Parasexual Genetics in *Dictyostelium discoideum*: Mitotic Analysis of Acriflavin Resistance and Growth in Axenic Medium

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SUMMARY

Drug resistant mutants of the slime mould *Dictyostelium discoideum* useful for mitotic genetic analysis have been studied. Mutations to acriflavin resistance are recessive and have been assigned to two genes (*acrA*, *acrB*) located in different linkage groups. One of these (*acrA*) confers resistance to the unrelated compounds methanol and thiabendazole. Ability to grow axenically (in the absence of bacteria) is determined by genes on two linkage groups, one carrying *acrA* and the other an established temperature sensitive mutation. Preliminary results for two linkage groups show that the sequence of genes on a linkage group can be determined by analysis of homozygous drug resistant diploids, obtained by drug selection from diploids heterozygous for recessive drug resistance markers.

INTRODUCTION

During the life-cycle of the cellular slime mould *Dictyostelium discoideum*, amoebae differentiate in response to starvation to form stalk and spore cells. Because of the relative simplicity of this differentiation and the ease with which the organism can be manipulated, it has been extensively studied by developmental biologists. Despite the availability of numerous mutants affecting the development of *D. discoideum*, no means of genetic analysis was available until recently to complement the biological and biochemical studies to which the organism is well suited. A complete sexual cycle has been postulated, but is not elucidated (Clark, Francis & Eisenberg, 1973; Erdos, Raper & Vogen, 1973). Genetic studies have therefore been based on parasexual analysis (Sinha & Ashworth, 1969; Katz & Sussman, 1972). The parasexual system exploits rare but spontaneous fusions of haploid, growth-temperature sensitive amoebae, differing in their temperature sensitive mutations, to form temperature resistant diploids. Once formed, these diploids can be maintained indefinitely. Haploid segregants may be obtained by including recessive drug resistance markers in the diploids, which makes possible the selection of drug resistant haploids arising through spontaneous haploidization. Because chromosomes assort randomly without a high frequency of genetic exchange between homologues (mitotic crossing-over) any genetic marker may be assigned to a linkage group. However, selection of the products of mitotic crossing-over permits the order and relative distance between markers on a linkage group to be determined (Pontecorvo & Kafer, 1958).

Good chromosomal markers are rare in *D. discoideum*. Since a defined medium for growth has not yet been developed, auxotrophic mutants are not available. Recessive muta-

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tions affecting pigmentation are known (Sussman & Sussman, 1963) and are useful for monitoring the stability of diploids. The use of these pigmentation markers to recover rare (10^{-3}) haploids without preselection on drugs is not practical in *D. discoideum*, although visual selection of haploid segregants on the basis of recessive colour mutations is used routinely in *Aspergillus nidulans* (Pontecorvo & Kafer, 1958). Hence other selective markers must be sought. Katz & Sussman (1972) characterized cycloheximide resistance as a recessive marker located on linkage group I. Complementation and linkage studies in this laboratory with three strains each containing an independently isolated cycloheximide resistance mutation suggest that there is only one gene in *D. discoideum* conferring a high level of resistance to cycloheximide (500 µg/ml). In response to a suggestion by Dr E. R. Katz, acriflavin resistance has been studied as a source of further recessive resistance mutations. Such mutations have been characterized in *Aspergillus nidulans* (Roper & Kafer, 1957). As a result we report two genes determining resistance to acriflavin located on linkage groups I and II (*acrB* and *acrA* respectively).

For biochemical studies the growth of *D. discoideum* on bacteria is a serious disadvantage, so axenic strains have been isolated (Sussman & Sussman, 1967). The isolation of such strains is very time-consuming and few have been reported (Schwalb & Roth, 1970; Watts & Ashworth, 1970; Loomis, 1971). For this reason we studied the genetic basis of axenic growth in the hope that the ability to grow axenically could be inserted into non-axenic strains (Williams, Kessin & Newell, 1974). From cytological evidence, *D. discoideum* is thought to have seven linkage groups (Brody & Williams, 1974). We report here the assignment of axenic genes to two of the seven linkage groups.

METHODS

Strains. The characteristics of the strains used are detailed in Tables 1 and 2. Nomenclature is based on that used in bacterial genetics (Demerec, Adelberg, Clark & Hartman, 1966). Growth-temperature sensitive mutants (NP2 and NP3) of the axenic strain AX3 were isolated following treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Ralph Emanuel Ltd, Wembley) and subsequent selection involving incorporation of bromodeoxyuridine and killing by near u.v. light (Kessin, Williams and Newell, unpublished). Spontaneous acriflavin resistant mutants were selected on *Aerobacter aerogenes* growing on nutrient agar plates containing acriflavin (100 µg/ml, Sigma, neutral) or methanol (2 %, v/v, Analar, Fisons). These concentrations were used in all experiments.

Maintenance of stocks and growth conditions. Stocks of all strains were maintained as spores in silica gel at 0 to 5 °C (Perkins, 1962). Strains being used routinely were maintained clonally in association with *A. aerogenes* on 1.5 % (w/v) nutrient agar plates at 22 °C, and cloned weekly (Sussman, 1966).

Formation of diploids. When two growth-temperature sensitive haploid strains were to be fused to form a temperature resistant diploid, 10^5 to 3×10^5 amoebae of each strain were plated on separate nutrient agar plates in association with *A. aerogenes*. The amoebae were harvested as they cleared the *A. aerogenes* after approximately 48 h at 22 °C, washed free of bacteria, and treated as described previously (Williams *et al.* 1974). Diploids appeared at the restrictive temperature (27 °C) at a frequency of about 1 in 10^5 amoebae plated.

Selection of haploid segregants. Haploids were selected by plating 5×10^4 diploid amoebae, which were heterozygous for a particular drug resistance marker, together with *A. aerogenes* on nutrient agar containing the drug. The concentrations of inhibitor used in the nutrient agar were: cycloheximide (Sigma), 500 µg/ml; acriflavin, 100 µg/ml; methanol, 2 % (v/v).

Table 1. Description of the haploid strains of *D. discoideum* used

Strain	Parent	Source*	Genotype						Relevant phenotype†									
			<i>whi</i>	<i>bwn</i>	<i>tsg</i>	<i>cyc</i>	<i>acr</i>	<i>axe</i>	<i>spr</i>	<i>Cy</i>	<i>Ac</i>	<i>Meth</i>	<i>Sp.Col.</i>	<i>Sp.Sh.</i>	<i>Pig</i>	<i>G.Res.</i>	<i>Ax</i>	
NC4		1	+	+	+	+	+	+	+	+	S	S	S	Y	E	NB	G	NG
TS12	NC4	2	<i>whi</i>	+	<i>DI2</i>	<i>AI</i>	+	+	+	+	R	S	S	W	E	NB	NG	NG
M28	NC4	2	+	<i>bwn</i>	<i>EI3</i>	+	+	+	+	+	S	S	S	Y	RD	B	NG	NG
AX3	NC4	4	+	+	+	+	+	+	+	+	S	S	S	Y	E	NB	G	G
FGR7	AX3	5	+	+	<i>F6</i>	+	+	<i>C4</i>	<i>AI, BI</i>	+	S	S	R	Y	E	NB	NG	G
NP2	AX3	3	+	+	<i>AI</i>	+	+	+	+	+	S	S	S	Y	E	NB	NG	G
NP3	AX3	3	+	+	<i>B3</i>	+	+	+	+	+	S	S	S	Y	E	NB	NG	G
NP12	NP3	3	+	+	<i>B3</i>	+	+	<i>A2</i>	<i>AI, BI</i>	+	S	R	R	Y	E	NB	NG	G
NP14	TS12	3	<i>whi</i>	+	<i>DI2</i>	<i>AI</i>	+	<i>AI</i>	+	+	R	R	R	W	E	NB	NG	NG
NP15	M28	3	+	<i>bwn</i>	<i>EI3</i>	+	+	<i>B3</i>	+	+	S	R	S	Y	RD	B	NG	NG
NP24	AX2	6	+	+	<i>-I4</i>	+	+	+	+	<i>spr</i>	S	S	S	Y	E	NB	NG	G
NP29	M28	3	+	<i>bwn</i>	<i>EI3</i>	+	+	+	+	<i>spr</i>	S	S	S	NS	NS	B	NG	NG
NP46	X2	3	+	<i>bwn</i>	<i>AI</i>	+	+	+	+	+	S	S	S	NS	NS	B	NG	G
NP56	X2	3	+	<i>bwn</i>	<i>AI</i>	+	+	<i>A6</i>	<i>AI, BI</i>	+	S	R	R	Y	E	B	NG	G
NP57	X2	3	+	<i>bwn</i>	<i>AI</i>	+	+	<i>A7</i>	<i>AI, BI</i>	+	S	R	R	Y	E	B	NG	G
NP58	X2	3	+	<i>bwn</i>	<i>AI</i>	+	+	<i>A8</i>	<i>AI, BI</i>	+	S	R	R	Y	E	B	NG	G
NP59	X2	3	+	<i>bwn</i>	<i>AI</i>	+	+	<i>A9</i>	<i>AI, BI</i>	+	S	R	R	Y	E	B	NG	G
NP60	X2	3	+	<i>bwn</i>	<i>AI</i>	+	+	<i>A10</i>	<i>AI, BI</i>	+	S	R	R	Y	E	B	NG	G
NP68	NP14	3	<i>whi</i>	+	<i>DI2</i>	<i>AI</i>	+	<i>AI</i>	+	+	R	R	R	W	E	NB	NG	NG
X2	DP3	3	+	<i>bwn</i>	<i>AI</i>	+	+	+	+	+	S	S	S	Y	E	B	NG	G
X9	DP4	3	<i>whi</i>	<i>bwn</i>	<i>DI2</i>	<i>AI</i>	+	<i>AI</i>	+	+	R	R	R	W	E	B	NG	NG
X11†	DP19	3	<i>whi</i>	<i>bwn</i>	<i>AI</i>	+	+	<i>AI</i>	<i>AI, BI</i>	+	S	R	R	W	E	B	NG	G
X20	DP35	3	+	<i>bwn</i>	<i>F6</i>	<i>AI</i>	+	<i>C4</i>	<i>AI, BI</i>	+	R	R	S	Y	E	B	NG	G

Strains produced in this laboratory designated NP are haploid, those designated DP are diploid (see Table 2), and x strains are haploid strains containing reassorted chromosomes from a diploid.

Genotypes are detailed in full in Table 4.

* (1) Raper (1935); (2) Katz & Sussman (1972), obtained from Dr E. R. Katz; (3) this laboratory; (4) Loomis (1971); (5) obtained from Professor F. G. Rothman, Brown University, Providence, Rhode Island 02912, U.S.A.; (6) obtained from Dr E. Gingold and Professor J. M. Ashworth, Leicester University. Formerly designated Ax-2g88.

† (Cy) cycloheximide; (Ac) acriflavin; (Meth) methanol; (Sp.Col.) spore colour; (Sp.Sh.) spore shape; (Pig) pigment; (G.Res.) growth at restrictive temperature, 27 °C; (Ax) axenic medium; (S) sensitive; (R) resistant; (Y) yellow; (W) white; (E) elliptical; (RD) round; (B) brown; (NB) not brown; (G) growth; (NG) no growth; (NS) no spores (strains NP29 and NP46 are developmental mutants).

‡ Strain X11 may contain *tsg Dir2* in addition to *tsg AI*.

Table 2. *Description of the diploid strains of D. discoideum used*

All of the diploid strains were derived in this laboratory. All mutant alleles detailed here are recessive, so the genotype and phenotype of diploids can be determined from Table 1.

Strain	Haploid parents	Strain	Haploid parents
DP2	TS12/NP2	DP35	X9/FGR7
DP3	M28/NP2	DP40	X20/TS12
DP4	M28/NP14	DP44	TS12/NP56
DP5	NP15/TS12	DP45	NP14/NP57
DP6	NP14/NP15	DP46	NP14/NP59
DP8	NP3/X9	DP47	NP14/NP60
DP19	NP46/NP14	DP49	TS12/NP59
DP30	X9/NP24	DP54	NP68/NP56
DP32	TS12/NP12	DP55	NP68/NP58
DP33	X9/NP12	DP59	TS12/NP58
DP34	NP29/NP12		

The plates were prepared by adding filter-sterilized inhibitor to molten agar immediately before pouring. After 4 to 5 days haploids and homozygous diploids resistant to the inhibitor were observed. These were differentiated on the basis of spore size and subsequent segregation of genetic markers (Katz & Sussman, 1972). Cycloheximide was used at a higher concentration (500 µg/ml) than that used previously (300 µg/ml; Katz & Sussman, 1972) since some axenic strains showed a partial resistance to cycloheximide at 300 µg/ml.

In some cases haploid segregants were selected from heterozygous axenic/non-axenic diploids after prolonged incubation in axenic medium. Ability to grow axenically is a recessive character (Williams *et al.* 1974). Such haploid segregants were not used in assigning markers to linkage groups since many of them may have been of clonal origin.

Characteristics of haploid segregants. Clones appearing on inhibitor plates were picked with a sterile toothpick on to nutrient agar plates previously spread with *A. aerogenes*. Because the haploidization procedure is a single colony isolation method, further single colony isolation was generally not necessary. Occasionally, mixed cultures were observed which when analysed invariably proved to be a mixture of haploids and diploids and could be purified by single colony isolation. Colour markers (brown pigment production, white or yellow spores) and ploidy (on the basis of spore size) were established. The ability to grow at 27 °C (restrictive temperature) and resistance to other inhibitors were determined by transferring with toothpicks on to appropriate agar plates spread with *A. aerogenes*.

Screening for axenic growth. Amoebae were transferred directly into 1 ml axenic medium (Watts & Ashworth, 1970) plus 250 µg dihydrostreptomycin sulphate (Sigma)/ml contained in sterile multiple-welled tissue culture dishes (Linbro FB-16-24-TC). It was possible to pick amoebae directly from bacterial plates into axenic medium because the bacteria (streptomycin sensitive *A. aerogenes*) were regularly cloned. The culture dishes were shaken in the dark at 150 cycles/min at 22 °C, and inspected with an inverted microscope (Olympus, Model CK, Tokyo, Japan) on at least two occasions approximately 3 and 7 days after inoculation. Axenic growers, defined as growing to more than 2×10^6 amoebae/ml at stationary phase, were easily observed. In some cases axenic growth was confirmed in 25 or 125 ml flasks, containing 5 or 25 ml of axenic medium shaken at 150 cycles/min at 22 °C.

Table 3. *Dominance and complementation analysis of acriflavin resistance mutations*

Either two acriflavin resistant haploids, or an acriflavin resistant and acriflavin-sensitive haploid, were fused to form a diploid. In all cases strains were suitably marked so that diploidy could be confirmed by inspection; e.g. all diploids were non-brown, but were heterozygous with respect to *bwn*, a recessive allele which results in the production of brown pigment. Diploidy was also confirmed by inspecting spore size.

Name of diploid	Acriflavin resistance mutations	Characteristic of the diploid*
DP33	<i>acrA1</i> , <i>acrA2</i>	r
DP54	<i>acrA1</i> , <i>acrA6</i>	r
DP45	<i>acrA1</i> , <i>acrA7</i>	r
DP55	<i>acrA1</i> , <i>acrA8</i>	r
DP46	<i>acrA1</i> , <i>acrA9</i>	r
DP47	<i>acrA1</i> , <i>acrA10</i>	r
DP8	<i>acrA1</i> , +	s
DP32	<i>acrA2</i> , +	s
DP44	<i>acrA6</i> , +	s
DP59	<i>acrA8</i> , +	s
DP49	<i>acrA9</i> , +	s
DP6	<i>acrB3</i> , <i>acrA1</i>	s
DP5	<i>acrB3</i> , +	s
DP35	<i>acrA1</i> , <i>acrC4</i>	s
DP40	<i>acrC4</i> , +	s

* r, Resistant, denotes the ability to grow in the presence of acriflavin (100 µg/ml); s, sensitive, denotes the inability to grow under the same conditions.

RESULTS

Acriflavin resistance mutations

Mutations to acriflavin resistance (100 µg/ml) located in two unlinked genes designated *acrA* and *acrB* were isolated and are detailed here.

Isolation of mutants. Strains carrying the allele *acrA* show cross-resistance to the unrelated compounds methanol (2 %, v/v) and thiabendazole (10 µg/ml, gift from Merck, Sharp & Dohme, New Jersey, U.S.A.). Spontaneous *acrA* mutants, which were not of clonal origin, were selected at a frequency of approximately 2×10^{-6} on nutrient agar plates containing acriflavin (strain NP14, containing *acrA1*), acriflavin followed by reselection on thiabendazole (strain NP12, containing *acrA2*), or methanol (strains NP56, 57, 58, 59, 60, containing *acrA6*, 7, 8, 9, 10 respectively).

Only a single mutant of *acrB* has been isolated (strain NP15, containing *acrB3*). It is distinguished from *acrA* mutants by showing no cross-resistance to the other compounds.

Dominance and complementation properties of mutants. Both *acrA* and *acrB* mutations are recessive since diploids heterozygous for these resistance markers are sensitive to acriflavin (Table 3), but give rise to acriflavin resistant haploid and diploid clones. Complementation tests on independent mutants containing *acrA* indicate that they probably involve the same gene, since diploids containing *acrA1* with either *acrA2*, 6, 7, 8, 9, or 10 in *trans* were all resistant to acriflavin (Table 3). All five strains isolated as resistant to methanol involved a mutation in *acrA*, hence it is possible that this is the only gene conferring resistance to methanol in *D. discoideum*. Strains containing *acrB* (e.g. NP15) complement those containing *acrA* (e.g. NP14) (Table 3), hence *acrA* and *acrB* are non-allelic.

Plating efficiency. Strains carrying *acrA* (e.g. NP14) give close to 100 % plating efficiency on nutrient agar plates containing acriflavin or methanol. Growth is slightly slower on plates containing methanol than on nutrient agar alone. Growth of strains containing *acrA*

Table 4. *Linkage groups in D. discoideum*

The linkage groups have been numbered. In order to standardize nomenclature, we suggest that gene symbols be altered as shown below to conform with the system of Demerec *et al.* (1966).

Linkage group*	Gene symbol	Previous symbol	Mutant phenotype	Reference†
I	<i>cycA</i>	<i>cy^r</i>	Growth in the presence of cycloheximide (500 µg/ml)	1
	<i>spr</i>	<i>sp^R</i>	Round spore	1
	<i>tsgE</i>	<i>t₂^s</i>	Temperature sensitive for growth	1
	<i>acrB</i>		Growth in the presence of acriflavin (100 µg/ml)	2
II	<i>whi</i>	<i>wh⁻</i>	White spore	1
	<i>tsgD</i>	<i>t₁^s</i>	Temperature sensitive for growth	1
	<i>acrA</i>		Growth in the presence of acriflavin (100 µg/ml) or methanol (2 %) or thiabendazole (10 µg/ml)	2
	<i>axeA</i>		Growth in axenic medium (provided that <i>axeB</i> is also present)	2
III	<i>tsgA</i>		Temperature sensitive for growth	3
	<i>axeB</i>		Growth in axenic medium (provided that <i>axeA</i> is also present)	2
IV	<i>bwn</i>	<i>br⁻</i>	Formation of brown pigment	1
	<i>tsgB</i>		Temperature sensitive for growth	3
V	<i>tsgC</i>		Temperature sensitive for growth	3

* The remaining two linkage groups have not been characterized.

† (1) Katz & Sussman, 1972; (2) this paper; (3) Kessin, Williams and Newell, unpublished.

on plates containing acriflavin is complicated because some grow poorly (e.g. NP56), while others grow poorly but 30 to 50 % of clones produce vigorous sectors (e.g. NP57, 58, 59, and 60).

Strain NP15 bearing *acrB3* has a plating efficiency of 100 % on plates containing acriflavin but produces small colonies when compared with its growth on nutrient agar.

Assignment of acriflavin resistance genes to linkage groups

acrA. This gene segregates with the associated parental allele at the *whi* locus and hence has been assigned to linkage group II (Table 4). The allele *acrA1* was selected in a strain containing the mutant white spore marker (NP14), and this strain was then fused to form a diploid with an acriflavin sensitive normal yellow spored strain (DP19: $\frac{acrA}{+} \frac{whi}{+}$). In addition, this diploid was heterozygous for markers on linkage groups I (*cycA*), III (*tsgA*) and IV (*bwn*) (Tables 1 and 2). Subsequent selection on acriflavin or methanol revealed that essentially all haploids had white spores. Haploids with yellow spores were sufficiently rare (4 in 940 segregants screened) to be due to either a mitotic crossover event before or coincident with haploidization (Pontecorvo & Kafer, 1958) or a mutation.

In contrast, when a diploid (e.g. DP44, DP49, DP59) was constructed with *acrA* in *trans* to the white spore marker ($\frac{acrA}{+} \frac{+}{whi}$), selection on nutrient agar plates containing acriflavin or methanol produced essentially all yellow haploids, although four white haploids were observed in 565 haploids screened from DP44. Independent segregation of *acrA* with markers on linkage groups I (*cycA*), III (*tsgA*), IV (*bwn*), and V (*tsgC*) was observed.

acrB. The *acrB3* mutation segregates with the associated parental allele at the *cycA* locus and hence has been assigned to linkage group I (Table 4). A diploid (DP5) was constructed with *acrB3* in *trans* with *cycA1* and *cis* with a spore shape marker and temperature sensitive mutation ($\frac{cycA}{+} \frac{+}{spr} \frac{+}{tsgE} \frac{+}{acrB}$). In addition, this diploid was heterozygous for markers on linkage groups II (*whi*) and IV (*bwn*) (Tables 1 and 2). Selection on nutrient agar plates containing cycloheximide produced only acriflavin sensitive, elliptical spored (*spr*⁺), temperature resistant (*tsgE*⁺) haploids among 67 screened. Selection on nutrient agar plates containing acriflavin produced only cycloheximide sensitive, round spored, temperature sensitive haploids among 92 screened.

Selection on nutrient agar plates containing both cycloheximide and acriflavin produced colonies at considerably less than 1 % the frequency on cycloheximide alone, whereas a diploid (e.g. DP44) with *cycA* and *acrA* in *trans* ($\frac{cycA}{+}; \frac{+}{acrA}$) produced haploid colonies at a frequency between 10 and 50 % of that on cycloheximide alone. Hence *cycA* and *acrB* are linked, and selection on nutrient agar plates containing both cycloheximide and acriflavin allows the growth of only rare haploids produced by sequential crossover and haploidization events, whereas *acrA* and *cycA* are unlinked and, in theory, 50 % of cycloheximide resistant haploids should also be acriflavin resistant. Independent segregation of *acrB* with markers on linkage groups II (*whi*) and IV (*bwn*) was observed.

Linkage analysis of the ability of strain AX3 to grow in axenic medium

Evidence for the involvement of mutations on linkage groups II and III in the determination of axenic growth in strain AX3 has been obtained. Linkage group II was also implicated in the axenic growth of strain AX2.

Involvement of linkage group II. When a diploid (e.g. DP19) was formed from a white spore forming, acriflavin resistant (*acrA*), non-axenic strain, and a yellow-spore-forming, acriflavin-sensitive, axenic strain ($\frac{acrA}{+} \frac{whi}{+} \frac{+}{axe}$), and haploids were selected on nutrient agar plates containing acriflavin, good axenic growers were very rarely obtained. A white, acriflavin-resistant, axenic strain (X11) was obtained only after screening more than 240 white, acriflavin resistant, haploid segregants of DP19. In contrast, when a diploid (e.g. DP34) was constructed from an acriflavin resistant (*acrA*), axenic strain and an acriflavin sensitive, non-axenic strain, such that the axenic genes and *acrA* were in coupling, then selection on acriflavin or methanol resulted in the recovery of axenic haploids with a high frequency. Of 47 resistant haploid clones, 25 grew axenically while 19 did not, and three were contaminated.

Hence it was suspected that linkage group II (Table 4) contained a gene required for growth in axenic medium. This was confirmed using diploids (e.g. DP8) that were heterozygous for both *acrA* and *whi* (linkage group II), and *cycA* (linkage group I)

$$\left(DP8: \frac{cycA}{+}; \frac{acrA}{+} \frac{tsgD}{+} \frac{whi}{+} \frac{+}{axe} \right).$$

Haploids were selected on cycloheximide and screened for the ability to grow axenically, and also for acriflavin resistance or spore colour markers, or both. This experiment has been repeated a number of times using different diploids, and a representative experiment using DP8 is shown in Table 5(i). No white spore forming, acriflavin resistant haploid grew

Table 5. *Linkage analysis of the ability of derivatives of strain AX3 to grow in axenic medium*

Only results of selection on nutrient agar plates containing cycloheximide (500 µg/ml) are shown here, since all haploids selected on acriflavin were unable to grow in axenic medium.

(i) DP8 = x9 (non-axenic) × NP3 (axenic)						
Linkage group...	I	II			III	IV
DP8 { x9	<u>cycA1</u>	<u>tsgD12 acrA1 whi +</u>			<u>+</u>	<u>bwn +</u>
NP3	<u>+</u>	<u>+</u>	<u>+</u>	<u>axeA1*</u>	<u>axeB1*</u>	<u>+ tsgB3</u>
Haploid segregants						No.
Non-axenic	<u>cycA1</u>	<u>tsgD12 acrA1 whi +</u>			<u>un</u>	<u>bwn un</u>
	<u>cycA1</u>	<u>tsgD12 acrA1 whi +</u>			<u>un</u>	<u>+ un</u>
	<u>cycA1</u>	<u>+</u>	<u>+</u>	<u>axeA1</u>	<u>+</u>	<u>bwn +</u>
	<u>cycA1</u>	<u>+</u>	<u>+</u>	<u>axeA1</u>	<u>+</u>	<u>+ tsgB3</u>
Axenic	<u>cycA1</u>	<u>+</u>	<u>+</u>	<u>axeA1</u>	<u>axeB1</u>	<u>bwn +</u>
	<u>cycA1</u>	<u>+</u>	<u>+</u>	<u>axeA1</u>	<u>axeB1</u>	<u>+ tsgB3</u>
						49 Haploids
						86 Diploids
						3 Mixed clones
						138 Total
(ii) DP2 = TS12 (non-axenic) × NP2 (axenic)						
Linkage group...	I	II			III	
DP2 { TS12	<u>cycA1</u>	<u>tsgD12 whi +</u>			<u>+</u>	<u>+</u>
NP2	<u>+</u>	<u>+</u>	<u>+</u>	<u>axeA1</u>	<u>tsgA1</u>	<u>axeB1</u>
Haploid segregants						No.
Non-axenic	<u>cycA1</u>	<u>tsgD12 whi +</u>			<u>un</u>	<u>un</u>
	<u>cycA1</u>	<u>+</u>	<u>+</u>	<u>axeA1</u>	<u>+</u>	<u>+</u>
Axenic	<u>cycA1</u>	<u>+</u>	<u>+</u>	<u>axeA1</u>	<u>tsgA1</u>	<u>axeB1</u>
						59 Haploids
						229 Diploids
						288 Total

un, Genotype not determined.

* The markers concerning axenic growth (*axeA*, *axeB*) represent the proposed axenic genotype; both are required to express the axenic phenotype.

axenically, while about 50 % of the yellow spore forming, acriflavin sensitive haploids were axenic growers.

Linkage group II is also involved in the ability of strain AX2 to grow axenically, since diploid DP30 ($\frac{+}{cycA}; \frac{+ + + + axe}{acrA tsgD whi +}$) (Tables 1 and 2) produced no haploid axenic growers among 125 screened using acriflavin as haploid selector, while yellow, acriflavin sensitive, but not white acriflavin resistant, axenic haploids were commonly recovered using cycloheximide to select segregants. This is not surprising since it has been suggested that the genetic basis of the ability of strains AX2 and AX3 to grow axenically may be the same (Williams *et al.* 1974).

Involvement of linkage group III. Because only about 50 % of the haploids containing linkage group II from an axenic strain (AX3) grew axenically, it was suspected that a second linkage group was involved in this characteristic. When diploids (e.g. DP2) with the genotype (II, $\frac{tsgD whi +}{+ + axe}$; III, $\frac{+ +}{tsgA axe}$) were studied, the only haploid axenic strains isolated were

temperature sensitive and yellow spored. No white spore formers, and no temperature resistant, yellow spored haploids were axenic growers. A representative experiment is shown in Table 5(ii). Hence it was suspected that linkage group III also contained a gene involved in the ability to grow axenically. In subsequent studies only yellow, temperature sensitive axenic strains, or yellow, temperature resistant non-axenic strains have been isolated from diploid DP2, which contains *tsgA1* from an axenic strain, despite the fact that more than a hundred yellow haploid segregants have been carefully screened. Other diploids gave the same result.

Since linkage groups I, IV (Table 5) and V (unpublished results) from non-axenic strains may be substituted into axenic strains without effect, they do not carry genes for axenic growth. Thus it seems almost certain that for strain AX3 such genes are located only on linkage groups II and III, although until markers are found on the remaining two linkage groups, VI and VII, their involvement cannot be rigorously excluded. The genes for growth in axenic medium on linkage groups II and III have been designated *axeA* and *axeB* respectively.

The method used to screen for axenic growth made it difficult to distinguish partial growth (to less than 5×10^5 amoebae/ml) from no growth, so no attempt has been made to determine whether a strain possessing one of the linkage groups II or III derived from an axenic strain has some ability to grow axenically.

Mitotic crossing-over analysis of linkage groups I and II

Preliminary studies are reported here of homozygous resistant diploids produced from heterozygous, sensitive diploids using selection on nutrient agar plates containing an inhibitor. In *Aspergillus nidulans*, homozygous diploids are most frequently formed as a result of mitotic crossing-over (Pontecorvo & Kafer, 1958).

Linkage group I. A diploid containing four markers on linkage group I has been constructed for studies on the sequence of markers, DP5 $\left(\frac{acrB \ spr \ tsgE}{+ \ + \ +} \frac{+}{cycA} \bigcirc \right)$. All diploids selected on nutrient agar plates containing cycloheximide were homozygous wild type for the other three markers. Diploids selected on nutrient agar plates containing acriflavin were often heterozygous for the other three markers. This result suggests that all four markers are on the same arm of linkage group I, and that *acrB* is the most distal, and *cycA* the most proximal marker to the centromere. The relative positions of *spr* and *tsgE* have not yet been established.

Linkage group II. The relative positions of three markers on linkage group II are being studied using the diploid DP8 $\left(\frac{acrA \ tsgD \ whi}{+ \ + \ +} \bigcirc \right)$. Selection of diploids homozygous for acriflavin resistance from DP8 results in three phenotypic classes – white spored, temperature sensitive; yellow spored, temperature sensitive; yellow spored, temperature resistant – indicating that the order on the chromosome is *acrA*, *tsgD*, *whi*, centromere. The results of two other groups investigating the sequence of markers on linkage group II are in agreement with those presented here (E. R. Katz and V. Kao, personal communication; E. B. Gingold and J. M. Ashworth, personal communication).

DISCUSSION

Acriflavin resistance mutations are proving a profitable source of selective markers for linkage analysis in *D. discoideum*. In addition to the mutants *acrA* and *acrB* described here,

a third mutant, *acrC*, which is unlinked to both *acrA* and *acrB*, has been isolated and characterized (F. G. Rothman and E. T. Alexander, personal communication).

In this study the selective markers for cycloheximide and acriflavin (or methanol) resistance have been used to obtain haploid segregants from heterozygous diploids in order to assign the genes for growth in axenic medium to linkage groups. It was assumed that the segregants arose by independent haploidization events, and in many cases 1:1 segregation of unlinked markers supported this assumption. However, sometimes segregation departed from a 1:1 ratio quite substantially, probably because in these instances many of the segregants were derived from a haploid segregant which arose spontaneously before plating under restrictive conditions. Diploid amoebae of *D. discoideum* spontaneously give rise to haploids (Sussman & Sussman, 1963; Brody & Williams, 1974). The only way to obtain segregants of certain independent origin is to analyse single segregants from different clones of a heterozygous diploid. This is tedious, and is unnecessary for assigning markers to linkage groups provided that experiments are repeated a number of times using different diploids. For the determination of mitotic map distances (work in progress) it is essential to consider the possible clonal origin of segregants.

Use of the acriflavin resistance mutations *acrA* and *acrB* has, for the first time in *D. discoideum*, made possible the determination of the sequence of genetic markers on linkage groups II and I respectively, by analysis of mitotic crossing-over. By studying homozygous (resistant) diploids arising from heterozygous (sensitive) diploids under restrictive conditions, the sequence of markers and mitotic map distances can be established. In *Aspergillus nidulans* such diploids arise (predominantly) as a result of mitotic crossing-over (Pontecorvo & Kafer, 1958). In the study on *D. discoideum* by Katz & Sussman (1972) it was suggested that cycloheximide resistant diploids obtained from a heterozygous (sensitive) diploid on nutrient agar plates containing cycloheximide may have resulted from a non-disjunctional rather than a crossover event, because simultaneous homozygosity for two other markers (*spr*, *tsgE*) was observed. We have confirmed that selection of diploids for cycloheximide resistance results in homozygosity for *spr* and *tsgE*. However, it appears that this is due to cycloheximide resistance (*cycA*) being the most proximal marker to the centromere on linkage group I, since heterozygosity for the markers *cycA*, *spr* and *tsgE* has been observed when diploids homozygous for *acrB* were selected on acriflavin. All of these results are consistent with the suggestion that diploid segregants arise primarily as a result of mitotic crossing-over.

Hence, using genetic methods based on mitotic analysis, both linkage analysis and mapping are now possible in *D. discoideum*.

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