Analysis of lysine-dependent yeast sporulation: a decrease in cyclic AMP is not required for initiation of meiosis and sporulation in *Saccharomyces cerevisiae*

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Cells of the yeast *Saccharomyces cerevisiae* sporulated in nutrient-rich medium containing L-lysine. Sporulation was specific to the presence of L-lysine and was initiated when the cellular content of this basic amino acid reached approximately 0.2–0.5 mmol (g cells)⁻¹, at early stationary phase. The formation of asci was most efficient at pH 7.0 and 50–100 mM L-lysine; in these optimum conditions, the sporulation frequency reached about 60% after 5 d incubation. The L-lysine-dependent sporulation system in nutrient-rich conditions was distinct from the currently used potassium-acetate-dependent system in nutrient-deficient conditions. Analysis of the L-lysine-dependent system indicated that, prior to entrance into meiosis and/or sporulation processes, the yeast cells change in shape, their pool sizes for L-cysteine and glutathione alter, and they synthesize a protein with a molecular mass of 15 kDa. A low level of cAMP was not required for the entrance into meiosis and/or sporulation.

Keywords: *Saccharomyces cerevisiae*, meiosis, sporulation, L-lysine, cyclic AMP

INTRODUCTION

When vegetative cells are transferred to nutrient-deprived medium containing potassium acetate, *a/α* diploid cells of the yeast *Saccharomyces cerevisiae* can enter into meiosis and subsequent sporulation processes. Nutrient-deprived medium containing potassium acetate has frequently been utilized for the analysis of meiosis and/or sporulation processes of yeast cells. However, *a/α* diploid yeast cells have been known to be able to sporulate even in medium with sufficient nutrients; nutritional conditions for sporulation have been extensively studied by Freese *et al.* (1982, 1984) and Olempska-Beer & Freese (1987). Our preliminary study (Kawado *et al.*, 1993) also indicated that *a/α* diploid yeast cells can enter into meiosis and sporulation processes on a nutrient-rich agar medium containing L- or D-lysine.

In the present study, we characterized a novel lysine-dependent sporulation system for *S. cerevisiae* in nutrient-rich conditions, and used the system to examine the hypothesis of Matsumoto *et al.* (1983, 1985) that initiation of meiosis requires a low level of cAMP.

METHODS

Yeast strains. The wild-type *a/α* diploid strains of *Saccharomyces cerevisiae* AKU 4011 (Kawado *et al.*, 1993), 2HY-1 and IFO 2034 were used. Strain AKU 4011 was obtained from the Laboratory of Industrial Microbiology, Department of Food Science and Technology, Kyoto University, Kyoto, Japan, and was used throughout the study. Strains 2HY-1 and IFO 2034 were from Gekkeikan Sake Co., Kyoto, Japan, and were used as controls.

Sporulation conditions. Cells of all three strains were pre-grown at 30 °C overnight in YPD nutrient medium [2.0% (w/v) glucose, 1.0% (w/v) Bactopeptone (Difco), 0.5% (w/v) yeast extract (Difco)]. The pH of the medium was adjusted to 5.0 with 0.1 M HCl. The cells were collected by centrifugation, washed twice in chilled water, and 0.1 ml of the cell suspension (approximately 10⁵ cells) was transferred to 20 ml nutrient-rich sporulation medium (NR medium) in a 50 ml Erlenmeyer flask. NR medium (pH 7.0) consisted of 1.0% (w/v) glucose, 0.3% (w/v) Yeast Nitrogen Base w/o amino acids (Difco), 0.01% (w/v) yeast extract and 100 mM PIPES. To induce sporulation in yeast cells, L-lysine was separately sterilized and added at 0.1 M to NR medium. The medium was shaken at 30 °C (3 cm stroke, 120 r.p.m.) for several days. Growth was determined by measuring OD₆₁₀ after dilution of cultures. The turbidity (OD₆₁₀) can be converted to cell number (*N*, ml⁻¹) using the

equation $N = OD_{610} \times 4 \times 10^6$. Formation of asci was microscopically confirmed and the sporulation frequency was determined by scoring the number of asci in a total cell population of at least 300. Washed YPD pre-grown cells were also transferred to 20 ml nutrient-deprived sporulation medium (ND medium) in a 50 ml Erlenmeyer flask. ND medium consisted of 1.5% (w/v) potassium acetate (pH 7.0) and initial cell concentration was adjusted to approximately 10^7 ml⁻¹. Incubations in ND medium were carried out as above.

Amino acid and glutathione assays. Cells of strain 4011 were washed twice in chilled water, resuspended in chilled water to give a cell concentration of 30 mg (wet wt) ml⁻¹, and 0.1 ml of the suspension was treated at 100 °C for 5 min. The cells were removed by centrifugation and the resulting supernatant was used for amino acid and glutathione assays. Amino acid composition was determined by an amino acid analyser (Hitachi L8500). Total glutathione (reduced and oxidized forms) was determined enzymically by the method of Tietze (1969).

Measurement of cAMP. The cAMP content of cells of strain 4011 was determined as described previously (Kano *et al.*, 1983). Briefly, the cells (0.1 g wet wt) grown in NR medium in the presence or absence of L-lysine were extracted for cAMP with 20 ml 6.0% (w/v) perchloric acid for 20 min at 4 °C. After neutralization with 1.5 M KOH, the mixture was made up to 5.0 ml, centrifuged at 3000 r.p.m. for 10 min, and the resulting clear solution was used for the radioimmunoassay of cAMP by using a cAMP assay kit (Yamasa Shoyu Co.). No appreciable loss or degradation of cAMP was observed during the assay period and duplicate determinations were made for each sample.

Protein analysis. After washing once in chilled water, cells of strain 4011 were suspended in 5.0 mM potassium phosphate buffer (pH 7.5) and disrupted at 5 °C for 4 min by a Braun homogenizer. The homogenate was centrifuged at 25000 g, 4 °C, for 30 min and proteins in the supernatant were analysed by SDS-PAGE (Laemmli, 1970).

RESULTS

Properties of the L-lysine-dependent sporulation system

The properties of L-lysine-dependent sporulation system were determined by using strain 4011.

Effect of pH. The effect of pH on sporulation was determined by varying the pH of NR medium with L-lysine at 0.1 M (Fig. 1). Growth was maximal at pH 6.0, and was completely arrested at pH 8.0. Formation of asci was greatest at pH 7.0, and decreased with the decrease in initial pH of NR medium.

Effect of L-lysine concentration. The concentration of L-lysine in NR medium (pH 7.0) was varied from 0 to 300 mM without significant growth inhibition (Fig. 2). The maximum formation of asci was attained at 100 mM L-lysine. The intracellular L-lysine content increased with extracellular L-lysine concentration and reached a plateau [$1.3\text{--}1.4$ mmol (g cells)⁻¹] when L-lysine was present at more than 100 mM.

Effect of carbon source. Sucrose (1.0%, w/v) was used as a replacement for glucose as carbon source, without any appreciable decrease in sporulation frequency or growth

Fig. 1. Effect of pH on sporulation. Cells were incubated in NR medium containing 0.1 M L-lysine, adjusted to pH 4.0, 5.0, 6.0, 7.0 and 8.0. The final pH of the medium after 5 d incubation is indicated in parentheses in the figure. Growth (OD₆₁₀) (○) and sporulation (●) were determined after 5 d incubation.

Fig. 2. Effect of L-lysine on sporulation. Cells were incubated in NR medium (pH 7.0) containing L-lysine at various concentrations. Growth (OD₆₁₀) (○), sporulation (●) and intracellular L-lysine level (■) were determined after 5 d incubation.

(data not shown). Fructose, maltose, mannose and galactose were also effective, although both growth and sporulation frequency were considerably lowered when these sugars were used at 1.0% (w/v) (data not shown). L-Lysine failed to induce sporulation in ND medium, when added in place of potassium acetate (Table 1). The use of L-lysine together with potassium acetate in ND medium resulted in a marked decrease in sporulation frequency.

Time course of ascus formation. Cells were grown in NR medium (initial pH 7.0) containing L-lysine at various

Table 1. Effect of potassium acetate and L-lysine on induction of sporulation

Cells were incubated at 30 °C for 5 d in nutrient-deprived ND medium (pH 7.0) supplemented with potassium acetate (AcK, 1.5 %, and/or L-lysine (0.1 M). The initial cell concentration was adjusted to approximately 10^7 ml⁻¹. Final pH, sporulation and types of ascus were determined.

Addition to ND medium	Final pH	Sporulation (%)	Types of ascus (%)			
			1-spored	2-spored	3-spored	4-spored
None	6.55	0	—	—	—	—
AcK	9.14	82.6	0	0	5.4	94.5
L-Lysine	7.27	0	—	—	—	—
AcK + L-Lysine	8.01	18.6	0	0	10.4	89.6

Fig. 3. Time course of ascus formation. (a) Cells of strain 4011 were incubated in NR medium (pH 7.0) containing L-lysine [solid lines: 0 (●), 50 (○), 100 (▲), 200 (■) and 300 (□) mM] or potassium acetate [dashed line: 1.5% (w/v) (Δ)]. (b) Cells of other strains [strains 4011 (●), 2HY-1 (○) and 2034 (■)] were also incubated in NR medium containing 0.1 M L-lysine. Growth (OD₆₁₀) (solid and dashed thin lines) and sporulation (solid and dashed thick lines) were determined.

concentrations from 0 to 300 mM (Fig. 3a). Irrespective of the L-lysine concentration, glucose in the medium was lost after 2 d incubation and the pH of the medium remained above 6.2 throughout the incubation period (data not shown). Turbidity of cultures in the medium with L-lysine initially increased, reached a maximum at early stationary phase, slightly declined just before the appearance of asci, and then increased at the final stage of growth. No asci were formed in the medium containing no added L-lysine, or when potassium acetate was used in NR medium in place of L-lysine. In the presence of 0.1 M L-lysine, sporulation reached about 60 % after 6 d incubation. As well as strain 4011, α/α diploid strains of *S. cerevisiae* 2HY-1 and 2034 were also found to sporulate in NR medium containing 0.1 M L-lysine (Fig. 3b). The time courses of sporulation of these strains were very similar to that of strain 4011.

Relations between sporulation and cellular components

A typical sporulation experiment for *S. cerevisiae* 4011 was conducted in NR medium (initial pH 7.0) with 0.1 M L-lysine, and the changes in cell shapes and cellular components were compared with those of cells similarly grown in NR medium in the absence of added L-lysine (Fig. 4). Irrespective of the presence or absence of L-lysine, glucose in the medium was lost after 2 d incubation, and the pH of the medium remained above 6.2 throughout the incubation period.

Sporulation and cell size. The cells in early stationary phase (incubation time 75 h in Fig. 4b) in L-lysine medium were round and larger in diameter than cells in the same growth phase (incubation time 75 h in Fig. 4a) in control medium without L-lysine (Fig. 5, top). The yeast cells sporulated only in the presence of L-lysine and asci, produced after about 6 d incubation (Fig. 4b), contained four spores (Fig. 5, bottom).

Amino acids. Amino acid concentration in cells before the entrance into sporulation in ND medium with L-lysine (culture time 50 and 75 h in Fig. 4b) was determined and compared with that in cells grown for 50 h (Fig. 4a) in the absence of L-lysine (Table 2). In the presence of L-lysine, cellular levels of L-serine, L-cysteine (or L-cystine) and L-lysine significantly increased, whereas those of L-threonine, L-glutamic acid, L-alanine and L-arginine decreased. The formation of asci began at the time when the intracellular L-lysine level reached about 0.5 mmol (g cells)⁻¹ (Fig. 4b), and continued during the period tested.

Total glutathione. A preliminary study indicated that glutathione was a prerequisite for sporulation in nutrition-deprived ND medium containing potassium acetate (Suizu *et al.*, 1994a). In the L-lysine-dependent sporulation system, the tripeptide rapidly disappeared just before the entrance into sporulation, and was almost completely absent during sporulation (Fig. 4b). In control medium

Fig. 4. Changes in cellular glutathione, L-lysine and cAMP levels during sporulation. Cells were incubated in NR medium with (b) and without (a) 0.1 M L-lysine. At the prescribed time, growth (OD₆₁₀) (▲), glucose (Δ), pH (+), sporulation (●) and intracellular concentrations of glutathione (□), L-lysine (○) and cAMP (■) were determined.

Fig. 5. Changes in cell shape during sporulation. Cells were incubated in the absence (left) and presence (right) of 0.1 M L-lysine in NR medium as indicated in Fig. 4. After incubation for 75 h (top) and 150 h (bottom), the cell shapes were microscopically examined. Bar, 10 μm.

Table 2. Change in cellular amino acid concentration during sporulation

Cells were grown in NR medium with and without L-lysine (0.1 M) for 50 and 75 h, which correspond to the culture times shown in Fig. 4. Amino acid composition was determined as described in Methods. ND, Not detected.

Amino acid	Amino acid concn [$\mu\text{mol (g cells)}^{-1}$]		
	Minus L-Lysine		Plus L-Lysine
	75 h	50 h	75 h
Asp	ND	7.33	11.0
Thr	307	151	86.0
Ser	30.7	43.0	107
Glu	483	210	136
Gly	41.7	16.7	12.3
Ala	180	72.3	37.7
Cys	56.0	142	416
Val	46.7	31.0	31.0
Met	ND	2.67	7.33
Ile	16.3	12.3	8.33
Leu	9.33	12.0	9.00
Tyr	7.33	11.7	9.67
Phe	10.7	6.33	14.0
Lys	77.3	430	1370
His	30.7	7.00	9.33
Arg	173	44.7	27.0
Pro	503	6.33	113

without L-lysine, glutathione concentration was slightly increased (Fig. 4a).

cAMP. Irrespective of the presence (Fig. 4b) or absence (Fig. 4a) of L-lysine in NR medium, no appreciable changes of intracellular cAMP level were observed, except for a transient decrease (not more than 15%) in cells grown with L-lysine.

Proteins. Proteins in cells grown in the absence and presence of L-lysine were analysed (Fig. 6). Cells in the early stationary phase in both cultures (incubation time 75 h in Figs 4a and b) gave very similar protein profiles, except for an additional 15 kDa protein in L-lysine-grown cells.

DISCUSSION

Nutrient-deficient medium containing potassium acetate has been utilized for analysis of meiosis and/or sporulation processes of *S. cerevisiae*. This nutrient-deficient medium may be suitable for the analysis of differentiation processes, since it is simple in composition. However, for induction of meiosis and/or sporulation in nutrient-deficient medium, yeast cells have to be transferred from nutrient-rich medium to nutrient-deficient medium. This

Fig. 6. The 15 kDa protein formed in the presence of L-lysine. Cells were grown in NR medium with (Fig. 4b) or without (Fig. 4a) 0.1 M L-lysine, and proteins in the cells were analysed by SDS-PAGE. Lane 1, standard proteins (from top): myosin (H-chain), 200 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; α -chymotrypsinogen, 25 kDa; β -lactoglobulin, 18 kDa; lysozyme, 14 kDa. Lane 2, cells grown for 50 h in the absence of L-lysine; lanes 3 and 4, cells grown for 50 and 75 h, respectively, in the presence of L-lysine (the 15 kDa protein is indicated by an arrow).

abrupt change in nutritional conditions inevitably gives rise to some irrelevant responses other than those specific to meiosis and/or sporulation. Furthermore, sporulation in nutrient-deficient medium is dependent on the concentration of cells transferred from nutrient-rich medium, and yeast cells cannot sporulate when the cell concentration is less than 10^5 ml^{-1} (Yamashita *et al.*, 1994). In contrast to the nutrient-deficient conditions used previously, the nutrient-rich medium supplemented with L-lysine enables yeast cells to enter into meiosis and subsequent sporulation processes in the same medium, and meiosis and/or sporulation events can be analysed continuously.

The analysis of the L-lysine-dependent yeast sporulation system in nutrient-rich conditions revealed some characteristic features of the system and processes of meiosis and/or sporulation.

Properties of the L-lysine-dependent sporulation system

(i) In the course of sporulation in nutrient-deficient medium containing potassium acetate, the pH of the medium increases, roughly from 7 to 9 (Table 1). Since the

yeast cells cannot grow at a pH above 8 (Fig. 1), it is an intriguing question whether the G1 arrest in nutrient-deprived medium is due to the nutrient-deficiency or the high pH value. On the other hand, in nutrient-rich medium with L-lysine, yeast cells can enter into differentiation processes at a physiological pH that allows growth (Figs 1 and 3).

(ii) Sporulation in nutrient-rich medium is completely dependent on the presence of L-lysine in the medium (Figs 2, 3 and 4). Among D- and L-amino acids, only D-lysine can be used in place of L-lysine (Kawado *et al.*, 1993). The sporulation of cells in nutrient-rich medium containing L-lysine is not a unique property of strain 4011, since similar sporulation kinetics were obtained with other, genetically unrelated, α/α diploid strains (Fig. 3b).

(iii) Sporulation in nutrient-rich medium is initiated when the intracellular L-lysine content reaches about 0.2–0.5 mmol [g (wet wt) cells]⁻¹ (Figs 2 and 4b).

(iv) Potassium acetate has no effect in nutrient-rich medium (Fig. 3), and L-lysine has no effect in nutrient-deficient medium (Table 1). This result indicates that the nutritional signal transduction necessary for meiosis and/or sporulation in nutrient-rich conditions is different from that in nutrient-deficient conditions.

Properties of meiosis and/or sporulation processes

(i) The yeast cells become large and round just before sporulation (Fig. 5). The irregular turbidity changes observed in the presence of L-lysine at various concentrations (Figs 3 and 4b) may either reflect the change in cell size during formation of asci, or be an optical effect caused by a change in the proportion of cells with buds in the population.

(ii) Before entrance into meiosis and/or sporulation in nutrient-rich medium with L-lysine, large amounts of L-cysteine were accumulated in addition to L-lysine (Table 2), although glutathione disappeared in association with the onset of sporulation (Fig. 4b). Glutathione has recently been proved to be indispensable for yeast sporulation (Suizu *et al.*, 1994a). The functions of L-cysteine and glutathione are not yet clear.

(iii) No marked changes in intracellular cAMP levels were observed before and after entrance into sporulation processes (Fig. 4b). Matsumoto *et al.* (1982, 1983, 1984, 1985) proposed that initiation of meiosis may require a low level of cAMP or absence of cAMP-dependent protein kinase. Our results obtained in the L-lysine-dependent system go against their hypothesis, and support the results of Olempska-Beer & Freese (1987) and Cameron *et al.* (1988). Olempska-Beer & Freese (1987) showed, in nutrient-rich conditions, that the intracellular cAMP level is irrelevant to meiosis and/or sporulation and that guanine nucleotides have a crucial role as differentiation signals. Cameron *et al.* (1988) also indicated that cAMP-independent mechanisms must exist for regulating sporulation processes, since sporulation of mutants with disruptions in *TPK1*–*TPK3*, genes for

catalytic subunits of cAMP-dependent protein kinase, is still regulated by nutrients. The observed change in cAMP level observed by Matsumoto and co-workers may be due to the stress adaptation of cells suddenly transferred to nutrient-deficient medium from nutrient-rich medium.

(iv) Although a more definitive conclusion on the function of the Ras/cAMP pathway in yeast differentiation processes is required, we attach importance to the function of Ca²⁺ in meiosis and/or sporulation. Ca²⁺ is indispensable for sporulation (Suizu *et al.*, 1994b) and is supplied through the influx of external Ca²⁺ rather than by release from internal calcium stores (unpublished). In this context, the 15 kDa protein that is specifically produced by the yeast cells grown in nutrient-rich medium with L-lysine (Fig. 6) may be significant, since a protein with the same molecular size has been identified as an endogenous substrate for a Ca²⁺/calmodulin-dependent protein kinase in *Fusarium oxysporum* (Hoshino *et al.*, 1992).

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