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Genome Sequencing of *Thermoplasma acidophilum* and Characterization of the Translation-Associated ATPase of 43 kDa (TAA43), a member of the AAA$^+$ Family

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Genome Sequencing of *Thermoplasma acidophilum* and Characterization of the Translation-Associated ATPase of 43 kDa (TAA43), a member of the AAA+ family

1. SUMMARY

Thermoplasma acidophilum (*T. acidophilum*), an obligatory acidophilic and thermophilic organism contains one of the smallest genomes (1.5 Mbp). Due to its simplicity, it is an excellent model for studying its proteins and for the comparison with corresponding proteins of higher organisms. The sequencing of its entire genome has provided an enormous amount of information: 55% of its chromosomal coding sequences have similarity to known proteins from all domains of life (archaea, bacteria, and eukarya), 29% have similarity to proteins of unknown function, and the remaining 16% contain no assignable information. Within the 29% of coding sequences without a known function, a set of proteins from the superfamily of ATPases associated with various activities (AAA+ family) was found. AAA+ proteins are a family of enzymatic machines involved in diverse cellular functions ranging from DNA repair and replication to organelle biogenesis, membrane trafficking, transcriptional regulation, and protein quality control. The *T. acidophilum* genome is lacking the presence of the AAA-regulatory particle of the 26S proteasome of eukaryotes, or the archaeal PAN in *Methanococcus jannaschii*, which are needed to unfold and disaggregate protein substrates and to facilitate their entry into the chamber which harbors the proteolytic active sites in proteasomes. Therefore, it was of particular interest to find out if one of the AAA+ proteins (VAT, Lon-2, and/or TAA43) found in *T. acidophilum* could participate directly or indirectly in the ATP- and proteasome-dependent protein degradation pathway. The *T. acidophilum* ORF 1175 (namely TAA43) was selected for such investigation. *In vitro* and *in vivo* interaction analyses provide a hint of AAA+ TAA43 protein as associated with proteins found in two major molecular machineries of the cell, the transcription and the translation apparatus, thus discarding the hypothesis proposed above. Analysis of the gene organization of *T. acidophilum* indicates the *taa43* ORF to be in close proximity to genetic elements resembling a superoperon (a cluster of genes composed by ribosomal proteins, a DNA-directed RNA polymerase subunit, and transfer RNAs, among others).

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2. INTRODUCTION

2.1. BACKGROUND

2.1.1. General features

Thermoplasma acidophilum (*T. acidophilum*) as its name indicates, is a thermoacidophilic organism that lives at an optimum temperature of 59°C and a pH 1-2. It does not grow at temperatures lower than 45°C or higher than 62°C, and it undergoes lysis at neutral pH. *T. acidophilum* was first isolated from a coal refuse pile where the mean temperature was 56°C (Darland et al., 1970). It was discovered later that the primary habitats of *T. acidophilum* are solfatara fields (Segerer et al., 1985).

T. acidophilum cells are pleomorphic spheres with a diameter between 0.3 and 2 μm, and no organelles or nuclei were found. Its internal pH is around 5.5 when analyzed at room temperature (Hsung and Haug, 1975). The growth of *T. acidophilum* cells is inhibited by the antibiotic novobiocin, an inhibitor for DNA gyrase.

Some of the characteristics that distinguishes the genus *Thermoplasma* morphologically from other organisms are the following: the cells are polymorphic spheres, during the exponential growth phase they show filaments, whereas the spherical forms are predominant in the stationary phase, and the cells are devoid of a cell wall. The latter characteristic was confirmed by inhibition studies, in which resistance to the cell wall inhibitors vancomycin and ristocetin was found. Thus *Thermoplasma* was first classified as a Mycoplasm, a cell-wall lacking bacterium, but later 16S rRNA sequence analysis showed that *Thermoplasma* belongs to the archaea (figure 2.1), the third domain of life (Fox et al., 1980). The *Thermoplasma* membrane contains a highly glycosylated membrane protein rich in mannosyl residues (Searcy et al., 1982).

Other characteristics that make *Thermoplasma* distinct are its physiological properties. *Thermoplasma* cells need yeast extract to grow. The presence of other nutrients like sucrose, glucose, mannose, galactose, and fructose was found to stimulate growth significantly (Segerer et al., 1988).

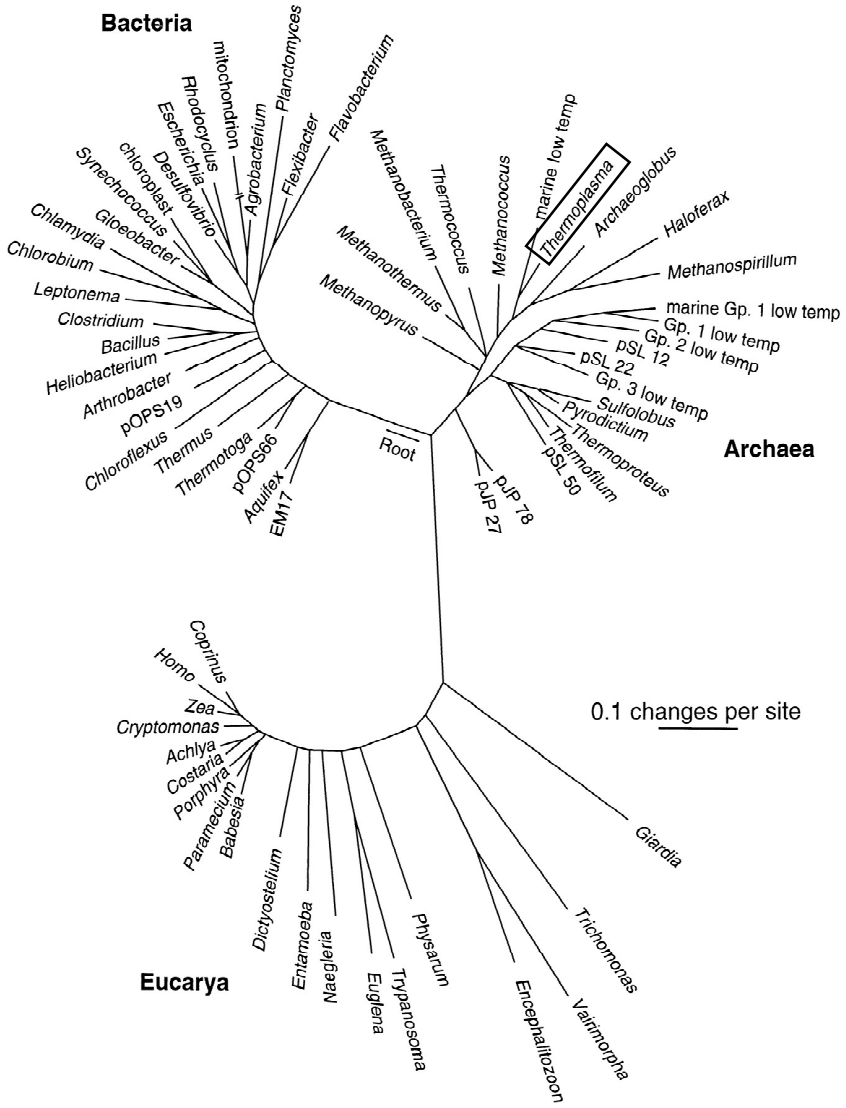


Figure 2.1. Universal phylogenetic tree based on small-subunit (SSU) rRNA sequences. Sixty four rRNA sequences representative of all known phylogenetic domains were aligned (Pace, 1997).

Up to now, five species of the genus *Thermoplasma* have been described: *T. acidophilum* (Darland et al., 1970), *T. volcanium* (Segerer et al., 1988), *Picrophilus oshimae* (Schleper et al., 1995), *Ferroplasma acidiphilum* strain Y^T (Golyshina et al., 2000), and *Ferroplasma acidarmanus* (Edwards et al., 2001).

The main reason for studying such organisms is their relative simplicity. Due to the fact that archaeal organisms contain small genomes, the information obtained from their DNA sequence could make it easier to understand complete cellular networks.

They contain many molecules found in higher organisms and can therefore be used as simple models to study the more complex eukaryotic homologs.
Many proteins from *T. acidophilum* have been described so far. They comprise, among other proteins, the 20S proteasome (Dahlmann et al., 1989), tricorn protease and its associated aminopeptidases (Tamura et al., 1996), (Tamura et al., 1998), the thermosome (Phipps et al., 1993), and VAT, a homolog of the eukaryotic CDC48/p97 vesicle-fusion proteins (Pamnani et al., 1997).

2.1.2. Description of ATPases

In general, molecular interactions result from conformational changes in proteins (enzymes) that occur in response to their binding of not only ATP (adenosine 5'-triphosphate) but also many other factors. ATPases that bind and hydrolyze ATP and other nucleotides (GTP, CTP, and, UTP), play an important role in converting chemically stored energy into a biological activity.
Two decades ago, a common motif was identified, the phosphate-binding loop (P-loop) of ATP- and GTP-binding proteins. This domain was originally found in α- and β-subunits of ATP synthases, the myosin head, the adenylate kinase and the RecA protein (Walker et al., 1982) and is now referred to as Walker A domain. Then, an increasing number of sequences were found to have this particular signature: GXXXXGK[TS]*, examples of these are kinases, the *ras* oncogene product p21, the heterodimeric G proteins and elongation factors, together with several proteins involved in active transport.
Later on –and more specifically in ATP-binding proteins- it was found that these proteins contain a second signature: DEXX. The sequence was termed Walker B motif. This region was discovered to bind metal ions, where aspartic acid was involved in the water-mediated binding of Mg^{++} and glutamic acid was involved in the actual catalysis, (Saraste et al., 1990) i.e. hydrolysis of the nucleotide.
A new class of the Walker-type ATPases –the AAA ATPases-, appeared to be different from the rest of the ATPases due to the presence of a distinctive sequence signature.

* X is any amino acid residue.

2.1.3. AAA family

The AAA (ATPases Associated with various cellular Activities) (Erdmann et al., 1991) protein family is characterized by the presence of the Walker A and Walker B boxes and a unique trait denominated second region of homology (SRH). As shown in figure 2.2 organisms from all domains of life (archaea: *Thermoplasma acidophilum* and *Methanococcus jannaschii*, eukarya: *Saccharomyces cerevisiae*, and bacteria: *Rhodococcus erythropolis*) contain proteins from the AAA family and share the typical AAA boxes. This particular amino acid sequence signature, distinguishes the AAA proteins from other ATPase families like the ion pumps, DEAD box-helicases, ABC transporters, etc. (Peters et al., 1990). The AAA family members contain one or two copies of this AAA cassette of about 230-250 amino acid residues.

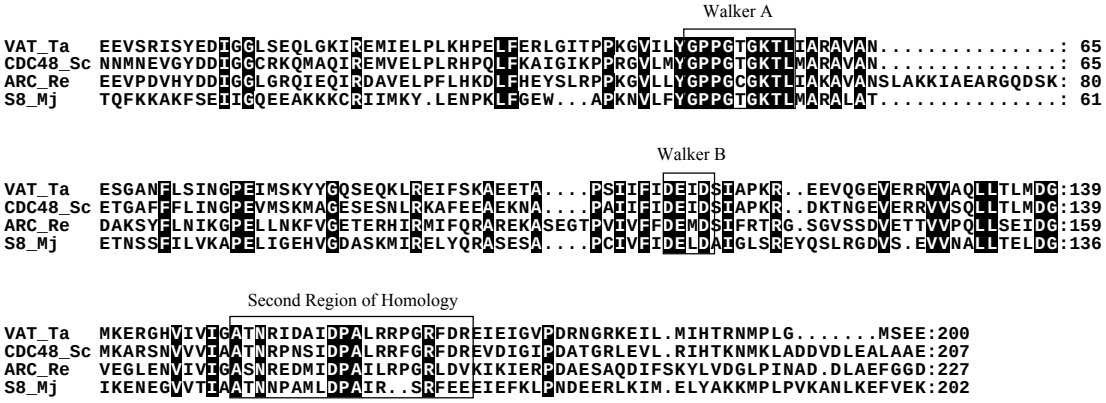


Figure 2.2. Multiple sequence alignments of members of the AAA family of proteins from the three domains of life. These members are typically identified by the presence of the Walker A and B and the second region of homology signatures. The unique AAA domain of the proteins ARC and S8, and the second domain of VAT and CDC48 where chosen for alignments. Ta, *T. acidophilum*; Sc, *Saccharomyces cerevisiae*; Re, *Rhodococcus erythropolis*; Mj, *Methanococcus jannaschii*. Accesion numbers: VAT_Ta (SWISS-PROT O05209), CDC48_Sc (SWISS-PROT P25694), ARC_Re (EMBL AF088800), S8_Mj (GenBank U67590).

AAA proteins are found in all kindoms of life (Archaea, Eubacteria, Eukaryota: Protista, Fungi, Plants, Animals) and participate in very diverse functions as in cell

cycle regulation, vesicle and organelle biogenesis, proteolysis, etc. All members of this family are mostly Mg^{++}-dependent ATPases and form several oligomeric states.
Historically, the porcine VCP (valosin containing protein) was the first AAA protein whose sequence was determined (Koller and Brownstein, 1987). It was found to have high similarity with proteins like p97 from *Xenopus laevis*, which participates in membrane fusion events (Peters et al., 1990), and CDC48 (cell division control protein) from *Saccharomyces cerevisiae* whose mutants show arrest in mitosis (Fröhlich et al., 1991). All these proteins contain two AAA domains. Moreover, structural studies showed that they form homo-hexameric rings. Later, several other homologues displaying the same characteristics were described, e.g. VAT from *T. acidophilum* (Pamnani et al., 1997). AAA proteins with a single AAA domain were also identified and the AAA family was divided into two groups: type I, containing a single copy of the AAA cassette (e.g., Vps4p, FtsH and katanin), and type II, with two AAA cassettes (e.g., p97, Cdc48 and NSF) (Confalonieri and Duguet, 1995).

2.1.4. AAA family tree

Since the first member of the AAA family was described in 1987, an increasing number of proteins belonging to it have been described. It was possible to classify them in a phylogenetic tree that could assign them to functional subfamilies. As mentioned before, AAA proteins are highly conserved and found in the three domains of life, Bacteria, Archaea, and Eukarya. Therefore, they can be analyzed from the evolutionary point of view. For that purpose, an AAA server was established (http://yeamob.pci.chemie.uni-tuebingen.de/) comprising all the known members according to their primary structure. The family tree that is displayed in figure 2.3 shows six functional categories: 1) meiosis/mitochondria, 2) secretion/neurotransmission, 3) peroxisome biogenesis, 4) subunits of the 26S proteasome, 5) cell division cycle/centrosome/ER homotypic fusion, and 6) membrane-associated proteases.
A new AAA family tree was released recently (Fröhlich, 2001). It resulted from the alignment of 345 AAA boxes from 316 AAA proteins and 92 species. The figure contains essentially the same classification as in figure 2.3.

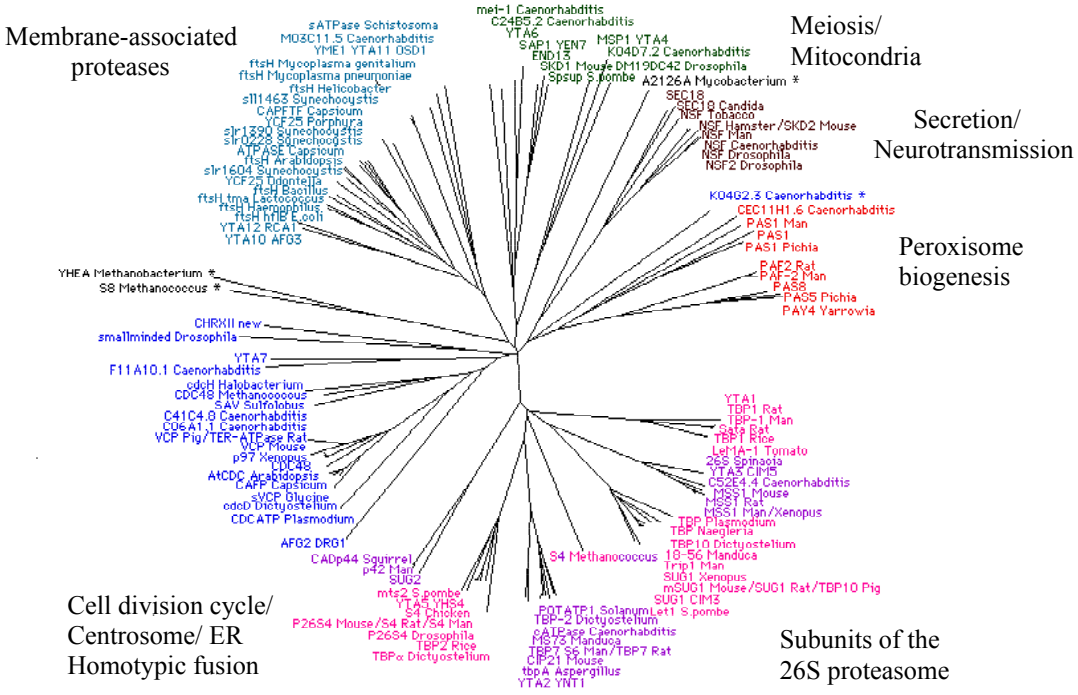


Figure 2.3. AAA phylogenetic tree. Taken from http://yeamob.pci.chemie.uni-tuebingen.de/AAA/Tree.html.

2.2. DESCRIPTION OF AAA SUBFAMILIES

2.2.1. Meiosis/mitochondria

Members of this subfamily contain a single copy of the AAA cassette. Representatives of this group are the microtubule-severing protein katanin participating in mitosis, neuronal differentiation and flagellar physiology (McNally, 2000), the intramitochondrial sorting protein MSP1 (Nakai et al., 1993), and mei-1 (Clark-Maguire and Mains, 1994), a gene that encodes a protein with similarity to the p60 catalytic subunit of the heterodimeric sea urchin katanin. Mei-1 is required for meiotic spindle organization in *C. elegans* (Srayko et al., 2000).

2.2.2. Secretion/neurotransmission

Vesicular transport is mediated in part by NSF (*N*-ethylmaleimide sensitive factor) in the secretion pathway (May et al., 2001) and serves to recycle membrane components and soluble proteins. The SNARE complexes (soluble NSF attachment protein receptors, found in both the vesicle and the target membranes) bind to the α-SNAP (soluble NSF attachment protein) and form a complex known as the 20S complex (different from the 20S proteasome). NSF and α-SNAP cooperate to disassemble the 20S complex through the ATPase activity of the NSF yielding free SNAREs. Thus, assembly and disassembly of SNARE complexes are important events for the subsequent membrane trafficking reactions (Wimmer et al., 2001).
This process is similar in neuronal cells; vesicles and target membrane containing the VAMP (SNARE) proteins attach to the target membrane containing syntaxin (NSF) and SNAP-25 (α-SNAP) thus releasing the neurotransmittor (Taubenblatt et al., 1999), (Chapman, 1994). A homologue of NSF has been described in yeast as Sec18p (Grote et al., 2000).

2.2.3. Peroxisome biogenesis

The peroxisome is an organelle that participates in the trafficking of newly folded and oligomeric polypeptides. The importance of peroxisome biogenesis was recognized when it was found to be associated to severe human anomalies (Moser et al., 1991). Peroxins are the proteins participating in the peroxisome biogenesis and protein import, 23 molecules of this type have been identified. Pex5p, a soluble peroxisomal matrix protein, is the main receptor in the traffic of molecules, it shuttles between the cytoplasm and matrix during the import cycle (Holroyd and Erdmann, 2001) via the peroxisomal targeting signal 1 (PTS1) (Smith and Schnell, 2001). From the 23 identified genes two, *PEX1* and *PEX6*, belong to the AAA family of proteins.

2.2.4. Subunits of the 26S proteasome

The 26S proteasome is a supramolecular protein complex with a molecular weight between 2 and 3 MDa. It participates in the degradation of cytosolic and nuclear proteins in eukaryotic cells and has two main components: the core subunit –the 20S proteasome-, and the regulatory complex (RC) –the 19S cap- (Coux et al., 1996).
The RC is associated with one or both ends of the cylindrical core complex (Peters et al., 1993). It has also been studied in several organisms like *Drosophila melanogaster*, bovine, and human (Glickman et al., 1999), (Holzl et al., 2000). The RC from *Saccharomyces cerevisiae* consists of 18 subunits (Rpn/Rpt subunits). Six of these subunits are recognized as ATPases from the AAA+ family. The importance of the RC relies in the recognition of ubiquitinated substrates, which are then deubiquitylated and prepared for the degradation in the 20S core (Glickman et al., 1998). In this context, the ATPases mediate the energy-dependent removal of folded and aggregated proteins, acting as reverse chaperones by unfolding and disaggregating protein substrates (Strickland et al., 2000) and facilitating their entry into the 20S core that contains the active sites for proteolysis (Braun et al., 1999).
According to the analyses of archaeal genome sequences only a single protein shares similarity with eukaryotic RC subunits, namely with the AAA-ATPases. This protein, originally identified as S4 in *Methanococcus jannaschii*, was found to stimulate the proteolytic activity of the 20S proteasome in an ATP-dependent reaction (Wilson et al., 2000). The ATPase complex was therefore renamed PAN, for proteasome-activating nucleotidase (Zwickl et al., 1999), (Wilson et al., 2000). Later PAN was shown to have protein unfolding activity (Benaroudj et al., 2001). However, it remains to be unravelled how proteins are recognized for their degradation in archaea, since these organisms lack the ubiquitin tagging system.

2.2.5. Cell division cycle/centrosome/ER homotypic fusion

The mammalian p97 and cdc48p from yeast are the most intensively studied proteins from the AAA family. They are involved in several cellular processes and have highly conserved homologues in many diverse species like *Xenopus laevis* p97 (Peters et al., 1990), *Arabidopsis* AtCDC48 (Feiler et al., 1995), and *T. acidophilum* VAT (Pamnani

et al., 1997). These proteins contain two copies of the AAA cassette and form hexameric rings. Some of the functions that have been proposed include homotypic membrane fusion, fusion of the endoplasmic reticulum in yeast (Latterich et al., 1995) and reassembly of Golgi cisternae in mammals (Rabouille et al., 1995). Another function proposed for the p97/cdc48p protein is a role in the ubiquitin-dependent protein degradation, implying a function in protein unfolding or disassembly of protein complexes (Patel and Latterich, 1998).

2.2.6. Proteases

The Zn-dependent metalloprotease FtsH (filamentation temperature-sensitive) is a bacterial member of the AAA family. FtsH is involved in the selective degradation of the membrane proteins SecY and the *a* subunit of the F_0 complex of the H^+-ATPase, and the soluble proteins σ^{32} (heat-shock sigma factor) and phage λ CII (transcriptional activator). In the eukaryotic organisms, homologues of FtsH are associated with mitochondria and chloroplasts (Schumann, 1999). FtsH is supposed to function as a chaperone and as a proteolytic enzyme. It contains a single AAA domain and the protein is anchored to the cytoplasmic membrane where it forms a complex. In general, it has been suggested that these ATP-dependent proteases are indispensable to maintain the quality control of protein turnover (Arsene et al., 2000).

2.3. AIMS OF STUDY

As mentioned briefly in the section of general features, elucidation of the complete genome of *T. acidophilum* was of great interest because of its model organism character. This Ph.D. work started when half of the genome had already been sequenced. The first objective therefore was the cloning and sequencing of the genome to completion, the editing of the data produced, and the assignment of the probable function via the BLAST search. During the annotation process of the genome (a work done by the Munich information center for protein sequences, MIPS), four genes with the typical characteristics of the AAA+ superfamily were found. The ORFs were the following: Ta0840 for VAT protein, Ta1081 for Lon protein, Ta0098 for Lon-2 protein, and Ta1175 for TAA43. Because so far, no protein had been found in *T. acidophilum* that could replace the activity of the regulatory complex found in eukaryotic organisms or PAN from *M. jannaschii,* the proteins coded by these ORFs are the likely candidates to provide this function. The investigation of the translated product from ORF Ta1175 (TAA43) was selected for this study. The aim of the second part of this work was therefore the full characterization of TAA43 protein: amino acid sequence analysis, biochemical, functional, and structural studies, and in addition, the detection of its natural substrate(s).

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Chemicals

ADP	Sigma
Agarose (SeaKem LE)	FMC
AMC and Suc-LLVY-AMC	Bachem
AMPPNP	Sigma
ATP	Pharmacia
Bromophenol blue	Serva
Casein	Sigma
Coomassie G 250	Serva
CTP	Pharmacia
L-Cystein	Merck
DMSO	Sigma
Dyalysis tubing Spectra/Pro 2 Membrane	Laguna Hills
Ethidium bromide	Sigma
N-Ethylmaleimide	Sigma
Filter unit	Nalgene
Gelatin	Sigma
GTP	Pharmacia
HEPES	Biomol
Malachite green	Sigma
ß-Mercaptoethanol	Merck
MES	Sigma
Millex-GS Filter Unit 0.22 µm	Millipore
Nickel-NTA-Agarose	Qiagen
dNTPs	Pharmacia
Optitran Nitrocellulose membrane	Schleicher & Schuell

Petri dishes	Greiner
Ponceau S concentrate	Sigma
Protein loading buffer 4x	Roth
ProtoGel	National diagnostics
Qiabrane Nylon Plus	Qiagen
Reacti-Bind^{TM} EIA Microwell Plates	Pierce, USA
Rhodanese	Sigma
Slide-A-Lyzer Cassette	Pierce
TEMED	Serva
Triton X-100	Roth
Tween 20	Calbiochem
UTP	Pharmacia
Whatman paper	Schleicher & Schuell
Xylene cyanol FF	Fluka

3.1.2. Bacterial culture media

Bacto-Agar	Difco
Bactotryptone	Difco
Yeast extract	Difco
MacConkey agar	Difco
Maltose	Difco

3.1.3. Antibiotics

Ampicillin	Sigma
Chloramphenicol	Sigma
Kanamycin	Sigma

3.1.4. Kits

BigDye Sequencing kit	Applied Biosystems
Bio-Rad protein assay kit	Bio-Rad
DIG Gel Shift Kit	Roche

DNA Ligation Kit	Takara Shuzo Co., Ltd.
ß-Gal Assay Kit	Invitrogen
Gigapack III XL-Packaging Extract	Stratagene
IgG assay kit	Pierce
Immunoprecipitation Starter Pack	Pharmacia
Prep-a-gene kit	Bio Rad
Qiagen plasmid isolation kit	Qiagen
Qiaquick PCR purification kit	Qiagen
QuikChange^{TM} Site-Directed-Mutagenesis kit	Stratagene
QIAprep Turbo Miniprep kit	Qiagen
QIAGEN Plasmid Midi kit	Stratagene
SuperCos 1 cosmid vector kit	Stratagene
Two-Hybrid in Bacteria	Hybrigenics
ZAP express vector	Stratagene

3.1.5. Enzymes and Antibodies

Alkaline Phosphatase	New England BioLabs
Pfu Turbo DNA polymerase	Stratagene
Restriction Endonucleases	New England BioLabs
Lysozyme	Sigma
Benzonase	Merck
X-Gal	Biomol
DNase I	Sigma
RNase A	Sigma
IPTG	Biomol
T4 DNA Ligase	New England BioLabs
Anti-rabbit IgG	Sigma
ImmunoPure Rabbit IgG	Pierce

3.1.6. Size markers

Lambda DNA/*Hind*III Marker	Fermentas

High molecular weight calibration kit	Pharmacia
SDS-PAGE molecular weight standards	Bio-Rad

3.1.7. Columns and chromatography media

MAbTrap G II	Pharmacia
MicroSpin^{TM} G-50	Pharmacia
Protein G SepharoseTM 4 Fast Flow	Pharmacia
Superose 12 HR 10/30	Pharmacia
Superose 6 HR 10/30	Pharmacia

3.1.8. Bacterial strains

Thermoplasma acidophilum DSM 1728 was obtained from the German Collection of Microorganisms and Cell Cultures, DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweige, Germany).

E. coli strain for amplification and isolation of plasmid DNA:
Epicurian Coli XL1-Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI^{q}ZΔM15 Tn10 (Tet^{r})]* from Stratagene, Heidelberg, Germany

E. coli strain for protein expression:
Epicurian Coli BL21 (DE3) *B F^{-} dcm omp T hsdS(r$_B^-$m$_B^-$) gal (DE3) [pLysS Cam^{r}]^{a}* from Stratagene, Heidelberg, Germany.

E. coli strain for the two-hybrid system:
BTH101 *F^{-}, cya-99, araD139, galE15, galK16, rpsL1 (Str^{r}), hsdR2, mcrA1, mcrB1* from Hybrigenics.

3.1.9. Vectors

pET28a(+)	T7 *lac* promoter, pBR322 origin, Kan^{r}	Calbiochem/ Novabiochem
pT25	p *lac* promoter, p15A origin, Cm^{r}	Hybrigenics

pUT18	p *lac* promoter, ColEI origin, Amp^r	Hybrigenics
pKT25-Zip	p *lac* promoter, p15A origin, Kan^r	Hybrigenics
pUT18C-Zip	p *lac* promoter, ColEI origin, Amp^r	Hybrigenics
pBK-CMV	*lac* promoter, f1 (-) origin, Neo^r/Kan^r	Stratagene

3.1.10. Primers (all 5'-->3')

Primers for the C-terminal $(His)_6$-tag TAA43 overexpression in *E. coli*

3C-His-sense	CATGCCATGGGCGTGTTGGATGACATCGACGAGAAG
4C-His-antisense	CCGCTCGAGGAATCCAGCGAATTCCCTGTATTTCG

Primers for mutagenesis in TAA43

K159Nsense	CCGGGCACAGGAAACACCTTCATAGTAAAGGCC
K159Nantisense	GGCCTTTACTATGAAGGTGTTTCCTGTGCCCGG
D212Esense	CAATAATATTCTTCGAAGAAATAGATGCCCTTGTGCCG
D212Eantisense	CGGCACAAGGGCATCTATTTCTTCGAAGAATATTATTG
D264Esense	CCCTGGGAGATAGAAGAAGCTATGCTGAGGCC
D264Eantisense	GGCCTCAGCATAGCTTCTTCTATCTCCCAGGG
R269Lsense	GGGAGATAGATGAAGCTATGCTGCTACCTGGCCGATTCG
R269Lantisense	CGAATCGGCCAGGTAGCAGCATAGCTTCATCTATCTCCC

3.2. METHODS

3.2.1. Cell culture methods

3.2.1.1. Growth of *T. acidophilum* (Christiansen et al., 1975)

A freeze-dried sample obtained from DSMZ was resuspended in 25 mL of the *T. acidophilum* media previously equilibrated to 59°C in an oil bath. The culture was grown at 59°C and 150 rpm for 3 days until reach an OD_{540nm} between 0.5 to 0.6.

***T. acidophilum* media** per 1 liter (final pH 1.5)

10 ml Solution A
100 ml Solution B
10 ml 10% (w/v) sterile Yeast extract
100 ml1 0% (w/v) sterile glucose
4 ml 50% H_2SO_4

Medium

Solution A per 1 liter

1.93 g $FeCl_3.6H_20$
0.18 g $ZnSO_4.7H_20$
0.45 g $MnCl_2.4H_20$
0.022 g $Na_2B_4O_7.10H_20$
0.005 g $CuCl_2.H_20$
0.003 g $NaMoO_4.2H_20$
0.003 g $VOSO_4.7H_20$
0.001 g $CoSO_4.7H_20$

Solution B per 1 liter

13.2 g $(NH_4)SO_4$
3.72 g KH_2PO_4
2.47 g $MgSO_4.7H_20$
0.74 g $CaCl_2.H_20$

3.2.1.2. Storage of *T. acidophilum* cells

To prepare a frozen stock of *T. acidophilum* cells an aerobic culture 1 day after inoculation with a low cell density (OD_{540nm}0.05) was used. The culture was allowed to cool down to room temperature and the pH was adjusted to pH 3.0 by adding 7% NH_4OH. Then, 0.15 g of sucrose per ml of culture was added and completly dissolved. The culture was divided into 1-ml aliquots and frozen at -70°C.

3.2.1.3. Growth and storage of *E. coli* cells

E. coli cells were grown in LB media (supplemented with antibiotics for selection of bacterial resistance when needed) at 30° or 37°C (depending on the protocol to follow) with sufficient aeration. Cultures were stored at -80°C after adding sterile glycerol to a final concentration of 15% (Sambrook et al., 1989).

LB (Luria Bertani broth) per 1 liter

10 g Bacto-tryptone
5 g Yeast extract
5 g NaCl

Agar medium LB + 1.5% (w/v) Bacto-Agar

3.3. MOLECULAR BIOLOGY

3.3.1. Basic techniques

3.3.1.1. Plasmid miniprep procedure

A single colony was picked with a sterile toothpick and transfered to 5 ml LB broth supplemented with the appropiate antibiotic. Incubation was done overnight with shaking at 37°C. Cells were pelleted at 4000 rpm at 4°C for 10 min. The QIAprep Spin Miniprep Kit Protocol in combination with a microcentrifuge was used for the procedure. Briefly, the pelleted cells were resuspended in buffer P1. To achieve the alkaline lysis of the bacteria, the lysate is incubated in buffer P2 and subsequently neutralized and adjusted to high-salt binding conditions in the presence of buffer N3. Purification was performed on a QIAprep silica-gel membrane with several washing steps and the plasmid was eluted with TE buffer.

Buffer P1	50 mM Tris-HCl pH 8.0 10 mM EDTA 100 µg/ml RNase A
Buffer P2	200 mM NaOH, 1% SDS
Buffer N3	3.0 M potassium acetate pH 5.5
TE buffer	10 mM Tris.HCl pH 8.0 1 mM EDTA

3.3.1.2. Determination of DNA concentration

DNA concentration was determined spectrophotometrically at OD_{260nm} by comparing serial dilutions. Calculation was performed assuming that an OD_{260nm} of 1 corresponds to 50 ng/µL of double stranded DNA.

3.3.1.3. Agarose gel electrophoresis

DNA samples were resuspended in 6X loading buffer and separated in a 1% agarose gel in 1X TAE buffer and run at 1-5 V/cm to visualize the DNA by UV-light. Gels were stained by soaking in a solution of 0.5 µg/ml ethidium bromide for 30 minutes at room temperature.

6X loading buffer	10 mM Tris/HCl pH 7.5
	50 mM EDTA
	10% Glycerol
	0.25% Bromophenol blue
	0.25% Xylene Cyanol FF
10X TAE buffer	2 M Tris-acetate
	0.05 M EDTA
	Adjusted to pH 7.2

3.3.1.4. DNA sequencing

All DNA sequencing was performed using dye terminator chemistry (Big Dye RR Terminator Cycle Sequencing kit, Perkin-Elmer). The annealing temperature for cycle sequencing (GeneAmp PCR system 2400, Perkin-Elmer) was at least 55°C. Primers were synthesized by Metabion, Munich, Germany, with a length of at least 20 nucleotides. Analysis of the DNA sequencing reactions were performed at two service facilities of the Max-Planck Institute for Biochemistry (M. Boicu and C. Czoppelt), which are equipped with an ABI 373 and an ABI 377 sequencer respectively, or by Medigenomix, Munich, Germany.

3.3.1.5. Polymerase chain reaction (PCR)

Amplification of DNA of interest was performed with *Pfu*Turbo DNA polymerase from *Pyrococcus furiosus* (Stratagene) using the GeneAmp PCR System 2400 Thermal Cycler (Perkin Elmer).

3.3.1.6. Restriction endonuclease digestion

To create compatible DNA ends for cloning PCR products or genomic DNA fragments into plasmids, DNA was cleaved in presence of 10-20 U of restriction enzyme/µg DNA. The concentration of DNA used in each experiment was variable depending on the molecular size of the DNA fragment. To obtain complete digestion, samples were incubated for 1-3 hours at the optimal reaction temperature (usually at 37°C).

3.3.1.7. Dephosphorylation

1 unit of Shrimp Alkaline Phosphatase (Amersham) per µg of digested double stranded plasmid was used to remove completely the terminal 5'-phosphate. The reactions were incubated at 37°C for at least 2 hours before the enzyme was completely inactivated at 65°C for 15 minutes.

3.3.1.8. Ligation

T4 DNA Ligation Kit (TaKaRa) was used in dephosphorylated restricted cloning vectors (plasmids). The reaction was performed overnight in presence of 1 mM of ATP at 16°C in a 1:10 molar ratio of vector to insert.

3.3.1.9. Preparation of competent cells using $CaCl_2$

E. coli cells were grown from a single colony at 37°C at 300 rpm until OD_{600} of 0.4-0.6 was reached. Cells were harvested and washed with ice-cold 0.1M $CaCl_2$ as described in Sambrook et al., 1989.

3.3.1.10. Transformation of *E. coli* cells

Plasmids (no more that 50 ng in a volume of 10 µL or less) were incubated with 200 µL of freshly thawed competent cells on ice for 30 min. Cells were heat shocked in a preheated water bath at 42°C for exactly 90 seconds. The reaction was chilled on ice

and 800 µL of SOC medium was added. To regenerate, the cells were shaken at 37°C for 1 hour before 100 µL of the suspension was plated on LB agar (containing the appropriate antibiotic) and incubated overnight at 30° or 37°C. 10 to 15 colonies were screened by isolation of the plasmid followed by restriction analysis.

SOB	per 1 liter
	20 g Bacto-tryptone
	5 g Yeast extract
	0.5 g NaCl
	2.5 mM KCl
	20 mM $MgCl_2$
SOC	SOB + 20 mM sterile glucose

3.3.1.10. Site-directed mutagenesis of conserved residues

Mutants in the His-tagged *taa43* gene were produced by PCR using the synthetic primers described in the materials section with the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Briefly, a PCR was done using the plasmid DNA containing the complete *taa43* gene with the His_6-tag, *Pfu* Turbo DNA polymerase and the corresponding primer pairs resulting in the desired mutation. The cycling parameters were adjusted depending on the type of mutation. The point mutation type was a change in a single nucleotide and the single amino acid mutation was a change in a different triplet (12 or 16 cycles respectively). The final product was treated with *Dpn I* endonuclease to digest the parental DNA template and to select for the mutation-containing synthesized DNA. Lastly, this DNA was transformed into XL1-Blue *E. coli* cells and sequenced to confirm the mutation. The expression and purification steps were performed in the same manner as with the wild-type protein (His_6-tag fused to the C-terminal end of the TAA43 protein).

3.3.2. Genome sequencing

3.3.2.1. Isolation of chromosomal DNA from *T. acidophilum*

Thermoplasma acidophilum cells were harvested from 1 liter when the log phase culture reached an OD_{540nm} of 0.6-0.7 by centrifugation at 6000xg for 15 minutes at room temperature. The cell pellet was resuspended in 8 ml of TNE solution (pH 7.5) and 1 ml of 10% N-lauroylsarcosine was added. After gentle mixing of the suspension 1 ml of 10% SDS was added, followed again by gentle mixing. Subsequently, 0.5 ml of proteinase K at 20 mg/ml were added and incubated for 3 hours at 50°C. After incubation, the lysate appeared translucent and viscous. 11 ml of TE-saturated phenol (pH 7.5) were added and stirred for 10 min. To separate the two phases (organic/aqueous) the sample was centrifuged at 5400 g for 10 minutes at 20°C (swing-out rotor). The aqueous layer was transfered into a new tube and the washing step was repeated twice. For the extraction of the DNA, 11 ml of chloroform/isoamyl alcohol (24:1, vol/vol) were added and stirred for 10 min. After separation of the two phases by centrifugation (5400 g for 10 min at 20°C in a swing-out rotor) the aqueous phase was divided in three ml aliquots. To each aliquot 8 ml of 95% ethanol was added and incubated for 1 hour at –20°C. To pellet the DNA, centrifugation was performed at 2000 rpm for 15 min at 4°C (swing-out rotor) and afterwards the pellet was resuspended in 3 ml of 70% ethanol and centrifuged at 1000 rpm for 10 min at 20°C. This washing step was repeated once. The pellet was air-dried for 2-3 minutes and completly resuspended in 2 ml of TE solution (pH 7.5). As a final step, 3 µL of 10 mg/ml DNAse-free RNase were added and the solution was incubated by agitation for 1 hour at 37°C. The DNA was stored at –20°C.

DNase-free RNase	10 mg/ml in TE buffer
TNE solution	100 mM Tris-HCl pH 7.5 100 mM NaCl 50 mM EDTA
TE solution	10 mM Tris-HCl pH 7.5 and 2 mM EDTA

3.3.2.2. Phagemid libraries

Four phagemid libraries were created by complete cleavage of genomic DNA with the restriction enzymes *BamH I*, *Bgl II*, *Sac I* and *EcoR I,* respectively. Another library was created by partially cleavage with *Sau3A I*. Subsequently, the DNA fragments were separated on 0.8% agarose gels. Gel slices with DNA fragments in the range from 3 kbp to 8 kbp were cut from the gel and isolated with the Prep-a-gene kit (Bio Rad). These DNA fragments were cloned into the Lambda ZAP express vector (Stratagene). For packaging of the phages for phagemid and cosmid libraries, the Gigapack III XL-Packaging Extract (Stratagene) was used. Construction of the phagemid libraries was performed according to the instructions of the manufacturer.

3.3.2.3. Cosmid libraries

A cosmid library was constructed using the SuperCos I cosmid vector Kit (Stratagene). *E. coli* cultures were always incubated at 30°C in order to avoid the occurrence of recombination events. Small scale isolation of cosmid DNA were performed with the QIAprep Turbo Miniprep kit (QIAGEN). Cosmid DNA was eluted with 200 µl TE buffer and later concentrated to a suitable volume. For large scale cosmid isolations the QIAGEN Plasmid Midi kit (Stratagene) was used.

3.3.2.4. Long range PCR

DNA fragments, ranging between 4.5 and 15.9 kbp, were amplified using long range PCR (Expand Long Template PCR System, Roche), performed according to the instructions of the manufacturer. The PCR fragments were directly sequenced by primer walking (Ruepp et al., 2000).

3.3.2.5. Sequencing the reverse strand of cosmids

The sequencing of the reverse strand of cosmid clones was performed with DNA templates from different sources in order to close the gaps. One method was using PCR fragments that were amplified from genomic DNA. Another method used was to

perform of sequencing of strand and reverse strand with different cosmids covering the same area on the genome.

3.3.2.6. Assembly and editing

Editing and assembly of the DNA sequencing reactions was performed using the Sequencher 3.0 software (Gene Codes Corporation, Ann Arbor, MI) on a MacIntosh G3 computer.

3.4. PROTEIN CHEMICAL METHODS

3.4.1. Expression and purification of recombinant His-tagged protein under native conditions

Growth of the expression culture and purification of the protein was done following the Ni-NTA Spin Handbook (Qiagen). Initally, a single colony was inoculated into 5 ml LB broth containing appropiate antibiotics and grown overnight at 37°C. The non-induced culture was diluted 1:60 with fresh media and antibiotic(s) and incubated until the OD_{600} reached 0.6. At this point, 1 mM of IPTG was added to the culture and shaken for additional 4 hours. Cells were harvested by centrifugation at 4000g for 15 minutes at 4°C. The cell pellet was resuspended in lysis buffer and incubated with 1 mg/ml of lysozyme for 30 minutes on ice. The cells were lyzed by sonication and then centrifuged at 10000g for 30 minutes at 4°C. The supernatant was then applied to a equilibrated Ni-NTA spin column and spun down for 2 minutes at 700g. The column was washed twice and the protein eluted twice with 200 µL elution buffer at 700 g for 2 min.

Lysis buffer	50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 10 mM imidazole
Wash buffer	50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 20 mM imidazole
Elution buffer	50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 250 mM imidazole

3.4.2. Determination of protein concentration (Bradford, 1976)

Protein concentration determination was done utilizing the Bradford reagent supplied by Bio-Rad. BSA was used for the standard curve and the absorption was measured at A_{595} nm.

3.4.3. Denaturing SDS-PAGE (Lämmli, 1970)

The analysis of the proteins was carried out under conditions that could ensure their dissociation into their monomeric polypeptide subunits and minimizing their

aggregation. Samples were mixed with 2x loading buffer and heated at 95°C for 5 minutes just before loading onto a 12.5% SDS polyacrylamide gel. Electrophoresis was subjected at a constant current of 2mA/cm at room temperature.

Resolving gel	12.5% Acrylamide
	0.375 M Tris-HCl pH 8.8
	0.1 % SDS
	0.05% APS and TEMED
Stacking gel	3% Acrylamide
	0.125 M Tris-HCl pH 6.8
	0.1% SDS
	0.05% APS and TEMED
Running buffer	25 mM Tris-base
	0.192 M Glycine pH 8.3
	0.1% SDS
2x Loading buffer	0.1 M Tris-HCl pH 6.8
	20% Glycerol
	4% SDS
	0.03% Bromophenol blue
	40 mM DTT

3.4.4. Native-PAGE

Native gels were used to check the state of oligomerization of proteins. 4-12% gradient native gels were purchased from Invitrogen and run at least for 24 hours at 4°C to reach equilibrium.

Gradient gel	4-12% Acrylamide
	90 mM Tris-HCl pH 8.6
	0.008 mM EDTA pH 8.3
	0.05% APS and TEMED

Running buffer 90 mM Tris-base
38 mM Glycine

4x Loading buffer 0.2 M Tris-HCl pH 6.8
20% glycerol
0.03% Bromophenol blue

3.4.5. Colloidal Coomassie Brilliant Blue G-250 staining (Neuhoff et al., 1988)

SDS- or native gels were first fixed in 12% TCA for 30 minutes and subsequently washed with several changes of water. The staining was done overnight at room temperature and the excess of stains was washed out with destilled water.

Staining solution 40 ml 10% $(NH_4)_2SO_4$ /2% H_3PO_4
10 ml Methanol
0.8 ml 5% Coomassie blue G-250

3.4.6. Preparation of antiserum

Recombinant TAA43 protein (2 mg) purified by Ni-NTA column was separated in 12.5% SDS polyacrylamide gel. The resulting protein band was excised from the gel and used to produce polyclonal antisera in rabbit (Eurogentec, Bel S.A.).

3.4.7. Protein transfer onto nitrocellulose

Separated proteins were transferred from SDS- or native gels onto nitrocellulose membranes using a Bio-Rad Semi-Dry Electrophoretic Transfer Cell. Pre-equilibration of the membrane was done for at least 15 minutes before, using the transfer buffer. The procedure was carried out at 15 V for 15 minutes.

Transfer buffer 39 mM Glycine
48 mM Tris-base
(0.037 % SDS in denaturing gels)
20 % Methanol

Successful transfer was detected by staining of the membranes with Ponceau S dye.
In order to detect quickly proteins eluted from a column or a ultracentrifugation step a dot-blot procedure was done. For this purpose the samples were applied directly to the nitrocellulose membrane using a dot-blot vacuum device.

3.4.8. Immuno-blotting with alkaline phosphatase

After the protein transfer, the membrane was washed with 1X TBS buffer and unspecific binding sites were blocked overnight with a solution containing 1-2% gelatin. After washing the membrane with washing solution (2 x 5 min), the membrane was incubated with the primary antibody (diluted 1:1000 in blocking solution) against the protein of interest for 1 hour. Excess of antibody was removed by several washing steps prior to the incubation with the second antibody (diluted 1:1000 anti-rabbit IgG, alkaline phosphatase conjugated from Sigma in blocking solution) again for 1 hour. Lastly, the membrane was washed and developed with 10 ml of developing solution containing 66 µL NTB (Sigma) and 33 µL BCIP (Sigma) until color appeared. The reaction was stopped with 10 ml stop solution.

1X TBS 10 mM Tris-HCl pH 7.5
150 mM NaCl

Blocking solution 1X TBS
1-2% gelatin
0.05% Tween 20

Washing solution 1X TBS
0.05% Tween 20

NTB (Nitrotetrazolium blue)
50 mg in 1 ml 70% DMF

BCIP (5-Brom-4-Chlor-Indolylphosphate)
50 mg in 1 ml 100% DMF

Developing solution	10 ml washing solution
	66 µL NTB
	33 µL BCIP
Stop solution	10 ml washing solution
	20 mM EDTA

3.4.9. Gradient ultracentrifugation

10-30% glycerol gradient ultracentrifugation was performed using 10 mg of crude extract from *T. acidophilum* and in parallel, 250 µg of the recombinant protein. For this purpose, a SW28 swinging bucket rotor was used in a L8M Preparative Ultracentrifuge (Beckman). The samples were centrifuged at 24000 rpm for at least 24 hours at 4°C. The gradient was fractionated and fractions were tested for their content of TAA43 by dot-blot.

3.4.10. Size exclusion chromatography

FPLC gel filtration was performed with these samples using a Superose 12 column (Pharmacia Biotech) with a LCC-500 Plus chromatography system monitored at 280 nm. The fractions collected were separated in a non-denaturing 4-20% gel, and analyzed by Western-blotting.

3.4.11. Characterization of ATPase activity

The optimal ATPase activity conditions were determined using the malachite green method as described by Lanzetta et al. In all cases, 10 µg of recombinant protein was added into 50 µL total reaction buffer. The reaction was carried out for 15 minutes 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 done in duplicates and measured at 640 nm. A standard curve was

measured in parallel each time, for that purpose, a 0.4 mM KH_2PO_4 stock solution was used to prepare serial dilutions containing 0 to 10 nmol of Pi (from the KH_2PO_4).

Reaction buffer 50 mM MES buffer pH 5.5
1 mM ATP
10 mM $MgCl_2$
2 mM L-Cystein

Color reagent 3:1 mixture of:
0.045% malachite green hydrochloride
4.2% ammonium molybdate in 4 N HCl
(containing 0.1% Triton X-100 in the total solution)

3.5. COMPUTER ANALYSIS

3.5.1. Phylogenetic tree classification of TAA43 and sequence analysis

Alignments of the sequences were done using the Clustal X application program. The TreeView program was used to display TAA43 within the AAA family tree. The sequence alignments were performed with the conserved domain of the most representative members of each subfamily (sequences were selected from the web site http://yeamob.pci.chemie.uni-tuebingen.de).

3.5.2. DNA and protein sequence analysis

GENETYX-MAC version 9.0 software was used to translate DNA sequences to the amino acid primary structure and to display the restriction sites present in any given DNA sequence.

3.6. FUNCTIONAL STUDIES

3.6.1. Foldase/unfoldase studies (Chaperonin-assisted protein folding of the enzyme rhodanese by TAA43)

Unfolded rhodanese (Sigma) was obtained by incubation at 25°C for at least 60 minutes in the denaturing buffer. The reaction contained 18.83 µL 1x buffer, 0.33 µL TAA43 at 30 µM, 0.2 µL rhodanese at 50 µM, 0.14 µL GroES at 137 µM and 0.5 µL ATP at 0.2 M. This mixture was incubated for different periods of time (0, 10, 20, 30, 40, and 50 minutes), and then 180 µL of the reaction solution were finally added. The reaction was incubated for 4 minutes and stopped with 100 µL of 15% formaldehyde. For the developing the color 300 µL of ferric nitrate reagent were added, mixed throughly and read at 460 nm within 30 minutes.

1x Buffer	20 mM MOPS pH 7.4 100 mM KCl 10 mM $MgCl_2$
Denaturing buffer	6M Guanidine-HCl 5 mM DTT
Reaction buffer	50 mM KCN 40 mM KH_2PO_4 50 mM NaS_2O_3 45 mM CDTA pH 8.0
Ferric nitrate reagent	92 mM $Fe(NO_3)_3.9H_20$ 8.6% HNO_3

3.6.2. Electrophoretic mobility shift assay (EMSA)

3.6.2.1. Labeling of oligonucleotides at the 3' end

A set of double stranded oligonucleotides with consensus sequences was purchased from Stratagene. A DIG Gel Shift Kit was used for the EMSA assay (Roche). The first step consisted in the annealing of the oligonucleotides at 95°C for 10 minutes, then, they were diluted to a final concentration of 3-4 pmol/µL. The labeling step was made in a final volume of 20 µL per oligonucleotide. Briefly, the digoxigenin-11-ddUTP solution was incubated with the oligonucleotide of interest in presence of 50 U of Terminal transferase at 37°C and precipitation was done in presence of 4 M LiCl and chilled ethanol at -70°C. After spinning and several washing steps, the pellet was resuspended at a final concentration of 0.155 pmol/µL. The labeled probes were then diluted to reach a working concentration of 15-30 fmol/µL.

3.6.2.2. Gel shift reaction and chemiluminescent detection

Three samples were prepared for the control reaction and for the analysis of TAA43:
1. 50 fmol of labeled oligonucleotide without TAA43
2. 50 fmol of labeled oligonucleotide with 100 ng of TAA43
3. same as in 2., but with an 123-fold excess of unlabeled oligonucleotide for specific competition.

All samples contained 1 µg of poly[d(I-C)] as DNA competitor and 0.1 µg poly L-lysine as protein competitor. Samples were incubated for 15 minutes at room temperature, chilled on ice and loaded to a pre-electrophoresed native gel. The gel was run at 8 V/cm and then electroblotted onto a positively charged membrane for 30 minutes at 400 mA. The oligonucleotides transfered onto the membrane were UV cross-linked for 3 min at 254 nm with a transilluminator.
Chemiluminescence detection was done as described in the following. The membrane was blocked for 30 minutes and then incubated with 75 mU/ml of anti-digoxigenin Fab fragments conjugated with alkaline phosphatase. The excess of unbound conjugate was removed by washing 2 times for 15 minutes. The membrane was

incubated with the DNA side down for 5 minutes in presence of the CSDP substrate (disodium 3-4(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-choloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate). The excess of liquid was dripped off the membrane using a sheet of Whatman 3MM paper. The membrane was sealed in a hybridazation bag, pre-incubated for 15 min at 37°C and exposed to X-ray film for 30 min at room temperature. Developing of the X-ray film was done using a CURIX 60 film processor (Agfa).

3.6.3. Immunodepletion assays

Depletion of molecules was carried out using affinity purified antibodies against the molecules of interest (MAbTrap G II column, Pharmacia). The reaction consisted of a mixture of 100 µg *T. acidophilum* crude extract plus 5-10 µg of specific purified antibody coupled to 500 µL of protein G Sepharose (Pharmacia). Each reaction was incubated for 1 hour at 4°C and then centrifuged at 12000 g for 20 seconds. The supernatant was recovered and subjected to peptidase and casein degradation assays. Each reaction was done in duplicates.

3.6.4. Determination of peptidase activity

For assaying peptidase activity, the fluorogenic peptide Suc-LLVY-AMC (Bachem) were added to a given reaction to a final concentration 100 µM (immunodepleted crude extract). Incubation was carried out at 60°C for 30 minutes and stopped using a SDS solution at final concentration of 0.1%. A 100% control containing 10 µM of AMC was prepared in 0.1 M HEPES buffer pH 8.0, and, as background control, 100 µM of the fluorogenic peptide Suc-LLVY-AMC was used. Fluorescence activity of released AMC was monitored fluorometrically at 360/460 nm in a spectrofluorometer (LS50B, Perkin-Elmer).

3.6.5. Casein degradation assay

25 µg FITC-casein were added to the immunodepleted samples. Reactions were incubated for 1 hour at 60°C and stopped with 3.5% TCA. A precipitation step followed i.e. chilling the reactions on ice for 30 minutes and subsequent

centrifugation at maximal speed (14000 rpm) for 10 minutes. The supernatant was saved and additional buffer was supplied to adjust a final volume of 700 µL. Samples were monitored by the release of the FITC at 490/525 nm in a spectrofluorometer.

3.6.6. Two-hybrid system in bacteria (2HB)

3.6.6.1. Plasmid library construction into pUT18 (pUT18-T.a. hybrid)

20 µg of *T. acidophilum* genomic DNA was digested with 20 U of *BamHI* restriction enzyme and in a parallel reaction 50 ng of plasmid pUT18 for 2 hours at 37°C. Cloning steps were performed as in the molecular biology section.

3.6.6.2. Cloning *taa43* into pT25 (pT25-taa43 hybrid)

A PCR reaction was performed to amplify the *taa43* gene and clone it into the pT25 using *Pfu* Turbo DNA polymerase kit (Stratagene). The PCR product was purified with the Qiaquick PCR purification kit (Qiagen) and digested with *BamHI* for 2 hours at 37°C. Cloning steps and transformation were done as in the protocol explained in the molecular biology section.

3.6.6.3. Preparation of BTH101 competent cells by $CaCl_2$ (containing pT25-taa43 hybrid)

A single colony containing the pT25-taa43 hybrid was grown in LB media with 30 µg/ml chloramphenicol. Cells were grown from a single colony at 37°C at 300 rpm until a OD_{600} of 0.4-0.6 was reached. Cells were harvested and washed with ice-cold 0.1M $CaCl_2$ as described in Sambrook et al., 1989.

3.6.6.4. Co-expression and selection of interacting hybrid proteins

400 µL of competent cells containing pT25-taa43, were transformed with serial dilutions of the pUT18-T.a. hybrid library (up to 100 ng). Transformants were plated on M63 minimal medium containing 30 µg/ml chloramphenicol, 100 µg/ml ampicillin, 0.5 mM IPTG and 40 µg/ml X-Gal. Growth was detected after 4-5 days of

incubation at 30°C and the blue colonies were selected. Characterization of positive clones was done using the ß-galactosidase assay and co-precipitation studies.

Synthetic media (M63) per 1 liter

2 g $(NH_4)_2SO_4$

13.6 g KH_2PO_4

0.5 mg $FeSO_4.7H_2O$

17 g agar

Adjust pH 7.0 with KOH, autoclave

1 ml 1M $MgSO_4.7H_2O$

15 ml 20% maltose

2 ml 0.05% vitamin B1 (thiamin)

3.6.6.5. ß-galactosidase assay

The microtiter plate assay was performed according to the instructions of the manufacturer (Invitrogen) for the analysis of the selected clones. For this purpose, a 5 ml overnight liquid culture was grown at 30°C with the appropriate antibiotics. A 10% glycerol stock was stored at -80°C from each culture. Samples were read at 420 nm. In all cases, a blank control (untransformed cells) and a positive interacting control (plasmids containing the leucine zipper domains supplied by Hybrigenics) were grown in parallel.

3.6.7. Co-precipitation studies

Physical interaction of proteins was performed as suggested by the Immunoprecipitation Starter Pack (Pharmacia). A pre-clearing step was done to avoid excess of antibody. 50 µL of protein G Sepharose (Pharmacia) were 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 hour, washed 3 times with HEPES buffer, and centrifuged at 12000 x g for 20 seconds. In the precipitation reaction, 100 µg of *T. acidophilum* crude extract was added to the protein G Sepharose coupled to the anti-TAA43 IgG and incubated once more for 1 hour at 4°C in 0.1 M HEPES buffer. To avoid non-specific binding single reactions were washed at least 5 times and

centrifuged at 12000 g for 20 seconds with 1% Tween 20 washing solution resuspended in 0.1 M HEPES pH 8.0.
After the washing steps, the final pellet of the reaction was resuspended in 30 µL SDS loading buffer, heated at 95°C for 3 minutes, and centrifuged at 12000 x g for 20 seconds. The supernatant was loaded on a 12.5% SDS polyacrylamide gel and staining of the gel was done with Coomassie brilliant blue.

3.6.8. Protein sequencing analysis

The amino acid sequence analysis was done in the Protein Chemistry group at the Max-Planck-Institute for Biochemistry, Martinsried.

3.6.9. Computer analysis

DNA and protein sequences were analyzed using the standard nucleotide-nucleotide and protein-protein BLAST program available in the web (http://www.ncbi.nlm.nih.gov/BLAST/). The submission of the DNA and protein sequences was done in a FASTA format, and then compared against the NCBI nucleotide and protein databases.

4. RESULTS

4.1. *T. ACIDOPHILUM* GENOME PROJECT

The first part of this thesis work resulted in the completion of the total genomic DNA sequence of *T. acidophilum*. A total of 400 phagemids, covering 850 kbp of the genome (54%), and 469 cosmids, covering 1533 kbp (98%), were partially or fully sequenced using the shotgun approach combined with primer walking. The genome contains a single circular chromosome with 1,564,905 bp. 1,509 ORFs were identified and comparative analysis of the proteins from archaea, bacteria, and eukaryotes disclosed that more than a third of the ORFs have homologs in the three domains of life as shown in figure 4.1 (Ruepp et al., 2000).
A concise description of the results obtained from the analysis of the genome is shown in Table 4.1.

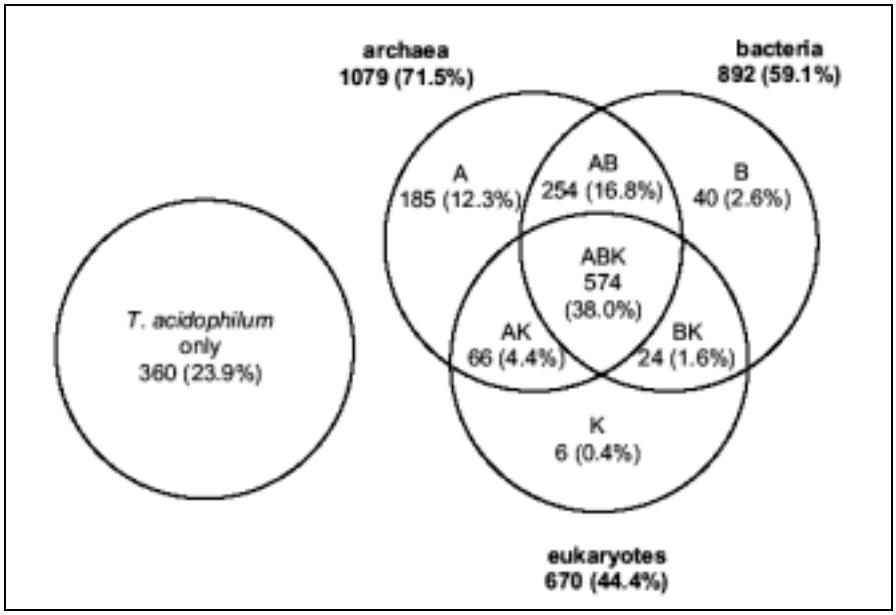


Figure 4.1. Comparison of the *T. acidophilum* protein complement with that of archaea, bacteria, and eukaryotes. Supplementary information taken from Ruepp et al., 2000.

Table 4.1. Summary of the analysis *T. acidophilum* ORFs*.

1507 entries in the database *T. acidophilum*
1203 sequences have homologues to known proteins (79.8%)
37 sequences have putative self-matches (2.5%)
410 sequences contain PROSITE patterns (27.2%): 204 PROSITE patterns present
1341 sequences contain sequence BLOCKS (89.0%): 337 sequence BLOCKS present
767 sequences contain PFAM domains (50.9%): 494 PFAM domains present
882 sequences contain superfamily assignments (58.5%): 901 superfamily assignments present
681 sequences contain PIR keywords (45.2%): 295 PIR keywords present
380 sequences contain EC numbers (25.2%): 309 EC numbers present
556 sequences are assigned to functional categories (36.9%): 128 functional categories present
153 sequences have signal peptides (10.2%)
28 sequences contain coiled-coil regions (1.9%)
383 sequences have at least one transmembrane region (25.4%)
268 sequences have at least two transmembrane regions (17.8%)
224 sequences have at least three transmembrane regions (14.9%)
37 sequences contain more than 20% of low complexity sequence (2.5%)
538 sequences contain non-globular regions (35.7%)
165 sequences are all-alpha proteins (10.9%)
54 sequences are all-beta proteins (3.6%)
901 sequences are alpha/beta proteins (59.8%)
4 sequences are irregular proteins (0.3%)
705 sequences have known or homologous 3D structure (46.8%)
643 sequences contain at least one SCOP domain (42.7%): 376 SCOP folds present

*Web site (http://pedant.GSF.DE/cgi-bin/wwwfly.pl?Set=Tacidophilum&Page=index)

4.2. TAA43 CHARACTERIZATION

4.2.1. Sequence analysis of TAA43

54% of the approximately 1500 proteins from *T. acidophilum* still lack functional characterization. One of these new proteins, encoded by ORF Ta1175, was identified by sequence comparison as a previously unknown member of the AAA protein family. The ORF Ta1175 with a length of 1128 bps and GC content of 48.9% is located in the genome from nucleotides 1,246,336 to 1,247,463. Translation of its DNA sequence using the GENETYX-MAC version 9.0 software (figure 4.2) results in a sequence of 375 amino acid residues. This protein, termed TAA43 has a calculated molecular weight of 41999.4 and a calculated isoelectric point of 6.2.

The TAA43 protein contains one copy of the AAA domain. The closest relative of TAA43 found in the *T. acidophilum* genome is the VAT protein (VCP-like ATPase containing two AAA domains). VAT is 745 amino acids large and contains -in contrast to TAA43- two AAA domains: from Ile^{187} to Val^{459} and from Val^{464} to Lys^{718}

(Pamnani et al., 1997). A sequence comparison with the complete deduced sequence of TAA43 using the GENETYX-MAC program revealed an amino acid sequence identity of 29% when aligned with the first AAA domain of VAT. The alignment of the second AAA domain yielded an identity of 39% (data not shown).

```
   ATGAGAGTGTTGGATGACATCGACGAGAAGATAGATAAAGCGACTAAGCTGCTTCAGGGA  60
 1  M  R  V  L  D  D  I  D  E  K  I  D  K  A  T  K  L  L  Q  G
   GGAATAAAGGAAGAATCCCTAGGCCACAAAGACAAGGCAAAGGAGTATTACTTCGCCGCT  120
21  G  I  K  E  E  S  L  G  H  K  D  K  A  K  E  Y  Y  F  A  A
   TACAGACTGATGCTGGAAGCCGCCAACGCGTCCCCCCCAGATCTCAAAAAGAAAAGACTG  180
41  Y  R  L  M  L  E  A  A  N  A  S  P  P  D  L  K  K  K  R  L
   GATCAGTGCAACATCATTCTAAGTGCCTACCAGCGCGTCAATGAAAACAGAACCGCACCA  240
61  D  Q  C  N  I  I  L  S  A  Y  Q  R  V  N  E  N  R  T  A  P
   GCTGAAGAGAAGACCAGCGACAGGATATCTGAGGGAGAGGCCCTGCTTGAAGAAATAGGC  300
81  A  E  E  K  T  S  D  R  I  S  E  G  E  A  L  L  E  E  I  G
   CTAGAGAAGCCAGAGATACCGAAGGTAACGTTTGAGGATGTTGCTGGGCTTGATGACGTT  360
101  L  E  K  P  E  I  P  K  V  T  F  E  D  V  A  G  L  D  D  V
   AAAAACGAGATCCTCGGGAAGATCGTGTATCCGATGAAATTCAAGGAGCTGTCCCAGGAA  420
121  K  N  E  I  L  G  K  I  V  Y  P  M  K  F  K  E  L  S  Q  E
   TACAATATTCAATTTGGAGGAGGCATGCTGTTGTACGGCCCTCCGGGCACAGGAAAAACC  480
141  Y  N  I  Q  F  G  G  G  M  L  L  Y  G  P  P  G  T  G  K  T
   TTCATAGTAAAGGCCATAGCCAATGAGGTCAGGGCGAGGTTCATCAATGTAAACCCGGCC  540
161  F  I  V  K  A  I  A  N  E  V  R  A  R  F  I  N  V  N  P  A
   AGCCTCTACAGCCAGTGGTTCGGTATGTTCGAGAAAAACATATCGAAGCTTTTCCGGGCT  600
181  S  L  Y  S  Q  W  F  G  M  F  E  K  N  I  S  K  L  F  R  A
   GCGGCTTTGCTCTCTCCTTCAATAATATTCTTCGATGAAATAGATGCCCTTGTGCCGAAA  660
201  A  A  L  L  S  P  S  I  I  F  F  D  E  I  D  A  L  V  P  K
   CGTGATACCTCAAACTCAGATGCGGCAAAACGGGGCGTTGCGCAACTGCTGAATGAGGTC  720
221  R  D  T  S  N  S  D  A  A  K  R  G  V  A  Q  L  L  N  E  V
   GGCGGCATAAATTCACAGAAGAACAAGAATCTGTTCATCATAGCCGCAACGAACAATCCC  780
241  G  G  I  N  S  Q  K  N  K  N  L  F  I  I  A  A  T  N  N  P
   TGGGAGATAGATGAAGCTATGCTGAGGCCTGGCCGATTCGATATCAAAATATACGTGCCG  840
261  W  E  I  D  E  A  M  L  R  P  G  R  F  D  I  K  I  Y  V  P
   CCCCCGGACATTATAGCCAGAAAGAAGATCTTTGAGCTGAACATGGCCAAGGTTAAGCAG  900
281  P  P  D  I  I  A  R  K  K  I  F  E  L  N  M  A  K  V  K  Q
   GCTGGCAATATTGATTACAATTTGCTTGCCCAGCAGACTGAGGGGTACAGCGGTGCAGAC  960
301  A  G  N  I  D  Y  N  L  L  A  Q  Q  T  E  G  Y  S  G  A  D
   ATAGAATTCATATGCAAGAAGGCCAGCCAGAACGTATTCATGGAAGCCGTTAAATCCGGA  1020
321  I  E  F  I  C  K  K  A  S  Q  N  V  F  M  E  A  V  K  S  G
   AAACAGAGGCAGGTGGAGACCAGGGATCTCCTCGACGTGATCGGTAGCATAAAACCGTCA  1080
341  K  Q  R  Q  V  E  T  R  D  L  L  D  V  I  G  S  I  K  P  S
   ATCGATGCCGATCTGCTCTCGAAATACAGGGAATTCGCTGGATTCTAA              1128
361  I  D  A  D  L  L  S  K  Y  R  E  F  A  G  F  *
```

Figure 4.2. DNA and deduced amino acid sequence obtained from *T. acidophilum* ORF Ta1175.

A BLAST search identified an AAA ATPase from *Thermoplasma volcanium* (VAT-2_Tv, NCBI NP_110901), as the closest homolog. The sequence identity between TAA43 and its homolog from *T. volcanium* is 79%.

Originally, TAA43 was termed VAT-2 because of the homology with the second domain of the *T. acidophilum* VAT protein (Ruepp et al., 2000). Later on, because of the biochemical and structural differences with VAT, (as shown later), it was renamed TAA43.

A multiple sequence alignment with other members of the AAA family is shown in figure 4.3 and clearly reveals the existence of the AAA domain. The AAA domain consists of the three highly conserved boxes, Walker A, Walker B and the Second Region of Homology.

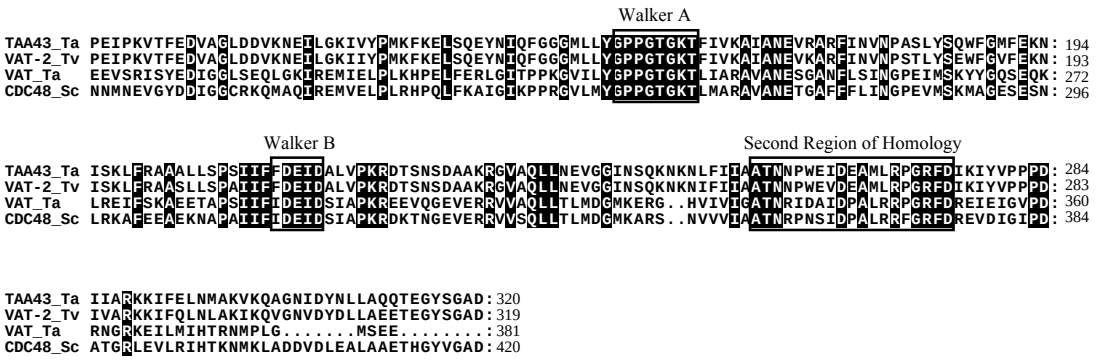


Figure 4.3. Sequence comparison of the amino acid sequence of TAA43* with *T. volcanium* VAT-2*; VAT-2_Tv (NCBI NP_110901), *T. acidophilum* VAT**; VAT_Ta (SWISS-PROT O05209), and *S. cerevisiae* CDC48**; CDC48_Sc (SWISS-PROT P25694). * single domain, ** second domain.

4.2.2. Phylogenetic tree classification of TAA43 within the AAA family

In order to classify the TAA43 protein within the AAA tree, it was necessary to compare it with a complete set of the conserved domains of representatives of the AAA superfamily from Bacteria, Eukarya and Archaea. For the analysis, this set included a selection of updated sequences taken from the AAA web site (http://yeamob.pci.chemie.uni-tuebingen.de). The phylogenetic distribution of this updated tree (Frohlich, 2001) subdivides the AAA family into at least seven cellular functions. Among them are the 1) meiosis, microtubule severing, mitochondrial functions, 2) homotypic fusion/cell division cycle/ polyubiquitination, 3) peroxisomal proteins, 4) ARC: AAA ATPase forming Ring-shaped Complexes, 5) subunits of the proteasome, 6) membrane-associated proteases, and 7) secretion proteins. Alignments

were carried out with the Clustal X multiple sequence alignment program (Thompson et al. 1997). The distance-based dendrogram is shown in figure 4.4.

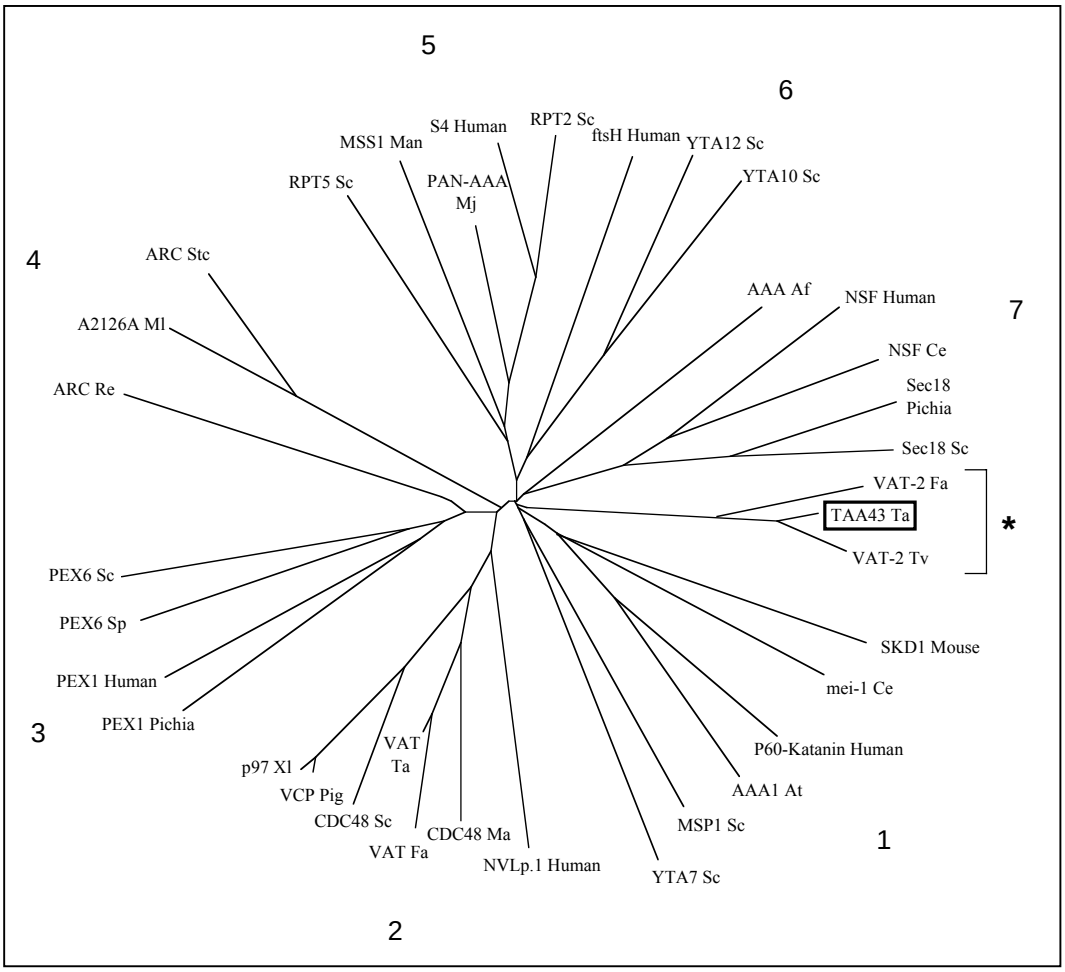


Figure 4.4. Phylogenetic classification of TAA43 within the AAA family. The tree was formed according to the distance-based sequence analysis of the highly conserved ATPase domains: aminoproximal modules of the secretion and homotypic fusion families and carboxyproximal module of the peroxisomal family. Clockwise description: 1) meiosis, microtubule severing, mitochondrial functions, 2) homotypic fusion/cell division cycle/ polyubiquitination, 3) peroxisomal proteins, 4) ARC: AAA ATPase forming Ring-shaped Complexes, 5) subunits of proteasomes, 6) metalloproteases, 7) secretion proteins and, *) new branch formed.
The abbreviations of the organisms for sequence alignments are the following: Af *Archeoglobus fulgidus*, At *Arabidopsis thaliana*, Ce *Caenorhabditis elegans*, Fa *Ferroplasma acidarmanus*, Ma *Methanobacterium thermoautotrophicum*, Mj *Methanococcus janaschii*, Ml *Mycobacterium leprae*, Pichia *Pichia pastoris*, Re *Rhodococcus erythropolis*, Sc *Saccharomyces cerevisiae*, Sp *Schizosaccharomyces pombe*, Stc *Streptomyces coelicolor*, Ta *Thermoplasma acidophilum*, Tv *Thermoplasma volcanium*, Xl *Xenopus laevis*. Sequences were taken from the list of AAA family proteins available at the AAA server (http://yeamob.pci.chemie.uni-tuebingen.de/AAA/List.html).

TAA43 and its homologues, VAT-2 from *T. volcanium* and VAT-2 from *Ferroplasma acidarmanus* form a new branch off the main root, which is not assignable to any of the existing subfamilies.

4.2.3. Sequence analysis of the N-terminal domain of TAA43

A secondary structure prediction for the N-terminal domain of TAA43 was performed together with the homologous sequences from *T. volcanium* and *F. acidarmanus* using the Jpred method (http://jura.ebi.ac.uk:8888/submit.html) (Cuff et al., 1998). The prediction shows that the three sequences seem to contain a four-helix bundle of the square type (figure 4.5). Four-helix bundles are common domain structures in alpha (α) helical proteins (Branden et al., 1991). Sequence comparisons were also done using PSI-Blast on the non-redundant database (NCBI/BLOSUM62 matrix). Similarity was not sufficiently significant to allow inference of homology, but it produced a fair degree of low-level sequence similarity with RNA-binding proteins (data not shown).

```
                          hhhhhhhhh              hhhhhhhhhhhhhhhhh
TAA43 Ta            MRVLDDIDEKIDKATKLLQGGIKEESLGHKDKAKEYYFAAYRLMLEAAN:49
VAT-2 Tv              MSDDVQNKIEKATKLLHAGIKEEENGHKDKAKEYYLVAYRVMLEAAN:47
VAT-2 Fa         MLMTRSSTMTETKIDHVSLLIRKAIEEESSGNKNEAARTYKAAYKEVLEIAS:52

                 hhhhhhhhhhhhhhhhh               hhhhhhhhhhh
TAA43 Ta  ASPPDLKKKRLDQCNIILSAYQRVnenrtapaeek-TSDRISEGEALLEEIGLEKPEI:106
VAT-2 Tv  DSPSDLKKKRLDQCALILNAYKRVsgertdftvkkqNDDEIVEGEALLEEIGIEKPEI:105
VAT-2 Fa  RSTGKIKQKRLEQAANLKKIYESIggeav-------EEDRISEGKKILGTLNIEPPEI:103
```

Figure 4.5. Secondary structure prediction of the N-terminal domain of TAA43 from *T. acidophilum* (TAA43 Ta, accession number CAC12300.1), VAT-2 from *T. volcanium* (VAT-2 Tv accession number NP_110901.1), and VAT-2 from *F. acidarmanus* (VAT-2 Fa with a gene finder in the *F. acidarmanus* genome project: Contig 157/Gene 81). The runs of letter h on top of the amino acid sequences in the figure indicate the predicted helices. The prediction was done by Andrei N. Lupas using Jpred (http://jura.ebi.ac.uk:8888/submit.html) (SmithKline Beecham Pharmaceuticals).

4.2.4. Cloning

Genomic *T. acidophilum* DNA was used to amplify the ORF for TAA43 by PCR, introducing a 5' *NcoI* site and a 3' *XhoI* site (figure 4.6), which changed an arginine in

position 2 to glycine. This allowed the subcloning of the gene into the reading frame of the pET28a(+) vector, resulting in the fusion of a $(His)_6$-tag at the COOH-terminus of the protein (figure 4.7).

a)

Nco I site:	5'-CATGCCATGGGCGTGTTGGATGACATCGACGAGAAG-3'
N-terminal sequence:	**M** G V L D D I D E K
Xho I site:	3'-GCTTTATGTCCCTTAAGCGACCTAAGGAGCTCGCC-5'
Complementary strand:	5'-CGAAATACAGGGAATTCGCTGGATTCCTCGAGCGG-3'
C-terminal sequence:	…-- K Y R E F A G F L E --- **HHHHHH**

b)

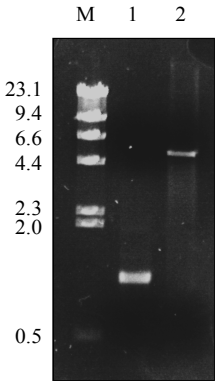
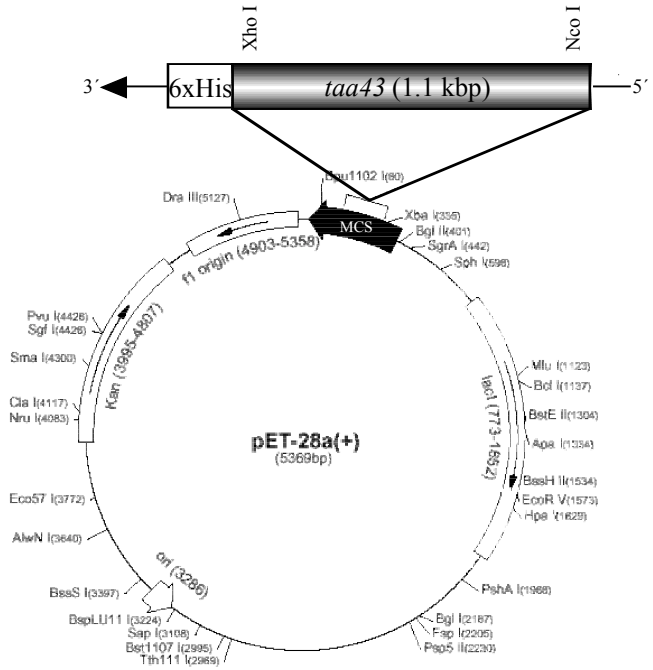


Figure 4.6. a) Modifications obtained with these specific primers to produce the gene according the COOH-terminal His_6-tagged TAA43. b) 1% agarose gel. M, molecular markers in kbp; 1, *taa43* amplified product; and 2, pET28a(+) vector (both containing the *NcoI/XhoI* sites).

Figure 4.7. Cloning of the *taa43* PCR product into the expression vector pET-28a(+). The PCR product was digested with the *NcoI/XhoI* endonucleases and cloned into the reading frame of pET-28a(+) vector to obtain the 6xHis affinity tag at the C-terminal end.



E. coli XLI-Blue cells were transformed with this ligation reaction and subsequently screened for positive clones by restriction analysis as shown in figure 4.8. Those plasmids containing the correct size of *taa43* (1128 bp) were selected for sequence analysis. Plasmids with the confirmed sequence were transformed into the expression strain *E. coli* BL-21 (DE3), leading to the expression of recombinant TAA43 protein with a total size of 383 amino acids: 375 amino acids from the original gene + 6 histidines + 2 extra amino acids to obtain the C-terminal His-tag.

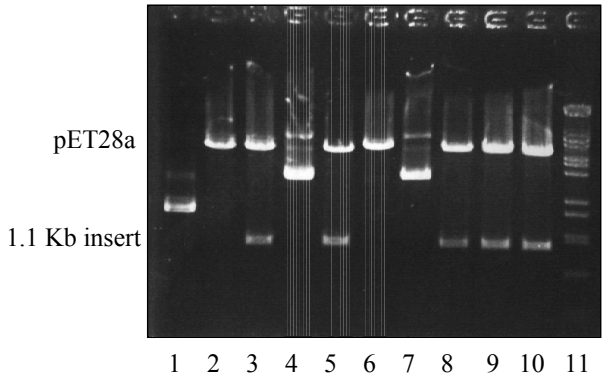


Figure 4.8. 1% agarose gel showing the analysis of the clones obtained after transformation with the pET-28a(+)-*taa43* ligation mixture. Plasmids from lanes 3, 5, 8, 9, and 10 were selected for sequencing and subsequent transformation.

4.2.5. Expression and purification of recombinant TAA43 protein in *E. coli*

C-terminal His_6-tagged TAA43 (TAA43-His_6) was expressed in small-scale cultures and was purified in a single step on a nickel-affinity column. Fractions containing the TAA43-His_6 were identified by SDS-PAGE (figure 4.9). The yield of the recombinant protein was 60 μg per ml of LB media. The purified recombinant protein was used to produce anti-TAA43 polyclonal antibodies in rabbit. The concentrated protein was soluble to at least 15 mg/ml in 20 mM Tris-HCl pH 8.0 and was routinely stored at 4°C at 1 mg/ml. Sequence confirmation of the protein was done by Edman degradation which generated the following N-terminal sequence: MGVLDDIDE (Lottspeich group). The recombinant TAA43 protein was soluble when purified under native conditions and apparently pure, as judged by Coomassie staining (figure 4.9).

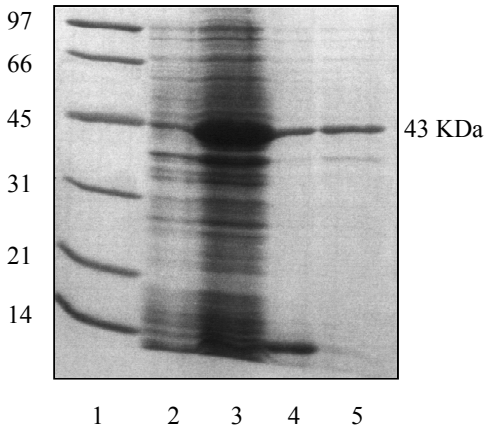


Figure 4.9. 12.5 % SDS PAGE of the 6xHis-tagged TAA43 overexpression and purification. Lane 1, molecular standards; 2, non induced *E. coli* cells; 3, cells induced with IPTG; 4 and 5, eluates from the Ni-NTA Sepharose column.

4.2.6. Detection of *in vivo* expression in *T. acidophilum*

T. acidophilum crude extract was subjected to 12.5% SDS-PAGE and the TAA43 molecule was detected by Western blot. In parallel different dilutions of the recombinant TAA43 were also probed by Western blot analysis (figure 4.10). The amount of TAA43 protein present in the cell corresponds to 0.05% of the total protein content. The antiserum was specific for the TAA43 protein and did not crossreact with any other *Thermoplasma* protein.

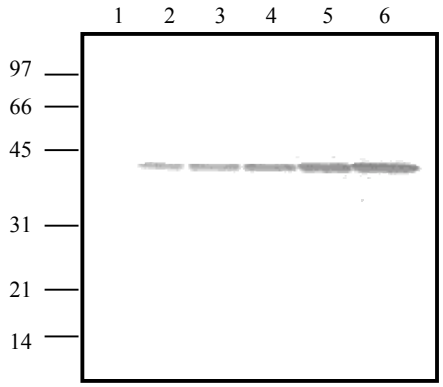


Figure 4.10. Detection of the native TAA43 protein in the *T. acidophilum* crude extract by Western blotting. Lanes 1 to 5, 0; 2.5; 5; 10 and 15 ng of recombinant TAA43 respectively. Lane 6, 1 µL of crude extract at 20 µg/µL. The approximate amount of constitutive protein present in *T. acidophilum* cells is 0.01 µg/µL.

4.2.7. Determination of relative molecular mass

Based on the amino acid sequence and the results obtained from the denaturing gel electrophoresis, the protein has a molecular weight of 43. In order to determine whether the recombinant TAA43 protein exists as a high molecular mass protein complex, several techniques were used to infer the molecular size in its native state. The recombinant TAA43 was subjected to a 4-20% native gradient gel electrophoresis. In native gel electrophoresis the migration behavior of the proteins depends solely on the molecular size and not on the charge of the protein. TAA43 migrated with an approximate molecular weight between 80-90 kDa (Figure 4.11). The electrophoresis was carried out for at least 24 hours at 125V at 4°C, to assure that the migration was completed.

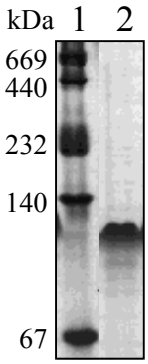


Figure 4.11. 4-20 % Tris-glycine gradient native PAGE. Lane 1, molecular standards; 2, recombinant TAA43. Electrophoresis was performed at 125V for 24 hours to reach equilibrium. Proteins were visualized by Coomassie staining.

In order to determine the molecular mass of the recombinant TAA43 protein by SEC (size exclusion chromatography), a Superose 12 column was used. For that purpose, it was necessary to calibrate the column with proteins of known molecular size (figure 4.12a). The elution volume calculated for TAA43 (figure 4.12b) corresponded to a molecular weight between 70 and 80 kDa. This data is in accordance with the results of native gradient gel electrophoresis. Both experiments suggest a dimeric form for the TAA43 molecule. In addition, the molecular mass of the recombinant TAA43 protein was determined by SEC in the presence of ATP, AMPPNP, low and high concentration of Mg^{++} (10 and 120 mM) and different pH buffers (pH 5.5, 7.0 and 8.0). No other oligomeric forms were found by these methods.

A Superose 6 column was used to determine the molecular size of TAA43 in its native form in *T. acidophilum* crude extract (figure 4.13). The calibration of the

column was done in a similar way as with the Superose 12 column. *T. acidophilum* crude extract was applied and when the fractions obtained were analyzed by Western blot they revealed a similar elution volume for the native TAA43 protein as compared to the recombinant protein (Figure 4.13d).

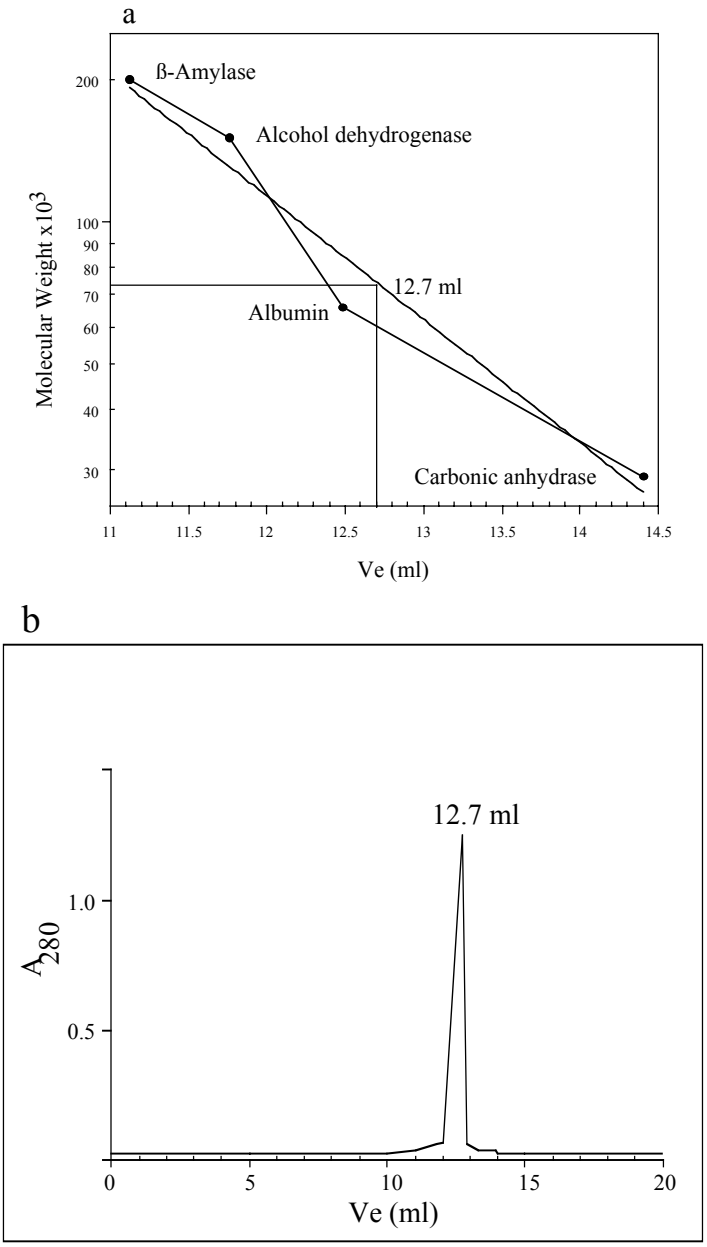


Figure 4.12. FPLC SEC. a) Superose 12 calibration. Molecular standards were used to determine the elution profile of recombinant TAA43 (70-80 kDa), ß-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; albumin, 66 kDa and carbonic anhydrase, 29 kDa. b) 1 mg of recombinant TAA43 was applied to the calibrated Superose 12 column eluting as a dimer. The flow rate selected for the running column was 0.4 ml/min and detector was settled at 1.0 AUFS at 280 nm.

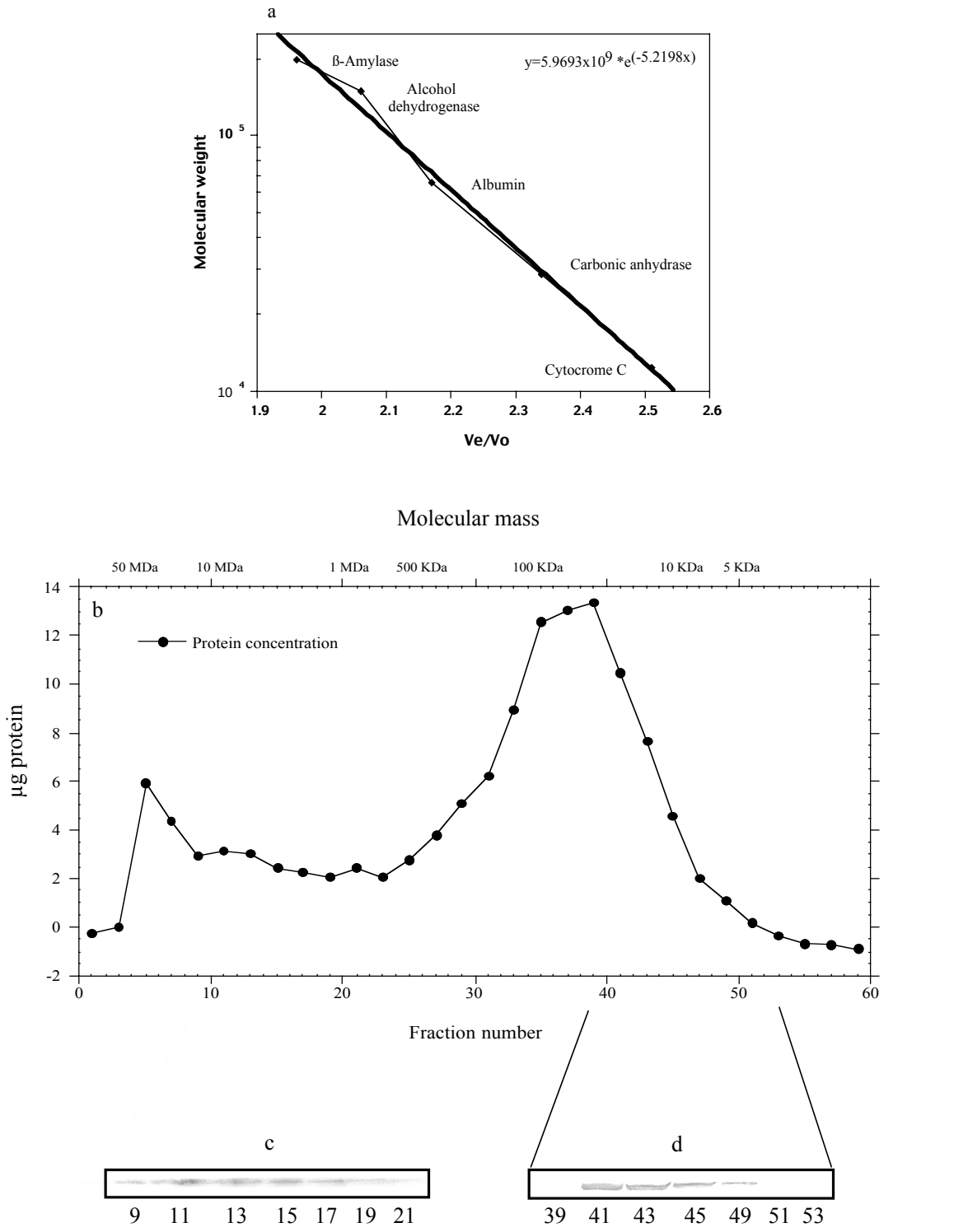


Figure 4.13. *T. acidophilum* cude extract fractionation by Superose 6 SEC. The flow rate was 0.25 ml/min and detector settled at 0.5 AUFS at 280 nm. a) Superose 6 calibration. b) Each fraction was analyzed for its content of protein. The assigned molecular mass shown on the top of the graph is derived from the equation obtained in graph a ($y=5.9693^{9}*e^{(-5.2198x)}$). The eluted fractions were analyzed by Western blot: c) fractions containing TAA43 of high molecular mass (2-10 MDa) and d) fractions containing the dimeric TAA43 (70-80 kDa).

The evaluation of the fractions by Western blot gave an unexpected result. The TAA43 protein eluted in fractions corresponding to a dimeric form (70-80 kDa). In addition, TAA43 was found to elute in fractions (figure 4.13c) corresponding to a large complex (between 2-10 MDa) in a 1:4 ratio when compared to the amount eluted in dimeric form as judged by densitometric evaluation of the bands shown in figure 4.13. The same fractions were analyzed by Western blotting using antibodies reacting against other molecules (VAT, Lon-2, and proteasome) producing a negative pattern (data not shown).

4.2.8. Enzymatic characterization

Purified TAA43-His_6 was used to measure the enzymatic hydrolysis of various nucleotides (ATP, GTP, CTP and UTP) by the malachite green assay. ATP was hydrolyzed in higher rates as compared for the other three nucleotides (figure 4.14d). The maximum activity was found at a temperature between 60 to 70°C (figure 4.14a), which is close to the optimal growth temperature of *T. acidophilum* (59°C). Thenceforward ATPase activity assays were carried out at 65°C.

For the determination of the optimal pH for the reaction the effect of pH on ATP hydrolysis was investigated. The pH of each buffer solution was measured at room temperature and at 65°C (ATPase assay) (Table 4.2) to investigate the temperature dependence of the pH.

Table 4.2 Buffers used to determine the pH optimum of the TAA43 ATPase activity. The assay was conducted at 65°C. Buffer solutions were chosen according to their pKa values.

Buffer	pH at room temperature (20°C)	pH at ATPase assay temperature (65°C)	Specific activity nmol Pi /mg min (65°C)
TCA	1.44	0.74	13.45
Formic acid	2.54	2.10	13.61
	3.00	2.72	13.18
	3.50	3.39	14.37
	4.00	4.00	15.01
MES	**5.50**	**4.82**	**44.70**
	6.00	5.39	39.12
	6.50	5.90	30.63
HEPES	6.50	5.75	30.47
	7.00	6.23	27.36
	7.50	6.50	29.61
	8.00	7.20	27.41
	8.50	7.83	33.26
	9.00	8.43	30.84
Boric acid	8.27	8.27	34.34
	8.55	8.55	32.19
	9.05	9.05	31.01
	9.50	9.50	28.94
	10.00	10.04	22.15

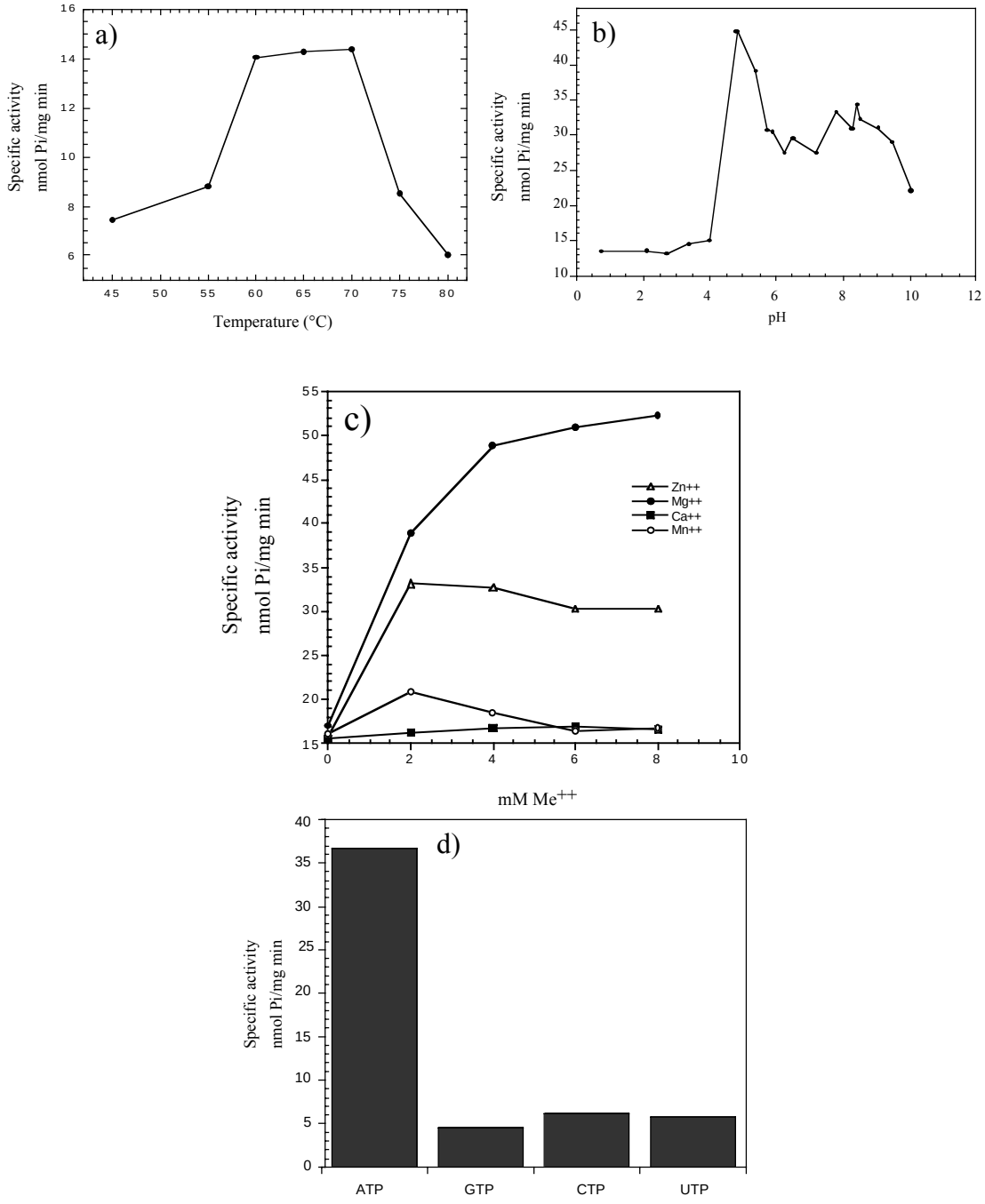


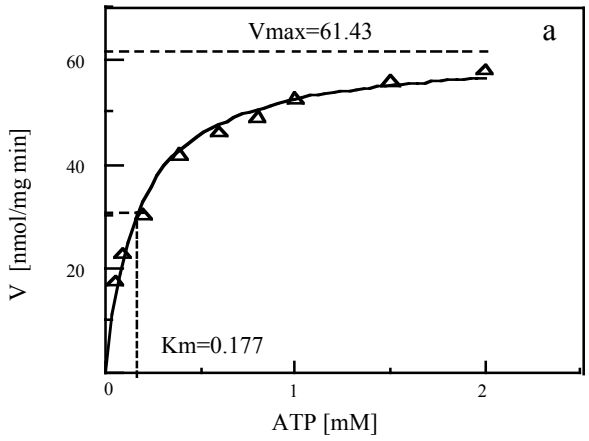
Figure 4.14. Enzymatic characterization of recombinant TAA43 by the malachite green method. a, temperature dependence; b, pH dependence; c, divalent metal ion specificity at 1 mM ATP; d, nucleotide specificity. Background rates were corrected for each reaction as non-enzymatic release of Pi from controls without TAA43.

As shown in figure 4.14b, the maximum ATPase activity at 65°C was found at pH 4.82 (corresponding to pH 5.5 at 20°C) using MES buffer at a concentration of 50 mM. This corresponds to the internal pH of *T. acidophilum* (Searcy, 1976). The activity of TAA43 was not affected by changes of the NaCl concentration in the range of 0-300 mM (data not shown).

To investigate the divalent metal ion specificity for TAA43 ATPase activity, $MgCl_2$, $ZnCl_2$, $CaCl_2$ and $MnCl_2$ were used. Figure 4.14c displays Mg^{++} as the preferred divalent cation of the reaction. Zn^{++} produced an ATP hydrolysis of 50 % when compared to the Mg^{++}. Virtually no ATP hydrolysis was detected when $CaCl_2$ or $MnCl_2$ were used.

4.2.9. 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/mg of protein min (figure 4.15). To determine the kinetics of the ATP hydrolysis the enzyme kinetics software version 1.0 was used (D.G. Gilbert, dog Star Software Bloomington Indiana & Biology Dept. Indiana Univ.).



Substrate concentration mM ATP	Velocity (V)
0.05	17.75
0.10	22.85
0.20	30.28
0.40	41.85
0.60	46.20
0.80	48.94
1.00	52.70
1.50	55.60
2.00	58.12

Figure 4.15. Kinetics of TAA43 ATPase activity. Velocity vs. substrate concentration plot for ATP hydrolysis.

N-Ethylmaleimide (NEM), an inhibitor of the H^+-ATPase activity (Brooker and Slayman, 1982), and sodium azide (NaN_3), an inhibitor of the F_0F_1 ATPase activity (Muneyuki et al. 1993), were tested for TAA43 inhibition. It has been shown that several AAA family members are inhibited by these two molecules (e.g. NSF and VAT). Figure 4.16 shows that neither NEM nor NaN_3 has an inhibitory effect on TAA43. ADP, as expected, and the non-hydrolyzable analog of ATP (AMPPNP), inhibited the reaction significantly.

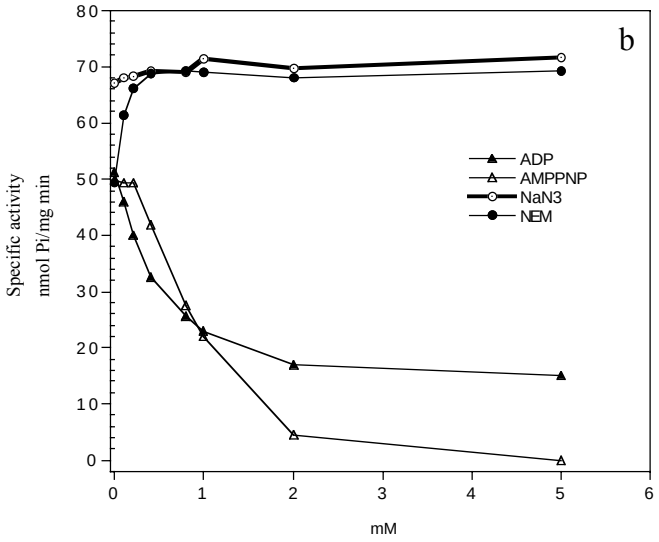


Figure 4.16. Effect of different compounds on the TAA43 ATPase activity. The samples were preincubated with the different inhibitors for 15 minutes.

4.2.10. Site-directed mutagenesis of conserved residues

Four different mutants of the recombinant TAA43-His_6 were constructed in order to identify residues involved in nucleotide hydrolysis (Figure 4.17 and 4.18). Highly conserved residues involved in ATP binding and hydrolysis were changed into closely related residues, instead of carrying out an alanine scanning mutagenesis, which would introduce drastic substitutions (Karata et al., 1999).

Specifically, lysine 159 (positively charged) in the Walker A motif was modified to asparagine (polar but uncharged), aspartic acid 212 (negatively charged) in the Walker B motif to glutamic acid (similarly negatively charged) and in the Second Region of Homology (SRH) aspartic acid 264 to glutamic acid, as well as arginine 269 (positively charged) to leucine (bulky and uncharged). ATPase activity was measured and compared to the wild-type TAA43 protein. Table 4.3 shows the specific

activities obtained. The Walker B mutant (D212E) was the only mutant that conserved its ATPase function.

Figure 4.17. Site directed mutagenesis of conserved residues in TAA43.

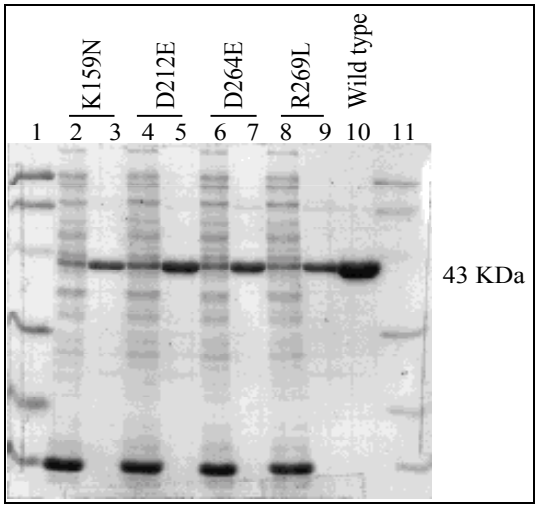


Figure 4.18. 12.5 % SDS-PAGE. Purification of 6xHis-tagged mutants. Lanes 2, 4, 6 and 8 show the crude extract lysate. Lanes 3, 5, 7 and 9 are Ni-NTA Spin column purified mutants. Lane 10, TAA43 wild type. Lanes 1 and 11, molecular markers.

Table 4.3. ATPase activity in mutants

Mutation	**Specific activity** nmol Pi/mg/min	**% activity**
Wild-type TAA43	61.43	100
K159N - Walker A	2.50	4
D212E - Walker B	68.40	111
D264E - SRH	3.00	4
R269L - SRH	12.00	19

4.3. FUNCTIONAL STUDIES

4.3.1. *T. acidophilum* genome analysis –the translation-associated ATPase (*taa43*) gene and its neighbours in the genome

The genome sequence determination of *T. acidophilum* has enriched the protein databases with approximately 1500 new protein sequences. However, 54% of them still lack a functional characterization.

In order to make a prediction for the function of a gene it is worth to consider how genes are ordered. Studies done in bacterial genomes have provided useful information about the existence of well-defined organization of genes –the operons-, they contain two kinds of constituents: structural genes and regulatory elements. This principle is based on the fact that genes belonging to the same operon invariably encode functionally associated proteins (Jacob and Monod, 1961). Nevertheless, in prokaryotic and archaeal genomes, the genetic organization is poorly conserved except for operons that encode physically interacting proteins of complexes like the ribosome, the H^+-ATPase or the DNA-dependent RNA polymerase.

The gene order in *T. acidophilum* compared to other archaeal genomes has been discussed recently (Koonin et al., 2001). The presence of a cluster of genes was described encoding protein complexes like RNAses, RNA-binding proteins, and helicases, which form a potential exosomal superoperon. In addition, genetic elements were present in this cluster constituting a COG (Cluster of Orthologous Groups of proteins), as the catalytic subunits of the proteasome, two ribosomal proteins, and a DNA-directed RNA polymerase subunit suggesting a connection between translation, RNA processing and protein degradation. The COG system allows the prediction of automatic functional and phylogenetic annotation of genes and gene sets (http://www.ncbi.nlm.nih.gov/COG/). To facilitate functional studies, the COGs have been classified into 17 broad functional categories by the biochemical activity. Each COG consists of proteins that likely share a common function or domain, which in turn has a role in a given cellular process (or

processes). The letters (function codes) in the table below represent these major cellular processes. In addition to the information given below, the functional categories in the web page indicate the number of COGs assigned to each group, the number of proteins or domains (since some proteins are divided) assigned to each group, and the number of pathways and functional systems, if any, that are part of each group.

Table 4.4. Functional categories of COGs*.

Information storage and processing	**Codes**
Translation, ribosomal structure and biogenesis	J
Transcription	K
DNA Replication, recombination, and repair	L
Cellular processes	
Cell division and chromosome partitioning	D
Cell envelope biogenesis, outer membrane	M
Cell motility and secretion	N
Posttranslational modification, protein turnover, chaperones	O
Inorganic ion transport and metabolism	P
Signal transduction mechanisms	T
Metabolism	
Energy production and conversion	C
Amino acid transport and metabolism	E
Nucleotide transport and metabolism	F
Carbohydrate transport and metabolism	G
Coenzyme transport and metabolism	H
Lipid metabolism	I
Poorly characterized proteins	
General function prediction only	R
Function unknown	S

* Web site (http://www.ncbi.nlm.nih.gov/COG/)

The TAA43 ORF (Ta1175) is located in a region of the genome not far from the predicted exosome ORFs (Ta1286 to Ta1295), or what can be considered the ribosomal operon (Ta1249 to Ta1271). TAA43 ORF was found not to be embodied in these operons, but located in a region between a cluster of genes that include the DNA-directed RNA polymerase, two ribosomal proteins, a tRNA intron endonuclease, and two tRNAs among other related elements, thus being reminiscent of the exosomal superoperon (figure 4.19).

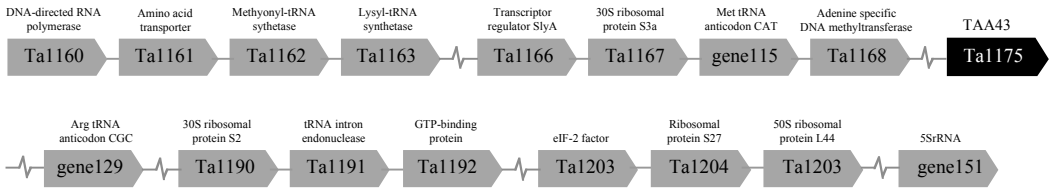


Figure 4.19. Organization of the genes in the T. acidophilum genome found in proximity to the TAA43 ORF. The proportion of the arrows in the figure does not correspond to the actual size of the genes, and the symbols ⊣⊢ are ORFs omitted (illustration adapted from Koonin et al., 2001).

The region containing the *taa43* gene shown in figure 4.19 was compared with archaeal COGs (*Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Sulfolobus solfataricus*, *Halobacterium* sp., *Methanobacterium thermoautotrophicum*, *Methanococcus jannaschii*, *Pyrococcus abyssii*, and *Pyrococcus horikoshii*). The comparison localizes this region between two COGs (COG1096 and COG1761). COG1096 contains 7 orthologous genes that code for a predicted RNA-binding protein (that consists of an S1 domain and a Zn-ribbon domain), and COG1761 consisting of 11 genes that code for subunit L of DNA-directed RNA polymerase (Koonin et al., 2001). These COGs are classified within the functional category J or K, respectively as shown in table 4.4: translation, ribosomal structure and biogenesis, and transcription (http://www.ncbi.nlm.nih.gov/COG/).

As described by Koonin et al., the archaeal genomes studied above had the gene belonging to COG1096 in the same area with the exception of *T. acidophilum*. In Figure 4.19, this gene (DNA-directed RNA polymerase) seems to be is localized at the beginning of the region studied for *taa43* gene and not at the end, suggesting a poor conservation of the gene order of *T. acidophilum*, although the genetic elements participating in the potential superoperon are present.

4.3.2. Chaperonin-assisted protein folding of the enzyme rhodanese by TAA43

In order to analyze whether TAA43 has a foldase/unfoldase function, a chaperonin-assisted refolding assay was performed with the enzyme rhodanese. Rhodanese catalyses the formation of thiocyanide from thiosulfate and cyanide in a two step mechanism:

$$Rho + S_2O_3^{2-} \text{ ---> } Rho\text{-}S + SO_3^{2-}$$

$$Rho\text{-}S + CN^- \text{ ---> } Rho + SCN^-$$

This enzyme has several advantages: it is a 33 kDa monomeric protein and does not need cofactors, prosthetic groups, or side chain modifications for its enzymatic activity. Thus, only its amino acid sequence is needed to conserve its structure, function, folding, and transport. The sulfur-bound rhodanese (Rho-S) is more stable than free rhodanese (Rho).

This procedure was suitably modified for attempting the refolding of rhodanese by the TAA43 protein. After denaturation of rhodanese by guanidinium hydrochloride, refolding was monitored by following the recovery of rhodanese activity during the time-course.

Refolding activity could not be detected in the presence of TAA43 (figure 4.20) since the increase in absorbance was comparable to the spontaneous refolding and the blank.

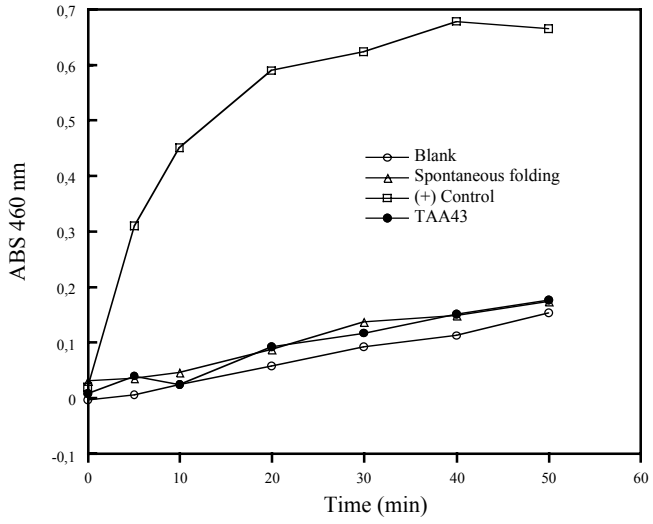


Figure 4.20. Refolding of rhodanese. The experiment included GroEL/GroES as positive control, a spontaneous refolding reaction containing native rhodanese as background control, and the blank. TAA43 did not show any chaperone activity.

4.3.3. Proteolytic degradation pathway "via proteasome"

It was considered that the TAA43 protein could substitute for the function of the archaeal PAN protein (Zwickl et al., 1999), which is not present in *Thermoplasma*, in the ATP-dependent degradation of proteins by the proteasome. Therefore, it was tested whether TAA43 or other *Thermoplasma* AAA ATPases participate directly or indirectly in proteasome-mediated protein degradation. To do this, it was necessary to remove the protein of interest from the cell lysate in order to allow the observation of the effect of depletion on protein degradation.

Complete depletion of TAA43, VAT, Lon-2 and proteasomes was achieved using specific affinity purified IgGs coupled to Sepharose G beads. For that purpose, serial dilutions of the recombinant proteins and *T. acidophilum* crude extract were carried out in the same fashion as described in section 4.2.6 (figure 4.21). The amount of protein present for each protein studied is shown in table 4.5.

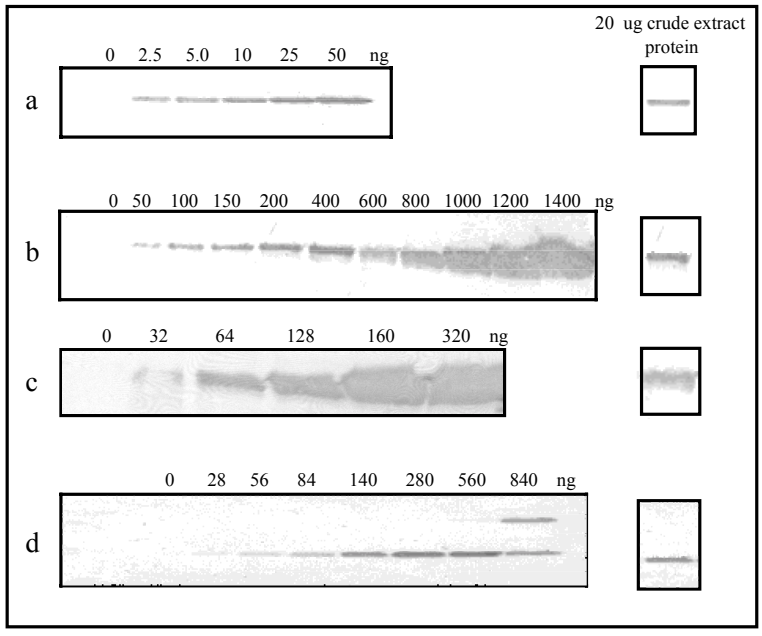


Figure 4.21. Expression levels of several proteins in *T. acidophilum* cells. Serial dilutions of recombinant protein were separated by SDS-PAGE and blotted. The amount of recombinant protein and *T. acidophilum* crude extract used is shown at the top of each blot. Rabbit Polyclonal antibodies against the different proteins were used for that purpose. a) TAA43, b) VAT, c) Lon-2, and d) proteasome. The calculated percentage of each protein resulting from densitometric analysis is shown in table 4.5.

Table 4.5. Content of molecules of interest in *T. acidophilum* cell extract

Molecule	µg of molecule/ µL crude extract	Total content in *T.a.* cytosol*
a) TAA43	0.01 µg/µL	0.05 %
b) VAT	0.20 µg/µL	1.00 %
c) Lon-2	0.10 µg/µL	0.50 %
d) Proteasome	0.28 µg/µL	1.40 %

*100% corresponds to the total amount of proteins in the cell crude extract as determined by the Bradford protein assay.

In order to remove each protein from the *T. acidophilum* crude extract an excess of specific IgG was used as shown in figure 4.22 for TAA43. Comparable patterns were observed for the other three enzymes.

The protease activity of the crude extract was measured in the presence and absence of ATP and $MgCl_2$. Furthermore, protease activities were tested in single or multiple depleted extracts. As shown, protease activity was only significantly reduced when proteasomes were precipitated (Figure 4.23, 3, 7, 8, 9, and 11). The same results were obtained when the aminopeptidase activity was assayed (data not shown).

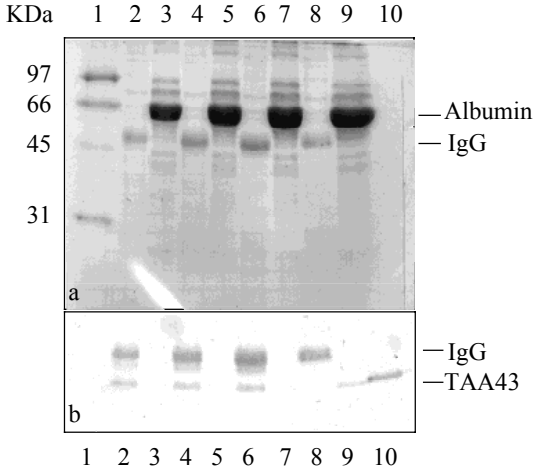


Figure 4.22. Immunodepletion experiments for TAA43. a) Coomassie staining of SDS-PAGE from 4 independent immunoprecipitation reactions. Lane 1, molecular marker, lanes 2, 4, and 6 show the immunoprecipitates. Lanes 3, 5 and 7 show the immunodepleted crude extract with the excess of albumin left from the reaction. Lane 8, negative control using non-immune serum; lane 9, immunoprecipitate reaction from 8; and lane 10, 5 ng of recombinant TAA43 as positive control. b)Western blot analysis of the same samples as shown in a).

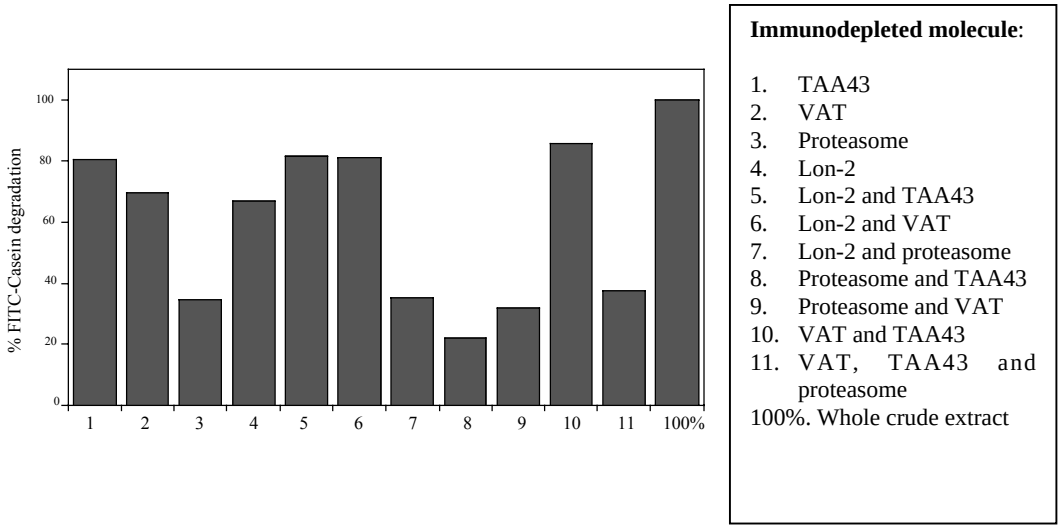


Figure 4.23. Protease activity tested by FITC-casein degradation in immunodepleted crude extracts from *T. acidophilum* cells. No significant difference was found when samples were tested in the presence or absence of ATP and Mg^{++} (data not shown).

4.3.4. DNA-binding assay

Some ATPases from AAA^{+} superfamiliy are known to interact with nucleic acids and seem to be involved in chaperone-like activities and in transcriptional processes (e.g. the MCM DNA-licensing factors, the bacterial DnaA, RuvB, and McrB proteins, the prokaryotic NtrC-related transcription regulators, etc.). Moreover, they seem to share distinct structural and mechanistic characteristics that distinguish them from other NTPases (Neuwald et al., 1999). In this context, it was of relevance to study the interaction of the TAA43 protein with specific consensus DNA sequences involved in transcriptional activities.

The formation of DNA-TAA43 complexes was analyzed by using a set of specific double stranded deoxyoligonucleotides (Stratagene) in a non-radioactive electrophoretic mobility shift assay (EMSA) kit (Roche). The principle of the method is very simple –DNA fragments or oligonucleotides and the protein of interest are mixed in a suitable buffer and binding is allowed to occur. The mixture is then separated by native PAGE; stable complexes of DNA and protein are generally significantly retarded in mobility in contrast to free DNA and the separated complexes are detected by an enzyme immunoassay.

NF1/CTF, SP1, AP_1, AP_2, AP_3, NF_kb, GRE, CREB, Oct-1 were the specific labeled oligonucleotides used in this experiment. They are known to bind motifs of regulatory transcription factors in eukaryotic cells (Lewin B, 1994). Digoxigenin labeled oligonucleotides were bound by anti-digoxigenin antibody Fab fragments coupled to alkaline phosphatase. Detection of alkaline phosphatase was performed by autoradiography of the chemiluminiscence produced during the enzymatic dephosphorylation of CSPD (disodium 3-4(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-choloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate) leading to a light emission at a maximum wavelenght of 477 nm. The chemiluminescence signals were recorded by exposure to an X-ray film.

As shown in figure 4.24, SP1 did not form a complex with TAA43. The presence of TAA43 did not induce a gel shift in any of the other oligonucleotides analyzed (NF1/CTF, AP_1, AP_2, AP_3, NF_kb, GRE, CREB, and Oct-1).

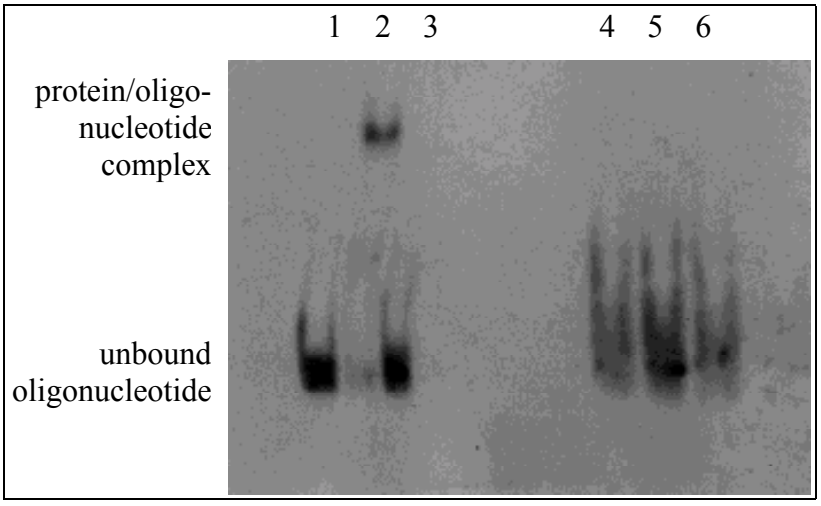


Figure 4.24. Detection of DNA-binding proteins by gel shift. The binding reactions were separated on a 6% native PAGE. Oligonucleotides were transferred onto a positively charged nylon membrane by electroblot. The membrane was exposed to an X-ray film at room temperature and detected by autoradiography. Lanes 1-3 are control reactions: 1, labeled 39-mer oligonucleotide without Oct2A factor; 2, labeled oligonucleotide with Oct2A; and 3, same as 2, but with an 125-fold excess of unlabeled oligonucleotide for specific competition. Lanes 4-6: 4, labeled SP1 oligonucleotide without TAA43; 5, labeled SP1 oligonucleotide with TAA43; and 6, same as 5, but with an excess of unlabeled SP1 oligonucleotide for competition.

4.3.5. *In vivo* analysis of protein-protein interactions

The technique used here -known as the two-hybrid system in bacteria- is based on the co-expression of two-hybrid proteins in *E. coli* cells. Interactions can be traced by the restoration of an enzymatic activity (a selective trait like the ß-galactosidase activity) as shown in figure 4.25.

The hybrid proteins are usually called the bait, a fusion protein containing the protein of interest (TAA43 in this study), and the prey, potential partners present in a cell (a library made of randomly fused polypeptides from *T. acidophilum*).

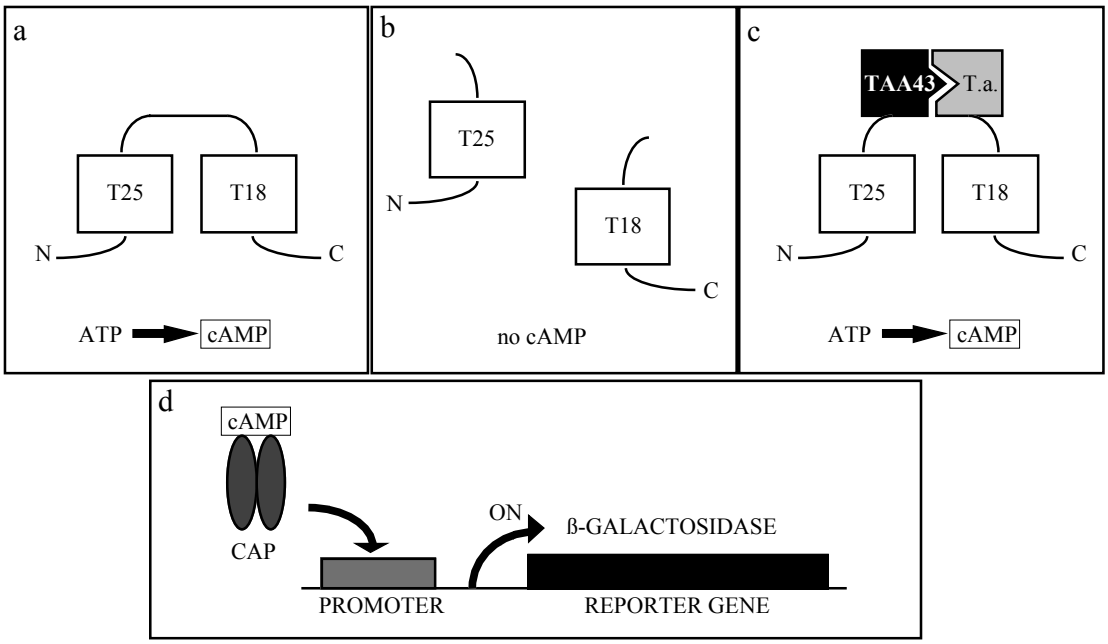


Figure 4.25. Principle of the *E. coli* two-hybrid system. a) T25 and T18 are fragments that constitute the domain of the *B. pertussis* adenylate cyclase. cAMP is synthesized when the fused protein is expressed. b) When the two fragments are expressed independently in the same cell, no cAMP synthesis occurs. c) When the two fragments are fused to interacting proteins (e.g. pT25-TAA43 hybrid and a given fragment from *T. acidophilum* library, pUT18-T.a. hybrid), cAMP synthesis occurs when the T25 and the T18 fusions are brought into close proximity. d) The cAMP synthesized binds to the catabolite gene activator protein (CAP), which recognizes the promoter of the ß-galactosidase gene -*lacZ*- and switches on the transcription of the gene.

4.3.5.1. Prey library construction with pUT18 (pUT18-T.a. hybrid)

In order to identify the interacting partner(s) of the TAA43 protein, it was necessary to construct a hybrid library covering the whole *T. acidophilum* genome. Several single colonies were selected randomly to confirm the correct cloning of DNA fragments from *T. acidophilum* genome into the pUT18 vector. They were grown in liquid media with antibiotic and the plasmid preparation was performed as usual with the Qiagen miniprep kit. Plasmids were subjected to restriction analysis and DNA sequencing.

As shown in figure 4.26, DNA fragments of different sizes were cloned successfully into the pUT18 vector.

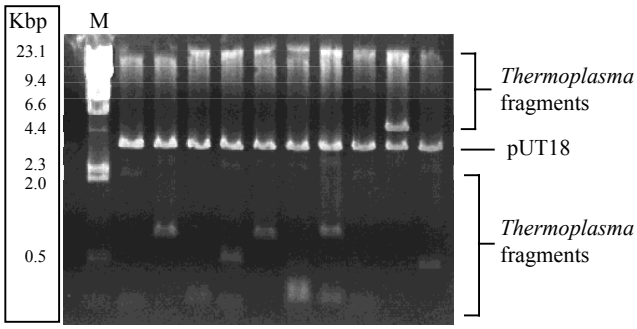


Figure 4.26. Restriction analysis of positive clones from the prey library.

4.3.5.2. Bait cloning: *taa43* into pT25 (pT25-taa43 hybrid)

Taa43 was cloned into the pT25 vector and correct cloning orientation was confirmed by restriction analysis and DNA sequencing (figure 4.27).

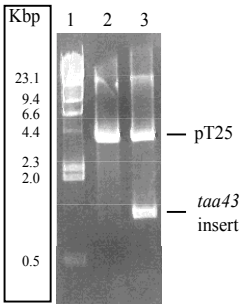


Figure 4.27. Construction of the bait. 1, Molecular markers; 2, pT25; 3, restriction analysis of the pT25 with the *taa43* gene inserted.

4.3.5.3. Screening of positive interacting colonies by the bacterial two-hybrid system

Co-transformed BTH101 cells containing the hybrids pT25-taa43 and pUT18-T.a. were analyzed with blue/white screening. Positive colonies where distinguished by the blue color on the indicator M63 plates containing X-Gal.

All the blue colonies were picked and grown in liquid media overnight at 30°C. One part of the culture was used for plasmid isolation and subsequent restriction analysis and DNA sequencing (figure 4.28). The other part was used for the quantification of the functional complementation between TAA43 and the interacting protein, as measured by ß-galactosidase activity.

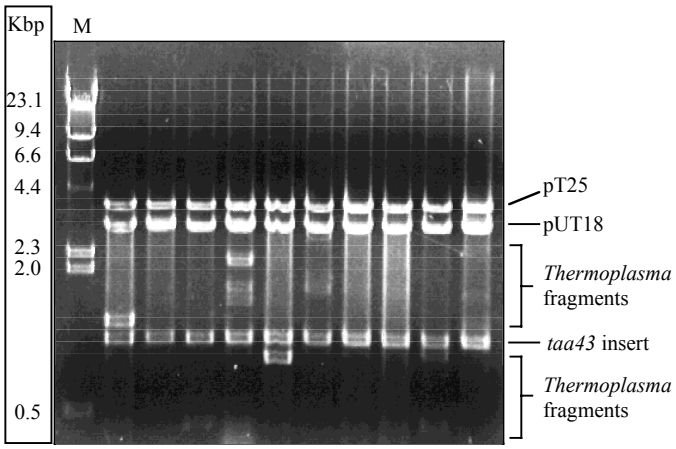


Figure 4.28. Restriction analysis of clones obtained by X-Gal blue/white selection.

From $7.8x10^4$ of the total blue and white colonies plated (100%), 300 blue colonies were collected (0.38%), retransformed, and grown. Subsequently, the insert-sequence of the isolated plasmid was determined and localized in the *T. acidophilum* genomic database.

The 300 blue colonies were tested twice in the ß-galactosidase assay in duplicate with positive and background controls. 47 of the 300 clones were positive for complementation. The residual clones were false positives or contained non-ORF encoding regions and were therefore discarded.

4.3.5.4. Positive interacting colonies

Results obtained from the DNA sequencing analysis of the positive colonies were compared against the *T. acidophilum* genomic database and identified as shown in table 4.5. Common motifs of the TAA43 interacting proteins are displayed by BLOCKS. BLOCKS are short multiply aligned ungapped segments corresponding to the most highly conserved regions of proteins. The BLOCKS database of the web server (http://blocks.fhcrc.org) assists for the detection and verification of protein sequence homology. The list of Blocks shown in table 4.6 was taken from the functional categories of the *T. acidophilum* genome database (http://pedant.GSF.DE/cgi-bin/wwwfly.pl?Set=Tacidophilum&Page=index).

Table 4.6. Interacting TAA43 proteins identified by the bacterial two-hybrid system

Clone #	Blast search result vs. *T. acidophilum* genome database	Classification according to BLOCKS
L6	Ta0672 Polyketide synthase chain 7 rel. protein	LYSYL OXIDASE COPPER-BINDING REGION
L15	Ta1188 Multidrug resistance protein NorA related protein	NC
L21	Ta0515 Probable signal recognition particle protein	RIBOSOMAL PROTEIN L31E
L29	Ta0415 Hypothetical membrane protein	POLY(ADP-RIBOSE) POLYMERASE ZINC FINGER DOMAIN
L32	Ta1371 Conserved hypothetical protein	NC
L33	Ta0542 Probable D-lactate dehydrogenase	RIBOSOMAL PROTEIN L31E
L36	Ta1308 H+/K+-transporting ATPase chain C related protein	Kinesin light chain repeat
L45	Ta0737 Conserved hypothetical protein	nc
L49	Ta0902 Conserved hypothetical membrane protein	nc
L56	Ta0611 Inosine-5'-monophosphate dehydrogenase related protein	RIBOSOMAL PROTEIN L31E
L57	Ta0202 FERRIC TRANSPORT ATP-BINDING PROTEIN AFUC related protein	RIBOSOMAL PROTEIN L31E
L68	Ta1454 Hypothetical protein	RIBOSOMAL PROTEIN L31E
L69	Ta0820 Conserved hypothetical protein	RIBOSOMAL PROTEIN L31E
L71	Ta0849 Alanyl-tRNA synthetase related protein	*
L74	Ta0290 Hypothetical protein	RIBOSOMAL PROTEIN L31E
L75	Ta1403 Thermopsin precursor related protein	nc
L81	Ta0777 Leucyl-tRNA synthetase related protein	POLY(ADP-RIBOSE) POLYMERASE ZINC FINGER DOMAIN
L83	Ta1496 Hypothetical protein	LYSYL OXIDASE COPPER-BINDING REGION
L84	Ta1163 Lysyl-tRNA synthetase (lysS) related protein	LYSYL OXIDASE COPPER-BINDING REGION
L90	**Ta1475 Ribonucleotide reductase**	RIBONUCLEOTIDE REDUCTASE LARGE SUBUNIT
L93	Ta0765 Aspartate transaminase related protein	43 Kd postsynaptic protein
L94	Ta0858 Glycerate dehydrogenase related protein	LYSYL OXIDASE COPPER-BINDING REGION
L98	Ta0907 DNA polymerase (PolB), large chain related protein	Kinesin light chain repeat
L106	Ta0664 Hypothetical protein	Class A bacterial acid phosphatases
L108	Ta0397 Conserved hypothetical protein	Granulins
L109	Ta0687 Conserved hypothetical protein	RIBOSOMAL PROTEIN L31E
L111	Ta0872 Iron-dependent transcription repressor related protein	Granulins
L112	Ta0451 Cell division control protein 6 related protein	LYSYL OXIDASE COPPER-BINDING REGION
L113	Ta0446 Translation elongation factor aEF-2 related protein	RIBOSOMAL PROTEIN L31E
L119	Ta0724 Conserved hypothetical protein	POLY(ADP-RIBOSE) POLYMERASE ZINC FINGER DOMAIN
L131	Ta0220 Aldehyde dehydrogenase, mitochondrial 3 precursor related protein	LYSYL OXIDASE COPPER-BINDING REGION
L143	Ta0166 Conserved hypothetical protein	LYSYL OXIDASE COPPER-BINDING REGION
L147	Ta1322 Surface antigen genes (Methanosarcina mazei) related protein	nc
L162	Ta0735 Hypothetical membrane protein	RIBOSOMAL PROTEIN L31E
L175	Ta0040 Valyl-tRNA synthetase related protein	*
L179	Ta1192 Hypothetical GTP-binding protein	RIBOSOMAL PROTEIN L15E

nc, not clear,

* and UPPER characters are proteins directly involved in RNA metabolism

4.3.6. *In vitro* analysis of protein-protein interactions

To confirm the results obtained with the two-hybrid system, it was necessary to study protein-protein interactions *in vitro* by a co-immunoprecipitation assay. Pre-cleared *T. acidophilum* crude extract was incubated together with Sepharose G beads coupled with IgG specific for TAA43 and washed with a 1% Tween 20 solution as described in methods. In order to have enough material for sequencing the reaction was done in excess as shown in figure 4.29a lane 3, the co-precipitated material was subjected to SDS-PAGE, the gel stained with Coomassie blue (figure 4.29a) and the bands sequenced.

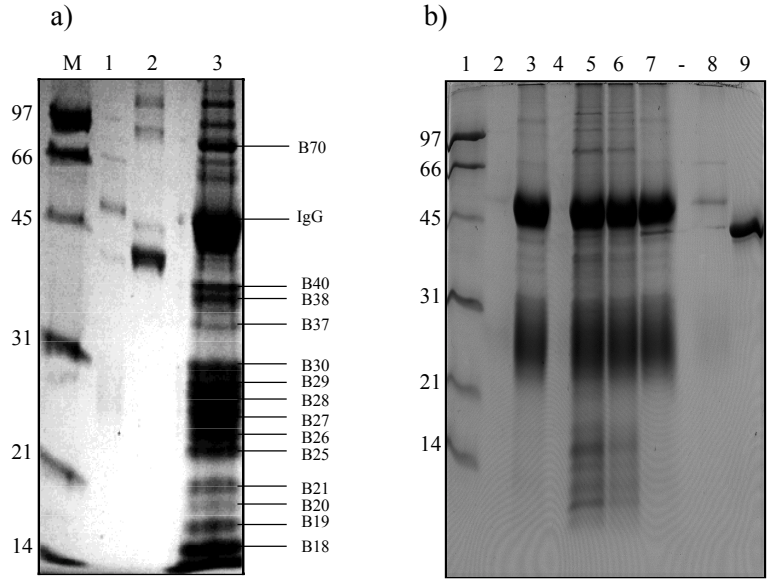


Figure 4.29. SDS-PAGE. a) Co-immunoprecipitation studies. Lanes: M, molecular markers in kDa; 1, antibody against TAA43; 2, recombinant TAA43; and 3, coprecipitated bands sequenced by mass spectrometry are shown on the right indicating the molecular mass in kDa (B70 to B18). b) Controls. Lanes: 1, Molecular marker in kDa; 2, Sepharose G beads (SGB); 3, SGB + anti-TAA43 IgG; 4, pre-treated SGB + *Thermoplasma* crude extract; 5, Complete co-precipitation reaction (pre-treated SGB + anti-TAA43 IgG + *Thermoplasma* crude extract); 6, Co-precipitation using fraction 11 from Superose 6 column (the high molecular mass fraction, see figure 4.13c); 7, (+) control: anti-TAA43 IgG + recombinant TAA43; 8, anti-TAA43 IgG; and 9, recombinant TAA43.

The 16 co-precipitated bands shown in the SDS-polyacrylamide gel (B70 to B18) were identified by mass spectrometry (MS) (Lottspeich group) and the results are described in table 4.7.

Controls are included in figure 4.29b. Lane 5 shows a co-precipitation experiment exactly as done for sequencing by MS for comparison with a co-precipitation performed with fraction 11 (lane 6) obtained from Superose 6 chromatography (see figure 4.13c). It is possible to identify some of the bands by MS from figure 4.29a as B70, B40, B38 and B37 in figure 4.29b lane 6, which are present in the supramolecular eluate from the Superose 6 (2-10 MDa).

Table 4.7. Proteins co-precipitated with TAA43 antibodies and identified by mass spectrometry.

Signature Band number	Protein name	Ta ORF
B70	**Ribonucleotide reductase**	Ta1475
B40	50S Ribosomal L3	Ta1271
B38	Conserved hypothetical protein	Ta1488
B37	Hypothetical protein	Ta0466
B30	50S Ribosomal L4, L2	Ta1270, 1268
B29	50S Ribosomal L4, L2	Ta1270, 1269
B28	50S Ribosomal L1	Ta0360
B27	50S Ribosomal L1	Ta0361
B26	30S Ribosomal S4	Ta1032
B25	30S Ribosomal S7	Ta0092
	50S Ribosomal L6	Ta1255
B21	30S Ribosomal S13	Ta1033
	50S Ribosomal L13	Ta0433
B20	Prob. conserved protein	Ta1149
	50S Ribosomal L18	Ta1252
B19	50S Ribosomal L30	Ta1250
	50S Ribosomal L19	Ta1253
	30S Ribosomal S19	Ta1267
B18	50S Ribosomal L22	Ta1131
	30S Ribosomal S14	Ta1266
	Hypothetical protein	Ta0817
	30S Ribosomal S9	Ta0432

In both experiments, i.e. the *in vivo* (two-hybrid system) and the *in vitro* (co-precipitation) analysis, proteins interacting with TAA43 are in their majority translation-associated. Ribonucleotide reductase (RNR) was the only protein identified with both experimental techniques.

5. DISCUSSION

5.1. BIOCHEMICAL CHARACTERIZATION OF TAA43

5.1.1. Conformational state

Several AAA proteins such as VAT, NSF, Vps4p and p97 are structurally characterized by their ability to assemble into hexameric rings. This organization is considered to be a key requirement for their mechanism of action. Nevertheless, there are other states of oligomerization such as in katanin, a substrate-dependent protein with a transient heterodimeric-homohexameric state or dynein, a heteroheptameric protein.
In this context, it was expected that TAA43 –like the hexameric protein VAT and many other AAA proteins- assembled into a hexameric structure. However, according to the results obtained from the molecular weight analysis (figures 4.11, 4.12 and 4.13), TAA43 is a dimer in its native state, independently of the presence or absence of ATP (or the non-hydrolyzable analog AMPPNP). Moreover, as depicted in figure 4.13, TAA43 co-elutes also with proteins of high molecular weight. Hence, we have to consider two possibilities:

1) either TAA43 remains in a dimeric state and is part of a big molecular complex or,
2) the dimer is rearranged once it finds its physiological partner(s) to form a different multimeric assembly (as mentioned for katanin above).

Several factors have been described that may affect the state of oligomerization in a protein, for instance different concentrations of a ligand, cofactors, substrate, or product generated, and switching to distinct functions (moonlighting proteins, Jeffery, 1999). A clear example is the nuclear uracil-DNA glycosylase activity in the monomeric form that switches to glyceraldehyde-3-phosphate dehydrogenase activity in a tetrameric form (Meyer-Siegler et al., 1991).

5.1.2. ATPase activity

As described in the results, the His-tagged version of TAA43 was used to carry out the enzymatic analyses. A comparative description of the ATPase activity of several members of the AAA family is given in Table 5.1.

Table 5.1. Biochemical features of several members of the AAA family.

Protein	Km µM	Vmax pmol Pi/mg/min	Ion dependance	Oligomeric state	Temp. °C	pH optimum	pI	NEM Inhib.	Nucleotide dependance
Ta TAA43*	177	61	Mg^{2+}/Zn^{2+}	dimeric	70	4.8	6.24	-	ATP
Ta VAT**	720	558	Mg^{2+}	hexameric	70	8.0	9.1	+	ATP[§]
CHO NSF**	650	67	Mg^{2+}	hexameric	37	9.0	--[§]	+	ATP[§]
Re ARC*	200	268	Mg^{2+}	hexameric	30	7.8	4.9	+	ATP/CTP
Sc CDC48**	550	1560	Mg^{2+}	hexameric	--[§]	9.0	5.7	+	ATP[§]
Ec FtsH*	83	$459x10^3$	Zn^{2+}	hexameric	37	--[§]	--[§]	+	ATP/CTP/GTP

*Group I, proteins containing one domain, **Group II, proteins containing two domains.
Abbreviations: Ta *Thermoplasma acidophilum*, CHO Chinese hamster ovary, Re *Rhodococcus erythropolis*, Sc *Saccharomyces cerevisiae*, Ec *Escherichia coli*.
[§] Not determined for other nucleotides.

One of the most interesting characteristics found for TAA43 is its selectivity to use ATP as source of energy. Other AAA proteins like ARC or FtsH protease are able to utilize other nucleotides like CTP or GTP in addition to ATP (keeping their highest affinity for ATP). Moreover, TAA43 did not show an inhibition of its ATPase activity by NEM, a characteristic shown by several AAA proteins (table 5.1). ATPase activity tested in the presence of NaN_3 had no inhibitory effect, in contrary to the findings with the ARC protein (Wolf et al., 1998). Additionally, TAA43 also accepts Zn^{2+} ions instead of Mg^{2+}. This characteristic is shared by proteins such as AAA proteases (e.g. FtsH) and DNA/RNA-binding proteins, which are activated in the presence of this divalent cation.

Altogether, TAA43 seems to be different from the other AAA ATPases, with respect to its oligomerization state and its nucleotide specificity.

5.1.3. Mutational analysis

The confirmation of the intrinsic ATPase activity of TAA43 was accomplished by site-directed mutational studies, which identified the residues that are catalytically important for the binding and hydrolysis of ATP. The change of amino acid residues in the Walker A motif (K159N) and in the SRH (D264E and R269L) resulted in the loss of ATPase activity. The conservative mutation generated in the Walker B motif (D212E) did not affect the activity (see figures 4.17 and 4.18, and table 4.3).

The above results are in accordance with the data obtained from the AAA protease FtsH (Karata et al., 2001), for which the mechanism of ATP hydrolysis and substrate translocation was analyzed by mutagenesis and modelling. In the model, the bound ATP makes a multitude of interactions with surrounding residues (e.g. N301, K201 and E255) which together form the ATP-binding pocket.

K201 in FtsH (K159 in TAA43) of the Walker A motif forms hydrogen bonds with the oxygens of both the ß- and γ-phosphate of the ATP, therefore a disruption in the ATP-binding pocket in TAA43 was generated by the substitution of K159 with an uncharged residue (N).

The invariant residues D307 and R312 in FtsH (D264 and R269 in TAA43 respectively) form a tight double intramolecular salt bridge which stabilizes the conformation of the SRH. Hence, the loss of ATPase activity and the functional activities of the FtsH protease were directly correlated (Karata et al., 2001). The question remains whether this salt bridge plays a role for the function of the TAA43 protein.

It was demonstrated that the catalytic function of the Walker B motif in FtsH is triggered by E255 instead of D254 (D212 in TAA43), which forms an important part of the ATP-binding pocket mentioned above. The D212E mutation did not affect the ATPase activity in TAA43, which can be explained by the use of a conservative substitution (E) that could mimic the actual spatial conformation.

5.2. FUNCTIONAL ANALYSIS

5.2.1. Two-hybrid system in bacteria

It was of great interest to use this new system, because of easier handling compared to the yeast system. The information obtained suggests a functional role for TAA43.

The two-hybrid system in bacteria (2HB) allowed the *in vivo* identification of interacting proteins with TAA43 (table 4.5). TAA43 interacts with many RNA-binding proteins and also some DNA-binding proteins. Clear examples are the tRNA synthetases (four of them were identified in this study), ribonucleotide reductase, the translation elongation factor aEF-2, the cell cycle control protein 6 and DNA polymerase. In addition, almost half of the proteins identified (table 4.6, BLOCK classification) contain a ribosomal protein domain (L31E). This data suggests that TAA43 may be involved in RNA metabolism.

It should be considered that although 2HB is a fast method of detection, there is still, potentially, a significant percentage of false positives. Therefore, an independent approach was adopted in addition to the 2HB.

The results were analyzed in reference to the work done in *H. pylori*, involving several steps of processing the raw two-hybrid results (Rain et al., 2001). First, positive prey fragments (blue/white screening) were selected and referred to as selected interacting domains (SID). Here, 2680 (19%) positive SID clones were selected from a total of 13,962 clones. Second, SIDs that did not code for part of a *H. pylori* ORF were discarded (41% of the 2680 selected clones).

The results obtained in the *T. acidophilum* study shows that from a total of 78,000 clones generated, only 300 clones (0.38%) were positive for complementation according to the blue/white screening. Of these 300, 47 clones (16%) were positive for complementation as monitored by the ß-galactosidase activity assay. The rest of the clones contained non-ORF encoding regions as analyzed by DNA sequence alignments against the *T. acidophilum* genomic database and were thus discarded (84% of the 300 clones).

5.2.2. Co-precipitation analysis

The second method of detection -an *in vitro* approach- was used to compare the results obtained from the *in vivo* interaction analysis (2HB). Two advantages of the co-precipitation method are that (1) native proteins from *T. acidophilum* crude extract are used, (2) conditions for the co-precipitation can be modified (such as the pH, buffers, temperature, etc.), and (3) all *T. acidophilum* proteins are present. Thus, interactions involving more that one protein may be detected. On the other hand, weak or transient interactions are practically undetected. The identified proteins co-precipitating with TAA43 gave interesting results (figure 4.28 and table 4.7). From the 14 bands sequenced, 10 corresponded to ribosomal subunits, 3 to hypothetical proteins without known function, and 1 to ribonucleotide reductase (detected also by the 2HB).
The majority of the proteins detected here were components of the small or large subunit of the ribosome. Localization of some of the individual proteins in the ribosome is shown in figure 5.1.

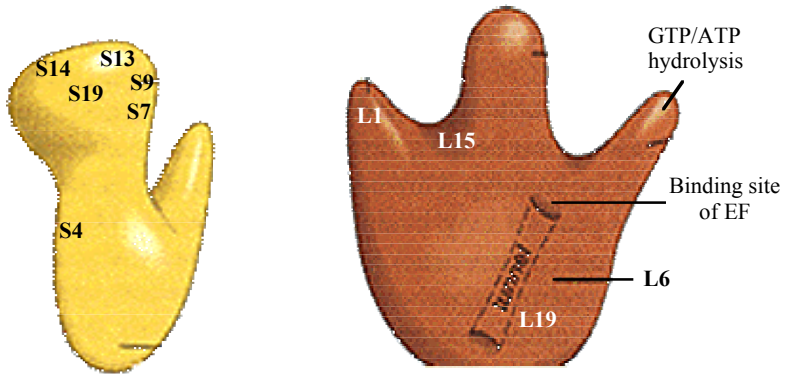


Figure 5.1. Localization of some of the ribosomal subunits co-precipitated with TAA43 and other proteins found by the two-hybrid system in bacteria (illustration taken from the web site http://ntri.tamuk.edu/cell/ribosomes.html).

The principal differences of the two approaches used are summarized in table 5.2.

Table 5.2. Characteristics of the methodology used in this study for the detection of protein-protein interactions.

Two-hybrid system in bacteria (2HB)	**Co-precipitation assay**
-*In vivo* assay	-*In vitro* assay
-Use of foreign cellular environment (*E.coli*)	-Virtually complete set of *T. acidophilum* proteins is present (crude extract)
-Fragment hybrid proteins expressed (polypeptides generated from a genomic library)	-Native proteins (complete protein complexes e.g. complete ribosomes)
-Weak and transient interactions may be detected	-Weak interactions not detected
-Conditions of the system cannot be changed (e.g. temperature, pH, salinity, etc.)	-Conditions of the assay can be modified
-Detection of specific domains	

Whereas the 2HB can detect protein-protein interactions *in vivo*, co-precipitation gathers interactions *in vitro*. Major disadvantages of the 2HB are the fact that only fused fragments of proteins are expressed while the co-precipitation assay uses native proteins from cell crude extract, or, the false positive results generated when non-encoding ORFs are expressed. Nevertheless, with the 2HB it is possible to detect weak and transient interactions and to identify the specific region of the polypeptide interacting with the bait protein (TAA43).

5.3. DESCRIPTION OF THE TAA43 INTERACTING PROTEINS

Although the present data do not permit the identification of the function of TAA43, initial hints have been obtained by using two different approaches.
Features of the proteins found in this study which are involved in RNA metabolism, translation and transcription will be summarized below and a speculation on the cellular role of TAA43 will be presented.

5.3.1. Ribosomes

Translation is the process in which individual amino acids are linked together into a polypeptide chain to form proteins. Translation is performed by subcellular particles called ribosomes and comprises the decoding of nucleotide sequence information carried on messenger RNAs (mRNA). Ribosomes in archaea and bacteria are ribonucleoproteins composed of the two subunits: the large 50S subunit and the small 30S subunit that form a 70S particle when assembled. Each of these subunits is associated with RNA (ribosomal RNA, rRNA) constituting two-thirds of the mass of the ribosome. The 30S subunit consists of 21 proteins and a 16S rRNA, and the 50S subunit contains 31 proteins associated with a 23S rRNA and a 5S rRNA. The function of the rRNAs is mainly structural, ribosomal subunits bind to rRNA at particular sites to obtain an orderer assembly. Evidence for interactions of rRNA with mRNA or tRNA at each stage of translation is documented. In addition, ribosomal proteins are necessary to maintain the rRNA in a structure in which it can perform the catalytic functions (e.g. a modification in the 23S rRNA is involved in peptide chain formation) (Lewin, 1994).
The ribosomes contain several active sites (figure 5.2), which are concentrated around the translational domain where proteins are synthesized. The major active sites described in *E. coli* are the P, A, and E site, the EF-Tu and EF-G binding site, the peptidyl transferase, and a mRNA-binding site (Rheinberger, 1991).
In summary, the ribosome is a very sophisticated macromolecular complex. The mode of interaction of a great number of components (ribosomal proteins, protein factors, rRNA, mRNA and tRNA) to form the ribosomal machinery is still a matter of analysis,

along with the study of the interaction of molecules in transit during protein synthesis. It is believed that the ancient ribosome contained only RNA, an idea supported by the presence of the rRNA in the assembly of the ribosomes and its role for their function (Wool, 1996). Hence, two possibilities have been considered while studying the origin of ribosomes: 1) the ribosomal proteins were designed specifically for the ribosome; or 2) they were co-selected from previously existent proteins with specific functions.

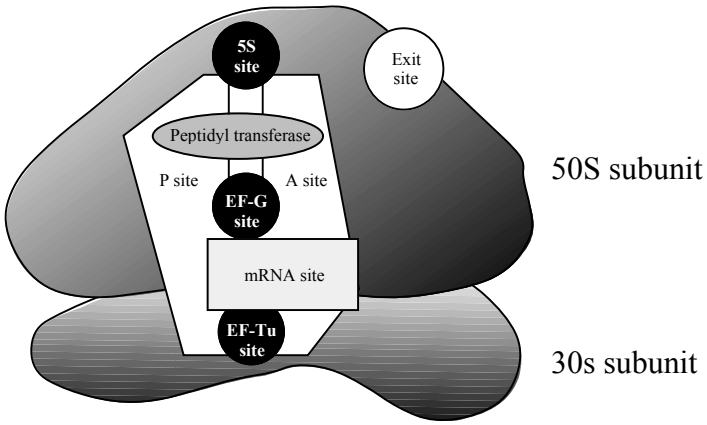


Figure 5.2. Illustration of the active centers in the translational domain of the ribosome (taken from Lewin, 1994).

Findings that ribosomal proteins show extraribosomal functions support the second scenario. Evidence for the existence of ribosomal proteins shared by the transcription and translation apparatus (Squires and Zaporojets, 2000) reinforce the latter theory.

5.3.2. Ribonucleotide reductase (Ta1475)

From all proteins detected as potential partners of TAA43, ribonucleotide reductase (RNR) was the only one detected by the *in vivo* and *in vitro* methods. RNRs are metallo-enzymes found in all organisms and their catalytic mechanism is based on the reduction of ribonucleotides to synthesize the analogous deoxyribonucleotides (dNTPs) needed for DNA synthesis and repair, using a protein radical as a cofactor (figure 5.3).

Among the three existing classes, *T. acidophilum* RNR belongs to class II RNRs which use adenosylcobalamin (a cysteinyl radical) as specific cofactor.

From the evolutionary point of view it has been assumed that RNA was the primordial molecule of life. The appearance of RNRs in all organisms was the milestone for the transition of RNA to DNA becoming the fundamental macromolecule of life (Jordan et al., 1997), (Jordan and Reichard, 1998). It has been an enigma why three classes of RNRs structurally different, uses distinct mechanisms to synthesize all dNTPs.

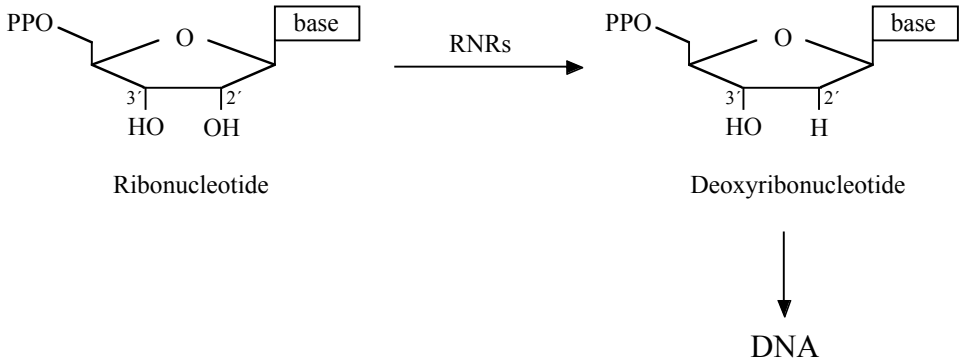


Figure 5.3. The reaction catalyzed by ribonucleotide reductases.

It has been reported that one of the most interesting properties of RNRs is their allosteric regulation. As shown in figure 5.4, the RNRs class I and III contain two allosteric sites, one of activating and one of inhibitory character, whereas the effectors in the reaction for RNR class II are just of activating character (Eliasson et al., 1999).

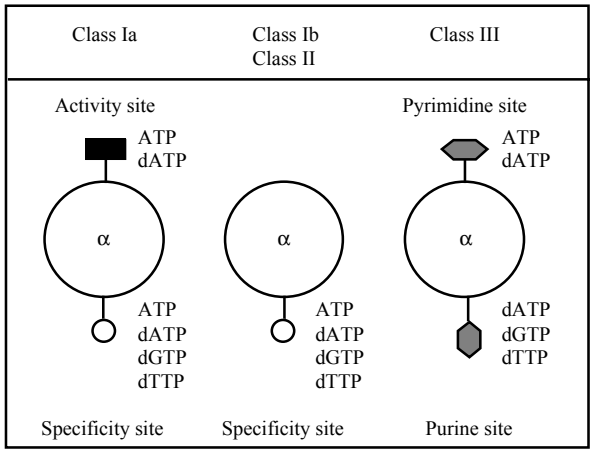


Figure 5.4. Illustration of the allosteric regulation of the three RNR classes. Taken from Fontecave, 1998.

5.3.3. Inosine-5´-monophosphate dehydrogenase (Ta0611)

Inosine-5´-monophosphate dehydrogenase (IMPD) catalyzes a key step in the *de novo* biosynthesis of guanine ribonucleotides, through the oxidation of IMP to xanthosine 5'-monophosphate (XMP) with the simultaneous reduction of NAD+ to NADH.

5.3.4. Aminoacyl-t-RNA synthetases (Ta0849, Ta0777, Ta1163, and Ta0040)

Aminoacyl-t-RNA synthetases (AARS) are in charge of coupling the correct amino acid to the corresponding transfer RNA (tRNA). The mechanism of action develops in a two step process: (1) the amino acid (AA) is activated via ATP hydrolysis, (2) the activated amino acid is transferred to its correspondent tRNA and AMP is released (figure 5.5). The aminoacyl-tRNA is then ready to be used by the ribosomal machinery for protein synthesis.

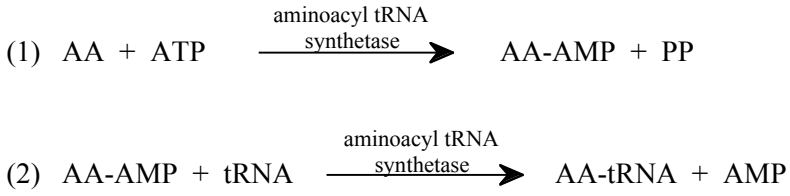


Figure 5.5. The two step mechanism of the t-RNA synthetases reaction.

There are 20 distinct AARS and they are classified into two highly conserved structural groups, classes I and II, each one with unrelated active site topologies. The specificity of a particular sustrate is almost completely conserved throughout evolution, which means that a particular AARS will always belong to the same class. Until now, the only exception is the lysyl-tRNA synthetase, which is a class I enzyme in most archaea and a few bacteria, but otherwise member of class II (Ibba and Soll, 2001).
The 4 AARS identified in this study comprise the alanyl-tRNA synthetase, leucyl-tRNA synthetase, lysyl-tRNA synthetase, and valyl-tRNA synthetase.

5.3.5. Translation initiation factor eIF-1A (Ta1454)

Initiation factors are proteins involved exclusively in the formation of the ribosomal initiation complex. The proposed function of eIF-1A in eukaryotes is the binding of 40S (small) ribosomal subunits and thus arresting the premature association with the 60S (large) ribosomal subunits.

5.3.6. Translation elongation factor aEF-2 (Ta0446)

EF-2 is a key element in translation. It is only present when the ribosomal subunits are assembled and it binds to each aminoacyl-tRNA (except for the initiator, Met-tRNA) to transport them into the A site of the ribosome (see figure 5.1). The reaction is dependent on GTP hydrolysis, and once the aminoacyl-tRNA binds the ribosome, EF-2 is released and prepared to bind in a cyclic manner the next aminoacyl-tRNA.

5.3.7. Iron-dependent transcription repressor (Ta0872) and the ferric transport ATP-binding protein AFUC (Ta0202)

The metabolism of most bacteria depends on iron. Iron is required in the redox centers of the heme and iron-sulfur proteins of the membrane-bound electron transport chains as well as in cytoplasmic enzymes like ribonucleotide reductase, nitrogenase, aconitase, oxygenases, hydrolases, synthases, and superoxide dismutase. The iron-dependent transcription repressors are able to detect the level of free iron (Fe^{++}) in the cell, which is extremely limited *in vivo*. At a low level of iron, the enzyme looses its affinity for certain regions of the DNA and allows the expression of iron-related genes –e.g. the ferric transport ATP-binding protein AFUC-, in order to import iron from the environment. Regulation of the intracellular iron concentration is of great importance because an excess of iron enhances the formation of oxygen and hydroxyl radicals, which in turn damage cells (Braun, 1997).

5.3.8. Cell division control protein 6 (Ta0451)

The cell division control protein 6 (cdc6) is a member of the AAA+ family and a protein essential for the regulation of the initiation of DNA replication in eukaryotic cells. Cdc6 binds transiently to the chromatin at replication origins during the G1 phase of the cell cycle. Together with other factors like ORC, Cdc45, Mcm and other unknown factors, it triggers the initiation of DNA synthesis.
Cdc6 is the only protein from all the interacting proteins detected by the 2HB system that belongs to the AAA+ family of proteins. The protein fragment expressed that interacts with the TAA43 protein contains part of the AAA domain; the Walker B and the SRH.

5.3.9. Polyketide synthase (Ta 0672)

Polyketide synthases (PKS) are large multienzyme systems (10-100,000 kDa) that catalyze the assembly of exceptionally complex natural products from simple precursors. The mechanism of action of the PKS resembles the process of the fatty acid biosynthesis. Substrates are 2-, 3-, and 4-carbon chains and products synthesized are the acetyl-CoA, propionyl-CoA and methylmalonyl-CoA. The PKSs are under intense study because of their pharmacological relevance as antimicrobials, antifungals, antiparasitics, antitumor agents, and immunosuppressors.

5.4. HYPOTHESIS FOR THE CELLULAR ROLE OF TAA43

The essence of the data obtained in this study is the following:

1. The gene organization up- and downstream of the *taa43* ORF in the *T. acidophilum* genome suggests the existence of a superoperon that contains a cluster of genes involved in translation and transcription (figure 4.19). As described by Jacob and Monod in 1961, genes belonging to the same operon invariably encode functionally associated proteins.

2. TAA43 is classified as an AAA+ class protein according to the analysis of the *T. acidophilum* genome (Ruepp et al., 2000).

3. TAA43 forms a dimeric complex, which differs from the hexameric pattern exhibited by many AAA+ class proteins (figures 4.11, 4.12, and 4.13), although substrate-induced oligomerization was described for katanin.

4. Sequence analysis of the N-terminal domain of TAA43 protein reveals a possible RNA-binding region of very low significance found as well in two identified homologues, VAT-2 from *T. volcanium* and from *F. acidarmanus* (figure 4.5).

5. Protein interaction studies indicate that TAA43 might be involved in the transcription and translation pathways. Interestingly, at least 10 of the 47 protein fragments detected by the 2HB (table 4.5) exhibit homology to the genuine ribosomal L31E protein domain but are not ribosomal proteins (e.g. the signal recognition particle, the D-lactate dehydrogenase, or the ferric transport ATP-binding protein AFUC).

6. An AAA+ protein which participates in DNA replication, cdc6, was also detected.

7. One single protein was scored as an interacting partner of TAA43 by two different methods: ribonucleotide reductase (RNR), a protein involved in DNA synthesis (figure 5.3). It may be speculated that TAA43 acts together with RNR involving an interaction either with RNA and/or DNA. In support of this idea is the fact that several ribosomal subunits are shared by the translational and the transcriptional machineries (Squires and Zaporojets, 2000) involved in RNA or DNA metabolism respectively.

8. Several proteins of the small and large subunit of the ribosome were identified as partners of TAA43 by co-precipitation analysis. If TAA43 binds to any ribosomal subunit, it is expected that the entire ribosomal complex will precipitate. However, the identification of co-precipitating proteins requires the presence of bands sufficiently distinct for amino acid sequence determination and the band pattern is quite complex. A recent identification and characterization of three proteins (2 in yeast and 1 in bacteria) showed a similar co-precipitation pattern as found for TAA43. The first example is the nuclear AAA-type ATPase, Rix7p (Gadal et al., 2001) required for biogenesis and nuclear export of 60S ribosomal subunits. The second example is the nuclear GTPase, Nug1p (Bassler et al., 2001), which co-precipitates with proteins of the 60S subunit, late precursors of the 25S and the 5.8S rRNAs and at least 21 nonribosomal proteins. Lastly, a ribosome-bound ATPase from *E. coli*, RbbA, was shown to copurify with 30S and stimulate the synthesis of polyphenylamine *in vitro* (Kiel et al., 1999), (Kiel and Ganoza, 2001).

All considerations presented above suggest a possible role for TAA43 in translation and transcription processes.

5.5. FUTURE WORK

A subject of further study will be the state of oligomerization. TAA43 was found to co-precipitate with a molecule corresponding to the molecular size of the ribosome (figure 4.13). Western blotting and electron microscopy can be used as immediate tools to prove the existence of the proposed complex between TAA43 and the ribosome.

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7. ABBREVIATIONS

2HB	Two-hybrid in bacteria
AAA	ATPase Associated with several Activities
ADP	adenosine 5'-diphosphate
AMPPNP	adenosine 5'-[ß,γ-imido] triphosphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase
bp	base pair
$CaCl_2$	calcium chloride
CTP	cytidine 5'-triphosphate
D	aspartic acid
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
E	glutamic acid
EDTA	ethlylendiamine tetraacetic acid
FITC	fluorescein isothiocyanate
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid
IgG	immunoglobulin G
IPTG	ß-D-isopropyl-thiogalactopyranoside
GTP	guanosine 5'-triphosphate
K	lysine
KDa	kilodalton
L	leucine
Mg^{++}	magnesium ion
$MgCl_2$	magnesium chloride

MDa	megadalton
MES	2-(N-morpholino)ethanesulfonic acid
Min	minute
MW	molecular weight
N	asparagine
NaCl	sodium chloride
NaOH	sodium hydroxide
Ni-NTA	nickel nitrilo-tri-acetic acid
OD	optical density
PAGE	polyacrylamide gel electrophoresis
Pi	released phosphate
R	arginine
RNA	ribonucleic acid
RNase	ribonuclease
Rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl-ethylendiamine
Tris	tris(hydroxymethyl)aminomethane
U	unit
UTP	uridine 5'-triphosphate
X-Gal	5-bromo-4-chloro-3-indolyl-ß-galactopyranoside

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