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Identification of novel genes conferring altered azole susceptibility in *Aspergillus fumigatus*

Paul Bowyer[1,*], **Juan Mosquera**[1], **Michael Anderson**[1], **Mike Birch**[2], **Michael Bromley**[1], and **David W Denning**[1]

[1]The University of Manchester, Manchester Academic Health Science Centre, NIHR Translational Research Facility in Respiratory Medicine, University Hospital of South Manchester NHS Foundation Trust, Manchester, M23 9LT United Kingdom.

[2]F2G ltd., Lancro Way, Eccles, Manchester, UK.

Abstract

Azoles are currently the mainstay of antifungal treatment both in agricultural and clinical settings. Although the target site of azole action is well studied the basis of azole resistance and the ultimate mode of action of the drug in fungi is poorly understood. In order to gain a deeper insight into these aspects of azole action restriction mediated plasmid integration (REMI) was used to create azole sensitive and resistant strains of the clinically important fungus *Aspergillus fumigatus*. Four azole sensitive insertions and 4 azole resistant insertions were characterised. Three phenotypes could be re-created in wild type AF210 by reintegration of rescued plasmid and a further 4 could be confirmed by complementation of the mutant phenotype with a copy of the wild type gene predicted to be disrupted by the original insertional event. Six insertions were in genes not previously associated with azole sensitivity or resistance. Two insertions occur in transporter genes that may affect drug efflux whereas others may affect transcriptional regulation of sterol biosynthesis genes and NADH metabolism in the mitochondrion. Two insertions are in genes of unknown function.

Introduction

Over the past few decades, the incidence of invasive aspergillosis has risen steadily. It is now the most common invasive mould infection worldwide [1-3]. At least 4 % of all patients dying in tertiary care hospitals in Europe have invasive aspergillosis [21-23]. Mortality is almost 100% if the disease is left untreated and high (50 to 100 %) even with therapy [1]. *Aspergillus fumigatus* is usually the most common etiologic agent, being responsible for up to 90% of human *Aspergillus* infections. As well as infecting humans fungi may also cause diseases of plants and are one of the most important causes of crop loss in temperate regions [4-6]. Both human and agricultural diseases are treated with azole antifungals. These compounds target 14 sterol demethylase and interfere with ergosterol production in the fungus [7-9].

[*]**Correspondence to**: Dr P Bowyer **paul.bowyer@manchester.ac.uk**.

None of the authors have any conflicts of interest to declare.

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Recent testing of *A. fumigatus* clinical isolates has identified cases of azole resistance [10-20, 21, 24-27] some of which have been shown to be resistant to treatment with itraconazole (ITR) in murine models of infection [28-30]. Long-term treatment of patients with ITR [28, 30-33] appears to lead to azole resistance in infecting fungi, although these cases were not always associated with ITR treatment failure. Two per cent of 913 *A. fumigatus* isolates in the literature published before 2000 were found to be resistant (28). Recent surveys have reported frequencies ranging from 1 to 6 % (total number of isolates surveyed was 357) [21, 24, 25, 27], although other surveys have reported no resistant isolates out of a total of 2100 isolates [34-41]. Recent estimates from our laboratory suggest higher levels of azole resistance [42] and similar studies in the Netherlands have also shown high levels of azole resistance.

A number of mutations in the cyp51A lanosterol 14α-demethylase gene have been associated with azole resistance [30, 31, 43-47]. Other mechanisms for azole resistance include increased expression of efflux pumps. The increased expression of an ATP-binding cassette (ABC) transporter (AtrF) in the presence of ITR has been shown in a clinical isolate with reduced drug accumulation [48] and possible transporters have been implicated in azole resistance of laboratory selected mutants [30, 45, 49].

Restriction enzyme-mediated integration (REMI) was employed, as it increases transformation efficiency and promotes single-copy, non-rearranged integrations of the transforming DNA [50-53]. This technique has been employed as a means of generating tagged insertional mutants in a variety of organisms [50, 52, 53]. REMI has previously been used in *Aspergillus* [51, 54] to identify genes required for in vivo growth or normal morphology. Osherov et al. [55] used an overexpression approach to isolate genes that give resistance to ITR in *A. nidulans* but only identified the P-450 14 DM gene, *pdmA*, as a mechanism of resistance. de Souza *et al.* [52] screened 1354 REMI insertional mutants to study azole resistance in *A. nidulans* of which 33 displayed sensitivity to ITR, however no molecular analysis of insertion sites was performed.

In this study we employed a restriction enzyme-mediated integration (REMI)-tagged insertional mutagenesis screen in order to identify transformants with increased ITR susceptibility in *A. fumigatus*. Since fungi display a basal resistance to azoles we also screened for isolates that were more susceptible to azoles as in this case inactivated genes would be involved in azole toxicity.

Materials and Methods

Strains, plasmids and chemicals

Aspergillus fumigatus clinical isolate AF210 (NCPF 7101) is susceptible to itraconazole and amphotericin B [56]. PyrG^{-} mutants were isolated by screening 10^7 to 10^8 spores on 1 % glucose agar plates containing Vogel's salts, 1 g/l of 5-fluoro-orotic acid (5-FOA), 0.02 M uracil and 0.1 M uridine. They (n=20) were subsequently checked for uracil and uridine auxotrophy and a low reversion rate to prototrophy. One of the mutants was selected and designated AF210.1.

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The pPyrG plasmid consists of the *A. nidulans pyrG* gene cloned into pUC19 [57]. ITR (Janssen Research Foundation, Beerse, Belgium), voriconazole (VOR) (Pfizer, Sandwich, UK), posaconazole (POS) (Schering-Plough Research Institute, Bloomfield, N.J., USA) and ravuconazole (RAV)(Bristol-Myers Squibb, Princeton, N.J., USA) were dissolved in DMSO and stored in aliquots at −20°C.

Protoplast transformation

AF210.1 conidia were inoculated into 100 ml of Sabouraud dextrose liquid medium containing 0.02 M uracil and 0.1 M uridine to a final concentration of 5×10^6/ml and incubated for 12 h on a rotary shaker at 37°C. 2 g (wet weight) of mycelium was digested at 30°C in 20 ml of 0.6 M KCl (pH 6.8) containing 5 % Glucanex® (Novo Nordisk Ferment, Dittingen, Switzerland) for 2 h. Protoplasts were filtered through Miracloth, washed twice with 0.6 M KCl and resuspended in 0.6 M KCl, 0.05 M $CaCl_2$ to a final concentration of 10^7/ml. 200 μg *Xho*I linearized pPyrG was added to 4 ml of protoplasts, followed by 160 U of *Xho*I and 2 ml of 0.05 M $CaCl_2$, 0.6 M KCl, 0.01 M Tris-Cl [pH 7.5], 40 % PEG 4000, and mixed. After incubation on ice for 20 min a further 40 ml of this buffer was added and mixed, followed by an additional 15 min incubation at room temperature. Six ml of the transformation mixture was then added to a liquid layer of 4 ml of RPMI containing 2 % glucose, Vogel's salts, 0.6 M KCl, penicillin (100 U) and streptomycin (100 ng/ml) on top of 9 cm RPMI agar plates containing 2 % glucose and Vogel's salts, incubated at 37°C. For transformations with the plasmids obtained by plasmid rescue, 20 times smaller volumes were used. Each plasmid was digested with the restriction enzyme that had been used for the plasmid rescue (*Xho*I or *Cla*I).

ITR susceptibility screening and azole MIC testing

After 24 to 48 h at 37°C, plates containing putative transformed colonies were overlayed with 4 mg/l ITR in RPMI, 1% agar. After 48 h, a differentiable new ring of growth was observable. The colonies that had a bigger or smaller ring than the majority were checked for their susceptibility to ITR by inoculating spores onto RPMIplates containing 2% glucose and 2% agar and either 0.50, 0.25 and 0.12 mg/l ITR. Mutants with ITR susceptibilities clearly different from the parental isolate were subsequently tested for their MICs to four azoles (Table 2) [56]. The MICs were read visually and were defined as the lowest drug concentration with no visible growth.

Fungal DNA extraction, PCR and Southern hybridization

Fungal DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Crawley, UK). The presence of the integrated pPyrG plasmid was confirmed by PCR using primers Cf and Gr directed against the Amp^R gene (Supp. Table 1).

Genomic DNA (3 μg) was digested to completion with *Xho*I, *Cla*I or *Nco*I, as appropriate, separated in 0.8% agarose, transferred onto a positively charged nylon membrane (Roche Diagnostics, Lewes, UK) and hybridized overnight at 42°C in DIG Easy Hyb (Roche) with a DIG-labelled probe consisting of the pUC19 DNA or the *Hin*dIII fragment of the pPyrG plasmid. Washing was done at 65°C in 0.5× SSC, 0.1% SDS with stringent washing using 0.1XSSC, 0.1%SDS.

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Plasmid rescue and sequencing

Plasmid rescue was carried out by digesting genomic DNA with *Xho*I or *Cla*I, separating the DNA in 0.8 % agarose and purifying DNA of ±1-2 kb of the estimated size according to the Southern hybridizations. DNA was ligated overnight at 16°C with T4 DNA ligase and electroporated into *E. coli* DH5α (Invitrogen, Paisley, UK) or SCS110 (Stratagene, Amsterdam, The Netherlands). The sequence flanking the pPyrG insertion site was determined using primers FOR and REV that hybridized 68 bp upstream and 88bp downstream of the *A. nidulans pyrG* XhoI site respectively.

Construction of clones for mutant complementation

Regions including ~1 Kb upstream and 1 Kb downstream of AFUA_5G07550, AFUA_2G11840, AFUA_2G11020, AFUA_4G10880 and AFUA_6G12570 were amplified by PCR using primers 5G07550F and 5G07550R:, 2G11840F and 2G11840R:, 2G11020F and 2G11020 R: , 4G10880F and 4G10880R: , and 6G12570F and R: 50 μl PCR reactions contained 25 μl 2X Phusion mastermix, 40 pM primers and 200 ng Af293 DNA according to the manufacturers instructions (New England Biolabs) and were subjected to 35 cycles at 96 °C for 15 s, 58 °C for 5 min and 72°C for 80 s followed by an extension step at 72°C for 5 min. Products were assessed by gel electrophoresis, gel purified using a Qiaex kit (Qiagen) and then cloned into pGEM-T (Promega). Plasmids were termed p5G07550, p2G11840, p2G11020, p4G10880 and p6G12570. and co-transformed into REMI strains as appropriate using 3 μg pAN7-1 as cotransforming plasmid with selection on 150 μg/ml hygromycin.

Computer analyses

Sequences were compared to *A. fumigatus* Af293 genomic sequence [58] using the BLAST function on the CADRE database [59]. Both flanking regions were located in the genomic sequence and used to pinpoint the insertion site.

Growth experiments

Colony radial growth experiments were carried out as described previously [60] using 2% glucose in agar plates containing Vogel's salts.

Results

Isolation and characterization of tagged insertional mutants

Four thousand transformants were isolated and screened for altered susceptibility to ITR. After overlay with ITR contining agar 19 transformants that displayed either continued or completely arrested growth were selected, of which eight had at least a 4-fold difference in ITR susceptibility relative to the parental strain (Table 1). All 8 transformants displayed normal growth rate colony morphology and sporulation compared to the parental strain. These eight transformants were selected for further analysis.

Analysis of REMI integration events

The eight transformants (termed REMI-11, -14D, -56, - 85, -101, -102, -103 and -116) were characterized further to determine the nature of the REMI insertion. PCR using primers

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directed against the Amp^R gene in pUC19 confirmed that all of them had at least one integrated copy of pPyrG. Restriction digestion followed by Southern hybridization with the pUC19 vector fragment of pPyrG was carried out in order to determine the nature of the plasmid integrations. *Xho*I digests established whether or not "perfect" REMI integrations that retained the XhoI sequence at the site of insertion had occurred: a single 4.8 kb hybridising band, which represents pPyrG, indicated such an event (Figure 1). REMI 11, 56 and 101 all give 4.8 Kb bands expected from a single insertion. REMI 85, 14D, 103 and 102 give single bands larger than 4.8 Kb and REMI 116 gives two bands. This data was combined with sequence from the insertion site and flanking regions (see below) to determine whether the REMI event had occurred at a genomic XhoI site. In REMI 85, 14D, 102, 104 and 116 the rescued plasmids had partial XhoI sites flanking the insertion suggesting that integration occurred in an imperfect manner. REMI 11, 56 and 101 all contained intact XhoI sites at the insertional locus. Combining the Southern blot data and the flanking sequence we were able to categorise the REMI insertion into perfect or imperfect (Table 1) and determine the insertional copy number. 7/8 RMI isolates had one single plasmid insertion in the genome, three which were perfect REMI integrations. One of them, 116, had multiple insertions and was not investigated further.

The site of plasmid insertion was successfully determined by plasmid rescue in all REMI transformants. The rescued genomic sequence on either side of the insertion was determined by plasmid sequencing and used to identify the locus of insertion by comparison to the Af293 genome.

Analysis of REMI insertion sites

Rescued plasmids were sequenced and analysed by restriction digestion to define the site of insertion in the genome. Insertion sites are shown in the cartoon in Figure 2.

In REMI-11 the insertion had occurred at a *Xho*I site 1820 bp downstream from the start codon of an ABC transporter family gene (AFUA_1G14330) and within the coding region. REMI-56 is an insertion upstream of a MFS transporter. The insertional mutation has occurred in the 619 bp intergenic region between two tandemly transcribed genes, 203 bp downstream of a putative sulphate transporter (AFUA_1G05020) and 416 bp upstream of a putative MFS transporter (AFUA_1G05010). The REMI insertion is close to the start codon of this ORF and to motifs associated with the core promoter in filamentous fungal genes. REMI-14D is an insertion in the coding region of triose phosphate isomerase. Sequence analysis of rescued plasmid shows that REMI-14D contains an insertion within the coding region of AFUA_2G11020, the single copy triose phosphate isomerase gene. The insertion is within the coding region, 452 bp from the start codon. REMI-85 and REMI-103 occur within the coding regions of two hypothetical genes (AFUA_5G07550 and AFUA_4G10880 respectively). These genes have no known function or homology with any gene of known function. AFUA_5G07550 is conserved in all *Aspergillus* species but poorly conserved in other fungi. The moderately azole resistant strain REMI-101 has an insertion within the gene coding for the mitochondrial 29.9 KD ubiquinone NADH oxidoreductase subunit of respiratory complex I (AFUA_2G10600). The insertion was shown to occur at a genomic XhoI site 534 bp downstream from the start codon of the gene. MICs for this strain were 1.0,

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0.50 and 4.0 μg/ml for itraconazole, posaconazole and ravuconazole, respectively versus 0.25, 0.12 and 1.0 μg/ml for AF210.

REMI-102 was determined to contain an insertion in the promoter region of the *A. fumigatus* cyc8 gene orthologue (AFUA_2G11840). The final REMI strain (REMI-116) was shown to have an insertion in the epsin 2 gene (AFUA_6G12570). However Southern blot analysis suggests that there are at least two insertional events in this strain and attempts to re-create the phenotype were unsuccessful. Therefore it seems likely that the insertion in the epsin2 gene and the observed phenotype may be unlinked.

Confirmation of the association between plasmid insertion and observed phenotype

Since uncharacterised secondary mutations may arise during transformation or REMIRescued plasmids were used to recreate the azole resistant phenotype in the parental strain. Since the rescued plasmids contain flanking regions of the original insertion site this procedure is functionally analogous to commonly used allele replacement methods and recombination between flanking regions and genomic DNA should regenerate the original REMI insertion in a new wild type or parental strain (Supp. Figure 1).

Plasmids were termed p11, p85 etc according to the REMI number of the parental isolate and were successfully rescued for all eight REMI strains. Flanking regions of genomic DNA in the rescued plasmids were mapped to genes by sequencing. The regions rescued are shown in Figure 2.

When AF210.1 was retransformed with p11, p56 and p101 the phenotype of the original REMI-11, -56 and -101 strains was reconstituted. Frequency of reconstruction of REMI insertions was 1 in 380 transformants for p11, 2 in 870 for p56, and 4 in 40 for p101. These transformants had the same azole susceptibilities as the original REMI strains. A Southern hybridisation carried out on genomic DNA isolated from these transformants confirmed that they had the same hybridisation pattern as the original transformants, indicating that the insertion had occurred at the same parts in the genome (Figure 3). We note that for retransformation of AF210 with p56 aberrant band sizes were obtained on a Southern blot after *Xho*I digestion although the expected sizes were obtained after *Cla*I digestion. We are unable to explain this banding pattern and REMI-56 was therefore included in the complementation experiments described below.

The plasmids used for the other reconstruction experiments lacked long flanking sequences on one side of the insertion and it is possible that the double recombination event required for reconstitution of the REMI was inefficient. A high number of transformants was tested in these cases (670 for p102, 3200 for p85, 540 for p14D and 420 for p103). In order to determine whether phenotype and insertion were linked it was decided to attempt to complement the mutations using genes amplified by PCR from Af293. Plasmids p5G07550, p1G05010, p2G11840, p2G11020, p4G10880 and p6G12570 were co-transformed into REMI-85, -56 -102, -14D, -103 and -116 respectively. Wild-type (AF210) or parental (AF210.1) levels of azole resistance were obtained from all transformations except that of REMI-116 (Table 3). Primers flanking the original insertion site were used to confirm that intact copies of complementing genes were present in strains where wild type phenotypes

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had been restored. In all cases restoration of parental AF210.1 phenotype as assessed by MIC^{ITR} corresponded to integration of an intact genomic copy of the appropriate gene.

Discussion

In this study we aimed to discover new genes and mechanisms involved in ITR resistance in *A. fumigatus.* Several insertional mutants were isolated from a REMI screen and characterized. Eight of 4000 mutants tested displayed altered azole sensitivity with 4 mutants showing increased sensitivity and 4 showing decreased sensitivity.

Two putative transporter genes were isolated in the screen as being involved in resistance to azoles (ie the insertions were more sensitive to azoles). One gene identified is an ABC transporter and is probably an orthologue of the *A. nidulans AtrG* and *Pmr*1 of *P. digitatum* [61]. Overexpression of multidrug resistance (MDR) efflux transporter genes of the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) classes is thought to play an important role in azole resistance in *Aspergillus*. In *C. albicans,* ABC transporter genes CDR1 and CDR2, and major facilitator efflux gene MDR1 have been shown to be involved in azole resistance [13-16]. The *A.fumigatus* orthologue of *C. albicans* CDR1 is AFUA_1G14330, the site of the insertion in REMI-11. Insertional inactivation of this protein would therefore be expected to lead to azole sensitivity.

The REMI-56 insertion is upstream of a putative MFS transporter (AFUA_1G05010). The closest *C. albicans* MDR1 orthologue in *A. fumigatus* is AFUA_2G16860 annotated as an MFS transporter, BLAST search of the *A.fumigatus* sequence with MDR1 does not identify AFUA_1G05010 (BLAST cut off score of 30, E value of 0.1). Comparison of AFUA_1G05010 with the *C. albicans* genome reveals similarity to XP_716751, one of a family of related potential transporter genes (XP_719316.1, XP_716470.1, XP_715705.1, XP_723465.1, XP_723276.1, XP_709949.1 and XP_712988.1) similar to *S.cerevisiae* YKR105C, YCL069W, SGE1 (YPR198W) and AZR2(YGR224W) MFS-MDR proteins involved in resistance to mutagens. The association of this class of MDR protein with azole resistance has not previously been reported. Given that the insertion in REMI-56 is in the promoter region of AFUA_1G05010 there is a formal possibility that the gene is overexpressed rather than down regulated. In this case the gene might be involved in azole uptake.

One insertion leading to azole sensitivity was found in the *A. fumigatus* cyc8 orthologue (AFUA_2G11840). If this protein is involved in repression of ergosterol biosynthesis in a manner similar to that observed in *S. cerevisiae* [67, 68] then insertional inactivation could lead to activation of the ergosterol biosynthetic pathway. This may lead to azole resistance by increasing levels of the target protein [28].

Two genes were identified where insertional mutagenesis resulted in an increase in azole resistance. This implies that these genes act to confer azole sensitivity in the wild-type isolate. These genes have never been associated with azole action and are at first sight unrelated. The first gene, a component of complex I of respiration is well studied in the context of complex I activity and activation in *N. crassa* and appears to be involved in a

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switch between active and less active forms of the complex [62-64]. This suggests that regulation of the enzymic activity of complex I may play an important role in azole action although the nature of this role remains to be determined. The second gene, triose phosphate isomerase, is also well studied as a model enzyme and encodes a glycolytic enzyme [65]. The insertional mutants from this REMI screen have no obvious aberrant phenotype and it appears that these two genes are dispensable for normal growth. The function of these genes may be linked or separate in their role in azole sensitivity but we suggest that the simplest explanation is that they may function in a related manner. One potential link between these two genes is that a substrate for triose phosphate isomerase is dihydroxy acetone phosphate. This compound is part of the glycerol phosphate shuttle [66] which regenerates NADH inside the mitochondrion as cytoplasmically derived NADH is unable to pass into this organelle. Thus these two seemingly disparate genes may be linked by utilisation and supply of NADH in the mitochondrion with the possibility that susceptibility to azoles functions via mitochondrial NADH metabolism or NAD/NADH redox stress. One issue raised by the involvement of complex I in azole sensitivity is the value of *S. cerevisiae* as a model system for study of azoles as this organisms lacks a functional complex I.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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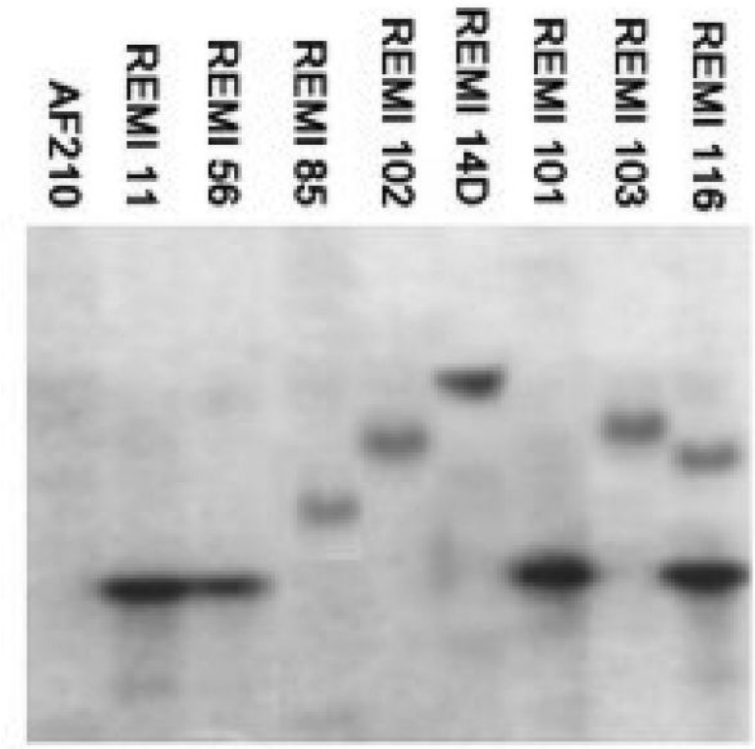

Figure 1. Southern analysis of REMI transformants

5 μg genomic DNA was digested overnight at 37°C with 20 U *Xho*I then run on an 0.8% agarose gel before blotting and probing with DIG labelled pUC19 DNA. For REMI insertions arising from a perfect *Xho*I site integration a 4.8 Kb band is expected.

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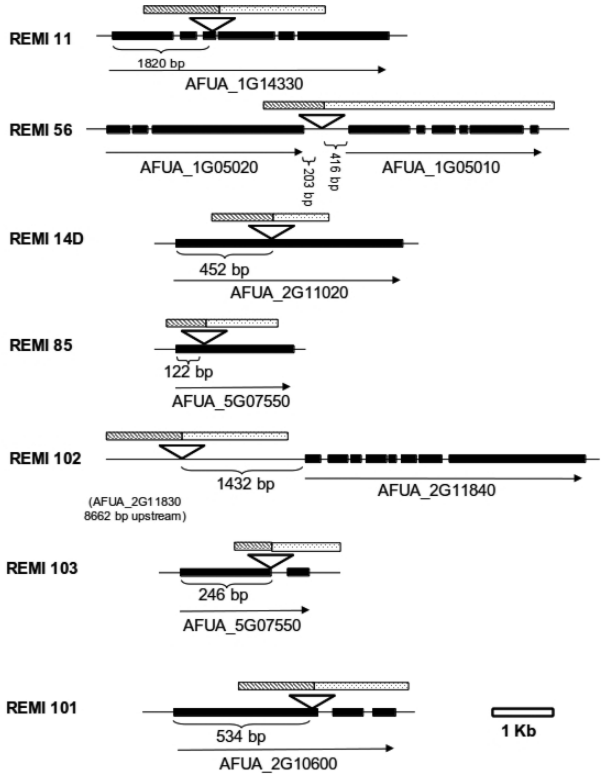

Figure 2. Schematic of REMI insertion sites showing rescued genomic regions

Gene maps and graphics are adapted from pages on the CADRE website (www.cadre-genomes.org). Insertion sites were determined by sequencing rescues plasmids. Stippled and striped boxes above the gene schematics show the regions of DNA rescued with stippled and striped areas representing different arms of the plasmid rescue.

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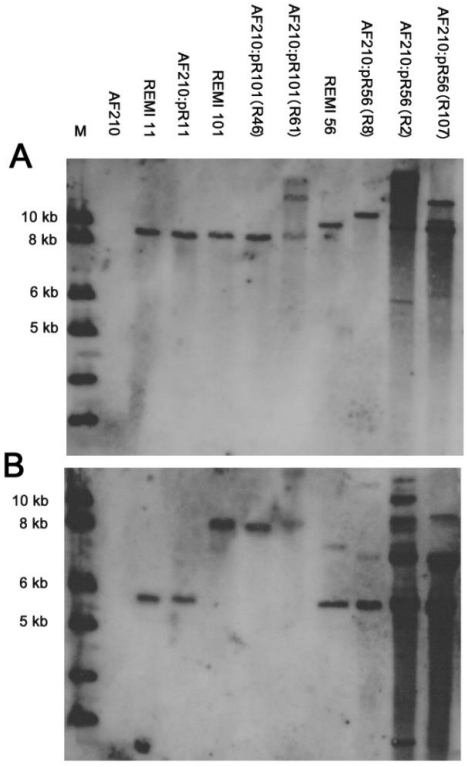

Figure 3. Southern analysis of plasmid rescue re-transformant strains

3 μg genomic DNA was digested overnight at 37°C with 20 U *Xho*I (panel A) or 20 U *Cla*I (panel B) then run on an 0.8% agarose gel before blotting and probing with DIG labelled *Hind*III digested pPyrG DNA. In cases where the retransformation results in recreation of the REMI insertion the size of the retransformant band should correlate with that obtained in the parental REMI strain. These appear correct for REMI-11 and -101 retransformants however the REMI-56 transformant and retransformants give abberant bands for the *Xho*I digestion.

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Table 1

Identities, insertion sites and MICs of AF210 and REMI transformants.

	Insertion site		(MIC mg/l)			Integration events
			ITR	POS	RAV	
AF210	-	-	0.25	0.12	1.0	-
REMI 11	ABC transporter	AFUA_1G14330	0.062	0.031	0.031	1 single REMI*
REMI 56	MFS transporter	AFUA_1G05010	0.062	0.031	0.25	1 single REMI*
REMI 85	Hypothetical gene	AFUA_5G07550	0.0078	0.031	0.062	1 single REMI#
REMI 102	Cyc8	AFUA_2G11840	0.062	0.031	0.25	1 single REMI#
REMI 14D	Triose phosphate isomerase	AFUA_2G11020	8.0	1.0	8.0	1 single REMI#
REMI 101	29.9KD NADH dehydrogenase	AFUA_2G10600	1.0	0.50	4.0	1 single REMI*
REMI 103	Hypothetical gene	AFUA_4G10880	1.0	0.50	4.0	1 single REMI#
REMI 116	Epsin2	AFUA_6G12570	1.0	0.50	4.0	2 single REMI

ITR, itraconazole, POS, posaconazole, RAV, ravuconazole

*insertion at intact XhoI site.

#insertion at degenerate XhoI site.

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Table 2

MIC values for gene disruption transformants derived from retransformation by rescued plasmids

Parent/transformant	Strain number	Azole resistance (MIC mg/l)			
		ITR	PCZ	RVZ	VCZ
AF210.1	AF210.1	0.25	0.12	0.5	0.5
REMI 11	REMI 11	0.12	0.06	0.12	0.12
AF210:pR11	R81	0.12	0.06	0.12	0.12
REMI 101	REMI 101	1	0.5	2	2
AF210:pR101	R46	1	0.5	2	2
	R61	1	0.5	2	2
REMI 56	REMI 56	0.12	0.03	0.25	0.12
AF210:pR56	R56(8)	0.12	0.06	0.25	0.25
	R56(2)	0.12	0.06	0.25	0.25
	R107	0.12	0.06	0.25	0.25

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Table 3

Complementation of REMI mutants with rescued plasmid

Parent	Transformant/strain	MIC^{ITR} (mg/ml)
AF210.1	AF210.1	0.25
REMI-103	REMI-103	1.0
	REMI-103: p4G10880-1	0.25
	REMI-103: p4G10880-2	0.25
REMI-116	REMI-116	1.0
	REMI-116: p6G12570-1	0.25
	REMI-116: p6G12570-2	0.25
REMI-85	REMI-85	0.0078
	REMI-85: p5G07550-1	0.25
	REMI-85: p5G07550-3	0.25
REMI-102	REMI-102	0.062
	REMI-102: p2G11840-1	0.25
	REMI-102: p2G11840-2	0.25
REMI-14D	REMI-14D	8
	REMI-14D: p2G11020-1	0.25
	REMI-14D: p2G11020-2	0.25
REMI-56	REMI-56	0.062
	REMI-56: p1G05020-1	0.25
	REMI-56: p1G05020-2	0.25