PilB and PilT are ATPases acting antagonistically in type IV pili function in

*Myxococcus xanthus*

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Abstract

Type IV pili (T4P) are dynamic surface structures that undergo cycles of extension and retraction. T4P dynamics center on the PilB and PilT proteins, which are members of the secretion ATPase superfamily of proteins. Here, we show that PilB and PilT of the T4P system in *Myxococcus xanthus* have ATPase activity *in vitro*. Using a structure-guided approach, we systematically mutagenized PilB and PilT to resolve whether both ATP binding and hydrolysis are important for PilB and PilT function *in vivo*. PilB as well as PilT ATPase activity was abolished *in vitro* by substitution of conserved residues in the Walker A and Walker B boxes involved in ATP binding and hydrolysis, respectively. PilB proteins containing mutant Walker A or Walker B boxes were non-functional *in vivo* and unable to support T4P extension. PilT proteins containing mutant Walker A or Walker B boxes were also non-functional *in vivo* and unable to support T4P retraction. These data provide genetic evidence that both ATP binding and hydrolysis by PilB are essential for T4P extension and that both ATP binding and hydrolysis by PilT are essential for T4P retraction. Thus, PilB and PilT are ATPases that act at distinct steps in the T4P extension/retraction cycle *in vivo*. 
Introduction

Type IV pili (T4P) are versatile, filamentous surface structures found in many Gram-negative bacteria. In *Myxococcus xanthus*, *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae* T4P mediate surface motility (27). T4P also mediate attachment and microcolony formation by human pathogens such as *Escherichia coli*, *N. gonorrhoeae*, *P. aeruginosa* and *Vibrio cholerae* on eukaryotic host cells (6). Moreover, T4P have important function in biofilm formation (22, 34) and DNA uptake by natural transformation (9). A hallmark of T4P compared to other filamentous surface structures is their dynamic nature, i.e. T4P undergo cycles of extension and retraction and it is during the retraction step that a force sufficiently large to pull a bacterial cell forward is generated (29, 51, 52).

T4P are thin (5-8 nm), flexible, helical filaments several microns in length, with high tensile strength (>100 pN) and typically only composed of the PilA pilin subunit (6). The protein machinery required for T4P biogenesis and function is highly conserved and encompass 17 proteins as defined for T4P in *N. meningitidis* (4). These proteins localize to the cytoplasm, inner membrane, periplasm and outer membrane (35). *In vitro* analyses and genetic analyses of T4P in *N. meningitidis* suggest that these proteins interact extensively and form a trans-envelope complex (4). Many of the proteins involved in T4P biogenesis and function share similarity with proteins found in type II secretion systems (T2SS) and archaeal flagella systems (35). Several of the proteins are phylogenetically related suggesting that the three machineries may share functional characteristics (35). Indeed, overexpression of pseudopilins from the T2SS in *Klebsiella oxytoca*, *Xanthomonas campestris* and *P. aeruginosa* results in the formation of pilin-like structures (10, 16, 45).
T4P dynamics includes two steps: Extension by polymerization in a process that involves the addition of pilin subunits from a reservoir in the inner membrane (31) to the base of the pilus (7), and retraction by depolymerization in a process that involves the removal of pilin subunits from the base and with the pilin subunits being transferred to the inner membrane (29, 31, 51, 52). The dynamic extension/retraction cycle of T4P centers on two members of the superfamily of secretion ATPases, PilB and PilT, which have been identified in all T4P systems. With the exception of the PilT protein, all T4P proteins analyzed including PilB are required for T4P extension (27, 55) whereas the PilT protein is specifically required for T4P retraction (29). T2SS only contains one ATPase, which are orthologs of the PulE protein in \textit{K. oxytoca}, and closely related to PilB (35, 36).

PilB, PilT and PulE belong to distinct subfamilies of the superfamily of secretion ATPases (35, 36). In addition to T4P systems and T2SS, secretion ATPases have been identified in type IV secretion systems (T4SS) as well as in archaeal flagella systems (35, 36). PilB and PulE orthologs contain a relatively well-conserved N-terminal region of 160-175 amino acids that is not present in PilT orthologs (35) (Fig. 1A). Structural analyses of six secretion ATPases (HP0525, which is part of the T4SS of \textit{Helicobacter pylori} (47, 61), EpsE, which is part of the T2SS in \textit{V. cholera} (40), XpsE, which is part of the T4SS of \textit{X. campestris} (5), VirB11 of the \textit{Brucella suis} T4SS (12), afGspE, which functions in protein secretion in \textit{Archaeoglobus fulgidus} (60), and PilT from \textit{Aquifex aeolicus} (44)) have shown that these 160-175 residues are followed by a region of 110-130 amino acids (Fig. 1AB), which is relatively well-conserved in secretion ATPases and folds into a structurally conserved domain referred to as the N-terminal domain. The N-terminal domain is followed by a highly conserved region of 190-240 amino acids (Fig. 1AB), which also folds into a structurally conserved domain referred to as the C-terminal domain encompassing the sequences associated with...
ATP binding and hydrolysis including four conserved sequence motifs: The Walker A box with the P-loop GX₄GK(S/T), the atypical Walker B box motif Dh₄GE (h stands for hydrophobic residue), the His-box and the Asp-box (Fig. 1AB) (12, 40, 44, 46, 47, 60, 61).

The unifying picture emerging from structural analyses of secretion ATPases is that they are dynamic hexameric assemblies that bind and hydrolyze ATP and with ATP binding and hydrolysis inducing major conformational changes that could facilitate the formation of macromolecular complexes (46). Several lines of evidence support this picture. Generally, Walker A box substitutions in PulE orthologs interfere with the normal functioning of these proteins in vivo (37, 38, 43, 53). Specifically, the PulE orthologs EpsE of V. cholerae (3) and XpsE of X. campestris (50) have been shown to have ATPase activity and to form oligomers in vitro. Moreover, substitution of the conserved lysine residue in the Walker A box leads to a reduction in ATPase activity in both proteins (3, 50) and mutants that are unable to support secretion (43, 50). For the PulE ortholog afGspE in A. fulgidus, substitution of the lysine residue in the Walker A box abolishes ATP binding (60). For PilB orthologs, PilQ, which is required for formation of thin conjugative pili in E. coli, (41) and BfpD, which is required for formation of bundle-forming pili in E. coli, were reported to have ATPase activity in vitro and to form homoctamers and homohexamers, respectively in vitro (8).

Moreover, in PilQ substitution of the conserved lysine residue in the Walker A box abolishes ATPase activity in vitro and results in the inability to assemble pili (41). For PilT orthologs, ATPase activity was reported in vitro for PilT of Synechocystis sp. PCC 6803 (33), PilT of Microcystis aeruginosa (32), and for hexameric PilT from A. aeolicus (13). Moreover, for the latter protein substitution of the conserved lysine residue in the Walker A box abolishes ATP hydrolysis (13). However, the relevance of the PilT ATPase activity in vivo remains to be demonstrated.
In order to systematically address biochemically and genetically the function of a pair of PilB and PilT proteins acting in the same T4P system, we analyzed PilB and PilT of the T4P system in *M. xanthus*. In *M. xanthus* T4P are unipolarly localized (20) to the leading cell pole of the rod-shaped cells (30) and S-motility, which is the equivalent of twitching motility in *P. aeruginosa* and *N. gonorrhoeae* (27), depends on T4P (20, 58). Deletions of *pilB* and *pilT* in *M. xanthus* abolish S-motility (58, 59). We report that PilB and PilT of *M. xanthus* display ATPase activity *in vitro*. Using a structure-based approach to substitute key residues predicted to be involved in ATP binding and ATP hydrolysis, we show that ATPase activity in both proteins depends on intact Walker A and Walker B boxes and that both motifs are required for PilB and PilT function *in vivo*. Our data show that PilB and PilT are ATPases that act at distinct steps in the T4P extension/retraction cycle and they suggest that ATP binding as well as ATP hydrolysis are essential for PilB and PilT function *in vivo*. 


Materials and Methods

Cell growth and motility assays. Bacterial strains and plasmids used are listed in Table 1. Sequences of primers used in this work are available from the authors upon request. *M. xanthus* was grown in CTT medium in liquid cultures or on CTT, 1.5% agar plates as described (14). Kanamycin was used for selective growth at concentrations of 40 µg/ml. *E. coli* strains were grown in LB broth in the presence of relevant antibiotics (42). Strains to be tested for motility were grown in CTT to a density of 5x10^8 cells/ml, harvested and resuspended in MC7 (10 mM MOPS pH 7.0, 1 mM CaCl_2) to a calculated density of 5x10^9 cells/ml. 10 µl aliquots of cell suspensions were spotted on a thin layer of 0.5% agar supplemented with 0.5% CTT (15, 49). After 24 hrs, colony morphology and colony edges were observed visually in a Leica MZ8 stereomicroscope and visualized using a Leica DFC280 CCD camera.

Strain and plasmid construction. Plasmids were propagated in *E. coli* Top10 (F- mcrA Δ(mrr-hsdRMS-mcrBC)80lacZΔM15ΔlacX74, deoR, recA1arsD139 Δ(ara-leu)7697, galU, galK, rpsL (Str^R) endA1, nupG (Invitrogen) unless otherwise stated. All plasmids generated by PCR were verified by sequencing. To generate pSL105, which contains *pilB* cloned downstream of the *pilA* promoter, the primers opilB1 and opilB2 were used to amplify *pilB*. Subsequently, the PCR product was cloned in the plasmid pSW105, which contains the phage Mx8 attP site. To generate pSL104, which contains *pilT* downstream of the *pilA* promoter, the primers opilT1 and opilT2 were used to amplify *pilT*. Subsequently, the PCR product was cloned in the plasmid pSW105. To generate mutations in the Walker A and Walker B boxes of *pilT* and *pilB*, one of the four mutagenic primers (opilTWalkerA, opilTWalkerB, opilBWalkerA and opilBWalkerB) was used in a first round of PCR with opilT4 or opilB4 and pSL104 or pSL105 as templates. Products from the first PCR reactions were used as primers in a second PCR reaction with the primers opilT1 or opilB1 and pSL104 or pSL105 as templates.
PCR products were cloned in pSW105 to create pSL104TWalkerA, pSL104TWalkerB, pSL105BWalkerA and pSL105BWalkerB. Plasmids were electroporated into wild type *M. xanthus* and ∆*pilT* or ∆*pilB* mutant strains (21) and correct clones verified by PCR.

To construct pSL5, which encodes the His<sub>6</sub>-*pilB* allele expressed from the *tac* promoter, *pilB* was amplified using the primers opilB3 (with an *Eco*RI site followed by six histidines codons and the first 22 nucleotides of *pilB*) and opilB4 (containing the last 19 nucleotides of *pilB* and a *Hind*III site). The PCR product was cloned in pUHE24-2 (24) to give pSL5. To construct pSL4, which contains the His<sub>6</sub>-*pilT* allele expressed from the *tac* promoter, *pilT* was cloned in pUHE24-2 as described for *pilB* except that the primers opilT3 (*Eco*RI site followed by codons for six histidines and the first 18 nucleotides of *pilT*) and opilT4 (*Hind*III site and the last 19 nucleotides of *pilT*) were used. Constructs overexpressing His<sub>6</sub>-PilB with substitutions in either the Walker A or Walker B box were created by *Eco*RV/*Hind*III excising a fragment containing the mutation from pSL105BWalkerA or pSL105BWalkerB followed by cloning in pSL5 to obtain pSL5BWalkerA and pSL5BWalkerB. The same strategy was used to generate pSL4TWalkerA and pSL4TWalkerB, which encode His<sub>6</sub>-PilT proteins containing substitutions in either the Walker A or Walker B box. Expression plasmids were propagated in JM109 (F<sup>−</sup> traD36, proA<sup>B</sup>B<sup>−</sup>/lacI<sup>q</sup> ΔlacZM15/Δ(lac-proAB)glnV44 e<sup>14</sup>, gyrA<sup>96</sup>, recA1, relA1 endA1, thi, hsdR17) (New England Biolabs) with the plasmid pMS421 (11).

**Cell fractionation and western blots.** Biochemical fractionation of cells was done as described (57). Briefly, 50 ml of exponentially growing *M. xanthus* cells of DK1622GFP, which expresses the green fluorescent protein (GFP) from the *pilA* promoter, were grown in CTT medium to a cell density of 5×10<sup>8</sup> cells per ml harvested and resuspended in 2.5 ml of buffer containing 20% sucrose (w/v), 50 mM Tris-HCl (pH 7.6) supplemented with Complete Mini Protease Inhibitor cocktail (Roche). After incubation for 10 min at 25°C, cells were harvested and osmotically shocked by
resuspension in 0.5 ml of 4°C 50 mM Tris-HCl (pH 7.6) with the protease inhibitor cocktail. After a short incubation at 4°C, spheroplast formation was verified microscopically. The supernatant (containing periplasmic proteins) was separated from spheroplasts by centrifugation at 4400xg for 10 min at 4°C. Spheroplasts were lysed by sonication and cleared from non-lysed cells by centrifugation at 16000xg for 10 min at 4°C. The cleared lysate was centrifuged at 100000xg for 1 hr at 4°C to separate soluble, cytoplasmic proteins from the insoluble, membrane enriched fraction. Proteins from all collected fractions (periplasmic, cytoplasmic and membrane) were precipitated with acetone at -20°C overnight, resuspended in SDS-PAGE loading buffer; separated by SDS-PAGE, and analyzed in Western blots. Western blots were performed using standard procedures (42) using polyclonal anti-PilT and anti-PilB antibodies and peroxidase conjugated goat-anti-rabbit immunoglobulin G as secondary antibodies as recommended by the manufacturer (Roche). As controls for proper fractionation, fractions were tested with antibodies against GFP in the cytoplasm, full-length CsgA protein in the outer membrane (23, 25), and the inner membrane protein PilC (V. Jakovljevic and L. Søgaard-Andersen, unpublished). In case of GFP, monoclonal anti-GFP mouse antibody (Roche) and peroxidase conjugated rabbit-anti-mouse secondary antibody (DakoCytomation) were used. Blots were developed using the Supersignal West Pico Chemiluminescence reagent (Pierce).

Purification of His<sub>6</sub>-PilB. JM109/pMS421 containing pSL5 was grown in 2xYT at 37°C (42) and His6-pilB expression induced by addition 0.1 mM IPTG. Cells were harvested at 5000xg for 20 min, resuspended in NLT buffer (300mM NaCl, 50 mM Tris-HCl, 10% Glycerol, 5 mM β-mercaptoethanol, 10 mM imidazole, pH 8.0), and lysed by sonication. The cell lysate was cleared at 13000xg for 30 min at 4°C and His6-PilB in the supernatant purified using Ni-NTA agarose (Qiagen) following the recommendations of the supplier. Protein concentrations were determined using the Bradford assay (Biorad). If necessary, purified His<sub>6</sub>-PilB was concentrated by
ultrafiltration through an Microcon filter with 30 kDa molecular weight cut-off (Millipore). Mutant His<sub>6</sub>-PilB proteins were purified using the same procedure.

**Purification of His<sub>6</sub>-PilT.** JM109/pMS421 containing pSL4 was grown in 2xYT at 37°C. Cells were harvested and lysed as described for PilB. After clearing of the cell lysate, the pellet containing His<sub>6</sub>-PilT was resuspended in DTB buffer (8 M Urea, 0.1 M Tris-HCl, pH 8.0), incubated for 1 hr at room temperature with shaking to dissolve inclusion bodies and centrifuged for 30 minutes at 13000xg at 4°C. The supernatant was incubated with Ni-NTA agarose (Qiagen) and denatured His<sub>6</sub>-PilT purified following the recommendations of the supplier. Protein concentrations were determined using the Bradford assay. To refold His<sub>6</sub>-PilT, 4 mg of denatured His<sub>6</sub>-PilT protein were added drop-wise to 80 ml of refolding buffer A (0.5 M L-arginine, 1 mM DTT, 0.1 M Tris-HCl pH 8.0) and gently stirred overnight at 4°C. Next day, 20 ml of the mixture was concentrated by ultrafiltration through an Amicon filter (Millipore) with 30 kDa molecular weight cut-off (Millipore). Buffer A was exchanged by addition of 10 ml of NLT buffer to the same filter and subsequent centrifugation at 4000xg to a final volume of 0.5-1ml. Mutant His<sub>6</sub>-PilT proteins were purified using the same procedure.

**Determination of ATPase activity of His<sub>6</sub>-PilB.** Phosphate release during ATP hydrolysis was measured using the EnzChek Phosphate assay kit (Molecular Probes). Briefly, each reaction contained 5 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 200 µM EnzChek MESG substrate (2-amino-6-mercapto-7-methyl-purine riboside) and 0.1 unit of PNP (purine nucleoside phosphorylase). In presence of inorganic phosphate MESG is converted via PNP to ribose-1-phosphate and 2-amino-6-mercapto-7-methylpurine leading to an increase in A<sub>360</sub>. To determine the specific ATPase activity of His<sub>6</sub>-PilB, A<sub>360</sub> was measured in 1 minute intervals during 60 minutes at room temperature. Reactions were started by addition of ATP to a final concentration of 0.5 mM and His<sub>6</sub>-PilB to a concentration of 5 µM in a total volume of 100 µl. Phosphate release was
determined by comparing $A_{360}$ to a standard curve prepared with $\text{KH}_2\text{PO}_4$ as described (48). The blank reaction was a reaction mixture including protein, but without ATP. The blank value was subtracted from reads of $A_{360}$, the release of free phosphate estimated from the standard curve, and specific activity calculated.

**Determination of ATPase activity of His$_6$-PilT.** 25 µl reactions were prepared containing 15 µM of His$_6$-PilT in NLT buffer supplemented with 50 mM KCl and 5 mM MgCl$_2$.

Reactions were started by addition of radioactively labelled $\alpha$-$^{32}$P-ATP (GE Heathcare) giving a specific activity of 3.2 MBq per ml and final ATP concentration of 0.2 mM. After 30 minutes incubation at room temperature, protein was removed by ultrafiltration using a Microcon filter with 30 kDa molecular weight cut-off (Millipore). Labelled adenosine phosphates were separated by thin layer chromatography (TLC) as described (39). Briefly, aliquots of 1.0 µl of the eluate were applied to a poly(ethyleneimine)-cellulose FTLC plate (Merck) with 2.4 M formic acid as the solvent system. The labelled nucleotides on the TLC plates were visualized and quantified by phosphorimaging and analyzed using the ImageQuant software. [$\alpha$-$^{32}$P]-ATP and [$\alpha$-$^{32}$P]-ADP were located on the TLC plates using the products of ATP hydrolysis by the apyrase ATPase (Sigma-Aldrich) as markers.

**Antibody generation.** To generate rabbit, polyclonal anti-PilT and anti PilB antibodies, His$_6$-PilT and His$_6$-PilB were overexpressed from pSL4 and pSL5, respectively and purified under denaturing conditions as described for His$_6$-PilT and used to immunize rabbits using standard procedures (42).

**Transmission electron microscopy of T4P.** TEM was used to visualize T4P essentially as described (54). Briefly, 50 µl of exponentially growing *M. xanthus* cultures were placed on parafilm. A small piece of carbon-coated mica was dipped into the drop for
30 s allowing cells to adsorb to the surface of the carbon film. The carbon film was picked with a copper grid, excess liquid soaked off, placed briefly on a drop of distilled water, excess liquid soaked off again, transferred on a drop of 2% uranyl acetate (w/v) for two seconds, and blotted dry. Transmission electron microscopy was performed on a Philips EM 301 electron microscope at calibrated magnifications.
Results

PilB and PilT of *M. xanthus* are ATPases

PilB and PilT of *M. xanthus* conform to the general description of secretion ATPases and contain all four conserved sequence motifs in the C-terminal domain (Fig. 1). These analyses, taken together with the observation that PilB and PilT of *M. xanthus* are required for T4P formation and retraction (58, 59), respectively, made these two proteins a good pair for systematically analysing the requirements for ATP binding and hydrolysis by PilB and PilT in T4P function.

It has previously been suggested that PilT is localized to the periplasm (19). A periplasmic localization of PilT would argue against ATPase activity being important for function. To determine the localization of PilB and PilT, we carried out fractionation experiments in which total cell extract of the strain DK1622GFP was separated into fractions enriched for cytoplasmic, periplasmic and membrane proteins and subsequently probed with antibodies against either full-length His$_6$-PilB or full-length His$_6$-PilT. In immunoblot analyses of total cell extract from DK1622GFP and DK10416, which carries an in-frame deletion of *pilB*, the anti-PilB antibodies recognized a protein with a size of 63 kDa in DK1622GFP, which was not present in DK10416 (Fig. 2A). The size of this protein is in good agreement with the calculated molecular mass of PilB of 63.2 kDa, thus, confirming that the antibodies are specific. Likewise, in immunoblot analyses of total cell extract from DK1622GFP and DK10409, which carries an in-frame deletion of *pilT*, the anti-PilT antibodies recognized a protein with a size of 39 kDa in DK1622GFP, which was not present in DK10409 (Fig. 2B). The size of this protein is in good agreement with the calculated molecular mass of PilT of 40.7 kDa, thus, confirming that the antibodies are specific. In the cell fractionation experiments, PilB and PilT fractionated with the cytoplasmic fraction (Fig. 2AB). Control experiments in which the fractions were tested with antibodies against the
cytoplasmic GFP, full-length CsgA protein in the outer membrane (23, 25), and the inner membrane protein PilC (V. Jakovljevic and L. Søgaard-Andersen, unpublished) demonstrated that the fractionation functioned properly (data not shown). Thus, we conclude that PilB and PilT are cytoplasmic proteins.

To test whether PilB of *M. xanthus* has ATPase activity, the *pilB* gene was cloned and PilB overexpressed in *E. coli* as an N-terminal hexahistidine fusion protein. His$_6$-PilB was purified under native conditions by Ni$^{2+}$ affinity chromatography. The apparent molecular mass of purified His$_6$-PilB was 62 kDa as calculated from SDS-PAGE and in good agreement with the calculated molecular mass of 63.8 kDa of His$_6$-PilB (Fig. 3A). The specific ATPase activity of purified His$_6$-PilB was measured using a coupled enzyme assay, which detects release of phosphate from ATP. As shown in Fig. 3C, we observed increased phosphate release with time when His$_6$-PilB was incubated with ATP, demonstrating that His$_6$-PilB possesses ATPase activity *in vitro*. We calculated the specific ATPase activity of His$_6$-PilB to $2.9 \pm 0.6$ nmol of phosphate min$^{-1}$ mg$^{-1}$ (mean ± standard deviation). As a positive control for ATPase activity, we used the enzyme apyrase, which was found to have a specific activity of $5.3 \pm 0.2$ µmol of phosphate min$^{-1}$ mg$^{-1}$ (data not shown) similarly to that previously observed (48).

We also sought to test whether His$_6$-PilB forms a homooligomer *in vitro* using gel filtration chromatography. Under all conditions tested, i.e. in the presence or absence of ATP, ADP, the non-hydrolyzable ATP homolog ATP-$\gamma$-S, and Mg$^{2+}$, His$_6$-PilB eluted as a sharp peak with an estimated molecular mass of ~60 kDa corresponding to the size of the His$_6$-PilB monomer (data not shown). Thus, we could not demonstrate that His$_6$-PilB forms an oligomer.

PilT has been previously purified from *A. aeolicus* as a soluble C-terminal hexahistidine fusion (13) and as soluble N-terminal hexahistidine fusion proteins in the
case of two cyanobacterial species (32, 33). We cloned \textit{pilT} of \textit{M. xanthus} and attempted to overexpress the protein with either an N-terminal hexahistidine tag or with a C-terminal hexahistidine tag. We only obtained expression of the N-terminal hexahistidine fusion protein (Fig. 3B). Under all expression conditions tested His\textsubscript{6}-PilT was found in inclusion bodies (data not shown). Consequently, we purified His\textsubscript{6}-PilT under denaturing conditions and renatured it as described in Materials and Methods. The apparent molecular mass of purified His\textsubscript{6}-PilT was 39 kDa as calculated from SDS-PAGE and in agreement with the calculated molecular mass of 41.3 kDa of His\textsubscript{6}-PilT (Fig. 3A, right panel). In order to test His\textsubscript{6}-PilT for ATPase activity, we initially used the coupled enzyme assay described above for His\textsubscript{6}-PilB. However, under all conditions tested His\textsubscript{6}-PilT formed precipitates in this assay, thus, precluding the determination of enzyme activity. Instead we used an enzyme assay in which the release of $\alpha^{32}\text{P}-\text{ADP}$ from $\alpha^{32}\text{P}-\text{ATP}$ was measured. His\textsubscript{6}-PilT remained soluble under the conditions used in this assay. Importantly, we observed a low but significant increase in accumulation of $\alpha^{32}\text{P}-\text{ADP}$ when His\textsubscript{6}-PilT was incubated with $\alpha^{32}\text{P}-\text{ATP}$ (Fig. 3D). However, the specific ATPase activity of His\textsubscript{6}-PilT could not be determined because ATP hydrolysis reached saturation within 5-10 min of incubation (data not shown). As positive control for ATPase activity in this assay we used apyrase, which was found to have a specific activity in this assay similar to that found under the conditions used to test His\textsubscript{6}-PilB (data not shown).

We also sought to determine whether His\textsubscript{6}-PilT forms a homooligomer \textit{in vitro} using gel filtration chromatography. The \textit{A. aeolicus} PilT protein was reported to form an oligomer in the absence of ATP and Mg\textsuperscript{2+} (13). The His\textsubscript{6}-PilT protein of \textit{M. xanthus} eluted as a sharp peak with an estimated molecular mass of $\sim$ 40 kDa corresponding to the size of a His\textsubscript{6}-PilT monomer under all conditions tested, i.e. in the presence or absence of ATP, ADP, the non-hydrolyzable ATP homolog ATP-γ-S, and Mg\textsuperscript{2+} (data
not shown). Thus, also for His<sub>6</sub>-PilT we could not demonstrate that it forms an oligomer.

**Substitutions in the Walker A and Walker B boxes of PilB and PilT abolish ATPase activity**

Based on the recently solved structure of several secretion ATPases, the conserved lysine residue in the Walker A box is important for ATP binding and the conserved glutamate residue in the Walker B box is important for ATP hydrolysis (12, 40, 44, 46, 47, 60, 61). To investigate the role of the Walker A and B boxes for PilB and PilT ATPase activity *in vitro*, the conserved lysine residue in the Walker A box and the conserved glutamate residue in the Walker B box were substituted individually with alanine in both proteins (Fig. 1).

The two mutant PilB proteins, PilB<sub>K327A</sub> and PilB<sub>E391A</sub>, were purified as N-terminal hexahistidine tagged proteins as soluble proteins as described for the wild type protein (Fig. 3A). The two mutant PilT proteins, PilT<sub>K137A</sub> and PilT<sub>E205A</sub>, were purified as N-terminal hexahistidine tagged proteins under denaturing conditions and then renatured as described for wild type His<sub>6</sub>-PilT (Fig. 3B).

The His<sub>6</sub>-PilB<sub>K327A</sub> and His<sub>6</sub>-PilB<sub>E391A</sub> proteins were strongly reduced in ATPase activity (Fig. 3C). Likewise, we were unable to detect ATP hydrolysis in the presence of the His<sub>6</sub>-PilT<sub>K137A</sub> and His<sub>6</sub>-PilT<sub>E205A</sub> proteins (Fig. 3D). These observations confirm that the ATPase activities measured for the wild type proteins were due to His<sub>6</sub>-PilB and His<sub>6</sub>-PilT rather than contaminating proteins. Moreover, these observations verify the importance of the conserved lysine residue in the Walker A box and the conserved glutamate residue in the Walker B box for PilB and PilT ATPase activity *in vitro*. Based on the structure of secretion ATPases and the analyses of mutant afGspE proteins (60), we suggest that the Walker A box substitutions (in His<sub>6</sub>-PilB<sub>K327A</sub> and His<sub>6</sub>-
PilT<sup>K137A</sup>) abolish ATPase activity indirectly by interfering with ATP binding and that the Walker B box substitutions (in His<sub>6</sub>-PilB<sup>E391A</sup> and His<sub>6</sub>-PilT<sup>E205A</sup>) abolish ATPase activity by directly interfering with ATP hydrolysis.

PilB and PilT ATPase activity is essential for T4P-dependent motility

In-frame deletion mutants of pilB and pilT of M. xanthus abolish T4P-dependent motility (58, 59). To determine genetically whether ATP binding and hydrolysis by PilB affect T4P-dependent motility in M. xanthus, we introduced the wild type pilB allele and the two mutant pilB alleles, pilB<sup>K327A</sup> and pilB<sup>E391A</sup>, into the strain DK10416, which carries an in-frame deletion of pilB. In the three plasmids containing the pilB alleles, the pilB genes were expressed from the constitutively active pilA promoter. The three plasmids were integrated by site-specific recombination at the phage Mx8 attB site on the chromosome giving rise to SA2021 (ΔpilB/pilB<sup>+</sup>), SA2414 (ΔpilB/pilB<sup>K327A</sup>) and SA2415 (ΔpilB/pilB<sup>E391A</sup>). To determine whether ATP binding and hydrolysis by PilT affect T4P-dependent motility, we used a similar strategy and introduced plasmids containing the wild type pilT allele and the two mutant pilT alleles, pilT<sup>K137A</sup> and pilT<sup>E205A</sup>, into the strain DK10409, which carries an in-frame deletion of pilT, giving rise to SA2020 (ΔpilT/pilT<sup>+</sup>), SA2412 (ΔpilT/pilT<sup>K137A</sup>) and SA2413 (ΔpilT/pilT<sup>E205A</sup>).

We analyzed the six strains for T4P-dependent motility defects on 0.5% agar, which favors motility by means of T4P using the wild type strain DK1622 as a positive control, and the strains DK10416 (ΔpilB), DK10409 (ΔpilT) and DK10410, which carries an in-frame deletion of the pilA gene that codes for the T4P subunit, as negative controls. As shown in Fig. 4A, the wild type DK1622 formed colonies with large rafts of cells at the edge typical of T4P-dependent motility whereas DK10416 (ΔpilB), DK10409 (ΔpilT) and DK10410 (ΔpilA) did not form rafts of cells at the edge, thus, confirming that pilB, pilT and pilA are required for T4P-dependent motility. Moreover, SA2021 (ΔpilB/pilB<sup>+</sup>) and SA2020 (ΔpilT/pilT<sup>+</sup>) displayed a motility
phenotype similar to that of the wild type DK1622. Thus, pilB\(^+\) and pilT\(^+\) expressed from the pilA promoter fully complemented the defects in T4P-dependent motility in the respective in-frame deletion mutants. Importantly, SA2414 (ΔpilB/pilB\(^{K327A}\)) and SA2415 (ΔpilB/pilB\(^{E391A}\)) displayed motility defects similar to those observed in DK10416 (ΔpilB). Thus, pilB\(^{K327A}\) and pilB\(^{E391A}\) were unable to complement the defect in T4P-dependent motility caused by the ΔpilB mutation. Also, SA2412 (ΔpilT/pilT\(^{K137A}\)) and SA2413 (ΔpilT/pilT\(^{E205A}\)) displayed motility defects similar to those observed in DK10409 (ΔpilT). Thus, pilT\(^{K137A}\) and pilT\(^{E205A}\) were unable to complement the defect in T4P-dependent motility caused by the ΔpilT mutation.

Immunoblot analysis with anti-PilB and anti-PilT antibodies confirmed that the PilB\(^+\), PilB\(^{K327A}\) and PilB\(^{E391A}\) proteins in SA2021, SA2414 and SA2415, respectively accumulated at levels similar to that observed for PilB\(^+\) in the wild type DK1622 and that the PilT\(^+\), PilT\(^{K137A}\) and PilT\(^{E205A}\) proteins in SA2020, SA2412 and SA2413, respectively accumulated at levels similar to that observed for PilT\(^+\) in DK1622 (Fig 4B). From these analyses we conclude that ATPase activity of PilB as well as of PilT is essential for T4P-dependent motility in M. xanthus. Moreover, our data suggest that not only ATP hydrolysis (likely directly abolished in PilB\(^{E391A}\) and PilT\(^{E205A}\)) but also ATP binding by PilB and PilT (likely directly abolished in PilB\(^{K327A}\) and PilT\(^{K137A}\)) are essential for T4P-dependent motility in M. xanthus.

PilB ATPase activity is essential for T4P extension and PilT ATPase activity is essential for T4P retraction

We used transmission electron microscopy to determine whether the lack of T4P-dependent motility in the Walker A and Walker B box mutants of PilB and PilT was due to the lack of T4P or due to non-functionality of T4P. Approximately 20 cells of each strain were analysed for the presence of T4P (Fig. 5 and Table 2). As previously
reported (20), the wild type strain DK1622 assembled T4P in a unipolar pattern. On the average nine T4P per cell assembled per pole. As previously reported (58, 59), DK10416 (ΔpilB) and DK10410 (ΔpilA) did not assemble T4P whereas DK10409 (ΔpilT) assembled T4P in a unipolar pattern. T4P formation was restored to wild type levels in SA2021 (ΔpilB/pilB') whereas SA2414 (ΔpilB/pilB^K327A) and SA2415 (ΔpilB/pilB^E391A) did not assemble T4P. SA2020 (ΔpilT/pilT'), SA2412 (ΔpilT/pilT^K137A) and SA2413 (ΔpilT/pilT^E205A) all formed T4P at wild type levels and in all cells analyzed the T4P were localized to one pole. These data taken together with the defects in T4P-dependent motility in the various mutants suggest that ATP binding as well as ATP hydrolysis by PilB are important for T4P extension and that ATP binding as well as ATP hydrolysis by PilT are not essential for T4P formation but essential for T4P retraction.
Discussion

Here, we present the first report in which the function of a pair of PilB and PilT proteins acting in the same T4P system have been analyzed systematically in vitro and in vivo. We showed directly in vitro that both PilB and PilT of M. xanthus have ATPase activity. PilB and PilT of M. xanthus both contain the conserved Walker A and Walker B boxes. The structures of five secretion ATPases (12, 40, 44, 46, 47, 60, 61) have revealed that the conserved lysine residue in the Walker A box is important for binding of ATP by making contacts to the β-phosphate and that the conserved glutamate residue in the Walker B box is pointing toward the γ-phosphate of ATP and appropriately positioned to activate a water molecule during hydrolysis. The five structures of secretion ATPases allowed us to use a structure-guided approach to substitute amino acid residues in PilB and PilT predicted to be involved in ATP binding or ATP hydrolysis, respectively. To determine the importance of ATP binding for PilB and PilT activity, we substituted the conserved lysine residues in the Walker B box to alanine. To determine the importance of ATP hydrolysis for PilB and PilT activity, we substituted the conserved glutamate residue in the Walker B box to alanine. As expected all four mutant proteins displayed loss of ATPase activity in vitro, confirming the importance of the substituted residues for ATPase activity suggested by the structural analyses. Importantly, when tested in vivo we observed that the Walker A and Walker B box mutants of PilB were unable to complement the defect in T4P dependent motility in a ∆pilB mutant. Moreover, a ∆pilB mutant containing the mutant pilB alleles did not assemble T4P. These data strongly suggest that ATP binding as well as ATP hydrolysis by PilB is essential for T4P assembly. For the two mutant PilT proteins we observed that the Walker A and Walker B box mutant were unable to complement the defect in T4P dependent motility in a ∆pilT mutant. However, the ∆pilT mutant containing the mutant PilT proteins still assembled T4P. These data strongly suggest that ATP binding as well as ATP hydrolysis by PilT is essential for
T4P retraction. The distinct phenotypes of insertion mutations in pilB and pilT previously lead to the suggestion by Whitchurch et al. (56) that PilB and PilT act at distinct steps in the T4P extension/retraction cycle and function antagonistically in T4P extension/retraction with PilB promoting the addition of pilin subunits to the growing pilus and PilT promoting their removal. Our data support this model with the addition that ATP binding and hydrolysis by PilB and PilT are essential for their antagonistic functions.

For His$_6$-PilB a specific ATPase activity of 2.9 nmol min$^{-1}$ mg$^{-1}$ was measured. This specific activity is similar to that previously reported for the PilB ortholog PilQ of the thin conjugal pilus system in E. coli (1 nmol min$^{-1}$ mg$^{-1}$) (41) but significantly lower than for the PilB ortholog BfpD of the bundle-forming pili in E. coli (62.2 nmol min$^{-1}$ mg$^{-1}$) (8). It has previously been reported that PilT proteins of mesophilic bacteria tend to be poorly soluble (1). Consistently, we observed that His$_6$-PilT of M. xanthus under all conditions tested formed inclusion bodies. His$_6$-PilT purified from the inclusion bodies, denatured and renatured formed precipitates under the assay conditions used to monitor ATPase activity of His$_6$-PilB. However, using an assay in which we monitored the conversion of α-$^{32}$P-ATP to α-$^{32}$P-ADP, we measured a low but significant ATPase activity of His$_6$-PilT. However, because His$_6$-PilT did not follow standard reaction kinetics, the specific ATPase activity could not be determined. The specific activities of two PilT proteins have been reported, i.e. PilT of A. aeolicus has a specific activity of 15.7 nmol min$^{-1}$ mg$^{-1}$ (13) and PilT of M. aeruginosa has a specific activity of 37.5 nmol min$^{-1}$ mg$^{-1}$ (32). In vivo several factors may contribute to increasing the ATPase activity of secretion ATPases, i.e. for BfpD it was reported that ATPase activity is stimulated 1200-fold by the interaction with cytoplasmic domains of the inner membrane proteins BfpC and BfpE (8), for EpsE of the TT2S in V. cholerae acidic phospholipids were reported to stimulate activity in combination with a cytoplasmic domain of the inner membrane protein EpsL (2), and for XpsE of the T2SS in X. campestris ATPase
activity is stimulated by interaction with the cytoplasmic domain of the inner membrane protein XpsL (50). Structural analyses of traffic ATPases strongly suggest that the active form of these proteins is hexameric and that intersubunit interactions in the hexamers would be important for ATPase activity (12, 40, 44, 47, 60, 61). We neither observed hexamer formation by His\textsubscript{6}-PilB nor by His\textsubscript{6}-PilT of \textit{M. xanthus}. We speculate that the active form of PilB and PilT \textit{in vivo} is hexameric and that the absence of hexamer formation \textit{in vitro} may contribute to the low ATPase activities observed for PilB and PilT of \textit{M. xanthus}.

The molecular mechanisms by which PilB and PilT function in the extension/retraction cycle of T4P remain unanswered. In principle, PilB as well as PilT could act as molecular motors that facilitate the incorporation of and removal of pilin subunits, respectively (28). According to this model, ATP binding by PilB followed by hydrolysis would provide the energy for the insertion of pilin subunits from a reservoir in the inner membrane into the growing pilus, and ATP binding by PilT followed by ATP hydrolysis would provide the energy for the removal of pilin subunit from the shrinking pilus into the inner membrane. A model for direct assembly of pilin subunits stimulated by PilB has been proposed in which ATP hydrolysis by PilB provides force to an unknown inner membrane protein, which in turn acts like a mechanical piston to push pilin subunits into a growing pilus (7). In the “power stroke” model, PilT was proposed to retract T4P by direct removal of pilin subunits from the base of the pilus (19). However, the power stroke model is based on the assumption that PilT is localized to the periplasm, which is in disagreement with our observations of the cytoplasmic localization of PilT.

However, other models are also possible for the function of PilB and PilT. Some biological fibers assemble and disassemble spontaneously (26). Merz and Forest (28) noticed that formally only one step in T4P function requires energy and with the
second process occurring spontaneously. Thus, if T4P assembly is energetically favourable and with pilin subunits melting spontaneously from the inner membrane into the growing T4P, then PilT could act as a molecular motor generating the energy required to retract T4P. In this model, PilB ATP binding and hydrolysis would have a regulatory function to stimulate the switch from retraction to assembly. Alternatively, assembly of T4P could be energy-dependent and with PilB acting as a molecular motor providing the energy required for the incorporation of pilin subunits into the growing pilus. In this model, PilT ATP binding and hydrolysis could have a regulatory function, which would serve to stimulate the switch from assembly to retraction. Interestingly, it was recently observed in *M. xanthus* that a two-fold decrease in the level of accumulation of the pilin subunit PilA resulted in a 10-fold decrease in the level of assembled T4P (17). These observations seem to support a spontaneous T4P assembly hypothesis. Clearly, our data do not allow us to distinguish between these models. However, our data show that irrespective of the function of PilB (molecular motor or regulator) and PilT (molecular motor or regulator) ATP hydrolysis are required by both proteins.
Acknowledgements

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15:255-257.

are dynamic hexameric assemblies: new insights into bacterial type IV


Legends to Figures

Figure 1. Domain structure of secretion ATPases.

A) Scheme of domain structure of PulE, PilB and PilT proteins. The conserved N-terminal region in PulE and PilB proteins, the N-terminal domain conserved in all secretion ATPases, and the C-terminal domain conserved in all secretion ATPases are indicated. Vertical grey bars in the C-terminal domain indicate the four conserved sequence motifs in secretion ATPases: Walker A box, Asp box, Walker B box and His box.

B) Alignment of PilB and PilT of *M. xanthus* with secretion ATPases. PilB (PilBMyxa) and PilT (PilTMyxa) of *M. xanthus* were aligned with PilB of *P. aeruginosa* (PilBPsea), PilF (a PilB ortholog) (PilFNe) of *N. gonorrhoeae*, EpsE of *V. cholerae* (EpsEVich), VirB11 of *B. suis* (VirB11Brsu), PilT of *A. aeolicus* (PilTAqae) and PilT of *P. aeruginosa* (PilTPsea). The conserved Walker A, Asp-, Walker B and His boxes are indicated. The conserved lysine in the Walker A box and the conserved glutamate in the Walker B box substituted in this study are indicated with asterisks. White on black residues are 100% conserved, white on grey residues are 80% conserved and black on grey residues are 60% conserved. Notice that the N-terminal extensions of PilB, EpsE, VirB11 and HP0525 are not included.

Figure 2. Subcellular localization of PilB and PilT in *M. xanthus*.

A) PilB is a cytoplasmic protein. Cells of the wild type DK1622GFP were separated into fractions enriched for periplasmic, cytoplasmic and membrane proteins. Following separation by SDS-PAGE, the fractions were subjected to immunoblot analysis with anti-PilB antibodies. As a control, total cell lysates of DK1622GFP and *ΔpilB* cells (DK10416) are included. Protein from 5 × 10⁶ cells was loaded per lane.

B) PilT is a cytoplasmic protein. The experiment was done as described in (A) except that anti-PilT antibodies were used and the control included *ΔpilT* cells (DK10409).
Figure 3. PilB and PilT of *M. xanthus* are ATPases.

A) Purification of wild type and mutant PilB proteins. *E. coli* JM109/pMS421 containing pSL5 was grown in the absence of IPTG (uninduced) and in the presence of 0.1 mM IPTG (induced). Soluble His$_6$-PilB was purified by Ni$^{2+}$ affinity chromatography as described in Materials and Methods. His$_6$-PilB$^{K327A}$ and His$_6$-PilB$^{E391A}$ proteins were purified using a similar procedure. After SDS-PAGE, proteins were stained with Coomassie Brilliant Blue. Molecular size markers (lane marked marker), with sizes in kDa, are included on the left.

B) PilT purification. *E. coli* JM109/pMS421 containing pSL4 was grown in the absence of IPTG (uninduced) and in the presence of 0.1 mM IPTG (induced). His$_6$-PilT was isolated from inclusion bodies and purified by Ni$^{2+}$ affinity chromatography as described in Materials and Methods. His$_6$-PilT$^{K137A}$ and His$_6$-PilT$^{E205A}$ proteins were purified using a similar procedure. After SDS-PAGE, proteins were stained with Coomassie Brilliant Blue. Molecular size markers (lane marked marker), with sizes in kDa, are included on the left.

C) Time dependence of the ATPase activity of purified His$_6$-PilB, His$_6$-PilB$^{K327A}$ and His$_6$-PilB$^{E391A}$ proteins. His$_6$-PilB proteins were incubated as described in Experimental procedures, $A_{360}$ followed over time and converted into P$_i$ released. Closed diamonds, wild type His$_6$-PilB; open boxes, His$_6$-PilB$^{K327A}$; closed triangles, His$_6$-PilB$^{E391A}$; crosses, incubation buffer.

D) ATPase activity of His$_6$-PilT, His$_6$-PilT$^{K137A}$ and His$_6$-PilT$^{E205A}$ proteins. Autoradiogram of labelled adenosine phosphates after incubation of [$\alpha$-$^{32}$P]-ATP with the indicated His$_6$-PilT proteins or apyrase followed by TLC. The positions of [$\alpha$-$^{32}$P]-ATP and [$\alpha$-$^{32}$P]-ADP are indicated.

Figure 4. T4P dependent motility of *M. xanthus* strains containing mutant *pilB* and *pilT* alleles.
A) T4P dependent motility of the indicated strains on 0.5% agar plates. Cells were incubated for 24 hrs on 0.5% agar supplemented with 0.5% CTT. Strain names and relevant genotype are indicated above each panel. Scale bar: 1 mm

B) Accumulation of PilB and PilT wild type and mutant proteins. Cells from exponentially growing cultures of the indicated strains were harvested, and total protein was separated by SDS-PAGE and analyzed by immunoblotting using anti-PilB (left panel) or anti-PilT (right panel) antibodies. Protein from 5x10^7 cells was loaded per lane.

Figure 5. Electron micrographs of M. xanthus strains.
Cells from exponentially growing cultures of the indicated strains were directly transferred to a grid, stained with 2% uranyl acetate (w/v) and visualized using transmission electron microscopy. Scale bar: 0.1µm.
Table 1. *M. xanthus* strains and plasmids used in this work

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Table 2. Type IV pili formation in *M. xanthus* mutants carrying substitutions in the Walker A and Walker B boxes of PilB and PilT.

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Figure 1: Jakovljevic et al
Figure 2: Jakovljevic et al. 2007
Figure 3: Jakovljevic et al. 2007

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Figure 3: Jakovljevic et al. 2007

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Figure 3: Jakovljevic et al. 2007

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Figure 3: Jakovljevic et al. 2007

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Figure 4: Jakovljevic et al 2007

A

DK1622 (wild type)  
DK10416 (Δ pilB)  
SA2021 (Δ pilB/pilB<sup>+</sup>)  
SA2414 (Δ pilB/pilB<sup>K327A</sup>)  
SA2415 (Δ pilB/pilB<sup>E391A</sup>)

DK10410 (Δ pilA)  
DK10409 (Δ pilT)  
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<th>SA2020 (Δ pilT/pilT&lt;sup&gt;+&lt;/sup&gt;)</th>
<th>SA2412 (Δ pilT/pilT&lt;sup&gt;K137A&lt;/sup&gt;)</th>
<th>SA2413 (Δ pilT/pilT&lt;sup&gt;E205A&lt;/sup&gt;)</th>
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<td>PilT</td>
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