrated gene regulatory networks reveal wide gaps in our current knowledge of the system (Balasubramanian *et al.*, 2013a,b). Understanding how AmpR and other regulators orchestrate the virulence and metabolic processes in *P. aeruginosa* in response to external signals is critical in dealing with infections caused by this successful opportunistic pathogen.

The microarray, RNA-Seq, and proteomic analysis of PAOΔ*ampR* mutant sufficiently established the role of AmpR as a key regulator of antibiotic resistance as well as acute and chronic infections (Fig. 3). Apart from the known pathways, AmpR regulon also includes small RNAs. rgRNAs have been shown to be extensively involved in gene regulation in *P. aeruginosa* and other bacteria (Wilderman *et al.*, 2004; Brencic and Lory 2009; Brencic *et al.*, 2009; Sonnleitner *et al.*, 2009, 2011;

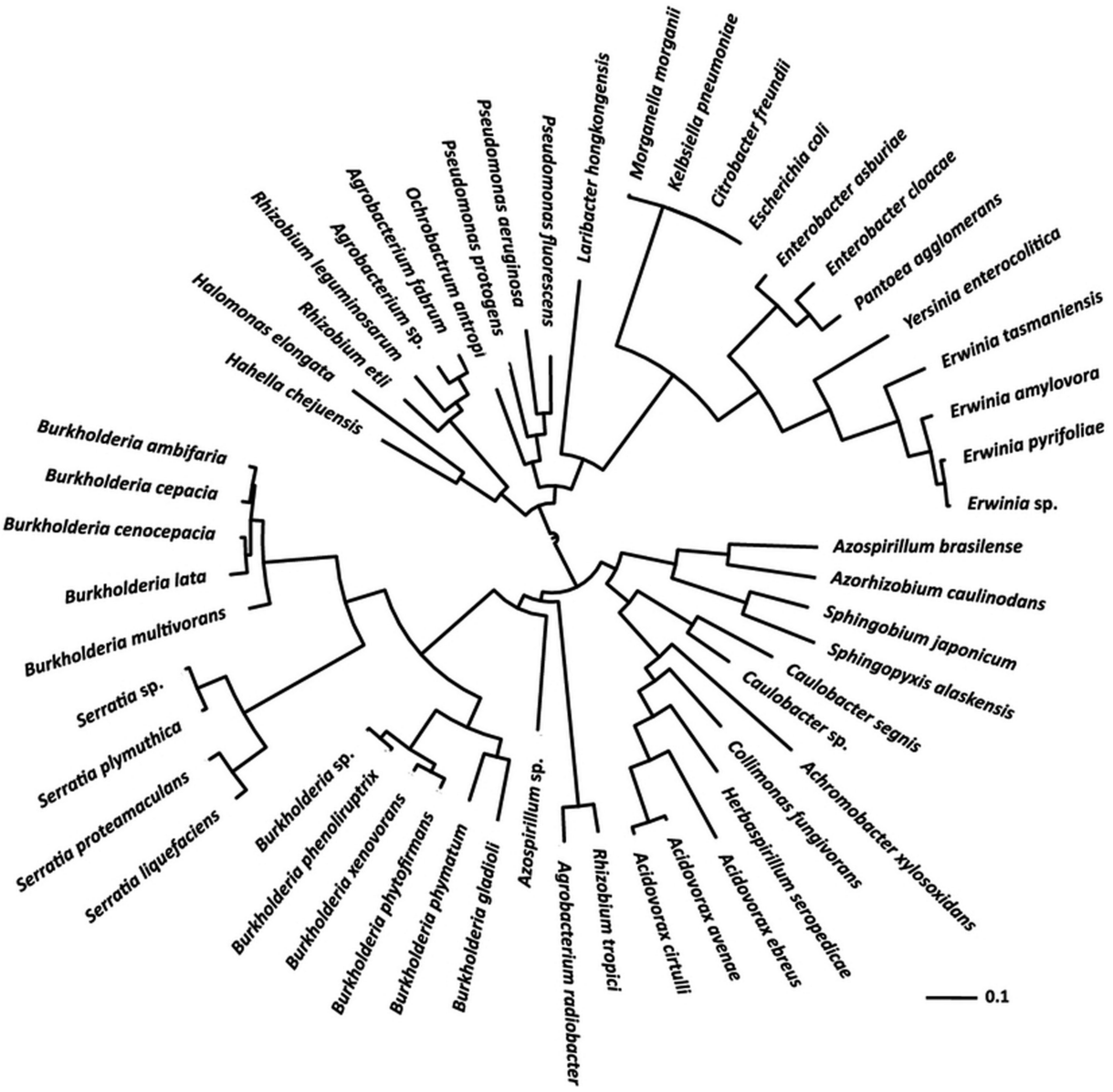

Figure 4. Prevalence and relatedness of AmpR in proteobacteria. The precomputed BLAST data for the AmpR (PA4109) amino acid sequence from the *Pseudomonas* Genome Database (Winsor *et al.*, 2011) were used to identify homologs in other bacteria. The cutoff score was set at 850 corresponding to 56% protein identity. For the sake of clarity, only the top hit identified in the sequenced genomes of each species was considered for further analysis. The matches that conform to these criteria were aligned using NCBI Constraint-Based Multiple Alignment Tool (Papadopoulos and Agarwala 2007), and the resulting alignment file was used to generate a phylogenetic tree (Dereeper *et al.*, 2008, 2010). The GI protein IDs in the Newick format of the tree were replaced with organism names (identified using the NCBI Batch Entrez), and the tree was visualized using the Interactive Tree of Life (Letunic and Bork 2007, 2011).

Wiedenheft *et al.*, 2012). The interplay between rgRNAs and transcriptional regulators in controlling critical functions in bacteria is being increasingly appreciated.

Although recent studies have identified over 500 novel sRNAs in *P. aeruginosa* (Dotsch *et al.*, 2012; Gomez-Lozano *et al.*, 2012), their function and regulation have not been elucidated. Given the important regulatory role of AmpR in *P. aeruginosa* virulence and metabolism, it is not surprising that rgRNAs, such as rgRsmZ, asPrrF1, rgP32, and rgRgsA, are AmpR-regulated (Fig. 3). It is possible that other sRNAs are AmpR-regulated. Future research on determining the AmpR-regulated sRNAs will provide valuable information to our current understanding. Given the many different ways in which sRNAs can modulate gene expression (Sonnleitner *et al.*, 2012) and potentially undiscovered ones, we can look forward to exciting new discoveries in bacterial gene regulation in the coming years.

Using a combination approach of transcriptomic, proteomic, and phenotypic assays, AmpR was determined to affect the expression of 2121 genes, 363 of which overlapped in at least two analyses (Balasubramanian *et al.*, 2012, 2014; Kumari *et al.*, 2014b). As AmpR occupies a nodal position in the regulatory network of *P. aeruginosa* that affects expression of diverse phenotypes (Balasubramanian *et al.*, 2013a), it makes for an attractive therapeutic target to combat the antibiotic resistance problem. Loss of *ampR*, in addition to rendering *P. aeruginosa* sensitive to many β-lactam antibiotics, also results in

reduced production of many acute virulence factors (Balasubramanian *et al.*, 2012). Small molecule inhibitors of AmpR can be potential therapeutic agents against *P. aeruginosa* in the clinical setting, thus reducing virulence and rendering the cells sensitive to β-lactam antibiotics, without causing selective pressure. Although PAOΔ*ampR* displays fluoroquinolone resistance, targeting AmpR is still a good proposition because the strains will become susceptible to β-lactams and have reduced virulence factor production. Moreover, use of the major fluoroquinolone ciprofloxacin in the clinical setting is on the decline owing to the development of high-level resistance (Hidron *et al.*, 2008).

In conclusion, understanding the regulatory network of *P. aeruginosa* in a holistic manner is imperative to compete with the evolving bacterial strategies against antibiotic use. With fewer new antibiotics being discovered, the focus should also be on developing new therapeutic strategies involving important players of resistance and virulence, such as AmpR.

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Author contribution

D.B. and H.K. contributed equally to this work.

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