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Minireview

Biogenesis of *Pseudomonas aeruginosa* type IV pili and regulation of their function

Tiffany L. Leighton,[1†] Ryan N. C. Buensuceso,[1†] P. Lynne Howell[2,3*] and Lori L. Burrows[1]**

[1]*Department of Biochemistry and Biomedical Sciences, Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, ON, Canada.*
[2]*Program in Molecular Structure & Function, The Hospital for Sick Children, Toronto, ON, Canada.*
[3]*Department of Biochemistry, University of Toronto, Toronto, ON, Canada.*

Summary

Type IV pili (T4P) are bacterial virulence factors involved in a wide variety of functions including deoxyribonucleic acid uptake, surface attachment, biofilm formation and twitching motility. While T4P are common surface appendages, the systems that assemble them and the regulation of their function differ between species. *Pseudomonas aeruginosa*, *Neisseria spp.* and *Myxococcus xanthus* are common model systems used to study T4P biology. This review focuses on recent advances in *P. aeruginosa* T4P structural biology, and the regulatory pathways controlling T4P biogenesis and function.

Introduction

Type IV pili (T4P) are long, thin (5–8 nm diameter) hair-like appendages found on bacterial and archaeal cell surfaces (Bradley, 1973; Pelicic, 2008). They play roles in surface attachment/adhesion, cell-cell aggregation, biofilm formation and motility (Bradley, 1980; Mattick, 2002; Burrows, 2005, 2012; Pelicic, 2008). Type IV pili are important for virulence, as mutants lacking T4P are impaired for host cell colonization and thus less infectious (Farinha *et al.*, 1994; Hahn, 1997). Type IV pili are assembled at the cytoplasmic membrane by intricate nanomachines whose molecular mechanisms and regulation are not yet fully understood. This review discusses recent advances in T4P biology in the model organism *Pseudomonas aeruginosa*, focusing on the interplay between structural and regulatory aspects of the system.

Type IV pili are divided into two major subfamilies, type IVa (T4aP) and type IVb (T4bP) pili, characterized by differences in the major and minor pilin subunits which make up the pilus fibre and among assembly system components (Burrows, 2012; Craig *et al.*, 2004). Unlike most other bacterial surface filaments with the exception of F-pili (Novotny *et al.*, 1974), T4aP are dynamic and can be rapidly retracted, producing forces in excess of 100 pN per filament (Merz *et al.*, 2000; Maier *et al.*, 2002). Through their ability to repeatedly extend, adhere and retract, T4aP confer unique locomotion modalities including twitching, swarming, walking and sling shot motilities (Yeung *et al.*, 2009; Gibiansky *et al.*, 2010; Jin *et al.*, 2011). These modalities use T4aP alone or in combination with other surface appendages such as flagella to achieve movement. Type IVa – the best-studied subfamily – are the focus of this mini-review and will henceforth be called T4P for simplicity.

There are over 40 genes, distributed throughout the genome, involved in the assembly and regulation of the T4P system in *P. aeruginosa* (Pelicic, 2008; Burrows, 2012). These genes can be divided into two groups: structural components that form the assembly machinery plus the pilus itself, and regulatory components which dictate when to move, and where. Interestingly, some proteins fit both categories (discussed below). This review describes the assembly and regulatory components and how they relate to global regulation of this system. Although the majority of the information presented, and the nomenclature used, is from the *P. aeruginosa* T4P system, insights from other T4P systems are mentioned where informative.

The assembly machinery: an overview

Four sub-complexes spanning the bacterial cell envelope make up the T4P assembly system in Gram-negative bacteria (Fig. 1). The outer membrane (OM) secretin sub-complex is composed of an oligomer of 12–14 subunits of

Received 13 February, 2015; revised 13 March, 2015; accepted 14 March, 2015. For correspondence. *E-mail howell@sickkids.ca; Tel. 416-813-5378; Fax 416-813-5379. **E-mail burrowl@mcmaster.ca; Tel. 905-525-9140 x22029; Fax 905-522-9033. †These authors contributed equally.

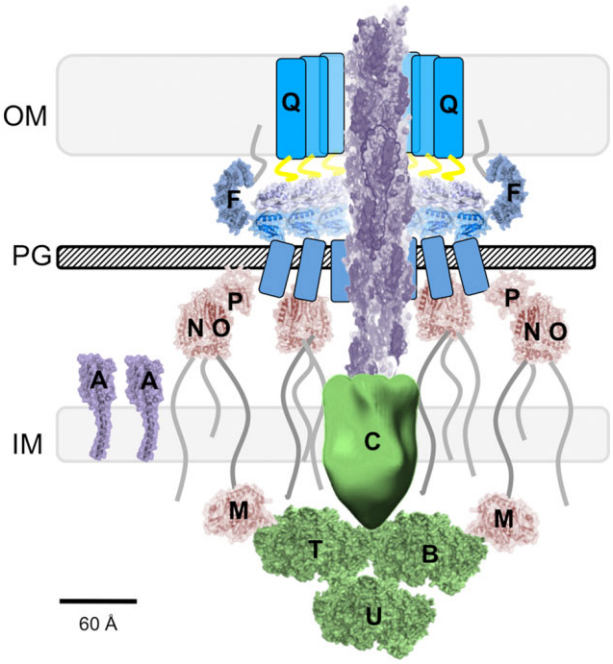

Fig. 1. Schematic of the type IVa pilus system in *P. aeruginosa*. Proteins are labeled according to the Pil nomenclature. Components include the outer membrane (OM) secretin sub-complex: pilQ [3EZJ] and pilF [2HO1] (blue), the inner membrane (IM) motor sub-complex: pilC, pilB, pilT [3JVU] and pilU (green), the IM alignment sub-complex: pilm [2YCH], pilN, pilO [2RJZ] and pilP [2LC4] (red), and the pilins and pilus fibre: pila [1OQW] (purple). Protein data bank (PDB) accession codes are shown in square brackets. GspD, the pilQ orthologue was used to model pilQ residues 280–445. The pilnO dimer (Sampaleanu *et al.*, 2009), the pilus fibre (Craig *et al.*, 2006) and pilC (Collins *et al.*, 2007) models were previously published. The pilT structure was used for pilB and pilU. All parts were drawn to scale with a scale factor of 60 Å as indicated by the black bar. Peptidoglycan (PG) is shown as striped bar. Not shown is FimV, which has cytoplasmic and periplasmic domains connected by a single transmembrane segment, and PA0020 (predicted tsap) an OM-associated protein with a PG-binding motif. Adapted with permission (Koo, 2012, p. 6).

PilQ (Collins *et al.*, 2001; Collins *et al.*, 2003; Burkhardt *et al.*, 2011) with its pilotin protein PilF, forming a pore through which the pilus is extruded (Koo *et al.*, 2008; Koo *et al.*, 2012;). TsaP, recently identified in *Neisseria gonorrhoeae* and *Myxococcus xanthus*, contains a peptidoglycan (PG)-binding LysM motif, and may help anchor the secretin to the bacterial cell wall (Siewering *et al.*, 2014). In *P. aeruginosa*, PA0020 encodes a TsaP homologue whose role has not yet been established. The inner membrane (IM) motor sub-complex is composed of an integral membrane 'platform' protein PilC, and three cytoplasmic ATPases, PilBTU, which provide the energy for extension and retraction of the fibre (Chiang *et al.*, 2005; Takhar *et al.*, 2013). The secretin and motor sub-complexes are linked by the alignment sub-complex, PilMNOP (Ayers *et al.*, 2009; Sampaleanu *et al.*, 2009), which may also include FimV – a large protein with periplasmic and cytoplasmic domains connected by a single transmembrane segment. Like TsaP, FimV has a peptidoglycan-binding LysM motif and is important for secretin formation (Wehbi *et al.*, 2011). FimV may help the multimeric secretin traverse the PG layer, and/or participate in regulation of pilus biogenesis (Fulcher *et al.*, 2010), as discussed below. The PilMNOP proteins span from the cytoplasm to the periplasm and connect with the PilQ secretin via PilP, potentially controlling secretin gating (Tammam *et al.*, 2013; Tammam *et al.*, 2011). Together, the motor and the alignment sub-complexes comprise the IM platform. The final sub-complex is the helical pilus fibre, composed of major (PilA) and minor (FimU, PilVWXE) pilin subunits along with a non-pilin protein, PilY1 (Rudel *et al.*, 1995; Mattick, 2002; Craig *et al.*, 2004; Nguyen *et al.*, 2010; Nguyen *et al.*, 2015;). Together with regulatory components (below), these sub-complexes form a functional T4P system.

The secretin: a gate to the outside

The type II secretion (T2S) and T4P system secretins belong to a large family of pore-forming proteins also involved in type III secretion (T3S) and the export of filamentous phage (Crago *et al.*, 1998; Opalka *et al.*, 2003; Tosi *et al.*, 2014). Secretins form a 50–80 Å diameter channel that allows the polymerized fibre to pass through the outer membrane (Korotkov *et al.*, 2011). In the absence of a pilus fibre, the secretin pore is closed by loops that are part of the secretin structure, or in some systems, by a plug protein that physically blocks the channel (Korotkov *et al.*, 2011; Disconzi *et al.*, 2014;). These components inhibit the release of periplasmic proteins while the secretin is in its resting state, unoccupied by pili (Disconzi *et al.*, 2014). Secretins consist of two regions; a variable N-terminal periplasmic region responsible for protein–protein and/or protein–peptidoglycan interactions and substrate recognition, and a highly conserved C-terminal domain that forms the physical pore in the membrane (Bouley *et al.*, 2001; Balasingham *et al.*, 2007; Tammam *et al.*, 2013). The periplasmic region of the T4P PilQ secretin is divided into four sub-domains, starting at the N terminus; SS1-SS2-N0-N1 (Fig. 2) (Tammam *et al.*, 2013). The N-terminal species-specific (SS) or beta domains of PilQ are structurally related to the Amidase N-terminal domain (AMIN) domain, found in peptidoglycan hydrolases (de Souza *et al.*, 2008; Berry *et al.*, 2012; Rocaboy *et al.*, 2013). The AMIN domain of AmiC, an *Escherichia coli N*-acetylmuramoyl-L-alanine amidase responsible for the final stages of daughter cell separation, binds PG and is proposed to target the protein to the septum where it participates in daughter cell separation (Rocaboy *et al.*, 2013). In PilQ, the AMIN domain could help to target the secretin subunits to

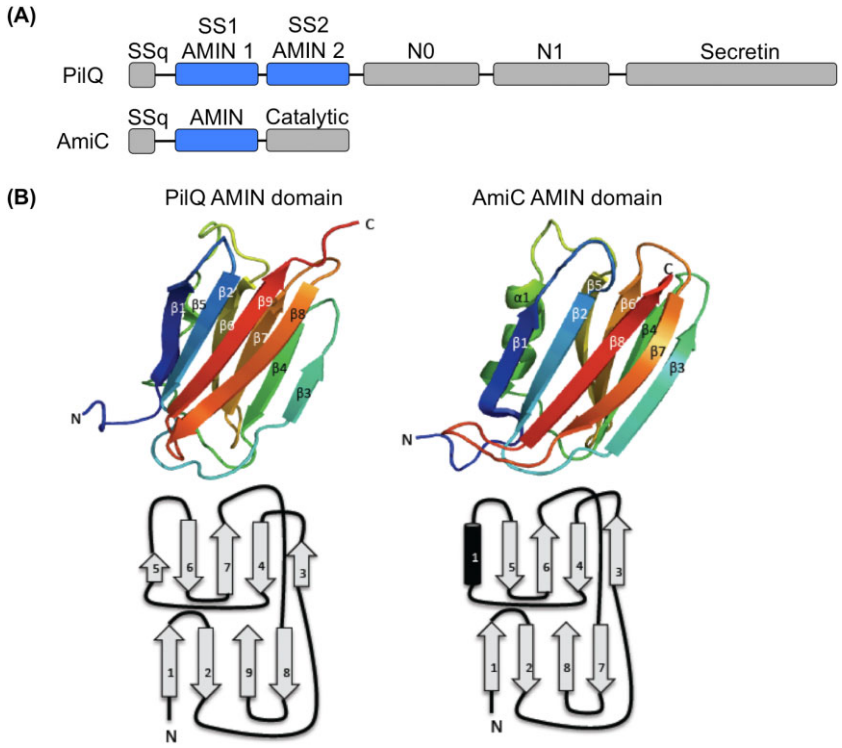

Fig. 2. Structural similarity between the AMIN domains of pilQ and amic.
A. pilQ has five domains: two species specific domains (SS1 and SS2) with structural similarity to the PG-binding AMIN domain of amic, the N0 domain that interacts with pilP, the N1 domain and the OM inserted secretin domain. The amic structure has an AMIN domain responsible for its localization to the septal ring, and a catalytic domain responsible for daughter cell cleavage. Both pilQ and amic have an N-terminal signal sequence (SSq).
B. Top: A model of the second AMIN domain of pilQ from *P. Aeruginosa* (modelled from the nuclear magnetic resonance structure of the B2 domain in *Neisseria Meningitidis* (Berry *et al*., 2012) [4AQZ]) and the corresponding domain from the crystal structure of the *N*-acetylmuramoyl-L-alanine amidase amic from *E. Coli* (Rocaboy *et al*., 2013) [4BIN]. Bottom: The topology of the two domains is nearly identical, but amic has an alpha helix in place of beta strand 5 in the corresponding domain of pilQ. Protein data bank accession codes are shown in square brackets.

nascent poles as cell division is occurring, to allow for incorporation of the large multimeric secretin into the cell wall as the PG is newly synthesized (Scheurwater *et al*., 2011; Burrows, 2013). This domain could also play a role in anchoring the secretin and/or assembly system into the cell wall, acting with FimV and/or the TsaP homologue PA0020 to brace the assembly system against forces generated by retraction of the pilus fibre (Wehbi *et al*., 2011; Siewering *et al*., 2014). The adjacent N0 region of PilQ plays an important role in protein–protein interactions, as it is the point of contact with the alignment sub-complex protein, PilP (Tammam *et al*., 2013). N0, plus the N1 and/or secretin sub-domains, may participate in the interaction between PilQ and its lipoprotein pilotin, PilF (Koo *et al*., 2013). Depending on the system, secretin localization to the OM and multimerization can occur independently or in concert with various accessory proteins such as pilotins (Koo *et al*., 2012). Trafficking of a PilQ-PilF pair by the lipoprotein sorting machinery (Lol pathway) is one mechanism by which PilQ monomers could be shuttled to the outer membrane (Koo *et al*., 2013). PilF is likely inserted into the OM by Lol machinery, while PilQ may be spontaneously co-inserted or require other cellular machinery (Nickerson *et al*., 2012; Koo *et al*., 2013). Evidence from studies of *M. xanthus* supports an outside-in pathway of T4P system assembly, where the OM secretin first oligomerizes, then recruits alignment sub-complexes, which subsequently recruit the IM motor sub-complex (Friedrich *et al*., 2014). Whether this scenario holds true for the T4P system of *P. aeruginosa*, and how the system ends up in its final configuration at the cell poles, has yet to be elucidated.

The pilus fibre: the rope and grappling hook

The pilus fibre contains hundreds of subunits in a helical filament that is capable of rapid assembly and disassembly. PilA is the predominant subunit, though other essential pilin-like proteins known as minor pilins are incorporated into the filament at low abundance (Strom *et al*., 1993; Giltner *et al*., 2010). A polycistronic operon encodes five minor pilins (FimU-PilVWXE) and two non-minor pilin proteins (PilY1 and PilY2) (Belete *et al*., 2008). The minor pilins are proposed to form an initiation complex for pilus assembly, leading to their incorporation into the fibre, likely at the tip (Winther-Larsen *et al*., 2005; Winther-Larsen *et al*., 2007; Giltner *et al*., 2010). Minor pilins PilVWX and the ~125 kDa PilY1 protein are proposed to form an IM sub-complex that recruits PilE, then interacts with PilA through both PilE and FimU (Nguyen *et al*., 2015). This pilus initiation structure (PilY1-PilVWX-PilE-FimU-PilA) is proposed to act as a primer for subsequent polymerization of hundreds of PilA subunits, forming a helical filament that elongates from its base. Minor pilin FimU was suggested to be a connector coupling the major pilin to a minor pilin–PilY1 complex

(Nguyen *et al*., 2015), similar to the role proposed for its T2S homologue GspH in connecting a minor pseudopilin complex to the major pseudopilin (Douzi *et al*., 2009). Structures for *P. aeruginosa* major and specific minor pilins (PilA, FimU, and PilE) have been solved and reveal a similar architecture (Craig *et al*., 2003; Nguyen *et al*., 2010; Nguyen *et al*., 2015) (PilE, PDB 4NOA). Pilins have a long, S-shaped, hydrophobic N-terminal α-helix, followed by a variable C-terminal region β-sheet composed of between four and seven antiparallel β-strands (Nguyen *et al*., 2010; Giltner *et al*., 2012). Most major (and some minor) pilins have two Cys residues in the C-terminal region, forming a disulfide bond that staples the C terminus of the proteins to the β-sheet (Giltner *et al*., 2012). The loop formed by this bond stabilizes the interaction between adjacent subunits, as mutating the Cys residues prevents assembly (Harvey *et al*., 2009) and treating assembled pili with a reducing agent causes their rapid disintegration (Li *et al*., 2012).

PilY1, encoded with the minor pilin genes, is essential for pilus assembly (Alm *et al*., 1996b). PilY1 was initially proposed to be an adhesin (Rudel *et al*., 1995) but its role in pilus-mediated adherence was hard to test, since *pilY1* mutants lack surface pili (Alm *et al*., 1996b; Heiniger *et al*., 2010). In a recent study, atomic force microscopy was used to test the contribution of PilY1 to pilus-mediated adherence. Overexpression of PilY1 in the wild type did not increase adherence (Beaussart *et al*., 2014), prompting the authors to conclude that PilY1 is not directly involved in surface adhesion; however, they did not test whether overexpression of PilY1 alone – without its minor pilin partners – changed the amount of PilY1 on the cell surface. Expressing PilY1 alone alters the stoichiometry between it and its partners PilVWX (Nguyen *et al*., 2015), which could affect PilY1 surface display. A more recent study (Luo *et al*., 2015) suggested that overexpression of PilY1 alone significantly increases the amount recovered in cell surface fractions, even in the absence of pili; however, the potential pilus-independent mechanism of PilY1 secretion remains unknown.

A crystal structure of the C-terminal domain of PilY1 revealed a beta propeller fold and a novel calcium-binding motif (Orans *et al*., 2010). When mutated to a configuration mimicking the calcium-bound state, PilY1 opposed retraction, while a calcium-free mimetic state increased the amount of retraction relative to control (Orans *et al*., 2010). A second calcium-binding site was identified in the N-terminal domain of PilY1, and pull-down experiments showed that integrin binding was regulated by both sites (Johnson *et al*., 2011). Integrin and integrin-like proteins are present on many different cell types in the plant, insect and animal kingdoms, and are common targets for bacterial pathogens (Huang *et al*., 2006; Julio *et al*., 2009). Binding of PilY1 to integrins expressed on the surface of the target cell could allow *P. aeruginosa* to sense attachment and pull itself towards the host. A recent study proposed a more general mechanosensory role for PilY1 in modulating virulence (Siryaporn *et al*., 2014). Shear forces generated upon attachment to a surface were suggested to deform the mechanosensitive Von Willebrand Factor-like domain in the N-terminal region of PilY1, signalling successful surface engagement and inducing a virulence program (Siryaporn *et al*., 2014). Interestingly, PilY1 was required for surface-activated virulence independent of pilus expression, as other pili-less mutants remained virulent as long as PilY1, PilW and PilX were present (Siryaporn *et al*., 2014). Even when not incorporated into the pilus, PilY1 participates in a surface-activated virulence mode, likely by acting upstream of the diguanylate cyclase (DGC) SadC, responsible for modulating cellular levels of 3′,5′-cyclic-di-guanylate (c-di-GMP) (Bohn *et al*., 2009; Kuchma *et al*., 2010; Ha *et al*., 2014). PilY1's potential regulatory roles are discussed in detail below. At this point, the exact role(s) of PilY1 in the T4P system of *P. aeruginosa*, and how they are integrated with its biology, remain areas of intense study.

The motor: powering the grappling hook

Like a molecular grappling hook, the T4P system functions through repeated rounds of pilus extension, adherence to a surface and pilus retraction, which winches the cell towards the point of attachment (Skerker *et al*., 2001). This motion can be jerky and erratic, hence the term twitching motility (Bradley, 1980). The pilus is responsible for attachment, but cytoplasmic ATPases provide the driving force behind its extension and retraction. *P. aeruginosa* has three hexameric motor ATPases, PilB, PilT and PilU (Whitchurch *et al*., 1991; Whitchurch *et al*., 1994; Mattick, 2002; Chiang *et al*., 2005; Chiang *et al*., 2008) that hydrolyse ATP to provide energy for pilus extension and retraction. It is likely that the ATPases contribute to assembly/disassembly of pilin subunits through their interactions with the IM platform proteins (PilMNOP and/or PilC) (Misic *et al*., 2010). Interaction between PilB and the N-terminal domain of PilC was demonstrated by co-affinity purification (Takhar *et al*., 2013). It was hypothesized in that study that the C-terminal cytoplasmic domain of PilC may interact with the retraction ATPases, PilT and/or PilU based on the phenotypes of PilC point mutants, though solubility issues with the C-terminal domain of PilC have made it difficult to test this hypothesis. The interaction between PilC and the hexameric ATPases (Herdendorf *et al*., 2002; Maier *et al*., 2002; Misic *et al*., 2010) is thought to transduce

mechanical energy from conformational changes occurring upon ATP hydrolysis through the IM platform, leading to the addition or removal of pilin subunits from the fibre (Py *et al*., 2001; Satyshur *et al*., 2007). The structure of PilT was solved to 2.6 Å resolution, with and without the non-hydrolyzable ATP analogue β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP) (Misic *et al*., 2010). Each subunit of PilT consists of N-terminal and C-terminal domains separated by a flexible linker, with the active site at the interface between these two domains, similar to other T4P-associated ATPases (Yeo *et al*., 2000; Robien *et al*., 2003; Hare *et al*., 2006; Satyshur *et al*., 2007;). Although no structures of *P. aeruginosa* PilB and PilU are yet available, both are predicted to have similar structural features, with sequence identity among the three ATPases ranging from 20–40%. A unique internal region of PilB, not functionally conserved with the other two ATPases, was recently shown to be the site of interaction for a D3112 bacteriophage-encoded protein, gp05 (also called twitching inhibitory protein – Tip) (Chung *et al*., 2014). Binding of Tip to PilB caused delocalization of PilB from the poles of the cell, resulting in disruption of surface pili and motility in *P. aeruginosa* (Chung *et al*., 2014). Although the mechanism was not determined, the delocalization of PilB by Tip could be caused by disruption of PilB hexamer formation or loss of its interaction with the IM platform protein PilC (Takhar *et al*., 2013).

Whereas most T4P systems function with two ATPases, all three are required for twitching motility in *P. aeruginosa* (Nunn *et al*., 1990; Whitchurch *et al*., 1991; Whitchurch *et al*., 1994). PilB and PilT function in extension and retraction, respectively, but the function of the third ATPase, PilU, remains enigmatic (Burrows, 2005). Unlike *pilB* and *pilT* mutants that have surface piliation phenotypes reflective of their role in T4P extension and retraction, respectively, *pilU* mutants are incapable of twitching, have at least wild-type levels of surface pili, and are sensitive to killing by pilus-specific bacteriophages (Whitchurch *et al*., 1994). These phenotypes suggest that *pilU* mutants assemble and retract surface pili to some degree. However, given that *pilU* mutants do not twitch, it is likely that PilU modulates PilT-mediated retraction. The link between PilU and PilT is strengthened by the idea that PilU arose through a gene-duplication event, as *pilT* and *pilU* are adjacent on the *P. aeruginosa* chromosome. In *N. gonorrhoeae*, there are multiple PilU homologues, all of which have been implicated in fine tuning of pilus retraction (Kurre *et al*., 2012).

In *M. xanthus*, the extension and retraction ATPases are primarily found at the leading and lagging poles respectively (Bulyha *et al*., 2009). During reversals in motility, the ATPases switch places, the lagging pole becomes the new leading pole, and motion proceeds in a new direction (Bulyha *et al*., 2009). This oscillation of the ATPases has been linked to the chemosensory system which regulates the reversals in *M. xanthus* (Keilberg *et al*., 2012). *P. aeruginosa* PilU is unipolar, unlike PilB and PilT that are found at both poles (Chiang *et al*., 2005). PilU might differentiate the leading and the lagging poles to control the direction of motility. Without PilU, *P. aeruginosa* may extend and retract pili at both poles simultaneously, resulting in the net-zero motility phenotype characteristic of a *pilU* mutant.

The alignment sub-complex: threading the needle

With the platform and the motor of the system located in the cytoplasm and IM, and the secretin in the OM, connection of these sub-assemblies is required for efficient pilus assembly. Four genes, *pilMNOP*, form a polycistronic operon with *pilQ*, and their products span the cytoplasm (PilM), inner membrane and periplasm (PilNOP), effectively linking the motor and the secretin. A crystal structure of the cytoplasmic protein PilM from *Thermus thermophilus*, bound to a short fragment of the N terminus of PilN, revealed structural similarities to cell division proteins FtsA and MreB, as well as to GspL from the T2S system (Abendroth *et al*., 2004a; Karuppiah *et al*., 2011). Similarly, *P. aeruginosa* PilM is bound to the N-terminus of PilN through a short, highly conserved sequence INLLP (Sampaleanu *et al*., 2009). PilN and PilO are predicted to be structurally similar proteins, with a single transmembrane segment (TMS), a pair of coiled coils and a core domain composed of two αββ repeats (Sampaleanu *et al*., 2009). A crystal structure of a PilO dimer with a N-terminal truncation of 68 residues (missing the TMS and part of the first coiled coil) revealed a high degree of structural similarity to orthologous proteins from the T2SS in *Vibrio*, including the modified ferredoxin fold (αββ) in the core domain (Abendroth *et al*., 2004b; Sampaleanu *et al*., 2009). Via its unstructured N-terminal region, the IM lipoprotein PilP binds the PilNO heterodimer (Balasingham *et al*., 2007; Tammam *et al*., 2011), while its C-terminal β sandwich domain (likely β strands 1–2 or 6–7) interacts with the N0 domain of the OM secretin (Korotkov *et al*., 2006; Tammam *et al*., 2013), creating a transenvelope sub-complex.

The alignment sub-complex has a number of proposed and complementary roles. Alignment of the IM motor with the OM secretin would allow for efficient extension of the pilus fibre through the pore, similar to threading a needle. It might transduce conformational changes from the cytoplasmic motor ATPases to the OM secretin, inducing ‘open’ or ‘closed’ states. In the T2SS of *Dickyea didantii*, the GspL and GspM proteins – functionally analogous to PilMN and PilO, respectively – undergo dramatic rotational rearrangements, switching between homodimeric

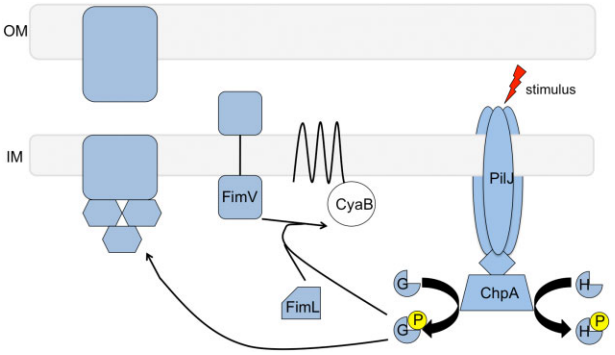

Fig. 3. Model of Chp-mediated regulation of CyaB activity. In response to a chemotactic stimulus, PilJ transmits a signal to ChpA, leading to phosphorylation of the response regulators, PilG (G) and PilH (H). PilH functions as a phosphate sink to control levels of downstream Chp signalling. Phosphorylated PilG activates the adenylate cyclase CyaB, through a complex involving FimV and FimL. In addition to activating the adenylate cyclase, phosphorylated PilG controls T4P function in a cAMP-independent manner.

or heterodimeric states (Lallemand *et al.*, 2013). 'Partner switching' has not been shown for the T4P system but the idea is intriguing, and could be a way to switch between the open and closed or assembly and disassembly states of the system. Finally, the alignment sub-complex may concentrate PilA subunits. Upon disassembly of the pilus fibre, PilA subunits are re-deposited into the IM at approximately 1000 subunits per second (Merz *et al.*, 2000; Maier *et al.*, 2002). Since PilA subunits are recycled (Skerker *et al.*, 2001), retaining them near the assembly platform via interactions with PilNOP (Karuppiah *et al.*, 2013; Tammam *et al.*, 2013) could increase local concentrations and efficiency of assembly. With the secretin predicted to contain 12–14 subunits of PilQ (Collins *et al.*, 2001; Collins *et al.*, 2003; Burkhardt *et al.*, 2011), and the organization of the *pilMNOPQ* operon, which suggests 1:1:1:1:1 stoichiometry, it is likely that there are 12–14 sets of PilMNOP proteins surrounding the pilus (Tammam *et al.*, 2013). In the absence of any of the alignment sub-complex proteins, pili are not expressed on the surface of the bacteria except in a retraction-deficient background (Ayers *et al.*, 2009; Takhar *et al.*, 2013). This finding indicates a basal level of pilus assembly occurs without PilMNOP, but with them, the system is more efficient (Takhar *et al.*, 2013).

Together, the four sub-assemblies (secretin, motor and alignment sub-complexes plus the pilus fibre) form the physical apparatus of the T4P system. However, a number of regulatory proteins that sense and respond to signals in the environment, and modulate pilus extension and retraction accordingly, are also required for function. These regulatory proteins and signals will be discussed in detail in the next section.

Regulation of twitching motility

Chemotactic control

The Pil-Chp cluster (hereafter referred to as the Chp cluster) is one of four putative chemotactic clusters in *P. aeruginosa*, and the only one that controls twitching motility (Darzins, 1994; Whitchurch *et al.*, 2004). The Chp system encodes components homologous to those of the well-studied *E. coli* Che system that controls flagellar rotation (Baker *et al.*, 2006) (Fig. 3). The Che system includes a number of methyl-accepting chemotaxis proteins (MCPs) that sample the cell's chemical environment. In response to appropriate stimuli, the MCPs undergo conformational changes, leading ultimately to autophosphorylation of a histidine kinase, CheA, linked to the MCPs via the adaptor, CheW. CheA phosphorylates the response regulator CheY, which then interacts with the flagellar motor to reverse the direction of flagellar rotation. Levels of phosphorylated CheY are controlled by the phosphatase CheZ. Adaptation of the system as the cell moves along a chemical gradient is mediated by differential methylation of the MCP by the CheR methyltransferase, and CheB methylesterase. By analogy, the Chp system of *P. aeruginosa* encodes one MCP, PilJ, whose ligand(s) is unknown but may include phosphatidylethanolamine (Kearns *et al.*, 2001). PilK and ChpB are homologous to CheR and CheB and are thus predicted to be responsible for methylation and demethylation respectively (Darzins, 1994; Whitchurch *et al.*, 2004; Luo *et al.*, 2015;). PilI and ChpC are both CheW homologues, although the role of each is unclear. Only deletion of PilI impairs twitching motility, but ChpC has been hypothesized to function as an adaptor to couple other MCPs to the Chp system (Whitchurch *et al.*, 2004). ChpA is a large, complex orthologue of CheA containing nine potential sites of phosphorylation, although not all are required for twitching motility (Leech *et al.*, 2006).

Instead of a single CheY-like response regulator and a CheZ-like phosphatase, the *chp* cluster encodes two potential response regulators, PilG and PilH, both of which may be phosphorylated by ChpA (Darzins, 1993; 1994). Based on mutant phenotypes, PilH was proposed to be a phosphate sink that could attenuate Chp signalling in lieu of a phosphatase (Fulcher *et al.*, 2010). A similar arrangement was reported in *Sinorhizobium meliloti*, where a two-response regulator system controls swimming motility (Scharf *et al.*, 2002). Signalling through the Chp pathway is required for twitching motility and surface piliation. Notably, deletion of *pilG, pilJ*, *chpA* or *pilI* results in significantly reduced levels of surface pili, suggesting all are required for pilus biogenesis (Darzins, 1993; 1994; Leech *et al.*, 2006; DeLange *et al.*, 2007).

The exact mechanism by which the Chp cluster controls twitching motility and surface piliation remains unclear,

but involves the second messenger molecule, cyclic adenosine monophosphate (cAMP; (Fulcher *et al*., 2010)). Signalling through the Chp system activates the major adenylate cyclase of *P. aeruginosa*, CyaB, leading to transcription of pilus genes regulated by the cAMP-binding protein, virulence factor regulator (Vfr) (Wolfgang *et al*., 2003). The Chp system has an additional, cAMP-independent role in regulating T4P retraction, although how it does so remains unknown. Whitchurch and colleagues (2004) hypothesized that the Chp system may function similarly to the *M. xanthus* Frz chemosensory system that controls reversals and motor ATPase localization in T4P-mediated social (S)-motility (Kaimer *et al*., 2013). Signalling through the Frz system results in a switch in polar distribution of the *M. xanthus* T4P motor ATPases, PilB and PilT (Bulyha *et al*., 2009). In *P. aeruginosa*, PilU is the only ATPase with unipolar localization – PilB and PilT are bipolar (Chiang *et al*., 2005). It is possible that Chp signalling serves to localize PilU to one pole of the cell, allowing for directed movement.

Small molecule regulation of motility

In *P. aeruginosa*, twitching motility and T4P function are regulated by a number of different signalling inputs. Both cAMP and c-di-GMP regulate motility, as well as virulence and biofilm formation (Wolfgang *et al*., 2003; Borlee *et al*., 2010). In turn, levels of cAMP and c-di-GMP are regulated by the coordinated signalling input from a number of proteins (Ryan *et al*., 2006; Fulcher *et al*., 2010; Ryan, 2013). In complex with the cAMP receptor protein homologue Vfr, cAMP regulates the expression of ~200 virulence-related genes – including the T4P alignment sub-complex (*pilMNOPQ*) and minor pilin (*fimU-pilVWXY1Y2E*) operons (Wolfgang *et al*., 2003).

The levels of intracellular cAMP control pilus extension, as mutants with low levels of cAMP (e.g. *cyaAB*, *pilG*) had little or no recoverable surface pili. Supplementation of the growth medium with cAMP restored wild-type levels of surface pili but not twitching to a *pilG* mutant, suggesting an additional cAMP-independent role of the Chp pathway in twitching motility, likely in pilus retraction (Fulcher *et al*., 2010). Interestingly, a *pilB* mutant had reduced levels of cAMP, suggesting that surface pili are required for cAMP biosynthesis. However, other components of the T4P assembly complex were not identified in the screen, suggesting that T4P-mediated cAMP regulation is not simply related to pilus assembly.

c-di-GMP plays a key role in the transition between motile and sessile lifestyles (Wolfe *et al*., 2008). At high c-di-GMP concentrations, *P. aeruginosa* transitions to sessile growth, leading to the formation of adherent biofilms (Simm *et al*., 2004). The intracellular c-di-GMP pool is maintained through the activity of DGCs and phosphodiesterases (PDEs) that synthesize and degrade c-di-GMP respectively. Diguanylate cyclases contain a characteristic GGDEF motif, while PDEs typically have EAL or HD-GYP motifs (Tal *et al*., 1998; Galperin *et al*., 2001).

In addition to several DGCs and PDEs, *P. aeruginosa* encodes a number of GGDEF and EAL domain-containing regulatory proteins without enzymatic function. Two have been identified as T4P regulators – PilZ and FimX. PilZ is required for pilus extension (Alm *et al*., 1996a), and while FimX is required for T4P assembly at low concentrations of c-di-GMP, it is dispensable at high c-di-GMP concentrations (Jain *et al*., 2012). *P. aeruginosa* PilZ is the original member of the 'PilZ domain' family – a large and diverse family of c-di-GMP binding proteins (Alm *et al*., 1996a; Amikam *et al*., 2006) – despite the fact that PilZ itself does not bind c-di-GMP. How PilZ functions remains unknown; however, in *Xanthomonas axonopodis* pv. citri, PilZ interacts with FimX and PilB (Guzzo *et al*., 2009). FimX is a GGDEF and EAL domain-containing c-di-GMP regulatory protein originally characterized as a sensor of environmental signals and their transduction to T4P (Huang *et al*., 2003). *fimX* mutants are retraction competent, but have low levels of surface pili, suggesting impaired pilus extension (Huang *et al*., 2003). Contrary to the original report, FimX was subsequently suggested to be catalytically inactive (Rao *et al*., 2008), but capable of binding c-di-GMP with high affinity (Qi *et al*., 2011). As research into c-di-GMP signalling continues, it is important to note that degeneracy of GGDEF and EAL domains will be a complicating matter in identification of novel DGCs and PDEs, as well as other c-di-GMP binding proteins that may affect motility.

While c-di-GMP levels can affect pilus function, the T4P protein PilY1 and the minor pilins PilW and PilX have been implicated in regulation of c-di-GMP-mediated control of swarming motility and biofilm formation (Kuchma *et al*., 2010; Kuchma *et al*., 2012). Impaired swarming motility in a mutant lacking the PDE BifA (which has high levels of c-di-GMP) was rescued by suppressor mutations in *pilY1*, *pilX* or *pilW*, consistent with the recent finding that the products of those genes likely form a sub-complex that also contains PilV (Nguyen *et al*., 2015), although interestingly, loss of the latter had no effect on swarming (Kuchma *et al*., 2010). The effect on swarming motility involved regulation of the activity of the DGC, SadC. Surprisingly, PilWXY1 exert this regulatory effect independently of their T4P-related functions; mutation of the leader sequence of PilX to prevent processing by the prepilin peptidase PilD, required for extraction from the inner membrane, blocked twitching motility but did not affect PilX's ability to modulate swarming. Further elaboration of the regulatory circuitry that connects the cAMP

and c-di-GMP regulons will help to shed light on the ancillary functions of the PilWXY1 proteins.

Luo and colleagues (2015) recently proposed a model that integrates cAMP and c-di-GMP signalling with T4P biology. In their model, PilY1 specifically – rather than the pilus itself – is the lynchpin for regulation of both pathways. They suggested that signalling by an unknown ligand(s) through the Chp system results in an increase in cAMP levels and thus Vfr-dependent transcription of the minor pilin operon, which includes *pilY1*. The resulting increase in PilY1 surface expression leads to signalling through the PilMNOP alignment sub-complex to activate SadC activity. Increased SadC activity leads to increased c-di-GMP levels, promoting sessility and downregulating motility.

The multifunctional protein, FimV

FimV was identified in a transposon screen for mutants deficient in twitching motility (Semmler *et al*., 2000). It is a large protein with an N-terminal periplasmic region containing a peptidoglycan-binding LysM motif and coiled-coil region, a single TMS and a highly acidic C-terminal cytoplasmic domain containing two predicted tetratricopeptide repeat (TPR) motifs separated by a long unstructured region (Wehbi *et al*., 2011). Together, these features suggest that FimV is involved in a number of protein–protein interactions. Its exact role in twitching motility remains unclear, although in-frame deletion of FimV's PG-binding LysM motif reduced motility and secretin levels, suggesting that it helps integrate PilQ into the cell wall (Wehbi *et al*., 2011). Both periplasmic and cytoplasmic domains are required for twitching, although they can be provided as physically separated fragments and still complement a *fimV* mutant, suggesting their functions are independent of one another.

In addition to twitching, FimV is required for secretion of T2S substrates on solid media (Michel *et al*., 2011) and positively regulates levels of intracellular cAMP (Fulcher *et al*., 2010). Therefore, FimV could be indirectly involved in pilus assembly via cAMP-dependent transcriptional regulation of T4P assembly components. Although not formally tested, the reported deficiencies in T2S in the *fimV* background were likely due to reduced levels of cAMP, as T2SS expression is Vfr dependent (Wolfgang *et al*., 2003). FimV's role in CyaB activation suggests that it may interact with the adenylate cyclase, either directly or through other cAMP-regulatory proteins.

The *Vibrio cholerae* HubP protein, while lacking significant sequence identity with FimV, shares similar domain organization (Yamaichi *et al*., 2012). HubP is required for polar localization of the *V. cholerae* chromosomal segregation and chemotaxis machineries. To date, there is no evidence that FimV affects localization of any T4P proteins, nor has a direct link between FimV and any of the other cAMP-regulatory proteins been observed. However, if FimV functions similarly to HubP, it may physically coordinate interactions among regulators of CyaB activity, including components of the Chp system.

FimL

FimL was originally identified as a gene product required for T4P biogenesis and function that intersected with the Vfr pathway (Whitchurch *et al*., 2005). Impaired twitching in a *fimL* mutant was subsequently shown to be rescued by a compensatory mutation in *cpdA*, the cAMP phosphodiesterase responsible for breakdown of cAMP (Inclan *et al*., 2011). Mutants lacking *fimL* are phenotypically similar to those lacking *cyaAB* – with decreased levels of intracellular cAMP and reduced twitching motility. Since the compensatory mutation in *cpdA* rescues the *fimL* phenotype, FimL likely functions exclusively in regulating CyaB activity and cAMP production, and not in any other T4P-specific behaviour.

FimL is homologous to the N terminal region of the Chp histidine kinase, ChpA, with two phosphotransfer-like domains. However, while ChpA has His and Thr phosphoacceptor residues in those domains, they are replaced with Gln in FimL (Whitchurch *et al*., 2005). Thus, it is unlikely that FimL plays a direct role in a phosphorelay. Instead, FimL may control CyaB function in a complex with other cAMP-regulatory proteins. Interestingly, Nolan and colleagues (2012) identified suppressor mutations that rescue twitching and intracellular cAMP levels in a *fimL* mutant but mapped outside the *cpdA* locus. The locations of the suppressors were not identified, but *pilG*, *pilH*, *vfr*, *cyaA* and *cyaB* were eliminated as possibilities, suggesting that there is at least one more component in the FimL branch of the cAMP-regulatory pathway.

The putative PocAB/TonB3 complex

In a screen to identify regulators of polar flagellum localization in *P. aeruginosa*, Cowles and colleagues (2013) identified two proteins, PocA and PocB, which regulate T4P levels and localization in conjunction with the previously identified TonB3 protein (Huang *et al*., 2004). TonB3, PocA and PocB are homologous to *E. coli* TonB, ExbB and ExbD, respectively, which form an inner membrane complex involved in siderophore uptake (Fischer *et al*., 1989; Karlsson *et al*., 1993). The exact mechanism of uptake is unclear, but ExbB and ExbD are hypothesized to use the proton motive force to promote TonB-mediated transport of siderophores across the periplasm (Noinaj *et al*., 2010; Krewulak *et al*., 2011). Like ExbB and ExbD, PocA and PocB could be co-purified, suggesting that they

form a complex, but neither PocA nor PocB were able to pull down TonB3 (Cowles *et al*., 2013), potentially due to its low abundance.

tonB3 and *pocB* deletion mutants were almost entirely non-piliated, while *pocA* mutants had an increased propensity to produce non-polar pili. TonB3 and PocB exert their effects on T4P biology in part at the transcriptional level, as *tonB3* and *pocB* – but not *pocA* – mutants had decreased transcription of most pilus genes. While only the *pocA* mutation resulted in increased amounts of non-polar T4P, mutation of any of *tonB3*, *pocA* or *pocB* mislocalized PilT and PilQ, both of which have been previously established as being polarly localized (Chiang *et al*., 2005; Cowles *et al*., 2010) and increased the proportion of cells with a non-polar flagellum. The TonB3-PocA-PocB signalling axis shows that signalling pathways regulating T4P are not limited to controlling transcription of T4P components or mechanics of T4P function. This is the first example of a signalling pathway that controls T4P localization, and *pocA* is the first non-cytoskeletal mutant background where non-polar pili have been isolated. The TonB3 and PocAB proteins are not localized to the poles, and the reasons that *tonB3* or *pocB* mutations have drastic effects on piliation remain unknown.

The interplay, if any, between the TonB3-PocA-PocB signalling axis and cAMP or c-di-GMP signalling axes has not yet been investigated, so it is unclear if decreased levels of pili in *tonB3* or *pocB* mutants are a result of changes in the amount of cAMP or c-di-GMP, or if the effects are mediated directly by TonB3 and PocB. It is also unclear why *pocA* and *pocB* mutants have different phenotypes, even though their products physically interact.

FimS-AlgR and AmrZ signalling

FimS-AlgR is a two-component system that controls twitching motility and alginate biosynthesis (Whitchurch *et al*., 1996; Yu *et al*., 1997). AlgR is a two-domain response regulator protein with an N-terminal CheY-like domain and a C-terminal DNA-binding domain. AlgR signalling was originally shown to be important for the production of mucoid colonies. However, AlgR was later implicated in control of twitching motility and T4P assembly (Whitchurch *et al*., 2002). Whitchurch and colleagues (1996) identified FimS (also called AlgZ; hereafter referred to as FimS) as the putative sensor kinase of the FimS-AlgR regulatory circuit. Interestingly, although AlgR was required for alginate biosynthesis, FimS did not play a role in alginate production, but was required for twitching motility (Whitchurch *et al*., 1996). Control of twitching by FimS and AlgR occurs at least in part through transcriptional activation of the minor pilin operon (Belete *et al*., 2008). Interestingly, Wolfgang and colleagues (2003) have shown that the minor pilin operon is also under regulation of the cAMP-Vfr regulatory circuit. This observation was extended by Jones and colleagues (2010) who showed that AlgR is involved in inhibition of Vfr signalling.

In contrast, Luo and colleagues (2015) propose an alternate model for FimS-AlgR regulation of the minor pilin operon. They propose that both Vfr and the Chp system MCP PilJ – through direct interactions with FimS – control transcription of the minor pilin operon. The product of this operon, PilY1, then closes this circuit, downregulating its own transcription by inhibiting Vfr and PilJ-FimS-AlgR dependent transcription of the minor pilin operon.

P. aeruginosa encodes another protein that was originally named AlgZ before being renamed AmrZ (Baynham *et al*., 2006). AmrZ is a ribbon-helix-helix DNA-binding protein that is required for twitching motility (Baynham *et al*., 2006) and functions as both a transcriptional activator and repressor of different genes (Pryor *et al*., 2012). However, although *amrZ* mutants do not twitch, RNA-seq experiments showed that AmrZ is actually a repressor of a number of different T4P-related genes, including those encoding the PilMNOPQ alignment proteins and secretin, PilHI (part of the Chp cluster) and the minor pilins PilVW (Jones *et al*., 2014). AmrZ likely does not exert these effects directly, as the complementary ChIP-seq experiments revealed no evidence that AmrZ binds directly to any of the three operons. Instead, their results showed that AmrZ is a transcriptional repressor of the DGC AdcA (Jones *et al*., 2014), suggesting that changes in twitching and T4P assembly in *amrZ* mutants may be a c-di-GMP-mediated phenomenon.

The big picture

pilA and *pilB* mutants, neither of which can assemble T4P, have decreased levels of cAMP, suggesting that surface-exposed pili may be involved in positive regulation of intracellular cAMP levels (Fulcher *et al*., 2010). Further, levels of T4P assembly proteins correlate with cAMP concentrations in the cell. This positive feedback loop may be indicative of a T4P-dependent mechanism of virulence control (Fig. 4). Increased cAMP levels lead to increased transcription of T4P components. The TonB3-PocA-PocB complex ensures that the requisite components are expressed and correctly localized. The resulting increase in piliation and expression of the mechanosensor PilY1 leads to further surface contact and increased levels of cAMP in a positive feedback cycle (Siryaporn *et al*., 2014). Luo and colleagues (2015) suggested that PilY1 can inhibit Vfr signalling. This inhibition may occur once the positive feedback loop has led to sufficient levels of surface-expressed PilY1 to inhibit Vfr signalling. High levels of cAMP generated through such a positive feedback loop increase transcription of the structural

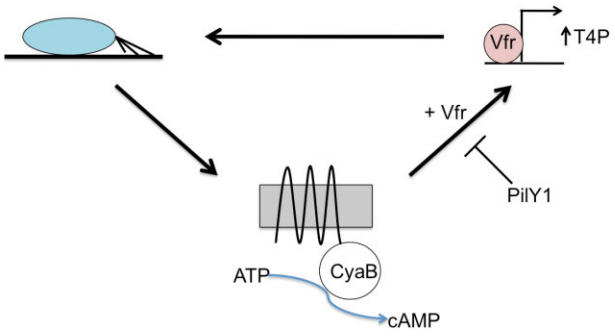

Fig. 4. Model for cAMP-T4P positive feedback loop. In response to surface attachment, T4P-mediated signalling activates the adenylate cyclase CyaB, increasing intracellular levels of cAMP. In response to increased cAMP, Vfr binds cAMP and increases transcription of Vfr-dependent genes, including T4P. Increased levels of T4P promote more attachment.

components of the virulence-associated T2S and T3S systems, the secreted protein ExoY and precursors of other secreted exotoxins (Wolfgang *et al.*, 2003). Conversely, transcription of flagellar structural components is decreased by high concentrations of cAMP (Wolfgang *et al.*, 2003). However, a screen for proteins that regulate cAMP levels failed to identify other components of the T4P assembly complex (Fulcher *et al.*, 2010), suggesting that the regulatory circuit is more complex than a simple feedback loop between CyaB and extended surface pili.

T4P-mediated attachment of *P. aeruginosa* to a surface leads to activation of CyaB and an increase in intracellular levels of cAMP (Fig. 5). This increase in cAMP would promote downregulation of swimming motility and upregulation of T4P structural components to further increase attachment and twitching. Increased cAMP would also lead to increased expression of the T2S and T3S systems, as the bacterium switches on its surface-associated virulence program.

Recently, it was suggested that surface attachment of *P. aeruginosa* induces its virulence program in a PilY1-dependent manner (Luo *et al.*, 2015; Siryaporn *et al.*, 2014). Given the central role of cAMP in controlling virulence factor expression, it is surprising that PilY1 was not detected in the screen for cAMP-regulatory proteins (Fulcher *et al.*, 2010), although this deficit supports the idea that there may be another unidentified members of this regulatory circuit. Interestingly, *pilB* and *pilC* mutants, both previously shown to lack surface pili (Nunn *et al.*, 1990) and were virulent (Siryaporn *et al.*, 2014). Contrary to existing literature, these results suggest that PilY1 itself is the lynchpin for virulence rather than T4P. Luo and colleagues (2015) proposed a mechanism where upon attachment to a surface, PilY1 expression is upregulated via AlgR transcriptional activation. PilY1 is then deployed onto the cell surface, where it signals through the T4P alignment sub-complex to adjust levels of cAMP and c-di-GMP and thus coordinate the virulence program. Combined with the proposed roles of PilWXY1 in controlling c-di-GMP levels via modulating activity of the DGC, SadC (Kuchma *et al.*, 2010; Kuchma *et al.*, 2012), PilY1 and the minor pilins appear to have complex effects on second messenger molecule regulatory circuits that regulate a switch in lifestyle modes.

Research into the cAMP-T4P signalling axis is still in its infancy. Although Fulcher and colleagues (2010) identified a number of key players, how FimV and FimL control CyaB activity and how they intersect with the Chp system is unclear. Given FimV's organizational similarity to *V. cholerae* HubP and the chaperone-like characteristics of its cytoplasmic domain (TPR motifs, unstructured segments and low pI), we suspect that FimV may bridge FimL and the Chp system to activate CyaB (Fig. 3).

Chp signalling was shown by Fulcher and colleagues (2010) to have a cAMP-independent role in controlling twitching. The observation that a *pilG* mutant grown on cAMP-supplemented media has normal levels of surface pili suggests that these mutants have a defect in retraction. An effect of Chp signalling on PilU localization or function is an attractive possibility, as its unipolar localization is consistent with a role in directional motility, and the phenotypes of a cAMP-supplemented *pilG* mutant and a *pilU* mutant – no twitching and near wild-type levels of surface pili (Whitchurch *et al.*, 1994; Fulcher *et al.*, 2010) – are similar.

Future perspective

The use of high-resolution structural techniques such as X-ray crystallography and nuclear magnetic resonance

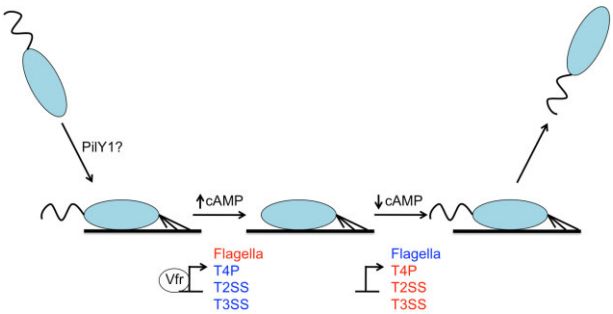

Fig. 5. Proposed model for T4P-mediated upregulation of virulence. Upon surface binding, increased levels of cAMP lead to increased transcription (blue text) of virulence factors, including T4P, and T2S and T3S systems. Upregulation of T4P and downregulation of flagella favours attachment and surface movement rather than swimming. Decreased levels of cAMP (stimulus unknown), lead to decreased virulence factor expression (red text), and increased flagella and swimming motility. Initial surface binding is proposed to be mediated by PilY1, a putative T4P-associated adhesin.

spectroscopy have helped unlock key information about T4P-related proteins, but how all the pieces in this complex system fit together to produce retractable pili is still largely a mystery. These uncertainties emphasize the need for use of lower resolution techniques such as cryo-electron microscopy, cryo-tomography and small angle X-ray scattering, whereby multi-component complexes can be imaged. High-resolution structures can then be fitted into the envelopes generated by these methods to help visualize the orientations of components with respect to one another. A combination of high and low-resolution techniques have worked well in other multi-component machineries such as the T3SS to help understand its spatiotemporal assembly and regulation (Galkin *et al*., 2010; Schraidt *et al*., 2010; Abrusci *et al*., 2014; Demers *et al*., 2014;). Understanding how the components interact and investigating the dynamics associated with the T4P system in *P. aeruginosa* will be essential information to move the field forward.

Similarly, there is still much room for advancement in our understanding of T4P regulatory pathways. While components of the T4P system have been implicated in both c-di-GMP and cAMP regulatory pathways, how these two pathways converge remains unclear. The coming years will almost certainly identify new proteins that are involved in one or both of the signalling pathways. Recent studies of cAMP signalling have shown independently that the Chp system, FimV and FimL all have roles in the same regulatory pathway but not yet how they are connected. The biggest hurdle moving forward will be identifying all the members of the T4P-regulatory signalling pathways. We suspect that in addition to new signalling proteins, previously identified proteins will be found to have multiple roles – as was observed for PilY1 (Siryaporn *et al*., 2014). Resolution of the cAMP and c-di-GMP signalling pathways will improve, and we envisage that these two pathways will be intimately linked.

With all the proteins in the assembly and the signalling systems identified, we will begin to understand how bacteria attach to surfaces via their T4P and then how this information is relayed throughout the bacteria such that all systems are correctly upregulated or downregulated (Fig. 5). Adhesion to a surface is generally regarded as the first step in a virulence program launched by bacteria, and the first step in biofilm formation (Stoodley *et al*., 2002). The virulence program is regulated in part by cAMP, and shortly after attachment, production of a biofilm begins through stimulation of extracellular polymeric substances (EPS) production to form a protective matrix in which the bacteria are embedded (Busscher *et al*., 2012). Investigations of T4P biology in *P. aeruginosa* to understand the initial stages of attachment and signalling are critical to find alternative antibacterial strategies to combat this opportunistic pathogen.

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