

# Primary Structure and Effect of pH on the Expression of the Plasma Membrane H<sup>+</sup>-ATPase from *Dunaliella acidophila* and *Dunaliella salina*<sup>1</sup>

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The plasma membrane H<sup>+</sup>-ATPase gene was cloned and sequenced from the extremely acidophilic green alga *Dunaliella acidophila* and from the extremely halotolerant *Dunaliella salina*. A special feature of the *Dunaliella* H<sup>+</sup>-ATPases is an extended C-terminal domain. The deduced amino acid sequences of the two proteins are 75% identical but differ in their C terminus. A hydrophilic loop within this domain in *D. salina*, which presumably faces the cell exterior, has a high ratio of acidic over basic amino acids, typical of halophilic proteins. The amount of the ATPase protein in plasma membranes and the level of its mRNA transcript in *D. acidophila* are far higher than in *D. salina*, suggesting that *D. acidophila* overexpresses the enzyme. A pH shift from 9.0 to 7.0 induces in *D. salina* a large increase in the level of the H<sup>+</sup>-ATPase mRNA and in the amount of the H<sup>+</sup>-ATPase protein. This suggests that the expression of the H<sup>+</sup>-ATPase in *D. salina* is pH-regulated at the transcriptional level. The implications of these findings are discussed with respect to the adaptive pressures imposed on these algal species by their exceptional environmental conditions.

The P-type H<sup>+</sup>-ATPases are the major primary ion pumps in the plasma membranes of plants and fungi. They provide the driving force for the uptake of nutrients and minerals, they are responsible for pH and volume regulation, and they play a role in signal transduction (Serrano, 1989; Briskin, 1990). It has been established that in higher plants PMHAs are encoded by a multigene family, the isoforms of which are expressed in different tissues and under specific conditions (Harper et al., 1989).

PMHAs from plants and fungi are made of a single catalytic 100-kD polypeptide. Sequence analysis reveals that approximately 20% of the protein is hydrophobic, comprising 8 to 10 putative TM domains, 4 to 6 of which are located in the C-terminal region. More than 70% of the enzyme is hydrophilic and faces the cytoplasmic surface. The large hydrophilic loop between TM domains 4 and 5 contains the conserved aspartyl phosphorylation and ATP-binding domains. The N- and C-terminal ends facing the cytoplasmic surface are the most divergent between the different isoforms of PMHA (reviewed by Serrano, 1989,

1990; Kasamo and Sakakibara, 1995). The C-terminal domain is believed to possess regulatory functions, since its deletion leads to activation of ATPase and proton-pumping activities and mimics activation by Glc in yeast or by fusicoccin and lysolecithin in higher plants (Serrano et al., 1992).

Little is known about the PMHAs from algae. Plasma membrane preparations that possess a vanadate-sensitive proton-pumping activity were demonstrated in only a few algal species (Sekler et al., 1991; Popova and Balnokin, 1992). Purification of the ATPase protein was conclusively demonstrated only in *Dunaliella acidophila* (Sekler and Pick, 1993). *Dunaliella*, a unicellular green alga, is a particularly attractive organism with which to study PMHA for several reasons. First, the species *D. acidophila*, which grows at extremely acidic pH, overproduces a potent PMHA that resembles in its catalytical and regulatory properties the higher plant PMHA (Sekler et al., 1991; Remis et al., 1992; Sekler and Pick, 1993; Sekler et al., 1994). The enzyme can be purified in a catalytically active state and thus provides an excellent model for the biochemical analysis of plant PMHA. Studies of plant PMHA are hampered by low abundance (Sussman, 1994), the existence of multiple isoforms (Palmgren and Christensen, 1994), and the instability of the purified enzyme. The second reason *D. acidophila* is a good species with which to study PMHA is that, when its cell membrane faces unusually low pH or high salt, it is expected that the hydrophilic parts of the enzyme that face the outer cell surface will exhibit unusual amino acid compositions. Therefore, PMHA from *Dunaliella* can provide an interesting model of protein structural adaptation.

Recently, a P-type ATPase gene was cloned from *Dunaliella bioculata*, which, on the basis of sequence similarity to ATPase genes from plants and fungi, was suggested to encode a PMHA (Wolf et al., 1995).

The purpose of this work was to clone and sequence the PMHA genes from *D. acidophila* and from *D. salina* and to determine whether the different adaptive capacities of these two species to extreme conditions are correlated with differences in the sequence and/or expression of the H<sup>+</sup>-ATPase gene.

Abbreviations: FITC, fluorescein isothiocyanate; pK, dissociation constant (negative log); PMHA, plasma membrane proton ATPase; TM, transmembrane; RACE, rapid amplification of cDNA ends.

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## MATERIALS AND METHODS

### Cell Cultures

*Dunaliella acidophila* (Kalina) Massjuk strain no. SAG 19.85 (from the algal collection of the Institute of Plant Physiology, University of Göttingen, Germany) was a generous gift from Dr. Schlösser and was cultured at pH 0.7 or 3.0 as previously described (Sekler et al., 1991). *Dunaliella salina* was obtained from the culture collection of Dr. W.H. Thomas (La Jolla, CA) and cultured in axenic culture as described previously (Ben-Amotz and Avron, 1973; Fisher et al., 1994).

### Plasma Membrane and ATPase Purification and Analysis

The PMHA from *D. acidophila* was purified, cleaved with endoprotease, and analyzed for the amino acid sequence of the proteolytic fragments, as described previously (Sekler et al., 1994).

Plasma membranes from *D. salina* cultured for 3 to 5 d under pH-controlled conditions (pH 7.0 or 9.0) were isolated as previously described (Weiss et al., 1991).

### SDS-PAGE and Western Blot Analysis

The polypeptide composition of plasma membranes (30–50  $\mu\text{g}$  of protein per lane) was analyzed on 10% (w/v) SDS-polyacrylamide gels (Laemmli, 1970). For western blot analysis, proteins were transferred to nitrocellulose membranes and reacted with rabbit polyclonal antisera prepared against synthetic peptides of  $\text{H}^+$ -ATPase. Peptides were synthesized on an automated peptide synthesizer (model 430B, Applied Biosystem) using solid-phase methods with Boc-amino acids. Cleavage of the peptide from the resin and removal of protecting groups were done with hydrofluoric acid. The peptides were conjugated to key-hole limpet hemocyanine through amide coupling using ethylene diaminopropyl carbodiimide. The blots were analyzed using the enhanced chemiluminescence technique as previously described (Fisher et al., 1994).

### Nucleic Acid Isolation and Synthesis

Total RNA was isolated with Tri Reagent (Molecular Research Center, Cincinnati, OH) according to the supplier's directions. The extracted total RNA was used for poly(A)<sup>+</sup> mRNA isolation or northern analysis without further purification. Poly(A)<sup>+</sup> mRNA was isolated using a PolyA Tract mRNA isolation kit (Promega). cDNA synthesis was carried out using a ZAP-cDNA synthesis kit (Stratagene). Oligonucleotide primers were synthesized using a DNA synthesizer (model 380B, Applied Biosystems) operated with a standard synthesis program and stored at  $-20^\circ\text{C}$ .

### PCR Amplification

PCR was carried out in a thermal cycler (MJ Research, Watertown, MA) using *Taq* polymerase (Beit-Haemek, Israel). All reactions were preheated for 5 min at  $95^\circ\text{C}$ . The temperature was then reduced to  $80^\circ\text{C}$  for 5 min while the

enzyme was added. The reactions were cycled 30 to 35 times for 1 min at  $95^\circ\text{C}$ , for 1 min at the annealing temperature (according to the primers used), and for 2 min at  $72^\circ\text{C}$ . PCR products were analyzed by electrophoresis on 1 to 1.5% agarose gels. Ethidium bromide-stained cDNA fragments were eluted by the GeneClean II kit (Bio 101, Vista, CA), ligated into the PGEM-T vector system (Promega), and used to transform JM109 *Escherichia coli* competent cells. Clones containing plasmids with inserts were isolated and plasmid DNA was prepared using the Wizard miniprep purification system (Promega). The 3'-end amplification of *D. acidophila* cDNA, using the 3'-RACE system of GIBCO-BRL, and the 5'-end amplification, using the 5'-AmpliFINDER RACE kit (Clontech, Palo Alto, CA), were performed essentially as described previously (Frohman, 1990).

Cloning of the *D. salina*  $\text{H}^+$ -ATPase gene was performed by screening a cDNA library prepared from poly(A)<sup>+</sup> mRNA in a  $\lambda$ Zap vector (Fisher et al., 1996) with a 600-bp DNA fragment obtained from the *D. acidophila*  $\text{H}^+$ -ATPase gene by PCR and labeled by the Random Primed DNA kit (Boehringer Mannheim).

DNA sequence analysis was performed by the dideoxy-sequencing method in a DNA sequencer (model 373A, Applied Biosystems). Sequences were analyzed using software from the Genetics Computer Group (Madison, WI).

### Southern Blot Analysis

Genomic DNA was prepared from *D. acidophila* cells as previously described (Lers et al., 1991). Ten-microgram samples of DNA were digested by restriction endonucleases, separated on 0.8% (w/v) agarose gels, and transferred to nitrocellulose filters. DNA probes were labeled using the Random Primed DNA kit (Boehringer Mannheim). Hybridization was performed at  $65^\circ\text{C}$  as previously described (Church and Gilbert, 1984).

### Northern Blot Analysis

Total RNA (20–30  $\mu\text{g}$ /lane) was analyzed on a 1% (w/v) formaldehyde agarose gel (Ausubel et al., 1995). DNA probe preparation and hybridization were as in Southern blot analysis.

## RESULTS

### Cloning the Plasma Membrane $\text{H}^+$ -ATPase Gene from *D. acidophila* and *D. salina*

We have previously reported the purification of an  $\text{H}^+$ -ATPase from the plasma membrane of *D. acidophila* (Sekler and Pick, 1993). Cleavage of the 105-kD ATPase polypeptide by the endoprotease V-8 yielded several proteolytic fragments, two of which (of 9 and 12 kD) were isolated and their N-terminal sequences were determined to be MLAV-LPMFDPPR and FAGPSGMV PANFSNP, respectively. The former sequence contains the DPPR motif, which is highly conserved in all P-type ATPases. The latter was essentially identical with a hydrophilic sequence in the C-terminal region of a putative  $\text{H}^+$ -ATPase gene recently cloned from

*Dunaliella bioculata* (Sekler et al., 1994; Wolf et al., 1995). A pair of degenerated oligonucleotide mixtures corresponding to PMFDPP (5' primer) and MVPANF (3' primer) were synthesized and utilized for PCR amplification of cDNA prepared from *D. acidophila* poly(A)<sup>+</sup> mRNA. A single 1.5-kb DNA fragment was reproducibly amplified by this procedure and was cloned and sequenced. The predicted amino acid sequence contained the exact N-terminal sequences of the 9- and 12-kD polypeptides and revealed a striking similarity to plant and fungal PMHA genes, indicating that it was a fragment of the *D. acidophila* PMHA gene. Sequencing of the complete ATPase gene from *D. acidophila* cDNA was carried out using 3'- and 5'-RACE PCR methods. The 3990-bp DNA sequence contained an open reading frame of 3309 bp, encoding a 1103-amino acid polypeptide and flanked by 177 bp on its 5' end and 496 bp on its 3' end attached to a poly(A) tail (Fig. 1).

The assignment of nucleotide 178 as the ATG starting codon was based on the fact that (a) it is the only in-frame ATG codon found upstream of the codons specifying the N-terminal sequence of the ATPase, and (b) the bases preceding the start codon (AnnATG) agree with the consensus of translation initiation in animals and plants (Lütcke et al., 1987).

The *D. salina* cDNA library was screened using a *D. acidophila* 600-bp DNA (nucleotides 1834–2426). It yielded four positive clones, which had identical 3'-end sequences, suggesting that they represent the same gene. The largest (4.8 kb) clone was sequenced and found to contain 4863 bp with an open reading frame of 3393 bp encoding a deduced protein of 1131 amino acids. Comparison of this open reading frame with the published sequence of the putative PMHA from *D. bioculata* showed more than 99.8% identity.

**Figure 1.** Nucleotide and deduced amino acid sequence of the *D. acidophila* plasma membrane H<sup>+</sup>-ATPase. Amino acid sequences matching two proteolytic fragments are underlined and in bold. The putative polyadenylation signal nucleotide sequence at the 3' end is also underlined.

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1 TCTAATTTTAAAGTGCCCTTAAACACTTTGATGCCCTAGTCCGCGCGCTGTGCATGCACCTAAGTTGGTAGTCAAGGCCATCGCTGGTT 90
91 TAGTTTGGCAGCCCGAGCTCTCGCTATTGCTCAAGCAGCTCTTGAAGGGCTGATCAGACCCCTGGAGCCGGCTCTGGCTCTAGAGGATG 180
181 AGTGGAAAGGAGAGGACCGAGGAAAATGGGGCCGTCAGCAGSACAGAAAGGAGCGTCAAGAAATCGGCCGACATGGAGATAAAGGC 270
S G K E R T E S N C A V K Q D T K E Q V K K S A D N G D R K G
271 GTGAGCAGAGTGGACTTGGCCAGAGTTGGCTGGAGATGCTCTCAAGATGCTGATTCGTCGAGCATGGCTCGAGCCCGCTGAAGCC 360
V D E V D F A K K I G L E D A F K Y L N C S E H G L S G A E A
361 GAAGCAGCGCTTAAACAGCAGCGGCTAATAGCTCCCTGACACTCCCGTAAACCAGTTCCTGGTAGCTTTGGGTACATGTGGAAACCC 450
E A R L K Q H G P N K L P D N S R N P V L V Y F G Y M W N P
451 TTGGCTTGGCCATGGAGCGGCTGATCATTCATTCATTCGCTTGGTGGATGGTGCAGACTTCGCGCTGATTTGGGCCCTGCTGATCATC 540
L A W A M E A A A I I A I A L V D D G A D F A L I V G L L I I
541 AATGCCACTATCAGTTTCTGTCGAGGAGGCAATGCTGATAAGGCTATCAAGGCTTCTGCAGCTGCCCTAGCACCCAAAGCCATGGCCCTA 630
N A T I S F V E E S N A D K K A I K A L S A A L A P K A M A L
631 CGAAATGGAGCATGGTGAACATGAGCCGTGGACTTTGGCCCGGGGATGTGATTTTGGATTCGGATCGTAAAGCTTTGGCCCGCGCAT 720
R N G A M V T D V F T I D A V T D L V F G D V F L R I G N V F A D
721 GTTAAATTTCTCGGACATGGCGGGATGACTATGAGACGCGCGCTGGACAGCTGCTGACAGGAGAGTCTTACAGGCC 810
V K L L P E H G A D D V E T P V Q I D Q A A L T C E S P A
811 AAGAATGTCACAGCAGCTGGCTTACGCGGTTCTACTGTCAAAACAGGAGAGGACAGCGTGTGTGTATGCCACCGGCTGAACACC 900
K K F T G N V A F S G S T V K Q G E R H A V V Y A T G V N T
901 TTCTTGGCCGCTGCTCGCTCATCATGAGGACTCACAACGTGAGCAATATTCAGCGTGTGATGAAACAGGATCGTGGCCCTTTGCTC 990
F P G R A A A L I S G T H N V A N I Q R V M N R I G L C L
991 ATCACCATTGAGATGGGCTGTCATGAAGTGCCTGCGAGTTTGCACATTAATAGCATTTCATGCTAGCTGGCAAGAGGGCTGCCCC 1080
I T I G V W V I E I E V P V Q P A H Y K H S C V A G K E G C P
1081 ACCCTACTCAACATGGTGTATTCATGCTGGTGGTGTATTCCTCATCGCCATGCCACTTGGCTGCTGAGTGGCTGGCCCTGGGAGCTTAC 1170
T L N W L V A L V G A H P T A H T V L S V T L A L G A
1171 AGCTTGCAGCTGAGGCTTATTTGACTCGGATGAGTCTGGAGAGATGGCAGGCTGGATCTGTATGCTCTGACAGAGCTGGA 1260
K L A R E G A C I A V T R M S A V E E M A G L D V L C S D K T G
1261 ACCTTGAACCTTAAACAAGTCAATCATGATGATGCAATGTTCCCTGTGGCAGATGGACATGCCAGAGGTTATGAAGTTCGGCCCT 1350
T L T L N K L S I D P S N V F P V G T M D I P E V M K F G A
1351 TTGCTGCCAATATCACTAGAGGAGCTCATGATGCTGTGGAGTACACCCTGAGCCGAGAAAATAAATCAGAGTACAAG 1440
L S A N I I T E E P I D M V L W E S Y P E R E K L K S E Y K
1441 CACACCAAGTACCTCCATCAACCCAAATGACAAGATCCACTCGCAACCGCTTGGAGATTGCCACTGGCCGCTGTTCGGAGTCCCTC 1530
H T K Y F P F N P N D K I T I A T V L E I A T G R V F R V L
1531 AAAGCTTCCCTCAGTGGTCTTGGCCAGGCAATGGATGGCCAGCCCTGGATGGCCCTGGAATGAAAGATAAAGAGTATGACAGCC 1620
K G F A I A G A G A G E K I A V N E I K E G
1621 AGAGCTTCCCTTCTTGGCCATTCGCTGGCAGGAGGGGATGGCAAGGACGGAACAAGTGGAGATGCTGGCCGCTGCTGCCATGTT 1710
R G F R S L G I A M A E G D G K D G T K W E M L A V L P M F
1711 GAGCCCTTCCGACGACACCAAGGAACCAATTGAGCGTGCATGAAGCAGGATATTCAGTCAAGATGGTCAAGCGGACCACTTGGT 1800
D P E R H D T K E T I E R C M K Q G I A V K M V T D H L L
1801 ATTGTAAAGAGATGCGAGATGCTGGCATGGTACTGGATGACCCAGTGGTGTGATCAAGCCCGCAATGGTATGGAG 1890
I G K E T A K M L G M G T E M Y P S E V L I K A R N G D V E
1891 GCGCCGCTGGTCAAAAACACTAGTGGCAATGGTGGAGCATGCAAGCCCTTGCACAGTCTTCCCTGAACAAGTTGAAATGTCA 1980
A P H G Y K N Y V A M V E A C N G F A Q V F P E H K F E I V
1981 GAAATCTGCA/ GAAGCCACCAGCTGTGGCATGACAGTGGAGCTGTAACGACCGCCCTGGCTCAAGAAGCCGATGTAGGTGTG 2070
E I L Q E A H R V G M T G D G V N D A P A L K K A H V G V
2071 GCTTGGCAGATGCCACAGTGGCCGCGAGGTCGCTGACATGCTCAGCCAGCCCTGGTCTATCAACCATTTGGACCGCTGCTATT 2160
A V D A T D A A R A B T A H T P V L S S T L V P A V I
2161 GCGCAGCAAGATCTTCAAGCAAGTCACTATGCAAGTACACACATTCGCTGACCTTCCGATGCGCTTACCTTGGGCTCCTC 2250
G A R K I F K R M T T Y A K Y T I S V T F R I A F T F G L L
2251 ACTGTGATCATGACGCTGATCTCCGACCATCTCATCGTCACTTGGCTGCTTAAATGATGGTCCATGATCGCCCTATCCAAGGAC 2340
T V I Y D W Y F P T I L I V I L A V F N D G A M I A L S X D
2341 CGTGTGGGCTCTGTGTGCTAGCACCTGGAACCTCGCCACCACTCTGACCGGCTTTTGTCTACGCAATGGCTGACTGCTCTCC 2430
R V V A S V L P S T W N L A T I F V P P G F V Y A M W L T L S
2431 TCCCTGGCAGTGTACAGTGGCCACATAGCAGCTCTTGAAGCGTGAACCCACTGCCATCACTCAACACCCAGCATGGGACTCTC 2520
S W A L Y Q V A T H S T F F R M T P L P S L N T Q H A T L
2521 ATATCTGGTGTGAGATGAGTCAAGCAGTGGGCTCATGCTCAGCTGCTGCGTGGCAGTATGCCATGCTGATGACGCTG 2610
T S H C E D E I S K L G V N P Q D S L C T V F S Y A D Q L
2611 ATGATGTAAGGCTCTGTGAGCTGAGCTCAAGGCTCCCTGGGCGCCACCAATTTGGATCAGTGGCTACTGAGCAGCGCTACAT 2700
N E C K G S V S L S S Q V P G V P T I L D Q C V T E Q R Y I
2701 CGAGATGCTTGCACAGTGGCTCAATACACCCACTCTGCTCTCGCCGCAAGCCGTTGTGTTGGTGGCAGCGCTGGCTTCTCT 2790
R D A L T R A L I Y T H L S V S G Q A V V F V V R T S G F S
2791 CTGAAGGAATGGCAGGCTCTCCACATGCTGCTTCTGCTCTTGGCCAGTGTGGCCACAATGTTGGCATCTTGGCTGGGAGGC 2880
L K E V A G V S T Y V A F A L A Q F G A T M F G I F G L G G
2881 TATACAGCCCGGACAGATTTTGCACACTGCCAGTCTCTGATTACTCACCATAATCGGCTGGCTCTTACTACTAGAGTGGAA 2970
Y N F Y F E D N C Q P C Q D V E T H N E V L F N S E V E
2971 CCTGCGCTGTACAGAACTGCTGACACTGCTTCTGATGGATGGGAGTATGATGATGCTGCTGGATCGGCTGGCTGGCTGGCT 3060
P R A G T E S V Y T A S V I G C G G Y V I V A W A L F
3061 TAGACTGCCGTGATCCCTGAAGTGGGCTTGTGATGGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 3150
Y T A L D P L K W G L M W I M N D D G F R D R H A W R K S N
3151 CAGCAGGCAATGAGCCGCTGAGCAGGAGCAGTGGACAACAAGAGTTCGCTGGCCCTCAGGCAATGGTGGTGGCAACTTCTCTAAC 3240
H E A M E R R S R E Q L D N K E F A G P S G M V P A N F S N
3241 CCTCTGGCCGCTCCATGCTCAAGCCGCTGCGCTTGGCTTGAACCGAAGTGTGCATCCTTGTGGCTATTAACCGCAGCTCCATG 3330
E L G R A S M S K P V S A L L D R K S A S L V A I N R S S M
3331 ACTGTGACGACCCCAACCATGCACTGAACTCGGACCGGCTCCATGATGGACCGCCCTCTGGTCTTGGGAGCAGCTCCCAAC 3420
T V S H D P N H A L N I G R R S M I G R P S G P L G R N S N
3421 ACCGGTCAAGCCACCCCTGACAGCTCTTCAAGTGGAGTCAAGCCGCTGCGCCACGAGGTTAAGAAAACCCATCAGTGGATGT 3510
T S G P K
3511 AAGGCACACTACTGTCAGAGGGATGTGGTACATGGCTGGTGTGCTGTGAGGAAAGTCTGGAGTGGCTATGTCATGGGCCC 3600
1601 TATTTCAAGTTTTCATGACAGCAAGCTGAGTGGTAAAGCTGGTGTCTGCTGCTGCTGGCCCTGGTTCCTGCTGCTTTTGGTGG 3690
1691 TAGACTTCAAAATGGCCCTGAGTGGTTTGGTGCACACTTGACATGATGATCAACATCGCAACTAAACTACATGATGATGATGATG 3780
1781 TGACTGCTTCTGACCACTTTTGGTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCT 3870
1871 CTGCTGCTCAAAATGCTGCTCTGCTGCTGCTTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 3960
1961 GTAAAGTCTTTTTCACAAAAAATAAATAA 3990
    
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Sequence Analysis

Alignment of the putative amino acid sequence of the ATPase genes from D. acidophila and D. salina with various P-type H+-ATPases revealed a high homology with PMHAs from higher plants and fungi, manifested in the localization of the TM I to TM VIII (Fig. 2; Table I). A particularly high homology was obtained in the two putative cytoplasmic loops that contained the six highly conserved amino acid motifs (darkened) common to all P-type ATPases and in the putative TM I, II, and IV. It is noteworthy that the part of the amino acids that was conserved between Dunaliella and plant genes was not conserved in fungi genes and vice versa

(Fig. 2, triangles and inverted triangles, respectively). The evolutionary position of Dunaliella, and possibly other algal PMHA genes, between fungi and the higher plants (Fig. 3) is consistent with these findings.

The largest divergence from plant and fungal PMHA genes was observed in the more extended C-terminal domain in both Dunaliella genes, which included two hydrophilic extended segments between TM VI to TM VII and TM VII to TM VIII (Wolf et al., 1995). A comparison of the D. acidophila and D. salina genes revealed that these C-terminal segments (IN-1, IN-2) also showed the lowest interspecies homology (less than 50% identity) compared

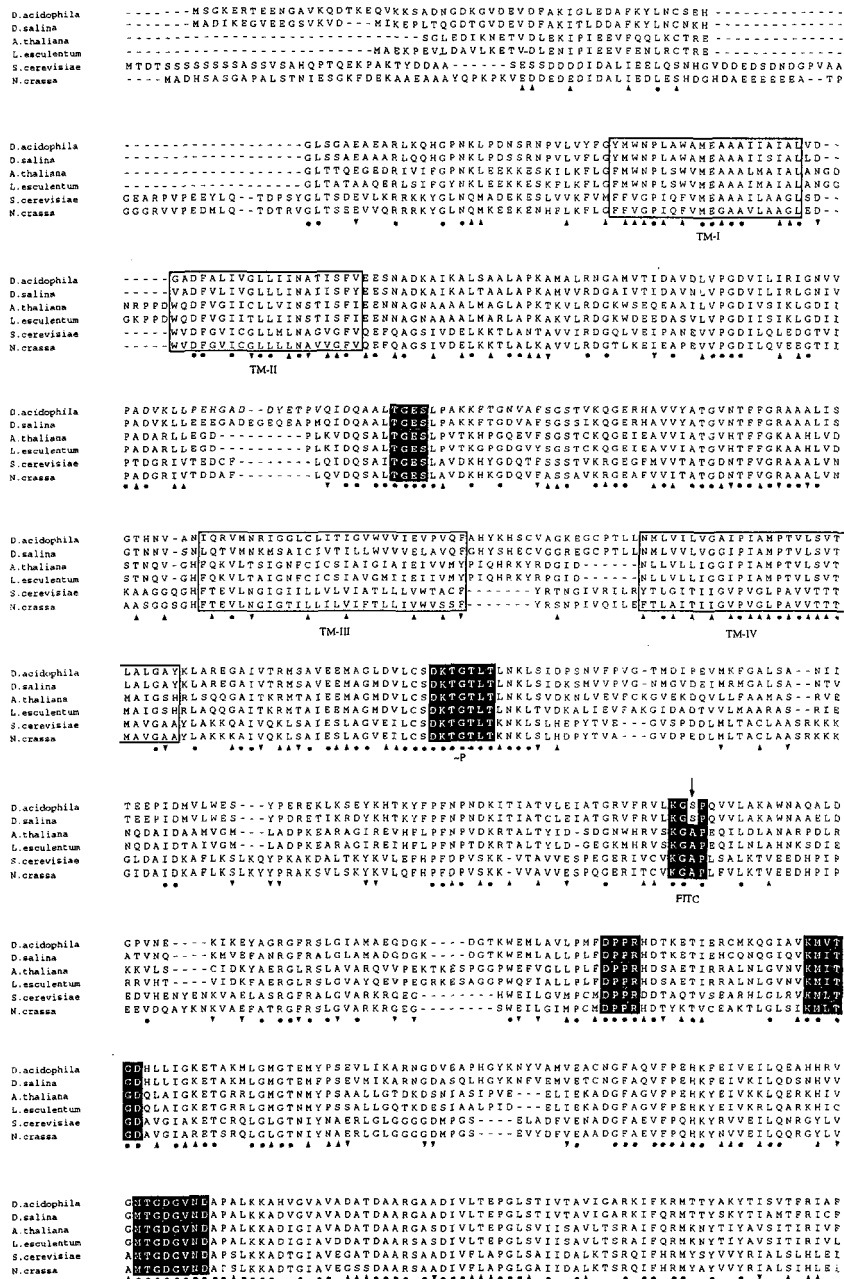
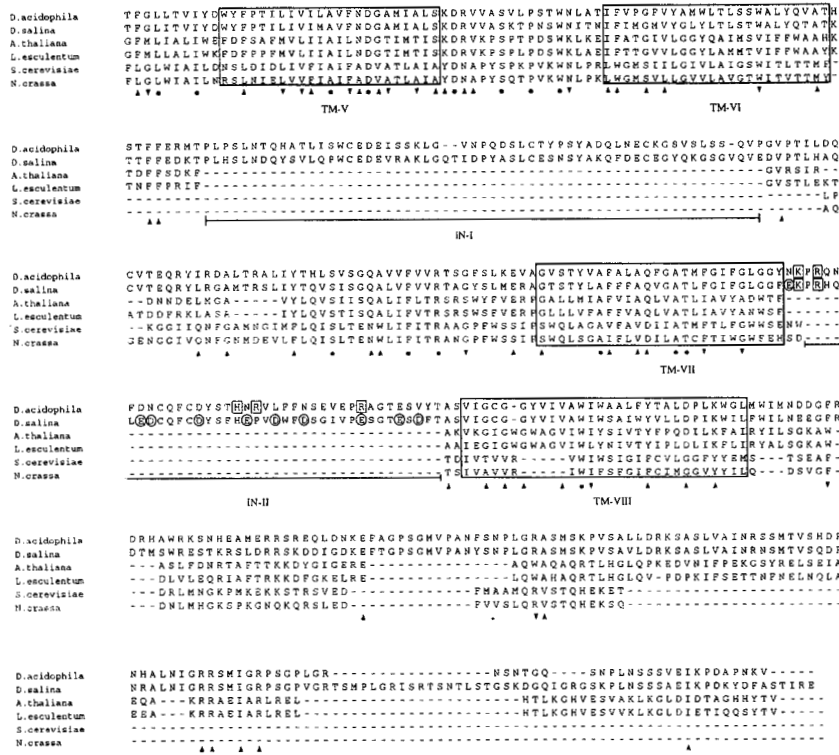


Figure 2. (Figure continues on following page.)



**Figure 2.** (Continued from previous page.) Alignment of *Dunaliella*, plant, and fungus plasma membrane H<sup>+</sup>-ATPases. Amino acid sequences are from *D. acidophila* (DaDHA1) and *D. salina* (DsDHA1), *Arabidopsis thaliana* (AtAHA2, Harper et al., 1989), tomato (*Lycopersicon esculentum*, LeLHA1, Ewing et al., 1990), *Saccharomyces cerevisiae* (ScPMA1, Serrano et al., 1986), and *Neurospora crassa* (NcPMA1, Hager et al., 1986). Conserved P-type ATPase motifs are shown in white on black-background blocks and putative TM segments are boxed. The arrow indicates the substitution in the FITC-binding site. IN-I and IN-II, Hydrophilic segments at the C-terminal part of *Dunaliella* ATPase genes that are absent in plant and fungus genes. Solid circles, Identical amino acids in *Dunaliella*, plants, and fungi. Solid inverted triangles, Identical amino acids in *Dunaliella* and plants but not in fungi. Solid inverted triangles, Identical amino acids in *Dunaliella* and fungi but not in plants. Circled and boxed amino acids, Negatively and positively charged amino acids, respectively, in IN-II (see text).

with more than 84% identity for the central cytoplasmic loops (L-1, L-2) and 75% identity for the N-terminal domain (Fig. 4). The possible relevance of the high variance in IN-2 is discussed below.

Another minor difference between the *Dunaliella* genes and plant and fungal H<sup>+</sup>-ATPase genes was the presence of a Ser instead of an Ala within the FITC-binding domain KG(A/S)P (Fig. 2, arrow), which is a highly conserved motif in most known P-type ATPases. In this respect it is interesting to note that the PMHA of *D. acidophila* was insensitive to inhibition by FITC (up to 100 μM at pH 9; I. Sekler, unpublished observations), suggesting that the Ala within the FITC motif may play a role in FITC binding.

**Reaction with Antibodies**

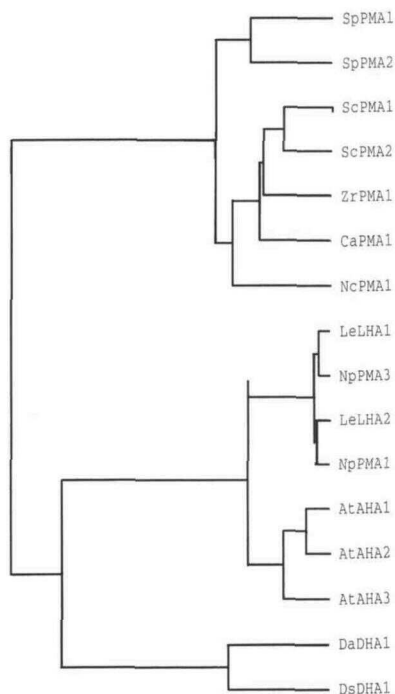
To conclusively verify the identification of the ATPase gene as a plasma membrane H<sup>+</sup>-ATPase, we prepared antibodies against synthetic peptides derived from putative sequences at the C- or N-terminal domains of the *D. acidophila* H<sup>+</sup>-ATPase, as well as against a central domain sequence conserved in the two species, and tested them against the purified protein. As shown in Figure 5, all three antibodies strongly cross-reacted with the previously char-

acterized 105-kD ATPase polypeptide (Sekler and Pick, 1993). These results indicate that the C- and N-terminal ends of the protein do not undergo proteolytic processing during the assembly into the plasma membrane.

To compare the level of expression of the ATPase protein in *D. salina* with that of *D. acidophila*, we tested the reactivity of the antibodies against the central domain with proteins in plasma membrane preparations. As demonstrated in Figure 6, the antibodies at this titer (1:30,000) cross-reacted strongly with the ATPase polypeptide only in *D. acidophila* plasma membranes. It should be noted that the

**Table I.** Sequence homologies between *D. acidophila* and other plasma membrane H<sup>+</sup>-ATPases

Organism	Identity
	%
<i>D. salina</i>	75
<i>A. thaliana</i>	43
<i>L. esculentum</i>	43
<i>S. cerevisiae</i>	37
<i>Schizosaccharomyces pombe</i>	35
<i>N. crassa</i>	35
<i>Leishmania donovani</i>	33

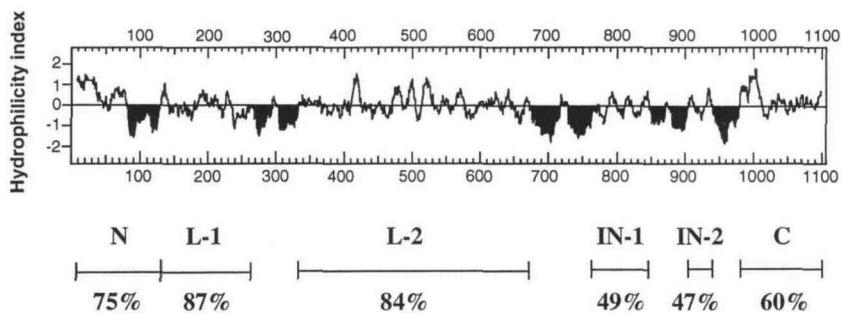


**Figure 3.** Graphical representation of relatedness of plasma membrane  $H^+$ -ATPases derived from amino acid sequences (Fig. 2; Table I). Sp, *S. pombe*; Sc, *S. cerevisiae*; Zr, *Zygosaccaromyces rouxii*; Ca, *Candida albicans*; Nc, *N. crassa*; Le, *L. esculentum*; Np, *Nicotiana plumbaginifolia*; At, *A. thaliana*; Da, *D. acidophila*; and Ds, *D. salina*.

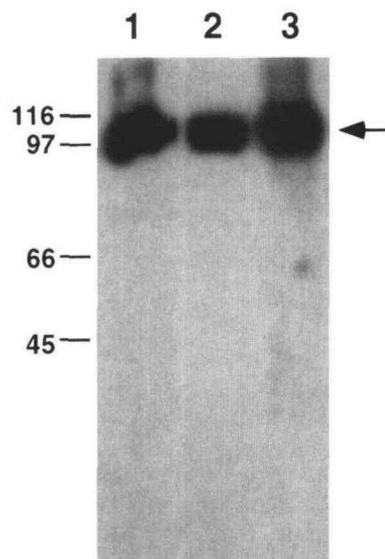
antibodies were raised against a 15-amino-acid sequence in the central domain of *D. salina* PMHA, which differs in one amino acid (L/V) from the gene of *D. acidophila*. Thus, the antibodies were expected to recognize the ATPase of *D. salina* at least as well as in *D. acidophila* plasma membranes. These results indicate that the level of expression of the protein in *D. acidophila* is far higher than that in *D. salina*.

#### Effect of pH on Expression

Since the unique characteristic of *D. acidophila* is its adaptation to extremely acidic pH, it seemed of interest to



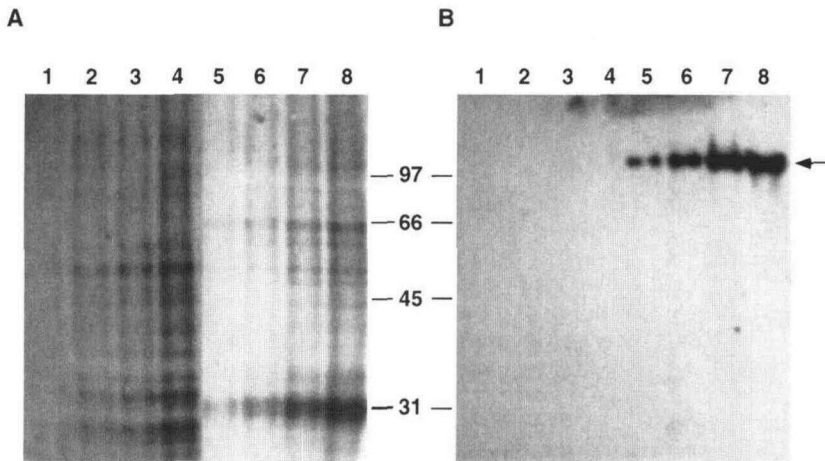
**Figure 4.** Hydrophilicity analysis (*D. acidophila*) and sequence identity of the different domains of the plasma membrane  $H^+$ -ATPase genes between *D. acidophila* and *D. salina*. N, N terminus, arbitrarily defined as the N to end of TM II; L-1 and L-2, central cytoplasmic loops; IN-1 and IN-2, C-terminal hydrophilic sequences between TM VI to TM VII and TM VII to TM VIII; C, C terminus, arbitrarily defined from the end of TM VIII to the end of the protein. Hydrophilicity was calculated according to the method of Hopp and Woods (1981).



**Figure 5.** Recognition of the  $H^+$ -ATPase protein in plasma membrane preparations of *D. acidophila* by antibodies raised against synthetic peptides of the N-terminal (lane 1), central (lane 2), and C-terminal (lane 3) domains. The synthetic peptide sequences are SGKERTEN-GAVKQ (N-terminal domain), EMLALLPLFDPPRHD (central domain) and CSVEIKPDAPNKV (C-terminal domain). The arrow denotes the 105-kDa protein.

check whether external pH has an effect on the expression of plasma membrane  $H^+$ -ATPase. The effect of external pH on the mRNA transcript level of the ATPase was determined by northern blot analysis of total RNA extracted from cells adapted to pH 0.7, 3.0, or 7.0 for several days. pH 0.7 and 3.0 are within the physiological growth range of this extreme acidophile (Gimmler and Weis, 1992). At pH 7.0 *D. acidophila* survived for several days but did not divide. As shown in Figure 7, the ATPase gene was transcribed at all three pH values (Fig. 7A), but its level was highest at pH 0.7, slightly lower at pH 3.0, and further decreased at pH 7.0 (Fig. 7B).

In contrast to the narrow pH range of *D. acidophila*, *D. salina* grows at a wider pH range, from 6.0 to 10.0. To test whether transcription of the ATPase in the latter is pH-



**Figure 6.** Comparison of levels of H<sup>+</sup>-ATPase protein expression in plasma membrane preparations from *D. salina* and *D. acidophila*. A, Coomassie blue-stained gels. B, Immunoblots reacted with antibodies against the central domain. Lanes 1, 2, 3, and 4, *D. salina* plasma membranes containing 3, 10, 20, and 30 μg of protein, respectively; lanes 5, 6, 7, and 8, *D. acidophila* plasma membranes containing 3, 10, 20, and 30 μg of protein, respectively.

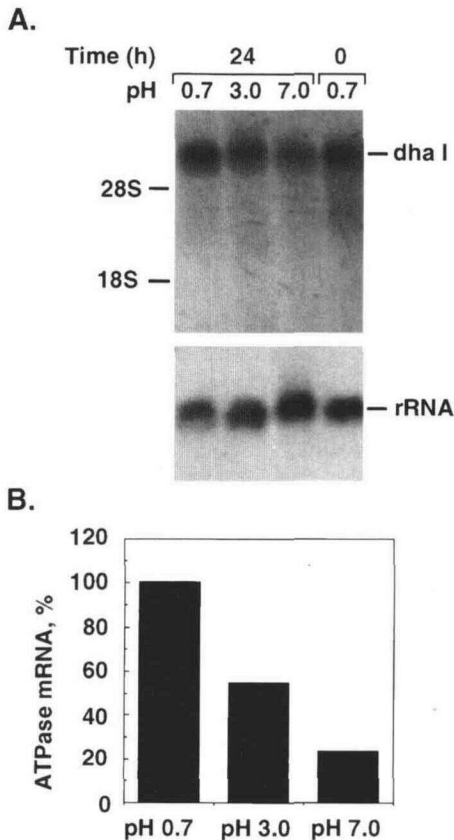
regulated, the mRNA transcript level was analyzed following a pH shift from 9.0 to 7.0. As shown in Figure 8, the transcript level in cells adapted to pH 9.0 was very low, greatly increased 12 h after the pH shift, and reached an intermediate level after about 48 h. To determine whether the ATPase protein level was also influenced by the pH of the growth medium, we probed plasma mem-

brane preparations from cells adapted to pH 9.0 or 7.0 with antibodies against the conserved central domain of the ATPase (titer of 1:2000) by western blot analysis (Fig. 9). The antibodies cross-reacted with a 108-kD polypeptide in the cell membranes adapted to pH 7.0, but the response was much weaker in membranes from pH 9.0 cells, indicating that the ATPase is preferentially expressed at the lower pH.

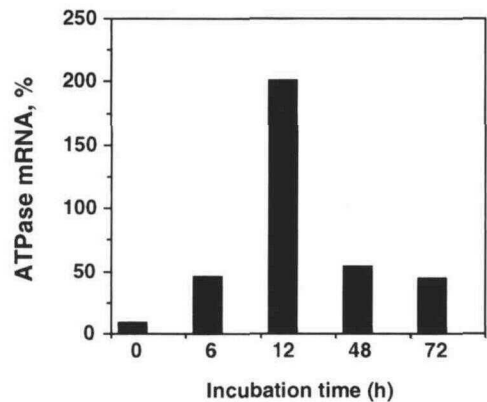
To test whether *D. acidophila* possesses more than one copy/isoform of the plasma membrane H<sup>+</sup>-ATPase, we probed the genomic DNA from the alga with a 600-bp probe coding for the central cytoplasmic domain by Southern blot analysis. The analysis showed only a single hybridizing fragment with different restriction enzymes (Fig. 10), consistent with a single copy of the ATPase gene.

**DISCUSSION**

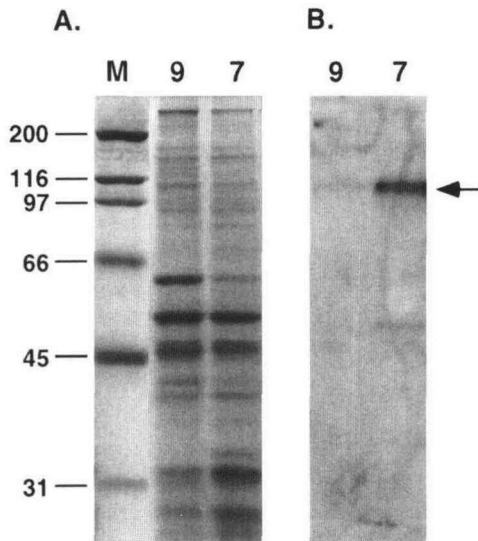
*Dunaliella* is the first alga for which a P-type H<sup>+</sup>-ATPase gene has been sequenced and, therefore, it is interesting to compare its sequence with that of ATPases from higher plants and fungi. The plasma membrane H<sup>+</sup>-ATPase gene



**Figure 7.** Effect of medium pH on *D. acidophila* H<sup>+</sup>-ATPase transcript levels. A, Total RNA (20 μg) derived from cells adapted to the indicated pH was analyzed with probes for the H<sup>+</sup>-ATPase (*dha 1*) and for rRNA (Lers et al., 1991). mRNA levels were determined by northern blot hybridization. B, Quantitative analysis of the H<sup>+</sup>-ATPase transcript level at different pH values with reference to rRNA.



**Figure 8.** Effect of a pH shift on transcription of *D. salina* H<sup>+</sup>-ATPase mRNA. *D. salina* cells were cultured for several days at pH 9.0 and then transferred to pH 7.0. RNA was extracted after the indicated incubation times at pH 7.0 and 30 μg of total RNA was applied per lane and analyzed by northern blot hybridization for mRNA transcript levels as in Figure 7.



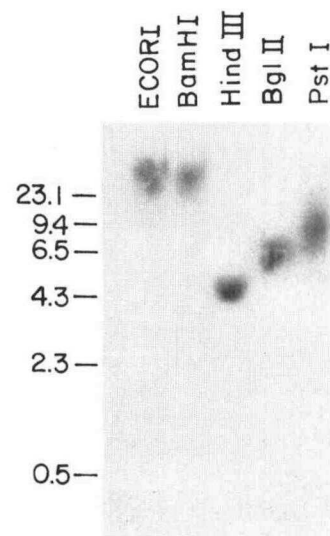
**Figure 9.** Effect of medium pH on expression of the  $H^+$ -ATPase protein in plasma membranes of *D. salina*. Purified plasma membrane protein (30  $\mu$ g) was separated by electrophoresis on 10% (w/v) SDS-polyacrylamide gels and either stained (A) or electroeluted to nitrocellulose paper (B) and reacted with polyclonal antibodies against the conserved central domain sequence (Fig. 5) at a dilution of 1:2000. M, Molecular weight markers; lanes 9 and 7, plasma membrane proteins of cells cultured at pH 9.0 or 7.0, respectively.

from *Dunaliella* is significantly larger than those in higher plants and fungi and, as noted above, the major difference in the protein structure is its more extended C-terminal domain (Wolf et al., 1995). Because of the absence of PMHA gene sequences from other algae, it is not known whether this is a general feature of algal enzymes or whether it is a unique structural characteristic of the *Dunaliella* PMHA.

A possible reason for the structural differences in the C-terminal domain of the enzyme in *Dunaliella* may be related to its regulatory roles. The *D. acidophila* enzyme resembles the plant PMHA in being activated by the fungus-derived toxin fusicoccin and in having a C-terminal autoinhibitory domain (Sekler et al., 1994, 1995). However, in contrast to the higher plant enzyme, *D. acidophila* PMHA is inhibited rather than activated by lysolecithin (I. Sekler, unpublished observations). Part of the structural differences in this domain may be related, therefore, to changes in the mode of regulation of the enzyme in *Dunaliella*.

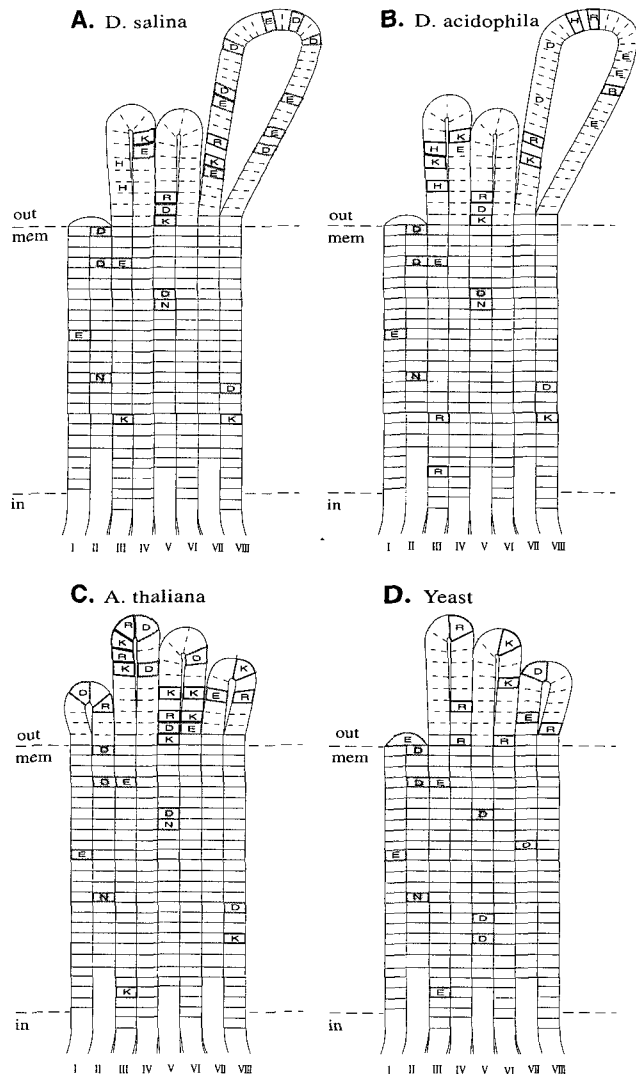
The presence of hydrophilic inserts in the sequence of the C-terminal domain in *Dunaliella* compared with PMHA genes of plant and fungi (Serrano, 1989, 1990; Kasamo and Sakakibara, 1995) may be related to adaptation to special environmental constraints. For example, the hydrophilic 40-amino-acid stretch between TM VII and TM VIII, which, according to the predicted model of the enzyme faces the cell exterior (Serrano, 1990), contains a 5:1 excess of acidic over basic amino acids in *D. salina* but not in *D. acidophila*. It may be noted that the spacing of charged amino acids in this stretch can be well accommodated for in an amphipathic  $\alpha$  helix, which may be oriented at the outer membrane surface (Fig. 11A). The abun-

dance of surface acidic amino acids and the introduction of extrinsic domains rich in carboxylates are characteristic of enzymes from extreme halophilic bacteria and are believed to stabilize protein structure in hypersaline environments by increasing their hydration shell (Dyn et al., 1995; Frolow et al., 1996); this was also found for two extrinsic salt-induced proteins in *Dunaliella* (Fisher et al., 1996; M. Weiss and U. Pick, unpublished observations). In *D. acidophila*, the same domain differs significantly in its amino acid composition, and at its physiological pH range of survival possesses a net charge of +5 (Fig. 2). The overall calculated net positive charge contributed by all putative extrinsic loops in *D. acidophila*  $H^+$ -ATPase is +11 (Fig. 11B). This is consistent with the net positive zeta potential of *D. acidophila* (Gimmler et al., 1991) and suggests that this major plasma membrane protein contributes to the overall positive surface charge of this organism. The fact that this domain is also the most variable region between the genes of the two *Dunaliella* species is consistent with the notion that it is associated with specific adaptations to the different living conditions of the two strains. The abundance of positive charge at the extrinsic domain may have two crucial consequences for survival at extremely acidic pH. First, the delocalized, positive surface charge can decrease the passive influx of protons into the cell and in this way protect the cells against cytoplasmic acidification. Second, the vicinity of surface positive charges to extrinsic membrane carboxyl groups may significantly lower their pK. It should be remembered that *D. acidophila*  $H^+$ -ATPase releases protons into a medium having pH values as low as 0. Thus, the proton-leaving group is expected to have a pK value of 0 or lower. This is several orders of magnitude lower than that of carboxyls in solution. We suggest, therefore, that the proton-leaving group of the *D. acidophila*  $H^+$ -



**Figure 10.** Genomic Southern blot analysis of *dha-1*. Total *D. acidophila* DNA was digested with the indicated restriction enzymes and the blot was analyzed by hybridization as described in "Materials and Methods." Numbers denote DNA molecular weight standards.





**Figure 11.** Putative model showing the distribution of charged amino acids in TM and extrinsic domains of plasma membrane (mem) H<sup>+</sup>-ATPases. A, *D. salina* (present study); B, *D. acidophila* (present study); C, *A. thaliana* (AtAHA2, Harper et al., 1989); D, *S. cerevisiae* (ScPMA1, Serrano et al., 1986). Negatively charged amino acids (including membrane asparagines) are circled, and the most highly conserved membrane groups are gray-shaded. Positively charged amino acids (including extrinsic His in *D. acidophila*) are boxed. TM-spanning domains are designated I to VIII.

ATPase is a carboxyl, located within the membrane close to its outer side and having an exceptionally low pK by virtue of its close proximity to positively charged extrinsic groups. Three to four conserved aspartate/glutamate residues exist in TM domains II, III, and V close to the outer surface (Fig. 11).

The extended hydrophilic cytoplasmic stretches at the C-terminal end and between TM VI and TM VII may have a role in the stabilization of the catalytic domains in the high-glycerol cytoplasmic environment characteristic of these halotolerant species (Ben-Amotz and Avron, 1973).

The following observations suggest that the PMHA in *D. acidophila* is overexpressed compared with *D. salina*: (a) a higher vanadate-sensitive ATPase and H<sup>+</sup>-pumping activity in plasma membrane preparations of *D. acidophila* (Sekler et al., 1991); (b) a far stronger cross-reaction in plasma membrane preparations with antibodies against a conserved domain in the central loop of the H<sup>+</sup>-ATPase (Fig. 6); and (c) a higher level of mRNA transcript of the ATPase gene of *D. acidophila* revealed by northern blot analysis. The observation that external pH has a small effect on the mRNA transcript level in *D. acidophila* suggests that the expression of the enzyme is largely constitutive in this species, probably as a means of ensuring a high capacity of H<sup>+</sup> extrusion from the cells and for maintenance of the large pH gradient across the plasma membrane.

In contrast, the expression of the H<sup>+</sup>-ATPase in *D. salina* appears to be strongly regulated by external pH, as indicated by the effect of pH on the protein and mRNA transcript levels. These results suggest that the enzyme is transcriptionally regulated by pH and probably plays a role in pH homeostasis.

The low level of expression of H<sup>+</sup>-ATPase in *D. salina*, especially at alkaline pH, is surprising in view of the fact that this is the major plasma membrane ATPase that serves as the primary active transport system in most nonmammalian eukaryotes. A possible reason for the low expression of the PMHA in *D. salina* is that an alternative system for pH homeostasis exists in this species. This possibility is consistent with the presence of a plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter in *D. salina* that is activated by cytoplasmic acidification (Katz et al., 1991). Thus, the antiporter apparently utilizes the large inwardly directed Na<sup>+</sup> gradient across the plasma membrane to drive the extrusion of protons upon internal acidification. It appears, therefore, that at alkaline pH the antiporter activity may be sufficient for keeping a cytoplasmic neutral pH, whereas at neutral or acidic pH, the H<sup>+</sup>-ATPase is also induced to assist in pH homeostasis.

The low level of expression of the PMHA in *D. salina* is at variance with previous demonstrations of a major vanadate-sensitive ATPase from plasma membrane preparations of *D. salina* (Weiss et al., 1991) and *D. bioculata* (Smahel et al., 1990), which were proposed to be H<sup>+</sup>-translocating ATPases. Both preparations, however, show characteristics not typical of PMHA: the *D. salina* enzyme was solubilized by a trypsin treatment, whereas the *D. bioculata* enzyme could be solubilized with Triton X-100, which does not solubilize the ATPase from *D. acidophila* (Sekler and Pick, 1993) or from plant plasma membranes (Vara and Serrano, 1982). Triton X-100 also solubilized two major vanadate-sensitive ATPase components from *D. salina* plasma membranes but not the protein that cross-reacts with antibodies against the conserved cytoplasmic domain of PMHA. Thus, it appears that the PMHA is a minor enzyme in *D. salina*, constituting about 2 to 10% of the total ATPase activity in the plasma membrane (U. Pick, unpublished observations). The major Triton-soluble enzymes may be a different vanadate-sensitive ATPase of an unknown nature.

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