Disruption of \textit{vma-1}, the Gene Encoding the Catalytic Subunit of the Vacuolar H\textsuperscript{+}-ATPase, Causes Severe Morphological Changes in \textit{Neurospora crassa}\textsuperscript{*}

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Emma Jean Bowman‡, Ryan Kendle, and Barry J. Bowman

From the Department of Biology, University of California, Santa Cruz, California 95064

By using the process of Repeat-induced Point mutation (Selker, E. U., and Garrett, P. W. (1988) \textit{Proc. Natl. Acad. Sci. U. S. A.} 85, 6870–6874), we inactivated \textit{vma-1}, the gene encoding subunit A of the V-ATPase of \textit{Neurospora crassa}. Two \textit{vma-1} mutant strains were characterized. One was mutated at multiple sites, did not make a protein product, and produced spores that only rarely germinated. The other had four point mutations, made a protein product, and produced viable spores. Neither strain had detectable V-ATPase activity. The \textit{vma-1} mutant strains did not grow in medium buffered to pH 7.0 or above or in medium supplemented with the cation Zn\textsuperscript{2+}. They were completely resistant to inhibition by concanamycin C, supporting our hypothesis that the V-ATPase is the \textit{in vivo} target of this antibiotic. Inactivation of the \textit{vma-1} gene had a pronounced effect on morphology and development of the organism. In the mutants tip growth was inhibited, and multiple branching was induced. The \textit{vma-1} mutant strains could not differentiate conidia or perithecia. They could grow slowly as mycelia and could donate nuclei in a sexual cross. A mutation in the plasma membrane ATPase, which suppressed the sensitivity of wild type \textit{N. crassa} to concanamycin, also proved effective in suppressing the sensitivity of a \textit{vma-1} null mutant to basic pH but did not correct the morphological defects.

Vacuolar H\textsuperscript{+}-ATPases (V-ATPases)\textsuperscript{1} are large, complex enzymes found on multiple members of the endomembrane system of eukaryotic cells and on the plasma membranes of many specialized cells (1, 2). They hydrolyze ATP and pump protons across membranes to acidify cellular compartments. They have been implicated in a number of cellular processes, including protein sorting, receptor-mediated endocytosis, zymogen activation, and pH and calcium homeostasis. Studies of V-ATPases in fungi have primarily focused on the process of vacuolar protein degradation and turnover, and as sequestration sites of potentially toxic ions, especially Ca\textsuperscript{2+}, as sites for basic amino acids and polyphosphate, as sites for protein degradation and turnover, and as sequestration sites of potentially toxic ions, especially Ca\textsuperscript{2+} (3–5).

Because of the central role of the V-ATPase in cellular metabolism, we anticipated that genes encoding it would be essential, and in animal cells that appears to be the case. In \textit{Drosophila melanogaster}, inactivation of \textit{tha55}, the gene encoding subunit B of the V-ATPase, resulted in a larval lethal phenotype (6). In \textit{Dictyostelium discoideum}, a slime mold, the failure to obtain a V-ATPase knockout strain combined with characterization of strains carrying antisense RNA for the proteolipid c subunit of the V-ATPase led to the conclusion that V-ATPase genes were probably essential in this organism (7). Similarly, we reported that \textit{vma-1}, the gene encoding the catalytic subunit A of the V-ATPase, was indispensable in the filamentous fungus \textit{Neurospora crassa}. In that study we generated a RIP/Rescue strain, which contained an inactivated \textit{vma-1} gene at the endogenous site and a functional second copy at an ectopic site. When the RIP/Rescue strain was crossed with the wild type, the mutated \textit{vma-1} gene behaved like a simple recessive lethal (8).

However, V-ATPase genes are clearly not essential in the yeast, \textit{Saccharomyces cerevisiae}, where deletion strains have been characterized in considerable detail. Perhaps the most defining and useful phenotype of VMA deletion strains in yeast is the response to pH; VMA delete strains grow reasonably well in acidic medium (pH 5.5) but fail to grow in basic medium (above pH 7) (9, 10). The strains are sensitive to high concentrations of Ca\textsuperscript{2+} (11) in the medium or of heavy metals such as Zn\textsuperscript{2+}, Mn\textsuperscript{2+}, and Cu\textsuperscript{2+} (12–14). Although not strictly \textit{pH}-dependent, inactivation of \textit{vma-1} results in a 

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‡ To whom correspondence should be addressed: Dept. of Biology, University of California, Santa Cruz, CA 95064. Tel.: 831-459-2245; Fax: 831-459-3139; E-mail: rbwman@biology.ucsc.edu.

1 The abbreviations used are: V-ATPase, vacuolar proton-translocating ATPase; CCC, concanamycin C; Hyg\textsuperscript{R}, resistant to hygromycin B; Hyg\textsuperscript{G}, sensitive to hygromycin B; VM, Vogel’s medium N; ccr, concanamycin resistant; pvn, putative vma null; CTC, chlorotetracycline; RIP, repeat-induced point.
Disruption of vma-1 in N. crassa

| Strain | Mating type | Genotype | Relevant phenotype |
|--------|-------------|----------|--------------------|
| 74     | A and a     | No mutation | Wild type          |
| 5883   | A           | arg12-    | Am                 |
| arg12  | a           | arg12-    | Arg                |
| bump   | A           | Unidentified | HygR Colonial growth |
| ccr4–5 | A and a     | pma-1     | CCC£a             |
| cvn1-121 | a         | amir3 19 | Am       |
| cvn2-53 | A and a     | arg12-    | Arg      |
| cvn2-53-19 | a       | vma-1-    | CCC£ slow         |
| RR24-7-48 | a       | vma-1-    | CCC£ slow         |
| vma-1RR1 | a         | vma-1-    | Am     |

£a CCC£ indicates resistant to concanamycin C.

TABLE I  N. crassa strains used in this study

The properties of the strains used in this work are summarized in Table I. Wild type and marker strains came from the Fungal Genetics Stock Center (FGSC, University of Kansas Medical Center, Kansas City, KS). Strain vma-1RR1 was described in Ferea et al. (18) and ccr4–5 in Bowman et al. (19). Strain RR24-7-48a was generated from a cross between vma-1RR1a and a strain with genetic markers for his-1, al-3, and m, linked genes on linkage group V near vma-1, given to us by Dr. Robert Metzenberg (Stanford University). Bump was constructed in collaboration with Dr. Metzenberg in an earlier attempt to inactivate vma-1 by sheltered RIP (20). We inserted a 2.4-kilobase pair SseI fragment of genomic DNA from vma-1-21 (21) into the plasmid pCSN43 (22), which has the hph gene that encodes hygromycin phosphotransferase and confers resistance to hygromycin B in N. crassa. The vma-1 fragment terminated at amino acid residue 484 (of 607) and presumably did not make a functional gene product. This plasmid, pRIP1, was introduced into the sheltered RIP host. Crossing of the transformant to wild type 74A resulted in recovery of the strain bump, which exhibited an extreme colonial growth habit. #a Genetic analysis showed that bump contained a wild type vma-1 gene at the endogenous locus on linkage group V and that the plasmid carrying the hph gene together with the partial vma-1 gene had integrated very near arg12 on linkage group II. Our hypothesis is that pRIP1 interrupted a gene of unknown function, causing the bump phenotype. Thus, fortuitously, bump had two copies of vma-1, a functional copy at the endogenous location and a non-functional copy at an exogenous location, making it a suitable strain for inactivation of the vma-1 gene by RIP as described under “Results.”

The origin of pvn (putative vma null) strains is described under “Results.”

Genetic Manipulations, Scoring of Phenotypes, and Growth Tests—For standard genetic analyses, i.e. crossing and analyzing progeny, the procedures of Davis and de Serres (23) were used. Because bump and pvn strains grow slowly and do not conidiate, they were maintained as heterokaryons with the sterile a+ m+ helper strain (FGSC 4564). The heterokaryons grew like wild type, but only the nuclei without the a− m− mutation could participate in a sexual cross (24). Typically, spores from a sexual cross were spread on agar plates, heated for 35 min at 60 °C to induce germination, and picked to 1 ml of liquid medium. After 5 days of growth at 30 °C and 5–7 days more at room temperature, phenotypes were tested. Progeny that grew well, i.e. not pvn strains, were spot tested on agar plates containing Vogel’s medium N (VM, a minimal medium salt solution) supplemented with 2% sorbose to induce colonial growth, 0.05% fructose, 0.05% glucose, and 0.025% insoluble and other supplements as needed. Slow-growing pvn strains were usually tested in 1 ml of liquid medium (VM with 2% sucrose plus supplements) or less frequently on agar plates with VM and 2% sucrose as the carbon source. Am− progeny were tested on medium supplemented with 0.2 mg/ml alanine at 1.5 mg/ml glycine; they grow on alanine but not glycine plates. Supplements for tests of Arg− or His− were 0.2 mg/ml arginine or histidine. Resistance to hygromycin B (HygR) was scored as the capacity to grow on medium containing 200 μg of hygromycin B/ml, added before autoclaving. The HygR phenotype was scored more easily in liquid medium than on agar plates. Resistance to concanamycin C was scored as the capacity to grow on agar plates with VM/sucrose-medium containing 0.2 μg/ml CCC, added after autoclaving, at pH 7.2 for non-pvn strains or at pH 5.8 for pvn strains.

For quantitative comparisons of the growth rates of wild type and pvn strains, we measured colony diameters on agar plates containing VM and 2% sucrose with supplements as indicated under “Results.” Plates were inoculated with the wild type from a conidial suspension, and plates were inoculated with the pvn strain from a plug of culture from a colony on an agar plate. Colony diameter was measured for an 8-h interval following the lag phase for the wild type and at 24-h intervals for the pvn strains during growth at 30 °C. To measure growth as the increase in mass, cells were grown in aerated liquid medium. Wild type cultures were inoculated with 106 conidia per ml. Because pvn strains have an unusual growth morphology and do not produce asexual conidia, an alternative procedure was necessary. Bits of mycelium were scraped off an agar surface or tweezed apart from a liquid tube culture and grown at 25 °C for 2–4 days in 25 ml of medium on a reciprocal shaker. The resultant balls of mycelium were suspended in fresh medium and macerated for 30 s in a Waring blender, yielding a uniform suspension of mycelia. An aliquot of this mycelial suspension was collected and extracted with acetone to determine the dry weight. Liquid cultures were inoculated with the suspension to give 0.025 mg dry wt of mycelium/ml of medium. To measure doubling times cells were grown at 25 °C in 4 liters of medium with forced air. Dry weights were determined on 50–100 ml aliquots taken during the exponential growth phase, between 0.1 and 1.2 mg/ml. In some experiments growth yield was compared as the dry weight of mycelium produced in 25 ml of liquid medium grown 2–4 days at 25 °C on a shaker.

Analysis of vma-1 in pvn Strains—To demonstrate the presence of a single, mutated copy of vma-1 in pvn strains, we used Southern blotting and DNA sequencing. Genomic DNA was extracted from N. crassa by the method of Oakley et al. (25), digested with BmiHI, electrophoresed, and transferred to Nytran using a TE-90 Transvac vacuum blotter (Schleicher and Schuell, Keene, NH). The blot was probed with a radioactive 1632-nucleotide BglII/BamHI fragment of vma-1 (21), using the method of Feinberg and Vogelstein (26). The endogenous copy of vma-1 was amplified by polymerase chain reaction with specific primers, 5'-CAATACCTCTACACCACCCC-3' and 5'-CAGAGACCCCATGCAT-CATGG-3', and subcloned to pCR2.1 from the Original TA Cloning Kit (Invitrogen, Carlsbad, CA). The cloned DNA was sequenced on a Perkin-Elmer ABI310 Sequencer, using the Big Dye dye terminator kit (Perkin-Elmer).

Arginine Content in Cells and ATPase Activity in Membranes—The arginine content in whole cell extracts was assayed as described (19). Vascular membranes, plasma membranes, and mitochondria were isolated as described (27) and modified (19). In addition, the supernatant remaining after pelleting plasma membranes at 49,000 × g was subjected to a final high speed centrifugation step (1 h at 40,000 rpm in a Beckman Ti60 rotor, 161,000 × g neat) to collect a “light membrane” fraction, expected to contain any membranes not previously pelleted and to be enriched in endoplasmic reticulum. Protein and ATPase activities were assayed as before (27).

Microscopy—To visualize the growing front of mycelium, cultures were grown as colonies on agar plates. Regions of hyphae were photographed directly by bright field microscopy. To examine vacuoles, mycelia were grown overnight in liquid and observed under Nomarski optics. We used Kodak TMY 400 print film and a Leitz Aristoplan microscope and camera.

RESULTS

Isolation of a vma-1 Null Strain from the RIP/Rescue Strain—To generate a strain of N. crassa that lacked a functional V-ATPase, we used the process of RIP (28). N. crassa is a haploid organism, which readily takes up DNA by transformation and incorporates it into the genome either by homologous recombination or more frequently by recombination at 2  E. J. Bowman, unpublished results.
random locations. When a second copy of an endogenous gene is introduced by transformation and the transformant is crossed to another strain, a unique process called RIP (Repeat-induced point mutation) scans the genome and introduces multiple point mutations into both copies of the duplicated regions of DNA. Because the efficiency of RIP varies, individual progeny from such a cross can have many, few, or no mutations in the duplicated gene. By using this procedure, we earlier generated a RIP/Rescue strain, vma-1RR1, in which the endogenous copy of vma-1 was inactivated by multiple point mutations and a second, functional copy was introduced on a different chromosome. The strain grew like wild type. Attempts to generate a strain that contained only the inactivated endogenous vma-1 gene by genetics were unsuccessful, leading us to propose that vma-1 was an essential gene in N. crassa (8).

A more recent cross gave a different result. We exploited the fact that the vma-1 gene is tightly linked (less than 1% recombination) to the am gene on chromosome V. The RIP/Rescue strain, vma-1RR1, is am+ and has a RIPed vma-1 gene. It also contains a functional vma-1 gene, introduced on a plasmid with an hph gene, which confers resistance to hygromycin, on a different, unidentified chromosome. Strain vma-1RR1 was crossed to the wild type strain 74A (Table II, Cross 1). Mature, germinated spores were picked to liquid medium. At first all crossed to the wild type strain 74A (Table II, Cross 1). Mature, different, unidentified chromosome. Strain vma-1RR1a was vma-1 contains a functional ectopic copy of vma-1, and the sequence of the endogenous gene from strain vma-1RR1 (Fig. 1B) and Ref. 8). The gene contained 42 point mutations, one of which converted amino acid residue 130 in the endogenous gene on chromosome V. Thus, bump was a good candidate to use in RIPing experiments, and it fortuitously led us to a

To determine whether the slow growth phenotype was caused by inactivation of the vma-1 gene, we used both genetic and biochemical tests. First, strain pvn1-121A was crossed to the wild type 74a to look for linkage of slow growth to Am (Table II, Cross 1). Many spores failed to germinate. Among spores that did germinate, 306 were Am+ and grew fast like wild type, and 7 were Am− and grew slowly like pvn1. Thus, slow growth cosegregated with am− and the adjacent vma-1 locus. The low recovery of pvn1 progeny and the high number of ungerminated spores further suggested to us that the mutation resulted in a low rate of germination, approximately 1% if we assume that most of the ungerminated spores had the pvn1 genotype.

In a second test we found that a functional ectopic copy of vma-1 rescued the slow growth phenotype in pvn1. In a genetic cross we replaced the chromosome V that carried the vma-1 RIP gene in strain vma-1RR1 with a chromosome V carrying wild type genes for am and vma-1 and mutant marker genes for his-1, al-3, and int loci. This new strain, RR24-7-48a, was crossed to pvn1-121A (Table III). All the progeny grew like wild type. Among the Am+ progeny, which were predicted to be vma-1+, half were HygS and half were HygR, indicating that they were able to grow with or without the ectopic vma-1 gene. The Am− progeny, predicted to be vma-1RIP, were all HygR+ thus able to grow like wild type when a functional ectopic copy of vma-1 was present. Twelve additional progeny, not included in Table II, were the result of crossover events between am and int loci, and strains with the vma-1RIP gene could grow when the functional ectopic copy was present. In this particular experiment none of the 86 isolates displayed the pvn1 phenotype, presumably because of the low germination rate of pvn1 spores.

Our interpretations of the genetic experiments were supported by Southern blot analysis and sequencing. Genomic DNA digested with BamHI and probed with vma-1 DNA revealed a single band in the wild type strain and two bands, corresponding to the endogenous and the ectopic genes, in the RIP/Rescue parent (Fig. 1A). pvn1-121A had a single band, of the size predicted for the endogenous gene on chromosome V. Finally we used the polymerase chain reaction to clone the endogenous vma-1 gene from strain pvn1-121A. The sequence was identical to that previously reported for the vma-1RIP gene in strain vma-1RR1 (Fig. 1C and Ref. 8). The gene contained 42 point mutations, one of which converted amino acid residue 130 (of 607) into a stop codon.

We concluded that pvn1-121A was indeed a viable vma-1 null strain of N. crassa. The pvn phenotype was tightly linked to am− on chromosome V. At slow growth, cosegregated with am− and the adjacent vma-1 locus. The slow growth recovered pvn1 progeny and the high number of ungerminated spores further suggested to us that the mutation resulted in a low rate of germination, approximately 1% if we assume that most of the ungerminated spores had the pvn1 genotype.

### Table II

| Strain | Genotype | Phenotype | Progeny from cross |
|--------|----------|-----------|-------------------|
| Cross 1 | am−vma-1RIP LGV | Am HygR Fast | Am HygR Fast | 61 |
| vma-1RR1a | hph vma-1− LG? | | Am HygS Fast | 21 |
| 74A | am−vma-1− LGV | Am HygR Slow | Am HygR Fast | 32 |

### Table III

| Strains: pvn1−121A × RR24−7−48a |
|---|---|---|
| Genotypes: am−vma-1RIP his-1− al-3− int− LGV × am−vma-1− his-1− al-3− int− LGV; hph vma-1− LG? |
| Progeny | HygS | HygR |
| am−vma-1− his-1− al-3− int− LGV | 35 | 23 |
| am−vma-1− his-1− al-3− int− LGV | 28 | 63 |

### Table IV

| Strains: pvn1−121A × RR24−7−48a |
|---|---|---|
| Genotypes: am−vma-1RIP his-1− al-3− int− LGV × am−vma-1− his-1− al-3− int− LGV; hph vma-1− LG? |
| Progeny | HygS | HygR |
| am−vma-1− his-1− al-3− int− LGV | 35 | 23 |
| am−vma-1− his-1− al-3− int− LGV | 28 | 63 |

**Isolation of a vma-1 Null Strain from the Mutant Strain Bump**—The strain bump is a tight colonial mutant that was generated by an alternative approach to inactivate vma-1 as described under “Experimental Procedures.” In essence, bump contains a normal endogenous vma-1 gene on chromosome V and a partial vma-1 gene, incorporated via a plasmid with an hph gene (confers resistance to hygromycin), on linkage group II near the arg12 locus. Thus, bump was a good candidate to use in RIPing experiments, and it fortuitously led us to a
second pvn strain, pvn2, which differs in certain properties from pvn1.

In an effort to map the chromosomal location of bump more precisely, we crossed it to an arg12 strain (Table IV, Cross 1). Fifty progeny had the Arg12 phenotype, 43 had the bump phenotype, and 3 were Arg, slow-growing strains reminiscent of pvn1 mutants in phenotype. We conducted further tests to determine if they were pvn1 mutants. In two strains, the slow growth phenotype was not linked to either am or arg12. However, the third strain showed clear linkage between the slow growth phenotype and am (Table IV, Cross 2) and exhibited the characteristic morphology of pvn1 on an agar plate, making it a promising candidate for a second vma-1 null strain. It was named pvn2. A genetic cross demonstrated that the pvn2 phenotype could be rescued by a functional, ectopic copy of vma-1 (Table IV, Cross 3). The cross was potentially confusing because the two pvn strains had different alleles of vma-1 (Table IV, Cross 1). The inheritance of one or the other allele was inferred by scoring the closely linked am locus; vma-1RIP in pvn1 was linked to am+, and vma-1RIP in pvn2 was linked to am-1. HygB served as the marker for the functional, ectopic vma-1+ gene. As before, pvn1 progeny grew only when the ectopic vma-1+ gene was present (22 isolates). pvn2 progeny grew like wild type in the presence of the ectopic vma-1+ gene (15 isolates), indicating rescue, and pvn2 progeny germinated and grew slowly in the absence of the ectopic vma-1+ gene (17 isolates). Thus, in contrast to ascospores of strain pvn1, ascospores of pvn2 showed no impairment in germination (Table III, Crosses 2 and 3).

Southern blot analysis confirmed that a single copy of vma-1 DNA was present at the endogenous locus in strain pvn2 (Fig. 1B), and sequence analysis confirmed that this DNA was mutated (Fig. 1D). RIPing in strain pvn2 was light. Four nucleotides within the coding region of the gene were mutated from G to A, predicting amino acid residue changes in subunit A of G170S, D266N, G375S, and G392S. No stop codons were introduced, suggesting that pvn2, unlike pvn1, could make a protein product. Whether the protein would be functional was harder to predict. All four of the affected residues are conserved among eukaryotic V-ATPases but not in the comparable residue of the homologous β subunits of F-ATPases. Only one, G170S, is conserved in both vacuolar and the related archaebacterial ATPases. This is an interesting residue because of its location within the “non-homologous” region of V-ATPases, the region which has no counterpart in F1β. The function of this region in V-ATPases remains unknown; however, its deletion from VMA1 in S. cerevisiae appeared to be lethal.3

We concluded that the slow growth phenotype of pvn2 was due to mutation of the vma-1 gene by RIP. In contrast to pvn1, the pvn2 spores germinated efficiently, and a protein product was likely to be made. Because the strain grows so poorly, it probably produces either no active V-ATPase or enzyme with minimal activity.

Growth Properties of pvn1 and pvn2—To examine the effect of gene inactivation on the growth of N. crassa we surveyed a number of conditions found to be inhibitory to VMA deletion strains in S. cerevisiae. In nature filamentous fungi grow primarily by apical extension of hyphae over a solid surface. For most of our growth experiments we measured the increase in colony diameter on an agar plate. The pvn strains grew slowly compared with wild type, but the visible growth on plates was

3 L. Taiz, University of California, Santa Cruz, personal communication.
However, a high concentration of NaCl (1.0 M) inhibited growth of wild type strains. 0.1 M NaCl, expected to inhibit, also had no effect. Inositol, reported to stimulate growth of a strain in complete medium (Table V), similar to the results reported for S. cerevisiae (11), had no effect on the N. crassa pvn strains. The sensitivity of the pvn strains from N. crassa to several other compounds is summarized in Table VI. Growth was measured as the acid-soluble arginine in whole cell extracts. Strains pvn1 and pvn2 accumulated 5.7 and 5.2 nmol of Arg/10 mg dry weight of cells grown in concanamycin A to inhibit the V-ATPase (19). These results indicated lack of a functional V-ATPase in either pvn1 or pvn2. By using our standard procedures (27), we isolated membrane fractions containing gradient-purified mitochondria, vacuolar membranes, plasma membranes, and “light” membranes (endoplasmic reticulum and others), and compared ATPase activities in strains 74A and pvn2-53-19A. We thought it necessary to collect all the membrane fractions because our procedure for isolation of vacuolar membranes depends on having osmotically active vacuoles that become heavy when cells are lyzed in 1 M sorbitol and pellet with the dense mitochondria. In the absence of V-ATPase activity the vacuoles do not accumulate the same amount of solutes (e.g. arginine and other basic amino acids) and probably do not behave as dense organelles in the fractionation procedure. Instead, isolated vacuolar membranes, which have a low density, would be expected to be in the light membrane fraction. In 74A the V-ATPase concentrations of these substances. Several ions have been reported to be potent inhibitors of growth in yeast VMA deletion strains (12–14). Only 4 mM Zn\(^{2+}\) was particularly potent against the N. crassa pvn strains. Mn\(^{2+}\) (3 mM) and Fe\(^{2+}\) (3 mM) were weakly inhibitory, and even high Ca\(^{2+}\) (100 mM) inhibited the pvn strains only 50% while also mildly affecting the wild type. Cu\(^{2+}\) appeared to stimulate growth of the pvn strains. Compared with the wild type, the N. crassa pvn strains grew reasonably well on non-fermentable carbon sources, 1.5% glycerol and 50 mM sodium acetate, as measured by linear extension. However, the mycelial density was considerably reduced in both wild type and pvn mutants on these media. When grown on a highly enriched, undefined medium (complete), all three strains grew marginally faster than the 100% controls on minimal medium, and the mycelial mats looked more filled in on the rich medium.

**Lack of V-ATPase Function in pvn1 and pvn2—Arginine**, together with other basic amino acids, is stored at high concentrations in fungal vacuoles. Accumulation of arginine is dependent on the proton gradient generated by the V-ATPase (29). Consequently, a convenient and straightforward method to assess *in vivo* function of the V-ATPase is to measure the acid-soluble arginine in whole cell extracts. Strains pvn1 and pvn2 accumulated 5.7 and 5.2 nmol of Arg/10 mg dry weight of cells, respectively, approximately 1/5th the amount found in 74A (28.7 nmol/10 mg) or the RIP/Rescue strain vma-1RR1 (26.7 nmol/10 mg) and similar to the amount found in cells grown in concanamycin A to inhibit the V-ATPase (19). These results indicated lack of a functional V-ATPase in either pvn1 or pvn2. By using our standard procedures (27), we isolated membrane fractions containing gradient-purified mitochondria, vacuolar membranes, plasma membranes, and “light” membranes (endoplasmic reticulum and others), and compared ATPase activities in strains 74A and pvn2-53-19A. We thought it necessary to collect all the membrane fractions because our procedure for isolation of vacuolar membranes depends on having osmotically active vacuoles that become heavy when cells are lyzed in 1 M sorbitol and pellet with the dense mitochondria. In the absence of V-ATPase activity the vacuoles do not accumulate the same amount of solutes (e.g. arginine and other basic amino acids) and probably do not behave as dense organelles in the fractionation procedure. Instead, isolated vacuolar membranes, which have a low density, would be expected to be in the light membrane fraction. In 74A the V-ATPase activity.

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**TABLE IV**

| Strain | Genotype | Phenotype | Progeny from cross |
|--------|----------|-----------|-------------------|
| Cross 1 | hph vma-1<sup>am</sup> arg12<sup>+</sup> LGII | Arg<sup>+</sup> Hgy<sup>a</sup> Fast, Colonial | Am<sup>+</sup> Hgy<sup>a</sup> Fast, Colonial |
| arg12a | arg12<sup>-</sup> LGII | Hgy<sup>a</sup> Fast | Hgy<sup>a</sup> Slow |
| Cross 2 | arg12<sup>-</sup> LGII | Am<sup>+</sup> Arg<sup>+</sup> Fast | Am<sup>+</sup> Arg<sup>+</sup> Fast, Colonial |
| amΔ132A | arg12<sup>-</sup> LGII | Am<sup>+</sup> Arg<sup>+</sup> Fast | Am<sup>+</sup> Arg<sup>+</sup> Slow |
| Cross 3 | am<sup>+</sup> vma-1<sup>RIP(2)</sup> LGV | Am<sup>+</sup> Hgy<sup>a</sup> Slow | Am<sup>+</sup> Hgy<sup>a</sup> Slow |
| vma-1RR1a | am<sup>+</sup> vma-1<sup>RIP(2)</sup> LGV | Am<sup>+</sup> Hgy<sup>a</sup> Fast | Am<sup>+</sup> Hgy<sup>a</sup> Fast, Colonial |

<sup>a</sup> ND, not determined.
specific activity (pH 7.5, CCC-sensitive) was 2.0 μmol/min/mg of protein in vacuolar membranes and less than 0.1 in the other membrane fractions. Strain pvn2-53-19A had no detectable V-ATPase activity in any membrane fraction, as expected if the strain produces no active V-ATPase.

Pvn Mutants Demonstrate Striking Differences in Morphology and Development—In N. crassa, the effects of disrupting the vma-1 gene on morphology and development were dramatic. Both pvn strains had a distinctive colony morphology on 2% agar (Fig. 2A). Although the wild type rapidly spreads across the surface, primarily by hyphal tip extension, and produces aerial hyphae that result in a cottony appearance, the pvn strains progressed slowly and produced few aerial hyphae. Then the colony filled in behind the growing front, resulting in a characteristic layered appearance. Comparison of the growth fronts at the microscopic level showed a clear difference in branching patterns (Fig. 3). Wild type grows primarily by apical extension and produces side branches at infrequent intervals, resulting in a rapid advance across the agar surface. In the pvn strains branching was primarily dichotomous and frequent, resulting in a crowded growth front that advanced forward slowly.

Vacuoles in VMA disruptants of S. cerevisiae (10) and A. gossypii (17) were reported to appear normal. This was not the case in the pvn strains of N. crassa. Compared with wild type vacuoles those in the two pvn strains were more irregular, often misshapen, and frequently appeared to be multilamellar (Fig. 4). Together, the cell fractionation results and microscopic analysis of vacuoles strongly suggested that vacuolar morphology was significantly altered by inactivation of vma-1 in N. crassa.

The pvn strains had also lost the capacity for some types of cellular differentiation. N. crassa has an interesting life cycle that requires the development of several different morphological structures (30). When nutrients are available, wild type N. crassa grows vegetatively as filamentous hyphae that fuse and intertwine to form a mycelial mat, the defining state of a filamentous fungus. Environmental conditions such as nutrient depletion or desiccation induce a conidiation pathway. Aerial hyphae form, followed by differentiation into asexual spores called conidia. Conidia can germinate and grow into a new mycelium or function as a male parent in a sexual cross. On crossing medium, which is low in nitrogen, N. crassa enters a sexual cycle. Hyphae of opposite mating types (A and a) fuse and form a complex fruiting body, the perithecium. Within this

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**TABLE V**

| Strain      | Growth rate | Doubling time |
|-------------|-------------|---------------|
|             | Colony diameter, VM | VM | Complete |
|             | mm/h | h |
| 74A         | 4.2   | 2.5  | 2.3 |
| pvn1–121A   | 0.50  | 4.6  | 3.2 |
| pvn2–53-19A | 0.63  | 3.2  | 2.6 |

**TABLE VI**

Effect of selected compounds on growth of pvn strains

| Carbon source | 2% sucrose | Wild-type 74A | pvn1–121A | pvn2–53-19A |
|---------------|------------|---------------|-----------|-------------|
| Addition      | %control   | %control     | %control  |
| None          | 100        | 63           | 100       |
| Alanine, 0.2 mg/ml | 104      | 100          | 103       |
| CCC, 0.3 μM   | 34         | 110          | 100       |
| Inositol, 2 mg/ml | 108     | 115          | 100       |
| 0.1 mM NaCl   | 108        | 112          | 97        |
| 0.3 mM NaCl   | 91         | 73           | 78        |
| 1.0 mM NaCl   | 32         | 0            | 13        |
| 4 mM ZnCl₂   | 110        | 19           | 13        |
| 3 mM MnCl₂   | 110        | 90           | 72        |
| 3 mM FeCl₂   | 110        | 96           | 75        |
| 1 mM CuCl₂   | 110        | 150          | 127       |
| 100 mM CaCl₂ | 87         | 47           | 57        |
| 200 mM CaCl₂ | 66         | 27           | 36        |
| 1% glucose   | 110        | 116          | 108       |
| 1.5% glycerol| 84         | 108          | 87        |
| 50 mM NaOAc  | 71         | 58           | 60        |
| Complete     | 110        | 121          | 105       |
structure the nuclei fuse to form the only diploid cell in the life cycle and immediately undergo meiosis. The four meiotic products divide, yielding eight nuclei, each of which develops into a large tough spore called an ascospore. The ascospores can lie dormant for years or be induced by heat shock to germinate and go through the vegetative life cycle.

pvn1 and pvn2 mutants were blocked at several stages of development in the life cycle. They produced only short aerial hyphae and never formed conidia, the asexual spores. They could not differentiate into a fruiting body and thus could not function as a female parent. However, they did function as a male parent, donating a nucleus in a sexual cross to a fertile strain such as 74A. In such a cross mature ascospores were produced. The wild type spore progeny germinated efficiently. Consistent with the results described above, pvn1 and pvn2 mutant spores differed significantly in germination rate. Spores carrying the pvn1 mutation germinated at low frequency (0.5–1.0%), but spores with the pvn2 mutation germinated as efficiently as wild type. Upon germination, both strains grew slowly, forming highly branched hyphae as described above.

By using the $a^{m1}$ helper strain, which carries a mating type mutation (24), we were able to investigate the consequences of crossing pvn mutants to themselves. We constructed heterokaryons containing nuclei from the $a^{m1}$ helper strain and from each of the pvn strains. These heterokaryons grew like wild type and made abundant conidia and protoperithecia, indicating that the $vma-1^{RIP}$ alleles were recessive. By using these heterokaryons, we made pvn1-121A × pvn1-121a and pvn2-53-19A × pvn2-53-19a crosses. Although the helper strain allows for formation of perithecia, it does not contribute to development of asci and ascospores. In both pvn crosses ascus development was arrested in meiotic prophase. In pvn1-121A × pvn1-121a the homologous chromosomes showed little or no pairing. In pvn2-53-19A × pvn2-53-19a arrest was somewhat later. Chromosomes did pair, and some anaphase stages and chromosome segregation were observed. However, in both crosses ascus aborted prior to development of individual ascospores, and no mature ascospores were formed.

In summary, inactivation of $vma-1$ resulted in multiple defects in morphogenesis of N. crassa. Pvn strains grew slowly, showed altered hyphal branching patterns, failed to differentiate conidia or perithecia, arrested in meiosis, and in the case of pvn1 spores germinated only rarely. These results point to an important role of the V-ATPase, either direct or indirect, in all of these processes.

Partial Suppression of the pvn Phenotype by a pma-1 Mutation and by Sorbitol—To begin to dissect the role of the V-ATPase in these processes, we sought conditions that could suppress the pvn phenotype. By having previously isolated and characterized mutants that could partially suppress sensitivity

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4 N. B. Raju, Stanford University, personal communication.
to concanamycin A (or C) (19), we first tested these mutants for the capacity to suppress the pvn phenotype. The ccr strains are mutated in pma-1, the gene encoding the plasma membrane H^+-ATPase. They allow N. crassa to grow in the presence of concanamycin at basic pH. The effect of the mutation is to change the kinetic behavior of the plasma membrane H^+-ATPase. In the ccr mutant strain the K_m for ATP is lower, and the curve that describes rate of hydrolysis versus ATP concentration is hyperbolic, as opposed to the sigmoid curve observed in the wild type. We have suggested that the mutants are resistant to concanamycin because the altered plasma membrane H^+-ATPase can use low levels of ATP more efficiently than the wild type enzyme. Thus, substances like calcium or divalent cations, released from the vacuole into the cytosol when the V-ATPase is inhibited by concanamycin, can be pumped out of the cell and prevented from attaining toxic concentrations in the cytosol.

We constructed double mutants of pvn2-53-19 and ccr-4-5 by a genetic cross. (We were unable to obtain pvn1, ccr-4-5 double mutants, presumably due to the deficiency in spore germination.) The most significant effect of the ccr mutation was suppression of the pH-sensitive phenotype. In the experiment shown in Fig. 5, pvn2-53 did not grow at pH 7.0 and above, but the double mutant grew over the same range of pH as the wild type. Growth of pvn2-53 and of pvn2-53, ccr-4-5 was also compared under the conditions given in Table V. In these tests the double mutant grew marginally better, 10–20%, than the pvn2 mutant (data not shown) and was completely resistant to concanamycin. The double mutant had the same morphological and developmental deficiencies as the pvn2 single mutant strain. Thus, the ccr mutation was effective in suppressing the sensitivity of pvn to basic pH yet had little effect on other phenotypes caused by inactivation of vma-1.

While testing growth of pvn strains on different media, we added 1 M sorbitol to the medium as a test of osmotic sensitivity. After several days of growth conidia were produced by the pvn2 strain (or pvn2, ccr-4-5 double mutant) but not the pvn1 strain. Thus, some signal brought about by the high concentration of sorbitol was able to overcome the deficiency of the pvn2 strain in forming conidia. We are investigating the explanation for this surprising phenomenon.

**DISCUSSION**

**Phenotype of vma-1 Null Mutants in N. crassa**—To our knowledge, deletion strains for genes encoding V-ATPase subunits have been described in only three organisms. Deletion of the gene encoding subunit B in D. melanogaster resulted in an embryonic lethal phenotype (6). In the yeast S. cerevisiae and the closely related filamentous fungus A. gossypii, deletion of V-ATPase genes was not lethal but did cause a number of growth defects. We have previously reported that vma-1, which encodes subunit A of the vacular ATPase, appeared to be essential in the filamentous fungus N. crassa (8). However, in the present paper we have succeeded in inactivating vma-1, and we show that the gene is not essential for vegetative growth but is necessary for normal morphological development.

Inactivation of the V-ATPase had pleiotropic effects on the growth of N. crassa. The vma-1 null strains showed clear pH sensitivity, growing best at pH 5.5 and not at all at pH 7.0 or above. Conditional lethality at basic pH has become a hallmark of VMA deletion strains in S. cerevisiae (9, 10) but, curiously, was not observed in A. gossypii (17). The basis of the pH sensitivity is not understood. Growth of the N. crassa vma-1 null strains was strongly inhibited by the divalent cation Zn^{2+} and weakly affected by Mn^{2+} and Fe^{2+}. We were surprised by the observation that 1 mM Cu^{2+} stimulated the radial expansion of colonies on agar plates. By contrast, in S. cerevisiae, Zn^{2+}, Mn^{2+}, and Fe^{2+} all strongly inhibit growth of a VMA deletion strain, and Cu^{2+} has given contradictory results (13, 14). Which divalent cations (other than Ca^{2+}) are stored in the vacuole of N. crassa has not been reported, but these results implicate the vacuole as a critical organelle for storage of Zn^{2+}. It was also important to observe that the pvn strains were completely resistant to inhibition by concanamycin C, thus strengthening our original finding that the V-ATPase is the cellular target for this powerful antibiotic.

The most important consequence to the cell of inactivating the V-ATPase may well be the effect on Ca^{2+} homeostasis. Electrophysiological experiments have demonstrated that the vacuole of N. crassa accumulates high concentrations of Ca^{2+} (300 μM) and is largely responsible for maintenance of low concentrations of Ca^{2+} in the cytosol (0.1 μM) (31). High concentrations (100 or 200 μM) of Ca^{2+} in the medium restricted growth of the N. crassa vma-1 null strains by approximately 50–60% but also affected the wild type to a lesser extent (10–20% inhibition). By comparison, high Ca^{2+} concentrations strongly inhibit growth of VMA disruptant strains of S. cerevisiae (11) and have little effect on the VMA1 disruptant in A. gossypii (17). Preliminary results of staining with chlorotetracycline (CTC) suggested that vacuolar Ca^{2+} stores were de-
pleted in our vma-1 null strains.\(^5\) CTC fluoresces when bound to Ca\(^{2+}\) in the vicinity of a membrane and is suited to visualizing Ca\(^{2+}\) accumulated in vesicles and organelles. It has been used to visualize Ca\(^{2+}\) gradients in the growing hyphal tips of filamentous fungi (32). When we added CTC to N. crassa cells, we saw brightly glowing hyphal tips in the wild type and detected no fluorescence in the vma-1 null strains. We have not yet attempted to measure Ca\(^{2+}\) concentrations in the cytosol of the vma-1 null mutants.

When growth was assessed as an increase in mass of an aerated liquid culture, the N. crassa vma null strains behaved similarly to VMA disruptants in S. cerevisiae and A. gossypii, i.e. growth was slower but not dramatically different from the wild type. In either minimal or complete medium, the two mutant strains of N. crassa had doubling rates of 1.3- and 1.8-fold those of the wild type. Similar to A. gossypii and different from S. cerevisiae, N. crassa vma-1 null strains grew on non-fermentable substrates. Because N. crassa is an obligate aerobe, the mitochondria must be functional in the vma null strains and not compromised as suggested by the petite phenotype reported in early studies of VMA deletion strains of S. cerevisiae (11).

**Inactivation of vma-1 Has Striking Effects on Cell Morphogenesis in N. crassa**—The most obvious and pronounced effects of inactivating the vma-1 gene in N. crassa were on cellular morphology and development. The primary effect appeared to be on the control of tip elongation and hyphal branching. In the vma-1 null strains the rate of hyphal extension at the apex was greatly decreased, and the frequency of branching was substantially increased. This change could account for many of the observed growth phenotypes, the slow progress of vma-1 strains across an agar surface, and the thickening of the mycelial mat on an agar surface, the formation of round balls in shaking liquid culture, and the failure of mycelia to reach the surface in standing liquid. It might also restrict the ability of the organism to form conidia, which differentiate on the ends of aerial hyphae, to form perithecia, which differentiate from a mass of mycelium, and to germinate from spores, where a tip must emerge from the spore body. Although similar phenotypes have not been observed in VMA disruptants of S. cerevisiae, a temperature-sensitive mutant of VMA4 exhibited abnormal morphologies including abnormally elongated or multiple buds (16). Development was also affected in the VMA1 disruptant of A. gossypii, where cells grew in pellets, which appeared more dense and less branched, and failed to produce generative spores (17).

How branching is controlled in filamentous fungi is not understood. Normal tip growth involves a number of processes including cell wall digestion, cytoplasmic streaming, and organelar distribution and depends on an organized actin cytoskeleton (reviewed in Refs. 32–35). Some of the agents that elicit increased branching affect the cell wall or the cytoskeleton as follows: for example the sugar t-sorbitol and several fungicides, all inhibitors of β-1-3-glucan synthetase (35); and cytochalasin A (36), an antiaecin drug. Other agents appear to disrupt calcium homeostasis as follows: for example A23187, a Ca\(^{2+}\) ionophore (37); verapamil, a Ca\(^{2+}\) channel blocker (38); and cyclosporin A, an inhibitor of calcineurin, a Ca\(^{2+}\)-calmodulin-dependent protein phosphatase (39). In addition, colonial mutants characterized by profuse branching are plentiful in fungi dependent protein phosphatase (39). In addition, colonial mutants characterized by profuse branching are plentiful in fungi.

A logical deduction is that the primary effect on cell metabolism of inactivating the V-ATPase is to raise the cytosolic free Ca\(^{2+}\), which results in the gross changes in morphogenesis. Ca\(^{2+}\) normally accumulates in vacuoles of N. crassa and other fungi, and this sequestration depends on the activity of the V-ATPase. In S. cerevisiae, deletion of a V-ATPase gene was shown to increase cytosolic Ca\(^{2+}\) concentrations (11).

**Vacular Morphology in vma-1 Null Strains Is Abnormal**—In VMA deletion mutants of both S. cerevisiae and A. gossypii the vacuoles had normal morphology. This was not the case in N. crassa. Vacuoles in the vma-1 null strains were highly variable when viewed under the light microscope. Some were small and round like wild type organelles, but others were large and distorted, frequently appearing as vacuoles within vacuoles. This would suggest a role for the V-ATPase in determining organelar structure within the endomembrane system, either direct or indirect via Ca\(^{2+}\) and the cytoskeleton.

**Phenotypes of pvn1 and pvn2 Are Not Identical**—In this work we inactivated the vma-1 gene by RIPing, which introduces point mutations into the genomic DNA sequence (28), rather than by gene disruption as used in S. cerevisiae and A. gossypii. Fortuitously, we isolated strains with a heavily RIPed gene (pvn1, 42 point mutations) or a lightly RIPed gene (pvn2, 4 point mutations). Both strains appeared to have non-functional V-ATPases. We detected no concanamycin-sensitive ATPase activity in membranes from the mutants, and both strains accumulated similar low levels of total cellular arginine, an indicator of lack of vacuolar function in whole cells. The two pvn strains had similar abnormalities in hyphal morphology, in vacuolar morphology, and in responses to basic pH or inhibitors.

They differed strongly in one phenotype, spore viability. Spores of pvn1 rarely germinated, demonstrating an important role of the V-ATPase in this process. Indeed, the V-ATPase might be essential for germination. Rare germings may have resulted from inclusion of maternal material with an active V-ATPase in the spore as happens with mitochondria. Spores of pvn2 germinated with high efficiency. The two strains showed small differences in two other properties. Pvn2 grew slightly faster than pvn1 on agar plates or in liquid cultures, and pvn2 arrested in meiosis at a later stage than pvn1. Because pvn2 probably makes a vma-1 protein product and pvn1 does not, two explanations for the phenotypic differences seem plausible. First, although we could not detect V-ATPase activity in pvn2, a very low activity, below the sensitivity of our assay, could be present and account for the observed differences. Second, the presence of the protein product itself may be required for spore germination. Possibly, subunit A of the V-ATPase has a second, unknown function in the cell.

**Selective Suppression of the vma-1 Null Phenotype**—If wild type N. crassa is grown in the presence of concanamycin, the cells exhibit grossly distorted hyphal morphology and are unable to grow at pH 7.2. We previously isolated concanamycin-resistant strains that had mutated alleles of pma-1, the gene that encodes the plasma membrane H\(^+\)-ATPase (19). The pma-1 mutations suppressed the toxicity of concanamycin at basic pH but did not suppress the morphological abnormalities. The mutations changed the kinetic behavior of the enzyme, lowering the K\(_m\) for ATP from 1.2 mM in the wild type to 0.2–0.6 mM in the mutants. We hypothesized that the concanamycin-resistant mutants partially suppressed the sensitivity of

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\(^5\) B. J. Bowman and E. J. Bowman, unpublished results.
signals for inducing conidiation in the culture, and desiccation is one of the environmental effects on other phenotypes, nor did we see conidiation in the presence of a high salt concentration (1.0M NaCl) or a 45 °C temperature shock treatment. 1.0M sorbitol is likely to desiccate the culture, and desiccation is one of the environmental signals for inducing conidiation in N. crassa (43). Perhaps sorbitol acts via the same pathway to induce conidiation in pvn2. The simplest postulate for why pvn2 conidates and pvn1 does not is that pvn2 does make a V-ATPase of very low activity and that the stress of desiccation induces expression of the vma-1 gene and synthesis of subunit A, resulting in sufficient V-ATPase activity to allow conidiation.

Conclusion—Inactivation of vma-1, the gene encoding subunit A of the V-ATPase, has multiple effects on N. crassa. In particular, these effects implicate the V-ATPase as involved in normal apical growth, perhaps regulating signals for branching and differentiation. Our findings also implicate the V-ATPase in the processes of germination and meiosis. As a relatively simple eukaryote, which nevertheless shows considerable morphological diversity, N. crassa is a good model organism to investigate the participation of the V-ATPase in these complex phenomena.

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REFERENCES
1. Nelson, N., and Harvey, W. B. (1999) Physiol. Plant. 105, 361–385
2. Stevens, T. H., and Forger, M. (1997) Annu. Rev. Cell Dev. Biol. 13, 779–808
3. Davis, R. H. (1986) Microbiol. Rev. 50, 380–313
4. Klionsky, D. J., Herman, P. K., and Emr, S. D. (1990) Microbiol. Rev. 54, 266–292
5. Jones, E. W., Webb, G. C., and Hiller, M. A. (1997) Molecular biology of the Yeast Saccharomyces cerevisiae, Vol. III, pp. 363–470, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
6. Davies, A. S., Goodwin, S. F., Kelly, D. C., Wang, Z., Sozen, M. A., Kaiser, K., and Dow, J. A. T. (1996) J. Biol. Chem. 271, 30677–30684
7. Xie, Y., Coulomb, M. B., and Gombar, Z. (1996) J. Cell Sci. 109, 1989–1997
8. Ferreira, T. L., and Bowman, B. J. (1996) Genes Dev. 10, 1217–1225
9. Nelson, H., and Nelson, N. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 3503–3505
10. Yamashiro, C. T., Kane, P. M., Wolczyk, D. F., Preston, R. A., and Stevens, W. H. (1990) Mol. Cell. Biol. 10, 3717–3734
11. Ohye, Y., Umemoto, N., Tanida, I., Ohba, A., Iida, H., and Anraku, Y. (1991) J. Biol. Chem. 266, 13971–13977
12. Bachhawat, A., Manolson, M. F., Murdock, D. G., Garman, J. D., and Jones, E. W. (1993) Yeast 9, 175–184
13. Eide, D. J., Brigham, J. T., Zhao, Z., and Mattoon, J. B. (1993) Mol. Gen. Genet. 241, 247–256
14. Ramsay, L. M., and Gadd, G. M. (1997) J. Gen. Microbiol. 143, 9442–9448
15. Supek, F., Supekova, L., and Nelson, N. (1994) J. Biol. Chem. 269, 26479–26485
16. Ziarat, J. W., Parra, J. K., Liu, J., and Kane, P. M. (1998) J. Biol. Chem. 273, 18470–18480
17. Förster, C., Santos, M. A., Ruffert, S., Kramer, R., and Revuelta, J. L. (1999) J. Biol. Chem. 274, 9442–9448
18. Drose, S., and Altendorf, K. (1997) J. Exp. Biol. 200, 1–8
19. Bowman, E. J., O'Neill, F. J., and Bowman, B. J. (1997) J. Biol. Chem. 272, 14776–14786
20. Harkness, T., Metzenberg, R. L., Schneider, H., Lill, R., Neupert, W., and Nargang, F. E. (1994) Genes Dev. 8, 107–118
21. Bowman, E. J., Tenney, K., and Bowman, B. J. (1988) J. Biol. Chem. 263, 13994–14001
22. Staben, C., Jensen, B., Singer, M., Pollock, J., Schechtman, M., Kinsey, J., and Selker, E. (1989) Fungal Genet. Newslett. 36, 79–81
23. Davis, R. H., and de Serres, F. J. (1970) Methods Enzymol. 17, 79–143
24. Perkins, D. D. (1984) Fungal Genet. Newslett. 31, 41–42
25. Oakley, C. E., Weil, C. F., Kretz, P. L., and Oakley, B. R. (1987) Gene (Amst.) 53, 293–298
26. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13
27. Bowman, E. J., and Bowman, B. J. (1988) Methods Enzymol. 157, 562–573
28. Selker, E. U., and Garrett, P. W. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 5670–5674
29. Zerres, C. R., Weiss, R. L., Franklin, C., and Bowman, B. J. (1986) J. Biol. Chem. 261, 8877–8882
30. Rauj, N. B. (1992) Mycol. Res. 96, 241–262
31. Miller, A. J., Vogg, G., and Sanders, B. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 9348–9352
32. Jackson, S. L., and Heath, I. B. (1993) Microbiol. Rev. 57, 367–382
33. Harford, J. W. (1994) in Growth, Differentiation, and Sexuality (Wessels, J. G. H., and Meinhardt, F., eds) Vol. 1, pp. 89–109, Springer-Verlag, New York
34. Heath, I. B. (1994) in Growth, Differentiation, and Sexuality (Wessels, J. G. H., and Meinhardt, F., eds) Vol. 1, pp. 85–104, Springer-Verlag, New York
35. Trinei, A. P. J., Wiebe, M. G., and Robson, G. D. (1994) in Growth, Differentiation, and Sexuality (Wessels, J. G. H., and Meinhardt, F., eds) Vol. 1, pp. 175–183, Springer-Verlag, New York
36. Riquelme, M., Beyna-Pena, C. G., Gierz, G., and Bartnicki-Garcia, S. (1998) Fungal Genet. Biol. 24, 101–109
37. Schmid, J., and Harbold, F. M. (1988) J. Gen. Microbiol. 134, 2623–2631
38. Dicker, J. S., and Turian, G. (1990) J. Gen. Microbiol. 136, 1413–1420
39. Proksch, H., Yarden, O., Diemer, M., Tropschug, M., and Bartelsme, I. B. (1997) Mol. Gen. Genet. 256, 104–114
40. Perkins, D. D., Radford, A., Newmeyer, D., and Bjorkman, M. (1982) Microbiol. Rev. 46, 420–570
41. Seiler, S., Nargang, F. E., Steinberg, G., and Schliwa, M. (1997) EMBO J. 16, 3025–3034
42. Yarden, O., Plamann, M., Ebbole, D. J., and Yanofsky, C. (1992) EMBO J. 11, 2159–2166
43. Springer, M. L., and Yanofsky, C. (1989) Genes Dev. 3, 559–571