A DMSO-sensitive conditional mutant of the fission yeast orthologue of the *Saccharomyces cerevisiae SEC13* gene is defective in septation

Deborah Poloni, Viesturs Simanis*

Cell Cycle Control Laboratory, Swiss Institute for Experimental Cancer Research (ISREC), 1066 Epalinges, Switzerland

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Abstract Dissection of complex processes using model organisms such as yeasts relies heavily upon the use of conditional mutants. We have generated a collection of fission yeast mutants sensitive to dimethylsulphoxide (DMSO). Among these we have found a mutant in the *Schizosaccharomyces pombe* orthologue of the *Saccharomyces cerevisiae SEC13* gene, which fails to cleave the division septum. Generation of a null allele demonstrates that the *S. pombe sec13* gene is essential.

Key words: DMSO; Fission yeast orthologue; Septation; *SEC13* gene; *Saccharomyces cerevisiae*

1. Introduction

Simple model systems such as yeasts have been used to dissect complex cellular processes through the analysis of conditional mutants. The majority of mutant hunts have used shifts in temperature to impose the restrictive condition, though other methods, for example altered sensitivity to drugs and pheromones, changes in ionic strength and so on, have also been used [1–12]. It has been observed that many genes are not identified by examination of heat-sensitive or cold-sensitive mutants [13]. This may be due to redundancy of gene function, though, in other cases, the structure of the protein may not lend itself to the kind of mutation required to produce a thermosensitive mutant. To circumvent the latter problem, modified growth media have been used to attempt to interfere with hydrogen bonding and thereby disrupt the structure of a mutant protein. For example, high percentages of deuterated water have been added to the growth medium [3]. An alternative to this has been the addition of organic solvents, for example ethanol and formamide, to the growth medium [1,2,7]. In this present study, we have used dimethylsulphoxide (DMSO)-containing media to identify conditional mutants of the fission yeast *Schizosaccharomyces pombe*. We describe a novel septum cleavage mutant that is an allele of the fission yeast orthologue of the *Saccharomyces cerevisiae SEC13* gene.

2. Materials and methods

2.1. Growth and manipulation of fission yeast

Standard methods were used throughout [14]. Cells were grown in complete (YE) medium, or supplemented minimal (EMM2) medium, as indicated. For growth in the presence of different percentages of DMSO, media were made without DMSO, then adjusted to the required final volume by addition of DMSO after autoclaving and cooling. Cells growing in medium without DMSO were collected by centrifugation and resuspended in prewarmed medium containing DMSO. In some experiments DMSO was added to 4% (v/v) directly to the growing culture. The results obtained were very similar to those using the protocol described above. When making plates, DMSO was added after autoclaving. To maximise consistency, plates containing DMSO were kept for less than 2 weeks, and were not stored at 4°C. For DAPI and Calcofluor staining, cells were fixed in ethanol as described [15]. Cell number was determined using a CASY cell counter.

2.2. Mutagenesis and genetic analysis

DMSO-sensitive mutants with division and mitotic defects were generated using the diploidisation enrichment protocol described by [16]. Briefly, the strain *leu1-32 mam2*$^{-}$ *h*^{90} was treated with 3% (v/v) ethyl-methyl sulphonate to kill 90% of cells. After mutagenesis, cells were grown for 5 h at 29°C in YE to recover, then the culture was incubated in 4% (v/v) DMSO for 3 h to allow expression of the DMSO-sensitive (ds) phenotype. Cells were then collected by centrifugation, and inoculated into minimal medium without NH_4Cl at 29°C in order to induce meiosis in cells that had increased in ploidy during incubation in the presence of DMSO. After 3 days at 29°C, cells were collected by centrifugation, resuspended in water and vegetative cells were eliminated by extensive digestion with 2 mg/ml lysing enzymes (Sigma), followed by treatment with 20% (v/v) ethanol. Spores were plated out at 29°C, and colonies that showed a DMSO-sensitive proliferation defect after replica-plating to media containing 4% (v/v) DMSO and phloxin B were examined further. All strains were outcrossed to wild-type at least twice before analysis. FACS analysis was performed using a Becton Dickinson FACScan according to the protocol of [17].

2.3. Cloning and analysis of the ds293 gene

The mutant *ds293 ura4-D18* was transformed to uracil prototrophy with a wild-type *S. pombe* genomic DNA library [18]. Colonies were allowed to form on minimal plates at 29°C and then replica-plated to minimal plates containing phloxin B and 5% (v/v) DMSO and incubated at 36°C. Plasmids were recovered from surviving colonies, and after confirming that they were capable of rescuing upon retransformation, they were sequenced.

2.4. Deletion of the sec13 gene

To delete the *sec13* gene, the *ura4*$^{+}$ gene was inserted between regions of DNA flanking the *sec13* gene. A *Nsi*I–*Sty*I fragment of cosmid SPBC215 (nt 34956–35902) was cloned into the *Pst*I and *Hin*dII sites of pDW232. A second *Bam*HI–*Sty*I fragment (nt 36501–37252) was cloned between the *Bam*HI and *Sma*I sites of pDW232. The resulting construction was cut with *Bam*HI, treated with DNA polymerase I Klenow fragment to render it blunt-ended, and the *ura4*$^{+}$ gene was inserted into the *Bam*HI site as a blunt-ended frag-

*Corresponding author. Fax: (41)-21-652 6933.
E-mail address: viesturs.simanis@isrec.unil.ch (V. Simanis).

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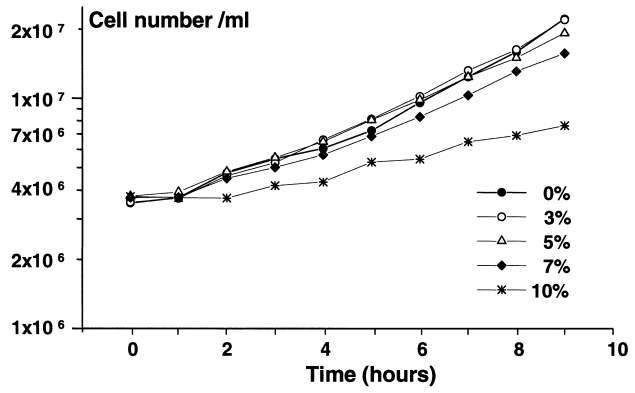

Fig. 1. The effect of DMSO upon the growth of fission yeast cells, and phenotypes of ds mutants. *leu1-32 mam2*$^{-}$ *h*^{90} cells were grown to mid-exponential phase in YE medium at 29°C, collected by centrifugation and resuspended in fresh medium containing the indicated percentage of DMSO. Cell number was determined at the indicated times after re-inoculation.

ment. This construction was digested with *Eco*RI and *Sph*I to liberate a 3.5 kb fragment, which was used for transformation of a diploid *ade6-M210/ade6M-216 leu1-3/leu1-32 ura4-D18/ura4-D18 h*$^{-}$*/h*$^{+}$ to uracil prototrophy. Deletion of one copy of the gene was confirmed by Southern blotting (not shown).

3. Results

3.1. The effect of DMSO concentration on the growth of fission yeast

In order to investigate the feasibility of using DMSO to impose the restrictive condition upon mutants, we tested the sensitivity of *S. pombe* cells to DMSO. At 29°C in YE medium, the doubling time of wild-type cells (approximately 2.5 h) was not significantly altered by addition of DMSO up to 5% (v/v). At 7% (v/v) DMSO, the doubling time increased to 3 h, while at 10% (v/v) DMSO the doubling time became approximately 6 h (Fig. 1). Cells plated on solid media containing 7% (v/v) DMSO and the vitality stain phloxin B were significantly darker than those on media containing 5%(v/v) DMSO or less, indicative of elevated numbers of dead cells. Since we planned to use the diploidisation selection screen described by [16] in order to enrich for mitotic and septation mutants, we also tested the effect of DMSO concentrations up to 5% (v/v) upon the number of spontaneous diploids generated following 4 h exposure to solvent-containing medium; no significant increase was noted (data not shown).

We also tested the effect of DMSO at different temperatures in the commonly used *leu1-32* and *ura4-D18* genetic backgrounds. No difference in the effect of DMSO at different temperatures was noted in either a wild-type or *leu1-32* background. However, it was found that a *ura4-D18* mutant was unable to grow on media containing 4% (v/v) DMSO at temperatures of 32°C or above. Cells stopped growing and lysed, producing a similar phenotype to that generated by starving *ura4-D18* cells of uracil. No deleterious effects were noted below 32°C. On the basis of these trials, analysis of mutants was standardised using 4% (v/v) DMSO as the restrictive condition.

3.2. Generation of ds mutants

Cells were mutagenised and the resulting spores plated at 29°C. Approximately 50 000 colonies were replica-plated to YE medium containing 4% (v/v) DMSO and phloxin B to identify those containing many dead cells. After restreaking, 108 ds mutants were retained for further analysis. Replica-plating to 19°C or 36°C showed that of these, 28 were also heat-sensitive and 17 were cold-sensitive. Full details of the screen will be presented elsewhere. Here, we present our analysis of one of the ds mutants, which displays a defect in cleavage of the division septum.

3.3. Analysis of mutant ds293

Mutant *ds293* appears wild-type at all temperatures (19–36°C) in the absence of DMSO. In the presence of 4% (v/v) DMSO a high percentage of cells accumulate a single division septum, which is not cleaved (Fig. 2A). A time course following addition of DMSO showed a first-cycle arrest, with approximately 80% of cells arresting with a single septum (Fig. 2B). All the septated cells were binucleate, with apparently normal post-mitotic nuclei (the chromatin domains of the nuclei face each other), suggesting that mitosis had been completed normally. FACS analysis showed that cells had a 4C DNA content after 6 h, indicating that cells entered the next round of DNA synthesis despite having failed to cleave the division septum (Fig. 2C).

Two classes of non-overlapping plasmids from a wild-type genomic DNA library were capable of complementing the ds phenotype of the *ds293* mutant. Sequencing and database searches indicated that the first lay adjacent to the *cdc25* gene on chromosome I, the second on chromosome II, close to *cdc23*. We therefore crossed *ds293* to *cdc25-22* and *cdc23-M36*. No linkage was observed between *ds293* and *cdc25-22*, indicating that this plasmid encodes a multicopy suppressor. In contrast, a cross of *ds293* to *cdc23-M36* produced 45 PD:4 TT:1 NPD in 50 tetrads, indicating linkage of the two genes. Subcloning indicated that open reading frame (ORF) SPBC215.15 (Sanger centre nomenclature: http://www.sanger.ac.uk/Projects/S_pombe/) was responsible for the complementation. Integration of this ORF with a *ura4*$^{+}$ marker, followed by crossing to *ds293*, indicated that it encodes the gene that is mutated in *ds293* (not shown). Database comparisons showed a strong homology of this ORF to *S. cerevisiae* Sec13p (Fig. 3A). The mutant will therefore be referred to hereafter as *sec13-ds293*. The multicopy suppressor near *cdc25* (ORF SPAC24H6.08, see Fig. 3B) does not display any significant homologies to proteins in the current databases.

3.4. The sec13-ds293 mutant loses viability at the time of septum formation

Addition of DMSO to asynchronous *sec13-ds293* cells produced a loss of viability (as assayed by colony formation) that was proportional to the percentage of septated cells in the culture (not shown). To determine at which cell cycle stage cells died, wild-type and *sec13-ds293* cells were synchronised by centrifugal elutriation and re-inoculated into medium containing 4% (v/v) DMSO. Cell viability (ability to form colonies on medium lacking DMSO), cell number and septation index were determined at intervals thereafter. Wild-type and *sec13-ds293* cells divided with similar kinetics in the absence of DMSO (Fig. 3C,E). In contrast, while the switch to

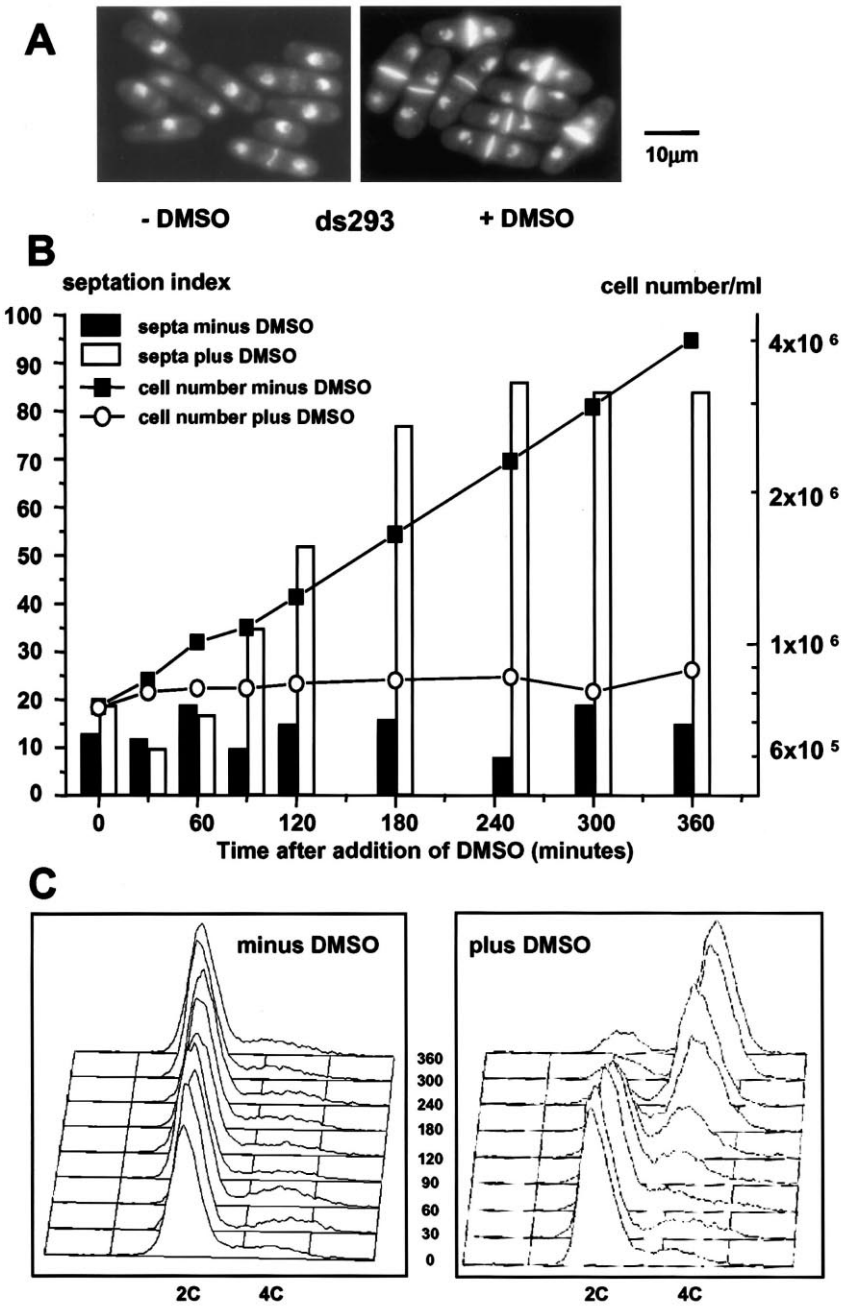

Fig. 2. Analysis of mutant *ds293*. A: Mutant *ds293* was grown to mid-exponential phase at 29°C and resuspended in medium containing 4% (v/v) DMSO. After 6 h, cells were fixed with ethanol and stained with DAPI and Calcofluor. B: Samples were removed at the indicated times from the culture in the experiment described above and the septation index and cell number were determined. C: Samples were removed at the indicated times from the culture in the experiment described in A and the DNA content was analysed by FACS.

DMSO-containing medium produced a minor division setback to wild-type cells (as is also the case for temperature shifts; see for example [12,19], no cell number increase was observed in the *sec13-ds293* culture during the course of the experiment (Fig. 3C). Nonetheless, these cells septated with similar kinetics to the controls (Fig. 3D), but the septum was not cleaved. Cell viability was high initially (indicating that the effect of addition of DMSO is reversible). However, when the septation index began to increase, *sec13-ds293* cells lost viability in the presence of DMSO (Fig. 3E). Therefore, the division septum produced by *sec13-ds293* cells in the presence of DMSO cannot be cleaved, and failure of an event at the end of the cell cycle may be responsible for the death of these cells.

3.5. The S. pombe sec13 gene is essential

Part of one copy of the *sec13* gene was replaced by the *ura4*$^+$ gene in a diploid. Dissection of tetrads indicated that initially all four members of the tetrad germinated and formed colonies. However, two were large, and two were very small (Fig. 4A). Replica-plating indicated that the large colonies were ura$^-$, while the small ones could not be propagated further. Examination of the cells in the microcolonies revealed that they contained 100–400 cells, suggesting that approxi-

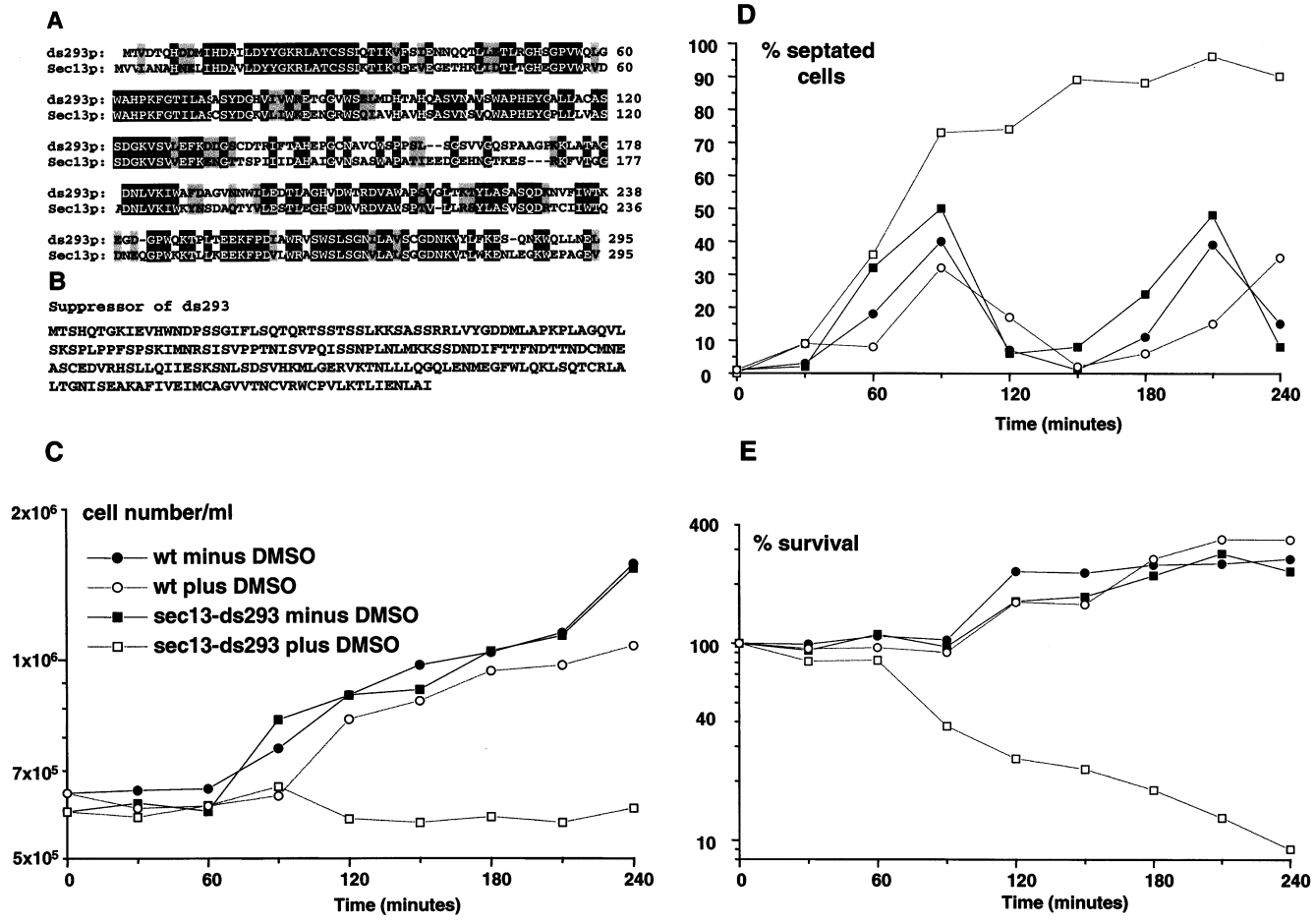

Fig. 3. The *ds293* mutant defines the *S. pombe* orthologue of the *S. cerevisiae SEC13* gene. A: Sequence comparison of the budding yeast *SEC13* gene and its putative fission yeast homologue. B: Sequence of the multicopy suppressor of *ds293*. C–E: Synchronous cultures of wild-type or *sec13-ds293* cells were generated by centrifugal elutriation. Cells were reinoculated into YE medium at 29°C in the presence or absence of 4% (v/v) DMSO. Samples were removed at intervals, and the parameter indicated in each panel was assayed. Colony formation with time is expressed as a percentage of the number of colonies obtained at $t=0$. Samples were removed and plated on medium without DMSO after appropriate dilution.

mately eight divisions had occurred after germination. The cells were larger than wild-type, mis-shapen, and often swollen at one end (Fig. 4B). These data show that the *S. pombe sec13* gene is essential.

4. Discussion

We have isolated a mutant of the *S. pombe sec13* gene among a collection of conditional mutants sensitive to DMSO. Orthologues of the *S. cerevisiae SEC13* gene are found from yeasts to man. Sec13p is part of the COPII coat that is involved in anterograde transport of proteins from the endoplasmic reticulum to the Golgi complex (reviewed by [20–22]. Why this mutant should produce predominantly a septation phenotype is not clear at present. However, it seems reasonable to assume that the cell cycle arrest is due to a failure to deliver proteins or membrane vesicles required for correct assembly and/or cleavage of the division septum. The *S. cerevisiae SEC13* gene is essential [23], and mutant alleles rapidly cease growing and arrest randomly throughout the cell cycle [22]. The difference between the phenotypes of the null and ds mutants in *S. pombe* is intriguing. The ability of the null to undergo multiple cycles of division after spore germination is most likely due to significant amounts of sec13p incorporated in the spore from the diploid. The *S. pombe sec13-ds293* mutant arrests in the first cycle after addition of DMSO, suggesting that loss of function is rapid. However, the uniformity of the arrest suggests that the *sec13-ds293* allele may be hypomorphic and that septum formation is the step in the cell cycle that is most sensitive to impaired protein trafficking. Consistent with the notion that other functions in addition to septation are affected in *sec13-ds293*, examination of a double mutant with *cdc25-22* (which arrests as a highly elongated cell in G2 prior to septum formation) showed that the cells elongated less than the *cdc25-22* mutant alone in the presence of 4% (v/v) DMSO (not shown), suggesting that the *sec13-ds293* cells also have a defect in growth at the tips.

The phenotype of a high percentage of binucleate cells with a single, uncleaved septum is also produced following addition of cycloheximide late in the cell cycle [25,26], suggesting that proteins synthesised after the initiation of mitosis are essential for cleavage or correct assembly of the division septum. This may explain why a mutation in the protein trafficking machinery can give rise to a defect in cleavage of the division septation. A collection of *S. pombe* mutants that arrest binucleate

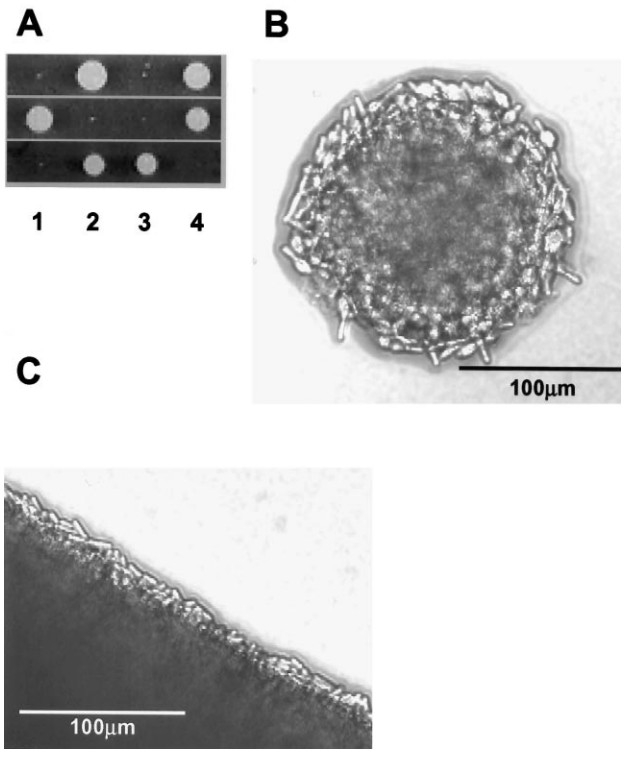

Fig. 4. The *sec13* gene is essential. A: A diploid heterozygous for the *sec13::ura4*$^+$ allele was sporulated and tetrads were dissected. Three tetrads are shown. Note the presence of two large and two small colonies in each row. B,C: Photograph of the *sec13::ura4*$^+$ microcolony (B) and the edge of a wild-type colony grown for the same length of time (C).

with a single septum has been described [24]. However, in contrast to *sec13-ds293*, these do not undergo the next round of DNA synthesis. At present it is not known whether any of these are allelic with *sec13*.

The irreversibility of the arrest once the septum has been formed is intriguing. Cells clearly do not die immediately, as they are able to undergo the next round of DNA synthesis. Accumulation of division septa is not per se lethal, since a *cdc16-116* mutant can recover with high viability ($>90\%$) after prolonged incubation at restrictive temperature when the cells have more than three septa each and have undergone the next round of DNA synthesis (V. Simanis, unpublished observations; [27]). It is possible that once perturbed by loss of COPII function, the protein sorting machinery cannot recover adequately to permit resumption of growth.

The sequence of the multicopy suppressor of *ds293* gives no clue as to its biological function. Further experiments will be required to determine whether it plays any role in protein sorting and secretion.

Sensitivity to DMSO should be useful for generating alternative conditional (ds) alleles in genes of interest, and perhaps for producing conditional mutations in proteins that are not readily amenable to the production of heat- and cold-sensitive mutants. The lab collection of septation and cell cycle mutants was tested for a ds phenotype at 25°C, and the majority were not sensitive (unpublished observations). This raises the possibility of performing reciprocal shifts and order-of-execution experiments between ds and thermosensitive mutants, complementing the cold-sensitive–heat-sensitive and pheromone–thermosensitive mutant methods [28,29]. We have also noted that many thermosensitive mutants arrest better at 36°C if 4% (v/v) DMSO is added to the medium. This may facilitate cloning of genes where only weak alleles are available.

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