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Genetics of Resistance to Itraconazole in *Aspergillus amstelodami*

Sahi J. Dhahi
Department of Biology
College of Science
Mosul University

Asia A. Mohammed
Department of Biology
College of Science
Dohuk University

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ABSTRACT

A sample of 24 spontaneous mutants resistant to the antifungal itraconazole were isolated in the brown strain A76 (18 mutants) and the white strain AZG131 (6 mutants) of the fungus *Aspergillus amstelodami* . Dominance tests, in heterokaryons, have shown that all mutants are recessive to their respective wild type alleles. Complementation tests, also in heterokaryons, among the mutants indicated that all mutantions belong to a single gene given the gene symbol *itzA* as it was the first gene of its kind to be identified in this fungus. There are nine (1-IX) linkage groups so far recognized in this fungus and haploidization analyses of diploids between one of the mutants and suitable master stains put the gene *itzA* outside groups I-VII, but its relationship to groups VIII and IX was not determined as no master strains carrying markers on these two groups were available at the time. Also, the biochemical function of *itzA* in relation to the three genes *cyp51A*, *cyp51B* and MDR, recognized to confer resistance to itraconazole in *Aspergillus* could not be determined.

Key words : Itraconazole, Resistance, Genetics, *Aspergillus amstelodami*

وراثة المقاومة للاتراكونازول في الفطر *Aspergillus amstelodami*

الملخص

لقد جرى عزل 24 طافرة تلقائية مقاومة للمضاد الفطري اتراكونازول في السلالة البنية A76 (18 طافرة) والسلالة البيضاء AZG131 (6 طافرات) من الفطر *Aspergillus amstelodami*. وقد اظهرت اختبارات السيادة باستخدام متباين النوى ان جميع الطافرات هي طافرات متنحية بالنسبة لاليلاتها البرية. كما اظهرت اختبارات التتام باستخدام متباين النوى ايضاً بين الطافرات المختلفة ان جميع الطفرات انما تعود لجين واحد اطلق عليه *itzA* بوصفه الجين الاول من نوعه الذي يشخص في هذا الفطر، وقد جرى تحديد تسع من مجاميع الارتباط (I-IX) في هذا الفطر لحد الان غير ان التحليلات الوراثية حسب مبدأ تنصيف السلالات المتباينة الزيجة والثنائية المجموعة الكروموسومية المتضمنة احدى الطافرات الحالية وبعض السلالات المرجعية الحاملة لعلائم وراثية على كروموسوماتها اظهرت ان الجين *itzA* لا يقع في أي من المجاميع السبع (I-VII) الاولى من هذا الفطر ولكن عدم توفر سلالات مرجعية مناسبة في حينها حال دون تحديد علاقة *itzA* بالمجموعتين الاخريتين الثامنة (VIII) والتاسعة (IX). كما لم يمكن تحديد

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العلاقة الكيمياوية للجين *itzA* بالجينات الثلاث الاخرى *cyp51A*, *cyp51B* و MDR والتي ذكر انها تحدد المقاومة للاتراكونازول في انواع اخرى من *Aspergillus*.

INTRODUCTION

Human fungal infections have increased in incidence and severity in recent years. This was mainly attributed to advancements made in surgery, cancer treatment, the HIV epidemics and use of immunosuppressive drugs, and the wide application of boad-spectrum antibiotics (Walverton, 2001; Sheppard and Lampiris, 2001). This led to the wide spread of fungal infections not only by the fungal pathogens such as *Candida* and *Cryptoccoccus* species but also by the opportunistic *Aspergillus* species (Chmel and Louria, 1980; Laurence and Bennett, 2001).

Diseases caused by species of the genus *Aspergillus* (aspergilloses) are gaining more and more prominence where more than 85% of the patients die (Bossche et al, 1989; Denning, 1998). Associated with that was the rise in the production and application of antifungal antibiotics (Chmel and Louria, 1980; papich et al, 2001). Among the most widely used anitfungals are polyenes (e.g. nystatin), griseofulvin and the synthetic azoles, both imidazoles (e.g. ketoconazole) and triazoles (e.g. itraconazole) (Laurence and Bennett, 2001). The spectrum of action of azoles is quite broad and itraconazole is the antifungal of choice in this respect as it can be used to treat a variety of fungal infections including those caused by *Aspergillus* species (aspergilloses) (Katzung, 2004).

Like bacteria (Franklin and Snow, 1975), however, fungal pathogens have also developed resistance to a variety of antifungal drugs (Bossche, 1997; Moore et al, 2000). Understanding the genetics and mechanism of antimicrobial resistance is an important step in controlling the resistant pathogens and developing more effective drugs (Sherris and Minchew, 1980). The aim of the present work is to isolate and genetically characterize spontaneous mutants resistant to the antifungal itraconazole in the fungus *Aspergillus amstelodami*. This was hoped to expand the genetic map of the fungus (Dhahi, 1996) and give some insight into the mechanism(s) of resistance that could help using or developing more effective antifungals against the resistant strains.

MATERIALS AND METHODS

1- Strains : The origins and genotypes of strains used in the present work are given in Table (1).

Table 1 : Origins and genotypes of strains of *Aspergillus amstelodami* used in the present work

Strain	Genotype	Reference
A76	*bwA1 nicA*	DeBertoldi and Caten (1979)
AZG131	*wA1 lysA azgA131*	AL-Hamdaney (1985)
A167	*bwA argA oclA dilA sC proA azgA*	Dhahi (1996)

arg, lys, nic and *pro* are genes representing requirements for arginine, lysine, nicotinic acid and proline respectively. s represents inability to utilize inorganic sulphates and growth requirement is satisfied with L-methionine (*met*). *w, bw, ocl* and *dil* are mutations affecting colour giving white conidia, brown conidia, orange cleistothecia and dilute conidial colour (green or brown) respectively. *azgA* represents a mutation conferring resistanse to 8-azaguanine (an analogue of the natural base guanine). *bwA argA* means that the two genes are linked on the same chromosome.

2- **Microbiological techniques :** Media, culturing conditions, and conidial suspension preparation were all as described by Caten (1979). Two basic media; the minimal (M) and the malt extract-salt (MTS) were used. When many separate discrete colonies per plate were needed from conidia, the two media were supplemented with the salt sodium deoxycholate (D) at a final concentration of 400 μg/ml to get the MD and MTSD media respectively. The M and MD are, chemically, well defined and critical tests were done on them. The MTS and MTSD, on the other hand, are less well defined and were used when rapid growth and heavy conidiation were needed (Caten, 1979). Incubation was done at 30oC, the optimal growth temperature of the fungus.

3- **Stock solutions :** Stock solutions of individual amino acids, vitamins, nitrogenous bases, and complete (C) supplement (containing a mixture of amino acids, vitamins and bases) were prepared as described by Caten (1979). A stock solution of the toxic base analogue, 8-azaguanine (Fluka, Switzerland), was prepared according to Hoffman and Malling (1974). A stock solution of the antifungal itraconazole was prepared in strile distilled water and used without further sterilization. Due to the inavailability of a pure sample of the antifungal, the pharmaceutical grade Sporanox (Janssen-Cilag, Belgium) was used. One capsule (100 mg active ingredient) was dissolved in 100 ml distilled water to get a stock solution containing 1000 μg/ml. Because of the incomplete solubility of itraconazole in water (Sheppard & Lampiris, 2001) this concentration could be over estimated.

4- **Determination of the minimal inhibitory concentration (MIC) of itraconazole :** This was done by making point inoculations of the two strains A76 and AZG131 on M (appropriately supplemented with nutritional requirements) containing ascending concentrations of the antifungal. The concentration that gave negative growth after 3 days incubation was considered the MIC for that strain.

5- **Isolation of resistant mutants :** Only spontaneous mutants were looked for. Heavy conidial suspensions containing 107 conidia/ml (haemocytometer count) were prepared in distilled water from 3-day old colonies. Resistant mutants were isolated in both parents A76 and AZG131 by spreading 0.5 ml of the conidial suspension of each strain onto 5 MD plates, appropriately supplemented for nutitional requirements and contained the antifungal at a concentration of 25 μg/ml which is higher than the MIC of both strains. Colonies found growing, after 3 days incubation, on such media were considered resistant. These were single-spored on the same selective medium and kept on CM (M containing the complete supplement) slants until further use.

6- **Genetical analysis :** Dominance and complementation tests were done in heterokaryons and assigning genes to linkage groups were done by hoploidisation analysis of heterozygous diploids (DeBertoldi & Caten, 1979; Dhahi, 1996). Haploidization was induced by the fungicide benlate (Hestie, 1970) at a final concentration of 0.3 μg/ml of the medium CMTS (MTS containing the C supplement at a concentration of 5% V/V).

RESULTS AND DISCUSSION

Both parental strains A76 and AZG131 gave poor growth on the concentration of 15 μg/ml and completely stopped growing on 20 μg/ml and 25 μg/ml itraconazole (Table 2).

Table 2 : Growth response of strain A76 and AZG131 on various concentrations of itraconazole

Itraconazole Con. (μg/ml)	Growth of A76 on		Growth of AZG131 on	
	M + nic	M + nic + itraconazole	M + lys	M + lys + itraconazole
0	+	+	+	+
5	+	+	+	+
10	+	+	+	+
15	+	±	+	±
20	+	-	+	-
25	+	-	+	-

+ full growth, ± partial growth, - no growth.

Therefore, operationally the MIC was considered to be 20 μg/ml and selection of mutants was done at 25 μg/ml itraconazole. Obviously, the real MIC could be well below 20 μg/ml as the antifungal dissolves poorly in water (Sheppard and Lampiris, 2001). A total of 24 itraconazole resistant (ITZ) mutants were isolated in parent A76 (ITZ1-ITZ18) and parent AZG131 (ITZ19-ITZ24). As these mutations were the first of their kind to be isolated in this fungus (Dhahi, 1978; DeBertoldi and Caten, 1979; Bloornfield, 1982), they were all given the mutation symbol *itz* (*itz1-itz24*)according to there commendations of Clutterbuck (1973) for naming new mutations and genes in *Aspergillus*. Apart from their resistant phenotype, all mutants have normal morphologies.

Heterokaryons of mutants ITZ1-ITZ18 with parent AZG131 and those of ITZ19-ITZ24 with parent A76 gave very poor growth on M containing 25 μg/ml itraconazole, almost similar to that of the heterokaryon between the wild type parents A76 and AZG131 which failed to grow on this medium. Therefore, all mutations (*itz1-itz24*) were considered recessive to their respective wild type alleles. Recessiveness is a common property of most mutations (Hartl and Clark, 1997). Heterokaryons of mutants ITZ1-ITZ18 (in the *bwA nicA* background) with mutant ITZ21 (*wA lysA azgA*) gave full growth on M containing the antifungal (25 μg/ml). This indicated that mutations *itz1-itz18* are allelic to mutation *itz21*. Similarly, heterokaryons of muatants ITZ19-ITZ24 with mutant ITZ6 (*bwA nicA*) gave full growth on the antifungal medium indicating that mutations Itz19-itz24 are allelic to mutation *itz6*. From the first set of heterokaryons, however, itz6 appeared allelic to *itz21*. Therefore, it was concluded that all 24 *itz* mutations are alleles of a single gene which was given the gene symbol *itzA* (Clutterbuck, 1973) as it was the first gene of its kind to be identified in *A. amstelodami* (Dhahi, 1978; DeBertoldi and Caten, 1979; Bloomfield, 1982).

A total of 108 independent haploid colour sectors were picked up from the heterozygous diploid ITZ21/A167 sectoring on CMTS medium containing the haploidizating agent benlate at a final concentration of 0.3 μg/ml. By visual inspection (for the colour markers) and by replication on various differential media (for other markers), *itzA* appeared to recombine freely (% recombinants ranged between 43.6 and 60.9) with all markers of the master strain A167 (Table 3). This indicated that *itzA21* is not linked in any of the first six linkage groups (I-VI) recognized in this fungus (DeBertoldi and Caten, 1979).

Table 3 : Reassortment of the *itzA21* gene with markers of the master strain A167 among haploid sectors from the heterozygous diploid ITZ21/A167

(Diploid : ITZ21 / A167 : + + *wA* *lysA* + + + + *azgA* *itza21* / *bwA* *azgA* + + *proA* *sC* *oclA* *dilA* *azgA* +)

Number of haploid segregants with genotype:

Linkage group in the master	**I**		**I**		**II**		**III**		**III**		**IV**		**V**		**VI**	
Marker in the linkage group	*bwA*		*argA*		*wA*		*lysA*		*proA*		*sC*		oclA		*dilA***	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
+ Gene *itzA21*	4*	14	12*	10	18	4*	12	10*	10*	12	18*	4	10*	2	14*	4
-	13	38*	40	6*	51*	35*	40*	46	46	40*	55	31*	50	6*	30	21*
Total	69		108		108		108		108		108		108		69	
% recombinants	60.9		53.7		50.9		46.3		46.3		25.8		43.6		50.7	

* recombinant class; ** segregation of this marker can be scored in wA^+ (brown and green) segregants only; + wild type allele of the respective gene, - mutant allele.
% Rec. = (Number of recombionants/Total) x 100 for each marker in the master.
Gene symbols are as in Table (1).

Table 4 : Reassortment of the *itzA21* gene with markers of haploid sectors from the heterozygous diploid ITZ21/A76

(Diploid : ITZ21 / A167 : + *wA* *lysA* + *azgA* *itzA21* / *bwA* + + *nic* + +)

Number of haploid segregants of genotype:

Linkage group	**I**		**II**		**III**		**IV**		**VI**	
Marker in linkage group	*bwA*		*wA*		*lysA*		*nicA*		*azgA*	
	+	-	+	-	+	-	+	-	+	-
+ Gene *itzA21*	12*	8	25	20*	32	13*	30*	15	17	28*
-	6	13*	14*	13*	18*	9	8	19*	3*	24
Total	39		72		72		72		72	
% recombinants	64.1		47.2		43.1		68.1		43.1	

* recombinant class; + wild type allele of the respective gene, - mutant allele.
% Recombinant as in Table 2.
Gene symbols are as in Table 1.

The linkage of *itzA21* in group VII defined by the marker *azgA* in this fungus (Dhahi and Caten, 1987) could not be determined from this diploid as both parental

strains carried this marker (Table 1). Therefore, another diploid involving ITZ21 and master strain A76 which is *azgA+* was synthesized. This diploid was haploidized as in the first diploid and a sample of 72 haploid sectors were checked for the segregation of *itzA21* with the markers of strain A76. *itzA21* recombined freely with all these markers including *azgA+* in group VII (Table 4) and hence should not be linked in any of the first seven (I-VII) linkage groups in this fungus. However, there are nine (I-IX) linkage groups so far recognized in *A. amstelodami* (Dhahi, 1996) but due to invailability, at the time, of master stains carrying markers in groups VIII and IX the linkage relationship of *itzA21* to these two groups could not be determined in the present work.

Mellado et al (2001) identified two genes (*cyp51A* and *cyp51B*) conferring resistance to itraconazole in *Aspergillus fumigatus* and other *Aspergillus* species. Both genes represented mutations in the cytochrome P450 enzyme 14-α demethylase that demethylates lanosterol to ergosterol, the major sterol component of fungal cell membrane. Nascimento et al (2003), on the other hand, found mutations outside the *cyp51* loci that conferred high resistance to itraconazole. These were found to increase the effluxing of drugs outside the cell and hence will be multidrug resistant (MDR). It is not clear, however, whether the *itzA* is a *cyp51* or an MDR gene.

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