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IDENTIFICATION AND DNA SEQUENCE OF A NEW H$^+$-ATPase IN THE UNICELLULAR GREEN ALGA *CHLAMYDOMONAS REINHARDTII* (CHLOROPHYCEAE)[1]

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Insertional mutagenesis was used to identify genes involved in mating and/or zygote formation in the unicellular green alga *Chlamydomonas reinhardtii* Dangeard. Approximately 800 insertionally mutagenized transformants were examined, and a single nonagglutinating mutant was identified. Plasmid rescue was used to clone a genomic fragment containing transforming DNA. This fragment was then used to identify the wild-type copy of the gene disrupted during mutagenesis. The wild-type gene is transcribed during all stages of the life cycle and, based on sequence similarity, encodes a P2-type proton transporting ATPase. The gene is referred to as *Pmh1* for plasma membrane H$^+$-ATPase. PMH1 displays the greatest sequence similarity to ATPases from two parasitic flagellates and a raphidophytic alga but not to the ATPase from a closely related green alga. We propose that PMH1 represents a distinct H$^+$-ATPase isoform expressed in flagellates.

***Key index words:* *Chlamydomonas*; DNA sequence; flagella; H$^+$-ATPase; mutagenesis**

The life cycle of the green alga *Chlamydomonas reinhardtii* has been described in the greatest molecular detail of any of the unicellular algae. Under nutrient-replete conditions, the haploid vegetative cells divide mitotically and are unable to mate. In response to nitrogen starvation (Sager and Granick 1954) and in the presence of light (Beck and Haring 1996), vegetative cells initiate gamete-specific gene expression (von Gromoff and Beck 1993, Kurvari et al. 1998) and reversibly differentiate into gametes of either mating-type plus (mt+) or mating-type minus (mt−). When gametes of the opposite mating type are mixed, they rapidly recognize one another and interact via recognition glycoproteins known as agglutinins, located on the surface of the flagella (Goodenough and Heuser 1999). This flagellar agglutination leads to a rise in cAMP levels (Pasquale and Goodenough 1987), a shedding of cell walls, activation of mating structures, and fusion of gametes to form diploid zygotes (reviewed in Goodenough et al. 1995). Within minutes of zygote formation, zygote-specific gene expression is initiated (Ferris and Goodenough 1987), required, in part, for formation of the zygote cell wall (Woessner and Goodenough 1989) and uniparental inheritance of chloroplast genomes (Armbrust et al. 1993, Armbrust 1998). After an obligate period of dormancy, during which time a wave of new gene expression occurs (Wegener and Beck 1991), and on return to favorable conditions, zygotes undergo meiosis and germination to form four haploid meiotic progeny that once again reproduce mitotically.

Differentiation from vegetative to gametic cells requires activation of two general categories of genes: those that are linked to the mating type locus and are expressed exclusively in gametes (Gillham 1969) and those that are unlinked to mating type but are also required for gamete-specific traits (e.g. Campbell et al. 1995, Goodenough et al. 1995, Vallon and Wollman 1995). This latter category of genes has been identified through mutational analysis, but none of these genes has been cloned and characterized at the molecular level. The goal of this study was to identify additional mutations that prevented mt− gamete formation and/or the ability of mt− gametes to fuse with mt+ gametes to form viable zygotes. The use of insertional mutagenesis resulted in the identification of a single mt− mutant that could not agglutinate with mt+ gametes. A null mutation was created in a gene encoding an H$^+$-transporting ATPase, normally expressed throughout the life cycle of cells of both mating types. Vegetative cells that carry this mutation appear unaffected by the loss of the ATPase. This ATPase is hypothesized to belong to a form of H$^+$-ATPases present in flagellated organisms.

MATERIALS AND METHODS

Strains and culture conditions. All products were purchased from Fisher Scientific (Atlanta, GA) unless stated otherwise. *Chlamydomonas reinhardtii* strains CC620 (mt+) and CC621 (mt−) were maintained in constant light on Tris-acetate-phosphate (TAP) (Gorman and Levine 1965) media solidified with

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1.5% agar; CC1618 (*cw15 arg7 mt−*) was maintained on TAP agar plates supplemented with 100 mg·L^{-1} arginine. Vegetative cells were grown with shaking in liquid TAP media under constant light. Gametes were generated by transferring cells maintained on TAP plates for at least 7 days (Martin and Goodenough 1975) to nitrogen-free high salt minimal media (N-free HSM) (Sueoka 1960) for 1–2 h. Zygotes were formed by mixing gametes of opposite mating types. The *pmh1* strain has been submitted to the Duke University *Chlamydomonas* Culture Collection and designated CC-3833. All strains used in this study can be obtained from the Duke University *Chlamydomonas* Culture Collection (Durham, NC).

Insertional mutagenesis and mutant screen. Insertional mutants were generated according to the methods of Tam and Lefebvre (1993, 1995). Briefly, CC1618 cells, which possess a defective cell wall, were transformed directly with circular pARG7.8 (Debuchy et al. 1989) using the glass bead technique (Kindle 1990) and plated onto TAP plates lacking arginine. Those colonies that grew on the selective plates were replica plated onto TAP plates and screened for mating deficiencies. Transformants were maintained on TAP plates for at least 7 days before resuspension in 100 μL N-free HSM for 1–2 h. Each transformant was mixed with 100 μL of wild-type CC620 gametes in individual wells of 96-well plates and allowed to mate overnight. The plate was then scanned visually to determine whether pellicle had formed, an indication of successful zygote formation (Harris 1989). Putative mutants that did not form pellicle when mated with wild-type CC620 gametes were screened a second time. A single mutant was isolated for further study.

Plasmid rescue. Total DNA was isolated from the mutant strain according to methods described by Weeks et al. (1986). Ten micrograms of DNA was restriction-digested with *Eco*RI and extracted with phenol/chloroform. The purified DNA was ligated overnight at 16°C in a final volume of 450 μL. The ligated DNA was ethanol precipitated and used to transform chemically competent *Escherichia coli* strain JM109 (New England Biolabs, Beverly, MA). Potential transformants were plated on Luria broth plates supplemented with 100 μg·mL^{-1} ampicillin (Sambrook et al. 1989).

Southern analysis. Plasmid DNA was isolated using the alkaline lysis miniprep method (Sambrook et al. 1989). Restriction-digested DNA was electrophoresed in 0.8% agarose gels and transferred to nitrocellulose (Schleicher & Schuell Inc., Keene, NH). ^{32}P-labeled probes (NEN Life Science Products, Inc., Boston, MA) were generated using the random prime labeling kit (Boehringer Mannheim, Roche Diagnostics Corporation, Indianapolis, IN). The blots were prehybridized and hybridized at 65°C according to the methods of Church and Gilbert (1984). The "pArg" probe was a 1.3-kb *Sal*I fragment from the cloned arginosuccinase gene (Debuchy et al. 1989); the "SK" probe was full-length pBluescript SK (Stratagene, La Jolla, CA) linearized with *Eco*RI.

Isolation of genomic clones. A 1-kb *Not*I/*Sal*I fragment from the rescued plasmid was random prime labeled with ^{32}P and used to isolate three overlapping phage clones (9-1J, 9-1H, and 10-1B) from a library containing partially *Sau*3AI-digested genomic DNA inserted into λEMBL3 (Stratagene), as described by Ferris (1989). Positive genomic clones were characterized by restriction enzyme mapping.

Northern analysis and isolation of partial cDNAs. Total RNA was isolated according to methods described by Kirk and Kirk (1985), electrophoresed through a formaldehyde/agarose gel, and transferred to nitrocellulose (Sambrook et al. 1989). The constitutively expressed 204 gene (Ferris and Goodenough 1987) was used as a probe to normalize RNA loading. A 2.2-kb *Sal* I fragment isolated from the genomic phage clone, 9-1J, was labeled with ^{32}P and used to probe the Northern blot. The same probe was used to screen a cDNA expression library in Uni-ZAPXR (Stratagene) prepared from 1-h zygotic RNA as described in Armbrust et al. (1993). The cDNA clones were characterized by restriction enzyme mapping.

DNA sequencing and analysis. Both single-stranded and double-stranded plasmid DNA was used for sequencing. Single-stranded DNA was generated (Sambrook et al. 1989) and used in dideoxy sequencing with the Sequenase kit (Amersham Pharmacia Biotech, Piscataway, NJ). Double-stranded plasmid DNA was isolated using the Qiagen (Valencia, CA) Mini Prep Kit and sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAq DNA polymerase (Applied Biosystems, Foster City, CA). DNA sequencing of the double-stranded plasmid DNA was performed on an Applied Biosystems 373A DNA sequencer. Sequence data were compiled and analyzed with the Wisconsin Package, version 10.0, of the Genetics Computer Group (Madison, WI). (Devereux et al. 1984). Intron locations within genomic sequence were predicted using the free GeneMark program (Lukashin and Borodovsky 1998). Homology between the predicted amino acid sequences and those present in the GenBank database was detected using BLAST 2 (Altschul et al. 1997). Multiple amino acid alignments were performed using the ClustalW 1.7 program (http://dot.imgen.bcm.tmc.edu: 9331/multi-align/multi-align.html.). Phylogenetic neighbor-joining trees were generated using the PAUPSEARCH feature of Genetics Computer Group. The *pmh1* cDNA sequence has been submitted to EMBL and given the accession number AJ300672.

RESULTS

An H+-ATPase null mutation was generated in a nonagglutinating mutant. Approximately 800 insertionally mutagenized mt− transformants were screened for their ability to successfully mate with wild-type mt+ gametes and form pellicle. A single nonmating mutant was identified. Microscopic examination of mutant cells indicated that they were able to swim and thus apparently possessed functional flagella, but nitrogen-limited mutant gametes were unable to agglutinate with either wild-type mt+ or mt− gametes. Rare quadriflagellated cells and gametes interacting via their mating structures, but not their flagella, were observed only if exogenous dibutyryl-cAMP (db-cAMP) was added to the mixture of mutant mt− and wild-type mt+ gametes. The addition of db-cAMP bypasses the need for the flagellar agglutination step in mating (Pasquale and Goodenough 1987). Mutant cells continued to swim normally when exposed to db-cAMP, which suggested that they were true gametes because vegetative cells retract their flagella in the presence of db-cAMP. No pellicle was formed after this treatment, however, and viable zygotes were not obtained. These data suggested that mutant cells were capable of undergoing gametogenesis but that zygote formation and/or germination were somehow prevented. This inability to generate viable zygotes meant that tetrad analysis could not be used to determine whether the mutant phenotype cosegregated with the inserted DNA.

Plasmid rescue was used to isolate directly the gene disrupted by the insertion event. A single genomic clone containing transforming pARG 7.8 DNA was plasmid rescued from mutant cells. A combination of restriction mapping and Southern blot analysis was used to determine that the genomic clone contained a fragment of *C. reinhardtii* genomic DNA flanking one side of inserted pARG7.8 sequence. The 1-kb *Sal*I/*Not*I genomic fragment closest to the site of insertion was used to probe a wild-type genomic library to isolate overlapping genomic DNA. A comparison of the

restriction maps of wild-type and mutant DNA indicated that the transforming DNA inserted near the *Sal*I site (Fig. 1A).

To determine whether this particular insertion event disrupted expression of a gene and thus could have created the nonagglutinating phenotype, a 2.2-kb *Sal* I wild-type genomic fragment that spanned the insertion site (Fig. 1A) was used to probe a Northern blot of total RNA isolated from different developmental stages of either wild-type or mutant cells. A single mRNA of 4400 nucleotides was observed in wild-type zygotes and in wild-type vegetative and gametic cells of both mating types. Steady-state levels of this mRNA were relatively high in wild-type vegetative cells and zygotes and at lower levels in wild-type gametes (Fig. 1B), suggesting that the protein encoded by this gene is likely required throughout the *C. reinhardtii* life cycle. This same mRNA was not observed in either the mutant vegetative or gametic cells (Fig. 1B), indicating that the insertion event generated a null mutation in this gene.

Disrupted gene encodes a P-type proton transporting ATPase. The 2.2-kb *Sal* I genomic fragment (Fig. 1A) was used to probe an early zygote cDNA library (Armbrust et al. 1993). The longest isolated cDNA that corresponded to the disrupted gene was only 2.2 kb, which was much shorter than the predicted 4400 nt mRNA. This partial cDNA was sequenced to completion and was found to consist of 1.2 kb of coding sequence and 1 kb of 3′ untranslated region. The predicted 400 amino acids encoded by the partial cDNA displayed 38% identity and 53% similarity to the carboxyl terminus of the proton transporting ATPase LDH1A from *Leishmania donovani* (Meade et al. 1987, 1989). This relatively high similarity at the carboxyl terminus was surprising because this region generally shows the greatest amount of divergence within the family of H^+-ATPases (e.g. Harper et al. 1989). Full-length amino acid sequence was obtained by sequencing the *Hind*III/*Bam*HI wild-type genomic fragment (Fig. 1A), which spans approximately 4.7 kb of genomic DNA upstream of the 5′-most end of the partial cDNA. The Gene Mark program (Lukashin and Borodovsky 1998) was used to identify the presence of introns within this genomic DNA. Nine introns were identified that ranged in size from a minimum of 60 base pair (bp) to a maximum of 288 bp. Only when the intron sequences were removed was an open reading frame of 3183 bp obtained that displayed the codon bias characteristic of *C. reinhardtii* (Silflow 1998). The first methionine was assumed to be the start codon of the open reading frame.

The predicted polypeptide (Fig. 2) is composed of 1061 amino acids with a molecular mass of 115 kD and an isoelectic point of 6.17. The polypeptide displays strong homology over its entire length to P-type plasma membrane proton transporting ATPases. In particular, the polypeptide is 47% identical and 59% similar to the entire amino acid sequence of the proton translocating P-type ATPase LDH1A from *L. donovani* (Meade et al. 1989) and 61% similar to a P-type ATPase from the unicellular alga, *Heterosigma akashiwo* (Wada et al. 1994). In addition, similar to other H^+-ATPases (Lutsenko and Kaplan 1995), a signal peptide sequence was absent and the N-terminus was highly charged. Ten putative transmembrane domains were identified (Fig. 2), which is consistent with experimental data for P-type ATPases (Auer et al. 1998, Campbell et al. 1992). The *C. reinhardtii* polypeptide also displayed four highly conserved domains observed in other ATPases: The sequence DKTGTLT, which contains the aspartate phosphorylated during ATP hydrolysis, is the defining motif for P-type ATPases (Swiss Institute for Bioinformatics 2001); the sequence TGES; the sequence TGDN, which is the putative ATP-binding domain; and the sequence MTGDGVNDAP, which corresponds to the MXGDGXNDXP (where X refers to any amino acid) motif that connects the ATP-binding domain to transmembrane segments involved in ion binding and translocation (Lutsenko and Kaplan 1995). The locations of these conserved domains relative to putative transmembrane domains indicated that the *C. reinhardtii* ATPase is a non-heavy metal-transporting P2-type ATPase (Maeda et al. 1998). Based on strong homology to other H^+-ATPases, the disrupted *C. reinhardtii* gene is referred to as *Pmh1* for plasma membrane H^+-ATPase. The subcellular location for PMH1 is unknown, but by convention plasma membrane is assumed in the absence of other evidence.

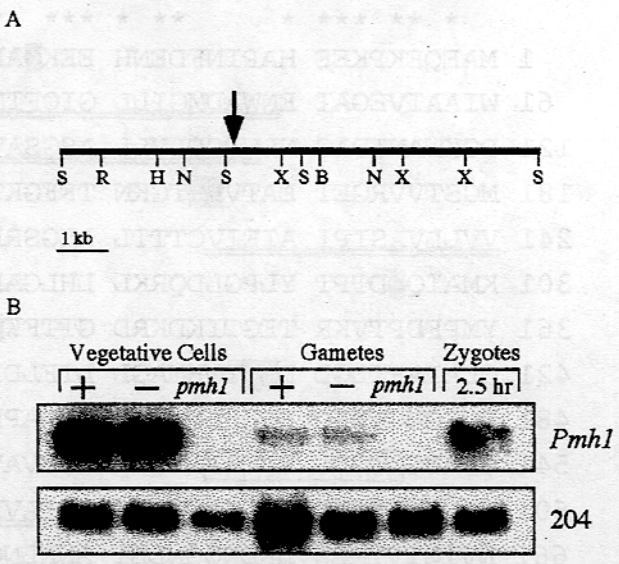

FIG. 1. (A) Restriction map of wild-type genomic DNA phage clone 9-1J showing the site of insertion of the transforming DNA (arrow). S, *Sal*I; R, *Eco*RI; H, *Hind*III; X, *Xho*I: B, *Bam*HI; N, *Not*I. (B) Northern blot analysis of steady-state mRNA levels of either *Pmh1* or the constitutively expressed 204 gene in wild-type or mutant vegetative cells, wild-type or mutant gametes, and wild-type zygotes. The wild-type 2.2-kb *Sal*I fragment that spans the site of insertion (A) was used to probe for *Pmh1* expression.

PMH1 displays domains shared by flagellated protists. An analysis of the phylogenetic relationship between PMH1

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      * ** *** *     ** * ***  **  *  * * * *** *       *
    1 MAEQEKPKEE HAPINFDENH EEKRAEELIK VHGRNELEEK HTPSWLIFLR QIYQPMPIMI
   61 WIAAIVEGAI ENWADMGILL GIQFINATLR LVGQAFETTK AGDAVAALKA SLKPLATAKR
  121 DGKWANIDAG NLVPGDLVLL ASGSAVPADC LINHGTVDID QAALTGESLP VTMHKGDSAK
  181 MGSTVVRGET EATVFVTGKN TFFGKTASML QQSGGELGHL QKILLTIMFV LVVTSFIFFT
  241 VVLLVASIPI AIEIVCTTTL ALGSRELSRH GAIVTRLAAI EDMAGMNMLC SDKTGTLTLN
  301 KMAIQFDTPT YLPGLDQRKL LHLGALAAKW HEPPRDALDT LVLTCETQDL SALDVYEQID
  361 YMPFDPTVKR TEGTIKDKRD GTTFKITKGA PHIILKLTHD ERIHHMVDET VAAFGQRGIR
  421 CLAIARTLGD DKNTWHMAGL LTFLDPPRPD TKDTIHKVMA YGVDVKMITG DNILIAKETA
  481 RVLGMGTNIQ DPKSLPTMDA EGKAPKDLGK KYGKIIMEAD GFAQVFPEHK YLIVEALRQN
  541 GFACGMTGDG VNDAPALKRA DVGVAVQGAT APLAPPPTIV LTEPGLSTIV HGIVTARCIF
  601 QRMKNFINYR IAATLQLLTF FFIAVFALPP IDYPQGMWPT CNTPAAVGSP TCCPETYTYD
  661 NVTSTVTMEW LRDNQGNDEI ANNLNGVCFT DGEPWPDFFK MPVLMLMLIT LLNDGTLISI
  721 GYDHVKPSAM PEKWNLPALF AISIVLGMVA CGSSLLLLWA ALDSWNTNGI FQKWGLGGMP
  781 YGKVTTIIYL KVSVSDFLTL FSARTHDGFF WSARPSPILM GAALLALSLS TILACVWPKG
  841 HTDKQLSMGL AYETDPHSNT LMPLWIWIYC VFWWFVQDFM KVAAYWMMHR YNWFDINTSM
  901 AINKRDANKV DDRHDPLARG SVGLVEGKLL AAKVEEAQAK VNAAIKHDQA TNLGRASANX
  961 GRVSANLKQA GMARHSGNPK GADVEGAAQT VENVLLHLDE ARGELDPKVQ QEIAPAIEGV
 1021 REAAEKLAAN TAAALGGANP EQTLAKISSK RHM
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FIG. 2. Deduced amino acid sequence of PMH1. Charged amino acids D, E, K, R, and H at the N-terminus are indicated by an asterisk. The P-type ATPase-defining motif (bold box) DKTGTLT is located at amino acid positions 292–298. Ten predicted transmembrane domains are double underlined, and three additional domains conserved in ATPases are boxed. Transmembrane regions 3 and 4 are contiguous, as are those for regions 7 and 8. The known junctions between adjacent exons are denoted by gray shading on the appropriate amino acids.

and other H+-ATPases (Fig. 3) indicated that PMH1 formed a distinct cluster with H+-ATPases from two parasitic protists, *L. donovani* (Meade et al. 1987, 1989) and *Trypanosoma cruzi* (Genbank accession number AF000161), as well as the raphidophytic alga *H. akashiwo* (Wada et al. 1994). Comparison of the amino acid sequences of four highly conserved transmembrane domains (domains 4, 6, 7, and 8; Fig. 4A) predicted to be critical for ion transport (Clarke et al. 1989, Auer et al. 1998, Zhang et al. 1998) indicated that the highest level of sequence identity in these regions was shared by these four protists (Fig. 4B). The *C. reinhardtii* PMH1 contained an insertion of 57 amino acids between transmembrane domains 5 and 6 relative to other known H+-ATPases (Fig. 4A); the *L. donovani* and *T. cruzi* ATPases both contain an insertion of 13 amino acids and the *H. akashiwo* ATPase contains an insertion of 11 amino acids in this same region. Interestingly, the *C. reinhardtii* PMH1 does not group with H+-ATPases isolated from two other chlorophytes (Fig. 3), *Dunaliella biculata* (Wolf et al. 1995) and *D. acidophla* (Weiss and Pick 1996). Thus, it appears likely that the group of ATPases defined by the *C. reinhardtii* clade represents an H+-ATPase distinct from the more commonly observed enzyme.

DISCUSSION

The *Pmh1* gene was uncovered in a mutational screen designed to identify genes necessary for *C. reinhardtii* mating and/or zygote formation. Based on amino acid sequence similarity, *Pmh1* is hypothesized to encode a P2-type ATPase expressed throughout the *C. reinhardtii* life cycle. All P-type ATPases use the energy released during ATP hydrolysis to transport cations against their concentration gradient (MacLennan et al. 1997); P2-type ATPases specifically transport protons. Norling et al. (1996) previously described a proton-transporting ATPase in *C. reinhardtii*. They used cell fractionation techniques to show that vanadate-sensitive ATPase activity and cross-reactivity to antibodies generated against an *Arabidopsis* H+-ATPase copurified with the plasma membrane fraction. The antibodies used in their study were raised against a peptide consisting of the highly conserved amino acids 6–51 in the *Arabidopsis* H+-ATPase, AHA3 (Ramon

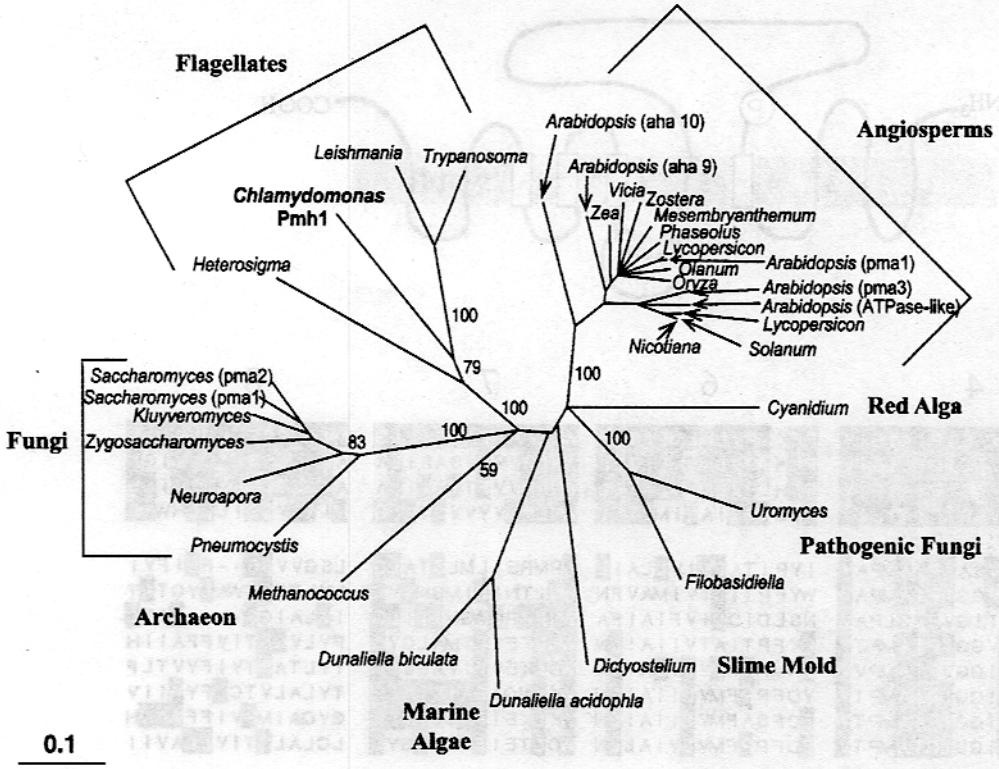

FIG. 3. Unrooted neighbor-joining tree of P-type ATPases from a variety of organisms. The percent of 1000 bootstrap replicates that support the branching order is shown near major nodes. The scale bar indicates 10% differences in amino acid sequence.

Serrano, Universidad Politechnica de Valencia, CSIC, personal communication). This same amino acid sequence is not present in PMH1, indicating that we have identified a second proton-transporting ATPase in *C. reinhardtii*. The redundancy of H^+-ATPases in *C. reinhardtii* likely explains why *pmh1* vegetative cells did not display obvious mutant phenotypes.

Possession of at least two P2-type ATPases is not uncommon for eukaryotes. What is intriguing about the newly identified *C. reinhardtii* PMH1 is that it displays limited sequence similarity to most ATPases, including those from closely related *Dunaliella* spp. (Wolf et al. 1995, Weiss and Pick 1996). Instead, PMH1 forms a distinct cluster with H^+-ATPases from the parasitic protists, *Leishmania* and *Trypanosoma* (Meade et al. 1987, 1989), and the unicellular alga *Heterosigma* (Wada et al. 1994). The only obvious characteristic shared by these four distantly related protists (Sogin and Silberman 1998) is that each is flagellated.

Both trypanosomes and *Heterosigma* appear to possess an H^+-ATPase that localizes to intracellular membranes in addition to the more commonly observed location of the plasma membrane. In *Heterosigma*, immunolocalization experiments localized an H^+-ATPase to distinct intracellular compartments, possibly ER-Golgi and related vesicles (Wada et al. 1994). In *T. cruzi*, cell fractionation studies indicated that a P-type H^+-ATPase acidified intracellular compartments (Scott and Docampo 1998). Taken together, this evidence suggests that PMH1 may be a member of a specialized group of H^+-ATPases, possibly specific to flagellates.

In contrast, the *C. reinhardtii* H^+-ATPase identified by Norling et al. (1996) likely represents the more ubiquitous form of plasma membrane-localized H^+-ATPases observed in a wide variety of plants and algae. Based on unique regions in the DNA sequences of *pmh1*-like ATPases, it should now be possible to develop clade-specific probes to identify further members of this family of H^+-ATPases.

In this study, we did not determine empirically whether PMH1 was able to function as a H^+-ATPase; therefore, it is formally possible that PMH1 may not function as an H^+-ATPase. It is unclear why the loss of PMH1 would result in the inability of flagellar agglutination. Perhaps PHM1 affects the pH of an internal compartment that plays a role in agglutinin posttranslational modification (e.g. glycosylation or proteolysis) or targeting to the flagellar membranes. Nevertheless, this is the first P2 ATPase cloned and sequenced from *Chlamydomonas*, and its sequence should prove useful in identifying other P-type ATPases in this model organism.

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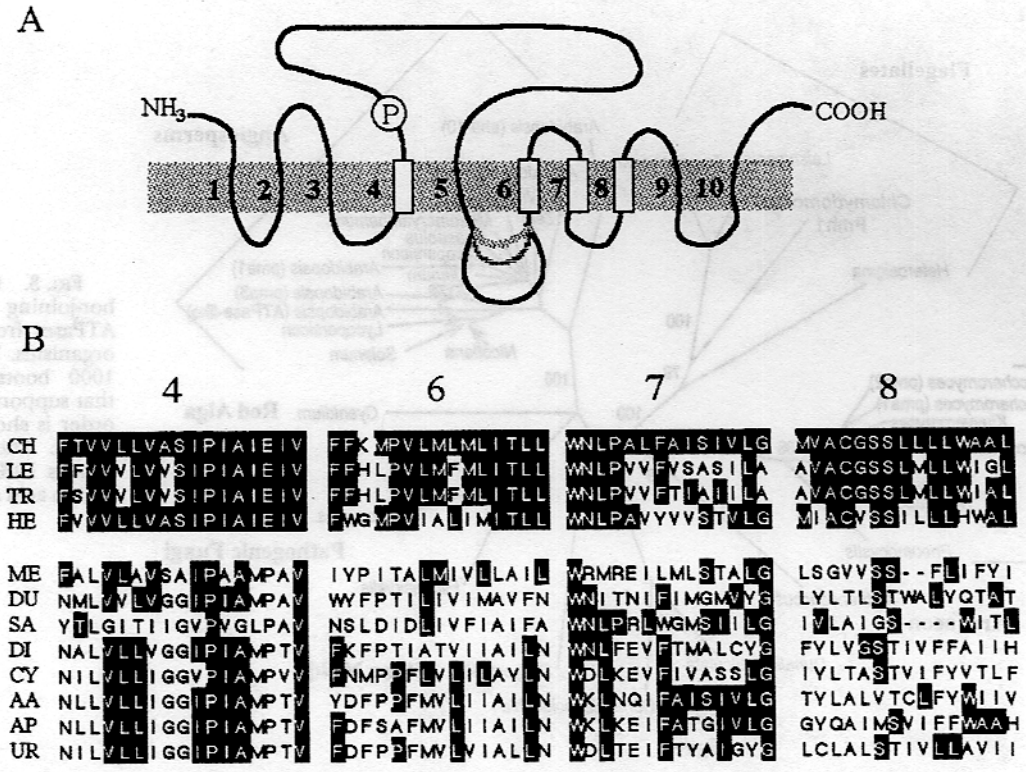

FIG. 4. Comparison of the overall structure and a portion of the amino acid sequence of P-type ATPases. (A) Schematic of the predicted PMH1 membrane spanning domains. The four boxed transmembrane domains are predicted to be involved in ion transport. The circled P indicates the location of the aspartate phosphorylated during ATP hydrolysis. The region between domains 5 and 6 indicate the location of the amino acid insertions in the *Chlamydomonas reinhardtii* (black line), *Trypanosoma cruzi* and *Leishmania donovani* (stippled line), and *Heterosigma akashiwo* (dotted line) ATPase. (B) Comparison of the amino acid sequence of transmembrane domains 4, 6, 7, and 8 from representative organisms from the phylogenetic tree shown in Figure 3. Identical amino acids are highlighted in black. Alignment gaps are indicated by dashes. CH, *Chlamydomonas reinhardtii*; LE, *Leishmania donovani*; TR, *Trypanosoma cruzi*; HE, *Heterosigma akashiwo*; ME, *Methanococcus jannaschii*; DU, *Dunaliella biculata*; SA, *Saccharomyces cerevisiae*; DI, *Dictyostelium discoideum*; CY, *Cyanidium caldarium*; AA, *Arabidopsis thaliana* AHA10; AP, *Arabidopsis thaliana* PMA1; UR, *Uromyces fabae*.

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