Insertional mutagenesis of pathogenic fungi

Jeremy S Brown and David W Holden*

Screening insertional mutants for loss of virulence is an effective method for investigating the molecular genetic basis of bacterial pathogenesis, but has only recently been applied to fungal pathogens. For many pathogenic fungi transformation with heterologous plasmid DNA results in complex integration events. This problem can now be circumvented for some species using restriction enzyme mediated integration. Insertional mutagenesis of *Fusarium oxysporum* using the naturally occurring fungal transposon *impala* has been described, but transposon tagging for other fungi has yet to be developed. Although insertional mutagenesis has recently identified important virulence determinants of fungal phytopathogens, the lack of suitable screening strategies has so far limited its applicability for fungal pathogens of humans.

Addresses
Department of Infectious Diseases, Imperial College School of Medicine, Du Cane Road, London W12 ONN, UK
*e-mail: dholden@rpms.ac.uk

Current Opinion in Microbiology 1998, **1**:390–394

http://biomednet.com/elecref/1369527400100390

© Current Biology Publications ISSN 1369-5274

Abbreviations
REMI restriction-enzyme-mediated integration
STM signature-tagged mutagenesis

Introduction

Classic genetic analysis using mutagens such as chemicals or ultraviolet light has yielded a wealth of knowledge on fungal physiology and development. These mutagens mainly generate base pair deletions or substitutions, which can result in the loss or alteration of gene function, and their relative lack of specificity allows saturation of a genome with mutations. Subsequent genetic analysis of mutant strains can, however, be time-consuming because it usually involves isolation of the mutated gene by complementation using a genomic DNA library from the wild-type strain. This partly explains the increasing use of insertional mutagenesis by chromosomal integration of transforming DNA. The presence of a selectable marker in the transforming DNA can be used to establish linkage between the insertion and the observed phenotype, and to recover DNA representing the mutated allele for cloning and subsequent analysis.

The economic importance of fungal phytopathogens and the increasing incidence of fungal infections in hospital patients [1] have stimulated research into the molecular genetics of fungal pathogenesis. Although insertional mutagenesis has been highly successful in identifying virulence determinants of bacterial pathogens [2], there are several problems in adopting this approach for fungi. Apart from the fact that some fungi, such as *Candida albicans*, do not have a haploid state that can be exploited for random insertional mutagenesis, the most obvious problem is the relative size of fungal genomes. Assuming that mutagenesis is completely random, the theoretical number of mutants necessary for saturation mutagenesis can be estimated from the size of the genome and the mean gene density. These are not known for pathogenic fungi, but for *Aspergillus nidulans* the genome size is approximately 31 megabases with a mean gene density of 1 per 3.2–3.8 kb [3•]. The mean length of 50 randomly chosen genes whose complete sequences are available in gene databases is 2.0 kb. A mutagenesis of *A. nidulans* will therefore require in the order of 15,000 mutants to have a mean mutation density of one per gene, and will require several times that number to be fully comprehensive; the comparable figure for some bacterial pathogens is 2000 mutants [4,5].

Approximately 3–4% of bacterial insertional mutants are reduced in virulence [6,7•,8], but for fungal phytopathogens this figure is markedly lower, varying from 0.3%–1.4% [9•,10,11]. Several fungal pathogens of humans are opportunistic and have not evolved ways of overcoming the normal mammalian immune system. In these species the proportion of attenuated insertional mutants is likely to be relatively low compared to that for fungal phytopathogens.

Once an interesting phenotype has been identified it is important to confirm that this is due to the insertion of the transforming DNA and not due to a secondary mutation. For bacteria this can be done quickly using conventional genetic methods such as phage transduction, and in some fungi linkage can be established by genetic crosses [11,12]. Pathogenic fungi, however, often lack a sexual cycle, so linkage has to be established by the more tedious process of cloning the affected gene and either complementing the mutant strain with the wild-type allele or reconstructing the mutant in a wild-type genetic background.

In this review we discuss current approaches for insertional mutagenesis of fungi, and methods for screening mutants to identify virulence determinants.

Methods for insertional mutagenesis

In order to exploit insertional mutagenesis for investigating the genetics of fungal virulence, an efficient method is needed for generating transformants containing single copy insertions of transforming DNA at different genomic sites. Several methods are being developed with this goal in mind.

Transformation with plasmid DNA

For some fungi, transformation with heterologous plasmid DNA carrying a selectable marker results in its single copy insertion [13,14]. Although the transformation frequency is generally low [9•,13], this method's simplicity is attractive, and has proved useful for the identification of virulence genes from the plant pathogens *Colletotrichum lindemuthianum* [9•] and *Ustilago maydis* [15]. For many fungi, however, plasmid DNA integrates in multiple copies, either at different genomic sites or in tandem at one genomic site [16–19], and alternative methods of insertional mutagenesis are necessary.

Restriction-enzyme-mediated integration

Restriction-enzyme-mediated integration (REMI) facilitates single copy integration of heterologous transforming DNA for several eukaryotes [12,20–23]. This process requires the addition of a restriction enzyme with a single site in the transforming plasmid, to the transforming reaction. REMI frequently increases transformation efficiency compared to conventional transformation [12,23,24]. How REMI works is unclear, but for several fungi integration of transforming DNA occurs at genomic restriction sites corresponding to those of the enzyme used for REMI [12,20]. The restriction enzyme presumably cleaves genomic DNA at some of its recognition sites, a proportion of which then provide targets for integration of the linear transforming plasmid. This results in genomic DNA containing a copy of the transforming plasmid flanked by restriction sites for the REMI restriction enzyme.

For many fungi, REMI provides a method of limiting how many copies of the transforming plasmid integrate into transformant genomic DNA [10,11,23,24]. As REMI insertions occur at genomic restriction enzyme recognition sites, the proportion of mutable genes per genome can be estimated from the genome size, G+C content and mean gene length. For example, the G+C content of *A. nidulans* is close to 50% [25]. Using a mean gene size of 2.0 kb (see above), saturation mutagenesis by REMI using a 6 bp recognition sequence restriction enzyme would result in insertions in approximately half of the total number of genes. Furthermore, insertions occurring near the 3′ ends of genes or in introns may not affect gene function. Southern analysis of mutants has not provided evidence of REMI hot-spots in the genomes of those fungi studied [10,11,20], but this possibility is difficult to exclude. Clearly, comprehensive mutagenesis of pathogenic fungi using REMI requires the use of at least two 6 bp recognition sequence restriction enzymes.

Despite these limitations, REMI mutant banks have been used successfully to isolate virulence genes for several fungal phytopathogens, including *U. maydis*, *Magnaportha grisea* and *Cochliobolus heterostrophus* [10–12] (Table 1). With some organisms REMI results in tandem insertions in a proportion of transformants [11,23,24]. These could arise from the formation of concatemers of the transforming plasmid before integration, or by repeated rounds of homologous recombination with a plasmid inserted into the chromosome. Dephosphorylation of the transforming plasmid to prevent concatemer formation during REMI transformation of *Penicillium paxilli* did not decrease the frequency of tandem insertions, suggesting that, at least for some fungi, the latter mechanism is predominant [24]. See Riggle and Kumamoto (this issue, pp 395–399) for further information on REMI.

Other methods

Bacterial transposons derived from Tn*5*, Tn*10* and Tn*917* have been used extensively for insertional mutagenesis of bacteria [2]. Numerous fungal transposons have been identified, including some which show evidence of active transposase function [26,27]. Recently, insertional mutants

Table 1

Examples of virulence determinants of pathogenic fungi identified by insertional mutagenesis.

Organism	Method of insertional mutagenesis	Method for screening for phenotype	Genes isolated	Gene product and phenotype	Reference
C. lindemuthianum	PT	Excised leaves	*clk1*	Serine/threonine kinase, involved in penetration of the cuticle by appressoria	[9•]
M. grisea	REMI	*In planta*	?	?	[11]
	REMI	*In planta*	*con4*, *con7*	Spore morphogenesis	[39•]
	REMI	*In planta*	*acropetal*	Defective appressorium development	[44]
U. maydis	REMI	*In planta*	?	Reduced virulence (13 mutants)	[10]
	PT	*In vivo* morphology	*myp1*	Defective for filamentous growth	[15]
C. heterostrophus	REMI	T-toxin assay	?	T-toxin deficient	[12]
C. glabrata	REMI	STM cell culture	?	An adhesin	†
*S. cerevisiae**	SM	*In vitro*	Several	Defective for filamentous growth	[31•]

**S. cerevisiae* is not a pathogen but provides a useful model of cell morphology switching for *C. albicans*. †B Cormack, personal communication. PT, plasmid transformation; SM, shuttle mutagenesis; STM, signature-tagged mutagenesis.

of *Fusarium oxysporum* have been generated using a strain which contains a copy of the naturally occurring transposon *impala* in the *NiaD* gene encoding nitrate reductase [28]. Selection for restoration of *NiaD* function was accompanied by excision of *impala* with transposition to new chromosomal loci, permitting the isolation of avirulent mutants [28]. This type of transposon would therefore appear to have considerable promise as a tool for insertional mutagenesis of fungi.

A few fungi, exemplified by *Saccharomyces cerevisiae*, recombine short lengths of their own DNA into chromosomal DNA very efficiently by homologous recombination. This ability has been exploited to produce two methods of insertional mutagenesis. The first method, internal fragment disruption [29], is based on the integration of small internal segments of plasmid-borne genes at their homologous chromosomal sites. For the second method, shuttle mutagenesis, transposon-mediated disruptions are introduced into a genomic DNA library in a bacterial host or *in vitro*, and then re-introduced into the host cell chromosome by homologous recombination [30]. Shuttle mutagenesis has been used to isolate genes involved in switching from the yeast form to filamentous growth morphology of *S. cerevisiae* [31•], which may provide a useful model for pathogens such as *C. albicans* or *U. maydis* where switching to filamentous growth is related to virulence [32•,33]. Although transposon insertion systems carrying markers of transformation for pathogenic fungi have been developed [34], the incidence of homologous recombination for many pathogenic fungi appears to be too low for either of these methods to be generally feasible [35–38].

Screening of mutants

The method used to screen mutants should obviously be relevant to the pathogen's disease process, the ideal being infection of the host species or, for human pathogens, an animal substitute. If previous research has suggested a particular phenotype is associated with virulence, then it may be possible to screen insertional mutants for loss of that phenotype by *in vitro* tests. For example, the *Tox1* locus responsible for T-toxin production by *C. heterostrophus* was discovered by screening 1310 REMI mutants for T-toxin production using a microbial assay [12]. For many plant pathogens large numbers of mutants can be tested for their inability to cause disease after inoculation of individual mutant strains onto the plant host or harvested fruit [10,11,33,38,39•]. Excised primary leaves or bulb layers maintained in petri dishes can offer a more manageable alternative to infecting whole plants [9•,38,40]. But for human pathogens inoculation of each mutant into a separate animal is simply not practicable and screening for virulence determinants requires different approaches.

Cell culture assays representing a stage known to be important for virulence can be a useful substitute for animal models of infection for some pathogens. Cell culture is particularly appropriate for assessing adhesion of pathogens to host cell surfaces [41] and for investigating intracellular pathogens, such as *H. capsulatum*, using a macrophage-like cell line [42]. Despite these advantages, cell culture can not replicate the cellular architecture of the target organ or the complex interactions between the immune system and an invading pathogen. Organ culture systems which represent the respiratory mucosa are available, and can partially circumvent the limitations of cell culture [43].

Signature-tagged mutagenesis (STM) enables mixtures of different mutants to be screened within one animal, and has been used successfully to identify virulence genes of some bacterial pathogens [6,7•,8]. In STM each insertional mutation carries a different DNA sequence tag which can be detected by hybridization analysis. The tags are flanked by invariant sequences which are used for co-amplification and labelling of the tags by PCR. The hybridization signals from tags in mutant strains recovered from the target organ of the infected animal are compared with those from the strains used as inoculum. Tags which are lost during infection represent mutants that are attenuated in virulence [6]. STM requires a method of insertional mutagenesis and an infection model capable of supporting growth of numerous different mutants. We have recently established an STM system for *Aspergillus fumigatus* and have validated it using an avirulent mutant auxotrophic for *para*-aminobenzoic acid (PABA) synthesis. When combined in an inoculum with 95 different insertional mutants, the PABA auxotroph is reliably identified as attenuated in virulence (JS Brown, abstract 64, Nineteenth Fungal Genetics Conference, 18-23 March 1997, Pacific Grove, California). Although large numbers of mutants have to be screened to identify small numbers of avirulent mutants, STM does appear to be a feasible method for investigating the genetics of fungal pathogenesis.

Conclusions

Insertional mutagenesis has only recently been used to investigate the genetics of virulence of fungal pathogens, and will require screening of large numbers of mutants in order to isolate relatively few virulence determinants. This strategy has already been successful in identifying novel virulence determinants of several fungal phytopathogens. In the absence of suitably developed transposons REMI provides a convenient means for carrying out insertional mutagenesis for several species of fungi. Investigation of the major fungal pathogens of humans by insertional mutagenesis presents specific technical challenges, including the development of valid screens for mutants with reduced virulence.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Beck-Sagué C, Jarvis WR: **Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. National Nosocomial Infections Surveillance System.** *J Infect Dis* 1993, **167**:1247-1251.

2. Hensel M, Holden DW: **Molecular genetic approaches for the study of virulence in both pathogenic bacteria and fungi.** *Microbiology* 1996, **142**:1049-1058.

3. • Kupfer DM, Reece CA, Clifton SW, Roe BA, Prade RA: **Multicellular ascomycetous fungal genomes contain more than 8000 genes.** *Fungal Genet Biol* 1997, **21**:364-372.

An analysis of all the open reading frames on a cosmid carrying 38.8 kb of *A. nidulans* DNA, from which the gene density, gene size, and likely total number of genes in the genome can be calculated.

4. Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM *et al*: **Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd.** *Science* 1995, **269**:496-512.

5. Tomb J-F, White O, Kerlavage A, Clayton R, Sutton G, Fleischmann R, Ketchum K, Klenk H, Gill S, Dougherty B *et al.*: **The complete genome sequence of the gastric pathogen *Helicobacter pylori*.** *Nature* 1997, **388**:539-547.

6. Hensel M, Shea JE, Gleeson C, Jones MD, Dalton E, Holden DW: **Simultaneous identification of bacterial virulence genes by negative selection.** *Science* 1995, **269**:400-403.

7. • Mei J-M, Nourbakhsh F, Ford CW, Holden DW: **Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis.** *Mol Microbiol* 1997, **26**:399-407.

Using signature-tagged mutagenesis (STM) a large number of genes required for infection of a mouse model by *S. aureus* were isolated. The STM method was modified [6] by pre-selection of signature tags to avoid colony hybridization. This modification should be useful when applying STM to fungal pathogens.

8. Chiang SL, Mekalanos JJ: **Use of signature-tagged transposon mutagenesis to identify *Vibrio cholerae* genes critical for colonization.** *Mol Microbiol* 1998, **27**:797-805.

9. • Dufresne M, Bailet JA, Dron M, Langin T: ***clk1*, a serine/threonine protein kinase-encoding gene, is involved in pathogenicity of *Colletotrichum lindemuthianum* on common bean.** *Mol Plant–Microbe Interact* 1998, **11**:99-108.

A good example of how insertional mutagenesis by simple transformation with heterologous plasmid DNA has been applied to a fungal phytopathogen to isolate a novel virulence determinant.

10. Bölker M, Böhnert HU, Braun KH, Görl J, Kahmann R: **Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI).** *Mol Gen Genet* 1995, **248**:547-552.

11. Shi Z, Christian D, Leung H: **Enhanced transformation in *Magnaporthe grisea* by restriction enzyme mediated integration of plasmid DNA.** *Phytopathology* 1995, **85**:329-333.

12. Lu S, Lyngholm L, Yang G, Bronson C, Yoder OC, Turgeon BG: **Tagged mutations at the *Tox1* locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration.** *Proc Natl Acad Sci USA* 1994, **91**:12649-12653.

13. Hogan LH, Klein BS: **Transforming DNA integrates at multiple sites in the dimorphic fungal pathogen *Blastomyces dermatitidis*.** *Gene* 1997, **186**:219-226.

14. Daboussi MJ, Djeballi A, Gerlinger C, Blaiseau PL, Bouvier I, Cassan M, Lebrun MH, Parisot D, Brygoo Y: **Transformation of seven species of filamentous fungi using the nitrate reductase gene of *Aspergillus nidulans*.** *Curr Genet* 1989, **15**:453-456.

15. Giasson L, Kronstad JW: **Mutations in the *myp1* gene of *Ustilago maydis* attenuate mycelial growth and virulence.** *Genetics* 1995, **141**:491-501.

16. Fincham JR: **Transformation in fungi.** *Microbiol Rev* 1989, **53**:148-170.

17. Wang J, Holden DW, Leong SA: **Gene transfer system for the phytopathogenic fungus *Ustilago maydis*.** *Proc Natl Acad Sci USA* 1988, **85**:865-869.

18. Worsham PL, Goldman WE: **Development of a genetic transformation system for *Histoplasma capsulatum*: complementation of uracil auxotrophy.** *Mol Gen Genet* 1990, **221**:358-362.

19. Varma A, Edman JC, Kwon-Chung KJ: **Molecular and genetic analysis of URA5 transformants of *Cryptococcus neoformans*.** *Infect Immun* 1992, **60**:1101-1108.

20. Schiestl RH, Petes TD: **Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*.** *Proc Natl Acad Sci USA* 1991, **88**:7585-7589.

21. Kuspa A, Loomis WF: **Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA.** *Proc Natl Acad Sci USA* 1992, **89**:8803-8807.

22. Black M, Seeber F, Soldati D, Kim K, Boothroyd JC: **Restriction enzyme-mediated integration elevates transformation frequency and enables co-transfection of *Toxoplasma gondii*.** *Mol Biochem Parasitol* 1995, **74**:55-63.

23. Granado JD, Kertesz-Chaloupkova K, Aebi M, Kües U: **Restriction enzyme-mediated integration in *Coprineus cinereus*.** *Mol Gen Genet* 1997, **256**:28-36.

24. Itoh Y, Scott B: **Effect of de-phosphorylation of linearised pAN7-1 and of addition of restriction enzyme on plasmid integration in *Penicillium paxilli*.** *Curr Genet* 1997, **32**:147-151.

25. Storck BS, Alexopoulos CJ: **Deoxyribonucleic acid of fungi.** *Bacteriol Rev* 1970, **34**:126-154.

26. Langin T, Capy P, Daboussi MJ: **The transposable element *impala*, a fungal member of the *Tc1-mariner* superfamily.** *Mol Gen Genet* 1995, **246**:19-28.

27. Kempken F, Kuck U: ***restless*, an active *Ac*-like transposon from the fungus *Tolypocladium inflatum*: structure, expression, and alternative RNA splicing.** *Mol Cell Biol* 1996, **16**:6563-6572.

28. Daboussi DJ: **Fungal transposable elements: generators of diversity and genetic tools.** *J Genet* 1996, **75**:325-339.

29. Shortle D, Haber JE, Botstein D: **Lethal disruption of the yeast actin gene by integrative DNA transformation.** *Science* 1982, **217**:371-373.

30. Seifert HS, Chen EY, So M, Heffron F: **Shuttle mutagenesis: a method of transposon mutagenesis for *Saccharomyces cerevisiae*.** *Proc Natl Acad Sci USA* 1986, **83**:735-739.

31. • Mosch HU, Fink GR: **Dissection of filamentous growth by transposon mutagenesis in *Saccharomyces cerevisiae*.** *Genetics* 1997, **145**:671-684.

Using shuttle mutagenesis the authors isolated 16 genes, disruption of which prevented *S. cerevisiae* switching from yeast to filamentous cell morphology. Homologues of these genes can now be sought in fungal pathogens, such as *C. albicans*, for which filamentous growth is associated with virulence.

32. • Lo H-J, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR: **Nonfilamentous *C. albicans* mutants are avirulent.** *Cell* 1997, **90**:939-949.

By searching in *C. albicans* for homologues of mitogen-activated protein kinase (MAPK) genes regulating morphology switching in *S. cerevisiae*, the authors isolated two MAPKs which, when disrupted, prevented filamentous growth and abolished virulence in a mouse model of invasive candidiasis.

33. Gold SE, Brogdon SM, Mayorga ME, Kronstrad JW: **The *Ustilago maydis* regulatory subunit of a cAMP-dependent protein kinase is required for gall formation in maize.** *Plant Cell* 1998, **9**:1585-1594.

34. Hamer L, Gilger S: **Bacterial transposons containing markers for fungal gene disruption.** *Fungal Genet Newslett* 1997, **44**:19-23.

35. Bird D, Bradshaw R: **Gene targeting is locus dependent in the filamentous fungus *Aspergillus nidulans*.** *Mol Gen Genet* 1997, **255**:219-225.

36. Odom A, Muir S, Lim E, Toffaletti DL, Perfect J, Heitman J: **Calcineurin is required for virulence of *Cryptococcus neoformans*.** *EMBO J* 1997, **16**:2576-2589.

37. Sweigard JA, Chumley FG, Valent B: **Disruption of a *Magnaporthe grisea* cutinase gene.** *Mol Gen Genet* 1992, **232**:183-190.

38. Crowhurst RN, Binnie SJ, Bowen JK, Hawthorne BT, Plummer KM, Rees-George J, Rikkerink EHA, Templeton MD: **Effect of**

disruption of a cutinase gene (*cutA*) on virulence and tissue specificity of *Fusarium solani* f.sp. *cucurbitae* race 2 toward *Cucurbita maxima* and *C. moschata*. *Mol Plant–Microbe Interact* 1998, **10**:355-368.

39. • Shi Z, Christian D, Leung H: **Interactions between spore morphogenetic mutations affect cell types, sporulation and pathogenesis in *Magnaporthe grisea*.** *Mol Plant–Microbe Interact* 1998, **11**:199-207.

Another example of isolation of virulence determinants of a fungal phytopathogen by insertional mutagenesis.

40. Xu J-R, Urban M, Sweigard JA, Hamer JE: **The *CPKA* gene of *Magnaporthe grisea* is essential for appressorial penetration.** *Mol Plant–Microbe Interact* 1997, **10**:187-194.

41. Gale CA, Bendel CM, McClellan M, Hauser M, Becker JM, Berman J, Hostetter MK: **Linkage of adhesion, filamentous growth and virulence in *Candida albicans* to a single gene, *INT1*.** *Science* 1998, **279**:1355-1358.

42. Eissenberg LG, West JL, Woods JP, Goldman WE: **Infection of P388D1 macrophages and respiratory epithelial cells by *Histoplasma capsulatum*: selection of avirulent variants and their potential role in persistent histoplasmosis.** *Infect Immun* 1991, **59**:1639-1646.

43. Tsang KW, Rutman A, Tanaka E, Lund V, Dewar A, Cole PJ, Wilson R: **Interaction of *Pseudomonas aeruginosa* with human respiratory mucosa *in vitro*.** *Eur Respir J* 1994, **7**:1746-1753.

44. Lau G, Hamer JE: ***Acropetal*, a genetic locus require for conidiophore architecture and pathogenicity in the rice blast fungus.** *Fungal Genet Biol* 1998, in press.