SHORT COMMUNICATIONS

A Cryobiological Method for the Enrichment of Fungal Mutants

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INTRODUCTION

In a previous abstract we reported the successful application of cryobiological techniques to the recovery of auxotrophic mutants of *Neurospora crassa* from a mixture of wild-type and mutant conidia (Leef & Gaertner, 1975). This communication presents in greater detail the methods used and the pertinent physiological observations which led to the development of the technique. We also show that by using cryobiological techniques it is possible to enrich for auxotrophs by means of the effects of either dehydration or the formation of intracellular ice.

METHODS

Neurospora crassa wild type (74-A) and a tryptophan auxotroph (48-R) were grown at 25 °C on 2% (w/v) Difco Bacto agar slants containing Vogel's minimal medium (VMM; Vogel, 1956) with and without 40 µg L-tryptophan ml⁻¹. Conidia were harvested from agar slants as described by Leef & Mazur (1978), resuspended in 50 ml VMM or VMM without sucrose in 125 ml Erlenmeyer flasks and shaken (100 rev. min⁻¹) at 37 °C in a gyratory water bath for up to 5 h. After various periods of incubation, 10 ml portions of the conidial suspension were centrifuged for 15 min at 50 g, washed twice in distilled water and resuspended in sterile distilled water. Samples (0.1 ml) of these suspensions were cooled at 500 °C min⁻¹ to –196 °C, held at that temperature for at least 15 min, and then warmed at either 1000 or 25 °C min⁻¹ as previously reported (Leef & Mazur, 1975, 1978). Viability was determined as previously reported (Leef & Mazur, 1978), except that tryptophan was added as required by the mutant strain. In a given enrichment experiment, equal numbers (as determined by haemocytometer counts) of wild-type and mutant spores were incubated in VMM and washed before being frozen as described above. The viability of the wild-type organisms was so low after freezing and thawing that it was necessary to inoculate unsupplemented plates with 10⁴ to 10⁶ conidia to determine their survival. Since the wild-type organism grew on both supplemented and unsupplemented agar but the mutant grew only on the supplemented plates, the viability of each strain was easily calculated.

To study the effects of dehydration alone, without freezing, conidia were harvested and incubated at 30 °C in VMM or distilled water for 5 h as described above. The suspensions were then concentrated to a dense slurry by centrifugation at 200 g for 15 min. The supernatant was removed by aspiration, and 0.1 ml portions of the dense cell suspension were transferred to plastic capsules (10 × 15 mm) with a Hamilton microlitre syringe. In a given experiment, one capsule was retained as an undried control. The remaining capsules were placed *in vacuo* at room temperature for 2 to 5 h, then diluted to the appropriate concentration and plated for viability as described above.

RESULTS

Approximately 90% of wild-type conidia germinated at 30 °C within 6 h in complete VMM, whereas conidia in VMM without sucrose, in 0.1 M-potassium phosphate (pH 7.0) or in distilled water did not germinate even after 48 h incubation. However, ungerminated conidia remained viable, since within 24 h after the addition of sucrose to the deficient

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Table 1. *Percentage survival of frozen and thawed wild-type conidia after incubation in Vogel's medium (complete or without sucrose)*

All preparations were washed by centrifugation and resuspension with water before freezing. Samples were cooled at $500\text{ }^{\circ}\text{C min}^{-1}$ and warmed at either $25\text{ }^{\circ}\text{C min}^{-1}$ or $1000\text{ }^{\circ}\text{C min}^{-1}$. Each viability estimate is the mean of at least three determinations; the range in standard deviations was ± 0.5 to ± 20 .

Incubation time (h)	Vogel's medium, complete		Vogel's medium without sucrose	
	$25\text{ }^{\circ}\text{C min}^{-1}$	$1000\text{ }^{\circ}\text{C min}^{-1}$	$25\text{ }^{\circ}\text{C min}^{-1}$	$1000\text{ }^{\circ}\text{C min}^{-1}$
0	40	68	40	68
1	3	53	35	63
2	3	29	41	86
3	0.3	7	47	111
5	0	0	80	122

medium, or after the addition of concentrated VMM to conidia in either phosphate buffer or water, essentially all the conidia germinated.

Table 1 shows the effects of freezing on the survival of wild-type conidia incubated for 0, 1, 2, 3 or 5 h in either VMM or VMM without sucrose before they were frozen. The results show that (i) conidia warmed at $1000\text{ }^{\circ}\text{C min}^{-1}$, regardless of the incubation time or the medium in which they were suspended during incubation, survived to a greater extent than did those warmed at $25\text{ }^{\circ}\text{C min}^{-1}$, and (ii) regardless of the warming rate, conidia incubated in VMM prior to freezing became progressively more sensitive to freezing as the incubation time was increased, while those incubated in VMM without sucrose prior to freezing became more resistant.

After incubation for 5 h in VMM prior to freezing, the survival of wild-type conidia was low (0.07%), whereas the survival of mutant conidia was 70.1%. A mixture of equal numbers of wild-type and mutant conidia gave 36.2% survival. This result is close to the value of 35.1% which would be expected if only the auxotrophic strain 48-R survived. These data indicate that the conidial population was enriched for mutants by a factor of about 1000.

The effect of drying (without freezing) on the survival of conidia was also studied. Conidia were dried *in vacuo* at about $25\text{ }^{\circ}\text{C}$ as described in Methods. After 2 h, 75 to 80% of the conidia incubated in water germinated, whereas only 20% of those incubated in VMM did so. Even after they were dried for 5 h, nearly 60% of the conidia incubated in water germinated, but less than 0.1% of those incubated in VMM were viable.

DISCUSSION

The fact that different types of cells have different requirements for cooling and warming rates to achieve optimum viability following freezing has been amply demonstrated (Mazur, 1965*b*, 1970; Mazur *et al.*, 1972; Meryman, 1966*a, b*; Mazur & Schmidt, 1968; Leibo *et al.*, 1970). It has also been shown that sensitivity to freezing of a given cell type varies with the stage of the growth cycle at the time of freezing (Meryman, 1966*a*; Mazur, 1965*a, b*, 1966; Koch *et al.*, 1970; McGann *et al.*, 1972). Leef (1974) and Leef & Mazur (1975, 1978) demonstrated that the response of fungal cells to given cooling and warming rates depended on their metabolic activity. We have used these cryobiological findings to develop a technique for selective mutant enrichment. Other investigators have used freezing and/or osmotic shock to enrich for and isolate fungal mutants (Leibo & Mazur, 1966; Ferenczy *et al.*, 1975; Leef & Gaertner, 1975; Peters & Sypherd, 1978).

Peters & Sypherd (1978) reported the use of freezing as a method for isolating auxotrophs in *Mucor racemosus*; however, it is likely that they selected mutants by the effects of dehydration rather than by the formation of intracellular ice. They cooled their cell suspensions slowly to $-20\text{ }^{\circ}\text{C}$ by placing them in a conventional freezer at $-20\text{ }^{\circ}\text{C}$; this slow cooling

rate (about $0.6\text{ }^{\circ}\text{C min}^{-1}$ for their 2 ml volumes) would result in cellular dehydration, but no intracellular freezing would occur. The technique we describe involves freezing the cells by cooling them very rapidly ($500\text{ }^{\circ}\text{C min}^{-1}$). Although both techniques involve freezing, the 'freezing damage' to each system results from different types of injury (Mazur, 1965*b*, 1966, 1970; Mazur *et al.*, 1972; Diller & Gravalho, 1973). At cooling rates less than optimum, cells are killed by 'solute effects' (Mazur *et al.*, 1972), including dehydration and concomitant changes in pH and changes in ionic strength, all of which result from the removal of pure water into ice. Conversely, at rates higher than optimum, cells are killed by 'intracellular ice' effects (Mazur *et al.*, 1972). The formation of intracellular ice is generally lethal, due, in part, to migratory recrystallization which takes place at storage temperatures below about $-60\text{ }^{\circ}\text{C}$ or during the thawing process (Bank, 1973).

The warming rate (thawing) is also an important factor in preserving or selectively destroying cells by freezing. In general, the warming rate is of less importance with cells which are frozen slowly at cooling rates much less than their optimum than with rapidly frozen cells in which intracellular ice may be present. We used a warming rate of $25\text{ }^{\circ}\text{C min}^{-1}$ because the resultant survival is much lower when rapidly frozen cells are warmed slowly than when identically cooled cells are warmed rapidly (Table 1). The reason for this is the destructive effect of migratory recrystallization discussed in detail by Leef & Mazur (1978) and Bank (1973). Therefore, it is important to use more than one cooling and warming rate when attempting cryobiological enrichment techniques. The rates we have selected were obtained from a study in which the effect of several cooling and warming rates on the viability of *N. crassa* conidia had been determined (Leef & Mazur, 1975, 1978).

Cryobiological techniques may thus be used to select for auxotrophic mutants by maximizing either solute effects or intracellular ice effects. Perhaps a higher enrichment could be achieved by subjecting conidia first to solute effects (dehydration with or without slow freezing), then to rapid freezing and slow thawing, or the reverse. Such a procedure may render a mixed population of mutant and wild-type cells essentially free of wild-type organisms.

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