Molecular Responses in *Aspergillus nidulans* to *Streptomyces*-produced Inhibitors of V-ATPases

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Abstract

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In nature filamentous fungi and bacteria compete for space and nutrients. Both kinds of organisms have evolved mechanisms such as synthesis of antibiotic secondary metabolites to defeat other microbes. Studies of synthesis of antibiotics and microbial ecology in general have important applications in both agriculture and medicine. This thesis deals with molecular responses in the filamentous fungus *Aspergillus nidulans* to inhibitors of V-ATPases, bafilomycin and concanamycin. These antibiotics are produced by various species within the bacterial genus *Streptomyces*. The main function of V-ATPases in fungi is to keep the vacuoles acidified. Inhibition of V-ATPases leads to fungal hyperbranching and extremely reduced radial growth. Changes at the molecular level were observed when the fungus was treated with the antibiotics. Using mRNA differential display, five genes with changed expression after treatment were identified. A proteomic approach was used to screen for affected proteins, and 20 proteins displayed changed abundance after antibiotic treatment. Five of these were successfully identified. Most of these gene products were previously unknown, but one could be directly linked to disrupted V-ATPases. The function of several others could not, at this point, be directly related to inhibited V-ATPases. In this thesis, two genes were further characterised. The first of them, *vmaA,* encodes a major subunit of the V-ATPase. Disruption of *vmaA* confirmed that the V-ATPase is the main target for bafilomycin and concanamycin in *A. nidulans*. This mutant strain promises to be a useful tool in further studies of the identified gene products. The most extensively studied gene in this work is *phiA*. This gene was identified by mRNA differential display, and was up-regulated by bafilomycin. Surprisingly, *phiA* was found to be essential for normal asexual development. This is intriguing, and several hypotheses can be formulated, of which the most likely is that the induced *phiA* expression after inhibited V-ATPases is due to secondary effects in the fungus, *i.e.* caused by triggering growth arrest. In this thesis, several molecular methods, *e.g.* differential display, proteomics, and immunohisto-chemistry, have been used successfully to study interactions between bacteria and filamentous fungi.

Keywords: Emericella nidulans, bafilomycin, concanamycin, mRNA differential display, proteomics, targeted gene disruption, immunohistochemistry.

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Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I.   Melin, P., Schnürer, J. and Wagner, E.G.H. 1999. Changes in *Aspergillus nidulans* gene expression induced by bafilomycin, a *Streptomyces*-produced antibiotic. *Microbiology* 145: 1115-1122.

II.   Melin, P., Schnürer, J., and Wagner, E.G.H. 2002. Proteome analysis of *Aspergillus nidulans* reveals proteins associated with the response to the antibiotic concanamycin A, produced by *Streptomyces* species. *Molecular genetics and genomics* 267: 695-702.

III.   Melin, P., Schnürer, J., and Wagner, E.G.H. 2002. Disruption of the gene for the V-ATPase subunit A results in inhibition of normal growth and abolished sporulation in *Aspergillus nidulans. Manuscript.*

IV.   Melin, P., Schnürer, J., and Wagner, E.G.H. 2002. Characterization of *phiA,* a gene essential for phialide development in *Aspergillus nidulans. Manuscript.*

Aim of the study

The main goal of this thesis was to study interactions between filamentous fungi and bacteria at the molecular level. We chose to study responses at the molecular level in the filamentous fungus *Aspergillus nidulans* when it is treated with bafilomycin and concanamycin. These two antibiotics are produced by several *Streptomyces* species and function as specific inhibitors of V-ATPases that reduce growth and alter the morphology of filamentous fungi. The more specific objective of this work was to identify gene products in the fungus involved in the response to these antibiotic secondary metabolites. A further objective was to compare available techniques in molecular biology that may be useful in studies of microbial interactions.

Introduction

Fungi and bacteria in the ecosystems

Fungi and bacteria frequently occupy similar niches

Filamentous fungi and bacteria often co-exist in a multitude of habitats, *e.g.* soil, plants and litter. Competition for nutrients and space is frequently observed among and between the two types of organisms. All fungi are heterotrophic organisms, *i.e.* require uptake of organic carbon sources. If the nutrient source is depleted most fungi can form one or several variants of spores, *e.g.* sexual spores in all major fungal groups. Fungi can also form one or two kinds of asexual spores like conidia, sporangiespores and chlamydospores. Several bacterial species also produce spores, *e.g.* in the genera of *Streptomyces* and *Bacillus*. The spores can survive in many different environmental conditions and can be dispersed over long distances. In the competition between microbes, *e.g.* fungi versus bacteria, several secondary metabolites have been evolved and produced to kill or stop growth and dispersal of other hostile microbes (Maplestone *et al.*, 1992).

Synthesis of secondary metabolites

Most studies concerning the competition between different microbes have been aimed at elucidating the synthesis of antibiotic secondary metabolites. The predominant hypothesis is that these secondary metabolites are synthesised to give the producing organism a competitive advantage by killing or inhibiting growth of other microbes. Alternative hypothesises have been proposed, *e.g.* the reduction of abnormally high concentrations of intermediate metabolites during growth arrest. One argument states that the concentrations of secondary metabolites in the field are not high enough to stop growth of other microbes (Gottlieb, 1976; Brian, 1949)**.** One strong reason why synthesis of these antibiotics is strictly related to the competitive advantage is that the genes encoding a specific antibiotic together with a resistance gene against it usually are located in the same gene cluster (Stone and Williams, 1992). More research is required to understand the ecological and

evolutionary aspects of secondary metabolites. There are also several important applications in both medicine and agriculture connected with the competition between microbes and the synthesis of secondary metabolites.

Using bacterial strains in biocontrol of filamentous fungi

Agriculture continually faces serious problem with fungal infections. Several fungi produce a wide spectrum of mycotoxins that contaminates food. Many fungi are plant pathogenic, *e.g. Botrytis*, *Fusarium* and *Magnaporte* whereas others preferentially infect the food during storage, *e.g. Penicillium* and *Aspergillus* (Samson *et al.*, 2000)*.* Since bacteria and filamentous fungi compete for space and nutrients, *e.g.* by producing substances to mutually combat each other, it is possible to use bacterial strains for biocontrol against fungi. The two most commonly used bacterial genera in biocontrol are *Bacillus* and *Pseudomonas* (Whipps, 2001). The use of fungal antagonistic bacteria in biocontrol has already become quite common and serves as an alternative or a complement to the use of pesticides. Naturally, there are biological, practical and legal problems associated with biocontrol (Gerhardson, 2002). However, this thesis will exclusively address problems connected with biology. Most importantly, since the bacteria probably will secrete toxic compounds to defeat the fungus, these substances should be harmless to mammals when used in biocontrol. Alternatively, toxins may be unstable and thus be degraded before consumption. The efficiency of the potential use of a biocontrol strain must also be measured both in terms of reduced fungal growth and reduction of toxic compounds. Since stress can trigger fungi to, in turn, produce toxic secondary metabolites, a reduced fungal growth does not necessarily result in a less toxic food product.

Aspergillus nidulans, the model organism

The Euascomycete *Emericella nidulans* (Eidam) Vuill., more often known by its anamorphic name *Aspergillus nidudans* (Eidam) Wint.*,* has been used as a genetic model organism for over 60 years. Most of the pioneering research outlined below has previously been reviewed (Pontecorvo *et al.*, 1953; Timberlake and Marshall, 1988; Timberlake, 1990; Martinelli and Kinghorn, 1994).

Several properties of *A. nidulans* make it a suitable model organism for studies of the genetics in fungi or other eukaryotes. One main advantage is the simplicity of culturing *A. nidulans,* and its ability to grow both as haploid and diploid. Strains of *A. nidulans* can be maintained by vegetative growth or by inoculating conidia, the asexual spores in Ascomycetes. The genetic regulation of asexual sporulation in *A. nidulans* has been studied in far more detail than in any other filamentous fungi (reviewed in Adams *et al.*, 1998). Hundreds of conidia are produced from a single *Aspergillus* conidiophore, the typical spore-containing structure of all Aspergilli species (Fig. 1). Several *Aspergillus* species, including *A. nidulans,* also have a teleomorph (*e.g. Emericella*) that produces ascospores. Unlike some other *Aspergillus,* species within the genus *Emericella* do not form specific sexual organs. *A. nidulans* is homothallic (has only one mating type), and can therefore initiate sexual development by itself, whereas *Emericella heterothallica*, by

contrast, has two mating types. It has been reported that *A. nidulans* strains prefer to mate with distantly related strains rather than with itself, a phenomenon called relative heterothallism (Hoffmann *et al.*, 2001). Sexual development is initiated by fusion of two hyphal compartments, whereupon two haploid nuclei can fuse to form a diploid nucleus. This diploid nucleus gives rise to one ascus containing eight haploid ascospores. Hundreds of asci are formed in a larger, closed fruitbody called a cleistothecium. Each cleistothecium is derived from a single hyphal fusion and each ascus is derived from a single nuclear fusion event.
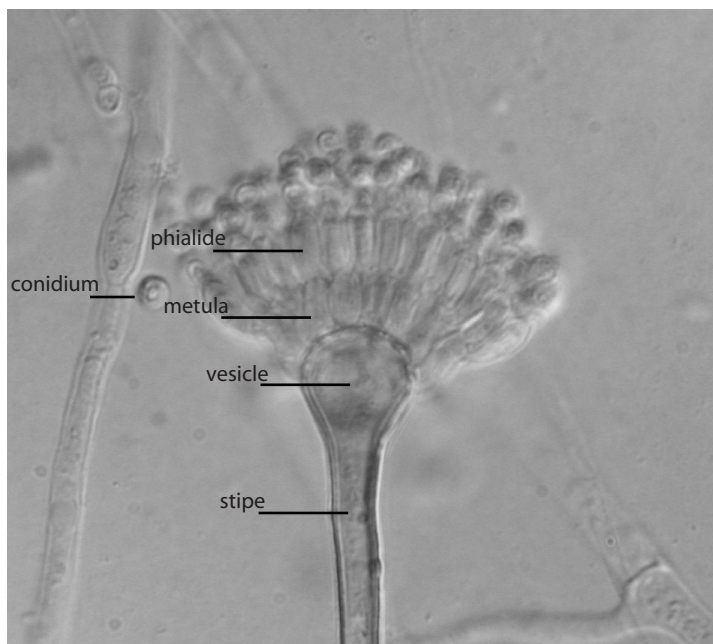

Figure 1. The conidiophore of *A. nidulans*. In contrast to some other *Aspergillus* species, *A. nidulans* is biseriate, *i.e.*, carries an extra cell layer, the metulae, which separates the vesicle from the flask-shaped phialide cell.

In *A. nidulans* a great number of mutant strains have been isolated. Most of these are auxotrophic for one or more amino acid(s), vitamin or nucleotide, *e.g.* arginine, biotin or uracil. *I.e.,* the fungus is unable to synthesise the substance in question, and growth in a minimal medium therefore requires the nutrient to be supplemented. There are also strains with mutations that result in morphological phenotypes such as reduced growth or a colour different from that of the normally green conidia. These mutations are useful as genetic markers and are ideal for selecting or crossing different strains. Another beneficial property of *A. nidulans* is its ploidy: the fungus is normally haploid, but diploid strains can easily be produced and maintained. To create a diploid strain mycelium of two strains with different selective markers are fused to create a heterokaryon (a strain with nuclei from at least two different genotypes). Spores from two strains are mixed and germinated in non-selective medium and then transferred to selective medium.

Stable heterokaryons will grow rapidly and are therefore easily isolated. Diploidisation of nuclei occurs spontaneously at a very low frequency (one nucleus in a thousand is diploid). Thus, single conidia can be transferred to selective media on which only heterozygous diploids can germinate. Diploid strains will degenerate spontaneously on non-selective media. A diploid *A. nidulans* strain can be treated with benomyl, a substance that induces haploidisation. Subsequently, haploid strains can be selected by their faster growth. It must be emphasised that this diploidy is different from the one prior to ascospore formation since in diploid strains all nuclei are diploid. These strains undergoes sexual development but at a lower frequency, and each ascus contains 16 haploid ascospores. The complete lifecycle of *A. nidulans* is shown in Figure 2.

The features mentioned above make *A. nidulans* a very useful tool in classical genetics. In modern molecular biology, it is also essential to manipulate the DNA molecule, which is feasible in *A. nidulans*. Foreign DNA can be integrated by heterologous recombination (the DNA is integrated in the genome at a non-specific site). This is an important tool for studies of gene function in *A. nidulans* or for expression of heterologous proteins (Timberlake and Marshall, 1989). Disruption of a specific gene can be performed in *A. nidulans* using homologuos recombination. *E.g.,* a mutated gene recombines with the wild-type gene and disrupts its function. Transformation of fungi is usually performed using an auxotrophic strain (Ballance *et al.*, 1983; Tilburn *et al.*, 1983; Yelton *et al.*, 1984), *e.g.* auxotrophic for arginine. Such as strain carries a deleted or mutated *argB* gene, and is transformed with DNA containing an intact *argB* gene to create arginine prototrophs (strains that can grow without addition of arginine into the medium). There is also a possibility to transform DNA with a gene that codes for resistance to a specific antibiotic, *e.g.* hygromycin B. Transformants can then be selected on plates containing the antibiotic (Punt *et al.*, 1987; Cullen *et al.*, 1987). However, this method has not been frequently used in *A. nidulans* since several suitable nutritional markers are available; the use of nutritional markers is generally more effective and much less expensive than the use of antibiotics.

The *A. nidulans* genome contains approximately 39 million base pairs (Kupfer *et al.*, 1997). This is approximately three times the size of the yeast genome but only about 1 % of the human genome. The genomes of *A. nidulans* and other fungi have only short regions of repetitive (or junk) DNA that make them suitable as model organisms within the eukaryotic domain. The estimated number of genes is 8000 (10-25 % compared to the current estimates of the number of genes in humans). The entire sequence of the *A. nidulans* genome has recently been determined by Cereon Genomics, and is now, with some restrictions, available to the research community. Although there are several reasons to choose *A. nidulans* as the model organism in this study, one alternative species, the heterothallic Euascomycete *Neurospora crassa*, was also considered. Both fungi have similar features but with advantages and drawbacks. The reason for choosing *A. nidulans* for the present study was that it is a close relative to other, more economically or medically important fungi, within the genera of *Aspergillus, e.g. A. flavus*, *A. fumigatus* and *A. oryzae*.
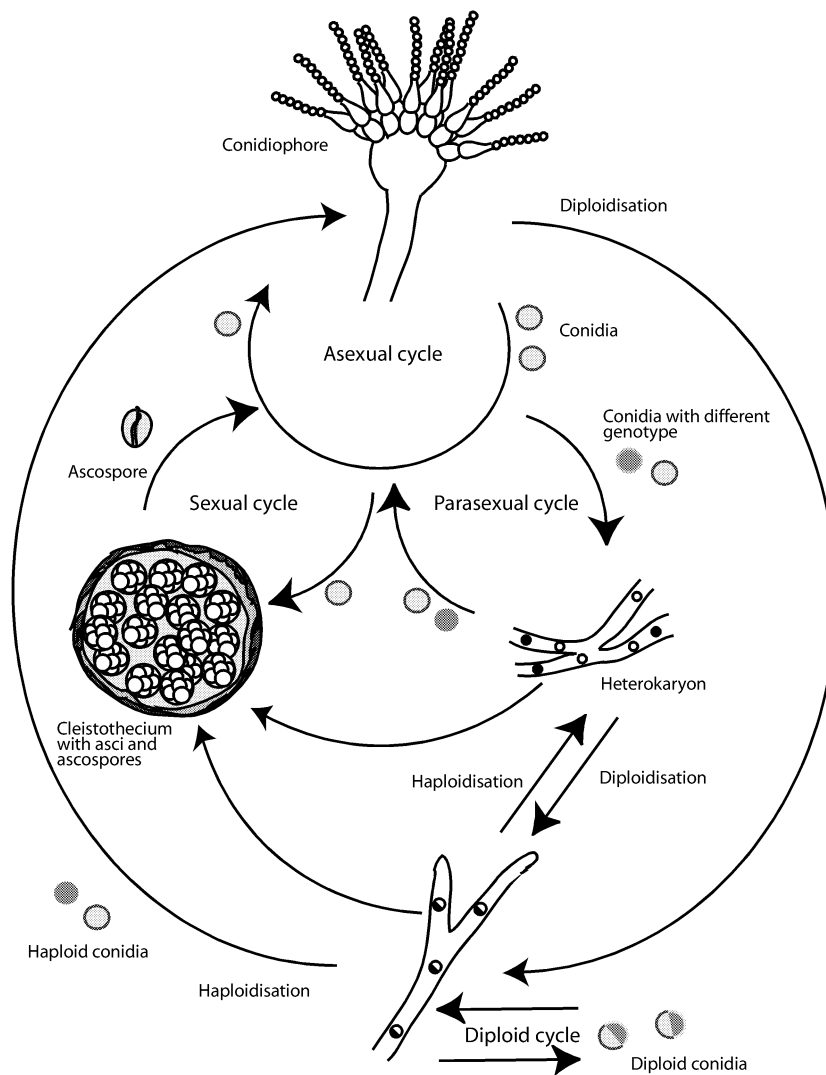
Figure2. The life cycle of *Aspergillus nidulans*. During the asexual cycle the fungus propagates with conidia. In the sexual cycle ascospores are formed in large fruit bodies (cleistothecia). In the parasexual cycle, including formation of diploids, genetic recombination occurs without meiosis.

Antibiotic secondary metabolites produced by bacteria

Streptomyces sp.

Most antibiotics known today are produced by bacteria within the genus *Streptomyces* (Demain, 1999). The *Streptomyces,* similar to filamentous fungi, form long filaments, and produce spores and a large variety of secondary metabolites. *Streptomyces* species and many filamentous fungi occupy similar ecological niches. Therefore, it can be expected that production of, and defence systems against, hostile secondary metabolites have evolved during competition between these two types of organisms. These filamentous bacteria are oligotrophic gram-positive bacteria usually habituating soil (Hodgson, 2000). Most antibiotic compounds produced by *Streptomyces* are associated with sporulation (Bennett, 1995). In May 2002, the complete genome of *Streptomyces coelicolor* was published (Bentley *et al.*, 2002). This genetic information will facilitate studies in the areas of antibiotic synthesis and *Streptomyces* biology in general.

Antifungal substances and their targets

Since fungi are eukaryotes, most of the antibiotics effective against fungi are also toxic for animals, including man. There are, however, some unique features in the fungal kingdom, *e.g.* the chitin-containing cell wall that might provide fungus-specific targets. Antibiotic compounds that target the fungal cell wall may be useful in treatment of fungal infections (Hector, 1993). Chitin synthases are inhibited by polyoxins and nikkomycins, both produced by various *Streptomyces* species (Li and Rinaldi, 1999). Another predominantly fungal substance is ergosterol, a sterol located in the cell membrane (Bowman *et al.*, 1987). The most commonly used antifungal component in medical treatments is amphotericin B, a *Streptomyces*-produced polyene. This antibiotic forms a complex with ergosterol that causes leaking of the membrane. Unfortunately, amphotericin B is nephrotoxic and its usage is therefore limited (Finquelievich *et al.*, 2000). Today, the improved methods used to treat immunosuppressed patients, *i.e.* in patients with cancer or HIV, have accentuated the problem of fungal infections. Therefore, there exists an urgent need to discover new antifungal compounds, and to develop new treatments.

The fungal vacuole

Form and function of fungal vacuoles

Vacuoles of filamentous fungi vary greatly in form and size. They can be both spherical and tubular (Weber *et al.*, 1999) whereas yeasts usually contain only one single large vacuole (Banta *et al.*, 1988). The fungal vacuole has its counterpart in plants (the plant vacuole) and in animals (the lysosome) but differs from these in both form and function. The functions of fungal vacuoles are many: storage of nutrients, digestion of organelles and polypeptides, and regulation of the concentrations of several ions (Klionsky *et al.*, 1990). Adjacent vacuoles are connected and involved in transport and allocation of nutrients throughout the

mycelium (Weber, 2002). The pH in the vacuoles is around six, and is regulated by the vacuolar ATPases that pump protons into the vacuole.

Vacuolar ATPases and its inhibitors, bafilomycins and concanamycins

The vacuolar ATPase (=V-type ATPase or V-ATPase) is present in all eukaryotes. It acidifies various organelles, *e.g.* endosomes and lysosomes in animals and vacuoles in fungi and plants (Forgac, 1989). Intensive research is currently carried out to study the form and the many functions of the V-ATPases. This is of great medical interest since this enzyme is proposed to have a role in tumour metastasis, entry of viruses such as HIV and mutations in V-ATPases can also cause deafness, (reviewed in Nishi and Forgac, 2002). In fungi the enzyme's main function is to keep the vacuole acidified, but additional functions may relate to various other cellular processes. In *N. crassa* and *Saccharomyces cerevisiae* the V-ATPases are composed of 13 different subunits (Margolles-Clark *et al.*, 1999; Stevens and Forgac, 1997; Fig. 3)**.**
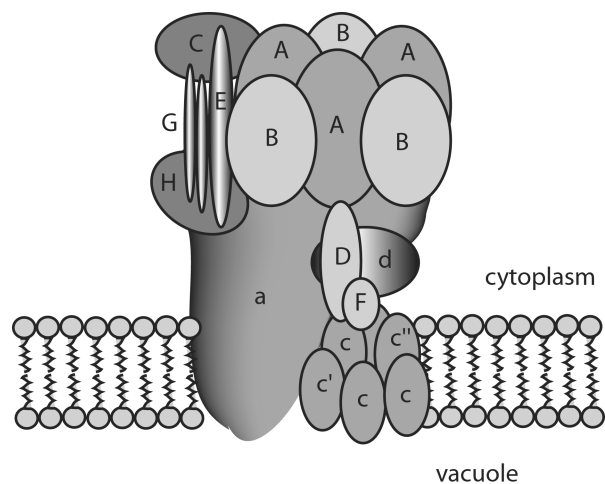

Figure 3. The V-ATPase. The enzyme consists of one cytoplasmic domain, subunits A to H and one transmembranic domain, subunits a, c and d, (adapted from Nishi and Forgac, 2002).

The use of specific inhibitors of the proton pumps aids in the elucidation of the architecture and functions of the V-ATPases. The two most potent specific inhibitors of V-ATPases are the bafilomycins and the concanamycins (Fig. 4). Bafilomycin (A-D) and concanamycin (A-F) belong to a class of macrolide antibiotics produced by several *Streptomyces* species (Dröse and Altendorf, 1997). Bafilomycin B is also known as setamycin (Omura, 1986), and concanamycin A as folimycin (Muroi *et al.*, 1993). The chemical structures of bafilomycin B1 and concanamycin A are shown in Figure 3. When filamentous fungi, *e.g. Penicillium*, *Aspergillus* and *Neurospora*, are treated with these antibiotics both growth inhibition and morphology changes are observed. The mycelium is hyperbranching and more swollen than in untreated fungi (Bowman *et al.*, 1997; Frändberg *et al.*, 2000). *Neurospora* strains with a disrupted V-ATPase subunit A also show this hyperbranching characteristic. These mutant strains are also totally resistant to concanamycin A, suggesting that the V-ATPase is the only target for the antibiotics (Bowman *et al.*, 2000). One hypothesis suggests that the increased branching occurs because the fungus with disrupted V-ATPases cannot control the Ca^{2+} concentration in the cytoplasm. It is well known that calcium plays an important role in the regulation of hyphal tip growth (Heath, 1995; Regalado, 1998).
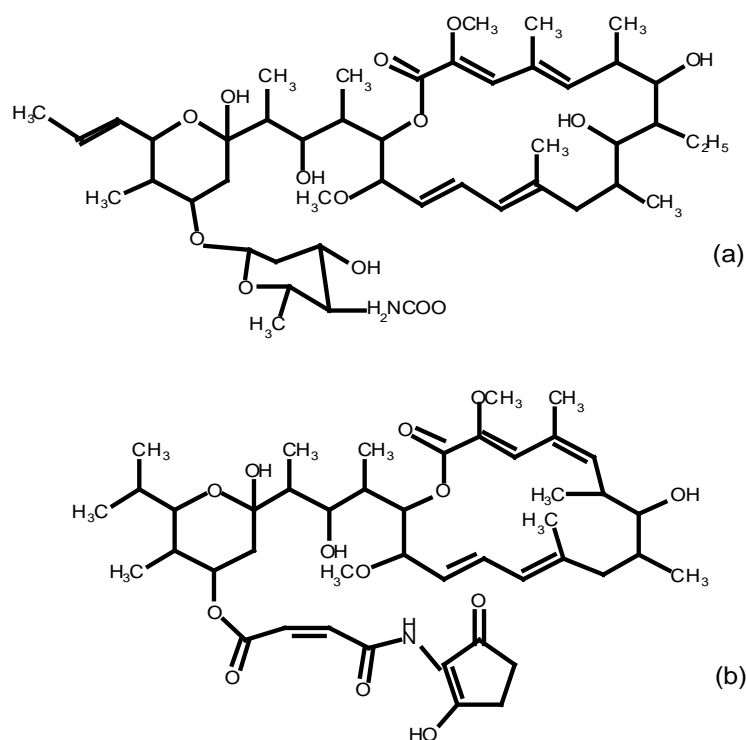

Figure 4. The chemical structures of (a) concanamycin A and (b) bafilomycin B1, the two V-ATPase inhibiting antibiotics used in this study.

How can we study the interactions between bacteria and filamentous fungi at the molecular level?

Relatively few studies of interactions between different kinds of microbes have been conducted at the molecular level. There are several methods available that may be useful in such studies. I chose here to distinguish between quantitative and qualitative approaches. Quantitative studies aim at monitoring gradual changes in gene expression and protein synthesis. This can be done both for specific genes /proteins or by screening for gene products that increases or decreases after encountering other microbes. Qualitative studies focus on the function of specific genes or proteins. Alternatively, these studies can be used to screen for and to identify genes that have a specific function. Figure 5 summarizes most of the described approaches in relation to the central dogma of molecular biology.

Quantitative approaches

These kinds of methods measure the relative amount of mRNAs, proteins or metabolites present in an organism, comparing at least two different conditions. In the organism the protein is usually the functional gene product, *e.g.* serving as an enzyme**,** but regulation occurs mostly at the transcriptional level (Lewin, 2000). Disregarding post-transcriptional control, measurements of mRNA synthesis give a good indication of the amount of protein synthesised. Small RNAs may also be active as regulatory components, *i.e.* by antisense mechanisms (reviewed in Wagner and Flärdh, 2002). However, in filamentous fungi no proof of natural antisense systems has to my knowledge so far been described.

Comparing the expression of a single gene under different environmental conditions

Depending on the changes in the environment, the expression of a gene may vary. Typically, a gene encoding a stress-induced protein is expressed under a condition of environmental stress. The standard method to measure such expression differences is using Northern blots. If the transcript is very rare it is possible to enhance detection by reverse transcription of the mRNA into cDNA, followed by quantitative RT-PCR (QRT-PCR; reviewed in Freeman *et al.*, 1999). Using a PhosphorImager it is easy to quantify differences in gene expression measured by Northern blot analysis or QRT-PCR. Differences in abundance at the protein level can be detected using Western blots.

The above methods do not explain the mechanisms behind the changed expression. Using a construct containing the promoter of the fungal gene of interest upstream of a reporter gene such as the *Escerichia coli lacZ* gene it is possible to easily detect changes at the transcription level (van Gorcom *et al.*, 1985). This method is also suitable to map promoters or other regulatory regions that are essential for the change in expression, *i.e.* binding sites for a transcription factor. This approach has been successfully employed in studies of *Aspergillus* development (Adams and Timberlake, 1990; Han and Adams, 2001) and penicillium synthesis (Brakhage and Brulle, 1995).

Differential screening

Differential screening has been used in filamentous fungi to screen for genes that are involved in the formation of asexual spores (Boylan *et al.*, 1987). A genomic library is dot-blotted onto, *e.g.* a nitrocellulose filter. Labelled cDNA from two different environmental or physiological conditions, *e.g.* vegetative growth and developed hyphae, are then hybridised to the filter. If a single dot only hybridises with cDNA from sporulating fungus it may contain a gene responsible for sporulation. The main problem with this approach is that in order to be able to screen the entire genome, the cosmids have to be numerous or very large. This approach tends to be very labour-intensive if a gene of interest is to be identified. With *A. nidulans* it is possible to purchase gene libraries separated in up to 38 chromosome-specific 96-well microtiter plates that may facilitate the screening process (Brody *et al.*, 1991). Another drawback with differential screening is that, similar to all RNA-based methods, this approach does not account for any post-transcriptional modifications. Also, due to the detection limits, low abundance transcripts will not be detected in any of the cDNA pools. Many variants of this approach have been described. The most sophisticated variant is, in my opinion, the microarray technique that will be described further below.

mRNA differential display

Since differential screening is time-consuming, several techniques have been developed to simplify detection of mRNA that is differentially expressed under different conditions. mRNA differential display (RNA-DD; Liang and Pardee, 1992) is based on the principle that two or more pools of RNA are reverse transcribed with primers that hybridise to the poly A tail of mRNAs (usually 12 thymidine bases and one or two additional bases at the 3´end which may be degenerated , *e.g.* T12MA, a primer consisting of 12 thymidine bases, followed by a G, A or C and a final A at the 3' end). The obtained pools of cDNAs are then used as templates for PCR. Short, usually decameric, arbitrary primers (AP) and the T12MN primers are used in this amplification. PCR products are subsequently separated on sequencing gels, and are visualised by autoradiography or silver staining. DNA fragments present exclusively, or more abundant, in lanes corresponding to one of the treatments, represent candidate genes that may in some way be involved in the response. RNA-DD has been used successfully with fungi, *e.g.* in *Gibberella* to monitor genes involved in synthesis of secondary metabolites (Appleyard *et al.*, 1995).

The main problem with RNA-DD is the great number of false positives often found using this technique. Even if duplicate and independent reverse transcriptions and PCR are performed, the majority of bands may represent false positives (Liang and Pardee, 1995). To confirm that the bands correspond to differentially expressed genes the relative change in expression has to be measured using Northern blots or QRT-PCR. The latter method is to prefer only if working with small amounts of RNA. To reduce the high frequency of false positives inherent in RNA-DD several similar methods have been developed, reviewed in (Matz and Lukyanov, 1998). Finally, RNA-DD only provides sequence information about a region close to the poly-A tail. In order to identify the entire gene the sequences can be blasted against published protein, cDNA, or genomic

databases, if available. If both the gene and the genome are unknown, the corresponding full-length mRNA sequence may be obtained using 5' RACE (Frohman *et al.*, 1988). Alternatively, the encoding DNA can be deduced using one of several PCR strategies that detect flanking DNA, *i.e.* inverse PCR (Ochman *et al.*, 1988) and step-down PCR (Zhang and Gurr, 2000).

Microarrays

Today, as entire genome sequences and expressed sequences for more and more species have been published it is possible to screen for, in principal, all differentially expressed genes in a single experiment. The main principle of microarrays is to attach up to several thousands nucleic acid sequences corresponding to different transcripts on a glass plate; whole cDNAs or oligonucleotide arrays corresponding to a single gene can be used to represent each transcript. A convenient way to screen for differentially expressed genes is to generate two cDNA pools generated from RNA of the organism, grown under the two different environmental conditions of interest. The cDNAs are labelled, each with a different fluorescent dye. The two pools of cDNA are then hybridised simultaneously to the glass plate. If the expression of a gene is higher in one condition than in the other, this is scored as an increase in the ratio of the dye-specific signals (Brown and Botstein, 1999; Duggan *et al.*, 1999).

Naturally, there are limitations of this method. Most of the drawbacks of the RNA-DD technique are also evident when using microarrays. Generally, micoarrays require greater amounts of total RNA than RNA-DD. Despite this, and the high initial costs required to produce the glass plates, microarray is a very promising technique and it will certainly be used more frequently when studying microbial interactions. As an example, it has been used in *Candida albicans* to detect genes whose expression was changed upon treatment of the fungus with itraconazole, an antifungal compound that stops the synthesis of ergosterol (De Backer *et al.*, 2001).

Proteomics

Differential screening, mRNA differential display and microarrays only measure changes at the mRNA level. However, even if the amount of a specific mRNA has changed one cannot be certain whether this mirrors a proportional change in the abundance of the encoded protein. Likewise, different splicing patterns of the transcript cannot easily be detected with these screening methods.

By using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) a significant part of the whole proteome can be displayed. Total proteins (from two or more conditions/cell types etc) are first separated in one dimension according to their iso-electric points. The gel with iso-electrically separated proteins is then transferred to an SDS-polyacrylamide gel, and the proteins are subsequently separated according to their size (second dimension). Proteins are visualised by autoradiography or staining (silver or Coomassie blue). Dependent on gel conditions, different parts of the proteome can be displayed. Protein spots of changed abundance under one set of conditions are likely to be directly or indirectly involved in the response to the new environment. However, it is

essential to pay attention to reproducibility since the gels and the detection methods are occasionally not perfectly matched between runs. There is computer software available to match and quantify proteins spots on series of proteomic gels. The next step is the identification of the protein. For several organisms, available proteomic databases facilitate the identification of the protein analysed. When working with an organism for which no such database has yet been established, or if the protein spot of interest has not previously been annotated, the protein spot must be extracted from the gel and identified. Several methods have been developed for determination of amino acid sequences (Hunkapiller *et al.*, 1984). The fastest and most sensitive way to identify the proteins is Mass Spectrometry (MS). Nanomolar amounts of proteins are digested, and the sequences of the resulting shorter peptides are derived by measuring their molecular masses, followed by calculations based on the known masses of amino acids (Wilm *et al.*, 1996).

Proteomics is a rapid and convenient method to screen for proteins involved in a certain response. The potential problem using 2D-PAGE lies in the identification of previously unknown proteins. If working with an organism for which a large set of Expressed Sequence Tags (EST) has been obtained, the full-length protein can be deduced by blasting the peptides against the EST database. Similarly, a genomic sequence database can be employed. However, the presence of introns in eukaryotic mRNAs occasionally renders this problematic. The sequenced peptides may be encoded, in part, by different exons, and therefore the matching score may not be high enough to identify the encoding DNA sequence. If the sequence of an organism in question, or that of a very closely related one, is not available, the sequence of the entire protein can be obtained using methods like Edman degradation. Alternatively, degenerated primers can be designed from the peptide sequences. These primers can then be used in PCR or for hybridisation in order to find the encoding DNA sequence. All of these approaches are time-consuming. Therefore, proteomics is not recommended unless EST or entire genomic sequences are available. So far, this approach has been used successfully in *A. fumigatus* and *Trichoderma reseei* for the identification of cell wall proteins (Bruneau *et al.*, 2001; Lim *et al.*, 2001).

Metabolomics

The last of the quantitative methods described here is metabolomics. In this case, gene products are not detected, but rather the compounds produced by them. The term metabolome denotes the sum of all metabolites present in and around an organism. The total number or metabolites is usually much lower than the number of different proteins and mRNAs in an organism. *E.g.,* in *S. cerevisiae* it has been reported that the entire metabolome consists of only ≈600 metabolites (Oliver *et al.*, 1998). It is likely that the pattern of metabolites produced by one organism is changed when it encounters a second one. Both the synthesis of primary and secondary metabolites may be affected. Changes in the metabolome can be detected by analytical methods such as Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS) or Liquid Cromatography-Mass Spectrometry (LC-MS). Multivariate statistics is generally required for data analysis (Raamsdonk *et al.*, 2001). The information obtained from a metabolomic study can be useful by itself, but a combination with other approaches, *e.g.* proteomics or microarrays, may be more instructive. With such a combined approach it should be possible to link gene products to their metabolites, *i.e.* the enzymes involved in the synthesis of specific metabolic compounds can be revealed.

Qualitative approaches

Overexpression of a specific gene and targeted gene disruption

None of the quantitative approaches mentioned above gives any direct information about the function of the identified gene or protein. *I.e.,* a RNA-DD approach may have revealed a gene that is overexpressed in one microbe when encountering a substance produced by another microbe. The objective is now to characterize the function of this gene. This can be accomplished as follows. The gene is overexpressed, and increased resistance against the substance is tested. Overexpression can be triggered by constructing a plasmid with the gene of interest fused with an inducible promoter, *e.g.* the *alcA* gene mentioned above (Adams *et al.*, 1988), after transformation into the organism. The opposite approach uses mutations or complete gene deletions, scoring for a phenotype. A convenient way to delete genes in an auxotrophic *Aspergillus* strain involves the construction of a vector initially containing the intact gene with its flanking regions. The plasmid-borne target gene is then replaced by a marker gene (a gene that restores prototrophy for a nutrient). Finally, this construct, in linearized form, is transformed into an auxotrophic strain (Fincham, 1989).

Random gene inactivation and overexpression

In order to find genes involved in resistance or sensitivity to another microbe or a compound, one can randomly disrupt genes and subsequently screen the resulting mutant strain against the organism/compound. One method involves the introduction of mutations, *e.g.* by exposure to UV light, followed by a search for resistant or hypersensitive strains. By transformation of the target organism with a gene library, and screening for complementation of the target gene, followed by retransformation of a complementing plasmid into *E. coli* cells, the gene encoding the desired complementing activity can be sequenced and identified (Johnstone *et*

al., 1985). By this method only point mutations are introduced. If a target gene carries such a mutation, one may for instance observe that the antibiotic can no longer interact with the gene product, which however may retain residual activity required to fulfil its role.

Genes can also be inactivated using Restriction Enzyme-Mediated Integration (REMI). Here, the organism is transformed with a mixture of a restriction enzyme (*e.g. Bam*HI) and a plasmid that has been linearized using the same enzyme. The plasmid will integrate between two *Bam*HIsites, randomly generated by cleavages of chromosomal DNA, and may occasionally disrupt a gene. Mutant strain isolates with desired traits carry the integrated plasmid flanked by the two segments of the disrupted gene. By rescue (digestion of genomic DNA with a second enzyme, ligated and retransformed into *E. coli*) the gene sequence can be obtained (Sanchez *et al.*, 1998; Schiestl and Petes, 1991). In *A. nidulans,* this strategy has been used to screen for genes involved in multidrug resistance (de Souza *et al.*, 2000)**.** A different approach giving similar results uses transposons, mobile DNA elements that can jump into and thereby disrupt genes. This approach has been implemented in fungi (reviewed in Kempken and Kuck, 1998). Both REMI- and transposon-mediated mutagenesis are useful due to the simplicity of target gene recovery. However, point mutations are not generated and, therefore, it is less likely that a gene disruption may increase the resistance against a hostile compound.

Screening for mutated genes that result in phenotypes of interests can also be performed by random overexpression of introduced genes. In *Aspergillus,* the *alcA* promoter has been used for controlled overexpression of genes integrated downstream; this promoter is activated by ethanol (Gwynne *et al.*, 1987). By constructing a library of genes preceded by the *alcA* promoter it is possible to screen for genes whose overexpression results in a phenotypic change (Marhoul and Adams, 1995).

Localisation of proteins or mRNAs of interests

The putative function of a gene product can also be approached by determination of its intracellular location. Probes can be used to detect in which hyphal compartments an mRNA is synthesized, or where a protein is located. mRNA can be visualised using radioactive or fluorescent probes that bind to the RNA. Proteins can be detected using immunohistochemistry. Polyclonal or monoclonal antibodies are raised against a specific protein. Secondary antibodies with fluorescent, radioactive, enzymatic or heavy metal markers can then be used to detect the protein-antibody complex. The choice of marker is dependent on the microscopic system used. An alternative localization method is based on hybrid proteins. A DNA construct, that encodes a fusion between the target protein and a marker protein, is transformed into the organism. The introduced protein can be a target for monoclonal antibodies or be fluorescent by itself. The green fluorescent protein (GFP) from jellyfish is often used for such fusion experiments. The use of a plant-adapted GFP has been successful in *Aspergillus* (Fernandez-Abalos *et al.*, 1998).
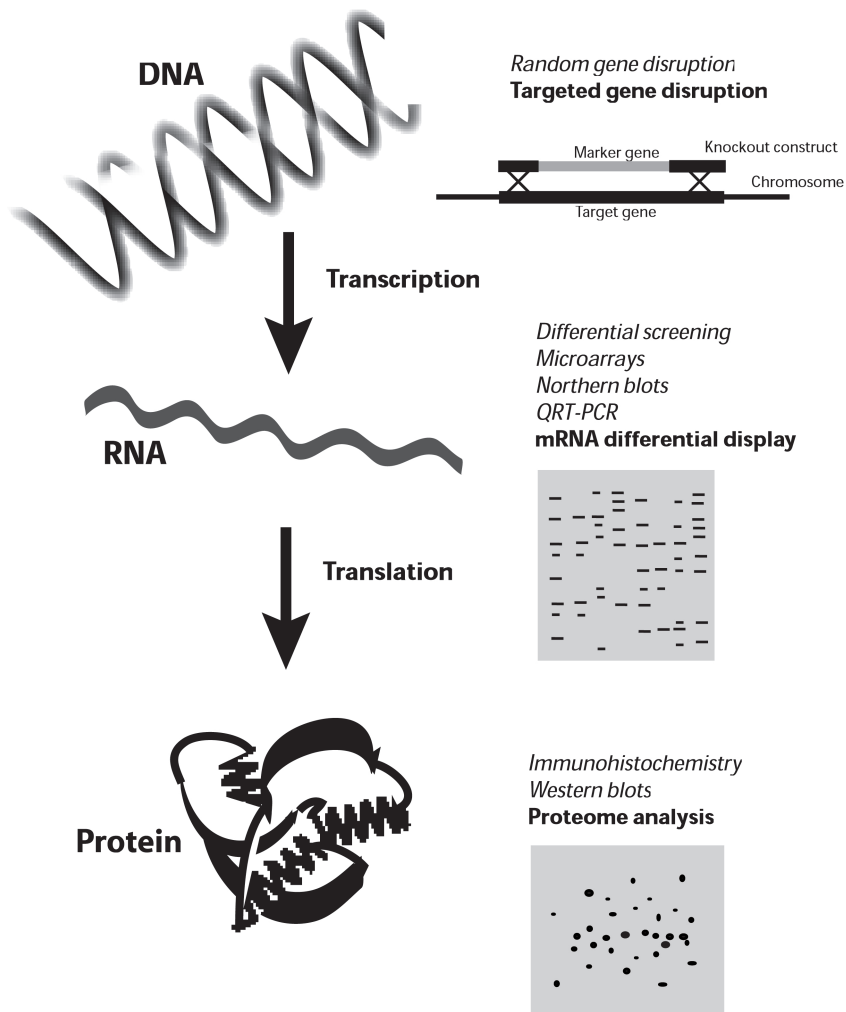
DNA

Random gene disruption
Targeted gene disruption

Marker gene   Knockout construct

Chromosome

Target gene

Transcription

RNA

Differential screening
Microarrays
Northern blots
QRT-PCR
mRNA differential display

Translation

Protein

Immunohistochemistry
Western blots
Proteome analysis

Figure 5. A graphical overview of methods discussed in this thesis. Left hand figures shows the central dogma; DNA-RNA-Protein. Right hand: Methods indicated by **bold** characters are illustrated.

Present investigation

mRNA differential display and proteomics to display genes   and proteins involved in response to *Streptomyces*-produced V-ATPase inhibitors (I,II)

Previously, in a search for fungal antagonistic bacteria with a potential use in biocontrol of filamentous fungi, a *Streptomyces halstedii* strain was isolated. This strain produces substances that reduce growth and increase the branching of filamentous fungi (Frändberg and Schnürer, 1998). These substances were identified as bafilomycin B1 and bafilomycin C1 (Frändberg *et al.*, 2000). The bafilomycins, and the similar concanamycins, are known as inhibitors of V-ATPases, but other targets may exist. We were interested in responses, at the molecular level, in the fungus, and wished to investigate whether other targets of the antibiotics could be identified. To answer these questions we chose to use the well-characterized filamentous fungus *A. nidulans* as a model organism. Figure 6 shows the morphology of *A. nidulans* treated with bafilomycin.
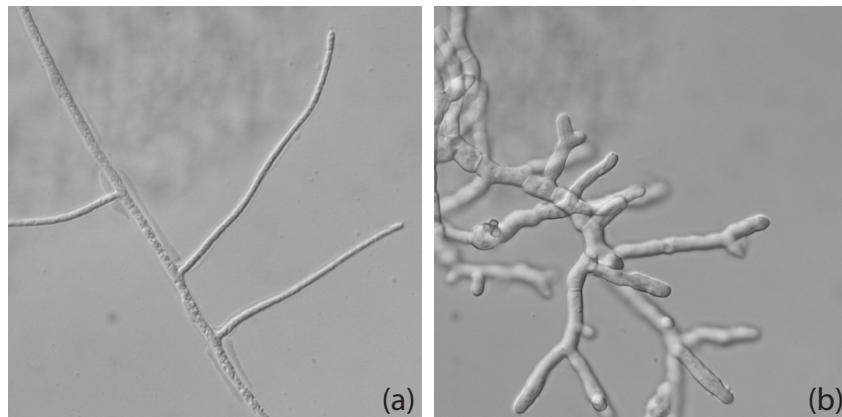

Figure 6. Bafilomycin changes the growth rate and morphology of *A. nidulans*. (a). Untreated *A. nidulans.* (b). *A. nidulans* treated with bafilomycin B1 (25 µg/ml).

Mimicking the fungal-bacterial interaction

Instead of using a system consisting of both bacterium and fungus we decided to simplify the system by growing the fungus in the presence of the antibiotic. To grow the fungus we inoculated conidia from *A. nidulans* in liquid media for 48 hours. Since the antibiotics severely inhibited spore germination, the compounds were added into medium after 24 hours of growth. No sporulation occurred in this submerged system. This observation may be of importance, since several gene products in *A. nidulans* are associated with sporulation. Removal of these gene products from the system probably resulted in a less complex transcriptome and proteome.

mRNA differential display

In the first study (**I**), we prepared total RNA from *A. nidulans* grown in the presence of bafilomycin B1. By RNA-DD, using 20 different primer combinations, a total of 20 bands, corresponding to putative target genes, were excised from the gels. After confirmation by QRT-PCR with sequence specific primers, five genes were unambiguously scored as being differentially expressed. Next, 5′ RACE was used to obtain full-length cDNA for each of the five genes.

Proteome analysis

In this study (**II**) we prepared proteins from *A. nidulans* grown in the presence of concanamycin A in Yeast Nitrogen base. The change from the non-specific malt extract to the more specific Yeast Nitrogen base was motivated by our desire to limit carbon and nitrogen sources to glucose and ammonium acetate only, and thus to repress as many proteins as possible. Another objective for this proteomic approach was to compare two different screening methods, RNA-DD at the transcription level, and 2D-PAGE at the protein level. In the first dimension, proteins were separated according to their pI values in the range from 3 to 10. In the second dimension we used both 10 and 15% PAGE in order to resolve as many proteins as possible. ^{14}C-labelled amino acids were added simultaneously with the antibiotic, in order to permit subsequent assessment of proteins synthesized. This procedure enabled monitoring of only the proteins synthesised subsequent to concanamycin treatment. In parallel, we also silver-stained some pairs of gels, to confirm reproducibility, and to facilitate excision of unlabelled protein spots for MS identification. Of a total of twenty proteins with changed abundance after treatment, six proteins were extracted and digested with trypsin, followed by MS analysis. For five of these we could deduce, by blasting against several databases, the entire amino acid sequence of the protein.

Summary of genes and proteins found

In the following, the identities of the discovered genes and proteins are discussed briefly. More information can be found in the included papers. However, I have obtained additional results regarding *breB* that were not available at the time of publication. *bre* stands for bafilomycin repressed gene, *bin* for bafilomycin induced gene, Cip for concanamycin induced protein, and Crp for concanamycin repressed protein.

breA, 20-fold down regulated mRNA expression after bafilomycin B1 treatment.

This gene had been previously identified in *A. nidulans* as *aspnd1*. It encodes a cell wall-associated protein involved in zinc uptake into the cytoplasm. Its expression is triggered when the concentration of Zn^{2+} decreases in the medium (Segurado *et al.*, 1999). Since disruptions in the V-ATPases in *N. crassa* and *S. cerevisisae* are hypersensitive to zinc, this down-regulation of *breA* may protect the organism from uptake of toxic Zn^{2+} (Bachhawat *et al.*, 1993; Bowman *et al.*, 2000; Eide *et al.*, 1993; Ramsay and Gadd, 1997). Treatment with concanamycin A resulted in an even higher decrease in expression, *i.e.* 100-fold.

breB, 10-fold down regulated mRNA expression.

The encoding cDNA of this gene does not seem to contain an ORF. In the 1642 base pairs long fragment obtained, the longest identifiable ORF had a length of 414 bp and lacked a putative ATG start codon. Thus, an mRNA is transcribed but an encoded protein-related function cannot be deduced. When I used concanamycin in paper **II**, repression was more severe, *i.e.* gave a 20-fold reduction in mRNA synthesis. Examination of the DNA sequence indicated it to be purine-rich and very repetitive. *E.g.,* it contains two AGGCAGAAGAACGCCAG repeats. By inverse PCR (Ochman *et al.*, 1988), we found that the sequence 5' of the transcription start of *breB* shares 133 base pairs of 96% similarity with the 5' region of a published teleomeric sequence consisting of TTAGGG repeats (Bhattacharyya and Blackburn, 1997). The region of sequence similarity is located entirely at the 5' end of the two sequences. The TTAGGG repeats is not included in the *breA* gene. At this point, we cannot assess the significance of these results.

binA, 60-fold up regulated mRNA expression.

The short mRNA encoded by *binA* contains an ORF, but neither the mRNA nor the deduced protein indicate any described homologies to proteins, or conserved motifs.

binB, 30-fold up regulated mRNA expression.

This gene is found in all filamentous Euascomycetes, of which abundant sequence information is available. This suggests that the encoded protein is conserved among this group. At the protein level, I found similarity to a stress-induced gene in *S. cerevisise* (van der Vaart *et al.*, 1995). Since this similarity is limited to the N-terminal halves of these proteins, they may not be, strictly speaking, homologues (in this region, approximately 50 % of the amino acids are identical or exchangeable). This gene is further characterised in (**IV**) under its new name *phiA*.

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binC, expression was only observed after treatment with bafilomycin.

The deduced amino acid sequence encoded by this gene suggests a short cysteine-rich, mostly hydrophobic protein. Despite these features, this protein does not fulfil the requirements of a hydrophobin (Wessels, 1996). Expression of *binC* was only detected after treatment with bafilomycin B1. When changing the growth conditions and the antibiotic to concanamycin, no expression was observed under any conditions.

CipA, up-regulated 2-fold.

This protein is homologous to a hypothetical protein in *Candida* whose expression is strongly up-regulated by cadmium (Hong *et al.*, 1998). It is also homologous to plant proteins involved in the synthesis of phytoalexins (Kuc and Rush, 1985). This similarity to a plant defence system is intriguing but may also be coincidental.

CipB, up-regulated up to 50-fold.

The abundance of this protein on 2D gels was variable. In some experiments, it was scored as very abundant on both treated and in treated gels, and sometimes it was present only in negligible amounts. However, in most pairs of proteomic gels the protein was almost only present after concanamycin treatment. In these cases it was perhaps the most abundant protein in the displayed proteome. CipB is homologous to LovC, a protein identified in *Aspergillus terreus,* which is incolved in the synthesis of lovastatin (Kennedy *et al.*, 1999), a secondary metabolite working as a cholesterol-decreasing agent (Alberts *et al.*, 1980). The ambiguous abundance of CipB may be due to the fact that it is only synthesised in the fungus after growth arrest. Concanamycin may, in some samples, have triggered growth arrest at an earlier time point.

CipC, up-regulated 6-fold

This protein was previously unknown, except for a homologue represented by an mRNA sequence in *Aspergillus niger*.

CrpA, down-regulated, 3-fold

The first of the down-regulated proteins had previously been identified as the *A. nidulans* CpcB, a Gβ-like protein involved in global amino acid control and initiation of sexual development (Hoffmann *et al.*, 2000).

CrpB, down-regulated 2-fold

The corresponding gene for this protein has previously been identified as *gpdA*, and encodes glyceraldehyde-3-phosphate dehydrogenase, a central protein in glycolysis (Punt *et al.*, 1988). The overall reduced growth rate may explain the decreased abundance of GpdA after treatment with concanamycin.

Remarks

In the proteomic study (**II**) an additional 15 proteins displayed changed abundance after treatment. We tried unsuccessfully to identify one other protein (CipD) using MS but we could not deduce the corresponding DNA sequences from the obtained peptide sequences. The remaining 14 protein spots were not further investigated, since their intensity indicated that identification by MS would be cumbersome. From the analysis presented, it is clear that overlap between genes/proteins identified in the two studies (**I, II**) was not found. It remains however possible that such overlaps may be present within the remaining set of 14 proteins.

At this point, few conclusions can be drawn about the identified gene products. The genes and proteins discussed above fall into three different categories; (*i*). Known genes or proteins with a function that can be related to inhibited V-ATPases, *i.e. breA* and, possibly, CrpB. (*ii*). Gene products that have previously been described, or have known homologues, but lack obvious connection to inhibited V-ATP. Perhaps the *binB* gene, and most certainly two of the identified proteins (CipA and CipB), may be correlated to physiological stress situations. (*iii*). Totally unknown gene products.

Disruption of the V-ATPase subunit A in *Aspergillus nidulans* (III)

In this work we decided to disrupt the gene encoding subunit A of the V-ATPase of *A. nidulans*. It had previously been shown in *N. crassa* that the V-ATPase is the main target of concanamycin (Bowman *et al.*, 2000). The morphology effect obtained after treatment of *A. nidulans* is however more severe, suggesting that the antibiotic affects the two filamentous fungi differently, or at least to different degrees. Another aim addressed by the disruption in *A. nidulans* was the construction of a strain that would be useful in further studies of the genes and proteins identified in papers **I** and **II**. Since most of these gene products were totally or mostly unknown it was of interest to investigate whether there were direct connections between the inhibited V-ATPases caused by bafilomycin and concanamycin, or whether changes were more indirectly linked. One of the previously identified genes, *breA* (expression down-regulated by bafilomycin), was previously identified in *A. nidulans* as *aspnd1*, encoding a zinc binding protein (Segurado *et al.*, 1999). Since Zn^{2+} is reported to be toxic in other fungi (Bachhawat *et al.*, 1993; Bowman *et al.*, 2000; Eide *et al.*, 1993; Ramsay and Gadd, 1997), we wanted to investigate if this also holds true in *A. nidulans.* If so, this would link the reduced expression of *breA* to a mechanism that prevents undesired uptake of toxic Zn^{2+}.

Growth, physiology and morphology of the vmaA1 *mutant*

The *vmaA* gene was disrupted by homologous recombination. The phenotype of the mutant strain is characterized by extremely slow growth and increased branching. This is reminiscent of the morphology in the fungus after treatment with bafilomycin or concanamycin (Fig. 7). This suggests that the V-ATPase is the only target for the antibiotics also in *A. nidulans*. The fungus grew at an extremely low rate. Only occasionally any form of developed hyphae were observed, but mature conidia were always lacking. From this severely affected mutant strain it was difficult to extract nucleic acids. In order to grow and maintain the strain, we had to harvest conidia from a heterokaryotic strain. In agreement with previous studies in *S. cerevisisae* and *N. crassa*, the mutant strain was unable to grow at basic pH and at high concentrations of Zn^{2+}.
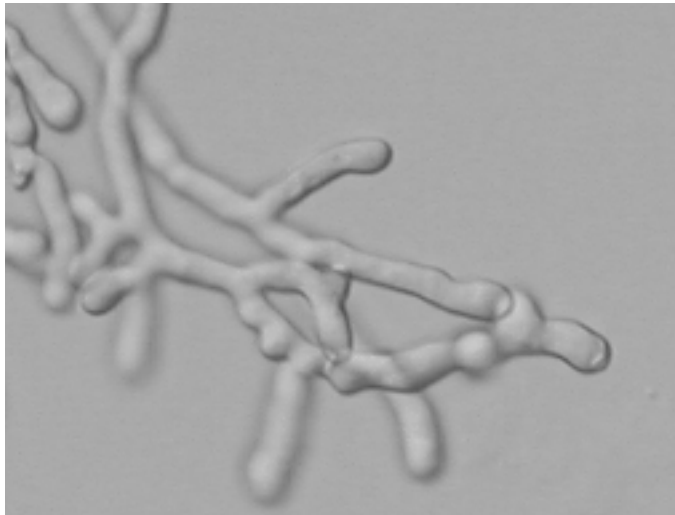

Figure 7. Morphology of the *vmaA1* mutant. See Figure 6 for a comparison with wild-type and bafilomycin-treated *A. nidulans*.

Characterization of the *phiA* gene in *Aspergillus nidulans* (IV)

In the previous studies (papers **I** and **II**) we found several unknown genes or proteins affected by bafilomycin and concanamycin. However, these studies remain inconclusive until the gene products are characterised. I decided to concentrate my efforts on one of the up-regulated genes, *binB*. The function of the corresponding protein was unclear, but some sequence similarity with a stress-induced protein in *S. cerevisiae* (van der Vaart *et al.*, 1995) was observed. This is intriguing since treatment of *A. nidulans* with bafilomycin should constitute a stress for the fungus. However, only some sequence segments of the two proteins are similar, and hence homologies cannot be unequivocally confirmed. Searching several DNA and EST databases I found homologous proteins in other filamentous Euacomycetes (>60 % amino acid identity or similarity). All filamentous fungi for which abundant sequence data are available (genomic or EST), encode *binB* homologues. Thus, even if this gene does not encode a general stress protein, it may encode a protein unique in filamentous Euascomycetes. Incidentally, when preparing paper **I,** we were not aware that the name *bin* already was used for a class of *A. nidulans* genes (Hynes, 1975). During preparation of this paper, we therefore renamed the gene to *phiA*, due to its presence and importance in phialide development (see below).

Morphology of the phiA1 *mutant*

If one wants to elucidate the function of a gene, it is useful to create a deletion mutant and monitor the resulting phenotype. This is only possible if the gene is not vital for the fungus. The *phiA* gene was disrupted by homologous recombination. An arginine auxotrophic strain was transformed with a plasmid in which part of the cloned *phiA* gene had been replaced by the *A. nidulans argB* gene. Arginine prototrophs were selected. Subsequent analysis showed that in some transformants *phiA* had been disrupted. The phenotype of these mutants is slow growth and reduced asexual development. Observation of the conidiophores revealed that the phialides were not developed properly. In some conidiophores the phialide formed a complete new hypha. Conidia were rare and occured sparsely, instead of in long chains as in the wild type (Fig. 8).

The functional link between treatment with bafilomycin and normal conidiophore development is not clear. It is possible that the PhiA protein carries out multiple functions. It is also possible that dysfunctional V-ATPases indirectly trigger sporulation and synthesis, and thereby also the synthesis of PhiA. A plausible hypothesis would suggest that an increased synthesis of PhiA might protect the fungus from uptake of bafilomycin. To test this, we treated the *phiA* mutant with bafilomycin and concanamycin. However, no signs of a drastic increase in sensitivity could be observed.

Immunohistochemistry

Since it was clear that PhiA had no protective role against the antibiotics, but rather was involved in sporulation, we decided to raise polyclonal antibodies against PhiA in order to study its localisation. Using both enzymatic and fluorescent labelling in the detection protocol, we found that PhiA was mainly located in the phialides, but also in the adjacent metulae and conidia (Fig. 8). This may explain the important role of *phiA* in the development of phialides and conidia, although the connection with bafilomycin and V-ATPases remains unclear.
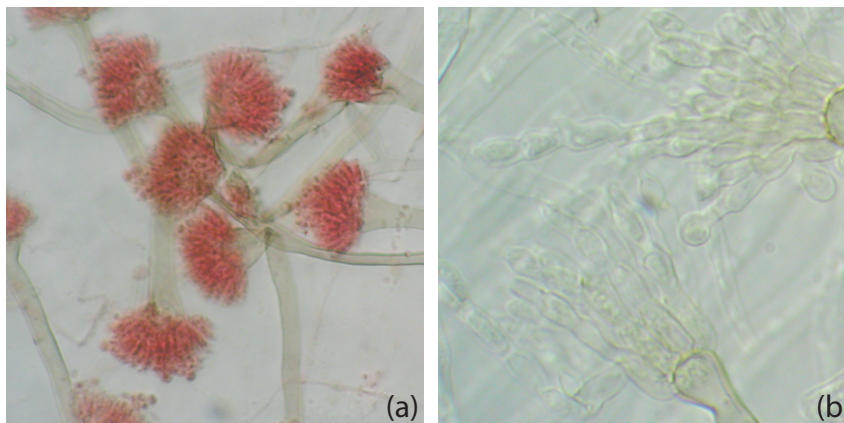
Figure 8. phiA is essential for normal conidiophore development and the encoded protein is mainly located in the phialide. (a). Immunohistochemistry of wild-type *A. nidulans*. The polyclonal antibodies bind to the phialides. (b). *phiA1* mutant strain. The phialides are not developed and sporulation is strongly reduced.

Discussion

In the present study I have demonstrated the usefulness of mRNA differential display and proteomics in the search for gene products involved in microbial interactions. Instead of investigating a direct interaction between bacteria and filamentous fungi I used the bacterial metabolites, *i.e.* bafilomycin and concanamycin. This approach is simpler and eliminates the complexity arising from the presence of other metabolites that may affect the fungus. However, in an ecologically relevant situation all competing microbes produce a wide spectrum of different metabolites involved in the struggle for space and nutrients. Therefore, strictly speaking, the gene products identified cannot unequivocally be related to a real fungal-bacterial interaction. On the other hand, the less complex system used here has the advantage that, in principal, all the gene products identified are directly connected to the V-ATPase inhibitors, bafilomycin and concanamycin.

In paper **I** we used RNA-DD and identified five genes with at least ten-fold change in expression when the fungus was treated with bafilomycin B1. Using 2D-PAGE (**II**) we found 20 proteins with changed abundance of at least two-fold. The differences in numbers and magnitudes of affected genes or proteins reflect the sensitivities but also limitations of each of the approaches. In RNA-DD, a majority of the detected DNA fragments turned out not to be differentially expressed, and were discarded as false positives. There might be some significant errors associated with the estimated ratios while measuring differential amounts of transcripts using QRT-PCR. By setting the threshold value relatively high, *i.e.* at least a 10-fold change in expression, we ascertained that all five identified genes indeed were differentially expressed. It is likely that we would have identified more genes if we had used additional sets of arbitrary primers in RNA-DD. In 2D-PAGE analyses, it is relatively straightforward to repeat experiments with high reproducibility. Therefore, it is possible to detect relatively small but significant changes in protein abundance. Admittedly, false positives are also encountered when using 2D-PAGE, but, in my experience, occur less frequently than in differential display. The main disadvantage with 2D-PAGE is that only quite prominent spots can be identified. However, this is a practical problem that can be overcome by multiple repeats with pooling of extracted proteins. More proteins can also be detected by changing the separating conditions, but a subset of the protein complement may, due to their composition, remain impossible to resolve.

It is clear that all affected genes or proteins cannot have been identified, since the different results in both approaches indicate that a search has not been saturated. However, we can draw some conclusions about the response in the fungus after encountering V-ATPase inhibitors. Treating the fungus with bafilomycin and concanamycin causes effects at both the mRNA and protein level. In this study I have identified 25 gene products with changed abundance. Likewise, I have also shown that the expression of most genes, and the abundances of most proteins, is not affected. Therefore, in spite of severe morphology and growth rate effects caused by inhibiting the V-ATPases, it is possible to conclude that treatments results in only moderate overall effects at the molecular level. This may not be surprising since the growth and morphology of filamentous fungi can

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be altered drastically if a carbon source is changed, if treated with specific substances, or if point mutations are induced (Harris *et al.*, 1999; Trinci, 1984)**.** Unfortunately, most of the identified genes and proteins were previously unknown, and for most of the known proteins no clear connection could be drawn from gene product to inhibited V-ATPases.

For only one gene, a connection with inhibited V-ATPases is suggested by experimental data. This gene, *breA*, encodes a Zn^{2+} binding protein involved in the uptake of zinc (Segurado *et al.*, 1999). In paper **III** we have shown that Zn^{2+} is toxic when the V-ATPase is disrupted in *A. nidulans,* as in other fungi. This indicates that the down-regulation of *breA* may serve to protect the fungus from toxic Zn^{2+}. In paper **III** we also characterized the V-ATPase of *A. nidulans* by disrupting *vmaA*, the gene encoding subunit A. The features of the *vmaA1* mutant confirm previous studies in *N. crassa* suggesting that the V-ATP is the only target for the antibiotics (Bowman and Bowman, 2000; Bowman *et al.*, 2000). However, the phenotype in *A. nidulans* is more extreme both in terms of morphology and growth. This may indicate that the V-ATPase or the vacuole plays a somewhat different role in *Aspergillus*. A more plausible explanation is that the observed phenotype of the *vma* mutant may reflect the generally slower growth of wild-type *A. nidulans* compared to *N. crassa.*

The most extensively studied of the previously unknown genes is *phiA*. In paper **IV** we characterized the gene both at the genetic level, by disruption, and at the protein level, by raising polyclonal antibodies to detect where the protein is located. The phialides in *phiA1* mutant strains cannot develop properly resulting in a severely reduced asexual sporulation. This peculiar morphology has, to my knowledge, not been described before. However, numerous abnormal morphologies of the conidial head have been described. *E.g.*, hyphal like metulae (Karos and Fischer, 1996) and bristle (Clutterbuck, 1969; Prade and Timberlake, 1993). Most likely, there is no causal connection between the *phiA* gene and disrupted V-ATPases. First, since a *phiA1* strain does not show increased sensitivity to the antibiotics, the encoded protein cannot have a protective role. I propose that the increase in gene expression may be due to the growth arrest caused by the antibiotics, and that an initiation of conidiophore development takes place within the fungal pellet. In conclusion, we have identified a new gene that is probably present in all Euascomycetes. It encodes a protein necessary for normal asexual development. Since it is present in the cell types that require an intact *phiA* gene to develop properly, PhiA probably has a direct functional role in the phialide and in the conidium.

For the remaining genes and protein we cannot, at this point, draw conclusions about why their expression or abundance is changed after treatment with bafilomycin or concanamycin. Their functions may become apparent when knockouts are created and protein localisation is shown by immunohistochemistry. The *vmaA* mutant strain, although very slow growing, may be instrumental in testing whether the other gene products are directly affected by a disrupted V-ATPase. This could be approached by creating a double mutant to investigate whether the *vmaA1* mutation can suppress another mutation, or vice versa. Hopefully, subsequent work will lead to identification of these gene products, or

their homologues, in a different functional context, and provides clues about their roles in fungal biology. In combination with such new results, it may be possible to make use of the information contained within this thesis, to learn more about the gene product in question, and possibly also to connect the presence of the gene product with inhibited V-ATPases. This also holds true for the already partially characterized genes or proteins in this thesis.

Concluding remarks

The main objective of the work described in this thesis was to use techniques from the expanding area of molecular biology, to study interactions between bacteria and filamentous fungi, focusing on gene expression in the fungus. More specifically, I wanted to study changes at the level of gene expression and protein synthesis in the filamentous fungus *A. nidulans* when its V-ATPases were inhibited or disrupted. In simple terms, I considered it useful to employ molecular biology to answer ecological questions.

   In the present work I used several different molecular methods; gene cloning and targeted gene disruption at the DNA level, RNA-DD and QRT-PCR at the RNA level, and 2D-PAGE and immunohistochemistry at the protein level. The results obtained can be divided into two categories. Firstly, I showed that both RNA-DD and 2D-PAGE are useful when screening for gene products, although both methods have their limitations. In these studies, I identified several new genes and proteins in *A. nidulans* that are in some way related to inhibited V-ATPases. I also report a more detailed characterisation of two genes; *vmaA*, the gene encoding subunit A of the V-ATPase in *A. nidulans,* and *phiA*, a gene essential for normal asexual sporulation. Although the description of these two genes is of interest in several respects, the main contribution of this work – in my own opinion – lies in the demonstration that several molecular techniques are useful for studies of interactions between microbes. I hope that this thesis can serve as a guide to other students or researchers attempting to answer similar questions.

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