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# Timing and Function of Chitin Synthesis in Yeast

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A temperature-sensitive mutant of *Saccharomyces cerevisiae*, L-2-42, is blocked at 37 C at a stage of the cell cycle prior to septum formation. When single cells of the mutant are allowed to bud at 37 C in a medium containing tritiated glucose, a large incorporation of radioactivity into chitin takes place. Thus, the synthesis of chitin, the major component of the primary septum, is initiated in a phase of the cell cycle which precedes septum closure. This early period of chitin synthesis is not required for emergence and growth of buds because, in the wild type, budding takes place normally in the presence of concentrations of polyoxin D that effectively and specifically prevent chitin formation. However, at a later time a majority of these cells lyse, presumably because of the inability to form a septum. Polyoxin D also prevents the appearance of enhanced fluorescence at the junction between mother cell and bud, as observed in the presence of a brightener. Therefore, the fluorescence is due to chitin and its presence at the base of very early buds indicates that chitin synthesis begins at or shortly after bud emergence. A scheme for chitin synthesis and primary septum formation which embodies these and other results is presented.

Previous work from this laboratory has established that chitin is the main and possibly the exclusive component of the primary septum of yeast (5). This specialized function of chitin is in agreement with its discontinuous synthesis during the yeast cell cycle (4), as measured in a synchronized culture. However, the difficulty in achieving perfect synchronization has precluded establishment of the precise time at which chitin synthesis begins. It has been suggested recently (9, 14) that this time is very close to that of bud initiation. This notion is based on the fact that a fluorescent ring has been observed at the junction between mother cell and bud in the presence of primulin, or "brighteners," at the time of bud emergence (9, 14). The fluorescence has been attributed to chitin (9), nevertheless conflicting views have been expressed (14) since fluorescent brighteners also bind to other polysaccharides (10).

The experiments reported in this paper were designed to answer two questions: (i) at what point in the yeast cell cycle does chitin synthesis begin? and (ii) is chitin synthesis required for the emergence and growth of buds or only for septum formation?

## MATERIALS AND METHODS

**Chemicals.** Polyoxin D and Calcofluor White M2R {disodium salt of 4,4'-bis[4-anilino-bis(*beta*-hydroxyethyl) amino-*s*-triazine-2-ylamino]-2,2'-stilbene disulfonic acid} were generous gifts of the Kaken Chemical Co., Tokyo, Japan, and of the American Cyanamid Co., Bound Brook, N.J., respectively. D-[6-$^3$H]glucose, specific activity 8.45 $\mu$Ci/$\mu$mol, and Aquasol were purchased from New England Nuclear. Chitinase was purified as described previously (2). Giemsa stain R66 was obtained from Biomedical Specialties, Santa Monica, Calif.

**Strains and growth conditions.** *Saccharomyces cerevisiae* strains S288C (ATCC 26108), X2180 (ATCC 26109, isogenic diploid of S288C), and L-2-42 were used. L-2-42 is a temperature-sensitive mutant derived from S288C by mutagenesis with ethyl methane sulfonate and isolated as a strain which yields lysed cells at 37 C but not at 25 C (3). The mutant grows well in both rich or minimal medium, with a growth rate about two-thirds that of the parent. In the experiments described below the yeast was grown in a minimal medium (1) at the temperature indicated in each experiment.

**Isolation of single cells and counting.** Unbudded cells were isolated from a logarithmic phase culture by centrifugation on a sucrose density gradient (12). The number of budding cells in the isolated fraction was less than 1%. The single cells were washed with water, suspended in 1% NaCl containing 50 $\mu$g of aureomycin per ml, and stored overnight in the refrigerator before use, except where otherwise indicated.

For counting, samples of cultures were diluted into 1.7% formaldehyde, and plastic tubes containing the resulting suspension were subjected to sonic oscillation in the cup of a Taytheon oscillator for 2 min at half-maximal intensity. This treatment was sufficient to break up all clumps with minimal lysis of cells. In the experiments with polyoxin D, the sonic treatment was omitted to avoid disrupting partially lysed cell

pairs (1). The clumps that formed when lysis was extensive were broken up by adding to each sample 0.05 volume of 20% sodium dodecyl sulfate and vortexing. Cells were counted in a hemocytometer. Any visible bud, however small, was counted as a cell. The percentage of abnormal cells produced in the presence of polyoxin D was determined as previously described (1).

**Labeling of cell wall polysaccharides.** In the experiments with mutant L-2-42, $2 \times 10^7$ counts/min of D[6-$^3$H]glucose was added to each milliliter of a suspension containing $1.5 \times 10^7$ unbudded cells per ml in minimal medium. Half of the suspension was incubated in a shaking bath at 24 C, the other half at 37 C. Samples were withdrawn at appropriate time intervals, and the radioactivity in mannan, glucan, and chitin was determined as previously described (1).

In experiments with *S. cerevisiae* X2180 and polyoxin D, the unbudded cells were incubated overnight at 30 C in the presence of 0.7% yeast nitrogen base (Difco) and 50 μg of aureomycin per ml. After centrifuging, they were resuspended at a concentration of about $10^7$ cells/ml, in minimal medium containing $2 \times 10^7$ counts/min of D[6-$^3$H]glucose per ml, and with or without 1 mg of polyoxin D per ml. Incubation was at 37 C in a shaking bath. After 5.5 h the cultures were removed, and radioactivity in the cell wall polysaccharides was measured as previously reported (1), except for omission of the separation between abnormal and apparently normal cells.

In every case, the size of the sample was sufficient to yield between 1,000 and 6,000 counts/min in actual countings, except for the diacetylchitobiose from cells inhibited with polyoxin D, where about 100 counts/min were found.

In all these experiments, cell growth and production of abnormal cells were monitored, as described above, in controls lacking the radioactive glucose.

**Observation of fluorescence.** Cells were suspended in 0.01% Calcofluor and centrifuged. The subsequent operations were carried out at 37 C, in a room maintained at that temperature. The cell pellet was resuspended in 0.01% Calcofluor containing 0.5% agar and 3 μl of the suspension was placed between slide and cover slip and sealed with Permount (Fisher Scientific Co.). The slides were allowed to cool at room temperature, whereby the agar solidified and the cells remained immobilized, thus facilitating photography. Observations were made with a Leitz Ortholux microscope with a Phloem Illuminator and an HBO 200/W4 light source. A red suppression filter (BG38), 2 excitation filters (KP 490), and a barrier filter at 530 nm were used.

**Nuclear staining.** Nuclei were stained with Giemsa according to Robinow (13), but using the ribonuclease treatment recommended by Hartwell (7) in place of hydrolysis in hydrochloric acid.

**Electron microscopy.** Samples were prepared for electron microscopy as described (1).

## RESULTS

**Chitin synthesis before septum closure.** To determine whether chitin synthesis precedes closure of the septum, a temperature-sensitive mutant was used that is blocked at a stage of the cell cycle prior to septum formation. This mutant, L-2-42, was isolated using a screening method that detects colonies which produce lysed cells at 37 C (3). Nevertheless, when the mutant was allowed to grow in liquid culture, little or no lysis was noticed until incubation was prolonged for 12 h or more. Instead, the cells collected as doublets in which the bud had reached the same size of the mother cell (Fig. 1), whereafter both cells continued increasing in

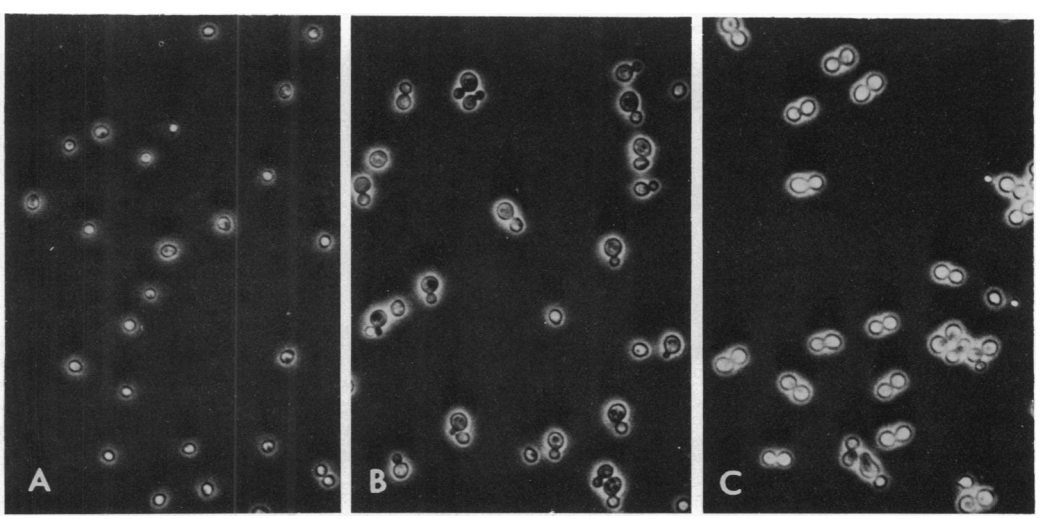


FIG. 1. *Morphology of S. cerevisiae mutant L-2-42. (A) Unbudded cells obtained by gradient centrifugation. (B) After growth for 10 h at 24 C. (C) After growth for 10 h at 37 C.* ×450.

volume. In these doublets, nuclear division had arrested at the stage where a single nucleus is at the neck between mother and daughter cells, in some cases partially protruding into the bud (Fig. 2). Observations in the electron microscope confirmed the absence of septa (Fig. 3). Only 7 out of 158 sagittal sections observed showed a septum. Thus, although no genetic study of this mutant was carried out, it appears that L-2-42 is akin to the nuclear division mutants isolated by Culotti and Hartwell (6).

To study the formation of chitin in the mutant, single, unbudded cells were isolated by centrifugation in sucrose density gradients (see above) and allowed to grow either at 24 or 37 C in a medium containing tritiated glucose (Fig. 4). After a 5-h lag, the cells began to bud. At 37 C the cell number doubled and then remained constant, whereas the total number of cell groups (singlets at the beginning and doublets later) remained essentially unchanged. At 24 C, growth was slower but continued after the first division, and the number of cell groups steadily increased. Some synchronization could be observed at this temperature. Samples were taken at the times shown in Fig. 4, and the

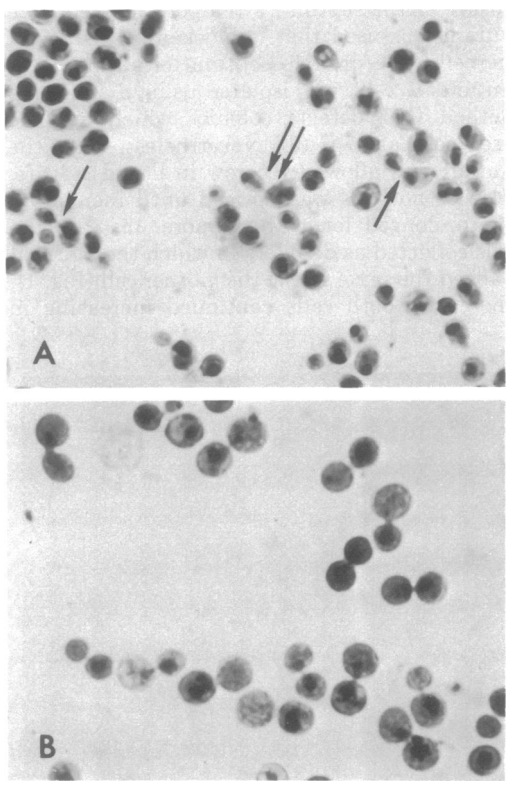


FIG. 2. *Giemsa staining of mutant L-2-42. (A) After growth at 24 C. Cells are seen in which nuclei are partially (arrows) or totally (double arrows) separated. (B) After growth at 37 C. Nuclei remain in the mother cell and are often beginning to enter into the bud. ×1060.*

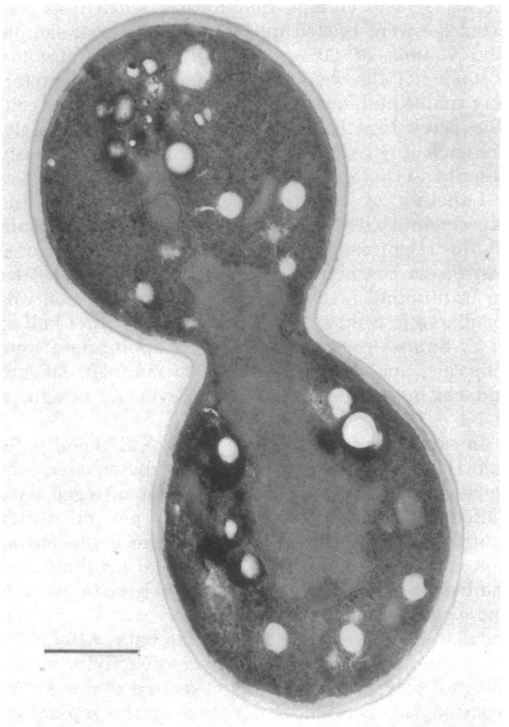

FIG. 3. *Electron micrograph of mutant L-2-42 blocked in cell division at 37 C. No septum is visible in the very large cells and the channel between mother cell and bud is quite wide. Scale line is 1 μm.*

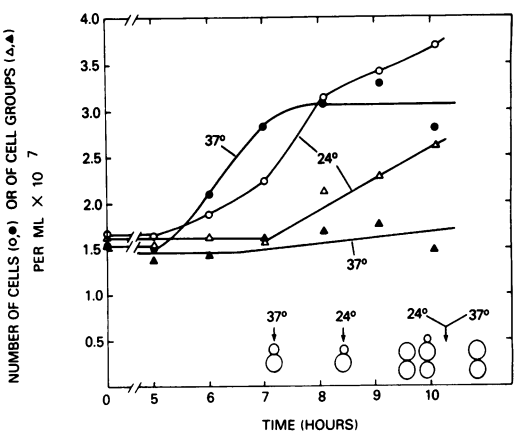


FIG. 4. *Growth of mutant L-2-42 at 24 and 37 C, beginning with a population of unbudded cells. Cells were counted as indicated in the text. Arrows indicate the time at which samples were withdrawn for the experiment of Table 1; the predominant morphology in each case is depicted.*

incorporation of tritium into mannan, glucan, and chitin was measured (see Table 1). The amount of radioactivity recovered in the first two polysaccharides was similar, although glucan tended to be more heavily labeled at 37 C. At 24 C, the ratio of chitin to glucan or mannan radioactivity resembled that found in the wild type (see below; Table 2). Surprisingly, the incorporation into chitin was even greater at 37 than 24 C, despite the absence of a septum at the higher temperature. The even larger value observed in the late sample of the 37 C culture may be due to the fact that, although division was blocked, the cells continued increasing in size (see above); this is also reflected in the higher radioactivity of mannan and glucan in the same sample (Table 1). In particular, the neck region between mother cell and bud, where chitin is presumably located, appears to be much wider after incubation at the nonpermissive temperature (see Fig. 3).

**Effect of polyoxin D on cell development.** According to the results described above, it would appear that the bulk of chitin synthesis takes place before the septum is closed. However, it was not clear whether formation of chitin was required for the emergence or growth of the bud. To answer this question, the effect on budding of the antibiotic polyoxin D, an inhibitor of chitin synthetase (1) was examined. In previous work with this antibiotic, a nonsynchronous culture was used and the specific effect on bud emergence could not be observed (1). In the present series of experiments, single, unbudded cells of the wild-type diploid, X2180, were incubated in growth medium in the presence of polyoxin D. The antibiotic did not prevent or reduce budding, as can be seen in Fig. 5. In fact, in several experiments the rate of the first budding was invariably as high as in the control culture (Fig. 6). Examination by electron microscopy of the cells budding in the presence of polyoxin did not reveal any obvious abnormality. Even that portion of the cell wall which is at the junction between mother cell and bud, a point where chitin would be normally deposited, did not show any visible change (see Fig. 7). However, at a later stage of the cell cycle, abnormal cells of the type already described (1) began to appear, and after 5 h, 60% of the population was found as "refringent" and "exploded" pairs (Fig. 6, lowest panel). Simultaneously, a sharp decrease in the rate of growth (upper and middle panel of Fig. 6) took place. Although a complete cessation of cell division was observed in this experiment, a sustained but very slow division rate was main-

TABLE 1. *Incorporation of radioactivity in cell wall polysaccharides of mutant L-2-42*[a]

| Cells analyzed[b] | Mannan counts/min per cell ($\times 10^6$) | Glucan counts/min per cell ($\times 10^6$) | Chitin counts/min per cell ($\times 10^6$) |
|---|---|---|---|
| Early sample at 25 C | 33 | 33 | 1.7 |
| Late sample at 25 C | 41 | 41.5 | 1.75 |
| Early sample at 37 C | 30 | 45 | 2.1 |
| Late sample at 37 C | 49 | 63 | 4.8 |

[a] For details of the procedure see the text.

[b] For the time at which the samples were withdrawn see Fig. 4.

TABLE 2. *Effect of polyoxin D on the incorporation of radioactivity from [$^3$H]glucose into cell wall polysaccharides*[a]

| Cells analyzed | Mannan counts/min per cell ($\times 10^6$) | Glucan counts/min per cell ($\times 10^6$) | Chitin counts/min per cell ($\times 10^6$) |
|---|---|---|---|
| Control | 71 | 67 | 2.0 |
| Polyoxin D | 67 | 51 | 0.2 |

[a] For experimental details see the text. At the time cells were withdrawn for analysis (5.5 hour of incubation) about 60% of them were abnormal.

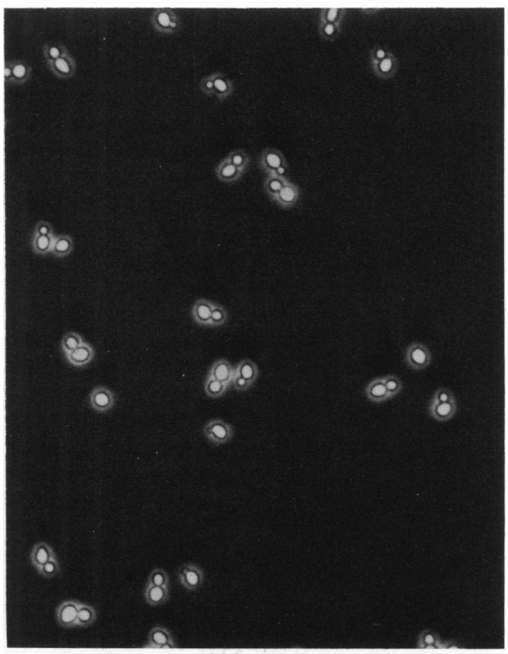

FIG. 5. *Cells of S. cerevisiae X2180 budding in the presence of 1 mg of polyoxin D per ml. The culture was begun with unbudded cells.* ×430.

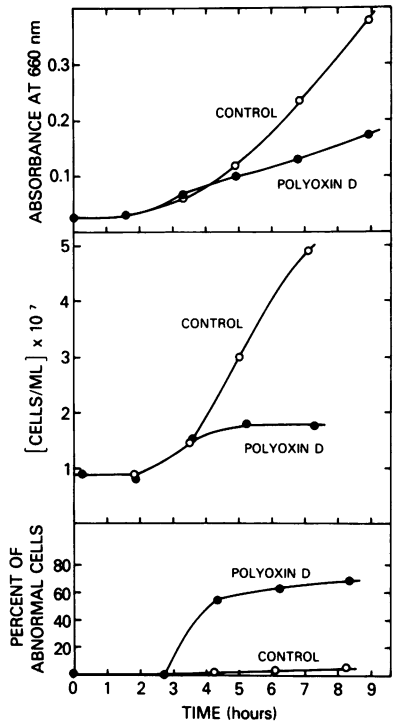


FIG. 6. *Growth of S. cerevisiae X2180 in the presence or absence of polyoxin D at 37 C. Cell number was determined as explained in the text.*

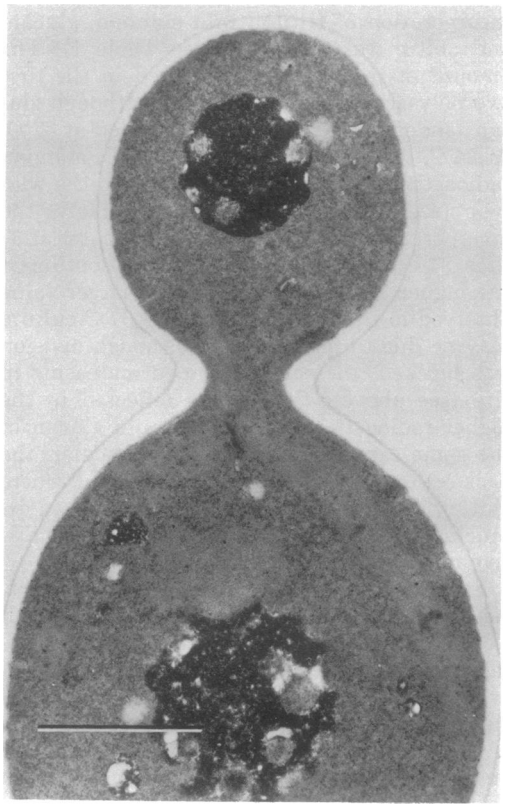

FIG. 7. *A section of a cell budding in the presence of polyoxin D. No obvious anomaly is noticed and the cell wall at the mother cell-bud junction appears to be normal. Scale line is 1 μm.*

tained in other instances. In every case, the surviving cells recovered and started growing again later, as already reported (1).

It remained to be determined how effectively the antibiotic had inhibited chitin synthesis. With tritiated glucose in the medium and in the presence of polyoxin D, the incorporation of radioactivity into the acetylglucosamine polysaccharide was 90% inhibited as compared to the control, whereas normal values were found for mannan and glucan (Table 2). In this case, incorporation was measured in the total population, without prior separation of "normal" from abnormal cells, as was done previously (1). However, the inhibition of incorporation into chitin was the same as measured earlier in isolated abnormal cells. It may be concluded that even the apparently "normal" cells in the culture were almost devoid of chitin. Thus, chitin synthesis does not appear to be required either for bud emergence or for subsequent growth of the bud. Its absence, however, leads to disastrous consequences at the time of cell division, the period during which the inward growth of the septum normally occurs.

**Effect of polyoxin D on the fluorescence caused by brighteners.** As shown in the preceding section, cells budded in the presence of polyoxin D are devoid of chitin while containing a normal amount of the other components of the cell wall. With the use of these cells it became possible to determine whether the enhanced fluorescence observed in the presence of certain dyes at the junction between mother cell and bud was indeed due to chitin.

The single cells of strain X2180 used for the experiment showed only a weak fluorescence in the presence of Calcofluor M2R. The fluorescence was uniformly distributed on the cell wall, with the exception of an occasional slightly brighter spot probably due to a birth scar (Fig. 8a). After budding in normal medium, a bright area at the neck between mother and daughter appeared (Fig. 8b). The fluorescent area could be already seen at the base of very small buds (Fig. 8b) as noted by Hayashibe and Katohda (9). The resolution of the method did not allow us to decide whether the fluorescence was concomitant with bud emergence.

At later stages in bud development the

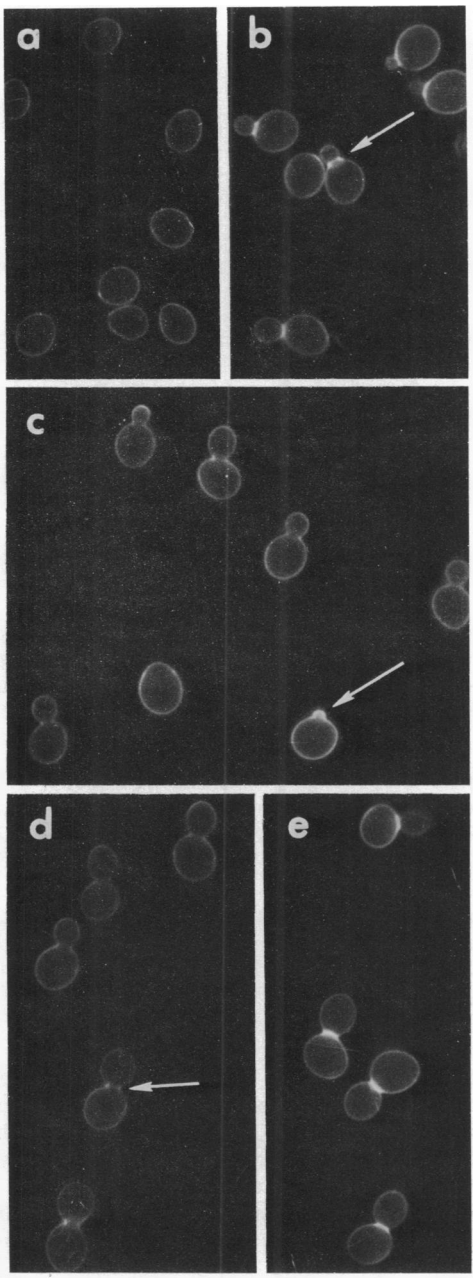


FIG. 8. *Cells of S. cerevisiae X2180, grown with and without 1 mg of polyoxin D per ml and observed in the fluorescence microscope in the presence of Calcofluor White M2R. (a) Unbudded cells used to start the culture. The cell wall shows a low and uniform fluorescence with an occasional brighter spot which may correspond to a birth scar. (b) Early budding in normal medium. A bright line is observed between mother cell and bud (arrow). By focusing at different levels it was possible to observe that the bright area is in the form of a ring around the constriction. (c) Early budding in medium containing 1 mg of polyoxin D per ml. No enhancement of fluorescence is visible at the base of the bud. A bright spot (arrow) is observable at the tip of a very young bud. (d) Late budding in polyoxin D-containing medium. In some cases, bright points can be seen on both sides of the constriction (arrow) but no fluorescent line is observed at the location of the septum. (e) Late budding in normal medium. The bright line between two cells of approximately equal size remained usually unchanged at various focusing levels, an indication that at this stage the primary septum had been probably completed.*

fluorescent zone appeared as a line completely dividing the two cells (Fig. 8e). Cells budding in the presence of polyoxin D lacked the fluorescent ring (Fig. 8c), although two bright spots could be seen occasionally on both sides of the neck between mother cell and bud, at the later stages of cell division (Fig. 8d). Curiously, a bright spot was occasionally observed at the very tip of an emerging bud in the presence, but not in the absence, of polyoxin D.

## DISCUSSION

The first question asked in the present investigation was when in the yeast cell cycle chitin synthesis begins. Since the available evidence indicated that chitin is the principal component of the primary septum (5), one would have expected chitin to be made just before cell separation. Nevertheless, strain L-2-42, at a temperature which does not allow septum completion, shows as much or more incorporation from [$^3$H]glucose into chitin as at the permissive temperature during budding. Therefore, it appears that the bulk of the chitin is synthesized before the septum closes. There remains the possibility that, in this mutant at 37 C, chitin was laid down at an abnormal location. We tried to look into this possibility by observing L-2-42 in the fluorescence microscope in the presence of Calcofluor, but the results were inconclusive, because the cell wall of the single cells used to start the culture was rather strongly fluorescent and the difference between the neck region and the remainder of the cell contour was largely obliterated.

On the other hand, the fluorescence at the junction between mother cell and bud was quite striking in the wild-type diploid. The suppression of this effect by polyoxin D, a reagent that specifically abolishes chitin synthesis, clearly indicates that the acetylglucosamine polymer is related to the localized enhancement of fluorescence, as proposed by Hayashibe and Katohda (9). Glucan may originate the less intense luminosity of the remainder of the cell wall (10).

A bright ring of fluorescence is observed with Calcofluor even in very young buds, thereby confirming the conclusion reached with mutant L-2-42 that chitin synthesis starts much earlier than septum formation.

Is this early synthesis required for the emergence and growth of the bud? The idea has been advanced by several laboratories (9, 14), including ours (2), that the early establishment of a rigid chitin ring around the constriction between mother cell and bud may contribute to the reinforcement of the channel and thereby help to maintain it open during growth of the bud. If the chitin ring has that function, it appears to be one which can be dispensed with, as spectacularly demonstrated by the observation that almost complete inhibition of chitin synthesis and disappearance of the fluorescent ring in the presence of polyoxin D does not affect the ability of cells to bud or that of buds to grow. Nevertheless, difficulties appear later in the division cycle for these cells, in that the lack of chitin synthesis prevents completion of the septum and, as a consequence, at least 70% of the population dies. Those cells which survive are later able to bud again, and after overnight incubation, the culture attains stationary phase with as many cells as in the absence of polyoxin. When the experiment was performed in the presence of tritiated glucose, the incorporation of label in chitin per cell in the overnight culture with polyoxin was identical to that of the control (not shown). This apparent recovery from the effects of polyoxin has been mentioned previously (1) and remains unexplained. It has been reported that peptides inhibit the action of polyoxin D on intact cells by an unknown mechanism (11) and it is possible that enough peptides are released from lysed cells in the presence of polyoxin to protect the remaining cells from the action of the antibiotic and permit their recovery.

The results obtained in the present and in previous studies (5) on the course of chitin synthesis and of septum formation are summarized diagrammatically in Fig. 9. In this scheme, only the primary septum is shown and the plasma membrane and secondary septa (1) have been omitted for reasons of clarity. As shown in Fig. 9a, a chitin ring is formed at the base of the bud very soon after its emergence. It is indeed possible, as proposed by Hayashibe and Katohda (9) and by Hasilik (8), that chitin synthesis is concomitant with bud emergence, but our results are insufficient to clarify this point. As growth of the bud progresses (Fig. 9b), a point is reached at which chitin extends both inwards and outwards (Fig. 9c, stippled areas)

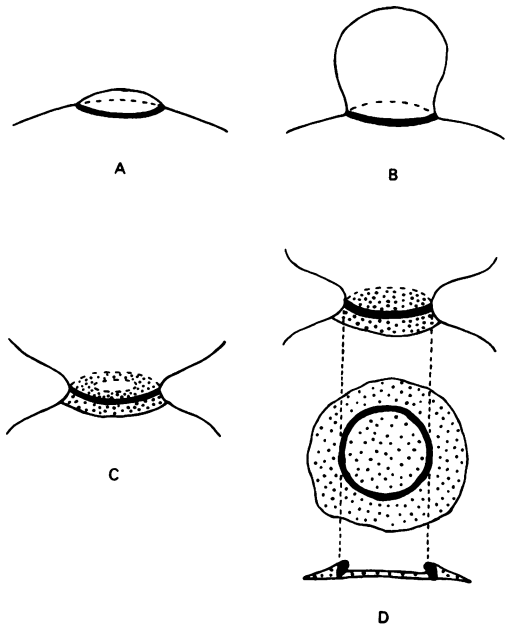


FIG. 9. *Scheme of chitin synthesis and formation of the primary septum. The dark ring and stippled areas represent chitin. In D, projections of the septum are included, to show how it would look from above or in section, after flattening on a horizontal plane.*

until the septum is closed (Fig. 9d). It is possible, although unlikely, that this last phase does not require continued synthesis of chitin, since it may be that the plasma membrane in closing inwards pulls with it the already accumulated chitin. Electron micrographs show the centripetally growing septum covered by plasma membrane (1) but do not permit conclusions as to which is "pushing" or "pulling" or whether both are growing independently in coordinated fashion.

Projections in Fig. 9d show the finished septum flattened on a plane and either seen from above or in section. These drawings resemble the electron micrographs of the chitin layer found inside the bud scar (see Fig. 3 and 5, reference 2), and illustrate the fact that the bulk of the chitin may be found in the ridge which represents the original ring of early buds. The concentration of material at the ridge is emphasized by its much higher electron density in unstained whole mounts of isolated bud scars (Fig. 4, reference 2). The high incorporation of label into chitin in strain L-2-42 thus may be explained if most of the polysaccharide is already present at the ring, before septum closure.

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