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Current status of systems biology in Aspergilli

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ABSTRACT

In this review, we present a comprehensive overview of the current status of genomics, transcriptomics, proteomics, metabolomics, and metabolic modeling in *Aspergillus* species. Currently, 13 *Aspergillus* genomes divided across seven species have been sequenced with more to come, and many applications of this information on a systems level have been published. More than 30 studies on global transcription analysis have been published, and 21 different platforms are available for *Aspergillus* transcription studies. Additionally, the fields of proteomics and metabolomics have, while still in their infancy, produced intriguing results and novel applications. Finally, multiple levels of *Aspergillus* metabolism have been reconstructed and modelled. Systems-level research of Aspergilli have in a few years added to our knowledge on processes relevant to evolution, central and secondary metabolism, cellular organization, pathogenicity and biotechnology and future efforts may lead to much improved understanding of regulation of key cellular processes in this important family of filamentous fungi.

1. Introduction

The complexity of the cellular machinery is a constant challenge for biological research. Redundancies and complex circuitries within the cell often counteract modifications introduced by the research. Additionally, regulation can occur on the transcriptomic, proteomic, and metabolomic level, and a more holistic view than a traditional reductionist approach is therefore often required. For this reason, the use of a system-based approach is gaining ground, both as a separate discipline, and as an aid for constructing new hypotheses.

In recent years, several studies have been published using systems-wide techniques in Aspergilli. The sudden burst in available fungal genomes (Nierman et al., 2005; Machida et al., 2005; Galagan et al., 2005; Pel et al., 2007; Fedorova et al., 2008) makes the system-wide approach even more appealing for the future. For these reasons, we present in this review an overview of genomic, transcriptomic, proteomic, metabolomic, and modeling studies in Aspergilli. In many of the applications, this work shows the possibilities and potential of systems biology, and the perspectives of developing new platforms and tools.

2. Genomics

Even though genome sequencing projects for the *Aspergillus* species *Aspergillus nidulans* and *Aspergillus oryzae* started as early as 1998 and 1996, respectively, the genomics era of filamentous fungi is generally agreed to have started in February 2001, when the Whitehead Institute Center for Genome Research released a draft version of the genome sequence of *Neurospora crassa*. The finished version of the genome sequence was published in 2003 (Galagan et al., 2003). As a testament to the importance of the work, a 107 page review of the impact of the *N. crassa* genome sequence was published by Borkovich et al. (2004).

In this chronology, *Aspergillus* genomics thus started in January 2003, where full public access was given to a threefold (3×) coverage *A. nidulans* genome sequence from Cereon genomics (Archer and Dyer, 2004). Two months later, this sequence was re-released by the Whitehead Institute/MIT Center for Genome Research with the addition of a 10× coverage sequence to generate a draft genome sequence with 13× coverage. Similarly, the Dutch company DSM announced the genome sequencing of an enzyme-producing strain of *Aspergillus niger* (CBS 513.88) in a press release in December 2001, but restricted access to collaborators. A publication on the *A. nidulans* genome sequence was released in December 2005 along with publications on the genome sequence of *A. oryzae* and the opportunistic pathogen *Aspergillus fumigatus*, the primary agent behind invasive aspergillosis, the most frequent fungal infection in the world (Galagan et al., 2005; Machida et al., 2005;

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Nierman et al., 2005), while the genome sequence of *A. niger* CBS 513.88 was made accessible to the public along with the publication of Pel et al. (2007).

Several *Aspergillus* genomes have been sequenced in parallel with or during the mile-stone publications of these four genomes, some by independent agencies, other as part of community efforts. One such effort is the Fungal Genome Initiative (FGI) (http://www-genome.wi.mit.edu/seq/fgi/) that since 2002 has released white papers suggesting fungi to be sequenced, including the 10x sequencing of *A. nidulans* which was a part of the proposal of the first white paper (Fungal Genome Initiative, 2002). So far four white papers have been released suggesting the sequencing of a total of 99 fungal species, including eight Aspergilli. Table 1 summarizes the current number of whole-genome sequencing projects on Aspergilli as well as a few key statistics on the genomes. These include the Aspergilli described above, as well as the following:

Aspergillus aculeatus, a plant pathogen also used for identification of new extracellular enzymes as in the study by Dalbøge (1997), where interesting genes were cloned from cDNA and expressed in *A. oryzae*.

Aspergillus carbonarius, an ochratoxin producer and involved in food spoilage, particularly of grapes. It is closely related to *A. niger* and the other black Aspergilli.

Aspergillus clavatus, an allergenic and rarely pathogenic fungus that is primarily being sequenced to gain a further understanding of the two closely related pathogenic Aspergilli *A. fumigatus* and *Aspergillus terreus*.

Aspergillus (Neosartorya) fischeri, a non-pathogenic close relative to the pathogenic *A. fumigatus*.

Aspergillus flavus, human pathogen producing the highly carcinogenic aflatoxin. It also causes economic losses due to grain infections.

Aspergillus parasiticus, a producer of aflatoxin.

Aspergillus terreus, used industrially to produce the cholesterol lowering agents pravastatin, simvastatin, and lovastatin and the organic acid itaconic acid (Bonnarme et al., 1995; Manzoni and Rollini, 2002), but has also been connected with aspergillosis (Birren et al., 2004).

As the list of Table 1 continues to grow, so does the need for organization. Resources like the Broad Institute *Aspergillus* comparative database (http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html), e-fungi (http://www.cs.man.ac.uk/~cornell/eFungi/database.html), and the central *Aspergillus* data repository (CADRE) (http://www.cadre-genomes.org.uk/ (Mabey et al., 2004), designed for the comparison of genomic data, will arguably be of even greater importance to researchers as the number of whole-genome sequenced Aspergilli increases and allows for more studies based on comparative genomics.

The original four mile-stone genome papers have catalyzed a wide range of genome-driven work, as can be reflected in a recent special issue of Studies in Mycology (no. 59) on "*Aspergillus* systematics in the genomic era", as well as the present issue of Fungal Genetics. As an overview of the status and potential of the genome-driven approach, the following sections will outline research on genomics in *Aspergillus* species, where such has been published. In the context of fungi, a recent definition of genomics has been "the determination and use of the genome sequence of organisms to identify genes and non-coding, but potentially functionally important, regions of the genome" (Archer and Dyer, 2004; Hofmann et al., 2003). This overview will focus on this type of studies, such as the publications on whole-genome sequencing or genome surveys; however, a few examples of systems-wide functional genomics or comparative genomics will be included to provide perspectives of genomics.

2.1. Aspergillus aculeatus genomics

The genome sequence of *A. aculeatus* is still in the sequencing and annotation pipeline of the Joint Genome Institute (JGI) (Scott Baker, personal communications), and no study of the genomics or the perspectives of these have been released at this time.

2.2. Aspergillus carbonarius genomics

As with *A. aculeatus*, the genome sequence of *A. carbonarius* has not yet been released from the JGI (Scott Baker, personal communications). Studies on genomics have been limited to a comparative study of the mitochondrial genome of different *A. carbonarius* strains Hamari et al. (1999), but it is based mainly on restriction mapping, and not on genome sequencing.

Table 1
Overview of whole genome sequence projects for *Aspergillus* species

Species	Strain	Sequencing	Contigs	Coverage (Fold)	Gene models	Size (Mb)	Publication
A. aculeatus	ATCC 16872[a]	DOE Joint Genome Institute	N/A	N/A	N/A	N/A	Scott Baker, Personal commun.
A. carbonarius	IMI 388653[a]	DOE Joint Genome Institute	N/A	N/A	N/A	N/A	Scott Baker, Personal commun.
A. clavatus	NRRL 1	TIGR	231	11.4	9125	27.86	Fedorova et al. (2008)
A. (Neosartorya) fischeri	NRRL 181	TIGR	1067	N/A	10,407	32.55	Fedorova et al. (2008)
A. flavus	NRRL 3357	TIGR	3388	10	13,071	36.3	Yu et al. (2005)
A. fumigatus	Af293	TIGR/Sanger Institute.	19	10.5	9887	29.4	Nierman et al. (2005)
A. fumigatus	A1163	TIGR/Celera Genomics/Merck & Co., USA	140	N/A	10,099	29.2	Fedorova et al. (2008)
A. (Emericella) nidulans	FGSC A4	Broad Institute	248	13	10,701	30.06	Galagan et al. (2005)
A. niger	CBS 513.88	DSM, The Netherlands	498	7.5	14,165	33.9	Pel et al (2007)
A. niger	ATCC 9029	Integrated Genomics	9510	4	N/A	N/A	
A. niger	ATCC 1015	DOE Joint Genome Institute	24	8.9	11,200	34.9	Baker (2006)[b]
A. oryzae	RIB40	NITE, Japan	24	9	12,074	37	Machida et al. (2005)
A. parasiticus	Unknown[a]	University of Oklahoma	N/A	N/A	N/A	N/A	
A. terreus	ATCC 20542	Microbia	N/A	N/A	N/A	N/A	
A. terreus	NIH 2624	Broad Institute	267	N/A	10,406	29.3	

This list was compiled from Jones (2007), the NCBI genome project webpage and Genomes OnLine Database (GOLD, http://www.genomesonline.org).

[a] Not yet released.

[b] This publication was not formally the genome analysis publication, which is in preparation (Scott Baker, personal communications), but is often used as a reference.

2.3. Aspergillus clavatus genomics

The genome sequence of *A. clavatus* strain NRRL 1 has recently been published by Fedorova et al. (2008) along with the genome sequences of *Neurospora fischeri* and a second strain of *A. fumigatus*, and is available from NCBI in its third version. Research on this fungus is sparse compared to other Aspergilli, and the contributions based on nucleotide sequence are limited to the cloning of clavin by Parente et al. (1996). Wortman et al. (2006) have anticipated that comparative genomics of *A. fumigatus*, *A. clavatus*, and *N. fischeri* will allow the identification of factors responsible for pathogenicity in *A. fumigatus*. Pathogenicity was indeed one of the objects of study in the genome paper by Fedorova et al. (2008) and will be discussed in more detail in the section on *A. fumigatus* genomics.

2.4. Neurospora fischeri genomics

The genome sequence of the eight chromosomes of *N. fischeri* has been analyzed and compared to the genomes of *A. fumigatus* Af293, A1163 and *A. clavatus* NRRL 1 in the work by Fedorova et al. (2008).

2.5. Aspergillus flavus genomics

A preliminary commentary on the publication of the *A. flavus* genome has been published by Yu et al. (2005), marking the availability of the sequence information. While the article does not contain the genomic analysis found in the publications of *A. fumigatus, A. nidulans*, *A. niger*, and *A. oryzae*, aflatoxin biosynthesis and the aflatoxin gene cluster were mentioned as targets of special interest for future analysis. This has indeed been analyzed further, as will be described in the section on transcriptomics.

2.6. Aspergillus fumigatus genomics

The sequencing of *A. fumigatus* Af293 was initiated in 2001, and a commentary was published by Denning et al. (2002), describing the anticipation of how the project would fuel research in anti-fungal drugs. The annotation and analysis of a 922 kb large region was published by Pain et al. (2004), allowing the early identification of factors putatively involved in pathogenicity and anti-fungal targets. However, this study was superseded by the publication of the analysis of the full genome sequence by Nierman et al. (2005), where the genome was examined for allergens and features that may be involved in pathogenicity. These analyses predicted nine proteins to be allergens, and identified 26 clusters that may be involved in the production of secondary metabolites.

Furthermore, a derivative of the clinical isolate strain *A. fumigatus* CEA10, *A. fumigatus* FGSC A1163, has been sequenced by Merck & Co and was subject to a preliminary analysis by Wortman et al. (2006) and a comparative analysis of *A. fumigatus* FGSC A1163 and *A. fumigatus* Af293 by Rokas et al. (2007). In the recent publication of the genome sequence of *A. fumigatus* FGSC A1163 along with the sequences of *A. clavatus* NRRL 1 and *N. fischeri* NRRL 181, the genome sequences were compared to identify genomic elements unique to *A. fumigatus* FGSC A1163, and to *A. fumigatus*, thus providing potential targets for further study of the cause of pathogenicity.

The research spurred by the genome sequence of *A. fumigatus* Af293 covers many aspects, as was described in a review by Ronning et al. (2005), focusing on a wide range of characteristics and comparative genomics to *A. oryzae* and *A. nidulans*. One use of the genome sequence in the context of basic research was the identification of mating-type-like genes in the asexual *A. fumigatus* (Pöggeler, 2002; Firon et al., 2002; Varga, 2003) (reviewed by Dyer et al. (2003)). The two mating types were finally confirmed by Paoletti et al. (2005), thereby suggesting an active sexual cycle. A review of the impact of the genome sequence of pathogenic fungi as well as identification of more putative pathogenic gene clusters has been put forth by Bowyer and Denning (2007).

2.7. Aspergillus nidulans genomics

As described in the introduction to *Aspergillus* genomics above, the genome sequencing of *A. nidulans* FGSC A4 was initiated by Cereon Genomics (Monsanto) in 1998 with the public release of the genome sequence in June 2003. After two years of annotation and research efforts, the genome paper by Galagan et al. (2005) was published. The *A. nidulans* genome was originally predicted to contain 9541 protein coding genes, but after an extensive manual curation of the gene predictions in March 2006, the number of predicted genes was revised to 10,701.

A recent paper by David et al. (2008) uses genomics as well as metabolic pathway reconstructions to provide functional assignments to 472 orphan genes.

2.8. Aspergillus niger genomics

Aspergillus niger CBS 513.88 was the first of so far three *A. niger* strains to be whole-genome sequenced. The genome sequencing was initiated by the Dutch company DSM in cooperation with Gene Alliance in July 2000, and was reported by DSM to be finished in December 2001. The strain is a progenitor of the industrial glucoamylase-producing strains currently in use and the genome sequence was analyzed by Pel et al. (2007). A major contribution of this genome publication was the annotation of a number of commercially relevant enzymes, including more than 200 putative proteases and approximately 170 hypothetical and previously characterized carbohydrate-active enzymes.

The second *A. niger* genome sequence is of *A. niger* ATCC 9029, an often used lab-strain. The genome was sequenced by Integrated Genomics and released in 2005 as a heavily fragmented (9510 contigs) draft sequence without annotation or gene predictions.

Finally, the Joint Genome Institute (JGI) under the funding support from the US Department of Energy (DOE) has completed and released the genome sequence of *A. niger* ATCC 1015, a citric acid producer originally described in 1917. The publication of Baker (2006) was the first study using and presenting the *A. niger* ATCC 1015 genome sequence, although not formally a publication of the genome sequence.

Other genomics performed in *A. niger* includes the sequencing of 12,820 ESTs by Semova et al. (2006) with the prediction of 5108 genes. This survey of the expressed genes provides a valuable resource that has value both separately and in conjunction with an available genome sequence. Furthermore, Juhász et al. (2008) sequenced the complete genome sequence of the 1a mtDNA type of *A. niger* and 2b mtDNA type of *Aspergillus tubingensis* and found the two mtDNAs to be almost identical.

The value of a genome sequence for an industrially relevant species has been demonstrated by DSM where it has been used in product development. Examples include a protease product called "Brewer's Clarex" that prevents chill-haze in beer (Lopez and Edens, 2005), a protease product to eliminate bitterness of protein supplement sport drinks (Edens et al., 2005), and "PreventASe", an asparaginase product aimed at reducing acrylamide formation in baked and fried foods (DSM Food Specialities, 2007).

2.9. Aspergillus oryzae genomics

While the whole genome sequencing of *A. oryzae* RIB40 was initiated in 1996 (Machida, 2002), the complete genome sequence was not publicly released until 2005. The genome paper by Mach-

ida et al. (2005) analyzed the genome and the predicted genes. An interesting finding is that compared to *A. nidulans* and *A. fumigatus*, the *A.* oryzae genome seems to have been enriched for genes involved in metabolism. Many of the enriched traits seem to be at least partially due to its long history of food production. A detailed review by Kobayashi et al. (2007) describes recent results of a number of genomics and functional genomics efforts.

The *A. oryzae* genome has special interest for enzyme processing of food stuffs, and as a parallel example to the *A. niger* asparaginase PreventASe described above, an *A. oryzae*-derived enzyme of a similar function and efficiency has been launched by Novozymes A/S under the name AcrylAway (Novozymes, 2007).

2.10. Aspergillus parasiticus genomics

A cosmid-based sequencing of the *A. parasiticus* genome has been undertaken by the University of Oklahoma. While the sequencing is still in progress, the first two cosmids (more than 70 kb of sequence in total) are publically available from the NCBI.

2.11. Aspergillus terreus genomics

The sequencing of *A. terreus* was suggested in February 2002 in the first white paper by FGI due to its commercial relevance. Another white paper by Birren et al. (2004) emphasized the potential in comparative analysis with the genomes of other pathogenic Aspergilli such as *A. clavatus*, *N. fischeri*, and *A. fumigatus* to improve the understanding of fungal evolution and pathogenesis.

In August 2005, the sequence of the clinical isolate strain NIH 2624 was released by the Broad Institute complete with automatic annotation, but no analysis of it has been published so far.

Furthermore, the genome sequence of one of the *A. terreus* industrial strains is in the process of being sequenced by Microbia (now Ironwood Pharmaceuticals). No details on this project are readily available, but the clone library was applied in the work by Askenazi et al. (2003), which will described in detail in the section on transcription analysis.

3. Transcriptomics

The free accessibility of the genome sequences has resulted in several transcriptome studies of Aspergilli. Since microarrays for expression analysis of Aspergilli have not yet been designed by commercial companies, the research is mainly driven by the efforts of university research groups that have designed arrays and often have made the technology available to other researchers. An overview of the different platforms that have been made available for transcription studies in Aspergilli as well as a comprehensive list of the studies using these arrays are found in Table 2.

As indicated in Table 2, at least 34 examples of *Aspergillus* transcriptomics using microarray techniques have been published. In addition, several studies based solely on EST sequencing have been reported, the first as early as 1996 (Lee et al, 1996). EST-libraries have been very useful prior to genome sequencing such as in the studies by Prade et al. (2001) and Han and Prade (2002), and in tandem with genome data such as in the study by Ray et al. (2004) described in detail below.

To provide an overview of the area of transcriptomics, examples of distinct types of studies will be presented in more detail. The main groupings of transcriptional studies are: annotation of gene functions (functional genomics), studies of carbon flow regulatory mechanisms, EST database mining for new gene targets, elucidation of pathogenicity, spatial differentiation in the hyphae and direction of metabolic engineering using association analysis.

3.1. Functional genomics using microarrays

With the increasing number of genomes of Aspergilli being sequenced and published, the number of potential targets for study is increasing rapidly. However, the speed of sequencing and the rel-

Table 2
Overview of *Aspergillus* transcriptome studies and microarray formats

Species	Gene models (%)	Type	References
A. nidulans	19.4	cDNA	Sims et al. (2004a), Pocsi et al. (2005)
A. nidulans	26.0	cDNA	Malavazi et al. (2006)
A. nidulans[a]	79.0	Oligo	Malavazi et al. (2007), Breakspear and Momany (2007)
A. nidulans	89.2	Nimblegen	Bok et al. (2006)
A. nidulans	30.6	Febit	Mogensen et al. (2006)
A. nidulans	16.7	cDNA	Ray et al. (2004)
A. nidulans	26.1	cDNA	Sims et al. (2005)
A. nidulans	88.3	Nimblegen	David et al. (2006, 2008)
A. nidulans	99.6	Affymetrix	Andersen et al. (2008b)
A. niger[b,c]	100.0	Affymetrix	MacKenzie et al. (2005), Martens-Uzunova et al. (2006), Levin et al. (2007a), Pel et al. (2007), Guillemette et al. (2007), Yuan et al. (2008a,b)
A. niger[c]	99.3	Affymetrix	Andersen et al. (2008b)
A. fumigatus	96.2	Oligo	Nierman et al. (2005)[d] Sheppard et al. (2005), da Silva Ferreira et al. (2006), Perrin et al. (2007)
A. oryzae	16.8	cDNA	Maeda et al. (2004)
A. oryzae	24.4	cDNA	Masai et al. (2006)
A. oryzae	97.7	Nimblegen	Kimura et al. (2008)
A. oryzae	99.7	Affymetrix	Andersen et al. (2008b)
A. oryzae	89.1	Oligo	Tamano et al. (2008)
A. oryzae	98.6	Oligo	http://www.fermlab.com[e]
A. flavus[f]	39.7	Oligo	Yu et al. (2007)[d], Price et al. (2006), O'Brian et al. (2007), Wilkinson et al. (2007a,b), Cary et al. (2007), Chang et al. (2007)
A. flavus	39.9	Oligo	Kim et al. (2008)
A. flavus	6.0	cDNA	O'Brian et al. (2003), Price et al. (2005)

The percentage of the predicted gene models from the genome that are included on the microarray is shown.
[a] This array is available from the Pathogen Functional Genomics Resource Center (PFGRC).
[b] This array is proprietary of DSM, the Netherlands.
[c] These two arrays are designed from two different sets of predicted genes.
[d] This array was presented and defined in this study.
[e] No publication was found using this recently designed array.
[f] This array was designed from the *A. flavus* genome sequence, but has also been used for studies of *A. parasiticus*.

atively smaller community for genetics in Aspergilli compared to yeast makes high-quality annotation for all of the genes hard to come by.

Automatic annotation is being improved, but still manual curation is needed for the less studied genes. One approach is using transcriptomics as done for *A. nidulans* in the studies by Sims et al. (2004a,b), who presented two case-studies with slightly different approaches. One study (Sims et al., 2004a), is elucidation of the malate dehydrogenase isoenzymes and the other (Sims et al., 2004b) concerns itself with examination of 20 secretion-related genes. However, the approaches could be used for any group of genes of interest with some *a priori* knowledge of the regulation in the examined organism or a related species.

For the identification and examination of malate dehydrogenases, the first three possible targets were found by using the three *Saccharomyces cerevisiae* MDH genes as "*in silico* probes" and comparing these to the *A. nidulans* genome using BLASTn. This identified three regions in the *A. nidulans* genome with sequence identity to the corresponding yeast genes. By translating the coding regions of the *A. nidulans* sequence and using hierarchical clustering, it was found that the three *A. nidulans* sequences each paired with a distinct yeast malate dehydrogenase. This functional assignment was confirmed by examining the regulation of the three *A. nidulans* genes, and it was indeed found, that the genes behaved according to the functional prediction in a glucose-shift experiment.

Another study by Sims et al. (2004b) used 20 known genes from *A. niger* related to the secretory pathway to identify homologous sequences in *A. nidulans*. The putative exons of these sequences were examined using a custom DNA microarray, and found to be regulated on the transcriptional level in a manner mirroring that of the behavior in *A. niger*. Additionally, hypothetical orthologues of the endoplasmatic reticulum (ER) chaperones were found to be up-regulated, which supported the functional assignment.

A number of studies have used a combination of bioinformatics tools such as protein family predictions or sequence homology combined with expression profiling to annotate genes encoding carbohydrate-active enzymes in *A. niger*. Martens-Uzunova et al. (2006) identified glycoside hydrolases of family 28 (pectinolytic functions), and used expression profiling to provide functional assignment to 12 previously un-described genes. A similar method was used by Yuan et al. (2008b) to examine alpha-glucan acting enzymes. In the work of Yuan et al. (2008a), putative transcription factors located near inulinolytic genes in the genome sequence were deleted in separate mutant strains, and one transcriptional activator, influencing growth on inulin (InuR), was characterized and a list of genes putatively induced by this protein was generated.

3.2. Examining regulation of carbon metabolism

Knowledge of the regulation of the central metabolism is useful for understanding the potential for improved product formation or examination of carbon repression mechanisms. Several applications of microarrays are studying this type of regulation in Aspergilli.

Maeda et al. (2004) performed a comparative study in *A. oryzae* with a special emphasis on the excreted hydrolytic enzymes. An EST library of >16,000 sequences was prepared from mRNA from mycelia grown under several different culture conditions, including glucose-rich and carbon-deprived medium. In this case, there were approximately 6000 non-redundant sequences. Of these, 2070 highly expressed sequences were used for the construction of cDNA microarrays. By comparing the expression profiles of cultures grown on different media, it was found that the transcription of genes encoding hydrolytic enzymes was largest on complex wheat-bran medium. By examining the transcription levels for genes that encode enzymes of the Embden–Meyerhoff–Parnas (EMP) pathway and the tri-carboxylic acid (TCA) cycle, it was found that both of the pathways were active when grown on glucose.

David et al. (2006) used oligo-nucleotide arrays covering the majority of the identified genes in the *A. nidulans* genome. Their analysis also involved a reconstruction of the metabolic network from genomic sequences, and in this process they annotated more than 500 metabolic genes. Through combination of the reconstructed metabolic network and transcription profiling during growth on three different carbon sources (glucose, glycerol, and ethanol), they identified co-regulated metabolic genes and mapped the operation of the metabolic network during growth on these three carbon sources.

The work of Mogensen et al. (2006) used a custom array for genes in *A. nidulans* to study carbon catabolite repression at the level of transcription by comparing a *creA* deletion strain to a wild type grown on either glucose or ethanol. The study found a number of indirect effects of the *creA* deletion, and showed that ethanol, which had been thought to be a non-repressing carbon source, still induces some of the carbon catabolite repression components.

3.3. Transcription-regulated pathogenicity

A large effort has been put in examining pathogenicity in Aspergilli, mainly in comparisons of aflatoxin-producing strains of *A. flavus* and *A. parasiticus* as well as general studies of virulence and morphology in *A. fumigatus*. One of the first examples of this is the work of O'Brian et al. (2003), where an *A. flavus* cDNA clone library was used to identify new genes from *A. parasiticus* associated with aflatoxin production. A very similar approach is used by Price et al. (2005), but here a clone library was applied to identify a gene which decreases aflatoxin production when over-expressed.

The functions of a number of regulators of secondary metabolism have been examined using comparisons of expression indices in the wild type to the deletion strain. AflR, the regulator of the aflatoxin biosynthetic cluster, was examined in this fashion in *A. parasiticus* grown under conditions favouring aflatoxin production, thereby identifying genes not in the gene cluster that are influenced by the deletion of the gene (Price et al., 2006). Other studies include deletion of the *A. fumigatus* virulence and morphology-regulating factor StuA (Sheppard et al., 2005), the global regulator of secondary metabolism LaeA in *A. fumigatus* (Perrin et al., 2007), and the toxin regulator VeA in *A. flavus* and *A. parasiticus* (Cary et al., 2007).

The transcriptional response to the exposure to anti-fungal compounds or suppressors of virulence has also been the target of study. da Silva Ferreira et al. (2006) performed time-course transcriptome analysis of *A. fumigatus* exposed to the anti-fungal drug voriconazole to study the development of drug resistance. Meyer et al. (2007) studied drug resistance through the transcriptional response of *A. niger* to sub-lethal doses of the anti-fungal drugs caspofungin and fenpropimorph, inhibitors of glucan synthesis and ergosterol synthesis, respectively. A main result of this study was that the cell compensates for the inhibited processes by up-regulation of genes with similar function and cell-wall strengthening properties. A very interesting result is that of Kim et al. (2008), where transcription analysis of cultures grown in the presence of the anti-oxidant caffeic acid showed that it decreases both aflatoxin production and the expression of the 29 genes of the aflatoxin gene cluster, suggesting that aflatoxin production is triggered by a response to oxidative stress.

A reciprocal approach to understanding aflatoxin production is that of Chang et al. (2007), where instead of examining drug resistance, *A. flavus* loss-of-aflatoxin production mutants were cultured

and analyzed. This confirmed the involvement of many of the genes detected in the studies above.

3.4. Spatial differentiation

An often overlooked phenomenon in the study of fungal metabolism is differentiation in the mycelium. More often than not, the cells grow as a multicellular organism with a distinct difference between hyphal tip and the center of a pellet or the base of a substratum, as well as a difference of age between center and rim of a fungal colony.

A transcript profiling study of hyphal differentiation in *A. oryzae* was done by Masai et al. (2006). By separating the mycelium from the solid substrate with a cellulose acetate membrane and inoculation at one end of a square agar plate, the mycelium was made to grow in uni-directionally on top of the membrane. This technique allowed expression analysis of three distinct sections of the colony, namely the base, the white mycelium and the hyphal tips.

An even more sophisticated method is the ring-plate system presented by Levin et al. (2007b) that allows the division of agar-plate-grown cultures into five concentric circles. Transcriptome analysis of these circles allows transcription profiling across a fungal colony in *A. niger* (Levin et al., 2007a). This study showed clear spatial differentiation in areas of metabolism such as nitrate assimilation, secretion and proteases, as well as distinct differences in expression patterns on maltose versus xylose cultivations.

3.5. EST mining

An example of the use of functional annotation based on the use of an EST library is presented by Chigira et al. (2002). Initially, two candidate chitin synthases were found in a database of *A. oryzae* ESTs and cloned from the available sequence. The genes were sequenced and characterized, and one was selected for further analysis. Afterwards, the transcription levels of the selected chitinase gene were compared to the levels of other chitin synthases using microarrays.

In the work of Ray et al. (2004), two *A. nidulans* EST libraries (one from glucose growth and one from growth on polysaccharide) were compared to find genes induced on the polysaccharides using a novel method called negative subtraction hybridization allowing the identification of unique ESTs in a library comparison. These results were validated using a microarray, thus presenting both a new method and a list of induced genes during growth on polysaccharide.

3.6. Association analysis: directing the metabolic engineering

Perhaps one of the best examples of the use of transcriptomics in Aspergilli, that is also applicable for metabolic engineering, was presented by Askenazi et al. (2003). The authors developed a method, referred to as association analysis, which has the potential to decode the relationship between gene expression and metabolite levels. The method was applied on lovastatin and (+)-geodin production in *A. terreus*.

Initially, 21 strains with different yields of lovastatin and (+)-geodin was constructed and their gene-expression was examined on microarrays containing approximately 21,000 random genetic elements with an average length of 2 kb. Additionally, metabolite-levels were determined using high performance liquid chromatography (HPLC) and mass spectrometry (MS). Association analysis was performed on the combined metabolic and transcriptional data. By reducing the complexity of the transcription data to up-, down-, or no significant regulation for the transcription data, and decrease/increase in level for the metabolite data, an algorithm was employed to determine which genetic elements were significantly associated with metabolite production.

The association analysis was able to successfully identify the lovastatin biosynthetic cluster as being associated with lovastatin production. Additionally, it identified several previously unknown genetic elements correlated with geodin production, including the polyketide synthase. In addition to functional assignment, association analysis also provides information on the regulation of central pathways relative to production of secondary metabolites.

In the context of metabolic engineering, association analysis is a valuable tool. In addition to the discovery of new targets to engineer, several other uses are proposed and tried. First, the information on regulation of the central pathways gives pointers to which regulatory elements it could be profitable to un-couple. Second, if the analysis is performed with the yield of an unwanted by-product, one could find targets for elimination, and thus potentially improve yields of the wanted compound, and make downstream processing less cumbersome. Third, as demonstrated by Askenazi et al. (2003), promoters from genes correlated with metabolite yields can be used for making efficient reporter systems.

3.7. Identification of secondary metabolite clusters

The study of Bok et al. (2006) examined the potential of *A. nidulans* to produce novel secondary metabolites, but the method can be applied to any filamentous fungus with a sequenced genome. Secondary metabolites differ from other metabolites in filamentous fungi on the genomic level in that the genes of the biosynthetic pathway are often arranged in clusters. By examining the up- or down-regulation as a function of the position of the genome, clusters that are co-regulated can be found. Bok et al. (2006) used this to find the cluster for the biosynthesis of a novel metabolite, terrequinone A.

4. Proteomics

A recent review of proteomics in filamentous fungi uses a definition of the proteome to be the "global set of proteins expressed in a cell at a given time and biological state" (Kim et al., 2007b). While the presence of a genome sequence allows the identification of a protein from information on the amino acid sequence, the limitation in proteomics is often the purification, separation, detection and quantification of the individual proteins. A number of general methods mainly developed in *Escherichia coli* and *S. cerevisiae* have been published for this, including, but not limited to, isotope-coded affinity tags (Gygi et al., 1999), protein arrays (Zhu et al., 2001), two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) combined with MS of excised proteins (Bader and Hogue, 2002), and yeast two-hybrid systems (Auerbach et al., 2002). However, a direct adaptation of the protocols from yeast is not possible due to more sturdy cell walls, making the cell-lysing step of the preparation limiting in filamentous fungi (Kniemeyer et al., 2006). Additionally, heavily glycosylated proteins, found especially in the extracellular fraction, often have varying masses and are difficult to separate clearly as the isoelectric point can vary with the glycosylation. Method-development in proteomics of Aspergilli is being actively pursued, and the review by Kim et al. (2007b) refers to four studies presenting methods for sample preparation in relation to Aspergilli. Even so, all but two of the *Aspergillus* studies presented here, were done using 2D-PAGE for separation and MS for identification.

The limitation in the experimental procedures, perhaps combined with the relative ease of transcriptomics, means that the number of studies on *Aspergillus* proteomics is rather low compared to transcriptomics. Often, what is identified is a small per-

centage of the proteome or a so-called subproteome, a more or less defined subset of the proteome (Cordwell et al., 2000). Of special relevance to filamentous fungi, both in the context of industrial and medical applications, is the intracellular and extracellular subsets as well as proteins present in the cell wall and/or membrane. This section will make an overview of the proteome studies in Aspergilli in a roughly chronological order, with a particular focus on the progression in the number of separated and identified proteins as well as the examined fractions. To reflect the progression of the efficiency of the technique, a timeline of the studies showing the statistics of separation and identification can be found in Fig. 1.

Perhaps the first proteome study in Aspergilli is that of Bruneau et al. (2001), where the subproteome of proteins glycosylphosphatidylinositol (GPI)-anchored to the cell wall of *A. fumigatus* is examined. Using a sophisticated combination of enzymatic cleavage of the anchor, polyclonal antibodies and 2D-PAGE/MS, this group of morphogenesis proteins was studied prior to the release of an *A. fumigatus* genome sequence by comparison to the *S. cerevisiae* genome sequence.

Following that is the work of Melin et al. (2002), which must be the first comparative proteomics study in Aspergilli. Here, more than 200 peptide spots from *A. nidulans* were separated and at least 20 of these were found to be differentially regulated in a comparison of cultures treated with the antibiotic concanamycin A to a reference culture. Five of these were identified.

The work of Medina et al. (2004) examined enzymes secreted from *A. flavus* when grown on the flavonoid rutin or potato dextrose. The method obtained separation of more than 110 peptides, and identified 15 rutin-induced proteins and 7 non-induced proteins. A continuation of this study (Medina et al., 2005) expanded the growth conditions to include a glucose medium and refined the separation and detection methods to identify 51 secreted proteins.

With a special focus on the drug resistance response, Ström et al. (2005) studied the effect of anti-fungal compounds secreted from *Lactobacillus plantarum* on *A. nidulans* by comparing to control cultivation. A systemic response could be observed on the gels, and two differentially regulated genes were identified.

Asif et al. (2006) examined the subproteome of the *A. fumigatus* conidial surface in search of potential antigens. Using 2D-PAGE/MS followed by liquid chromatography (LC) and tandem MS/MS, 26 proteins were identified, among these a known allergen, thus proving the validity of the method.

A study of protein secretion in *A. oryzae* by comparative proteomics of extracellular proteins from solid-state and submerged cultures was conducted by Oda et al. (2006). Eighty-five peptide spots from the solid-state culture and 110 spots from the submerged culture were analyzed and 29 of them identified. A similar analysis of the subproteome of the cell wall bound fraction allowed a mapping of the secretion/retention pattern of the identified proteins, indicating that some proteins were expressed but retained in the cell wall in one culture while being excreted in the other.

Kniemeyer et al. (2006) evaluated a number of different methods of sample preparation for 2D-PAGE/MS and the best resulted in separation of more than 800 peptides from the intracellular fraction of proteins from *A. fumigatus*. The best method was used to examine the protein fractions detectable in cultures grown on glucose or ethanol, where 37 differentially regulated proteins were identified.

In a study of glutathione transferases from *A. fumigatus*, Carberry et al. (2006) examined these potential mediators of resistance to oxidative stress and anti-fungal drugs or xenobiotics. In a survey of the proteome, 180 peptides were separated and 54 intracellular proteins were identified. Using chromatography, the authors also performed a study of the subproteome consisting of glutathione binding proteins. From this, 10 proteins were separated and four of them identified followed by the characterization of one of the proteins.

The latest general proteome study and the one with the highest resolution is that of Kim et al. (2007c), where osmoadaptation in *A. nidulans* was investigated by comparative proteomics on a normal liquid culture medium versus growth in the same medium with 0.6 M KCl. Nine hundred and twenty-seven distinct protein spots were identified and 90 of these were determined to be differentially expressed, of which 30 were identified.

In a very applied and focused example of the potentials of *Aspergillus* proteomics, Kim et al. (2007a) used a novel combination of 2D-PAGE of secreted enzymes from *A. fumigatus* with an in-gel assay for beta-glucanase activity to identify expressed beta-glucanases. The detected beta-glucanases were identified using MS, and a novel heat-resistant beta-glucanase was characterized.

5. Metabolomics

The study of the full set of metabolites in the cell (metabolomics) is, compared to genomics, transcriptomics, and proteomics, a more complex task due to the large variation in the physical and chemical properties of the metabolites. Excluding sample-preparation, which can require several fractions and/or derivatization such as the method presented by Villas-Boas et al. (2003), combinations of analytical methods are required to separate and identify the metabolites. This has lead to the development of a number of high-throughput tandem methods for quantitative analysis including gas chromatography–mass spectrometry (GC–MS), gas chromatography–time-of-flight mass spectrometry (GC–TOF), and liquid chromatography–mass spectrometry (LC–MS) (Villas-Boas et al., 2005), as well as direct infusion mass spectrometry (diMS), a metabolome profiling technique developed specifically for fungi (Smedsgaard and Nielsen, 2005). While the potential is

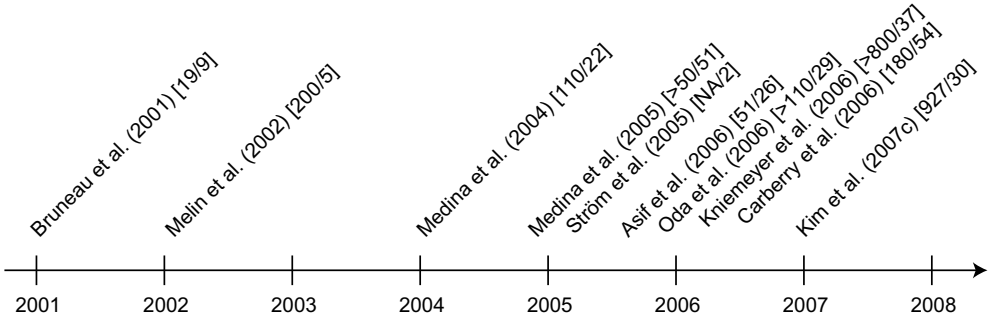

Fig. 1. Timeline of proteomics studies in Aspergillus species. Numbers in sharp brackets are [number of separated peptides/number of identified peptides]. "NA" denotes that the information was not available.

thus present (Fernie et al., 2004), very few research papers have been published doing large-scale quantitative studies in Aspergilli. One such is the work of Askenazi et al. (2003) discussed in the section on transcriptomics, where the combined analysis of metabolome and transcriptome were employed to improve the yield of lovastatin and identify new genes.

Another very recent example, and perhaps the best example of metabolomics in an *Aspergillus* species, is the work of Kouskoumvekaki et al. (2008), where metabolic profiles based on more than 450 metabolites from *A. nidulans* were used to identify seven biomarkers indicative of the genotype of the strain. This method can identify mutants producing the model polyketide 6-methylsalicylic acid in high-yields.

6. Modeling

Generally, two main methodologies of systems-wide analysis of metabolism have been applied in microbial systems (Nielsen, 1998). One is metabolic flux analysis (MFA) or flux balance analysis (FBA) characterized by the calculation of carbon fluxes using a list of bio-chemical reactions available to the cell (a stoichiometric model) and applying mass balances for each metabolite. Typically, carbon fluxes into and out of the cell are estimated by measuring extracellular concentrations and biomass and used to calculate the fluxes in the cell. Stoichiometric models for the central metabolism and full genome-scale of *A. niger* (David et al. (2003), Melzer et al. (2007), Andersen et al. (2008a)) and a genome scale model for *A. nidulans* (David et al., 2006) have been published previously, and a genome-scale model for *A. oryzae* is currently in review (Vongsangnak et al., personal communications).

The other main approach is kinetic modeling. One framework for this is metabolic control analysis (MCA). This approach focuses on quantifying the control the individual enzyme activities in a pathway exert on the flux through the pathway. This is done by two sets of coefficients, the so-called elasticity coefficients and the flux control coefficients (FCC) (Fell, 1992). The first expresses the sensitivity of the individual reaction rates to changes in the metabolite concentrations and the FCC express the sensitivity of the flux towards the enzyme activities in the metabolic network. In order to derive the elasticity coefficients and the FCCs, it is necessary to either titrate the enzyme level inside the cell or to set up a kinetic model for the individual enzymatic reactions in the metabolic network under study. The latter requires extensive information on the kinetics of the individual enzymes as well as solid data on the intracellular metabolite levels, and is therefore laborious to obtain. To circumvent this problem, it has been proposed to use generalized kinetic expression, the so-called S-system, where the kinetics of each reaction in the network is described as a power-law function of all the metabolites in the network (Voit, 2005).

6.1. Stoichiometric modeling

The use of FBA has several useful properties for understanding metabolism. The main strength is the power to elucidate the distribution of carbon fluxes and identifying key branch points in the cellular pathways. One also has the possibility of examining whether the insertion of new enzymes or entire pathways has the potential to improve the yield of a given product. Yet another use is the calculation of the maximum theoretical yields (Nielsen, 1998). This allows for estimation of the commercial relevance of production of a given metabolite/protein in the modelled organism. Price et al. (2004) describe a toolbox available to analysis of stoichiometric models of microbial cells.

Several stoichiometric models exist for *A. niger*. An early model for *A. niger* central carbon metabolism was presented by David et al. (2003). In this case, the model was applied to search for strategies to improve succinate production. When testing all combinations of two gene deletions, it was found that a fruitful strategy might be a deletion of ATP:citrate oxaloacetate-lyase and pyruvate decarboxylase giving a yield of at least 1.12 mol succinate per mol glucose. A smaller model of *A. niger* metabolism has been presented by Melzer et al. (2007) based on the model of David et al. (2003) and other information, and this model was used to predict metabolic fluxes through pathways of central metabolism in cultivations on two different media and at varying levels of ambient pH.

A recently published comprehensive model of *A. niger* metabolism and the validation and analysis of it was presented by Andersen et al. (2008a). This study calculated the maximum theoretical yield of several industrial products as well as the model was used to simulate the operation of the oxidative pathways during production of citrate in high yields.

For *A. nidulans*, a stoichiometric model has been developed by David et al, and applied for studies in the publications of David et al. (2006) and David et al. (2008). The model was used to improve the functional annotation of 472 genes as well as aiding in the interpretation of transcriptome data.

6.2. Kinetic modeling

Torres et al have addressed the kinetic modeling of glucose metabolism in *A. niger* in several articles (Torres et al., 1993, 1998; Torres, 1994a,b). In the publications of Torres et al. (1993) and Torres (1994a), the model was presented and tested for stability. Torres (1994b) analyzed the system, and came to the conclusion that an up-regulation of the hexokinase transporter would be beneficial for improving citrate production; however this analysis used lumped expressions for the largest part of glycolysis. A further analysis with newer algorithms (Torres et al., 1998) found the optimal production of citrate while still holding the metabolite pools approximately constant. However, this solution required the simultaneous modulation of seven or more enzymes.

The model was further improved with an increased complexity and used in the study by Alvarez-Vasquez et al. (2000). This model represents the main pathways involved in citric acid production including the mitochondrial reactions, which were not included in the study by Torres et al. (1998). When applied to the optimization of citric acid production, it was found that the maximum potential was not yet reached. However, the further increase would require the modification of a minimum of 13 enzymes. The rate of citrate production was determined to be able to increase 3- to 50-fold.

Another dynamic pathway modeling in *A. niger* was performed by de Groot et al. (2005) where MCA was employed to examine the potential to grow on L-arabinose. The goal was to find targets for metabolic engineering so that organic waste may be used for production processes involving *A. niger*. By examining the FCC's it was found that the first three enzymes of the pathway, L-arabinose reductase, L-arabitol dehydrogenase and L-xylulose dehydrogenase would be the most profitable targets. A technique to insert the L-arabinose pathway in fungal species and thus expanding their production range has been patented by Londesborough et al. (2002).

7. Future perspectives

With the number of *Aspergillus* species still in the sequencing pipeline, it is clear that the genomic era of Aspergilli has only just started. The future release of more sequences as well as more thorough comparative genomics studies holds promises for unravelling many different new mechanisms and identification of elements that define the individual species as well as are conserved through-

out the genus. Especially for research in pathogenicity, biotechnology and eukaryotic cell physiology, future genome studies may have a substantial impact.

The sequencing of *Aspergillus* genomes, both the currently available and the ones to be released in the future will continue to drive the progress of the other levels of systems-wide studies. As seen in Table 2, transcriptome platforms have already been developed for five different Aspergilli species, and with this increased availability of platforms, the road is paved for more studies on transcriptional regulation. As described in the sections on transcriptional regulation of carbon metabolism and pathogenicity, this tool may be a powerful aid in unraveling the role of the hundreds of transcriptional regulators present in the *Aspergillus* species, especially in the conjunction with large-scale deletion studies such as what has been conducted in *S. cerevisiae* (Winzeler et al. 1999).

While the discipline of proteomics in Aspergilli is only in its infancy, the perspectives and potentials of the method are evident. Especially the studies taking advantage of the protein separation, such as that of Oda et al. (2006) or Kim et al. (2007a) are producing results that would be difficult to obtain using other techniques. A recent publication by Daly et al. (2008) using novel statistical methods and an LC/MS methodology to perform high-throughput analysis of the proteome of *Trichoderma reesei* and the identification of 1546 proteins, shows the potential of systems-level proteomics in filamentous fungi.

However, the largest potential in the application of the genome-scale techniques lies in the combination of genome data with data sets from several disciplines to amount in multi-level systems biology. While this is often done in modeling approaches, this approach also represents the addition of value to the data, as multiple levels—correctly interpreted—provide the researchers with novel insights to the biology of Aspergilli. The first steps down this road have already been taken and new insights are many, thus holding great promises for the future of systems biology in *Aspergillus*. We therefore foresee that as the systems biology toolbox of *Aspergilli* is expanded, it will be possible to further advance our understanding of how some of these species impose pathogenicity and also further advance the use of *Aspergilli* as cell factories for sustainable production of chemicals.

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