uvsH//uvsH diploid strain favors an efficient method to evaluate the recombinagenic effect of chemical and physical agents in *Aspergillus nidulans* (Ascomycetes)

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ABSTRACT. Ascomycete *Aspergillus nidulans* is an excellent system for mitotic crossing-over studies. This is due to the fact that much of its cell cycle is passed in G2 and presents *uvs* mutations that increase frequencies of normal mitotic recombinations (*uvsF* and *uvsH*). The aim of this research was to obtain a new diploid strain of *A. nidulans* with proper characteristics for recombinagenesis investigations, or rather, heterozygous for nutritional markers and conidia coloration and homozygous for *uvsH* mutation. Higher sensitivity of diploid *uvsH//uvsH* in the monitoring of mitotic recombination events was shown by higher indexes of the diploid's spontaneous mitotic recombination when compared with diploid *uvsH+//uvsH+*. New strain is a versatile tool that may be used in different studies on mitotic recombination in *A. nidulans*.

Key words: mitotic crossing-over, loss of heterozygozity, DNA repair, homozygosity index, chemical genotoxicity.

RESUMO. Utilização de linhagens diplóides *uvsH//uvsH* de *Aspergillus nidulans* (Ascomycetes) para a avaliação do potencial recombinagênico de agentes químicos e físicos. O ascomiceto *Aspergillus nidulans* apresenta-se como um excelente sistema para o estudo da recombinação somática, por passar grande parte de seu ciclo celular em G2 e por apresentar mutações *uvs* que promovem aumento das freqüências normais de recombinação mitótica (*uvsF* e *uvsH*). O presente trabalho teve como objetivo obter uma nova linhagem diplóide de *A. nidulans,* com características apropriadas para estudos da recombinagênese, tais como: hetererozigose para marcadores nutricionais e de coloração de conidios e homozigose para a mutação *uvsH*. A maior sensibilidade do diplóide *uvsH//uvsH* no monitoramento de eventos de recombinação mitótica foi demonstrada através dos mais altos índices de recombinação mitótica espontânea por ele apresentados, em comparação com o diplóide *uvsH+//uvsH+*. A nova linhagem apresenta-se como uma ferramenta versátil, podendo ser utilizada em diferentes estudos relacionados à recombinação mitótica em *A. nidulans*.

Palavras-chave: crossing-over mitótico, reparo do DNA, índice de homozigotização, genotoxicidade de substâncias químicas.

Engendering of antibody diversity frequently occurs through the somatic recombination of small genic segments. Recombination method partakes in other cell processes, such as DNA repair, chromosome stability and antigenic variations (Osman *et al.,* 1991). Mitotic crossing-over participates in oncogenic mechanism too and causes loss of heterozygosity in tumor suppressor loci. Process was first noted in retinoblastomas (Zimmermann, 1966, 1971, 1992; Hagstrom and Dryja 1999).

Due to the importance of somatic recombination in the maintenance of cell and systemic homeostasis and to the fact that numberless chemical and physical agents, UV light, ionizing radiations and DNA synthesis inhibitors, such as Mitomycin C and Norfloxacin, act as inductors of mitotic crossing-over, studies have been developed to identify possible recombinagenic agents (Morpurgo 1963; Espósito and Holliday, 1964; Pires and Zucchi 1994; Franzoni *et al.,* 1997).

The filamentous fungus *Aspergillus nidulans* stands out among the organisms in which mitotic

crossing-over occurs. This is chiefly due to the fact that mitosis in the ascomycete has many common characteristics with mitosis of higher eukaryotes. *A. nidulans*, *Saccharomyces pombe* and cells of mammals have most of their cell cycle in G2 (Osman *et al.* 1993). This is significantly propitious for mitotic recombination since chromosomes are duplicated in this phase (Osman *et al.,* 1993; Kafer and May 1999).

Mitotic recombination in *A. nidulans* has been monitored by efficient methodologies, namely, the determination of Homozygosity Index (HI) for a specific nutritional marker and the analysis based on the occurrence of recombination in specific genic intervals (Pires and Zucchi 1994; Baptista and Castro-Prado, 1997; Franzoni *et al.,* 1997).

Analysis of intergenic mitotic recombination in *A. nidulans* may be greatly facilitated by the use of *uvs* mutants (sensitive to UV radiation) which change the normal frequencies of recombination. Although the role of these genes in the DNA repair pathway has not yet been completed, preliminary studies have identified the four epistatic groups of *uvs* mutants: *UvsF*, *UvsB*, *UvsC* and *Uvsl* (Kafer and Mayor, 1986; Chae and Kafer 1993). The first group has a direct link with recombinagenesis events.

The product of *uvsH* gene, integrating the epistatic group *UvsF*, works within the DNA post-replicational repair pathway. It is homologous with protein RAD18 of *Saccharomyces cerevisiae*. RAD18 belongs to epistatic group RAD6 of *S. cerevisiae* and apparently works in the post-replicational repair pathway, associated with genes RAD5 and RAD6 (Prakash *et al.,* 1993; Johnson *et al.,* 1992). *uvsH* mutants are sensitive to MMS (Methyl Methane-Sulfonate) and show increase in frequencies of UV-induced mutations and decrease in frequency of meiotic recombination. High levels of mitotic recombinations are also found in homozygous diploids *uvsH//uvsH*. They may thus be used in the monitoring of mitotic recombinagenesis.

Current research work integrates many characteristics of fungus *A. nidulans* for the analysis of mitotic crossing-over and describes a sensitive system for the evaluation of the recombinagenic potential of chemical and physical agents. Diploid strain, homozygous for *uvsH* mutation and heterozygous for various nutritional genetic markers, and for conidia coloration, was employed. Diploid may be used in analyses based on intergenic mitotic recombination and in the monitoring of mitotic crossing-over events by HI methodology.

Material and methods

Aspergillus nidulans strains and media. *A. nidulans* strains are described in Table 1. Complete (CM) and minimal medium (MM) were described previously (Pontecorvo *et al.* 1953, Van De Vate and Jansen 1978); selective medium (SM) was MM supplemented with the nutritional requirements of each strain. Solid medium contained 1.5% agar. Compact colonies were obtained in SM plus Triton X-100 (0.01%) (TSM).

Table 1. Genotype and origin of strains

Strains	Genotype	Origin
A837	*pabaA1, uvsH77, pyroA4, choA1, chaA1*	FGSC
A610	*pabaA1, γA2*	FGSC
UT448	*riboA1, pabaA124, biA1, AcrA1*	Utrecht (Holland)
A757	*YA2, methA17, pyroA4*	FGSC
B520	*pabaA124, biA1, methA17*	A757 X UT448

Mutant alleles produced the following phenotypes: *riboA1, pabaA124, biA1, me*thA17, *pyroA4, cho*A1: requirements for riboflavin, ac. p-aminobenzoic, biotin, methionine, pyridoxine and choline respectively; *y*, *w* and *cha*: yellow, white and *chartreuse* coloration of conidia, respectively; *uvs*, sensibility to UV light; *AcrA1*, resistance to Acriflavine. FGCS: Fungal Genetic Stock Center (University of Kansas Medical Center, Kansas)

Genetic Techniques. The general methodology followed previous reports (Pontecorvo *et al.*, 1953). The diploid strains were obtained by Roper's method (Roper, 1952). Heterokaryons were prepared in liquid MM plus 2.0% CM. Cleistothecia were obtained from heterokaryons after 21 days of incubation in sealed plates containing solid supplemented MM, according to the requirements of the crossed strains.

Determination of UV sensitivity. Conidia were collected with Tween 80 (0.001%). The suspension was filtered, washed by centrifugation and stored at 5°C in NaCl (0.85%) before treatments. The density of the suspension was determined by haemocytomer counts. Germinant conidia were pre-incubated for 6h before mutagen exposure. Conidia were spread on TSM media and irradiated. The UV dose rate reached 1.4 ergs.sec^{-1}. Results represent the mean ± SEM of 4 experiments carried out in dark or red light to exclude photoreactivation.

Obtaining diploid segregants. Conidia of diploids UT448//A757 (*uvsH$^+$//uvsH$^+$*) and A837//B211 (*uvsH//uvsH*) were inoculated in petri dishes with MM and incubated for 6 days at 37°C.

Each diploid produced visible mitotic sectors considered dominant homozygotes (+/+) or heterozygotes (+/- or -/+). They were not recessive homozygotes (-/-) for the nutritional markers, since the latter did not grow in MM. Diploid sectors were

haploidized spontaneously in CM after purified in MM.

Mitotic segregants obtained after haploidization were purified in CM and their mitotic stability were evaluated. Only segregants that did not give rise to secondary sectors were chosen for the recombinagenesis tests (Franzoni *et al.* 1997).

Calculation of Homozygosity Index (HI).

Conidia of every haploid sector were individually transferred to 25 positions in CM plates. After 48 hours of incubation at 37°C, colonies were replicated in MS for phenotype analysis of haploid segregants.

When one takes into account that mitotic crossing-over induces homozygotization of genes in a heterozygous condition, a crossing-over towards a particular nutritional marker of diploids in MM will produce only heterozygous (+/- or -/+) or homozygous (+/+) segregants. After haploidization of recombinant diploids in CM the phenotype analysis of haploids produces the ratio 4+:2-. This is the opposite of what occurs when there is no crossing-over, or rather, when ratio of haploid segregants is 4+:4- (Figure 1).
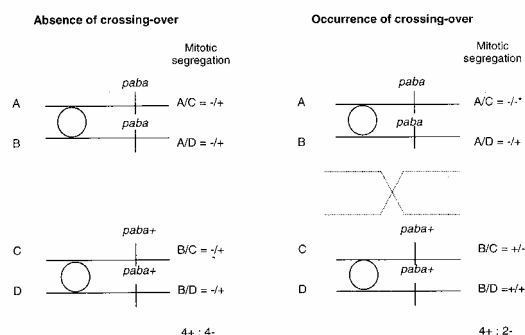

Figure 1. Origin of heterozygous (-/+ and +/-) and homozygous (+/+) diploids by mitotic crossing-over between gene *paba* and centromere. ★Homozygous diploid (-/-) will not be chosen in MM plates

A recombination event occurs when HI (number of prototrophic segregants / number of auxotrophic segregants) from a certain gene is 2.0. HIs higher than 2.0 indicate the occurrence of more than one crossing-over event for a given gene. On the other hand, HIs less than 2.0 indicate absence of crossing-over (Pires and Zucchi, 1994; Baptista and Castro-Prado 1997). Genes for conidia coloration are not selected in MM.

HIs for diploid UT448//A757 were established for genes *riboA*, *pabaA*, *bioA*, *methA* and *pyroA* and *pabaA*, *bioA* and *methA* for diploid A837//B211.

Results and discussion

A diploid strain of *A. nidulans* monitoring the recombinagenic potentiality of chemical and physical compounds was prepared. Strain would have the following genotypic characteristics: (i) heterozygosity of nutritional markers in different chromosomes, allowing an analysis of mitotic recombinagenesis by HI method; (ii) adequate genetic intervals in distinct chromosomes for the analysis of intergenic recombination frequency; (iii) homozygosity for *uvsH*77 mutation, giving greater sensitivity to strain in mitotic recombination events when compared to normal strains *uvsH*⁺//*uvsH*⁺.

Many sexual crosses were undertaken between *uvsH* and wild-type strains to obtain a proper haploid strain, complementary to strain A837 (*uvsH*).

The strains UT448 and A837 were crossed meiotically. After incubation period, hybrid cleistothecia were analyzed, while 75 segregants of offspring were chosen and underwent phenotype analysis. Segregant F1, called B210, with genotype *paba, y⁺, bi⁺* (I); *Acr, w, meth⁺* (II); *pyro* (IV) and *cho⁺* (VII) was chosen (Figure 2). UV sensitivity test showed *uvsH* mutation in the genome of strain B210 (Figure 3).
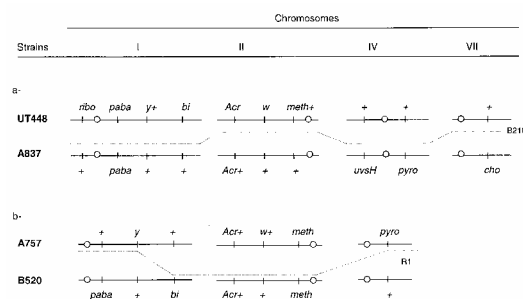

Figure 2. a- Segregant F1, named B210, obtained from cross UT448 X A837; b- Meiotic segregant, named R1, obtained from A757 X B520 cross
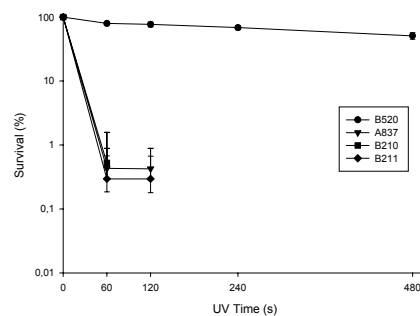

Figure 3. Survival of segregants B210 and B211 from cross A837 x UT448 after UV radiation with 6 hours of pre-incubation. Controls: B520 (*uvs*⁺) and A837 (*uvsH*77)

Meiotic cross between strains A757 and B520 was undertaken as above. Segregant called R1, bearer of genotype *paba*[+], *γ, bi* (I); *Acr*[+], *w*[+], *meth* (II); *pyro* (IV) and *cho*[+] (VII), was selected from offspring (Figure 2). Later it underwent meiotic cross with strain B210. A segregant bearing markers *paba*[+], *γ, bi* (I); *Acr, w, meth* (II); *uvsH* (IV) and *cho*[+] (VI) was isolated among the progeny of R1 x B210 cross. Due to the short meiotic distance that separates genes *wA* and *methA* (1.3 cM) (Clutterbuck, 1994), 400 previously selected segregants such as *w* (white) were analyzed to obtain the proper recombinant *w, meth*. Analyses on nutritional needs and Acriflavine sensitivity were done in selected white segregants. Only one segregant, called B211 (Figure 4), had the desired qualifications. This includes *uvsH* mutation in its genoma (Figure 3).
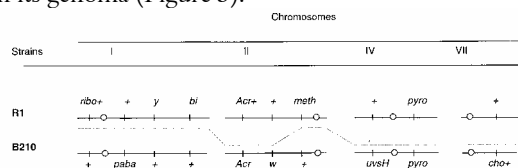

Figure 4. Segregant B211 obtained in the R1 x B210 cross

Segregant B211 made feasible the construction of a diploid bearing heterozygosity for genes *pabaA, γA* and *biA* (I), *AcrA, wA* and *methA* (II) and *choA* (VII); genetic intervals *paba-γ, paba-bi, γ-bi* (I) and *Acr-w, Acr-meth, w-meth* (II) and homozygosity for *uvsH* mutation (IV). Homozygosity for nutritional marker *pyro* (IV) has been obtained too, since the diploid is auxotrophic for pyridoxine (Figure 5).
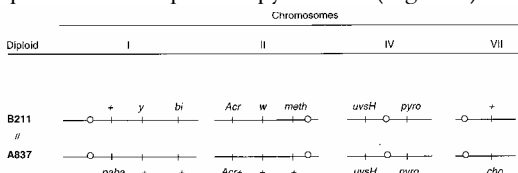

Figure 5. Schematic diagram of chromosomes I, II, IV and VII of *Aspergillus nidulans* B211//A837 (*uvsH//uvsH*) diploid strain

HI values for diploid A837//B211 were significantly higher than HI values for wild-type diploid strain (UT448//A757) for *pabaA, bioA* and *methA* markers (Tables 2 and 3). It was impossible to compare HI of *riboA* and *pyroA* markers in diploids UT448//A757 and A837//B211, since the latter was homozygous to above markers. Diploid A837//B211 caused higher frequency of spontaneous mitotic segregants in CM. Therefore, determination of HI was made easier for the markers under analysis (Figure 6).
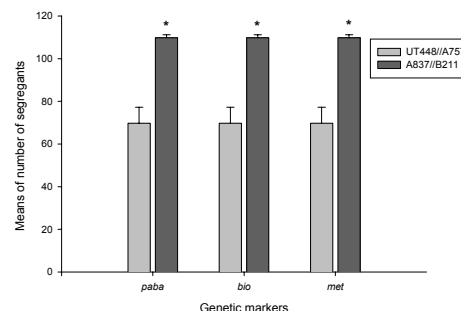

Figure 6. Means of number of spontaneous haploid segregants obtained in CM from diploids UT448//A757 and A837//B211. ⋆ Significantly different from control when P<0.05 (t-test, Sigma Stat program)

Table 2. Homozygosity Index (HI) of several markers of D1, D2, D3 and D4 diploids obtained from the UT448//A757 diploid strain grown in MM

	D1		D2		D3		D4	
	N° Seg.	HI(%)	N° Seg.	HI(%)	N° Seg.	HI(%)	N° Seg.	HI(%)
paba +	40	1.17	38	1.26	34	1.30	39	1.02
paba –	34		30		26		38	
bio +	45	1.55	42	1.61	33	1.22	43	1.26
bio –	29		26		27		34	
meth +	40	1.17	38	1.26	33	1.22	43	1.26
meth –	34		30		37		34	
pyro +	32	0.76	34	1.00	31	1.06	41	1.13
pyro –	42		34		29		36	
ribo +	42	1.31	37	1.19	33	1.22	44	1.33
ribo –	32		31		27		33	

N° Seg, number of haploid mitotic segregants. HI, Homozygosity Index

Table 3. Homozygosity Index (HI) of several markers of D5, D6, D7 and D8 diploids obtained from the A837//B211 diploid strain grown in MM

	D5		D6		D7		D8	
	N° Seg.	HI(%)	N° Seg.	HI(%)	N° Seg.	HI(%)	N° Seg.	HI(%)
paba +	71	⋆1.87	75	⋆2.08	72	⋆1.84	73	⋆2.08
paba –	38		36		39		35	
bio +	72	⋆1.94	71	⋆1.77	69	⋆1.64	68	⋆1.70
bio –	37		40		42		40	
meth +	71	⋆1.86	69	⋆1.64	72	⋆1.84	71	⋆1.92
meth –	38		42		39		37	

N° Seg, number of haploid mitotic segregants. HI, Homozygosity Index; ⋆Significantly different from control at P<0,05 (t-test, Sigma Stat Program)

Diploid obtained was adequate for the evaluation of recombinagenesis by HI. However, its genotype constitution does not constrain it to this methodology. In fact, the diploid is a useful tool for other evaluation methods of intergenic mitotic recombinagenesis in the ascomycete. Results show that homozygous strain for *uvsH* mutation (A837//B211) is a sensitive system for the evaluation

of the recombinagenesis potential of chemical and physical agents in *Aspergillus nidulans*.

Acknowledgments

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