Physiological Consequence of Disruption of the VMA1 Gene in the Riboflavin Overproducer Ashbya gossypii*

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The vacuolar ATPase subunit A structural gene VMA1 of the biotechnologically important riboflavin overproducer Ashbya gossypii was cloned and disrupted to prevent riboflavin retention in the vacuolar compartment and to redirect the riboflavin flux into the medium. Cloning was achieved by polymerase chain reaction using oligonucleotide primers derived from conserved sequences of the Vma1 proteins from yeast and filamentous fungi. The deduced polypeptide comprises 617 amino acids with a calculated molecular mass of 67.8 kDa. The deduced amino acid sequence is highly similar to that of the catalytic subunits of Saccharomyces cerevisiae (67 kDa), Candida tropicalis (67 kDa), and Neurospora crassa (67 kDa) with 89, 87, and 60% identity, respectively, and shows about 25% identity to the β-subunit of the F1F0-ATPase of S. cerevisiae and Schizosaccharomyces pombe. In contrast to S. cerevisiae, however, where disruption of the VMA1 gene was conditionally lethal, and to N. crassa, where viable disruptants could not be isolated, disruption of the VMA1 gene in A. gossypii did not cause a lethal phenotype. Disruption of the AgVMA1 gene led to complete excretion of riboflavin into the medium instead of retention in the vacuolar compartment, as observed in the wild type.

Vacuolar H+-ATPases (V-ATPases) belong to a highly conserved family of proton pumps that provide the energy required for transport processes in the vacuolar system of eukaryotic cells (1–5). V-ATPase activity generates and maintains an acidic pH inside the organelles of the central vacuolar system, including lysosomes, endosomes, the Golgi apparatus, secretory vesicles, and clathrin-coated vesicles (4). Its action is also responsible for Ca2+ and pH homeostasis of the cytosolic compart-ment (6). The V-ATPase is similar in structure and subunit composition to that of the catalytic subunits of the F1F0-ATPase of the inner mitochondrial membrane (7). In yeast, the V-ATPase is composed of at least 10 subunits, which range in mass from 17 to 100 kDa (8). It is composed of a V0 complex of integral membrane proteins forming the proton translocating channel (9–13) and a catalytic V1 complex of hydrophilic, peripherally associated proteins facing the cytoplasm. The V1 subunit is composed of the 69-kDa (14) and 60-kDa (15) proteins representing the catalytic and regulatory subunits of the V-ATPase and additionally contains the 54-, 42-, 32-, and 27-kDa subunits (11, 16, 17).

Yeast mutants lacking any of these vacuolar membrane ATPase subunits (with exception of the 100-kDa subunit; Ref. 18) display the characteristic vma-phenotype (6). This phenotype is conditionally lethal and cells have a dysfunctional vacuole (15–17, 19–23). The mutation affects growth, e.g. increased calcium sensitivity as well as the inability to grow on nonfermentable carbon sources or in medium buffered at neutral pH (6). Isolated vacuolar membranes from Saccharomyces cerevisiae vma mutants lack ATPase activity, suggesting an essential role of vacuolar acidification in growth. In Neurospora crassa, attempts have been made to inactivate the VMA1 and VMA2 genes by repeat-induced point mutations (26, 27). Strains lacking a functional copy of the VMA1 or VMA2 gene were not viable. Data on the phenotype of filamentous fungi lacking a functional V-ATPase are therefore missing.

The filamentous fungus Ashbya gossypii (28) is used for industrial riboflavin production. Biosynthesis (29), regulation, and production parameters (30) have been studied, resulting in well established fermentation processes with a reported maximum yield of 15 g/liter (30). Riboflavin production by A. gossypii starts in the late growth phase when septa are formed in the hyphae and vacuoles become visible. Concomitantly, vacuoles begin to accumulate large amounts of riboflavin. The vacuolar compartmentation of metabolites, e.g. amino acids, plays a major role in the regulation of metabolism in fungi in general (31), and the retention of riboflavin in the vacuolar compart-ment is a crucial factor in riboflavin production by A. gossypii. The present work addresses the compartmentation of metabolites in A. gossypii VMA1 disruptants and the resulting redirection of riboflavin fluxes.

MATERIALS AND METHODS

Strains and Media—A. gossypii strain ATCC 10895 mycelium was grown overnight in liquid medium (MA2) consisting of 2 g/liter yeast extract, 20 g/liter peptone, 0.6 g/liter myo-inositol (Sigma), and 10 g/liter glucose, pH 6.8, at 28 °C in shaking flasks on a rotary shaker at 120 rpm. For compartmentation analysis under production conditions, the producing strain ItaGS01 (30) was grown in 10 g/liter soybean oil, 10 g/liter yeast extract, and 6 g/liter glycine under the same conditions.

General Molecular Biology Techniques—Restriction endonucleases, T4 DNA ligase, Klentov DNA polymerase I fragment, and other enzymes were purchased from either Boehringer Mannheim, New Eng-land Biolabs, or Amersham Pharmacia Biotech.

DNA preparation, restriction enzyme digestions, ligation of DNA fragments, Southern blots, etc., were carried out according to standard techniques (32). Radioactive labeling of DNA was performed with [α-32P]dCTP and the Klenow DNA polymerase I fragment (32). Transformation of A. gossypii spores was done by electroprotecor (33). For

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PCR,1 a mixture of oligonucleotide primers was used derived from conserved amino acid regions from different Vma1 proteins (see Fig. 1). Oligonucleotides CF1a (5′-ATCGCCTATGTCGCTG-3′) and CF1b (5′-ATCGCCTATGTCGCTG-3′) were purchased from MWG Biotech (Ebersberg, Germany). PCR (30 s at 94 °C, 60 s at 52 °C, and 60 s at 72 °C for 36 cycles) was carried out using Taq polymerase (Boehringer Mannheim) buffer conditions as recommended by the manufacturer, 8 μM primer CF1a, 4 μM primer CF1b, and the Gene Amp® PCR System 9700 (Applied Biosystems). Chromosomal A. gossypii DNA as template was prepared according to standard techniques (32).

Isolation of the VMA1 Gene—The VMA1-PCR fragment amplified using primers CF1a and CF1b was used to screen an A. gossypii prepared according to standard techniques (32). radioactive labeling of DNA with [3H]cosmid clones containing homologous regions of DNA, we performed cosmid library in the cosmid vector SuperCos1 (Stratagene). To identify conserved amino acid regions from different Vma1 proteins (see Fig. 1).

buffer conditions as recommended by the manufacturer, 8

mM tetramethylammonium chloride and 12% (v/v) acetonitrile at a flow rate of 1 ml/min.

Translation of the VMA1 open reading frame reveals that it codes for an acidic protein (calculated pI, 5.21) of 429 amino acids and has a predicted molecular mass of 47,806 daltons. The ATP-binding site motif P (P-loop) is well conserved in the catalytic subunits of vacuolar ATPases. This motif (AG-X4-Y-G-K-(S/T)) appears at positions 257–264 in the predicted AgVma1 polypeptide. Genetic and biochemical studies using Vma1p from S. cerevisiae have identified several amino acid residues involved in either enzyme assembly or catalysis. Two cysteine residues (Cys284 and Cys539), one tyrosine residue (Tyr343), and one glycine residue (Gly250) that are conserved in all A. subunits sequenced so far and have been shown to be important for the correct folding or stability of the A subunit are also present in the Ashbya Vma1p protein (45, 46). Likewise, the essential aromatic residues located at the catalytic site Phe452, Tyr532, and Phe538, as well as the acidic Glu286 residue proposed to participate in the hydrolysis of ATP, are all conserved in AgVma1p (46, 47).

Disruption of the VMA1 Gene—To study the properties of cells lacking the VMA1 gene product, the VMA1 gene was disrupted by replacing a 0.25-kb fragment with a genetic resistance marker controlled by promoter and terminator sequences of the A. gossypii TEF gene (kan’ cassette G418) (36). The existence of only one copy of the VMA1 gene in A. gossypii was determined by restriction analysis with several restriction enzymes.

RESULTS

Isolation and Sequence Analysis of the AgVMA1 Gene—To isolate the AgVMA1 gene, degenerate oligonucleotide primers were derived from two highly conserved regions of Vma1p encoding sequences of Saccharomyces cerevisiae (15), Candida tropicalis (41), Schizosaccharomyces pombe (42), and N. crassa (43) (Fig. 1). Using these primers in a PCR reaction with chromosomal DNA from A. gossypii as template, a 585-bp DNA fragment was amplified, purified, and cloned into Smal-digested pUC19 DNA for sequence determination. This fragment

was found to contain sequences encoding a polypeptide that exhibited strong similarity to vacuolar ATPase A subunits from different organisms.

To isolate a fragment encompassing the entire AgVMA1 gene, the radiolabeled 585-bp PCR fragment was used to probe an A. gossypii cosmid library. The PCR probe hybridized to four independent clones. Restriction analyses with BamHI and an second hybridization with the 585-bp PCR fragment revealed four different sets of overlapping inserts. Two hybridizing BamHI subfragments of approximately 1 and 7 kb were isolated from one of the positive cosmids and cloned into Bluescript SK+ to generate pJRI796 and pJRI797. Its nucleotide sequence was determined in both strands. The nucleotide sequence of a 3440-bp BamHI-SphI fragment contained part of an unidentified open reading frame, AgURF1 (600 bp) and a complete open reading frame (1854 bp), capable of encoding a protein of 617 amino acids. The deduced amino acid sequence of the 1854-bp open reading frame showed 89% identity with the ScVma1p, 87% identity with the CtVma1p, and more than 60% identity with the SpVma1p and NcVma1p, suggesting that this open reading frame encodes subunit A. We thus designated the gene AgVMA1. Direct evidence for the expression of AgVMA1 came from Northern experiments. Total RNA from exponentially growing cells was fractionated by electrophoresis, transferred to a nylon membrane, and hybridized to the radioactive labeled 585-bp PCR fragment. A single transcript of approximately 2.1 kb, sufficient to accommodate the predicted 617-amino acid polypeptide, was detected (data not shown).

Analysis of the DNA sequence upstream of the AgVMA1 gene for transcriptional signals showed that the 5′-flanking region does not contain the consensus TATAAA sequence, although one copy of the functional variant TATAATA was identified beginning at position −237. Downstream of the open reading frame, there were several motifs commonly observed in the 3′ noncoding region of S. cerevisiae and thought to be necessary for transcription termination, processing of the 3′ end, or addition of poly(A) at the 3′ terminus. Thus, two copies of the hexanucleotide TACATA and one copy of the hexanucleotide TATATA that have been implicated in mRNA 3′ end formation in yeast can be recognized 6, 18, and 81 bp after the stop codon (44).

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enzymes and subsequent Southern blotting under conditions of decreasing stringency, using the 585-kb PCR fragment as a probe (data not shown). For the disruption experiment, the NcoI-SpeI fragment of the amplified 585-bp PCR fragment was isolated from pJR1685 and cloned into the polylinker site of a modified pGEM®T vector lacking the PstI site. The gene was disrupted on the plasmid by replacement of the BamHI-PstI fragment (0.25 kb) with the 2-kb TEF-G418 fragment (36) (Fig. 2A). The vma1::G418 fragment was liberated from the plasmid by NcoI-SpeI digestion, and A. gossypii ATCC 10895 spores were transformed by electroporation (33), thereby inducing DNA integration by homologous recombination. Homokaryotic G418-resistant transformants (vma1::G418) were obtained after sporulation and clonal selection of the primary heterokaryotic G418-resistant colonies and subjected to Southern analysis to confirm that the correct gene disruption occurred (Fig. 2B).

Agvma2 Phenotype—For S. cerevisiae it has been shown that disruption of the VMA1 gene severely affects growth (20). The resulting mutants only grow within a narrow pH range around 5.5. Furthermore, mutants did not grow on nonfermentable carbon sources, and sensitivity to high extracellular calcium concentrations has been detected (48). In A. gossypii, the growth parameters and the final biomass obtained were compared for wild type and disruptant strain with respect to growth on different media (Table I). Generally, the mutant cells had a distinct phenotype. As observed for the parental strain, cells grew in pellets but more dense and less branched (data not shown). Disruption of the VMA1 gene caused an increase in the lag phase from 5 to 12 h, but growth, however, was still possible on MA2 complete medium, pH 6.8. The growth rate of VMA1 disruptant cells was decreased by about 50% (Table I). Growth on the nonfermentable carbon source soybean oil was possible and did not exhibit distinct growth parameters (Table I). Because the V-ATPase has an established role in acidifying the vacuolar system, we studied the effect of external pH on growth of the A. gossypii VMA1 mutants (Table I). Surprisingly, mutant cells grew better under neutral pH conditions than under acid conditions. Nevertheless, no particular pH value could be found that fully restored growth of the mutant cells. In contrast to the data reported for yeast (6), cells were only slightly sensitive to elevated Ca2+ concentrations (100 mM) (Table I). We did not observe formation of generative spores in the disruptant cells, irrespective of using widely ranging pH values and Ca2+ concentrations in the media used, of carbon source supplied, neither in liquid nor in solid media.

A qualitative assay of vacuolar pH was employed to prove the acidification defect of the VMA1 disruptants. Cells were labeled with acridine orange, a fluorescent dye that is trapped within acid compartments (49). The failure to acidify vacuoles in the disruptant strains was indicated by the lack of acridine orange accumulation in vacuoles as detected by fluorescence microscopy (Fig. 3). By phase contrast microscopy it could be shown...
that vacuoles of the VMA1 disruptants are of the same size and number as vacuoles of the wild type. To further confirm the loss of V-ATPase activity in vacuolar membranes of the VMA1 disruptants, we isolated a membrane fraction from highly purified vacuoles. Severe osmotic shock was employed to lyse the vacuoles. Disrupted as described. Compartmentation analysis showed that, in the disruptant strains, the redirection was found to be even more pronounced: 90% of the glycine, 96% of the lysine, and 100% of the vacuolar arginine were found in the medium, even more pronounced: 90% of the glycine, 96% of the lysine, and 100% of the vacuolar arginine were found in the medium, respectively. Our results thereby confirm an essential role of the V-ATPase in the vacuolar compartmentation of amino acids.

During riboflavin production, effective accumulation of this vitamin into the vacuole, partly forming riboflavin crystals in this compartment, could be observed by fluorescence microscopy (data not shown). From a total production of 140 μmol/mg deionized water, 55 μmol/mg deionized water was retained in the vacuolar compartment in the parental strain ItaGS01 (Fig. 4). By inhibition of the vacuolar ATPase with concanamycin A, redirection of riboflavin fluxes could be detected, and the entire product was excreted into the medium (Fig. 4). To construct a production strain with a dysfunctional V-ATPase, the VMA1 gene of the riboflavin producer strain ItaGS01 (30) was disrupted as described. Compartmentation analysis showed that, as described for amino acids, an effective redirection of riboflavin fluxes toward the medium took place (Fig. 4).

DISCUSSION

The vacuolar system is a vital metabolic compartment in fungal cells. The physiological function of the H⁺-ATPase in the vacuolar membrane is not yet entirely understood. In yeast, its action gives rise to a low vacuolar pH value of 5.5–6.2 and an electrochemical proton potential of approximately 180 mV.
The energy of the proton potential is used for the vacuolar compartmentation of metabolites, e.g. basic amino acids (53), as well as for other energy requiring processes, e.g. protein sorting (54). Disruption of the genes encoding vacuolar membrane ATPase subunits has been shown to be lethal in *N. crassa* (26, 27) and conditionally impaires cell growth in *S. cerevisiae* (15–17, 19–23). *S. cerevisiae vma* mutants still grow in a medium of pH 5.5, which led to the suggestion that acidification of the vacuolar system by equilibration with the external medium occurs by fluid phase endocytosis (55). Consequently, the phenotype of cells lacking a functional vacuolar ATPase has only been examined in *S. cerevisiae*.

In the present work, with *A. gossypii* VMA1 disruptants we describe the first viable VMA disruptant in yeast and fungi that does not display the conditionally lethal *vma* phenotype. Several results indicate that AgVMA1 encodes subunit A, namely (i) the similarity of the amino acid sequence deduced from the gene to other Vma1 proteins, (ii) the observation that disruption of the chromosomal VMA1 gene resulted in a dys-

![Image](220x243 to 554x732)

**Table II**

| Compartmentation of amino acids | Compartmentation of amino acids |
|---------------------------------|---------------------------------|
|                                 | Wild type | Wild type, 5 μM concanamycin A | VMA1::G418 |
|                                 | % of total content | % of total content | % of total content |
| Glycine                         | 8.5 ± 2.5 | 20.0 ± 2.3 | 12 ± 1.7 |
| Cytosol                         | 47.9 ± 2.3 | 9.5 ± 1.2 | 5 ± 1.3 |
| Vacuole                         | 43.6 ± 2.8 | 70.5 ± 5.0 | 83 ± 4.8 |
| Medium                          | 9.1 ± 1.3 | 11.1 ± 1.6 | 14 ± 4.0 |
| Lysine                          | 31.3 ± 0.5 | 1.3 ± 0.3 | 2.0 ± 0.7 |
| Vacuole                         | 59.6 ± 0.8 | 87.6 ± 1.0 | 84 ± 0.4 |
| Medium                          | NDb | ND | ND |
| Arginine                        | 37 ± 7.0 | ND | ND |
| Vacuole                         | 63 ± 13.0 | 100 ± 10.0 | 100 ± 8.0 |

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*Values referred to dry weight.

b ND, below detection limit of 1 μM.*
functional vacuole, and (iii) the fact that no vacuolar H⁺-ATPase activity was determined in vacuolar membranes from vma− strains.

The predicted amino acid sequence of subunit A was found to be highly similar to those of the catalytic subunits of other yeast and fungi. The construction of a dendrogram of vacuolar membrane ATPase subunit A proteins (Vma1p) shows that Vma1p from A. gossypii is the subunit A most closely related to Vma1p from S. cerevisiae. Lower but still significant similarity to β-subunits F₂,F₁-ATPases is also observed. The molecular mass of subunit A was calculated to be about 68 kDa, which corresponds to the size of other Vma1 proteins (56). Homologous regions in Vma1p from S. cerevisiae contain several residues that have been proven to be important for correct folding or stability of the A subunit and to participate in the hydrolysis of ATP in S. cerevisiae, respectively (56). Even with high identity to ScVma1p, the gene encoding A. gossypii subunit A does not comprise an intein sequence as observed in S. cerevisiae (57) and C. tropicalis (58).

Strikingly, the vacuolar ATPase proved not to be essential for viability in A. gossypii, i.e. cells could adapt to the loss of V-ATPase function. In disruptant strains the vacuolar accumulation of metabolites, e.g. amino acids, was bypassed by excretion into the growth medium. This might also be valid for the sequestration of toxic substances and for the accumulation of calcium. An important physiological role of the vacuole is protein turnover and targeting; consequently, the vacuole contains a large number of membrane-bound and soluble hydrolases (58). Proteins are delivered to the vacuole through an endosomal intermediate (54, 60). Nevertheless, it is known for a subset of proteins that transit to the vacuole via the secretory pathway that their secretion is achieved by an alternative subset of proteins that transit to the vacuole via the secretory pathway, bypassing the endosome (61). It may be thus concluded in view of the adaptation of A. gossypii to the loss of V-ATPase activity that an alternative pathway of proteins into the vacuole might be present, too. Although growth was possible, we never observed sporulation in the A. gossypii disruptant strains. From studies with S. cerevisiae it is known that sporulation requires the formation of a prospore membrane (62). The synthesis of this membrane takes place in a developmentally regulated branch of the secretory pathway in the vacuolar system in yeast (9, 62), thus rendering VMA disruptants unable to sporulate.

We further studied compartmentation of amino acids between the cytosol and the vacuolar space in A. gossypii wild type strain in the absence and presence of concanamycin A as well as in VMA1 disruptants. Similar to yeast (51), compartment analysis in A. gossypii cells showed significant vacuolar amino acid pools. In filamentous fungi, the confinement of amino acids into different organelles might have an important regulatory role, e.g. to avoid catabolism of these compounds when biosynthetic need exists (63). Even though excretion of amino acids in the disruptant strains deprives the cell of reusable storage of these compounds, it might be crucial for the ability of cells to adapt to the loss of V-ATPase function.

As a biotechnologically important product, A. gossypii excretes riboflavin. At the same time, a significant part of the product is retained in the vacuolar compartment. Inhibition of the vacuolar ATPase with concanamycin A, as well as disruption of the VMA1 gene, prevented accumulation of riboflavin in the vacuoles of A. gossypii. Instead, the product was completely excreted into the medium. These results strongly indicate that the vacuolar accumulation of riboflavin depends on the action of the V-ATPase by using the electrochemical proton potential across the vacuolar membrane. With the disruptant cells from strain ItaGS01, a new production strain with an effective redirection of riboflavin flux into the medium has therefore been designed.

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