ASPECTS OF BUDDING IN THE YEAST, SACCHAROMYCES CEREVISIAE, AS STUDIED THROUGH THE USE OF CELL-DIVISION-CYCLE AND KARYOGAMY-DEFICIENT MUTANTS.

by

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BUDDING IN YEAST USING MUTANTS AS PROBES

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ABSTRACT

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Aspects of budding in the yeast, Saccharomyces cerevisiae, as studied through the use of cell-division-cycle and karyogamy-deficient mutants.

Cell-division-cycle and karyogamy-deficient mutants were used as probes to study certain aspects of budding. Fluorescent staining with FITC-Con A, for mannan, or with calcofluor and DAPI, for chitin and nuclei, revealed interesting relationships. Mutant cdc-24-1 grown at 37°C and kar- crosses both produce bi- or multinucleate cells. In most binucleate cells, the two nuclei lie close together and divide into the same bud and in a few, the nuclei are far apart, and most often produce one or two buds, proximal to a nucleus which will divide into it. In cdc-24-1 cells, the former type gives a typical "haploid" budding pattern even in large cells. The proximity of daughter nuclei in most blocked cdc-24-1 cells suggests a role for the CDC-24 product in spindle elongation. The relationship between the nuclei and the location and number of buds suggests a preponderant role for the nucleus in budding. Although buds develop preferentially in regions of low chitin content in kar- crosses, the ability of cdc-24-1 cells to bud even with a uniformly high content of chitin and mannan suggests a secondary role for the cell wall in determining the sites of bud emergence. The chitin ring is not needed for bud emergence, but seems to play a role in normal bud development and in septum formation.

RESUME

M.Sc. Mario Lachapelle Plant Science

Aspects of budding in the yeast, Saccharomyces cerevisiae, as studied through the use of cell-division-cycle and karyogamy-deficient mutants.

Des mutants déficients dans leurs cycles de division cellulaire, ou en karyogamie, ont été utilisés pour sonder certains aspects du bourgeonnement. L'application des teintures fluorescentes FITC-Con A, pour les mannans ou calcofluor et DAPI, pour la chitine et les noyaux, ont permis la révélation d'intéressantes relations. Le mutant cdc-24-1 cultivé à 37°C et les croissements kar- produisent tous deux des cellules à noyaux doubles ou multiples. Dans la plupart des cellules binuclées, les deux noyaux sont accolés et peuvent se diviser dans un bourgeon unique. Moins fréquemment, les noyaux sont éloignés et peuvent produire un ou deux bourgeons, chacun à proximité d'un noyau pouvant se diviser dans celui-ci. La proximité des noyaux filles dans la majorité des cellules cdc-24-1 soumises au bloque suggère un rôle pour le produit CDC 24 dans l'élongation du fuseau. La relation existante entre les noyaux et le nombre et la location des bourgeons suggère un rôle prépondérant pour le noyau lors du bourgeonnement. Quoique les bourgeons émergent de préférence dans des régions pauvres en chitine chez les croissements kar-, l'habilité des cellules cdc-24-1 à bourgeonner dans des régions à contenu élevé en chitine et mannan suggère un rôle secondaire de la paroi cellulaire dans la détermination des sites de bourgeonnements. L'anneau de chitin n'est pas nécessaire à l'émergence du bourgeon mais semble jouer un rôle dans le développement normal de celui-ci ainsi que dans la formation du septum.

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I. INTRODUCTION

Mitosis and budding are two processes which are capable of occurring separately in the yeast Saccharomyces cerevisiae (Hartwell, Mortimer, Culotti and Culotti, 1973), but must be coordinately regulated if vital cellular functions are to be maintained (Johnston, Pringle and Hartwell, 1977).

Specifically, mitosis is a process, most often though not necessarily accompanied by cell division, in which the daughter nuclei possess the same chromosomal constitution as the parental one. Budding, a typical asexual reproductive process of yeast, involves the formation of a new cell, the bud, as an outgrow of the mother-cell. Furthermore, only a limited number of daughter cells are produced by each parental cell (Mortimer and Johnston, 1959).

In yeast, the formation of a bud is not a random event, and must be the result of complex processes involving a large number of subcellular structures. For instance, the location of the nucleus and its associated structures, the 10-nm "microfilament" ring, the chitin ring, the CDC 24 gene product and the competency of the cell wall, have all been more-or-less implicated in the selection of a budding site, one of the first steps in the formation of a daughter-cell. However, not until more is known about the relationships existing between these various components will it be possible to fully comprehend their relative importance. Indeed, the discovery of relationships might indicate the essentiality of certain

components of budding. This would lead to a better understanding of how morphogenetical processes are controlled and regulated.

Clues indicating the importance of studying such relationships have accumulated during the past two decades. Some of them will be briefly described here.

While studying the budding process of S. cerevisiae, Byers and Goetsch (1975) suggested that associated structures of the nucleus such as the spindle pole bodies (SPBs) and extranuclear microtubules played a crucial role in the emergence of the bud. Studies of temperature-sensitive cell-division-cycle (ts-cdc) mutants, however, had shown that although essential; SPB material duplication alone was not sufficient to explain bud emergence (Byers and Goetsch, 1974).

Furthermore, since bud scar pattern appears to be genetically influenced by the mating-type locus (Hicks, Strathern and Herskowitz, 1977; Sloat, Adams and Pringle, 1981), the CDC 24 locus (Sloat, Adams and Pringle, 1981), and the homothallism/heterothallism locus (Hicks, Strathern and Herskowitz, 1977), these genes must be somehow implicated in budding. That the ploidy level is also an important factor has been shown by Winge (1934), Freifelder (1960), Streiblová (1970), and Hicks, Strathern and Herskowitz (1977).

Apart from the participation of intracellular components and of gene products, the cell wall, an extracellular structure, may play an active or passive part in the selection of a bud site. Changes in the cell wall of mother cells at the onset of budding have been observed (Seichertová,

Beran, Holan and Pokorný, 1975). Assuming that budding pattern is not random and that a bud cannot form twice at the same site, Byers and Goetsch (1975) questioned the competency of certain cell wall regions to respond to budding signals. Additional evidence provided by cdc-24 mutant yeast strains (Sloat and Pringle, 1978; Sloat, Adams and Pringle, 1981) strengthens the probability that competent cell wall regions are involved in the budding process since these mutants are unable to bud at restrictive temperature and have in their cell wall extensive amounts of chitin.

Considering first that the inability of the above mutants to bud might be attributed to a failure to organize chitin into normal rings (Sloat, Adams and Pringle, 1981), and second that the latter seems to precede bud emergence (Hayashibe and Katohda, 1973; Sloat and Pringle, 1978), some investigators have assumed a major role for the chitin ring in the process of budding. This assumption is, however, difficult to reconcile with studies involving chitin inhibitors (Bowers, Levin and Cabib, 1974; Cabib and Bowers, 1975) where budding was observed without ring formation. The methods employed in these studies have been criticized (Sloat and Pringle, 1978) and now it is widely believed that the chitin ring is essential for budding. Results presented in this thesis challenge this position and suggest other functions for the ring.

Certain aspects of budding were investigated by taking advantage of characteristics of temperature-sensitive cell-division-cycle (cdc-24; Hartwell, Culotti and Reid, 1970) and karyogamy-deficient (kar-1; Conde and Fink, 1976) mutant strains. Mutant cdc-24-1 grown at restrictive

temperature and kar-1 crosses both produce bi- or multinucleate cells whose mitotic behavior in relation to budding and changes in the pre-budding cell wall was followed and compared. The karyogamy-deficient mutant system, which mimics some of the nuclear changes produced by the cdc-24-1 system without corresponding cell wall modification, provides a useful control. In this way, it has been possible to see how the difference in the location and synchrony of nuclei within the same cell might influence near-by cell wall and consequently the budding pattern.

Mutants are now considered as classical tools for learning about various processes such as budding. However, numerous workers are concerned about the validity of the use of ts-cdc mutants in block-release experiments and are cautious about the conclusions that might be drawn from such studies. Their doubt is legitimate in as much as a mutant cell displaying a terminal phenotype at restrictive temperature might not show a "normal" behavior when returned to permissive temperature. Data presented in this thesis tend, au contraire , to support the usefulness and validity of such block-release experiments.

Ultimately, one would like to establish whether the budding pattern is under the sole control of nuclear (and other intracellular) events or is also under the influence of competent regions of the cell wall. It is for this reason that this thesis was initiated.

II. LITERATURE REVIEW

1. The intracellular components of budding: the nucleus, spindle pole bodies, extranuclear microtubules, 10-nm "microfilament" ring, vesicles and non-structural components

i. The nucleus and its associated structures

Several pieces of evidence point to the role played by the nucleus and its associated structures in the process of bud emergence;

a) Position and behavior of the nucleus during the cell-cycle

Although it was thought that the nucleus was randomly positioned during interphase (Freifelder, 1960), it was subsequently reported in the review of Mattle, Moor and Robinow (1969) to be located between the vacuole and the bud in vegetatively growing cells, except following division when it migrated to the opposite pole of the parental cell. This observation, though never noted before, is apparent in the micrographs of Robinow and Marak (1966). Byers and Goetsch (1975) have reported that zygotes have their nuclei proximal to early emerging buds. Further evidence for a spatial relationship between these two structures was obtained by Hungate and Byers (cited in Byers, 1981), who displaced nuclei in budding zygote cells by centrifu-

gation and observed a direct correlation between the position of the bud and the pole to which the nucleus had been relocated.

b) Spatial, temporal and physical association of the double spindle pole body with the budding site

Structures and types of spindle pole bodies: Byers and Goetsch (1974, 1975) found three stages of spindle pole body (SPB) formation during the S. cerevisiae cell cycle:

1) the single SPB, which is seen at early G1, consists of one outer plaque bearing extranuclear microtubules, one spindle plaque from which emerge short intranuclear microtubules, and one half-bridge.

2) the modified SPB, seen at later G1 and in cells preparing to conjugate, bears an electron-dense satellite proximal but not continuous with the half-bridge. Additionally, extranuclear microtubules are evident on the latter.

3) the duplicated or double SPB, observed at early bud emergence and at initiation of DNA synthesis (all events occurring within 2% of cell cycle), is characterized by having a complete bridge replacing the half-bridge. Each structure is duplicated except for the satellite which is no longer seen.

The complete spindle, seen at termination of DNA synthesis, bears a single pole body at each extremity.

In all cases, the spindle plaque and bridge, either half or

complete, are located on the persistent nuclear membrane but the satellite and outer plaque lie near-by in the cytoplasm.

King, Hyams and Luba (1982) were successful in isolating the mitotic spindle of a ts cdc-6.1 mutant yeast cell defective in medial nuclear division at restrictive temperatures. Electron-microscopic examination following positive or negative staining has revealed that the SPB is a quadrilaminar structure of alternating light and dark material, where extranuclear microtubules attach to the dark outermost layer and intranuclear microtubules connect to the dark internal layer. The proximal ends of both types of microtubules were rounded unlike their distal ends which were opened.

Associations of the double SPB with the budding site: Although double SPBs have different origins in vegetative cells and in zygotes, they have been associated in each case, and in various ways, with the sites of budding. For instance, Byers and Goetsch (1975) have demonstrated that the double SPB faces in the general direction of the site of bud emergence. In addition, they noted a temporal association between SPB duplication and bud initiation, though the exact order of occurrence has not been determined with certainty. Moreover, double SPB and budding site were physically connected by extranuclear microtubules emerging from the bridge. The microtubules project towards the neck which is filled with vesicles. The same spatial associations were observed by Tanaka and Hayashibe (cited in Hayashibe, 1975). However, mere associations between bud sites and

double SPBs do not necessarily imply causal relationships and it is still unknown whether or not one of these structures might be the cause or consequence of the other. Nevertheless, if there is any causal relationship between double SPB and bud emergence, Hayashibe (1975) has predicted a change in the location of the single SPB, before bud emergence, in unipolar budding yeast to explain its budding pattern. On the basis of his assumption, one would expect the reorientation of a single SPB to result from either a rotation of the nucleus or migration of the single SPB through a fluid nuclear membrane prior to SPB duplication and bud emergence.

ii. Role of the microtubules in budding and in nuclear processes

Three types of microtubules have been described in S. cerevisiae which are associated with SPBs (Robinow and Marak, 1966; Peterson and Ris, 1976; King, Hyams and Luba, 1982). They are: the extranuclear microtubules; the pole-to-pole intranuclear microtubules, also called polar or continuous microtubules; and the chromosomal or discontinuous microtubules. Postulated roles of microtubules in the budding process may be studied by the use of microtubule inhibitors. Among the latter, erythro-9-[3-(2-hydroxynonyl)] adenine (EHNA) (Beckerle and Porter, 1982; Cande, 1982), vanadate (Cande, 1982), benomyl (Oakley and Morris, 1980), and Colcemid (Byers and Goetsch, 1975) have all been used successfully.

a) Colcemid, extranuclear microtubules and bud emergence

For Byers and Goetsch (1975), Sloat and Pringle (1978) and Sloat, Adams and Pringle (1981), the SPBs and extranuclear microtubules arising from them seem essential components for the selection of the budding sites and the eventual emergence of buds from these sites. Assuming in addition, that these microtubules might transport vesicles necessary for the formation of daughter cells at specific sites of the cell wall (Olmsted and Borisy, 1973), Byers and Goetsch (1975) have suggested that destruction of the extranuclear microtubules should prevent bud emergence. After applying Colcemid, a derivative of colcichine, to cells emerging from stationary-phase, they have noticed a delay in the appearance of the first buds, as would be expected if extranuclear microtubules act as a guiding system for the transport of vesicles.

b) Vanadate, EHNA, extranuclear microtubules and translocation of pigment granules

Beckerle and Porter (1982), in a study of intracellular motility based on cytoplasmic microtubules in erythrophores, have proposed that a dynein-like molecule may be a component associated with this type of microtubules. Erythrophores were isolated from squirrelfish and are cells capable of translocating pigments along their cytoplasmic microtubules. Increasing micro-

injections of vanadate (an inhibitor of dynein ATPase activity in vitro preventing microtubule-based motility in cilia, flagella, and the mitotic spindle) and EHNA (a protein carboxyl-methylation inhibitor which prevents flagellar beat and dynein ATPase activity) were correlated with an increasing inhibition of translocation. This provides a second example of the possible involvement of extranuclear microtubules in transportation.

c) Benomyl, extranuclear microtubules and nuclear migration

Oakley and Morris (1980) inhibited microtubule function by applying benomyl to Aspergillus nidulans, a euascomycete. Nuclear behavior was followed using light microscopy. When microtubule formation was inhibited so was nuclear migration. By repeating the experiment using mutants having genetic lesions in β-tubulin, they were eventually able to demonstrate the dependence of nuclear transport on that subunit. It seems, therefore, that extranuclear microtubules might have more than one function.

d) EHNA, intranuclear microtubules and spindle elongation

Cande (1982) has investigated the effect of EHNA on chromosomal movement in permeabilized mammalian PtK1 cells (a cell line derived from the kidney epithelium of a rat kangaroo). In these mitotic cells, anaphase can be subdivided into stage A and B. Chromosomal microtubules shorten during anaphase A whereas polar

microtubules rearrange and lengthen in the B stage. Cande found that EHNA blocks anaphase B but not anaphase A suggesting the involvement of a dynein-like ATPase activity in spindle elongation.

iii. Existence of cdc mutants

Hartwell, Culotti and Reid (1970) were the first to report on the existence of temperature-sensitive cell-division-cycle mutants. Up to now, more than 50 genes are known to be involved in cdc events (Pringle and Hartwell, 1981). Byers and Goetsch (1974) and Byers (1981) have studied the terminal phenotypes of most of these mutants using electron-microscopy. When arrested at specific stages in their life cycles, they can provide valuable information about morphogenetic processes and interrelationships of the structures involved in these processes (Hartwell, Mortimer, Culotti and Culotti, 1973).

a) cdc-4 mutants

At restrictive temperature, the mutants have the capacity for repeated budding in the absence of nuclear division. They are defective in both the initiation of DNA synthesis and in separation of the double SPBs, although the double SPB is present. Therefore SPB duplication seems, at first glance (see below), to be essential for bud emergence but DNA synthesis is not (Byers and Goetsch, 1974).

b) cdc-31 mutants

At restrictive temperature, these mutants exhibit a single SPB but are capable of budding. The single SPB is not typical though, displaying twice the usual number of extranuclear microtubules and being larger than expected. In these mutants, bud emergence is not related to a double SPB per se, as implied above, but to doubling of an event or substance(s) associated with the SPB(s) (Byers, 1981).

c) cdc-24 mutants

At restrictive temperature, these mutants continue cell growth (Johnston, Pringle and Hartwell, 1977; Sloat and Pringle, 1978), DNA synthesis (Sloat and Pringle, 1978) and nuclear division (Byers and Goetsch, 1974; Sloat and Pringle, 1978) but are defective in bud emergence (Byers and Goetsch, 1974; Sloat and Pringle, 1978; Sloat, Adams and Pringle, 1981) and chitin ring formation (Sloat and Pringle, 1978; Sloat, Adams and Pringle, 1981). The cell wall contains large amounts of delocalized phosphatase, a manno-protein (Field and Schekman, 1980), mannan (Sloat, Adams and Pringle, 1981) and chitin (Sloat and Pringle, 1978; Sloat, Adams and Pringle, 1981).

Sloat and Pringle (1978), and Sloat, Adams and Pringle (1981) have postulated that the function of the CDC-24 gene product might be to guide vesicles that contain digestive enzymes (Matile, Moor and

Robinow, 1969; Cortat, Matile and Wiemken, 1972; Schekman and Brawley, 1979), chitin activating factors (Cabib, Duran and Bowers, 1978), or cell wall precursors (Matile, Moor, and Robinow, 1969; Byers and Goetsch, 1976a; Schekman and Brawley, 1979).

Sloat, Adams and Pringle (1981) have also proposed other roles for this gene product in cellular morphogenesis:

1) Selection of a site where the daughter-cell will initiate
2) Deposition of a ring of chitin at the above site
3) Localization of bud cell wall material at the chosen site
4) Regulation of the appropriate amounts of cell wall material needed for normal bud shape.

iv. Functions of the 10-nm "microfilament" ring during budding

There is a highly ordered ring of 10-nm filaments on the interior surface of the plasma membrane (Byers and Goetsch, 1976a) the absence or abnormality of which might be responsible for the failure of bud emergence in cdc-24 mutants at restrictive temperature (Sloat and Pringle, 1978). The ring is normally found within the bud neck. It forms at early bud emergence or slightly before and disappears at early cytokinesis (Byers and Goetsch, 1976a; Byers, 1981). Various functions have been proposed by Byers and Goetsch (1976a), they are:

1) limitation of "surface expansion to dimensions appropriate for budding".

2) counteraction of "the normal outward curvature of the cell surface".

3) "deposition of specific components in the overlying region of the cell wall".

4) inhibition of "vesicle fusion until the appropriate phase of the cell division process".

Since Byers and Goetsch (1976b) found that the filament ring is never seen in cytokinesis-defective mutants, due to its loss, it seems probable that the ring functions also during cytokinesis.

2. The cell wall as an extracellular component of budding

i. Yeast cell wall morphology and chemical composition

Moat (1979), in his book "Microbial Physiology", mentions the existence in the yeast cell wall of at least two layers of different chemical composition: one layer is composed mainly of the polysaccharide glucan and the other of a combination of mannan and proteins. When the cell wall of S. cerevisiae is removed and analyzed, the following compounds are found: glucan (30 to 34%), mannan (30%), proteins (6 to 8%), lipids (8.5 to 13.5%), and a smaller proportion of chitin (1 to 2%) (cited in Pelczar, Reid and Chan, 1977). Free galactose, glucose and mannose have also been detected (cited in Moat, 1979), together with glucosamine (cited in Pelczar, Reid and Chan, 1977).

In their review, Matile, Moor and Robinow (1969) mention the existence of a three-layer cell wall in which the intermediate layer is more electron-transparent than the others. According to them, the outer layer contains a mixture of mannan and proteins, the middle layer glucan, and the innermost layer is more proteinaceous in nature.

It seems that the molecular composition of the cell wall varies with culture conditions (Hayashibe, 1975), the mating-type locus (Brock, 1959) and the age of the culture (Beran, 1968; Matile, Moor and Robinow, 1969).

As mentioned earlier, cdc-24 mutants when shifted to restrictive temperatures, produce changes in their cell wall structure that are reflected, among other things, by an increased delocalization of chitin (Sloat and Pringle, 1978; Sloat, Adams and Pringle, 1981). Return to a permissive temperature involves the formation of a bud cell wall poorer in chitin than its mother-cell (Sloat and Pringle, 1978; Sloat, Adams and Pringle, 1981). This raises the question whether there is any modification of cell wall components in the parental cell wall at the site of emergence, prior to bud formation. Modification of compounds might be restricted to certain cell wall regions (competent cell wall regions) and chitin is a likely candidate to study. Since mannan behavior parallels chitin behavior in blocked mutant cells (Sloat, Adams and Pringle, 1981), it constitutes a second component whose fate should be followed. In this respect,

the structure, distribution and behavior of these cell wall components during the yeast cell-cycle is described below.

a) Chitin

Chitin is a polymer of N-acetyl-glucosamine (GlcNAc) which can be found in such diverse organisms as Protozoa, Fungi, Insects and Crustaceans. Chitin, in S. cerevisiae and in many fungi, is a high molecular weight polysaccharide, that can be synthesized as follows (Gooday, 1977):

$$\text{UDP-GlcNAc}+(\text{GlcNAc})n \xrightarrow[\substack{\text{chitin}\\\text{synthetase}}]{mg^{+2}} [\beta(1\rightarrow4)\text{-N-acetylglucosamine}]_{n+1}+\text{UDP}$$

The enzyme, chitin synthetase (EC.2.4.1.16) is activated by the substrate, uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).

Distribution of chitin in the yeast cell wall and septum wall formation: Chitin in the genus Saccharomyces is mostly restricted to bud scars (Molano, Bowers and Cabib, 1980), that is the distinctive marks left in the parental cell wall after bud detachment (Barton, 1950) which result from the formation of a primary septum. In budding yeasts, this developmental process occurs in two stages (Cabib and Bowers, 1975), the first of which involves the formation of the chitin ring, an annular structure seen at the junction between the daughter and mother-cell

(Hayashibe and Katohda, 1973). The second stage occurs before cell division, together with a second deposition of chitin (Cabib and Bowers, 1975), and is characterized by the centripetal growth of material to form a disk-shape cross wall between the two cells. More than 90% of chitin in the yeast cell wall is restricted to the primary septum (Molano, Bowers and Cabib, 1980). The rest is distributed randomly in lateral wall (Hayashibe and Katohda, 1973; Horisberger and Vonlanthen, 1977; Sloat and Pringle, 1978; Molano, Bowers and Cabib, 1980; Sloat, Adams and Pringle, 1981). Some authors do not make the distinction between chitin rings and bud scars (Hayashibe and Katohda, 1973; Sloat and Pringle, 1978; Sloat, Adams and Pringle, 1981), however the structures should be regarded as different since their morphology (Cabib and Bowers, 1975) and chemical composition (Molano, Bowers and Cabib, 1980) are different. Although some authors believe that cell wall chitin stained with calcofluor, a fluorescent dye, shows maximum fluorescence in regions of circular arrangement (Streiblová and Beran, 1965; Beran, 1968; Seichertová, Beran, Holan and Pokorný, 1975), not all studies provide evidence for such a behavior (Sloat and Pringle, 1978). Field and Schekman (1980), for their part, believe that randomly distributed chitin, in lateral walls, plays a role in compartmentalization and segregates regions of different enzymatic activity.

Role of the chitin ring: The chitin ring can be easily detected by fluorescence microscopy (Streiblová and Beran, 1963a, 1963b, 1965; Streiblová, 1970; Hayashibe and Katohda, 1973; Seichertová, Beran, Holan and Pokorný, 1973; Cabib and Bowers, 1975; Sloat and Pringle, 1978; Sloat, Adams and Pringle, 1981) or as an electron transparent material using transmission electron microscopy (Cabib and Bowers, 1971; Seichertová, Beran, Holan and Pokorný, 1975; Cabib, Duran and Bowers, 1978). In fluorescence microscopy, the chitin ring has been revealed by brighteners such as Calcofluor White M2R (CFW) or CFW New (Hayashibe and Katohda, 1973; Seichertová, Beran, Holan and Pokorný, 1973; Cabib and Bowers, 1975; Sloat and Pringle, 1978; Sloat, Adams and Pringle, 1981), or by the fluorochrome primulin, a monoazo-dye (Streiblová and Beran, 1963a, 1963b; Streiblová, 1970; Seichertová, Beran, Holan and Pokorný, 1973; Byers and Goetsch, 1974).

Although authors agree on the chemical nature and location of the chitin ring, controversy exists concerning its time of appearance and function during the cell-cycle (Hayashibe and Katohda, 1973; Cabib and Bowers, 1975; Sloat and Pringle, 1978). Whether it is essential for budding is contentious and has been the center of debate (Cabib and Bowers, 1975; Sloat and Pringle, 1978). The matter arose in 1973, when Hayashibe and Katohda were able to demonstrate, prior to bud emergence, the appearance of a

ring of chitin, within which the incipient bud initiated, suggesting a possible interrelationship between the two. Soon after, Bowers, Levin and Cabib (1974) and Cabib and Bowers (1975) using an inhibitor of chitin synthetase, polyoxin D, showed that budding could occur without the formation of a chitin ring. It seems, therefore, that there is no causal relationship between chitin ring formation and bud emergence, although they are sequentially related. Additionally, the aforementioned authors demonstrated the early synthesis of chitin and its function during cytokinesis. Three years earlier, it was proposed that the ring was a rigid structure having two main functions (Cabib and Bowers, 1971; Cabib and Farkaš, 1971): to protect the channel between mother-cell and bud allowing the passage of organelles and to prevent the new cell wall of the bud to grow "backwards" i.e. into the parental cell. In 1978, Sloat and Pringle using a diploid homozygous cdc-24-1 mutant strain, obtained results similar to Hayashibe and Katohda (1973) relative to chitin ring formation prior to bud emergence. Both groups isolated small unbudded cells devoid of bud scars and allowed them to reproduce; more "rings" than buds were formed initially. They also showed that every bud without exception was accompanied at its base by a chitin ring. For them, no budding without rings seemed to be the rule. Based on their results and those of Hayashibe and Katohda (1973), Sloat and Pringle (1978) have suggested "the necessity

for a reinforcing ring of chitin if cell wall expansion is to result in bud formation, rather than in generalized expansion". This, obviously, conflicts with the results obtained by Cabib and Bowers (1975) and Bowers, Levin and Cabib (1974). Sloat and Pringle (1978), based on a review paper by Gooday (1977), have criticized the method used by Cabib and Bowers (1975), saying that the concentration of Polyoxin D employed was too high and applied outside the normal growth range temperatures. They have, however, attempted to explain both results by suggesting that their cdc-24-1 mutant might be "defective in forming...the microfilament ring...necessary both for localized deposition of chitin and for budding", an explanation which according to them, would solve this "Gordian knot".

b) Mannan

Mannan is a branched homopolymer having a non-reducing terminal residue with side chains (α-D1$\rightarrow$2, α-D1$\rightarrow$3) attached to the α1$\rightarrow$6 backbone by α1$\rightarrow$2 linkages (Tkacz, Cybulska and Lampen, 1971). Composed of repeated units of D-mannose, it represents one of the two major polysaccharydes found in the cell wall of S. cerevisiae, along with glucan (cited in Pelczar, Reid and Chan, 1977). Concanavalin A (Con A), a lectin isolated from the jack bean Canavalia ensiformis, has been successfully conjugated to fluorescein isothiocyanate (FITC) to reveal the presence of

mannan in the yeast cell wall (Tkacz, Cybulska and Lampen, 1971; Tkacz and Mackay, 1979; Sloat, Adams and Pringle, 1981). Since Con A combines with α-linked mannose homopolymers and since these polymers are found only in the form of mannan in the yeast cell wall, the binding of FITC-Con A to mannan is considered specific: Con A does not bind to glucan or chitin (Tkacz, Cybulska and Lampen, 1971).

Distribution: When stained with FITC-Con A, wild type yeast cells growing at 24°C or 36°C (Tkacz, Cybulska and Lampen, 1971; Sloat, Adams and Pringle, 1981) or cdc-24-4 mutant yeast cells growing at 24°C (Sloat, Adams and Pringle, 1981) show extensive uniform fluorescence for mannan in both budded and unbudded cells. In budded cells, the mother cells and the buds are equally fluorescent (Tkacz, Cybulska and Lampen, 1971; Sloat, Adams and Pringle, 1981). In budding cells, however, localized incorporation of new mannan is restricted to tip of growing buds (Sloat, Adams and Pringle, 1981).

Behavior: Numerous investigators followed the behavior of mannan in blocked cdc-24 cells (Sloat, Adams and Pringle, 1981), in wild type cells of opposite mating-type in preparation for conjugation (Lipke, Taylor and Ballou, 1976; Tkacz and Mackay, 1979), and in zygotes (Tkacz and Mackay, 1979). Their results are summarized here.

Sloat, Adams and Pringle (1981) have shown that cdc-24-4

mutant cells growing at 36°C increase the mannan content of their cell wall in a randomly delocalized manner. In addition, Tkacz and Mackay (1979) have demonstrated that when G1 stage cells of the a mating-type were treated with α-factor, and then stained with FITC-Con A, there was an increase in fluorescence for mannan at the tip of the newly formed "schmoo" (pear-shaped cell). The reciprocal treatment of α cells with a-factor also yielded the same result but the increased fluorescence was not as great as in the first case (Tkacz and Mackay, 1979). The increased fluorescence observed at the tip of the schmoos seems, at first glance, in contradiction with results reported by Lipke, Taylor and Ballou (1976), who studied the morphogenic effect of α-factor on a cell by electron microscopy and by following incorporation of radioactive precursors in the yeast cell wall. They found that treated cells have a thinner mannan coat in the vicinity of the tip and incorporated less radioactive mannan precursors than control cells. Less mannan in this region but greater fluorescence suggests either a greater accessibility of FITC-Con A or a chemical modification of the less numerous mannans making them appear more fluorescent. Lipke, Taylor and Balou (1976) were able to demonstrate that mannan of α-factor treated a cells have shorter side chains and contain an increased number of unsubstituted backbone mannose units, but no firm conclusion can be made concerning the above hypotheses.

ii. Changes in the cell wall and in cytoplasmic organization at the onset of budding

An accumulation of sulphydryl compounds (Nickerson, 1963) and vesicles (Matile, Moor and Robinow, 1969; Sentandreu and Northcote, 1969; Byers and Goetsch, 1975) had been found in the region of bud formation prior to bud emergence. Sentandreu and Northcote (1969) and Matile, Moor and Robinow (1969) were among the first to report the existence of such vesicles. Their location was clearly described by Sentandreu and Northcote (1969): "vesicles accumulate at the site of bud emergence in the mother-cell and are also found in the growing bud during the growth of its wall". Byers (1981), who in his recent review ignores the contribution of the latter authors and seems to give sole credit to Matile, Moor and Robinow, gives the following description: "During the early portion of the cell-division-cycle, many vesicles (ca. 30-40 nm in diam.) accumulate first at the site where the bud will emerge and are later seen...within the early bud". These vesicles are thought to be required for the enzymatic modification of the cell wall (Byers, 1981) and micrographs published by Byers and Goetsch (1975) clearly show them at the above mentioned sites. Moor (1967) using freeze-etched techniques suggested that the endoplasmic reticulum is inducing bud formation by the localized production of vesicles that might contain enzymes or cell wall precursors needed for cell wall softening and bud wall growth. This was an alternative to the proposed model of Nickerson (1963) where

enzymatic vesicles involved in budding are not directed to specific sites. Other evidences also point to the involvement of enzymes in this process such as glucanase (Cortat, Matile and Wiemken, 1972) and protein-disulphide reductase (Nickerson, 1963; Moor, 1967).

Freifelder (1960) has looked at the budding region using various microscopic techniques and commented: "the presumptive bud region has been examined prior to budding by phase-contrast, polarization, and interference microscopy but no particular differentiation has been evident". For Belin (1972) buds appear as a slight protuberance on the surface of the mother-cell by an evagination of the cell wall that shows no sign of rupture. A small mucilaginous ring appears at the base of the protuberance. Seichertová, Beran, Holan and Pokorný (1975) have reported, using transmission electron microscopy, a disruption of the innermost cell wall layer before chitin ring formation but no such change has been detected in the outermost layer before bud emergence. Thus, in summary, it seems likely that changes at the prebudding site, at the onset of budding, occur primarily on the cytoplasmic face of the cell wall where enzymatic vesicles might be specifically directed.

iii. Sites and sequence of budding

The position of buds in yeasts has been found to be non-random (Winge, 1934; Nickerson, 1942; Barton, 1950; Bartholomew and Mittwer, 1953; Freifelder, 1960; SentheShanmuganathan and Nickerson, 1962;

Nickerson, 1963; Streiblová and Beran, 1965; Streiblová, 1970; Belin, 1972; Hayashibe, 1975; Hicks, Strathern and Herskowitz, 1977; Sloat, Adams and Pringle, 1981). The budding pattern seems to vary with 1) the ploidy level (Winge, 1934; Freifelder, 1960; Streiblová, 1970; Hicks, Strathern and Herskowitz, 1977; Sloat, Adams and Pringle, 1981), 2) the mating-type locus (Hicks, Strathern and Herskowitz, 1977; Sloat, Adams and Pringle, 1981), 3) the homothallism/heterothallism gene locus (Hicks, Strathern and Herskowitz, 1977), 4) the CDC-24 gene product (Sloat, Adams and Pringle, 1981), 5) culture conditions (Senthe Shanmuganathan and Nickerson, 1962; Hayashibe, 1975), 6) species (Senthe Shanmuganathan and Nickerson, 1962; Streiblová and Beran, 1965; Belin, 1972), and 7) incompetency of certain cell wall regions to support further budding (Barton, 1950; Mortimer and Johnson, 1959; Freifelder, 1960; Nickerson, 1963; Streiblová and Beran, 1965; Byers and Goetsch, 1975).

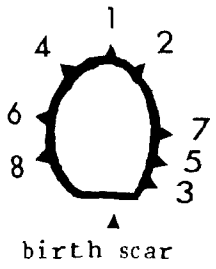
a) Ploidy level

As early as 1934, Winge reported differences in budding behavior between haploid and diploid strains. Haploids were found to start budding "proximally" at the partition between mother and daughter cells (i.e. birth scar end) producing small, spherical cells. By contrast, diploids were more elongated and larger, with buds set out "distally" at the bud scar end. In both cases, budding occurred in rows but not necessarily sequentially or

unidirectionally.

Sixteen years later, Barton (1950) studying budding in baker's yeast found the pattern shown in Figure 1.

Figure 1. Position of buds one to eight in Saccharomyces cerevisiae Hansen var. ellipsoideus grown in liquid culture.



The first bud to arise was always positioned at the opposite pole from the birth scar. The ploidy level was not indicated but, since each scar is approximately 2 μm in diameter and corresponds to the size expected for a diploid bud scar (Streiblová, 1970), it seems likely that the budding pattern of a diploid strain was investigated.

Barton's results, however, were not supported by Bartholomew and Mittwer (1953), who used the same strain, since they found a tendency for bud scars to be located on opposite ends of the cell. The first and second buds did not emerge at the opposite pole from the birth scar, but rather laterally to it. The ploidy level was not specified, and the authors suggested

that differences might be due to culture conditions.

Freifelder (1960) was the first to show clearly that, "the position of the first bud of a new cell is invariant but dependent on ploidy", occurring at the birth end for haploids and at the opposite end (bud scar end) for polyploids. "Some regularity in the position of the buds produced successively by a single mother-cell" was observed as far as the third or fourth buds. Later buds appeared randomly.

Reviewing molecular bases of form in yeasts, Nickerson (1963) stated that, "the location of a site of bud formation is not random" and, based on Barton's finding (1950), suggested that a bud emerges from a parental cell (diploid cf. Streiblová, 1970), at the point of maximum curvature, through permitted loci:

1) first at the apex,
2) then at one or more sites proximal to the distal apex and forming a ring around it,
3) subsequently at sites adjacent to the proximal apex and forming a ring around it,
4) finally at lateral sites.

According to him, "selection of sites of bud formation is governed by purely physical limitations".

Streiblová and Beran (1965) confirmed the above pattern for multipolar budding yeast (diploid cf. Streiblová, 1970): "The bud scars are successively formed, usually first in the region oppo-

site to the birth scar, then at the pole around the birth scar and at last in the central area of the cell surface".

Five years later Streiblová (1970), studying haploid strains, confirmed the results of Freifelder (1960) that for most cells the first bud was formed adjacent to the birth scar. In addition, there was a certain percentage of cells (not given) where the position of the first bud varied. Her results for diploids agreed with those of Barton (1950) and Nickerson (1963): budding sites occurred "as a progression through permitted loci of maximal curvatures". In zygotes, however, Streiblová (1970) found that diploid buds were formed mainly on the conjugation tube of the zygote in the region of minimal curvature of the cell wall. She concluded that the model proposed by Nickerson (1963) is not adequate: budding sites are not selected only by physical considerations, at least not in zygote cells. Her observations though, could be accounted for by the model of Moor (1967) where budding is related to a local activity of the endoplasmic reticulum.

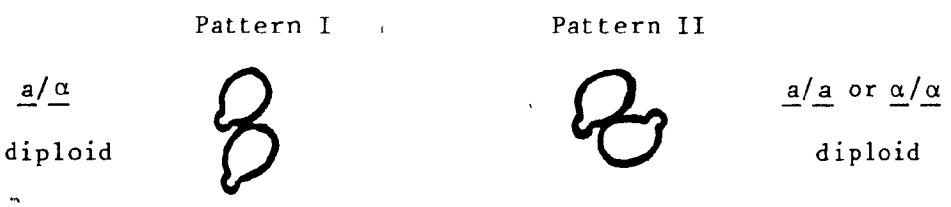
Sloat, Adams and Pringle (1981), briefly summarized the situation by saying that, "the bud scars of multiparous cells are clustered at the poles of the cells, generally at one pole on haploid cells and at both poles on diploid cells".

b) Mating-type and homothallism gene loci

The mode of budding has been found by Freifelder (1960) not to be related to the mating-type locus, since within haploid and diploid strains having different mating types, budding patterns are consistent regardless of mating-types. On this basis, Freifelder concluded that the budding pattern was not of genetic character. This idea that diploids homozygous or heterozygous for the mating-type locus have the same budding pattern is in contradiction with observations made by Hicks, Strathern and Herskowitz (1977) and Sloat, Adams and Pringle (1981).

In 1977, Hicks, Strathern and Herskowitz described two budding patterns for diploids, depending on the mating-type locus, which are illustrated in Figure 2.

Figure 2. The polar and medial patterns of diploid strains having different mating types



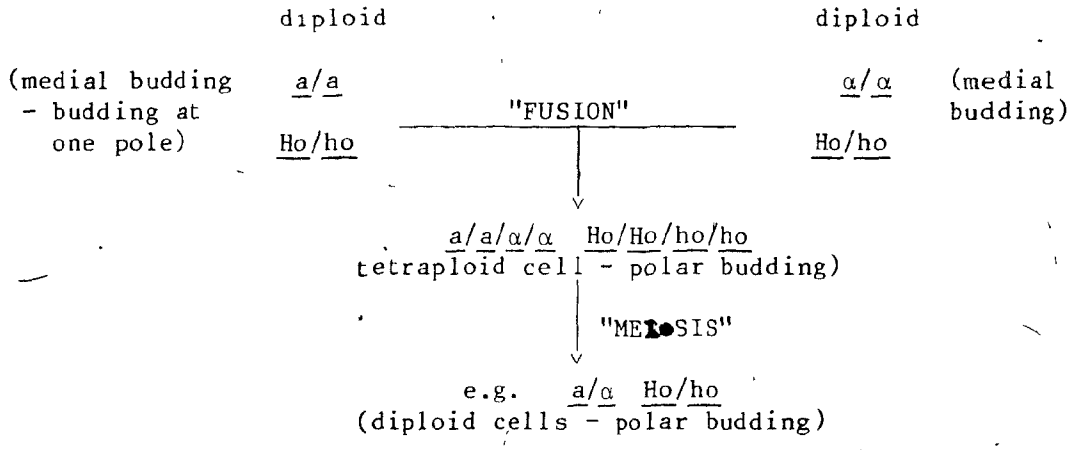
Pattern II resembles the haploid budding mode described by Winge (1934) and Freifelder (1960).

Therefore, as was later confirmed by Sloat, Adams and

Pringle (1981), the diploid strains homozygous for mating type display the typical budding of haploid strains while heterozygous diploid strains display a different budding pattern which is identical to strains having higher ploidy levels (Freifelder, 1960).

Interestingly, Hicks, Strathern and Herskowitz (1977) reported on the interconversion of yeast mating types by the action of the homothallism gene (HO) in cells homozygous for the mating-type locus. Due to the presence of the HO gene, some diploid a/a cells are converted to the opposite mating type. If these diploid cells are growing together, they can eventually fuse and then undergo meoisis to produce diploid cells heterozygous for the mating-type locus which exhibit polar budding (budding at both poles). This phenomenon is illustrated in Figure 3.

Figure 3. Interconversion of yeast mating types by the action of the homothallism gene (HO)



Since the allele for homothallism (HO) is dominant over the allele for heterothallism (ho), HO strains are unstable relative to their mating-type locus which can be interconverted as often as each cell division.

c) CDC 24 gene locus

Sloat, Adams and Pringle (1981) found that an effect of the cdc-24-3 and cdc-24-4 mutant alleles is to change the budding pattern in haploids and homozygous diploids from bud scars clustered at one pole to bud scars randomly scattered over the surface of the cells (at permissive temperature). This effect seems to be recessive in heterozygous diploids. Subsequently, it was found that the abnormal pattern of budding sites segregated with the temperature-sensitivity in crosses. Further support for the involvement of this gene product in the determination of the budding pattern was provided by the existence of spontaneous haploid revertants, exhibiting a recovery to the normal budding pattern.

d) Culture conditions

Hayashibe (1975), referring to an earlier paper written in Japanese [Hayashibe, 1968], has examined position and sequence of budding in various baker's yeasts under shaking and slide culture methods. He found variation even for the same strain: "Notable

differences in the budding position were seen between cells cultured by shaking and on agar medium". In shaking cells, the daughter-cell first bud did not influence the position of the next bud as it might in agar culture.

e) Species

The comparative study of the budding pattern of yeasts as diverse as Saccharomyces cerevisiae, Saccharomyces ludwigii, Schizosaccharomyces pombe (Streiblová and Beran, 1965), Trigonopsis variabilis (Sentheshanmuganathan and Nickerson, 1962), Saccharomyces uvarum Beijerinck (among which is included Saccharomyces carlsbergensis) (Belin, 1972), has demonstrated that the sites of bud emergence are not random, but dependent on species.

f) Incompetent cell wall regions

Little work has been done on whether or not there are truly such regions in the yeast cell wall. It is, however, known that buds cannot appear twice at the same site (Barton, 1950; Mortimer and Johnson, 1959) presumably because the cell wall region becomes ultimately incompetent to further answer budding signals (Byers and Goetsch, 1975). Eventually, when a region of the mother cell wall is filled with bud scars, other regions seem to respond to the signals (Freifelder, 1960; Nickerson, 1963; Streiblová and Beran, 1965). The nature of the budding signal is

unknown and competency may not be restricted to regions having bud scars.

3. Karyogamy-deficient mutants

Conde and Fink (1976) have reported on a single nuclear mutation called kar 1-1 which causes a defect in nuclear fusion. Normally, haploid strains of opposite mating-type may under appropriate conditions fuse to produce a diploid zygote which may reproduce by vegetative means to give buds having a single diploid nucleus (Sena, Radin and Fogel, 1973). In the kar 1-1 mutant strain, the defect prevents the two haploid nuclei from fusing together after cytoplasmic fusion has occurred. Consequently, a binucleate zygote is formed which will subsequently reproduce to give heteroplasmons and heterokaryons. Heteroplasmons are cells containing the original cytoplasm of both parents but only the haploid nucleus of one of the parental cells. By contrast, a heterokaryon is a cell containing nuclei from different strains (Conde and Fink, 1976). In the sexual cycle of S. cerevisiae, the a cell-type and α cell-type are both capable of producing a pheromone which would arrest a cell of the opposite-mating type at stage G1 of its life cycle (Hartwell, 1973). For instance, when the a mating-type cells are arrested with α-factor at a susceptible stage of their life cycle (G1), the cells change shape becoming pear-shaped (Lipke, Taylor and Balou (1976). The pear-shaped cells are often referred to as "schmoos" (Field and Schekman, 1980). Chitin has been found to be

delocalized at the tip of the schmoos (Schekman and Brawley, 1979), as well as mannans (Tkacz and Mackay, 1979) and acid phosphatase (Field and Schekman, 1980). After schmoo formation the cells are first capable of cytoplasmic fusion as a consequence of the fusion of receptors at the cell surface (Thorner, 1981) followed by nuclear fusion. Byers and Goetsch (1975) have followed the fusion process using transmission electron microscopy. After cytoplasmic fusion, the nuclei moved towards each other with each SPB facing the other and meeting at the isthmus. Fusion occurs only between modified spindle pole bodies in which the satellite regions first fuse, followed by outer plaques and spindle plaques. The process is the same in diploid cells except that the SPBs are larger than those in haploid cells. Nuclear movement at this stage is prevented by the narrow orifice found between the two fused cells before the isthmus fully enlarges. After staining with Giemsa, the first bud was found to arise from the isthmus nearest the location of the fused SPBs. This relationship however, decreases with an increase in ploidy level and corresponding increase in isthmus size, probably because there is less constraint on nuclear movement. Extranuclear microtubules and vesicles were found at the base of the newly emerging bud.

More recently, quantitative cytological examination of Giemsa-stained budding zygotes by Dutcher and Hartwell (1982) has revealed that the first buds to be produced are of two types: medial and terminal. Medial buds emerge on the conjugation tube (isthmus) whereas

terminal buds initiate at the poles. They followed bud position in 500 zygotes heterozygous for the kar- mutation: fifty-eight percent (58%) of the buds were medial and forty-two percent (42%) terminal. Eighty-nine percent (89%) of the medial buds were found to be binucleate and ninety-three percent (93%) of the terminal buds uninucleate. Finally, Lipke, Taylor and Balou (1976) reported that in a cells treated with α-factor the nucleus is seen near the tip of the schmoo, where the bud will eventually emerge. These two observations strengthen the argument for a relationship between nuclear position and bud emergence.

III. MATERIALS AND METHODS

Yeast strains, culture conditions, and media

All strains were obtained from the Yeast Genetic Stock Center, Berkeley, California. The temperature-sensitive (ts) cell-division-cycle (cdc) mutant strain, the two karyogamy-deficient (kar-) strains, and the wild-type strain are described in Table 1.

Table 1. Yeast strains used in this study

Strain[1]	Genotype
182-6.3	cdc-24-1 a ural tyr1 arg4 thr4 ade his trp gal
JC7	kar1 a leu1
JC25	kar1 α ade2-1 his4 Δ15
X-2180-1A	wild type a SUC2 mal gal2 CUP1

[1] Yeast Genetic Stock Center designation.

Upon receipt, strains were grown on YEPD plates and stored at 4°C. Cultures were grown on a rotary shaker (New Brunswick Scientific, 115 cycles/min.) in 125 mls. erlenmeyer flasks containing approximately 25 mls. of YEPD medium. Each culture was subcultured twice at room

temperature (23°C) prior to sample collection. Log-phase cultures were grown to about 1 x 10^6 cells/ml., and stationary-phase cultures to approximately 4 x 10^8 cells/ml. Cell densities were determined using a hemacytometer following sonication (Sonifier Cell Disruptor, model W185D, Branson Sonic Power Co., L.I., N.Y. Output control to 1, time of sonication: 15 secs. by bursts). Budding cells were recorded as single cells.

YEPD medium consists of 1.0% yeast extract (Difco), 2.0% peptone (Difco), and 2.0% dextrose (Byers and Goetsch, 1975). For plates, 1.5% bacto-agar (Difco) was added. YED 3.0% contains 0.5% yeast extract (Difco) and 3.0% dextrose (Sena, Radin, Welch and Fogel, 1975); pH was adjusted to either 4.5 or 8.5 with IN HCl or NaOH respectively.

Fixation

One-ml. samples were centrifuged (Sorvall GLC-1, 3,000 rpm, 1 min.) and supernatants discarded. Cells were fixed in three mls. of saline-formaldehyde (3.0% formaldehyde, 50 mM CH_3COOK, 0.9% NaCl) to which was added one ml. of Gurr's buffer (BDH), pH = 6.8. Both the fixative and the buffer were added at room temperature, and cells were fixed for 60 minutes at 23°C or for longer times at 4°C.

Giemsa-stained samples

Yeast nuclei can be revealed by staining with the following Giemsa method which represents a modified version of the technique employed by

Sherman, Fink and Hicks (1979):

1. Following fixation, centrifuge cells and discard supernatant.
2. Rinse twice with two mls. of Gurr's buffer pH = 6.8.
3. Centrifuge cells and resuspend them in five mls. of Gurr's buffer pH = 6.8 for sonication.
4. After sonication, centrifuge cells and discard supernatant.
5. Rinse cells twice in two mls. of alcohol 70%, centrifuging and discarding supernatants each time.
6. Rinse cells with two mls. of Gurr's buffer pH = 6.8.
7. Collect cells, remove supernatant, and resuspend in two mls. of 1.0% NaCl at 60°C for 90 minutes.
8. After collecting cells, rinse them with two mls. of Gurr's buffer pH = 6.8.
9. Recollect cells, remove supernatant, and add two mls. of IN HCl at 60°C for 10 minutes.
10. Centrifuge cells, discard supernatant, and rinse twice with two mls. of Gurr's buffer pH = 6.8.
11. Resuspend cells in Giemsa stain (BDH) diluted 1:20 in Gurr's buffer pH = 6.8 for 5 to 20 minutes.
12. Mount directly onto slides. Seal and store at 4°C until examination (maximum storage time used, 48 hours).

DAPI- calcofluor-stained samples

Cells can be simultaneously stained with calcofluor, for chitin, and

DAPI[1], for DNA (hence nuclei), by using the following technique:

1. Rinse fixed cells twice with distilled water (dH_2O).
2. Centrifuge cells, discard supernatant, and resuspend in dH_2O.
3. Sonicate cells and centrifuge.
4. Discard supernatant, add 0.1% calcofluor White-M2R (Polysciences) in dH_2O for five minutes.
5. Centrifuge cells, discard calcofluor, and rinse once with dH_2O.
6. Resuspend centrifuged cells in 0.2M PBS[2] pH = 7.3 ($Na_2HPO_4 \cdot 7H_2O$, 20.7 g/l; $NaH_2PO_4 \cdot H_2O$, 3.2 g/l; NaCl, 0.9 g/l), add stock solution of DAPI (10 µg/ml. dissolved in PBS pH = 7.3, stored in dark at 4°C) to get a final ratio of 10 to 1. Keep cells in that solution for 10 minutes.
7. Centrifuge cells, discard supernatant, and rinse once with PBS pH = 7.3.
8. After centrifugation, discard supernatant, and resuspend cells in PBS pH = 7.3 to low density. Transfer cells onto slides, seal, and keep in dark until examination (maximum storage time used, 2 hours).

DAPI-stained samples

To stain with DAPI alone, omit steps 4 and 5 above.

[1] DAPI, 4'-6-diamidino-2-phenylindole.

[2] PBS, phosphate-buffer saline.

Calcofluor-stained samples (Sloat and Pringle, 1978)

To stain with calcofluor alone, omit steps 6 and 7 above.

FITC-Con A[1]-stained samples (modified after Tkacz, Cybulska and Lampen, 1971).

Mannan in yeast cell walls can be detected by staining with FITC-Con A as follows:

1. Rinse 2 x 10^7 fixed cells with 0.2 M PBS buffer pH = 7.2 ($Na_2HPO_4 \cdot 7H_2O$, 19.3 g/l.; $NaH_2PO_4 \cdot H_2O$, 3.9 g/l.; NaCl, 0.9 g/l.).
2. Centrifuge cells and discard supernatant.
3. Resuspend cells in PBS pH = 7.2 and sonicate.
4. Collect cells and discard supernatant.
5. Add 30 µg (0.1 ml.) of unlabelled Con A[2].
6. Add 138 µg (1.5 ml.) of FITC-Con A[3] for 10 minutes.
7. Centrifuge cells, remove supernatant, and rinse cells once with PBS pH = 7.2.
8. Centrifuge cells, remove supernatant, and resuspend in PBS pH = 7.2 to low density. Transfer cells onto slides, seal, and keep in dark until examination (maximum storage time used, 2 hours).

[1] FITC, fluorescein isothiocyanate; Con A, concanavalin A.

[2] To prepare unlabeled Con A: add 30 mg of Con A (Sigma) to 100 mls. of PBS buffer pH = 7.2.

[3] To prepare FITC-Con A (92 µg/ml.): add 5 mg of FITC-Con A (Sigma) to 54.35 mls. of PBS pH = 7.2.

Fluorescence microscopy

Cells prepared for fluorescence microscopy were examined under incident light fluorescence with a Leitz orthoplan microscope (total mag. 1000X). The microscope was equipped with Ploem Pak 2 and HBO 100 ultra-stable (for excitation) systems. All photographs were taken with a Leica M2 camera using a Visoflex Leica system and TRI-X films.

Bright-field microscopy

Cells prepared for bright-field microscopy were examined using a Wild M 11 (total mag. 900X) or Zeiss research photomicroscope (total mag. 1000X).

Testing for volutin granules

Cells, extracted or not with perchloric acid (PCA), were stained with an aqueous solution of 0.5% toluidine blue to localize volutin granules. After staining for two minutes, cells were washed with distilled water and examined by bright-field microscopy. Extraction of the granules was done in ice cold 2.0% PCA for one hour, as recommended by Coleman (1979).

Block

A log-phase culture of 182-6.3 a cdc-24-1 mutant haploid cells grown in YEPD medium at 23°C (permissive temperature), was blocked at 37°C for eight hours. One-ml. samples were collected hourly (cells from each sample referred to as B=0, B=1, B=2, B=3, B=4, B=5, B=6, B=7, B=8) and fixed in

saline-formaldehyde. Cells from hourly samples were examined by bright-field microscopy after staining with Giemsa, for nuclei, or by fluorescence microscopy after staining with DAPI and calcofluor, for nuclei (DNA) and chitin respectively. Samples collected at B=0 and B=8 were also analyzed by fluorescence microscopy after staining with FITC-Con A, for mannan. The number of nuclei and buds, as well as the distribution of the cell wall chitin and mannan was then followed in all of the above samples. A minimum of 100 cells was analyzed in each sample. Cell density was estimated at the beginning and end of the block using a hemacytometer.

Release

After the eight-hour block, the above culture was released (returned to permissive temperature, 23°C) for 20 hours. Samples were collected at various times (R=0.5, i.e. half-an-hour after release; R=1.0; R=1.5; R=2.0; R=2.5; R=3.0; R=3.5; R=4.0; R=5.0; R=6.0; R=7.0; R=20.0 D; R=20.0 ND) and fixed as before. At R=7.0, the remainder of the culture was divided into two flasks. One was diluted with fresh YEPD medium, added at room temperature, to early log-phase density (1×10^6 cells 1 ml.; R=20.0 D), the other was not (R=20.0 ND). Collected samples were then stained with a combination of DAPI, for nuclei (DNA), and calcofluor, for chitin, and examined by fluorescence microscopy. Samples R=0.5 and R=5.0 were also examined by the latter technique after staining with FITC-Con A for mannan. The budding, nuclear, and cell wall chitin and mannan behavior was followed in all of the above samples. Approximately 100 cells were analyzed in each sample.

Cell diameter determination

The first 25 unbudded cells of the following fixed cultures were recorded for their minimal and maximal length using a calibrated micrometer and a Zeiss research photomicroscope (total mag. 1200X):

1) an unblocked log-phase culture (T_0),
2) a blocked log-phase culture (T_1, B=8),
3) an unblocked stationary-phase culture (T_2),
4) a blocked stationary-phase culture (T_3, B=24),
5) a blocked stationary-phase culture whose cells have been resuspended at the same density (B=24), in fresh YEPD medium, and which was further blocked for eight hours (T_4),
6) a blocked stationary-phase culture whose cells have been resuspended to a log-phase density (1×10^6 cells/ml.; B=24), in fresh YEPD medium, and which was further blocked for eight hours (T_5).

Volume determination

The volume of each of the above cells was calculated using the formula employed by Johnston, Pringle and Hartwell (1977), i.e.:

$$V = \left(\frac{\pi}{6}\right) \; l \; w^2$$

where, V = volume
l = maximal length of an individual cell
w = minimal length of an individual cell

This equation assumes prolate spheroid yeast cells.

Statistical analysis

The significance of differences between minimal length, maximal length, and volume of individual cells from each population was tested using a one-way variance analysis (Table 35) and the Duncan's new multiple-range test (Tables 32, 33, 34). The description of these analytical tests is given by Steel and Torrie (1960).

Karyogamy-deficient crosses (after the method described by Sena, Radin, Welch and Fogel, 1975).

Cells were grown in YEPD medium to early stationary-phase (5×10^7 cells/ml.). Then 5×10^7 cells/ml of α and *a kar-* cells were mixed together, centrifuged and incubated for 60 minutes in a 30°C water bath (with rotation), in 3.0% YED medium, pH = 4.5. Cells were then centrifuged, the supernatant discarded, and resuspended in 3.0% YED medium, pH = 8.5, for 60 minutes in the 30°C water bath (with rotation). At the end of that period, cells were pelleted and resuspended in 3.0% YED medium, pH = 4.5. After sonication, cells were diluted with fresh 3.0% YED medium, pH = 4.5, to a density of 1×10^7 cells/ml., centrifuged and incubated as a pellet for 30 minutes at 30°C. Then, the pellet was disrupted by gentle shaking, and after an additional 30 minutes, the mixture was shaken again and incubated without shaking at 30°C. That time corresponded to M=3.0 or three hours after initial mixing. Samples were collected before mixing (unmixed *a* and α *kar-* cells) or at M=3.0, M=4.0, M=5.0, M=6.0, M=6.5, M=7.0 and M=8.5 and fixed with saline-formaldehyde in Gurr's buffer pH = 6.8.

Cells were then analyzed for:

1) their budding behavior (number of budded and unbudded zygotes, location of buds),
2) the location and number of nuclei (DAPI- calcofluor-stained samples),
3) their chitin behavior (DAPI- calcofluor-stained samples),
4) their mannan behavior (FITC-Con A-stained samples, only sample M=3.0 and unmixed *a* and *α kar-* samples examined).

IV. OBSERVATIONS AND RESULTS

1. The eight-hour block

i. Giemsa-stained samples

A log-phase culture of the mutant haploid strain 182-6.3 *a cdc-24-1* was blocked at 37°C for eight hours. Samples were collected hourly, fixed and stained with Giemsa prior to examination by bright-field microscopy.

The nuclear and the budding behavior of cells contained in the above culture were observed as it progressed through the block. Results are illustrated in Table 2. The percentage of budding cells decreased progressively from 36.5% at B=0 (n=200) to 2.2% at B=8 (n=405). Most cells had ceased budding after two hours at restrictive temperature to reach a basic level of 6.5% or less. Binucleate cells appeared as soon as one hour after the temperature shift, to attain a level, when combined with multinucleate cells, of 28.9% by B=8 (n=405).

The consistency of the results obtained in duplicate experiments (Table 2, samples B=6 to B=8), indicates the reliability of the analysis and of the system when the experiment is repeated and samples analyzed by a second investigator.

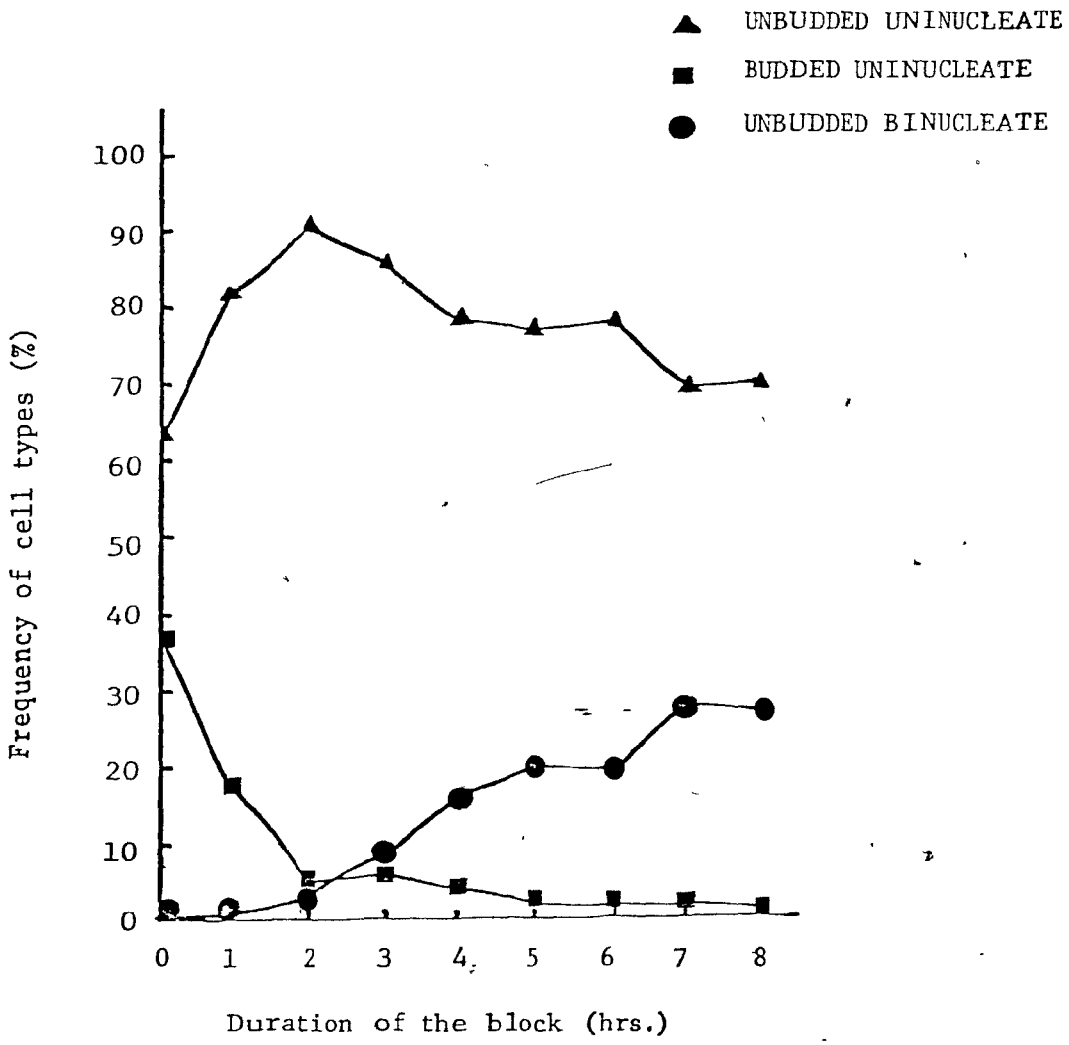
Figure 4 provides a graphical illustration of the behavior of the three most common types of cells found in Table 2, and shows a

Table 2. Characteristics of cdc-24-1 haploid strain 182-6.3 a stained with Giemsa during an eight-hour block at 37°C; frequency of cell types.

Number of hours of block[4]	B=0	B=1	B=2	B=3	B=4	B=5	B=6			B=7			B=8		
							M	M	C	M[1]	R[2]	C[3]	M	R	C
Sample size	200	200	200	200	200	200	200	200	400	200	238	438	200	205	405
Cell type															
	127 63.5%	164 82.0%	181 90.5%	171 85.5%	156 78.0%	154 77.0%	158 79.0%	154 77.0%	312 78.0%	135 67.5%	168 70.6%	303 69.2%	140 70.0%	144 70.2%	284 70.1%
	73 36.5%	35 17.5%	10 5.0%	12 6.0%	8 4.8%	4 2.0%	1 0.5%	6 3.0%	7 1.8%	2 1.0%	6 2.5%	8 1.8%	1 0.5%	2 1.0%	3 0.7%
		1 0.5%	6 3.0%	17 8.5%	32 16.0%	40 20.0%	39 19.5%	39 19.5%	78 19.5%	61 30.5%	62 26.1%	123 28.1%	52 26.0%	57 27.8%	109 26.9%
			3 1.5%		4 2.0%	2 1.0%	2 1.0%	1 0.5%	3 0.8%	1 0.5%	1 0.4%	2 0.5%	5 2.5%	0	5 1.2%
										1 0.5%	0	1 0.2%	1 0.5%	1 0.5%	2 0.5%
											1 0.4%	1 0.2%	1 0.5%	0	1 0.3%
														1 0.5%	1 0.3%

[1] Sample analyzed by the author (M)
[2] Sample analysed by Dr. E.R. Boothroyd (R)
[3] Combined results (C)
[4] Unblocked sample (B=0), sample blocked for one hour (B=1), ... etc.

Figure 4. Budding and nuclear behavior of the three most common types of cells observed during the block



clear inverse relationship between the number of uninucleate budded cells and the number of unbudded binucleate cells.

ii. DAPI- and calcofluor-stained samples

A log-phase culture of the same mutant strain brought to the same density and subcultured the same number of times as Giemsa-stained material, was blocked at 37°C for eight hours. Samples were collected hourly and fixed as before, but cells were stained with DAPI combined with calcofluor or with calcofluor alone prior to examination by fluorescence microscopy.

a) Budding behavior

Table 3 shows the number of budding cells recorded at various stages of the block. By three hours of block, budding was reduced to 12% or less (cf. nuclear and budding behavior of blocked cells stained with Giemsa in part b) of this section). When the data in Table 3 are plotted as the percentage of budded cells versus the duration of the block (Fig. 5), it is found that the decrease in budding ability is noticeable within one hour at restrictive temperature, as observed with cells stained with Giemsa. A smaller slope between B=0 and B=1 is indicative that some time might be needed at the start for the culture to reach 37°C. An experiment has therefore been designed to estimate the time needed for a log-phase culture to reach effective blocking

temperature (Table 4, Fig. 6). The reported effective blocking temperature for this mutant is 36°C (Hartwell, Culotti and Reid, 1970; Hartwell, Mortimer, Culotti and Culotti, 1973; Sloat and Pringle, 1978; Sloat, Adams and Pringle, 1981). This temperature was reached in all flasks within four minutes at 37°C (water bath temperature). When the temperature of the medium is plotted against time, the average time for a flask to reach 36°C is 220 seconds (Fig. 6).

The distribution of the relative volume of the buds remaining on mother cells, as the block progresses, is represented in Table 5. Unblocked (B=0) or early blocked cells (B=1) display a nearly normal distribution of budded cells, with about 50% of the budding population having buds smaller than half the relative volume of their mother cells. Later (B=2, B=3), fewer cells with large buds are observed. After three hours of block, little can be said of significance.

In addition to apparent diminishing size of remaining buds relative to mother cells during the block, a decrease in the relative size of buds to mother cells when nuclei enter buds has been observed (Table 6, Figs. 9, 10). Nuclear division is occurring in unblocked cells when the bud is about half the volume of the mother-cell (Table 6, Fig. 9).

The loss of budding ability through the block can also be demonstrated by recording cell density at the beginning and end

of the block period and by comparing the obtained values with a wild-type strain of the same ploidy and mating-type whose budding behavior is not affected by a switch to the higher temperature. Table 7 shows that cell density, recorded using a hemacytometer, has increased for the mutant cells by nearly 60% for an eight-hour block and by approximately 1310% for the wild type X-2180-1A a under the same growing conditions, even if started at a 10 fold higher density.

During the block, there was a continuous growth of the ts mutant cells (see section 5, cf. Fig. 11 with Figs. 9 and 12).

Occasionally, a few cells escape the block to produce buds that render the mother cells pear-shaped (see section 5, and Fig. 12). Among the few cells successful in escaping the block over an eight-hour period, only one bibudded cell, whose number of nuclei is unknown, has been observed.

A few "small cells" containing little cytoplasm and one nucleus (one exception at B=8) were seen connected to large blocked cells (Table 8; seventeen small cells were recorded, but more were seen during scanning, see Fig. 13). Small cells are a class of cells differing from other classes by being smaller than one-quarter the relative volume of their mother cells, and by being somehow attached to the latter at one point (sonication will not disrupt the connection). In most cases, these small cells contain one nucleus. They are also totally absent prior to a two-hour block period.

Table 3. Budding behaviour of log-phase 182-6.3 a cdc-24-1 cells blocked for eight hours.

Stage	Sample Size	No. of Budded Cells (%)	No. of Unbudded Cells (%)
B = 0	100	55 (55.0)	45 (45.0)
B = 1	102	44 (43.1)	58 (56.9)
B = 2	100	25 (25.0)	75 (75.0)
B = 3	100	7 (7.0)	93 (93.0)
B = 4	100	12 (12.0)	88 (88.0)
B = 5	100	12 (12.0)	88 (88.0)
B = 6	100	4 (4.0)	96 (96.0)
B = 7	103	1 (1.0)	102 (99.0)
B = 8	101	7 (6.9)	94 (93.1)

Figure 5. Frequency of budded cells throughout the block

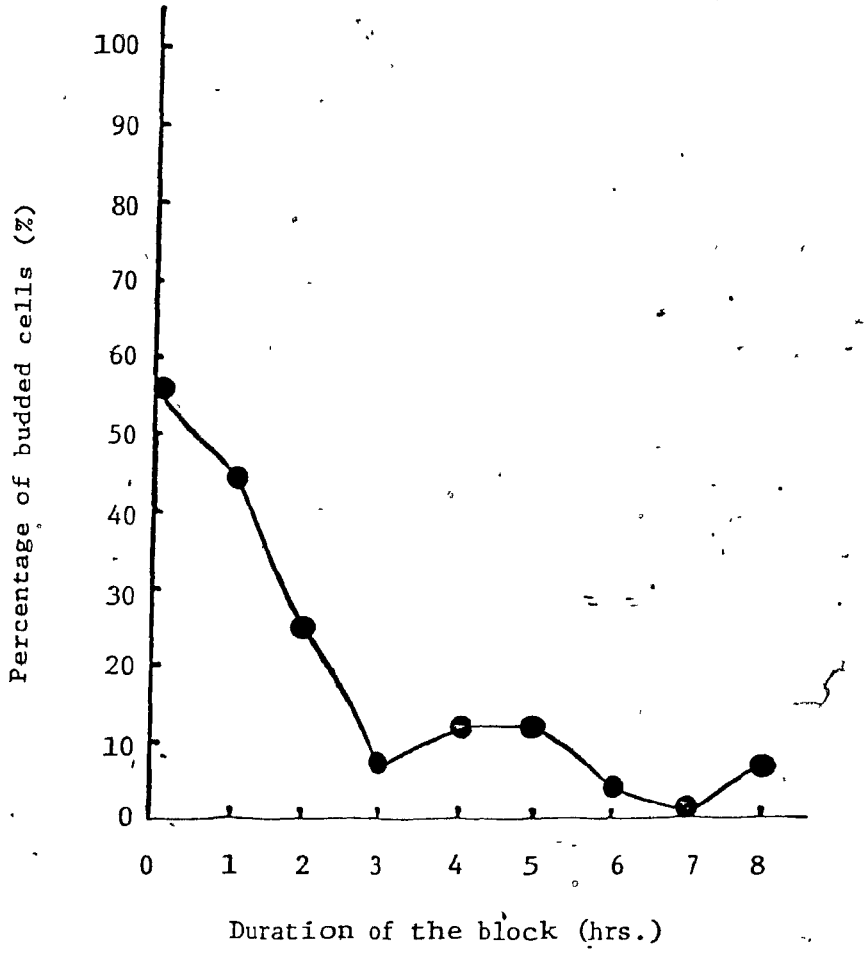


Table 4. Time needed for YEPD culture medium to increase from room temperature to 37°C.

	TEMPERATURE (°C)[3]			
Time (Secs)	Exp. I	Exp. II	Exp. III	Ave[2]
0	24.0	23.5	23.0	23.5
60	NR[1]	30.0	29.5	30.0
120	34.5	34.0	33.0	34.0
180	35.5	35.5	35.0	35.5
240	36.5	36.0	36.0	36.0
300	37.0	36.5	36.5	36.5
360	NR	37.0	37.0	37.0
420	NR	37.0	37.0	37.0

1 NR, not recorded

2 Averaged to the closest half of degree

3 Recorded to the closest half of degree

Figure 6. Average flask temperature (^{o}C) as incubation time increases (secs.)

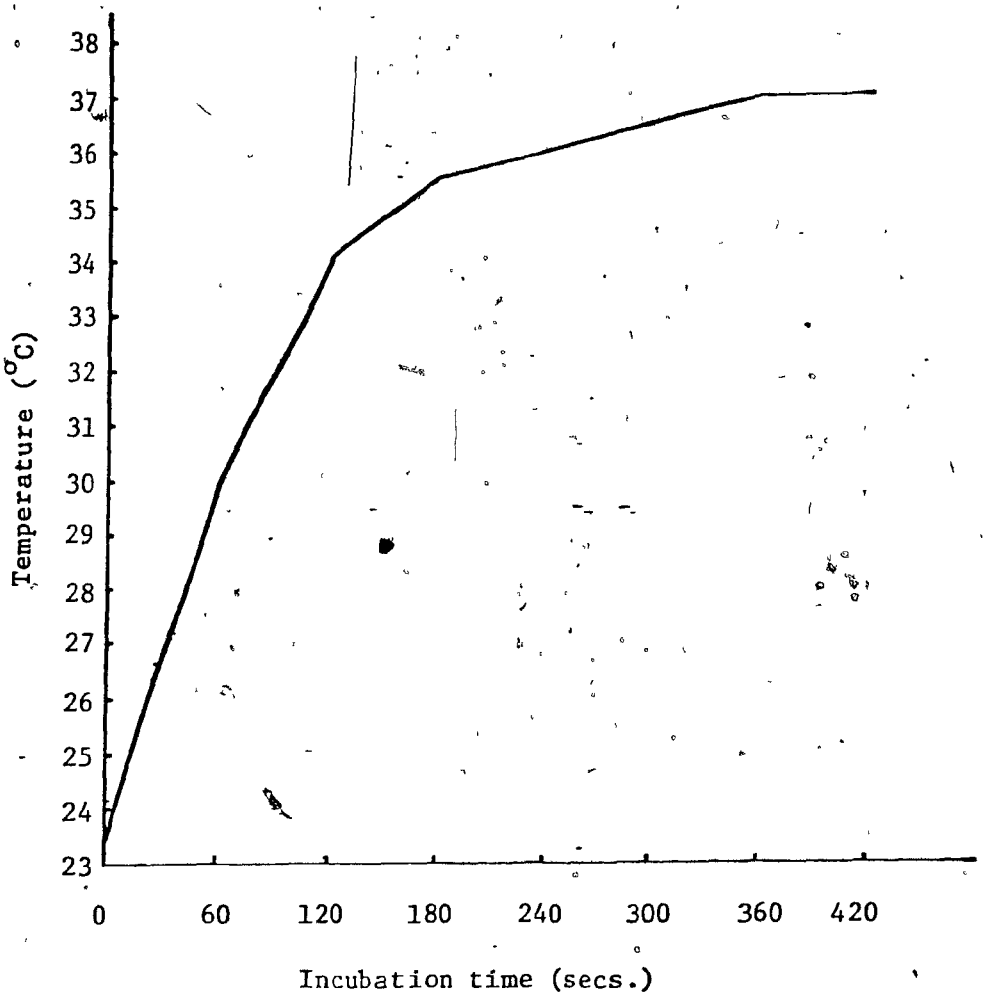


Table 5. Distribution of bud size greater than or equal to half the relative volume of their mother cells during the block.

Stage	No. of recorded budded cells	No. of budded cells with buds ≥ 1/2 volume of mother cells	Percentage (%)
B=0	55	24	43.6
B=1	44	21	47.8
B=2	25	4	16.0
B=3	7	1	N.S.[1]
B=4	12	3	N.S.
B=5	12	6	N.S.
B=6	4	0	N.S.
B=7	1	1	N.S.
B=8	7	0	N.S.

[1] N.S., not significant

Table 6. Volume of buds relative to mother cells during nuclear division and when nuclei enter buds.

	During Nuclear Division		When Nuclei enter Buds		
Stage	Size of buds Relative to Mother Cells at Nuclear Division (Volume)	No. of Cases Observed	Relative Bud Size (Expressed As a Fraction of Mother Cell Volume)	Proportion of buds Having one or more Nuclei	Estimated Stage at which Nuclear Division is occurring (Relative bud/ Mother Cell Volume)
B=0	1/2	2	3/4	10/10	
			1/2	8/14	1/2
			≤ 1/3	0/27	
B=1	1/2	1	1:1	4/4	
			3/4	13/13	
			1/2	4/4	≤ 1/2
			≤ 1/3	0/23	
B=2	1/4	4	1:1	1/1	
			3/4	3/3	
			1/4	8/10	≤ 1/4
	1/8	1	1/8	2/7	
			≤ 1/16	0/3	
B=3	-	-	3/4	1/1	
			1/4	1/1	< 1/4
			≤ 1/32	0/5	
B=4	1/4	1	3/4	2/2	
			1/2	1/1	
			1/4	2/2	
			1/8	2/2	≤ 1/4
			1/16	1/2	
			< 1/16	2/2	
B=5	-	-	3/4	5/5	
			1/2	1/1	
			1/8	1/1	< 1/8
			1/16	2/5	

* After stage B=5 buds too small and rare to yield reliable data

Table 7. Cell density at the beginning and end of the block period of mutant strain 182-6.3 a cdc-24-1 and wild-type strain X-2180-1A a.

Strain	182-6.3 a			X-2180-1A a		
Cell density (cells/ml)	B=0 $X10^6$	B=8 $X10^6$	Percentage increase(%)	B=0 $X10^7$	B=8 $X10^8$	Percentage increase(%)
Assay I	1.1000	1.8125	65	1.5250	2.0750	1261
Assay II	1.1250	1.7500	56	1.6500	2.4000	1355
Average	1.1125	1.7813	60.5	1.5875	2.2375	1308

Table 8. Number of small cells recorded at various stages of the block.

Stage	Relative size of the bud (volume)	Frequency	Total	Number of nuclei per small cell
B=2	1/8	1	1	1
B=3	1/4	1	1	1
B=4	1/4	1	5	1
	1/8	2		1
	1/16	1		1
	< 1/16	1		1
B=5	1/8	1	3	1
	1/16	2		1
B=6	< 1/4	1	1	1
B=7	1/16	2	3	1
	< 1/16	1		1
B=8	1/8	1	3	1
	1/16	1		1
	1/32	1		0

b) Nuclear behavior

Despite budding cessation, DNA synthesis and nuclear division proceed, as reflected by the appearance of bi- and multinucleate cells (Table 9, Fig. 7). The number of cells having two or more nuclei increases steadily after a one-hour period to reach a value close to 90% at B=8.

Fewer budding and multinucleate cells have been detected with Giemsa staining and bright-field microscopy than with DAPI/ calcofluor staining and fluorescence microscopy. Although the total magnification was slightly higher in the latter (1000X) than in the former (900X), this effect alone is not expected to generate such a difference.

There are two types of binucleate cells appearing during the block (Table 10); those which have their nuclei far apart (Figs. 14 and 15) and those whose nuclei are lying side by side i.e. less than one half nucleus diameter apart. At B=8, these cell types represent respectively 10.3% (8/78) and 89.7% (70/78) of the total binucleate population. In addition to these 78 binucleate cells, 9 uninucleates, 4 trinucleates, 1 tetranucleate and 9 unknown were observed (n=101, Table 9).

In most cases, nuclei tend to be somewhat larger than at permissive temperature (Range for B=0: Figs. 16 and 17, for B≠0: Figs. 14 and 15).

During the block, nuclei are pushed against the cell wall

Table 9. Nuclear behavior of the mutant strain 182-6.3 a cdc-24-1 during the eight-hour block.

Stage	Sample Size n=	Number of Unbudded Cells (%)	Number of Nuclei (N)[2]	Percentage of Sample Size (%)	Number of Budded Cells (%)	Number of Nuclei (N)	Percentage of sample size (%)	Total No. of bi- or multinucleate cells in sample[1] (%)
B=0	100	45(45.0)	1N=45	45.0	55(55.0)	1N=38 1dN= 2 1N,1N=15	38 0 2.0 15.0	0 (0.0)
B=1	102	58(56.9)	1N=58	56.9	44(43.1)	1N=23 1dN= 1 1N,1N=20	22.5 1 0 19.6	0 (0.0)
B=2	100	75(75.0)	1N=66 2N= 9	66.0 9.0	25(25.0)	1N=11 1dN= 5 1N,1N= 8 2N,2N= 1	11.0 5.0 8.0 1.0	10 (10.0)
B=3	100	93(93.0)	1N=72 1dN= 3 2N=18	72.0 3.0 18.0	7(7.0)	1N=2 1N,1N= 2 2N= 3	2.0 2.0 3.0	21 (21.0)
B=4	100	88(88 0)	1N=58 1dN= 2 2N=28	58.0 2.0 28.0	12(12.0)	1N= 2 1dN= 1 1N,1N= 4 2N,1N= 5	2.0 1.0 4.0 5.0	33 (33.0)
B=5	100	88(88.0)	1N=48 1dN= 1 2N=36 3N= 2 UNK.= 1	48.0 1.0 36.0 2.0 1.0	12(12.0)	1N,1N= 5 1N,2N= 1 2N= 3 2N,1N= 2 2N,2N= 1	5.0 1.0 3.0 2.0 1.0	44 (44.0)
B=6	100	96(96.0)	1N=23 1dN= 2 2N=70 3N= 1	23.0 2.0 70.0 1.0	4(4.0)	2N= 2 2N,1N= 1 2N,2N= 1	2.0 1.0 1.0	75 (75.0)
B=7	103	102(99.0)	1N=17 1dN= 1 2N=84	17.0 1.0 84.0	1(1.0)	2N,1N= 1	1.0	85 (82.5)
B=8	101	94(93.1)	1N= 8 2N=74 2N,1dN=1[3] 3N= 3 4N= 1 UNK.= 7	8.0 74.0 1.0 3.0 1.0 7.0	7(6.9)	1N= 1 2N= 3 2N,1N= 1 UNK.= 2	1.0 3.0 1.0 2.0	83 (90.2)

[1] Excluding unknown cells

[2] One nucleus=1N, one dividing nucleus=1dN, one nucleus in mother-cell and on nucleus in bud= 1N,1N,...etc.

[3] This unbudded cell is classified as having three nuclei (among which one is dividing).

Figure 7. Appearance of bi- and multinucleate cells during the eight-hour block

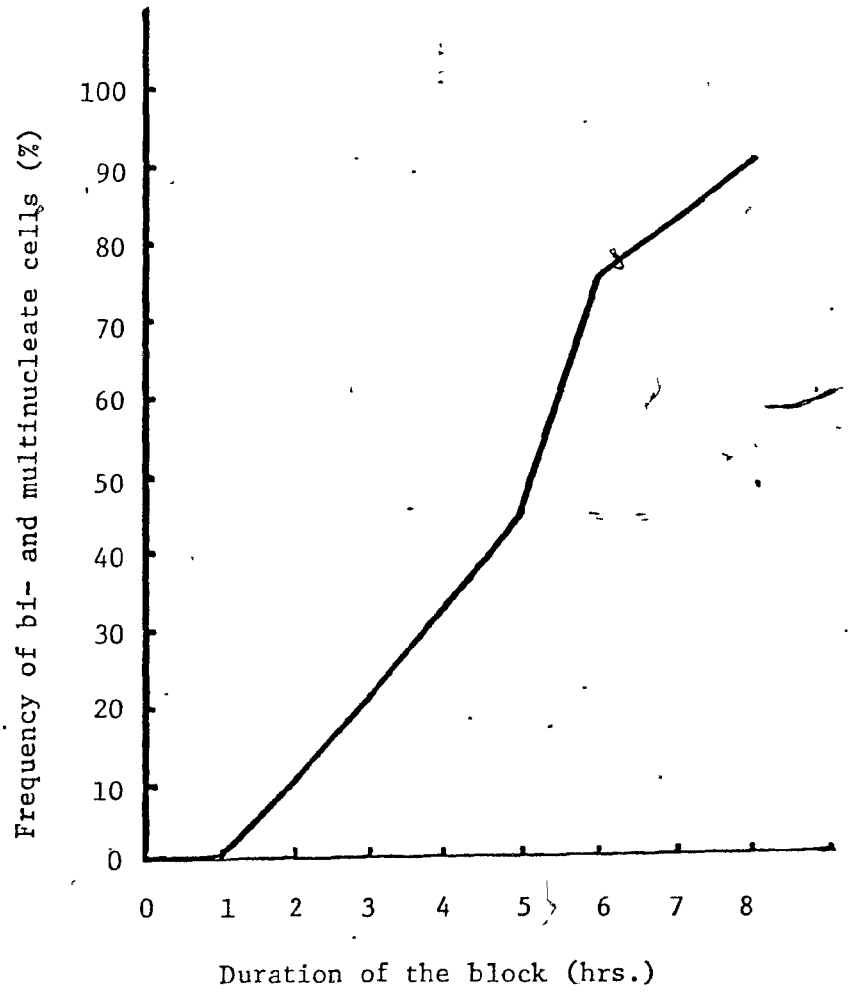


Table 10. Frequency and type of bi- and multinucleate cells observed during the block.

Stage	Number of nuclei (N)	Distribution of nuclei (Type)		Frequency[1] of type among bi- and multinucleate cells (%)
B=2	2	UNB.	8 close	80.0
			1 apart	10.0
		BUD.	1 apart	10.0
B=3	2	UNB.	18 close	75.0
			3 apart	12.5
		BUD.	2 close	8.3
			1 apart	4.2
B=4	2	UNB.	28 close	80.0
			2 apart	5.7
		BUD.	4 close	11.4
			1 apart	2.9
B=5	2	UNB.	35 close	77.8
			2 apart	4.4
			3 close	6.7
		BUD.	3 apart	6.7
	3	UNB.	2 apart	4.4
B=6	2	UNB.	59 close	76.6
			13 apart	16.9
		BUD.	3 close	3.9
			1 apart	1.3
	3	UNB.	1 apart	1.3
B=7	2	UNB.	73 close	84.9
			12 apart	14.0
		BUD.	1 apart	1.2
B=8	2	UNB.	66 close	79.5
			8 apart	9.6
		BUD.	4 close	4.8
	3	UNB.	4 apart	4.8
	4	UNB.	1 apart	1.2

[1] may not add to 100.0%, ± 0.1%

Table 11. Number and position of nuclei in multinucleate cells.

Stage	Number of nuclei	Position of nuclei	Frequency
B=5	3		2
B=6	3		1
			3
B=8	3		1
	4		1

(Fig. 15) by enlarging vacuoles (Figs. 16, 18 (B=0); Figs. 19, 20 (B=8)). Vacuoles are becoming conspicuous at B=6 due to their large size. They could be best seen with Nomarski interference contrast microscopy at B=8 (Fig. 20).

When the distribution of nuclei is followed in multinucleate cells, one observes that nuclei are grouped most often two by two (one exception at B=6; Table 11).

c) Chitin behavior

There are two kinds of chitin fluorescence appearing during the block: localized (Table 12, Fig. 22) and general (Table 13, Figs. 23 and 24). When cells are blocked, the first two hours of an eight-hour block show that chitin is not only randomly deposited in the cell wall (general fluorescence) but also spreads from localized areas such as the chitin ring and bud scars (localized fluorescence). Localized spread of chitin appears sooner than general fluorescence which is prominent at B=2 and is observed in nearly all cells by B=3 (Tables 12 and 13, Fig. 8). The sequence of chitin distribution in blocked cells can be followed by examining Figs. 21 (B=0), 22 (B=1), 23 (B=5) and 24 (B=8).

Leaky cells, many of which are pear-shaped, have less fluorescent "buds" with no ring or sometimes a weak or ill-defined line (partial ring) at the mother cell-bud junction

Table 12. Frequency of blocked cells showing localized spread of chitin fluorescence.

Stage	Sample size	Frequency of unbudded cells showing localized spread[1]	Percentage (%)	Frequency of budded cells showing localized spread[2]	Percentage (%)	Total (%)
B=0	100	0/45	0.0	0/55	0.0	0 (0.0)
B=1	102	7/58	12.1	20/44	45.4	27(26.4)
B=2[3]	100	6/75	8.0	8/25	32.0	14(14.0)

[1] cells must show chitin "diffusing" from buds scars or from discrete sites in the cell wall. Cells may show low general chitin fluorescence.

[2] mother cells must show chitin "diffusing" from bud scars or chitin rings or from discrete sites in the cell wall. Cells may show low general chitin fluorescence.

[3] General chitin fluorescence is becoming too strong, and covers too much of the surface of the cell wall to allow the detection of localized spread of chitin.

Table 13. Frequency of blocked cells showing general chitin fluorescence.

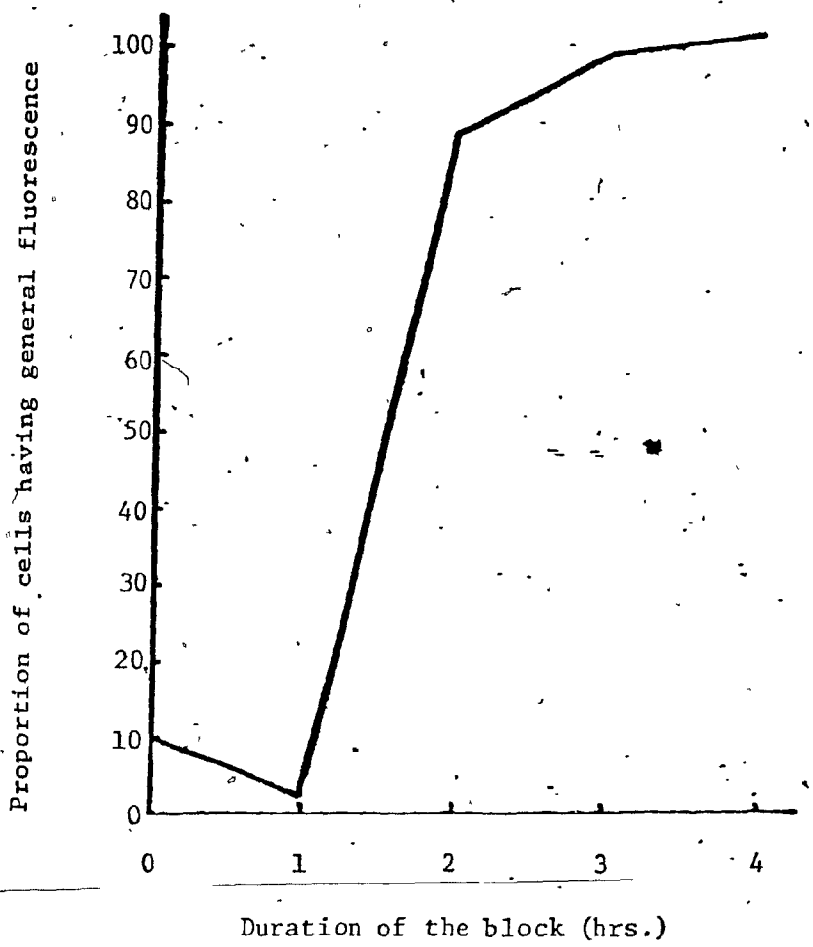
Stage	Sample size	Frequency of unbudded cells showing general fluorescence[1]	Percentage (%)	Frequency of budded cells showing general fluorescence[2]	Percentage (%)	Total (%)
B=0	100	4/45	8.9	5/55	9.1	9 (9.0)
B=1	102	1/58	1.7	2/44	4.5	3 (2.9)
B=2	100	67/75	89.3	22/25	88.0	89 (89.0)
B=3	100	92/93	98.9	7/7	100.0	99 (99.0)
B=4[3]	100	88/88	100.0	12/12	100.0	100(100.0)

[1] cells must be entirely fluorescent, with or without sites of higher fluorescence.

[2] mother cells must be entirely fluorescent, with or without sites of higher fluorescence.

[3] later samples similar to B=4.

Figure 8. Frequence of blocked cells showing general chitin fluorescence



(Fig. 12, see also section 4 on the role of the chitin ring).

Sometimes, abnormal chitin fluorescence is detected (Fig. 23) which might correspond to cell wall abnormalities observed with electron microscopy (Boothroyd, unpublished results).

Moreover, throughout the eight-hour block, bud scars seem hidden by the general fluorescence and do not appear to be dismantled by the increasing size of the mother-cell, an observation which suggests that they are stable structures (Fig. 25, further evidences given in section 2).

iii. FITC-Con A -stained samples

Samples were prepared as described in part ii, except that cells were stained with FITC-Con A instead of DAPI and calcofluor, and that only stages B=0 and B=8 were investigated.

When the mutant was blocked for eight hours at 37°C, cells were more fluorescent at B=8 (Fig. 27) than at B=0 (Fig. 26), showing that mannan, like chitin, becomes widely distributed as it is synthesized throughout the block. At B=0, there is already a rather large amount of accumulated mannan (Fig. 26) even in budded cells (Figs. 28, 29, 30), as compared with chitin (Fig. 21). At B=8, however, the fluorescence for mannan (Figs. 27, 31, 32) is not as strong as for chitin (Fig. 24), suggesting that despite a higher initial fluorescence (B=0), the accumulation for mannan is not as great in an eight-hour period.

Interestingly, regions of less fluorescence were seen in the cell wall (Fig. 31). In addition, in unblocked cells, very small buds (ca. $\leq$ 1/16 volume of mother cells) were less fluorescent than their mother cells (3/3 cases), but soon afterwards as they increased in size they became as fluorescent (Figs. 28, 29, 30; 56/56 cases), [n=100, 59 budding cells[1]].

Again, pear-shaped cells were observed at B=8 and these leaky cells have less fluorescence at their tips (3/3 cases). In other cells, some abnormal fluorescence was seen (Fig. 33) which again might represent regions of cell wall abnormalities. There were also a few cells with large buds showing more fluorescence at neck region (Fig. 32). This observation is equally valid for unblocked budding cells (Figs. 28 and 30), although in that case the fluorescence is evenly distributed over the whole neck region. Regions of higher mannan fluorescence were also seen in unblocked cells which might correspond to either small regions of cell wall abnormalities or recent areas of bud detachment, i.e. fresh bud scars (Figs. 26 and 30).

[1] This figure is consistent with the number of budding cells (55%) in the unblocked sample stained with DAPI- calcofluor.

iv. Miscellaneous observations

In the unblocked mutant, most bud scars appeared in a row (Table 14) and all cells showed a basic level of chitin fluorescence, albeit weak (range Figs. 16 and 17). It was common for cells having many bud scars to have a higher basic level of chitin fluorescence in their walls than cells with no scars.

Furthermore, "orange bodies" appeared inside vacuoles during the block (Table 15, Fig. 34). Upon exposure to ultra-violet light, these bodies show a strong orange fluorescence but the emission disappears quickly, within approximately 10 seconds. They are frequent at B=3, but not before, and become more yellowish at later stages (B=6 and longer). The number of fluorescent granules, of nuclei, and the budding state of blocked cells having orange bodies can be seen in Table 15. The number of orange bodies per cell displaying them ranges from one to four, and seems unrelated to the number of nuclei contained in these cells. After three hours of block, 9.4% (53/561) of unbudded cells possess at least one orange body, while 2.3% (1/43) of the budded population displays a corresponding trend. Thus, the tendency is for unbudded cells to have orange bodies.

Similar fluorescent particles have been observed in algal cells stained with DAPI or 33258 Hoechst (Coleman, 1978). However, since these particles were not seen in rapidly growing or phosphate-limited cells, Coleman suggested that they might be polyphosphate granules

(volutin). She demonstrated that these granules stained metachromatically with toluidine blue (Coleman, 1978) and can be extracted in ice-cold 2% perchloric acid (Coleman, 1979), two observations which are consistent with their polyphosphate nature.

In order to learn more about the nature and behavior of orange bodies in yeast cells, similar treatments were applied to the blocked and unblocked mutant (Table 16). Experiments I to VI show that DAPI is responsible for the fluorescence of orange bodies which arise during the block and are located exclusively inside vacuoles. Experiments VII to VIII show that when cells are stained with toluidine blue, metachromatically-stained granules are visible in unblocked cells, although they are rather small and randomly distributed. At B=8, their behavior parallels that of orange bodies in that they are larger and less numerous than at B=0, and located at the same sites as orange bodies. When mutant cells are extracted for one hour with perchloric acid (experiments IX to XII), orange bodies and metachromic granules tend to diminish or disappear. To make sure that orange bodies and metachromic granules do not show such a behavior because of the increase in temperature instead of the mutation effect, a wild type strain unaffected by the ts mutation (same ploidy level and mating type), was submitted to identical treatments (experiments XIII to XVI). The results indicate that orange bodies do not appear after eight hours at 37°C, nor do metachromatically-stained granules behave differently than at B=0.

Table 14. Bud scar distribution in unblocked mutant cells.

No. of bud scars on mother cells[1]	Frequency	Distribution
2	4	in a row
3	4	in a row
4	4	in a row
5	1	in a row
unknown but > 2	4	in a row
unknown but > 4	1	in a row
4	1	3 in a row + 1 adjacent
4	1	random[2]

[1] Include the chitin ring in budding cells

[2] In addition cell show high general chitin fluorescence

Table 15. Frequency and type of mutant cells with orange bodies at various stages of the block.

Stage	No. of cells with orange bodies	No. of orange bodies per cell	Frequency	Nuclei Number	Frequency	Budding state[1]
B=0	0/100	-	-	-	-	-
B=1	0/102	-	-	-	-	-
B=2	0/100	-	-	-	-	-
B=3	25/100	1	15	1	11	UNB
				2	3	UNB
				2	1	BUD[2]
		2	8	1	7	UNB
				UNKNOWN	1	UNB
		3	1	UNKNOWN	1	UNB
		4	1	UNKNOWN	1	UNB
B=4	4/100	1	4	1	4	UNB
B=5	7/100	1	6	1	6	UNB
		3	1	1	1	UNB
B=6	9/100	1	4	2	4	UNB
			3	1	3	UNB
		2	2	2	2	UNB
B=7	0/103	-	-	-	-	-
B=8	9/101	1	3	1	3	UNB
		2	3	UNKNOWN	2	UNB
				1	1	UNB
		3	3	2	3	UNB

[1] UNB, unbudded; BUD, budded

[2] Bud has one orange body

Table 16. Behavior of orange bodies and metachromatically-stained granules in mutant and wild-type cells after various treatment.

Experiment No.	Strain used	Stage	Treatment	Observations
I	182-6.3 a	B=0	DAPI-Calcofluor	"Orange bodies" not seen
II	182 6.3 a	B=8	DAPI-Calcofluor	Large "orange bodies" seen in vacuoles
III	182-6.3 a	B=0	Calcofluor	as for I
IV	182-6.3 a	B=8	Calcofluor	as for I
V	182-6.3 a	B=0	DAPI	as for I
VI	182-6.3 a	B=8	DAPI	as for II
VII	182-6.3 a	B=0	Toluidine Blue D	Small reddish and bluish granules observed either inside or at periphery of the cell
VIII	182-6.3 a	B=8	Toluidine Blue D	Granules are observed in vacuoles, never at periphery. They are larger than at B=0, and have no color or are bluish. Total number of granules has decreased
IX	182-6.3 a	B=0	DAPI + PCA[1]	as for I
X	182-6.3 a	B=0	Toluidine Blue D + PCA	Fewer and smaller granules seen than in VII
XI	182-6.3 a	B=8	DAPI + PCA	Very few "orange bodies" were observed inside vacuoles. They faded faster than in VI and II and were smaller
XII	182-6.3 a	B=8	Toluidine Blue D + PCA	Granules inside vacuoles are smaller, less numerous, and less stainable than in VIII
XIII	X-2180-1A a	B=0	DAPI	as in I
XIV	X-2180-1A a	B=0	Toluidine Blue D	Most granules associated with vacuoles
XV	X-2180-1A a	B=8	DAPI	as in I
XVI	X-2180-1A a	B=8	Toluidine Blue D	as in XIV

[1] PCA, perchloric acid

PLATE I. Budding behavior during the block

Figures 9 to 13. 182-6.3 a cdc-24-1 mutant cells stained with calcofluor and DAPI (Figs. 9 and 10) or with calcofluor alone (Figs. 11, 12 and 13). 1500X.

Fig. 9. In unblocked cells nuclei enter buds about half the volume of the mother-cell (B=0).

Fig. 10. Nucleus has divided into bud about one quarter the relative volume of the mother-cell (B=2).

Fig. 11. Blocked cells become larger, unbudded and more spherical (B=8).

Fig. 12. Example of a pear-shaped cell. The bud is less fluorescent than the mother-cell and has a partial ring at its base (B=5).

Fig. 13. Example of a small cell. The less fluorescent small cell seems connected to its progenitor (B=8).

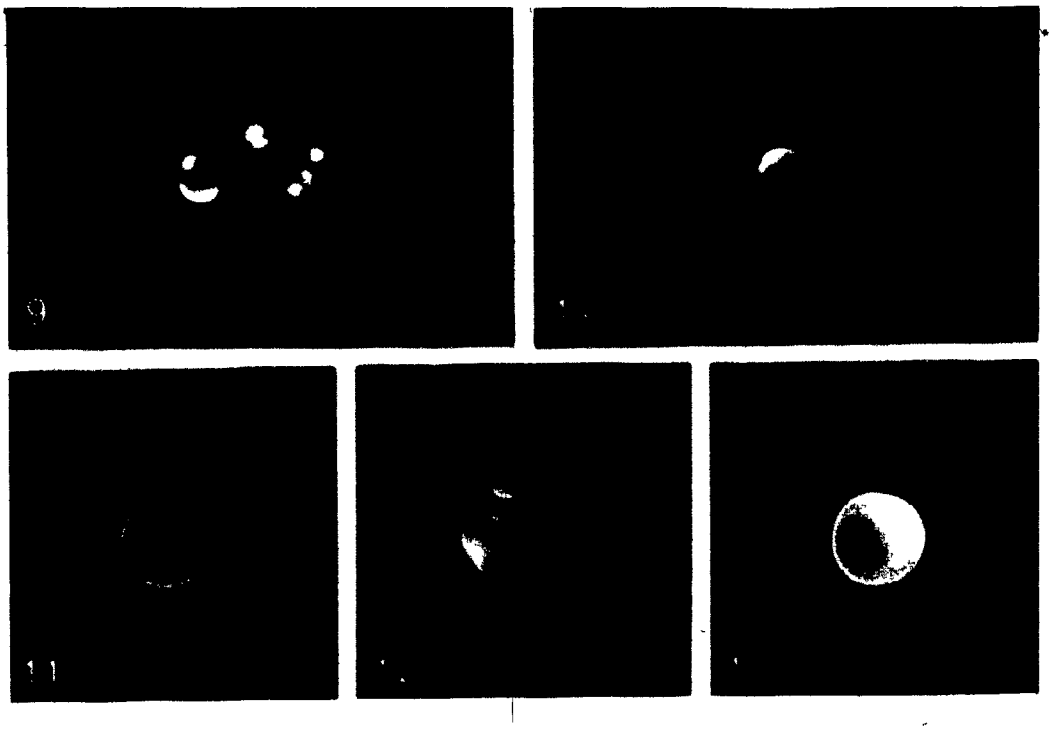


PLATE II. Nuclear behavior during the block

Figures 14 to 20. 182-6.3 *a* *cdc-24-1* mutant cells stained with calcofluor and DAPI (Figs. 14, 15, 16 and 17; 1500X), or calcofluor alone (Fig. 19; 1500X), or unstained (Figs. 18 and 20; 1200X).

Fig. 14. Budded binucleate cell with nuclei far apart. Bud (out of focus) is less fluorescent than mother cell (B=3).

Fig. 15. Unbudded binucleate cell with nuclei far apart showing more fluorescence at one cell wall region. Nuclei are close to the cell wall (B=7).

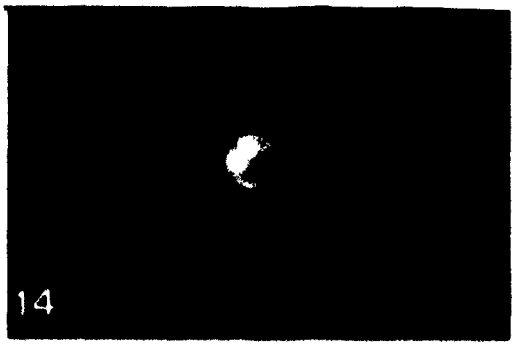
Fig. 16. Unblocked budded uninucleate cell. Note that less fluorescent bud has a chitin ring at its base, and the presence of a small vacuole in mother-cell near bud neck region (B=0).

Fig. 17. Unblocked budded uninucleate cell with nucleus larger than in Fig. 16 (B=0).

Fig. 18. Unblocked budded uninucleate cell observed with Nomarski interference contrast. Note vacuole and numerous granular inclusions (B=0).

Fig. 19. Unbudded cell with very large vacuole near cell wall (B=8).

Fig. 20. Cluster of unbudded cells observed with Nomarski interference contrast. Note very large vacuoles and their numerous granular inclusions (B=8).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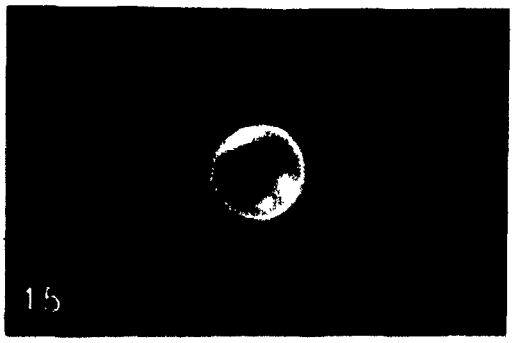
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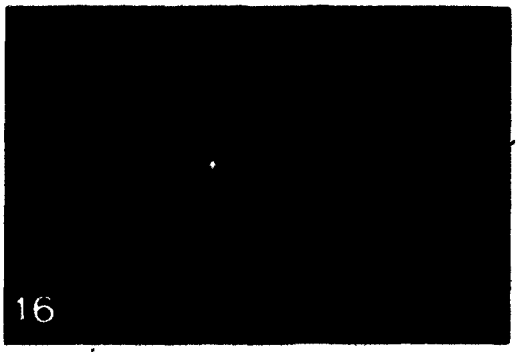
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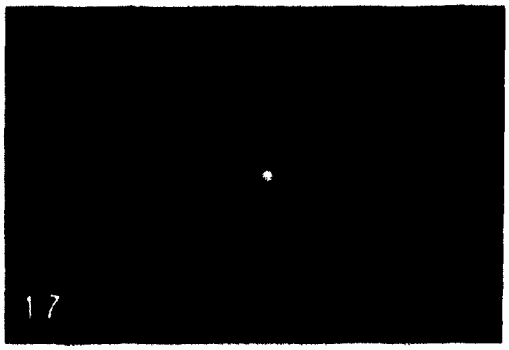
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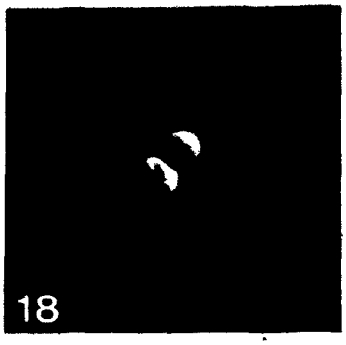
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PLATE III. Chitin behavior during the block

Figures 21 to 25. 182-6.3 a cdc-24-1 mutant cells stained with calcofluor; 1500X.

Fig. 21. Unblocked budded cell with a ring of chitin at mother cell-bud junction (B=0).

Fig. 22. Budded cells showing spreading of fluorescence from the mother cell-bud junction and increasing general fluorescence (B=1).

Fig. 23. Unbudded cells with general fluorescence. One cell has a site of higher fluorescence (B=5).

Fig. 24. Unbudded cell with high general fluorescence (B=8).

Fig. 25. One cell with three bud scars in a row and general chitin fluorescence (B=5).

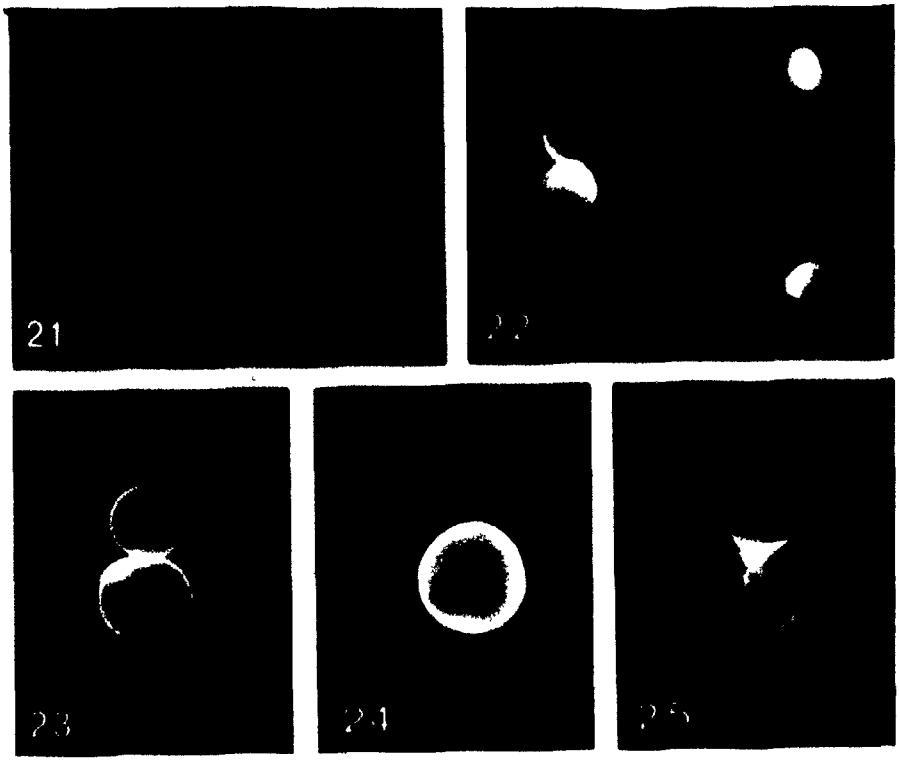
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PLATE IV. Mannan behavior during the block

Figures 26 to 33. 182-6.3 *a* *cdc-24-1* mutant cells stained with FITC-Con A; 1500X.

Fig. 26. Unblocked unbudded cell showing basic level of mannan fluorescence. Note region of higher fluorescence (B=0).

Fig. 27. Unbudded cell with extensive level of mannan fluorescence (B=8).

Fig. 28. Unblocked budded cell with small bud slightly less fluorescent than its mother cell. Higher mannan fluorescence at mother cell-bud junction (B=0).

Fig. 29. Unblocked budded cell with larger bud than in Fig. 28, being as fluorescent as its parent. Note higher general fluorescence of both the bud and mother-cell (B=0).

Fig. 30. Unblocked cell with large bud as fluorescent as its parent. Higher mannan fluorescence in bud neck and lateral walls (B=0).

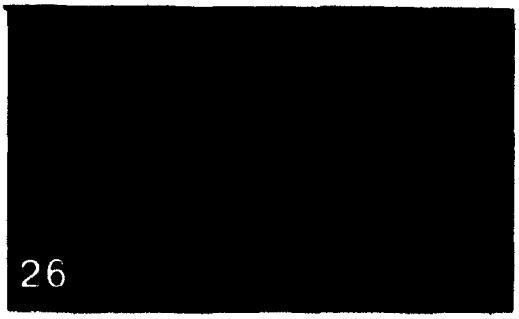
Fig. 31. Unbudded cell with region of less fluorescence (B=8).

Fig. 32. Large cell with connected bud. Note unequal strong fluorescence at the bud junction (B=8).

Fig. 33. Large cell with extensive cell wall abnormalities (B=8).

PLATE V. Occurrence of orange bodies during the block

Figure 34. 182-6.3 *a* *cdc-24-1* mutant cells stained with DAPI and calcofluor; 1500X. Cells with granules fluorescing for a short time with an orange color (B=6).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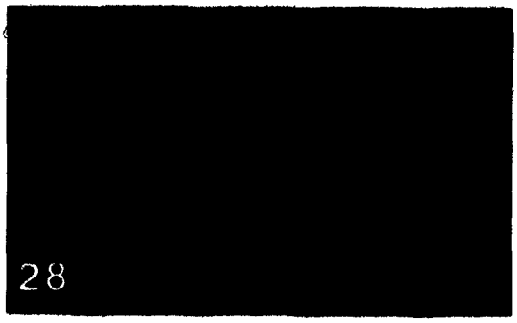
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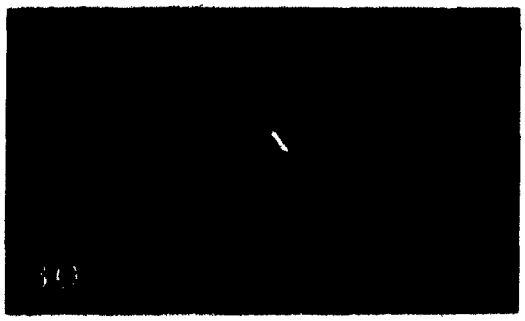

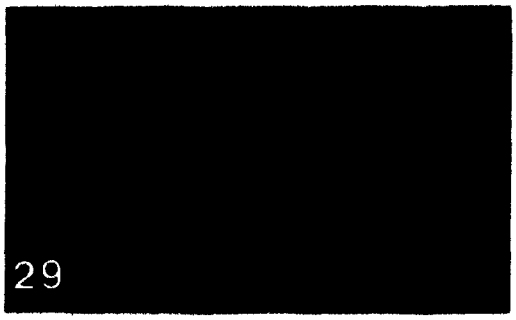
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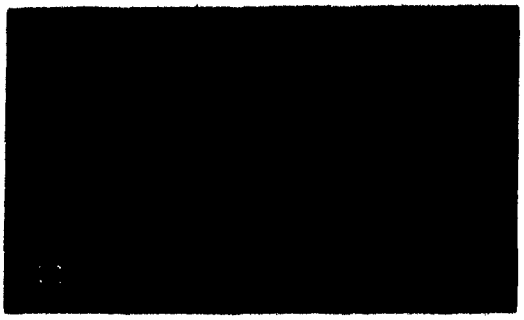

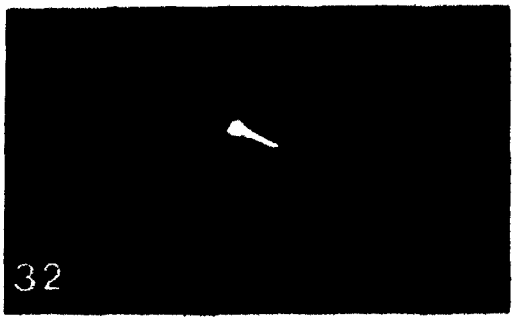
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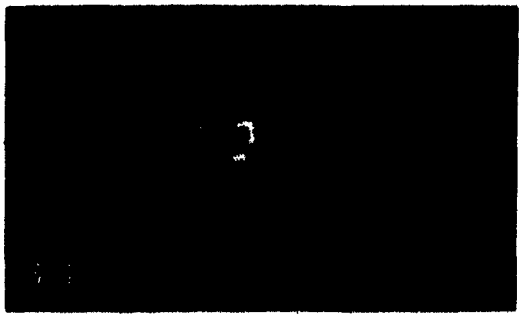

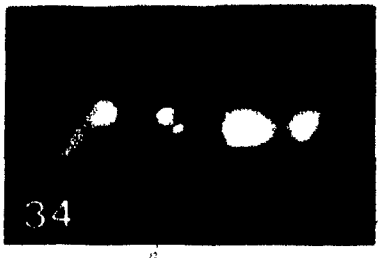
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2. The release

i. Time needed for YEPD cultures to cool

The time taken by blocked cultures to reach permissive temperature might be long and influence the exact timing of first bud emergence. Thus, an experiment has been conducted to determine the period of time needed for YEPD cultures (37°C) to cool to non-blocking temperatures. Sloat, Adams and Pringle (1981) reported an effective permissive temperature of 30°C for cdc-24 mutants. This temperature is reached in all flasks 600 seconds after the shift (Table 17). Additionally, when the drop in temperature is plotted against time, the average time necessary for a flask to attain 30°C is 570 seconds (Fig. 35).

ii. DAPI- and calcofluor-stained samples

Cells described in Section 1, part ii, were transferred from 37°C to 23°C. Samples were collected at various times, fixed, and stained with a combination of DAPI and calcofluor prior to examination by fluorescence microscopy, as previously described.

a) Budding behavior

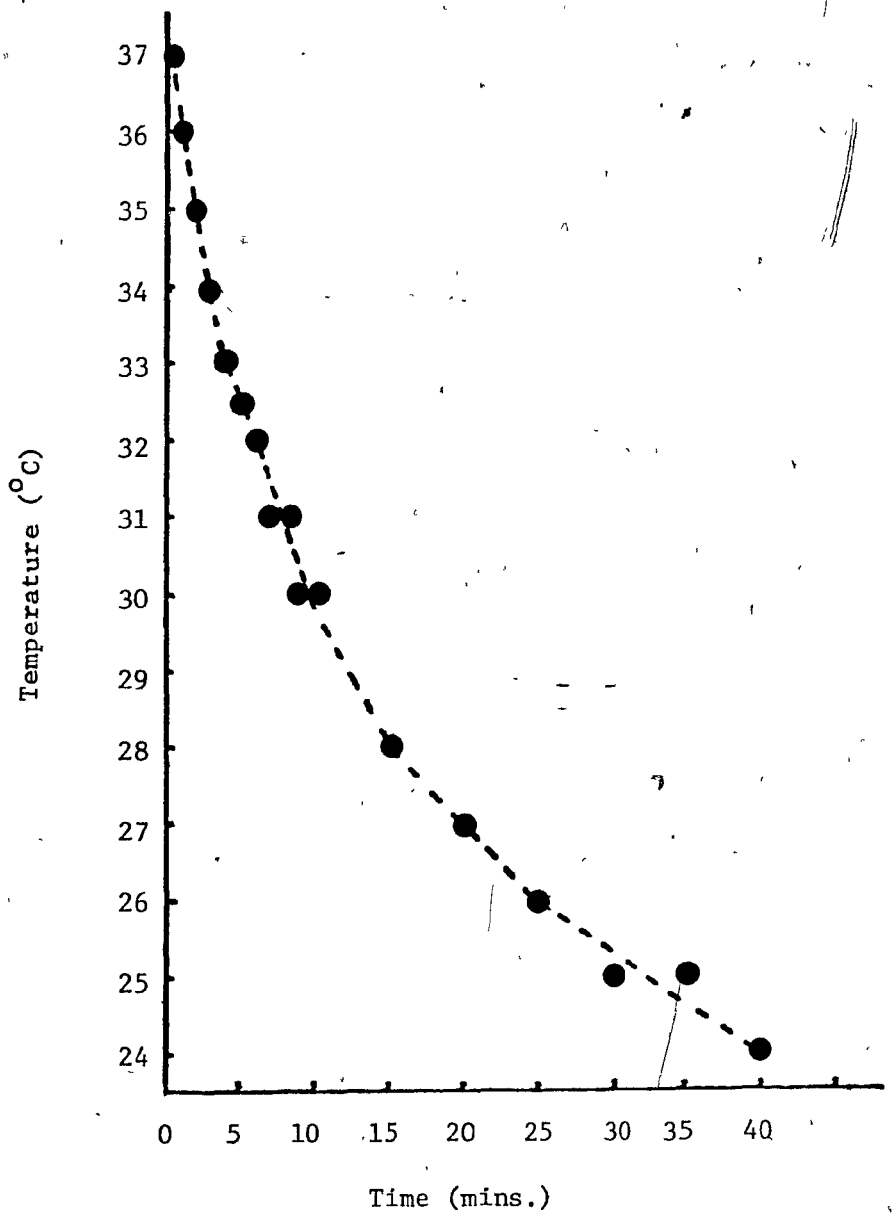
When returned to permissive temperature, cdc-24-1 cells, blocked for eight hours, resume budding within 0.5 hour (Table 18). As soon as 1.0 hour after release, cells producing more

Table 17. Time needed for YEPD culture medium to cool from 37°C to room temperature.

Time (secs.)	Exp.I [1](°C)	Exp.II [1](°C)	Exp.III [1](°C)	Ave. (°C)
0	37.0	37.0	37.0	37.0
60	35.5	36.0	36.0	36.0
120	34.5	35.0	35.0	35.0
180	33.5	34.0	34.0	34.0
240	32.5	33.0	33.0	33.0
300	32.0	32.5	32.5	32.5
360	31.5	32.0	32.0	32.0
420	31.0	31.0	31.5	31.0
480	30.5	31.0	31.0	31.0
540	30.0	30.0	30.5	30.0
600	29.5	30.0	30.0	30.0
900	28.0	28.0	28.0	28.0
1200	26.5	27.0	27.0	27.0
1500	25.5	26.0	26.0	26.0
1800	25.0	25.0	25.0	25.0
2100	25.0	25.0	25.0	25.0
2400	24.0	24.0	24.0	24.0

[1] ± 0.5°C

Figure 35. Time needed for YEPD cultures (37°C) to drop to room temperature



than one bud were detected. This is reflected by the total number of buds which is greater than the number of budding cells. Multibudded cells are more numerous at intermediate times of release (between R=3 and R=7)[1] and tend to disappear at later stages (R=20), to approach the behavior of early released cells (R=0.5), i.e. budding cells possess a single bud. Three types of multibudded cells were seen, one type is characterized by the simultaneous emergence of its buds (buds have identical sizes and are at the same stages of their life cycles), another type by the asynchronous emergence of its buds (they have different sizes, Fig. 38), and the last one by the failure of previous buds to detach (buds are at different stages of their life cycles, Fig. 39). Buds that have failed to separate might themselves produce buds. In such a case, the first undetached bud is considered both as a mother-cell and a bud.

During release, 71 cases of an undetached bud producing at least one other bud (total 76 buds) have been observed out of a total of 1,314 released cells. Such buds appearing on a bud are most often oriented towards or located adjacent to the first mother-cell (66 buds, Figs. 39 and 40) or less often positioned distally to it (the remaining 10 buds). When second or third

[1] Thirty minutes after release, R=0.5; one hour after release, R=1.0; ...etc.

buds emerge on the first mother-cell (58 cases), they are usually proximal to the first undetached bud (46 cases, Fig. 39). More rarely, the second bud arises at the opposite side (6 cases, Fig. 38). If many adjacent buds occupy a certain region, new buds appear at the opposite side of the cell (4 cases). In the event where these two opposite regions are filled with buds and bud scars, new buds form laterally (2 cases).

Normal-looking cells appeared as soon as 1.5 hour after release but their frequency did not significantly increase before an additional 3.5 hours (Table 19, Fig. 41). An average of two generations is needed to generate such normal-looking cells, i.e. they do not develop directly from blocked cells but are produced by the buds of the latter (Fig. 42). Normal-looking cells are usually uninucleate or binucleate and do not show any chitin fluorescence except for the ring and bud scars. Uninucleate normal-looking cells have the same size as unblocked mutant cells (cf. Fig. 41 with 9, 16, 17). Binucleate normal-looking cells are more elongated and slightly larger than the former. At late release (R=20 ND[1]), normal-looking cells form the majority of the total cell population (80.4%), although their proportion can be increased (90.0%, R=20 D[1]), if released cells are diluted with fresh YEPD medium at R=7.0 (Table 19).

[1] See materials and methods: ND, non-diluted; D, diluted.

Table 18. Budding behavior of released cdc-24-1 mutant cells.

Stage (no. of hours after release)	Sample size n =	No. of budding cells	Percentage (%)	No. of unbudded cells	Percentage (%)	Total No. of buds
0.5	102	34	33.3	68	66.7	34
1.0	101	63	62.4	38	37.6	64
1.5	101	73	72.3	28	27.7	75
2.0	99	82	82.8	17	17.2	85
2.5	100	71	71.0	29	29.0	75
3.0	100	73	73.3	27	27.0	93
3.5	100	76	76.0	24	24.0	110
4.0	105	82	78.1	23	21.9	119
5.0	103	82	79.6	21	20.4	130
6.0	100	59	59.0	41	41.0	83
7.0	101	74	73.3	27	26.7	120
20.0 ND	102	52	51.0	50	49.0	59
20.0 D	100	53	53.0	47	47.0	56

Table 19. Time of appearance and chitin distribution of normal-looking cells produced during release.

Stage	Sample size n =	No. of normal looking cells	Percentage	No. of normal looking cells with higher than basic chitin
0.5	102	0	0	0
1.0	101	0	0	0
1.5	101	1	1.8	0
2.0	99	4	4.0	0
2.5	100	3	3.0	0
3.0	100	4	4.0	0
3.5	100	6	6.0	0
4.0	105	2	1.9	1
5.0	103	11	10.7	2
6.0	100	26	26.0	0
7.0	101	24	23.8	1
20.0 ND	102	82	80.4	1
20.0 D	100	90	90.0	5

b) Nuclear behavior

Table 20 demonstrates that the frequency of any cell-type relative to its number of nuclei does not significantly change at any stage of release, as there is approximately the same proportion of cells containing none, one, two, three, four, five or more nuclei, at early, middle and late release stages. Binucleate constitutes, by far, the most abundant class (not less than 68.0%) and two types of such cell exist (Table 21); those which have their nuclei far apart (Figs. 43 and 44) and those whose nuclei lie side by side (Fig. 45). Although the total number of cells having two nuclei does not markedly change during release, the type of binucleate cell does. After six hours and more, binucleate cells with nuclei far apart tend to be replaced by those whose nuclei are close together.

The two types of binucleate cells give rise to two main budding patterns. In most binucleate cells, the two nuclei lie close together (ave. 87.6%) and divide into the same bud (Fig. 36), and in a few, the nuclei are far apart (ave. 12.4%) and may produce either one or two buds, each proximal to a nucleus which will divide into it (Fig. 37). The former type generates binucleate cells with nuclei close to each other while the latter type is responsible for the production of uninucleate buds. If this is so, one would expect the proportion of uninucleate cells to increase as release progresses, but this is clearly not the

Figure 36. Typical nuclear division in released binucleate cdc-24-1 cells with nuclei close together

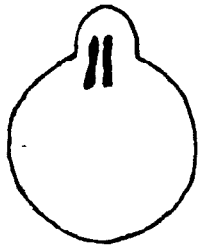
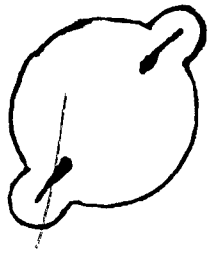


Figure 37. Typical nuclear division in released binucleate cdc-24-1 cells with nuclei far apart



case, and it seems in contradiction with the data presented in Table 20. Two phenomena might explain this apparent disparity;

1) Certain cell types might bud faster than others. For instance, the generative capacity of binucleate cells having close-together nuclei might be greater than that of uninucleate cells. One might speculate that there is an additive effect of correctly oriented microtubules where twice as many vesicles will reach the same site. Unfortunately, the present form of analysis cannot support or contradict this hypothesis, and leaves us with an open question. A study at the electron-microscopic level with early released samples should resolve this issue.

2) Second, and not exclusive of one, there might be some kinds of "compensatory mechanisms" which are responsible for the generation of certain cell types. For instance, a binucleate cell having its nuclei far apart might give rise to a

uninucleate bud, but if, as the bud grows, there is migration of one of the parental nuclei to the bud and if the bud nuclei lie close together, a binucleate budding pattern might be regenerated. The fate of this bud and of its successive generations has been changed from uninucleate cells to binucleate cells by nuclear migration, an example of "compensatory mechanisms".

In order to verify the existence of such mechanisms and consequently the validity of Tables 20 and 21, released cells were classified in various categories according to the number of nuclei observed either in mother cells or buds, and nuclear behavior was followed in relation to the budding behavior. Released cells and/or their buds were pooled and separated into the following categories:

1) budded or unbudded cells having no nuclei
2) budded or unbudded cells or buds having one nucleus
3) as 2) but with two nuclei
4) as 2) but with three or more nuclei

Hypotheses are then made on how a cell-type might have arisen from a previous stage.

Budded or unbudded cells having no nuclei: The following phenomena or mechanisms might be responsible for such cell types:

1) Migration of the nucleus or nuclei from the mother cells to the buds. Buds having one, two or three nuclei were seen

where the mother cells have none (36 cases).

2) Destruction of the DNA of mother cells by the action of DNAases. Mother cells did not stain because they contain no DNA. Budded and unbudded cells were observed which did not fluoresce with DAPI (32 cases).

3) Loss of the nucleus from the cell. A few unbudded cells (5 cases) were observed where the single nucleus was being "lost" through the cell wall. This might not represent a true phenomenon but some fixation artifact (Fig. 46).

4) There is no combination of the stain with DNA because cells might be dead prior to fixation and the entry of the stain through the plasmalemma is impeded. This possibility is supported by the fact that in cells having no fluorescent nucleus or nuclei, mitochondrial DNA fluorescence was also absent.

Furthermore, in the buds of a few cells where the mother cells had no detectable nuclei, a single very large nucleus was seen, suggesting that nuclear fusion might have occurred (5 cases).

Cells or buds with one nucleus: The following processes or cell types can yield uninucleate cells or buds:

1) Uninucleate cells (131 cases). Budding uninucleate cells produce uninucleate daughter cells.

2) Binucleate cells with nuclei far apart (135 cases) or

multinucleate cells having at least one of their nuclei apart from the others (27 cases). When two nuclei (Figs. 37 and 47) or more are separated by a distance greater than the diameter of a nucleus they behave independently, i.e. each nucleus can give rise to a uninucleate cell.

3) Nuclear migration (20 cases).

4) Nuclear fusion (46 cases, Fig. 43).

5) Binucleate cells showing asynchrony in nuclear division (10 cases). Even if a binucleate cell has its two nuclei lying side by side, only one nucleus might divide into the single bud if they are at different stages of their life cycles. In such a case, a uninucleate cell is generated.

Cells or buds with two nuclei: The following processes or cell types might be responsible for the generation of binucleate cells or buds:

1) Binucleate cells having their nuclei residing side by side (1,007 cases, Figs. 36 and 49) or multinucleate cells having a group of two nuclei close to each other and away from other nuclei. The group of two nuclei will divide into the same bud (20 cases).

2) Nuclear migration (22 cases).

3) Nuclear division without budding. Unbudded uninucleate cells that have their nucleus dividing without bud formation or modification of cell wall fluorescence (3 cases).

Cells or buds with more than two nuclei: The following processes might generate multinucleate cells or buds:

1) Nuclear division without budding (2 cases).

2) Nuclear migration (7 cases).

Thus, it seems likely that the conversion of certain cell types is the result of mechanisms such as nuclear migration, nuclear division without budding, nuclear fusion, loss of nuclear synchrony,...etc., to name a few. These mechanisms might be sufficient to explain the observed frequency of each cell type throughout the first twenty hours of release (Table 20).

Furthermore, in early released binucleate cells having adjacent nuclei, nuclear migration was observed prior to division (Table 22, Figs. 48 to 51) and the bud always emerged at the site opposite to the original nuclei location (Fig. 48). This appears to be valid only for the first nuclear division following release since this phenomenon was not observed in subsequent divisions. Binucleate cells with nuclei far apart seem not to show that behavior.

c) Chitin behavior

Within the first 0.5 hour at permissive temperature, there is a noticeable decrease in general fluorescence, which no longer masks the old bud scars. The first buds to emerge are not located sequentially to the row or rows of old bud scars and

Table 20. Number of nuclei in mother cells during release.

Number of nuclei in mother-cell[1]	R=0.5	R=1	R=1½	R=2	R=2½	R=3	R=3½	R=4	R=5	R=6	R=7	R=20D	R=20ND
0	2 2.0%	7 6.9%	2 2.0%	12 12.0%	7 6.9%	6 6.0%	2 1.9%	4 3.3%	8 6.3%	7 6.5%	6 5.0%	6 5.8%	2 2.0%
1	6 5.9%	1 1.0%	4 4.0%	12 12.0%	16 15.8%	21 21.0%	3 2.8%	15 12.4%	14 11.1%	3 2.8%	19 16.0%	11 10.7%	6 5.9%
2	89 87.3%	90 89.0%	95 94.1%	69 69.0%	77 76.2%	68 68.0%	96 89.7%	97 80.2%	98 77.8%	95 88.8%	92 77.3%	82 79.6%	94 92.2%
3	4 3.9%	3 3.0%	- -	5 5.0%	1 1.0%	5 5.0%	4 3.7%	3 2.5%	6 4.8%	1 0.9%	1 0.8%	3 2.9%	- -
4	1 1.0%	- -	- -	2 2.0%	- -	- -	1 0.9%	2 1.7%	- -	1 0.9%	- -	- -	- -
5	- -	- -	- -	- -	- -	- -	1 0.9%	- -	- -	- -	1 0.8%	- -	- -
5	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -		1 1.0%	- -
Total number of mother cells	102	101	101	100	101	100	107	121	126	107	119	103	102

[1] The total number of mother cells is not necessarily equals to the sample size since an undetached bud which is budding might be considered both as a mother-cell and a bud.

Nucleus undergoing division was counted as one (1 dN=1N).

Totals may not add to 100.0% since percentages were rounded up to the closest 0.1%.

Table 21. Frequency of the two types[1] of released binucleate cells.

Stage	No. of mother cells with 2 nuclei	No. of mother cells with 2 nuclei apart	Percentage	No. of mother cells with 2 nuclei together	Percentage
0.5	89	18	20.2	71	79.8
1.0	90	14	15.6	76	84.4
1.5	95	12	12.6	83	87.4
2.0	69	24	34.8	45	65.2
2.5	77	14	18.2	63	81.8
3.0	68	11	16.2	57	83.8
3.5	96	10	10.4	86	89.6
4.0	97	10	10.3	87	89.7
5.0	98	15	15.3	83	84.7
6.0	95	3	3.2	92	96.8
7.0	92	4	4.3	88	95.7
20.0ND	82	0	0	82	100.0
20.0D	94	0	0	94	100.0

1 - Apart means that there is more than half a nuclear diameter separating the two nuclei.

- Together means that the two nuclei are separated by a distance which is less than half a nuclear diameter.

Table 22. Nuclear location in early released budding binucleate cells with nuclei close together.

Stage	Total No. of budded binucleate cells with nuclei side by side	No. of binucleate cells with their nuclei in lower halves of mother cells	No. of binucleate cells with their nuclei in upper halves of mother cells	No. of binucleate cells with their nuclei centrally located
R=0.5	33	20	10	3
R=1.0	54	3	46	5

appear without rings (Figs. 52 and 53; further evidence given in section 4). The mother cells which produce these buds tend to be pear-shaped, as they lack the reinforcement of the ring, with buds emerging at the narrow end (Fig. 53). A few unbudded cells are seen which have one or two regions in their cell wall which are less fluorescent than adjacent regions. Although buds might appear in these regions of low fluorescence, they are not actually seen emerging from such regions because 1) there might be no relationship between these two events, or 2) as the bud emerges such a low fluorescent region becomes as fluorescent as adjacent regions (as is the case in budding zygotes). Regions of stronger localized fluorescence are evident in the wall of some cells (Fig. 47) which might correspond to structural anomalies seen at the electron-microscope level, as well as irregularities in the neck region (also observed in diploid cdc-24-2 cells; Boothroyd, personal communication). Most early buds (R=0.5 to R=2.0) show slow development of chitin rings and of septum formation (Figs. 52 and 54) but later (R=2.5 and longer), new buds have normal rings at their bases. Some quite abnormal buds can then be seen. Abnormal buds are frequently elongated and they may show chitin deposition and wall thickening around the middle of the side walls with less fluorescence or thickening towards the base and throughout the tip region (Fig. 55). When buds without normal rings at their bases are capable of

separating, a flat dark surface on the cell wall of the mother-cell and on the corresponding bud is observed (Fig. 56). Prior to separation, a fluorescent plaque develops at the base of such buds (Fig. 43).

iii. FITC-Con A -stained samples

Cells released for either 30 minutes or five hours were fixed, and subsequently stained with FITC-Con A. The distribution of mannan in the cell wall was studied using fluorescence microscopy, as previously described.

Table 23 shows that buds produced during early release are often less fluorescent than their parents (30 buds on 46) but later they become as fluorescent (64 buds on 73). By looking at the size of buds at these two stages of release, it becomes evident that small buds regardless of the stage of release are less fluorescent than their mother cells (Fig. 57) but as they grow they become as fluorescent (Fig. 58). Again, a few unbudded cells are seen which have one or two regions in their cell walls which are less fluorescent than adjacent regions. However, as for chitin, small buds were not actually seen emerging from these low fluorescent regions.

Some early released budding cells (6 cases) have a diffuse concentration of mannan around the bud neck region which might reflect the deposition of material needed for septum wall formation.

Table 23. Budding index and mannan fluorescence of buds of mutant cells released for 0.5 and 5.0 hours.

Stage (hr. of release)	R=0.5	R=5.0
Sample size, n=	103	101
No. of unbudded cells	59 (57.3%)	40 (39.6%)
No. of budded cells	44 (42.7%)	61 (60.4%)
No. of buds observed	46	73
No. of buds less fluorescent than parents	30 (65.2%)	9 (12.3%)
No. of buds equally fluorescent with parents	16 (34.8%)	64 (87.7%)

It is known that the chitin ring is absent at that stage (part ii). The same type of cells can be seen in the later sample but in higher frequency (45 cases) and mannan is more concentrated. Cells defective in cytokinesis show strong accumulation of mannan at the base of the large undetached bud (6 cases), which often is unequally distributed. Undetached buds show a plaque of chitinous material and newly produced buds a ring (part ii). Thus, mannan and chitin seem to be deposited at the same sites. This observation is confirmed by the existence, in cells released for five hours, of circular surfaces, the size and shape of chitin rings, which are devoid of mannan fluorescence. These surfaces are absent in early released cells which are also known to lack chitin rings. The regions adjacent to the rim of these darkly circular scars show an intense mannan fluorescence which decreases with increasing distance (4 cases, Fig. 59).

iv. Miscellaneous observations

Survival rate of eight-hour blocked cells: Is the eight-hour block responsible for the selective death of a part of the starting population (B=0) and can observations made on released cells be related to the whole initial culture or only to a fraction of it? To answer that question, the percentage of survival of an eight-hour blocked culture was determined by recording the ability of cells of this culture to form colonies on agar plates. Cells blocked for

eight hours at 37°C were transferred from liquid YEPD medium and plated at known density on YEPD-agar plates. After several days of growth at room temperature, the number of colonies was counted. The percentage of blocked cells able to form colonies was 76.5 (range 63.0% to 90.0%). This represents a minimal average value, since no sonification was used (sonification, at B=8, increases the percentage of lysed cells).

Orange bodies and metachromatically-stained granules: A total of 1,314 released cells (total sample size) and 1,103 buds (total no. of buds) were stained with DAPI and calcofluor, and examined by fluorescence microscopy. None (one exception at R=20 D) showed orange bodies, despite their relatively high frequency at late block.

When cells released for 0.5, 5.0, and 20.0 hours (diluted) were stained with toluidine Blue D and observed with bright-field microscopy, the following observations were made:

1) R=0.5 hour. Metachromatically-stained granules were absent. However, some tiny particles were seen randomly distributed (dusty appearance), except for some aggregation in the vicinity of the newly emerging bud.

2) R=5.0 hours. Metachromatically-stained granules were seen located randomly in the cytoplasm with more near the periphery of the cell. Granules were not seen in vacuoles.

3) R=20.0 (D). Metachromatically-stained granules were larger and mostly associated with vacuoles, the cell wall, or were found at periphery of the cell.

Thus, these granules seem to be synthesized in the cytoplasm and to accumulate in vacuoles as time after release increases.

PLATE VI. Budding behavior during release

Figures 38 to 42. 182-6.3 *a cdc-24-1* cells blocked for eight hours, released for various times, and either unstained (Fig. 42; 1200X) or stained with DAPI and calcofluor (Figs. 38, 39, 40 and 41; 1500X).

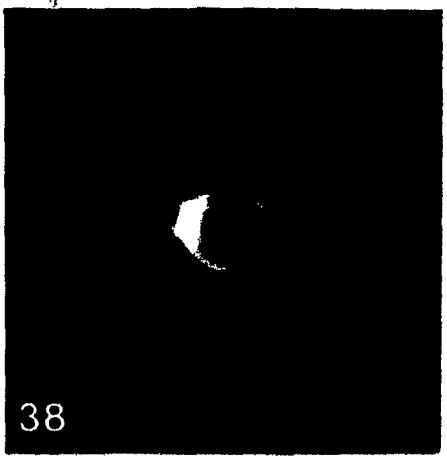
Fig. 38. Bibudded cell with buds of different sizes emerging on opposite sides of the mother-cell (R=3.0).

Fig. 39. Multibudded cell with buds that failed to separate. The largest mother-cell shows a bud producing a bud which is less fluorescent than its progenitor and appears uninucleate. This bud is oriented towards the first mother-cell which has also produced a second bud that appears binucleate and which is proximal to the first emerged bud (R=5.0).

Fig. 40. Mother-cell displaying a bud on a bud. Nuclear division is occurring into that latter bud which is oriented towards the first mother-cell, and appears less fluorescent than its parent . Nuclear division is occurring in the least fluorescent bud and its mother-cell (R=5.0).

Fig. 41. Uninucleate normal-looking cell with one bud scar. Note basic fluorescence of the cell wall and mitochondrial fluorescence (R=20.0 ND).

Fig. 42. Large cell with large vacuole, typical of blocked cell; intermediate sized cell, apparently with two buds; small, normal-looking cell with bud (Nomarski interference contrast; R=7.0).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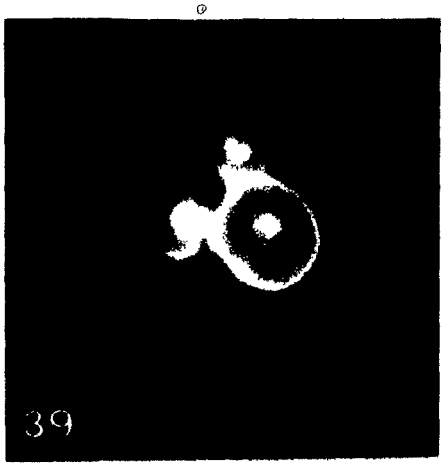
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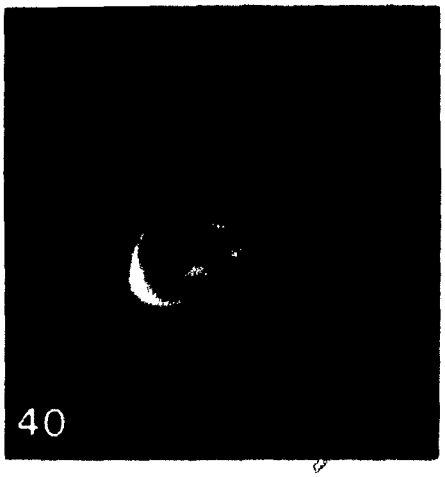
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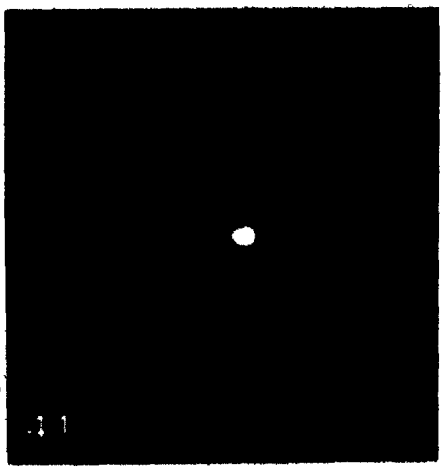
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PLATE VII. Nuclear behavior during release

Figures 43 to 51. 182-6.3 a cdc-24-1 cells blocked for eight hours, released for various times, and stained with DAPI and calcofluor; 1500X.

Fig. 43. Budded binucleate cell with nuclei far apart. The bud contains a single large highly fluorescent nucleus. Note the fluorescent plaque at the base of the bud (R=2.5).

Fig. 44. Unbudded binucleate cell with nuclei far apart (R=2.0).

Fig. 45. Unbudded binucleate cell with nuclei close together (R=7.0).

Fig. 46. Unbudded cell losing its nucleus through the cell wall (R=2.5).

Fig. 47. Budded binucleate cell with nuclei far apart. The less fluorescent bud is uninucleate. Note circular region of higher fluorescence at base of mother cell wall (R=5.0).

Figures 48 to 51. Early released binucleate cells showing nuclear migration prior to division. The bud emerged at the opposite side to the original nuclear location. (Fig. 48, R=0.5). As the bud enlarges, nuclei approach (Fig. 49, R=0.5; Fig. 50, R=1.0; Fig. 51, R=1.0).

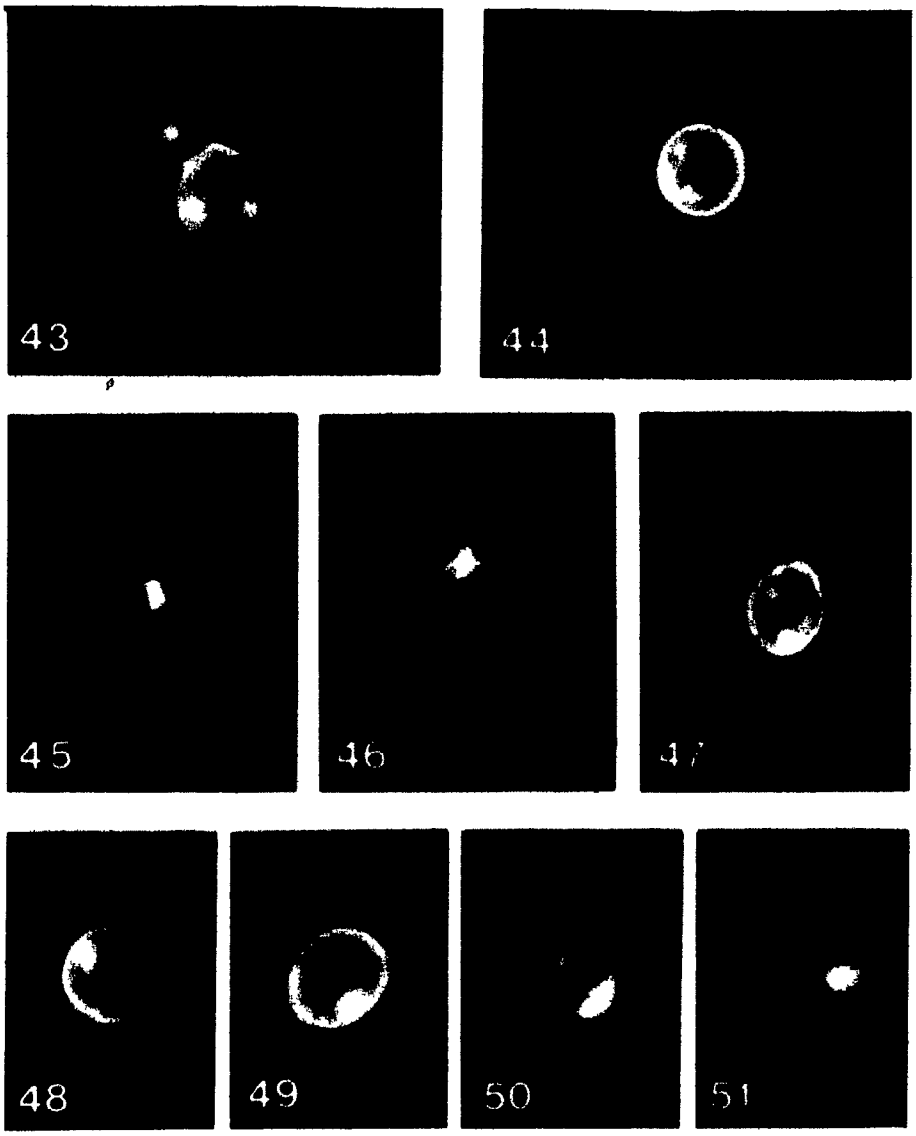
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PLATE VIII. Chitin and Mannan behavior during release

Figures 52 to 56. 182-6.3 a cdc-24-1 cells blocked for eight hours, released for various times, and either stained with calcofluor alone (Figs. 52, 53, 54 and 55) or with DAPI combined to calcofluor (Fig. 56). 1500X.

Fig. 52. Early released bibudded cell. Buds appeared without rings at widely separated sites on the mother-cell. Their cell walls are practically non-fluorescent. Note the absence of constrictions at the bases of the buds (R=0.5).

Fig. 53. Pear-shaped cell with a less fluorescent bud emerging at the narrow end without a ring. The bud is not constricted at its base (R=1.0).

Fig. 54. Budded cell showing a partial ring at the base of its medium sized bud. This bud is less fluorescent than its mother cell and is somewhat constricted at its base (R=2.0).

Fig. 55. Elongated bud with fluorescence around mid-region of side walls; some fluorescence at neck (R=2.0).

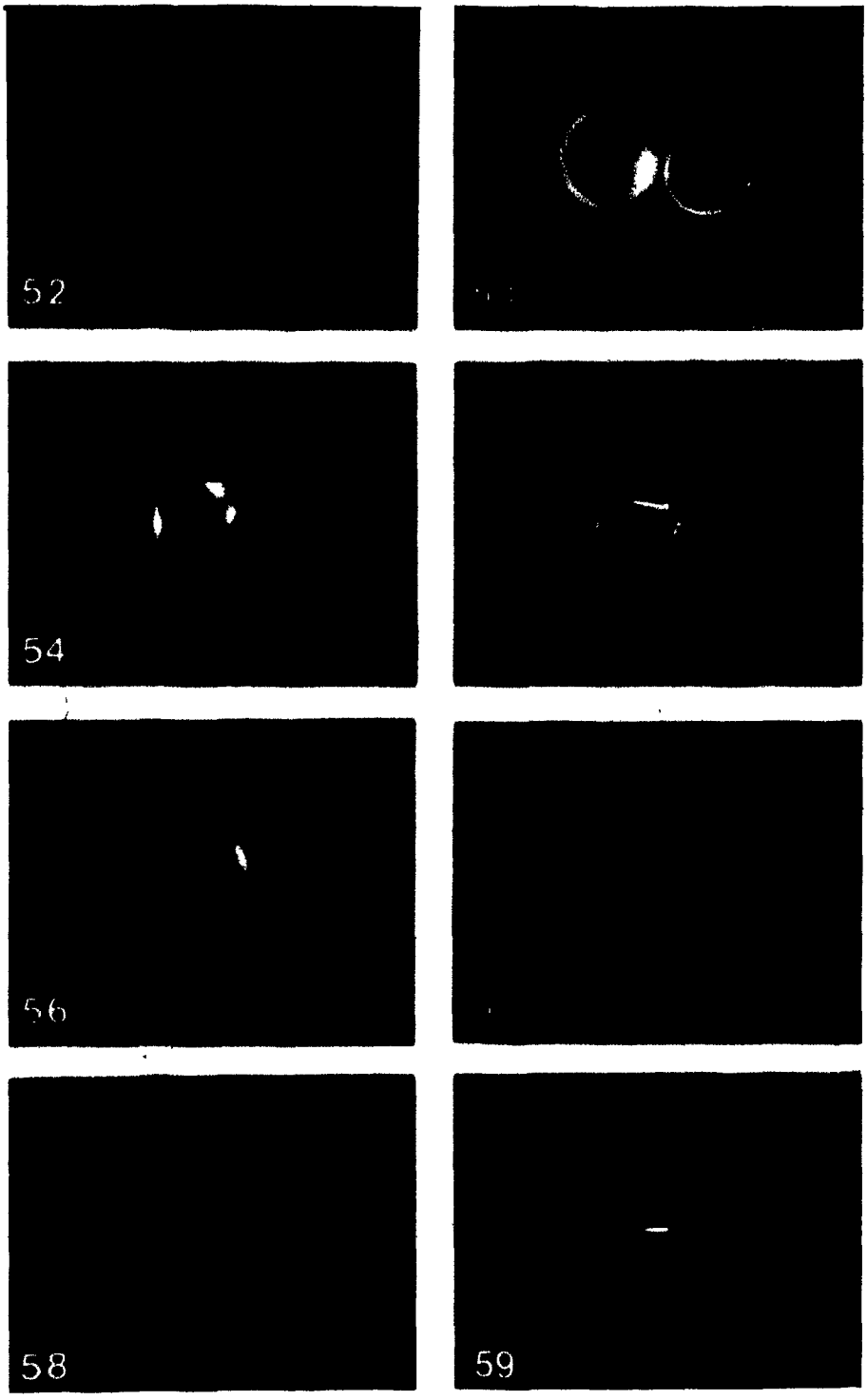
Fig. 56. Released cell showing a less fluorescent flat surface on its cell wall (R=5.0).

Figures 57 to 59. 182-6.3 a cdc-24-1 blocked for eight hours, released for 0.5 or 5.0 hours, and stained with FITC-Con A; 1500X.

Fig. 57. Early released budded cell with less fluorescent bud (R=0.5).

Fig. 58. Late released budded cell with larger and more fluorescent bud (R=5.0).

Fig. 59. Example of a "mannan bud scar" (R=5.0).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3. Karyogamy-deficient crosses

Equal numbers of cells opposite in mating-type and deficient in karyogamy were mixed. Samples were taken before (unmixed strains JC-25α, JC-7a) and three, four, five, six, six and a half, seven, and eight and a half hours after mixing (respectively referred to as M=3.0 to 8.5), and fixed as reported before. The budding, nuclear, and chitin and mannan cell wall behavior of the zygotes and of their buds were studied following methods described for the cdc-system. Mannan distribution was followed only in the two pre-mixed samples and three hours after mixing.

Throughout the kar- mating experiment, 85 zygotes were recorded, among which 27 were unbudded and 58 were budded, bearing a total of 65 buds. These 65 buds had the following location and chitin distribution: 45 had rings with 28 of these buds being terminal (Figs. 60 and 61) and 17 central; 12 had no ring with 3 of these buds being terminal and 9 central; 8 had an incomplete ring (partial) with 3 of these buds being terminal, and the remaining 5 central (Table 26). Budded zygotes with terminal buds can be seen in Figures 60 to 63, while those with central buds can be seen in Figures 64 to 67. These buds appeared in regions of low fluorescence of chitin (Figs. 60 and 61). There were three such regions in fused cells; in the central portion of the conjugation tube (Figs. 61 and 68) and at the extremities of the zygote (Figs. 60, 61 and 68). The bud emerged in

the low fluorescent region closest to the nucleus or nuclei (Figs. 60 and 61). As the bud enlarged, the region in which it had emerged became as fluorescent in chitin as adjacent regions (Figs. 62, 64, 65 and 66). If a bud has emerged without a ring, one might form later when the daughter-cell enlarges. This deduction is based on the following observations:

1) More buds with rings were seen at late stages (M=6.0 to 8.5) than at early stages (M=3.0 to 4.0), 27 buds out of 28 versus 13 buds out of 28.

2) The number of buds with partial rings decreased during the same period, 7 buds out of 28 versus none out of 28.

The absence of partial rings in buds of the late samples and the corresponding high frequency of buds with normal rings suggests a conversion of the former into the latter.

Small buds emerging on unfused cells were usually less fluorescent in chitin and mannan than their parent cells.

Two types of bi- and multinucleate cells were observed following mating. Those with nuclei or groups of nuclei far apart and those with nuclei close together. Again, if two or more nuclei were close to each other, i.e. separated by less than half a nuclear diameter, they divided synchronously into the same bud, otherwise each nucleus or isolated group of nuclei divided into the closer forming bud and acted independently of the other. Most buds produced from the zygotes were binucleate (17 buds on 21, Fig. 64, 70 and 71) with nuclei close

together (13 buds on 17, Fig. 70). Chitin in these cells was restricted to the ring and bud scars, if present (Fig. 70). The buds resembled diploids in being larger and more elongated than haploid uninucleate cells and bipolar budding seemed very common. They were also larger and more elongated (Figs. 70 and 71) than normal-looking binucleate cells produced in the cdc-system following return to permissive temperature. As a general rule, the volume of a cell tended to increase as the number of nuclei increased (uninucleate, Fig. 60; binucleate, Figs. 70 and 71; trinucleate, Fig. 72; tetranucleate, Fig. 73).

Two phenomena encountered in the cdc-system were also observed here, namely nuclear division without budding (there were more multinucleate cells at M=8.5 than at M=3.0), and the presence of large nuclei which might represent nuclear fusion in spite of the kar- mutation.

PLATE IX. Karyogamy-deficient zygotes

Figures 60 to 68. Kar- zygotes observed at various times following mating, and stained either with DAPI and calcofluor (Figs. 60, 61, 62, 64, 65, 66 and 68) or with FITC-Con A (Figs. 63 and 67). 1500X.

Fig. 60. Multinucleate zygote apparently with three nuclei. Two of the nuclei (out of focus) are at the base of the less fluorescent terminal bud. The bud has a ring and is located in a cell wall region poor in chitin (M=3.0).

Fig. 61. Zygote with a terminal bud having a ring of chitin at its base. The bud is located in one of the two cell wall regions poor in chitin. Apparently, two nuclei (out of focus) are at the base of the bud (M=3.0).

Fig. 62. Zygote with a less fluorescent terminal bud. The zygote seems trinucleate with two of its nuclei near the base of the bud (M=6.5).

Fig. 63. Zygote with a terminal bud (FITC-Con A-stained, M=3.0).

Fig. 64. Binucleate zygote with a less fluorescent central bud having two nuclei far apart. Note the highly fluorescent cell wall at the base of the large bud and the less fluorescent extremities of the zygote (M=4.0).

Fig. 65. Zygote with a uninucleate central bud. The bud and the two extremities of the zygote are less fluorescent than other cell wall regions. The cell wall at the base of the large bud is highly fluorescent. Note the presence of a nucleus in one of the zygote extremities (M=4.0).

Fig. 66. Zygote with less fluorescent central bud. The extremities of the zygote are less fluorescent than its central portion (M=8.5).

Fig. 67. Zygote with less fluorescent central bud (FITC-Con A -stained, M=3.0).

Fig. 68. Unbudded zygote with nuclei far apart. One extremity of the zygote as well as its central part are less fluorescent than adjacent regions (M=4.0).

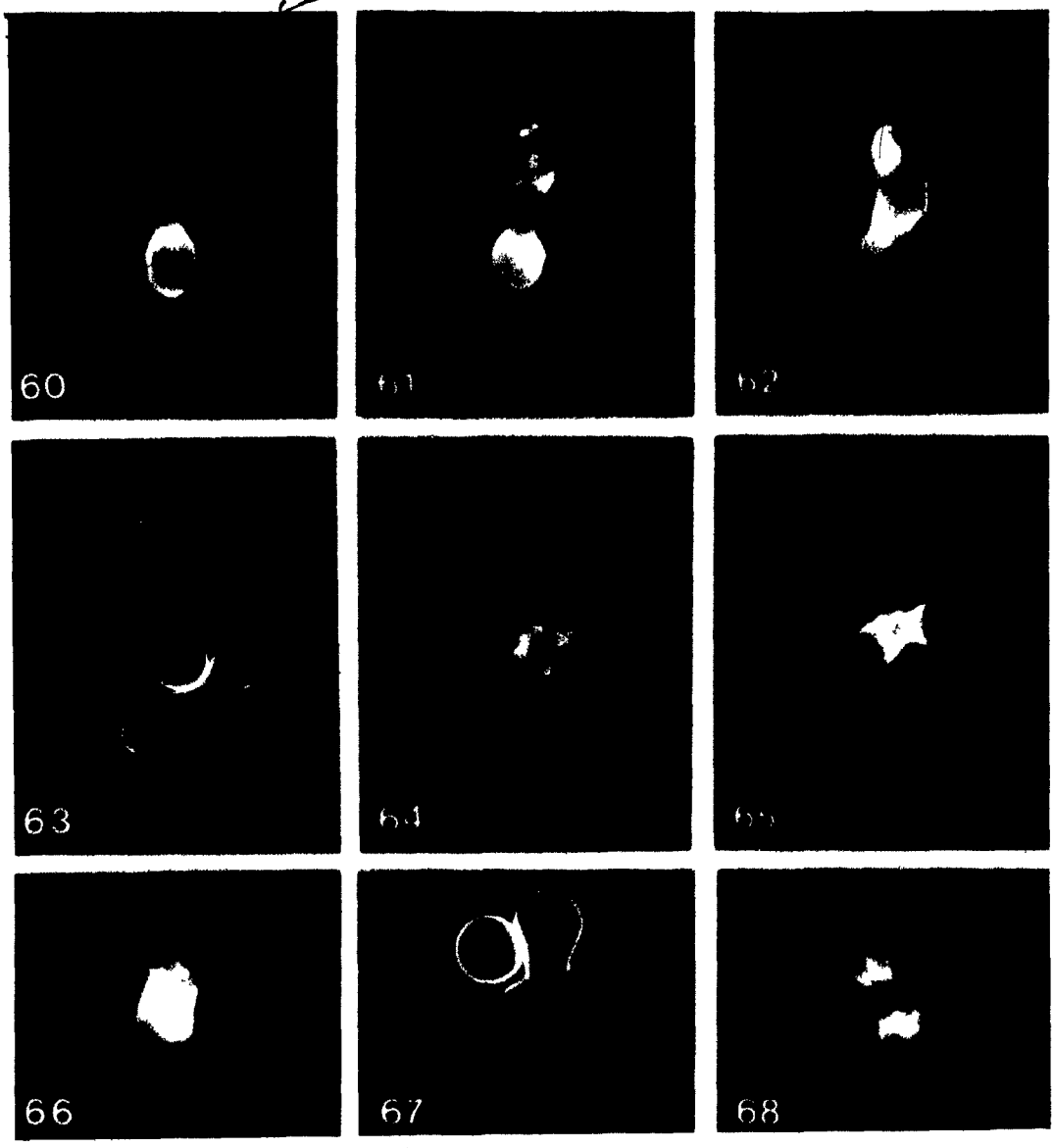
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PLATE X. Cells produced by budding zygotes and unfused kar- cells

Figures 69 to 73. Cells produced by zygotes (Figs. 70, 71, 72 and 73) or unfused kar- cells (Fig. 69) stained with DAPI and calcofluor). 1500X.

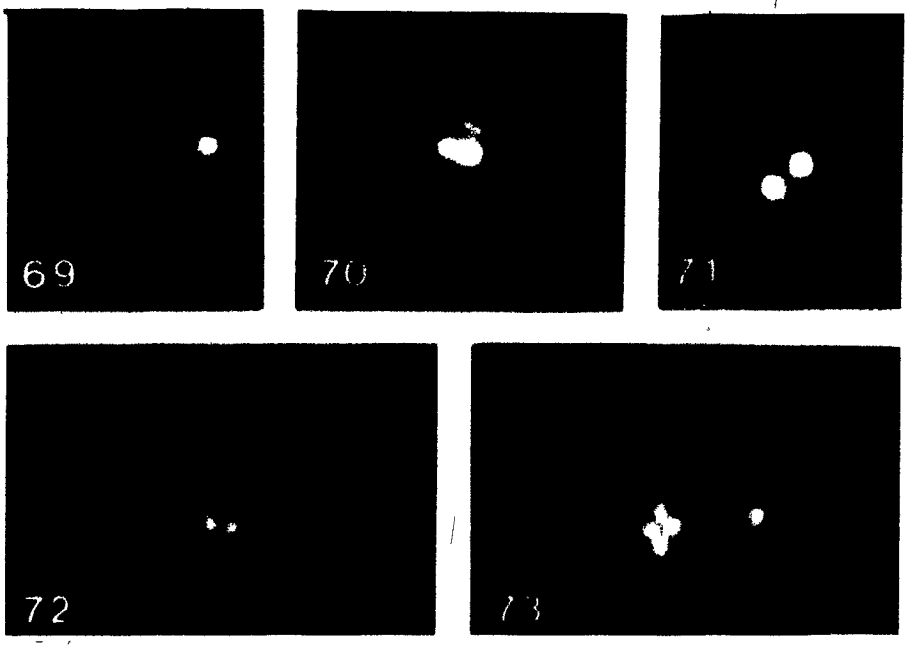
Fig. 69. Uninucleate unfused kar- cell. The unbudded cell is relatively spheroid.

Fig. 70. Budded binucleate cell with nuclei close together and chitin ring at the base of the bud. Note elongated shape and increased volume as compared with Fig. 69 (M=6.0).

Fig. 71. Unbudded binucleate cell with nuclei far apart. The cell is more elongated and larger than in Fig. 69 (M=7.0).

Fig. 72. Trinucleate cell with nuclei close together. Bud scars can be seen at the extremities of the elongated larger mother-cell (M=8.5).

Fig. 73. One tetranucleate cell with nuclei close together. The cell is elongated and very large (M=8.5).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4. Role of the chitin ring

i. Chitin ring formation and bud emergence

No essential relationship was found between the formation of a ring of chitin and the emergence of a bud, since many buds without rings were seen in both blocked and released cdc-24-1 cells and in kar- budding zygotes. All observations were made on cells that were stained either with a combination of DAPI and calcofluor, or with calcofluor alone.

a) Released cells

The uncoupling of chitin ring formation and bud emergence implies a lack of a causal relationship: during early release, buds first appeared without rings (Table 24). This indicates that the ring is not needed for bud initiation and must then have other functions. Buds with partial rings were seen as early as one hour following release, and less often afterwards. Buds with typical rings appeared 1.5 hour after the temperature shift. One might then ask whether budding cells displaying partial rings are cells depositing their rings in their already emerged buds or cells whose buds possess that particular type of ring on emergence? If the latter hypothesis is valid, one would expect, at R=1.5, to see most very early buds with partial rings, but if the former hypothesis is valid, to observe a wider range of buds

Table 24. Frequency of released budded cells without, with partial, or with complete rings.

Stage of Release	Sample size	No. of budding cells	Total no. of buds	No. of buds with no rings	No. of buds with partial rings	No. of buds with rings	Unknown cases
R=0.5	102	34	34	34	0	0	0
R=1.0	101	63	64	50	14	0	0
R=1.5	101	73	75	54	10	11	0
R=2.0	99	82	85	17	7	59	2
R=2.5	100	71	75	4	1	70	0
R=3.0	100	73	93	4	2	86	1
R=3.5	100	76	110	11	5	88	6
R=4.0	105	82	119	5	5	108	1
R=5.0	103	66	130	7	1	121	1
R=6.0	100	59	83	3	0	80	0
R=7.0	101	74	120	2	2	115	1
R=20.0D	102	52	59	3	2	54	0
R=20.0ND	100	53	56	0	1	55	0

of different sizes having partial rings. At R=1.5, eleven buds with partial rings were seen, among which two were smaller than one sixteenth the relative volume of their mother cells, and nine larger. These nine buds ranged in size from one eighth to seven eighths the relative parental volume. It seems therefore, that early released budded cells were able to deposit a ring of chitin at the base of their buds after a short lag-period.

b) Blocked cells

Occasionally, a few leaky cells escaped the block and produced buds that rendered the mother-cell pear-shaped. Leaky cells had buds with no rings or sometimes a weak and ill-defined ring (partial ring, Table 25, Fig. 12).

c) Budding zygotes

Approximately 20% of the buds produced on zygotes did not display rings of chitin at their bases (12 buds out of 65, Table 26). Furthermore, two abnormally elongated buds were observed on binucleate cells that had no rings. Again, there seems to be no relationship between the ability of such cells to lay down a ring and to bud, nor was there any relation between the position of the bud on a zygote and the presence of a ring (Table 26).

Table 25. Type of chitin ring seen at the base of a bud in leaky mutant cells blocked up to eight hours.

Stage	Sample size	No. of budding cells	Budding cells with rings	Budding cells with partial rings	Budding cells with no ring	Unknown cases
B=0	100	55	55	0	0	0
B=1	102	44	44	0	0	0
B=2	100	25	24	0	0	1
B=3	100	7	0	3	2	2
B=4	100	12	0	0	3	9
B=5	100	12	2	0	2	8
B=6	100	4	0	1[1]	0	3
B=7	103	1	0	0	0	1
B=8	101	7[2]	3	0	3[1]	2

[1] pear-shaped

[2] one budding cell has two buds

Table 26. Position of buds and type of chitin ring observed on budding kar- zygotes.

Total no. of zygotes	No. of budding zygotes	Total No. of buds	No. of buds with rings			No. of buds with partial rings			No. of buds with no ring		
			Total	Position T[1]	Position C[2]	Total	Position T	Position C	Total	Position T	Position C
85	58	65	45	28	17	8	3	5	12	3	9

[1] T, terminal bud

[2] C, central bud

ii. Chitin ring formation and normal bud shape

The chitin ring seems important for the development of the normal bud shape. In the absence of a complete ring the buds were abnormally shaped, both in blocked and released cdc-24-1 cells and in budding kar- zygotes.

a) Released cells

Cells that have no chitin rings have abnormally-shaped buds with no constriction at their bases: during release, 194 buds without rings were seen, none of them showed a constriction at mother cell-bud junction (Table 27). Mother cells tended to be pear-shaped at early bud emergence (34 buds on 34, Table 24, Fig. 53), but when the ring was deposited later (R=1.5 and longer, Table 24, Fig. 54), constriction at the bases of the buds was observed (at R=2.5, 75 buds were analyzed for presence of a ring and constriction; 70 buds with rings were constricted, one with partial ring was somewhat constricted, and four with no ring showed no constriction).

It is still unknown whether the ring plays a passive or active role in constricting the mother cell-bud junction, i.e. whether the ring sets boundaries upon which cell wall expansion of the bud is limited or if it is actively compressing the neck region on appearance.

Cells that were budding with no ring at stage R=2.5 or

later (40 cases) were pear-shaped (19/40 cases), or had their buds with a large base (13/40 cases), elongated (4/40 cases), or even curved (4/40 cases).

Additionally, a few abnormal buds with normal rings were seen that were constricted at their bases (20 cases).

b) Blocked cells

As mentioned before, cells that were escaping the block and budding without rings were pear-shaped. Among the ten cases observed at various stages of the block, all were displaying such a morphology (Table 25). In addition, budding cells having a partial ring at the base of the bud (4 cases) were also pear-shaped but were slightly more constricted than if no ring was present (Fig. 12).

c) Budding zygotes

Following mating, 12 zygotes were able to bud without a chitin ring (Table 26). There was no constriction at the base of these buds (12 buds on 12), making them look larger than normal at their bases. Buds that had developed on zygotes with a ring showed constriction at their bases (45 cases on 45). There were eight buds with partial rings; four had a large base or were abnormally shaped. Furthermore, there were two budding cells produced from the zygotes but not attached to them which had abnormally elongated buds and no chitin rings.

Table 27. Frequency of buds with no ring at various stages of release.

Stage	No. of buds	No. of buds with no ring	Percentage
R=0.5	34	34	100.0
R=1.0	64	50	78.1
R=1.5	75	54	72.0
R=2.0	85	17	20.0
R=2.5	75	4	5.3
R=3.0	93	4	4.3
R=3.5	110	11	1.0
R=4.0	119	5	4.2
R=5.0	130	7	5.4
R=6.0	83	3	3.6
R=7.0	120	2	1.7
R=20ND	59	3	5.1
R=20D	56	0	0.0

iii. Chitin ring formation and bud separation

The chitin ring seems to be important for formation of the septum needed for bud detachment. Buds formed without rings fail to separate from their mother cells until a complete ring is formed and grow larger than their parents.

This statement is supported by the observation of the following cell types arising sequentially during cdc-24-1 release (Table 24):

1) At R=0.5, all buds emerged without rings (34 cases on 34; Figs. 52 and 53).
2) Later (R=2.0 and longer), most buds showed a ring of chitin at their bases, but a few failed to deposit a complete ring, and had large bases (Fig. 43).
3) The buds of these latter cells grew large and started to produce buds of their own (71 cases; Figs. 40 and 41).
4) Eventually, a complete chitinous plaque or system is deposited between the first mother-cell and its bud (43 cases).
5) Cell separation is then possible, since a few of the above cells in late release were seen with a large non-fluorescent flat side where a "budding bud" was previously attached (15 cases, Fig. 56).

Good evidence for failure of detachment in the absence of a complete ring or plaques was provided by budding cells having buds larger or as large as their mother cells and partial rings or

plaques[1] at their bases (17 cases on 22; Table 28). However, five buds were seen that were larger than their mother cells and yet had a ring. The presence of a ring in very large buds might be explained by the following :

1) the ring had formed late, when the bud was already very large.
2) the ring was abnormally functioning but structurally normal-looking.
3) the ring was necessary for septum formation but was not sufficient.

When the chitin distribution of undetached buds producing buds is looked at (Table 29), it is found that buds that have failed to separate from the original mother cells may display a partial ring (8/76 cases), a plaque (48/76 cases), a ring (6/76 cases), or no rings (4/76 cases). The complete absence of a ring strengthens the possibility of a direct relationship between chitin ring formation, septum formation and cell separation.

Further evidence exists in budding zygotes that the absence of a complete ring is responsible for the failure of bud separation. Of the 12 buds developing without rings, six were larger than all the buds emergings with a ring (45 cases).

[1] A plaque seems to represent a later stage of development than a partial ring and buds at that stage might soon separate.

Table 28. Released mutant cells: type of chitinous material found at the base of buds larger than their parents.

Stage	No. of cells with bud larger than parent	No. of large buds		
		with rings	with partial rings	with plaques
R=3.0[1]	2	1	1	0
R=3.5	3	0	3	0
R=4.0	5	0	2	3
R=5.0	5	0	0	5
R=6.0	3	1	0	2
R=7.0	1	0	0	1
R=20D	2	2	0	0
R=20ND	1	1	0	0
TOTAL	22	5	6	11

[1] Before R=3, all buds are smaller than their parent

Table 29. Chitin distribution of released cells of the type "bud on a bud"; type of chitinous material found at the base of the first bud.

Stages	Total No. of cases observed	Chitin Distribution of First Bud			
		No Ring	Ring	Partial Ring	Plaque
R-2.0	1	0	0	1	0
R-2.5	1	0	0	1	0
R-3.5	7	0	2	2	3
R-4.0	17	0	7	1	9
R-5.0	22	3	2	1	16
R-6.0	7	0	2	0	5
R-7.0	18	1	2	1	14
R-20 ND	1	0	1	0	0
R-20 D	2	0	0	1	1
TOTAL	76	4	16	8	48

5. A comparative study of log- and stationary-phase mutant cells

Sloat, Adams and Pringle (1981) reported that stationary-phase diploid cdc-24-1/cdc-24-1 and cdc-24-4/cdc-24-4 mutant cells shifted to restrictive temperature (36°C) without a change of medium do not show delocalized chitin deposition even after a 24 hour-block. Is such a behavior ploidy- or strain-specific? Will the haploid cdc-24-1 mutant used throughout this study behave correspondingly? In order to answer these questions, mutant cells were grown at room temperature until stationary-phase was reached. Then, cells were blocked for 24 hours, at which time a sample was collected, fixed, and examined by fluorescence microscopy following staining with calcofluor. No difference in chitin distribution was found between stationary-phase cells blocked for 24 hours (T_3) and log-phase unblocked cells (T_0, Table 30). In both cases, chitin was restricted to bud scars and chitin rings.

Would any other characteristics of a log unblocked state be found in stationary-phase blocked mutant cells? For instance, will the shape, volume, budding index, nuclear number, chitin and mannan content of cells be similar in both populations?

These questions can be answered by comparing the aforementioned characteristics in cells of both populations. Change in cell shape has been detected by measuring the minimal and maximal length of individual cells (n=25 for each population). In turn, these measurements have

been used to determine the cell volumes. The number of nuclei per cell has been revealed by staining with DAPI, while the chitin and mannan content of the cell wall have been determined by staining respectively with calcofluor and FITC-Con A. The percentage of budding cells has been recorded during analysis of cells stained with calcofluor and FITC-Con A. Results can be seen in Table 30. Stationary-phase cells blocked for 24 hours possess only one nucleus, as do log-phase unblocked cells, but they differ from the latter in their mannan content and their budding index. Moreover, there is no difference between the volume of these two types of cells (Table 34; T_3, T_0).

Are these differences reflecting changes in age of the population (log/stationary) or temperature (blocked/unblocked)? To answer that question, two additional types of cultures need to be studied:

1) a log blocked culture (B=8, designated as T_1),

2) a stationary unblocked culture (B=0, designated as T_2).

Comparisons between the log unblocked culture (T_0) and the log blocked culture (T_1) should indicate differences due to the block. Comparisons between the log unblocked culture (T_0) and the stationary unblocked culture (T_2) should point out differences due to age. Similarly, differences due to the block might be revealed by comparing the stationary unblocked culture (T_2) to the stationary blocked culture (T_3). If results obtained in such a case do not parallel those obtained between cultures T_0 and T_1, some insight is provided concerning the expression of the CDC 24 product at different stages of

the growth cycle. Tables 30 and 31 provide a summary of such a study (T_0, T_1, T_2, T_3). A large amount of mannan was observed in unblocked stationary cells (T_2) and at this stage, most cells were unbudded. Mannan accumulation and unbudding seem to be a consequence of the age of the population and not of the temperature since cells are at permissive temperature. When a similar culture was blocked for 24 hours (T_3), almost no phenotypic changes were observed due to the prolonged block: no new chitin or mannan had accumulated, most cells were still unbudded and uninucleate, and the volume of cells had not significantly changed (Table 34). However there was a highly significant difference between the minimal and maximal length of each type of cell (Table 32, 33). This suggests that although cells are not more voluminous, they have changed shape due to the block.

Unblocked log-phase cells (T_0) are characterized as previously mentioned by a cell wall relatively poor in chitin and mannan, by being uninucleate and mostly budding. However, when an identical population was blocked for eight hours (T_1), tremendous changes occurred. The level of both mannan and chitin greatly increased, cells became mostly binucleate and unbudded, and their minimal and maximal diameters and volume augmented highly significantly. Thus the temperature has caused both the shape to change and volume of the cells to increase, plus an accumulation of cell wall components such as mannan and chitin. In addition, the temperature was responsible for budding cessation that rendered cells bi- or multinucleate. By comparing changes brought by

temperature in T_0, T_1 cells to changes occurring in T_2, T_3 cells, one could see that the only common change was in the shape of the cells. That is to say that whether a cell is in log-phase or in stationary-phase, a restrictive temperature would affect its shape, making it more rounded. The other changes brought by the temperature shift, i.e. accumulation of chitin or mannan, DNA synthesis, or volumetric change, are phase-dependent.

Which factors might be responsible for the absence of blocked phenotype in stationary-phase cells? Is the observed phenotype due to medium depletion or turning off of the CDC 24 product, or to high cell density, accumulation of toxic products or absence of positive growth,...etc?

First, the effect of quality medium (old or fresh liquid YEPD) was tested at permissive temperature to determine whether stationary medium free of cells would still be able to support active cell growth at permissive temperature. If this is so, one might assume that the CDC 24 product is turn off in stationary-phase because of ageing, otherwise the aforementioned other possibilities hold. Clearly log-phase growing cells were unable to further reproduce in the old medium, but did well in fresh medium (Fig. 74).

The next variable looked at was cell density. Mutant cells were grown up to stationary-phase and blocked for 24 hours. The medium was then replaced by fresh YEPD medium (added at 37°C), but cell density was not changed. This culture was blocked for a further eight hours

(T_4) to see to what extent the unblocked phenotype of stationary-phase cells will change to a blocked log-phase phenotype (Tables 30 and 31). In terms of their volumes and sizes, cells were showing a phenotype intermediate between unblocked and completely blocked log-phase stages (Table 31). In addition, a majority of cells had two nuclei and high chitin cell wall content, two characteristics of a blocked log-phase phenotype. The mannan content of the cell wall did not seem to be significantly affected by this treatment, nor was the budding index. When a similar experiment was repeated but in addition cells were diluted at the end of the 24 hour-block to a log-phase density (approximately 1 x 10^6 cells/ml.) the results were more pronounced (T_6, Tables 30 and 31). Cells tended to assume a near perfect log-phase blocked phenotype except for their minimal and maximal length and volume which were slightly but significantly inferior.

Therefore, blocked stationary-phase cells could approach closely the phenotype of blocked log-phase cells if a change of medium is made and is accelerated by a low cell density. High cell-density by itself does not prevent cells from blocking when a change of medium occurred, but remains a key factor to determine the rate at which cells will block. However, low cell density and change of medium are not totally sufficient, but seem to be the main factors, for blocked stationary-phase cells to show the typical blocked log-phase phenotype.

Figure 74. Growth curve of unblocked log-phase cdc-24-1 cells grown in old or fresh medium at 23°C

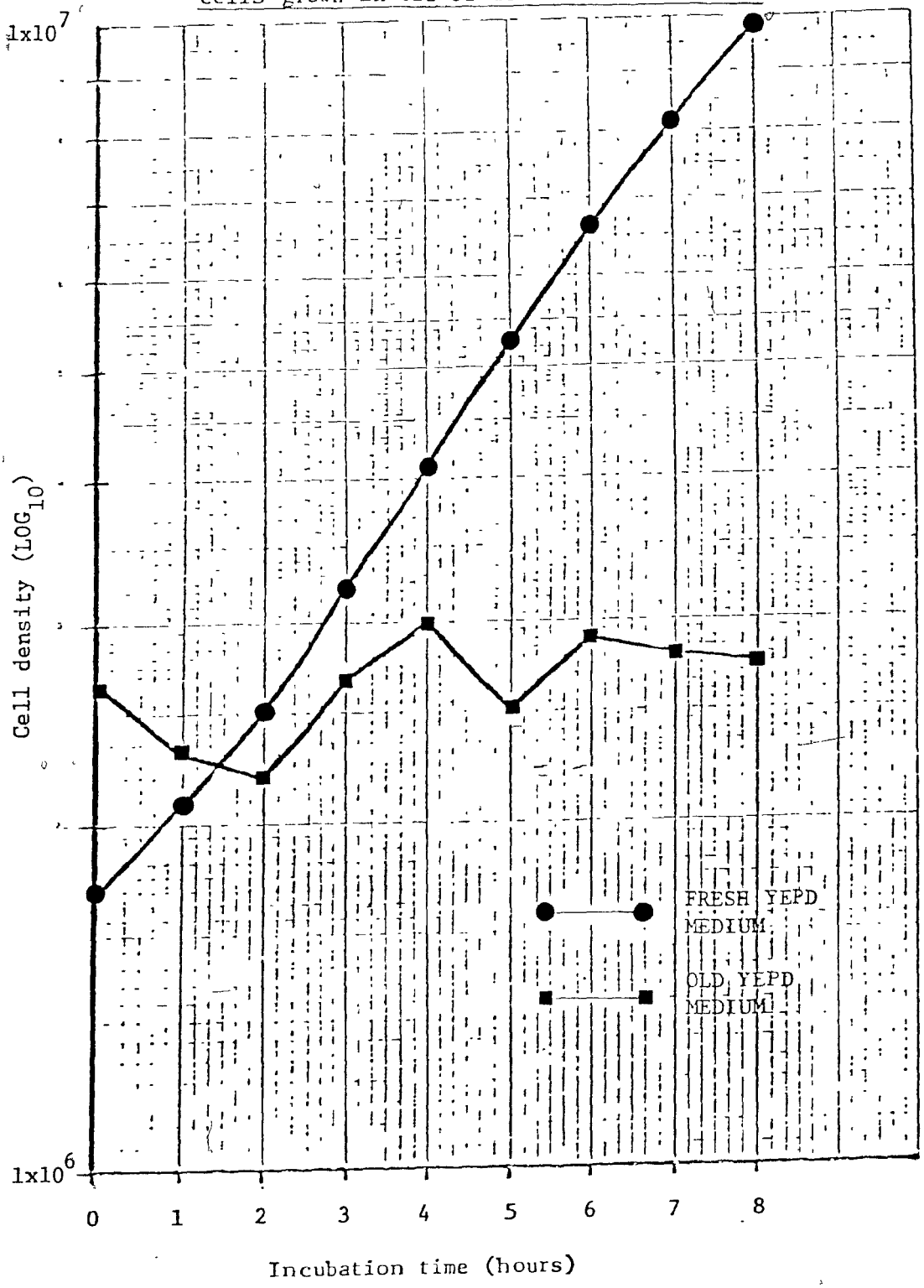

Table 30. Effect of different treatments on the mannan and chitin distribution, budding index and nuclear number of log- and stationary-phase cdc-24-1 mutant cells.

Culture	Mannan Distribution	Chitin Distribution	Budding Index			Number of nuclei per cell	Initial density
Log-phase UNBLOCKED cells (T_0)	+	+	55.0% (n=100)	59.0% (n=100)		1 (100%, n=100)	1.1×10^6 cells/ml.
Log-phase BLOCKED cells (B=8, T^1)	++	+++	6.9% (n=101)	14.0% (n=100)		2 (90.2%, n=101)	1.8×10^6 cells/ml.
Stationary-phase UN-BLOCKED cells (T_2)	++	+	7.5% (n=67)	17.0% (n=100)		1 (100%, n=100)	4.2×10^8 cells/ml.
Stationary-phase BLOCKED cells (B=24, T_3)	++	+	10.6% (n=66)	4.0% (n=100)		1 (100%, n=100)	4.1×10^8 cells/ml.
"HIGH DENSITY" stationary-phase cells (T_4)	++	++	13.2% (n=58)	10.0% (n=100)	12.0% (n=50)	2 (70%, n=100)	3.6×10^8 cells/ml.
"LOW DENSITY" stationary-phase cells (T_5)	++	+++	2.7% (n=37)	2.0% (n=100)	6.0% (n=50)	2 (94%, n=100)	0.9×10^6 cells/ml.

Table 31. Effect of different treatments on the minimum and maximum length and volume of log- and stationary-phase cdc-24-1 mutant cells.

Variable		Treatment Applied T_0	T_1	T_2	T_3	T_4	T_5
Maximum length (μm) (n=25)	MEAN	3.048	6.156	3.344	4.164	4.924	5.448
	ST. DEV.[1]	± 0.409	± 1.034	± 0.496	± 0.763	± 0.793	± 1.050
	ST.E.M.[2]	± 0.082	± 0.207	± 0.099	± 0.153	± 0.159	± 0.210
	MAX	4.2	7.9	4.2	5.8	6.6	7.5
	MIN	2.5	3.7	2.1	2.9	3.3	2.5
	RANGE	1.7	4.2	2.1	2.9	3.3	5.0
Minimum length (μm) (n=25)	MEAN	2.820	5.948	2.868	3.744	4.592	5.088
	ST. DEV.	± 0.327	± 0.938	± 0.475	± 0.774	± 0.759	± 0.995
	ST.E.M.	± 0.065	± 0.188	± 0.095	± 0.155	± 0.152	± 0.199
	MAX	3.3	7.5	3.7	5.4	5.8	6.6
	MIN	2.1	3.7	1.7	2.5	2.9	2.5
	RANGE	1.2	3.8	2.0	2.9	2.9	4.1
Volume (μm^3) (n=25)	MEAN	17.752	122.000	17.704	33.760	58.328	83.536
	ST. DEV.	± 8.988	± 49.524	± 5.415	± 19.494	± 27.201	± 40.287
	ST.E.M.	± 1.798	± 9.905	± 1.083	± 3.899	± 5.440	± 8.057
	MAX	40.3	232.7	30.1	88.6	116.3	171.1
	MIN	5.8	26.5	3.2	10.8	14.0	8.2
	RANGE	34.5	206.2	26.9	77.8	102.3	162.9

[1] ST. DEV. = standard deviation

[2] ST.E.M. = standard error of the means

Table 32. Duncan's new multiple-range test for variable maximal length.

Variable	ST.E.M. for n=25 (within variation)	Degree of Freedom	Type of Combination	Number of means	Difference between means	Level of Significance
Maximal length	0.159	144	T_1 T_5	2	0.708	.01[1]
			T_1 T_4	3	1.232	.01
			T_1 T_3	4	1.992	.01
			T_1 T_2	5	2.818	.01
			T_1 T_0	6	3.108	.01
			T_5 T_4	2	0.524	.05[2]
			T_5 T_3	3	1.284	.01
			T_5 T_2	4	2.104	.01
			T_5 T_0	5	2.400	.01
			T_4 T_3	2	0.760	.01
			T_4 T_2	3	1.580	.01
			T_4 T_0	4	1.876	.01
			T_3 T_2	2	0.820	.01
			T_3 T_0	3	1.116	.01
			T_2 T_0	2	0.296	N.S.[3]

[1] L.S. = .01, highly significant difference

[2] L.S. = .05, significant difference

[3] N.S. = not significant at P=.05

Table 33. Duncan's new multiple-range test for variable minimal length.

Variable	ST.E.M. for n=25 (within variation)	Degree of Freedom	Type of Combination	Number of means	Difference between means	Level of Significance
Minimal length	0.150	144	T_1 T_5	2	0.860	.01[1]
			T_1 T_4	3	1.356	.01
			T_1 T_3	4	2.204	.01
			T_1 T_2	5	3.080	.01
			T_1 T_0	6	3.128	.01
			T_5 T_4	2	0.496	.05[2]
			T_5 T_3	3	1.344	.01
			T_5 T_2	4	2.220	.01
			T_5 T_0	5	2.268	.01
			T_4 T_3	2	0.848	.01
			T_4 T_2	3	1.724	.01
			T_4 T_0	4	1.772	.01
			T_3 T_2	2	0.876	.01
			T_3 T_0	3	0.924	.01
			T_2 T_0	2	0.048	N.S.[3]

[1] L.S. = .01, highly significant difference

[2] L.S. = .05, significant difference

[3] N.S. = not significant at P=.05

Table 34. Duncan's new multiple-range test for variable volume.

Variable	ST.E.M. for n=25 (within variation)	Degree of Freedom	Type of Combination	Number of means	Difference between means	Level of Significance
			T_1 T_5	2	38.46	.01[1]
			T_1 T_4	3	63.67	.01
			T_1 T_3	4	88.24	.01
			T_1 T_0	5	104.2	.01
			T_1 T_2	6	104.2	.01
Volume	5.946	144	T_5 T_4	2	25.20	.01
			T_5 T_3	3	49.77	.01
			T_5 T_0	4	65.78	.01
			T_5 T_2	5	65.83	.01
			T_4 T_3	2	24.56	.01
			T_4 T_0	3	40.57	.01
			T_4 T_2	4	40.62	.01
			T_3 T_0	2	16.00	N.S.[2]
			T_3 T_2	3	16.05	N.S.
			T_0 T_2	2	0.048	N.S.

[1] L.S. = .01, highly significant difference

[2] N.S. = not significant at P=.05

Table 35. One-way analysis of variance for each variable.

Variable	Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F Value
Maximal length	Between Treatment	184.4290	5	36.88583	58.31[1]
	Within Treatment	91.08911	144	0.632560	
	Total Variation	275.5183	149	—	—
Minimal length	Between Treatment	197.0247	5	39.40495	70.04[1]
	Within Treatment	81.02087	144	0.562640	
	Total Variation	278.0456	149	—	—
Volume	Between Treatment	213587.6	5	42717.52	48.33[1]
	Within Treatment	127281.8	144	883.9018	
	Total Variation	340869.5	149	—	—

[1] highly significant at .01 level

V. DISCUSSION

The role played by the nucleus and the cell wall in specifying bud site was investigated in two multinucleate systems: in one system, the cell wall was under the influence of a ts mutation, and in the other it was not.

In 1975, Byers and Goetsch proposed the hypothesis that the orientation of nucleus-associated structures, and hence the nucleus, was primordial in restricting the location of budding. That is, the double SPB and its extranuclear microtubules might, by their orientation, control the budding process by interacting with a nearby region of the cell wall. Whether or not a bud would form at that selected site would depend on the cell wall responsiveness to the budding signal. Under such a proposal, an incompetent cell wall region will ignore the budding stimulus, while a competent region will respond to it. Up to now, incompetent cell wall regions have been reported to be limited to bud scars rich in circularly-arranged chitin microfibrils (Cabib and Bowers, 1971).

The results reported in this thesis show that in most binucleate cells formed in both systems, the two nuclei lie close together and divide into the same bud. In a few, the nuclei are far apart, and either one or two buds will form, each proximal to a nucleus which will divide into it. The synchronous and coordinated nuclear division into a single bud as well as the short distance separating the nuclei are consistent with SPBs and extranuclear microtubules facing and sending common budding signals to the same cell wall region. The asynchronous and/or uncoordinated nuclear

behavior of binucleate cells with nuclei far apart as well as the long distance separating these nuclei are consistent with each double SPB and its corresponding extranuclear microtubules processing its respective budding signal to a different cell wall region which it is facing.

The relationship observed between the distance separating the nuclei and the location and number of buds suggests a preponderant role for the nucleus in budding and reinforces the hypothesis of Byers and Goetsch on the control of bud emergence by a proper orientation of the double SPB and extranuclear microtubules.

Usually, in both systems, buds develop in regions closest to the nucleus or nuclei, but in early released cdc-24-1 cells with nuclei close together, they are located at the opposite side of mother-cell from early emerging bud. The lack of a consistent relationship between the location of nuclei and the sites of bud emergence suggests that nuclear position is of less importance than the orientation of its double SPB and extranuclear microtubules in the selection of a budding site. That the pattern of nuclei far from the budding site was also observed in several diploid binucleate cells homozygous for the cdc-24-2 mutation confirms this view. In favorable sections of two such cells examined by electron-microscopy, the orientation of at least one of the two SPBs and their extranuclear microtubules was clearly toward the distant bud site (Boothroyd, personal communication).

Thus, the orientation of the nucleus-associated structures more than

the location of the nucleus determines where a daughter-cell will appear. The fact that a nucleus does not need to be proximal to a pre-budding site for bud emergence to occur, constitutes a modification of the hypothesis of Byers and Goetsch, who proposed that both the orientation and location of the nucleus were important in determining budding pattern.

Boothroyd and Byers (1979) reported that, often, nuclei of diploid binucleate cells homozygous for the cdc-24-2 mutation divided synchronously and coordinately into a single bud, as do most haploid cdc-24-1 binucleate cells in this study. This common nuclear and budding behavior is, therefore, ploidy- and allele- independent.

In budding kar- zygotes, buds developed preferentially in regions of low chitin content. There are usually three such regions in zygotes, i.e. at both poles and on the conjugation tube. Buds appeared in these poor chitin containing regions closest to where a nucleus or nuclei were positioned. Since the zygote cell wall contained a similar amount of mannan at all stages of budding, that component did not seem to influence bud emergence. In contrast, released cdc-24-1 cells contained high levels of both chitin and mannan in their cell wall which did not prevent buds from emerging. The ability of cdc-24-1 cells to bud even with a uniformly high content of these components suggests a secondary role for the cell wall in determining the sites of bud emergence unless some other factor is involved. It seems as though the absolute amount of chitin or the relative amount of chitin and mannans are not key factors in preventing bud initiation. Therefore, the incompetence of cell wall regions, like those

filled with bud scars, are probably due to the arrangement of chitin fibrils rather than to their absolute amount. Enzymes might be unable to digest efficiently these circularly-arranged fibrils. Furthermore, observations made on budding zygotes indicate that when buds are given the preference of appearing in cell wall regions of different chitin content, they do so in regions containing less chitinous material. All these findings suggest that there are on the one hand, true regions of incompetence such as those rich in circularly-arranged chitin fibrils, and on the other hand, preferred regions of budding, i.e. those with lesser chitin content. In the latter case, a region poor in chitin will be preferred to an adjacent richer region, perhaps because it is easier or faster for such a region to be softened by digestive enzymes.

It appears that the inability of mutant cdc-24-1 cells to bud at restrictive temperature is not due to their high cell wall content of chitin and mannan, but rather to their unresponsiveness to the budding signal (or absence of or inadequate signal). In fact, the accumulation of chitin and mannan seems secondary to the nuclear ts mutation effect. Byers and Goetsch (1974) examined blocked cdc-24 cells at the electron microscope level, and found that they possessed SPBs at various developmental stages, as expected with continuous nuclear divisions. Several SPBs were duplicated and bore normal-looking extranuclear microtubules pointing normally towards the cell wall. Thus, the inability of blocked mutant cells to bud is not related to unduplicated SPB or apparent lack of extranuclear microtubules, but rather to a malfunctioning of the latter.

One decade ago, Olmsted and Borisy (1973) suggested that extranuclear microtubules were involved in transporting vesicles to a selected cell wall site. The vesicles contain, among other things, digestive enzymes (Nickerson, 1963; Moor, 1967; Matile, Moor and Robinow, 1969; Cortat, Matile and Wiemken, 1972), cell wall precursors (Matile, Moor and Robinow, 1969; Byers and Goetsch, 1976a; Schekman and Brawley, 1979; Field and Schekman, 1980) and probably chitin activating factors (Cabib, Duran and Bowers, 1978). If the function of extranuclear microtubules is impeded at restrictive temperature, these vesicles might not be properly directed to the selected budding site. Instead, they might become more-or-less randomly distributed with, maybe, an expected higher initial frequency in the vicinity of extranuclear microtubules since their distal ends lie at the base of the bud (Byers and Goetsch, 1975), and since vesicles were shown by freeze-etching (Moore, 1967) and thin sectioning (Schekman and Novick, 1982) to be produced by the nearby endoplasmic reticulum. In early blocked budded cells, the spreading of chitin occurred first in the vicinity of the chitin ring located at the base of the bud. As the block progressed, cells increased in volume and cell wall abnormalities developed. These cell wall abnormalities might very well correspond to abnormal regions of chitin and mannan fluorescence or to irregular regions seen by thin sectioning and electron-microscopy in blocked cdc-24-1 cells by the author, and in cdc-24-2/cdc-24-2 cells by Boothroyd (personal communication). All these observations (spreading of chitin, increase in volume, cell wall abnormalities) can be explained by the following

hypothesis. It is possible that upon transfer to restrictive temperature, the budding cdc-24-1 cells cannot transfer their vesicles normally along their extranuclear microtubules to the base of the early emerging bud. Consequently, vesicles would reach the cell periphery more randomly, creating abnormalities if they contain digestive enzymes or their activators, preferential growth of the mother cell if they contain cell wall precursors needed for bud growth (as observed by Sloat, Adams and Pringle, 1981, when they inactivated the CDC 24 product in budded cells), and localized activation of chitin synthetase (observed as spreading chitin fluorescence) if the vesicles contain the chitin synthetase activating factors.

Furthermore, most proteins with substituted amino acids are known to denature easily at relatively lower temperature than unsubstituted proteins. The observations reported in this thesis are consistent with a CDC 24 gene product being a microtubule-associated protein helping in vesicle-extranuclear microtubule interactions. At restrictive temperature, this "map" would be inactivated (e.g. unfold) and be incapable of guiding or transporting vesicles along microtubules to the appropriate cell wall site. The CDC 24 product has not been isolated and characterized yet, but based on the above hypothesis, isolation of extranuclear microtubules and their "maps" might make possible the biochemical characterization of this gene product.

The proximity of daughter nuclei in most blocked cdc-24-1 cells suggests a further role for the CDC 24 product in spindle elongation. In Saccharomyces cerevisiae, spindle elongation follows separation of each

chromosomal set (Peterson and Ris, 1976). It seems that in blocked cells, chromosome segregation was not followed by spindle elongation, and binucleate cells with nuclei close together appeared. In the event that the level of activity of the CDC 24 product was high enough to allow spindle elongation but not budding, binucleate cells with nuclei far apart would have been generated. The generation of two types of binucleate cells during the block is consistent with a CDC product involved in intranuclear microtubule-microtubule interactions. Further support for this hypothesis is provided by the existence of grouping of nuclei two by two in multinucleate cells. For instance, during the block a few cells might contain higher level of CDC 24 activity and the first nuclear division would yield binucleate cells with nuclei far apart (this activity would be, however, too low to allow budding). At the next nuclear division, these binucleate cells, now containing lower level of active CDC 24 product due to the prolonged temperature inactivation effect, would be unable to achieve spindle elongation, and tetranucleate cells with two separated pairs of nuclei close together would be generated.

It is, however, difficult to reconcile a dual role for the CDC 24 product in transport and in spindle elongation. Superficially, though, microtubules are involved in each case, and the product seems to act as a link between interacting structures.

Besides, the cell-cycle stage at which the block is applied might be responsible for the cell type produced at restrictive temperature (Hartwell, Mortimer, Culotti and Culotti, 1973). During the block, three

major cell types were observed, namely unbudded, budded with small cells or buds, or with large buds. The most common type, unbudded, can be explained if the block is applied before commitment to budding has occurred, or alternatively after all the vesicles necessary for bud growth and septum formation have reached the appropriate cell wall site. The second cell-type, bearing small cells or buds, can develop if the block is applied after some vesicles needed for bud growth have reached the budding site, but before those containing septum wall precursors did. In such cases, not all bud growth vesicles would have reached the selected site due to the concurrent CDC 24 product inactivation. Larger buds would have received more cell wall precursors and smaller buds less. The last cell type, displaying large buds, can arise if the block is applied after all vesicles needed for bud growth have reached the incipient bud, but before septum wall precursor vesicles did. All the aforementioned cell types can be explained by transportation of vesicles along extranuclear microtubules being somehow interrupted by transfer to a non-permissive temperature.

Budding without chitin rings was first reported by Bowers, Levin and Cabib (1974) and Cabib and Bowers (1975) who used an inhibitor of chitin synthetase, polyoxin D. But this idea was not well received in terms of the results obtained by Hayashibe and Katohda (1973) and Sloat and Pringle (1978). That bud emergence can occur without ring formation in both the cdc-24-1 and kar- systems, and without the use of inhibitors, confirms a minor role for the chitin ring in bud initiation. Interestingly, and in support for such a non-essential role, a micrograph published by Piggott,

Rai and Carter (1982) clearly shows the absence of rings at the bases of budded cdc 28-1N mutant cells transferred to restrictive temperature and stained with DAPI and calcofluor. One should realize that although non-essential, the chitin ring is a normal feature of bud emergence. Furthermore, some relationship between ring formation and normal bud shape might be assumed since in the absence of a complete ring, buds tend to be abnormal. In its presence, though, the bud shape tends to be normal with buds being constricted at their bases. Thus, the chitin ring seems to act as a reinforcement device. However, since a few abnormal buds were seen with normal-looking rings, it appears that the ring might not be sufficient for normal bud shape, although these buds were constricted at their bases. In fact, the chitin ring seems essential for bud constriction (a characteristic of normal bud shape), but might be insufficient to ensure normal bud shape, especially if laid down long after bud emergence. Additionally, since most buds without rings fail to separate from their parents, and grow very large, the chitin ring might also play a role in septum formation needed for bud detachment.

Since the ring appears not essential for bud emergence, but might be necessary for bud shape and septum formation, one may wonder if the 10-nm filament ring, found below the chitin ring just inside the plasmalemma of the mother-cell and having the same timing of appearance as the chitin ring, might not be an essential structure needed for bud emergence (Sloat and Pringle, 1978; Soll and Mitchell, 1983).

In the cdc-24-1 system, early released binucleate cells with nuclei

close together give a rather typical haploid budding pattern in large as well as in smaller cells. This pattern is revealed by the existence of buds developing on the above cells and that failed to separate. While still attached, these buds can produce new buds. The latter buds are mostly oriented towards the first mother-cell, a characteristic of a typical haploid budding pattern (Winge, 1934; Freifelder, 1960; Streiblová, 1970). Furthermore, when the original mother cells of such undetached buds are producing additional buds, they form adjacent to the first emerged bud. This constitutes a further characteristic of the haploid budding pattern, though in that latter case, buds are relatively closer. In addition, since these characteristics of a haploid budding pattern are seen both in large and small cells, size seems not to be a factor involved in the determination of a budding pattern. All these findings validate the use of block-release experiments for learning about normal processes.

The last point of discussion in this thesis is the absence of blocked phenotype in stationary-phase cells. The CDC 24 product seems to be turned off in these cells, but it can be turned on again by a change to fresh medium and this process can be accelerated by a low cell density. The turn off of this gene product in stationary-phase cells is consistent with a low metabolic rate. As an example, blocked stationary-phase cells would not become binucleate presumably because DNA synthesis proceeds at a minimum rate, maybe due to a low availability of necessary precursors. Similarly, chitin would not accumulate in the cell wall because few vesicles, if any, containing the needed precursors are synthesized. The absence of vesicles

(Matile, Cortat, Wiemken and A. Frey-Wyssling, 1971), multiple nuclei, budding as well as the lack of accumulation of chitin and mannan are consistent with an absence or very low level of the CDC 24 product in stationary-phase cells.

A complementary approach to the use of cell-division-cycle mutants is provided by the existence of temperature-sensitive secretory mutants (sec mutants) which are defective in bud growth at restrictive temperature (Sheckman and Novick, 1982). Indeed, these mutants appear useful and promising for further study of budding in yeasts, and they should receive more attention.

VI. SUMMARY

1. Role of the nucleus in budding

In most binucleate cells formed in the cdc-24-1 and kar- systems, the two nuclei lie close together and divide into the same bud. In a few, the nuclei are far apart, and either one or two buds will form, each proximal to a nucleus, which will divide into it. The relationship observed between the distance separating the nuclei and the location and number of buds suggests a preponderant role for the nucleus in budding.

Usually, in both systems, buds develop in regions closest to the nucleus or nuclei, but in early released cdc-24-1 cells with nuclei close together, they are located at the opposite side of the mother-cell from the emerging bud. The lack of a consistent relationship between the location of the nuclei and the sites of bud emergence suggests that nuclear position is not of primary importance in the selection of a budding site.

2. Role of the cell wall in budding

In budding kar- zygotes, buds develop preferentially in regions of low chitin content. The zygote cell wall contains a similar amount of mannan at all stages of budding. cdc-24-1 cells are able to bud even with a uniformly high content of these compounds, high enough to obscure chitin rings. The absolute amount of cell wall chitin or the

relative amount of chitin and mannan are not key factors in preventing bud initiation. This suggests a secondary role for the cell wall in determining the sites of bud emergence. The incompetency of certain cell wall regions, like those filled with bud scars, is probably due to the arrangement of chitin fibrils rather than their absolute amount.

3. Roles of the CDC 24 product

There are two types of cdc-24-1 binucleate cells appearing during the block; those which have their nuclei far apart, and those whose nuclei are lying side by side, i.e. less than one half nucleus diameter apart. After eight hours of block, these cell types represent respectively 10.3% (8/78) and 89.7% (70/78) of the total binucleate population. The proximity of daughter nuclei in most binucleate blocked cdc-24-1 cells suggests a role for the CDC 24 product in spindle elongation.

There are two kinds of chitin fluorescence appearing during the block: localized and general. When cells are blocked, the first two hours of an eight-hour block show that chitin is not only randomly deposited in the cell wall (general fluorescence) but also spreads from localized areas such as the chitin ring and bud scars (localized fluorescence). The spreading of chitin occurs first in the vicinity of the chitin ring, located at the base of the bud, where the distal ends of the extranuclear microtubules are also found. These observations are consistent with a CDC 24 product being a microtubule-associated

protein (map) involved in vesicle-extranuclear microtubule interactions. One might speculate that at restrictive temperature, this map would be inactivated and be incapable of guiding or transporting vesicles along microtubules to the appropriate cell wall site.

The CDC 24 product seems to be turned off in stationary-phase cells.

4. Role of the chitin ring in budding

Budding is possible in both systems in the absence of a chitin ring. In the absence of a complete ring, the buds are abnormally shaped, both in blocked and released cdc-24-1 cells and in budding karzygotes. Cells that are budding with no ring are pear-shaped or have large based, elongated, or even curved buds. These buds never show constrictions at their bases. Buds formed without rings fail to separate from their mother cells until a complete ring is formed and grow larger than their parents. No essential relationship is found between the formation of a ring of chitin and the emergence of a bud. Instead, the ring seems to play a role in normal bud development, at least in constriction of the base, and in septum formation needed for bud detachment.

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