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A thermostable K^+-stimulated vacuolar-type pyrophosphatase from the hyperthermophilic bacterium *Thermotoga maritima*

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Abstract Current evidence suggests the occurrence of two classes of vacuolar-type H^+-translocating inorganic pyrophosphatases (V-PPases): K^+-insensitive proteins, identified in eukaryotes, bacteria and archaea, and K^+-stimulated V-PPases, identified to date only in eukaryotes. Here, we describe the functional characterization of a thermostable V-PPase from the anaerobic hyperthermophilic bacterium *Thermotoga maritima* by heterologous expression in *Saccharomyces cerevisiae*. The activity of this 71-kDa membrane-embedded polypeptide has a near obligate requirement for K^+, like the plant V-PPase, and its thermostability depends on the binding of Mg^{2+}. Phylogenetic analysis of protein sequences consistently assigned the *T. maritima* V-PPase to the K^+-sensitive class of V-PPases so far only known for eukaryotes. The finding of a K^+-stimulated V-PPase also in a member of a primitive eubacterial lineage strongly supports an ancient evolutionary origin of this group of pyrophosphate-energized proton pumps.

Key words: Heterologous expression; K^+-stimulation; Vacuolar-type H^+-pyrophosphatase; *Saccharomyces cerevisiae*; *Thermotoga maritima*

1. Introduction

Inorganic pyrophosphate (PPi) has been suggested to be the ancestor of ATP in the early stages of evolution and an alternative as 'energy currency' in the bioenergetics of some modern cells [1]. Membrane-bound proton-translocating inorganic pyrophosphatases (H^+-PPases or vacuolar-type inorganic pyrophosphatase (V-PPase) – for vacuolar-type PPases: EC 3.6.1.1) belong to a recently identified category of proton pumps which, unlike ATPases, utilize the energy of PPi hydrolysis as the driving force for H^+ movement across biological membranes [2]. V-PPases have been shown to occur in higher plants, parasitic protists, photosynthetic bacteria and archaea [3–7]. The information currently available suggests the occurrence of two types of V-PPases depending on their sensitivity to K^+. K^+-stimulated V-PPases have been found only in eukaryotes [3,6,7], whereas K^+-independent V-PPases have been identified both in eukaryotes [8] and prokaryotes [4,9]. The presence of a gene encoding a putative V-PPase in the hyperthermophilic bacterium *Thermotoga maritima* has been reported previously [10] and, more recently, the full sequence became available with the completion of the genome project of this organism [11]. Here, we report the functional characterization of the protein heterologously expressed in *Saccharomyces cerevisiae*. *T. maritima* V-PPase (TVP) has an optimal temperature of around 70°C and its activity is stimulated six-fold by K^+. To our knowledge, this is the first eubacterial K^+-stimulated V-PPase characterized to date. Moreover, the protein seems to be resistant to yeast proteases and its thermostability depends on the binding of magnesium ions. Phylogenetic analysis utilizing sequences of V-PPases from *T. maritima* and other organisms have been performed and the evolutionary implications are discussed.

2. Materials and methods

2.1. Strains and media

S. cerevisiae strain W303-1A (MAT**a**, *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) was used for transformation with autonomously replicating plasmids containing the *URA*3 gene as a selectable marker. Cells were grown at 30°C in medium containing 2% glucose, 0.67% yeast nitrogen base without amino acids, tryptophan (0.5 mM), histidine (0.4 mM), adenine (0.2 mM), leucine (1 mM) and uracil (0.2 mM). Uracil was omitted for selection of yeast transformants and glucose was replaced by galactose in order to induce the expression of foreign proteins.

2.2. Isolation of the genes encoding TVP and Rhodospirillum rubrum V-PPase (RVP)

The coding sequence of a putative V-PPase was amplified from genomic DNA of *T. maritima* MSB8 (strain DSM 3109) by high-fidelity polymerase chain reaction (PCR) using *Taq*Plus DNA polymerase (Stratagene). This 2.2-kb long DNA fragment had been suggested to encode a V-PPase during the course of the *T. maritima* genome project (GenBank accession number AE001702, protein id: AAD35267.1; [11]). Oligonucleotides corresponding to positions 1–21 and 2164–2184 of the mentioned sequence and containing artificial *Sal*I sites at their respective 5′-ends were utilized as primers. The coding sequence of RVP was amplified by high-fidelity PCR, utilizing as template the original 4.5-kb long cDNA clone, obtained as previously described [5]. Oligonucleotides were designed as above considering the newly proposed starting codon for RVP [12] and contained artificial *Xho*I sites for cloning purposes.

2.3. Plasmid constructions

Plasmid pJR1 was constructed in our laboratory by ligation of a 0.75 kb *Eco*RI–*Xho*I DNA fragment containing the *S. cerevisiae* galactokinase (*GAL*1) promoter, a 1.2-kb *Xho*I–*Hin*dIII fragment containing the transcription termination region of yeast *PMA*1 gene [13]

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Abbreviations: PPi, inorganic pyrophosphate; PCR, polymerase chain reaction; RVP, *Rhodospirillum rubrum* V-PPase; TVP, *Thermotoga maritima* V-PPase; V-PPase, vacuolar-type inorganic pyrophosphatase

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and the yeast shuttle vector YEp352 [14] digested with *Eco*RI and *Hin*dIII.

The PCR-amplified fragment from *T. maritima* was inserted into pGEM-T (Stratagene) and the resulting plasmid was subsequently digested with *Sal*I, yielding a 2.2-kb long DNA fragment that was inserted with the right orientation into the unique *Xho*I site of plasmid pJR1, thus obtaining plasmid pTVP. The PCR-amplified ORF of RVP was inserted in pJR1 by using the same approach, yielding plasmid pRVP.

2.4. Yeast transformation and culture

S. cerevisiae cells were transformed with plasmids pJR1, pRVP and pTVP by the LiOAc/PEG method [15] and selected by growth on 2% agar plates in culture medium without uracil. Transformants were liquid-grown in selective medium up to stationary phase, sedimented by centrifugation at 2000 rpm in a bench centrifuge for 5 min, washed thoroughly with water and resuspended in 2% galactose in glucose-free selective medium. After 24 h with gentle agitation, yeast cells were collected, washed with water, resuspended in working buffer (50 mM Tris–HCl, pH 8; 0.3 M sucrose, 2 mM DTT, 2 mM EDTA) and homogenized by shaking vigorously with glass beads [16]. The homogenate was centrifuged for 10 min at 2400 rpm (Sorvall SS-34 rotor) to remove beads and debris and the supernatant was centrifuged for 30 min at 40 000 rpm (Beckman 60 Ti rotor) to sediment the 'total membrane fraction'. This pellet, after resuspension in working buffer, homogenization and storage at −20°C, was used for subsequent experiments. EDTA was omitted and a protease inhibitor cocktail (Sigma) was added to the working buffer where indicated.

2.5. PPase activity assay, Western analyses and protein estimation

PPase activity was assayed at 70°C as previously described [17]. Membrane fractions of pJR1-transformed yeast cells were utilized as negative controls. Both samples and controls were subjected to a 5 min preincubation at 70°C in assay buffer in order to destroy any endogenous yeast PPase activity. Immunoblot (Western blots) assays were performed as reported elsewhere [18] using two antibodies: antibody PAB_{HK} [4] and an antibody against RVP. Protein was estimated by the Bradford method [19] with ovalbumin as a standard.

2.6. Sequence comparison

Multiple alignments of TVP sequence and other full-length V-PPase sequences were generated, and phylogenetic trees calculated by a Neighbor-Joining method, using the program CLUSTAL X [20]. The other gene-deduced V-PPase sequences used were: *Arabidopsis thaliana* K^+-activated V-PPase (AVP1, AB015138) and K^+-insensitive V-PPase (AVP2, AF182813); *Beta vulgaris* (BVP1, AAA61609); *Nicotiana tabacum* (TVP5, S61422); *Oryza sativa* (OVP1, BAA08232); *Hordeum vulgare* (HVP1, BAB18681); *Zea mays* (AAA80347); *Chara corallina* (AB018529); *Pyrobaculum aerophilum* (AF182812); *R. rubrum* (AAC38615.1); *Chlamydomonas reinhardtii* 21gr (AJ304836, J.R. Pérez-Castiñeira, R.L. López-Marqués and A. Serrano, unpublished results); *Leishmania major* strain 252 (J.R. Pérez-Castiñeira, L.M. Ruiz and A. Serrano, unpublished results); *Plasmodium falciparum* 1 (AAD17215) and 2 (preliminary data from The Institute of Genomic Research, TIGR, Sanger Centre and Stanford DNA Sequencing and Technology Center); *Streptomyces coelicolor* (CAB38484); *Chloroflexus aurantiacus* (contig 1051), *Methanosarcina barkeri* (contig 1693), *Magnetospirillum magnetotacticum* (contig 3815), *Magnetococcus* sp. MC1 (contigs 209 and 114), *Nitrosomonas europaea* and *Rhodopseudomonas palustris* (preliminary data from the DOE Joint Genome Institute, JGI); *Carboxydothermus hydrogenoformans* (contig 2356), *Caulobacter crescentus* (contig gcc 492), *Chlorobium tepidum* (contig gct 12), *Geobacter sulfurreducens* (contig 1280), and *Treponema denticola* (contig 10145) (preliminary data from TIGR).

3. Results and discussion

Two different antibodies against V-PPases recognized a protein of around 71 kDa in membrane preparations of yeast cells transformed with a plasmid containing the TVP gene under the control of *S. cerevisiae GAL*1 promoter. Consistently, the occurrence of TVP was strictly dependent on growing the yeast transformants in the presence of galactose and absence of glucose (Fig. 1A). Biochemical characterization of V-PPase genes heterologously expressed in yeast has been reported elsewhere demonstrating the suitability of *S. cerevisiae* for heterologous expression and characterization of V-PPases [3,4,8].

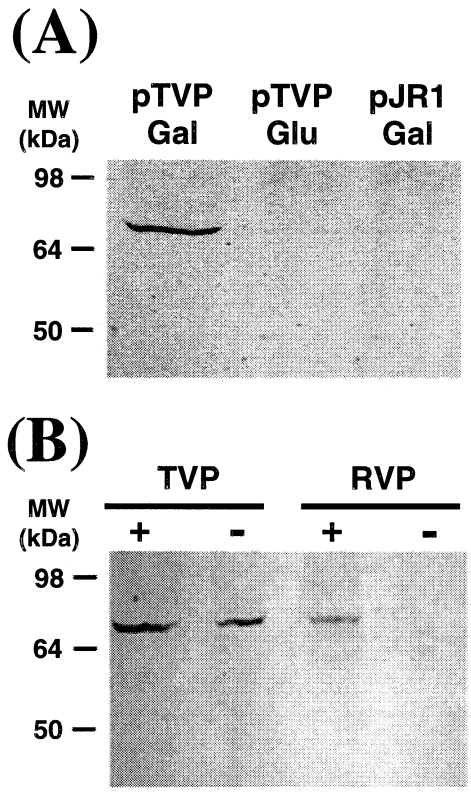

Fig. 1. A: Immunoblot analysis with antibody against RVP of total membrane fractions obtained from yeast cells transformed with plasmid pTVP, grown in galactose and glucose, and plasmid pJR1, grown in galactose. B: Immunoblot analysis of total membrane fractions from yeast cells transformed with plasmids pTVP and pRVP grown in galactose. Yeast membranes were obtained as described in Section 2 in the presence (+) or absence (−) of protease inhibitors. About 70 μg of protein per lane were loaded in both cases. Blots immunodecorated with antibody against RVP are shown, but identical results were obtained with antibody PAB_{HK}.

Protease-deficient strains and protease inhibitors are routinely utilized when isolating heterologously expressed proteins in *S. cerevisiae* [3,4,8] to avoid degradation by proteases during the purification procedure [21]. TVP was expressed in a yeast strain whose hydrolytic machinery was intact and it was observed in membrane fractions obtained in the absence of any protease inhibitor (Fig. 1B). Moreover, both the amount of TVP (Fig. 1B) and PPase activity (not shown) were not significantly affected by the presence of protease inhibitors along the isolation procedure. In order to check whether this was specific for TVP, another V-PPase (RVP), was expressed in yeast and isolated under identical conditions. In this case, the presence of protein in the samples was strictly dependent on the inhibition of yeast proteases (Fig. 1B). This was also the case for all other K^+-insensitive V-PPases of normophilic bacteria expressed in yeast in our laboratory (J.R. Pérez-Castiñeira and A. Serrano, unpublished results).

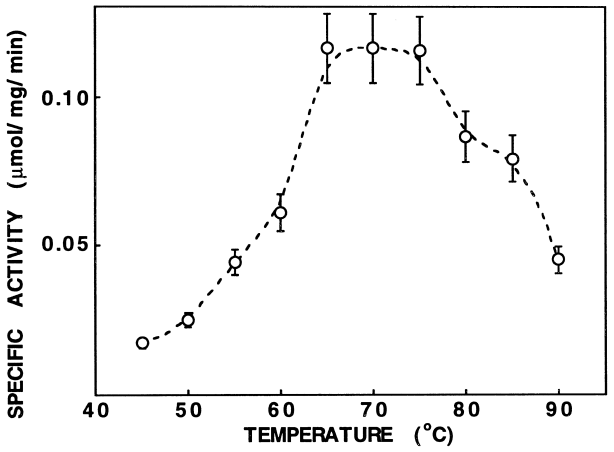

Fig. 2. Temperature dependence of TVP-mediated PPi hydrolysis. Membrane fractions of pTVP-transformed *S. cerevisiae* W303 cells were incubated at 70°C for 5 min in assay medium containing 1 mM $MgCl_2$ and 100 mM KCl. Assays were then performed at the indicated temperatures and started by addition of 0.5 mM PPi. Values are means ± S.E.M. obtained with three independent clones.

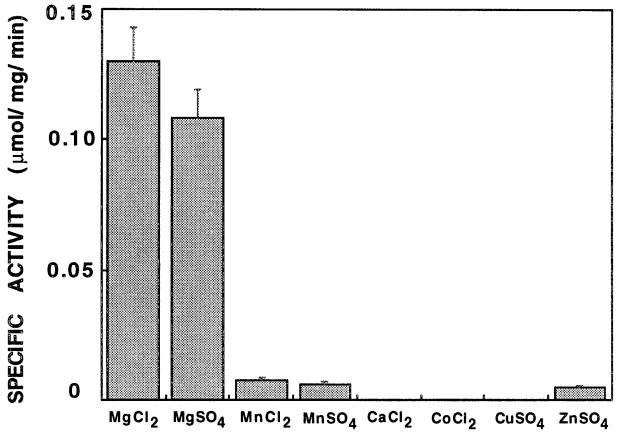

Fig. 4. Divalent cations dependence of PPi hydrolysis by TVP. Same conditions as in Fig. 2 except that reaction media contained 100 mM KCl in all cases plus 1 mM of the indicated divalent cation salt. Samples were obtained in the presence of EDTA and 200 μM EDTA was present during preincubation and assay. Values are means ± S.E.M. as in Fig. 2.

More studies on this characteristic of TVP and its possible link to thermophilicity are currently under way.

PPase activity was observed in membrane preparations obtained from pTVP-transformed galactose-grown yeast cells when assays were performed at temperatures higher than 50°C, the optimum being around 70°C (Fig. 2). This temperature is somewhat lower than that obtained for another V-PPase from a hyperthermophilic organism, the archaeon *P. aerophilum* [4], which is consistent with the optimal growth temperatures reported for *T. maritima* and *P. aerophilum*: 80 and 100°C, respectively [22,23]. A 5-min preincubation at 70°C was sufficient to destroy any intrinsic PPase activity from yeast, as demonstrated with appropriate controls. The activity remaining after preincubation was fluoride insensitive (not shown).

TVP-mediated PPi hydrolysis increased more than six-fold in the presence of KCl and NH_4Cl in comparison with the activity measured in the absence of any monovalent cation; NaCl produced a slight increase, whereas other salts such as lithium and choline chlorides were not effective (Fig. 3). The use of nitrates instead of chlorides made no difference (not shown), thereby suggesting that stimulation of the PPase activity was a cationic effect. This is a remarkable characteristic of TVP and is a major difference with respect to the K^+-insensitive V-PPase from *P. aerophilum*, the other hyperthermophilic protein characterized to date [4]. Although K^+-insensitive V-PPases have been shown to occur in both eukaryotes and prokaryotes [4,8,9], TVP is, to our knowledge, the first case of a K^+-stimulated V-PPase from a prokaryotic organism reported so far.

TVP showed maximal PPase activity with magnesium pyrophosphate, the physiological substrate of the protein,

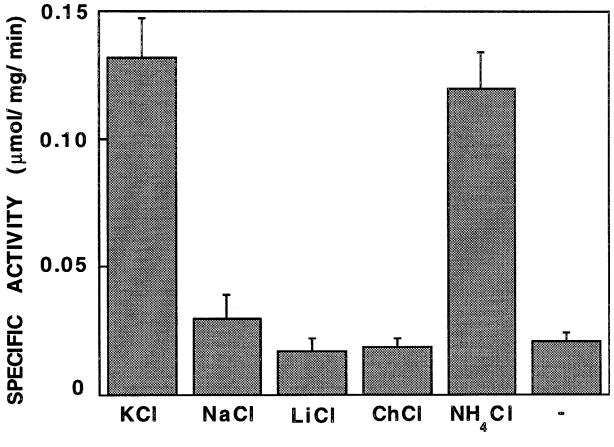

Fig. 3. Monovalent cations dependence of PPi hydrolysis by TVP. Assays were performed as in Fig. 2 except that KCl was replaced by the indicated monovalent cation salt. Values are means ± S.E.M. as in Fig. 2.

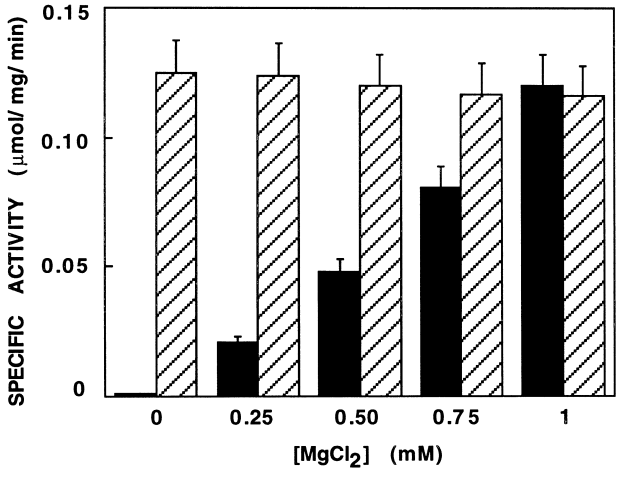

Fig. 5. Dependence of TVP-mediated PPi hydrolysis on the presence of magnesium during preincubation at 70°C. Membrane preparations from pTVP-transformed yeast cells were obtained in the absence (striped columns) or in the presence (black columns) of EDTA and incubated at 70°C for 5 min in assay medium containing the indicated concentrations of $MgCl_2$; assays were then started by addition of saturating amounts of $MgCl_2$ (1 mM) and PPi (0.5 mM). EDTA concentration both in the preincubation and the assay was 200 μM in all cases. Values are means ± S.E.M. as in Fig. 2.

whereas the rate of PPi hydrolysis was very low with manganese and zinc pyrophosphates and negligible with other divalent cation pyrophosphates (Fig. 4). The insensitivity of TVP to yeast proteases allowed us to obtain samples in the absence of EDTA, an inhibitor of metalloproteases. Under these conditions, TVP could be preincubated in assay medium for 5 min at 70°C without addition of any divalent cation (even in the presence of EDTA) before starting the assay. When samples were obtained in the presence of EDTA, the activity of TVP was lost during the preincubation unless magnesium

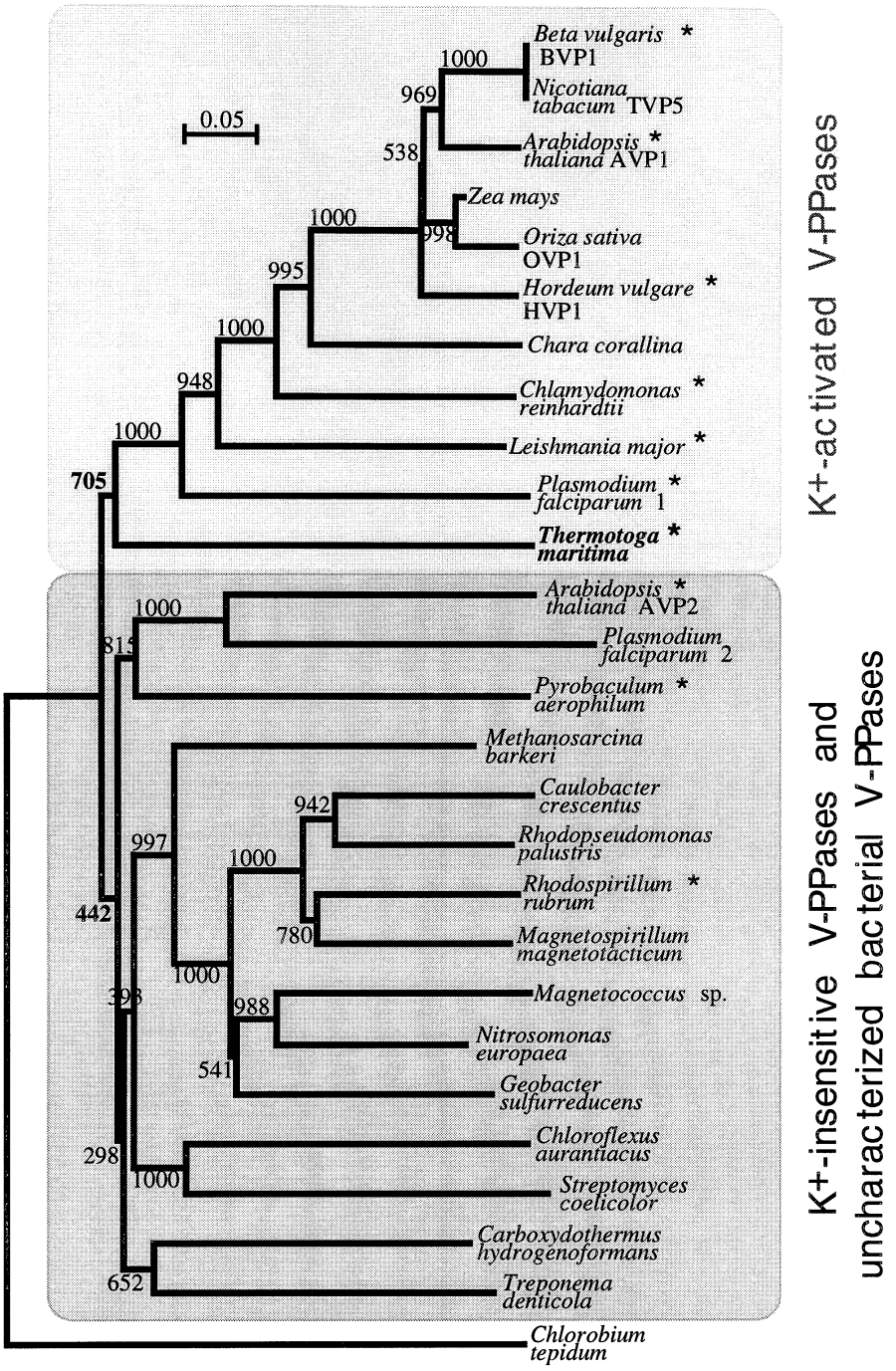

Fig. 6. Phylogenetic tree showing two main groups of V-PPases. Complete amino acid sequences deduced from genes obtained from databases and genome projects (see text for further details) were aligned and a Neighbor-Joining method used to calculate a phylogenetic tree by the CLUSTAL X program [20]. Scale bar represents 5% estimated difference in protein sequences. Two deeply rooted main groups of proteins are well supported by bootstrap analysis (number of times a particular branch topology was found in 1000 bootstrap replicates; values are indicated above the branches). One of them contains K^+-stimulated V-PPases representative of diverse groups of eukaryotic organisms and *T. maritima* TVP. The K^+-insensitive V-PPases from eukaryotes, archaea and bacteria, as well as a number of putative uncharacterized V-PPases from representative strains of the main eubacterial taxonomic groups, fall in the other main separated group. The V-PPase from the green sulfur bacterium *C. tepidum* has the most divergent sequence and was placed as an outgroup. Asterisks indicate proteins that have been functionally characterized.

was added to the medium in concentrations higher than that of EDTA present (Fig. 5). These results suggest that TVP strongly binds magnesium ions to acquire the right conformation and that it is isolated in a stable magnesium-containing form, unless cations are removed by a harsh treatment with a potent chelator. The thermostability of the soluble PPases from *Escherichia coli* and *S. cerevisiae* is also increased by magnesium [24–26]; this evidence, along with the fact that TVP has similar relative affinities for divalent cations as soluble PPases, suggests that membrane-bound and soluble PPases might have more similar conformations than previously thought. Actually, a tentative molecular outline of the active site of V-PPase has been proposed based on sequence similarities found between the well-characterized active site of soluble PPases and conserved motifs of vacuolar-type PPases (A. Schultz, J.R. Pérez-Castiñeira, M. Baltscheffsky and H. Baltscheffsky, unpublished results). On the other hand, it has been suggested that subunit interactions can be important for the thermostability of soluble PPases and that magnesium ions could be implicated in these interactions [24]. Radiation inactivation of V-PPases suggests that these proteins are dimers [27]; based on this evidence it is tempting to speculate that magnesium could also be involved in thermostability of V-PPases by enhancing the interaction between monomers.

Alignment of the TVP amino acid sequence with those deduced from other genes encoding V-PPases using the CLUSTAL X program resulted in a Neighbor-Joining phylogenetic tree which displays two major deeply rooted lineages of V-PPases well supported by bootstrap analysis (number of times a particular branch topology was found in a series of bootstrap replicates) [20]. Consistently with the experimental data presented in this report, TVP was assigned to one group together with the other well-characterized K^+-stimulated V-PPases (all of them from eukaryotic organisms) employed in the phylogenetic analysis (Fig. 6) [3,4,8,9]. The other deeply rooted group includes the second K^+-insensitive V-PPase of eukaryotes (plants and protists) and the archaeal K^+-insensitive V-PPase, both of them recently characterized [4,8], in addition to the well-known K^+-insensitive V-PPase of purple photosynthetic bacteria [5] and a number of yet uncharacterized putatives V-PPases from diverse prokaryotes representative of the main bacterial groups, including Proteobacteria, Gram-positive bacteria, green sulfur and non-sulfur bacteria and Spirochaetes (Fig. 6). Therefore, both biochemical and phylogenetic analyses indicate that the TVP is a K^+-stimulated V-PPase, being the first bacterial member of this class described so far. Moreover, since *T. maritima* is a member of the order Thermotogales, one of the deepest and most slowly evolving lineages in the bacteria, this finding has also a most important evolutionary significance. Thus, K^+-stimulated V-PPases seem to be as ancient as their K^+-insensitive counterparts, which are, on the other hand, distributed among eukaryotes, archaea and bacteria. Although more experimental evidence will be needed in order to elucidate the roles and occurrence of the two types of V-PPases, functional and phylogenetic analyses like those presented here will help to deepen the knowledge of the role of PPi metabolism in living organisms as well as the evolutionary origin of these proton pumps.

4. A note on TVP-mediated H^+-translocation

No significant proton-translocation activity could be assayed in the total membrane fractions of pTVP-transformed yeast cells. This is probably due to the fact that we are dealing with a hyperthermophilic enzyme whose activity is negligible below 50–55°C. Above 50°C the high passive conductance of yeast membranes to protons makes it impossible to establish a reasonable pH gradient as demonstrated with V-PPases that hydrolyze PPi at these temperatures. This situation has been reported and commented in detail elsewhere [4].

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