Sensitivity to *Bacillus subtilis*: A Novel System for Selection of Heterozygous Diploids of *Dictyostelium discoideum*

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INTRODUCTION

Genetic analysis of the cellular slime mould *Dictyostelium discoideum* makes use of the parasexual cycle. In this cycle haploid amoebae of two strains occasionally fuse to form heterozygous diploids that slowly and spontaneously revert to the haploid state with random segregation of the two sets of chromosomes (Sinha & Ashworth, 1969; Brody & Williams, 1974). This cycle allows complementation and linkage studies to be made (Pontecorvo & Käfer, 1958; Katz & Sussman, 1972). Spontaneous fusion to form heterozygous diploids, however, is a rare event, diploids being recovered at a frequency of about 1×10^{-5} in a population of starving amoebae over a 17 h period (Williams, Kessin & Newell, 1974*a, b*).

To overcome this problem, Loomis (1969) devised a selective method involving haploid strains carrying complementing (non-allelic) temperature-sensitive growth mutations (*tsg*). Rare diploids formed between these strains were selected by growth at the non-permissive temperature (27 °C). While this procedure has been successfully and routinely adopted by several laboratories, it has two major disadvantages that make the finding of an alternative system desirable. First, any particular *tsg* mutation is not generally distinguishable from any other. It is therefore difficult to ascertain by inspection whether haploid segregants from a diploid selected by the Tsg method possess one, or other, or both *tsg* mutations. Instead linkage or complementation analysis is necessary to identify the *tsg* markers (Kessin, Williams & Newell, 1974). This is tedious for routine work and not all *tsg* mutations are satisfactorily linked to known markers. Secondly, the possession of *tsg* mutations by haploid strains makes it technically difficult to introduce developmental temperature-sensitive mutations. Because *tsg* mutations are rare it is also not routinely feasible to select the *tsg* mutation after the developmental temperature-sensitive mutation.

A possible alternative selection procedure, using drug-resistant mutations has been tried by several groups. Unfortunately all reports so far, except one (Fukui & Takeuchi, 1971), indicate that resistance to a wide range of drugs tested is recessive (Sinha & Ashworth, 1969; Katz & Sussman, 1972; Loomis, 1971; Williams *et al.*, 1974*b*; Wright, Williams & Newell, unpublished). Diploids formed from pairs of haploids, each resistant to different drugs, are consequently sensitive to both drugs and while this system has been successfully used for selection of haploids from heterozygous diploids (Katz & Sussman, 1972; Williams *et al.*, 1974*b*), it cannot generally be used for the initial selection of the diploids. We therefore decided to look for mutants that were sensitive to drugs to which the wild type was resistant (rather than the converse) and to see if complementation of pairs of such mutants could be used to select diploids.

This paper shows that drug-sensitive mutants can, indeed, be used for diploid selection.

In addition we report an extension of this principle to selection using sensitivity of slime mould growth to the bacterium *Bacillus subtilis*. This 'bsg' system has proved valuable for routine diploid isolation and can be used in conjunction with *tsg* mutations to avoid the two difficulties of double *tsg* selection mentioned above.

METHODS

Strains. All strains of *Dictyostelium discoideum* are derived from the NC4 isolate of Raper (1935). Strain NP20 is a spontaneous mutant resistant to 500 µg cycloheximide ml⁻¹ (*cycA1*). Strain AX3 is a mutant capable of growth axenically (Loomis, 1971). Strain NP194 is a *Bacillus subtilis*-sensitive derivative of NP20 (*cycA1*, *bsg*). Strain X22 is a growth temperature-sensitive strain (*tsgD*, *tsgE*) capable of growth on 2 % (v/v) methanol (*acrA*) and produces white, round spores (*whi*, *sprA*) (Williams & Newell, 1976). The *Enterobacter* (formerly *Aerobacter*) *aerogenes* strain has been described previously (Williams & Newell, 1976). *Bacillus subtilis* 36.1 is a non-sporulating mutant showing a very low reversion rate, obtained from Professor J. Mandelstam and Dr M. Young (University of Oxford).

Isolation of drug-sensitive mutants. Drug concentrations that caused about a 10 % reduction in the rate of expansion of clones of AX3 on plates were used for mutant selection: these were 0.3 % (v/v) for methanol and 100 µg ml⁻¹ for cycloheximide. Drug-sensitive mutants were found by screening amoebae mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) to 10 % survival (Kessin *et al.*, 1974).

Isolation of mutants sensitive to Bacillus subtilis 36.1. Strain NP20 was mutagenized with NTG to 20 % survival and, after dilution and plating with *E. aerogenes* to produce 30 clones per plate, about 10⁴ amoebae from each surviving clone were transferred manually using toothpicks on to replica plates inoculated with either *E. aerogenes* or *B. subtilis* 36.1. To prevent carry-over growth of *E. aerogenes* on the latter, 0.1 ml dihydrostreptomycin sulphate solution (2 mg ml⁻¹) was routinely added with the *B. subtilis* inoculum. *Enterobacter aerogenes* was completely inhibited by the final concentration of antibiotic but *B. subtilis* 36.1 was unaffected.

Fusion of strains bearing the bsg marker with strains bearing tsg markers. Amoebae of both strains to be fused were separately harvested from the edges of clones growing on lawns of *E. aerogenes* on nutrient SM agar plates (Mosses, Williams & Newell, 1975) using a sterile platinum wire loop, and suspended in 5 ml sterile salts solution (SS) (Sussman, 1966). The bacteria were removed by differential centrifugation at room temperature using three rapid spins of 1000 rev. min⁻¹ for 2 min. Amoebae were then resuspended in SS to 2 × 10⁷ ml⁻¹. Portions (0.5 ml) of these suspensions were added to wells of Linbro tissue culture dishes (Linbro FB-16-24-TC; Biocult, Paisley, Renfrewshire) either as separate suspensions (controls) or mixed suspensions of strains (for fusion) together with 0.5 ml per well of fusion mixture, to give a cell density of 1 × 10⁷ ml⁻¹, and shaken at 170 rev. min⁻¹ in an orbital shaker at 22 °C for 17 h. Fusion mixture contained: 60 mM-KH₂PO₄/K₂HPO₄ buffer, pH 7.5, 100 ml; dextran (66000 mol. wt), 15 g; and dihydrostreptomycin sulphate, 200 mg. The solids were gently dissolved in the buffer and filter-sterilized. The dextran seemed to promote a consistent rate of fusion probably by its action in preventing big clump formation. After incubation, 0.1 ml samples of the amoebal suspension were plated directly on to SM nutrient agar plates together with a 0.1 ml inoculum of *B. subtilis* 36.1 (prepared by suspending, in 5 ml SS, five loopfuls of bacteria from an overnight culture grown on a nutrient plate at 37 °C) and 0.1 ml of filter-sterilized dihydrostreptomycin sulphate (2 mg ml⁻¹). The plates were incubated at 27 ± 0.2 °C for 4 days or until the diploid clones were large

enough to be subcultured on to *E. aerogenes* plates for further testing. Some strains of *D. discoideum* did not develop on *B. subtilis* plates, showing instead an aggregateless phenotype; thus *D. discoideum* should not be kept as stocks on these plates for longer than a few days. Fusion of haploid strains without *bsg* by double *tsg* complementation can also be very satisfactorily performed using the fusion mixture described above and *B. subtilis* plates incubated at 27 °C. In this way, conditions of fusion and diploid selection can be standardized for all *bsg/tsg* and *tsg/tsg* fusions.

RESULTS

Isolation of drug-sensitive mutants and their use for diploid isolation. It seemed wise at the outset to use drugs with known effectiveness on the slime moulds and at concentrations that were not greatly inhibitory for the wild type. For this reason, methanol and cycloheximide were chosen initially. Mutants of AX3 that were sensitive to these drugs were found by screening clones of mutagenized amoebae. From about 1000 clones tested three methanol-sensitive mutants and two cycloheximide-sensitive mutants were isolated. Inocula of 10^4 amoebae of the mutants formed no visible growth in 6 days in the presence of the drugs, but formed 10 to 20 mm diam. clones in their absence. None of the mutants showed sensitivity to both methanol and cycloheximide.

The dominance or recessiveness of the mutants was determined by incubating pairs of methanol-sensitive and cycloheximide-sensitive strains together under conditions promoting cell fusion, using the method described previously (Williams *et al.*, 1974a), and then plating on to medium containing both methanol and cycloheximide at a density of 10^6 amoebae per plate. Controls indicated that the parental strain AX3 plated with 100 % efficiency on this double-drug medium but that none of the sensitive mutants grew under these conditions. Amoebae from mixed incubations of methanol-sensitive and cycloheximide-sensitive mutants yielded doubly drug-resistant clones on methanol plus cycloheximide plates in all four combinations attempted. The diploid character of the clones was confirmed by determination of the shape and size of the spores produced by the growing clones; the diploids possessed characteristically curved spores which were approximately twice the volume of the haploids. We conclude that the mutations to methanol and cycloheximide sensitivity are both recessive to their wild-type alleles.

Isolation of mutants sensitive to Bacillus subtilis. Although the methanol-sensitive and cycloheximide-sensitive mutants indicated that the principle of diploid selection using sensitive mutants was tenable, these drugs were not suitable for routine diploid selection (see Discussion). Thus a search was carried out for other types of sensitive mutants.

Sensitivity to *Bacillus subtilis* 36.1 was investigated because it was noticed that this bacterium was used as a food source at a slightly lower rate than *E. aerogenes*, *Escherichia coli* B or *Salmonella typhimurium* LT2. After testing 8750 mutagenized clones for sensitivity to *B. subtilis*, six mutants were isolated that were able to grow on *E. aerogenes* but failed to grow on *B. subtilis* 36.1. (Controls indicated that the sensitivity was not due to other components such as the dihydrostreptomycin added with the *B. subtilis* inoculum). One of these mutants (NP194) seemed highly suitable for genetic work because of the absence of any slow leaky growth on the selection plates and its low reversion frequency ($< 2 \times 10^{-7}$).

Selection of diploids with B. subtilis-sensitive mutant NP194. The recessive nature of the mutation for *B. subtilis* sensitivity (phenotype, Bsg; locus, *bsg*) and its use for selection of heterozygous diploids were tested by an experiment involving fusion of strains NP194 (*cycA1*, *bsg*) and x22 (*tsgE*, *sprA*; *whi*, *acrA*, *tsgD*). After 17 h fusion, using the improved fusion technique (see Methods), amoebae were spread on to *B. subtilis* plates and incubated

Table 1. *Genotypes and frequencies of haploid segregants of diploid DP746*

Diploid DP746 was formed by fusion between strain NP194 carrying the *bsg* marker and strain x22 carrying a *tsg* marker using the selective technique described in Methods.

Linkage group...	I			II			Unassigned	No.
DP746 { NP194 x22	<i>cycA1</i>	+	+	+	+	+	<i>bsg</i>	
	+	<i>tsgE</i>	<i>sprA</i>	<i>whi</i>	<i>acrA</i>	<i>tsgD</i>	+	
Haploid segregants	<i>cycA1</i>	+	+	+	+	+	<i>bsg</i>	10
	<i>cycA1</i>	+	+	+	+	+	+	5
	<i>cycA1</i>	+	+	<i>whi</i>	<i>acrA</i>	<i>tsgD</i>	<i>bsg</i>	33
	<i>cycA1</i>	+	+	<i>whi</i>	<i>acrA</i>	<i>tsgD</i>	+	31
	+	<i>tsgE</i>	<i>sprA</i>	<i>whi</i>	<i>acrA</i>	<i>tsgD</i>	<i>bsg</i>	35
	+	<i>tsgE</i>	<i>sprA</i>	<i>whi</i>	<i>acrA</i>	<i>tsgD</i>	+	16
Total								130

at 27 °C for 4 to 8 days. Controls using amoebae of either x22 or NP194 incubated separately in fusion mixture did not form clones on any of 10 plates bearing 10^6 amoebae per plate, even after 8 days. Amoebae from mixed incubation of x22 and NP194, however, consistently showed about 20 clones per plate on each of 20 plates inoculated with 10^6 amoebae per plate, indicating a frequency of diploid formation of 2×10^{-5} . Several such clones were isolated, recloned and their ploidy confirmed by analysis of spore shape and size. [The heterozygous diploid (*sprA*/+) has a characteristically large oval shape.] Amoebae from one of these clones (DP746) were then plated at 5×10^3 amoebae per plate on to nutrient agar containing methanol (2 %, v/v) or cycloheximide ($500 \mu\text{g ml}^{-1}$) to resegment haploids (Williams *et al.*, 1974a). In the 130 segregant clones examined, sensitivity to *B. subtilis* segregated independently of resistance to cycloheximide or methanol/acriflavin (Table 1).

We conclude that the *bsg* marker is recessive, readily allows heterozygous diploid selection in amoebae in combination with *tsg* markers and segregates independently of markers on linkage groups I and II.

DISCUSSION

The initial postulate that drug-sensitive mutations used in pairs might enable diploids to be selected, was borne out by the isolation of doubly resistant diploids on plates containing both methanol and cycloheximide. While this provided the necessary impetus to search for other types of sensitive mutant, it was not (nor was it intended to be) a routinely workable system. Most of the other laboratory stocks of *D. discoideum* used for genetic studies carry markers for resistance to cycloheximide or methanol/acriflavin. Furthermore the mutants isolated, particularly those for methanol sensitivity, were all slow-growing strains that showed a partially defective developmental ability. They were not, therefore, good parental stocks in which to introduce developmental mutations. In contrast, strains bearing the *bsg* marker showed none of these undesirable qualities. They were generally indistinguishable from their parental strain in growth rate and they developed normally.

The use of haploids carrying the *bsg* marker, together with *tsg* bearing haploids, to select diploids, has the following advantages. First, the low reversion rate of Bsg strains gives a negligible background of revertants on *B. subtilis* plates. Diploids have been selected in over 50 separate fusion experiments using a variety of Tsg haploids as fusion partners. The *B. subtilis* lawn is very opaque and the growing clones produce neat transparent plaques in

about 4 days, hence diploids can be selected earlier and more easily than with the *tsg/tsg* fusions using *E. aerogenes* plates at 27 °C. Secondly, diploids synthesized in this way carry only one *tsg* mutation and there is therefore no ambiguity as to which *tsg* mutation is present in subsequently segregated haploids. Thirdly, Bsg strains are wild type with regard to their response to temperature, and temperature-sensitive mutations that are specific for development may therefore be readily selected. Genetic studies can then be performed by fusing the Bsg strains carrying developmental mutations to a well-marked master strain carrying a single *tsg* marker. Such a master strain is currently being produced.

The *bsg* marker can also be used to perform other genetic manipulations that are otherwise difficult or impossible. For example, if a temperature-sensitive marker such as *tsgE* or *tsgD* is not required in a certain strain while adjoining markers (such as *sprA*, *whi*, *acrA*) are needed, it is generally difficult to eliminate the *tsg* marker on its own. Simple reversion of the *tsg* marker would render the resultant strain temperature-resistant and consequently useless for subsequent diploid formation. If the *bsg* marker is also present, however, the revertant strain isolated can subsequently be fused to other strains and the markers adjoining the reverted *tsg* mutation used in other crosses. In addition, an interesting type of apparent revertant of *tsgD* has been found that retains the *tsg* marker but gains an unlinked suppressor mutation; such suppressors may be conveniently analysed with the aid of the *bsg* fusion system.

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