Functional role of conserved residues in the characteristic secretion NTPase motifs of the *Pseudomonas aeruginosa* type IV pilus motor proteins PilB, PilT and PilU

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Type IV pili are retractable protein fibres used by many bacterial pathogens for adherence, twitching motility, biofilm development and host colonization. In *Pseudomonas aeruginosa*, PilB and PilT are bipolar proteins belonging to the secretion NTPase superfamily, and power pilus extension and retraction, respectively, while the unipolar PilT paralogue PilU supports pilus retraction in an unknown manner. Assay of purified 6×His-tagged PilB, PilT and PilU from *P. aeruginosa* showed that all three proteins have ATPase activities *in vitro*. Conserved residues in the Walker A (WA), Walker B (WB), Asp Box and His Box motifs characteristic of secretion NTPases were mutated, and complementation of twitching motility was tested. Mutation of conserved WA or WB residues in any of the three ATPases abrogated twitching motility, and for the WA mutant of PilT caused loss of polar localization. The requirement for three invariant acidic residues in the Asp Box motif, and for two invariant His residues in the His Box motif varied, with PilB being the least tolerant of changes. In all three proteins, the third acidic residue in the Asp Box and the second His of the His Box were crucial for function; mutation of these residues caused loss of PilT ATPase activity *in vitro*. Modelling of the effects of these mutations on the crystal structures of *Aquifex aeolicus* PilT and *Vibrio cholerae* EpsE (a PilB homologue) showed that the critical Asp Box and His Box residues contribute to a catalytic pocket that surrounds the ligand. These results provide experimental evidence differentiating widely conserved Asp and His Box residues that are essential for function from those whose roles are modulated by specific local environments.

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INTRODUCTION

Gram-negative pathogens use ATP-hydrolysing proteins belonging to the ubiquitous P-loop NTPase superfamily to energize the type II secretion (T2S), type III secretion (T3S), type IV secretion (T4S) and type IV pili (T4P) systems, all of which are important for virulence in plant, animal and human hosts (Hobbs & Mattick, 1993). Of these, the ATPases of the T2S and T4P systems are more closely related to one another than to the T3S or T4S

ATPases; the phylogeny of T2S and T4P protein families is reviewed by Planet *et al.* (2001). The T4P system is typically associated with adherence and surface-associated ‘twitching’ motility, but in some bacteria has also been shown to participate in protein secretion (Forsberg & Guina, 2007; Hager *et al.*, 2006; Kirn *et al.*, 2003; Kirn & Taylor, 2005; Kennan *et al.*, 2001; Han *et al.*, 2007).

Unlike the T2S and T4S systems, which have a single motor ATPase, the T4P system has two, and sometimes three, motor ATPases (Burrows, 2005). The two-protein systems typically have an extension ATPase (pilin polymerase) and a retraction ATPase (pilin depolymerase). In three-protein systems, such as that found in the opportunistic and nosocomial pathogen *Pseudomonas aeruginosa*, the typical

Abbreviations: ASCE, additional strand, catalytic E; PNP, purine phosphorylase; T2S, type II secretion; T3S, type III secretion; T4S, type IV secretion; T4P, type IV pili; WA, Walker A; WB, Walker B; YFP, yellow fluorescent protein.

extension and retraction ATPases (PilB and PilT, respectively, in *P. aeruginosa*) are accompanied by a PilT paralogue called PilU (Mattick, 2002; Whitchurch *et al.*, 1991; Whitchurch & Mattick, 1994; Turner *et al.*, 1993). The larger (62 kDa) PilB protein is most similar to T2S ATPases, while the smaller PilT (38 kDa) and PilU (42 kDa) proteins are peculiar to T4P systems (Planet *et al.*, 2001). In many bacteria, including *P. aeruginosa*, T4P mediate twitching motility, a form of surface-associated translocation in which movement occurs via cycles of pilus assembly and disassembly (Skerker & Berg, 2001), thought to require ATP hydrolysis by PilB and PilT, respectively. The role of the PilT paralogue PilU in twitching motility is not clearly understood, but loss of this protein results in the unusual phenotypic combination of pilus-specific bacteriophage susceptibility (typically associated with functional, retractable pili) and loss of twitching motility (typically associated with loss of pilus extension or pilus retraction) (Whitchurch & Mattick, 1994).

The bacterial secretion NTPases are members of the P-loop NTPase family, which can be separated into two principal classes: the kinase-GTPase (KG) division and the additional strand, catalytic E (ASCE) division (Iyer *et al.*, 2004). ASCE division members, including the bacterial secretion NTPases, use a conserved glutamic acid residue to abstract a proton from water for nucleophilic attack on the γ -phosphate of the ATP ligand. ATP hydrolysis results in conformational changes within the protein, translating chemical energy into mechanical events that result in folding/unfolding or assembly/disassembly of protein complexes. All P-loop NTPases are characterized by highly conserved Walker A (WA; GxxGxGKT/S) and Walker B (WB; hhhhDE) motifs (Walker *et al.*, 1982). The WA residues form an extensive hydrogen-bonding network with the phosphate tail of the ATP substrate, while the WB motif participates in the coordination of the Mg^{2+} co-factor required for ATP hydrolysis (Robien *et al.*, 2003; Yeo *et al.*, 2000).

Secretion NTPases of the T2S and T4P systems, including PilB, PilT and PilU, function as toroidal homohexamers and, in addition to WA and WB motifs, contain unique Asp Box and His Box motifs (Planet *et al.*, 2001). Site-directed mutagenesis of the WA motif has been used before to inactivate PilB and PilT from *P. aeruginosa* (Aukema *et al.*, 2005; Turner *et al.*, 1993), but similar studies of PilU have not, to our knowledge, been reported. While the functions of the WA and WB motifs are well characterized, those of the Asp and His Box motifs remain largely unexplored, despite their broad conservation in the secretion NTPase family. Based on limited structural and mutagenesis data, residues of the Asp and His Boxes are likely to contribute to nucleotide binding and/or sensing as well as subunit-subunit interactions (Satyshur *et al.*, 2007; Robien *et al.*, 2003). In this work we demonstrate that the three motor proteins in *P. aeruginosa* are bona fide ATPases, and we use a site-directed mutagenesis approach to show that for their function all three require specific invariant residues within

the characteristic secretion NTPase motifs. Due to their wide conservation among these proteins, we focussed specifically on the three acidic residues within the Asp Box motif, and the two His within the eponymous His Box motif, providing experimental support for hypotheses regarding the function of these residues.

METHODS

Strains, plasmids and media used in this study. Bacterial strains and plasmids used in this work are summarized in Table 1. Both *Escherichia coli* and *P. aeruginosa* were grown on 1.5 % Luria agar plates or in Luria broth, containing, where appropriate, antibiotics at the following concentrations: tetracycline, 15 $\mu\text{g ml}^{-1}$ for *E. coli* and 50 $\mu\text{g ml}^{-1}$ for *P. aeruginosa*; gentamicin, 15 $\mu\text{g ml}^{-1}$ for *E. coli* and 30 $\mu\text{g ml}^{-1}$ for *P. aeruginosa*. For twitching assays, the agar concentration was reduced to 1 %.

Protein expression constructs. The genes for *pilB*, *pilT* and *pilU* were cloned by PCR from the *P. aeruginosa* PAK strain into an AccepTor vector (plasmid pSTBlue, Novagen) and sequenced. The primers used for cloning were designed to include the *NdeI* and *BamHI* (*pilT* and *pilU*) or *EcoRI* (*pilB*) restriction sites to facilitate subsequent subcloning into the pET28a expression vector (Novagen). Prior to subcloning, site-directed mutagenesis (QuickChange mutagenesis kit, Stratagene) was used to inactivate the internal *NdeI* site (position 1240) present in *pilB*. The pET28a vector allowed expression of PilB, PilT and PilU proteins with an N-terminal 6 $\times$ His tag using a T7 RNA polymerase system.

Protein expression and purification. The pET28a expression vectors were transformed into *E. coli* BL21 Codon Plus (Stratagene) and used to inoculate a 5 ml LB-Kan (50 $\mu\text{g kanamycin ml}^{-1}$) culture. The overnight culture was used to inoculate 1 l LB-Kan and the cells were allowed to grow at 37 °C until OD₆₀₀ reached 0.6–0.7, at which time protein expression was induced by adding IPTG to a final concentration of 0.5 mM. The temperature was then decreased to 16 °C and, after overnight incubation, the cells were harvested by centrifugation at 18 000 g for 30 min at 4 °C. Cells were stored at –20 °C until required.

The frozen cells were resuspended in 40 ml buffer A (20 mM Tris/HCl, pH 8.5, 150 mM sodium citrate) and one Complete EDTA-free protease inhibitor cocktail tablet (Roche) was added prior to cell lysis. The cell suspension was sonicated for seven 30 s pulses, with 60 s cooling on ice between each pulse, then centrifuged at maximum speed in a microcentrifuge for 30 min at 4 °C. The supernatant was mixed with 2 ml Ni-NTA agarose (Qiagen) at 4 °C for 2 h. The resin was then packed into a column and washed twice, first with buffer A containing 5 mM imidazole and subsequently with the same buffer containing 20 mM imidazole. The protein was eluted from the column in two fractions. The first fraction, eluted with buffer A and 100 mM imidazole, contained some protein impurities, while the second fraction, eluted with buffer A and 300 mM imidazole, yielded protein of higher purity, as estimated by SDS-PAGE (not shown). The second fraction was dialysed overnight at 4 °C in buffer A and concentrated to a final volume of approximately 1 ml. The protein concentration was determined using the Bradford assay (Pierce) with BSA (Pierce) as the standard. The protein was used immediately in the ATPase assays. To facilitate the interpretation of the results, the same purification protocol and buffer conditions (20 mM Tris/HCl, pH 8.5, and 150 mM sodium citrate) were used for all proteins.

ATPase assays. ATP hydrolysis was monitored spectrophotometrically using the EnzCheck phosphate assay kit (Invitrogen). The

Table 1. Strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i> DH5 α	F ⁻ Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _k ⁻ , m _k ⁺) <i>phoA supE44 thi-1 gyrA96 relA1 λ</i> ⁻	Invitrogen
<i>E. coli</i> BL21	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ , m _B ⁻) <i>gal dcm</i>	Novagen
<i>P. aeruginosa</i> PAK	Wild-type strain	J. Boyd, Dalhousie University
<i>P. aeruginosa</i> R364	Tn5-B21 insertion in <i>pilT</i>	Whitchurch <i>et al.</i> (1991)
<i>P. aeruginosa</i> S34	Tn5-B21 insertion in <i>pilU</i>	Whitchurch & Mattick (1994)
<i>P. aeruginosa</i> PAK-B Ω	Ω insertion mutant of <i>pilB</i>	Koga <i>et al.</i> (1993)
Plasmids		
pUCP20Gm	Shuttle vector with <i>Sma</i> I-flanked Gm cassette inserted into <i>Scal</i> site within <i>bla</i>	Chiang & Burrows (2003)
pUCP20Gm- <i>pilB</i>	PAK <i>pilB</i> construct	Chiang <i>et al.</i> (2005)
pUCP20Gm- <i>pilB</i> (G331S)	Gly331 in WA of PilB replaced with Ser	This work
pUCP20Gm- <i>pilB</i> (E354Q)	Glu354 in Asp Box of PilB replaced with Gln	This work
pUCP20Gm- <i>pilB</i> (D355N)	Asp355 in Asp Box of PilB replaced with Asn	This work
pUCP20Gm- <i>pilB</i> (E358Q)	Glu358 in Asp Box of PilB replaced with Gln	This work
pUCP20Gm- <i>pilB</i> (E396Q)	Glu396 in WB of PilB replaced with Gln	This work
pUCP20Gm- <i>pilB</i> (H414A)	His414 in His Box of PilB replaced with Ala	This work
pUCP20Gm- <i>pilB</i> (H421A)	His421 in His Box of PilB replaced with Ala	This work
pUCP20Gm- <i>yfp-pilT</i>	YFP-PilT fusion construct	Chiang <i>et al.</i> (2005)
pUCP20Gm- <i>yT</i> (G135S)	Gly135 in WA of PilT replaced with Ser	This work
pUCP20Gm- <i>yT</i> (E159Q)	Glu159 in Asp Box of PilT replaced with Gln	This work
pUCP20Gm- <i>yT</i> (D160N)	Asp160 in Asp Box of PilT replaced with Asn	This work
pUCP20Gm- <i>yT</i> (E163Q)	Glu163 in Asp Box of PilT replaced with Gln	This work
pUCP20Gm- <i>yT</i> (E204Q)	Glu204 in WB of PilT replaced with Gln	This work
pUCP20Gm- <i>yT</i> (H222A)	His222 in His Box of PilT replaced with Ala	This work
pUCP20Gm- <i>yT</i> (H229A)	His229 in His Box of PilT replaced with Ala	This work
pUCP20Gm- <i>yfp-pilU</i>	YFP-PilU fusion construct	Chiang <i>et al.</i> (2005)
pUCP20Gm- <i>yU</i> (G135S)	Glu135 in WA of PilU replaced with Ser	This work
pUCP20Gm- <i>yU</i> (E159Q)	Glu159 in Asp Box of PilU replaced with Gln	This work
pUCP20Gm- <i>yU</i> (D160N)	Asp160 in Asp Box of PilU replaced with Asn	This work
pUCP20Gm- <i>yU</i> (E163Q)	Glu163 in Asp Box of PilU replaced with Gln	This work
pUCP20Gm- <i>yU</i> (E204Q)	Glu204 in WB of PilU replaced with Gln	This work
pUCP20Gm- <i>yU</i> (H222A)	His222 in His Box of PilU replaced with Ala	This work
pUCP20Gm- <i>yU</i> (H229A)	His229 in His Box of PilU replaced with Ala	This work
pET28a	IPTG-inducible expression vector, T7 promoter	Novagen
pET28a-PilB	PilB expression construct	This work
pET28a-PilT	PilT expression construct	This work
pET28a-PilU	PilU expression construct	This work
pET28a-PilT G135S	Gly135 in WA of PilT replaced with Ser	This work
pET28a-PilT E163Q	Glu163 in Asp Box of PilT replaced with Gln	This work
pET28a-PilT E204Q	Glu204 in WB of PilT replaced with Gln	This work
pET28a-PilT H229A	His229 in His Box of PilT replaced with Ala	This work

inorganic phosphate released during ATP and 2-amino-6-mercapto-7-methyl-purine riboside (MESG) hydrolysis are converted by purine phosphorylase (PNP) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine, which results in an absorbance shift from 330 to 360 nm. The assay was performed in triplicate for each protein. The 500 μ l reaction mix contained between 9 and 95 μ g purified protein, 25 μ l 20 $\times$ reaction mix (1 M Tris/HCl, pH 7.5, 20 mM MgCl₂) supplemented with 5 mM MgCl₂, 0.2 mM MESG, 1 U PNP and 1 mM ATP. After incubation for 30 min at 24 °C the A₃₆₀ was determined. The results were normalized for non-enzymic ATP degradation by subtracting the reading from that of a control reaction containing ATP but no ATPase. BSA was used as a negative control.

The total amount of phosphate released was then determined by comparing the normalized absorbance of the protein sample to a phosphate standard curve.

Site-directed mutagenesis of conserved residues in PilB, PilT and PilU. Single amino acid replacement of conserved residues in the WA (G135S), Asp Box (E159Q, D160N, E163Q), Walker B (E204Q) and His Box (H222A, H229A) motifs of PilT, using our previously generated *yfp-pilT* construct (Chiang *et al.*, 2005) as a template, were performed with the QuikChange mutagenesis kit (Stratagene) as recommended by the manufacturer. Oligonucleotide sequences used are summarized in Table 2. Briefly, all PCR reactions were performed

Table 2. Oligonucleotides used in this study

Primer name	Sequence
PilB-1	5'-CTAGTCTAGAATGAACGACAGCATCCA
PilB-2	5'-CTAGTCTAGATTAATCCTTGGTCACGCGGT
PilB G331S-1	5'-GGCCCCACCGGCTCGAGCAAGACGGTATCGCTG
PilB G331S-2	5'-CAGCGATACCGTCTTGCTCGAGCCGGTGGGGCC
PilB E354Q-1	5'-AACATTTCCACCGCCCAGGACCCGGTGGAGATC
PilB E354Q-2	5'-GATCTCCACCGGGTCTTGGGCGGTGGAAATGTT
PilB D355N-1	5'-ATTTCCACCGCCGAAAACCCGGTGGAGATCAAC
PilB D355N-2	5'-GTTGATCTCCACCGGGTTTTCGGCGGTGGAAAT
PilB E358Q-1	5'-GCCGAAGACCCGGTGCAGATCAACCTGGAAGGC
PilB E358Q-2	5'-GCCTTCCAGGTTGATCTGCACCGGGTCTTCGGC
PilB E396Q-1	5'-GTGATCATGGTCGGCCAGATCCGCGACCTGGAG
PilB E396Q-2	5'-CTCGAGGTCGCGGATCTGGCCGACCATGATCAC
PilB H414A-1	5'-GCGGCGCAGACCGGGCGATGGTGTATGCCACC
PilB H414A-2	5'-GGTGGACATCACCATCGCCCCGGTCTGCGCCGC
PilB H421A-1	5'-GTGATGTCCACCCTGGCCACCAACAGCGCCGCCGAG
PilB H421A-2	5'-GGCGGCGCTGTTGGTGGCCAGGGTGGACATCAC
PilT G135S up	5'-TCACCGGGCCGACCGGTTTCGAGCAAGTCCACCACCTGGCGGGC
PilT G135S dn	5'-CGCCGCCAGGGTGGTGGACTTGCTCGAACCGGTCCGCCCGGTGA
PilT E159Q up	5'-CAAGTACCACCACATCCTCACCATCCAGGACCCGATCGAATTCGTCCACGAATCGAAG
PilT E159Q dn	5'-CTTCGATTCTGGACGAATTCGATCGGGTCTTGGATGGTGAGGATGTGGTGGTACTTG
PilT D160N up	5'-ATCCTCACCATCGAGAACCCGATCGAATTCGTC
PilT D160N dn	5'-GACGAATTCGATCGGGTTCTCGATGGTGAGGAT
PilT E163Q up	5'-TCACCATCGAGGACCCGATCCAGTTCGTCCACGAAT
PilT E163Q dn	5'-ATTCTGGACGAACCTGGATCGGGTCTCGATGGTGA
PilT E204Q up	5'-ATCATCCTGGTCGGCCAGATGCGCGACCTGGAA
PilT E204Q dn	5'-TTCCAGGTCGCGCATCTGGCCGACAGGATGAT
PilT H222A up	5'-GCGGCGGAGACCGGCGCCCTGGTATTCGGCACC
PilT H222A dn	5'-GGTGCCGAATACCAGGGCGCCGCTCTCCGCCGC
PilT H229A up	5'-GGTATTCGGCACCCCTGGCCACCACCTCGGCGG
PilT H229A dn	5'-CCGCCGAGGTGGTGGCCAGGGTGCCGAATACC
PilU G135S up	5'-TCGTCGGCGCCACCGGCACCAGCAAGTCCACCTCGTGGCGGC
PilU G135S dn	5'-GCCGCCAGCGAGGTGGACTTGCTGGTGCCGGTGGCGCCGACGA
PilU E159Q up	5'-CACATCATCTCCATCCAGGACCCGATCGAGTAC
PilU E159Q dn	5'-GTACTCGATCGGGTCTTGGATGGAGATGATGTG
PilU D160N up	5'-GGCACATCATCTCCATCGAGAACCCGATCGAGTACATCCACCA
PilU D160N dn	5'-TGGTGGATGTACTCGATCGGGTTCTCGATGGAGATGATGTGCC
PilU E163Q up	5'-ATCGAGGACCCGATCCAGTACATCCACCAGCAC
PilU E163Q dn	5'-GTGCTGGTGGATGTACTGGATCGGGTCTTCGAT
PilU E204Q up	5'-GTGATCATGATCGGCCAGGTGCGCTCGCGGGAG
PilU E204Q dn	5'-CTCCCGCGAGCGCACCTGGCCGATCATGATCAC
PilU H222A up	5'-TTCGCCGAGACCGGCGCCCTGTGCCTGGCGACC
PilU H222A dn	5'-GGTCGCCAGGCACAGGGCGCCGCTCTCGGCGAA
PilU H229A up	5'-TGCTGGCGACCCTGGCCGCAACAACGCCAAC
PilU H229A dn	5'-GTTGGCGTTGTTTCGCGGCCAGGGTCGCCAGGCA

at 55 °C annealing for 50 s and 68 °C extension for 20 min, over a total of 18 cycles. Similar constructs with mutations G135S, E159Q, D160N, E163Q, Q167E, E204Q, H222A and H229A were made in the PilU component of the previously generated yellow fluorescent protein (YFP)–PilU fusion construct (Chiang *et al.*, 2005).

A previously constructed YFP–PilB construct did not complement a *pilB* mutant (Chiang *et al.*, 2005), precluding functional analyses of the fusion. Therefore, an untagged version of PilB expressed from the same vector as the YFP–PilT and YFP–PilU fusions was mutated at the equivalent WA (G331S), Asp Box (E354Q, D355N, E358Q), WB (E396Q) and His Box (H414A, H421A) residues. The integrity of each

mutant construct was verified by DNA sequence analysis. Plasmid constructs were transformed into chemically competent *E. coli* for amplification and purification and subsequently introduced into relevant *P. aeruginosa* strains (mutant and wild-type) by electroporation (see below). For assays of the ATPase activity of mutant PilT proteins, the same mutagenic primers were used to mutagenize the PilT pET28a construct (described above), and the mutant proteins were expressed, purified and assayed as described above.

Complementation of motor protein mutants. Wild-type or mutant strains of *P. aeruginosa* were streaked for single colonies on

LB agar plates. Three to five colonies were washed and resuspended in 1 ml sterile distilled water, and 200 µl of the suspension was immediately transformed with 100–150 ng DNA using a 0.2 cm path-length electroporation cuvette (EquiBio cat. no. ECU-102) and the *E. coli* Pulser device (Bio-Rad). Transformants were selected on LB plates containing appropriate antibiotics at 37 °C overnight.

Twitching motility assays. Assessment of twitching motility was performed as described elsewhere (Semmler *et al.*, 1999), and the resulting zones of twitching motility were visualized by carefully removing the agar and staining the bacteria adhering to the polystyrene Petri plate with 1% crystal violet for 10 min at room temperature, followed by a brief rinse with tap water to remove unbound dye. ImageJ software (NIH) was used to measure and calculate average areas of the resulting twitching zones to acquire quantitative comparative data.

Western blot analysis to assess protein stability. Overnight cultures of the *P. aeruginosa* *pilB*, *pilT* or *pilU* mutants carrying the cognate complementation constructs were standardized to OD₆₀₀=0.6. A 200 µl volume of each of the suspensions was centrifuged at maximum speed in a microfuge to harvest the cells. The cells were lysed in 150 µl 1× SDS-PAGE sample buffer and 5 µl lysate was separated on a 12.5% SDS-PAGE minigel, then transferred to nitrocellulose. A second gel was stained with Coomassie brilliant blue to verify that equivalent amounts of lysate were loaded in each lane. The blots were blocked with 5% skimmed milk in sterile PBS, pH 7.4, at 4 °C overnight. The proteins of interest were detected using a rabbit polyclonal antibody to PilB or a mAb to GFP (JL-8, Clontech) for the YFP–PilT and YFP–PilU fusions. Both primary antibodies were used at a 1:5000 dilution in PBS for 1 h at room temperature. The secondary antibodies were goat anti-rabbit or goat anti-mouse alkaline phosphatase conjugates, also used at 1:5000 dilution in PBS for 1 h at room temperature. The blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, as recommended by the manufacturer (Sigma).

Analysis of sheared surface proteins. Surface proteins (mainly flagella and pili) were isolated using the methods of Castric (1995), with modifications. Bacteria were streaked in a grid pattern on LB agar plates containing 30 mg gentamicin l⁻¹ and 0.2% (w/v) L-arabinose, and incubated overnight at 37 °C. One or two plates per sample were used. The bacteria were gently scraped from the agar surface using a sterile coverslip and resuspended in 2 ml sterile PBS (pH 7.4) per plate, and surface proteins were sheared by vigorous vortexing for 30 s. The suspension was transferred to two 1.5 ml microcentrifuge tubes and centrifuged for 5 min at maximum speed to pellet the cells, which were retained for whole-cell-lysate Western immunoblots (below). The supernatant was transferred to a new tube and centrifuged for an additional 25 min at maximum speed at room temperature. To precipitate the sheared proteins, one-tenth volume each of 5 M NaCl and 30% (w/v) PEG (molecular mass ~8000 Da) were added to the supernatant, and the samples were incubated on ice for 60 min. Samples were centrifuged at maximum speed in a microcentrifuge for 25 min at 4 °C. After discarding the supernatant, the resulting pellets were resuspended in 2× SDS-PAGE loading dye [125 mM Tris, pH 6.8, 2% (w/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.001% (w/v) bromophenol blue, 4% (w/v) SDS], boiled for 5 min and resolved on a 15% 1D SDS-PAGE minigel with a pre-stained Benchmark Protein Ladder (Invitrogen). The proteins were visualized using Coomassie blue dye.

Fluorescence microscopy. Single colonies were streaked on 1.5% LB agar plates with the appropriate antibiotics then incubated for 18 h at 37 °C. The samples were prepared by mixing a single colony with 10 µl PBS (pH 7.1) on a glass slide using a sterile toothpick. Polylysine-coated coverslips (Sigma Diagnostics P8920) were used to

mount the cells for photography. Using fluorescence illumination, slides were viewed at ×100 magnification (oil immersion) with a Zeiss Axiovert 200M microscope. Open Lab V 3.1.3 (Improvision) software was used to collect fluorescence images. Images were processed with PhotoShop 7.0.1 (Adobe).

Bacteriophage susceptibility assay. Bacterial strains to be tested for phage susceptibility were streaked in a single line on an LB plate and 1 µl of a PO4 bacteriophage suspension, containing approximately 10⁹ p.f.u. ml⁻¹, was spotted onto the centre of the streak. The plates were subsequently incubated at 37 °C for 18 h. Phage-resistant strains showed no disruption of growth above and below the point of phage inoculation, while susceptible strains showed no growth in the centre of the streak, due to lysis of the cells by the bacteriophage.

RESULTS

ATPase activity of the T4P motor proteins

Previously, we have shown that despite the high level of amino acid similarity (~75% over 240 aa) between the C-terminal ATPase domains of PilT and PilU, a stable bipolar chimera containing the N terminus of PilT fused to the C terminus of PilU could not complement twitching in either *pilT* or *pilU* mutants (Chiang *et al.*, 2005). This result suggested that the C terminus of PilU does not contain a functional ATPase domain. Although PilT proteins from *Aquifex aeolicus*, *P. aeruginosa* and *Neisseria gonorrhoeae* have been demonstrated elsewhere to have ATPase activity (Aukema *et al.*, 2005; Herdendorf *et al.*, 2002; Maier *et al.*, 2002), the ATPase activities of PilB and PilU have not been assayed previously. Therefore, we sought to test the ATPase activities of purified PilB, PilT and PilU *in vitro*. *P. aeruginosa* PilB, PilT and PilU were N-terminally tagged with 6×His, expressed in *E. coli* and purified by affinity chromatography. The amount of protein obtained in the soluble fraction was sufficient to allow their enzymic characterization using an assay similar to the one performed for *Legionella pneumophila* DotB (Sexton *et al.*, 2004). PilB, PilT and PilU all exhibited low levels of ATPase activity, with the highest specific activity, 10.79 ± 1.09 nmol Pi min⁻¹ (mg protein)⁻¹, observed for PilB, and the lowest, 5.02 ± 0.78 nmol Pi min⁻¹ (mg protein)⁻¹, for PilU. PilT had specific activity of 7.43 ± 0.14 nmol Pi min⁻¹ (mg protein)⁻¹, intermediate between PilB and PilU (Table 3). These *in vitro* activities are consistent with those reported for other purified bacterial secretion ATPases, including EspE at 5.6 nmol min⁻¹ (mg protein)⁻¹, PilT from *Aquifex* at 15.7 ± 0.9 nmol min⁻¹ (mg protein)⁻¹, DotB from *L. pneumophila* at 6.4 nmol min⁻¹ (mg protein)⁻¹ and TrwD from plasmid R388 at 4.5 nmol min⁻¹ (mg protein)⁻¹ (Sexton *et al.*, 2004; Aukema *et al.*, 2005; Herdendorf *et al.*, 2002; Rivas *et al.*, 1997; Camberg & Sandkvist, 2005).

Effect of mutations in conserved secretion NTPase motifs on pilus-associated functions

Until recently, structures of the T4P motor proteins were not available, in part because the poor solubility of these

Table 3. ATPase activities of wild-type and mutant T4P motor proteins

Protein	Mutation	Specific activity [nmol Pi min ⁻¹ (mg protein) ⁻¹]*
PilB	None	10.79 ± 1.09
PilT	None	7.43 ± 0.14
PilU	None	5.02 ± 0.78
PilT	G135S	Below detection limit
PilT	E163Q	2.75 ± 0.54
PilT	E204Q	Below detection limit
PilT	H229A	1.08 ± 0.11

*Results are the mean ± SD for protein samples measured in triplicate.

proteins makes them challenging to purify and crystallize. However, the structure of PilT from *A. aeolicus* has recently been published (Satyshur *et al.*, 2007), allowing us to use it as a model for the *P. aeruginosa* motor proteins. The *A. aeolicus* PilT protein (PilT_{Aa}), as well as related T2S ATPases EpsE (*Vibrio cholerae*) and HP0525 (*Helicobacter pylori*), have a characteristic structure composed of globular N- and C-terminal domains connected by a highly flexible linker domain (Satyshur *et al.*, 2007; Robien *et al.*, 2003; Savvides *et al.*, 2003). The C-terminal domains containing the NTPase motifs are highly conserved among the motor proteins, allowing us to use the ATP-bound form of PilT_{Aa} and the AMP–PNP-bound form of EpsE as structural models with confidence. Examination of the seven genomes of *P. aeruginosa* currently available revealed that the PilT and PilU proteins are 100 % identical at the amino acid level in every strain (not shown). The PilB proteins are also highly conserved (94–100 % identical to PilB from laboratory strain PAO1), with most amino acid differences in the N-terminal domain.

To dissect the roles of the secretion NTPase motifs in motor protein function, site-directed mutagenesis of conserved residues in WA, WB, Asp and His Boxes (indicated in Fig. 1a) was performed on YFP–PilT, YFP–PilU and untagged PilB, and the ability of the mutant proteins to complement twitching motility in the cognate *P. aeruginosa* mutants was tested. Use of the mutated YFP–PilT and –PilU fusion proteins for complementation of their respective mutants allowed the simultaneous detection of protein localization and function *in vivo*. Since the YFP-tagged PilB construct used previously was not functional (i.e. could not complement a *pilB* mutant for twitching motility; Chiang *et al.*, 2005), we performed mutagenesis on an untagged version of the protein. Of particular interest were the three acidic residues within the Asp Box motif, and the two invariant His residues within the eponymous His Box motif. The WA Gly residue selected for mutation has been shown previously to inactivate both PilB and XcpR, the T2S NTPase in *P. aeruginosa* (Turner *et al.*, 1993), while the WB Glu selected

for mutagenesis is the key catalytic residue for ASCE family NTPases (Iyer *et al.*, 2004). The positions of the targeted residues in the *P. aeruginosa* ATPases relative to the ligand are indicated in Fig. 1(b).

The PilB, YFP–PilT and YFP–PilU mutant proteins were stable and expressed at levels comparable to the wild-type, as determined by Western immunoblot using specific antisera (Fig. 2), with the single exception of the PilB H414A mutant, which appeared to have a small deletion or truncation not due to DNA sequence alterations, and was therefore not further examined. The results of twitching motility and polar localization assays (for PilT and PilU) of *pilB*, *pilT* and *pilU* mutant strains complemented with their respective wild-type or mutated constructs are summarized in Table 4.

The WA motif is involved in ATP binding, while the WB motif is involved in the hydrolysis of ATP (Robien *et al.*, 2003; Yeo *et al.*, 2000). In contrast, the functions of the conserved Asp and His Box motifs are less clear. The role of these motifs in the *in vivo* function of the *P. aeruginosa* ATPases was tested using a twitching motility assay. Mutation of the WA or WB motif resulted in loss of the ability to restore twitching motility for all three proteins (Fig. 3a).

The Asp Box motif contains three invariant acidic residues, labelled as Asp Box 1, 2 and 3 in Fig. 1(b). Mutation of the first residue (Glu) affected the function of all three proteins, as determined by decreased twitching motility. The PilB E354Q mutant had the most severe deficit, with twitching areas reduced to 34 % of those of the positive control (unmodified protein), while the PilT and PilU mutants were less affected, with 62 and 46 % of the control twitching level, respectively (Fig. 3b). Mutation of the conserved Asp residue (Asp Box 2) had a limited effect on the function of PilB, twitching areas corresponding to 82 % of that of the control. This residue is naturally mutated to Asn in the PilB protein of *P. aeruginosa* PA7, which is able to twitch (not shown), supporting the minor effect of this mutation on function. In contrast, mutation of this Asp residue significantly impaired the function of PilU, with only 18 % of control twitching (Fig. 3b). Twitching motility in the strain complemented with the PilT D160N mutant was approximately half that of the control. The third of three conserved acidic residues, corresponding to E176 of PilT_{Aa} and E298 of EpsE (Fig. 1b), was essential for twitching motility in all three proteins (Fig. 3).

Mutating the first of two invariant His residues in the His Box motif resulted in PilB truncation when examined by Western blotting (Fig. 2), and this mutant was not further evaluated. PilT and PilU mutant proteins were stable but not markedly affected by this mutation; twitching in the YFP–PilT H222A mutant was reduced by approximately 50 % relative to the positive control (Fig. 3a). In contrast, the second His residue was essential for twitching motility in all three T4P motor proteins (Fig. 3a).

Loss of twitching motility can arise from lack of pilus assembly, or from loss of pilus retraction. SDS-PAGE analysis of sheared surface protein preparations from each mutant was performed to determine whether the twitching motility defects observed could be correlated with changes in the levels of surface piliation (Fig. 4). The results for the PilB mutants correlated well with the observed twitching phenotypes; of the seven mutations generated, only the D355N mutant retained significant twitching motility (Fig. 3), and only this strain had levels of surface pilin comparable to those of the strain complemented with the unmodified *pilB* allele. The PilT and PilU mutants are

P. aeruginosa strain PAK is susceptible to killing by the pilus-specific bacteriophage PO4, which attaches to the pili and is brought into contact with the cell surface upon pilus retraction (Bradley, 1974). Complementation of the phage-resistant *pilB* and *pilT* mutants with modified proteins that

Fig. 2. Stability of PilB, YFP-PilT, YFP-PilU and site-directed mutants thereof. Standardized whole-cell lysates of *P. aeruginosa* PAK wild-type and *pilB* Δ null mutant, as well as the *pilB* mutant complemented with an unmodified or mutant copy of *pilB* *in trans*, were probed with a rabbit polyclonal antibody to PilB. The levels of PilB expressed from the plasmid are similar to those observed in the wild-type. YFP-PilT, YFP-PilU and mutant variants thereof were probed with a mAb to YFP (Clontech). M, 72 kDa molecular mass marker.

supported twitching motility resulted in concomitant restoration of phage susceptibility in all cases (Table 4). Similarly, complementation of the *pilB* and *pilT* mutants with constructs that did not restore twitching resulted in continued phage resistance. The complemented *pilU* mutants exhibited phage susceptibility regardless of their twitching phenotype, consistent with the fact that the non-twitching *pilU* mutant continues to be phage-susceptible.

Effect on ATPase activity of mutations that abrogate twitching in all three ATPases

To determine whether those specific mutations that caused complete loss of twitching motility in all three motor proteins were associated with loss of ATPase activity *in vitro*, we used PilT as a model protein. Site-directed mutagenesis was used to introduce the G135S (WA), E163Q (Asp Box 3), E204Q (WB) and H229A (His Box 2) mutations into the *E. coli* expression construct encoding His-tagged *P. aeruginosa* PilT. The mutated proteins were expressed and purified, and their ATPase activities tested as described above. Compared to the wild-type PilT protein, all four mutant proteins showed reduced or undetectable ATPase activities (Table 3), confirming that loss of complementation *in vivo* was correlated with reduced ATPase activity *in vitro*.

Subcellular localization of mutant YFP fusions

We have demonstrated previously that YFP-PilT exhibits bipolar localization, while the localization of YFP-PilU is unipolar (Chiang *et al.*, 2005). All mutant fusion proteins tested retained their characteristic pattern of polar localization, with one exception. The WA mutant of YFP-PilT exhibited delocalized fluorescence in the *pilT* mutant background, while the same mutation in YFP-PilU

Table 4. Localization, motility and phage sensitivity of T4P ATPase mutants

NA, Not applicable. PilB fusions to YFP were not functional.

Strain and complementing construct	Subcellular localization of fusion	Twitching motility	PO4 bacteriophage susceptibility
PAK wild-type	NA	+	+
<i>PilB</i> ⁻ mutant	NA	-	-
+ <i>PilB</i>	NA	+	+
+ BG331S (WA)	NA	-	-
+ BE354Q (Asp Box)	NA	Reduced	+
+ BD355N (Asp Box)	NA	+	+
+ BE358Q (Asp Box)	NA	-	-
+ BE396Q (WB)	NA	-	-
+ BH421A (His Box)	NA	-	-
<i>PilT</i> ⁻ mutant	NA	-	-
+ YFP-PilT	Bipolar	+	+
+ YTG135S (WA)	Diffuse	-	-
+ YTE159Q (Asp Box)	Bipolar	Reduced	+
+ YTD160N (Asp Box)	Bipolar	Reduced	+
+ YTE163Q (Asp Box)	Bipolar	-	-
+ YTE204Q (WB)	Bipolar	-	-
+ YTH222A (His Box)	Bipolar	Reduced	+
+ YTH229A (His Box)	Bipolar	-	-
<i>PilU</i> ⁻ mutant	NA	-	+
+ YFP-PilU	Unipolar	+	+
+ YUG135S (WA)	Unipolar	-	+
+ YUE159Q (Asp Box)	Unipolar	Reduced	+
+ YUD160N (Asp Box)	Unipolar	-	+
+ YUE163Q (Asp Box)	Unipolar	-	+
+ YUE204Q (WB)	Unipolar	-	+
+ YUH222A (His Box)	Unipolar	+	+
+ YUH229A (His Box)	Unipolar	-	+

did not affect unipolar localization in the *pilU* mutant background (Fig. 5). When the WA mutant of YFP-PilT was introduced into the wild-type, the non-functional fusion protein was localized to both poles, similar to the wild-type pattern (Fig. 5). These results are consistent with those for the truncated constructs we reported previously (Chiang *et al.*, 2005), which retained their specific patterns of polar localization even when the secretion NTPase motif regions were deleted. Because these data suggest an interaction between active and inactive subunits, all mutated constructs unable to complement twitching motility in their cognate mutant background were introduced into the PAK wild-type strain to test for dominant-negative effects. None of the mutant constructs impaired twitching motility in the wild-type strain (data not shown).

DISCUSSION

This study demonstrated that the three motor proteins of *P. aeruginosa* have low, but quantifiable, ATPase activity *in*

Fig. 3. (a) Twitching motility of strains complemented with wild-type (wt) or mutant PilB, PilT and PilU proteins. Toothpick marks represent cells that have grown but were unable to twitch. Twitching strains appear as stained circles representing cells that have moved away from the point of inoculation. Complementation of the PilB mutant with *pilB* in *trans* did not restore twitching to 100 % of wild-type levels. Bar, 1 cm. (b) Residual twitching motility in Asp Box 1 and 2 mutants. While mutation of Asp Box 3 (see Fig. 1b) in any of the three proteins caused loss of motility, mutation of Asp Box 1 or 2 reduced motility to various degrees, depending on the specific ATPase. Shown are the average twitching zone areas (12 zones each, four technical replicates from three independent plates) for unmodified and mutant proteins.

vitro. The low activity of these proteins *in vitro* likely reflects the lack of their normal interaction partners. Recent studies of the T4P ATPase BfpD (a homologue of PilB) from the bundle-forming pilus system of *E. coli* have shown that its *in vitro* activity can be markedly enhanced (up to three orders of magnitude) in the presence of zinc, and upon allosteric interactions with specific regions of its partner proteins, including BfpE, a PilC homologue (Crowther *et al.*, 2005). Zinc would not be predicted to enhance the activity of the *P. aeruginosa* PilT and PilU proteins, as they lack the tetracysteine motif required for its coordination (Crowther *et al.*, 2005; Robien *et al.*, 2003).

We have shown previously that PilB requires PilC for polar localization (Chiang *et al.*, 2005); however, it has not yet been determined which region(s) of *P. aeruginosa* PilC interact with PilB, nor have potential partners for PilT and PilU yet been identified.

The demonstration that PilB, PilT and PilU have ATPase activity *in vitro* was consistent with the loss of function of each protein (as measured by the inability to complement twitching motility, and for PilT, loss of activity) when either of the WA or WB motif was mutated. WA mutations in the related proteins PilQ of the R64 thin pilus system

Fig. 4. Surface piliation of ATPase mutants. Surface proteins were sheared from each strain as described in Methods, and the resulting preparations analysed by Coomassie-stained 15% SDS-PAGE. null, mutant lacking the indicated protein; comp, mutant complemented *in trans* with the wild-type protein.

Fig. 5. Fluorescence microscopy of the WA mutants of YFP-PilT and YFP-PilU. (a) Bipolar localization of wild-type YFP-PilT in the *pilT* mutant; (b) loss of bipolar localization of YFP-PilT G135S in the *pilT* mutant; (c) expression of the YFP-PilT G135S mutant in wild-type PAK cells restores bipolar localization. The same mutation in YFP-PilU (G135S) does not affect its typical unipolar localization (d). Magnification, $\times 100$. Bar, 4 μm .

and DotB of *Legionella pneumophila* (T4S) have been shown to be dominant-negative, whereas VirB11 (T4S) WA mutants have not (Bhattacharjee *et al.*, 2001; Sakai *et al.*, 2001; Stephens *et al.*, 1995). Turner *et al.* (1993) have shown that a WA mutant of XcpR (*P. aeruginosa* T2S ATPase) causes dominant-negative effects on secretion in the wild-type, but that the identical mutation in PilB does not affect the presence of external T4P in a wild-type strain, as assessed by electron microscopy. This finding was confirmed by our work, as neither WA nor WB mutants of PilB impaired twitching motility when expressed in the wild-type. Similarly, PilT and PilU proteins with mutations in these motifs did not affect twitching in wild-type transformants.

These results suggest that only a subset of monomers in any of the three ATPases need be functional for pilus extension and retraction to occur. Recent studies of BfpD have shown that while all six members of a BfpD hexamer bind ATP, only alternating subunits interact with the BfpE peptide (Crowther *et al.*, 2005), a finding that is consistent with current models of the pilus as a three-start helix (Ramboarina *et al.*, 2005; Craig *et al.*, 2004, 2006). Structural studies of *A. aeolicus* PilT have revealed an interesting intermediate quaternary structure that contains a combination of subunits in partially open, open and closed conformations (Satyshur *et al.*, 2007), showing that heterogeneity in the conformational state of individual subunits within the hexameric assembly is likely to be a common feature of these proteins.

The WA mutant of YFP-PilT, but not YFP-PilU, delocalized from the cell pole. Although the factors required for the bipolar localization of PilT are unknown, it is conceivable that substrate binding is required for

correct interaction with the hypothetical polar retention protein or complex. Shiue *et al.* (2006) have found that binding of ATP by the T2S ATPase XpsE (a PilB homologue) is necessary for its oligomerization and subsequent interaction with its membrane anchor protein, XpsL. In the absence of ATP, or upon introduction of mutations that abrogate the binding of ATP, XpsE is monomeric and does not associate with XpsL. Other ATPases in the T4S family, such as HP0525 or TrbB (from conjugative plasmid RP4), have been observed to undergo ATP-independent oligomerization (Savvides *et al.*, 2003; Krause *et al.*, 2000), suggesting that this step can precede ligand binding, allowing the formation of localization-competent hexamers. It is possible that substrate binding is required for efficient oligomerization of PilT and that the delocalized cytoplasmic fluorescence arose from non-interacting monomers, though this was not tested directly. YFP-PilT WA mutant proteins expressed in the wild-type were correctly localized (Fig. 5c), suggesting that formation of stable mixed oligomers occurs despite the lack of dominant-negative effects on twitching motility, supporting the premise that only a subset of monomers need be active for twitching motility to occur.

Although the Asp and His Box motifs are widely conserved in the secretion NTPase family, their specific contributions to protein function have not been extensively investigated. In this work, we focused on the invariant acidic residues in the Asp Box motif, and on the invariant His residues in the His Box motif. We examined the molecular environment of the corresponding conserved residues in the crystal structures of the *A. aeolicus* PilT protein (Satyshur *et al.*, 2007), and the T2S ATPase EpsE (Robien *et al.*, 2003). The first invariant Asp Box 1 residue is E172 in PilT_{Ad} and E292

in EpsE. In EpsE, this amino acid is less than 3.2 Å (0.32 nm) from several residues, including V307, Q304 and Y275. Interestingly, all three of these residues are conserved in PilB but not PilT or PilU, and mutation of Asp Box 1 has more profound effects on PilB than on the other two proteins. The Y275 residue is close enough to form a hydrogen bond with the main chain nitrogen of the essential E296 residue (Asp Box 3 in Fig. 1b).

The second conserved Asp residue (Asp Box 2; D173 in PilT_{Aa}, D293 in EpsE, Fig. 1b) is required in all three twitching ATPases for normal twitching motility, although PilU function is most severely affected by its mutation. Site-directed mutagenesis of the corresponding Asp residue in the T2S protein PulE results in reduced secretion, while mutation of the Asp residue in R64 PilQ reduces both ATPase activity and mating efficiency in liquid (Sakai *et al.*, 2001; Possot *et al.*, 1992). Examination of the EpsE structure shows that the D293 residue forms a salt bridge to R336, stabilizing its guanidinium moiety. This Arg, which is conserved among all three twitching ATPases, interacts with the carboxylate of E334 in the WB motif, which was shown in this work to be essential for the function of all three ATPases. Disruption of the orientation of the WB Glu could cause the observed reductions in activity, with differences in the local environments in the three proteins modulating the severity of the mutation.

The third of three invariant acidic residues in the Asp Box (corresponding to E176 in PilT_{Aa} and E296 in EpsE, Fig. 1b) was essential for twitching motility in all three T4P AAA + proteins, and its mutation markedly reduced the *in vitro* ATPase activity of PilT. The corresponding residue in the related RecA protein (Glu96) has been proposed to activate an attacking water molecule during ATP hydrolysis (Story & Steitz, 1992). In both EpsE and PilT, this Glu is closest to the γ -phosphate of the ligand (AMP-PNP or ATP, respectively), while in the ADP-bound structure of PilT, there is a water molecule in the approximate position of the γ -phosphate, less than 4 Å (0.4 nm) from E176

(Satyshur *et al.*, 2007). The position of this residue and its requirement for the functioning of all three proteins tested suggest that it participates in catalysis.

Mutation of the first of two invariant His residues in the His Box motif resulted in a truncated PilB protein, precluding further analysis of its function in that protein. The YFP-PilT H222A mutant had twitching motility reduced by approximately 50 % relative to the unmodified protein (Fig. 3a), while YFP-PilU was not significantly impaired by this mutation. An H222R mutation in PilT_{Pa} has been shown by Satyshur *et al.* (2007) to abrogate twitching motility altogether. This residue is oriented away from the ligand-binding site and probably participates in subunit-subunit interactions (Fig. 6). Because the interaction surfaces of each ATPase in its hexameric state are formed in part by the less-conserved N-terminal domains Robien *et al.* (2003), mutation of this residue may have less impact in some proteins than others due to differences in local environment. The smaller, non-polar Ala side chain substituted here appears less perturbing to PilT activity than the large positively charged Arg side chain used by Satyshur *et al.* (2007).

In contrast, the second His (H242 in PilT_{Aa}, H359 in EpsE) was essential for twitching in all three T4P proteins, and for the *in vitro* ATPase activity of PilT. Examination of the EpsE structure shows that the imidazole nitrogen of H359 is predicted to be within 2.7 Å (0.27 nm) of the terminal phosphate oxygen atom of the ligand in a preferred side-chain conformation. This conformation, with the imidazole ring rotated towards the ligand, can be visualized in the ATP-bound PilT_{Aa} structure (Fig. 1b). This proximity makes it possible for His Box 2 to act as a phosphate sensor, as has been proposed for other ATPases such as RecA (Story & Steitz, 1992), with rotation of the side chain occurring upon hydrolysis. Mutation of the corresponding residue in plasmid R64 PilQ (a PilB homologue) reduced ATPase activity and DNA transfer efficiency; the other His residue was not mutated in that study (Sakai *et al.*, 2001).

Fig. 6. Relative positions of the His Box 1 and 2 residues in EpsE and PilT_{Aa}. The His Box 1 residue is solvent-exposed and probably affects subunit-subunit interactions, while the His Box 2 residue is positioned in the ligand-binding cleft where it could act as a phosphate sensor.

Of the three T4P motor proteins (PilB, PilT and PilU) in *P. aeruginosa* that are required for twitching motility, the function of PilU remains the most enigmatic. From previous studies of the function of PilB and PilT in *P. aeruginosa* and other bacteria, it is clear that they play antagonistic roles in the extension and retraction of T4P. In contrast, PilU and its homologues are less commonly identified in T4P-expressing bacteria, suggesting that it is not a universal component of the T4P apparatus (Chiang *et al.*, 2005). In *P. aeruginosa*, *pilU* mutants appear hyperpiliated, similar to *pilT* mutants; therefore, PilU is thought to be involved in pilus retraction. The inability of WA and WB mutants of PilU to complement twitching motility in a *pilU* mutant is consistent with *in vitro* assays that show that purified PilU has ATPase activity. Therefore, we conclude that *P. aeruginosa* requires three distinct, functional ATPases for twitching motility to occur. However, the continued sensitivity of *pilU* mutants to phage killing suggests that unlike *pilT* mutants, they are capable of some level of pilus retraction (mediated by PilT), although it is not sufficient to enable twitching motility to occur.

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