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Developing *Aspergillus* as a host for heterologous expression

David Lubertozzi [a], Jay D. Keasling [a,b,c,*]

[a] Department of Chemical Engineering, University of California, Berkeley, CA 94720, United States
[b] Department of Bioengineering, University of California, Berkeley, CA 94720, United States
[c] Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley CA 94720, United States

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ABSTRACT

Filamentous fungi have long been used for production of a range of valuable products; with the advent of molecular biology, it became apparent that these fungi possess considerable potential as expression hosts for the production of heterologous proteins and small molecules. *Aspergillus* is an important genus, including well known species of economically significant molds, and widely used for basic genetic research. The development of a genetic engineering "toolkit" for *Aspergillus*, such as those existing for the simpler yeasts and bacteria, was delayed due to the added complexity of the filamentous fungi, and also to the lesser resources devoted to their study. History of the development of *Aspergillus* as an expression host, current state of the art and future directions are reviewed, touching on related research in other fungi when discussing the areas of greatest potential for future biotechnological applications, focusing on the large and diverse families of fungal secondary metabolites.

Contents

1. Introduction

The Aspergilli are a large and diverse genus (~180 species) of filamentous fungi including several well known species with substantial commercial value (*A. oryzae* and *A. niger*) and medically significant molds; both as pathogens (*A. parasiticus* and *A. fumigatus*)

* Corresponding author. Berkeley Center for Synthetic Biology, 717 Potter Street, Building 977, Mail code 3224, University of California, Berkeley, CA 94720-3224, United States. Tel.: +1 510 495 2620; fax: +1 510 495 2630.
E-mail address: keasling@berkeley.edu (J.D. Keasling).

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and toxin-producing contaminants of food and feed (*A. flavus*), as well as lesser-known but valuable species such as *A. terreus*, source of the cholesterol lowering agent lovastatin. *A. nidulans* has been one of the most widely used filamentous fungi for basic genetic research, and continues to be used a model organism for the study of common eukaryotic cellular functions. *Aspergillus* will grow at a wide range of temperatures (10–50 °C), pH (2.0–11.0), and osmolarity (from nearly pure water up to 34% salt (Kis-Papo et al., 2003)). It is also capable of continuous excretion of large amounts of metabolites into the culture medium (e.g., up to 200 g/L of citric acid (Magnusun and Lasure, 2004)), and its filamentous morphology allows separation of cells by simple filtration, simplifying recovery of valuable products. Filamentous fungi have evolved a prodigious enzyme secretion ability in their natural saprobic growth habit, often on solid substrates at low water activity, down to A_w 0.40 (Nigam and Robinson, 2004); compared to 0.85 minimum for yeast (Beuchat, 1981). They are widely used for solid-state fermentation of grain-based foods (*miso*, *shoyu*) (Murooka and Yamshita, 2008) and beverages (*sake*, *shochu*) (Kitamoto et al., 1991; Minetoki and Kitamoto, 1997) and for industrial enzyme production, especially for their high titers of native hydrolytic enzymes, in particular amylases and proteases (e.g., 30 g/L of glucoamylase from *A. niger* (Withers et al., 1998)).

Despite its obvious utility and potential, the number of researchers working with *Aspergillus* is small compared to the yeast *Saccharomyces*; still, a great deal of work has been done. A complete physical map of the *A. nidulans* genome is finished (Clutterbuck, 1997) and in 2001 the whole genome was sequenced by Cereon (Monsanto, 2001), which was later updated with improved coverage and annotation and made public by the Broad Institute (Galagan et al., 2005). Genome sequences for *A. oryzae* (Machida et al., 2005), *A. fumigatus* (Nierman et al., 2005b) and *A. niger* (Pel et al., 2007) followed shortly and are at or nearing completion of annotation and public availability; genome sequences for *A. clavatus*, *A. flavus*, *A. parasiticus*, and *A. terreus* are also in various stages of development in public and private efforts (Jones, 2007). Over 300 genes have been cloned, mostly from *A. nidulans*, many by complementation of random mutants, which provides today's researcher with several hundred mutant strains to work with. Discovery of the penicillin and aflatoxin biosynthetic pathways in several *Aspergillus* species has spurred extensive investigation into their secondary metabolism, revealing important links with developmental processes and environmental influences such as nutrient source and host plant response (Wilson et al., 2004).

The Aspergilli, in common with *Saccharomyces* and other fungi, possess large genomes with many genes sharing homology to higher organisms. At ~30 Mbp, the *A. nidulans* genome is over twice the size of *Saccharomyces*; although both are ascomycetes, many genes appear to have no homologs in yeast (Bennett, 1997). The *A. fumigatus* genome is similarly sized at 29 Mbp; the *A. niger* and *A. oryzae* genomes are larger at ~36 Mbp and ~37 Mbp each, respectively. The greater number of genes they have (roughly 10,000 for *A. nidulans* and *A. fumigatus* (Broad Institute, 2003a; TIGR, 2005), ~12,000 for *A. ozryzae* (DOGAN, 2005) and perhaps over 14,000 for *A. niger* (Integrated Genomics, 2006) could be accounted for by their adaptation to utilize such diverse carbon and nitrogen sources as formamide and quinic acid (Bennett, 1997). *A. oryzae* appears to be especially enriched for genes involved in biomass degradation, primary and secondary metabolism (Kobayashi et al., 2007). The metabolic diversity of the Aspergilli presents an opportunity for expanding the range of possible heterologous metabolites in this host organism. *Aspergillus* is especially promising as a host for recombinant proteins, the fastest growing class of new therapeutics. Many are currently produced in mammalian cells, which have the cellular machinery for protein translation, folding, and post-translational modification that bacteria lack; the fungi also have this machinery, while sharing with prokaryotes much of their ease of culture.

The GRAS (generally recognized as safe) status of *A. niger* and *A. oryzae* makes them attractive as expression hosts, since this status should facilitate approval of new food or drug products. *A. nidulans*, unlike the others, possesses the distinct advantage of a sexual reproduction phase (upon discovery of which it was properly assigned to the genus *Emericella*, but the former imperfect name is still in common use). This allows genetic analysis *via* crossing and trait selection by conventional breeding. A parasexual phase also allows the creation of forced heterokaryotic diploid strains (Clutterbuck, 1996), especially useful in analyzing gene function in cases where a mutation may be lethal. Since many of its genes are functional in its relatives and *vice versa*, *A. nidulans* can serve as a surrogate research organism for species that are difficult or hazardous to work with such as *A. fumigatus*. *A. nidulans*, unlike some of the other species, also has uninucleate asexual spores (conidia), which simplifies the selection of homokaryotic strains.

While *A. nidulans* is perhaps the most studied, it is a toxigenic species, producing the less toxic sterigmatocystin rather than the notorious and highly toxic aflatoxins found in *A. flavus* and others. The genetics behind production of these toxins has been intensively studied for over two decades (reviewed in Bhatnagar et al., 2006). Investigation into aflatoxin biosynthesis in *Aspergillus* revealed that many commercial strains of *koji* molds (*A. oryzae*, *A. sojae* and even some *A. flavus*) possess aflatoxin pathway genes (Tominaga et al., 2006). In these strains the aflatoxin gene cluster is either incomplete or inactive due to various mutations that are still not fully characterized (Matsushima et al., 2001), thus, there is some concern for toxigenic potential in these species (van den Broek et al., 2001). Population genetic studies provide evidence for some occurrence of sexual reproduction in *A. fumigatus* (Dyer and Paoletti, 2005), and the *Aspergillus* genome data shows that several of the Aspergilli possess many of the genes known to be involved in mating in *A. nidulans* (Scazzocchio, 2006), so discovering or possibly engineering a sexual phase in one of the supposedly asexual species is not ruled out. This might also imply that wild toxigenic strains could cross with domestic non-toxigenic ones. Ultimately the safest solution may be to engineer *Aspergillus* host strains with all toxigenic genes removed.

Historically, strain improvements in filamentous fungi have been by a trial-and-error process, the development of penicillin production strains being archetypal: random mutation and screening to select for increased production of a single metabolite (or lack thereof, as with aflatoxin). Given our increasing knowledge of the molecular genetics of *Aspergillus*, it is now becoming more feasible to introduce rationally designed metabolic changes through genetic manipulation.

2. Transformation of *Aspergillus*

The development of transformation techniques for *Aspergillus* and other filamentous fungi lagged behind those for *E. coli* and *S. cerevisiae*, complicated by their multicellular morphology, thick chitinous cell walls, and lack of plasmids. The first reports of gene cloning (Kinghorn and Hawkins, 1982) and transformation in *Aspergillus* (Ballance et al., 1983; Tilburn et al., 1983) were in 1982 and 1983, respectively. Several laboratory methods for *Aspergillus* transformation are currently in use. The most popular technique, adapted from that originally developed for yeast, is the chemical treatment of nucleated protoplasts produced by enzymatic digestion of the fungal cell wall (see Fig. 1). Refinements of this method have increased transformation efficiencies several orders of magnitude since its conception, from <10 transformants/μg DNA to several hundred or thousand for some systems (Dawe et al., 2000). Other methods include electroporation (Ward et al., 1989; Sanchez and Aguirre, 1996) and a biolistic technique (Herzog et al., 1996; Barcellos et al., 1998). The plant transformation workhorse *Agrobacterium tumefaciens* has been used to transform *Aspergillus* and other fungi (Gouka et al., 1999), including