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## Selective Killing of Vegetative Cells in Sporulated Yeast Cultures by Exposure to Diethyl Ether

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*Summary.* Ascospores of *Saccharomyces cerevisiae* are considerably less sensitive to ether than vegetative yeast. Sporulated cultures grown on either liquid or solid media can be treated with ether to selectively kill vegetative organisms. This differential sensitivity of vegetative cells can be exploited to advantage in a number of genetic manipulations. These include: random spore analysis; selecting mutants affected in the developmental processes of sporulation and spore germination; detection of sporulating revertants or recombinants from non-sporulating diploids; determination of the incidence of spores present in cultures at frequencies as low as 1 per  $10^6$  viable organisms; and, complementation testing of developmental mutations.

### Introduction

During bacterial sporulation, the resistances to heat and various organic solvents acquired by the spore are useful for studying biochemical changes occurring during sporulation, in selecting sporulation and germination mutants and in estimating the extent of sporulation of a culture (Balassa, 1971; Murrell, 1967; Vary and Kornberg, 1970).

For the yeast *Saccharomyces cerevisiae* the finding of a similar differential resistance of spores to a chemical or physical treatment would have considerable impact in simplifying genetic manipulation. For example, separation of haploid ascospores from non-sporulated diploids involves either tedious physical procedures (Emsis and Gutz, 1958; Resnick, Tippetts and Mortimer, 1967; Rousscau and Halvorson, 1969), or for random spore analysis, the use of a recessive resistance mutation (*can<sup>r</sup>*) in heterozygous condition (Sherman and Roman, 1963). Moreover, in *S. cerevisiae*, selecting sporulation-defective mutants or sporulating revertants or recombinants from non-sporulating strains has proven a tedious task since every clone had to be inspected microscopically (Esposito and Esposito, 1969).

In the present study it is shown that exposure of yeast cultures on plates to diethyl ether vapour, or liquid cultures to ether, buffer biphasic mixtures causes rapid loss in viability of vegetative yeast organisms, whereas ascospores are resistant to ether and retain viability. This differential sensitivity of vegetative cells to ether can be applied in a number of genetic procedures:

1. Selectively killing vegetative cells during random spore analysis.
2. Selecting for mutations affecting the development processes of sporulation and spore germination.

3. Measuring the extent of sporulation in a culture over a wide range of ascospore incidence ( $10^{-6}$  to 1).

4. Complementation tests of mutations affecting sporulation or spore germination. This includes testing for mating type in the absence of appropriate selective markers.

### Materials and Methods

*Yeast Strains.* Strains of *Saccharomyces cerevisiae* used, and their genotypes are summarised in Table 1. The homothallic diploid S41 was obtained from the collection of H. O. Halvorson. Strain ID-14D is a non-sporulating diploid derived from haploids with non-complementing recessive mutations to asporogeny.

*Media.* Vegetative cultures were routinely obtained by incubating in YEPD medium (10 g yeast extract, 20 g bactopectone and 20 g glucose per litre). Sporulation was carried out according to one of the procedures of Fast (1973). Cultures were grown to log phase in YPA medium (YEPD with glucose replaced by 10 g potassium acetate) and transferred, after centrifugation and washing with distilled water, to sporulation medium (SM; 20 g potassium acetate per litre, pH 7). SM cultures were aerated at 30° C for at least 48 h for sporulation to occur. Solid sporulation medium contained per litre: 10 g potassium acetate, 1 g yeast extract, 0.25 g glucose and 20 g agar. Sporulated cultures were prepared on plates by preincubating on YEPD agar medium and replica plating directly onto sporulation plates.

*Ether Treatment.* For liquid cultures, organisms were centrifuged and resuspended in the same volume of 0.2 M potassium acetate buffer (pH 5.5). An equal volume of diethyl ether was added to each sample and the mixture contained in a 1 oz vial was emulsified by rolling on a Voss roller for an appropriate time at ambient temperature. The two phases were then allowed to separate and the lower aqueous layer removed by pipette. For cultures sporulated on plates ether treatment was carried out by replica plating onto YEPD in glass petri dishes, exposing these to ether vapour for 2.5 h at 23° C or 90 min at 30° C in a sealed vessel followed by incubation to detect survivors. It should be noted that the boiling point of diethyl ether under standard conditions is 34.5° C. Precautions must be taken to ensure that vessels containing ether at 30° C are perfectly sealed and capable of withstanding some positive internal pressure.

### Results

*Ether Resistance of Yeast Ascospores and Vegetative Cells.* A difference in survival was noted on shaking vegetative and sporulated cultures of yeast suspended in potassium acetate buffer (KAc) with an equal volume of diethyl ether at ambient temperature. Sporulated cultures lost viability slowly, whereas vegetative cultures underwent a sudden and marked decrease in concentration of viable organisms. Kinetics of these changes are shown in Fig. 1. The concentration of cells in a stationary phase vegetative culture of strain S41 resuspended in acetate buffer was reduced nearly one million-fold within five minutes of adding ether. In the same interval a sporulated culture of S41 containing approximately 50% asci showed 50% survival. Prolonged exposure of sporulated cultures led to a further slight decrease in survivors, such that 45 minutes in the presence of ether led to an overall 10% survival of viable units based on asci present in the original culture.

While these results were indicative of a useful and important difference in sensitivity of vegetative cells and ascospores of *S. cerevisiae* to ether, it remained to be shown that survival was an inherent characteristic of the sporulated state and not a reflection of the conditions of growth used in preparing the sporulated culture. This latter possibility can be rejected from a survey of the survival

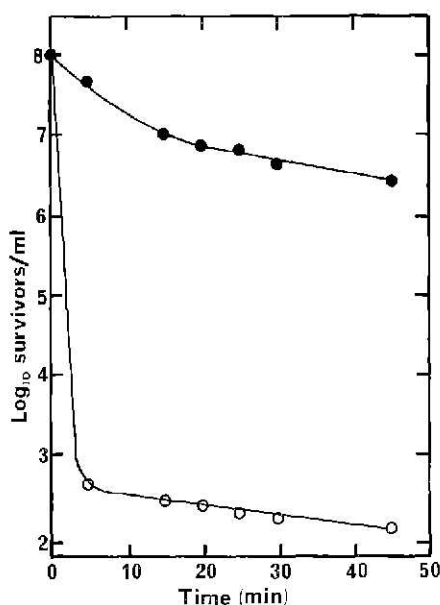


Fig. 1. Effect of exposure to ether of sporulated and vegetative cultures of strain S41. Cultures were resuspended in KAc buffer and an equal volume of ether added. The mixtures were shaken continuously and at appropriate intervals survivors in the aqueous phase were scored by plating on YEPD medium. Sporulated cultures were prepared by the YPA procedure of Fast (1973), vegetative cultures by growth to stationary phase in liquid YEPD medium.   
 ○—○ vegetative; ●—● sporulated culture

after ether treatment of various haploid and diploid strains grown under identical conditions. Data are summarised in Table 1 for the extent of survival of various strains cultured in rich medium (YEPD) in which normal diploids do not produce spores detectable by microscopic examination, and under conditions known to lead to sporulation of a high proportion of diploids heterozygous for the mating-type allele (Fast, 1973). The only cultures in which survivors were detected at a level greater than 1 in  $10^6$  were those of sporulating diploid strains grown under conditions known to induce sporulation. Haploids, an  $\alpha/\alpha$  diploid, and a normal diploid homozygous for a sporulation deficiency mutation grown under the same conditions do not sporulate nor do they survive ether treatment.

*Survivors of Ether Treatment: Random Spore Analysis.* In order to confirm that the high level of survival of sporulated cultures was due to resistance of spores to ether, and with a view to incorporating the technique into random spore analyses in which selection against non-sporulated diploids is essential, the genotypes of survivors of an ether-treated sporulated culture of strain IH-30D were determined. To minimise the problem of diploids forming by mating of spores within an ascus after ether treatment, the populations were treated with *Helix pomatia* lytic extract followed by mild sonication to prepare free spores (Johnston and Mortimer, 1959; Magni, 1963). This treatment gave similar results regardless of whether given before or after ether exposure, and no significant

Table 1. Loss of viability of various *Saccharomyces cerevisiae* strains after exposure of vegetative and sporulated cultures<sup>a</sup> to ether/KAc buffer mixtures

Strain nomenclature	Genotype	Growth conditions	Cell concentration		Fraction survivors
			Before ether (viable organisms/ml)	After ether	
ID-1	a <i>ade</i> <sub>1</sub>	vegetative	7.1 × 10 <sup>7</sup>	<10	<10 <sup>-7</sup>
		sporulation	1.0 × 10 <sup>7</sup>	<10	10 <sup>-6</sup>
ID-13	α <i>ade</i> <sub>5</sub>	vegetative	2.0 × 10 <sup>8</sup>	<10	<10 <sup>-7</sup>
		sporulation	6.3 × 10 <sup>8</sup>	<10	10 <sup>-6</sup>
IH-30D	a <i>ade</i> <sub>1</sub> + α + <i>ade</i> <sub>5</sub>	vegetative	6.6 × 10 <sup>7</sup>	<10	10 <sup>-7</sup>
		sporulation	2.1 × 10 <sup>8</sup>	4.5 × 10 <sup>4</sup>	2 × 10 <sup>-2</sup>
S41	a HO <sub>a</sub> HM arg <sub>4-1</sub> cyh <sub>1</sub> α HO <sub>a</sub> HM arg <sub>4-1</sub> cyh <sub>1</sub>	vegetative	7.8 × 10 <sup>6</sup>	<10	10 <sup>-7</sup>
		sporulation	2.8 × 10 <sup>8</sup>	6.1 × 10 <sup>5</sup>	2 × 10 <sup>-1</sup>
ID-14D	a <i>ade</i> <sub>1</sub>  · <i>ura</i> <sub>1</sub> <i>spo</i> <sub>78</sub> α + <i>ade</i> <sub>5</sub> <i>ura</i> <sub>1</sub> <i>spo</i> <sub>98</sub>	vegetative	5.2 × 10 <sup>6</sup>	<10	10 <sup>-6</sup>
		sporulation	2.7 × 10 <sup>7</sup>	2 × 10	<10 <sup>-6</sup>
ID-15D	α <i>ade</i> <sub>1</sub> + α + <i>ade</i> <sub>5</sub>	vegetative	6.4 × 10 <sup>7</sup>	<10	10 <sup>-6</sup>
		sporulation	2.3 × 10 <sup>8</sup>	<10	10 <sup>-6</sup>

<sup>a</sup> Vegetative cultures were prepared by growth on YEPD medium at 30° C; sporulation cultures according to the method of Fast (1973). Prior to ether treatment all cultures were resuspended in KAc buffer.

loss of viability was noted by either treatment in either order. Results of this random spore analysis of ether survivors from sporulated cultures of strain IH-30D are given in Table 2.

These data do not differ significantly ( $\chi^2$  test at 0.05 probability level) from that expected for segregation of three unlinked markers (mating, *ade*<sub>1</sub> and *ade*<sub>5</sub> loci). Moreover while in the untreated culture there were roughly 50% unsporulated diploids none of 200 survivors tested for mating response was found to be

Table 2. Genetic analysis of survivors of an ether-treated culture of strain IH-30D (a/α, *ade*<sub>1</sub>/+ , +/*ade*<sub>5</sub>) grown under sporulation conditions

Genotype	Frequency
a <i>ade</i> <sub>1</sub> +	11
α <i>ade</i> <sub>1</sub> +	8
a  · <i>ade</i> <sub>5</sub>	15
α + <i>ade</i> <sub>5</sub>	10
a + +	15
α + +	17
a <i>ade</i> <sub>1</sub> <i>ade</i> <sub>5</sub>	9
α <i>ade</i> <sub>1</sub> <i>ade</i> <sub>5</sub>	10
Other	0
	95

Expected frequency: 11.9  $\chi^2 = 6.55$  for 7 degrees of freedom.

diploid, indicating the applicability of ether selection for haploids in random spore analysis. Since this sample contained a relatively large number (25%) of prototrophic recombinants which are difficult to test for mating by conventional means, a modification to the ether selection technique was used to detect mating; this is discussed below.

For random spore analysis there is another useful feature of the pattern of ether resistance seen in Fig. 1. On prolonged exposure to ether, spores are slowly inactivated such that after 45 minutes approximately 10% of the original viable units survive. Under these conditions the chance that more than one spore per ascus survives to produce a colony is low, so that enzymic digestion of the ascus wall and subsequent sonication steps are no longer necessary.

When very concentrated ( $10^9$ /ml) vegetative cultures of strains IH-30D and S41 grown on YEED were treated with ether to determine the extent of kill of vegetative cells it was noted that a variable but low proportion (1 in  $10^6$ ) survived (Table 1). Survival in vegetative culture may have been due to one or more of the following possibilities: (i) sporulation of 1 in  $10^6$  cells during growth on YEED, (ii) the presence of ether resistant mutants, (iii) phenotypic variation within a population conferring temporary resistance on a low proportion of cells, (iv) physical protection of a low proportion of cells from lethal effects of ether due to high cell concentrations or cell clumping.

The first of these possibilities was not the main reason for survival under conditions given in Fig. 1, since only six of approximately 1000 survivors from strain IH-30D showed any pink colony trait expressed by organisms lacking a functional *ade<sub>1</sub>* gene product. This 0.6% of survivors, if spores, represents about 2.4 spores per  $10^8$  untreated cells (since only 25% spores give rise to pink colonies and survival was 1 in  $10^6$ ). This sets the upper limit for sporulation in YEED medium. Since all pink survivors were haploid and none were found in an untreated control culture the estimate is accurate unless ether induces haploidisation.

It seems likely that the majority of survivors from ether treatment of vegetative diploid cultures under the conditions used to obtain Fig. 1 is due to some form of protection of cells at the high cell concentration used to assess the extent of kill. This can be seen from Table 3 in which data are given for ether kill at different cell concentrations in the aqueous phase. When a suspension containing  $10^9$  viable organisms/ml is treated with an equal volume of ether there is about one survivor per  $10^6$ . At an initial concentration of  $10^7$ /ml no survivors were detected at a sensitivity limit of  $1/10^8$  cells, approaching the upper limit for spores in the parent culture.

Table 3. Cell concentration affecting survival during ether treatment of vegetative *S. cerevisiae* strain IH-30D

Concentration of organisms during ether treatment (no./ml)	Fraction survivors
$9.4 \times 10^8$	$1.0 \times 10^{-6}$
$9.4 \times 10^7$	$1.2 \times 10^{-7}$
$9.4 \times 10^6$	$< 1 \times 10^{-8}$

When attempting to assess the extent of sporulation of a culture at frequencies below  $10^{-5}$  per viable unit it is clear that the initial culture should contain less than  $10^7$  organisms/ml and some form of concentration may be necessary prior to plating for survivors. In addition, heterozygous *ade*<sub>1</sub> or *ade*<sub>2</sub> diploids are very useful in providing a rapid test of whether or not survivors are the products of meiosis and sporulation.

*Detection and Complementation Testing of Development Mutants.* A convenient method for detecting development mutants using a spore resistance character entails treating colonies on a plate with an agent lethal to vegetative cells, followed by replica plating onto a medium capable of supporting spore germination and outgrowth. In this way clones can easily be recognised for their inability to undergo one of the developmental processes of sporulation, spore germination or outgrowth.

For plate cultures the easiest method of treating with ether is exposure of open plates to ether vapour in a sealed container. Disposable plastic plates are not suitable, glass providing a less soluble solution to the problem.

An effective method of determining the length of treatment needed to distinguish sporulating from non-sporulating organisms is illustrated in Fig. 2. Haploid adenine-requiring tester strains of opposite mating type were cross-stamped onto a YEPD plate and left at 30° C overnight for mating to occur. This plate was replicated onto a number of sporulation (KAc) plates and a minimal plate as control. After sufficient incubation for diploids to form spores (5 to 6 days at 30° C) the KAc plate contents were replicated onto YEPD plates. These were exposed to ether vapour at 30° C for various times and subsequently incubated to allow survivors to grow. Under these conditions the optimal period of exposure to ether vapour was 90 minutes. Shorter doses did not kill all haploids while longer led to extensive killing of spores.

Figure 2 illustrates another aspect of ether treatment of plate cultures useful in sporulation or germination studies. The above routine is that which would be followed in assigning different recessive developmental mutations into complementation groups. If suitably complementary auxotrophic markers are included then each cross can also be tested to eliminate false negatives due to failure in mating. An obvious extension of this complementation test is the direct determination of mating-type of a haploid when other selective techniques fail, as used in the testing of prototrophic haploids while collecting data for Table 2.

*Ascospore Resistance to Other Solvents.* Four other organic solvents were tested on liquid cultures to determine whether any were similar to ether in selectively killing vegetative cells. None of these proved suitable. Octanol and toluene killed neither vegetative cells nor spores within a reasonable time, while chloroform was equally effective against both. Solutions of ethanol in water in the concentration range of 20% to 50% (v/v) were tested since *Schizosaccharomyces pombe* spores are differentially resistant to 30% ethanol (Leupold, 1957) and *S. cerevisiae* spores are reported to survive 33% ethanol exposure for 30 minutes (Zakharov and Ingo-Vechtomov, 1964). At a concentration of 30% ethanol there was some differential survival of ascospores compared with vegetative organisms but to a lesser degree than with ether treatment since spores were almost as sensitive as vegetative cells and a high proportion of survivors were petite. Even

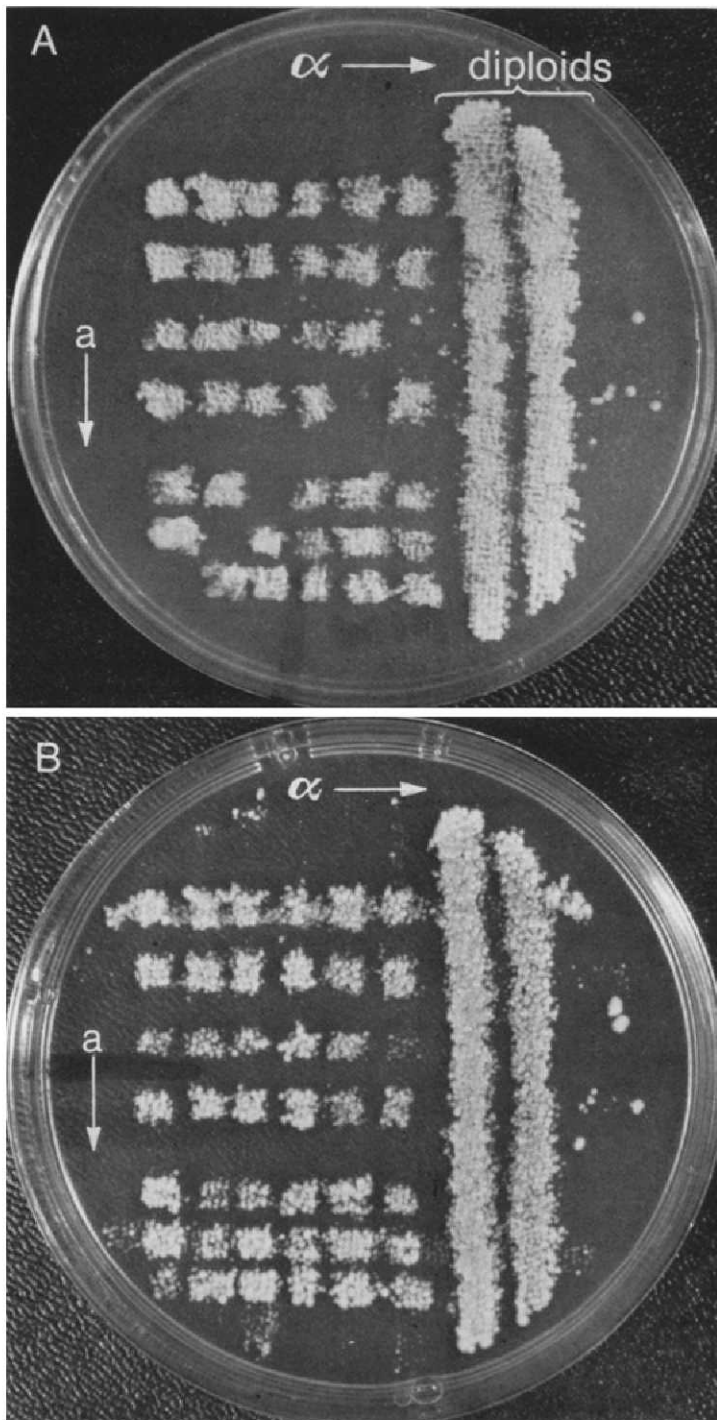


Fig. 2A and B. Plate test for sporulating diploids. Haploid *ade* tester strains (a *ade*<sub>1</sub> to *ade*<sub>3</sub> and *ade*<sub>5</sub> to *ade*<sub>8</sub>;  $\alpha$  *ade*<sub>1</sub> to *ade*<sub>6</sub>) and two sporulating diploids were crossed on a YEPD plate and left to mate. This master plate was replicated to minimal medium (Fig. 2A) and to sporulation medium. After tetrads had formed the sporulation plate was exposed to ether vapour for 90 min at 30° C and replicated onto YEPD (Fig. 2B)



were ethanol solutions as effective as ether, controlled exposure of sporulated plate cultures to ethanol is not possible.

### Discussion

A variety of procedures has been proposed for selecting ascospores of *S. cerevisiae* from populations containing vegetative diploids. These include either straightforward physical separation of spores by partition between immiscible liquids (Emeis and Gutz, 1958) or electrophoresis (Resnick, Tippetts and Mortimer, 1967), or selectively inactivating vegetative diploids by heat (Fowell, 1969) or ethanol (Zakharov and Inge-Vechtomov, 1964). Physical methods are appropriate in isolating large spore batches for physiological studies, but are not suitable for rapid genetic manipulation. Heat treatment has been used in hybridisation of various yeast strains, but suffers from the disadvantage that spores are only slightly more resistant than vegetative cells and some of the latter survive. Moreover different strains have different sensitivities necessitating control experiments for each diploid (Fowell, 1969). In routine random spore analysis none of the above is used to inactivate diploids since the technique of introducing a recessive resistance mutation in heterozygous condition usually suffices.

Ether treatment has advantages which make it very suitable for genetic procedures requiring diploid inactivation. First, it is rapid and convenient and can be applied to any sporulating diploid regardless of its genotype. For example, in isolating temperature-sensitive sporulation mutants, haploid spores of homothallic strains are mutagenised so that diploids resulting on outgrowth can express recessive mutations. In this case the introduction of a *can<sup>r</sup>* marker in heterozygous condition is not a simple task.

For *S. cerevisiae* genetics the most useful aspect of ether sensitivity is that plate cultures can be exposed to ether vapour, thereby selecting on plates for clones that are capable of sporulating. By standard replicating procedures many classes of developmental mutants can be quickly isolated; this includes asporogenous or spore germination and outgrowth mutants. Moreover sporulating revertants or recombinants from asporogenous strains can be scored and recessive developmental mutants easily assigned to complementation groups. Appropriately, ether resistance is a late event in yeast meiosis and sporulation, appearing considerably later than the first appearance of recognisable tetrads (Dawes and Hardie, manuscript in preparation). Hence if yeast asporogenous mutants exhibit the unidirectional pleiotropy seen in bacterial sporulation studies (Waites, Kay, Dawes, Wood, Warren and Mandelstam, 1970) almost the whole of the yeast sporulation sequence is open to more rapid genetic analysis.

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