USE OF NYSTATIN-RESISTANT MUTATIONS IN PARASEXUAL GENETIC ANALYSIS IN *DICTYOSTELIUM DISCOIDEUM*

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ABSTRACT

Nystatin-resistant mutations exhibit extreme sensitivity to 1.3 mM coumarin. The mutations fall into three complementation groups so it is possible to select for nonallelic mutations conferring sensitivity to coumarin by selection on nystatin-containing nutrient agar plates. Complementation between such coumarin-sensitive mutations allows the selection of diploids on coumarin-containing nutrient agar. Two of the nystatin resistance genes, *nysB* and *nysC*, have been mapped tentatively to the previously unmarked linkage group V.

IN *Dictyostelium discoideum* complementation and linkage studies use the parasexual cycle (SUSSMAN and SUSSMAN 1962, 1963; KATZ and SUSSMAN 1972; LOOMIS 1969; SINHA and ASHWORTH 1969). This involves the isolation of spontaneously occurring diploids that arise as a result of cell fusion and karyogamy in a population of starving haploid amoebas. Diploid formation is a rare event occurring at a frequency of 1×10^{-5} over a period of 17 hr. A selective system for isolating the diploids is, therefore, essential. The parasexual cycle is completed when the diploid subsequently haploidizes by random chromosome loss to give a population of haploid amoebae with reassorted chromosomes. The haploids so formed may be isolated by selecting for recessive genetic markers (e.g., recessive alleles for drug resistance) for which the diploid is heterozygous. Therefore, to do routine genetic analysis it is crucial to have selective systems first, for the isolation of diploids from a population of haploid cells and, second, for the isolation of haploid segregants among diploid cells. Several selective systems exist (LOOMIS 1969; NEWELL *et al.* 1977; WILLIAMS 1978). All have in common that they require three loci to carry out the selections, two complementing mutations to select for the diploid and a third mutation to select the haploids. We report a system that requires only two mutations. These mutations may be obtained as spontaneous mutants in a simple selective system.

A previous report from our laboratory described a direct selection of spontaneous nystatin-resistant mutants on nutrient agar plates containing nystatin (SCANDELLA, ROONEY and KATZ 1980). The mutations were assigned to three complementation groups. All of the mutations behaved as recessives in diploids with nys^+, and nystatin could be used to recover haploids from nys/nys^+ diploids. Recently, WELKER and WILLIAMS (1980, 1982) reported the effects of coumarin on growth and development in *D. discoideum* and the isolation of six

mutations conferring growth inhibition in the presence of 1.3 mM coumarin. The mutations were assigned to six complementation groups, and coumarin was used in the isolation of complementing diploids. We now report that nystatin-resistant mutants show extreme sensitivity to coumarin as a second phenotype and, therefore, provide the first Dictyostelium markers that can be used for the selection of both diploids and haploids.

MATERIALS AND METHODS

Chemicals and media: For linkage analysis SM agar plates (SUSSMAN 1966) containing inhibitors were used. Methanol (3% v/v), filter-sterilized cycloheximide (400 μg/ml), filter-sterilized cobaltous chloride (Sigma, 200 μg/ml) or nystatin (Sigma, 100 μg/ml) were added prior to pouring (KATZ and KAO 1974; WILLIAMS and NEWELL 1976; SCANDELLA, ROONEY and KATZ 1980). Coumarin (Sigma, 1.3 mM) was added before autoclaving (WELKER and WILLIAMS 1980). For the α-mannosidase assay (FREE, SCHIMKE and LOOMIS 1976) ρ-nitrophenyl-α-mannoside was obtained from Calbiochem. Benlate was generously supplied by DR. R. H. KESSIN. All other chemicals were obtained from Sigma Chemical Company.

Strains and growth conditions: The origins and genotypes of the haploid strains used are given in Table 1. The strains are all derivatives of NC4 (RAPER 1935). The strains were grown on lawns of *Enterobacter aerogenes* at 22° on SM plates. For growth in the presence of 200 μg/ml of cobaltous chloride a spontaneous mutant of *E. aerogenes* resistant to cobaltous chloride was used.

Genetic analysis: Diploids were selected on the basis of complementation of temperature sensitivity mutants (*tsg*) (LOOMIS 1969; KATZ and SUSSMAN 1972). The restrictive temperature used was 27°. Spores of isolates were analyzed on a Coulter counter equipped with a Channelyzer to confirm that the spore size was that of a diploid. For linkage analysis, haploid segregants were obtained from heterozygous diploids using drug selections (KATZ and KAO 1974) or benlate was used to promote haploidization (WILLIAMS and BARRAND 1978). The haploids were purified on drug plates prior to phenotypic scoring.

The *whi*, *bwn* and *frt* markers were scored visually. *tsg* alleles were scored by the ability of the clones to grow on bacterial lawns at 27°. The presence or absence of *cyc*, *acr*, *cob*, *nys* and *cou* were scored on appropriately supplemented drug SM agar plates. The *manA* marker was scored as described by FREE, SCHIMKE and LOOMIS (1976).

RESULTS

Nystatin-coumarin selection: We report a serendipitous observation that our nystatin-resistant strains are extremely sensitive to coumarin. As can be seen in Table 2 the nystatin-resistant strains (*nysA*, *nysB* and *nysC*) did not grow in the presence of 1.3 mM coumarin. To ensure that the coumarin sensitivity was not due to an interaction of the nystatin mutations with other mutations in the particular strains used, we isolated new nystatin-resistant mutations from a wild-type background. For axenic strains of Dictyostelium (LOOMIS 1971) 80 μg/ml of nystatin was used in this selection as compared with 100 μg/ml for nonaxenic strains. The fraction of nystatin-resistant mutants that were coumarin sensitive varied considerably and seemed to be quite strain dependent (data not included). In all strains, however, a minimum of 10% of all nystatin-resistant mutations displayed sufficient sensitivity to growth on coumarin to make genetic analysis feasible. We have successfully used coumarin selection for diploids to show, by complementation, that all of the new nystatin-resistant-coumarin-sensitive mutants that have been tested fall into the *nysB* or *nysC* cistrons.

TABLE 1

Genotypes of haploid strains of Dictyostelium discoideum

Strain	Parent	*acr*	*axe*	*bwn*	*cob*	*cou*	*cyc*	*frt*	*man*	*nys*	*spr*	*tsg*	*whi*	Reference[a]
Ts12m	Ts12	*A*	+	+	+	+	*A1*	+	+	+	+	*D12*	*A1*	1
M28	bwn	+	+	*A1*	+	+	+	+	+	+	*A1*	*E13*	+	1
HK4	DdB	+	+	+	+	+	+	+	+	*A201*	+	+	+	2
HK5	Ts12m	*A*	+	+	+	+	*A1*	+	+	*B203*	+	*D12*	*A1*	2
HK6	M28	+	+	*A1*	+	+	+	+	+	*B204*	*A1*	*E13*	+	2
HK7	M28	+	+	*A1*	+	+	+	+	+	*B205*	*A1*	*E13*	+	2
HK8	M28	+	+	*A1*	+	+	+	+	+	*B206*	*A1*	*E13*	+	2
HK9	M28	+	+	*A1*	+	+	+	+	+	*B207*	*A1*	*E13*	+	2
HK10	M28	+	+	*A1*	+	+	+	+	+	*C208*	*A1*	*E13*	+	2
HK11	M28	+	+	*A1*	+	+	+	+	+	*B209*	*A1*	*E13*	+	2
HK12	Ts12m	*A*	+	+	+	+	*A1*	+	+	*C210*	+	*D12*	*A1*	2
HK13	Ts12m	*A*	+	+	+	+	*A1*	+	+	*B211*	+	*D12*	*A1*	2
HK14	Ts12m	*A*	+	+	+	+	*A1*	+	+	*A212*	+	*D12*	*A1*	2
HK15	Ts12m	*A*	+	+	+	+	*A1*	+	+	*C213*	+	*D12*	*A1*	2
HK16	Ts12m	*A*	+	+	+	+	*A1*	+	+	*B214*	+	*D12*	*A1*	2
HU335	HU227, NP15	+	*B1*	*A1*	*A353*	+	*A1*	+	*A1*	+	+	*A1*	+	3
HU1183	DU1624	*B3*	+	*A1*	+	*A357*	+	*B353*	+	+	*A1*	*E13, K21*	*B513*	3

Phenotypes of mutations at these loci are *acrA*, resistance to 100 μg of acriflavin ml^{-1} or 2% methanol; *axe*, ability to grow in axenic media, *bwnA* production of brown pigment during development; *cobA*, resistance to 300 μg of cobaltous chloride ml^{-1}; *couA* sensitivity to 1.3 mM coumarin; *cycA*, resistance to 500 μg of cycloheximide ml^{-1}; *frtB* distribution of the fruiting bodies in concentric rings; *manA*, α-mannosidase-1 deficient; *nys*, resistant to 100 μg of nystatin ml^{-1}; *sprA*, round spores; *tsg*, temperature-sensitive for growth; *whi* absence of normal yellow pigment. *A* + denotes the wild-type allele.

[a] 1 = KATZ and SUSSMAN (1972); 2 = SCANDELLA, ROONEY and KATZ (1980); 3 = K. L. WILLIAMS, unpublished.

TABLE 2

Plating of nys *mutants on 1.3 mM coumarin agar*

	Parental strain	Mutant strain	E.o.p. of amoebas
I	DdB		0.95
		HK4 (*nysA201*)	0.38
II	M28		0.79
		HK6 (*nysB204*)	1.0×10^{-6}
		HK7 (*nysB205*)	1.0×10^{-6}
		HK8 (*nysB206*)	1.67×10^{-6}
		HK9 (*nysB207*)	9.52×10^{-7}
		HK10 (*nysC208*)	8.9×10^{-5}
		HK11 (*nysB209*)	3.75×10^{-6}
III	ts12M		0.96
		HK5 (*nysB203*)	$<10^{-7}$
		HK12 (*nysC210*)	1.18×10^{-7}
		HK13 (nysB211)	1.11×10^{-7}
		HK14 (*nysA212*)	2×10^{-6}
		HK15 (*nysC213*)	1.6×10^{-7}
		HK16 (*nysB214*)	$< \times 10^{-6}$

Amoebas were harvested from plates and suspended in SS. The efficiency of plating (E.o.p.) was calculated as the ratio of the number of plaques on coumarin agar to plaques on SM.

Since the three complementation groups associated with nystatin resistance all confer coumarin sensitivity, it was of considerable interest to study the nystatin-resistant-coumarin-resistant strains that were produced. In all cases we have found these mutants to be unstable. That is, when such strains are propagated in the absence of nystatin they rapidly revert to nystatin sensitivity. We are currently investigating the nature of this transient nystatin resistance.

Mapping studies: Linkage analysis of nystatin-resistant genes was undertaken by crossing *nysB* and *nysC* mutants to multiply marked strains and then analyzing haploid segregants obtained from the diploids. Linkage of a *nys* gene to one of the known linkage groups would be demonstrated by the lack of independent assortment with a phenotypic marker on that linkage group. As can be seen in Tables 3 and 4 both *nysB* and *nysC* appear to segregate independently of markers on all known linkage groups. They also appear to be linked to each other. We have examined more than 200 haploid segregants that were obtained from diploids constructed between *nysB* and *nysC* mutations. All were nystatin resistant, indicating a lack of independent assortment. The simplest interpretation consistent with these data is that both the *nysB* and *nysC* genes are located on the previously unmarked linkage group V. WALLACE and NEWELL (1982) have tentatively identified a streamer mutation on linkage group V, but difficulty in scoring makes this marker unsuitable in linkage analysis experiments. Our assignment should be considered tentative until other markers can be found on this linkage group.

Mapping the *nysA* mutations has proved to be difficult because haploid selection results in unusually intense skewing, i.e., the recovery of predomi-

TABLE 3

Pattern of phenotypes of haploid segregants selected from diploids heterozygous for the nystatin mutation nysB

Diploid	Parental haploids	nys/nys$^+$ phenotype	Linkage groups											
			I		II		III		IV		VI		VII	
			cycA		*acrA*		*whiB*		*bwnA*				*frtB*	
			+	−	+	−	−	+	−	+			−	+
DK1*	HU1183	+	7	29	11	24	5	6	40	5			18	16
	HK5	−	3	45	13	33	6	7	5	41			7	34
							tsgA				*manA*		*cobA*	
							+	−			+	−	+	−
DK2	HU335	−					50	24			8	0	31	42
	HK6	+					23	3			41	1	11	14
									bwnA					
									+	−				
DK3	Ts12M	+							1	20				
	HK7	−							3	32				

By convention the recombinant segregants lie on the lower left-upper right diagonal in each set of four figures so that zeros on these diagonals indicate linkage. + = wild type; − = mutant.

* The presence of the *whiB* mutation was scored only in segregants that did not carry the *acrA* mutation.

TABLE 4

Pattern of phenotypes of haploid segregants selected from diploids heterozygous for the nystatin mutation nysC

Diploid	Parental haploids	nys/nys+ phenotype	Linkage groups											
			I		II		III		IV		VI		VII	
							tsgA							
							+	−						
DK4	Hu335	−					13	6						
	HK10	+					18	8						
									bwnA		*manA*		*cobA*	
									+	−	+	−	+	−
DK5	Hu335	−							14	15	41	23	35	29
	HK15	+							3	33	18	15	19	16
			cycA		*acrA*		*whiB*		*bwnA*				*frtB*	
			−	+	−	+	+	−	+	−			+	−
DK6*	Hu1183	−	42	5	1	68	14	0	25	24			34	2
	HK12	+	18	18	15	85	10	23	7	27			27	39

By convention the recombinant segregants lie on the lower left-upper right diagonal in each set of four figures so that zeros on these diagonals indicate linkage. + = wild type; − = mutant.

* The presence of the *whiB* mutation was scored only in segregants that did not carry the *acrA* mutation.

nantly one class of haploids where equal numbers of two reciprocal classes would be expected. The reasons for this are unclear, but it should be noted that mutations in this gene appear to be different from mutations in either *nysB* or *nysC*. In the previous study (SCANDELLA, ROONEY and KATZ 1980) it was shown that unlike the *nysB* and *nysC* genes, *nysA* was not associated with a membrane sterol alteration. In addition, the two *nysA* alleles, *nys201* and *nys212*, differ in their sensitivity to coumarin over six orders of magnitude, whereas all alleles in *nysB* and *nysC* show a relatively uniform sensitivity to coumarin (Table 2). Finally, in the course of our genetic analysis we have isolated more than 20 new nystatin mutations, and none of them are in *nysA*. It appears, therefore, that the frequency with which spontaneous mutations arise in the *nysA* gene is lower than in either the *nysB* or *nysC* genes. We are continuing our attempts to characterize *nysA* genetically.

DISCUSSION

The results described in this report have wide applications to routine parasexual genetic analysis in Dictyostelium. The ability to readily select nystatin-resistant-coumarin-sensitive mutations makes mutations selected in a wild-type background immediately usable in genetic crosses with all other strains containing a *nys*-coumarin mutation in a different complementation group. This is useful both in complementation analysis as well as linkage studies.

The general applicability of the dual selection system depends on the frequency with which stable nystatin-resistant mutations occur. We have observed considerable variation in this frequency between strains. However, screening for coumarin-sensitive mutants is simple since it involves coumarin-agar streak testing of a relatively small number of nystatin-resistant isolates. Although, all of the nystatin-resistant isolates that are found to be coumarin resistant were also unstable, we cannot rule out the possibility that some stable nystatin-resistant-coumarin-resistant mutations will be found. The success of the dual selection also depends on the relative frequencies with which nonallelic mutations can be isolated. We have found strain variations in this parameter as well. The reasons for such variations are unknown.

In addition to *D. discoideum*, we believe that the nystatin-coumarin selection system can be used for gentic studies in other cellular slime molds since we have been able to obtain nystatin-resistant-coumarin-sensitive mutants in both *Dictyostelium purpureum* and *Polysphondelium violeceum*.

In addition to diploid selection, the nystatin-coumarin selection system gives us the possibility of doing fine structure mapping in Dictyostelium. We are currently investigating this possibility.

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