THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 37, pp. 27052–27062, September 15, 2006

Analysis of Strains with Mutations in Six Genes Encoding Subunits of the V-ATPase

*EUKARYOTES DIFFER IN THE COMPOSITION OF THE V_0 SECTOR OF THE ENZYME**

Received for publication, April 24, 2006, and in revised form, June 26, 2006 Published, JBC Papers in Press, July 20, 2006, DOI 10.1074/jbc.M603883200

Christopher Chavez, Emma Jean Bowman, Jack C. Reidling, Kimberly H. Haw, and Barry J. Bowman[1]

From the Department of Molecular, Cell, and Developmental Biology, Sinsheimer Laboratories, University of California, Santa Cruz, California 95064

To address questions about the structure of the vacuolar ATPase, we have generated mutant strains of *Neurospora crassa* defective in six subunits, C, H, a, c, c′, and c″. Except for strains lacking subunit c′, the mutant strains were indistinguishable from each other in most phenotypic characteristics. They did not accumulate arginine in the vacuoles, grew poorly at pH 5.8 with altered morphology, and failed to grow at alkaline pH. Consistent with findings from *Saccharomyces cerevisiae*, the data indicate that subunits C and H are essential for generation of a functional enzyme. Unlike *S. cerevisiae*, *N. crassa* has a single isoform of the a subunit. Analysis of other fungal genomes indicates that only the budding yeasts have a two-gene family for subunit a. It has been unclear whether subunit c′, a small proteolipid, is a component of all V-ATPases. Our data suggest that this subunit is present in all fungi, but not in other organisms. Mutation or deletion of the *N. crassa* gene encoding subunit c′ did not completely eliminate V-ATPase function. Unlike other V-ATPase null strains, they grew, although slowly, at alkaline pH, were able to form conidia (asexual spores), and were inhibited by concanamycin, a specific inhibitor of the V-ATPase. The phenotypic character in which strains differed was the ability to go through the sexual cycle to generate mature spores and viable mutant progeny. Strains lacking the integral membrane subunits a, c, c′, and c″ had more severe defects than strains lacking subunits C or H.

Vacuolar ATPases (V-ATPases)[2] are ATP-driven proton pumps that acidify intracellular compartments in eukaryotic cells, including the vacuoles of fungi and plants, lysosomes, endosomes, the Golgi, and secretory vesicles. Acidification of these organelles is important for numerous cellular processes, including receptor-mediated endocytosis, zymogen activation, intracellular trafficking, protein degradation, coupled transport of small molecules, and viral entry (1–3). V-ATPases are also located at the plasma membrane in a subset of cells in which they function in processes such as renal acidification, bone resorption, and pH homeostasis (4–6).

Much of what is known about V-ATPases comes from studies of fungal and bovine enzymes. The V-ATPase is composed of two sectors that form a ball and stalk structure (7, 8). The V_1 sector protrudes above the surface of the membrane. It has a molecular mass of ~570 kDa and is composed of eight types of subunits, A through H. ATP hydrolysis occurs at the interface between the A and B subunits. The ~260-kDa V_0 sector of the enzyme, embedded in the membrane, is composed of five or six types of subunits, ac(c′)c″de (1, 2). The V-ATPases are closely related to the F_1F_0-ATP synthases (F-ATPases) of bacteria, mitochondria, and chloroplasts, which synthesize ATP (9). The composition and stoichiometry of the F_1F_o-ATP synthase from *Escherichia coli* is $\alpha_3\beta_3\gamma\delta\epsilon$ for the F_1 and b_2ac_{10} for the F_0; the F_0 contains a ring of 10 identical c subunits that contain the proton-binding site (10, 11). Protons move across the membrane as the c subunits rotate past the single a subunit (12–14).

In several ways the V-ATPase has a more complex structure than the F-ATPase. It is significantly larger and has subunits (*e.g.* C, H and d) with no obvious counterparts in the F-ATPase (1, 2). However, genetic experiments with *Saccharomyces cerevisiae* have provided strong evidence that these subunits are essential components of the V-ATPase (15–17). The integral membrane part of the rotor is composed of two or three different types of proteolipids (the c, c′, and c″ subunits). The a subunit, which interacts with the proteolipids, is nearly 4-fold larger than its counterpart in the F-ATPase. Characterization of *S. cerevisiae* strains that lack each of the 14 subunits has provided strong evidence that every subunit is essential for the synthesis of a functional V-ATPase (1). Such a comprehensive study of V-ATPase mutants has not been possible in metazoans because the loss of the V-ATPase causes embryonic lethality (18–20).

Strains with a null mutation in a V-ATPase gene are viable in the filamentous fungus *Neurospora crassa.* Loss of the V-ATPase gives rise to a distinctive morphological change in the hyphae, loss of vacuolar function, and defects in development (21). In this report we have used this phenotypic complexity to ask whether strains of *N. crassa* lacking different V-ATPase subunits have identical phenotypes and to address specific questions concerning the subunit composition of the V-ATPase.

Subunits C and H have sometimes been viewed as accessory subunits that are not part of the core catalytic mechanism (22–25). Subunit H interacts with other proteins such as Nef in human immunodeficiency virus, type 1 (26) and yeast ectoapyrase (27),

* This work was supported in part by National Institutes of Health Grants GM28703 and GM58903. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed. Tel.: 831-459-2245; E-mail: bowman@biology.ucsc.edu.

[2] The abbreviations used are: V-ATPase, vacuolar ATPase; F-ATPase, F_1F_0-ATP synthase; RIP, repeat-induced point mutation.

perhaps facilitating the association of the V-ATPase with other enzymes or membranes. Subunit C appears to readily dissociate from the V-ATPase. Purified preparations of the enzyme from *N. crassa* were initially reported to lack this subunit (28). Here we ask, do strains lacking the C and H subunits have a typical V-ATPase null phenotype?

In *S. cerevisiae*, *vph1* mutants (*i.e.* lacking subunit a) are able to grow, although slowly, in medium that has high calcium or alkaline pH, conditions that completely prevent the growth of strains lacking other V-ATPase subunits (29). An isoform of the a subunit, encoded by the *STV1* gene, can partially substitute for the loss of Vph1p. Later work reported that in the wild type the *STV1*-encoded subunit functions in the Golgi, whereas the *VPH1*-encoded subunit is targeted to the vacuole (30). Our preliminary findings suggested that *N. crassa* has only one gene for this protein. In this report we ask, are multiple genes for the a subunit characteristic of fungal species?

Some investigators have suggested that V-ATPases from animals lack the c′ subunit, shown to be essential in *S. cerevisiae* (31). In this yeast c and c′ are similar proteins with 53% sequence identity (32, 33). Identification of the c′ subunit and characterization of strains with mutations in the gene encoding it have not been reported from other organisms. We have found a candidate gene for subunit c′ in *N. crassa* and have characterized strains with mutations in this gene. We have also analyzed genome sequence data bases to ask what types of organisms have genes that may encode a c′ subunit?

EXPERIMENTAL PROCEDURES

Protein Alignment and Phylogenetic Analysis—Sequences were obtained from the Neurospora Genome Project (34). Protein sequences were aligned with ClustalW (www.ebi.ac.uk/clustalw/) (35). Phylogenetic analyses were performed using the program PAUP (Phylogenetic Analysis Using Parsimony; Sinauer, Sunderland, MA). Phylogenetic trees were constructed with JALVIEW (36).

Mutation of vma Genes by Repeat-induced Point Mutation—The procedure of repeat-induced point mutation (RIP) is an efficient means of inactivating genes in *N. crassa* (37). Briefly, an ectopic copy of the gene to be mutated is introduced by transformation. The strain is crossed, and before nuclear fusion occurs a DNA silencing mechanism changes many of the C nucleotides to T in both the endogenous and ectopic copies of the introduced gene. If the size of the duplicated gene is 2 kb or larger, 5–50% of the progeny are typically mutated (38). Progeny from the cross are screened either by direct analysis of the DNA or by the appearance of a new phenotype. We used PCR to amplify the *vma-5*, *vma-11*, *vma-13*, and *vma-16* genes. The primers used and the sizes of the fragments generated are shown in Table 1. Each of the genes was then subcloned into the plasmid pCR2.1 TOPO from the TA cloning kit (Invitrogen). Gene fragments were excised from the pCR2.1 plasmids by digestion with either EcoRI (*vma-5* and *vma-13*) or NotI and SpeI (*vma-11* and *vma-16*) and subcloned into the vector pBM61 (39).

The resulting plasmids, pBM61/vma5, 11, 13, and 16, were used to transform the *his-3* strain of *N. crassa* (FGSC#6103) by electroporation. The pBM61 vector contains a truncated form of the *his-3* gene designed to facilitate the targeted integration of plasmid DNA at the *his-3* locus (39). We selected transformants that were histidine prototrophs. Because conidia typically have multiple nuclei, these primary transformants were usually heterokaryons. By crossing the transformants to the *his-3* strain and selecting *his*+ progeny, we obtained homokaryotic strains. Integration of the plasmid was confirmed by PCR using the T3 and T7 primer pair that flanks the cloning site in pBM61. The homokaryotic his^+ transformants were then crossed again to the wild type strain 74A. For the *vma-11* and *vma-16* genes, we isolated transformants of both mating types and crossed them to each other. Thus, all of the nuclei in these crosses had a gene duplication and were susceptible to the RIP process. The ascospores produced from each cross were germinated on agar plates containing Vogel's salts (40), 2% sorbose, 0.05% glucose, and 0.05% fructose. After 24 h at 30 °C colonies with defective V-ATPase were identified by the morphological abnormality shown in Fig. 1. For all putative mutant strains, genomic DNA was prepared from 20 mg of lyophilized mycelia, using the DNeasy Plant kit (Qiagen), and amplified by PCR, using the primers shown in Table 1. Sequencing was performed by the DNA Sequencing Facility at the University of California, Berkeley.

TABLE 1
PCR primers used to generate mutant strains

Gene	Primer pair	Fragment generated
		kb
vma-3 pair A	5′-cctggatccttgtgtgtttctgtcc-3′ 5′-cttggatcccgctttcgtactgccac-3′	4.9
vma-3 pair B	5′-ggaggcctgaatacgcaaagaac-3′ 5′-gcttacgcgtgggatgatgcgtgttg-3′	7.0
vma-5	5′-atggcgccgacaactcgc-3′ 5′-actaaaacctacccatcaagg-3′	1.4
vma-11 pair A	5′-ggagtgcgtcgttgcgtcggctgtctc-3′ 5′-gattgaattctgggatggttgcccctc-3′	1.2
vma-11 pair B	5′-tcagcttgcgatggggagatc-3′ 5′-ggtctagctaggttagct-3′	3.0
vma-11 pair C	5′-ctggacgtcgactgaatcagaatgttggcgacg-3′ 5′-agtactagcgagttgagttttcgaggggc-3′	5.9
vma-13	5′-atgtcccttgatccgcc-3′ 5′-tcatcacttggtgtcaaagc-3′	1.7
vma-16	5′-ctcggaattcccggctagcacctaaac-3′ 5′-cttcgaattcgtacaaaaaggatttag-3′	1.5
inl	5′-tcgacgcgtcctcacgagaggctaatg-3′ 5′-gatacgcgtggaatgatagtacctgagg-3′	2.4

The *vph-1* gene was originally cloned in 1993, before sequences were available from many organisms. Briefly, we obtained from Dr. Morris Manolson (University of Toronto) 2 primers designed for the *S. cerevisiae VPH1* gene (41). With these we used PCR to generate a 141-bp fragment of the *vph-1* gene from *N. crassa* genomic DNA. We screened a *N. crassa* λZAP cDNA library with this fragment and isolated several cDNA clones, one of which was used to screen the Orbach/Sachs cosmid library (Fungal Genetics Stock Center, Kansas City, MO). A 4.2-kb KpnI fragment containing the coding region and flanking sequences of *vph-1* was inserted into the SK vector to create pFO2B#1A. The cDNA and genomic DNA sequences were submitted to GenBank™ with accession number U36396. These sequences are identical to those obtained by the Neurospora Genome Project.

We used the RIP procedure to inactivate the *vph-1* gene. A 3.9-kb SpeI fragment with the *vph-1* coding region and flanks

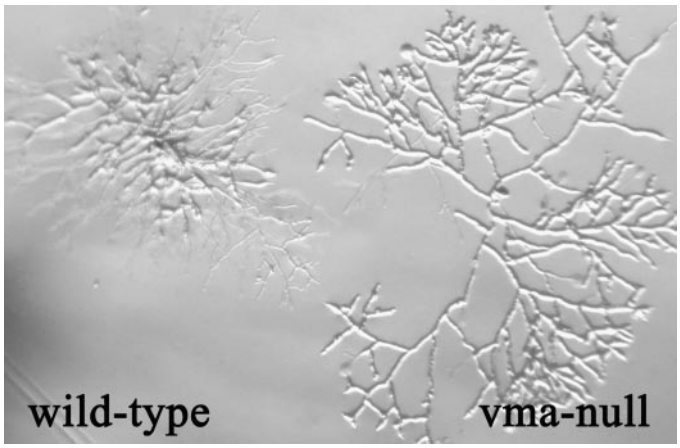

FIGURE 1. **Morphology of wild type and V-ATPase-deficient mutant strains.** Ascospores were germinated on solid medium containing 2% sorbose (see "Experimental Procedures") and grown for 20 h at 30 °C. The diameter of the mutant colony is ~1.5 mm. Much of the growth of the wild type strain is below the agar surface.

from pFO2B#1A was subcloned into the pBM61 vector and transformed into the *his-3* strain. By genetic crosses to a *his-3*$^-$ strain and then to the wild type 74A, we obtained one rare isolate that was *vph-1* null with no ectopic copy of the *vph-1* gene.

Inactivation of vma Genes by Homologous Gene Replacement—Despite numerous attempts, we were not able to isolate a strain with a null mutation in the *vma-3* gene by using the RIP procedure. Although much less efficient, we decided to use homologous integration to replace the *vma-3* protein-coding region with the *inl* gene, which encodes an enzyme required for inositol synthesis. Using primers that contained BamHI sites (Table 1, pair A), we amplified a 4.9-kb fragment that contained the *vma-3* protein-coding region plus a ~2-kb flanking region on each side. This fragment was digested with BamHI and cloned into the BamHI site in the Bluescript SK$^+$ vector (Stratagene) to produce pVMA3/5kb. We then used primers containing MluI and StuI sites (Table 1, pair B) to initiate replication on each side of the open reading frame, oriented such that the flanking regions and the vector were amplified, but not the open reading frame. Phosphate was added to the ends of the PCR product with T4 polynucleotide kinase, and subsequent ligation produced pVMA3noORF. Primers containing an MluI site (Table 1) were used to amplify the *inl* gene, and the gene was cloned into the MluI site in pVMA3noORF. This plasmid, named pVMA3/inl, had a functional *inl* gene flanked by the 5′ and 3′ noncoding regions of *vma-3*. It was used to transform the *inl* mutant strain by electroporation. Prototrophic transformants were selected. Essentially all of the transformants grew with normal hyphal morphology, indicating that the plasmid had integrated at an ectopic location, as is typical for *N. crassa*. However, one transformant had the same growth defects we had observed in other *vma* null strains. PCR analysis indicated that the *inl* gene had replaced the *vma-3* gene. The strain with the *vma-3*::*inl*$^+$ gene, had no detectable *vma-3* protein-coding region by Southern blot analysis. Introduction of the pVMA3–5kb plasmid, which contains a functional copy of *vma-3*, restored growth (data not shown).

As described under "Results" analysis of the phenotype of vma-11-RIP strains suggested they contained some residual V-ATPase activity. For this reason we used homologous integration to construct a strain in which the coding region of the *vma-11* gene was replaced by the *bar* gene, which confers resistance to the fungicide bialaphos (42). We used the same strategy as with pVMA3/inl described above. A 3-kb fragment of the *vma-11* gene containing ~1 kb upstream and 1 kb downstream of the protein-coding region was generated by PCR (Table 1, primer pair B). It was subcloned into the plasmid pCR2.1, generating pCR2.1bar. We then amplified this plasmid with primer pair C (Table 1, pair C). The resulting linear PCR product contained 1 kb of both upstream and downstream regions of *vma-11* as well as the pCR2.1 plasmid. The fragment was treated with T4 polynucleotide kinase and ligated, generating the plasmid pCR2.1vma11noORF. A 2.25-kb AatII fragment containing the *BAR* gene was isolated from the plasmid pBARGEM7–1 and cloned into the AatII site in pCR2.1vma11noORF at the junction of the *vma-11* flanking DNA generating the plasmid pCR2.1vma-11bar.

pCR2.1vma-11bar was introduced into the strain *mus-52 inl a* by electroporation. Strains with the *mus-52* mutation have recently been shown to have higher rates of homologous integration (43). Colonies that grew in the presence of 200 μg of bialophos/ml (Duchefa Biochemie, Haarlem, The Netherlands) were selected. PCR analysis of a colony with a morphology similar to that observed in other *vma* null strains indicated that the *vma-11* gene had been replaced by the *bar* gene. This strain, named *vma-11*::*bar*, had no detectable *vma-11* protein-coding region by Southern blot analysis (data not shown).

Generation of Anti-subunit H Antibody—A cDNA for *vma-13* was generated by PCR and cloned into the EcoRI-HindIII sites of pQE-9 (Qiagen), generating the plasmid pVMA-13–6His. The plasmid was transformed into the *E. coli* strain DH5α. Transcription of *vma-13* was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside. A cell lysate containing the expressed H subunit was then purified using a Ni$^+$ column. The H subunit was verified by Western blot using the His_6 antibody. The H subunit protein from an acrylamide gel was mixed with Freund's adjuvant and injected into a rabbit. Serum was collected and used to visualize the H subunit in Western blots prepared from *N. crassa* cell fractions.

Methods for Measuring Growth Rates, Spore Germination, and Arginine Content of Cells—The procedures used for composition of media, growth of strains, and genetic analysis were those of Davis (44). Vogel's medium contains salts (40) plus 2% sucrose. The rate of growth was determined by inoculating at the edge of agar plates containing Vogel's medium. The plates were incubated at 30 °C, and the position of the growth front of the colony was marked at 8- and 16-h intervals. To assess the production of viable progeny from crosses, ascospores were spread on plates containing Vogel's salts, 2% agar, 2% sorbose, 0.05% glucose 0.05% fructose, and 0.15 mg of *myo*-inositol/ml. Germination was induced by heat shock (30 min at 60 °C). The spores were either left on the plates or transferred individually to tubes with 1 ml of medium (2% Vogel's salts, 2% sucrose, 0.15

TABLE 2
Genes encoding subunits of the *N. crassa* V-ATPase

Gene name	Subunit	Molecular mass	Amino acids	Chromosomal location	Number of introns	Accession number
		kDa				
vma-5	C	44.55	392	IR	3	XP_329645
vma-13	H	54.07	481	IL	2	XP_331945
vph-1	a	98.01	856	IIL	4	CAD21112
vma-3	c	16.33	161	VR	4	CAC18222
vma-11	c′	17.06	167	IR	5	AAK13465
vma-16	c″	20.32	200	IR	3	XP_330397
vma-9	e	8.58	75	IVL	2	XM957230

mg of inositol/ml). The arginine content in whole cell extracts was assayed as described (21).

RESULTS

Comparison of Genes Encoding V-ATPase Subunits in N. crassa and Other Fungi—We have previously described the *N. crassa* V-ATPase subunits A, B, D, E, F, G, c, and d (45–50). Using the amino acid sequences of *S. cerevisiae* V-ATPase subunits, we searched the *N. crassa* genome and identified subunits C, H, a, c′, c″, and e (Table 2). The *N. crassa vma* genes are scattered randomly throughout the genome. We did no further analysis of subunit e.

One interesting finding was that, unlike *S. cerevisiae*, *N. crassa* appeared to contain only a single gene encoding subunit a. We examined the genomes of other fungi for which complete sequences are available and constructed a phylogenetic tree of all genes encoding subunit a (Fig. 2). Other filamentous ascomycetes, such as *Magnaporthe grisea* and *Aspergillus nidulans*, had a single gene, closely related to the *N. crassa* gene. *Cryptococcus neoformans*, a basidomycete, and *Schizosaccharomyces pombe*, a fission yeast, also had only one gene. Only *S. cerevisiae* and other budding yeasts had two genes. The *VPH1* and *STV1* genes of *S. cerevisiae* are less similar to each other than either one is to its apparent ortholog in other budding yeasts. Both mouse and human cells appear to have four genes encoding subunit a, which are equally divergent in sequence from the fungal *VPH1* and *STV1* genes. As in the budding yeasts, each human isoform is more similar to an isoform from mouse than to other human isoforms.

A second interesting finding concerned subunits c′ and c″. Like yeast, *N. crassa* contains orthologs of both the *vma-11* and *vma-16* genes, encoding subunits c′ and c″ (Table 2). These are small, hydrophobic proteins (proteolipids) with four or five transmembrane helices. We found orthologs to these genes in all of the completed fungal genomes. Subunits c and c′ have similar sequences, but within fungi they are clearly distinct types of proteins. The *N. crassa* c′ subunit has a higher degree of protein sequence identity to the *S. cerevisiae* c′ subunit (66.5%) than it does to the *N. crassa* c subunit (59.5%). Fig. 3 shows the alignment of amino acid sequences of c and c′ subunits from two filamentous fungi, two budding yeasts, and one fission yeast. The subunit c from mouse is also included. The helical regions of the proteins are most highly conserved, especially the fourth helix, which contains the glutamate (Glu^{138} in *N. crassa* subunit c) that is the probable proton-binding site. However, a few regions are unique to subunit c or c′. The loop connecting helices 2 and 3 is longer in the c′ subunits and has conserved

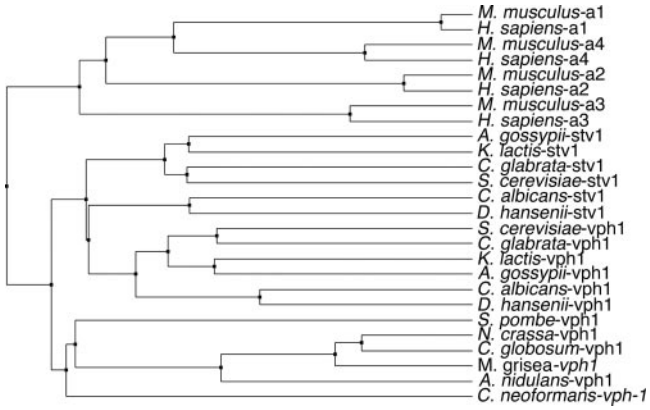

FIGURE 2. **Phylogenetic relationships of genes encoding subunit a.** The phylogenetic tree was generated using Clustal W and Jalview (36). Genes encoding subunit a from *Homo sapiens* were a1, AAH32398; a2, AAH68531; a3, Q13488; and a4, AAG11415. The mouse genes were a1, AAH71182; a2, NPO35726; a3, AAF37193; and a4, AAH46979. The sequences identified as vph-1 were *S. cerevisiae*, NP_014913; *Candida glabrata*, CAG58212; *Kluyveromyces lactis*, XP_452533; *Ashbya gossypii*, AAS52097; *Debaryomyces hansenii*, XP_458057; *Candida albicans*, XP_712251; *N. crassa*, CAD21112; *Chaetomium globosum*, EAQ86925; *M. grisea*, XP_361473; *A. nidulans*, XP_663210; *C. neoformans var. neoformans* JEC21, AAW42964; and *S. pombe*, NP_594219. Sequences identified as stv-1 were *S. cerevisiae*, NP_013770; *D. hansenii*, XP_461842; *C. albicans*, XP_712308; *C. glabrata*, CAG58212; *K. lactis*, XP_456260; and *A. gossypii*, AAS52047.

proline and histidine residues (marked with *arrows* in Fig. 3). Four residues in the loop between helices 3 and 4 (SFML in *N. crassa*) are consistently different in c′ subunits. The subunit from mouse is more similar to subunit c than to c′.

In a phylogenetic tree the c and c′ subunits of fungi cluster in two distinct groups (Fig. 4). The tree clearly suggests a closer relationship of the mammalian orthologs to fungal subunit c than to c′. For *Caenorhabditis elegans* the relationship is less clear. This organism has two "c-like" subunits that appear to have diverged from subunits in the other organisms but are still closer to subunit c (51). The plant *Arabidopsis thaliana* has five genes (not shown) encoding c subunits, all with nearly identical protein sequences that differ only in the first four residues (52). Overall, the analysis of the genome data bases was consistent with the conclusion that a distinct c′ subunit is found only in the fungi.

Although it is typical for *N. crassa* genes to contain introns, the c and c′ subunit genes have more than average. The *vma-3* and *vma-11* genes encode very small proteins yet contain 4 and 5 introns, respectively. Between the start and stop codons ~50% of the sequences are in introns, which is unusual for fungal genes. The positions of the introns are not conserved (data not shown). They break the coding regions into segments, the largest of which is 260 bp. An intriguing possibility is that long regions of high sequence similarity could make *vma-3* and *vma-11* susceptible to RIP (37). Without introns the nucleotide sequence of the coding regions would be 65% identical. The addition of introns lowers the sequence identity to 46%.

In contrast to the c′ subunit, the c″ subunit is highly conserved among *N. crassa*, other fungi and mammals (Fig. 5). This subunit differs from c and c′ by virtue of a putative additional helical region at the N terminus. The *S. cerevisiae* protein has at least 10 more residues in this region than the filamentous fungi; however, the N terminus of all the proteins is long enough for a transmembrane helix.

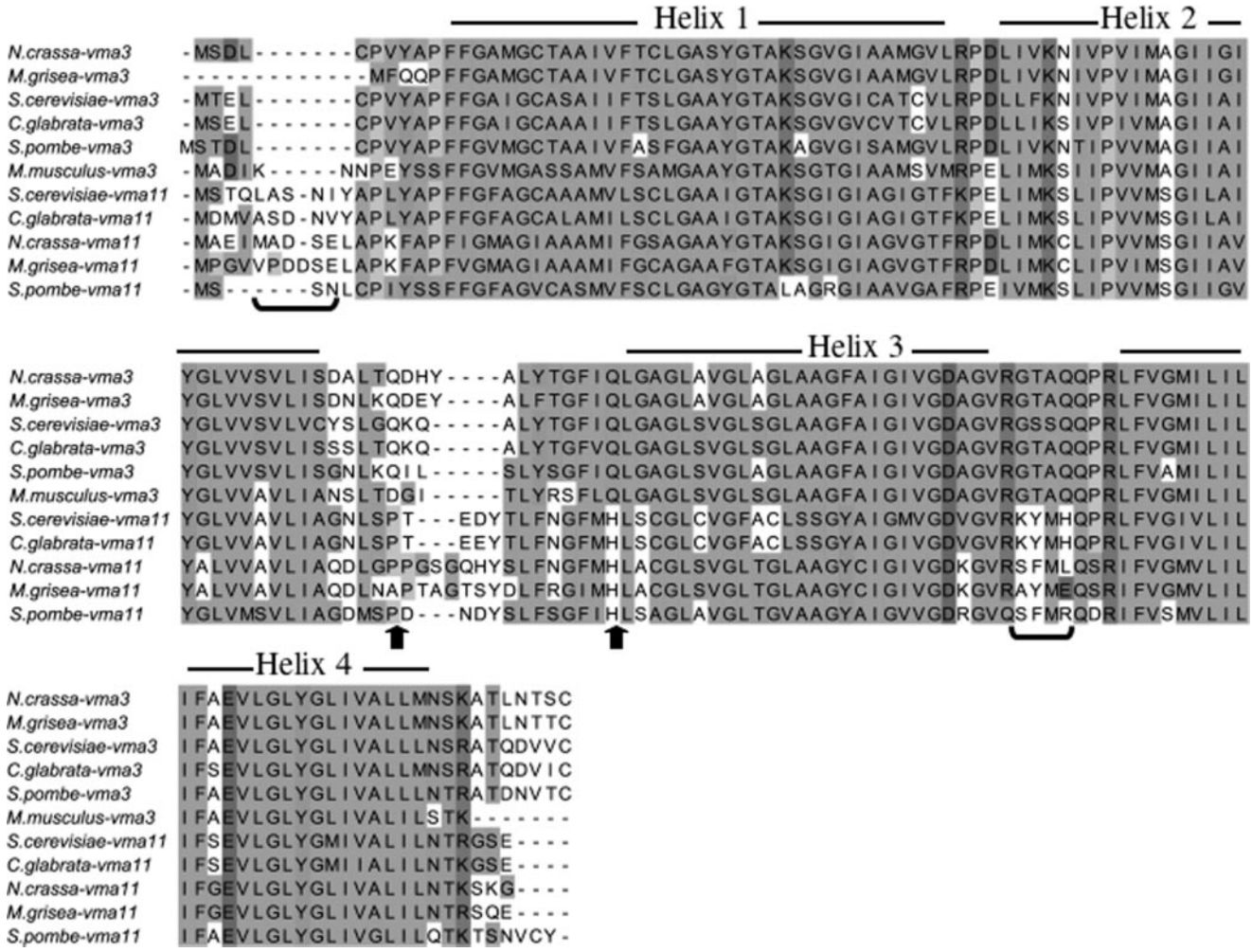

FIGURE 3. **Alignment of amino acid sequences of genes encoding subunits c and c′.** Regions in which subunits c and c′ are consistently different are marked by *arrows* and *brackets*.

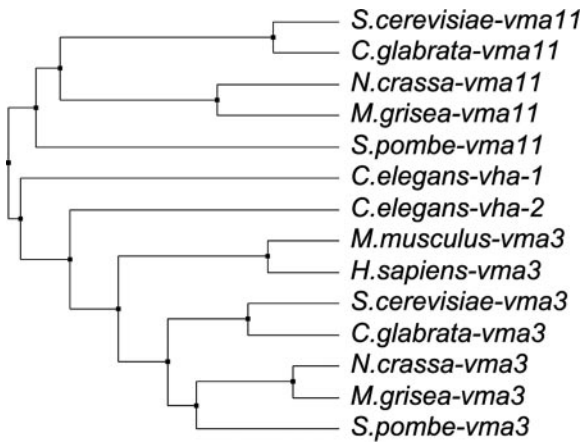

FIGURE 4. **Phylogenetic relationships of genes encoding subunits c and c′ in fungi.** The sequences identified as vma3 were *Mus musculus*, AAH83129; *H. sapiens*, AAH07759; *S. cerevisiae*, CAA33249; *S. pombe*, CAA42572; *M. grisea*, XP_365764; *N. crassa*, AAA19974; and *C. glabrata*, CAG60258. Sequences identified as vma11 were *S. cerevisiae*, BAA01367; *C. glabrata*, CAG58878; *N. crassa*, AAD45120; *M. grisea*, XP_366989; and *S. pombe*, CAB62424. The vha sequences are *C. elegans vha1*, BAA22595, and *C. elegans vha2*, BAA22596.

Analysis of Strains with Mutations in Genes Encoding Subunits C H, a, c, c′, and c″—We previously reported that disruption of *vma-1*, encoding subunit A of the *N. crassa* vacuolar ATPase, gives rise to a distinctive phenotype (21). The strain cannot grow on alkaline medium, growth on acidic medium is much slower than for the wild type, and the morphology of hyphae is severely altered. The strain does not form conidia (asexual spores) but is fertile in a cross if the other parent has a functional V-ATPase. When hyphae of opposite mating type fuse they form a multicellular fruiting body, the perithecium. Several hundred cells are generated, each with one nucleus from each parent. These nuclei fuse, immediately undergo meiosis, and then divide again to yield eight nuclei. Each nucleus develops into a separate heavily melanized spore. If a nucleus contains a mutant gene that disrupts development of that spore, an immature white spore is produced. The viability of ascospores that contain a *vma-1* null gene is low, less than 1%. In the mycelia, levels of cellular arginine are reduced by ~85% in the *vma-1* null strain. In the wild type strain almost all of the cellular arginine is stored in the vacuole (53). Concanamycin, a specific inhibitor of the V-ATPase and a potent inhibitor of growth of the wild type strain (54, 55), has no effect on the growth of the null strain. We have used this complex phenotype to evaluate the roles of previously uncharacterized V-ATPase subunits in *N. crassa*.

Strains with Mutations in Genes Encoding Subunits C and H—As described under "Experimental Procedures," the RIP proce-

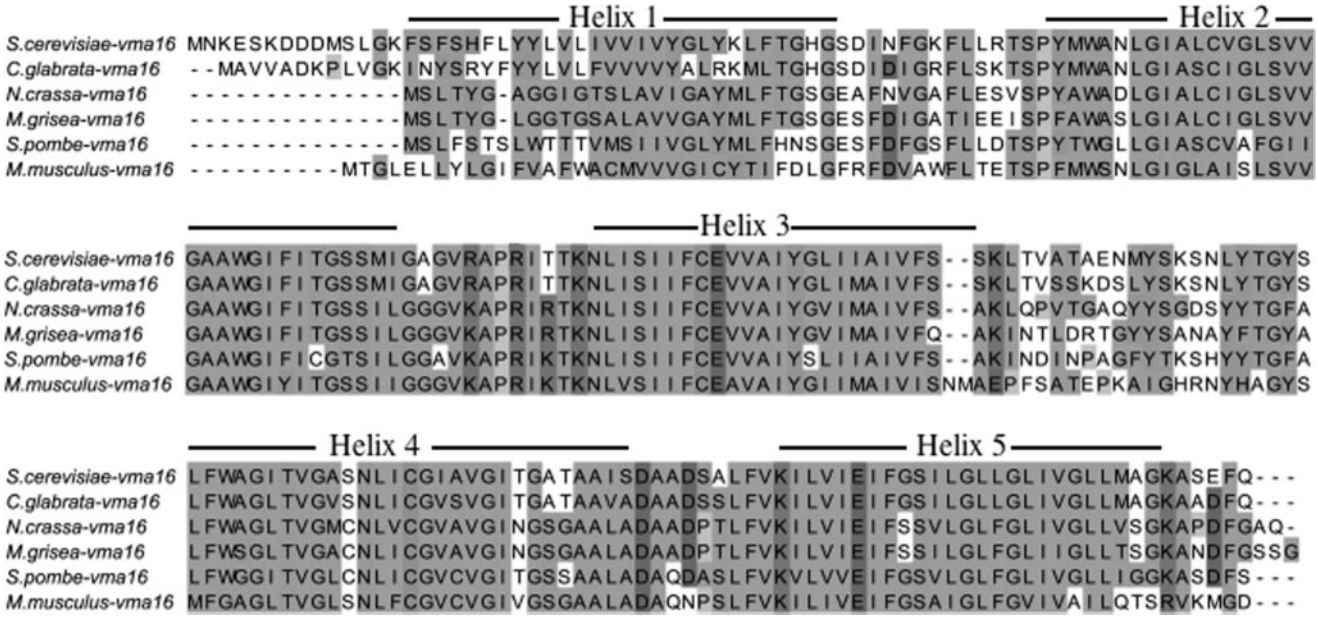

FIGURE 5. **Alignment of amino acid sequences of fungal genes encoding subunits c″.**

dure was used to inactivate the *vma-5* and *vma-13* genes. Sequencing showed that stop codons had been introduced into the first half of both genes (Table 3). The phenotypes of these mutant strains were nearly indistinguishable from those of the vma-1-RIP strain characterized previously. They were unable to grow at pH 7.2 and had the same severe morphological changes as the vma-1-RIP strain when grown at pH 5.8, which is optimal for *N. crassa* (Fig. 6). The mutant strains did not form conidia (asexual spores). The *vma-5* and *vma-13* mutant strains had only 15% the amount of arginine seen in the wild type (Table 4), the same as in the vma-1-RIP strain. Thus, the vma-5-RIP and vma-13-RIP strains had the V-ATPase null phenotype, indicating that subunits C and H were essential for generating a functional enzyme *in vivo*.

We observed one difference in the phenotypes of vma-5-RIP and vma-13-RIP strains as compared with the pvn1 strain (vma-1-RIP). Like the vma-1-RIP strain, vma-5-RIP and vma-13-RIP strains were fertile in a cross if the other parent had a functional V-ATPase. The crosses produced a high proportion, 22–30%, of white spores. However, the crosses also produced viable vma-5-RIP and vma-13-RIP progeny at significantly higher rates than the cross with the pvn-1 strain (Table 5).

Does N. crassa Contain Multiple Forms of Subunit H?—We previously reported that preparations of the *N. crassa* V-ATPase often appeared to have two polypeptides of ~54 kDa, the size of subunit H (47). The V-ATPase of clathrin-coated vesicles from bovine cells contains two polypeptides of 50 and 57 kDa that co-purify with the enzyme. These proteins, termed SFD (sub-fifty-eight-kDa dimer), activate ATPase activity of the enzyme and couple ATPase activity to proton pumping (25). The proteins turned out to be two forms of the H subunit with the 57-kDa species containing a 54-base pair insert in the open reading frame corresponding to an 18-amino acid insert in the protein (56). We generated a polyclonal antibody against subunit H and used Western blots to visualize the protein in different cell fractions. The subunit H protein was

TABLE 3
Mutations in vma RIP strains in *N. crassa*

Strain name	No. of nucleotide changes	Introduced stop codons	No. of amino acid changes
vph-1	177	Glu^{5a}	26
vma-5	10	Gly^{152}	7
vma-13	24	Gly^{71}	9
vma-16	16	Trp^{131}	19
vma-11–16	41	Glu^{82}	7
vma-11–25	47	0	5
vma-11–27	41	0	18

[a] vph-1 has another 11 introduced stop codons.

concentrated in the vacuolar membrane fraction, where it appeared as a single band of ~54 kDa (data not shown). Although it is possible that there are two subunits of nearly identical mobility, it appears that *N. crassa*, like *S. cerevisiae*, contains only one form of the subunit H protein.

Strains with Mutations in the vph-1 Gene, Encoding Subunit a—The RIP procedure yielded one viable strain with mutations in the *vph-1* gene. Sequencing showed 177 nucleotide changes and 12 in-frame stop codons in the first 2221 bp. No protein was detected in cell fractions on Western blots (data not shown), consistent with its being a null mutant strain. In *S. cerevisiae*, VPH1 deletion strains give a partial *vma* phenotype because *STV1* can partially compensate for the absence of *VPH1* (29). By contrast, the phenotype of the *N. crassa* vph-1-RIP strain was indistinguishable from that of the vma-1-RIP strains. On pH 5.8 medium the vph-1-RIP strain was slow growing and had altered morphology, and it failed to grow on alkaline medium (Fig. 6). It was defective in sequestering arginine in the vacuole (Table 4). Cell fractions had no detectable V-ATPase activity (data not shown). Like the pvn1 strain (vma-1-RIP) fewer than 1% of the viable progeny from crosses contained the *vph-1* RIP gene (Table 5). These data confirmed that *N. crassa* has a single gene encoding subunit a, in agreement with the analysis of the genome data base discussed above.

Strains with Mutations in the vma-3 Gene, Encoding Subunit c—We were unable to generate a strain with a null mutation in the

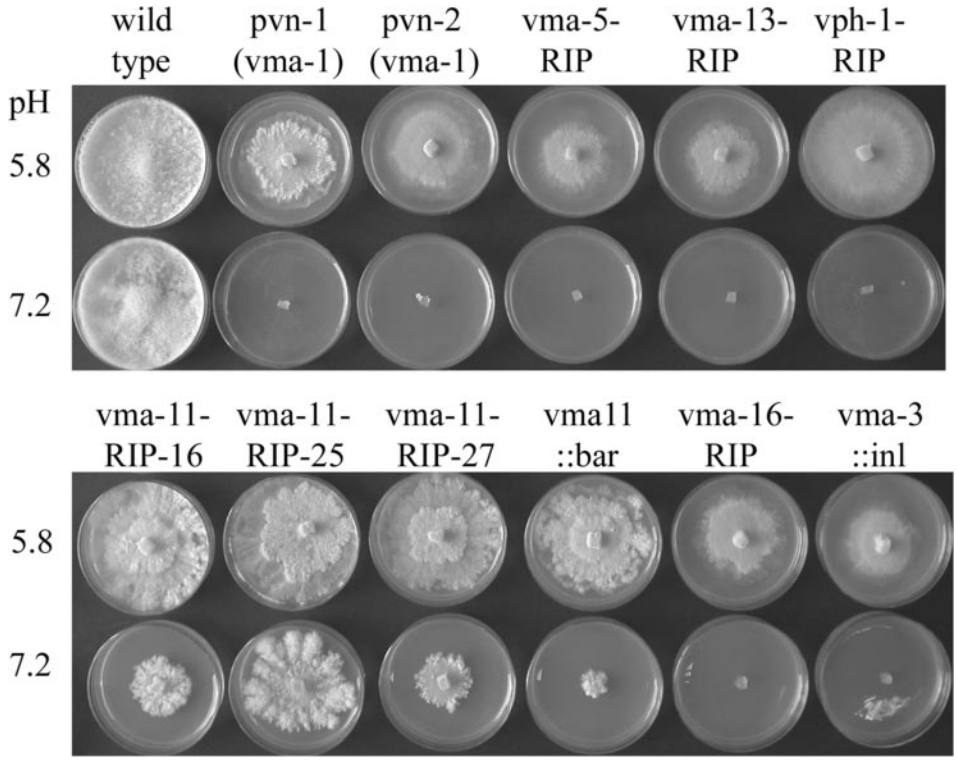

FIGURE 6. **Growth of wild type and mutant strains at pH 5.8 and 7.2.** Strains were grown on Vogel's medium (pH 5.8) or on Vogel's medium with 20 mM HEPES, pH 7.2 (21) for 4 days at 30 °C. Each plate was inoculated with a plug of the same size and age from cultures growing on agar plates, pH 5.8. The wild type strain grew much more quickly and covered the plate after 1 day.

TABLE 4
Arginine content of cell extracts

Strain	Arginine
	nmol/mg
Wild type 74A	22.4
vma-1, pvn1	2.3
vma-1, pvn2	2.5
vma-5-RIP	3.1
vma-13-RIP	2.8
vph-1-RIP	2.7
vma-3::inl	2.5
vma-11-RIP-16	2.3
vma-11-RIP-25	5.6
vma-11-RIP-27	2.9
vma-11::bar	2.0
vma-16-RIP	2.2

TABLE 5
Development of ascospores and formation of heterokaryons

Strain	White spores	Mutant vma progeny	Heterokaryon formation
	%	%	
Wild type 74A	6	0	Yes
vma-1, pvn1	14	<1	Yes
vma-1, pvn2	6	39	Yes
vma-3::inl	46	0	No
vma-5-RIP	43	11	Yes
vma-11::bar	31	<1	No
vma-11-RIP-16	59	7	Yes
vma-11-RIP-25	15	36	Yes
vma-11-RIP-27	37	11	Yes
vma-13-RIP	22	4	Yes
vma-16-RIP	10	<1	Weak
vph-1-RIP	29	<1	Yes

vma-3 gene using the RIP procedure. However, we were able to delete the protein-coding region of *vma-3* by homologous replacement with the *inl* gene as described under "Experimental Procedures." The *vma-3*::*inl*$^+$ strain had the same phenotype as vma-1-RIP with regard to growth, morphology, and cellular arginine (Fig. 6 and Table 4). It differed from other *vma* null strains in one characteristic. Although *vma-3*::*inl*$^+$ was fertile when mated with a *vma*$^+$ strain and produced *vma*$^+$ progeny, we never observed viable progeny that inherited the *vma-3*::*inl*$^+$ gene. Disruption of the *vma-3* gene appeared to completely prevent the development of viable spores (Table 5).

Strains with Mutations in the vma-16 Gene, Encoding Subunit c″—With the RIP procedure we generated a strain mutated in the *vma-16* gene. The phenotype was indistinguishable from that of the pvn1 (vma-1-RIP) strain (Fig. 6 and Table 4). Mutant progeny were obtained from crosses, but at very low rates, fewer than 1% of the viable progeny.

Strains with Mutations in the vma-11 Gene, Encoding Subunit c′—The RIP procedure readily generated strains with mutations in the *vma-11* gene. To our surprise the growth phenotypes of these strains were subtly different from the strains mutated in any of the other *vma* genes. Three vma-11-RIP strains had 5–18 amino acid changes, and the vma-11-RIP-16 strain had a stop codon at residue 82 (Fig. 7). Despite these extensive changes in the c′ subunit, the vma-11-RIP strains grew slightly better than other *vma* mutant strains on pH 5.8 medium; they also grew on medium at pH 7.2 (Fig. 6). These results suggested that the vma-11-RIP strains were not completely null for V-ATPase function.

To generate a strain that completely lacked the *vma-11* gene, we used homologous replacement as described under "Experimental Procedures." The resulting mutant strain, *vma-11*::*bar*, had a more severe growth defect than the vma-11-RIP strains but still grew marginally better than other *vma* null strains on pH 5.8 medium. Like the other *vma-11* mutant strains, it produced more aerial hyphae and grew slowly but significantly on alkaline medium (Fig. 8). To compare growth rates we measured radial growth on solid medium, pH 5.8 (Fig. 9). The wild type strain grew much faster than any of the *vma* mutant strains, completely covering the agar plate in 48 h. The vma-11-RIP-25 strain, which had 5 amino acid changes, grew at less than half the rate of wild type but faster than any other mutant strain. The *vma-11*::*bar* strain grew slightly faster than the strains with mutations in other *vma* genes. For example, at 100 h *vma-11*::*bar* had grown ~25% further than the others.

To determine whether the *vma-11* mutant strains retained residual V-ATPase activity, we tested the effects of concanamycin, a specific V-ATPase inhibitor, on growth. At pH 5.8, 0.2 μM concanamycin causes slow, morphologically abnormal growth

of the wild type strain (55), similar to that observed in *vma* null strains. All of the *vma* mutant strains, except *vma-11*, grew the same in the presence or absence of concanamycin (Fig. 10). The *vma-11* mutants, including *vma-11*::*bar*, grew more slowly in the presence of concanamycin and failed to produce aerial hyphae. In the presence of concanamycin the *vma-11* strains became indistinguishable from the other *vma* mutant strains. In general the amount of arginine in *vma-11* mutant strains was similar to that of other *vma* mutants. Strain vma-11-RIP-25, which grew significantly better than the others (Fig. 9), had approximately twice as much arginine as the other *vma-11* mutant strains.

The ability of the *vma-11* mutant strains to produce viable ascospores corresponded roughly to the severity of the mutation (Table 5). vma-11-RIP-25 produced fewer white spores and a higher proportion (36%) of viable mutant progeny. *vma-11*::*bar* produced more white spores and only a few viable mutant progeny per thousand spores. vma-11-RIP-16 and vma-11-RIP-27, which are intermediate in phenotype, also produced many white spores, but ~10% of the progeny were viable mutants.

The differences in the phenotypes of *vma-11* mutant strains prompted us to try another test for the ability to produce conidia. In our previous investigation we found that the pvn-2 allele of *vma-1*, which has only 4 altered amino acids, produces abundant conidia if high concentrations of sorbitol are added to the medium (21). We repeated this experiment, using 1.5 M sorbitol, with the new *vma* mutant strains. We found that the *vma-11*::*bar* strain also produced conidia, whereas strains mutated in other *vma* genes did not conidiate (data not shown).

The inability of the *vma* null strains to produce conidia makes it more difficult to store these strains and to transform them. For most aconidial stains of *N. crassa* this problem can be overcome by forming a heterokaryon with a "helper strain." For example, if a vma-1-RIP strain is co-inoculated with an adenine-requiring strain (*ad3-B*), the two can fuse, and each will complement the deficiency of the other. Most of the vma-RIP strains formed vigorously growing heterokaryons with helper strains. However, strains with null mutations in genes encoding subunits c, c′, and c″ did not (Table 5).

```
Wild type MAEIMADSELAPKFAPFIGMAGIAAAMIFGSAGAAYGTAKSGI
RIP-16    ------------------------------------V-----F--
RIP-25    -----------------------------------V--------
Rip-27    I-----------------SI------I--S^--TT-S------

Wild type GIAGVGTFRPDLIMKCLIPVVMSGIIAVYALVVAVLIAQDLGP
RIP-16    ----------------------------------------V*----
RIP-25    ------------------------------------------------
Rip-27    -------------I----------------------------------

Wild type PGSGQHYSLFNGFMHLACGLSVGLTGLAAGYCIGIVGDKGV
RIP-16    L--------------------L----F--------------
RIP-25    -L---Y--------------L-----F--------------
Rip-27    -----------S-I---YS--I--------------------

Wild type RSFMLQSRIFVGMVLILIFGEVLGLYGLIVALILNTKSKG
RIP-16    -----------------G----------------------
RIP-25    ------------------------------------------
Rip-27    ------------II------------S--I-----------
```

RIP-16 – 40 nucleotides, 7 amino acids changed, stop codon at residue 82
RIP-25 – 40 nucleotides, 5 amino acids changed
Rip-27 – 37 nucleotides, 18 amino acids changed, 3'splice site in intron after residue 31 changed (CAG→CAA)

FIGURE 7. **Mutations in vma-11-RIP strains.** For each vma-11-RIP strain, the amino acids that were changed are shown below the corresponding amino acid in the wild type strain.

DISCUSSION

Disruption of a gene encoding a subunit of the *N. crassa* vacuolar ATPase gives rise to a distinctive phenotype (21). The strain cannot grow on alkaline medium, growth on acidic medium is much slower than for the wild type, and the morphology of hyphae is severely altered. The strains do not form conidia. Levels of cellular arginine, which is normally stored in vacuoles, are reduced by ~85%. With the exception of strains mutated in *vma-11*, all of the strains we tested with putative null mutations in a V-ATPase subunit had this phenotype. The vma-5-RIP (subunit C) and vma-13-RIP (subunit H) strains were indistinguishable from the other *vma* null strains. Although these subunits do not appear to have counterparts in the F-ATPase, our data agree with observations made with *S. cerevisiae* that subunits C and H are essential for formation of a functional V-ATPase (1, 2).

A significant difference between *N. crassa* and *S. cerevisiae* is the presence of one *versus* two genes encoding subunit a. Analysis of fungal genomes for which a complete sequence is available finds a two-gene family, *VPH1* and *STV1*, only in the budding yeasts. Disruption of the *vph-1* gene in *N. crassa* gives a complete null phenotype. These results argue strongly that *N. crassa*, and probably most other fungi, have only one gene encoding subunit a. This raises the interesting question of whether different forms of subunit a, or some other V-ATPase subunit, occur generally in Golgi *versus* vacuoles as observed in *S. cerevisiae* (30). Plants and animals have multiple genes for subunit a, expressed at different levels in different types of cells (3, 57, 58). In animals there is good evidence that specialized proton-secreting cells have a specific isoform of the a subunit in the plasma membrane (3, 59), and cells in the kidney have another isoform targeted to the endosomes (60).

In eukaryotic cells V-ATPases have at least two types of proteolipid

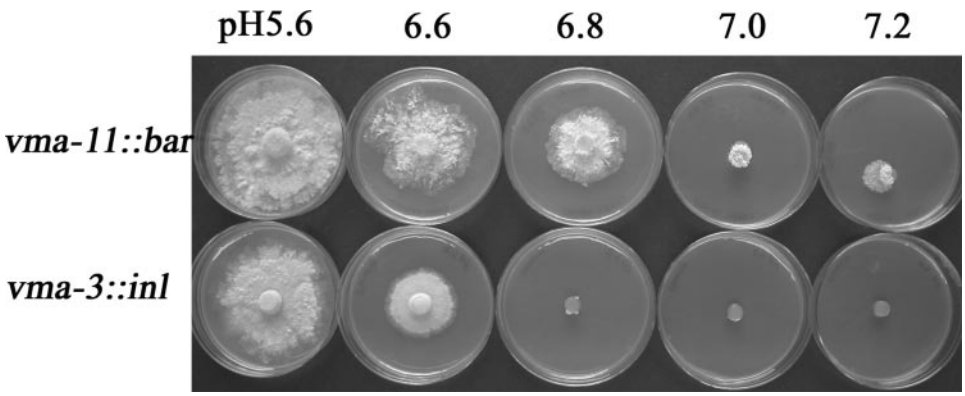

FIGURE 8. **The effect of pH on the growth of *vma-3*::*inl*$^+$ and *vma-11*::*bar*.** The strains were grown for 4 days at 30 °C. The medium was Vogel's plus 20 mM HEPES, adjusted to the indicated pH.

subunits, c (*vma-3*) and c″ (*vma-16*), both similar to the c subunit in F-ATPases. The c″ subunit is distinguished by additional residues at the N terminus that form an extra transmembrane helix. The function of this region is not known, and it can be deleted in *S. cerevisiae* without affecting the function of the V-ATPase (61). As expected, *N. crassa* and other fungi have genes encoding orthologs of c and c″. Disruption of these genes in *N. crassa* gave rise to strains with a typical *vma* null phenotype. The N-terminal region of c″ is shorter in *N. crassa* and other filamentous fungi than in *S. cerevisiae* but still of sufficient length to encode a fifth membrane helix.

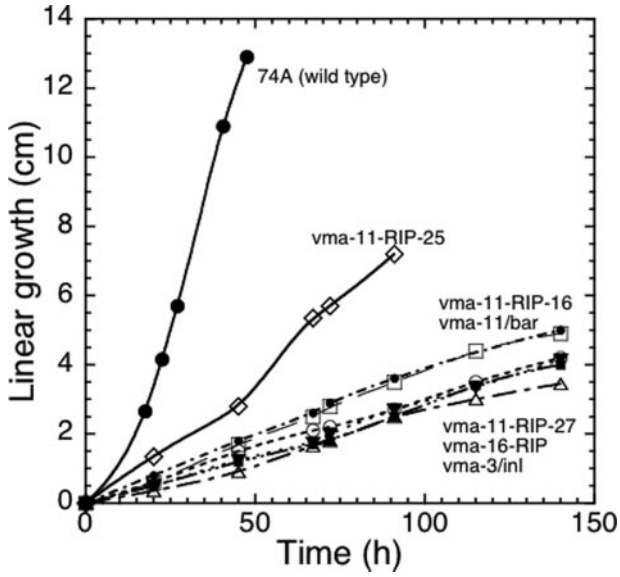

FIGURE 9. **Rates of linear growth for wild type and V-ATPase mutant strains.** Each strain was inoculated 1 cm from the edge of a Petri dish containing Vogel's medium, pH 5.8. The position of the edge of the mycelium was measured at 8- and 16-h intervals.

Whether V-ATPases of animals and plants have a c′ subunit orthologous to that encoded by the *VMA11* gene in *S. cerevisiae* is unclear. All of the sequenced fungal genomes have this subunit. The human and mouse genomes have only two "c-like" subunits, which appear to correspond to c and c″ (Fig. 3). Plants have multiple proteolipid subunits, but they also are most similar to either c or c″ (52). Thus, our analysis suggests that subunit c′ may be unique to fungi. Therefore, it was interesting to observe that strains with mutations in the *N. crassa vma-11* gene had different phenotypes from any of the other *vma* mutant strains. The *N. crassa* V-ATPase was unusually tolerant of mutations in the *vma-11* gene. We isolated strains with 5–18 amino acid changes, most in highly conserved positions. These mutant strains retained some V-ATPase function. They grew better than any other *vma* mutant strain, and the growth was inhibited by concanamycin, which is specific for the V-ATPase. The strain in which the *vma-11* gene was deleted also grew better than the other *vma* null strains and showed some sensitivity to concanamycin. This strain was also able to produce conidia. These results suggest that a small amount of functional V-ATPase can be made in the absence of subunit c′. Malkus *et al.* (62) reported that subunit c′ has a unique role in the assembly of the V-ATPase in *S. cerevisiae*. It mediates the binding of Vma21p, which appears to coordinate the assembly of V_o subunits in the endoplasmic reticulum and to help package them into transport vesicles. A possible explanation of the phenotype of the *N. crassa* mutant strains is that the V-ATPase in *vma-11* RIP strains retains some ability to interact with vma-21p and allows the assembly of a small amount of enzyme. In the *vma-11*::*bar* strain a smaller amount of enzyme may assemble in the absence of subunit c′. Subunits c and c′ are so similar that vma21p could possibly recognize subunit c, although with lower affinity. In summary, we speculate that other organisms make the V-ATPase without subunit c′ and that an *N. crassa* strain lacking subunit c′ can also generate some functional V-ATPase.

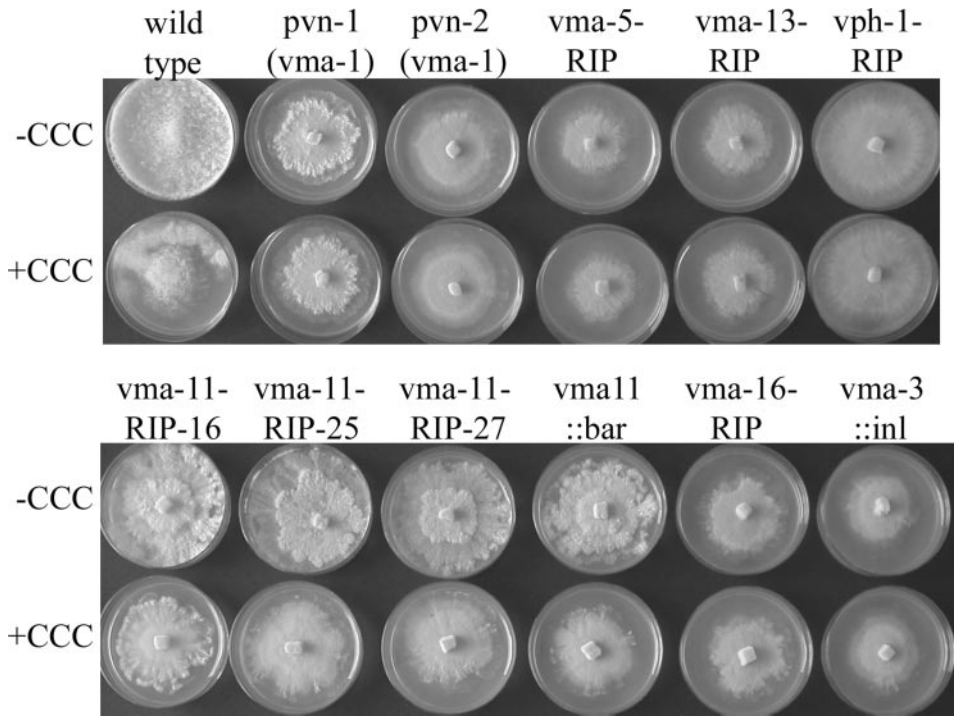

FIGURE 10. **The effect of concanamycin C on the growth of wild type and V-ATPase mutant strains.** Strains were grown on Vogel's medium, pH 5.8, with or without concanamycin C (*CCC*) for 4 days at 30 °C. After 1 day the wild type strain covered the plate that lacked concanamycin C, but it grew much less, with altered morphology, on the concanamycin C-containing plate (21).

Except for *vma-11*, the only phenotypic difference in strains lacking different V-ATPase subunits was in the ability to develop viable ascospores. Strains with the pvn2 allele of *vma-1* form mature viable spores almost as well as the wild type (21). vma-5-RIP, vma-13-RIP, and the vma-11-RIP strains produced more immature spores and had a lower germination rate than pvn2; nevertheless, 4–11% of the viable progeny were *vma* mutants. Strains with the

pvn1 allele of *vma-1*, vph-1-RIP, vma-16-RIP, and *vma-11*::*bar* produced only a few viable mutant progeny /thousand spores, and *vma-3*::*inl*$^{+}$ produced none. We have not been able to formulate a hypothesis to explain all of these differences. The pvn2 strain has only 4 altered amino acids and, conceivably, has V-ATPase activity not detectable in our assays. It is also possible that strains lacking subunits C or H retain some function associated with the V-ATPase. These subunits appear to be on the periphery of the enzyme and have no counterparts in F-type ATPases. Subunit C easily dissociates from the *N. crassa* enzyme. In *S. cerevisiae VMA13* deletion mutants (lacking H) are able to assemble the rest of the enzyme (17). The most severe effect on *N. crassa* spore development was observed in strains lacking the A, a, c, c′, and c″ subunits. Because the a, c, and c″ subunits form the core of the V_0 sector, the severity of the phenotype is not surprising. Likewise, subunit A forms most of the ATP-binding site and is thus a key component of the V1 sector (63). The nearly complete loss of ability to form spores in the *vma-11*::*bar* strain is somewhat surprising because that strain retained some V-ATPase activity. We can only speculate that the amount of enzyme in this strain is too small to form viable spores.

Acknowledgments—We thank Ryan Kendle, Forest O'Neill, Tri Vo, Amber Rivera, and Julio Escobar for technical assistance.

REFERENCES

1. Kane, P. M. (2006) *Microbiol. Mol. Biol. Rev.* **70,** 177–191
2. Nishi, T., and Forgac, M. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 94–103
3. Sun-Wada, G. H., Wada, Y., and Futai, M. (2004) *Biochim. Biophys. Acta* **1658,** 106–114
4. Wagner, C. A., Finberg, K. E., Breton, S., Marshansky, V., Brown, D., and Geibel, J. P. (2004) *Physiol. Rev.* **84,** 1263–1314
5. Gluck, S. L., Lee, B. S., Wang, S. P., Underhill, D., Nemoto, J., and Holliday, L. S. (1998) *Acta Physiol. Scand. Suppl.* **643,** 203–212
6. Farina, C., and Gagliardi, S. (2002) *Curr. Pharm. Des.* **8,** 2033–2048
7. Dschida, W. J., and Bowman, B. J. (1992) *J. Biol. Chem.* **267,** 18783–18789
8. Wilkens, S., Vasilyeva, E., and Forgac, M. (1999) *J. Biol. Chem.* **274,** 31804–31810
9. Gogarten, J. P., Kibak, H., Dittrich, P., Taiz, L., Bowman, E. J., Bowman, B. J., Manolson, M. F., Poole, R. J., Date, T., Oshima, T., Konishi, J., Denda, K., and Yoshida, M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 6661–6665
10. Senior, A. E. (1990) *Annu. Rev. Biophys. Biophys. Chem.* **19,** 7–41
11. Stock, D., Leslie, A. G., and Walker, J. E. (1999) *Science* **286,** 1700–1705
12. Fillingame, R. H., Angevine, C. M., and Dmitriev, O. Y. (2002) *Biochim. Biophys. Acta* **1555,** 29–36
13. Futai, M., Omote, H., Sambongi, Y., and Wada, Y. (2000) *Biochim. Biophys. Acta* **1458,** 276–288
14. Ueno, H., Suzuki, T., Kinosita, K., Jr., and Yoshida, M. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 1333–1338
15. Bauerle, C., Ho, M. N., Lindorfer, M. A., and Stevens, T. H. (1993) *J. Biol. Chem.* **268,** 12749–12757
16. Beltran, C., Kopecky, J., Pan, Y. C., Nelson, H., and Nelson, N. (1992) *J. Biol. Chem.* **267,** 774–779
17. Ho, M. N., Hirata, R., Umemoto, N., Ohya, Y., Takatsuki, A., Stevens, T. H., and Anraku, Y. (1993) *J. Biol. Chem.* **268,** 18286–18292
18. Inoue, H., Noumi, T., Nagata, M., Murakami, H., and Kanazawa, H. (1999) *Biochim. Biophys. Acta* **1413,** 130–138
19. Allan, A. K., Du, J., Davies, S. A., and Dow, J. A. (2005) *Physiol. Genomics* **22,** 128–138
20. Oka, T., and Futai, M. (2000) *J. Biol. Chem.* **275,** 29556–29561
21. Bowman, E. J., Kendle, R., and Bowman, B. J. (2000) *J. Biol. Chem.* **275,** 167–176
22. Keenan Curtis, K., and Kane, P. M. (2002) *J. Biol. Chem.* **277,** 2716–2724
23. Parra, K. J., Keenan, K. L., and Kane, P. M. (2000) *J. Biol. Chem.* **275,** 21761–21767
24. Puopolo, K., Sczekan, M., Magner, R., and Forgac, M. (1992) *J. Biol. Chem.* **267,** 5171–5176
25. Xie, X. S., Crider, B. P., Ma, Y. M., and Stone, D. K. (1994) *J. Biol. Chem.* **269,** 25809–25815
26. Lu, X., Yu, H., Liu, S. H., Brodsky, F. M., and Peterlin, B. M. (1998) *Immunity* **8,** 647–656
27. Zhong, X., Malhotra, R., and Guidotti, G. (2000) *J. Biol. Chem.* **275,** 35592–35599
28. Bowman, B. J., Vazquez-Laslop, N., and Bowman, E. J. (1992) *J. Bioenerg. Biomembr.* **24,** 361–370
29. Manolson, M. F., Wu, B., Proteau, D., Taillon, B. E., Roberts, B. T., Hoyt, M. A., and Jones, E. W. (1994) *J. Biol. Chem.* **269,** 14064–14074
30. Kawasaki-Nishi, S., Bowers, K., Nishi, T., Forgac, M., and Stevens, T. H. (2001) *J. Biol. Chem.* **276,** 47411–47420
31. Whyteside, G., Gibson, L., Scott, M., and Finbow, M. E. (2005) *FEBS Lett.* **579,** 2981–2985
32. Umemoto, N., Ohya, Y., and Anraku, Y. (1991) *J. Biol. Chem.* **266,** 24526–24532
33. Hirata, R., Graham, L. A., Takatsuki, A., Stevens, T. H., and Anraku, Y. (1997) *J. Biol. Chem.* **272,** 4795–4803
34. Galagan, J. E., Calvo, S. E., Borkovich, K. A., Selker, E. U., Read, N. D., Jaffe, D., FitzHugh, W., Ma, L. J., Smirnov, S., Purcell, S., Rehman, B., Elkins, T., Engels, R., Wang, S., Nielsen, C. B., Butler, J., Endrizzi, M., Qui, D., Ianakiev, P., Bell-Pedersen, D., Nelson, M. A., Werner-Washburne, M., Selitrennikoff, C. P., Kinsey, J. A., Braun, E. L., Zelter, A., Schulte, U., Kothe, G. O., Jedd, G., Mewes, W., Staben, C., Marcotte, E., Greenberg, D., Roy, A., Foley, K., Naylor, J., Stange-Thomann, N., Barrett, R., Gnerre, S., Kamal, M., Kamvysselis, M., Mauceli, E., Bielke, C., Rudd, S., Frishman, D., Krystofova, S., Rasmussen, C., Metzenberg, R. L., Perkins, D. D., Kroken, S., Cogoni, C., Macino, G., Catcheside, D., Li, W., Pratt, R. J., Osmani, S. A., DeSouza, C. P., Glass, L., Orbach, M. J., Berglund, J. A., Voelker, R., Yarden, O., Plamann, M., Seiler, S., Dunlap, J., Radford, A., Aramayo, R., Natvig, D. O., Alex, L. A., Mannhaupt, G., Ebbole, D. J., Freitag, M., Paulsen, I., Sachs, M. S., Lander, E. S., Nusbaum, C., and Birren, B. (2003) *Nature* **422,** 859–868
35. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
36. Clamp, M., Cuff, J., Searle, S. M., and Barton, G. J. (2004) *Bioinformatics* **20,** 426–427
37. Selker, E. U., and Garrett, P. W. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 6870–6874
38. Selker, E. U. (1990) *Annu. Rev. Genet.* **24,** 579–613
39. Margolin, B. S., Freitag, M., and Selker, E. U. (1997) *Fungal Genet. Newsletter* **44,** 34–36
40. Vogel, H. J. (1964) *Am. Nat.* **98,** 435–446
41. Manolson, M. F., Proteau, D., Preston, R. A., Stenbit, A., Roberts, B. T., Hoyt, M. A., Preuss, D., Mulholland, J., Botstein, D., and Jones, E. W. (1992) *J. Biol. Chem.* **267,** 14294–14303
42. Pall, M. L. (1993) *Fungal Genet. Newsletter* **40,** 58
43. Ninomiya, Y., Suzuki, K., Ishii, C., and Inoue, H. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 12248–12253
44. Davis, R. H. (2000) *Neurospora: Contributions of a Model Organism*, pp. 283–303, Oxford University Press, New York
45. Bowman, B. J., Allen, R., Wechser, M. A., and Bowman, E. J. (1988) *J. Biol. Chem.* **263,** 14002–14007
46. Bowman, E. J., Tenney, K., and Bowman, B. J. (1988) *J. Biol. Chem.* **263,** 13994–14001
47. Margolles-Clark, E., Tenney, K., Bowman, E. J., and Bowman, B. J. (1999) *J. Bioenerg. Biomembr.* **31,** 29–37
48. Hunt, I. E., and Bowman, B. J. (1997) *J. Bioenerg. Biomembr.* **29,** 533–540
49. Sista, H., Wechser, M. A., and Bowman, B. J. (1994) *Mol. Gen. Genet.* **243,** 82–90
50. Melnik, V. I., and Bowman, B. J. (1996) *Biochim. Biophys. Acta*

51. Oka, T., Yamamoto, R., and Futai, M. (1998) *J. Biol. Chem.* **273,** 22570–22576
52. Sze, H., Schumacher, K., Muller, M. L., Padmanaban, S., and Taiz, L. (2002) *Trends Plant Sci.* **7,** 157–161
53. Davis, R. H. (1986) *Microbiol. Rev.* **50,** 280–313
54. Bowman, E. J., Graham, L. A., Stevens, T. H., and Bowman, B. J. (2004) *J. Biol. Chem.* **279,** 33131–33138
55. Bowman, E. J., O'Neill, F. J., and Bowman, B. J. (1997) *J. Biol. Chem.* **272,** 14776–14786
56. Zhou, Z., Peng, S. B., Crider, B. P., Slaughter, C., Xie, X. S., and Stone, D. K. (1998) *J. Biol. Chem.* **273,** 5878–5884
57. Kluge, C., Seidel, T., Bolte, S., Sharma, S. S., Hanitzsch, M., Satiat-Jeunemaitre, B., Ross, J., Sauer, M., Golldack, D., and Dietz, K. J. (2004) *BMC Cell Biol.* **5,** 29
58. Nishi, T., and Forgac, M. (2000) *J. Biol. Chem.* **275,** 6824–6830
59. Manolson, M. F., Yu, H., Chen, W., Yao, Y., Li, K., Lees, R. L., and Heersche, J. N. (2003) *J. Biol. Chem.* **278,** 49271–49278
60. Hurtado-Lorenzo, A., Skinner, M., El Annan, J., Futai, M., Sun-Wada, G. H., Bourgoin, S., Casanova, J., Wildeman, A., Bechoua, S., Ausiello, D. A., Brown, D., and Marshansky, V. (2006) *Nat. Cell Biol.* **8,** 124–136
61. Nishi, T., Kawasaki-Nishi, S., and Forgac, M. (2003) *J. Biol. Chem.* **278,** 5821–5827
62. Malkus, P., Graham, L. A., Stevens, T. H., and Schekman, R. (2004) *Mol. Biol. Cell* **15,** 5075–5091
63. MacLeod, K. J., Vasilyeva, E., Baleja, J. D., and Forgac, M. (1998) *J. Biol. Chem.* **273,** 150–156