Disruption of the gene encoding the V-ATPase subunit A results in inhibition of normal growth and abolished sporulation in *Aspergillus nidulans*

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The authors have previously reported on molecular responses of *Aspergillus nidulans* to bacterial antifungal metabolites, e.g. bafilomycins and the related concanamycins. These compounds are known inhibitors of V-ATPases and cause dramatic effects on mycelial growth and morphology. In *Neurospora crassa*, studies have shown that disruption of the gene encoding subunit A of the V-ATPase results in morphological changes and reduced growth similar to those observed after addition of concanamycin. This phenotype, and the fact that this mutation confers resistance to concanamycin, suggests that V-ATPase is the main (or only) target for the antibiotics. However, growth inhibition and morphology changes in, for example, *A. nidulans* and *Penicillium roqueforti* are more severe, and thus other targets are possible. In this study, the *vmaA* gene of *A. nidulans*, encoding the subunit A of V-ATPase, was disrupted by homologous recombination. The resulting *vmaA¹* mutant strain displayed extremely slow growth and failed to produce asexual spores. Furthermore, an altered morphology similar to that caused by addition of V-ATPase inhibitors, i.e. bafilomycin or concanamycin, was observed, indicating that V-ATPase is the main target for the antibiotics also in *A. nidulans*. The *vmaA¹* mutant was not viable at pH values above 7 and was highly sensitive to high Zn²⁺ concentrations, in agreement with previous results from studies of *Saccharomyces cerevisiae* and *N. crassa*.

INTRODUCTION

The fungal vacuole is involved in several functions, e.g. macromolecular degradation, storage, and regulation of metabolites and ions (Klionsky et al., 1990; Weber, 2002). The pH of fungal vacuoles is regulated by vacuolar ATPases (V-ATPases) working as proton pumps. V-ATPases are present in all eukaryotes, in vacuoles in fungi and plants, and in several animal organelles such as endosomes and lysosomes (Forgac, 1989).

In a previous study we identified a strain of *Streptomyces halstedii* which produces compounds that both reduce the growth and dramatically alter the morphology of filamentous fungi. This effect was severe in all filamentous fungi tested but varied between species, e.g. *Penicillium roqueforti* is more sensitive than *Aspergillus fumigatus* (Frändberg & Schnürer, 1998). These substances were identified as bafilomycin B1 and bafilomycin C1 (Frändberg et al., 2000). The bafilomycins, and the related concanamycins, are known inhibitors of V-ATPases at nanomolar concentrations (Bowman et al., 1988; Dröse et al., 1993). Studies in *Neurospora crassa* showed that a similar growth inhibition and morphology change triggered by concanamycin treatment was obtained after disruption of *vma-1*, the gene encoding subunit A of the V-ATPase. These mutant strains were also resistant to concanamycin, suggesting that the V-ATPase is the only target for the antibiotic (Bowman et al., 2000). The inhibitory effect caused by bafilomycins is more severe in *Aspergillus* than in *Neurospora* (Werner & Hagenmaier, 1984). A further increase in antibiotic concentration results in increased swelling of *Aspergillus mycelium*, and growth is almost completely inhibited (unpublished observations). This indicates that a disruption of the *Aspergillus* V-ATPase gene results in a more severe phenotype. Alternatively, there may be additional, so far unidentified, targets for the antibiotics. Thus, it has been reported that bafilomycin is active against P-ATPases at micromolar concentrations (Bowman et al., 1988).

To investigate fungal responses to these antibiotics, two previous studies have employed mRNA differential display and proteomics approaches, respectively, to search for genes and proteins in *A. nidulans* involved in the response (Melin et al., 1999, 2002). These studies have so far only identified...
a small number of genes/gene products. One of these, breA (down-regulated in response to bafilomycin treatment), had previously been identified as aspn1, encoding a cell-wall-associated zinc-binding protein involved in zinc uptake into the cytoplasm (Segurado et al., 1999).

The objective of this study was to investigate the V-ATPase of Aspergillus, in particular with respect to the question whether additional targets for bafilomycins and concanamycins could be present. Here, we report the phenotypic effects of a disruption of the gene encoding one of the subunits of the A. nidulans V-ATPase. We anticipate that the construction of this mutant strain will be instrumental in further studies of the previously identified gene products whose abundance is affected by antibiotic treatment of the fungus.

**METHODS**

**Growth conditions.** The A. nidulans strains used in this study are summarized in Table 1. Strains were cultured on minimal medium supplemented as described by Kaminskyj (2001). A. nidulans genetic methods followed standard protocols (Pontecorvo et al., 1953). For cloning and plasmid propagation Escherichia coli DH5α was used (Sambrook et al., 1989).

**Preparation of DNA.** The procedure for growing and maintaining strains for DNA preparation varied. To extract DNA from the vmaA1 mutant strains (PM6 and PM7), fractions of the colony were crushed using a toothpick, and DNA was extracted with CTAB as described by Talbot (2001). Prior to digestion, the genomic DNA was purified by phenol/chloroform extraction.

### Table 1. A. nidulans strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source*</th>
</tr>
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<tbody>
<tr>
<td>A4</td>
<td>Wild-type</td>
<td></td>
</tr>
<tr>
<td>PW1</td>
<td>biA1; argB2; methG1; veA1</td>
<td>FGSC</td>
</tr>
<tr>
<td>A851</td>
<td>pabaA1; ya2; ArgB::trpCAR; veA1; trpC801</td>
<td>P. Weglenski</td>
</tr>
<tr>
<td>PM6</td>
<td>biA1; argB2; methG1; veA1; vmaA1::argB</td>
<td>This study</td>
</tr>
<tr>
<td>PM7</td>
<td>pabaA1; veA1; vmaA1::argB†</td>
<td>This study</td>
</tr>
</tbody>
</table>

*FGSC, Fungal Genetic Stock Center, Kansas City, KS, USA; P. Weglenski, Institute of Genetics, Warsaw University, Poland.
†This strain may also carry the mutations argB2 and ya2.

**Cloning and disruption of the vmaA gene.** A 2 kb fragment containing the vmaA gene was PCR-amplified (forward primer 5'-GTA TGG ATC CCA CTG TGG GA-3'; reverse primer, with an introduced Xbal site, 5'-CTC TTC TAG ACG ACC AGA AAC CTG-3') using pfu turbo polymerase (Stratagene). The DNA fragments were digested with BamHI and Xhol and subcloned into a modified (disrupted Xhol and Sal sites) pBS SK+ vector plasmid (Stratagene). Next, the construct was digested with Xhol to replace a 112 bp fragment, which includes the translation initiation codon, by the complete A. nidulans argB gene. The vmaA disruption construct was transformed as previously described (Melin et al., 2003). DNA used for transformation was PCR-amplified (primers as above) using taq+ polymerase (Stratagene) and purified with QiaQuick (Qiagen). The gene disruption was confirmed by Southern blot analysis, using a vmaA-specific probe which was 32P-labelled by random priming, according to standard methods (Fig. 1; Sambrook et al., 1989).

**Viability tests.** Tests for sensitivity to bafilomycin B1 and concanamycin A were performed as previously described (Melin et al., 2002), with the exception that ascospores (equivalent to approximately 200 c.f.u. ml⁻¹) were used instead of conidia. To test the viability of the vmaA1 strain, ascospores (2000 c.f.u. ml⁻¹) from the cross between vmaA1 and A851 were plated on minimal plates containing various additions, supplemented with p-aminobenzoic acid, and incubated at 30 °C. To increase the pH, plates were buffered with 0·2 M K₂HPO₄. To decrease the pH, the plates were buffered with 20 mM MES. Zn²⁺ concentrations were raised from the standard 0·15 mM by adding ZnSO₄. To increase the standard iron concentration (3·6 nM Fe²⁺) we used either FeSO₄ or Fe₃(SO₄)₂.

**RESULTS AND DISCUSSION**

**Identification of the vmaA gene.** The only identified V-ATPase subunit in A. nidulans is subunit B. The gene encoding it, vmaB, was identified by
its location directly downstream of the tryptophan synthase gene, *trpB* (Eckert et al., 2000). However, since subunit A is the catalytic subunit, and gene disruptions in both *Saccharomyces cerevisiae* and *N. crassa* had been characterized, we wanted to compare the obtained phenotypes and therefore decided to disrupt the corresponding *vmaA* gene in *A. nidulans*. The amino acid sequence of subunit A in *N. crassa* (accession no. J03955) was blasted against the *A. nidulans* Sequence Database (http://www.broad.mit.edu/annotation/fungi/aspergillus/index.html) to obtain the corresponding DNA sequence in *A. nidulans*. After retrieval of the *vmaA* candidate gene sequence, it was observed that the predicted two homologous proteins showed a very high similarity, and that, like the *Neurospora* gene, the *Aspergillus* gene lacked a canonical transcription initiation site. The *A. nidulans* *vmaA* gene is predicted to contain two introns, and has a total length of approximately 2100 bp.

**A vmaA disruption phenotype is similar to that caused by bafilomycin or concanamycin**

Gene disruptions in *A. nidulans* can be created by homologous recombination, though high frequencies of non-homologous recombination can be expected to result in random integrations. Thus, the transformants obtained may not contain a disrupted target gene (Fincham, 1989). However, the correct *vmaA* mutation can be expected to result in a characteristic slow-growing and hyperbranching phenotype. PCR-amplified DNA from a vector plasmid containing the *argB* marker gene inserted within an internally deleted partial *vmaA* gene was transformed into an *argB* deletion strain (Fig. 1; see Methods). Two out of the approximately 200 arginine-prototrophic transformants showed the expected phenotype. One of these was later discarded since Southern blot analysis failed to confirm the correct mutation (data not shown). The other mutant strain (PM6) was crossed with a strain that completely lacked the *argB* gene to ascertain that the obtained phenotype was due to *vmaA* disruption. Cleistothecia were collected, separated from conidia and vegetative mycelia, and ascospores (all wild-type-like) were released. When selective medium was used, all viable ascospores showed the expected *vmaA1* mutant phenotype (here exemplified by PM7). Fig. 2 shows a Southern blot analysis confirming integration into the *vmaA* gene. An interesting observation is that the band position is different for all strains. The additional variation between the two mutants indicates an introduced position is different for all strains. The additional variation is due to methionine prototrophy in submerged cultures, the ascospores did germinate but the growth rate was even lower than on solid media. Therefore, DNA extraction was carried out from mycelia that had been collected from cellophane-covered agar plates. In cultures grown in liquid medium on a shaker (100 r.p.m.) growth of the fungus was arrested. This close-to-lethality of the *vmaA* mutation under normal conditions rendered further studies difficult. Establishment of growth conditions able to suppress or compensate for the mutation would thus be desirable. In *S. cerevisiae*, it has been reported that the growth rate of cells carrying a mutated V-ATPase subunit can be restored by adding 5 mM Fe$^{2+}$ or Fe$^{3+}$ (Eide et al., 1993). When 2 days. After 3 days, the mean colony diameter was 2 mm (wild-type colonies normally grow to 40 mm after 3 days). Another general characteristic is the hyperbranching phenotype with swollen hyphae, which could be observed both in the *vmaA* mutant strain and after treatment with bafilomycin or concanamycin. In both situations the morphology showed some variations, i.e. the morphology was more severely affected in some hyphal tips than in others (Fig. 3). The most dramatic changes were obtained when the fungus was treated with at least 40 μg bafilomycin B1 ml$^{-1}$ or 20 μg concanamycin A ml$^{-1}$. Upon exposure of the mutant strain to higher concentrations of the antibiotics, no additional change in growth and morphology was observed. Thus, this phenotype strongly suggests that the V-ATPase is the only target also in *A. nidulans*; the severely inhibited growth and altered morphology caused by the antibiotics is consistent with inhibition of the V-ATPase. Even after prolonged incubation (2 weeks) the diameter of the *vmaA1* mutant strains did not increase beyond 1 cm (PM6) or 2 cm (PM7). In addition, the mutant was unable to form differentiated hyphae and produce spores. In contrast, the mutant strain formed large vacuoles in older mycelia that were indistinguishable from wild-type (data not shown). A slower growth rate after prolonged incubation might have been due to lack of essential nutrients or evaporation of water on the plate. The slightly higher growth rate of strain PM7 is probably due to methionine prototrophy.
Fig. 3. Light microscopic images of the mycelial phenotype of the vmaA1 mutant strain, and that of wild-type A. nidulans, untreated and after bafilomycin treatment. (a) Wild-type A. nidulans (48 h incubation); (b) vmaA1 (48 h); (c) wild-type A. nidulans (48 h) treated with bafilomycin B1 (50 μg ml⁻¹); (d) vmaA1 (48 h); (e) Wild-type A. nidulans (48 h) treated with bafilomycin B1 (50 μg ml⁻¹); (f) vmaA1 (48 h) treated with bafilomycin B1 (50 μg ml⁻¹). Bars, 20 μm.
tested in A. nidulans, the addition of concentrations up to 5 mM of either Fe\(^{2+}\) or Fe\(^{3+}\) failed to restore normal growth rate of the vmaA1 mutants but instead, at the highest concentrations, caused a further reduction in the growth rate; the same effect was seen in the wild-type strain. In addition, the number of germinated ascospores was decreased. Furthermore, we could not detect any difference in toxicity between Fe\(^{2+}\) and Fe\(^{3+}\) (Table 2).

**Alkaline pH is lethal for the vmaA1 strain**

V-ATPases keep vacuoles acidified by pumping protons from the cytoplasm into the vacuole. In both N. crassa and S. cerevisiae, V-ATPase mutants fail to grow at alkaline pH (Bachhawat et al., 1993; Bowman et al., 2000). To test whether this also holds true for A. nidulans, we inoculated ascospores from the PM6 × A851 cross on plates at five different pH values (4-9, 5-8, 6-5, 7-5 and 8-5). The mutant strain was non-viable at pH 7-5 and 8-5, whereas no significant growth-rate effects were detected at the three lowest pH values (Table 2). The same lethality was observed irrespective of whether parts of vmaA1 mutant mycelium, or ascospores, were transferred to the plates. These results indicate that, in agreement with reports from both S. cerevisiae and N. crassa, the A. nidulans V-ATPase subunit A is essential for the fungus at alkaline pH, probably because of the additional activity required to maintain an acidified vacuole interior.

**Sensitivity to zinc is increased in the vmaA1 mutant**

By mRNA differential display, we previously identified a gene that displayed 20-fold downregulated expression upon treatment with bafilomycin B1. This gene has previously been described as aspnd1 and is a zinc-binding protein involved in zinc uptake (Segurado et al., 1999). Since high zinc concentrations have been reported to be toxic for V-ATPase mutants in N. crassa and S. cerevisiae (Bachhawat et al., 1993; Bowman et al., 2000; Eide et al., 1993; Ramsay & Gadd, 1997), the same toxicity could be expected for the vmaA1 mutant in A. nidulans. This is because a down-regulated expression of aspnd1 in the fungus might reduce the uptake of toxic zinc ions. We tested this conjecture by asking whether vmaA1 mutant ascospores were able to germinate in the presence of toxic zinc ions. We tested this conjecture by asking whether vmaA1 mutant ascospores were able to germinate in the presence of toxic zinc ions. We tested this conjecture by asking whether vmaA1 mutant ascospores were able to germinate in the presence of toxic zinc ions. We tested this conjecture by asking whether vmaA1 mutant ascospores were able to germinate in the presence of toxic zinc ions.

**Table 2. Growth of the vmaA1 mutant strain in minimal medium at different pH values and concentrations of metal ions**

<table>
<thead>
<tr>
<th>pH value</th>
<th>vmaA1</th>
<th>Wild-type</th>
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</thead>
<tbody>
<tr>
<td>pH 4.9</td>
<td>+++</td>
<td>+ + +</td>
</tr>
<tr>
<td>pH 5.8</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>pH 6.5 (standard)</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>pH 7.5</td>
<td>−</td>
<td>+ + +</td>
</tr>
<tr>
<td>pH 8.5</td>
<td>−</td>
<td>+ + +</td>
</tr>
<tr>
<td>0.5 mM Zn(^{2+})</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>2 mM Zn(^{2+})</td>
<td>+</td>
<td>+ + +</td>
</tr>
<tr>
<td>5 mM Zn(^{2+})</td>
<td>−</td>
<td>+ +</td>
</tr>
<tr>
<td>0.1-3 mM Fe(^{2+})/Fe(^{3+})</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>4-5 mM Fe(^{2+})/Fe(^{3+})</td>
<td>+</td>
<td>+ +</td>
</tr>
</tbody>
</table>

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REFERENCES


