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ORIGINAL PAPER

Identification and characterization of a unique, zinc-containing transport ATPase essential for natural transformation in *Thermus thermophilus* HB27

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Abstract *Thermus thermophilus* is a model strain to unravel the molecular basis of horizontal gene transfer in hot environments. Previous genetic studies led to the identification of a macromolecular transport machinery mediating DNA uptake in an energy-dependent manner. Here, we have addressed how the transporter is energized. Inspection of the genome sequence revealed four putative transport (AAA) ATPases but only the deletion of one, PilF, led to a transformation defect. PilF is similar to transport ATPases of type IV and type II secretions systems but has a unique N-terminal sequence that carries a triplicated GSPII domain. To characterize PilF biochemically it was produced in *Escherichia coli* and purified. The recombinant protein displayed NTPase activity with a preference for ATP. Gel filtration analyses combined with dynamic light scattering demonstrated that PilF is monodispersed in solution and forms a complex of 590 ± 30 kDa, indicating a homooligomer of six subunits. It contains a tetracysteine motif, previously shown to bind Zn^{2+} in related NTPases. Using atomic absorption spectroscopy, indeed Zn^{2+} was detected in the enzyme, but in contrast to all known zinc-binding traffic NTPases only one zinc atom was bound to the hexamer. Deletion of the four cysteine residues led to a loss of Zn^{2+}. Nevertheless, the mutant protein retained ATPase activity and hexameric complex formation.

Keywords DNA transporter · AAA-ATPase · Metalloproteins · Molecular motor · *T. thermophilus*

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Introduction

Comparative genome analyses provide evidence that horizontal gene transfer is the major driving force for bacterial adaptation to different, often extreme environments and bacterial genome evolution. Among the microorganisms thriving in extreme habitats, thermophiles and hyperthermophiles clearly stand out in terms of interdomain DNA transfer such as 24 and 16.2% of the genes in the hyperthermophilic bacteria *Thermotoga maritima* and *Aquifex aeolicus*, respectively, are suggested to be transferred from archaeal hyperthermophiles (Aravind et al. 1998; Nelson et al. 1999). Many of the transferred genes are thermophilic traits that are essential for survival under extreme conditions.

Among the principal mechanisms of DNA transfer natural competence clearly stands out for its ability to transfer naked DNA. *Thermus thermophilus* has become a model system to study natural transformation in thermophiles (Averhoff 2009; Pantazaki et al. 2002). It is known to exhibit the highest natural transformation frequencies, an extraordinary broad substrate specificity and a high-efficiency

DNA uptake (Schwarzenlander and Averhoff 2006). These features of the DNA translocator suggest that the natural transformation system of *T. thermophilus* is of major importance for thermoadaptation and interdomain DNA transfer in hot environments.

A genetic screen identified 16 competence genes organized in seven distinct loci (Friedrich et al. 2001, 2002, 2003). Biochemical analyses together with genetics and bioinformatics culminated in a model for the DNA translocator in *T. thermophilus*. In its overall assembly it is similar to DNA translocators from other Gram-negative bacteria sharing similarity with type IV and type II protein secretion systems (T4SS and T2SS) and type IV pili (T4P) (Chen and Dubnau 2003; Averhoff 2004; Tonjum and Koomey 1997; Hobbs and Mattick 1993; Averhoff 2009). At least three distinct substructures are present: a pilus-like rod that extends from the cytoplasm through the periplasm to the outer membrane, a shaft, made by multiple copies of the secretin protein, that guides the rod through the outer membrane, and a cytoplasmic motor domain that powers assembly of the rod.

In terms of function it has been suggested that DNA translocators are highly dynamic structures (Averhoff 2004; Chen et al. 2005a; Fussenegger et al. 1997; Collins et al. 2005; Chen et al. 2006). DNA binds to the tip of the pilus-like rod and then the translocator retracts, thus transporting DNA into the periplasm where it is bound by DNA-binding proteins and guided to the cytoplasmic membrane. The last and least understood process is how the DNA crosses the cytoplasmic membrane that may involve the large polytopic inner membrane proteins (ComEC and ComA) (Draskovic and Dubnau 2005; Facius and Meyer 1993; Friedrich et al. 2001; Averhoff 2009).

Here, we have addressed the question how the movement of the DNA translocator is energized. Apart from the previously recognized motor AAA-ATPase PilF, which is suggested to be involved in transport of DNA through the outer membrane (Schwarzenlander et al. 2009), none of the other putative AAA-ATPases encoded by *T. thermophilus* is apparently involved in transformation. Therefore, PilF appears to be the key motor component of the DNA transporter of *T. thermophilus*. We have produced the recombinant PilF and studied its oligomeric state, function, metal content and metal-binding site that gave first insights into a hitherto unique motor component of the DNA translocator.

Materials and methods

Construction of overexpression vectors/strains

E. coli DH5α and *E. coli* BL21 (DE3) were used for gene expression and protein purification. We engineered two strains, one producing the mature PilF protein and one producing a PilF missing the first 268 residues of the mature protein. The primers used in this study are shown in Table 1. The portion of the *pilF* gene encoding residues 805–1695 was amplified from the genomic DNA and cloned in the pMAL-c2 vector (New England Biolabs, Ipswich, MA, USA). Expression in *E. coli* DH5α cells was induced by adding 1 mM IPTG at an OD_{600} of 0.6. The fusion protein comprising of ΔPilF and maltose binding protein was purified on amylose resin (New England Biolabs) and used for antibody generation.

The complete *pilF* gene was amplified from the genomic DNA and ligated into a pET28a expression vector (Stratagene) adding a N-terminal His-tag. Expression in *E. coli* BL21 (DE3) cells (Novagen, Inc.) was induced by adding 1 mM IPTG at an OD_{600} of 0.6. Heat precipitation of *E. coli* proteins was carried out at 65°C for 20 min and soluble proteins were separated from the cell debris by centrifugation at $10000 \times g$ for 35 min at 6°C. The supernatant containing soluble His_6-tagged AAA-ATPase was further filtrated (Millipore filters, 0.45 μm) and incubated with Ni–NTA resins for 1.5 h at 6°C under constant mixing. After incubation, His_6-tagged AAA-ATPase that was bound to the Ni–NTA matrix was gradually eluted in batch using 25–400 mM imidazole in buffer A. Fractions containing desired protein were identified by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) (Laemmli 1970), pooled, diluted to 50 mM Tris/HCl (pH 7.5) and 125 mM

Table 1 Oligonucleotides used

Name	Use	Sequence
forTTC1844	PCR of TTC1844	GCGGCCCGAATTCCTCTG
reTTC1844	PCR of TTC1844	AGGCCGATCTAGAGCTGG
forTTC1621	PCR of TTC1621	GGGATGAATTCCCTGAGGG
revTTC1621	PCR of TTC1621	CCAGGTGGATCCTTTTCCC
for*ΔpilF*	PCR of *Δpil*	GACGTGGAATTCCGGCTTTC
rev*ΔpilF*	PCR of *Δpil*	GGTGCAGATCTCGAAACCG
for*pilF*	PCR of *pilF*	GGTGTGACCATATGAGCGT
rev*pilF*	PCR of *pilF*	CCTCCTTTAGTCGACGGTACGCGC

NaCl and applied to anion exchange Resource Q (GE Healthcare, 6 ml) using Buffer A (50 mM Tris/HCl, pH 7.5) at a flow rate of 3.5 ml/min, followed by a gradient program of 0–30% buffer B (Tris/HCl, pH 7.5 and 1 M NaCl (not shown)). The His_6-tagged protein was eluted using a linear salt-gradient of 0–300 mM NaCl. PilF eluted at around 215–230 mM NaCl. Acquired fractions were analyzed by SDS-PAGE and fractions containing desired protein were pooled together and subsequently applied on to a size exclusion column (Superdex 200 HR 10/30 GE Healthcare) equilibrated in 50 mM Tris HCl, 300 mM NaCl, pH 7.5.

Dynamic light scattering

The purified protein was centrifuged at $10000 \times g$ for 10 min prior to dynamic light-scattering analysis; all measurements were carried out at 20°C in a 90 Plus particle size analyzer (Brookhaven Instruments Corporation).

Construction of plasmids for mutant generation

All recombinant DNA manipulations were carried out by standard methods. The primers used in this study are shown in Table 1. For cloning of *pilT*-homologues from *T. thermophilus* HB27 primers were synthesized based on the sequences of TTC1621 and TTC1844. The *orfs* were amplified by PCR using *T. thermophilus* chromosomal DNA as the template with the set of primers. The amplified fragments were cloned into *Eco*RI-/*Bam*HI- and *Eco*RI-/*Xba*I-digested pBluescriptIISK/KS (Stratagene), respectively, yielding pAF333 and pKB-PilT (Fig. S1). Mutations were introduced by inserting a thermostable kanamycin resistance marker (*kat* gene) into unique sites of TTC1621 and TTC1844. To insert the marker in TTC1621, plasmid pAF333 was digested with *Aat*II and *Nco*I, followed by Klenow treatment and religation. This resulted in the deletion of a 223-bp fragment near the 3-terminal end of TTC1621 (Fig. S1). Retransfer of the mutant loci into *T. thermophilus* HB27 resulted in TTC1621 mutants. To generate TTC1844 mutants, the kanamycin marker was inserted in the unique *Nco*I site of pKB-PilT (Fig. S1). Transformation studies of the resulting mutants were performed with chromosomal DNA of a spontaneous streptomycin-resistant mutant (Friedrich et al. 2002).

Functional analyses of the PilF tetracysteine motif

To investigate the role of the four cysteine residues of PilF in zinc binding, pET28-PilF, was used to exchange the highly conserved C-terminal cysteine residues Cys-781, Cys-784, Cys-816 and Cys-819 to alanins using the Phusion-site directed mutagenesis protocol from Finnzymes (Espoo, Finland). The purified PCR products were digested with *Dpn*I to eliminate hemimethylated template DNA.

Subcellular localization studies

Membrane isolation and subcellular fractionation of inner and outer membranes were performed by ultracentrifugation of HB27 cell lysates followed by *N*-laurylsarcosine precipitation as described recently (Rumszauer et al. 2006). The periplasmatic fractions were isolated by temperature shock (Hoshino and Kageyama 1980). Western blot analyses were carried out by using a BM Chemiluminescence Blotting Substrate Kit as recommended by the supplier (Roche Diagnostics GmbH, Mannheim, Germany). The purity of outer and inner membranes was verified by Western blot analyses of membrane fractions with antibodies directed against the S-Layer protein (outer membrane) and with antibodies directed against the inner membrane protein PilN of *T. thermophilus* HB27.

ATPase assay

160 μg protein in 1 ml of TMC buffer (50 mM Tris, 50 mM MOPS, 50 mM CHES, 50 mM Glycin, 200 mM KCl, 5 mM $MgCl_2$, 5% Glycerin, pH 9.5) was incubated at 70°C for 3 min before the reaction was started with ATP (2.5 mM ATP final concentration in the test) and incubated at 70°C. Samples were taken at different time points mixed with 40 μl of 30% CCl_3COOH and 1 ml AAM (0.5 ml acetone, 0.25 ml 2.5 M H_2SO_4, 0.25 ml 10 mM $(NH_4)_6Mo_7O_{24}$). The absorbance was measured after 10 min of incubation at a wavelength of 355 nm (Heinonen and Lahti 1981). To analyze the effect of different divalent cations on ATPase activity, 5 mM $MgCl_2$ was exchanged for 5 mM $MnCl_2$ or 5 mM $CaCl_2$. To study the effect of nucleotides on ATPase activity, ATP was exchanged for 2.5 mM GTP, CTP or UTP. To study the effect of zinc and/or DNA on ATPase activity, 160 μg purified PilF/ml TMC was dialyzed for 16 h against increasing amounts of $ZnCl_2$ (10–100 μM $ZnCl_2$ in TMC buffer). The ATPase activity of the dialyzed protein was determined in the absence or presence of 10 μM $ZnCl_2$. To analyze the effect of ssDNA and dsDNA on ATPase activity, PilF (160 μg of protein in 1 ml TMC buffer) was preincubated in TMC buffer with 4 μg ssDNA or 4 μg dsDNA at room temperature for 10 min. To study the effect of DNA and $ZnCl_2$, PilF was preincubated in TMC buffer with ssDNA or dsDNA and 10 μM $ZnCl_2$.

Analysis of metal content

Determination of the metal content was obtained by electrothermic atomic absorption with the Zeeman effect

(Hitachi Z2000) using hollow single-element cathode lamps. Before the measurements, the sample was diluted in water, injected in the pyrolytic graphite tube, which was heated according to the thermal program of the analyzed element. The elements Fe, Ni, Cu, Co, Mn and Zn were analyzed in these experiments. For zinc analyses, the following program was used: 100°C for the dry step (40 s), 300°C for the pyrolysis step (20 s) and 2000°C for the atomisation step (5 s). The analytical conditions were lamp constant current 5 mA; slit width 1.3 nm; wavelength 213.9 nm. The calibration curve was obtained by addition of increasing amounts of a standard zinc solution (linear standard curve in range of 10–50 nM of Zn) in water. Each value was the average of three measurements. The absorbance of the buffer was subtracted from the measured values of the protein sample (protein concentration of 20 nM to 40 nM). Overnight dialysis against a Zn^{2+} containing buffer has been done using a 10-kDa Spectra/Por Dialysis Membrane.

Circular dichroism spectroscopy

Steady-state CD spectra were measured in the far UV-light (180–260 nm) using a CHIRASCAN spectropolarimeter (Applied Photophysics). Spectra were collected in a 60 µl quartz cell (Hellma) with a path length of 0.1 mm, at 20°C and a step resolution of 1 nm. The readings were average of 2 s at each wavelength and the recorded milli-degree values were the average of three determinations for each sample. CD spectroscopy of PilF (2.0 mg/ml) was performed in a buffer of in 50 mM Tris/HCl, 250 mM NaCl, pH 7.5. The spectrum for the buffer was subtracted from the spectrum of the protein. CD values were converted to mean residue molar ellipticity in units of degree $cm^2\ dmol^{-1}$ using the software Chirascan Version 1.2, Applied Photophysics. This baseline-corrected spectrum was used as input for computer methods to obtain predictions of secondary structure. In order to analyze the CD spectrum, program package Dicroprot (Deleage and Geourjon 1993) and NeuralNet (Böhm et al. 1992) have been used.

Results

Absence of antagonistically acting motor NTPases in *T. thermophilus* HB27

PilF is a member of the PilF/PilB subfamily, which is suggested to control pilus extension in different bacteria such as *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa* and *Myxococcus xanthus* (Freitag et al. 1995; Chiang et al. 2008; Friedrich et al. 2002; Jakovljevic et al. 2008). In these model organisms, retraction AAA-ATPases of the PilT subfamily act antagonistically to PilF/PilB in T4P movement. Blast searches on the genome of *T. thermophilus* with gonococcal PilF and PilT as a bait revealed in total four homologous proteins: one is the already known PilF/PilB related AAA-ATPase PilF (TTC1622, formerly *orf332*), which is essential for natural transformation (Friedrich et al. 2002); the second, TTC1844 (formerly *orf830*), is similar to PilB/PilF-type ATPases, whereas two, TTC1621 (formerly *orf333*) and TTC1415 (formerly *orf1388*), are similar to PilT-type ATPases (Friedrich et al. 2002). The latter has already been shown to be not essential for natural transformation (Friedrich et al. 2002). To analyze the role of TTC1844 and TTC1621 in natural transformation, the genes were mutated by gene disruption (supplementary figure S1). The genotype of the mutants was verified by Southern blot and PCR analyses. Subsequently, the mutants were analyzed with respect to their piliation and transformation phenotype. Interestingly, mutants Tt29 (TTC1621::*kat*) and Tt16 (TTC1844::*kat*) were unaffected in transformation, such as the mutant strains and the HB27 wild-type strain exhibited analogous transformation frequencies of $\sim 1 \times 10^{-4}$ transformants/living count. Both mutants exhibited long pilus structures on the surface. This finding indicates that TTC1621 and TTC1844 are neither essential for transformation or piliation, although it has to be noted that small quantitative differences in piliation cannot be excluded completely by the electron microscopic studies. Taken together, these analyses corroborate the hypothesis that PilF (TTC1622) is the only AAA^+-ATPase essential for natural transformation of *T. thermophilus* HB27.

Unusual primary structure of PilF

Sequence analyses and alignment of PilF with members of the PilF/PilB and GspE (general secretion pathway, gsp) subfamily of motor ATPases revealed that PilF contains conserved Walker A and B boxes, involved in ATP binding and hydrolysis and conserved Asp Box and His Box motifs (Fig. 1). Interestingly, a multiple alignment revealed that the N-terminus of PilF is approximately 300–350 amino acids longer than the N-termini of members of the PilF/PilB or GspE subfamilies. The extended N-terminus of PilF comprises three copies of a conserved GSPII domain (aa 44–147, aa 201–300 and aa 369–478) present in secretion ATPases of the GspE subfamily, such as EpsE of *Vibrio cholerae*, PulE in *Klebsiella oxytoca*, XpsE of *Xanthomonas campestris*, and the PilB subfamily such as PilF and PilB of the T4P systems in *N. gonorrhoeae*, and *P. aeruginosa*, respectively.

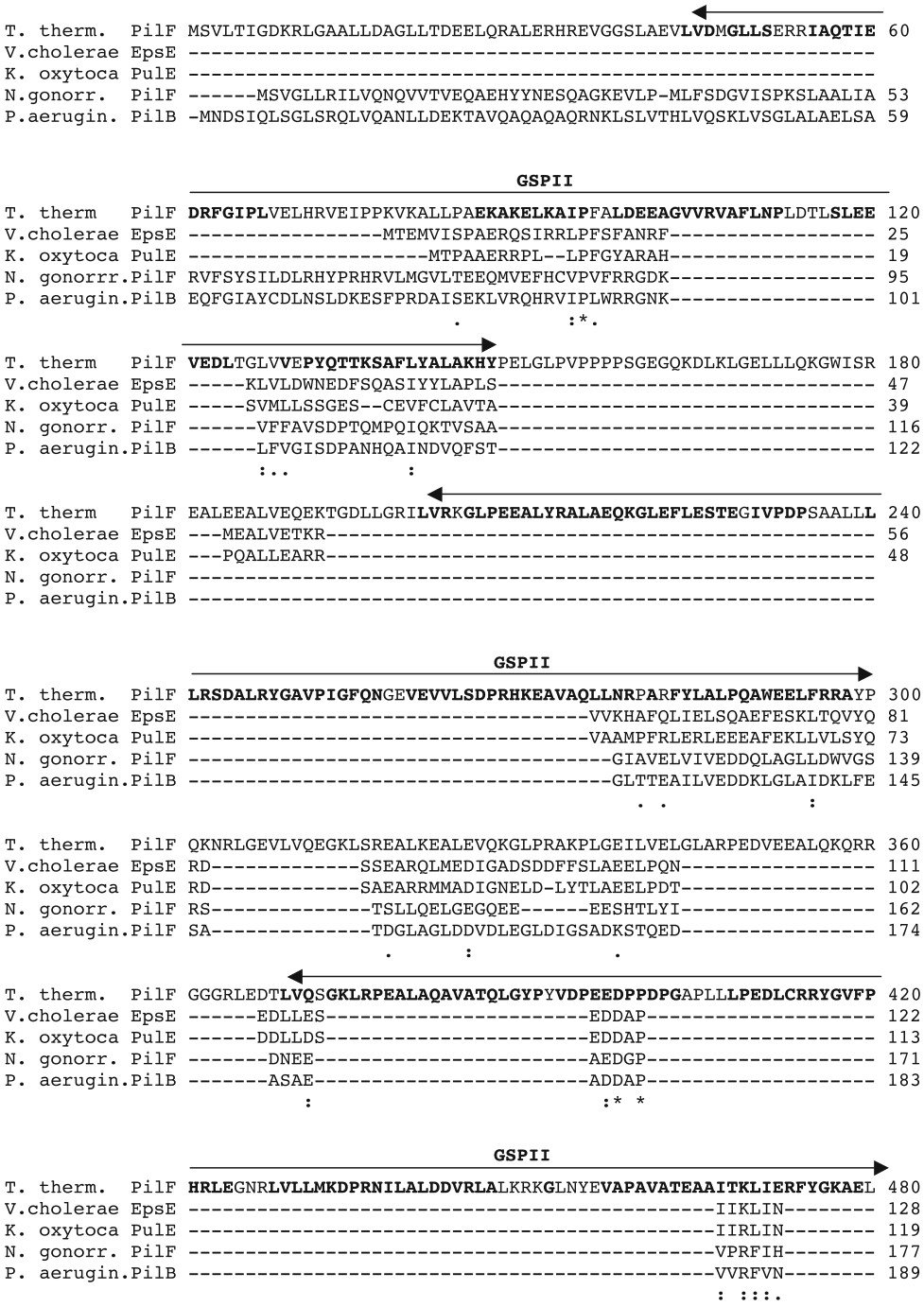

Fig. 1 Sequence alignment of PilF from *T. thermophilus* and members of the PilB/PilF and GspE subfamilies of AAA-ATPases. *V. cholerae, Vibrio cholerae; K. oxytoca*, *Klebsiella oxytoca*; *T. therm*., *Thermus thermophilus*; *N. gonorr*., *Neisseria gonorrhoeae*; *P. aerugin*., *Pseudomonas aeruginosa*. The *bold letters* indicate the conserved Walker A, Walker B, His-Box, Asp-Box and tetracysteine motifs. The *arrows* indicate the three conserved GSPII motifs at the N-terminus

Major amounts of PilF are localized in the cytoplasm

PilF had neither apparent regions of hydrophobicity nor a signal sequence, indicating that it was likely to be located in the cytoplasma. However, as a proposed motor of a membrane-bound macromolecular complex it should localize to the membrane via interaction with subunits of the translocator. To address the cellular localization of PilF in *T. thermophilus*, polyclonal antibodies against an N-terminal truncated version of PilF were generated and applied to crude extracts and subcellular fractions of *T. thermophilus* HB27. As can be seen from Fig. 2, the antibody reacted with a 99-kDa protein in the wild type that was absent in the *pilF* mutant. The apparent molecular mass correlated nicely with the predicted molecular mass of 98.21 kDa for PilF comprising of 889 amino acids. A second protein species with a molecular mass of about 60 kDa was detected in both, wild-type and *pilF*-mutant cells, and therefore, is suggested to result from an unspecific reaction of the PilF antibodies.

Fig. 1 continued

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T. therm.  PilF SEIAKEFAKKQAEEEVPSPLELDESAAQKFVKQVIREAFLQDASDIHIEPRQNDVQVRLR 540
V.cholerae EpsE ---------------------------------AMLGEAIKEGASDIHIETFEKTLSIRFR 156
K. oxytoca PulE ---------------------------------AMLTEAIKEKASDIHIETYERHLQIRFR 147
N. gonorr. PilF ---------------------------------KTLSDALRSGASDIHFEFYEHNARIRFR 205
P. aerugin.PilB ---------------------------------KMLLDAIKGGSSDLHFEPYEKIYRVRFR 217
                                                  : :*:   :**:*:*  :.   :*:*


T. therm.  PilF IDGALRPYSTLPKGALNAVISVVKIMGGLNIAEKRLPQDGRVRYR--EGAIDVDLRLSTL 598
V.cholerae EpsE VDGVLREVLAPSRKLSSLLVSRVKVMAKLDIAEKRVPQDGRISLR--IGGRAVDVRVSTM 214
K. oxytoca PulE VDGVLREILRPQRRLAALLISRIKVMASLDIAEKRIPQDGRMALR--IGGRAVDVRVSTL 205
N. gonorr. PilF VDGQLREVVQPPIAVRGQLASRIKVMSRLDISEKRIPQDGRMQLTFQKGGKPVDFRVSTL 265
P. aerugin PilB TDGMLHEVAKPPIQLASRISARLKVMAGLDISERRKPQDGRIKMRVSKT-KSIDFRVNTL 276
                ** *:            : : :*:*. *:*:*:* *****:          :*.*:.*:


                                                                  Walker A
T. therm   PilF PTVYGEKAVMRLLKKASDIPEIEDLGFAPGVFERFKEVISKPYGIFLITGPTGSGKSFTT 658
V.cholerae EpsE PSSHGERVVMRLLDKNATRLDLHSLGMTAHNHDNFRRLIKRPHGIILVTGPTGSGKSTTL 274
K. oxytoca PulE PSSYGERVVLRLLDKNSVNLDLLTLGMTPALLRQVDGLIARPHGIVLVTGPTGSGKSTTL 265
N. gonorr. PilF PTLFGEKVVMRILNSDAASLNIDQLGFEPFQKKLLLEAIHRPYGMVLVTGPTGSGKTVSL 325
P. aerugin.PilB PTLWGEKIVMRILDSSSAQMGIDALGYEEDQKELYLAALKQPQGMILVTGPTGSGKTVSL 336
                *:  **: *:*:*.. :    :  **            : :* *:.*:********: :


                                  Asp Box                                 Walker B
T. therm.  PilF FSILKRIATPDKNTQTIEDPVEYEIPGINQTQVNPQAGLTFARALRAFLRQDPDIIMVGE 718
V.cholerae EpsE YAGLQELNSSERNILTVEDPIEFDIDGIGQTQVNPRVDMTFARGLRAILRQDPDVVMVGE 334
K. oxytoca PulE YAALSRLDARERNIMTIEDPIEYELEGIGQTQVNAKVDMTFARGLRAILRQDPDVVLVGE 325
N. gonorr. PilF YTCLNILNTESVNIATAEDPAEINLPGINQVNVNDKQGLTFAAALKSFLRQDPDIIMVGE 385
P. aerugin.PilB YTGLNILNTTDINISTAEDPVEINLEGINQVNVNPRQGMDFSQALRAFLRQDPDVIMVGE 396
                :: *. : : . *  * *** * :: **.*.:** : .: *: .*:::******:::***


                                 His Box
T. therm.  PilF IRDSETAKIATEAALTGHLVIATLHTNDAAQAITRLDEMGVEPFNISAALIGVLSQRLVR 778
V.cholerae EpsE IRDLETAQIAVQASLTGHLVMSTLHTNTAVGAVTRLRDMGIEPFLISSSLLGVLAQRLVR 394
K. oxytoca PulE IRDGETAQIAVQASLTGHLVLSTLHTNSALGAISRLQDMGVEPFLLSTSLLAVMSQRLVR 385
N. gonorr. PilF IRDLETADIAIKAAQTGHMVFSTLHTNNAPATLSRMLNMGVAPFNIASSVSLIMAQRLLR 445
P. aerugin.PilB IRDLETAEIAIKAAQTGHMVMSTLHTNSAAETLTRLLNMGVPAFNLATSVNLIIAQRLAR 456
                *** ***.** :*: ***:*::***** *  :::*: :**: .* :::::  :::*** *


                  CXXC                                    CXXC
T.therm.   PilF RVCEHCKVEVKPDPETLRRLGLSEAEIQG--ARLYKGMGCERCGGTGYKGRYAIHELLVV 836
V.cholerae EpsE TLCPDCKEPYEADKEQRKLFDS--KKKEP--LILYRATGCPKCNHKGYRGRTGIHELLLV 450
K. oxytoca PulE RLCPHCRQQEPANADTAHQMEI--APG----TALWQPRGCAECGFTGYRGRTGIHELLLV 439
N. gonorr. PilF RLCSSCKQEVERPSASALKEVGFTDEDLAKDWKLYGAVGCDRCRGQGYKGRAGVYEVMPI 505
P. aerugin.PilB KLCSHCKKEHEVPRETLLHEG-FPEDKIG-TFKLYSPVGCDHCKN-GYKGRVGIYEVVKN 513
                 :*  *:                              *:   ** .*   **:** .::*::


T. therm.  PilF DDEIRHAIVAGKSATEIKEIARRKGMKTLREDGLYKALQGITTLEEVLARTIE------ 889
V.cholerae EpsE DDALQELIHSEAGEQAMEKHIR-ATTPSIRDDGLDKVRQGITSLEEVMRVTKES----- 503
K. oxytoca PulE DDRVRMAIHRGENEVTLIQQLG-TDYMTLRRAGREKALAGITSWQEVLRVTEQPIAEAC 497
N. gonorr. PilF SEEMQRVIMNNGTEVGILDVAYKEGMVDLRRAGILKIMQGITSLEEVTANTND------ 558
P. aerugin.PilB TPALQRIIMEEGNSIEIAEQARKEGFNDLRTSGLLKAMQGITSLEEVNRVTKD------ 566
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To address its subcellular localization experimentally, the periplasm was prepared by hyperosmotic/temperature shock and cytoplasmic membranes and outer membranes were extracted with *N*-laurylsarcosine extraction followed by ultracentrifugation separation. After separation on an SDS gel, the subcellular fractions were subjected to Western blot analyses using the anti-PilF antibody. PilF was found predominantly not only in the cytoplasm (90%) but also in the cytoplasmic membrane (10%), but not in the periplasm (Fig. 2). These results confirm the predicted localization of PilF.

Purification of a monodisperse PilF complex

To address the structure–function relationship of PilF, the *pilF* gene was cloned under the control of a strong promotor and fused to a His-Tag at the N-terminus, and the protein was overproduced in *E. coli*. PilF was present in the soluble fraction and first purified by affinity chromatography on a Ni^{2+}–NTA resin column. An imidazole-gradient (25–400 mM) in buffer consisting of 50 mM Tris/HCl (pH 7.5), 250 mM NaCl, 2 mM Pefabloc^{SC} (Biomol) and 2 mM PMSF was used to separate His_6–PilF from other proteins. PilF eluted at 250 mM imidazole, was collected and subsequently applied to a RESOURCE^{TM} Q column. Fractions containing PilF protein were eluted with 215–270 mM NaCl (Fig. 3a). A monodisperse PilF could be isolated by subsequent application of this protein sample onto a size exclusion column (Superdex 200 HR 10/30), equilibrated in 50 mM Tris/HCl (pH 7.5) and 300 mM NaCl (Fig. 3a, b). After purification a concentration of 0.5 mg pure protein per liter culture medium was obtained.

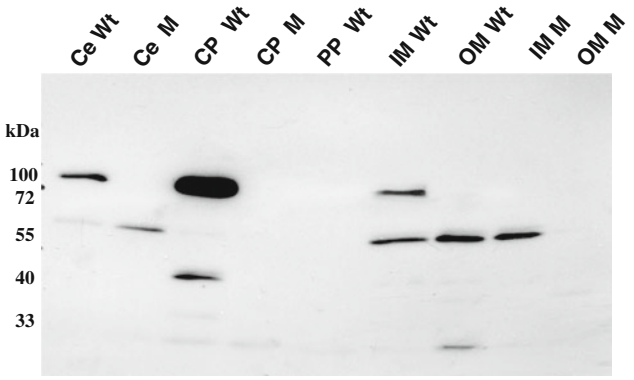

Fig. 2 Detection and cellular localization of PilF. Cells were harvested in the exponential growth phase, resuspended in lysis buffer and disrupted by sonification. Soluble fractions and membrane fractions were separated by ultracentrifugation prior to separation of inner and outer membrane fractions by *N*-laurylsarcosine precipitation. The resulting fractions were analyzed by SDS-PAGE and Western blotting using specific antisera against PilF. Ce, crude extract, CP, cytoplasm; PP, periplasm; IM, inner membrane; OM, outer membrane; Wt, wild type; M, *pilF* mutant

Analysis of the isolated protein by MALDI mass spectrometry revealed the high purity of PilF, and that the protein had the sequence-based predicted mass of 99.191 Da.

PilF is a NTPase

Although AAA-ATPases are widely assumed to energize motors in macromolecular transport machineries as well as pilus assembly by NTP hydrolysis, activity of secretion NTPases has actually been proven biochemically for several members of the type II secretion NTPase such as members of the GspE subfamily, and members of the PilT subfamily (Sexton et al. 2004; Chiang et al. 2008; Rivas et al. 1997; Camberg and Sandkvist 2005). NTPase activities of PilF/PilB NTPases implicated of natural transformation systems have not been analyzed so far. Therefore, the function of PilF as an NTPase was addressed. To analyze substrate saturation, ATP concentrations from 50 µM to 5 mM were used. Maximal activities were obtained with 2.5 mM ATP. This concentration was used throughout all following assays. The purified PilF hydrolyzed ATP with an activity of 81 nmol Pi mg protein^{-1} min^{-1}. By comparison, the nucleotides CTP, UTP and GTP were also hydrolyzed, but activities were considerably lower with 57-, 44- and 35 nmol Pi mg protein^{-1} min^{-1}, respectively (Fig. 4a). PilF had a requirement for Mg^{2+} although other divalent ions as Mn^{2+} (92%) and Ca^{2+} (35%) could substitute Mg^{2+} (Fig. 4b). ATPase activity was detected over a wide pH range from 6.5 to 11 with optimal activity at pH 9.5 (Fig. 4c). The enzyme was practically inactive at 37°C and the hydrolysis rate increased steadily with temperature (Fig. 4d). Maximal activity was observed at 75°C; thereafter ATPase activity declined again. The presence of ssDNA or dsDNA did not affect the ATPase activity (data not shown). Taken together, these data clearly demonstrated NTPase activity of PilF.

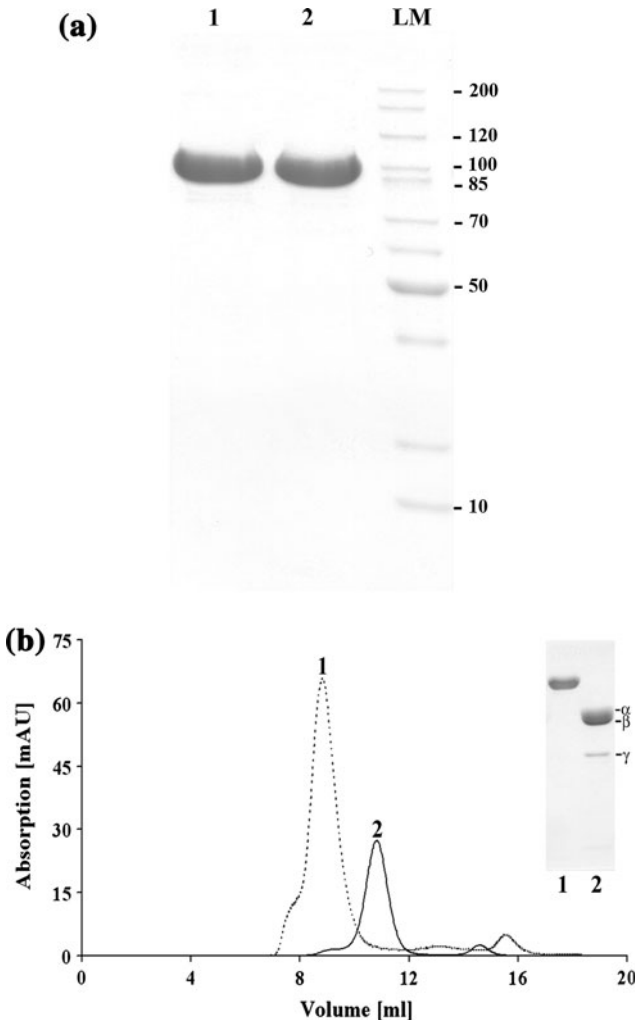

Fig. 3 Purification of recombinant PilF from *T. thermophilus*. **a** SDS-PAGE of PilF (*lane 1*) and its mutant protein (*lane 2*) after elution from the RESOURCE^{TM} Q column. *LM* molecular mass standard. **b** After ion-exchange chromatography, the protein sample was applied to Superdex HR200 gel filtration analysis as described in "Material and methods". The diagram shows the elution of PilF (*peak 1*) in comparison with the $\alpha_3\beta_3\gamma$ complex of the thermophilic F_1 ATPase from *Bacillus PS3* (*peak 2*). The inset represents the SDS gel of PilF and the $\alpha_3\beta_3\gamma$ complex in *lane 1* and *lane 2*, respectively

PilF in solution is a homooligomer comprising of six subunits

The secondary structure of the purified PilF was determined by circular dichroism (CD) spectroscopy (Fig. 5a). The overall spectrum is characteristic for a protein with mixed α/β-structure. The secondary structure content of this construct was calculated to be 51% α-helix and 39% β-sheet. The overall spectrum is in agreement with the

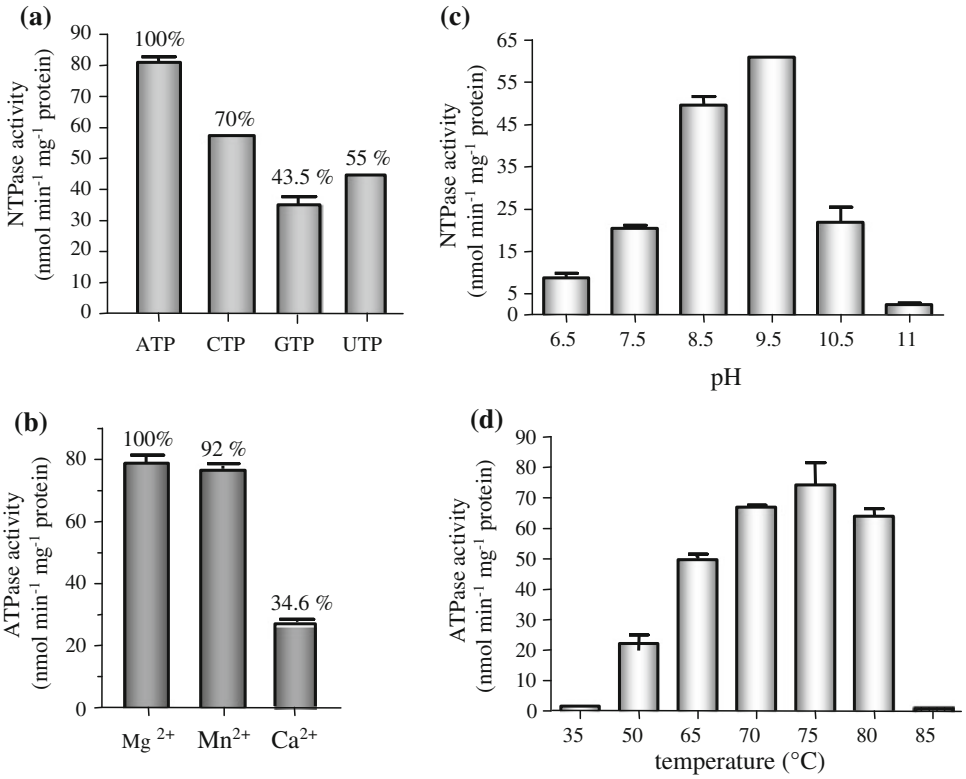

Fig. 4 ATPase activity of purified PilF. The activity was analyzed in Tris–MOPS–CHES–glycin–buffer with 160 µg protein depending on **a** nucleotides, **b** divalent cations, **c** pH, and **d** temperature as described in "Materials and methods". The ATP (CTP, GTP or UTP) concentration was 2.5 mM. Each *point* represents three replicates

predicted secondary structure of the protein based on its amino acid sequence, indicating correct folding of the protein. In order to determine the native molecular mass of PiIF, a Superdex 200 gel filtration column was calibrated by determining the K_{av} values for a set of standard proteins of known molecular mass. Comparison of the K_{av} for subunit PiIF versus the standard proteins suggests the native molecular mass of approximately 590 ± 15 kDa. The same value could be determined, when the $\alpha_3\beta_3\gamma$ complex of the thermophilic F_1 ATPase has been used as a standard (Fig. 3b). Dynamic light-scattering (DLS) experiments and first solution X-ray scattering data indicate that the protein is monodisperse in solution with an estimated molecular mass of 590 ± 30 kDa (data not shown), in agreement with the results of the gel filtration chromatography. PilF has a hydrodynamic diameter of 26.8 nm as determined by DLS. These data are consistent with a PilF forming a homooligomer of six subunits.

PilF contains one Zn^{2+} per hexamer

The UV–visible spectra of the recombinant PilF revealed a maximum absorbance at 356 nm and a second maximum at 476 nm, indicating a possible occupation of a cation, not bound via heme groups (Fig. 5b). To search for a metal bound to PilF, atomic absorption spectroscopy was performed and a dilution series of $Zn(NO_3)_2$, $Fe(NO_3)_3$, $Ni(NO_3)_3$ or $Co(NO_3)_2$ was used as a standard. These measurements clearly identified Zn^{2+} but not Fe, Ni or Co in the protein. When PilF was incubated with 10 mM of EDTA, no optical change could be observed, suggesting a tight binding of Zn^{2+} to the protein complex. In order to exclude that Zn^{2+} dissociated from the complex during the process of protein purification, PilF was dialyzed against Zn^{2+} containing buffer with a molar ratio of cation to protein of 100 to 1. Afterwards, the sample was dialyzed against a Zn^{2+}-free buffer to remove unbound cations and used in atomic absorption spectroscopy measurements. Again, a stoichiometry of one Zn^{2+} per PiIF complex was determined (supplementary figure S2B). The ATPase activity of PilF was not affected by the presence of exogenous zinc (10 µM) (data not shown).

Quantitative analyses revealed a rather interesting stoichiometrie: Only one Zn^{2+} was bound per hexamer (supplementary figure S2A). Some related AAA-ATPases have been shown to bind Zn^{2+} via a conserved tetracysteine binding motif that is also present in PilF (Fig. 1).

To study the role of the conserved tetracysteine motif in zinc binding the four conserved cysteines at the C-terminus of PilF were exchanged to alanines. Analyses of the purified mutant protein by atomic absorption revealed that the mutant PilF protein did not contain any zinc. Comparison of the secondary structure of the mutant compared with the wild type protein revealed a slight decrease of 2% in the α-helical content of the mutant protein (Fig. 5a). Furthermore, no alteration in the elution of the mutant protein

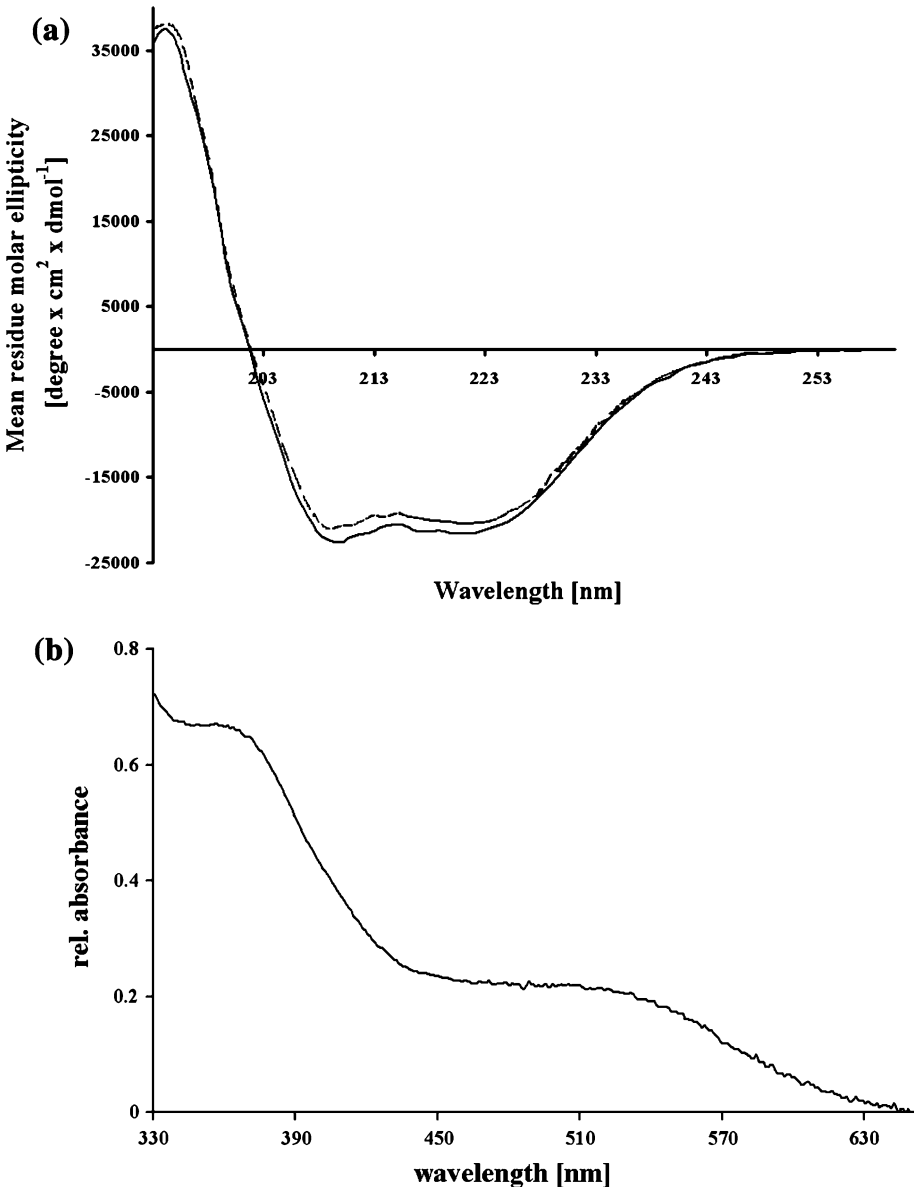

Fig. 5 **a** CD-spectra of PilF of purified wild type PilF (*solid lines*) and mutant PilF (*dashed lines*) (each 2 mg/ml) determined using a CHIRASCAN spectropolarimeter. **b** UV–vis spectrum of recombinant wild type (WT) PilF from *T. thermophilus* (2.0 mg/ml)

could be observed during size exclusion chromatography, reflecting that the cysteine to alanine substitution did not change the hexameric complex formation of the enzyme. Finally, the tetracysteine mutant exhibited ATPase activities comparable to those obtained for the PilF wild type protein with a slight drop of about 8%, implicating that zinc binding is not essential for hydrolytic activity.

Discussion

Retraction and elongation of the pilus-like rod of the DNA translocator is suggested to be one of the key elements in DNA transport across the outer membrane. The mechanism by which pilins are polymerized and depolymerized remains to be an enigma, but motor AAA-ATPases (ATPases associated with various activities) are suggested to energize pilus and DNA translocator extension and/or retraction (Chiang et al. 2008; Jakovljevic et al. 2008; Herdendorf et al. 2002; Maier et al. 2002). Genetic screens suggest a role for two different AAA-ATPases, one that presumably catalyzes retraction, a second catalyzes extension of the rod (Freitag et al. 1995; Wolfgang et al. 1998; Turner et al. 1993) and even the involvement of a third AAA-ATPase in energization of rod movement has been described (Whitchurch and Mattick 1994). In contrast to all other DNA translocators of Gram-negative bacteria, *T. thermophilus* apparently has only one, PilF, that may catalyze both extension and retraction. The same is apparently encountered in the natural transformation system of Gram-positive bacteria, such as ComGA that is the only motor ATPase of the DNA translocator in *Bacilus*

subtilis (Planet et al. 2001; Chen and Dubnau 2004). Obviously, the question how the cells coordinate retraction and extension with only one motor ATPase is a challenging task for future studies.

PilF is similar to phosphate-loop (P-loop) ATPases (AAA-ATPases) of type 2 secretion and type 4 secretion systems, conjugation and transformation machineries and type IV pili. Despite the similarities, clear distinctions are made among the secretion NTPases, which are differentiated into distinct subfamilies, such as VirB11, TrbB, TraG, GspE, PilB, PilT and ComG1 subfamilies (Planet et al. 2001). PilF of *T. thermophilus* belongs to the PilF/PilB subfamily (Planet et al. 2001). Members of this subfamily are characterized by conserved Walker A (P-loop) and Walker B boxes, unique Asp and His Box motifs as well as a conserved tetracysteine motif (Planet et al. 2001). They are envisaged to power type IV pilus rod extension, and the so-called PilT proteins, members of a different NTPase subfamily, act antagonistically in rod retraction (Maier et al. 2002; Clausen et al. 2009). In the case of *T. thermophilus* HB27, such a rod retracting motor component was not identified in the genetic screen so far.

PilF indeed catalyzed ATPase hydrolysis, consistent with its similarity to other motor ATPases. Interestingly, it has by far the highest ATPase activity reported to date with 81 nmol of Pi mg protein^{-1} min^{-1} which exceeds those reported for purified secretion ATPases, including *P. aeruginosa* PilT, PilU and PilB with 5–10.8 nmol Pi mg protein^{-1} min^{-1}, DotB from *Legionella* with 6.4 nmol Pi mg protein^{-1} min^{-1}, EpsE from *Vibrio* with 5.6 nmol Pi mg protein^{-1} min^{-1}, and TrwD from plasmid R388 with 3.3 nmol Pi mg protein^{-1} min^{-1} (Sexton et al. 2004; Chiang et al. 2008; Rivas et al. 1997; Camberg and Sandkvist 2005). Like other members of the PilT and PilF/PilB subfamily, PiIF has no specificity of purines over pyrimidines. PilF is the thus the first established motor ATPase purified and characterized from a thermophile with extraordinary high catalytic activity and may be useful in future structure–function analyses.

PilF differs from all other motor ATPases in having a unique extended hydrophilic N-terminus that contains three unique copies of GSPII domains, which are present in only single copies at the N-termini of motor proteins of T2S and T4P systems. Clues to the function of a triplicated GSPII domain in the *T. thermophilus* PilF protein may be derived from the X-ray structure of a complex of the N-terminal domain of EpsE, a member of the GspE subfamily ATPases, and the cytoplasmic domain of the inner membrane protein EpsL of *Vibrio cholerae* both being part of the T2SS complex. The GSPII domain of EpsE is suggested to be essential for stable interactions of EpsE and EpsL (Abendroth et al. 2005). In analogy, the three conserved GSPII N-terminal domains of PilF might be essential for stable multiple interactions of the PilF complex with inner membrane proteins of the DNA transporter in the thermophilic bacterium *T. thermophilus* HB27. A role of GSPII domains in subunit interactions is also supported by deletion analyses of the N-terminal residues of XpsE, a secretion ATPase of the *X. campestris* T2SS, that indicate that these N-terminal residues are required for exoprotein secretion by mediating the interaction of XpsE and the cytoplasmic membrane protein XpsL (Chen et al. 2005b). The finding that major amounts of PilF are in the cytoplasm whereas only little amounts were present in inner membrane fractions indicates loose interactions of PilF with inner membrane proteins disrupted by cell breakage and membrane preparation.

PilF apparently forms a hexameric complex and this is the first report on a hexameric structure in the PilB/PilF subfamily of traffic NTPases. Hexameric complexes, in which the homooligomer forms a threefold symmetrical ring structure, have been observed before for members of other subfamilies of the T2S/T4S secretion systems such as PilT from *Aquifex aeolicus*, the PulE homologe HP0525 of the *Helicobacter* T4S system, which is a member of the VirB11 subfamily of secretion ATPases, EpsE of the T2S system in *V. cholerae*, belonging to the GspE subfamily of traffic ATPases, BfpD, a PulE homologue of the enteropathogenic *E. coli* bundle-forming pilus biogenesis machinery, and for those of the RP4 and R388 plasmid conjugative system, TrbB and TrwD, respectively (Herdendorf et al. 2002; Forest et al. 2004; Savvides et al. 2003; Robien et al. 2003; Krause et al. 2000a, b; Crowther et al. 2005). Two of these ATPases (EpsE and PulE) apparently have zinc bound via a conserved tetracysteine motif (Planet et al. 2001; Camberg and Sandkvist 2005; Possot et al. 1992). The same motif is conserved in PilF and as expected, mutation of the motif led to a loss of Zn^{2+} binding. In a preparation of EpsE that was more than 93% monomeric (Camberg and Sandkvist 2005) 0.8 mol of zinc per monomer was obtained indicating an approximate 1:1 stoichiometry of zinc to protein. The crystal structure analysis of a truncated histidine-tagged EpsE demonstrated the presence of a tetrahedrally coordinated divalent metal but the site was not fully occupied (Robien et al. 2003).

In contrast, the PilF hexamer has only one zinc bound despite the presence of a binding site in each protomer. How this apparent asymmetry is achieved and whether the oligomeric state contributes to this switch remain to be established, but it should be noted that zinc generates an asymmetry inside the zinc uptake regulator FurB from *Mycobacterium tuberculosis* (Donaldson et al. 2003). Such an asymmetry has the advantage of defining one subunit in the hexameric PilF that might interact either directly with DNA or with DNA transporter components.

Since Zn^{2+} could not be removed from the enzyme by EDTA but only by denaturating the protein and since

tetragonal ZnS4 clusters are by far the preferred motifs for structural zinc sites (Robien et al. 2003; Krause et al. 2000a), it is likely, that the zinc-binding site in PilF serves a structural role. For ClpX, which has been described as a trimer of dimers, zinc is suggested to play a role in hexamer formation by forming the dimers via two interacting zinc-binding domains (Donaldson et al. 2003; Banecki et al. 2001). Consistent with a structural role of Zn^{2+} is the absence of a significant effect of zinc on ATPase activity. By comparison, treatment of zinc-bound AAA-ATPase subfamilies such as the *E. coli* ClpX, a member of the ATP-dependent protease complex ClpP/ClpX and the *E. coli* secretion NTPase SecA with a zinc chelator, resulted in a loss of hydrolytic activity which could be restored by addition of $ZnCl_2$ (Banecki et al. 2001; Camberg and Sandkvist 2005; Fekkes et al. 1999). Although no effect of zinc on the ATPase activity of *Thermus* PilF has been detected yet, an effect of zinc on ATPase activity cannot be ruled out since stimulation might depend on synergistic effects mediated by the presence of additional proteins of the natural transformation system. A synergistic stimulation of ATP hydrolysis has already been demonstrated for EpsE, a cytoplasmic component of the type II secretion system in *V. cholerae*, which is stimulated by EpsL and acidic phospholipids (Camberg et al. 2007). Analogously, the ATPase activity of BfpD, a member of the PulE superfamily, implicated in the enteropathogenic *E. coli* bundle-forming pilus system, was stimulated significantly in the presence of the N-termini of two Bfp proteins, such as BfpC and BfpE, plus zinc (Crowther et al. 2005).

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References

Abendroth J, Murphy P, Sandkvist M, Bagdasarian M, Hol WG (2005) The X-ray structure of the type II secretion system complex formed by the N-terminal domain of EpsE and the cytoplasmic domain of EpsL of *Vibrio cholerae*. J Mol Biol 348:845–855

Aravind L, Tatusov RL, Wolf YI, Walker DR, Koonin EV (1998) Evidence for massive gene exchange between archaeal and bacterial hyperthermophiles. Trends Genet 14:442–444

Averhoff B (2004) DNA transport and natural transformation in mesophilic and thermophilic bacteria. J Bioenerg Biomembr 36:25–33

Averhoff B (2009) Shuffling genes around in hot environments: the unique DNA transporter of *Thermus thermophilus*. FEMS Microbiol Rev 33:611–626

Banecki B, Wawrzynow A, Puzewicz J, Georgopoulos C, Zylicz M (2001) Structure-function analysis of the zinc-binding region of the ClpX molecular chaperone. J Biol Chem 276:18843–18848

Böhm G, Muhr R, Jaenicke R (1992) Quantitative analysis of protein far UV circular dichroism spectra by neural networks. Protein Eng 5:191–195

Camberg JL, Sandkvist M (2005) Molecular analysis of the *Vibrio cholerae* type II secretion ATPase EpsE. J Bacteriol 187: 249–256

Camberg JL, Johnson TL, Patrick M, Abendroth J, Hol WG, Sandkvist M (2007) Synergistic stimulation of EpsE ATP hydrolysis by EpsL and acidic phospholipids. EMBO J 26:19–27

Chen I, Dubnau D (2003) DNA transport during transformation. Front Biosci 8:544–556

Chen I, Dubnau D (2004) DNA uptake during bacterial transformation. Nat Rev Microbiol 2:241–249

Chen I, Christie PJ, Dubnau D (2005a) The ins and outs of DNA transfer in bacteria. Science 310:1456–1460

Chen Y, Shiue SJ, Huang CW, Chang JL, Chien YL, Hu NT, Chan NL (2005b) Structure and function of the XpsE N-terminal domain, an essential component of the *Xanthomonas campestris* type II secretion system. J Biol Chem 280:42356–42363

Chen I, Provvedi R, Dubnau D (2006) A macromolecular complex formed by a pilin-like protein in competent *Bacillus subtilis*. J Biol Chem 281:21720–21727

Chiang P, Sampaleanu LM, Ayers M, Pahuta M, Howell PL, Burrows LL (2008) Functional role of conserved residues in the characteristic secretion NTPase motifs of the *Pseudomonas aeruginosa* type IV pilus motor proteins PilB, PilT and PilU. Microbiology 154:114–126

Clausen M, Jakovljevic V, Sogaard-Andersen L, Maier B (2009) High-force generation is a conserved property of type IV pilus systems. J Bacteriol 191:4633–4638

Collins RF, Frye SA, Balasingham S, Ford RC, Tonjum T, Derrick JP (2005) Interaction with type IV pili induces structural changes in the bacterial outer membrane secretin PilQ. J Biol Chem 280:18923–18930

Crowther LJ, Yamagata A, Craig L, Tainer JA, Donnenberg MS (2005) The ATPase activity of BfpD is greatly enhanced by zinc and allosteric interactions with other Bfp proteins. J Biol Chem 280:24839–24848

Deleage G, Geourjon C (1993) An interactive graphic program for calculating the secondary structure content of proteins from circular dichroism spectrum. Comput Appl Biosci 9:197–199

Donaldson LW, Wojtyra U, Houry WA (2003) Solution structure of the dimeric zinc binding domain of the chaperone ClpX. J Biol Chem 278:48991–48996

Draskovic I, Dubnau D (2005) Biogenesis of a putative channel protein, ComEC, required for DNA uptake: membrane topology, oligomerization and formation of disulphide bonds. Mol Microbiol 55:881–896

Facius D, Meyer TF (1993) A novel determinant (*comA*) essential for natural transformation competence in *Neisseria gonorrhoeae* and the effect of a *comA* defect on pilin variation. Mol Microbiol 10:699–712

Fekkes P, de Wit JG, Boorsma A, Friesen RH, Driessen AJ (1999) Zinc stabilizes the SecB binding site of SecA. Biochemistry 38:5111–5116

Forest KT, Satyshur KA, Worzalla GA, Hansen JK, Herdendorf TJ (2004) The pilus-retraction protein PilT: ultrastructure of the biological assembly. Acta Crystallogr D Biol Crystallogr 60:978–982

Freitag NE, Seifert HS, Koomey M (1995) Characterization of the *pilF-pilD* pilus-assembly locus of *Neisseria gonorrhoeae*. Mol Microbiol 16:575–586

Friedrich A, Hartsch T, Averhoff B (2001) Natural transformation in mesophilic and thermophilic bacteria: identification and characterization of novel, closely related competence genes in *Acinetobacter* sp. strain BD413 and *Thermus thermophilus* HB27. Appl Environ Microbiol 67:3140–3148

Friedrich A, Prust C, Hartsch T, Henne A, Averhoff B (2002) Molecular analyses of the natural transformation machinery and identification of pilus structures in the extremely thermophilic bacterium *Thermus thermophilus* strain HB27. Appl Environ Microbiol 68:745–755

Friedrich A, Rumszauer J, Henne A, Averhoff B (2003) Pilin-like proteins in the extremely thermophilic bacterium *Thermus thermophilus* HB27: implication in competence for natural transformation and links to type IV pilus biogenesis. Appl Environ Microbiol 69:3695–3700

Fussenegger M, Rudel T, Barten R, Ryll R, Meyer TF (1997) Transformation competence and type-IV pilus biogenesis in *Neisseria gonorrhoeae*. Gene 192:125–134

Heinonen JK, Lahti RJ (1981) A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. Anal Biochem 113: 313–317

Herdendorf TJ, McCaslin DR, Forest KT (2002) *Aquifex aeolicus* PilT, homologue of a surface motility protein, is a thermostable oligomeric NTPase. J Bacteriol 184:6465–6471

Hobbs M, Mattick JS (1993) Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. Mol Microbiol 10:233–243

Hoshino T, Kageyama M (1980) Purification and properties of a binding protein for branched-chain amino acids in *Pseudomonas aeruginosa*. J Bacteriol 141:1055–1063

Jakovljevic V, Leonardy S, Hoppert M, Sogaard-Andersen L (2008) PilB and PilT are ATPases acting antagonistically in type IV pilus function in *Myxococcus xanthus*. J Bacteriol 190:2411–2421

Krause S, Barcena M, Pansegrau W, Lurz R, Carazo JM, Lanka E (2000a) Sequence-related protein export NTPases encoded by the conjugative transfer region of RP4 and by the cag pathogenicity island of *Helicobacter pylori* share similar hexameric ring structures. Proc Natl Acad Sci USA 97:3067–3072

Krause S, Pansegrau W, Lurz R, de la Cruz F, Lanka E (2000b) Enzymology of type IV macromolecule secretion systems: the conjugative transfer regions of plasmids RP4 and R388 and the cag pathogenicity island of *Helicobacter pylori* encode structurally and functionally related nucleoside triphosphate hydrolases. J Bacteriol 182:2761–2770

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Maier B, Potter L, So M, Long CD, Seifert HS, Sheetz MP (2002) Single pilus motor forces exceed 100 pN. Proc Natl Acad Sci USA 99:16012–16017

Nelson KE, Clayton RA, Gill SR, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Nelson WC, Ketchum KA, McDonald L, Utterback TR, Malek JA, Linher KD, Garrett MM, Stewart AM, Cotton MD, Pratt MS, Phillips CA, Richardson D, Heidelberg J, Sutton GG, Fleischmann RD, Eisen JA, White O, Salzberg SL, Smith HO, Venter JC, Fraser CM (1999) Evidence for lateral gene transfer between archaea and bacteria from genome sequence of *Thermotoga maritima*. Nature 399: 323–329

Pantazaki AA, Pritsa AA, Kyriakidis DA (2002) Biotechnologically relevant enzymes from *Thermus thermophilus*. Appl Microbiol Biotechnol 58:1–12

Planet PJ, Kachlany SC, DeSalle R, Figurski DH (2001) Phylogeny of genes for secretion NTPases: identification of the widespread tadA subfamily and development of a diagnostic key for gene classification. Proc Natl Acad Sci USA 98:2503–2508

Possot O, d'Enfert C, Reyss I, Pugsley AP (1992) Pullulanase secretion in *Escherichia coli* K-12 requires a cytoplasmic protein and a putative polytopic cytoplasmic membrane protein. Mol Microbiol 6:95–105

Rivas S, Bolland S, Cabezon E, Goni FM, de la Cruz F (1997) TrwD, a protein encoded by the IncW plasmid R388, displays an ATP hydrolase activity essential for bacterial conjugation. J Biol Chem 272:25583–25590

Robien MA, Krumm BE, Sandkvist M, Hol WG (2003) Crystal structure of the extracellular protein secretion NTPase EpsE of *Vibrio cholerae*. J Mol Biol 333:657–674

Rumszauer J, Schwarzenlander C, Averhoff B (2006) Identification, subcellular localization, and functional interactions of PilM-NOWQ and PilA4 involved in transformation competency and pilus biogenesis in the thermophilic bacterium *Thermus thermophilus* HB27. FEBS J 273:3261–3272

Savvides SN, Yeo HJ, Beck MR, Blaesing F, Lurz R, Lanka E, Buhrdorf R, Fischer W, Haas R, Waksman G (2003) VirB11 ATPases are dynamic hexameric assemblies: new insights into bacterial type IV secretion. EMBO J 22:1969–1980

Schwarzenlander C, Averhoff B (2006) Characterization of DNA transport in the thermophilic bacterium *Thermus thermophilus* HB27. FEBS J 273:4210–4218

Schwarzenlander C, Haase W, Averhoff B (2009) The role of single subunits of the DNA transport machinery of *Thermus thermophilus* HB27 in DNA binding and transport. Environ Microbiol 11:801–808

Sexton JA, Pinkner JS, Roth R, Heuser JE, Hultgren SJ, Vogel JP (2004) The *Legionella pneumophila* PilT homologue DotB exhibits ATPase activity that is critical for intracellular growth. J Bacteriol 186:1658–1666

Tonjum T, Koomey M (1997) The pilus colonization factor of pathogenic neisserial species: organelle biogenesis and structure/function relationships. Gene 192:155–163

Turner LR, Lara JC, Nunn DN, Lory S (1993) Mutations in the consensus ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in *Pseudomonas aeruginosa*. J Bacteriol 175:4962–4969

Whitchurch CB, Mattick JS (1994) Characterization of a gene, *pilU*, required for twitching motility but not phage sensitivity in *Pseudomonas aeruginosa*. Mol Microbiol 13:1079–1091

Wolfgang M, Lauer P, Park HS, Brossay L, Hebert J, Koomey M (1998) PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae*. Mol Microbiol 29:321–330