Acetate Utilization and Macromolecular Synthesis During Sporulation of Yeast

MICHAEL S. ESPOSITO,1 ROCHELLE E. ESPOSITO,3 MARYVONNE ARNAUD, AND H. O. HALVORSON

Laboratory of Molecular Biology and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 14 July 1969

Acetate utilization and macromolecule synthesis during sporulation (meiosis) of Saccharomyces cerevisiae were studied. When diploid cells are transferred from glucose nutrient medium to acetate sporulation medium at early stationary phase, respiration of the exogenously supplied acetate proceeds without any apparent lag. At the completion of ascospore development, 62% of the acetate carbon consumed has been respired, 22% remains in the soluble pool, and 16% is incorporated into lipids, protein, nucleic acids, and other cell components. Measurements of the rate of protein synthesis during sporulation reveal two periods of maximal synthetic activity: an early phase coincidental with increases in deoxyribonucleic acid, ribonucleic acid, and protein cellular content and a later phase during ascospore formation. Experiments in which protein synthesis was inhibited at intervals during sporulation indicate that protein synthesis is required both for the initiation and completion of ascus development.

Under appropriate conditions, sporulation can be induced in nearly all of the members of a diploid yeast population. The process is complex and involves meiosis and subsequent ascospore formation. At present, there is little biochemical information on the differences between meiosis and mitosis and the regulatory events which determine whether a diploid cell will initiate one or the other of these processes. The recent demonstration of numerous recessive conditional mutants in S. cerevisiae (8a; M. S. Esposito and R. E. Esposito, Genetics, vol. 60, Abstr., p. 176, 1968) and in the fission yeast S. pombe (2), which prevent meiosis and sporulation but which permit mitotic division, suggests that these two modes of nuclear division are under separate genetic control and hence may differ biochemically in a number of respects.

The present paper summarizes experiments designed to define the physiological stages of sporulation of homothallic strains of S. cerevisiae which permit isolation of meiotic conditional mutants (8a). To characterize the physiological changes associated with sporulation, the course of macromolecular synthesis and acetate utilization during sporulation were investigated.

MATERIALS AND METHODS

Yeast strains. The following homothallic diploid strains of Saccharomyces were employed in this study:

\[ \begin{align*}
S41 & \quad D \quad arg-4 \quad acr-1 \\
S & \quad D \quad arg-4 \quad acr-1 \\
z140-9A & \quad D \quad lys-2 \\
\alpha & \quad D \quad lys-2
\end{align*} \]

The D gene confers a homothallic phenotype by virtue of its action during the mitotic divisions following ascospore germination. It causes directed mutations of either the \( \alpha \) or \( \alpha \) mating type allele to its opposite form; mating ensues and the spore colony produced consists primarily of diploid cells (D. C. Hawthorne, Proc. 11th Intern. Congr. Genetics, Vol. 1, Abstr., p. 34, 1963). Symbols are as follows: \( a, \alpha \), mating type alleles; \( D \), diploidization gene (16); \( arg \), arginine auxotroph; \( acr \), actidione (cycloheximide) resistance; \( lys \), lysine auxotroph.

Media. The amounts of the various ingredients are those required for the preparation of 1 liter of medium. Yeast extract peptone (YPE) consisted of 20 g of dextrose, 20 g of Bacto-peptone, and 10 g of yeast extract. Sporulation medium I consisted of 20 g of potassium acetate and 1 g of yeast extract. Sporulation medium II consisted of 20 g of potassium acetate and 75 mg of l-arginine hydrochloride. Sporulation

1 Present address: Department of Biology, University of Chicago, Chicago, Ill.
2 Present address: Collegiate Division of Biology, University of Chicago, Chicago, Ill.

180
medium III consisted of 20 g of potassium acetate and 75 mg of L-lysine hydrochloride. To solidify any of these media, 15 g of agar was added.

**Sporulation and counting procedure.** Diploids to be sporulated were inoculated into liquid YEP medium at an initial cell density of $5 \times 10^6$ cells/ml and incubated with shaking at 30 C. The cells for this initial inoculum were obtained from 2-day-old colonies grown on solid YEP medium. The cells were harvested by filtration after 24 to 26 hr of growth in YEP (early stationary phase), washed twice with sterile distilled water, and suspended in sporulation medium at ca. $5 \times 10^7$ cells/ml. Tetracycline (20 mg/liter) was routinely added to sporulating cultures to retard bacterial contamination. The kinetics of the appearance of ascis in these cultures was determined by hemacytometer counts. For these estimates, no attempt was made to distinguish buds from cells; each was counted as a cell. This procedure was adopted because it eliminates subjective judgments regarding the total cell number which must be made when budded cells are classified as one or two cells, depending upon the size of the bud in relation to the mother cell. The sporulation frequencies obtained by this counting procedure are minimal estimates of the percentage of cells which have sporulated, since not all buds contain nuclei. Sporulating cultures which could not be counted immediately were fixed in 4% Formalin and refrigerated. Increases in cell number due to vegetative division did not occur in the sporulation media employed.

**Measurement of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein.** DNA was assayed colorimetrically with the diphenylamine reagent (3); DNA from Mann Chemical Co. served as a standard. DNA was extracted from samples of $4 \times 10^6$ to $10 \times 10^6$ whole cells by the method of Ogur and Rosen (12). Nucleic acid was extracted from each sample at least twice to insure total recovery of DNA. RNA was assayed by the orcinol method described by Ceriotti (4); yeast RNA was used as a standard. Colorimetric determinations of protein content were performed with the Folin phenol reagent as described by Lowry et al. (10), with bovine serum albumen used as a standard.

**Isotope incorporation.** Acetate uptake by sporulating cells was determined by using $1^4$C-acetate. For each sample, approximately $4 \times 10^6$ cells which had been exposed to labeled sporulation medium were washed twice and resuspended in 2 ml of water. A sample of this cell suspension was then introduced directly into scintillation fluid for counting. From these samples, the soluble pool, lipids, nucleic acids, and proteins were extracted. The soluble pool was extracted by boiling the 2-ml water suspensions for 10 min. The samples were centrifuged, and a portion of the supernatant fluid was removed for counting. Lipids were extracted from the pellet by heating in 2 ml of ethyl alcohol-ether (3:1) for 3 min at 60 C. Two ethyl alcohol-ether extractions were performed, and the supernatant fluids were combined and counted. Nucleic acids were extracted by 10 min of boiling in 2 ml of 5% trichloroacetic acid; a sample of the supernatant fluid obtained from this extraction was also counted. Proteins were extracted from the remaining material by overnight incubation in 2 ml of 0.1 M NaOH at 37 C. A portion of each of these final supernatant fluids was counted to measure incorporation of acetate carbon into protein. The residue of cellular debris was then collected on membrane filters (0.45 um pore size; Millipore Corp., Bedford, Mass.), dried overnight at 65 C, and suspended in scintillation fluid for counting. CO2 evolution resulting from acetate utilization was measured by trapping the CO2 evolved by sporulating cells in 0.3 M NaOH solutions; a sample was then introduced into scintillation fluid for counting.

Measurements of the incorporation of $H$ or $1^4$C or $1^4$C-amino acids as an index of protein synthesis were done as described by Rodenberg et al. (13). Preparation of unbudded cell fractions. To obtain a single cell fraction, approximately $1.5 \times 10^6$ cells, obtained from early stationary-phase growth in YEP medium, were washed twice in sterile distilled water and suspended in 3 ml of 12% sucrose. This suspension was layered on the surface of a 40-ml, 12 to 40% linear sucrose gradient which was centrifuged at room temperature for 4 min at 1,600 x g. The top 20 ml of the gradient was discarded, and the next 10 ml was collected and washed twice in sterile distilled water. Whereas unfractionated cell populations routinely have approximately 37% single unbudded cells, the fractions so prepared contain greater than 80% single unbudded cells.

**Dry weight measurements.** The dry weight of cells was determined by collecting a known number of cells on tared filters (Millipore). The filters were dried in a vacuum oven at 85 C for 48 hr and weighed.

**Materials.** The chemicals were obtained from the following suppliers: acetate-$L-1^4$C (13.7 mc/mmmole), acetate-$Z-1^4$C (34 mc/mmmole), $H$-$L$-lysine (252 mc/mmole), $L$-lysine (175 mc/mmole), and $1^4$C-$L$-phenylalanine (355 mc/mmmole) from Schwarz Bio-Research Inc., Orangebury, N.Y.; actidione from The Upjohn Co., Kalamazoo, Mich.; and tetracycline from K & K Laboratories, Jamaica, N.Y.

**RESULTS**

**Fregrowth and sporulation.** To determine the period during vegetative growth when cells transferred to sporulation medium produce ascis rapidly and to a maximal level, cells were removed at intervals from a culture of diploid S41 growing in YEP medium and were transferred to sporulation medium. The growth curve of S41 in YEP medium is shown in Fig. 1. The percentage of ascis present after 24, 48, and 72 hr of incubation is summarized in Table 1. Cells transferred to sporulation medium during early log phase (sample 1) did not yield ascis by 24 hr, though ascis eventually appeared. As early stationary phase is approached (samples 2 through 5), the rapidity with which ascis are formed and the final percentage of ascis observed reached maximal levels.

**Biochemical changes during sporulation.** Changes in the DNA, RNA, and protein content
TABLE 1. Effect of physiological age on sporulation

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Hr in growth medium</th>
<th>Cells/ml in growth medium</th>
<th>Percentage of asci at 24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19.5</td>
<td>7.2 \times 10^7</td>
<td>0</td>
<td>12</td>
<td>63</td>
</tr>
<tr>
<td>2</td>
<td>21.5</td>
<td>1.4 \times 10^8</td>
<td>38</td>
<td>59</td>
<td>68</td>
</tr>
<tr>
<td>3</td>
<td>23.5</td>
<td>1.6 \times 10^8</td>
<td>51</td>
<td>69</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>25.5</td>
<td>1.7 \times 10^8</td>
<td>52</td>
<td>70</td>
<td>78</td>
</tr>
<tr>
<td>5</td>
<td>27.5</td>
<td>1.7 \times 10^8</td>
<td>52</td>
<td>70</td>
<td>78</td>
</tr>
</tbody>
</table>

* See Fig. 1 for collection of samples.
* Percentage of sporulation at 24, 48, and 72 hr in sporulation medium of diploid S41 withdrawn from growth medium at time indicated.

of cells undergoing sporulation are summarized in Fig. 2A. DNA synthesis occurred from T = 11 to T = 15 and achieved a percentage increase equivalent to the total ascus production (Fig. 2B). The RNA and protein content per cell increased to maximal levels at T = 15 and subsequently declined. By 50 hr, the protein cell content returned to its initial value, whereas the RNA cell content remained approximately 20% higher than the value observed at T = 0. During sporulation, the dry weight of cells increased 60% over the initial value by T = 15 and slowly declined thereafter (Fig. 2B).

Before the appearance of asci, the pH of the supernatant medium increased from 7.0 to 8.5.

Acetate metabolism. At the period of growth in nutrient medium, when transfer of diploid S41 to sporulation medium results in rapid ascus formation, the population consisted of approximately 37% single unbudded cells and 67% budded cells and higher multiples. It was of interest to inquire whether a single cell fraction prepared from the total population would demonstrate the same rate of acetate consumption and ascospore development as an unfractioanted population. A single cell fraction obtained by sucrose density centrifugation, as previously described, and an unfraccionated cell population were compared with respect to the rate of CO₂ evolution upon transfer to sporulation medium containing acetate-\^1^4C (Fig. 3). The single cell fraction and the unfractioanted cells sporulated at the same rate and to the same level (65%).

Evolution of CO₂ from acetate oxidation began without any apparent lag (Fig. 3 insert). The initial rate of CO₂ evolution of both populations was identical, indicating that a single cell fraction is not different from the overall population with respect to ability to respire acetate upon transfer to sporulation medium. With time (after T = 15),
however, the rate at which acetate is respired by the single cell fraction decreased, so that by T72
only 380 μmoles of acetate-derived CO₂ evolved
in comparison to 500 μmoles by the unfractio-
nated cells.

The utilization of acetate during sporulation
has been studied by utilizing acetate-2-¹⁴C. For
these experiments, cells from an early stationary-
phase population of diploid S41 were introduced
into sporulation medium containing the isotope
and the evolution of ¹⁴C-CO₂, and the incorpo-
ration of acetate by cells was followed (Fig. 4). The
evolution of CO₂ proceeds linearly through the
completion of ascus development, whereas the
total incorporation of acetate by cells achieves a
maximal level by T₃₄. The distribution of the ace-
tate incorporated by cells (Fig. 4) into the soluble
pool, lipids, proteins, nucleic acids, and residue
of cellular debris is shown in Fig. 5. The kinetics
of incorporation of acetate-2-¹⁴C carbon into
protein and nucleic acids closely follow the bulk
increases in cell content of these macromolecules.
The incorporation of acetate into lipids proceeds
in two stages. The first stage is completed by
T₁₀, while the second phase occurs from T₂₃
to T₃₈. Exclusive of the soluble pool, the incorpo-
ration of acetate-derived carbon into the residue
fraction is proportionately greater than into other
cell components.

FIG. 3. Evolution of CO₂ during sporulation of
strain S41. Abscissa is micromoles of CO₂ evolved per
6.4 × 10⁶ cells in sporulation medium II containing
acetate-1-¹⁴C (1.33 μc per 20 mg per ml); (O) single
cells, (●) unfractinated cells. Insert: short-term
 evolution of CO₂. (The rate of ascus formation of the
unfractinated cells and of the single cell fraction was
identical to that shown for diploid S41, Fig. 2B.)

Protein turnover and protein synthesis during
sporulation. Cells of diploid Z140-9A were grown
in YEP containing ³H-L-lysine and then trans-
ferred to unlabeled sporulation medium. After
an initial incorporation of ³H-L-lysine (from the
soluble pool) into proteins, the ratio of ³H-lysine
counts per minute at Tₓ/To (Fig. 6) steadily
decreased from T₁₃ to T₃₈ and then reached a
plateau (Fig. 6A). This result is compatible with the
net changes in protein content of cells during
sporulation (Fig. 2A).

To determine whether the rate of protein
synthesis varies during sporulation, sporulating
cells were pulse-labeled with ¹⁴C-L-lysine through-
out the sporulation cycle. At intervals, a sample
of sporulating cells was withdrawn from a single
sporulating culture, isotope was added, and sam-
ple were removed for counting at 0, 1.5, and 3
hr after addition of isotope. The results of this
experiment are summarized in Fig. 6B. Two pe-
riods of maximal protein synthesis were observed
at T₄ to T₇ and T₂₃ to T₃₇. The latter peak period
of protein synthesis is coincidental with the second
phase of acetate-2-¹⁴C incorporation into lipids
(Fig. 5).
Inhibition of protein synthesis during sporulation. As shown in Fig. 6, although protein synthesis occurs throughout sporulation, its rate varies considerably. To determine whether continuous protein synthesis is required for sporulation, actidione (cycloheximide), an inhibitor of protein synthesis, was added to sporulating cultures and its effect on ascospore production was observed.

When actidione is added to cells (sensitive to this inhibitor) in sporulation medium, the onset of protein synthesis as measured by the incorporation of $^{14}$C-L-lysine into protein is prevented (Table 2). To ascertain the effect of inhibiting protein synthesis at various times during sporulation, a series of sporulating cultures of diploid Z140-9A, prepared from the same starting inoculum, were exposed to actidione at several different times after introduction into sporulation medium. The results of this experiment are summarized in Fig. 7. When protein synthesis is inhibited before $T_{14}$, no asci are formed. When actidione is added after asci have begun to appear, ascus formation proceeds for a short time and then ceases. The total percentage of asci present at

![Fig. 5. Incorporation of acetate-2-$^{14}$C into the soluble pool, lipid fraction, nucleic acid, protein, and residue extracts during sporulation of strain S41. Medium: sporulation medium II containing acetate-2-$^{14}$C (1.33 μC per 20 mg per ml).](image)

![Fig. 6. Protein synthesis and turnover during sporulation of strain Z140-9A. (A) Protein turnover. The culture was grown in YEP medium containing 2.5 μC of $^{3}$H-L-lysine/ml. When the culture reached the early stationary phase, the culture was harvested, washed, and suspended in sporulation medium III. The trichloroacetic acid-precipitable counts were measured initially ($T_{0}$) and at intervals ($T_{1}$) after the culture was transferred to sporulation medium. The ratio of $T_{2}/T_{0}$ is plotted as a function of time during sporulation. (B) Rate of protein synthesis. At intervals during sporulation in medium III, lysine-$^{14}$C-s. (0.9 μC/ml) was added and the rate of isotope incorporation was followed into hot trichloroacetic acid-precipitable material over a 3-hr period. The bars refer to the average rate of lysine incorporation over the 3-hr period for each sample.](image)

**TABLE 2. Effect of actidione on protein synthesis during sporulation**

<table>
<thead>
<tr>
<th>Hr in sporulation medium</th>
<th>Percentage of asci</th>
<th>Incorporation of lysine$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−Actidione</td>
<td>+Actidione$^a$</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>36.3</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>49.7</td>
<td>0</td>
</tr>
<tr>
<td>32</td>
<td>70.0</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>71.0</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ Nanomoles of lysine incorporated into trichloroacetic acid-precipitable material per 7.8 $\times$ 10$^9$ cells

$^b$ Cells of Z140-9A were placed in sporulation medium III containing $^{14}$C-L-lysine (0.9 μC/ml) and, where indicated, actidione (50 μg/ml).
T₂₅ in cultures exposed to actidione (at the times indicated by the arrows) is indicated by the dashed line in Fig. 7. Continued ascus formation after the addition of actidione is an index of the fraction of cells that had already completed the protein synthesis required for ascus formation at the time actidione was added.

**DISCUSSION**

Studies on meiosis in yeast reported here and elsewhere (6, 7) lead to the suggestion that sporulation is dependent upon acetate metabolism and on the physiological age of the cells when transferred to sporulation medium. The experiments reported here demonstrate that, when cells are transferred to sporulation medium at the optimal period of growth for sporulation, evolution of HCO₃⁻ from acetate oxidation proceeds without any apparent lag. These results indicate that glucose-grown cells at the appropriate physiological age for sporulation readily respire acetate. The data are in agreement with previous studies of the initial Qₒ₂ of cells transferred to acetate sporulation medium, which suggested that sporulation competency is dependent in part upon the development of the oxidative ability which appears during the latter stages of growth in glucose medium (6-8). Further evidence in behalf of this interpretation comes from the observation that logarithmic-phase cells grown in acetate nutrient medium display sporulation competency upon transfer to sporulation medium, whereas logarithmic-phase glucose-grown cells of the same strains sporulate slowly or not at all (14).

The incorporation of acetate-²⁻¹⁴C carbon into macromolecular fractions indicates that the acetate consumed is an important source of metabolites for the biosynthetic reactions which occur during sporulation. At T₁₀, when ascus development is essentially complete, 62% of the acetate consumed has been respired as CO₂, 22% is present in the soluble pool, and 16% has been incorporated into macromolecules or cellular debris. The residue of cellular debris which remains after extraction of lipids, nucleic acids and proteins retains approximately 21% of the acetate incorporated into cells. The nature of this material remains to be elucidated.

Meiosis and sporulation in yeast, like sporulation in bacteria (1, 9, 11, 15), is associated with extensive protein turnover. As shown here, the protein content of sporulating cells first increases and then declines as sporulation proceeds. Experiments with H-lysine-labeled cells (Fig. 6) clearly demonstrate that the fluctuations in protein content are a combination of protein synthesis and turnover. Synthesis is continuous throughout sporulation and shows two maxima, one before ascii appear (T₅₀) and a second during ascospore development (T₂₂). The observation (Fig. 7) that the inhibition of protein synthesis at any time before the completion of ascus development reduces the frequency of ascii produced indicates that the protein synthesis observed is necessary for sporulation. These data suggest that the proteins required are continually produced in individual cells until the last stages of ascospore assembly. Further experiments are needed to test this hypothesis and to determine whether the essential gene products are produced in an ordered or random sequence.

The physiological changes (effect of physiological age on the capacity to sporulate and the change in DNA, RNA, protein, and dry weight) which accompany meiosis and ascus formation of homothallic diploids of Saccharomyces are similar to the events which have been described for heterothallic diploids (5-7). Thus, the use of homothallic diploids which facilitate study of the genetic control of meiosis do not appear to introduce additional complexities into this system.

**ACKNOWLEDGMENTS**

This investigation was supported by Public Health Service research grants AI-0459 from the National Institute of Allergy and Infectious Diseases and GM-34,380 and GM-34,344 from the National Institute of General Medical Sciences. An NIH Research Career Professorship was awarded to one of us (H.O.H.).
LITERATURE CITED