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Thermoplasma acidophilum TAA43 is an archaeal member of the eukaryotic meiotic branch of AAA ATPases

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Abstract

Sequencing of the *Thermoplasma acidophilum* genome revealed a new gene, *taa43*, which codes for a 43-kDa protein containing one AAA domain; we therefore termed it *Thermoplasma* AAA ATPase of 43 kDa (TAA43). Close homologs of TAA43 are found only in related Thermoplasmales, e.g. *T. volcanium* and *Ferroplasma acidarmanus*, but not in other Archaea. A detailed phylogenetic analysis showed that TAA43 and its homologs belong to the 'meiotic' branch of the AAA family. Although AAA proteins usually assemble into high-molecular-weight complexes, native TAA43 is predominantly dimeric except for a minor fraction eluting in the void volume of a sizing column. Wild-type and mutant TAA43 proteins were overexpressed in *Escherichia coli*, purified as dimers and characterized functionally. Since the canonical proteasome activating nucleotidase is not present in Thermoplasmales, TAA43 was tested for stimulation of proteasome activity, which was, however, not detected. Interestingly, immunoprecipitation analysis with TAA43 specific antibodies found a fraction of native TAA43 associated with *Thermoplasma* ribosomal proteins.

Keywords: Archaea; proteasome activating nucleotidase; proteasome; VAT; VAT2.

Introduction

The members of the AAA family (ATPases associated with various cellular activities) (Erdmann et al., 1991) are found in all kingdoms of life (Archaea, Bacteria and Eukarya) (Beyer, 1997; Swaffield and Purugganan, 1997; Neuwald et al., 1999; Frickey and Lupas, 2004a; Iyer et al., 2004). Commonly, the proteins assemble into hexameric rings, which function as molecular machines in many cellular processes as diverse as DNA repair and replication, organelle biogenesis, membrane trafficking, transcriptional regulation, and protein quality control (Confalonieri and Duguet, 1995; Vale, 2000; Langer et al., 2001; Ogura and Wilkinson, 2001; Lupas and Martin, 2002).

Protein quality control involves disassembly of protein complexes and unfolding of proteins for subsequent proteolytic degradation (Wickner et al., 1999). In the archaeon *Methanococcus jannaschii* the AAA ATPase proteasome activating nucleotidase (PAN) prepares substrates in an energy-dependent reaction for proteasome-mediated degradation (Zwickl et al., 1999; Wilson et al., 2000). PAN is found in most archaeal genomes and is the evolutionary precursor of the six distinct ATPase subunits present in the 19S regulator of the eukaryotic 26S proteasome (Zwickl, 2002). Although *Methanococcus* PAN can activate the *Methanococcus* and the *T. acidophilum* proteasomes in an energy-dependent reaction, a homolog of PAN has not been found in *T. acidophilum* (Ruepp et al., 2000) or in the closely related Thermoplasmales *T. volcanium* and *Ferroplasma acidarmanus*. We were therefore interested in identifying the protein exerting the function of PAN in *T. acidophilum*. The *T. acidophilum* genome contains only two AAA ATPases: VAT, which contains two AAA domains and is homologous to eukaryotic Cdc48 and p97, and a single-domain representative, originally dubbed VAT2, now called TAA43 (Ruepp et al., 2000, 2001). Here we report on our studies of TAA43 in cell extracts from *T. acidophilum* and as recombinant protein isolated from *E. coli*. The protein forms dimers under most conditions tested (rather than hexamers, like PAN, VAT and most AAA proteins) and failed to activate the proteolytic activity of the *T. acidophilum* proteasome. Unexpectedly, a portion of cellular TAA43 protein was found in high-molecular-weight fractions associated with ribosomal proteins. This may suggest a unique role for TAA43 in ribosome function in Thermoplasmales.

Results

Phylogenetic classification of TAA43 within the AAA family

Translation of ORF Ta1175 (*taa43* gene) from *T. acidophilum* yields a protein of 375 amino acid residues, with a molecular weight of 42 000 and a calculated isoelectric point of 6.2. The TAA43 protein contains an AAA domain preceded by an N-terminal region of unknown function. BLAST searches revealed that the close relatives of *T. acidophilum*, *Thermoplasma volcanium* and *Ferroplasma acidarmanus*, which belong to the Thermoplasmales, contain a gene homologous to *taa43*; however, among other Archaea, TAA43 homologs are rare and only distantly related. Phylogenetic analysis of a huge set of AAA

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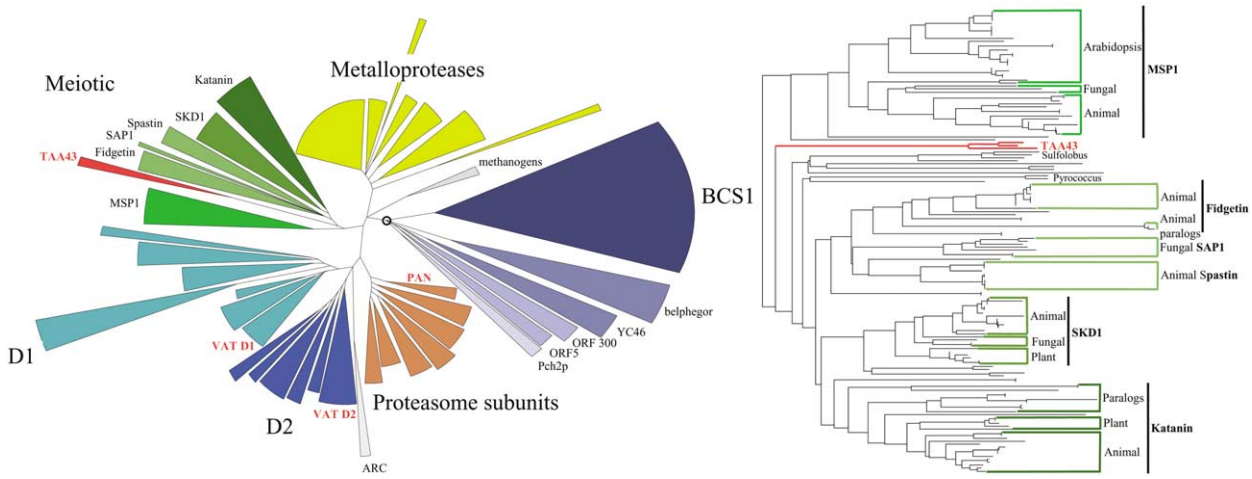

Figure 1 Phylogenetic tree showing the position of TAA43 relative to other sequences of the AAA family.
Major clades (D1, D2 of two ATPase domain AAA proteins, proteasome subunits, metalloproteases, BCS1 and a 'meiotic' group containing TAA43) are assigned separate colors and a black circle highlights the presumed root of the tree, found basal to BCS1. The angle and length of the triangles represent the number of sequences and evolutionary speed of that clade. The right panel shows a detailed phylogenetic representation for the sequences of the 'meiotic' group.

proteins classified TAA43 within the 'meiotic' branch of the AAA family (Figure 1), which is characterized by a gapped second region of homology (SRH), i.e. two amino acids have been deleted directly preceding the 'arginine finger' (canonical SRH: RPGR; gapped SRH: R--R). However, although most sequences of the 'meiotic' group have a gapped SRH, TAA43 and its two homologs from Thermoplasmales share a canonical SRH.

The alignments appear mutationally saturated due to the long divergence time and large differences in the substitution rates between alignment positions. The resulting large number of homoplasies can mask the 'true' phylogenetic signal and thus lead to erroneous branching patterns. We tried to take this into account by using AsaturA, a program that can correct for mutational saturation. A group of long-branching sequences consistently appears basal to a meiotic subgroup (i.e. the fidgetin, spastin, SKD1, and katanin branches) but clearly within the meiotic group (i.e. the MSP1 branch is even more basal). The monophyly of that group is unsure; it is possible that long-branch attraction is the cause for falsely grouping sequences from *Sulfolobus* (Su.so. gi|15897796 and relatives), *Pyrococcus* (Py.ho. gi|14591092 and relatives), *Plasmodium* (Pl.yo. gi|23489729 and relatives), and *Giardia* (Gi.la. gi|29249357) with the Thermoplasmales TAA43 homologs. In this context it should also be noted that AAA sequences from *Sulfolobus*, *Pyrococcus*, *Giardia* and *Plasmodium* are frequently found basal and connected via long branches to the major and minor clades throughout the phylogenetic tree.

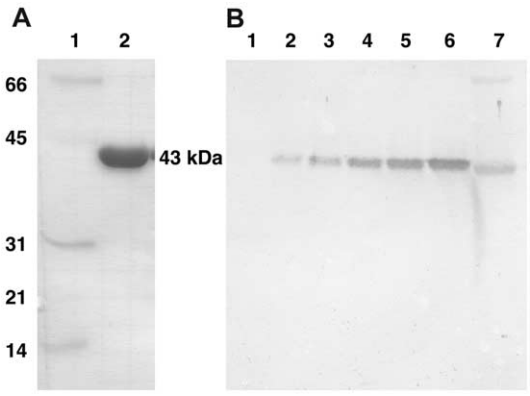

Figure 2 Expression of recombinant and native TAA43 protein.
(A) 12.5% SDS PAGE of the 6×His-tagged TAA43 purification. Lane 1, molecular mass markers; lane 2, purified 6×His-tagged TAA43 eluted from the Ni-NTA Sepharose column. (B) Detection of the native TAA43 protein in the *T. acidophilum* crude extract by Western blotting. Lanes 1–6: 0, 2.5, 5, 10, 15, and 25 ng of recombinant TAA43, respectively. Lane 7, 1 μl of crude extract at 20 μg/μl. The approximate amount of constitutive protein present in *T. acidophilum* cells is 0.01 μg/μl.

Production of recombinant TAA43 protein

C-terminally His_6-tagged TAA43 (TAA43-His_6) was produced in small-scale cultures and was purified in a single step on a nickel-affinity column to apparent homogeneity with a yield of 60 μg/ml of LB media (Figure 2A). The N-terminus of the TAA43-His_6 protein was determined by Edman degradation and corresponded to the deduced sequence of the *taa43* gene (MRVLDDIDE). Purified TAA43 was used to generate a polyclonal antiserum in rabbits. *T. acidophilum* crude extract and serial dilutions of TAA43-His_6 protein were subjected to 12.5% SDS-PAGE and detected by Western blotting with the specific TAA43 antiserum (Figure 2B). The estimated amount of TAA43 in *T. acidophilum* cells corresponds to 0.05% of the total protein.

Determination of relative molecular mass

TAA43-His_6 migrated with an approximate molecular weight of 80 000–90 000 when subjected to 4–20% native gradient gel electrophoresis (data not shown). The molecular weight was determined independently by size exclusion chromatography (SEC) using a Superose 12 column. The elution volume corresponded to a molecular weight of 70 000–80 000. Thus, both experiments suggest a dimeric form for TAA43-His_6. SEC was also per-

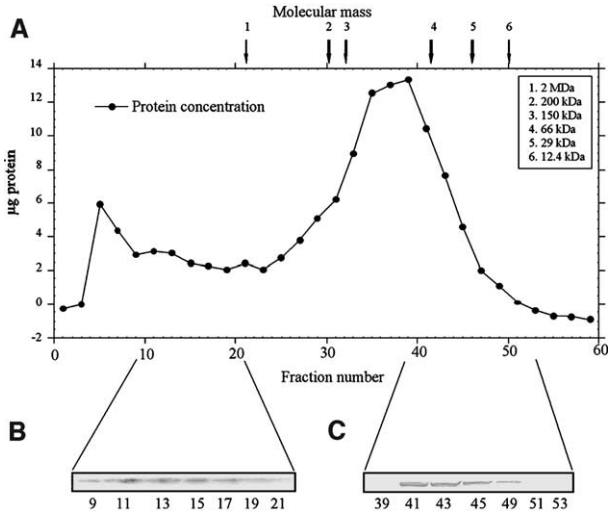

Figure 3 *T. acidophilum* crude-extract fractionation on Superose 6 SEC.
(A) Each fraction was analyzed for its protein content. The flow rate was 0.25 ml/min and the eluted fractions were analyzed by Western blotting. (B) High-molecular-weight fractions containing TAA43 (>2 million); and (C) fractions containing the dimeric TAA43 (70–80 kDa).

formed in the presence of ATP, AMP-PNP, low and high concentrations of Mg^{2+} (10 and 120 mM) and with buffers of different pH (pH 5.5, 7.0 and 8.0). Under all conditions described, the recombinant TAA43 protein retained the same elution volume.

A Superose 6 column was used to determine the molecular size of native TAA43 in *T. acidophilum* crude extract (Figure 3A). Evaluation of the distribution by Western blotting gave an unexpected result. The native TAA43 protein eluted in fractions corresponding to the dimeric form (Figure 3C) as expected; however, native TAA43 was also found to elute in high-molecular-weight fractions (>2 million) (Figure 3B). Densitometric evaluation of the bands shown in Figure 3B and 3C indicated that 20% of the total TAA43 protein eluted in high-molecular-weight fractions and 80% as dimer. The fractions probed for the TAA43 protein did not contain proteasomes as analyzed by Western blotting with anti-proteasome antibodies (see below).

Enzymatic characterization

The enzymatic hydrolysis of various nucleotides (ATP, GTP, CTP and UTP) by TAA43-His_6 was measured with the malachite green assay (Lanzetta et al., 1979). ATP was hydrolyzed at higher rates when compared to the other three nucleotides. The optimum temperature for ATP hydrolysis was 70°C, which is close to the optimal growth temperature of *T. acidophilum* (59°C). Thenceforward, ATPase activity assays were carried out at 65°C to minimize non-enzymatic ATP hydrolysis. The optimum pH for ATP hydrolysis at 65°C was pH 4.8, which is close to the internal pH of *T. acidophilum*, determined as pH 5.5 (Searcy, 1976). NaCl ranging from 0 to 300 mM did not affect the ATPase activity of TAA43-His_6. Different divalent metal ions, i.e. Mg^{2+}, Zn^{2+}, Ca^{2+} and Mn^{2+}, were tested as co-factors in the ATPase activity assay. Mg^{2+} was the preferred divalent cation, whereas Zn^{2+} supported ATP hydrolysis at half the rate and virtually no ATP hydrolysis was detected in the presence of Ca^{2+} or Mn^{2+}.

Kinetics and inhibition analysis

ATP hydrolysis followed Michaelis-Menten kinetics with a K_m for ATP of 0.177 mM and a V_{max} at 65°C of 61.43 nmol of ATP hydrolyzed per mg of TAA43 per min. *N*-Ethylmaleimide (NEM), an inhibitor of the H^+-ATPase activity, and sodium azide (NaN_3), an inhibitor of the F_0F_1 ATPase activity, were tested in ATPase assays with TAA43-His_6. Although it has been shown previously that some AAA family members are inhibited by these compounds (e.g. NSF and VAT), neither NEM nor NaN_3 exerted an inhibitory effect on TAA43.

Site-directed mutagenesis of conserved residues

Three conserved residues were exchanged in order to test their role in nucleotide hydrolysis. Mutant proteins were purified and ATPase activity was measured and compared to the activity of wild-type TAA43-His_6. As expected, exchange of Lys159, the nucleotide-binding residue in the Walker A box, for asparagine inactivated the ATPase activity of TAA43; the role of this residue in coordinating the β- and γ-phosphates of ATP is well understood and mutations at this position have been shown to inactivate many AAA proteins, including NSF (Whiteheart et al., 1994), the six proteasomal ATPases (Rubin et al., 1998), FtsH (Karata et al., 1999) and *Thermoplasma* Lon protease (Besche et al., 2004). Arg269 corresponds to the position 2 arginine or the so-called 'second arginine finger', which interacts with the γ-phosphate of the nucleotide bound to the neighboring subunit (Ogura et al., 2004). Mutation of the second arginine finger leads to a loss of ATPase activity in FtsH (Karata et al., 1999), or loss of function but not ATPase activity in NSF (Matveeva et al., 2002). In the case of TAA43 the Arg269Leu mutation reduced the ATPase activity to 20% of the wild-type enzyme. Two different crystal structures of the AAA ATPase p97 revealed that the position 2 arginine and a second nearby arginine, which corresponds to the position 1 arginine or 'arginine finger', might have overlapping function in hydrolysis of the ATP bound to the adjacent subunit (Ogura et al., 2004). Thus, in the TAA43 Arg269Leu mutant the respective position 1 arginine, i.e. Arg272, might mediate the residual ATPase activity.

Asp264 represents the only conserved polar residue in the second region of homology and it has been proposed that it interacts with the position 4 arginine, a further invariant arginine residue of AAA proteins (Ogura et al., 2004). The position 4 arginine (Arg221 in TAA43) is located C-terminally to the Walker B motif, and it has been suggested that it creates an intramolecular ring of salt bridges that stabilize the hexameric ring of FtsH (Karata et al., 2001). Separate mutations of both residues, arginine and aspartate, in FtsH yielded inactive mutants, except for arginine-to-lysine and aspartate-to-glutamate substitutions, for which residual activity was retained (Karata et al., 2001). However, in TAA43, even the Asp264Glu mutation results in loss of ATPase activity. A similar mutation of the first polar residue in the Walker B

motif, namely Asp212Glu, did not impair the ATPase activity. This Walker B aspartate residue is involved in the coordination of the β- and γ-phosphates of a bound nucleotide via an Mg^{2+} ion. Interestingly, all AAA proteins of the BCS1 group (see Figure 1) have a glutamate instead of an aspartate at the respective Walker B position. This indicates that an aspartate at the first polar Walker B position is not mandatory for ATP hydrolysis. All mutant proteins described were, like wild-type TAA43, dimeric, as shown by Superose 12 gel filtration chromatography.

Activation of the proteolytic activity of the proteasome

As *Thermoplasma* does not encode a homolog of PAN (Zwickl et al., 1999), the canonical activator of the proteasome, we assayed recombinant TAA43 for proteasome activation. However, neither the hydrolysis of small peptides nor the degradation of the loosely folded casein by *Thermoplasma* proteasomes was activated in the presence of TAA43. Moreover, TAA43 could not unfold the GFP-ssrA protein, a substrate of *Methanococcus* PAN, which is efficiently unfolded and translocated into the proteasome for degradation (Benaroudj et al., 2003). Therefore, we conclude that TAA43 is not a functional homolog of archaeal PAN. This is consistent with the fact that the TAA43 protein forms a dimer, whereas all characterized activators of protein degradation, e.g. PAN, ClpA, ClpX, and HslU, form hexameric complexes.

Immunoprecipitation of TAA43

In order to identify proteins interacting with TAA43 we performed an immunoprecipitation analysis. Pre-cleared *T. acidophilum* crude extract was immunoprecipitated with anti-TAA43 IgG coupled to Sepharose G beads. The precipitates were subjected to SDS-PAGE, the gel was stained with Coomassie blue and the 14 bands labeled were identified by mass spectrometry (MS) (Figure 4A). A total of 21 proteins was identified, 16 of which were ribosomal proteins, 10 from the large 50S and 6 from the small 30S subunit (Table 1). A similar pattern of bands was obtained, when fraction 11 from the Superose 6 separation shown in Figure 3 was used for immunoprecipitation (Figure 4B). Although ribosomal proteins are known to contaminate affinity purified proteins, the number of identified ribosomal proteins allows us to conclude that a fraction of cellular TAA43 is associated with ribosomes.

Discussion

During sequencing of the *T. acidophilum* genome a new single-domain AAA ATPase, ORF1175, was identified. Originally, the protein was called VAT2 because of its sequence similarity with the second AAA domain of *Thermoplasma* VAT (Ruepp et al., 2000). However, as shown in this communication, the TAA43 protein is structurally (dimeric complex) and functionally (no chaperone activity) divergent from VAT, and was thus renamed *Thermoplasma* AAA ATPase of 43 kDa (TAA43). BLAST searches identified homologs of TAA43 in the genomes of the closely related Thermoplasmales *Thermoplasma volcanium* and *Ferroplasma acidarmanus*. A comprehensive phylogenetic analysis showed that these three proteins belong to the meiotic branch within the AAA family and

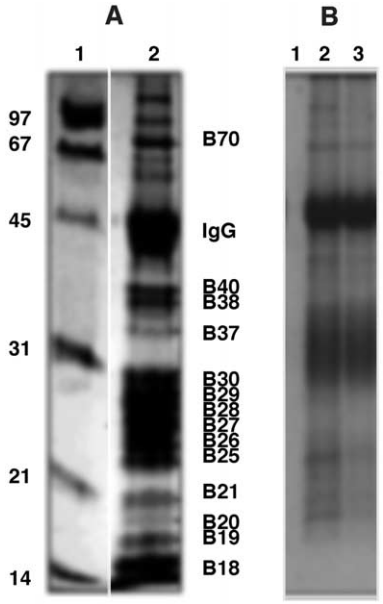

Figure 4 SDS-PAGE analysis of immunoprecipitates obtained with TAA43-specific antibodies and *T. acidophilum* lysate.
(A) Lane 1, molecular mass markers; lane 2, co-immunoprecipitated proteins; bands sequenced by mass spectrometry are indicated by their molecular mass in kDa (B70-B18) and are listed in Table 1. Protein bands without labels could not be identified. (B) Lane 1, control, i.e. Sepharose G beads incubated with *T. acidophilum* lysate; lane 2, immunoprecipitate from *T. acidophilum* lysate as shown in panel (A); lane 3, immunoprecipitate obtained with fraction 11 of the Superose 6 column separation shown in Figure 3.

Table 1 Proteins co-precipitated with TAA43 antibodies and identified by mass spectrometry.

Band number	Protein	Ta ORF
B70	Ribonucleotide reductase	Ta1475
B40	50S ribosomal L3	Ta1271
B38	Conserved hypothetical protein	Ta1488
	50S ribosomal L10P	Ta0359
B37	Hypothetical protein	Ta0466
B30	50S ribosomal L4, L2	Ta1270, 1268
B29	50S ribosomal L4, L2	Ta1270, 1268
B28	50S ribosomal L1	Ta0360
B27	50S ribosomal L1	Ta0360
B26	30S ribosomal S4	Ta1032
B25	30S ribosomal S7	Ta0092
	50S ribosomal L6	Ta1255
B21	30S ribosomal S13	Ta1033
	50S ribosomal L13	Ta0433
B20	ssDNA binding protein	Ta1149
	50S ribosomal L18	Ta1252
B19	50S ribosomal L30	Ta1250
	50S ribosomal L19	Ta1253
	30S ribosomal S19	Ta1267
B18	30S ribosomal S15P	Ta1131
	50S ribosomal L22	Ta1266
	Acetyltransferase subunit	Ta0817
	30S ribosomal S9	Ta0432

classify separately from *Thermoplasma* VAT and proteasome activators, e.g. PAN (Figure 1).

However, since no homolog of the canonical proteasome activator PAN, a single-domain AAA ATPase, was identified in the genomes of *T. acidophilum* and its close relatives, we envisioned TAA43 as a functional homolog. But activation of the proteolytic activity of the *T. acidophilum* proteasome was not detected in *in vitro* assays, indicating that TAA43 most likely does not activate proteasome activity *in vivo* as well. Thus, the endogenous *T. acidophilum* proteasome activator remains to be identified.

TAA43 is a minor protein and comprises only 0.05% of the total *T. acidophilum* proteins. Size exclusion fractionation of *T. acidophilum* cell lysate showed that approximately 80% of the TAA43 protein exists in a dimeric form, with the residual 20% eluted in high-molecular-weight (HMW) fractions (>2 million; Figure 3B). Recombinant TAA43 behaves as a dimeric protein according to native gel electrophoresis and gel filtration analysis. This is rather unusual for AAA ATPases, since most of these proteins assemble into hexameric complexes. However, the AAA ATPase katanin, belonging to the meiotic branch of the AAA family (Figure 1), was shown to undergo a substrate-induced conversion from a dimeric into a hexameric complex (Hartman and Vale, 1999). Remarkably, TAA43 specific antibodies precipitated several ribosomal proteins from the crude lysate. This finding might indicate that the TAA43 protein interacts with the *Thermoplasma* ribosome. Whether or not TAA43 has a different oligomeric state when associated with the ribosome remains to be investigated. Interestingly, in eukaryotes the AAA ATPases Rix7p and Rea1p were found to be required for biogenesis and nuclear export of 60S ribosomal subunits (Gadal et al., 2001; Nissan et al., 2002). Such a general function cannot be predicted for TAA43 due to its limited distribution in Archaea; however, a specific role in the biogenesis of ribosomes from *Thermoplasmales* is possible.

Materials and methods

Bacterial strains and plasmids

Thermoplasma acidophilum DSM 1728 was obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; DSMZ). Cloning and expression was performed with *E. coli* XL1-Blue and *E. coli* BL21(DE3) strains (Stratagene, La Jolla, USA), respectively.

Cloning, expression, purification, and mutagenesis

The *taa43* gene (ORF Ta1175) was amplified by PCR with the primer pair 3C-His-sense and 4C-His-antisense (Table 2) from genomic *T. acidophilum* DNA and cloned into the *Nco*I and *Xho*I sites of pET28a(+), resulting in a C-terminally His_6-tagged TAA43 protein. The TAA43 protein was purified with Ni-NTA spin columns (Qiagen GmbH, Hilden, Germany). The N-terminus of the purified protein was determined by Edman degradation. TAA43 mutant proteins (primer pairs are listed in Table 2) were obtained with the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and purified as described for the wild-type protein.

Preparation of antiserum and Western blotting

Recombinant TAA43 protein (2 mg) purified on a Ni-NTA column was separated on a 12.5% SDS polyacrylamide gel. The resulting protein band was excised from the gel and used to produce a polyclonal antiserum in rabbit (Eurogentec, Seraing, Belgium). *T. acidophilum* crude extract and purified recombinant TAA43 protein were blotted from SDS or native gels onto nitrocellulose. Membranes were probed first with rabbit antiserum against TAA43 as the primary antibody (diluted 1:1000), second with an anti-rabbit IgG-alkaline-phosphatase-conjugated antibody (diluted 1:1000) (Sigma, St. Louis, USA), and developed with NTB and BCIP (both from Sigma).

Size-exclusion chromatography

FPLC gel filtration was performed using a Superose 12 and a Superose 6 column (Amersham Biosciences, Little Chalfont, UK). The fractions collected were separated in non-denaturing 4–20% gels, and TAA43 was detected by Western blotting.

Characterization of ATPase activity

The optimal ATPase activity conditions were determined using the malachite green method as described by Lanzetta et al. (1979). In short, 10 μg of recombinant protein was added into 50 μl of total reaction buffer. The reaction was carried out for 15 min at 65°C and stopped by adding 800 μl of color reagent (3:1 mixture of 0.045% malachite green hydrochloride and 4.2% ammonium molybdate in 4 N HCl, containing 0.1% Triton X-100) and 100 μl of 34% sodium citrate solution. Each experiment was carried out in duplicate and the optical density (OD) was measured at 640 nm. A standard curve obtained using 0–10 nmol of P_i was measured in parallel. To determine the kinetics of the ATP hydrolysis, Enzyme Kinetics Software version 1.0 was used (D.G. Gilbert, Dog Star Software, Bloomington, Indiana and Biology Department, Indiana University, USA).

Preparation of *Thermoplasma* crude extract lysate

Cultured cells were harvested and resuspended in 5 volumes of lysis buffer [50 mM Tris-HCl, pH 8.0; 1 mM DTT; DNase II (10 μg/ml)] and incubated at room temperature for 30 min before lysis by sonication. The lysate was centrifuged for 1 h at 61 700 *g*, glycerol was added to the supernatant (final concentration 20%) and stored at -80°C.

Co-immunoprecipitation studies

Physical interaction of proteins was determined as suggested by the Immunoprecipitation Starter Pack (Amersham Biosciences). A pre-clearing step was carried out to avoid excess antibody. A 50-μl aliquot of protein G-Sepharose (Amersham Biosciences) was incubated with 70 μg of affinity purified anti-TAA43 IgG in 0.1 M HEPES buffer, pH 8.0. This reaction was incubated at 4°C for 1 h, washed three times with HEPES buffer, and centrifuged at 12 000 *g* for 20 s. In the precipitation reaction, 100 μg of *T. acidophilum* crude extract lysate was added to the protein G-Sepharose coupled anti-TAA43 IgGs and incubated once more for 1 h at 4°C in 0.1 M HEPES buffer. To avoid non-specific binding, single reactions were washed at least five times and centrifuged at 12 000 *g* for 20 se with 1% Tween 20 washing solution resuspended in 0.1 M HEPES, pH 8.0.

After washing, the final pellet was resuspended in 30 μl of SDS loading buffer, heated to 95°C for 3 min, and centrifuged at 12 000 *g* for 20 s. The supernatant was loaded onto a 12.5% SDS polyacrylamide gel and stained after electrophoresis with Coomassie Brilliant Blue. The identities of the co-precipitated proteins were determined by mass spectrometry (Protein Chem-

Table 2 Bacterial strains, vectors and primers.

Strain, vector or primer	Characteristics	Source or reference
E. coli strain		
Epicurian coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F′ proAB lacI^{q}ZΔM15 Tn10 (Tet^r)*]	Stratagene
Epicurian coli BL21 (DE3)	*B F$^-$ dcm omp T hsdS($r_B^-m_B^-$) gal (DE3)* [*pLysS Cam^r*]	Stratagene
Vector		
pET28a(+)	T7 *lac* promoter, pBR322 origin, Kan^r	Calbiochem/ Novabiochem
Primer (5′→3′)		
3C-His-sense	CATGCCATGGGCGTGTTGGATGACATCGACGAGAAG	This study
4C-His-antisense	CCGCTCGAGGAATCCAGCGAATTCCCTGTATTTCG	This study
K159N sense	CCGGGCACAGGAAACACCTTCATAGTAAAGGCC	This study
K159N antisense	GGCCTTTACTATGAAGGTGTTTCCTGTGCCCGG	This study
D264E sense	CCCTGGGAGATAGAAGAAGCTATGCTGAGGCC	This study
D264E antisense	GGCCTCAGCATAGCTTCTTCTATCTCCCAGGG	This study
R269L sense	GGGAGATAGATGAAGCTATGCTGCTACCTGGCCGATTCG	This study
R269L antisense	CGAATCGGCCAGGTAGCAGCATAGCTTCATCTATCTCCC	This study

istry Group, Max-Planck-Institute of Biochemistry, Martinsried, Germany).

Phylogenetic analysis of AAA proteins

AAA sequences were selected from the non-redundant protein sequence database (nr) at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) by seeding PSI-BLAST (Altschul et al., 1997) with the AAA domain alignment of the SMART database (Schultz et al., 1998; smart.embl-heidelberg.de) and extracting all sequences with expect values (E-values) of 10 000 or less. These were subsequently searched with HMMer (hmmer.wustl.edu) using a hidden Markov model (HMM) derived from regions alphaA to alphaE of the AAA alignment published by Lupas et al. (1997). Sequences that matched the HMM at E-values of 1 or less were extracted and clustered using CLANS (Frickey and Lupas, 2004b). CLANS, a JAVA application comparable to BioLayout (Enright and Ouzounis, 2001), uses probability values (*p* values) of high-scoring segment pairs (HSPs) obtained from an $N \times N$ BLAST search to compute attractive and repulsive forces for each sequence pair. Sequences are then seeded randomly in three-dimensional space and iteratively moved until convergence according to the force vectors resulting from all pairwise interactions. Clustering of these sequences using BLAST *p* values of 10^{-10} or less revealed a clearly delineated cluster for members of the AAA family, which were subsequently extracted and used for further analysis.

A HMM derived from regions αA to αC of the alignment of Lupas et al. (1997) was used to identify ATPase domains within the AAA sequences. After the removal of visibly degenerate AAA domains, all sequence regions with E-values of 10 or less were extracted and aligned using ClustalW (Thompson et al., 1994). The alignment used for phylogenetic tree construction was manually refined and insert regions present in less than 1% of the sequences were deleted. Phylogenies were inferred using the AsaturA software (Van de Peer et al., 2002) and the substitution matrices jtt (Jones et al., 1992) and mtrev (Adachi and Hasegawa, 1996). Due to greatly varying rates of evolution in the various branches, trees and subtrees calculated using different saturation correction scenarios were later combined to best reflect the actual branching pattern of the tree.

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Note added in proof

While this manuscript was under review, the genome sequence of *Picrophilus torridus*, a thermoacidophilic archaeon that belongs to the order Thermoplasmales, was published [Proc. Natl. Acad. Sci. USA *101* (2004), 9091–9096]. Neither the published annotation nor our analysis of the *P. torridus* genome sequence did reveal a homolog of the *T. acidophilum taa43* gene. This might indicate that the occurence of the *taa43* gene is limited to a subgroup of species within the order Thermoplasmales.

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