28. Extracellular tachyzoites were fixed for 1 hour on ice in 4% PFA in PBS containing 0.5% sodium azide, and then for 12 hours at 4°C in 8% PFA-buffered formaldehyde. The cell suspension was embedded in 10% gelatin, incubated for 2 hours at 4°C in PBS containing 2.5 M sucrose, and frozen by plunging into liquid nitrogen. Sections of the frozen samples were freshly prepared before each hybridization experiment. Cryosections were transferred to grids and digested for 40 min at 37°C in 2× SSC containing 0.5% proteinase K. Cellulase D. digested for 12 hours at 4°C in a humidified chamber in 5 μl of hybridization mix containing 10 to 20 ng/ml of DNA probe (28), washed three times for 5 min at 25°C in 4× SSC, twice for 3 min at 37°C in 50% formamide (in 2× SSC), twice for 5 min at 25°C in 2× SSC, and kept in 4× SSC at 25°C before staining. Hybridized probe was detected with polyclonal sheep anti-digoxigenin, followed by a secondary rabbit antibody directed against sheep immunoglobulin G (Proc) and Protein A conjugated to 10-nm particles of gold. Immunogold-labeled sections were blocked for 20 min at 25°C in 3× SSC containing 0.5% blocking reagent and were incubated in a monoclonal antibody against DNA followed by a rabbit anti-mouse secondary antibody and protein A conjugated to 5-nm gold particles. To improve the quality of the immunogold staining, sections were stained hybridized cryosections on ice for 10 min in 0.3% aqueous uranyl acetate plus 2% methylcellulose. Grids were air-dried on loops and examined with a Philips EM400 microscope.

30. The tentacle binding domain against DNA used in Fig. 2A probably recognizes both endogenous DNA and the digoxigenin-labeled probe. Similarly, the 5-nm gold-protein A conjugate used to visualize this antibody (by means of a secondary rabbit antibody) is potentially able to recognize any anti-digoxigenin that remained unbound. Comparable staining with antibody against DNA was observed even in the absence of a DNA probe, however (Fig. 2C), when control plasmid was used as a probe. Cryosections labeled with antibody to DNA before the application of anti-digoxigenin also showed no localization of large and small gold particles. The apparent clustering of labeling in Fig. 2. A and B, may be an artifact of in situ hybridization conditions, because antibody directed against DNA labels the organelle uniformly (Fig. 3C).

31. Infected cultures were fixed for 45 min in 4% PFA in PBS containing 0.5% sodium azide, and then for 12 hours at 4°C in PBS containing 0.5% glutaraldehyde and then for 12 hours at 4°C in 8% PFA-buffered formaldehyde. The cell suspension was embedded in 10% gelatin, incubated for 2 hours at 4°C in PBS containing 2.5 M sucrose, and frozen by plunging into liquid nitrogen. Sections of the frozen samples were freshly prepared before each hybridization experiment. Cryosections were transferred to grids and digested for 40 min at 37°C in 2× SSC containing 0.5% proteinase K. Cellulase D. digested for 12 hours at 4°C in a humidified chamber in 5 μl of hybridization mix containing 10 to 20 ng/ml of DNA probe (28), washed three times for 5 min at 25°C in 4× SSC, twice for 3 min at 37°C in 50% formamide (in 2× SSC), twice for 5 min at 25°C in 2× SSC, and kept in 4× SSC at 25°C before staining. Hybridized probe was detected with polyclonal sheep anti-digoxigenin, followed by a secondary rabbit antibody directed against sheep immunoglobulin G (Proc) and Protein A conjugated to 10-nm particles of gold. Immunogold-labeled sections were blocked for 20 min at 25°C in 3× SSC containing 0.5% blocking reagent and were incubated in a monoclonal antibody against DNA followed by a rabbit anti-mouse secondary antibody and protein A conjugated to 5-nm gold particles. To improve the quality of the immunogold staining, sections were stained hybridized cryosections on ice for 10 min in 0.3% aqueous uranyl acetate plus 2% methylcellulose. Grids were air-dried on loops and examined with a Philips EM400 microscope.

32. A total of 65 sequences, including nearly all available bacterial sequences and representative plastid sequences, were aligned using PILEUP (Genetics Computer Group, Madison, WI [1991]), with manual refinement on the basis of secondary structural information. Maximum likelihood analysis was performed with fastDNAml v1.0.6 [G. J. Olsen, H. Matsuda, R. Hagstrom, R. Overbeek, J. Felsenstein, CABIOS 12, 1489 (1996)] using nucleotide data from the first and second codon positions, and bootstrapping was carried out using 100 replicates with random addition sequences (where appropriate). LogDet distances are not directly comparable to standard distances but yield additive distances under any Markov model when sites are evolving independently and at the same rate (29).

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Evidence for a Family of Archaeal ATPases

The analysis by Carol J. Bult et al. of the Methanococcus jannaschii genome included families of paralogous proteins that did not seem to have counterparts in the current sequence databases (1). The largest of such families consists of 13 chromosomal and three plasmid-encoded proteins, which were found to be highly similar to one another [figure 6 in (1)], but did not show statistical significant similarity to any proteins, thus escaping functional prediction. Our inspection of the alignment, however, indicates that two of the conserved sequence blocks correspond to well-characterized functional motifs: namely, the phosphate-binding P-loop and the Mg\(^{2+}\)–binding site that are conserved in a vast variety of ATPases and GTPases (Fig. 1 and 2–4). Even though most commonly used methods for database search such as BLASTP (5) showed only marginally significant similarity to several ATPases, a new version of the BLASTP program that constructs local alignments with gaps (6) indicated a high probability of matching by chance between \(10^{-4}\) and \(10^{-6}\) for some of these proteins with the new archaeal family and bacterial DNA-proteins; the conservation was particularly notable in the two ATPase motifs (Fig. 1). Thus, even though these 16 proteins comprise a novel family that is so far represented only in archaea, they appear to belong to a known broad class of proteins, and we predict that they possess ATPase activity.

Screening of the nonredundant protein sequence database at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD), with a bipartite pattern representing the specific forms of the two ATPase motifs conserved in the M. jannaschii family—namely, hhhhhGx\(_2\)-GK[T][S][h][h][h][h][h][D][E] (h indicates a bulky hydrophobic residue), selected 271 proteins, all of which are either known to possess ATPase activity or are highly similar to ATPases. In addition to DnaA, this list includes a number of members of the so-called AAA ATPase family (7); the similarity between these proteins and DnaA has been noted before (4). Many of the AAA family proteins possess chaperone-like activity and, in particular, are involved in ATP-dependent proteolysis; examples include bacterial proteins ClpA, ClpB, ClpX, FtsH, and HslU; proteasome components; and yeast HSP78 (7). Members of the novel archaeal protein family could also perform chaperone-like functions. This is particularly plausible, because M. jannaschii does not encode several molecular chaperones that are ubiquitous and highly conserved in bacteria and eukaryotes—namely, members of the HSP70, HSP90, and HSP40 families. It remains to be seen how typical is this situation in archaea.

Finally, the family of putative ATPases contains a third strikingly conserved motif with two invariant histidines and one invariant cysteine (Fig. 1). Even though this motif did not show statistically significant similarity to any proteins in the database, this may be a specific metal-binding site, and some resemblance of the divalent cation-binding motif in bacterial FtsH proteins that are metal-dependent transcription regulators (8) could be detected (Fig. 1). Two observations seem relevant: (i) One of the chaperone ATPases, FtsH, contains a metal-binding motif conserved in its bacterial and eukaryotic homologs and is a Zn-dependent protease (9). (ii) Methanococcus jannaschii encodes at least two other putative ATPases, namely, the predicted proteins MJ0578 and MJ0579 that also contain a metal-binding site, in these cases a ferredoxin-like domain (10).

Thus, analysis of conserved motifs and application of additional methods for sequence database search yields specific functional predictions for archaeal proteins that initially appeared to comprise a unique family. There is little doubt that further exploration of the M. jannaschii genome sequence will bring more interesting findings.

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Aquaporins and Ion Conductance

A. J. Yool et al. studied membrane conductance in Xenopus laevis oocytes injected with aquaporin1 complementary RNA (AQP1 cRNA) and concluded that the conductance observed represented an intrinsic property of the protein (1). We have reevaluated our own studies of the AQP1 protein and have conducted new experiments; our results support our earlier conclusion that AQP1 transports water, but does not conduct ions (2).

As measured in our laboratory (2), oocytes injected with water (as a control) or with AQP1 cRNA exhibited similar, low conductances, while their permeabilities to water differed greatly [coefficient of osmotic water permeability (Pf) = 10 μm/s and ~200 μm/s, respectively]. Because we had not previously investigated effects of forskolin on membrane conductance, we used a two-electrode voltage clamp. Yool et al. (1) describe a rising scale of currents (measured in 100 mM NaCl, 2 mM KCl, 4.3 mM MgCl2, and 5 mM Hepes; pH 7.3) with water-injected oocytes rose 2.9 μA/V; AQP1 oocytes, 8.6 μA/V; and AQP1 oocytes with forskolin, 63 μA/V. In contrast, standard conditions (frog Ringers solution: 115 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 5 mM Hepes; pH 7.4), we did not see any significant difference in membrane conductance when water-injected oocytes (n = 7) or AQP1 oocytes (n = 20) were compared before or after forskolin treatment (<1 μA/V for all measurements).

We obtained similar results when measurements were conducted in 100 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 5 mM Hepes; pH 7.4, and when CaCl2 was omitted or when 0.5 mM 4,4'-diisothiocyanato-stilbene-2,2'-disulfonate (DIDS, Sigma, St. Louis, MO) was added to the solutions. Because AQP1 does not contain a classic cyclic AMP (cAMP)-dependent protein kinase A (PKA) phosphorylation consensus site, we performed experiments to evaluate oocytes that express AQP5 (which contains a classic PKA motif), but found that forskolin treatment also did not increase membrane currents of AQP5 oocytes (<1 μA/V in all measurements, n = 16).

The differences in membrane behaviors observed in our laboratories are not a result of the AQP1 cDNA, but of the construct used by Yool et al. We analyzed these oocytes in their laboratory using their technique, forskolin-induced ion currents were observed (3). The explanation for this discrepancy is unknown.

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