Novel strategies in antifungal lead discovery

Bo Jiang*†, Howard Bussey§ and Terry Roemer*‡

There have been significant developments in fungal genomics over the past year. The recently released genome sequences of *Aspergillus fumigatus* and *Cryptococcus neoformans* have provided unprecedented opportunities for comparative genomics studies of many clinically relevant fungal pathogens. Emerging experimental analysis tools, such as fitness profiling and protein microarrays, have greatly enhanced our ability to conduct genome-wide functional studies.

Addresses
*Elitra Canada, 225 President Kennedy West, Suite 2550, Montreal, Quebec, H2X 3Y8, Canada
†e-mail: bjiang@elitra.com
‡e-mail: troemer@elitra.com
§Department of Biology, McGill University, Stewart Biology Building, Room W5/19, 1205 Dr Penfield Avenue, Montreal, Quebec, H3A 1B1, Canada; e-mail: hbusse@po-box.mcgill.ca

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Abbreviations

CaM	calmodulin
Erα	estrogen receptor α
PI	phosphoinositide
STM	signature-tagged mutagenesis
TAP	tandem-affinity purification
TOR	target of rapamycin

Introduction

There is an acute need to develop new drugs to treat fungal infections. The urgency stems from the therapeutic limitations of the two current antifungal drug classes, polyenes (such as amphotericin B) and azoles (such as fluconazole and itraconazole), which display toxicity and a limited spectrum of efficacy, respectively. Compounding this need is an increasing incidence in life-threatening fungal infections associated with immunosuppressed and neutropenic individuals, as well as the emerging trend for drug-resistant isolates in the clinic. Recent progress has been made in the development of third-generation azoles [1] and, significantly, in 2001 — for the first time in over 30 years — a member of a new therapeutic class of antifungal drugs, Caspofungin™ [2,3], entered the clinic. However, to preclude wide-spread drug resistance and to provide safer and more efficacious treatment of fungal infections, a wider repertoire of antifungal drugs and new approaches for their identification and development is still required.

The application of fungal genomics offers an unparalleled opportunity to develop novel antifungals. The release of the genome sequence of *Candida albicans* in 2000 started a post-genomics era, and the subsequent release of the sequences of two additional fungal pathogens and the emergence of new genome-wide characterization tools have further improved its application. In this review, recent advances in fungal genome sequencing and strategies for research tool development in the area of fungal genomics are discussed, with special emphasis on their impact on drug discovery.

Fungal genome sequences

The year 2001 saw the release of the nearly complete, unannotated genomes of the human pathogens *Aspergillus fumigatus* and *Cryptococcus neoformans*, and this contributed greatly to the understanding of the degree of nucleotide sequence conservation in these clinically relevant pathogens. The *A. fumigatus* sequence assembly is available from The Institute for Genomic Research (TIGR) [4], with additional sequence available at the Sanger Institute [5]. For *C. neoformans*, the nucleotide sequences obtained from two strains are available from TIGR [6] and the Stanford Genome Technology Center [7]. Although both the *A. fumigatus* and *C. neoformans* sequencing projects are now in the final phase, there sadly appears to be no clear plan to complete sequencing of the entire eight-chromosome *C. albicans* genome, whose latest diploid assembly is represented by 270 contigs [8]. To date, eight complete or near-complete fungal genome sequences are publicly available [4–13], covering members of both ascomycete and basidiomycete subphyla, and providing a wealth of comparative information. The annotation of the human genome continues and the recent release of the mouse genome sequence [14] should accelerate human proteome annotation. Thus, the identification of the global set of broad-spectrum antifungal drug targets absent from humans, seemingly a dream a decade ago, is now an attainable goal.

Bioinformatics-based target prioritization has traditionally demanded that targets be conserved amongst fungal pathogens and lack a human counterpart. Although target conservation amongst fungal pathogens is obviously required for broad-spectrum drugs, examination of antibacterial and antifungal targets suggests that the need for the absence of a human counterpart is less clear. For example, the cell wall *murA* gene encodes the drug target of fosfomycin. Although the *murA* gene product has no clear human homolog (the closest related human gene product displays a BLAST Expect score of 5.0), the drug fosfomycin is poorly tolerated. (BLAST (Basic Local Alignment Search Tool) is a set of computer programs designed to conduct similarity searches against protein or DNA sequence databases. The Expect value [E] is a parameter that describes the number of hits one can 'expect' to see just by random chance in a particular search. For example, an E value of 5 assigned to the best murA hit can be interpreted as meaning that one might expect to see five matches with a similar alignment just by random chance,

indicating the match has NO statistical significance at all.) Conversely, azoles that inhibit *ERG11* (which encodes lanosterol 14α-demethylalase) are regarded as highly safe and non-toxic, despite a high degree of amino acid sequence conservation between the fungal (for example, *C. albicans*) and human *ERG11* proteins (BLAST Expect score of E-77 [i.e. the observed sequence similarity between yeast and human *ERG11* has very high statistical significance]). Therefore, target prioritization based on bioinformatic analyses is necessary but may not be sufficient or fool-proof in predicting the spectrum or toxicity of drugs developed against selected targets.

In view of this caveat, the wealth of genomic information now applicable to antifungal drug discovery shifts the challenge from target identification to validating and prioritizing targets among large potential target sets for those best suited for compound screening. Strategies are needed that functionally interrogate these 'in silico' target sets, and innovations in *Saccharomyces cerevisiae* functional genomics continue to rise to this challenge.

Genome-wide gene disruptions in *Saccharomyces cerevisiae*

The *Saccharomyces* Genome Deletion Project, in which an international consortium constructed individual deletion mutants in 5900 genes [15••], offers the only nearly complete collection of null mutants for any eukaryote. This directed, global-mutant approach offers several significant advantages over traditional genetic methods. Null-mutant phenotype screens are now possible, using the entire collection of non-essential mutants, for genes involved in a wide range of processes. Functional annotation of the genome is immediately revealed, as mutants displaying phenotypes under the condition of the screen identify participating genes. In contrast to classic mutagenesis, 'saturated' mutant screens may now be performed, with an accounting of which genes are involved, and which are not, in a particular phenotype. It is now up to the creativity of the researcher to develop novel screens to interrogate the deletion set.

Pertinent to antifungal lead discovery, the *S. cerevisiae* genome deletion project uncovered the global set of essential genes; 1105 genes were identified as essential for growth on rich medium at 30°C [15••]. Although this number is unlikely to exactly reflect the essential gene set in *S. cerevisiae*, it does 'filter' target sets to a subset that meets the traditional criteria of a drug target, which are that it be essential for cell growth and viability *in vitro*. The degree to which functional information from *S. cerevisiae* can be extended to fungal pathogens is unknown and large-scale gene disruption projects in fungal pathogens will be needed to address this important comparative question.

Chemogenomics

Recent reports have used the *S. cerevisiae* deletion set to understand global responses of cells challenged by chemical insults. For example, Chan *et al.* [16] studied global genetic interactions with target of rapamycin (TOR), which is encoded by the *TOR1* and *TOR2* genes. Rapamycin is an immunosuppressive antibiotic that, when complexed with its intracellular receptor *FKBP12*, binds and blocks TOR function. Complete loss-of-function mutations in the yeast *TOR1* gene result in rapamycin sensitivity, whereas a specific point-mutation, S1972I, leads to dominant rapamycin resistance. To better understand TOR function, Chan *et al.* examined the relative sensitivity of 2216 haploid non-essential gene deletion mutants to rapamycin, and identified 73 hypersensitive mutants and 27 resistant mutants. They showed that the rapamycin-hypersensitive phenotype was a direct effect of inhibition of TOR function, as none of the 73 mutants were hypersensitive to rapamycin in the presence of the S1972I dominant-resistant *tor1* allele. In total, 100 genes that display rapamycin 'chemotypes' as deletion mutants were identified, allowing construction of a genetic interaction map for TOR. The rapamycin sensitivity of 50 diploid strains containing heterozygous deletions of essential genes was also examined, and six rapamycin-hypersensitive diploid strains were identified, demonstrating that the approach is equally applicable to essential genes.

Furthermore, Desmoucelles *et al.* [17] screened 4787 non-essential gene deletion mutants for their relative growth in the presence of mycophenolic acid (MPA), a candidate immunosuppressive drug. The authors identified 100 new genes that affect MPA resistance, in the areas of transcription, ergosterol biosynthesis, vacuole biogenesis and glycosylation. These results suggest possible ways to potentiate a drug and to limit its natural detoxification.

Chemical genomics is thus a powerful tool in the understanding of gene function and is one amenable to mode-of-action studies for drug discovery. As drug sensitivity screening uses relative cell growth as a read-out, the procedure could be readily automated using, for example, the recently developed high-density cell microarray [18], which contains more than 5000 distinct yeast deletion strains.

Fitness profiling

A major advantage of the *Saccharomyces* deletion mutant set is the ability to examine phenotypes of all deletion strains simultaneously in a single culture [15••]. This is achieved by the introduction of two unique 20-nucleotide tags, or 'bar codes', that are internally assigned to the disruption allele in each deletion strain. These bar codes can be used as hybridization probes against a high-density oligonucleotide microarray containing the complementary tag sequences. The resulting hybridization intensities are then used to deduce the relative growth rate, or fitness, of each strain within the pool according to their relative abundance in the pool (that is, presence or absence, or under- or over-representation) after growth under a particular condition. Several papers attest to the utility of this 'parallel deletion analysis' or 'fitness profiling' approach. For instance,

Birrell *et al.* [19] treated a pool of 4627 deletion strains with low doses of UV radiation and successfully identified most of the previously known UV-sensitive strains present in the pool, as well as three deletion strains identifying genes not previously known to be UV-responsive. Using a pool containing 4757 deletion mutants, Giaever *et al.* [15••] performed fitness profiling studies under six different growth conditions: high salt, sorbitol, galactose, altered pH, minimal medium and nystatin treatment. Again, both known and novel genes required for optimal growth under each of these well-studied conditions were identified.

In other studies, Tong *et al.* [20] used the deletion mutant set to perform global synthetic lethal interaction studies. Millennium Pharmaceuticals (Cambridge, MA, USA) has elegantly used the fitness test assay, in conjunction with transcript profiling, to confirm the mechanism of action and cellular consequences of a new class of proteasome inhibitors [21•].

Although the *S. cerevisiae* deletion mutant set is a valuable resource, it has limitations. The deletion sets used in the above large-scale studies usually lack the essential genes, which are the preferred targets for antifungal lead discovery. Furthermore, achieving its full potential in the antifungal lead discovery area will require use of the heterozygous deletion strains covering both essential and non-essential genes [16,22]. Finally, *S. cerevisiae* is not a human fungal pathogen, which has significant drawbacks. Thus, the creation of a comprehensive deletion mutant set in a human fungal pathogen, such as *C. albicans*, may provide a better understanding of the genes that encode clinically relevant drug targets for human antifungal lead discovery.

Transcript profiling

An established approach in drug discovery is the use of transcript profiling, and two recent papers illustrate its extension to antifungal targets. Lorenz and Fink [23] studied the effects of phagocytosis on yeast cells by co-culturing *S. cerevisiae* cells and mammalian macrophages, isolating the phagocytosed yeast cells, and performing DNA microarray analysis. After phagocytosis, 11 out of the 15 most highly upregulated genes encode proteins related to the glyoxylate cycle, through which two-carbon compounds are assimilated into the tricarboxylic acid (TCA) cycle. The authors focused on two genes, *ICL1* (which encodes isocitrate lysase) and *MLS1* (which encodes malate synthase), whose activities are both specific and limited to the glyoxylate cycle. Northern analysis confirmed that both genes are upregulated by macrophage ingestion in both *S. cerevisiae* and *C. albicans*. They examined *C. albicans*, using a homozygous *Δicl1/Δicl1* deletion mutant, and showed that this mutant was less virulent in mice. These results identify the glyoxylate cycle enzymes, which are not found in mammals, as potential antifungal target candidates.

Lane *et al.* [24] used DNA microarrays containing PCR products from ~700 *C. albicans* genes. Despite their limited coverage, these microarrays allowed transcript profiling studies in the pathogen. By monitoring expression profiles under normal and hypha-inducing growth conditions, many virulence factor genes were found to be co-regulated by three transcriptional pathways. De Backer *et al.* [25] performed a more comprehensive transcript profiling using the Incyte *C. albicans* microarray to examine the effect of itraconazole, a broad-spectrum antifungal drug. A global upregulation of ergosterol-related genes was observed, with most of the genes that exhibited the highest level of upregulation encoding ergosterol biosynthetic enzymes. Interestingly, the target gene *ERG11* was not the only gene highly responsive to itraconazole treatment, precluding its unique identification.

Although these studies demonstrate the utility of transcript profiling analysis in target identification and mode-of-action studies, they also show that transcript profiling has limitations. One is lack of specificity, with transcript profiling analyses typically identifying simultaneous expression changes in hundreds of genes. Also important is the correlation between changes in gene expression in response to a particular condition and the function of such genes. Several recent studies uncover surprisingly little correlation between genes shown by transcript profiling to be upregulated in response to a particular condition and mutants of such genes showing any observable phenotypes under identical conditions. Giaever *et al.* [15••] compared fitness profiling results with transcript profiling data for all conditions tested (see above), and found that less than 7% of genes showing significant upregulation in transcript levels displayed a reduced fitness under the same growth conditions. Birrell and colleagues [26••] also found little overlap between the set of genes important for cell survival to DNA-damaging agents with those whose transcription is increased after exposure to these agents. These observations urge caution in interpreting transcript profiles for prioritizing antifungal targets for lead discovery. Part of the explanation for these counterintuitive observations probably rests in the redundancy of cellular genetic networks. In any event, target identification and mode of action studies will require additional, independent experimentation to distinguish the direct and specific responses from the numerous indirect ones.

Proteomics

Over the past year, significant advances have occurred in the field of proteomics that are applicable to antifungal lead discovery. For example, Zhu *et al.* [27••] fused the GST-HisX6 tags to the amino-terminals of 5800 genes, individually purified these fusion proteins, and robotically arrayed these proteins to glass slides. As more than 80% of the fusion proteins were successfully expressed, these high-density protein arrays allow enzymatic or binding assays to be performed simultaneously on ~80% of the *S. cerevisiae* proteome. In a calmodulin (CaM)-binding assay, most of the known CaM-binding proteins present on the chip were successfully identified, as were many new

CaM-binding proteins. Furthermore, Zhu *et al.* probed the protein microarray with liposomes containing five different types of phosphoinositides (PIs), and identified several PI-binding proteins with various specificities towards different PI molecules. The biochemical screening capacity offered by the protein microarray makes it a promising tool in antifungal lead discovery.

Two reports described large-scale analyses of protein complexes in *S. cerevisiae*. Gavin *et al.* [28•] inserted a tandem-affinity purification (TAP) tag at the 3′ end of nearly 30% of the yeast genome. The TAP-tagged gene set was expressed with the TAP-tagged proteins serving as 'bait' to fish out the respective *in vivo* protein complexes. Complex proteins were subsequently identified by SDS-PAGE and mass spectrometry. After processing 1739 3′-TAP-tagged genes, 589 protein complexes comprising 1440 distinct gene products were successfully obtained, defining 232 non-redundant multiprotein complexes that contained between two and 38 protein components. This study also identified 304 proteins with previously unknown functions in complexes with proteins of known function, providing 'guilt-by-association' evidence for their cellular roles. Using a similar approach, Ho *et al.* [29] constructed 725 'bait' proteins by using the Flag-epitope tag. From these bait proteins, 3617 interacting proteins were identified, representing 1578 different gene products. Of these, 531 corresponded to previously uncharacterized proteins. These studies herald the construction of a proteome-wide protein interaction map.

Signature-tagged mutagenesis

Antifungal drug discovery has focused on compounds that display cidal (such as amphothericin B) or static (such as fluconazole) activity towards the pathogen. Drugs that rapidly kill a pathogen are preferred when treating life-threatening infections. A long-term weakness of this approach is a strong selection for drug-resistant strains, especially as such drugs are increasingly being used prophylactically. An alternative strategy is to use virulence factors that contribute to pathogenesis as drug targets. For example, pathways contributing to bacterial pathogenesis, including secretion, two-component signaling and quorum sensing, are being leveraged for novel targets (see [30] and references therein). Resistance to drugs developed against virulence targets may be less likely, as selective pressure is reduced on non-essential targets required only to colonize host environments.

A major limitation, however, to identifying virulence genes is the need for genomic manipulations to be performed directly in the desired pathogen. Although the power of *S. cerevisiae* may often be usefully 'bent' toward this endeavour [23,31,32], only a limited coverage of the genes involved in pathogenesis can be obtained because substantial genomic and physiological non-overlap exists between the model organism and the pathogen. Therefore, verification of candidate genes contributing to virulence must be made directly in the pathogen of choice.

One potential solution to this problem is to use signature-tagged mutagenesis (STM). This method was first developed in bacteria to identify genes required for growth *in vivo*. STM involves creation of a mutant strain collection that contains unique strain-identifying tags. The resulting strain collection is pooled and used to infect a host animal. By comparing the relative abundance of individual strains in the pool before and after infection, mutants required for *in vivo* growth and/or virulence may be identified. Again, analogous to the *S. cerevisiae* bar code and DNA microarray technologies, the unique tags may be used to deconvolute which strains fail to grow *in vivo* and, hence, which gene products are required for virulence. STM offers an advantage over *in vivo* expression methods because a phenotype for a gene is identified directly. Although, in principle, STM can be performed on a genome-wide scale, it is currently restricted to non-essential genes, and several technical issues, such as pool and inoculum size and route of injection, can confound the interpretation of results.

STM has now been applied to several haploid pathogenic fungi, including *Candida glabrata* [33], *A. fumigatus* [34], and *C. neoformans* [35]. These studies lay down a technical framework for performing STM, but have implicated only a limited assortment of mutants defective for *in vivo* growth. Most notable is a convincing demonstration that the *A. fumigatus* gene, *pabaA*, which encodes para-aminobenzoic acid synthetase, is required for virulence. Validation that *pabaA* is necessary for *in vivo* growth was demonstrated by independent gene disruption and *in vivo* re-confirmation of the mutant phenotype. As the nucleotide sequence of major fungal pathogen genomes are largely complete [4–8] and as additional genomes become available, systematic application of STM may be increasingly possible. However, as *C. albicans* is an asexual diploid, use of the STM technique appears more problematic.

Assay development

To exploit the avalanche of novel drug targets, the development of suitable high-throughput assays to screen targets against chemical libraries becomes critical. Moreover, as the precise molecular function of many targets is unknown, novel assays are needed to solve this bottleneck in lead discovery. To this end, Tucker and Fields developed a novel assay, which detects the binding of small-molecule ligands to their protein targets by a simple growth assay in *S. cerevisiae* [36•]. This assay is based on two basic observations. The first relates to the common phenomenon that ligand–protein interaction often leads to conformational alteration or stabilization in the protein. The second observation is that for some yeast mutants harboring temperature-sensitive mutations, subtle alterations in the activity or stability of the temperature-sensitive proteins usually result in detectable changes in growth rate. To build their assay, Tucker and Fields replaced the endogenous yeast *DHFR* gene (which encodes dihydrolate reductase) with two different temperature-sensitive (*ts*) alleles of the mouse *DHFR* (*mDHFR*) gene.

One *mDHFR ts* allele contains the human *FKBP12* (FK506-binding) domain, whereas the other allele contains the human Erα (estrogen receptor α) ligand-binding domain. Both ligand-binding domains were inserted into the mouse *DHFR* protein at residue 107, where introduction of exogenous protein fragments are well tolerated without abolishing the catalytic activity. As a proof of principle, Tucker and Fields assayed the effects of the FK506–*FKBP12* binding on the growth of the corresponding temperature-sensitive strain, and observed that FK506 improved the growth rate of the temperature-sensitive strain specifically and in a dose-dependent manner. Similar results were also obtained for the estrogen–*Erα* interaction. Furthermore, the authors screened these two temperature-sensitive strains against a panel of 20 compounds, and were able to successfully identify ligands that bind to *FKBP12* or *Erα*. Although this study only tested two different ligand-binding domains, in principle, this assay could be used to study ligand–protein interactions for any protein fragment (including those without known functions) that could be inserted into the *mDFHR* gene. This report clearly demonstrated the utility and potential of this whole cell assay in various high-throughput screens for lead discovery.

Another novel assay that has promising potentials in antifungal lead discovery is the small-molecule microarray approach developed by Schreiber and colleagues. Kuruvilla *et al*. [37] constructed small-molecule microarrays by immobilizing 3780 structurally diverse chemical compounds on glass slides. These small-molecule microarrays allow protein-binding assays to be performed simultaneously against 3780 compounds by probing the microarrays with any fluorescently labeled protein. Using this approach, the authors identified a compound that specifically bound to the yeast protein *Ure2p*. Furthermore, Kuruvilla *et al*. demonstrated that this compound also inhibited the *Ure2p* function *in vivo*. Being highly scalable, the small-molecule microarray method could potentially be applied in many future antifungal high-throughput screens.

Conclusions

Fungal genomics is poised to play a central role in antifungal drug discovery. It is directly applicable to target identification and validation, to target prioritization and assay development, and when compounds are discovered, in confirming their mechanism of action and in toxicity-related issues. Critical analyses of the resulting data followed by independent experimental confirmation by genetic and/or biochemical means are paramount. Furthermore, combining genomic strategies (for example, transcript profiling and fitness tests [15••,21•]) probably provides deeper insights than either alone. Whether or not the promise of a rational drug discovery process fully reliant on genomic strategies can successfully accelerate novel anti-infective drug discovery remains to be demonstrated by their introduction into the marketplace. However, as this process may take a decade to complete, and both fungal genomics and its application to antifungals are relatively recent, its confirmation will probably *only* require patience and commitment.

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