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sepB: an *Aspergillus nidulans* gene involved in chromosome segregation and the initiation of cytokinesis

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In *Aspergillus nidulans* conidia, cytokinesis (septation) is delayed until three rounds of nuclear division have been completed. This has permitted the identification of essential genes that are involved in the coordination of cytokinesis with nuclear division. Conditional mutations in the *sepB* gene block septation but allow germinating spores to complete the first three rounds of nuclear division at restrictive temperature. *sepB3* mutants demonstrate transient delays in M-phase, accumulate aneuploid nuclei and show defects in chromosome segregation. Molecular analysis of the *sepB* gene reveals that it is essential and possesses limited similarity to the *CTF4* gene of *Saccharomyces cerevisiae*. Using temperature-shift analysis we show that *sepB* is required after the first nuclear division but before the onset of cytokinesis. A failure to execute the *sepB* function results in a block to nuclear division and leads to cell death at a time when wild-type cells would be undergoing cytokinesis. Finally, we demonstrate that *sepB* is also required for the uninucleate cell divisions of developing conidiophores. Our results suggest that *sepB3* mutants accumulate specific nuclear defects that do not arrest mitosis, but block the initiation of septum formation. Thus, proper chromosome segregation and a functional *sepB* gene are required to initiate cytokinesis.

Keywords: *Aspergillus*/cell cycle/cytokinesis/nuclear division/septation

Introduction

Cytokinesis is coordinated with mitosis and involves the directed assembly of the division apparatus at a specified site on the cellular cortex. The location of the site is believed to be determined by signals emanating from the mitotic spindle (Rappaport, 1986). Recent evidence suggests that cytokinesis may be spatially regulated by chromosomal passenger proteins such as the INCENPs [inner centromere proteins (Cooke *et al.*, 1987)], novel structures such as the telophase disc (Margolis and Andreassen, 1993) and/or by calcium waves (Earnshaw and Cooke, 1991; Fluck *et al.*, 1991). Other studies have shown that the timing of cytokinesis may be controlled by the central regulator of mitosis, $p34^{cdc2}$ (Satterwhite *et al.*, 1992; Yamakita *et al.*, 1994).

Genetic studies in the fission yeast *Schizosaccharomyces pombe* have identified a number of gene products that function in the regulation of septum formation and cytokinesis (Fankhauser and Simanis, 1994a). The *cdc16* gene product acts to ensure that only one septum is made per cell cycle and may be required to maintain high levels of $p34^{cdc2}$ kinase activity before the onset of anaphase (Fankhauser *et al.*, 1993). The activity of the *cdc16* gene product may be regulated in part by the protein kinase encoded by the *cdc7* gene (Fankhauser and Simanis, 1994b). Overexpression of *cdc7* results in a phenotype identical to that seen in the absence of *cdc16*; multiple rounds of septum formation occurring in the absence of mitosis (Minet *et al.*, 1979; Fankhauser and Simanis, 1994b). The *cdc14* gene product has also been implicated in the coordination of mitosis and septum formation in *S.pombe* (Fankhauser and Simanis, 1993). Despite the progress made in all of these studies, the specific component(s) of the nuclear division machinery that is monitored by these regulatory proteins remains unidentified.

The timing of cytokinesis during spore germination in *Aspergillus nidulans* has permitted the identification of a novel class of mutants with a distinct temperature-sensitive (Ts) arrest phenotype that suggests a defect in the coordination of cytokinesis with nuclear division. In wild-type strains the formation of the first septum is delayed until at least three rounds of nuclear division have been completed and an elongated germ tube has formed (Harris *et al.*, 1994). Passage through the third mitotic division is required to initiate cytokinesis. A particular class of early-acting *sep* mutants (*sepB*, *sepE*, *sepI* and *sepJ*) are able to successfully complete three or four rounds of nuclear division at restrictive temperature before arresting growth as aseptate cells (Harris *et al.*, 1994). Later-acting cytokinesis mutations (*sepA*, *D*, *G* and *H*) produce a more typical cytokinesis defect; namely, large multinucleate cells lacking septa at restrictive temperature.

A feature that distinguishes early-acting from late-acting *sep* gene mutations, is that late-acting mutations permit the assembly of septa at correct positions when cells are shifted from restrictive to permissive temperature. This result suggests that these mutants have marked sites for septum formation (septal primordia) during growth at restrictive temperature and are blocked at a later stage in septum assembly (Trinci and Morris, 1979; Harris *et al.*, 1994). In contrast, early-acting mutants fail to assemble septa following a return to permissive growth temperatures, suggesting they are defective in an earlier step in septum formation (Harris *et al.*, 1994). At restrictive temperature, early-acting mutations permit completion of the first three or four nuclear divisions but arrest cell and nuclear division at a time coincident with the onset of cytokinesis. Based on these phenotypes we have proposed that a regulatory mechanism exists in *A.nidulans* to prevent the further progression of nuclear division unless cytokinesis has

been properly initiated. The initiating event in *A.nidulans* appears to be the demarcation of sites in the cell cortex for septum assembly (Harris *et al.*, 1994).

Here, we describe a possible role for the early-acting *sepB* gene. Upon germination at restrictive temperature, *sepB* mutants are able to complete the first three rounds of nuclear division but arrest and die before initiating cytokinesis. We show that the *sepB3* mutant exhibits a transient delay in passage through M-phase and accumulates aberrantly shaped nuclei with an appearance characteristic of aneuploidy. A combination of phenotypic analysis and temperature-shift experiments shows that *sepB* function is required after the first nuclear division but well before the onset of cytokinesis. The *sepB* gene is essential and possesses limited similarity to a non-essential *Saccharomyces cerevisiae* gene implicated in chromosomal DNA metabolism (*CTF4*). Using a colony sectoring assay we show that *sepB* mutants produce defects in chromosome segregation. We propose that the *sepB* gene product performs an essential function during cell division in *A.nidulans* and that the *sepB3* mutation defines a novel cell cycle arrest point. Furthermore, our results show that *sepB* mutants fail to initiate cytokinesis due to incomplete or aberrant chromosome segregation events associated with early nuclear divisions.

Results

Genetic analysis of the sepB locus

The single Ts *sepB* mutant (*sepB3*) was originally identified by its inability to make septa at restrictive temperature while retaining the ability to complete early nuclear divisions (Morris, 1976). Further genetic analysis demonstrated that the *sepB3* mutation failed to recombine with another mutation originally designated *sepC4* and heterozygous diploids formed between *sepB3* and *sepC4* failed to complement. Thus, *sepC4* was renamed as *sepB4*. Additional septation mutants with similar phenotypes have recently been isolated (Harris *et al.*, 1994). Careful microscopic observations with nuclear and cell wall stains failed to reveal any difference between strains possessing the *sepB3* or *sepB4* alleles. Thus all phenotypic characterization was performed on mutant strains containing the *sepB3* allele. Genetic analysis of the *sepB3* mutant demonstrated that all of the associated phenotypes co-segregate and were due to a recessive mutation in a single gene (Morris, 1976; Harris *et al.*, 1994). Parasexual genetic analysis positioned *sepB* on chromosome V (Morris, 1976). Using a chromosome V mapping strain, we found that the *sepB* locus maps 37 map units centromere proximal to the *lysB* locus (Figure 1), thus placing it close to the chromosome V centromere.

Cloning of the sepB gene

Using pools of cosmids from a chromosome V-specific library (Brody *et al.*, 1991), we identified a single cosmid, L30D12, that was able to complement all phenotypes associated with the *sepB3* and *sepB4* mutations. Additional transformation experiments using sub-clones derived from L30D12 resulted in the identification of a 4.4 kb *Hin*dIII–*Xho*I fragment that contained complementing activity (Figure 1).

We showed that the bona fide *sepB* gene had been cloned using an approach described by O'Connell *et al.* (1992). Plasmid pSH7, containing the 4.4 kb *Hin*dIII–*Xho*I fragment and an *argB* gene (see Materials and methods), was transformed into the *sepB3* mutant, ASH15. We identified transformants in which homologous recombination between the resident *sepB3* allele and the *sepB* gene on pSH7 generated a tandem duplication of the *sepB* gene flanking the integrated *argB* gene. Two transformants were allowed to self-cross and Arg^- segregants were identified. These segregants arise as a result of recombination between the mutant and wild-type copies of *sepB* that flank the *argB* gene. For both transformants, many Arg^-, Ts^+ segregants were recovered, suggesting that the *sepB* gene had been cloned. Furthermore, when a single Arg^- Ts^+ segregant from each transformant was crossed to a wild-type strain, no Ts^- progeny were obtained (>200 ascospores tested).

Northern analysis using the 4.4 kb *Hin*dIII–*Xho*I fragment as a probe identified a low abundance 2.5 kb mRNA (S.Harris and J.Hamer, unpublished data). The same fragment was used to probe a cDNA library and two positive plaques were identified which were found to contain identical inserts. Co-transformation experiments showed that the cDNA was able to complement the *sepB* mutation (Figure 1).

sepB is an essential gene

The heterokaryon rescue technique (Oakley and Osmani, 1993) was used to determine if *sepB* is an essential gene in *A.nidulans*. This technique is based on the observation that transformed *A.nidulans* protoplasts are heterokaryotic, containing both transformed and untransformed nuclei. Conidiation of the heterokaryotic mycelium results in the production of uninucleate spores, of which a fraction will contain transformed nuclei. These spores can then be recovered by plating on the appropriate selective media. Plasmid pSH12 was constructed such that most of the *sepB* gene was replaced with the *argB* gene (Figure 1). This plasmid was used to transform both haploid and diploid strains to arginine prototrophy. Even though Arg^+ heterokaryons were recovered when haploid strain A850 was transformed with pSH12, we were unable to recover any Arg^+ conidia. Examination of three transformed Arg^+ heterokaryons by Southern analysis confirmed that the predicted gene replacement event had occurred in each case (data not shown). This result suggests that *sepB* is essential and is required in a cell autonomous manner during conidiation in *A.nidulans*.

Transformants heterozygous for the predicted gene replacement event were recovered when plasmid pSH12 was used to transform diploid strain A852 (data not shown). Random mitotic segregants were obtained from two transformants (T3-12 and T4-10) on complete media (Hastie, 1970). Of 47 segregants from transformant T3-12, 10 were Arg^- haploids that possessed the unaltered copy of the *sepB* gene based on the analysis of Southern blots. The remaining 37 segregants were Arg^+, of which five were able to grow on minimal media and were thus diploid. The other 32 segregants appeared to be disomes, as Southern blot analysis demonstrated that they remained heterozygous for the *sepB* gene replacement. A total of 23 mitotic segregants from transformant T4-10 were similarly analyzed and all were found to be Arg^- haploids.

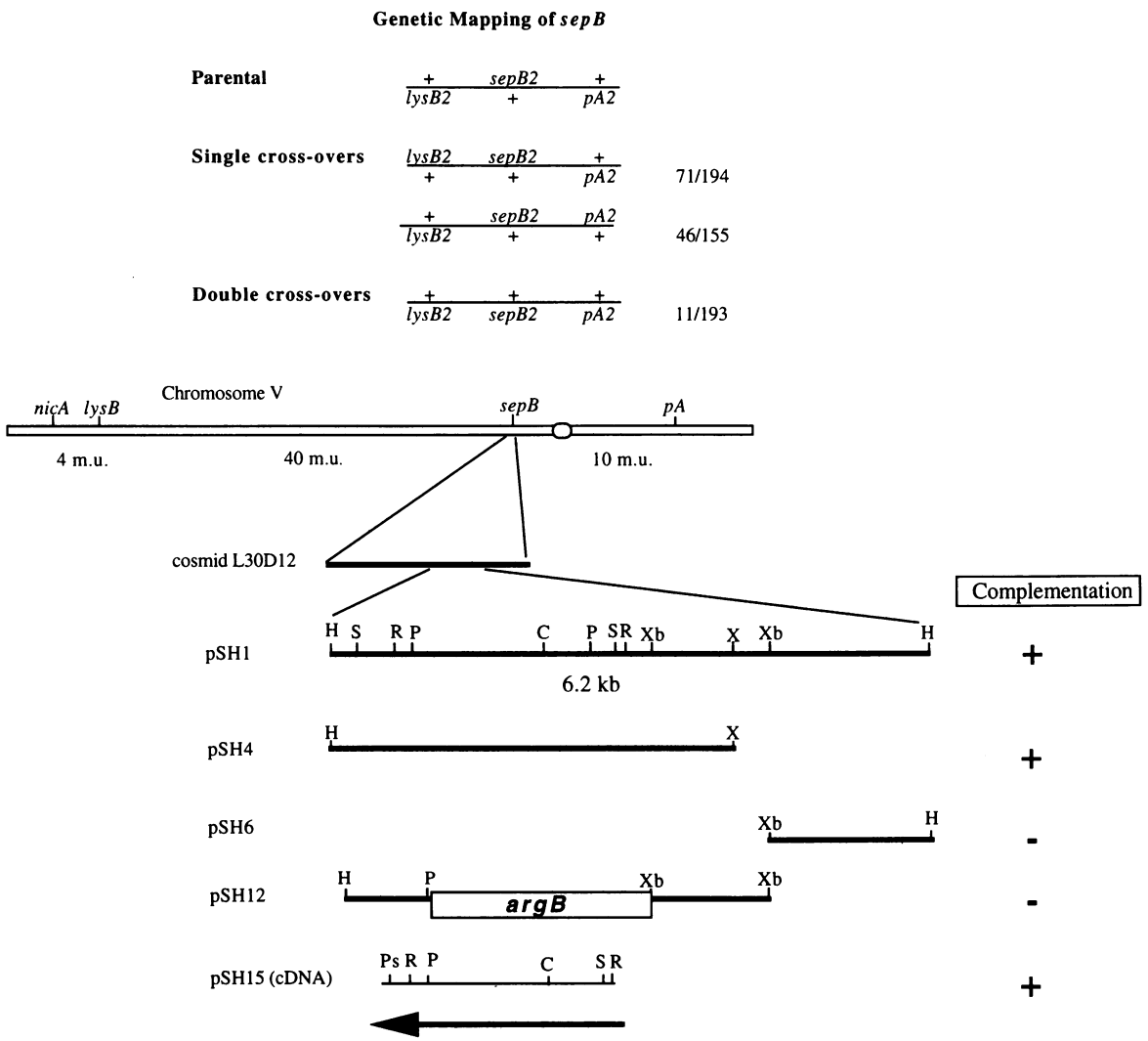

Fig. 1. Genetic and molecular analysis of the *sepB* gene. Genetic data were generated from the cross ASH15×A495. Genetic nomenclature and map distances for intervals *nicA–lysB* , *lysB*–centromere and centromere–*pA* are from Clutterbuck (1987). For certain progeny the 'pale conidia' phenotype could not be scored due to poor sporulation. The isolation of cosmid L30D12, the construction of sub-clones, and the isolation of the *sepB* cDNA are described in Materials and methods. A positive complementation result indicates restoration of growth at 42°C, the ability to make septa, and a normal nuclear morphology. C, *Cla*I; H, *Hin*dIII; P, *Pvu*II; Ps, *Pst*I; R, *Eco*RI; X, *Xho*I; Xb, *Xba*I; S, *Sal*I.

The inability to recover, from two independent transformants, haploid Arg^+ segregants harboring the gene replacement further demonstrates that the *sepB* gene is essential.

sepB exhibits sequence similarity to the yeast CTF4 gene

The complete *sepB* cDNA was sequenced on both strands (see Materials and methods). Analysis of the sequence revealed the presence of a single open reading frame (ORF) capable of encoding a 710 amino acid protein with an estimated molecular weight of 80 kDa and an estimated pI of 5.0. The N-terminal region of the putative *sepB* protein contains a single consensus WD-40 repeat and an additional near-consensus repeat (Figure 2A and C). One or more copies of this repeat are found in a variety of proteins involved in cell regulatory events (Neer *et al.*, 1994).

Searches of the available databases revealed that the predicted *sepB* protein possesses significant similarity to the *CTF4* gene from *S.cerevisiae*. The region of relatedness is distributed uniformly across the *sepB* protein and is at the level of 29% identity and 50% similarity (Figure 2B). The *S.cerevisiae CTF4* gene product is believed to play a role in monitoring chromosomal DNA metabolism (Kouprina *et al.*, 1992; Miles and Formosa, 1992b). Mutations in *CTF4* cause increases in chromosome loss, primarily due to defective DNA replication. However, in contrast to *sepB*, the *CTF4* gene product is dispensable and plays no apparent role in cytokinesis.

Despite the high overall similarity, there are significant amino acid sequence motifs that distinguish *CTF4* from *sepB* (Figure 2C). In particular, the N-terminus of the predicted *CTF4* protein possesses three potential zinc-finger motifs which may define DNA-binding domains (Kouprina *et al.*, 1992). In addition, the C-terminal region possesses a helix–loop–helix domain that is essential for *CTF4* function. No sequence matching the consensus for either the zinc-finger motif or the helix–loop–helix domain is present in *sepB* (Figure 2A and C). Moreover, the *CTF4* gene does not contain the WD-40 repeat that is found in *sepB*. These differences suggest that although the *CTF4*

A

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mgaedgtvwqyevksgrmdklltrtalavrdiaitkdngwvavasdeltvklvniedmtk    60
vkymreqtkgtkhitfdpngryvavsctdgivylysmdteepelarkldgvirrlepede   120
atarvvwhpdgtafatadasrdialfsvgewkkemsfsgghngditamswspngalmvta   180
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kfhciilkggdrypyfprpmlsefdfqipisdkpkpqeedeegstarndsmrleesfvrg   660
nlllslfqdllsathatatqraelsrkeleldkvllqllaiecregedga             710
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B

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sepB   1 mgaedgtvwqyevksgrmdklltrtalavrdiaitkdngwvavasdeltv 50
CTF4  70 mttmqgdalrynidssq.eellarfalplrdccvihsgkmavfegddlel 118
sepB  51 klvniedmtkvkymreqtkgtkhitfdpngryvavsctdgivylysmdte 100
CTF4 119 illelddethkkhaikideqvsqisynsqmnilavsmingkvqifsltst 168
sepB 101 epe.....larkldgvirrlepedeata..... 123
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CTF4 219 nvadpefcaanrictrvawhpkglhfalpcaddtvkifsikgyslqktls 268
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CTF4 269 tnlsstkahfidlqfdplrgtyiaavdlnnkltvwnwetseihytrefkr 318
sepB 204 nvinlaw....hptkngvslttsdgeififdgfvpkdyqallqkplqaap 249
CTF4 319 kitniawkiqadsktldlvlgtwsgsiaivqnlaesvvsnipdqsvaess 368
sepB 250 ifpgalteisdnv.....qrplasrpkea.....lrrgsid 280
CTF4 369 tkhglfvdsesdlenlegnddinksdklfsditqeanaedvftqthdgps 418
sepB 281 slddilgyd.qdmedfvedddgagyvedvngfgkrtnkhlgdieghmdkr 329
CTF4 419 glsekrkynfedeedfidddd gagyis.....gkkphnehsysrvhkths 463
sepB 330 tltsfpkpkihp..plqpgstpwr.gnrrylclnltgavwtvdqethntv 376
CTF4 464 fpislantgkfrympfspagtpfgftdrryltmnevgyvstvknseqysi 513

sepB 377 tvefydrelhrdfhftdpflydraclnengalfsnnpvddspatifyrph 426
CTF4 514 tvsffdvgrfreyhfedlfgydlcflnekgtlfgqsktgq....iqyrph 559
sepB 427 etwttradwkttlpkgehiralalsdsyivavttkdyyrvytlygtpfrv 476
CTF4 560 dsihsnwtkiiplqageritsvaatpvrvivgtslgyfrsfnqfgvpfav 609
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CTF4 610 ektspivaltaqnyrvfsvhysqfhglsyslselgtsskryykrecplpm 659
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CTF4 660 slpninsdmkkdanldyynfnpmgikslffssygdpcifgsdntllllsk 709
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CTF4 760 khiwpefplplpsemeirmpvfvksklleenkailnkkneigadteaeeg 809
sepB 640 deegstarndsmrleesfvrgnlllslfqdllsathatatqrae.lsrke 688
CTF4 810 eedkeiqipvsmaaeeeylrskvlselltdtlendgemygnenevlaaln 859
sepB 689 leldkvllqllaiecregedg 709
CTF4 860 gaydkallrlfasacsdqnve 880
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C

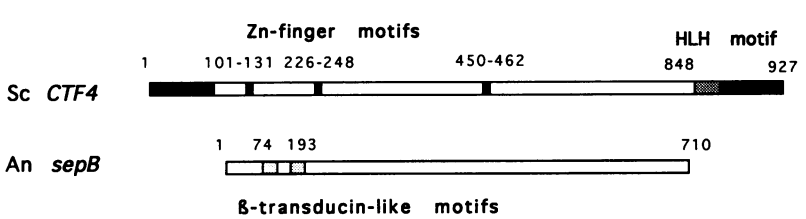

Fig. 2. *sepB* exhibits relatedness to the *CTF4* gene from *S.cerevisiae*. (**A**) The predicted amino acid sequence of the *sepB* gene. The Genbank-EMBL accession number for the DNA sequence is X86399. The single consensus (residues 158–193) and the near-consensus (residues 74–99) WD-40 repeats are indicated in bold-type. These repeats were identified using the Motifs program supplied with the GCG package and by visual inspection. (**B**) Homology between *sepB* and *CTF4*. The alignment was performed using the Bestfit program supplied with the GCG package. Identical amino acids are marked with an asterisk. Similar amino acids are marked with a dot. (**C**) A schematic comparison of the predicted *sepB* and *CTF4* gene products. The white area indicates the shared region of overall similarity. Stippled boxes indicated approximate positions of various motifs. In *CTF4* these are three Zn-finger motifs and an HLH motif. In *sepB* there are two β-transducin-like motifs. The black areas are unique to *CTF4*.

and *sepB* gene products may share a common evolutionary origin, they are unlikely to play identical roles in chromosomal DNA metabolism and cell division.

sepB3 mutants exhibit an M-phase delay and aberrant nuclei

The *sepB* mutants demonstrate a strikingly uniform arrest phenotype when examined after overnight incubation at 42°C (Figure 3A–C). The cells lack septa and arrest with 8–16 nuclei that possess an aberrant morphology (Figure 3C). In wild-type strains stained with DAPI, mitotic nuclei strain brightly and appear compact due to the condensed state of the chromatin. Interphase nuclei appear ellipsoid and contain a darker, poorly staining nucleolar region encompassed by a brighter chromatin-containing region (Figure 3D). In *sepB* mutants, interphase nuclei contain chromatin-staining regions that appear to be elongated (Figure 3E), in some cases extending a considerable length of the germ tube and occasionally even traversing a branch site. The nucleolar region appears normal. This type of nuclear morphology was apparent by both DAPI and Hoechst 33285 staining, and was rescued by introduction of the wild-type *sepB* gene.

The aberrant nuclear phenotype could be caused by a number of defects including failure to assemble a mitotic spindle, to partition chromosomes, or to complete some unknown aspects of karyokinesis. Immunofluorescence microscopy using anti-α-tubulin antibodies showed that while an asynchronous population of *sepB3* germlings grown at 42°C contained aberrant nuclei, they possessed typical interphase microtubule arrays and the spindle mitotic index was not elevated (data not shown). Staining with anti-γ-tubulin antibodies revealed that while *sepB3* germlings shifted from 30°C to 42°C and incubated for 3 h, had aberrantly shaped nuclei and failed to form septa, their interphase nuclei possessed a single spindle pole body (SPB) similar to cells held at 30°C. Thus *sepB* mutants do not appear to possess extra SPBs. However, we observed that *sepB3* germlings incubated at 42°C possessed slightly larger SPBs (Figure 3E and G) than cells held at 30°C (Figure 3D and F). This phenotype is suggestive of mis-scheduled enlargement or duplication (without separation) of the SPB. We surmise that the nuclear morphology changes that accompany imposition of the restrictive temperature in *sepB* mutants may affect nuclear-associated structures, such as the SPB.

To define more precisely the nature of the defect in *sepB* mutants we examined the early rounds of nuclear division at the restrictive temperature. Blocks to the nuclear division cycle in *A.nidulans* can be detected as an increase in the chromosome mitotic index [CMI (Oakley and Osmani, 1993)]. Conidia from a wild-type diploid and a *sepB* homozygous diploid were allowed to germinate on coverslips at 42°C. The progression of nuclear division and septum formation was monitored by staining with Hoechst 33258 and Calcofluor. As expected, wild-type germlings underwent nuclear division at intervals of ~80 min, and exhibited a CMI that ranged from 1 to 5% (Figure 4A and B). In *sepB* germlings, nuclear division occurred at intervals of ~100 min (Figure 4A). Furthermore, we noted a transient increase in the CMI just before each nuclear division in the *sepB* germlings (Figure 4B). Since nuclear division was still completed, this observation suggests that *sepB* mutants are transiently delayed in passage through mitosis. In contrast, typical mitotic mutants of *A.nidulans* maintain a high CMI (>50%) and fail to complete a single nuclear division (Morris, 1976). These results indicate that the effects of the *sepB3* mutation are detectable in the early nuclear divisions that accompany spore germination; however, this defect is subtle and fails to induce a mitotic arrest until the time of septum formation (Harris *et al.*, 1994).

Accompanying the M-phase delay, we noted that nuclei with aberrant morphology were apparent following the completion of the first nuclear division in *sepB* germlings at 42°C. Morphological alterations became increasingly obvious at the four-nuclei stage (Figure 4C and D). Despite these alterations in morphology, we were clearly able to distinguish interphase (decondensed) nuclei from mitotic (condensed) nuclei in the *sepB* mutant (germlings 2 and 3 versus germling 1, Figure 4C). As nuclear division progressed to the third division, nuclear morphology became even more aberrant (see Figure 3B). Cells contained elongated strands of chromatin stretched between interphase nuclei. The results from this and other temperature-shift experiments suggest that alterations in nuclear morphology rapidly accompany the loss of *sepB* function, and appear to be restricted to late anaphase or interphase nuclei.

sepB function is required for chromosome segregation

We previously noted that the aberrant nuclear morphology exhibited by the *sepB3* mutant bears a striking resemblance to that observed in *A.nidulans* aneuploids (Harris *et al.*, 1994: Upshall and Mortimore, 1984). This observation, and the observed similarity to the yeast *CTF4* gene, led us to employ a colony sectoring assay to test directly whether mutations in *sepB* result in defects in chromosome segregation.

A.nidulans forms stable diploids that can be returned to the haploid state by inducing aneuploidy. Aneuploids undergo chromosome loss in a random manner to produce stable haploid cells. To test the role of *sepB* in chromosome segregation, a diploid strain (ASH63) homozygous for the *sepB3* mutation and heterozygous for a number of easily scorable recessive visual markers was constructed. In this strain, chromosome loss events are detected by the appearance of sectored colonies expressing the recessive markers (*yA* = yellow conidia, *wA* = white conidia, and *argB* = auxotrophic growth and poor conidiation). Wild-type diploids containing the same heterozygous markers are very stable (Figure 5) and require treatment with microtubule depolymerizing agents such as benomyl to induce sectoring (Hastie, 1970). We asked if the *sepB3* mutation alone was able to cause sectoring, thus providing evidence that the *sepB* gene product functions in chromosome segregation.

Conidia from strains ASH63 (*sepB3/sepB3*), ASH64 (*sepB3/+*) and a control strain, A852 (+/+) were patched onto CM plates and incubated at semi-permissive temperature (37°C) for 48 h, after which they were shifted to permissive temperature for 72 h to allow colony formation. The incubation at semi-permissive temperature presumably resulted in a partial disabling of *sepB* function without completely preventing cell growth. Only colonies produced

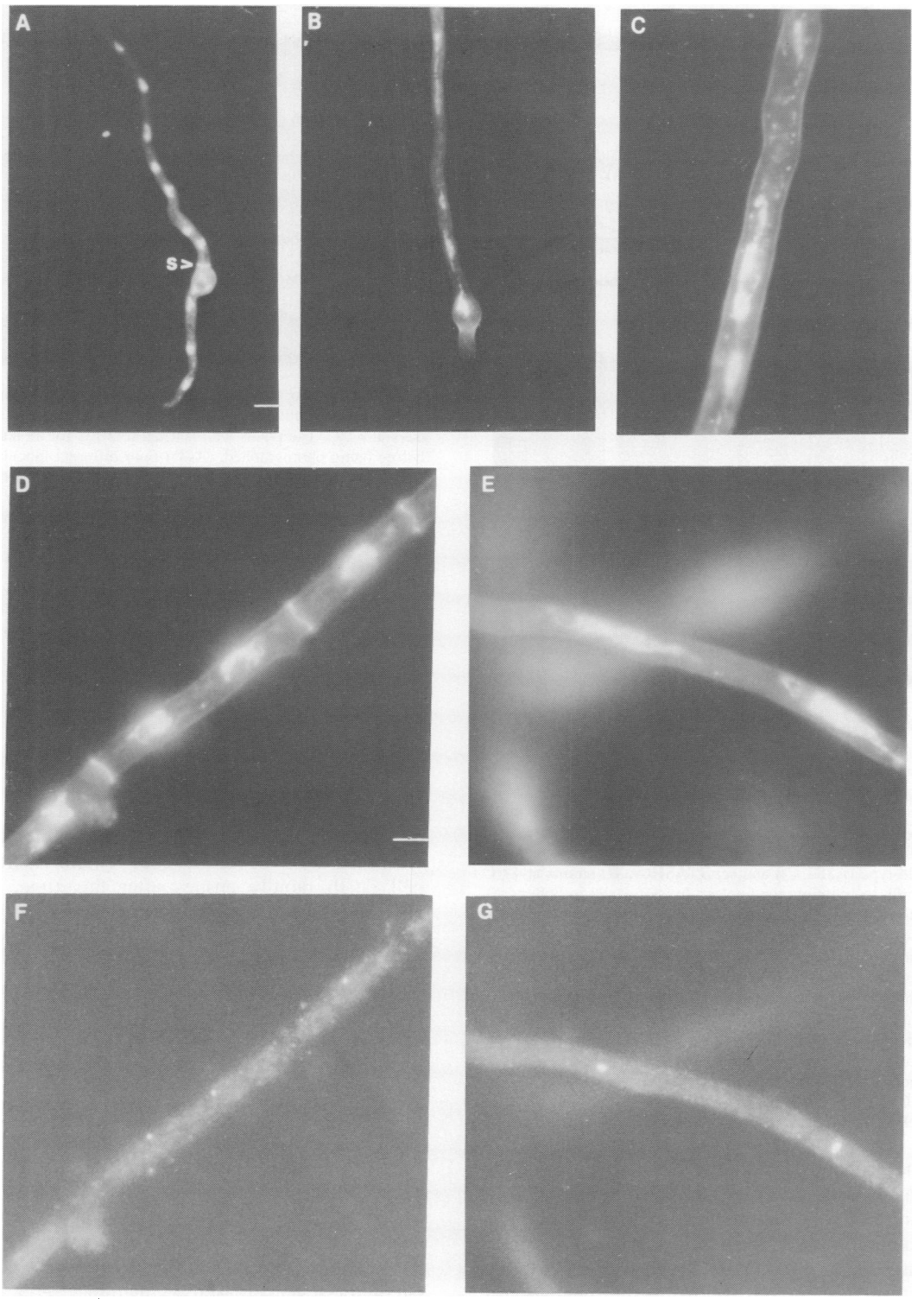

Fig. 3. *sepB* mutants possess an aberrant nuclear morphology and enlarged SPBs. (**A**) ASH60 germlings were incubated at 30°C and stained with Calcofluor and DAPI to visualize septa (s) and nuclei, respectively. The nuclei exhibit a characteristic interphase appearance. (**B** and **C**) ASH60 germlings were incubated at 42°C and stained with Calcofluor and DAPI. Note the absence of septa (B) and the aberrant nuclear morphology (C). (**D** and **F**) ASH63 germlings were incubated for 12 h at 30°C and stained with Calcofluor, Hoechst 33258, and a rabbit anti-γ-tubulin polyclonal antibody to visualize SPBs. A typical hyphal element is shown containing interphase nuclei and delimited by septa (D). Each interphase nucleus possesses a single SPB (F). (**E** and **G**) ASH63 germlings incubated for 9 h at 30°C were shifted to 42°C for 3 h and stained with Calcofluor, Hoechst 33258, and a rabbit anti-γ-tubulin polyclonal antibody. Typical germlings lack septa and the nuclei exhibit an aberrant morphology (E) along with enlarged SPBs (G). Scale bars in panels (A) and (D) represent 2 μm.

by strain ASH63 were sectored (Figure 5). This demonstrates that perturbation of *sepB* function is sufficient to induce chromosome loss.

In a separate experiment, conidia from strain ASH72/16 were diluted, spread onto CM plates and incubated at 37°C for 12 h followed by a shift to 30°C and continued

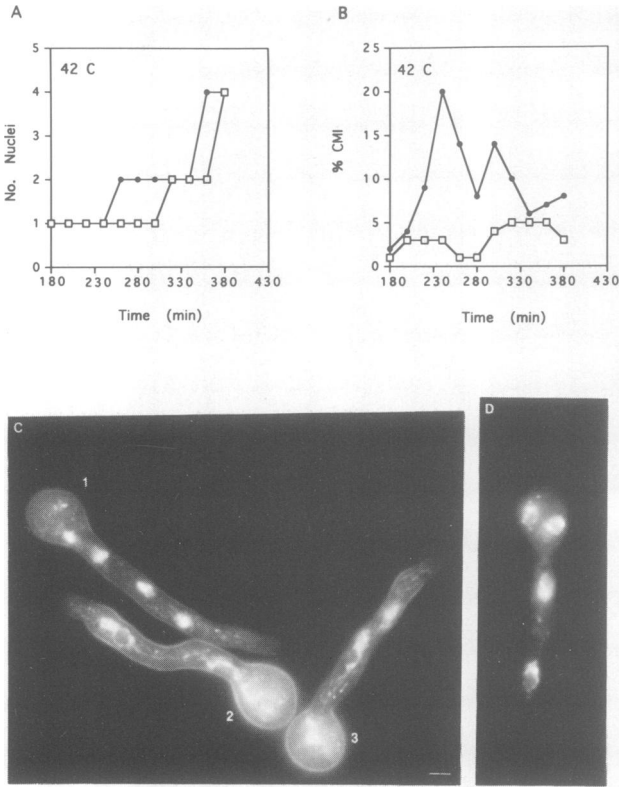

Fig. 4. *sepB* mutants are transiently delayed but not blocked in mitosis. Kinetics of nuclear division and cellular mitotic indices (CMI) during germination at 42°C in strains A852 (*sepB+*) and ASH63 (*sepB3*). Diploid strains were used because their larger size allowed more accurate determinations of CMI and nuclear counts. Nuclear counts and CMI determinations were monitored by staining with Hoechst 33258 as outlined in Materials and methods. (**A**) Nuclear division in wild-type (A852, □) and *sepB* (ASH63, ●) strains at 42°C. It takes ~100 min to complete the nuclear division cycle in both strains. The offsetting of the two curves is due to the longer period of time required to break dormancy in A852. (**B**) CMI in wild-type (A852, □) and *sepB* (ASH63, ●) strains at 42°C. ASH63 exhibits a delay in passage through mitosis based on the transient increase in the CMI that is observed preceding each nuclear doubling. (**C**) Representative germlings from strain ASH63 grown at 42°C and stained with Hoechst 33258 to visualize nuclei. Germling 1 possesses four chromatin masses identifying two nuclei in late anaphase. Germlings 2 and 3 possess interphase nuclei with aberrant morphology. (**D**) Representative wild-type germling with four interphase nuclei. Bar = 3 μm.

growth. Since colonies formed by *A.nidulans* aneuploids have a characteristic morphology (Kafer and Upshall, 1973), the number of abnormal colonies on these plates could be compared with the number on control plates incubated at 30°C. Approximately 55% (315/572) of the colonies appeared aneuploid following the incubation at 37°C, a 19-fold increase compared with the numbers observed following the control incubation [2.9% (18/607)]. Furthermore, since ASH72/16 contains recessive markers (*yA* and *pyrG*) on each arm of chromosome I, we examine whether mitotic recombination or non-disjunction had occurred. Conidia from 17 yellow sectors were purified and tested for the presence or absence of the *pyrG* marker. We found that 8/17 were Pyr^- and had thus lost a copy of chromosome I, while the remainder had undergone mitotic recombination and were still Pyr^+. We conclude that yellow sectors arose by both non-disjunction and mitotic recombination.

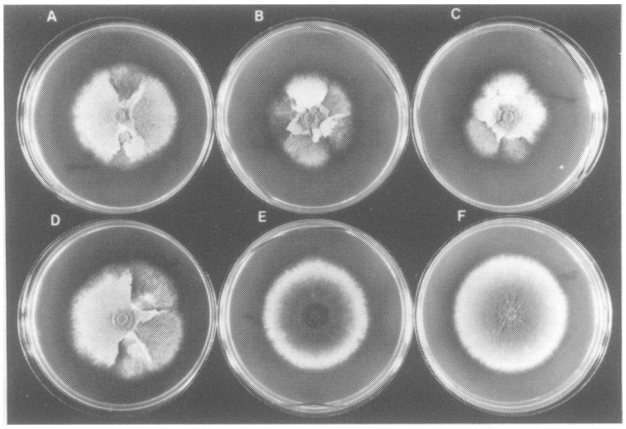

Fig. 5. The *sepB3* mutation causes defects in mitotic chromosome segregation. Conidia were patched onto CM plates and incubated for 2 days at 37°C. The plates were shifted to 30°C for an additional 3 days before being photographed. (**A–D**) Four different inoculations of strain ASH63 (*sepB3/sepB3*). (**E**) A852 (wild-type). (**F**) ASH64 (*sepB3/+*). Chromosome loss is indicated by colony sectoring. The expression of different recessive conidial color markers results in differential shading of the colonies.

Inviability and growth arrest in sepB mutants

During various temperature-shift experiments we noted that the viability of strains carrying *sepB* mutations decreased (as measured by colony forming units). To characterize the onset of cell death in *sepB* mutants at restrictive temperatures we incubated conidia from a strain containing the *sepB3* mutation on plates at non-permissive temperature for various lengths of time and then shifted the plates to 30°C (see Materials and methods). Figure 6A shows that cell viability does not decrease linearly with time at 42°C. Rather, cells remain viable for 8 h and cell death rapidly ensues after this time. Microscopic analysis showed that the majority of cells after 8 h at 42°C have 8–16 nuclei (data not shown). We conclude that *sepB3* is a temperature-sensitive lethal mutation. A failure to perform *sepB* function limits cells to three to four nuclear divisions, after which cell growth, nuclear division and cytokinesis cease and cell death occurs. During these experiments we observed an increasing proportion of aneuploid colonies ($n+1$) with increasing incubation times at 42°C (Figure 6B). The number of aneuploid colonies increased from 0.2% at the earliest time points (n = 590) to 32% (n = 22) at the later time points. This result is consistent with our earlier findings that the *sepB3* mutation causes defects in chromosome segregation.

Growth arrest and cell death in *sepB3* mutants could occur for several reasons. Functional *sepB* gene product accumulated during sporulation at permissive temperature could decay slowly at 42°C. Alternatively, cells may arrest growth and die due to a failure to execute a critical cell cycle-dependent step. Because we could not visualize germinating spores carrying null mutations in *sepB*, we performed a cell cycle block and release on *sepB3* germlings at restrictive temperature (Table I). Strain ASH13 was germinated in the presence of hydroxyurea (HU) at 42°C for 6 h. Arrested spores (42 + HU) fail to undergo nuclear division while spores germinated in the absence of HU for 6 h underwent one to two nuclear divisions. Control cells germinated at 30°C in the presence of HU

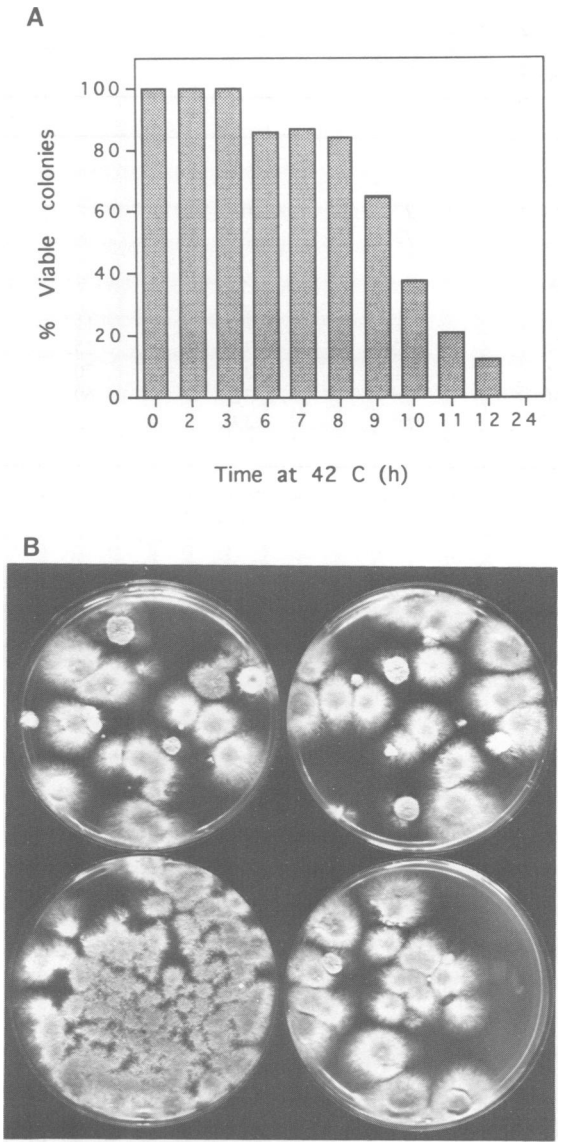

Fig. 6. The onset of lethality in *sepB3* mutants. (**A**) Conidia from strain ASH60 were diluted and plated for single colonies on CM plates. Plates were incubated at 42°C for the indicated time period and then shifted to 30°C. The zero time point plate was held at 30°C. Viability remains high until 8 h at 42°C. At 8 h the vast majority of cells have completed three mitotic divisions. An increase in the production of aneuploid colonies coincided with the onset of lethality. (**B**) Examples of the colonies produced by *sepB* mutants after 10 h at 42°C followed by a return to 30°C. The plate at bottom left is from the zero time point.

(30 + HU) also remained blocked. Cells germinated at 42°C in the presence of HU were washed free of HU and transferred to fresh media lacking HU and allowed to continue growth at 42°C for a further 5 h (total 11 h). Control cells germinated at 30°C in presence of HU were also washed free of the drug and allowed to continue growth at 30°C. If the Ts growth arrest in ASH13 is induced by a time-dependent decay of the *sepB* gene product, we expect that a significant fraction of the cells would fail to continue nuclear divisions when released from the HU block at restrictive temperature. However, Table I shows that following a release from the cell cycle block at 42°C, the vast majority of *sepB3* cells continue to undergo nuclear division at restrictive temperature. We conclude that passage through several nuclear division cycles is required for *sepB3*-induced arrest and that *sepB3* germlings arrest as a result of failing to perform a critical *sepB*-dependent step.

sepB functions at an early step in cytokinesis

We used temperature-shift experiments in an attempt to define the block in *sepB3* mutants and the thermosensitive period for *sepB* function. Earlier results from shift-down experiments led us to propose that the *sepB3* mutant is defective at an early step in septum formation (Harris *et al.*, 1994). We repeated these experiments except that germlings were now monitored at 2 h intervals for a period of 8 h following a shift down from 42°C to 30°C. During this period, only 10–20% of the observed cells were able to make septa and the remaining 80–90% were inviable (Table II). In the viable fraction of cells, nuclear division resumed and nuclear morphology returned to normal; however, septum formation did not occur until 6 h after the shift-down. In addition, the majority of these cells (16/22) appeared to make septa solely in regions of new growth (new germ tubes and branches) produced after the shift-down. We conclude that cells recovering from the *sepB3* block, must undergo new rounds of nuclear division and growth before being competent to form septa.

To determine the thermosensitive period for *sepB* function, strain ASH13 was grown at permissive temperature (30°C) and shifted to restrictive temperature (42°C) at hourly intervals during the first three nuclear divisions (Figure 7). Following a subsequent period of growth at restrictive temperature (8 h), we measured the extent of septum formation. In this analysis we found no change in the fraction of cells able to undergo septation following shifts to restrictive temperature before the 6 h time point. Thus, <5% of the *sepB3* cells are able to form septa when grown at restrictive temperature or when shifted to restrictive temperature after 6 h of growth at permissive temperature. When a majority of *sepB3* cells had traversed the first nuclear division at permissive temperature (8 h at 30°C) there was no dramatic change in the ability of cells to septate at restrictive temperature. As cells proceeded to complete the second and third nuclear divisions (9, 10 and 11 h time points at 30°C) a significant fraction of the cells (>40%) were able to form septa following the shift to restrictive temperature. This occurred despite the fact that *sepB3* mutants lose viability at restrictive temperature. These results could only be obtained if the critical *sepB* function had been completed before the shift to restrictive temperature. In wild-type cells, or *sepB3* mutants held at permissive temperature, the first septum is not made until cells possess at least eight nuclei (Harris *et al.*, 1994). The thermosensitive period for *sepB* function appears to occur before this time (approximately the four-nuclei stage). We conclude that the *sepB3* gene functions after the completion of the first nuclear division, but before the onset of septation. Completion of this function permits the execution of cytokinesis at the restrictive temperature.

sepB is required for cytokinesis during asexual development

Whereas vegetative hyphae undergo cell division by septation events that constrict the protoplasm, a mode of cytokinesis reminiscent of budding is employed during

Table I. Nuclear division block and release in *sepB3* mutants at 42°C

Time (h)			%GT[b]	Percentage of cells with *n* nuclei[a]			
				n = 1	*n* = 2	*n* = 4–8	*n* = 9–16
6	42 + HU	expt 1	11	100			
		expt 2	27	95	5		
	42	expt 1	51	13	77	10	
		expt 2	76	2	72	26	
	30 + HU	expt 1	0	100			
		expt 2	0	100			
11	42 – HU	expt 1	94	0	11	78	11
		expt 2	97	0	12	73	15
	42	expt 1	100	0	1	29	70
		expt 2	100	0		31	69
	30 – HU	expt 1	48	5	56	39	
		expt 2	64	1	45	54	

[a]200 cells were counted for each time point, under each condition, and the cells were sorted into categories depending on the numbers of nuclei they contained. Nuclei were observed by DAPI staining. Data from two experimental trials are given. A third trial produced similar results.
[b]Percentage of cells containing viable germ tubes.

asexual spore formation (conidiation) (Timberlake, 1991). While we anticipate that many gene products will be required for septum formation during both patterns of cell division, it is possible that some gene products will be uniquely required for one mode. Moreover, during conidiation uninucleate cell divisions accompany morphogenesis, suggesting a tighter coupling of cytokinesis and mitosis (Mirabito and Osmani, 1994). Thus, we were interested in assessing the role that the *sepB* gene plays during conidiation. A vegetative culture of strain ASH5 was synchronously induced to conidiate as described in Materials and methods. Following induction, plates were shifted from 30°C to 42°C at 2 h intervals, thereby inactivating *sepB* at different times during conidiation. Conidial yields were calculated following 48 h at restrictive temperature. Table III shows that early shifts (2 h) to restrictive temperature completely abolished conidial yields, while later shifts (4 h and 6 h) caused dramatic reductions in spore forming ability (~6500- to 3500-fold). At later times, inactivation of sepB still resulted in a significant decrease (~80-fold) in conidial yields. These results demonstrate a requirement for the *sepB* gene during conidiation and suggest that imposition of the restrictive temperature brings about a rapid arrest to cell division progression and morphogenesis.

Conidiophores produced following inactivation of *sepB3* undergo an abrupt growth arrest. Figure 8 shows a conidiophore from strain ASH5 shifted to 42°C during the formation of the metula and phailide cells (6 h post-induction). For comparison, a diagram of wild-type morphogenesis events associated with conidiation is shown above the micrograph. In wild-type strains metula cells bud from the surface of the apical vesicle and contain a single nucleus. The sporogenous phailide cells bud from the apical tip of the metula cells during division of the metula nucleus and a daughter nucleus migrates into the phailide cell. The phailide cells give rise to chains of asexual spores (Timberlake, 1991). In *sepB3* mutants shifted after 6 h at permissive temperature, phailide cell division is halted and the metula cells contained predominantly single nuclei. Furthermore, these cells were found to be elongated when compared with wild-type (12–15 mm for *sepB3* versus 5–5.5 mm for wild-type; see Clutterbuck, 1969). Most strikingly, we noted that multinucleate and binucleate metula cells were uncommon, and nuclei were not aberrantly shaped. Thus, imposition of restrictive temperature in these uninuclear cell divisions appears to cause an immediate cessation of nuclear division and also produces elongated cells suggestive of blocks to cytokinesis. We also observed abnormal patches of delocalized Calcofluor staining at junctions between presumptive phailides and conidia. This developmental phenotype contrasts with the hyphal phenotype observed in *sepB3* germlings grown at restrictive temperature. We propose that the altered *sepB3* mutant phenotype arises as a consequence of the uninuclear cell divisions that accompany sporulation. This phenotype is consistent with our earlier findings that a failure to execute the *sepB* step arrests further progression of the cell cycle.

Table II. Septum formation in *sepB* mutants following a 42–30°C shift[a]

Time at 42°C (h)	Time at 30°C (h)	No. of septa			% germlings with septa
		n	0	1	
12	0	200	200	0	0
12	2	125	120	5	4
12	4	154	146	8	5
12	6	174	158	16	9
12	8	112	90	22	20
20	0	153	146	7	5

[a]Cells were grown at 42°C for times indicated and shifted to 30°C. At times indicated between 100 and 200 cells were counted and the number of cells with septa determined by Calcofluor staining.

Discussion

For cytokinesis to be successful, DNA replication must be completed and chromosomes faithfully transmitted to daughter cells. We have taken advantage of the behavior of germinating conidia of *A.nidulans* to identify genes that are involved in regulating the onset of cytokinesis (Harris *et al.*, 1994). In *sepB3* mutants at restrictive temperature, cells complete early rounds of nuclear division but arrest growth and subsequently die at a time

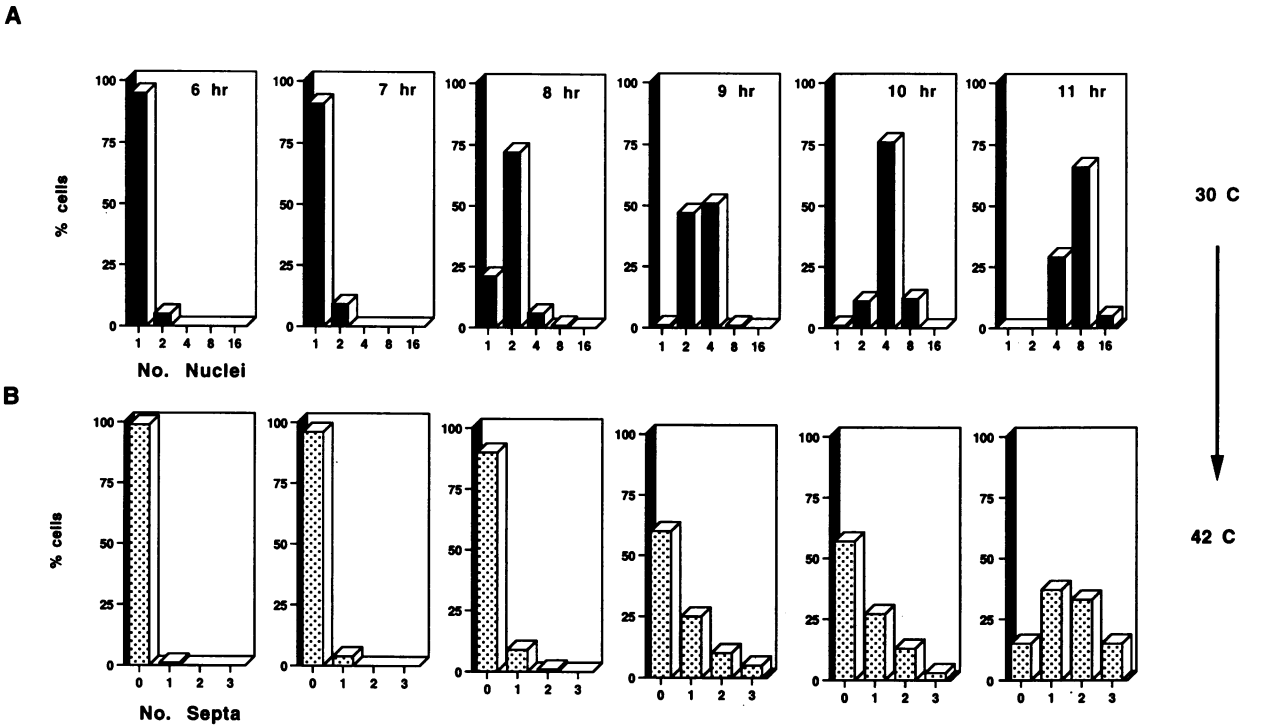

Fig. 7. Temperature shift-up experiments indicate an early requirement for the *sepB* gene in septum formation. Conidia from strain ASH13 were incubated on coverslips at 30°C. At the indicated time points, one coverslip was removed and processed for microscopy and a second one was shifted to 42°C. The second coverslip was incubated for 8 h at 42°C before being processed for microscopy. Coverslips were stained with DAPI and Calcofluor. (**A**) The percentage of cells with 1, 2, 4, 8 or 16 nuclei at the time of the shift to restrictive temperature. (**B**) The percentage of cells with 0, 1, 2 or 3 septa following an additional 8 h incubation at restrictive temperature. In a control culture held at 42°C for 18 h, 6.5% of germlings were able to make septa.

coincident with the onset of cytokinesis. The evidence presented here suggests that *sepB* performs an essential function early in the process of cytokinesis.

sepB function in nuclear division and the initiation of cytokinesis

A variety of genes have been shown to be involved in mitotic spindle assembly and chromosome segregation in *A.nidulans* (Morris and Enos, 1992). Mutations in all of these genes arrest mitosis and thus germinating spores are incapable of progressing through more than a single nuclear division under restrictive conditions. In contrast, the *sepB3* mutation permits cells to complete three to four rounds of nuclear division. During these nuclear divisions, there is a distinct but transient delay in M-phase. Interphase nuclei become aberrantly shaped and take on a characteristic aneuploid appearance. These observations suggest that *sepB3* mutants may accumulate defects in mitotic chromosome metabolism that result in increasingly aberrant nuclei. The similarity of *sepB* to the *S.cerevisiae CTF4* gene, as well as the instability of diploids homozygous for the *sepB3* mutation, suggests that these defects may be related to chromosome segregation. However, in contrast to mutations in tubulins, kinesins or other genes involved in chromosome segregation, the absence of *sepB* function is insufficient to arrest mitosis. Furthermore, we did not detect major defects in the mitotic spindle. Thus *sepB3* nuclei are capable of dividing and can thus accumulate segregation defects. Although we cannot rule out the accumulation of other kinds of chromosomal defects (i.e. unreplicated DNA), *sepB3* mutants are not hypersensitive to ultraviolet light or methyl methane sulfonate (S.Harris and J.Hamer, unpublished data).

Table III. Conidial yields during temperature shift-up experiments

Incubation conditions[a]	Conidial yield (per g dry weight)
42°C	0
30°C; 2 h → 42°C	0
30°C; 4 h → 42°C	1.3×10^5
30°C; 6 h → 42°C	2.4×10^6
30°C; 8 h → 42°C	1.1×10^7
30°C	8.5×10^8

[a]See Materials and methods.

The mitotic delay caused by the *sepB3* mutation may be due to the activation of a cell cycle checkpoint associated with DNA replication or spindle assembly. The checkpoint systems that monitor progression through the cell cycle in *A.nidulans* are not well characterized. A negative regulatory system, of which the *bimE* gene product is a component, acts to prevent entry into mitosis until DNA replication has been completed (Osmani *et al.*, 1988). A checkpoint governing the departure from mitosis has not been identified in *A.nidulans*, but the phenotypes exhibited by certain *bim* mutants (endoreplication) suggests that even if one does exist, it is capable of only temporarily halting the cell cycle (Enos and Morris, 1990; May *et al.*, 1992). Similarly, the checkpoint controls that respond to DNA damage are only able transiently to delay progression through the cell cycle (Denison and May, 1994). Thus, an M-phase checkpoint which is activated

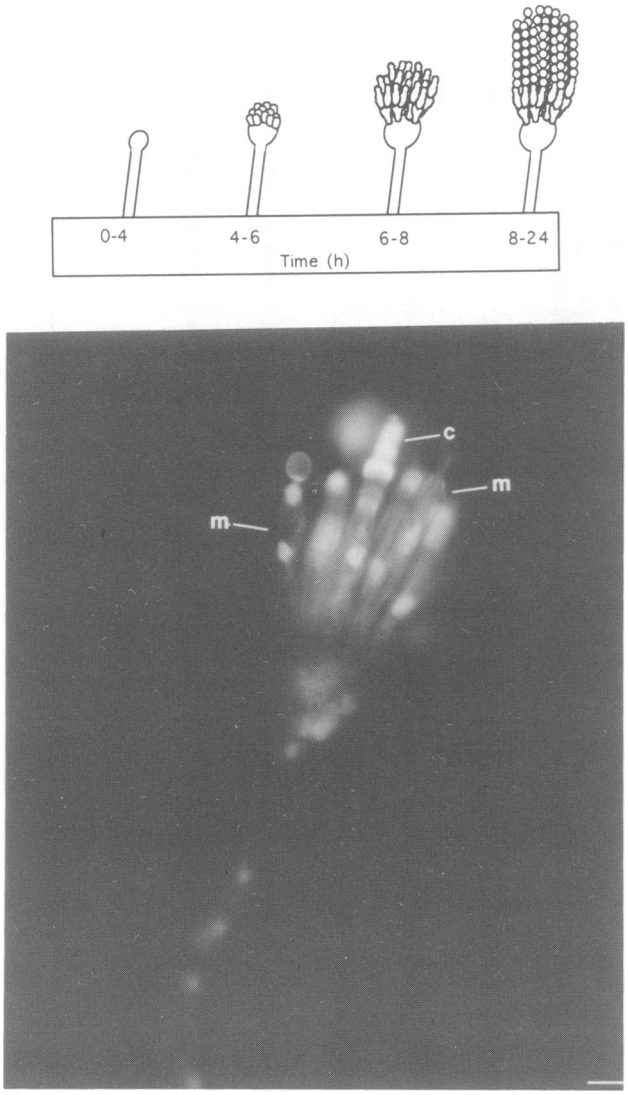

Fig. 8. *sepB* function is required for cell division during conidiophore development. Upper panel shows a diagrammatic representation of wild-type conidiation. Lower panel shows a photomicrograph of a *sepB3* conidiophore arrested at 42°C after 6 h of development at 30°C (see Materials and methods). Conidiophores were fixed and stained with Hoechst 33258 and Calcofluor. m, metula; c, abnormal patches of Calcofluor staining.

in response to the *sepB*-induced defect(s) may only temporarily slow passage through mitosis, resulting in the observed transient increases in the CMI.

One interpretation of the *sepB3* phenotype is that an accumulation of nuclear division defects results in a failure to initiate cytokinesis. Temperature shift-down experiments show that *sepB* mutants undergo cytokinesis in new regions of hyphal growth once nuclear division has resumed at permissive temperature. Presumably this is because properly segregated chromosomes (or undamaged nuclear divisions) are required to initiate cytokinesis. Temperature shift-up experiments demonstrate that the *sepB* gene product functions early in the process of cytokinesis. The completion of the first mitotic division at permissive temperature is insufficient to allow cytokinesis at the restrictive temperature. However, the completion of the second division (four-nuclei stage) permits cytokinesis at the restrictive temperature. One interpretation of this result is that more than one nuclear division at restrictive temperature is required to accumulate sufficient *sepB3*-induced damage to block cytokinesis. Alternatively, the *sepB* gene product may play a more direct role in initiating the onset of cytokinesis at a specific time following the completion of the first nuclear division.

Could accumulating chromosomal segregation defects (aneuploidy) block cytokinesis? In most eukaryotic cells aneuploidy does not block cytokinesis. For example, chromosomal segregation defects do not block septation in either *S.cerevisiae* or *S.pombe*. In fact, in *S.pombe* delays in anaphase generally produce a 'cut' phenotype as a result of cytokinesis occurring before chromosome segregation is complete (Hirano *et al.*, 1986). In addition, if accumulating chromosome segregation defects *per se* were the sole effect of the *sepB3* mutation, we might anticipate that at least a larger fraction of cells would be able to form septa at restrictive temperature. However, cells remain aseptate at restrictive temperature even though up to 20% of the cells can progress to the 16-nuclei stage or beyond (Harris *et al.*, 1994). Finally, during the uninucleate cell divisions associated with sporulation, the *sepB3* defect does not cause aberrantly shaped nuclei and does not appear to require an accumulation of defective cell divisions. Rather, cell division and morphogenesis appear to be abruptly halted.

Taken together, these results suggest two hypotheses concerning the function of *sepB*. First, the product of the *sepB* gene could have a specific role in monitoring or signaling the onset of cytokinesis. In this capacity the *sepB* gene product would participate in chromosome segregation, perhaps monitoring successful segregation events and eventually signaling the activation of a division site for septum formation. Alternatively, the absence of the *sepB* gene could trigger the formation of a particular kind of nuclear or DNA damage. This type of damage would not be monitored by the normal mitotic checkpoints, and thus could accumulate during the early nuclear divisions. Such *sepB* damage could also activate a novel checkpoint that would halt cytokinesis, and prevent damaged DNA or nuclei from being segregated to daughter cells. Further investigation of the *sepB* gene product and the nuclear morphology defect in *sepB* mutants may help to clarify its precise role.

DNA sequence analysis revealed that the *sepB* ORF has intriguing similarity to a *S.cerevisiae* gene, *CTF4*, which is involved in chromosomal DNA metabolism. Although the similarity between *sepB* and *CTF4* is dispersed throughout the ORFs, it does not include putative functional protein motifs. *sepB* lacks the essential DNA-binding and protein dimerization domains of *CTF4* (Kouprina *et al.*, 1992). Instead, the *sepB* ORF contains one or two WD-40 repeats. The prevalence of these repeats among proteins that perform a regulatory function (Neer *et al.*, 1994) suggests that *sepB* may possess a similar role for cytokinesis. The punctuated similarity of the *sepB* and *CTF4* genes in these divergent organisms (Taylor *et al.*, 1993) suggests that these genes may represent members of a family of cell cycle proteins that ensure the fidelity of chromosome replication during mitosis, perhaps by specific interactions with different multimeric complexes involved in replication and/or segregation. The cytokinetic

Table IV. *Aspergillus nidulans* strains

Strain	Genotype
ASH10	*sepB3 yA2*
ASH13	*sepB3 wA2*
ASH15	*sepB3 argB2 wA2*
ASH60	*sepB3 pabaA6 yA2*
ASH63	*sepB3 yA2 wA2 argB2 pabaA1 / sepB3 yA$^+$ wA$^+$ argB$^+$ pabaA$^+$*
ASH72/16	*sepB3 yA2 pabaA6 pyrG4 wA2 argB2 / sepB3 yA$^+$ pabaA$^+$ pyrG$^+$ wA$^+$ argB$^+$*
A495[a]	*lysB5 nicA2 pA2*
A850[a]	*biA1 ΔargB::trpCΔB methG1 trpC801*
A852[a]	*ΔargB::trpCΔB trpC801 biA1 pabaA1 yA2 methG1 / ΔargB::trpCΔB trpC801 biA$^+$ pabaA$^+$ yA$^+$ methG$^+$*

[a]Obtained from Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, KS 66160-7420.

defect in *sepB* mutants suggests that the *sepB* gene product has additional interactions with the cytokinetic machinery.

The sepB mutation reveals a novel arrest point for cell cycle progression

Two lines of evidence suggest that *sepB* mutants arrest growth and die in response to a specific block in cell cycle progression. First, *sepB* mutants arrest with a uniform cell morphology (Harris *et al.*, 1994; also this study). Second, cell death in *sepB3* mutants occurs only after incubations at non-permissive temperature beyond the time at which *sepB* gene function is required (four- to eight-nuclei stage). These results suggest that *sepB* mutants could die in response to an intolerable level of accumulated nuclear or DNA damage. However, we have been unable to detect direct evidence for DNA damage other than aneuploidy in *sepB3* mutants. Further, we suspect that aneuploidy is unlikely to cause an abrupt onset of lethality, especially in an organism with multinucleate cells. Alternatively, the *sepB3* mutation may reveal a specific commitment point in the growth of *A.nidulans*, namely a transition from unicellular to multicellular growth. A failure to initiate an early step in cytokinesis may bring about a halt to further nuclear division and growth.

An interdependency between early steps in cytokinesis and nuclear division as revealed by the *sepB3* mutation may be a unique feature of an organism such as *A.nidulans* that undergoes cytokinesis to produce multinucleate cells. However, *sepB* is also required for the uninucleate divisions associated with asexual development in *A.nidulans*. Loss of *sepB* function during sporulation blocks both nuclear division and cell division. Thus, the cytokinetic defects caused by a mutation similar to *sepB* may be less obvious in organisms that only grow in a uninucleate, unicellular state. *sepB* is one of four genes identified thus far in *A.nidulans* that cause a similar arrest phenotype (Harris *et al.*, 1994). Further studies of these genes may reveal how specific processes in nuclear division are monitored and coordinated with the early steps in cytokinesis.

Materials and methods

Strains, media, and growth conditions

All strains used in this study are listed in Table IV. Media used [complete media (CM), minimal media (MN) and yeast extract glucose media (YEG)] and general growth conditions were as described previously (Harris *et al.*, 1994). Cell cycle block and release experiments with hydroxyurea were performed on coverslip cultures as previously described (Harris *et al.*, 1994). The temperature shift-up experiments were initiated by growing strain ASH13 on coverslips at 30°C in YEG. At the indicated time points, two coverslips were removed and one was processed for microscopy. The second coverslip was transferred to fresh YEG prewarmed at 42°C and incubated at this temperature for 8 h before being processed for microscopy. Temperature shift-down experiments were initiated by growing ASH13 on coverslips at 42°C for 12 h. At this point, one coverslip was removed and processed for microscopy while the remainder were transferred to fresh YEG at 30°C. Over the subsequent 8 h period, coverslips were removed at 2 h intervals and processed for microscopy. The nuclear division time-course experiments were conducted by growing either strain ASH63 or A852 on coverslips at 42°C in YEG. Following an initial 3 h incubation, coverslips were removed at 20 min intervals over the next 5 h and processed for microscopy. Each experiment was repeated three times with essentially identical results. For all time points, 100–200 germlings were scored for the presence of septa and/or the number of nuclei that they contained. Data points in Figure 4A represent the modal average of the distribution of values for the corresponding time point. For example, if the majority of the germlings at a particular time point contained four nuclei, the data point was plotted at four. However, it should be noted that a minority fraction of the counted germlings at that time point could have contained two or eight nuclei.

Genetic and recombinant DNA manipulations

Methods for the genetic analysis of *A.nidulans* were as described previously (Kafer, 1977; Harris *et al.*, 1994).

All molecular biology procedures used have been described previously (Sambrook *et al.*, 1989). Standard procedures were employed for the isolation of RNA and DNA from *A.nidulans* mycelia and for the transformation of *A.nidulans* (Timberlake, 1980; Dobinson *et al.*, 1993; Oakley and Osmani, 1993).

Cloning of the sepB gene

Strain ASH15 was co-transformed with pSalArgB along with eight different pools of cosmid DNA from a chromosome V-specific cosmid library (Brody *et al.*, 1991). Transformants were selected on MN plates at 42°C, following a 16 h period of recovery at 28°C. Cosmids from a single pool were able to complement *sepB*. This pool was sub-divided and transformation experiments repeated until a single cosmid, L30D12, able to complement all aspects of the *sepB* phenotype was identified. The *sepB* complementing region on cosmid L30D12 was identified using the approach described by Timberlake *et al.* (1985). A single 6.2 kb *Hin*dIII fragment was to be able to complement the *sepB3* mutation.

Plasmid constructions

Plasmids pSH1 and pSH3 were constructed by sub-cloning the 6.2 kb *Hin*dIII fragment from cosmid L30D12 into the vectors pBC KS– and pBS KS– (Stratagene), respectively. Plasmid pSH4 was constructed by digesting pSH3 with *Xho*I and religating. Plasmid pSH7 was constructed by sub-cloning a 1.8 kb *Bam*HI fragment containing the *argB* gene from pSalArgB into *Bam*HI digested pSH4. Plasmid pSH17 was constructed by sub-cloning a 1.1 kb *Bam*HI fragment containing a truncated version of the *argB* gene from pSalArgB1.1 into *Bam*HI digested pSH4.

Plasmid pSH15 was constructed by sub-cloning a 2.0 kb *Eco*RI fragment derived from the *sepB* cDNA into pBS KS+. Plasmid pSH9 was constructed by sub-cloning a non-overlapping 0.5 kb *Eco*RI fragment derived from the cDNA into pBS KS–.

The *sepB* gene replacement vector, pSH12, was constructed in three steps. First, a 1.1 kb *Hin*dIII–*Pvu*II fragment from the 3′ flanking region of the *sepB* gene (this fragment contains the carboxy-terminal 393 bp of the predicted *sepB* open reading frame) was sub-cloned into pBS KS– to form pSH10. Next, a 1.2 kb *Xba*I fragment from the 5′ flanking region of the *sepB* gene was sub-cloned into pSH10 to form pSH11. Digestion with *Eco*RI and *Xho*I was used to confirm that the two fragments derived from the regions flanking *sepB* were correctly oriented with respect to each other in pSH11. Finally, the 1.8 kb *Bam*HI fragment containing the *argB* gene was sub-cloned into the unique *Bam*HI site of pSH11 to create pSH12.

Sequencing of the sepB gene

Both strands of the *sepB* cDNA were completely sequenced using the Sequenase Version 2.0 DNA Sequencing Kit (US Biochemical). Clones for sequencing were generated either by sub-cloning or by using exonuclease III to create nested sets of deletions (Henikoff, 1984). The sequence at sites used for sub-cloning was confirmed using overlapping fragments derived from genomic DNA. Gaps in the sequence were filled using oligonucleotide primers synthesized by the Laboratory for Macromolecular Structure, Purdue University. Analysis of the DNA sequence was performed using the Genetics Computer Group (GCG) of the University of Wisconsin (Devereux *et al.*, 1984) package provided by the AIDS Center Laboratory for Computational Biochemistry at Purdue University. The screening of DNA sequence databases was performed using the BLAST algorithm (Altschul *et al.*, 1990) at the NCBI or the GCG program FASTA. Amino acid alignments were made using the program BESTFIT.

Microscopy

Coverslips with adherent germlings were processed for microscopy and stained as described previously (Harris *et al.*, 1994). Chromosome mitotic indices were calculated as described by Oakley and Osmani (1993). Immunofluorescence microscopy was performed as described previously, except that the 10 min immersion in absolute methanol at –20°C was omitted. Primary antibodies used were the mouse anti-α-tubulin DM 1A monoclonal (Sigma Immunochemicals) at 1:500 and a rabbit anti-γ-tubulin polyclonal (kindly provided by Drs Katherine Jung and Berl Oakley, Ohio State University) at 1:200. Secondary antibodies used were FITC-conjugated anti-mouse and FITC-conjugated anti-rabbit (Sigma Immunochemicals) at 1:200.

Chromosome loss experiments

Conidia from strains ASH63, ASH64 and A852 were patched onto CM plates and incubated at 37°C (semi-permissive temperature for *sepB3* mutants). After 2 days, the plates incubated at 37°C were shifted to 28°C, and all plates were incubated for two further days before being photographed.

For quantitation of chromosome loss, conidia from strain ASH72/16 were diluted and spread for single colonies onto CM plates. The plates were incubated at 37°C for 12 h followed by a shift to 30°C. Control plates were incubated at 30°C for the duration of the experiment. *A.nidulans* aneuploids characteristically produce colonies that display abnormal morphologies and/or are sectored (Kafer and Upshall, 1973). At 3 days after plating, the number of abnormal and/or sectored colonies was determined and expressed as a fraction of the total number of colonies.

For the viability/chromosome loss experiment, conidia from strain ASH60 were diluted and plated for single colonies on CM plates. Plates were incubated at 42°C for the indicated time period and then shifted to 30°C. Control plates were incubated at 30°C.

Conidiation experiments

Conidia from strain ASH10 were inoculated into liquid CM and grown overnight at 30°C with shaking. Mycelia were harvested onto Whatman filter paper and washed with sterile distilled water. The mycelium was cut into six wedges and each wedge was carefully placed onto a CM plate. Four plates were incubated at 30°C for the indicated period of time before being shifted to 42°C. In addition, a single control plate was incubated at 42°C and another at 30°C. Plates were incubated for 2 days to allow conidiophores to develop and then each wedge was removed from its plate and placed in a Falcon tube. The mycelial weight was determined and 20 ml of 0.01% Tween 80 added. The tubes were vigorously vortexed to suspend conidia and the conidial yields determined by counting in a hemocytometer. Yields are expressed as the total number of conidia per g mycelia. The experiment was repeated three times with similar results and results from a single trial are given.

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