

Relationship between Sporulation Medium and Germination Ability of *Mucor racemosus* Sporangiospores

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Asexual sporangiospores of *Mucor racemosus* produced on a minimal sporulation medium (M-spores) germinated only if glucose, mannose or a complex substrate such as peptone, yeast extract or Casamino acids was present. Once germinated, growth was supported by a wide range of substrates including amino acids, carbohydrates or organic acids. Sporangiospores produced on a nutritionally complex sporulation medium (C-spores) germinated on a wide range of carbon sources. C-spore phenotype was pleiotropic in that sporangiospores capable of germinating on cellobiose could always germinate on glutamate or xylose; but C-spores capable of germinating on xylose or glutamate did not always germinate on cellobiose. There was a hierarchy of substrates capable of initiating germination with glucose = mannose > xylose > glutamate > cellobiose. C-spores also differed from M-spores by initiating germination in the presence of the non-metabolizable glucose analogue 3-O-methylglucose. These results suggest that at least two sporangiospore phenotypes are produced depending upon the concentration and type of ingredients present in the sporulation medium.

INTRODUCTION

The dimorphic phycomycete *Mucor racemosus* has been employed as a model system to study the control mechanisms involved during the yeast to hyphal differentiation process. The success of these studies resides in the fact that a single, controllable change in culture conditions leads to a predictable and synchronous morphological transition (Bartnicki-Garcia & Nickerson, 1962; Bartnicki-Garcia, 1968; Schulz *et al.*, 1974). Anaerobically, under CO₂ or with a high flow rate of 100% nitrogen (Mooney & Sypherd, 1976), vegetative growth occurs as budding yeast: low flow rates of nitrogen results in mycelial growth. A fermentable hexose is required for anaerobic growth of both forms (Larsen & Sypherd, 1974).

Aerobically, growth occurs as hyphae and is supported by a wide range of substrates including carbohydrates, amino acids and organic acids (Bartnicki-Garcia & Nickerson, 1962). On solid medium, mycelia form aerial hyphae which differentiate into sporangiospores. By 3–5 d the sporangiospores appear dark and contain mature, asexual sporangiospores. These sporangiospores remain dormant when harvested and held in distilled water; however, when placed into a suitable nutrient medium they undergo a morphological transition, initiate germination and develop from an elliptical sporangiospore into a spherical vegetative cell prior to either germ tube or bud emergence. This series of morphological events is amenable to biochemical analysis.

The components necessary for the initiation of germination have been reported to be present in many fungal spores. However, the mechanism by which the dormant state is maintained is unknown.

Most biochemical studies of *Mucor* described previously have been carried out in a nutritionally complex medium. We have initiated studies employing the defined minimal medium of Peters & Sypherd (1978) to determine the chemical requirements for sporangiospore germination. These studies led to the observation that the ability to germinate and the rate of germination of *M. racemosus* sporangiospores on a given carbon source is directly related to the nutritional complexity of the sporulation medium.

METHODS

Organism and culture conditions. *Mucor racemosus* (*M. lusitanicus*) ATCC 1216B was used in all experiments. Asexual sporangiospores were produced primarily on two types of media. Sporangiospores designated C-spores were produced on a complex sporulation medium (CSM) consisting of 1% (w/v) peptone (Difco lot no. 54219 except where noted otherwise), 0.3% (w/v) yeast extract (Difco), 2% (w/v) agar (Difco) and 2% (w/v) D-glucose. Sporangiospores designated M-spores were produced on a minimal sporulation medium (MSM) consisting of 0.05% (w/v) yeast nitrogen base without amino acids (Difco), 10 mM-(NH₄)₂SO₄, 10 mM-L-glutamate, 2% (w/v) agar (Difco) and 2% (w/v) D-glucose. The minimal germination medium (MGM) was a modified MSM in which L-glutamate, agar and glucose were omitted. The various carbon sources were reagent grade (Sigma) and usually supplied in MGM at 100 mM, a concentration giving the optimum germination rate, except the disaccharides which were supplied at 50 mM. The pH of all media was adjusted to 5.0 with H₂SO₄.

The sporulation media were spread-inoculated with 0.05 ml of a stationary phase culture of yeast (growing in 0.3% yeast extract, 1% peptone, 1% glucose, under 100% CO₂) and incubated at 22 °C. Sporangiospores were harvested 5 d after inoculation, washed twice in distilled water, and counted with either a haemocytometer or a Coulter counter (sporangiospores from 3, 4, 5, 6 and 7 d plates all had similar germination ability). Portions (10 ml) of MGM contained in 50 ml flasks were inoculated to a final concentration of 1×10^6 sporangiospores ml⁻¹ (inocula of 10⁴, 10⁵, 5×10^5 , 10⁶ and 2×10^6 sporangiospores ml⁻¹ germinated at essentially identical rates, whereas inocula of 10⁷, 2×10^7 and 10⁸ sporangiospores ml⁻¹ germinated at decreasing rates) and incubated at 25 °C in a gyratory water bath incubator shaking at 150 rev. min⁻¹. All sugars were autoclaved separately from the sporulation medium and added aseptically. Freshly prepared solutions of sugars were routinely added to the minimal germination test medium (MGM) unsterile to eliminate degradation. Identical results were obtained with carbohydrates sterilized by filtration.

Chemicals. The non-metabolizable glucose analogue 3-O-methyl-D-glucose was routinely used at 20 mM. Adenosine 3':5' cyclic monophosphate (cyclic AMP) was used at 10 mM. Both were reagent grade and supplied by Sigma.

Analysis of germination. *Mucor racemosus* sporangiospore germination involves a swelling phase prior to germ tube emergence. Coulter counter analysis has been reported to be the method of choice for the analysis of spore swelling (Barnes *et al.*, 1966; Barnes & Parker, 1967; Mandels & Darby, 1953; Mandels, 1965). In these studies, the swelling phase was monitored by measuring sporangiospore volume at various time intervals with a Coulter counter (model ZB1) coupled to a 100-channel pulse-height analyser (Channelyzer) and an XY plotter. The aperture tube employed throughout this study was 100 µm. Routinely, a sample of the germination mixture was diluted in Isoton (Coulter Electronics, Hialeah, Florida, U.S.A.), a counting fluid which both dispersed the sporangiospores and stopped the swelling process. (*Mucor racemosus* cells placed in Isoton stop developing immediately and remain at the volume attained at the time they were placed in the Isoton solution for at least 24 h.) For each time point, 8000–15 000 sporangiospores were counted and sized. The average volume of synchronously germinating sporangiospore populations was calculated from the XY plots. Monosized polystyrene beads (72.25 and 20.10 µm³; obtained from Coulter Electronics) were used as described by the manufacturer to calibrate the Coulter counter. An external check on the calibration was made by microscopic measurements of the beads and the sporangiospores. The volumes determined by both methods were essentially identical. The proportion of the sporangiospore population swelling from 20 to >45 µm³ was recorded from the integrator and is reported as the percentage of the sporangiospore population initiating germination. This allowed monitoring of asynchronously germinating sporangiospore populations.

RESULTS

Size distribution of M- and C-spores during germination

Mucor racemosus sporangiospores produced on either complex (C-spores) or minimal (M-spores) sporulation medium were found by Coulter sizing analysis to be similar in size ranging from 15 to 60 µm³, with the major portion averaging 20 µm³ when harvested in

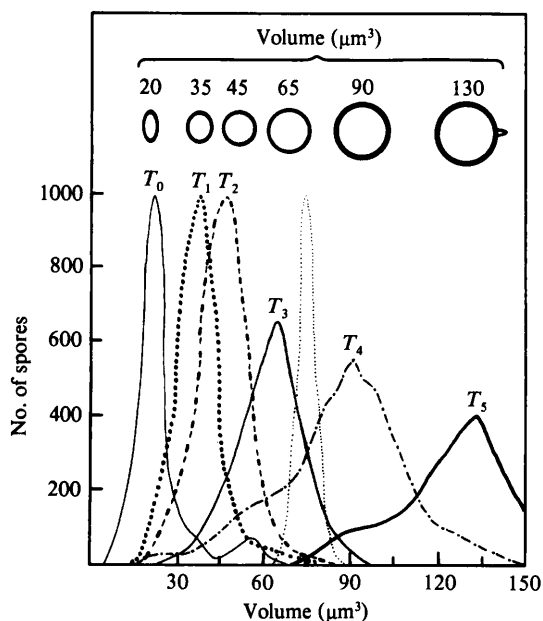


Fig. 1

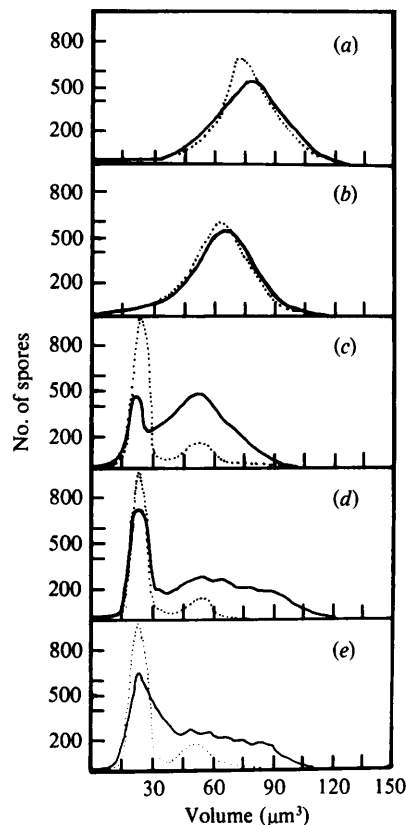


Fig. 2

Fig. 1. Germination of M-spores of *M. racemosus* in MGM/glucose (100 mM) was monitored at 1 h intervals (T_0 , T_1 , T_2 etc.) by measuring the size distribution of the swelling sporangiospores by Coulter sizing analysis. Monosized polystyrene beads ($72.25 \mu\text{m}^3$) were used as a sizing standard (unlabelled peak).

Fig. 2. Typical Coulter plots depicting the size distribution for M-spores (····) and C-spores (—) after 4 h incubation in MGM containing 100 mM of one of the following single substrates: (a) glucose, (b) mannose, (c) xylose, (d) maltose or (e) glutamate.

distilled water. This range is represented by a typical Coulter plot of the zero-time (T_0) sample shown in Fig. 1. The sporangiospores remained at this volume for several days in the absence of a suitable carbon source. The addition of 100 mM-glucose to the MGM resulted in a rapid increase in volume with the elliptical sporangiospores taking on a spherical appearance after 1 h accompanied by an increase in volume from 20 to about $35 \mu\text{m}^3$. The swelling rate was determined by monitoring the mode volume of the sporangiospore population with time. Figure 1 represents a series of Coulter tracings made at 1 h intervals from a typical experiment where M-spores were placed into MGM containing 100 mM-glucose as the sole carbon source. Only after the sporangiospores had swollen to $130 \mu\text{m}^3$ (Fig. 1) were germ tubes observed microscopically. By plotting the log of the average sporangiospore volume (as determined by the mode of each curve) against time, a straight line relationship was found (Fig. 3, $r = > 0.99$ for any given experiment). The results from several experiments indicated that the swelling rate for C-spores (slope 0.1502) was slightly greater than that for M-spores (slope 0.1447) germinating on glucose at the same concentration. This swelling phase (from 20 to $130 \mu\text{m}^3$) prior to germ tube emergence was considered to be the germination phase.

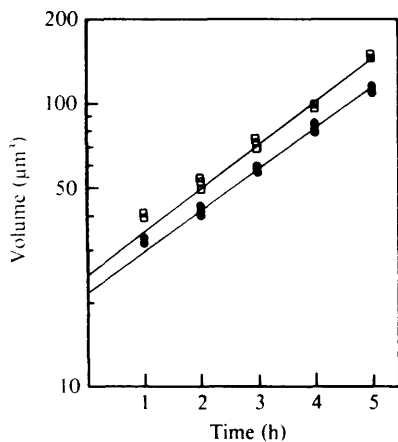


Fig. 3

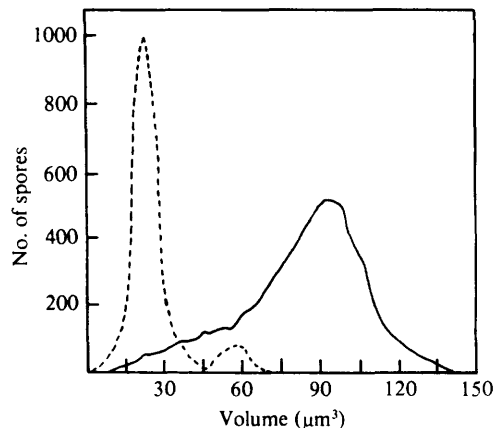


Fig. 4

Fig. 3. Typical plot of log sporangiospore volume against time for C-spores (□) and M-spores (●) germinating in MGM/glucose (100 mM). Each point represents the average of two replicates per experiment. T_{130} (i.e. the time for the average population to swell to $130 \mu\text{m}^3$) was calculated from the lines to be 5.3 h for M-spores and 4.8 h for C-spores.

Fig. 4. Typical Coulter plots of the size distribution for M-spores (---) and C-spores (—) after 24 h incubation in MGM containing 3-O-methylglucose (20 mM) in the absence of an exogenous energy source. The M-spore population microscopically appeared elliptical, ungerminated and identical to the MGM control (no substrate). The C-spore population upon developing to about $90 \mu\text{m}^3$ stopped short of germ tube emergence.

Hence, the time for the average sporangiospore population to swell to $130 \mu\text{m}^3$, denoted T_{130} is analogous to the time it takes the average sporangiospore in a given population to germinate. Thus, the germination rate of sporangiospores produced on various sporulation media can be easily compared. The T_{130} for C-spores (4.8 h) was consistently lower than that for M-spores (5.3 h).

Analysis of germination synchrony

Mucor racemosus C-spore germination was asynchronous in MGM containing a non-hexose such as glutamate as the sole carbon source (Fig. 2e). Hence, it was not possible to analyse germination by monitoring the movement of a single peak representing the entire sporangiospore population. Instead, germination was analysed by monitoring the proportion of the sporangiospores which initiated germination and swelling to some arbitrary size, i.e. $45 \mu\text{m}^3$. Sporangiospores which initiated germination in the presence of a suitable carbon source and swelled to $45 \mu\text{m}^3$ continued to germinate if left in the same medium. The Coulter integrator allowed determination of the number of sporangiospores present within a given size range. Since 90% of the sporangiospore population ranged from 0 to $45 \mu\text{m}^3$ in the absence of a suitable germinant, that proportion of the sporangiospore population which swelled to $>45 \mu\text{m}^3$ was operationally defined as having initiated germination. It should be stressed that this is only an operational definition. The proportion of the sporangiospore population larger than $45 \mu\text{m}^3$ at time zero varied from about 5 to 15% and has been subtracted (corrected) from all readings in order to make comparisons between sporangiospore preparations.

Effect of sporulation media on sporangiospore germination

Sporangiospores produced on complex sporulation medium (C-spores) were compared with sporangiospores produced on a minimal sporulation medium (M-spores) with respect to their ability to initiate germination on a variety of carbon sources. Both C- and M-spores

Table 1. Germination ability of *M*- and *C*-spores on various carbon sources

Sole carbon source in MGM	Percentage of spores >45 μm^3 by 8 h	
	M-spores*	C-spores†
Glucose (100 mM)	>95	>95
Mannose (100 mM)	>95	>95
Xylose (100 mM)	<5	70 (63–76)
Arabinose (100 mM)	<5	18 (18–26)
Cellobiose (50 mM)	<5	24 (15–45)
Maltose (50 mM)	<5	35 (21–45)
Glutamate (100 mM)	<5	54 (47–78)
L-Alanine (100 mM)	<5	18 (18–28)
Difco peptone (1%)	>95	>95
Difco yeast extract (1%)	>95	>95
Casamino acids, vitamin-free (1%)	>95	>95
No carbon source – control	<5	<5

* Typical results.

† Data obtained for a single batch: values in parentheses show the range obtained for several separate batches.

Table 2. Differential effects of 3-*O*-methyl-D-glucose and cyclic AMP on sporangiospore germination

Addition(s) to MGM*	Percentage of spores >45 μm^3 by 8 h	
	M-spores	C-spores
3- <i>O</i> -Methylglucose (no carbon source)	<5	69 (4.0)†
3- <i>O</i> -Methylglucose + glutamate	>95 (4.3)†	>95 (3.0)
Glucose	>95 (2.7)	>95 (2.4)
Glucose + glutamate	>95 (2.4)	>95 (2.1)
Cyclic AMP (no carbon source)	<5	<5
Cyclic AMP + glutamate	<5	76 (2.7)
Glutamate	<5	74 (4.0)

* Concentrations: 3-*O*-methylglucose, 10 mM; glutamate, 100 mM; glucose, 10 mM; cyclic AMP, 10 mM.

† T_{50} , i.e. calculated (linear regression analysis) time in hours for 50% of a sporangiospore population to swell from 20 to >45 μm^3 .

germinated synchronously on glucose or mannose (Fig. 2*a, b*). A variable proportion of C-spores germinated asynchronously on xylose, maltose or glutamate (Fig. 2*c, d, e*); in contrast, none of the M-spores germinated on the three non-hexoses. The percentage of each C- and M-spore population initiating germination and swelling to greater than 45 μm^3 by 8 h on a variety of substrates is given in Table 1. After 24 h incubation, little or no growth was observed in culture flasks containing M-spores and a non-hexose; and upon microscopic examination only elliptical, ungerminated sporangiospores could be seen with the exception of an occasional mycelial micro-colony. The contents of the flasks containing MGM/cellobiose and MGM/glutamate were filtered through Whatman no. 1 filter paper to remove germinated cells and then recounted. Essentially all the M-spores could be accounted for, and averaged 20 μm^3 . The variation from experiment to experiment in the ability of C-spores to germinate on non-hexoses is indicated in Table 1. This variability was between C-spore preparations and not experimental error within an experiment (usually < 5% between replicates).

Differential effects of 3-O-methylglucose and cyclic AMP on sporangiospore germination

It was found that the non-metabolizable glucose analogue 3-*O*-methylglucose would initiate swelling of C-spores but not M-spores in a defined medium without an exogenous carbon

Table 3. *Effect of complex components of the sporulation medium on sporangiospore germination ability*

Basic sporulation medium	Addition(s) to sporulation medium	Percentage of spores >45 μm^3 by 6 h			
		MGM/ glutamate (100 mM)	MGM/ cellobiose (50 mM)	MGM/ 3-O-methylglucose (20 mM)	MGM/ glucose (100 mM)
MSM	None	<5	<5	<5	>95
MSM	Peptone* (1%)	<5	<5	36	>95
MSM	Yeast extract (0.3%)	19	14	57	>95
MSM	Peptone* (1%)	48	41	62	>95
MSM	Yeast extract (0.3%)				
CSM	None	57	41	62	>95
MSM	Casamino acids, vitamin-free (1%)	20	16	49	>95

* Difco peptone lot 54219.

source (Fig. 4). However, the combination of 3-O-methylglucose and one of several carbon sources led to the germination of more than 95% of both C- and M-spores (Table 2).

It was reported (Larsen & Sypherd, 1974) that a significant rise in intracellular cyclic AMP occurs during spore germination. An explanation of why M-spores do not germinate on non-hexoses could lie in the inability of the sporangiospore to increase intracellular cyclic AMP levels in the absence of glucose and thus its inability to initiate germination. To examine this possibility we added cyclic AMP (10 mM) to M-spores and, as a control, to C-spores in MGM/glutamate. Cyclic AMP did not trigger the germination of M-spores but did stimulate the rate of C-spore swelling (Table 2).

Carbohydrate effect on sporangiospore phenotype

Glucose is the primary carbon source in MSM. Sporangiospores were produced on either complex or minimal sporulation media in which glucose was replaced by other substrates. When 2% glucose was replaced by 2% (w/v) glutamate or alanine, poor sporulation resulted in both complex and minimal media. These sporangiospores were not tested for their germination ability due to the low number obtained. When glucose was replaced by 2% (w/v) galactose, cellobiose, maltose, xylose or ribose, a high level of sporulation resulted, similar to that obtained with glucose. Less than 5% of the sporangiospores produced on MSM germinated in MGM/glutamate or MGM/cellobiose regardless of whether glucose, galactose, cellobiose, maltose, xylose or ribose was used as the primary carbon source in the MSM. These same sporangiospore preparations all germinated at similar rates ($T_{G50} = 2.3$ h) in MGM/glucose with more than 95% germinating by 6 h. Replacement of glucose in CSM by another hexose (i.e. galactose) had no effect on the C-spore phenotype.

Effect of nutritional components of the sporulation medium on sporangiospore phenotype

To examine the effects of the components of CSM on sporangiospore germination ability, the following mixing experiments were performed. Each component of CSM was added singly or in combination to MSM and the subsequent sporangiospore phenotype was tested (Table 3). Only the addition of both yeast extract and peptone to MSM resulted in a sporangiospore population equivalent to those produced on CSM.

Effect of yeast extract/peptone (YP) concentration on sporangiospore phenotype

Sporangiospores were produced on MSM containing the normal (1 \times) YP concentration (0.3% yeast extract, 1% peptone) found in CSM, or 0.01, 0.1, 2 or 5 \times that concentration.

Table 4. Effect of yeast extract/peptone (YP) concentration on sporangiospore germination

YP concn (relative to CSM) in MSM	Percentage of spores $>45 \mu\text{m}^3$ by 8 h			
	MGM/ glutamate	MGM/ cellobiose	MGM/ 3-O-methylglucose	MGM/ glucose
None (MSM)	3 (s.e. 5)*	4 (s.e. 5)	2 (s.e. 5)	>95 (2.2)†
0.01 ×	5 (s.e. 6)	8 (s.e. 6)	17 (s.e. 16)	>95 (1.9)
0.1 ×	6 (s.e. 1)	7 (s.e. 7)	17 (s.e. 8)	>95 (1.8)
1 × (normal)	74 (s.e. 13)	21 (s.e. 2)	65 (s.e. 1)	>95 (1.7)
2 ×	64 (s.e. 4)	44 (s.e. 11)	46 (s.e. 9)	>95 (2.4)
5 ×	41 (s.e. 6)	49 (s.e. 10)	40 (s.e. 10)	>95 (3.2)

* Standard error of the mean (s.e.); average of two experiments, three replicates per experiment.

† T_{G50} , i.e. calculated (linear regression analysis) time in hours for 50% of a sporangiospore population to swell from 20 to $>45 \mu\text{m}^3$.

As shown in Table 4, sporangiospores produced on MSM or on MSM supplemented with 0.01 × YP or 0.1 × YP were unable to initiate germination on MGM containing either glutamate, cellobiose or 3-O-methylglucose. The concentration of the nutritionally complex components was apparently insufficient for the production of the C-spore phenotype. At YP concentrations above normal (1 ×), the fraction of sporangiospores initiating germination in MGM/glutamate and MGM/3-O-methylglucose correspondingly decreased, while increased germination occurred in MGM/cellobiose. Surprisingly, the rate of sporangiospore germination in MGM/glucose also reflected the concentration of YP present in MSM demonstrated by the correlation between T_{G50} and YP concentration shown in Table 4: germination rates with different YP concentrations decreased in the order 1 × $>$ 0.1 × $>$ 0.01 × \gg MSM $>$ 2 × \gg 5 ×.

Effect of peptone type and lot in CSM on sporangiospore germination

The portion of a population of C-spores germinating on non-hexose substrates was consistent within experiments but varied considerably from experiment to experiment. The type and lot of peptone used in the CSM influenced the proportion of the sporangiospore population capable of initiating germination in MGM/glutamate. For example, with Difco peptone lot no. 54219, 59% of C-spores had germinated by 4 h in MGM/glutamate medium, whereas with lot no. 660308 only 28% of the spores had germinated by 4 h. Other peptones tested were Difco proteose peptone lot no. 512347 (34% of the spores germinated by 4 h), BBL polypeptone lot no. 8012 (33%) and several other Difco peptones which gave intermediate results.

DISCUSSION

The synchrony, reproducibility and rate of germination of a sporangiospore population must be known for the specific conditions under which they are to be studied. In previous studies (Bartnicki-Garcia & Nickerson, 1962; Borgia & Sypherd, 1977; Orłowski & Sypherd, 1978), *M. racemosus* sporangiospores were routinely produced on a nutritionally complex sporulation medium (YPG) with the subsequent germination studies carried out in similar liquid medium. The present studies employing a Coulter counter for monitoring germination demonstrated that complete ($>95\%$) and synchronous germination of C-spores (produced on the nutritionally complex sporulation medium) occurs in complex media containing yeast extract and/or peptone, or in a minimal medium containing glucose or mannose. The rate of germination varied with the carbon source(s). In contrast, C-spore germination was found to

be asynchronous in MGM containing a single non-hexose carbon source, with a proportion of the population remaining ungerminated after several days of incubation. In a sampling of one C-spore batch, 70% were able to germinate on xylose, but only 54% on glutamate, and 24% on cellobiose, with variable proportions germinating on the other non-hexoses (Table 1). These C-spores were not defective as greater than 95% germinated synchronously by 6 h on glucose, mannose, yeast extract or peptone.

Sporangiospores produced on a defined minimal sporulation medium (MSM; M-spores) did not germinate on any one carbon source except glucose or mannose, but did germinate on complex substrates. The addition of either yeast extract or peptone to MSM did not significantly increase the portion of sporangiospores capable of germinating on glutamate or cellobiose (Table 3), but when they were added in combination the normal C-spore phenotype was observed. This suggests that MSM does not contain an inhibitor of germination. To our knowledge this is the first report where the nutritional complexity of the sporulation medium has been shown to have a direct influence on the subsequent sporangiospore phenotype in terms of germination ability.

The literature is replete with reports linking the nutritional composition of the culture environment to gene expression in both prokaryotic and eukaryotic cells. O'Connor *et al.* (1978) have reported that extracellular protease synthesis in *Bacillus amyloliquefaciens* is directly related to the level of amino acids present in the culture medium. Reid *et al.* (1980) reported that peptone induces extracellular collagenase synthesis in *Vibrio alginolyticus*. From these studies it cannot be ruled out that one specific chemical in the CSM is influencing *M. racemosus* sporangiospore phenotype. The observation that both yeast extract and peptone are required in combination, and Casamino acids cannot replace the yeast extract and peptone in CSM, along with the observation that various lot numbers and types of peptone influence the C-spore phenotype, all taken together suggest that the sporulation medium is a dynamic environment closely regulating sporangiophore gene expression. This is further supported by the observation that when glucose is replaced by an equal concentration of an amino acid, or omitted from CSM, a low level of sporulation results.

Additional evidence demonstrating a relationship between sporulation medium and sporangiospore germination ability was obtained from experiments where sporangiospores were produced on MSM supplemented with the normal YP concentration found in CSM or various multiples of that concentration (Table 4). Concentrations of 0.1 × YP and below resulted in sporangiospores with the M-spore phenotype. The most surprising phenotypic difference between these sporangiospore preparations was manifested in the extremes in the germination rates observed on glucose. Sporangiospores produced on 1 × YP germinated almost twice as fast as those produced on 5 × YP. From a practical standpoint these observations demonstrate a need to monitor the germination rate of a given sporangiospore preparation in relationship to the evaluation of biochemical parameters which may be chronologically related to germination.

It has been demonstrated in this study that glucose can initiate germination of both M- and C-spores. In contrast, essentially no M-spores and only a proportion of a C-spore population can germinate on various non-hexoses. One explanation could be that some C-spores have the machinery necessary to convert certain non-hexoses to glucose or another hypothetical signal molecule. Hence, to initiate germination on cellobiose, a C-spore may only require a β-glucosidase, but on glutamate, an intact, functional gluconeogenic pathway may be needed.

These studies have led to a better understanding of the germination requirements of *M. racemosus* sporangiospores. The mechanism by which a compound initiates germination in a dormant bacterial endospore has been the subject of numerous hypotheses (Dring & Gould, 1975; Prasad *et al.*, 1972; Vary, 1978; Woese *et al.*, 1968), but has not been elucidated. By employing a defined minimal medium in these studies we have been able to produce a homogeneous minimal (M-spores) sporangiospore system which is particularly suited for exploring the mechanism(s) involved in the initiation of fungal spore germination.

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