REVIEW ARTICLE

Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa*

W. James Gooderham & Robert E. W. Hancock

Centre for Microbial Diseases and Immunity Research, Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada

Correspondence: Robert E.W. Hancock, #232-2259 Lower Mall Research Station, Vancouver, BC, Canada, V6T 1Z4. Tel.: +604 822 2682; fax: +604 827 5566; e-mail: bob@cmdr.ubc.ca

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Abstract

The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* ubiquitously inhabits soil and water habitats and also causes serious, often antibiotic resistant, infections in immunocompromised patients (e.g. cystic fibrosis). This versatility is mediated in part by a large repertoire of two-component regulatory systems that appear instrumental in the regulation of both virulence processes and resistance to antimicrobials. Major two-component regulatory system proteins demonstrated to regulate these diverse processes include PhoP–PhoQ, GacA–GacS, RetS, LadS, and AlgR, among others. Here, we summarize the current body of knowledge of these and other two-component systems that provides insight into the complex regulation of virulence and resistance in *P. aeruginosa*.

Introduction

The bacterial world is dynamic. Bacterial survival under changing environmental conditions requires the capability to sense and quickly respond to many different stimuli. Often, these physiological responses are mediated at the level of gene expression and accomplished by regulatory proteins. One class of regulators, the bacterial two-component regulatory systems, are designed to sense diverse stimuli and enact an appropriate and rapid adaptive physiological response that can involve the altered transcription of a substantial number of genes.

Two-component regulatory systems classically comprise an inner membrane-spanning sensor histidine kinase and a cytoplasmic response regulator (Stock *et al.*, 2000). The detailed mechanisms of two-component signal transduction systems have been reviewed previously (Stock *et al.*, 2000; Mascher *et al.*, 2006; Laub & Goulian, 2007). Functionally coupled sensor kinase and response regulator genes are often encoded adjacent to one another in the genome, forming an operon, and this arrangement is generally indicative of a cognate system which enacts a response to a specific signal detected by the sensor kinase, although in many cases the precise signal is unknown. The functional mode of both sensor kinases and response regulators is determined by reversible phosphotransfer reactions and ensuing protein conformational changes (Stock *et al.*, 2000). In the archetypical system, the sensor kinase contains an N-terminal periplasmic input domain that detects a specific stimulus and a C-terminal cytoplasmic transmitter domain that binds ATP and has histidine kinase activity. The cognate response regulator contains a conserved aspartate receiver domain and a variable output domain that often binds DNA.

Classically, a membrane-bound dimeric sensor kinase detects an environmental stimulus/ligand in the periplasm via its input domain, and then undergoes *trans*-autophosphorylation at a conserved histidine residue in its transmitter domain (Mascher *et al.*, 2006). This phosphoryl group is then transferred to and catalyzed by the conserved aspartate-containing receiver domain of the response regulator. Phosphorylation of the response regulator receiver domain often modifies the activity of the output domain, of which there are many types (Galperin, 2006; http://www.ncbi.nlm.nih.gov/Complete_Genomes/RRcensus). The output domain is most frequently a helix–turn–helix DNA-binding domain (e.g. OmpR, NarL, and NtrC subfamilies),

but other types include those with enzymatic functions (e.g. GGDEF diguanylate cyclases, EAL c-di-GMP phosphodiesterases, and CheB-like methylesterases). In the former case, phosphorylation of the receiver domain changes the response regulator's affinity for specific DNA elements so as to modify gene expression and initiate the corresponding cellular response. Dephosphorylation of the response regulator by the sensor kinase serves to return the system to its preactivation state.

Structural and functional modifications of this basic functionality do exist. Sensor kinases that contain an aspartate-containing receiver domain at their C-terminus, analogous to that of the response regulator, are termed 'hybrid' sensor kinases. To accomplish histidine to aspartate phosphotransfer, it is therefore thought that these hybrid systems utilize small separate proteins that contain histidine phosphotransfer (Hpt) domains to facilitate phosphotransfer from the sensor's histidine to the terminal response regulator's aspartate (His → Asp → His → Asp; Rodrigue *et al.*, 2000). Conversely, other nonclassical sensor histidine kinases are termed 'unorthodox' and contain an additional Hpt domain fused N-terminal to their receiver domain and are thought to participate in complex phosphorelay networks. Both types of systems may enable added flexibility relative to signal transduction mediated by the classical systems. In this review, we generally refer to each two-component system with the response regulator preceding its cognate sensor kinase.

Pseudomonas aeruginosa

Pseudomonas aeruginosa inhabits diverse environments that include soil and water habitats, and can also infect plants, insects, nematodes, and animals (Stover *et al.*, 2000). Like other Pseudomonads, it exhibits nutritional and metabolic versatility. Together, this remarkable adaptability is driven by sophisticated and coordinated regulation of gene expression. *Pseudomonas aeruginosa* is also a major opportunistic human pathogen of immunocompromised patients. It is the third most common nosocomial (hospital) pathogen in North America, and a leading cause of chronic pulmonary infections and mortality in cystic fibrosis (CF) patients, ventilator-associated pneumonia, and sepsis in burn patients (Fridkin *et al.*, 1999). A major reason for therapeutic failure in the treatment of these infections is its high intrinsic resistance to multiple classes of antibiotics due to low outer-membrane permeability and active antibiotic efflux systems (Hancock & Speert, 2000). The steady rise in adaptive and mutational resistance is increasingly impacting on therapeutic success and new antimicrobial therapeutic options are needed for resistant strains, some of which have developed resistance to virtually every type of antibiotic and have thus become hospital 'superbugs' (Falagas & Bliziotis, 2007).

Multideterminant virulence and high antibiotic resistance are two hallmarks of *P. aeruginosa* infections. A large number of two-component regulatory systems encoded in its genome have equipped *P. aeruginosa* with a sophisticated capability to regulate diverse virulence and resistance processes. In this review, we focus on the rapidly expanding recent body of knowledge regarding the roles of *P. aeruginosa* two-component regulatory systems participating in virulence and antibiotic resistance processes.

Pseudomonas aeruginosa virulence and antimicrobial resistance

An arsenal of virulence factors have been reported and appear to impact on the pathogenicity of this organism. Included among these are cell-associated factors such as lipopolysaccharide, flagella and type IV pili, the exopolysaccharide alginate, and secreted factors including toxins, elastase and other proteases, phospholipases and small molecules such as phenazines, rhamnolipids and cyanide. Quorum-sensing, type III secretion and many other regulatory systems are important for virulence and, as they have been reviewed in detail elsewhere, will not be a specific focus of this review (Kirisits & Parsek, 2006; Venturi, 2006; Yahr & Wolfgang, 2006).

The single polar flagellum of *P. aeruginosa* is an important and highly regulated virulence factor involved in both motility and surface attachment. Isolates from long-term chronic CF infections often lack flagella. One hypothesis addressing this is that these nonmotile cells are at some stage of a biofilm-like adaptation. Biofilm formation follows three distinct stages: surface attachment, microcolony formation, and differentiation into a mature biofilm community encased in an extracellular matrix (Kirisits & Parsek, 2006). Type IV pili-dependent twitching and flagellar-based swimming motility are important for colonization and initial surface attachment (O'Toole & Kolter, 1998), and many genes associated with these motility appendages are necessary for proper biofilm formation (Kirisits & Parsek, 2006). In addition, we demonstrated recently that there is a strong, but not obligate, correspondence in gene requirements between biofilm formation and swarming motility, which is dependent on both motility appendages and represents a complex adaptation to viscous environments (Overhage *et al.*, 2007, 2008). Type III secretion system-mediated cytotoxicity is also considered to be a key virulence property of *P. aeruginosa*, while type II secretion mediates the secretion of a broad range of toxins and other virulence-related enzymes; interestingly, the structures involved in these secretion systems resemble flagella and pili, respectively.

Pseudomonas aeruginosa is capable of causing both chronic and acute infections and appropriately regulates the virulence determinants required for each of these types

of infection (Furukawa *et al.*, 2006). Chronic persistent infections can be microcolony or biofilm-like in nature, such as those present in CF lung infections (Singh *et al.*, 2000), and on urinary catheters and other indwelling medical devices. Conversely, acute *P. aeruginosa* infections are typified by bacterial penetration of the host epithelium and systemic spread, such as in severe burn wound infections.

Antibiotic resistance is similarly complex. In addition to classical mutational or acquired resistance, *P. aeruginosa* antibiotic resistance can be triggered by environmental factors (a subset of adaptive resistance). Subinhibitory concentrations of antibiotics and other molecules can themselves induce resistance to subsequent exposure to otherwise lethal concentrations of antibiotics. During human infections, *P. aeruginosa* is likely exposed to endogenous β-defensin and cathelicidin host defense (antimicrobial) peptides at epithelial surfaces. Further, polymyxins (peptide antibiotics) and other types of conventional antibiotics also interact with *P. aeruginosa* when therapeutically administered. Of note, natural and synthetic antibacterial peptides are being pursed as novel therapeutics (Marr *et al.*, 2006). However, adaptive resistance to cationic antimicrobial peptides and polymyxin B is known to occur in response to limiting extracellular concentrations of divalent Mg^{2+} and Ca^{2+} cations, but this is unlikely to be clinically meaningful as the body contains 1–2 mM divalent cations. However, as noted below peptides themselves are able to induce peptide resistance mechanisms through two-component regulation.

Overview of two-component systems in *P. aeruginosa*

The *P. aeruginosa* PAO1 genome encodes one of the largest complements of regulatory proteins found in bacteria representing just under 10% of all genes (Stover *et al.*, 2000), and a major subset are the genes encoding 64 sensor kinases and 72 response regulators (Tables 1 and 2; Rodrigue *et al.*, 2000; Chen *et al.*, 2004; Galperin, 2006). These include four CheA-type sensor kinases and four CheB-type methylesterase response regulators that are part of chemosensory gene clusters. The most common type of gene organization (29 systems) comprises a response regulator upstream of its cognate sensor kinase. Almost all operons with this organization comprise an OmpR-type response regulator, and these systems likely represent coevolution through duplication from an ancestral OmpR-sensor kinase pairing (Chen *et al.*, 2004). Twenty-one systems contain sensor kinases upstream of their cognate response regulator-encoding genes (separated by three or less genes). NarL-type response regulators can be associated with both classical and hybrid or unorthodox sensors and therefore, likely represent evolutionary recruitment during cognate pair formation (Chen *et al.*, 2004). A further 13 sensors and 15 regulators are termed 'orphans' and are not physically linked to any other two-component gene, thus making the identification of cognate function pairs difficult (except GacA and GacS that are very well characterized). Additionally, there are three separate genes encoding HPt domains [*hptA* (PA0991), *hptB* (PA3345), and *hptC* (PA0033)] that possibly interact with hybrid kinases to form complex phosphorelay signaling networks (Rodrigue *et al.*, 2000). HptB was recently shown to be capable of interacting with several hybrid kinases in a multistep phosphorelay pathway (Hsu *et al.*, 2008). Complex CheA-type chemosensory signal transduction proteins such as ChpA (PA0413), which is involved in twitching and swarming motility, virulence and cytotoxicity (D'Argenio *et al.*, 2001; Whitchurch *et al.*, 2004; Leech & Mattick, 2006), will not be discussed in this review.

PhoP–PhoQ

The PhoP–PhoQ system mediates in part the adaptive response to low extracellular Mg^{2+} concentrations in *P. aeruginosa* (Fig. 1; McPhee *et al.*, 2006). PhoP–PhoQ controls resistance to aminoglycosides, polymyxin B, and antimicrobial peptides, albeit likely in different fashions (Macfarlane *et al.*, 1999, 2000). In response to low external

Table 1. *Pseudomonas aeruginosa* virulence- and antibiotic resistance-associated classical-type response regulators and sensor histidine kinases

PA number*	Gene product†	Brief functional description	References‡
PA 0034		Repressed during CF sputum medium *in vitro* growth. Directly upstream to *hptC* (PA0033)	Palmer *et al.* (2005)
PA 0408	PilG	Chemosensory pili (Pil–Chp) system, twitching motility	Darzins & Russell (1997)
PA 0409	PilH		
PA 0463/4	CreB–CreC	Catabolism. Swarming and swimming motility	Wagner *et al.* (2007)
PA 0929/30	PirR–PirS	Iron acquisition	Vasil & Ochsner (1999)
PA 1099/8	FleR–FleS	Flagellar motility, adhesion to mucin. FleS likely cytoplasmic sensor	Ritchings *et al.* (1995); Dasgupta *et al.* (2003)
PA 1157/8		PA1157 essential in rat chronic lung infection	Potvin *et al.* (2003)
PA 1179/80	PhoP–PhoQ	Low Mg^{2+} signal. Polymyxin, antimicrobial peptide and aminoglycoside resistance. Virulence, swarming motility and biofilm formation	Ernst *et al.* (1999); Macfarlane *et al.* (1999, 2000); Ramsey & Whiteley (2004); McPhee *et al.* (2006)

Table 1. Continued.

PA number*	Gene product†	Brief functional description	References‡
PA 1135/6		PA1136 encodes immunogenic peptides to cystic fibrosis serum	Beckmann *et al*. (2005)
PA 2523/4	CzcR–CzcS	Metal and imipenem resistance	Hassan *et al*. (1999); Perron *et al*. (2004)
PA 2586	GacA	GacA–GacS system. Multihost virulence, quorum-sensing-dependent regulation of exoproducts and virulence factors, biofilm formation and antibiotic resistance, swarming motility, type III secretion	Reimmann *et al*. (1997); Rahme *et al*. (2000); Parkins *et al*. (2001); Soscia *et al*. (2007)
PA 2686/7	PfeR–PfeS	Iron acquisition	Dean *et al*. (1996)
PA 2809/10	CopR–CopS	Metal and imipenem resistance	Teitzel *et al*. (2006); Caille *et al*. (2007)
PA 3045	RocA2	RocA2–RocS2. Fimbriae adhesin gene regulation	Kulasekara *et al*. (2005)
PA 3192/1	GltR	Glucose transport, type III secretion cytotoxicity	Wolfgang *et al*. (2003)
PA 3204/6		PA3206 encodes highly immunogenic peptides to cystic fibrosis serum	Beckmann *et al*. (2005)
PA 3346		HptB-mediated phosphorelay, swarming motility and biofilm formation	Hsu *et al*. (2008)
PA 3702/4	WspR–WspE	Wsp chemosensory system. Biofilm and cyclic-di-GMP level regulation, autoaggregation. WspR contains GGDEF output domain, WspE is CheA-type sensor	D'Argenio *et al*. (2002); Hickman *et al*. (2005); Kulasekara *et al*. (2005)
PA 3879/8	NarL–NarX	Nitrate sensing and respiration. Biofilm formation, swimming and swarming motility	Van Alst *et al*. (2007)
PA 3947	RocR (SadR)	RocS1/R/A1 (SadA/R/S) system. Cytotoxicity, virulence, regulation of fimbriae adhesins, type III secretion and biofilm formation. RocA1 contains EAL output domain, RocR is RocA1 antagonist	Kulasekara *et al*. (2005); Kuchma *et al*. (2005)
PA 3948	RocA1 (SadA)		Kulasekara *et al*. (2005); Ruer *et al*. (2007).
PA 4296/3	PprB–PprA	Outer-membrane permeability and aminoglycoside resistance. PprA sensor likely cytoplasmic	Wang *et al*. (2003); Wagner *et al*. (2007)
PA 4396		Overexpression impairs type III secretion-mediated cytotoxicity. GGDEF output domain	Kulasakara *et al*. (2006)
PA 4547/6	PilR–PilS	Type IV fimbriae expression, twitching and swarming motility, biofilm formation	Ishimoto & Lory (1992); Hobbs *et al*. (1993); Goodman *et al*. (2004); Overhage *et al*. (2007)
PA 4726/5	CbrB–CbrA	Carbon and nitrogen storage, cytotoxicity, swarming motility, nematode virulence	Gallagher & Manoil (2001); Rietsch *et al*. (2004); Wagner *et al*. (2007)
PA 4776/7	PmrA–PmrB	Induced by low Mg^{2+} and cationic antimicrobial peptides. Polymyxin B and antimicrobial peptide resistance	McPhee *et al*. (2003); Moskowitz *et al*. (2004).
PA 4959	FimX	Phosphodiesterase (GGDEF and EAL domains), twitching motility, reduced *in vitro* cytotoxicity	Huang *et al*. (2003); Kulasakara *et al*. (2006); Kazmierczak *et al*. (2006)
PA 5261/2	AlgR–FimS (AlgZ)	Virulence, alginate biosynthesis, twitching and swarming motility, biofilm formation, cyanide production, cytotoxicity and type III secretion system gene expression	Deretic & Konyecsni (1989); Whitchurch *et al*. (1996); Yu *et al*. (1997); Lizewski *et al*. (2002); Wu *et al*. (2004); Morici *et al*. (2007); Overhage *et al*. (2007); Belete *et al*. (2008).
PA 5360/1	PhoB–PhoR	Phosphate level regulation, low phosphate signal, quorum sensing	Filloux *et al*. (1988); Jensen *et al*. (2006)
PA 5483/4	AlgB–KinB	Alginate biosynthesis	Wozniak & Ohman (1991); Goldberg & Dahnke (1992); Leech *et al*. (2008)
N/A	PvrR	Phenotypic variation, antibiotic resistance, biofilm formation	Drenkard & Ausubel (2002)

*Annotated gene identification number (PA) and protein names according to the PAO1 genome at http://www.pseudomonas.com. Response regulator-encoding genes are listed before their cognate sensor kinase-encoding genes; thus, this does not necessarily represent transcriptional direction.
†Other classical-type RR and SK include PA0179/8, PA0267, PA0600/1, PA0756/7, PA1335/6 (*aauR/aauS*), PA1397/6, PA1437/8, PA1456/8 (*cheY/cheA*), PA1785 (*nasT*), PA1799/8, PA1978 (*agmR*)-PA1979/80, PA1637/6 (*kdpE/kdpE*), PA1785, PA1980/79, PA2376, PA2479/80, PA2572/1, PA 2657/6, PA2798, PA2881/2, PA2899, PA3077/8, PA3604, PA3714, PA4032/6, PA4080, PA4101/2, PA4117 (*bphP*), PA4296/7, PA4381/0 (*colR/colS*), PA4493/4, PA4781, PA4843, PA4885/6 (*irlR*), PA4983/2 (*dmsR*), PA4983/2, PA5124/5 (*ntrC/ntrB*), PA5166/5, PA 5200/9 (*ompR–envZ*), PA5364, PA 5511/2. Additional chemosensory-type systems: 4 CheB-like methylesterases: PA0173, PA0414 (*chpB*), PA1459, PA3703 (*wspF*). 4 CheA-like histidine kinases: PA0178, PA0413 (*chpA*), PA1458 (*cheA*), PA3704 (*wspE*).
‡Selected references generally do not contain original gene identification reference if this was unrelated to virulence or resistance.

Table 2. *Pseudomonas aeruginosa* virulence-associated hybrid and unorthodox sensor histidine kinases

PA number*	Sensor kinase	Brief functional description†	Reference
PA 0928	GacS	GacA–GacS system. Plant, insect, animal virulence. Motility, biofilm, secondary metabolite regulation; aminoglycoside resistance. HPt domain	Rahme *et al*. (2000); Brinkman *et al*. (2001); Davies *et al*. (2007)
PA 1611		PA3346–HptB-PA1611 phosphorelay	Lin *et al*. (2005); Hsu *et al*. (2008)
PA 3044	RocS2	RocA2–RocS2 system. Regulation of fimbriae adhesins. HPt domain	Kulasekara *et al*. (2005)
PA 3946	SadS (RocS1)	SadA–SadR–SadS system (RocS1–RocR–RocA1). Biofilm maturation, fimbrial genes, type III secretion; *C. elegans* virulence; HPt domain	Kulasekara *et al*. (2005); Gallagher & Manoil (2001); Kuchma *et al*. (2005)
PA 3974	LadS	Virulence, biofilm formation, type III secretion/cytotoxicity	Ventre *et al*. (2006)
PA 4112		Encodes immunogenic peptides towards cystic fibrosis serum. HPT domain	Beckmann *et al*. (2005)
PA 4856	RetS	Virulence, biofilm formation, type III secretion/cytotoxicity	Goodman *et al*. (2004); Laskowski *et al*. (2004)
PA 4982		Encodes peptides highly immunogenic to cystic fibrosis serum. HPt domain. Putative PA4983–PA4982 system	Beckmann *et al*. (2005)

*Annotated gene identification number (PA) and hybrid/unorthodox sensor kinase name according to the PAO1 genome at http://www.pseudomonas.com. Additional/uncharacterized hybrid sensors include PA1243, PA1396, PA1976, PA1992, PA2177, PA2583, PA2824, PA3271, and PA3462.
†Where available each hybrid or unorthodox sensor is written following its cognate signaling protein(s) (e.g. response regulator). Sensors which include an HPt domain are unorthodox sensor kinases.

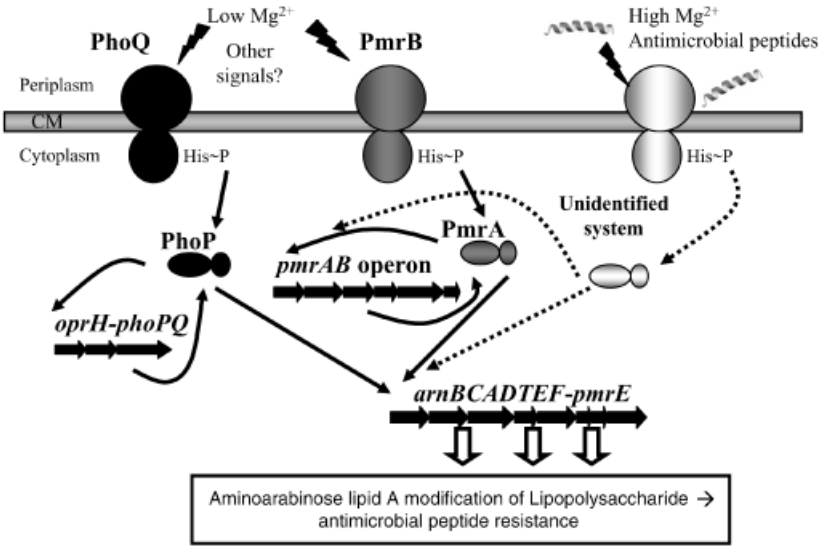

Fig. 1. A model for the *Pseudomonas aeruginosa* PhoP–PhoQ and PmrA–PmrB regulatory networks in resistance to cationic antimicrobial peptides. Mg^{2+} limitation leads to the activation (phosphorylation) of the PhoP and PmrA response regulators which positively autoregulate the transcription of their respective operons, as well as the *arnBCADTEF* operon. The ArnBCADTEF pathway modifies Lipid A with aminoarabinose, reducing the net negative charge on lipopolysaccharide, and consequently decreasing self-promoted uptake across the outer membrane, increasing resistance to polymyxins and cationic antimicrobial peptides. Conversely, under high Mg^{2+} conditions, PhoP and PmrA are dephosphorylated and presumed inactive (not shown). However, during growth in high Mg^{2+} plus subinhibitory antimicrobial peptides, an unidentified regulatory system is proposed to promote activation of *arnBCADTEF* and *pmrAB* operons, consequently increasing resistance to antimicrobial peptides. See text for details of these systems. Other genes regulated by PmrA and PhoP are not shown.

concentrations of Mg^{2+} that are detected by the PhoQ sensor kinase, the PhoP response regulator regulates polymyxin B and antimicrobial peptide resistance mainly through upregulation of *arnBCADTEF* (Fig. 1). This operon encodes a pathway for addition of 4-aminoarabinose to lipid A of lipopolysaccharide, thereby reducing the net negative charge of lipopolysaccharide and limiting its interaction with polycationic antibiotics such as polymyxin B and cationic peptides (McPhee *et al*., 2003; Moskowitz *et al*., 2004). Lipid A plays an important role in the pathogenesis of many Gram-negative bacterial infections by acting as a signature of such infections, activating the innate immune system, triggering the synthesis of host defense peptides, cytokines, clotting factors, and other immunostimulatory molecules. CF *P. aeruginosa* isolates contain lipid A structures that are equivalent to those formed upon activation of

PhoP–PhoQ, and appear to generate increased host inflammatory responses (Ernst *et al.*, 1999). A *phoP* response regulator mutant displays wild-type polymyxin B resistance in low Mg^{2+} media and remains sensitive when grown under high Mg^{2+} conditions (in contrast to the situation in *Salmonella*); however, a *phoQ* mutant displays super-resistance to polymyxin B and antimicrobial peptides in both low (wild-type resistance) and high Mg^{2+} (a constitutive resistance phenotype). These findings suggest that in noninducing high Mg^{2+} conditions, PhoQ dephosphorylates PhoP (inactivating it), and conversely in the *phoQ* mutant this inactivation is lost and possibly some other protein/mechanism allows PhoP to be activated through phosphorylation. Therefore, as for wild-type cells grown in low Mg^{2+}, the *phoQ* mutation leads to massive upregulation of the *arnBCADTEF* operon and resistance to polymyxin B and cationic peptides (McPhee *et al.*, 2003; W.J. Gooderham & R.E.W. Hancock, unpublished data). The role of PhoP in this resistance appears to be significant as the overexpression of PhoP in a *phoP* mutant gives rise to *phoQ*-like constitutive polymyxin B resistance (Macfarlane *et al.*, 1999). Another group found that a *phoP* mutant displayed intrinsic super-susceptibility to polymyxin B but, in contrast, not to C18G antimicrobial peptides (Ernst *et al.*, 1999). Consistent with PhoPQ Mg^{2+}-dependent regulation of lipopolysaccharide modifications, lipid A from a *phoP* mutant was not modified with aminoarabinose and palmitate (Ernst *et al.*, 1999). In addition, cells become resistant to gentamicin under limiting Mg^{2+} conditions when induction of PhoP–PhoQ occurs (Hancock *et al.*, 1981; Macfarlane *et al.*, 1999). However, this regulation is quite complex as, under high Mg^{2+} conditions, *phoQ* (and polar *phoP* mutants also deficient in *phoQ*) demonstrated increased resistance to the aminoglycoside antibiotics streptomycin, kanamycin, and amikacin (Macfarlane *et al.*, 2000; McPhee *et al.*, 2006).

The presence of polyamines in the growth medium has been shown to increase *P. aeruginosa* resistance towards multiple antibiotics, including polymyxin B, colistin, aminoglycosides, and quinolones (Kwon & Lu, 2006). This is linked to the ability of the polyamine spermidine to induce both the *phoPQ* and *arnBCADTEF* operons (no effect is observed on *pmrAB*). The PhoP response regulator was necessary for this spermidine-induced resistance as a *phoP* mutant exposed to spermidine no longer showed increased resistance to cationic peptides, polymyxin B and quinolone antibiotics. Extracellular Mg^{2+} ion concentrations had no effect on polyamine-mediated antibiotic resistance. Further complicating the situation, spermidine uptake mutants lost spermidine-induced resistance to aminoglycosides and quinolones but retained resistance to the cationic peptide antibiotics. PhoQ also appears to be involved in quinolone resistance as a *phoQ* mutant displayed low-level quinolone super-susceptibility (Kwon & Lu, 2006). It is possible that the large number of two-component systems in *P. aeruginosa*, and their potential for cross-talk, allows a different response towards polyamines as polyamine-induced polymyxin B resistance was not observed in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (Kwon & Lu, 2007).

The sensor kinase PhoQ is also required for biofilm formation when using a dynamic (greater aeration) biofilm assay, and also for the maturation of biofilms in flow cells (Ramsey & Whiteley, 2004). Our unpublished observations have revealed a role for PhoQ in regulating twitching motility, which may help explain the biofilm phenotype, and *phoQ* mutants are swarming impaired, while in contrast a *phoP* mutant is a super-swarmer (Brinkman *et al.*, 2001). Furthermore, *phoQ* mutants are less cytotoxic towards mammalian cells. Consistent with these observed phenotypes related to virulence, a *phoQ* mutant is significantly less virulent in a neutropenic mouse model (Macfarlane *et al.*, 1999) and our recent observations indicate a major role for PhoQ in chronic rat lung infection.

PmrA–PmrB

PmrA–PmrB is another two-component system that responds to low Mg^{2+} and regulates resistance to polymyxin B and antimicrobial peptides (Fig. 1; McPhee *et al.*, 2003, 2006; Moskowitz *et al.*, 2004). Interestingly, the *pmrAB* operon was also shown to be strongly induced by a variety of cationic antimicrobial peptides, including polymyxins, bovine indolicidin, human LL-37, and other synthetic peptides (McPhee *et al.*, 2003), in stark contrast to *Salmonella* where the binding of peptides to PhoQ mediates peptide resistance induction (Bader *et al.*, 2005). The lipopolysaccharide aminoarabinose modification operon *arnBCADTEF* is also strongly induced by peptides, and this activation was at least partially dependent on PmrA–PmrB (McPhee *et al.*, 2003). Transposon mutations in either the *pmrAB* or *arnBCADTEF* operons result in supersusceptibility to both polymyxin B and cationic antimicrobial peptides (Lewenza *et al.*, 2005). Interestingly, peptide-induced activation of the *pmrAB* operon appeared to be independent of both the PhoP–PhoQ and PmrA–PmrB systems (McPhee *et al.*, 2003), which indicates that another two-component regulator likely responds to cationic peptides (Fig. 1).

PmrA–PmrB was also identified by isolating spontaneous mutants with increased resistance to polymyxin B (Moskowitz *et al.*, 2004). These mutations mapped to the PmrB H-box motif, likely allowing PmrB to constitutively activate PmrA by increasing its net phosphorylation. Thus under high Mg^{2+}, the regulation of resistance to a variety of structural classes of cationic antimicrobial peptides and the addition of aminoarabinaose to Lipid A was shown to be dependent on *pmrAB* (Moskowitz *et al.*, 2004).

CzcR–CzcS and CopR–CopS

Cross-resistance between metals and antibiotics exists and has raised concerns about overlaps in environmental and clinical resistance (Baker-Austin *et al.*, 2006). The CzcRS (also termed CzrRS) two-component system was originally described in the context of regulation of the CzcBCA resistance-nodulation-division efflux pump and the control of intrinsic cadmium and zinc resistance (Hassan *et al.*, 1999). Subsequently, coregulation of resistance to trace heavy metals and the carbapenem antibiotic imipenem was demonstrated (Perron *et al.*, 2004). Activation of the CzcS sensor kinase led to imipenem and zinc resistance. It was demonstrated that exposure of *P. aeruginosa* to sublethal zinc ion concentrations activates *czcRS* transcription, which in turn leads to transcriptional activation of the *czcCBA* RND efflux pump system and zinc resistance. Concurrently, the increased levels of CzcR negatively regulate OprD levels, the porin that is the primary route by which carbapenems like imipenem enter the cell, thus explaining cross-resistance to imipenem (Perron *et al.*, 2004). This is in agreement with findings from another study which found that zinc eluted from siliconized latex urinary catheters exerted a negative effect on the expression of OprD and carbapenem resistance (Conejo *et al.*, 2003).

Copper treatment induces resistance to both copper and zinc. However, in a *czcRS* mutant, copper exposure was still capable of decreasing OprD levels and inducing imipenem resistance, indicating the presence of another regulatory system that responds to copper and negatively effected *oprD* expression. This new system involved the CopR response regulator that was demonstrated to link zinc, copper, and imipenem resistance by activating the *czcRS* operon, which consequently led to CzcR-mediated negative regulation of OprD levels and resistance to imipenem (Caille *et al.*, 2007). Transcription of *copRS* was induced in copper-shocked and copper-adapted cells, and was also found to regulate copper tolerance (Teitzel *et al.*, 2006).

GacA–GacS

The GacS–GacA two-component system forms one of the best characterized virulence regulatory pathways. Multihost *P. aeruginosa* pathogenicity studies identified the GacA response regulator as a master regulator of virulence and revealed conservation amongst virulence mechanisms in different hosts (Rahme *et al.*, 2000). Both *gacA* and *gacS* mutants were demonstrated to be required for pathogenicity in plant (*Arabidopsis thaliana*), nematode (*Caenorhabditis elegans*), insect (*Drosophila melanogaster, Galleria mellonella, Bombyx mori*), and mouse models of acute infection (Rahme *et al.*, 1997; Mahajan-Miklos *et al.*, 1999; Tan *et al.*, 1999; Jander *et al.*, 2000; Brinkman *et al.*, 2001; Chieda *et al.*, 2005). GacA also contributes to chronic infection as demonstrated in a CF murine model (Coleman *et al.*, 2003). Both GacA and the GacS sensor kinase are required for activation of multiple extracellular virulence factors as well as chronic persistence genes, including those required for biofilm formation and *N*-acyl-homoserine lactone autoinducers (Reimmann *et al.*, 1997; Parkins *et al.*, 2001). GacS mutants are also impaired in swarming motility (Brinkman *et al.*, 2001).

The signal transduction pathway from GacA to individual virulence genes varies, as recently reviewed by Lapouge *et al.* (2008). Activated (phosphorylated) GacA is thought to positively regulate the transcription of two small (untranslated) regulatory RNAs, RsmZ and RsmY, which appear to act in a parallel and redundant posttranscriptional fashion to jointly antagonize RsmA, a small RNA-binding protein that itself negatively regulates the expression of certain quorum sensing signals and several extracellular products (Fig. 2; Reimmann *et al.*, 1997; Pessi *et al.*, 2001; Kay *et al.*, 2006). Sequestration of RsmA by RsmY and RsmZ leads to derepression of RsmA-repressed promoters (Heurlier *et al.*, 2004).

RsmA normally negatively regulates *N*-butanoyl-homoserine lactone quorum sensing signal molecules and consequently a variety of extracellular products including pyocyanin (*phz*), hydrogen cyanide (*hcnABC*), elastase (*lasB*), and a lectin (*lecA*). RsmA also positively regulates the production of lipase (*lipAH*) and rhamnolipid (*rhlAB*). The RsmA/RsmZ system is thus able to positively control swarming motility, rhamnolipid and lipase biosynthesis (Heurlier *et al.*, 2004).

The GacA–GacS system also appears to be involved in antibiotic resistance. An early study showed that, relative to wild-type, a *gacS* mutant displayed supersusceptibility to the aminoglycosides, gentamicin and amikacin, and chloramphenicol (Brinkman *et al.*, 2001). Subsequently it was shown that transcription of the sensor kinase-encoding *gacS* gene is repressed during growth in the presence of subinhibitory concentrations of three different classes of antibiotics, represented by tobramycin, ciprofloxacin, and tetracycline (Linares *et al.*, 2006). This is apparently mediated through the RsmA/RsmZ system as *rsmA* was found to be transcriptionally induced (threefold) when *P. aeruginosa* cells were grown in the presence of subinhibitory concentrations of these three antibiotics (Linares *et al.*, 2006). The mechanism(s) of resistance regulated by GacAS are poorly understood, and susceptibility to other antibiotics and cationic peptides are apparently unaffected by *gacS* mutations. However, mutation of *gacS* leads to an increased occurrence of antibiotic resistant small-colony variant cells in *P. aeruginosa* biofilms and it was suggested that in, some instances, GacS may be necessary for reversion of these variants to a wild-type state (Davies *et al.*, 2007). Biofilms comprised of *gacS* mutant cells also produce phenotypically stable small colony

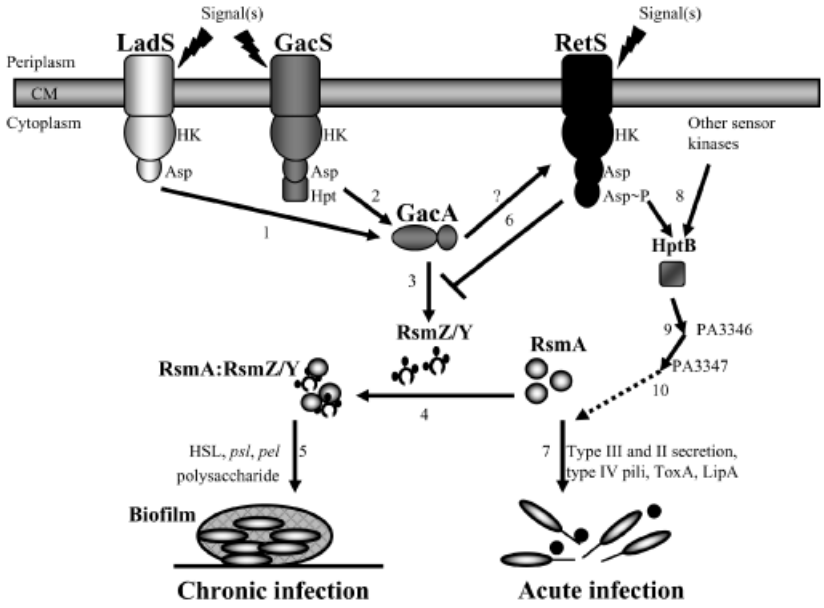

Fig. 2. A model for the opposing influence of the RetS and LadS on the GacA–Rsm regulatory network in *Pseudomonas aeruginosa*. Signals that activate RetS repress the activation of GacA (possibly by dephosphorylation; step 6) and the expression of small noncoding regulatory RNAs RsmZ/Y (step 6), leading to free RsmA and promotion of the production of virulence factors associated with acute infections (step 7; and biofilm repression). Conversely, signals that activate GacS and LadS lead to phosphorylation of GacA (steps 1 and 2) which increases the levels of the noncoding RsmZ/Y RNA molecules (step 3) and these RNAs bind and sequester RsmA (step 4). The decrease in free RsmA results in an increase in the expression of the biofilm matrix polysaccharides, Psl and Pel, and the consequent promotion of biofilms (step 5) and repression of acute virulence factors. See text for details of this regulatory network. Unknown signals also activate several sensor kinases (including RetS) to transfer phosphoryl groups to HptB (step 8) and initiate the HptB-PA3346-PA3347 phosphorelay pathway (steps 9 and 10) to ultimately repress biofilm formation (step 10) and increase swarming, through an as-yet unknown mechanism. This figure is based on models proposed in the literature (Goodman *et al.*, 2004; Ventre *et al.*, 2006; Hsu *et al.*, 2008).

variants with increased frequency when exposed to stresses such as hydrogen peroxide, human serum, or certain antibiotics (e.g. tobramycin, amikacin, oxacillin, piperacillin or rifampicin). These small-colony variants produce thicker biofilms and exhibited increased antimicrobial resistance compared to wild-type or parental *gacS* strains (Davies *et al.*, 2007). Consistent with this, mutation of *gacA* leads to moderate, biofilm-specific decreased resistance, relative to wild-type, towards multiple antibiotics including azythromycin, chloramphenicol, erythromyin, piperacillin and tetracycline (Parkins *et al.*, 2001).

RetS

The GacA/GacS/RsmZ pathway has been linked to two important two-component sensor/regulators of virulence, RetS and LadS (Fig. 2). RetS (regulator of exopolysaccharide and type III secretion) was identified during the initial screening of a panel of regulatory mutants for simple biofilm formation phenotypes (Goodman *et al.*, 2004). This hybrid sensor kinase contains one sensor kinase domain and two tandem response regulator receiver domains. In a model of *P. aeruginosa* virulence adaptation, the hybrid sensor RetS was proposed to act in an opposite fashion to GacS (Goodman *et al.*, 2004). Relative to wild type, a *retS* mutant led to massive increases in early biofilm formation that was linked to activation of the matrix exopolysaccharide *psl* operon. This phenotype correlated with hyperadhesion to mammalian CHO cells, but not increased cytotoxicity, as the *retS* mutant displayed impaired expression of the type III secretion system and associated effectors ExoS, ExoT and ExoY. In a mouse acute pneumonia model, the *retS* mutant was impaired in establishing lung infection and less able to disseminate systemically relative to wild type. Microarray analysis demonstrated the downregulation of several genes known to be required for acute infection, such as the type III secretion system, type II *xcp* secretion system, lipase (*lipA*), exotoxin A (*toxA*), and type IV pili genes. Conversely, the *retS* mutant was found exhibit derepression of specific genes associated with chronic infection such as the *psl* and *pel* exopolysaccharide-encoding operons. Thus, RetS acts normally to repress certain genes required for biofilm formation and activate expression of genes required for acute infection. The signal detected by RetS that governs this switch is as yet unknown. Screens and analysis of suppressor transposon mutants of *retS* mutant phenotypes revealed an important role for the GacS/GacA/RsmZ pathway in RetS signal transduction.

Another group similarly demonstrated that mutation of *retS* resulted in abolished expression of the type III secretion effectors ExoT and ExoU and a concomitant decrease in cytotoxicity towards epithelial cells and attenuated virulence in an acute infection model (Laskowski *et al*., 2004; Laskowski & Kazmierczak, 2006). This study however observed no differences between the *retS* mutant and wild type in epithelial cell attachment (Laskowski *et al*., 2004). Detailed mutational analysis of RetS indicated that the two tandem receiver domains are not equal and that the primary function of RetS may be to act as a phosphatase, as opposed to a kinase (Laskowski & Kazmierczak, 2006). This suggests a strong possibility of cross-talk with other sensor kinases.

Lost adherence sensor (LadS)

LadS was identified during the initial screening of transposon mutants (in a *pilA* mutant strain) for mutants with variant biofilm formation phenotypes (Ventre *et al*., 2006). Like RetS, LadS is a hybrid sensor kinase that shares similar domain organization with RetS, but unlike RetS it does not contain a second C-terminal response regulator receiver domain. A *ladS* mutant was unable to form biofilms and it appears LadS regulates both the early and later maturation stages of biofilm formation. This was traced to an ability of LadS to positively regulate the *pel* exopolysaccharide operon; indeed the opposite biofilm phenotypes of *retS* and *ladS* mutants likely reflect opposing influences on *pel* operon transcription (Fig. 2). Furthermore, transcriptional activation of *exoT* and resulting ExoT oversecretion in the *ladS* mutant correlated with increased cytotoxicity relative to wild type. Interestingly, a *ladS retS* double mutant displays enhanced biofilm formation and reduced cytotoxicity, phenotypes more reminiscent of a *retS* mutant, and possibly indicative of a signaling hierarchy in which LadS is upstream of RetS. Like RetS, LadS was found to be a component of the GacS/GacA/RsmZ signaling network in that deletion of *ladS* significantly decreased expression of *rsmZ*, whereas mutation of *retS* increased *rsmZ* transcription (Ventre *et al*., 2006). This has given rise to a model that attempts to explain the difference between acute infections (defined by expression and activation of the type III secretion system) and chronic infections (defined by a biofilm state), in which these three sensors are central in directing the expression of key virulence features (Fig. 2). In this model, LadS and GacS positively regulate *rsmZ* transcription leading to the chronic infection state, whereas RetS negatively regulates *rsmZ* transcription to promote an acute infection state. However, it is quite likely that this model understates the complexity of the switches involved.

It is not mechanistically known how LadS and RetS sensors accomplish signal transduction to modulate *rsmZ* and *rsmY* transcription. A simple explanation could be that RetS acts as a phosphatase to reverse the phosphorylation of GacA, whereas LadS acts to phosphorylate GacA (Laskowski & Kazmierczak, 2006) although this remains to be demonstrated experimentally and exactly how GacA modulates RsmZ levels is unknown. It is possible that Hpt proteins and other response regulators might be involved in the LadS and RetS modulatory pathways. Highlighting the prospective importance of these systems in controlling chronic infections, RetS and LadS regulate the HcpI secretion island I, which is required for chronic rat lung infection and thought to play an important role in chronic *P. aeruginosa* infections in CF by actively secreting HcpI (Potvin *et al*., 2003; Mougous *et al*., 2006). Discovery of the stimuli to which RetS, LadS, and GacS individually respond would provide substantial insight into how *P. aeruginosa* chronic and acute infection states are regulated. Interestingly, RetS and LadS share related 7TMR-DISMED2 periplasmic-sensing domains that have been predicted to bind carbohydrates, suggesting that they either bind host or bacterial-derived carbohydrates. We are unaware of any studies examining *retS* and *lads* mutants for possible antibiotic resistance phenotypes. These experiments would be of interest as these sensors are thought to be capable of influencing the GacA–GacS system, which has been implicated in antibiotic resistance and small-colony phenotypes (as discussed above).

SadA–SadR–SadS (RocA1–RocR–RocS)

Another system that appears to be a player in the acute/chronic infection switch is the SadA–SadR–SadS system, which regulates late biofilm maturation and the type III secretion (Kuchma *et al*., 2005). This tripartite system comprises a single sensor kinase (SadS) that interacts with two response regulators, SadA, a DNA-binding response regulator, and SadR, which contains an EAL output domain. Although they show no differences in twitching or swimming motility, or even in initial stages of biofilm formation, mutations in any one of *sadA*, *sadR*, or *sadS* lead to altered mature biofilm architecture. Furthermore, SadARS represses the type III secretion system specifically under conditions promoting biofilm formation. Again the nature of the signal involved is unknown and the *sadARS* locus itself does not appear to be induced during biofilm formation. Transposon mutants in *sadS* resulted in loss of virulence in a *C. elegans*-killing model (Gallagher & Manoil, 2001).

The *sadARS* genes were also simultaneously identified as *rocS1–rocR–rocA1* and found to contribute to regulation of the *cupB* and *cupC* genes, which encode fimbrial adhesion factors involved in biofilm and pellicle formation (Kulasekara *et al*., 2005; Ruer *et al*., 2007). Examination of *cupB* and *cupC* expression indicated that the SadS sensor kinase and

SadA response regulator form a cognate pairing to activate *cup* expression, whereas SadR represses *cup* expression by antagonizing SadAS signaling. *Pseudomonas aeruginosa* contains another *rocS1–rocA1* (*sadAS*) paralog, *rocS2–rocA2*, and both systems share significant homology to the *bvgAS* system of *Bordetella* (Kulasekara *et al*., 2005).

AlgR–FimS (AlgZ)

In *P. aeruginosa*, the AlgR response regulator (Deretic *et al*., 1989) regulates several important virulence factors including alginate, lipopolysaccharide, and hydrogen cyanide production (Carterson *et al*., 2004), and twitching (Whitchurch *et al*., 1996) and swarming motility (Overhage *et al*., 2007). AlgR likely forms a cognate system with the atypical sensor FimS (=AlgZ) which regulates alginate production (Yu *et al*., 1997). Highlighting the importance of these factors in virulence, an *algR* mutant displayed attenuated pathogenesis relative to the wild type in acute septicemia (neutropenic mouse model), but was cleared more rapidly in a murine pneumonia model (Lizewski *et al*., 2002).

Alginate production (mucoidy) has been proposed to bestow a selective advantage on *P. aeruginosa* during chronic CF lung infections by protecting it from phagocyte killing. AlgR regulates mucoidy (Deretic & Konyecsni, 1989) and is an activator of the *algD* and *algC* promoters. AlgC is involved in the production of alginate, lipopolysaccharide, and rhamnolipids whereas *algD* is the first gene in the alginate biosynthetic operon.

In addition to AlgR, the AlgB response regulator regulates alginate production by activating *algD* transcription through binding to sites in the promoter (Wozniak & Ohman, 1991; Goldberg & Dahnke, 1992; Leech *et al*., 2008). AlgB apparently forms a cognate system with the KinB sensor kinase (Ma *et al*., 1997). Interestingly, neither AlgB nor AlgR appear to require phosphorylation of conserved aspartate residues in their receiver domains to positively regulate alginate production (Ma *et al*., 1998). Activation of these systems may be a response to stress, since exposure of nonmucoid *P. aeruginosa* to cell wall-damaging antibiotics (e.g. D-cycloserine) caused strong activation of *algD* transcription (Wood *et al*., 2006). This stress effect was shown to be dependent on the regulators AlgB and AlgR, but independent of KinB, partially mimicking the transcriptional effects resulting from mucoidy in *P. aeruginosa* (Wood *et al*., 2006).

AlgR also regulates biofilm formation. AlgR was shown to have a role in early biofilm formation because an *algR* mutant was deficient in static biofilm formation (Whitchurch *et al*., 2002). In contrast, flow-cell analysis showed no difference in biofilm formation by this mutant relative to wild type at 24 h, but by days 3 and 6 the *algR* mutant exhibited reduced biofilm suggesting a possible role for AlgR in regulating biofilm maturation in this model system (Morici *et al*., 2007). Gene expression analysis of an *algR* mutant indicated that in mature continuous culture biofilms, but not in planktonic *algR* cells, AlgR selectively represses Rhl, but not Las quorum sensing (Morici *et al*., 2007).

Both *algR* and *fimS* mutants display defective twitching motility phenotypes, and phosphorylation of AlgR is necessary for twitching motility (Whitchurch *et al*., 1996, 2002). Similarly, AlgR mutants demonstrate reduced swarming motility (Overhage *et al*., 2007). Activated AlgR positively regulates the *fimU-pilVWXY1Y2E* prepilin cluster during the stationary phase of growth by physically binding to this promoter (Lizewski *et al*., 2002; Belete *et al*., 2008). Activation of this prepilin operon leads to the assembly and export of a functional type IV pilus. However, although complementation with the *fimTU-pilVWXY1Y2E* operon was able to restore twitching in an *algR* mutant, this operon was unable to complement the mature biofilm deficiency, indicating that twitching does not play a role in the observed *algR* mature biofilm defect (Morici *et al*., 2007). Instead the *algR* biofilm defect could be linked to AlgR-mediated derepression of Rhl quorum sensing, which in turn resulted in increased rhamnolipid production and active motility (Morici *et al*., 2007). Therefore, normal development into mature biofilms appears to require AlgR-mediated repression of the Rhl quorum-sensing system during growth as biofilms.

FleR–FleS and PilR–PilS

Microarray analysis has provided an insight into flagella gene regulation. At the top of the hierarchy is FleQ, the master regulator of flagellar biosynthesis that controls regulation of various promoters due to differential binding affinity during various stages in flagella assembly (Dasgupta *et al*., 2003). FleQ is NtrC-like, but is missing the key conserved aspartate in the receiver domain. FleQ can transcriptionally regulate the FleS–FleR two-component system, which also regulates the flagella gene transcriptional network. Together with the alternative sigma factor RpoN, activated FleR positively regulates class III genes in mucoid strains (Dasgupta *et al*., 2003). The FleS–FleR system also controls both flagellar synthesis (motility regulation) and adhesion to mucin (Ritchings *et al*., 1995). We demonstrated recently that FleS mutants are modestly supersusceptible to ciprofloxacin and have a substantial defect in biofilm formation and swarming and swimming motility compared with the wild-type and a complemented mutant, indicating a role for this sensor in intrinsic antibiotic resistance (Breidenstein *et al*., in press).

The PilR–PilS system was first identified as capable of transcriptionally activating the pilin gene *pilA* and

consequently controlling the expression of type IV pili (Ishimoto & Lory, 1992; Hobbs *et al.*, 1993; Boyd & Lory, 1996). Interestingly, the inner membrane-embedded PilS sensor kinase was shown to be retained at both poles of the cell, despite localization of pili to one cell pole, which suggests an added role for PilS in the maintenance of cell polarity after cell division (Boyd, 2000). More recently, purified PilS was paired with a panel of response regulators in order to identify potential acceptor proteins in phosphorelay. In addition to its cognate regulator, PilR, both the conventional PA4843 and the hybrid PA2824 response regulators were identified as putative PilS substrates (Labaer *et al.*, 2004). Although preliminary in nature, this suggests that *P. aeruginosa* histidine kinases can phosphorylate alternative response regulator substrates at rates similar to their cognate substrates, a phenomenon known in the field as cross-talk.

PvrR and PprB–PprA

The *P. aeruginosa* phenotypic variant response regulator PvrR plays a role in the formation of small colony variants that are hyper-adherent and antibiotic resistant (Drenkard & Ausubel, 2002). In the presence of high concentrations of the aminoglycoside kanamycin, *pvrR* mutation increases the frequency of appearance of these small colony variants (Drenkard & Ausubel, 2002). Interestingly, PvrR contains an EAL output domain that possesses bis-(3′,5′)-cyclic-dimeric-guanosine monophosphate (c-di-GMP) phosphodiesterase activity. This type of domain, together with the reciprocal activity of the GGDEF diguanylate cyclase c-di-GMP-synthesizing domain, regulates the cellular level of the second messenger c-di-GMP. To note, PvrR resembles the SadR (RocR) response regulator in terms of their common EAL output domain architecture. Phenotypic analysis of a variety of *P. aeruginosa* mutants in genes possessing GGDEF and EAL domains has strongly implicated c-di-GMP metabolism in virulence (Kulasakara *et al.*, 2006)

The PprA–PprB two-component system regulates cell membrane permeability and influences susceptibility to antibiotics (Wang *et al.*, 2003). The PprA sensor kinase was initially identified as a spontaneous mutant displaying increased resistance to aminoglycoside antibiotics. In a similar manner, introduction of a plasmid overexpressing the *pprB* response regulator into clinical isolates increased their susceptibility to aminoglycosides (Wang *et al.*, 2003).

NarL–NarX

As the CF lung is supplied with a low but continuous level of oxygen, *P. aeruginosa* might preferentially respire in this environment using oxygen. However in regions deep within the lungs of CF patients that contain thick mucous it is likely that *P. aeruginosa* respires anaerobically using nitrate. Nitrate metabolism has been linked to motility, biofilm formation, and virulence. The NarX sensor kinase is necessary for swarming motility, although in contrast, the *narL* response regulator mutant displayed a super-swarming phenotype that was proposed to result from overproduction of rhamnolipids (Van Alst *et al.*, 2007). Although the individual *narX* and *narL* mutants displayed normal swimming motility, a *narXL* double mutant, displayed reduced swimming motility, consistent with the possibility that both the sensor kinase and response regulator are jointly required for flagella-dependent movement. Furthermore, the hyper-biofilm phenotype displayed by a *narXL* double mutant implies that altered swimming might lead to impaired dispersal from the biofilm (Van Alst *et al.*, 2007). Thus, the NarX–NarL regulon likely includes genes that are able to modulate motility and biofilm formation.

Concluding remarks

As discussed in this review, *P. aeruginosa* contains a variety of two-component signal transduction systems that regulate complex antibiotic resistance and virulence processes. There are still, however, many putative two-component systems encoded in the genome that await detailed characterization. Given the important functions of those two-component systems already characterized, it is likely that some of these uncharacterized systems have interesting roles in antibiotic resistance and virulence processes, and our preliminary screening supports this possibility. However as *P. aeruginosa* is environmentally ubiquitous, some of the signals and physiological adaptations regulated by these uncharacterized systems may be related to niche-specific environmental adaptations and might therefore be tricky to fully elucidate.

Presently, all phenotypically characterized *P. aeruginosa* hybrid kinases have been shown to have roles in virulence and associated processes (Table 2). This could be due to the enormous emphasis on understanding the regulation of virulence in this organism. In contrast, to date only *hptB*, one of three Hpt phosphorelay proteins, has been ascribed a preliminary signal transduction role in interconnecting the hybrid sensors PA1611, PA1976, PA2824, and RetS with the response regulator PA3346 (Hsu *et al.*, 2008). The other two-phosphorelay proteins HptA and HptC, that are presumably required by a subset of hybrid sensors, have not been as well studied. For example it was observed that the expression of *hptC* was found to be transcriptionally repressed during growth *in vitro* in CF sputum medium (Palmer *et al.*, 2005), but the consequences thereof were not further investigated. It will be interesting to see which hybrid sensor kinases and response regulators form phosphorelay networks with HptA and HptC as well as the adaptations that are regulated by these signaling modules. Elucidating the cognate sensor pairings for orphan response regulators

would greatly facilitate our understanding of these systems and would shed light on the cross-talk that is likely to occur.

There is an apparent deficiency in our understanding of the signals/ligands sensed by the well-characterized histidine kinases, reflecting a general problem in the field. For example, the input signals for major regulators of virulence like GacS, LadS, and RetS, are unknown. Moreover, some of these signals might act indirectly with respect to transcriptional activation of promoters of two-component systems. For example, cationic antimicrobial peptides and low Mg^{2+} transcriptionally activate the *pmrAB* operon, enabling lipopolysaccharide modifications and resistance to antimicrobial peptides (McPhee *et al*., 2003). However, it seems likely that another regulator accomplishes the transcriptional activation of *pmrAB* (Fig. 1). When the specific inducing signal or ligand is unknown it makes phenotypic analyses of uncharacterized two-component systems extremely difficult in basic high-throughput screens. For example, PhoP and PmrA mutants do not demonstrate any observable antibiotic resistance phenotype, possibly due to redundancy; however knowing that Mg^{2+} deficiency is the inducing signal permits one to observe a role for these regulators in antimicrobial peptide supersusceptibility.

It is known that *P. aeruginosa* strains present at the later stages of CF infections differ remarkably in phenotype from their early environmental colonizer strain predecessors. Some of the most commonly observed phenotypes include antibiotic resistance, loss of motility, appearance of small-colony variants, increased mutation rate, and decreased virulence factor production. Whole-genome analysis documenting *P. aeruginosa* genetic adaptations in CF airways has demonstrated that the virulence factors required for acute infections are often mutated (i.e. selected against) in chronic CF infections (Smith *et al*., 2006). Intriguingly no two-component system was found to be selected against (apart from FleQ, which lacks a key aspartate residue). Conversely, there is evidence that certain two-component systems might mediate the persistent adaptations required for chronicity, as described above for RetS, LadS and SadARS. This, therefore, lends added support to the idea that these systems could function as adaptive factors required to establish and maintain chronic infections. In this regard, the development of improved chronic infection models, and the increased use of existing models, should help in the elucidation of how *P. aeruginosa* establishes persistence in CF chronic infections.

It appears evident that two-component regulatory systems, and especially their conserved histidine and aspartate features, could represent novel therapeutic targets due to their widespread distribution in prokaryotic genomes (Stephenson & Hoch, 2002). Although this avenue of research has received substantial interest, antimicrobial therapeutics for human use that harness this insight are yet to be developed.

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References

Bader MW, Sanowar S, Daley ME, Schneider AR, Cho U, Xu W, Klevit RE, Le Moual H & Miller SI (2005) Recognition of antimicrobial peptides by a bacterial sensor kinase. *Cell* **122**: 461–472.

Baker-Austin C, Wright MS, Stepanauskas R & McArthur JV (2006) Co-selection of antibiotic and metal resistance. *Trends Microbiol* **14**: 176–182.

Beckmann B, Brittnacher M, Ernst R, Mayer-Hamblett N, Miller SI & Burns JL (2005) Use of phage display to identify potential *Pseudomonas aeruginosa* gene products relevant to early cystic fibrosis airway infections. *Infect Immun* **73**: 444–452.

Belete B, Lu H & Wozniak DJ (2008) *Pseudomonas aeruginosa* AlgR regulates type IV pilus biosynthesis by activating transcription of the *fimU-pilVWXY1Y2E* operon. *J Bacteriol* **190**: 2023–2030.

Boyd JM (2000) Localization of the histidine kinase PilS to the poles of *Pseudomonas aeruginosa* and identification of a localization domain. *Mol Microbiol* **36**: 153–162.

Boyd JM & Lory S (1996) Dual function of PilS during transcriptional activation of the *Pseudomonas aeruginosa* pilin subunit gene. *J Bacteriol* **178**: 831–839.

Breidenstein EB, Khaira BK, Wiegand I & Hancock RE (in press) A complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. *Antimicrob Agents Chemotherapy*, in press.

Brinkman FS, Macfarlane EL, Warrener P & Hancock RE (2001) Evolutionary relationships among virulence-associated histidine kinases. *Infect Immun* **69**: 5207–5211.

Caille O, Rossier C & Perron K (2007) A copper-activated two-component system interacts with zinc and imipenem resistance in *Pseudomonas aeruginosa*. *J Bacteriol* **189**: 4561–4568.

Carterson AJ, Morici LA, Jackson DW *et al*. (2004) The transcriptional regulator AlgR controls cyanide production in *Pseudomonas aeruginosa*. *J Bacteriol* **186**: 6837–6844.

Chen YT, Chang HY, Lu CL & Peng HL (2004) Evolutionary analysis of the two-component systems in *Pseudomonas aeruginosa* PAO1. *J Mol Evol* **59**: 725–737.

Chieda Y, Iiyama K, Yasunaga-Aoki C, Lee JM, Kusakabe T & Shimizu S (2005) Pathogenicity of *gacA* mutant of *Pseudomonas aeruginosa* PA01 in the silkworm, *Bombyx mori*. *FEMS Microbiol Lett* **244**: 181–186.

Coleman FT, Mueschenborn S, Meluleni G, Ray C, Carey VJ, Vargas SO, Cannon CL, Ausubel FM & Pier GB (2003)

Hypersusceptibility of cystic fibrosis mice to chronic *Pseudomonas aeruginosa* oropharyngeal colonization and lung infection. *Proc Natl Acad Sci USA* **100**: 1949–1954.

Conejo MC, Garcia I, Martinez-Martinez L, Picabea L & Pascual A (2003) Zinc eluted from siliconized latex urinary catheters decreases OprD expression, causing carbapenem resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **47**: 2313–2315.

D'Argenio DA, Gallagher LA, Berg CA & Manoil C (2001) *Drosophila* as a model host for *Pseudomonas aeruginosa* infection. *J Bacteriol* **183**: 1466–1471.

D'Argenio DA, Calfee MW, Rainey PB & Pesci EC (2002) Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. *J Bacteriol* **184**: 6481–6489.

Darzins A & Russell MA (1997) Molecular genetic analysis of type-4 pilus biogenesis and twitching motility using *Pseudomonas aeruginosa* as a model system – a review. *Gene* **192**: 109–115.

Dasgupta N, Wolfgang MC, Goodman AL, Arora SK, Jyot J, Lory S & Ramphal R (2003) A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. *Mol Microbiol* **50**: 809–824.

Davies JA, Harrison JJ, Marques LL, Foglia GR, Stremick CA, Storey DG, Turner RJ, Olson ME & Ceri H (2007) The GacS sensor kinase controls phenotypic reversion of small colony variants isolated from biofilms of *Pseudomonas aeruginosa* PA14. *FEMS Microbiol Ecol* **59**: 32–46.

Dean CR, Neshat S & Poole K (1996) PfeR, an enterobactin-responsive activator of ferric enterobactin receptor gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* **178**: 5361–5369.

Deretic V & Konyecsni WM (1989) Control of mucoidy in *Pseudomonas aeruginosa*: transcriptional regulation of *algR* and identification of the second regulatory gene, *algQ*. *J Bacteriol* **171**: 3680–3688.

Deretic V, Dikshit R, Konyecsni WM, Chakrabarty AM & Misra TK (1989) The *algR* gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. *J Bacteriol* **171**: 1278–1283.

Drenkard E & Ausubel FM (2002) *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* **416**: 740–743.

Ernst RK, Yi EC, Guo L, Lim KB, Burns JL, Hackett M & Miller SI (1999) Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science* **286**: 1561–1565.

Falagas ME & Bliziotis IA (2007) Pandrug-resistant Gram-negative bacteria: the dawn of the post-antibiotic era? *Int J Antimicrob Agents* **29**: 630–636.

Filloux A, Bally M, Soscia C, Murgier M & Lazdunski A (1988) Phosphate regulation in *Pseudomonas aeruginosa*: cloning of the alkaline phosphatase gene and identification of *phoB*- and *phoR*-like genes. *Mol Gen Genet* **212**: 510–513.

Fridkin SK, Steward CD, Edwards JR *et al.* (1999) Surveillance of antimicrobial use and antimicrobial resistance in United States hospitals: project ICARE phase 2. Project Intensive Care Antimicrobial Resistance Epidemiology (ICARE) hospitals. *Clin Infect Dis* **29**: 245–252.

Furukawa S, Kuchma SL & O'Toole GA (2006) Keeping their options open: acute versus persistent infections. *J Bacteriol* **188**: 1211–1217.

Gallagher LA & Manoil C (2001) *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *J Bacteriol* **183**: 6207–6214.

Galperin MY (2006) Structural classification of bacterial response regulators: diversity of output domains and domain combinations. *J Bacteriol* **188**: 4169–4182.

Goldberg JB & Dahnke T (1992) *Pseudomonas aeruginosa* AlgB, which modulates the expression of alginate, is a member of the NtrC subclass of prokaryotic regulators. *Mol Microbiol* **6**: 59–66.

Goodman AL, Kulasekara B, Rietsch A, Boyd D, Smith RS & Lory S (2004) A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev Cell* **7**: 745–754.

Hancock RE & Speert DP (2000) Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug Resist Updat* **3**: 247–255.

Hancock RE, Raffle VJ & Nicas TI (1981) Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **19**: 777–785.

Hassan MT, van der Lelie D, Springael D, Romling U, Ahmed N & Mergeay M (1999) Identification of a gene cluster, *czr*, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa*. *Gene* **238**: 417–425.

Heurlier K, Williams F, Heeb S, Dormond C, Pessi G, Singer D, Camara M, Williams P & Haas D (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.

Hickman JW, Tifrea DF & Harwood CS (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc Natl Acad Sci USA* **102**: 14422–14427.

Hobbs M, Collie ES, Free PD, Livingston SP & Mattick JS (1993) PilS and PilR, a two-component transcriptional regulatory system controlling expression of type 4 fimbriae in *Pseudomonas aeruginosa*. *Mol Microbiol* **7**: 669–682.

Hsu JL, Chen HC, Peng HL & Chang HY (2008) Characterization of the histidine-containing phosphotransfer protein B-mediated multi-step phosphorelay system in *Pseudomonas aeruginosa* PAO1. *J Biol Chem* **283**: 9933–9944.

Huang B, Whitchurch CB & Mattick JS (2003) FimX, a multidomain protein connecting environmental signals to twitching motility in *Pseudomonas aeruginosa*. *J Bacteriol* **185**: 7068–7076.

Ishimoto KS & Lory S (1992) Identification of *pilR*, which encodes a transcriptional activator of the *Pseudomonas aeruginosa* pilin gene. *J Bacteriol* **174**: 3514–3521.

Jander G, Rahme LG & Ausubel FM (2000) Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *J Bacteriol* **182**: 3843–3845.

Jensen V, Lons D, Zaoui C, Bredenbruch F, Meissner A, Dieterich G, Munch R & Haussler S (2006) RhlR expression in *Pseudomonas aeruginosa* is modulated by the *Pseudomonas* quinolone signal via PhoB-dependent and -independent pathways. *J Bacteriol* **188**: 8601–8606.

Kay E, Humair B, Denervaud V, Riedel K, Spahr S, Eberl L, Valverde C & Haas D (2006) Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J Bacteriol* **188**: 6026–6033.

Kazmierczak BI, Lebron MB & Murray TS (2006) Analysis of FimX, a phosphodiesterase that governs twitching motility in *Pseudomonas aeruginosa*. *Mol Microbiol* **60**: 1026–1043.

Kirisits MJ & Parsek MR (2006) Does *Pseudomonas aeruginosa* use intercellular signalling to build biofilm communities? *Cell Microbiol* **8**: 1841–1849.

Kuchma SL, Connolly JP & O'Toole GA (2005) A three-component regulatory system regulates biofilm maturation and type III secretion in *Pseudomonas aeruginosa*. *J Bacteriol* **187**: 1441–1454.

Kulasakara H, Lee V, Brencic A *et al.* (2006) Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3′-5′)-cyclic-GMP in virulence. *Proc Natl Acad Sci USA* **103**: 2839–2844.

Kulasekara HD, Ventre I, Kulasekara BR, Lazdunski A, Filloux A & Lory S (2005) A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial *cup* genes. *Mol Microbiol* **55**: 368–380.

Kwon DH & Lu CD (2006) Polyamines induce resistance to cationic peptide, aminoglycoside, and quinolone antibiotics in *Pseudomonas aeruginosa* PAO1. *Antimicrob Agents Chemother* **50**: 1615–1622.

Kwon DH & Lu CD (2007) Polyamine effects on antibiotic susceptibility in bacteria. *Antimicrob Agents Chemother* **51**: 2070–2077.

Labaer J, Qiu Q, Anumanthan A *et al.* (2004) The *Pseudomonas aeruginosa* PA01 gene collection. *Genome Res* **14**: 2190–2200.

Lapouge K, Schubert M, Allain FH & Haas D (2008) Gac/Rsm signal transduction pathway of *Gammaproteobacteria*: from RNA recognition to regulation of social behaviour. *Mol Microbiol* **67**: 241–253.

Laskowski MA & Kazmierczak BI (2006) Mutational analysis of RetS, an unusual sensor kinase-response regulator hybrid required for *Pseudomonas aeruginosa* virulence. *Infect Immun* **74**: 4462–4473.

Laskowski MA, Osborn E & Kazmierczak BI (2004) A novel sensor kinase-response regulator hybrid regulates type III secretion and is required for virulence in *Pseudomonas aeruginosa*. *Mol Microbiol* **54**: 1090–1103.

Laub MT & Goulian M (2007) Specificity in two-component signal transduction pathways. *Annu Rev Genet* **41**: 121–145.

Leech AJ & Mattick JS (2006) Effect of site-specific mutations in different phosphotransfer domains of the chemosensory protein ChpA on *Pseudomonas aeruginosa* motility. *J Bacteriol* **188**: 8479–8486.

Leech AJ, Sprinkle A, Wood L, Wozniak DJ & Ohman DE (2008) The NtrC family regulator AlgB, which controls alginate biosynthesis in mucoid *Pseudomonas aeruginosa*, binds directly to the *algD* promoter. *J Bacteriol* **190**: 581–589.

Lewenza S, Falsafi RK, Winsor G, Gooderham WJ, McPhee JB, Brinkman FS & Hancock RE (2005) Construction of a mini-Tn5-luxCDABE mutant library in *Pseudomonas aeruginosa* PAO1: a tool for identifying differentially regulated genes. *Genome Res* **15**: 583–589.

Linares JF, Gustafsson I, Baquero F & Martinez JL (2006) Antibiotics as intermicrobial signaling agents instead of weapons. *Proc Natl Acad Sci USA* **103**: 19484–19489.

Lizewski SE, Lundberg DS & Schurr MJ (2002) The transcriptional regulator AlgR is essential for *Pseudomonas aeruginosa* pathogenesis. *Infect Immun* **70**: 6083–6093.

Ma S, Wozniak DJ & Ohman DE (1997) Identification of the histidine protein kinase KinB in *Pseudomonas aeruginosa* and its phosphorylation of the alginate regulator *algB*. *J Biol Chem* **272**: 17952–17960.

Ma S, Selvaraj U, Ohman DE, Quarless R, Hassett DJ & Wozniak DJ (1998) Phosphorylation-independent activity of the response regulators AlgB and AlgR in promoting alginate biosynthesis in mucoid *Pseudomonas aeruginosa*. *J Bacteriol* **180**: 956–968.

Macfarlane EL, Kwasnicka A, Ochs MM & Hancock RE (1999) PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. *Mol Microbiol* **34**: 305–316.

Macfarlane EL, Kwasnicka A & Hancock RE (2000) Role of *Pseudomonas aeruginosa* PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides. *Microbiology* **146**: 2543–2554.

Mahajan-Miklos S, Tan MW, Rahme LG & Ausubel FM (1999) Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa–Caenorhabditis elegans* pathogenesis model. *Cell* **96**: 47–56.

Marr AK, Gooderham WJ & Hancock RE (2006) Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Curr Opin Pharmacol* **6**: 468–472.

Mascher T, Helmann JD & Unden G (2006) Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev* **70**: 910–938.

McPhee JB, Lewenza S & Hancock RE (2003) Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. *Mol Microbiol* **50**: 205–217.

McPhee JB, Bains M, Winsor G, Lewenza S, Kwasnicka A, Brazas MD, Brinkman FS & Hancock RE (2006) Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg^{2+}-induced gene regulation in *Pseudomonas aeruginosa*. *J Bacteriol* **188**: 3995–4006.

Morici LA, Carterson AJ, Wagner VE, Frisk A, Schurr JR, Honer zu Bentrup K, Hassett DJ, Iglewski BH, Sauer K & Schurr MJ (2007) *Pseudomonas aeruginosa* AlgR represses the Rhl quorum-sensing system in a biofilm-specific manner. *J Bacteriol* **189**: 7752–7764.

Moskowitz SM, Ernst RK & Miller SI (2004) PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. *J Bacteriol* **186**: 575–579.

Mougous JD, Cuff ME, Raunser S *et al.* (2006) A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* **312**: 1526–1530.

O'Toole GA & Kolter R (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* **30**: 295–304.

Overhage J, Lewenza S, Marr AK & Hancock RE (2007) Identification of genes involved in swarming motility using a *Pseudomonas aeruginosa* PAO1 mini-Tn5-*lux* mutant library. *J Bacteriol* **189**: 2164–2169.

Overhage J, Bains M, Brazas MD & Hancock RE (2008) Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J Bacteriol* **190**: 2671–2679.

Palmer KL, Mashburn LM, Singh PK & Whiteley M (2005) Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. *J Bacteriol* **187**: 5267–5277.

Parkins MD, Ceri H & Storey DG (2001) *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. *Mol Microbiol* **40**: 1215–1226.

Perron K, Caille O, Rossier C, Van Delden C, Dumas JL & Kohler T (2004) CzcR-CzcS, a two-component system involved in heavy metal and carbapenem resistance in *Pseudomonas aeruginosa*. *J Biol Chem* **279**: 8761–8768.

Pessi G, Williams F, Hindle Z, Heurlier K, Holden MT, Camara M, Haas D & Williams P (2001) The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J Bacteriol* **183**: 6676–6683.

Potvin E, Lehoux DE, Kukavica-Ibrulj I, Richard KL, Sanschagrin F, Lau GW & Levesque RC (2003) *In vivo* functional genomics of *Pseudomonas aeruginosa* for high-throughput screening of new virulence factors and antibacterial targets. *Environ Microbiol* **5**: 1294–1308.

Rahme LG, Tan MW, Le L, Wong SM, Tompkins RG, Calderwood SB & Ausubel FM (1997) Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors. *Proc Natl Acad Sci USA* **94**: 13245–13250.

Rahme LG, Ausubel FM, Cao H *et al.* (2000) Plants and animals share functionally common bacterial virulence factors. *Proc Natl Acad Sci USA* **97**: 8815–8821.

Ramsey MM & Whiteley M (2004) *Pseudomonas aeruginosa* attachment and biofilm development in dynamic environments. *Mol Microbiol* **53**: 1075–1087.

Reimmann C, Beyeler M, Latifi A, Winteler H, Foglino M, Lazdunski A & Haas D (1997) The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol* **24**: 309–319.

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.

Ritchings BW, Almira EC, Lory S & Ramphal R (1995) Cloning and phenotypic characterization of *fleS* and *fleR*, new response regulators of *Pseudomonas aeruginosa* which regulate motility and adhesion to mucin. *Infect Immun* **63**: 4868–4876.

Rodrigue A, Quentin Y, Lazdunski A, Mejean V & Foglino M (2000) Two-component systems in *Pseudomonas aeruginosa*: why so many? *Trends Microbiol* **8**: 498–504.

Ruer S, Stender S, Filloux A & de Bentzmann S (2007) Assembly of fimbrial structures in *Pseudomonas aeruginosa*: functionality and specificity of chaperone-usher machineries. *J Bacteriol* **189**: 3547–3555.

Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ & Greenberg EP (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407**: 762–764.

Smith EE, Buckley DG, Wu Z *et al.* (2006) Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci USA* **103**: 8487–8492.

Soscia C, Hachani A, Bernadac A, Filloux A & Bleves S (2007) Cross talk between type III secretion and flagellar assembly systems in *Pseudomonas aeruginosa*. *J Bacteriol* **189**: 3124–3132.

Stephenson K & Hoch JA (2002) Two-component and phosphorelay signal-transduction systems as therapeutic targets. *Curr Opin Pharmacol* **2**: 507–512.

Stock AM, Robinson VL & Goudreau PN (2000) Two-component signal transduction. *Annu Rev Biochem* **69**: 183–215.

Stover CK, Pham XQ, Erwin AL *et al.* (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406**: 959–964.

Tan MW, Rahme LG, Sternberg JA, Tompkins RG & Ausubel FM (1999) *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. *Proc Natl Acad Sci USA* **96**: 2408–2413.

Teitzel GM, Geddie A, De Long SK, Kirisits MJ, Whiteley M & Parsek MR (2006) Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. *J Bacteriol* **188**: 7242–7256.

Van Alst NE, Picardo KF, Iglewski BH & Haidaris CG (2007) Nitrate sensing and metabolism modulate motility, biofilm formation, and virulence in *Pseudomonas aeruginosa*. *Infect Immun* **75**: 3780–3790.

Vasil ML & Ochsner UA (1999) The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry, and virulence. *Mol Microbiol* **34**: 399–413.

Ventre I, Goodman AL, Vallet-Gely I, Vasseur P, Soscia C, Molin S, Bleves S, Lazdunski A, Lory S & Filloux A (2006) Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci USA* **103**: 171–176.

Venturi V (2006) Regulation of quorum sensing in *Pseudomonas*. *FEMS Microbiol Rev* **30**: 274–291.

Wagner VE, Li LL, Isabella VM & Iglewski BH (2007) Analysis of the hierarchy of quorum-sensing regulation in *Pseudomonas aeruginosa*. *Anal Bioanal Chem* **387**: 469–479.

Wang Y, Ha U, Zeng L & Jin S (2003) Regulation of membrane permeability by a two-component regulatory system in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **47**: 95–101.

Whitchurch CB, Alm RA & Mattick JS (1996) The alginate regulator AlgR and an associated sensor FimS are required for twitching motility in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **93**: 9839–9843.

Whitchurch CB, Erova TE, Emery JA, Sargent JL, Harris JM, Semmler AB, Young MD, Mattick JS & Wozniak DJ (2002) Phosphorylation of the *Pseudomonas aeruginosa* response regulator AlgR is essential for type IV fimbria-mediated twitching motility. *J Bacteriol* **184**: 4544–4554.

Whitchurch CB, Leech AJ, Young MD *et al.* (2004) Characterization of a complex chemosensory signal transduction system which controls twitching motility in *Pseudomonas aeruginosa*. *Mol Microbiol* **52**: 873–893.

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.

Wood LF, Leech AJ & Ohman DE (2006) Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: roles of sigma (AlgT) and the AlgW and Prc proteases. *Mol Microbiol* **62**: 412–426.

Wozniak DJ & Ohman DE (1991) *Pseudomonas aeruginosa* AlgB, a two-component response regulator of the NtrC family, is required for *algD* transcription. *J Bacteriol* **173**: 1406–1413.

Wu W, Badrane H, Arora S, Baker HV & Jin S (2004) MucA-mediated coordination of type III secretion and alginate synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **186**: 7575–7585.

Yahr TL & Wolfgang MC (2006) Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. *Mol Microbiol* **62**: 631–640.

Yu H, Mudd M, Boucher JC, Schurr MJ & Deretic V (1997) Identification of the *algZ* gene upstream of the response regulator *algR* and its participation in control of alginate production in *Pseudomonas aeruginosa*. *J Bacteriol* **179**: 187–193.