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Initiation of Yeast Sporulation by Partial Carbon, Nitrogen, or Phosphate Deprivation

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In this paper we show that partial deprivation of a carbon source, a nitrogen source, or phosphate in the presence of all other nutrients needed for growth initiates meiosis and sporulation of *Saccharomyces cerevisiae* homothallic strain Y55. For carbon deprivation experiments, cells were grown in synthetic medium (pH 5.5) containing an excess of one carbon source and then transferred to the same medium containing different concentrations of the same carbon source. In the case of transfer to different acetate concentrations, the log optical density at 600 nm increased at the previous rate until the cells had used up all of the acetate, whereupon the cells entered a stationary phase and did not sporulate. The same was observed with ethanol. In contrast, at different concentrations of dihydroxyacetone or pyruvate, cells grew at different rates and sporulated optimally at intermediate concentrations (50 to 75 mM). The response to galactose was similar but reflected the presence of a low-affinity galactose transport system and the induction of a high-affinity galactose transport system. Cells could also sporulate when a glucose medium ran out of glucose, apparently because they initiated sporulation during the subsequent lag period and then used the produced ethanol as a carbon source. For phosphate deprivation experiments, cells growing with excess ethanol or pyruvate and phosphate were transferred to the same medium containing limiting amounts of phosphate. First, they used up the intracellular phosphate reserves for rapid growth, and then they sporulated optimally when an intermediate concentration (30 μM) of phosphate had been added to the medium. For nitrogen deprivation experiments, cells grown with excess acetate, ethanol, or pyruvate and NH_4^+ were transferred to the same medium from which all nitrogen had been removed. These cells sporulated well in acetate medium but poorly in ethanol and pyruvate media. However, the sporulation frequency in the latter media could be increased greatly by adding intermediate concentrations (1 mM) of the slowly metabolizable amino acids glycine, histidine, or phenylalanine. If one assumes that the sporulation response to partial deprivation of carbon-, nitrogen-, or phosphorus-containing compounds reflects control by a single metabolite, the intracellular concentration of this metabolite may decide at the START position (G1 phase) of the cell cycle whether **a**/α cells enter mitosis or meiosis.

The yeast *Saccharomyces cerevisiae* can use both glycolytic (glucose, fructose) and gluconeogenic (acetate, ethanol, pyruvate) carbon sources for growth. Sporulation, which occurs only in **a**/α cells, is usually initiated by the transfer of cells (somehow adapted to allow gluconeogenesis) to "sporulation medium," which simply consists of potassium acetate, sometimes supplemented with a buffer (4, 7, 23, 24, 29). Yeast can also sporulate, although usually not as well, in a solution containing other carbon sources, such as dihydroxyacetone, pyruvate, or galactose (24, 25). In most *S. cerevisiae* strains that have been studied, internal carbon sources do not suffice to allow sporulation (7, 25, 33). Glycogen accumulates only after sporulation has been initiated by cell transfer to acetate; eventually, the acetate can be removed, and glycogen be used as a carbon source (15, 16). A carbon source is needed to maintain ATP (2) and to allow the synthesis of new macromolecules needed for meiosis and sporulation; some new proteins made during sporulation on potassium acetate have been identified (18, 20). Other necessary precursors, including nitrogen- and phosphate-containing compounds, are usually available inside the cells during sporulation on acetate. These compounds can be produced by

de novo synthesis from simple precursors, such as amino acids or accumulated allantoin, or by turnover of existing macromolecules, such as polyphosphate, RNA, and protein (7, 32). Polyphosphate disappears during sporulation on acetate (7). The addition of NH_4^+ or rapidly metabolizable amino acids suppresses sporulation in acetate, whereas the addition of phosphate does not (7, 24). In contrast to a hypothesis (26) proposing that NH_4^+ inhibits sporulation directly, experiments by Vezinhet et al. (33) and the experiments described here indicate that this suppression results from a nitrogenous metabolite. Therefore, the transfer of cells to acetate alone allows sporulation because it decreases the synthesis of a nitrogen-containing suppressor of sporulation and provides a needed carbon source.

In a systematic approach to analyze the biochemical conditions under which sporulation can be initiated, we wanted to determine whether not only the deprivation of nitrogen but also the deprivation of carbon or phosphorus sources could lead to sporulation in the presence of the other nutrients. To avoid complicated interpretations, we wanted to find specific conditions that allowed growth as long as the test compound was present in excess in a growth medium but enabled sporulation when its concentration was reduced in medium that was otherwise the same.

Our experiments were guided by the results obtained with *Bacillus subtilis*, which showed that partial but not complete deficiencies of certain nutrients allowed sporulation (8). In these experiments, the following problem had to be overcome. Since most nutrients are actively transported by bacteria, with apparent K_m values in the micromolar range, the intracellular concentrations of the nutrients remained high, whereas the extracellular concentrations decreased due to cell multiplication, until the nutrients were essentially used up. Subsequently, growth stopped, but the cells could not sporulate because the required nutrients were so deficient that the cells could not produce the new mRNA and protein molecules needed for differentiation. To control the intracellular concentration of a nutrient, it was necessary to use conditions that circumvented the active transport of the nutrient. If this was done, *B. subtilis* sporulated well when the extracellular concentration and, therefore, the intracellular concentration of the nutrient were low enough to allow only slow growth, as measured by the increase) in optical density at 600 nm (OD_{600}).

In this paper we demonstrate that partial deprivation of an intracellular metabolite(s) is also necessary to enable meiosis and sporulation of yeast. This occurs if intracellular carbon-, nitrogen-, or phosphorus-containing compounds are available only at a low rate.

MATERIALS AND METHODS

Strain and media. Homothallic and prototrophic *S. cerevisiae* strain Y55 was obtained from H. O. Halvorson and was kept frozen at −70°C in 25% glycerol. The basic medium (MN) contained the following (final concentrations): 200 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) adjusted to pH 5.5 with KOH and 6.7 g of yeast nitrogen base without amino acids (Difco Laboratories) per liter. For phosphate limitation experiments, we reconstructed the "yeast nitrogen base without amino acids" but omitted the usual amount of potassium phosphate (N_{-P}) and then added different amounts of potassium phosphate (pH 5.5). The amount of potassium added together with the phosphate did not affect the behavior of cells in the final medium because the phosphate-free medium (MN_{-P}) already contained a high concentration of potassium (about 25 mM). For nitrogen limitation experiments, we used a medium (MN_{-N}) which contained yeast nitrogen base without amino acids but no ammonium sulfate (Difco). The solutions that contained compounds to be added were adjusted to pH 5.5 with KOH or HCl, except where noted otherwise.

Growth and sporulation conditions. We grew the cells for 24 h or longer on plates containing 1% yeast extract, 2% peptone, and 1% glucose (29) and inoculated them at an OD_{600} of about 0.05 into Erlenmayer flasks containing one-fifth or less of the volume of MN supplemented with different carbon sources, such as glucose (100 mM), potassium pyruvate (300 mM), potassium acetate (100 mM), ethanol (100 mM), dihydroxyacetone (150 mM), or galactose (100 mM). We did not use dihydroxyacetone and ethanol at concentrations higher than 150 and 100 mM, respectively, because such concentrations slightly inhibited growth. The flasks were shaken in a water bath shaker at 130 strokes per min and 30°C. When the OD_{600} was 0.50 to 1.0 (an OD_{600} of 1 corresponded to 10^7 cells per ml), we collected the cells on a membrane filter (pore size, 0.45 μm), washed them twice with 100 mM MES buffer (pH 5.5) at room temperature, and suspended them in twice-concentrated MN, MN_{-P}, or MN_{-N} or another buffer containing N. We then distributed 5-ml portions into 125-ml prewarmed (30°C) flasks to which we had added 5-ml portions containing different amounts of carbon sources (in the case of MN), a carbon source plus different amounts of potassium phosphate (in the case of MN_{-P}), or a carbon source plus different amounts of a nitrogen source (in the case of MN_{-N}). The time of this cell distribution was designated zero time. We followed growth by the increase in OD_{600}; when the OD_{600} was too high for measurement, a sample was diluted in water to obtain an OD_{600} of less than 0.6. At different times, we also measured the pH with a pH meter, or we centrifuged samples in a microfuge (Eppendorf) and used the supernatant to determine the remaining concentration of the added carbon compound. Under a phase-contrast microscope, we determined the frequency of asci per cell (cells included asci, mother cells, and buds counted separately); at least 400 and usually 1,000 cells were evaluated for each sample.

Carbohydrate assays. Pyruvate was determined by the method of Friedemann and Haugen (9), acetate

was determined by the method of Holz and Bergmeyer (14), and ethanol was determined by the method of Bernt and Gutmann (1). Glucose was estimated by using Diastix (Ames Co.).

RESULTS

To determine the growth of strain Y55 on different carbon sources, we first adapted the cells to each carbon source by overnight growth from a low inoculum in MN medium (MES buffer, pH 5.5, and yeast nitrogen base) supplemented with a concentration (100 mM or more) of the desired carbon source which was high enough to prevent sporulation during the initial growth (see above). When the culture reached an OD_{600} of 1, we washed and suspended the cells in MN medium without a carbon source and added samples to flasks containing different amounts of the same carbon source with which the cells had been grown previously. The growth curves showed two distinct types of behavior, depending on the carbon source used. On limiting acetate or ethanol, the cultures grew at rates (doubling times for acetate and ethanol, 3.5 and 4.5 h, respectively) which did not depend on the concentration of the carbon compound until it was essentially used up; subsequently, the OD_{600} remained constant (Fig. 1a and b). In contrast, the cultures grew at different exponential rates with different concentrations of pyruvate or dihydroxyacetone (Fig. 1c and d). (When cells, adapted to grow in dihydroxyacetone, were transferred to MN alone, the OD_{600} decreased slowly; presumably, such cells contained unusually weak walls, which tended to rupture [lyse].)

The growth behavior on limiting galactose was intermediate (Fig. 1e). At low galactose concentrations (<2 mM), the OD_{600} increased at rates that depended on the galactose concentration; the increase stopped within 3 h. At higher galactose concentrations, the OD_{600} initially increased at a rate (doubling time, 2.5 h) which did not depend on the concentration; when the galactose concentration had decreased to a low value, the growth rate decreased, and growth stopped within 2 h.

With different concentrations of glucose, the cultures showed typical biphasic growth (22,

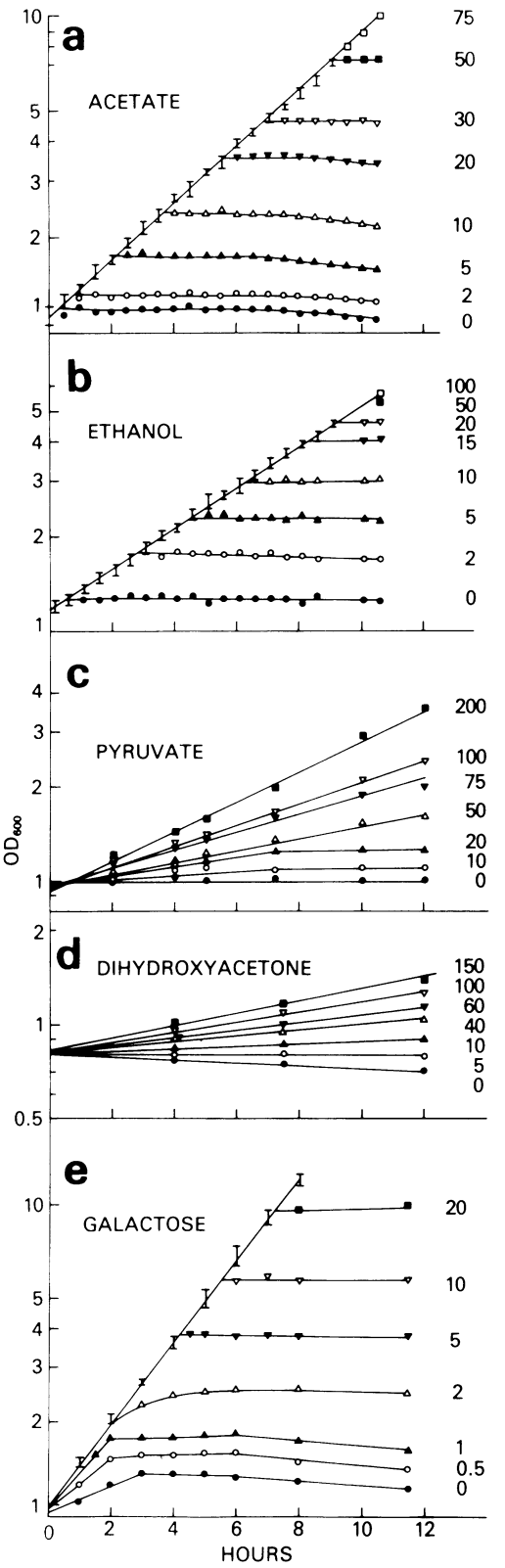

FIG. 1. Growth on different carbon sources. Strain Y55 was grown from a low inoculum in MN supplemented with 100 mM carbon source (300 mM for pyruvate and 150 mM for dihydroxyacetone). At an OD_{600} of about 1, the cells were washed twice on a membrane filter with MES buffer (pH 5.5) and then transferred to MN containing test concentrations of same carbon sources; the concentrations of the carbon sources tested are indicated on the right (millimolar) (see text).

27). They grew in MN supplemented with glucose at a high rate (doubling time, 1.5 h) as long as glucose was present. The growth curve then leveled off and, after a lag of 2.5 h, increased again at a slower rate (doubling time, 5 h) until the accumulated ethanol, to which the cells had adapted, was also used up (Table 1). The presence of up to 100 mM ethanol in cultures containing 10 mM glucose did not affect the initial rapid growth rate, and the cultures stopped growing at an OD_{600} of 3, as if they had received only glucose; subsequently, the cultures resumed slow growth, which continued as long as ethanol remained (Table 1).

Sporulation at different concentrations of acetate and ethanol. For scientists who are used to seeing high sporulation frequencies in potassium acetate, we should emphasize that we did not use a rich presporulation medium, which would have produced well-nourished cells that were adapted to enable maximal sporulation (29). Under these conditions it would have been difficult to interpret the results because many nutrients would have been absent after cell transfer to a sporulation medium. The following data enable a comparison. If we grew cells in MN supplemented with 100 mM acetate to an OD_{600} of 1 and then transferred them to 100 mM potassium acetate alone, we observed 20 to 30% asci per cell after 22 h. If we grew the cells in yeast extract peptone containing 100 mM potassium acetate to an OD_{600} of 1 and transferred them to potassium acetate, we observed 60 to 70% asci per cell after 22 h. Thus, our cells had two to three times less "sporulation potential" on acetate than cells grown in yeast extract peptone containing acetate.

TABLE 1. Sporulation in MN containing 50 mM acetate at different pH values[a]

pH	Buffer	Asci/cell (%) after:	
		21 h	46 h
5.5	MES	<0.1	<0.1
6.0	MES	<0.1	<0.1
6.5	PIPES	<0.1	<0.1
6.8	MOPS	<0.1	<0.1
7.0	MOPS	<0.1	0.7
7.15	MOPS	<0.1	3.4
7.2	HEPES	<0.1	6.5
7.3	HEPES	1.3	35.0
7.5	HEPES	<0.1	2.5

[a] Strain Y55 was grown in MN supplemented with 100 mM acetate to an OD_{600} of 1. After washing, the cells were transferred to N supplemented with 0.2 M buffer and 50 mM potassium acetate. The buffers used were MES, piperazine-*N*,*N'*-bis(2-ethanesulfonate) (PIPES), 3-(*N*-mor-pholino)propanesulfonate (MOPS), and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES). The media were adjusted to the desired pHs with KOH and were sterilized by membrane filtration. The pHs were maintained within 0.1 pH unit by adding HCl when needed.

When we transferred cells grown in MN plus acetate to MN supplemented with different acetate concentrations, we observed no spores (<0.1% asci per cell) at any time thereafter. At 20 h after cell transfer, the cultures contained no detectable acetate (<2 mM), even if the initial acetate concentration had been 150 mM (data not shown).

Because acetate has a pK value of 4.76, 26 times more acetate molecules are uncharged at pH 5.5 than at pH 7 (15 versus 0.57%). Transport studies in membrane vesicles have shown that the membrane can be passed by neutral acetic acid molecules much more readily than by negatively charged ions (28). Therefore, the uptake rate of acetate molecules into cells should be reduced by increasing the pH, and at a sufficiently low uptake rate, the cells may be able to sporulate. Because other transport mechanisms and, thus, cell metabolism are impaired at high pH values, sporulation would at best be expected somewhere between the extremes of too much acetate uptake and too much inhibition of general metabolism. Therefore, we measured sporulation in yeast nitrogen base (N) supplemented with acetate at different pH values, using different buffers (to keep the pH relatively constant during the experiment) and different acetate concentrations at each pH value; during the experiment, we adjusted the pH by adding HCl, maintaining it within 0.1 pH unit of the original value. A typical pH dependence of sporulation (for 50 mM acetate) is shown in Table 1. The cells sporulated optimally at a pH of about 7.3. Because we detected no acetate 20 h after cell transfer, all sporulating cells must have entered the developmental phase before that time.

After cell transfer from MN supplemented with 100 mM ethanol to MN supplemented with different concentrations of ethanol, we observed very little sporulation (<0.5% after 24 h) at low (about 1 mM) concentrations of ethanol and no sporulation (<0.1%) at higher ethanol concentrations (up to 46 h) (data not shown). Increasing the pH did not improve sporulation.

Sporulation at different concentrations of dihydroxyacetone, pyruvate, and galactose. In contrast to the results with acetate and ethanol described above, we observed good sporulation 20 h after cell transfer to MN supplemented with intermediate concentrations of pyruvate or dihydroxyacetone (Fig. 2a and b). At the sporulation optimum, the concentration of pyruvate was still 45 mM after 20 h. We did not measure the concentration of dihydroxyacetone, but much of this compound must still have been present at

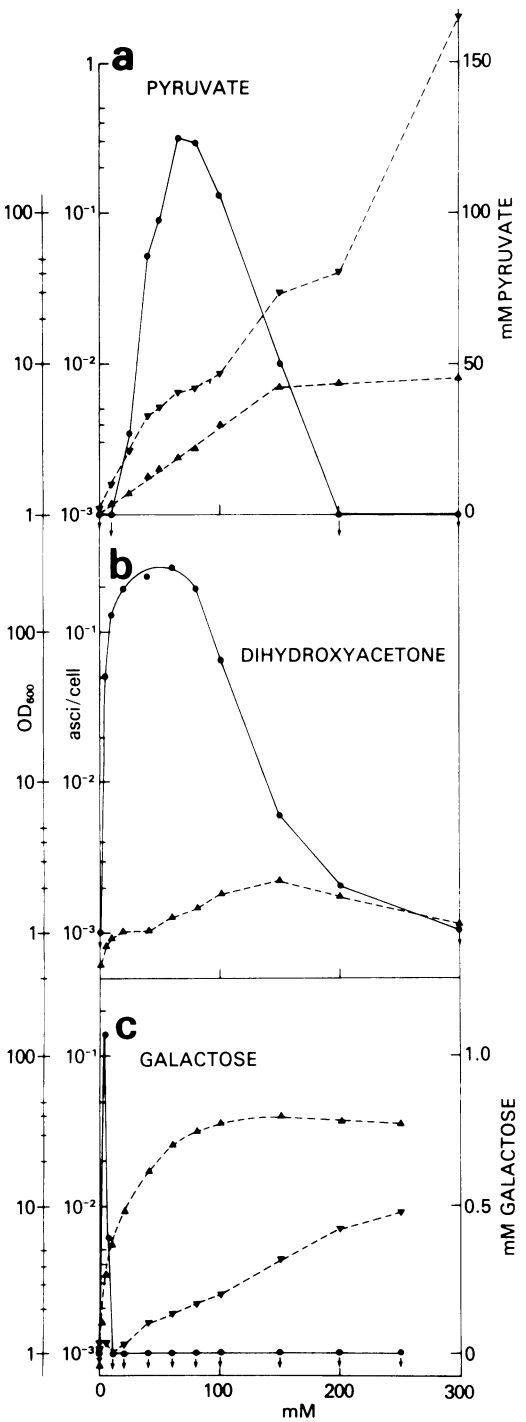

FIG. 2. Sporulation on different carbon sources. Cells were grown and transferred as described in the legend to Fig. 1. At 20 h after transfer, the OD_{600} (▲), the frequency of asci per cell (●), and the remaining carbon source (▼) were measured. In the corresponding experiments with MN supplemented with acetate (0 to 150 mM), no sporulation (<0.1% asci per cell) was observed after 20 h, and all of the acetate was used up at that time. In experiments with MN supplemented with ethanol (0 to 300 mM), no sporulation was observed, except when the initial ethanol concentration was 5 to 25 mM, when the maximal frequency of sporulation was 0.4% asci per cell; all of the ethanol was used up after 20 h.

the time of sporulation because the cells grew on this carbon source as slowly as or more slowly than they grew on pyruvate (Fig. 1).

Like growth, the sporulation response to galactose limitation was intermediate between the two types described above (Fig. 2c). At 20 h after transfer, we observed optimal sporulation in the cultures that initially contained 1 mM galactose and no sporulation in the cultures that initially contained 5 mM or more galactose. At later times, the cultures that initially contained 20 mM or more galactose also sporulated to some extent (Fig. 2c). The remaining galactose concentrations reflected the biphasic behavior of growth and sporulation; after 20 h, the galactose concentration was 80 to 120 μM in cultures which initially contained 1 mM galactose. Paradoxically, the galactose concentration was lower (less than 40 μM) in the cultures whose initial concentrations were somewhat higher (3 to 10 mM). Only if the initial galactose concentrations were higher than 10 mM did significant galactose concentrations remain after 20 h (Fig. 2c).

Sporulation after the consumption of glucose. When we transferred strain Y55 cells (OD_{600}, 0.6) from MN supplemented with 100 mM glucose to MN supplemented with different concentrations of glucose, we observed significant sporulation at intermediate initial glucose concentrations (Table 1). In cultures in which sporulation was observed, the glucose was used up within a few hours. For example, when the initial glucose concentration was 50 mM, glucose was used up after 7 h; after adaptation, the culture continued slow growth on ethanol, and we observed maximal sporulation after about 29 h. When the initial glucose concentration was 100 mM, sporulation was delayed, and when the concentration was 200 mM, no spores were seen (up to 36 h); in the latter case the culture stopped growth before glucose was used up and did not resume growth for 25 h (Table 2).

Because secondary growth on the ethanol produced ceased early when the initial glucose concentration was low (<15 mM), it seemed likely that sporulation of these cultures was prevented simply because they ran out of a gluconeogenic carbon source. Therefore, we also examined sporulation in cultures that initially contained 10 mM glucose and different concentrations of ethanol. As Table 2 shows, the higher amount of ethanol that remained after

TABLE 2. Growth and sporulation in media containing different initial concentrations of glucose and ethanol[a]

Initial glucose concn (mM)	Initial ethanol concn (mM)	OD_{600} at which rapid growth stopped	Lag period before resumption of growth (h)	OD_{600} at which subsequent slow growth stopped	Sporulation		Glucose concn remaining after 13 h (mM)
					Asci/cell (%)	Time (h)	
200	0	9.2	>25.0		<0.1	36	>50
100	0	9.0	5.5	27.0	16.5	36	0
50	0	8.3	4.5	24.0	18.0	36	0
10	0	3.3	3.0	6.5	<0.1	36	0
5	0	2.0	2.9	3.6	<0.1	36	0
10	2	3.1	2.5	7.0	<0.1	33	0
10	10	3.1	2.5	8.3	<0.1	33	0
10	20	3.2	2.5	9.5	0.6	33	0
10	30	3.2	2.5	11.5	0.8	33	0
10	50	3.2	2.5	16.0	2.0	33	0
10	100	3.1	2.5	24.0	11.4	33	0

[a] Cells were grown in MN supplemented with 100 mM glucose to an OD_{600} of 0.8. After washing with MES buffer, the cells were suspended at the same OD_{600} in MN supplemented with different concentrations of glucose and ethanol. The cultures went into lag phase at the stated OD_{600} and resumed growth at a lower rate after the stated lag period, except for the culture containing 200 mM glucose, which did not use up the glucose within the time of the experiment and did not resume growth.

glucose was used up at an OD_{600} of 3 allowed prolonged growth and enabled some sporulation.

Growth and sporulation at different phosphate concentrations. To measure growth and sporulation at different concentrations of phosphate, we prepared phosphate-free yeast nitrogen base (N_{-P}). We grew cells overnight in normal MN supplemented with a carbon source to an OD_{600} of 0.4, washed them extensively with MES buffer, suspended them in MN_{-P} supplemented with a carbon source (300 mM potassium pyruvate, pH 5.5, or 100 mM ethanol), and distributed samples into flasks containing different amounts of potassium phosphate (pH 5.5). After this cell transfer, the OD_{600} continued to increase at fourfold the original rate in all cultures (Fig. 3a). Subsequently, the increase in OD_{600} stopped soon if the new medium contained no phosphate. When phosphate was added, growth continued to a higher OD_{600}, but the growth rate eventually decreased, depending on the phosphate concentration in the new medium (Fig. 3a).

In the ethanol-containing cultures, we observed optimal sporulation for initial phosphate concentrations of 30 to 50 μM. However, when 10% of the cells contained asci, only 1 mM ethanol was left in the 50 μM phosphate culture, and no ethanol remained in the cultures that contained more phosphate (data not shown). To assure in subsequent experiments that sporulation did not result from carbon deprivation, we measured the ethanol concentration throughout the experiment and kept it between 70 and 100 mM by adding ethanol when needed. Figure 3b shows that there was a sporulation optimum at 30 μM phosphate. (When the initial ethanol concentration was 150 or 200 mM, which made it easier to avoid complete ethanol consumption, we observed a sporulation optimum at the same phosphate concentration; however, the extent of sporulation was reduced [maxima of 5 and 2%, respectively] because ethanol inhibited the general cell metabolism, as observed for growth [see above].).

When we transferred pyruvate-grown cells to MN_{-P} supplemented with 300 mM potassium pyruvate and different concentrations of phosphate, we observed after 26 h optimal sporulation (2% asci) for the culture containing initially 5 μM phosphate; after 32 h the optimum (12%) had shifted to 20 μM initial phosphate (data not shown). The pyruvate concentration decreased at a rate that increased with the phosphate concentration, but after 32 h it was still higher than 120 mM for phosphate concentrations up to 5 mM.

We observed no sporulation for up to 42 h when we transferred glucose-grown cells to MN_{-P} supplemented with 100 mM glucose and phosphate at any concentration. (We had to add glucose occasionally to maintain its concentration between 50 and 150 mM.)

Sporulation resulting from nitrogen deprivation. To measure sporulation after nitrogen removal, we transferred cells grown in MN plus a carbon source to the same medium but without $(NH_4)_2SO_4$ (MN_{-N} plus a carbon source); i.e., the medium did not contain any nitrogen source (it still contained excess sulfate). In acetate medium, the cells sporulated quite well after the

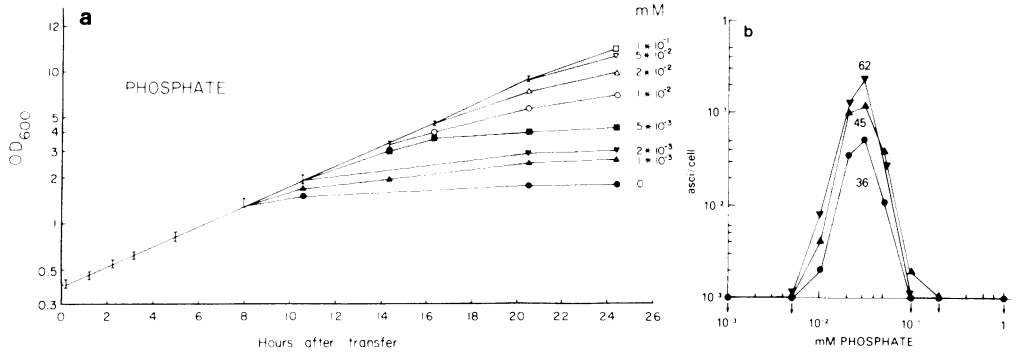

FIG. 3. Growth and sporulation at different phosphate concentrations. Cells were grown in MN supplemented with 100 mM ethanol to an OD_{600} of 0.4. After washing, the cells were transferred to MN_{-P} supplemented with 100 mM ethanol and different concentrations of potassium phosphate (pH 5.5). (a) Growth. The initial phosphate concentrations (millimolar) are shown on the right. (b) Sporulation. When needed, ethanol was added to maintain the concentration between 70 and 100 mM. The frequency of asci was determined 36 h (●), 45 h (▲), and 62 h (▼) after cell transfer. In pyruvate medium, similar results were observed, but the curves were broader and had maxima of 0.2% asci per cell after 26 h and 10% asci per cell after 32 h (initial phosphate concentration in both cases, about 10 μM).

cell transfer (Table 3). However, in ethanol or pyruvate medium, less than 1% of the cells produced spores within 22 h (Table 3).

To determine whether sporulation could be improved by the addition of a nitrogen source, we transferred ethanol-grown cells to MN_{-N} supplemented with 100 mM ethanol and different amounts of $(NH_4)_2SO_4$, KNO_3, alanine, glycine, histidine, lysine, or phenylalanine. The rate of subsequent growth increased with the concentration of $(NH_4)_2SO_4$ or alanine, up to the normal rate (doubling time, about 4 h) at a concentration of either compound of 1 mM or more (Table 4). At lower concentrations the growth curve eventually leveled off at a reduced OD_{600}, indicating that the compound was used up. We observed only a few asci at any concentration of $(NH_4)_2SO_4$ (Table 4). The same was true with alanine at a concentration of 1 mM or less. However, with 2 to 5 mM alanine, the cells eventually stopped their rapid growth and sporulated (Table 4). Because alanine is metabolized via pyruvate, this sporulation could be ascribed to partial carbon deprivation which resulted, after ethanol exhaustion, from the slow metabolism of alanine. KNO_3 was so poor a nitrogen source that it enabled neither growth nor sporulation in MN_{-N} containing ethanol.

Glycine and histidine were poor nitrogen sources and enhanced the residual OD_{600} increase only slightly (Table 4 and Fig. 4). Phenylalanine was a better nitrogen source (Table 4) but also allowed growth only at a low rate. All three of these compounds improved sporulation significantly when they were present at intermediate concentrations (about 1 mM) (Table 4 and Fig. 4); at high concentrations, they suppressed sporulation. Lysine at concentrations of 1 to 10 mM inhibited growth and reduced the frequency of sporulation below the background value; low concentrations (optimal concentration, 0.1 to 0.2 mM) increased the sporulation frequency above the background value by a factor of two at most (data not shown).

When ethanol was replaced by 100 mM glucose (and this concentration was maintained), we observed no sporulation at any concentration of phenylalanine, regardless of whether the cells were grown initially in MN containing 100 mM glucose or in MN containing 100 mM ethanol.

TABLE 3. Sporulation after nitrogen removal[a]

Carbon source before and after cell transfer	Sporulation	
	Asci/cell (%)	Time after cell transfer (h)
Acetate (100 mM)	27.0	22
	35.0	26
Ethanol (100 mM)	0.1–0.5	22
	0.5–1.5	26
Pyruvate (300 μM)	3.7	22
	4.2	26

[a] Cells were grown in MN supplemented with a carbon source to an OD_{600} of 1. After collection on a membrane filter and washing twice with MES buffer, they were suspended in MN_{-N} supplemented with the same carbon source.

TABLE 4. Growth and sporulation with different nitrogen sources[a]

Nitrogen source	Concn (mM)	OD_{600} after 22 to 24 h	Asci/cell (%) after 26 to 28 h	Ethanol concn after 26 to 28 h (mM)
None		1.0–1.7	0.5–1.5	70
KNO_3	1.0	1.0	2.5	72
	10.0	1.2	2.5	70
$(NH_4)_2SO_4$	0.2	1.6	2.5	66
	0.5	4.0	0.4	58
	2.0	8.6	0.2	32
L-Alanine	0.05	1.7	0.3	ND[b]
	0.1	1.7	1.5	ND
	0.5	2.5	2.0	ND
	1.0	2.8	3.5	ND
	2.0	5.8	7.9	ND
	5.0	10.5	11.0	ND
	10.0	19.0	1.0	ND
	20.0	25.0	0.1	ND
L-Histidine	0.01	1.5	0.7	ND
	0.05	1.8	1.7	ND
	0.1	2.0	6.0	ND
	0.5	2.4	10.7	ND
	1.0	2.3	14.5	60
	2.0	2.3	3.7	ND
	5.0	2.0	0.7	ND
	10.0	1.8	<0.1	68
Phenylanine	0.01	2.5	0.4	53
	0.05	2.4	1.7	53
	0.1	2.4	3.0	52
	0.5	3.0	13.0	48
	1.0	4.4	21.5	43
	2.0	5.8	25.0	42
	5.0	9.8	0.2	35
	10.0	11.0	<0.1	32

[a] Cells were grown in MN supplemented with ethanol (100 mM) to an OD_{600} of 1. After collection on a membrane filter and washing twice with MES buffer, they were suspended at the same OD_{600} in MN_{-N} supplemented with different concentrations of the stated nitrogen sources.
[b] ND, Not determined.

DISCUSSION

S. cerevisiae strain Y55 sporulated in a synthetic growth medium (MN plus carbon) if the concentration of the carbon source, the nitrogen source, or phosphate was lowered or if the nitrogen source was replaced by a slowly metabolizable nitrogen source. In all cases, the transfer medium allowed only slow cell metabolism (as measured by the slow increase in OD_{600}). For example, the cells could sporulate in the presence of excess NH_4^+ if the concentration of pyruvate or phosphate was limiting. Therefore, the previously observed suppression of sporulation by NH_4^+ (7, 24) occurs only if the intracellular supply of metabolizable carbon and phosphorus atoms is not limiting. To our knowledge, our results represent the first definite demonstration that sporulation of a yeast can be produced not only by nitrogen deprivation but also by carbon or phosphate deprivation.

After the transfer of cells grown in MN plus different carbon sources to MN alone, the OD_{600} increase stopped abruptly, and all O_2 consumption disappeared within 1 h (data not shown). No sporulation occurred, apparently because the cells did not contain sufficient amounts of a stored carbon source. At the other extreme, when all nutrients were present at concentrations that enabled normal growth, sporulation was also prevented. The intermediate condition of partial carbon deprivation could be obtained only with certain carbon sources. Acetate (at pH 5.5) and ethanol were taken up and metabolized so rapidly that cells grew rapidly in media containing limiting amounts of these carbon sources; growth stopped abruptly when the carbon source was used up, and no sporulation occurred. When acetate growth medium was used at pH 7.3, some sporulation was observed, presumably because at this pH acetate uptake was so slow that the suppression of sporulation was relieved. In contrast, dihydroxyacetone or pyruvate was transported or metabolized so slowly (even at pH 5.5) that the rate at which intracellular metabolites were available depend-

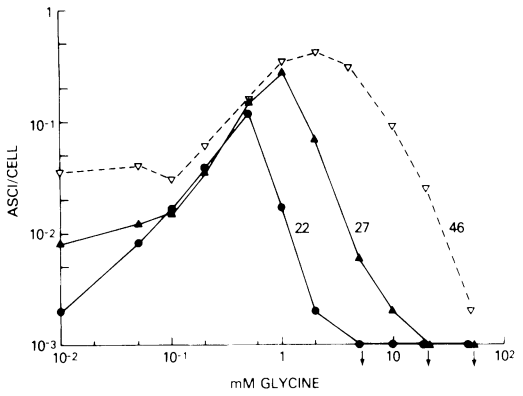

FIG. 4. Sporulation with different concentrations of glycine as the nitrogen source. Strain Y55 was grown in MN supplemented with 100 mM ethanol to an OD_{600} of 1. After being washed, the cells were transferred to MN_{-N} supplemented with 100 mM ethanol and different concentrations of glycine. After 46 h, the concentration of ethanol was still higher than 50 mM in all cultures. The frequency of asci was determined at 22 h (●), 27 h (▲), and 46 h (▽). Sporulation with other nitrogen sources is shown in Table 4.

ed on the extracellular concentration of the compound, as shown by the dependence of the growth rate on the concentration (Fig. 1). At intermediate concentrations of these compounds, a significant fraction of cells sporulated.

The growth and sporulation behavior with galactose was anomalous. After transfer to low galactose concentrations (<2 mM), the cultures grew at rates that depended on the galactose concentration and sporulated (after 20 h) optimally when the initial galactose concentration was 1 mM. At high galactose concentrations (>2 mM), the cultures continued to grow at the original rate until most of the galactose was used up and then entered a stationary phase; during this phasing out of growth, some cells apparently entered the sporulation process and then had a carbon source available to complete ascus formation. Between the low and high galactose concentrations there was a range in which galactose was used up rapidly, and the frequency of sporulation was very low. This biphasic behavior on galactose suggests that a high-affinity galactose transport system (or some metabolic reaction in the galactose pathway) was (partially) repressed at high galactose concentrations and had to be derepressed before cells could efficiently metabolize the galactose present at low concentrations. *S. cerevisiae* is known to have both low-affinity (and perhaps constitutive) and inducible high-affinity transport systems for galactose and certain other sugars (3, 17). Both of these systems seem to enable only facilitated transport (3). The inducible system is missing in *gal2* mutants, which can grow on 2% galactose but not on 0.5% galactose (5), and it is repressed in and inactivated by the presence of glucose (21). The genes coding for the enzymes that metabolize galactose are located at different chromosomal sites than the gene controlling the high-affinity galactose transport system (6); therefore, these genes may be controlled separately. Our results suggest that at least in strain Y55, the low-affinity transport system is so efficient in enabling rapid metabolism of galactose present at high (100 mM) concentrations that the inducible transport system remains repressed. Thus, with respect to galactose transport, strain Y55 cells may respond to high galactose concentrations as they respond to glucose. Before they can efficiently metabolize galactose at low concentrations (less than 2 mM), they apparently have to induce the high-affinity galactose transport system. However, during this adaptation, sporulation development is initiated in a fraction of the cells.

As long as the growth medium contained glucose, the cells could not sporulate, no matter how high the cell density was. However, if the cells were grown in a medium (MN) containing limiting amounts of glucose, they consumed all of the glucose and then adapted, within a few hours, to grow at a lower rate on the ethanol produced by fermentation, as observed previously (22, 27). A fraction of cells entered sporulation during this adaptation process and then used ethanol as a carbon source.

Optimal sporulation was obtained when the initial concentration of glucose was 50 mM (Table 1). When the initial glucose concentration was too low (≤10 mM), so little ethanol was produced that it was used up before the cells could complete spore production. We validated this interpretation by transferring glucose-grown cells to MN containing 10 mM glucose and different concentrations of ethanol; the frequency of subsequent sporulation increased with the ethanol concentration (100 mM was optimal) (Table 2). Because ethanol-adapted cells could not sporulate on ethanol, the partial carbon deprivation that occurred during the lag between the growth on glucose and the slower growth on ethanol apparently was essential for the initiation of sporulation.

Whereas cells growing exponentially in glucose medium can adapt readily to grow in ethanol medium, they cannot do so after transfer to acetate medium (2). It is also known that yeast growing exponentially in glucose-containing media sporulate poorly and do so only several days after transfer to potassium acetate (4, 7, 29). The much slower adaptation to acetate than to ethanol has been attributed to the lack of metaboliza-

ble electron donors (NADH) after glucose removal, causing a deficiency of ATP in acetate medium (2). To obtain good sporulation on potassium acetate as a sporulation medium, either cells have to be grown in glucose medium until all of the glucose is used up (4, 7) and until they have adapted to ethanol, or they have to be grown in an acetate-containing (29) or ethanol-containing glucose-free medium. In both cases acetate can then be used efficiently as a carbon source for sporulation because gluconeogenesis has been enabled.

Miller (24) observed that cells sporulated well after transfer to buffer (0.05 M phthalate, pH 5) containing both 10 mM $(NH_4)_2SO_4$ and 20 mM dihydroxyacetone; in contrast, the presence of $(NH_4)_2SO_4$ reduced sporulation in buffer containing any other carbon source, such as acetate, which by itself allowed sporulation. Sporulation observed with the latter carbon sources apparently resulted from nitrogen limitation, whereas our results indicate that sporulation with dihydroxyacetone in the presence of NH_4^+ resulted from carbon limitation.

After the removal of all of the phosphate from the growth medium, the OD_{600} continued to increase at the original rate by a factor of four, indicating the presence of rapidly available intracellular phosphate (e.g., phosphate stored as polyphosphate) (7, 13). Subsequently, growth stopped, and no sporulation occurred, presumably because further metabolism was severely blocked due to a severe deficiency of phosphate. In contrast, extracellular phosphate at a concentration of 20 to 30 μM apparently was taken up sufficiently slowly that it allowed the initiation of differentiation, and it remained available for a sufficiently long time that ascus development could go to completion.

When the nitrogen source (NH_4^+) was removed from the growth medium, strain Y55 sporulated to varying degrees depending on the carbon source. For example, when cells were transferred to MN_{-N} containing acetate, 27% contained asci after 22 h. This frequency was lower than that previously observed (29) when cells were transferred from yeast extract peptone acetate medium to potassium acetate alone. The much more severe nutritional shock may have initiated sporulation more efficiently than the removal of NH_4^+ alone; for *B. subtilis*, it has been observed that generally a greater shock produces better sporulation (8). Alternatively, in the richer growth medium a larger fraction of the cells may be in a state (e.g., larger cell size) that enables good sporulation on acetate (10); the following results suggest that this state may simply be a larger intracellular pool of nitrogen sources.

After transfer to nitrogen-free medium containing a carbon source other than acetate, only a few cells sporulated. Potassium acetate was selected previously as a sporulation medium because it empirically allowed better sporulation than other carbon sources (7, 25). This optimal effect of acetate does not imply a specific role of acetate in sporulation, but reflects an optimal rate of uptake and metabolism of acetate compared with other carbon sources. We could improve sporulation in ethanol (and pyruvate [data not shown]) if we added one of the slowly metabolizable amino acids glycine, histidine, and phenylalanine (optimal concentration, about 1 mM). Because these amino acids are metabolically unrelated, they presumably increased sporulation by slowly supplying amino groups (by transamination). Thus, even when sporulation is initiated by nitrogen deprivation, the rate at which the available carbon source can be metabolized and the conditions of prior growth (size and pools of cells and their cell cycle distribution) determine how much of a slowly metabolizable extracellular nitrogen source is required for good sporulation.

Vezinhet et al. (33) have described an interesting mutation (*spd1*), which in the homozygous condition enables a diploid yeast strain to sporulate in the presence of excess NH_4^+ and HPO_4^- when the carbon source is 3% glycerol or 1% lactate or pyruvate. These authors concluded that the *spd1* mutation probably decreases a central metabolic function concerned with the utilization of nonfermentable carbon sources. Our results suggest that this metabolic deficiency may shift the extracellular carbon concentration required for optimal sporulation in the presence of NH_4^+ to a higher value than the one observed for wild-type yeast.

Studies with mutants blocked in the cell division cycle (*cdc*) have shown that yeast cells decide in the G1 phase whether to enter mitosis or meiosis or to stop development (11, 30). If nutrition is too scarce (e.g., no carbon source left), the cells remain at the "START" position. If nutrition is abundant, the cells resume mitosis, to which they are committed once the doubled spindle pole bodies have separated before mitotic DNA synthesis (12). If cells are pregrown on gluconeogenic carbon sources and transferred to potassium acetate, they sense a deficiency in nitrogen and enter meiosis, to which they are committed after premeiotic DNA duplication has started and the spindle pole bodies have duplicated (31). Our results indicate that the probability with which cells remain at the START position or enter either meiosis or mitosis may depend on the intracellular concentration of a compound containing carbon, nitrogen, and phosporus atoms, possibly a nucleotide.

The different methods now available for initiating sporulation (by carbon, nitrogen, or phosphate deprivation) provide a test system, in addition to the often used comparison of α/α, a/a, and a/α cells, to determine which cellular changes occur during meiosis and sporulation under all conditions and therefore may be required for this differentiation and which changes are limited to specific conditions, such as the deficiency of nitrogen, and are therefore not absolutely necessary for differentiation. The use of different sporulation media may also enable sporulation of some of the yeast strains which cannot sporulate in acetate alone.

Because we have not been able to obtain sporulation by partial nitrogen or phosphate deprivation in the presence of glucose (or fructose), such rapidly metabolizable glycolytic carbon sources may prevent sporulation by two metabolic effects. One effect is the suppression of sporulation, which is also observed in gluconeogenic growth media, as described above. The other effect may be related to the catabolite repression of the synthesis of many cell components, such as mitochondria and gluconeogenic enzymes. One or more of these components may be required for sporulation. Thus, the control of sporulation in yeasts is more complex than that in *B. subtilis*; in the latter organism sporulation can be induced in the presence of glucose while several inducible enzymes remain repressed (8, 19). This difference might be related to the fact that *B. subtilis* is a strict aerobe which does not produce cyclic AMP, whereas yeasts are a facultative anaerobes which do produce cyclic AMP. The glycolytic carbohydrates that prevent yeast sporulation are also the compounds that allow anaerobic growth and repress the synthesis of the components needed for aerobic growth on gluconeogenic carbon sources.

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