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Sporulation in *Hansenula wingei* Is Induced by Nitrogen Starvation in Maltose-Containing Media

MARJORIE CRANDALL* AND L. J. LAWRENCE

Thomas Hunt Morgan School of Biological Sciences, University of Kentucky, Lexington, Kentucky 40506

The sexually agglutinative yeast *Hansenula wingei* lives in association with bark beetles that inhabit coniferous trees. This yeast was induced to sporulate by malt extract, which contains a high percentage of maltose (50%) and a low percentage of nitrogen (0.5%). A solution of 1.5% maltose without any growth factors also induced ascosporogenesis in *H. wingei*. Thus, only a carbon source is required for sporulation as in *Saccharomyces*. However, potassium acetate did not induce sporulation in *H. wingei* as it does in *S. cerevisiae*. Instead, disaccharides (such as maltose, sucrose, or cellobiose) promote sporulation better than either monosaccharides (such as dextrose, fructose, or mannose) or respiratory substrates (such as ethanol or glycerol). The specificity of disaccharides in promoting sporulation in *H. wingei* may be considered an adaptation since these disaccharides are present in the natural environment of this yeast. In addition, the specificity of disaccharides may be related to the induction of the disaccharidase because cells precultured on dextrose sporulate well on maltose, but cells precultured on maltose sporulate poorly on maltose. When $(NH_4)_2SO_4$ was added at a low concentration (3 mM) to synthetic sporulation medium (1.5% maltose solution), sporulation was abolished, whereas other salts and nitrogen sources inhibited to a lesser extent and vitamins and trace elements had no effect. Oxygen was required for sporulation, as expected for an obligate aerobe. Maximal sporulation was achieved in 2% malt extract broth at high cell density (10^9 cells per ml), pH 5, and 25°C. By using these optimal physiological conditions and hybrid strains selected from an extensive genetic breeding program, about 30% asci (10% tetrads) were obtained routinely. Thus, the genetics of cell recognition in this yeast can now be studied.

Opposite mating types of the yeast *Hansenula wingei* agglutinate immediately upon mixing (29). The cell surface recognition factors responsible for this sexual agglutination reaction are glycoproteins (5) that form a neutralized complex in vitro (7; reviewed in 4). However, genetic studies of the synthesis of these recognition factors in the haploids and their repression in the diploid have been impeded by poor sexual sporulation. Therefore, physiological conditions were sought that would maximally stimulate ascosporogenesis in *H. wingei*.

Sporulation in yeast is induced by high respiratory activity and the absence of a nitrogen source (reviewed in 24). A variety of nitrogen-poor conditions will induce ascosporogenesis in yeasts, including vegetable wedges, V8 juice agar, gypsum blocks, malt extract agar, and acetate agar (reviewed in 27). *Hansenula* species sporulate on malt extract agar (28) but different natural isolates of *H. wingei* sporulate poorly (0.1%) to only moderately (up to 15%) after 1 to 2 weeks. It was possible to increase this frequency of sporulation to about 30% (or more in some strains) by a combined genetic and physiological approach. Genetic crosses were performed and haploid spore clones were selected from good sporulating hybrids (M. Crandall and D. Iovannisci, manuscript in preparation). By using a good sporulating diploid, the following physiological parameters of sporulation in malt extract medium were studied: (i) solid versus liquid medium, (ii) aeration, (iii) temperature, (iv) light, (v) pH, (vi) age of the preculture, (vii) malt extract concentration, (viii) washing of the preculture, (ix) composition of the preculture medium, (x) cell density during sporulation, and (xi) metal ion requirement. Once standard sporulation conditions in malt extract medium were established, then the components of malt extract medium responsible for inducing sporulation were sought. The best synthetic sporulation medium that was developed contained only a carbon source (maltose). A comparative study of various carbon sources that trigger sporulation in defined medium was performed with strains of both *Saccharomyces cerevisiae* and *H. wingei*. All of these results have led to the devel-

opment of optimal conditions for sporulation in *H. wingei*, making genetic analysis feasible.

MATERIALS AND METHODS

Yeast strains. *H. wingei* diploid strain NRRL Y-2340 was isolated from the frass of bark beetles living in conifer trees in the western United States (29). Two spore clones of opposite mating type, strains 5-9A and 21-9D, from one tetrad of Y-2340 were isolated by Alberta I. Herman. Since the hybrid 5-9A × 21-9D sporulates poorly (about 0.1%), it was necessary to outcross these haploid strains to wild-type strains V-1A and V-1B derived from a diploid (NRRL YB-4662) that sporulates reasonably well (about 15%). These strains were also obtained from Alberta I. Herman. Hybrids between mating type 5 and V-1A or between mating type 21 and V-1B gave rise to recombinant spores that yielded good sporulating diploids after additional outcrosses and backcrosses (M. Crandall and D. Iovannisci, manuscript in preparation). For most of the experiments in this paper, diploid strain D119 was used; D119 is a hybrid between D92-54C and V-1B. The genotype of haploid strain D92-54C is *mat1-21 ade1 ade4 his1* (i.e., mating type 21 with a requirement for adenine and histidine). In some experiments, a subclone of D119 was used (D119e) because it sporulated with a slightly higher frequency. For later experiments, diploid strain D268 was used because of its increased sporulation and spore viability. The genotype of D268 is D191-18C (*mat1-21 met1 leu1 stn1*) × D135-23A (*mat1-5 cyh1 lys1 ade4 his1*). A diploid hybrid (SCD-1) of *Saccharomyces cerevisiae* was studied as a control. This is a prototroph isolated from a cross between the following two haploids: XP300-29B (*mat1* **a** *ade2-1 trp5-18 his6 lys1 gal2*) (obtained from James Mead) and 4G5α (*mat1* α *ade8-18 his4-4 thr1 ura3 met13*) (obtained from Seymour Fogel).

Media. The preculture medium (YKDB) for sporulation was a broth containing 0.7% yeast extract (Difco), 0.5% KH_2PO_4, and 2% dextrose. This medium was autoclaved at 121°C for 15 min. The pH of YKDB was 5.6 (unadjusted). The sporulation medium (MEB) was a broth containing 2.0% malt extract (Difco) reduced from 2.5% (5). Autoclaving MEB resulted in precipitation, but this had no effect on induction of sporulation. The pH of MEB was 4.7 to 5.0 (unadjusted).

Microscopic counts of sporulation percentage. After 4 days of aeration in MEB, samples of the sporulated culture (1.0 ml) were added to 0.1 ml of 37% formaldehyde and refrigerated. To facilitate cell counts, sporulated cultures were diluted 0.1 ml/2.0 ml of saline. Wet mounts were observed under phase contrast at 400×, and a differential count of all of the sporulated and unsporulated cells in each microscopic field was performed. Budded cells were counted as one cell. An unruptured ascus was counted as one sporulated diploid cell. A bud on an ascus was not counted because these ascal buds were never seen to sporulate. A group of two, three, or four spores, liberated as a result of spontaneous ascal rupture, was counted as one sporulated cell. Individual free spores were not counted. The sporulation percentage was equal to the number of asci plus spore groups × 100 divided by the total (asci + spore groups + unsporulated cells). Sporulation in *H. wingei* characteristically produces three-spored asci (22, 28). The frequency of tetrads in now-improved strains is about one out of five asci (M. Crandall and D. Iovannisci, manuscript in preparation).

Standard sporulation procedure. Fresh cultures were grown overnight in YKDB and then diluted 0.02 ml/2 ml of YKDB in a culture tube (18 by 150 mm with Morton closures). These precultures were aerated on a roller drum (model TC-6 New Brunswick Scientific Co., New Brunswick, N.J.) at room temperature (23 to 27°C) for 16 h. The cell density at this time was about 10^8 cells per ml, which is referred to as 1×. The cells were harvested by centrifugation for 5 min at 280 × *g* (1,000 rpm) in the original culture tubes in the HG-4 swinging-bucket rotor of the Sorvall RC-3 centrifuge (Dupont Co., Instrument Products Division, Newtown, Conn.). Up to 40 tubes may be centrifuged in one run with the 440 reducing adaptors. The cell pellets were washed once in sterile 0.9% saline and then resuspended at 1× cell concentration in 2 ml of MEB in the same culture tube. These cultures were aerated on the roller drum at room temperature. Sporulation was near completion after 2 days. The asci rupture spontaneously after 3 to 4 days. Sporulation in defined medium took longer (6 to 8 days), and a lower frequency of tetrads was obtained. Therefore, MEB was used routinely for tetrad analysis (M. Crandall and D. Iovannisci, manuscript in preparation).

RESULTS

To increase the sporulation frequency of *H. wingei* diploids, the following physiological parameters were studied with malt extract sporulation medium.

Solid versus liquid medium. Poorly sporulating strains will not sporulate in broth and will sporulate on agar only after 1 to 2 weeks. Cells streak purified on MEA (2.5% malt extract–3.0% agar) sporulated best in the area of the small, crowded colonies. Cells plated on MEA sporulated best when there were between 500 and 5,000 cells per plate. In contrast, good sporulating strains sporulated sooner (2 to 4 days) and to a greater percentage in MEB than on agar.

Aeration. Since *H. wingei* is an obligate aerobe (30) and since respiratory activity is required for sporulation in *S. cerevisiae* (8; reviewed in 24), cultures were always grown in a tube or flask with a small volume of broth compared with the capacity of the vessel, and these were well aerated on a roller drum or rotary shaker. In one experiment, a culture in MEB was allowed to sit on the bench top without aeration. This reduced sporulation from the control level of 25% to 0% without aeration.

Temperature. Sporulation is optimal at 25°C (Fig. 1). When cells were placed under conditions of diurnal variation in temperature as would

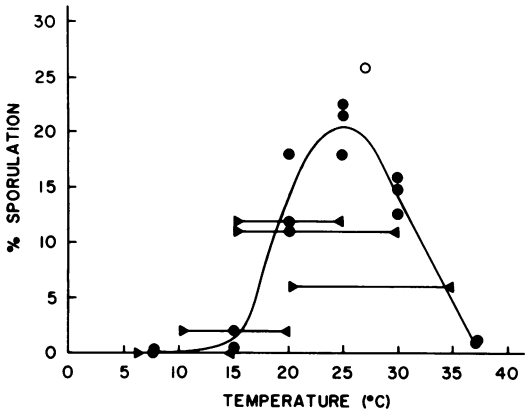

FIG. 1. *Effect of temperature on sporulation in MEB. D268 was precultured overnight in YKDB and then inoculated with 2 ml/200 ml of YKDB in a 1-liter Erlenmeyer flask. After 20.5 h of aeration at room temperature, the cells were harvested, washed twice with saline, and resuspended at the same cell density in 2% MEB. Aliquots of this cell suspension (10 ml) were added to Petri plates (15 by 100 mm); three replicates at each temperature. The plates were wrapped in aluminum foil to prevent evaporation and to exclude light and then were placed in constant temperature incubators (at 7.5, 15, 20, 25, 30, and 37°C) (●) or in alternating temperature incubators (12 h/12 h at 15°C/6°C, 20°C/10°C, 25°C/15°C, 30°C/15°C, and 35°C/20°C) (▶—◀). As a control, 10 ml of the cell suspension in MEB was aerated in a 50-ml flask at 27°C (○). After 5 days, cells were counted.*

occur in nature, the sporulation was decreased (see bars on Fig. 1). The narrow temperature optimum for sporulation was also noted by Stock and Black (22).

Light. Cultures grown in MEB in continuous light were compared to those grown in continuous darkness (flasks or plates covered with aluminum foil). In addition, cultures grown under conditions of diurnal alternation of light and temperature were compared with cultures under alternating temperatures in continuous darkness. No effect of light was observed. This finding is in contrast to the inhibitory effect of light on sporulation reported for *S. cerevisiae* (10).

pH. The optimal pH for sporulation was between 4.2 and 5.0 (unadjusted) (Fig. 2). This was true for MEB as well as for synthetic sporulation medium (see below).

Age of the preculture. Cells from early stationary phase (16 h) in a rich medium (YKDB) sporulated the best (Fig. 3). At this phase of growth the population was between 60 and 85% budded (down from 95% budded at 12 h). The peak of sporulation at 16 h has been confirmed in replicate experiments comparing 16-h and 20-h precultures. The cell concentration at 16 h was about 10^8 cells per ml.

Malt extract concentration. Maximal sporulation occurred between 1.5 and 3.0% MEB medium (Fig. 4). Routinely, 2% MEB was used.

Washing of the preculture. In one experiment, cells were precultured in YKDB, harvested, washed from one to five times or not at all in saline, and then resuspended in MEB. There was no effect of the number of washings on the sporulation percentage.

Composition of preculture medium. A rich preculture medium such as YKDB was best for sporulation; cells precultured in minimal medium (yeast nitrogen base [Difco] plus 2% dextrose or yeast carbon base [Difco] plus 0.5% asparagine) did not sporulate as well. Interestingly, if cells were first adapted to maltose in a preculture medium (YKMB, 0.7% yeast extract, 0.5% KH_2PO_4, 2% maltose broth) and then transferred to either MEB or synthetic sporulation medium (see below), sporulation was decreased compared with the control (YKDB) with dextrose as a carbon source in the preculture medium (Table 1). Another rich medium, YPDB, used for preculturing gave poorer sporulation than YKDB. The composition of YPDB is 1% yeast extract, 1% peptone (Difco), and 2% dextrose. Thus, substituting peptone for phosphate lowered sporulation. Addition of caffeine to the preculture medium increases sporulation in *S. cerevisiae* (26) but had no effect in *H. wingei*.

Cell density during sporulation. If cells from the preculture were diluted 1/100 into MEB, a period of extensive growth followed. This growth delayed sporulation and lowered the final percentage of sporulation. However, if cells were harvested from early stationary phase (16 h) and resuspended at the same cell density (10^8 cells per ml) in MEB, then maximal sporulation was obtained. This high cell density probably ensures that cells will rapidly exhaust the limited nitrogen reserves in the medium and become arrested at G1 of the cell cycle. The cells grew after transfer from YKDB to MEB. After 25 h in MEB, the cell density of D119e was 8.6 $\times$ 10^8 cells per ml (as determined by viable counts).

Metal ion requirement. The chelating agent disodium EDTA, when added at a concentration of 5 mM to 2.5% MEB, inhibited sporulation by 50%. Several trace metals (Fe, Mn, and Zn) were tested for their effect on sporulation in a limiting concentration of MEB. A small but reproducible stimulation of sporulation occurred when 0.1 μM $ZnSO_4$ was added to 0.9% MEB (from 10 $\pm$ 1 to 18 $\pm$ 3% sporulation).

Once the optimal physiological conditions for

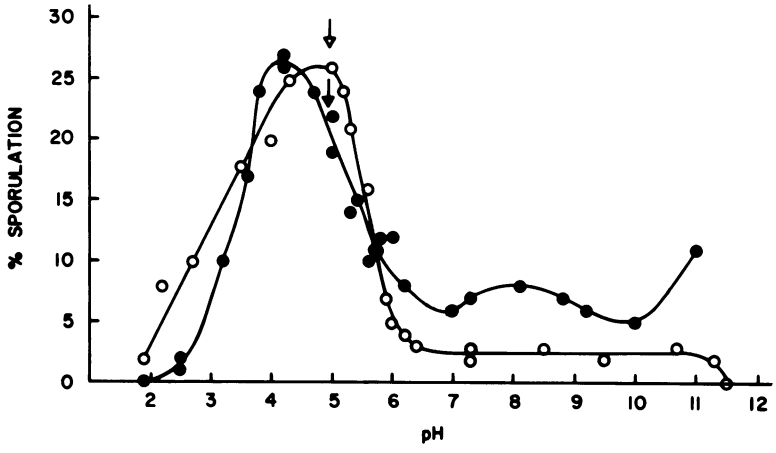

FIG. 2. *Effect of pH on sporulation in MEB and SSM. D119e was precultured overnight in YKDB and then inoculated with 0.25 ml/25 ml of YKDB in a 125-ml flask. After 20 h of aeration, the cells were harvested, washed twice with saline, and resuspended at the same cell density in either 2.5% MEB (●) or 1.5% maltose (○). To this cell suspension was added 1 M KH_2PO_4 to a final concentration of 10 mM. Aliquots (2 ml) of this cell suspension were pipetted into culture tubes, and the pH of each suspension was adjusted by adding 0.1 or 1.0 M KOH or HCl. Drops of the adjusted suspensions were tested for pH with ColorpHast Indicator Sticks from EM Laboratories Inc., Elmsford, N.Y. Tubes were aerated for 4 days (●) or 6 days (○) and then counted. In both experiments, the unadjusted pH is indicated by an arrow.*

sporulation in MEB had been determined, it was of interest to learn what substance(s) in malt extract induced ascospore formation in *H. wingei*. Difco Laboratories (Technical Research Division, Detroit, Mich.) supplied the following analysis of malt extract: maltose, 50.0%; dextrose, 27.0%; dextrin, 15.0%; moisture, 3.0%; ash, 1.5%; nitrogen, 0.5%. The two important aspects of the composition of malt extract are the high content of maltose and the low content of nitrogen. Based on this information, a defined medium was prepared containing maltose as a carbon source, a low concentration of Casamino Acids as a source of nitrogen, and the salts, vitamins, and trace elements used in Difco yeast nitrogen base. This first attempt at preparing a synthetic sporulation medium (SSM) was successful, but only a small percentage of sporulation was obtained. Then each time a component was eliminated from the SSM, the percentage of sporulation went up. Eventually it was found that diploid cells would sporulate best in a medium containing only maltose. After this discovery, the optimal conditions for sporulation in SSM were studied. These include: (i) maltose concentration, (ii) pH, (iii) cell density during sporulation, (iv) effect of nitrogen sources, (v) effect of salts, (vi) effect of required vitamins, (vii) effect of trace elements, and (viii) comparison of different carbon sources.

Maltose concentration. Maximal sporulation in SSM was obtained in a solution of 1.5% maltose. In Fig. 4, the concentration of various carbon sources in SSM is compared to the malt extract control. It can be seen that disaccharides (maltose and sucrose) are better carbon sources for sporulation than monosaccharides (dextrose and fructose) because disaccharides stimulate sporulation at lower concentrations (wt/vol) and to a greater extent than monosaccharides. Malt extract contains both maltose and dextrose and, interestingly, if the curves of these two sugars in SSM are added, they would approximate the MEB curve. Not shown in Fig. 4 are results for cellobiose, melezitose, and maltotriose; all three yielded maximal sporulation in SSM at 1.5% also.

pH. Just as with MEB, the unadjusted pH (pH 5) gave the best sporulation in SSM (Fig. 2).

Cell density during sporulation. Just as with MEB, the best sporulation is obtained when cells are harvested, washed, and resuspended at the same cell density (1×) (Fig. 5).

Effect of nitrogen sources. Ascosporogenesis in yeast is a response to nitrogen starvation conditions (24). Ammonium ion (at 1 mM) is a powerful inhibitor of sporulation in *S. cerevisiae* (17, 18). In *H. wingei*, complete inhibition by $(NH_4)_2SO_4$ occurred at 3 mM, whereas higher concentrations of other nitrogenous compounds such as asparagine (at 15 mM) were required for the same degree of inhibition (Fig. 6). In a separate experiment, lysine (at 10 mM)

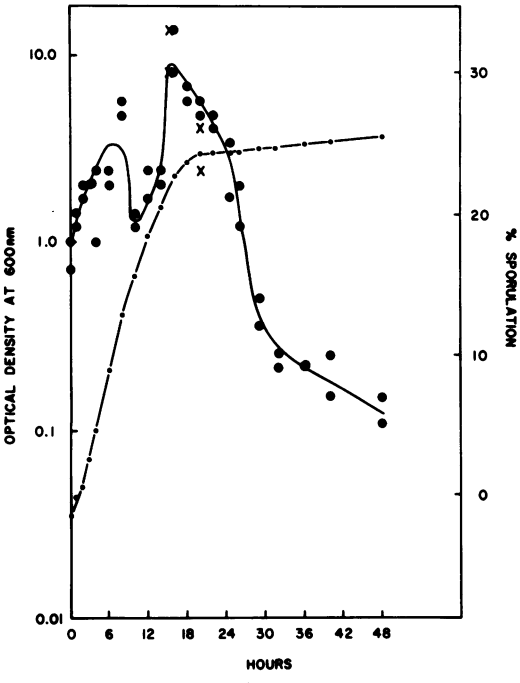

FIG. 3. *Relationship between preculture age and sporulation ability. D268 was precultured in YKDB for 21 h, and then diluted 20 ml/2 liters of YKDB at zero time. Immediately 200 ml (in duplicate) was removed, and the cells were harvested, washed, and resuspended in 2.0 ml of 2% MEB for sporulation (●). The preculture was aerated at 150 rpm at 25°C initially in two Fernbach flasks (2.8-liter capacity). When the cell density increased, the culture was transferred to four smaller flasks (less than 200/liter flask) to prevent foaming. At each time point, the flasks were pooled before the culture was sampled. The optical density (—·—) was read in a Turner (model 350) spectrophotometer at 600 nm. As the cell density increased, less volume was harvested for sporulation according to the formula: initial optical density × 200 ml/current optical density. For example, at 16 h, 3.1 ml of culture was harvested, washed, and resuspended in 2.0 ml of MEB, but at 20 h, only 2.3 ml of culture was sampled. In replicate experiments (×), 2.0-ml precultures (in duplicate) were grown for 16 and 20 h and then transferred to 2.0 ml of MEB; the same sporulation maximum at 16 h was obtained.*

inhibited sporulation completely, whereas glutamic acid (at 10 mM) only partially inhibited (20% of control).

Effect of salts. The salts present in yeast nitrogen base (Difco) inhibit sporulation in the following order: $CaCl_2 > KH_2PO_4 > NaCl \cong MgSO_4$. Since there was no enhancement of sporulation at concentrations below those in growth medium, salts were omitted from SSM.

Effect of required vitamins. *H. wingei* requires biotin, thiamine, and pyridoxine (30). Additions of these vitamins to SSM at various concentrations had no effect. Thus, the cytoplasmic pool of these vitamins from the preculturing medium must be sufficient for sporulation.

Effect of trace elements. The metal ions present in yeast nitrogen base had no effect when added to SSM. The zinc effect on sporulation observed in limiting concentrations of MEB was not observed in SSM. The conclusion drawn from the above studies is that sporulation proceeds by utilizing internal pools of nitrogen, salts, vitamins, and metal ions requiring only an exogenous source of carbon and energy.

Comparison of different carbon sources. In Table 2, the effect of various carbon sources

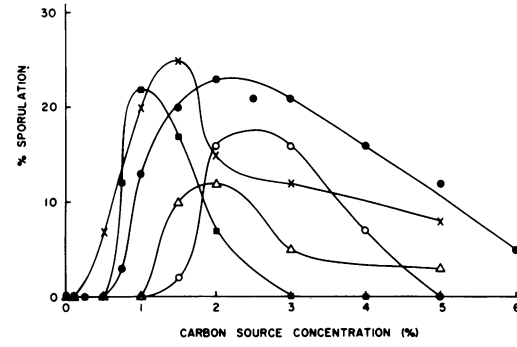

FIG. 4. *Concentration of various carbon sources for optimal sporulation. D119 was precultured in YKDB for 20 h, harvested, washed twice, and resuspended in 2.0 ml of MEB at varying concentrations. For carbon sources in SSM, cells of D119e were washed three times in saline and resuspended at 2× cell density in sterile distilled water. To each tube was added 1 ml of cell suspension at 2× and 1 ml of a filter-sterilized sugar solution at 2× the final concentration. Symbols: malt extract, ●; maltose, ×; sucrose, ■; dextrose, △; fructose, ○.*

TABLE 1. *Influence of the carbon source in the preculture medium on sporulation*[a]

Preculture medium	Sporulation medium	Sporulation[b] (%)
Dextrose	Dextrose	5, 5
Maltose	Maltose	3, 9
Maltose	Dextrose	2, 3
Dextrose	Maltose	28, 30

[a] Cells of D119e grown overnight in YKDB were diluted 0.02 ml/2 ml of either YKDB or YKMB (0.7% yeast extract, 0.5% KH_2PO_4, and either 2% dextrose or maltose). After 20 h of aeration at room temperature, cells were harvested, washed twice in saline, and resuspended at the same cell concentration in 2 ml of either 1.5% dextrose or maltose.

[b] Sporulation cultures (in duplicate) were aerated for 6 days at room temperature and then counted as described in the text.

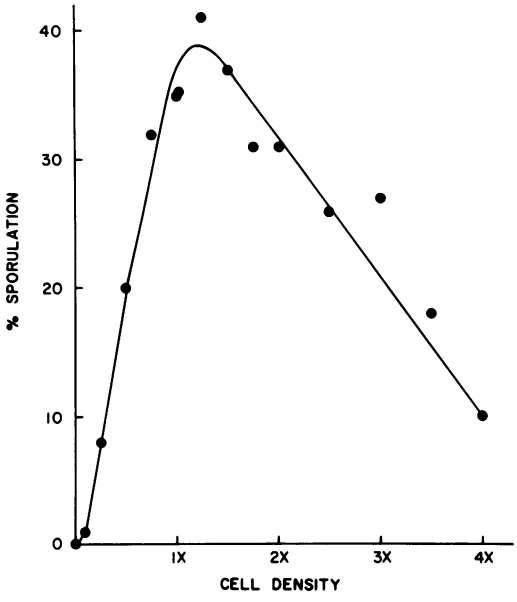

FIG. 5. *Cell density during sporulation. D119e was precultured in YKDB for 21 h, harvested, washed twice in saline, and then resuspended at 1× cell density in saline. Different volumes of cell suspension were pipetted into cultures tubes (the control is 2.0 ml). The cells were harvested again, and all pellets were resuspended in 2.0 ml of 1.5% maltose solution. Tubes were aerated at room temperature for 6 days and counted for sporulation.*

on sporulation in *S. cerevisiae* and *H. wingei* is compared. Even though *H. wingei* is an obligate aerobe and *S. cerevisiae* is a facultative anaerobe, both fermentative and respiratory substrates can serve as inducers of sporulation in both yeasts. However, catabolite-repressing sugars (such as glucose, fructose, and mannose) will not support sporulation in *S. cerevisiae* because these substrates will repress mitochondrial function. Galactose does not repress the synthesis of mitochondrial enzymes (23), and it therefore can serve as a carbon source for sporulation. Since *H. wingei* is not catabolite repressible (3), it can sporulate even on glucose. However, a finding that is not understood is why *H. wingei* does not sporulate on acetate even though it assimilates acetate, its metabolism is obligately respiratory, and it sporulates on other respiratory carbon sources. It can also be seen from Table 2 that yeasts will not sporulate on a carbon source that cannot be assimilated, as expected. However, a quite unexpected finding was derived from this experiment—*H. wingei* sporulates better on disaccharides than on monosaccharides. A hypothesis explaining why disaccharides are better inducers of sporulation is presented in the Discussion.

After this paper was completed, a good sporulating diploid strain (D571) was isolated from a backcross of a spore clone, D402-71A (*mat1-5 ade4*), to a mutant derived from one of its parents, D334-7A (*mat1-21 ino1 met1*) *asp1 his4*. Diploid D571 sporulates with a frequency of 65% (15% tetrads) (Fig. 7) (M. Crandall and D. Iovannisci, manuscript in preparation).

DISCUSSION

The trigger for sporulation in yeast is nitrogen starvation (reviewed in reference 24). The nitrogen content of malt extract medium is low relative to the amount of carbohydrate present, and this medium induces ascosporogenesis in many yeasts, including *H. wingei* (27, 28). The major carbohydrate in malt extract is maltose; this sugar promotes sporulation in *H. wingei* in the absence of any other growth factor or nutrient. Maltose and other disaccharides such as sucrose and cellobiose are better carbon sources for sporulation in *H. wingei* than are monosaccharides or respiratory substrates. An explanation for this observation is offered here. To use disaccharides for sporulation, the cell probably has to undergo enzyme induction to synthesize either maltase, invertase, or cellobiase de novo. In contrast, the enzymes for the oxidation of monosaccharides and respiratory substrates are probably constitutive in this obligately aerobic yeast. Induction

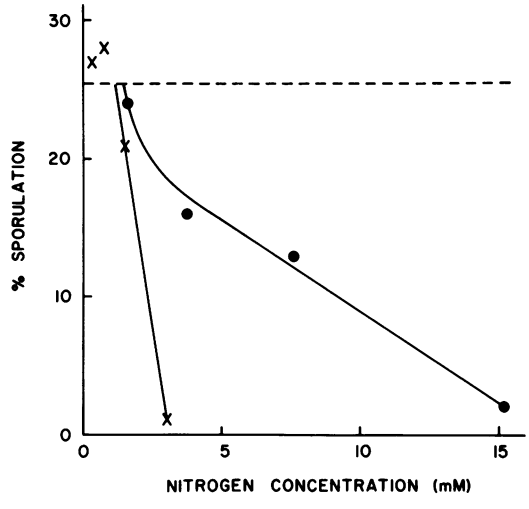

FIG. 6. *Effect of nitrogen sources on sporulation. D119 was precultured in YKDB for 20 h, harvested, washed, and resuspended at 2× cell density in water. To each culture tube was added 1.0 ml of 2× cell suspension, 0.15 ml of 20% maltose, and varying amounts of 2% asparagine (●) or 0.2% ammonium sulfate (×) or water (- - -). Tubes were aerated on a roller drum at room temperature for 5 days and then counted for sporulation.*

of a new enzyme requires an additional source of amino acids. Thus, when cells of *H. wingei* are transferred to a medium containing a substrate of an uninduced enzyme and no source of nitrogen, the cell will respond by synthesizing the enzyme utilizing its internal pool of amino acids. This additional nitrogen stress might enhance the effect of exogenous nitrogen limitation. In agreement with this hypothesis, it was found that if cells are precultured on maltose (rather than on glucose), then the sporulation percentage on maltose is lowered. Information pertaining to the turnover of macromolecules during induction of sporulation in *S. cerevisiae* further supports this hypothesis. When diploid cells of *Saccharomyces* are transferred to a medium lacking a nitrogen source, proteinase and RNase levels increase (1, 25). Presumably, this increased synthesis of degradative enzymes is an adaptive response to lowered internal pools of amino acids and nucleotides, the end products of the reactions catalyzed by these enzymes. These nitrogen-containing building blocks would then be used to synthesize spore structural proteins and mRNA molecules and enzymes that are sporulation specific such as the glycogenolytic enzyme (2) and an RNase (26).

TABLE 2. *Effect of various carbon sources on sporulation in an aerobic and a facultative yeast*[a]

Carbon source	*H. wingei*		*S. cerevisiae*	
	Assimilation[b]	Sporulation[c] (%)	Assimilation	Sporulation (%)
Fermentable				
Malt extract	+	27 ± 6	+	0
Dihydroxyacetone	+	23 ± 8	−	0
Monosaccharides				
Dextrose	+	10 ± 8	+	1
Fructose	+	14 ± 10	+	0
Mannose	+	5 ± 5	+	0
Galactose	−	0	+	24
N-Acetyl-D-glucosamine	−	0	−	0
Disaccharides				
Maltose	+	24 ± 4	−	0
Sucrose	+	22 ± 13	+	1
Cellobiose	+	20 ± 3	−	1
Melibiose	−	0	−	0
Trehalose	−	0	−	1
α-Methyl-glucoside	−	0	−	0
Trisaccharides				
Maltotriose	+	13 ± 5	−	0
Melezitose	+	18 ± 10	−	0
Raffinose	−	0	−	2
Nonfermentable				
Ethanol	+	2 ± 1	+	0
Glycerol	+	8 ± 5	−	0
Lactate	+	0	+	0
Citrate	+	0	−	0
Acetate (Na)	+	0	+	13
Inositol	−	0	−	0

[a] *H. wingei* diploid strain D119e and *S. cerevisiae* diploid strain SCD-1 were used.

[b] For assimilation, carbon sources were added at a final concentration of 2% in 0.67% YNB. This broth (2 ml) was inoculated with 0.02 ml of a washed cell suspension prepared from an overnight preculture grown in YNB–2% glucose. Growth tubes were aerated on a roller drum at room temperature and scored for growth after 2 days. Strain SCD-1 grew slowly on galactose.

[c] For sporulation, cells were precultured overnight in YKDB then inoculated 0.25 ml/25 ml of YKDB. After 20 h of aeration at room temperature, cultures were harvested, washed twice with saline, and resuspended at the same cell density in sterile distilled water (25 ml). Then, aliquots of this cell suspension were added to tubes. Concentrated carbon source solutions (filter sterilized) were added to a final concentration of 1.5% in 2 ml/tube. The malt extract control was prepared by resuspending cells in 2 ml of 2% MEB. Tubes were aerated at room temperature for 6 days and then counted. Values are the mean ± standard deviation of between 4 and 9 replicates.

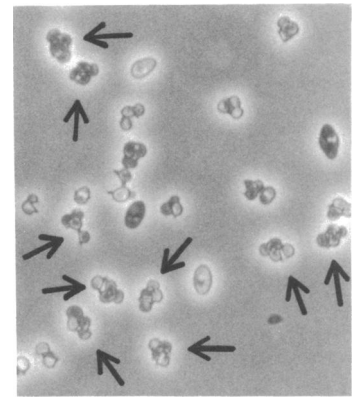

FIG. 7. *Spore tetrads in H. wingei. D571 was precultured in 2.0 ml of YKDB for 16 h, harvested, washed once in saline, resuspended in 2.0 ml of 2.0% MEB, and aerated for 4 days at room temperature. Tetrads are indicated by arrows. Notice also the brims on the ascospores.*

The prominent role played by disaccharides in promoting sporulation in *H. wingei* can be related to the sugars found in its ecological niche. *H. wingei* grows on the sap of coniferous trees in association with bark beetles (reviewed in 5). The larvae of the beetles eat the yeast, and in turn the beetles transfer the yeast from tree to tree, inoculating the yeast under the bark thereby ensuring that a new population of yeast will be propagated. Sucrose is the most common sugar in the phloem of most plants and the one usually found in the largest quantity (16). Hexoses or reducing sugars in general do not occur in sieve tubes. In addition to sucrose, cellobiose and maltose would also be found in the yeast's environment because the beetles feed on the phloem and digest the wood polysaccharides, hexosans, to disaccharides (21). Thus, the fact that *H. wingei* sporulates best on the disaccha-

rides sucrose, cellobiose, and maltose may be considered an adaptation to its natural environment.

Other findings concerning the in vitro induction of sporulation in *Hansenula* include (i) inhibition by low concentrations of nitrogenous compounds, (ii) requirement for oxygen, (iii) pH optimum at 5, (iv) temperature optimum at 25°C, (v) sensitivity to temperature fluctuations, (vi) stimulation by high cell density, and (vii) stimulation by unknown factors present in malt extract medium. Each of these findings will be discussed below.

It is clear that the proper conditions required for sporulation can be achieved when the yeasts are growing on the sap of coniferous trees. (i) Phloem exudate contains a high content of sugar relative to nitrogenous compounds. Sucrose is present at 80 to 106 mg/ml, whereas amino acids are present at only 5.2 mg/ml, ammonium is present at 0.029 mg/ml, and nitrate is absent (16). This small amount of nitrogen could be consumed in a microenvironment under the bark of a tree allowing the yeasts to sporulate using sucrose as a carbon and energy source. It is interesting to note that ammonium ion, the most powerful inhibitor of sporulation (17, 18, 20), is present at a low concentration in the plant. Ammonium ion plays a primary role in nitrogen metabolism in general by controlling the amino acid pool (13) but also appears to affect sporulation (secondary metabolism) specifically (19) by blocking the normal increases in proteases that occur in yeast cells after transfer to sporulation medium (15). Ammonium ion also inhibits RNA and protein synthesis during sporulation (9) as well as sporulation-specific glycogen degradation (11) and induction of glyoxylate enzymes during adaptation to acetate in *Saccharomyces* (12).

Returning now to the discussion of sporulation in *Hansenula* under natural conditions, (ii) oxygen is available in the phloem for respiration of the plant as well as the yeasts. Interestingly, most yeasts living in association with bark beetles are poor fermenters (21). Apparently, the conditions under which these yeasts grow are more favorable for an oxidative type of metabolism. *H. wingei* is an obligate aerobe (30), and in this paper oxygen has been found to be necessary for sporulation, as expected. (iii) The pH of sap is about 8.0 to 8.2 (16). A second minor pH optimum for sporulation in *H. wingei* was noted at pH 8.1 to 8.3 in MEB. (iv) Ambient temperatures in the summer would be about 25°C, the optimum for sporulation. (v) However, it is not so easy to understand the dramatic decreases in sporulation found in this study caused by variations in temperature as would occur between day and night. Extrapolating from these laboratory data, sporulation in nature would be expected to occur only in the summer when the nights remained balmy. (vi) The stimulatory effect of high cell densities on sporulation in *H. wingei* which might occur in a microenvironment under the bark of a tree may be explained in terms of arresting cells at G1 of the cell cycle. It is at G1 that a diploid cell becomes committed either to another round of mitosis, or if nutrients are limiting, to meiosis (14). When cells of *H. wingei* are transferred from growth medium to MEB sporulation medium at high cell density (10^8 cells per ml), the cells grow to a limited extent (increasing 10-fold), and they consume the small amount of nitrogen present and probably become arrested at G1 in a synchronous fashion. On the other hand, if cells are transferred from growth medium to MEB sporulation medium at low cell density (2×10^6 cells per ml), they undergo many rounds of cell division and the final sporulation percentage is decreased. This decrease in sporulation may be explained if there is no cell cycle synchrony in growth arrest. The advantage of studying sporulation at high cell density is that sufficient cell numbers are available for biochemical analysis. (vii) Finally, it is reported here that although *H. wingei* will sporulate in a solution of 1.5% maltose without any other growth factors added, there is a stimulation of sporulation by other substances also present in malt extract medium that leads to a higher percentage of tetrads. These same substances may be present in the sap of coniferous trees, which is the natural substrate for sporulation of this yeast.

The purpose of this study was to find conditions that optimized the sporulation process. This goal was achieved. The percentage of tetrads in sporulated cultures has been raised from essentially zero to between 10 and 20% by a combined approach involving physiological studies and an extensive genetic breeding program (M. Crandall and D. Iovannisci, manuscript in preparation). Haploid strains have been synthesized that (i) exhibit a strong sexual agglutination reaction, (ii) mate with a high frequency, (iii) are multiply marked, (iv) sporulate with a high frequency in crosses, and (v) give rise to spores from such crosses that have high viability. Thus, *H. wingei* may now be considered a new genetic organism. A preliminary genetic map is being constructed (M. Crandall and D. Iovannisci, manuscript in preparation), and part of this map has been published (6). Therefore, it is now possible to pursue studies of the genetic regulatory mechanisms governing cell

recognition and mating in this yeast. Future studies will focus on the genetics of the repression of the haploid cell recognition factors in the diploid.

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