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The *Aspergillus nidulans sepA* gene encodes an FH1/2 protein involved in cytokinesis and the maintenance of cellular polarity

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Cytokinesis (septation) in the fungus *Aspergillus nidulans* occurs through the formation of a transient actin ring at the incipient division site. Temperature-sensitive mutations in the *sepA* gene prevent septation and cause defects in the maintenance of cellular polarity, without affecting growth and nuclear division. The *sepA* gene encodes a member of the growing family of FH1/2 proteins, which appear to have roles in morphogenesis and cytokinesis in organisms such as yeast and *Drosophila*. Results from temperature shift and immunofluorescence microscopy experiments strongly suggest that *sepA* function requires a preceding mitosis and that *sepA* acts prior to actin ring formation. Deletion mutants of *sepA* exhibit temperature-sensitive growth and severe delays in septation at the permissive temperature, indicating that expression of another gene may compensate for the loss of *sepA*. Conidiophores formed by *sepA* mutants exhibit abnormal branching of the stalk and vesicle. These results suggest that *sepA* interacts with the actin cytoskeleton to promote formation of the actin ring during cytokinesis and that *sepA* is also required for maintenance of cellular polarity during hyphal growth and asexual morphogenesis.
Keywords: *Aspergillus*/cellular polarity/cytokinesis/FH1/2 protein/septation

Introduction

Cytokinesis requires assembly of the division apparatus at a specified cortical site. In animal cells, a contractile ring forms at the division site and then acts to cleave the cell such that two daughter cells are produced (reviewed by Satterwhite and Pollard, 1992). Amongst the major components of the contractile ring are actin and myosin, whose interaction provides the mechanical forces necessary for contraction of the ring and cell cleavage (reviewed by Fishkind and Wang, 1995). Although signals emanating from the mitotic spindle are known to specify the location of the division site (Rappaport, 1986), the mechanisms which lead to organization of the actin cytoskeleton at the selected site remain poorly understood. Initial insights into the nature of these mechanisms may be drawn from the observation that small GTP binding proteins of the *rho* class are required for cytokinesis in a number of cell

types (Kishi *et al.*, 1993; Mabuchi *et al.*, 1993; Larochelle *et al.*, 1996). This class of GTPases (including *rho*, *rac* and *cdc42*) controls the re-organization of the actin cytoskeleton required for formation of cellular extensions in fibroblasts (Chant and Stowers, 1995; Ridley, 1996). Conceivably, activated *rho*-related GTPases may trigger recruitment of proteins required for nucleation and polymerization of actin filaments to the division site. Proteins such as the septins (Longtine *et al.*, 1996) and the actin-associated proteins anillin and radixin (Sato *et al.*, 1991; Field and Alberts, 1995) may then act in a downstream manner to control formation of the contractile ring.

The actin cytoskeleton also plays a critical role in cytokinesis in fungal cells. In particular, the division apparatus is thought to organize an actin ring at the incipient division site (Raudaskoski, 1970; Hoch and Howard, 1980; Kilmartin and Adams, 1984; Marks and Hyams, 1985). Presumably, the actin ring directs vesicle-mediated delivery of cell wall biosynthetic precursors to this site. Ultimately, this leads to cell division via the centripetal formation of a cross-wall, termed a septum. Genetic screens in both the fission yeast *Schizosaccharomyces pombe* and the budding yeast *Saccharomyces cerevisiae* have resulted in identification of a number of genes required for septum formation. In particular, six different genes have been shown to be required for assembly of the actin ring in *S.pombe* (Chang *et al.*, 1996). Two of these genes, *cdc3* and *cdc8*, encode the actin-associated proteins profilin and tropomyosin respectively (Balasubramanian *et al.*, 1992, 1994). A third gene, *cdc4*, may function in a calcium-dependent fashion to regulate actin ring formation (McCollum *et al.*, 1995). In *S.cerevisiae*, the septins (encoded by the *CDC3*, *CDC10*, *CDC11* and *CDC12* genes; reviewed by Longtine *et al.*, 1996) may play a role in the re-organization of the actin cytoskeleton which precedes septum formation (Adams and Pringle, 1984). Despite the successful application of genetic approaches to identify numerous gene products required for septum formation in yeast cells, it is not yet clear how the actin ring is assembled.

We have used a genetic approach to identify gene products required for septum formation in the filamentous fungus *Aspergillus nidulans*. Previous studies have demonstrated that a transient actin ring forms at the incipient division site in *A.nidulans* and that loss of functional microfilaments prevents septum formation (Harris *et al.*, 1994). Amongst a group of eight genes required for cell division in *A.nidulans*, four (*sepA*, *sepD*, *sepG* and *sepH*) appear to encode products which function late in septum formation (Harris *et al.*, 1994). These gene products could be components of the division apparatus or factors which regulate its assembly. Evidence supporting this notion comes from the observation that unlike the 'early acting' *sep* mutants (Harris and Hamer, 1995),

temperature-sensitive (Ts) mutations in any of these genes do not affect cellular growth or nuclear division (Harris *et al.*, 1994). Moreover, the septation defects caused by these mutations are readily reversible. Shifting *sepA*, *sepD*, *sepG* or *sepH* mutants from the restrictive to the permissive temperature leads to the formation of septa with rapid kinetics and proper spatial distribution throughout the previously aseptate hyphae (Harris *et al.*, 1994). These results suggest that these mutations do not block early events required for septum formation (i.e. specification of the division site).

Mutations in *sepA* confer additional defects which may reflect a failure to properly organize the actin cytoskeleton at sites of localized cell wall deposition. In particular, *sepA* mutants display mild defects in establishment and maintenance of hyphal polarity, such that mutant hyphae are wider than wild-type and exhibit an aberrant dichotomous branching pattern (Harris *et al.*, 1994). Cell wall deposition at growing hyphal tips is similar to septum formation in that it also requires recruitment of actin to specific cortical sites (Mulholland *et al.*, 1994; Gow, 1995). The observation that both septum formation and polarized growth have gone awry in *sepA* mutants suggests that the *sepA* gene product has a general role in promoting formation of actin-rich structures. We have investigated this possibility by pursuing a detailed phenotypic analysis of *sepA* mutants and by cloning and characterizing the *sepA* gene. Here we report that *sepA* is a member of the expanding FH1/2 class of proteins and that the previously described *figA* gene is actually an allele of *sepA*. Furthermore, we show that *sepA* is required for actin-related functions in *A.nidulans* and that it acts post-mitotically to promote septum formation. Finally, we show that *sepA* function is necessary for maintenance of cellular polarity during asexual development.

Results

Cloning of the sepA gene

The *sepA* locus was originally defined by three Ts mutations (*sepA1*, *sepA2* and *sepA3*) which cause defects in septum formation at restrictive temperatures and also lead to aberrant hyphal branching patterns as well as increased hyphal width at both restrictive and permissive temperatures (Morris, 1976; Harris *et al.*, 1994). Microscopic observations of mutant hyphae using both cell wall (Calcofluor) and nucleic acid (Hoechst 33258) stains revealed that all three mutations cause similar defects. Genetic analyses indicated that each mutation is recessive and that all associated phenotypes co-segregate with the mutation. Parasexual genetic analysis was used to show that the *sepA* locus resides on chromosome I. Furthermore, meiotic mapping experiments revealed that *sepA* is closely linked to the *lysF* locus near the chromosome I centromere (Figure 1).

Linkage between the *sepA* and *lysF* loci was exploited to clone the *sepA* gene. Amongst the cosmids known to contain the cloned *lysF* gene (S.Harris, unpublished results), coslys12 was found to complement all of the phenotypes caused by the *sepA1* mutation. Additional co-transformation experiments using fragments derived from this cosmid showed that the complementing activity was located on a *Hin*dIII fragment (S.Harris, unpublished

results). Since restriction mapping revealed that coslys12 may contain rearrangements, pooled *Hin*dIII fragments from this cosmid were used to screen a chromosome I-specific cosmid library (Brody *et al.*, 1991). Two of the cosmids identified, L29G03 and W12E03, were shown to possess *sepA*-complementing activity when transformed into either *sepA1* or *sepA3* mutants (Figure 1). Additional experiments revealed that the complementing activity resided on a 6.1 kb *Hin*dIII–*Sph*I fragment (pON48 and pKES1, Figure 1). RNA blot analyses indicated that the sequences contained on this fragment hybridized to a single transcript of ~6.0 kb in vegetative mRNA (L.Hamer, unpublished results).

sepA is a member of the FH1/2 family of proteins

Both strands of the 6.1 kb *Hin*dIII–*Sph*I fragment contained on pKES1 were sequenced. Analysis of the DNA sequence revealed the presence of two open reading frames (ORFs) of 4617 and 753 bp interrupted by a 52 bp intron. The presence of the intron was confirmed by the isolation and sequencing of a 2.2 kb cDNA clone which encompasses the spliced region. Since repeated attempts to recover cDNA clones spanning additional regions of the ORF were unsuccessful, we cannot rule out the possibility that other small in-frame introns exist. The combined ORFs are capable of encoding a 1790 amino acid residue protein (Figure 2A). A pronounced feature of the predicted *sepA* protein is the presence of an ~100 amino acid region containing numerous clusters of consecutive proline residues (underlined in Figure 2B). This region contains two
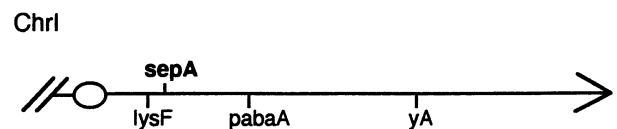
Fig. 1. Genetic and molecular analysis of the *sepA* gene. (**A**) Genetic mapping of *sepA*. Mapping data were generated from the crosses ASH20×A28 and ASH51×A376. The following map distances were obtained: *sepA–yA*, 26 mu; *sepA–pabaA*, 8 mu; *sepA–lysF*, 3 mu. Details of genetic mapping in *A.nidulans* are summarized in Clutterbuck (1987). (**B**) Cloning of the *sepA* gene. Isolation of cosmids coslys12, L29G03 and W12E03, as well as descriptions of plasmids pON48, pKES1 and pKES2, are presented in the text. A positive result for complementation of *sepA* indicates restoration at 42°C of ability both to grow and to form septa. A positive result for complementation of *lysF* indicates ability to grow on supplemented MN plates without lysine. H, *Hin*dIII; X, *Xba*I; S, *Sph*I. nd, not determined.

clusters of 12 consecutive proline residues and additional strings of nine and seven consecutive prolines. The predicted *sepA* protein contains two regions which are predicted to adopt a coiled-coil conformation (Figure 2B),

A

B

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                MPTSDKSRQTSAGKSFFGRKLHKERPVEDRWDAHGSWESL
                APPSSAAGSRSSRYSKRSSIQSVDFGADIDPSLLSTSAGP
                ITSIPFESLSTDTQSPIPVDYLSKAETSPRKEPSPGHLAK
                GVGDFHQYPAWDPSAMRNHQQFCTQRGPDHHHNAAGVAMS
                SSATGDKGARYQQWGRPGSSAGNAGLSHHSSSTVDSSTNS
                RMSIDQDSIHSSLSSNTRGSSYISTDGSSRTTLPSHSNDR
                SNYYAAMNSGRGSSAQGAIPPAQPRVQNTEQYLTRPRDDR
                VVDQLFLELMQKRGWQNLPEQARRQMMAYPASKKWTLVHQ
                DRLTELQGEQKRKQKARETHGYDGPSGILERADEEGSPEW
                YVKKVMDDTITSKQLASLSVSLRTQPISWVKAFVEAQGQI
                ALTNVLVKINRKKVTGPVPAPPSGDKDLDREYDIVKCLKA
                LMNNKYGADDALAHQQIIVALISSLLSPRLNTRKLVSEVL
                TFLCHWAEGQGHERVLQAMDHVKNHQGETGRPFDAWMRIVE
                VTIDGRGKMGSLVGASEEYRSGGIGMENLLMEYAVSTMIL
                INMLVDAPENDLQLRCHIRAQFISCGIKRLLSKMEGFQYE
                VIDKQIEHFRENEAIDYEDLLQRESSSTKDSIEGEVKDMT
                DPLQITDAIASRLNGTRAHDYFLSALQHLLLIRENSGEDG
    |   |       LRMYQLVDAMLSYVAMDRRLPDLDLRQGLTFTVQSLLDRL
    |   |       HTDAEARRAYDESLEARQIAEAALAERDEMKAQVELGADG
                LVRKLQKQIEEQTGIIELQSRQNEMLKAELADVQRLRAQE
                LQRNELETRELYLMLRDAQDIAASNAKKSNMGEAETDPAH
                MRGILDREKLLTRLEKQLERTKTQFKLEGKVWGQHDPSDR
                LRELREQMDGDAGPREAFEEQARLNLSLNPVGSVYRKKTY
                IQGMEDTATEELGQTDDEVVYAKARLVDLHRPRMDPEQAT
                GLLGEIAAKVPKIDADDAKDEGKPTESEQPAEGAATKGDE
                QGVDDTVAVDKATAAPPPPPPPPPAHPGLSGAAPPPPPPP
                PPPPPGAGAAPPPPPPPPPPPPPGGLGGPPPLPSPSSTGWF
                WWTPAASPSTWWFWRSTSAAAASYWWCICVPPPPPPPPGTV
                IGGWRANYLASQGAPSHAIPVMSSIRPKKKLKALHWDKVD
                TPQVTVWATHGTTPQEKEEKYVELAKRGVLDEVERLFMAK
                ETRIFGGGVAAKQRKDKKQIISNDLSKNFQIALSKFSQFP
                AEEVVRRIIHCDAEILDNMVVMEFLQRDEMCTVPENVSKL
                MAPYSKDWTGPDAANTEREQDPSELTREDQIYLYTAFELN
    |           HYWKARMRALALTRSFEPDYEHISAKLREVVRVSESLRDS
    |           VSLMNVLGLILDIGNFMNDANKQAQGFKLSSLARLGMVKD
                DKNETTFADLVERIVRNQYPEWEDFTEQISGVIGLQKLNV
                DQLRTDAKKYIDNIKNVQASLDAGNLSDPKKFHPQDRVSQ
    |           ITQRSMKDARRKAEQMQLYLEEMVKTYDDIMVFYGEDNTD
    |           DGARRDFFAKLAAFLQEWKKSKEKNIALEEARRRTEASLA
                RKRINVGLANGAGAAGDAPVSPATSGAMDSLLEKLRAAAP
                QAKDQRDRRRRARLKERHQVRVASGQKIPDLEGAEAPGSG
                GQNRGATDTNATDSSLLSPTIQEPEGGSSPIASQSEDVAD
                RAASMLQDMLRNSPDPERTRRRRESAEEERRKRRLRRRNG
                ATSGSKDSNDTTPLSPVTEPTSTQGESAEPENLSLSSPPN
                GEDPTLNPPTIVLSSDASDTPDDEHRPSTS
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coils paircoil

C

	FH1+FH2		FH2	
	% IDENTITY	% SIMILARITY	% IDENTITY	% SIMILARITY
FIGA	98	98	99	99
BNI1	43	65	49	67
CDC12	30	57	39	60
FUS1	33	56	35	58
DIAPHANOUS	25	54	39	57
CAPPUCCINO	31	50	30	48
FORMIN4	28	46	29	44

indicating that *sepA* may oligomerize or be part of a multiprotein complex.

Searches of the available databases revealed that *sepA* is a member of the FH1/2 family of proteins (Figure 2A and C). The FH1 and FH2 domains of *sepA* possess significant levels of identity to the *BNI1* gene from *S.cerevisiae* (Jansen *et al.*, 1996; Zahner *et al.*, 1996; H.Fares and J.Pringle, personal communication), the *fus1*[+] and *cdc12*[+] genes from *S.pombe* (Petersen *et al.*, 1995; Chang *et al.*, 1997), the *diaphanous* and *cappuccino* genes from *Drosophila melanogaster* (Castrillon and Wasserman, 1994; Emmons *et al.*, 1995) and the *formin 4* gene from *Mus musculus* (Woychik *et al.*, 1990). The similarities between *sepA* and the other members of this family are mainly confined to the FH1 and FH2 domains, which reside within the C-terminal region of each protein. An additional feature shared by *sepA* and most of the FH1/2 proteins is the presence of regions capable of adopting a coiled-coil conformation (Lupas *et al.*, 1991; Berger *et al.*, 1995).

figA is a dominant negative allele of *sepA*

Database searches showed that the C-terminus of the predicted *sepA* protein (residues 563–1539) is identical to an *A.nidulans* sequence previously designated *figA* (Figure 2C). The *figA* sequence was identified in a screen for *A.nidulans* genes which cause dominant negative inhibition of growth when over-expressed (Marhoul and Adams, 1995). A number of observations indicate that *figA* represents a dominant negative allele of the *sepA* gene. First, all cosmids and plasmids which possess *sepA*-complementing activity (L29G03, pON48 and pKES1) also contain the entire *figA* sequence. Second, DNA blot analysis using a probe spanning the entire *sepA* gene demonstrates that the *figA* transformant (TTAP22) contains an over-expression plasmid integrated at the *sepA* locus (L.Hamer, unpublished results). As a result, TTAP22 possesses an intact *sepA* gene and a truncated version missing sequences from the 5′-end. Over-expression of the truncated allele is controlled by the inducible *alcA* promoter and over-expression leads to inhibition of growth on inducing media (Marhoul and Adams, 1995; Figure 3B). Notably, the region of *sepA* which prevents growth when over-expressed includes both the FH1 and FH2 domains. Hereafter we refer to *figA* as *sepA5*[figA].

Additional evidence demonstrating that *sepA5*[figA] is an allele of *sepA* comes from transformation experiments

Fig. 2. *sepA* is a FH1/2 protein. (**A**) Structure of the *sepA* gene. A restriction map of the *sepA* gene is shown on the top line. Shown underneath is a schematic drawing of the *sepA* gene product. The thin grey line represents the *sepA* coding region, with the FH1 and FH2 domains depicted as square black boxes. X, *Xba*I; Sp, *Spe*I; B, *Bam*HI; E, *Eco*RI; S, *Sph*I. (**B**) The predicted amino acid sequence of the *sepA* gene product . The DDBJ/EMBL/GenBank accession No. for the DNA sequence is U83658. The FH1 (polyproline) and FH2 domains are indicated by underlining. Regions of *sepA* capable of adopting a coiled-coil conformation (Lupas *et al.*, 1991; Berger *et al.*, 1995) are depicted by lines drawn in the left column. (**C**) Levels of sequence similarity between *sepA* and the other FH1/2 proteins. FH1+FH2 refers to the sequence spanning the FH1 and FH2 domains and including the intervening region. FH2 refers to the sequence encompassed solely by the FH2 domain. Comparisons were made using the software provided in the XREFdB package (Bassett *et al.*, 1995).
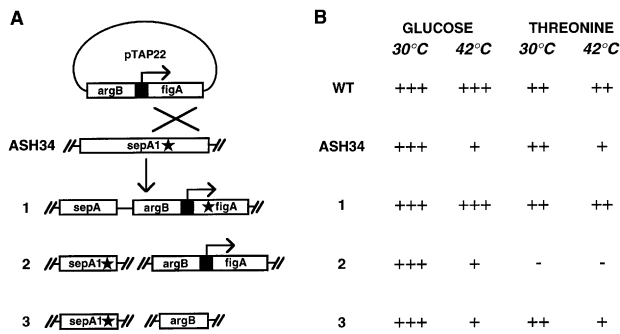
Fig. 3. *figA* sequences can repair the *sepA1* mutation. (**A**) The different classes of transformants obtained when pTAP22 is transformed into ASH34. The open boxes represent either the *argB* gene, *figA* sequences or the *sepA* gene and are labeled accordingly. The star represents the *sepA1* mutation. The stippled box depicts the *alcA* promoter. (**B**) The colony growth phenotypes of the three different classes of transformants. The extent of growth on glucose or threonine plates at either 30 or 42°C is shown. +, ++ and +++ represent increasing extents of colony growth. – indicates failure to form a colony.

which show that *sepA5*[figA] sequences can repair the *sepA1* mutation by homologous recombination. The three classes of transformants recovered in these experiments and their growth phenotypes are shown in Figure 3. Two classes of transformants remain Ts due to the presence of the *sepA1* mutation. One of these classes (class 2) is unable to grow on media which lead to induction of the *alcA* promoter and is due to ectopic integration of *sepA5*[figA]. The second class (class 3) is not inhibited for growth on inducing media and represents conversion of the *argB2* mutation present in the recipient strain by the plasmid-borne *argB* gene. The third class of transformants (class 1) are distinctive in that they are no longer Ts and are also able to grow under *alcA*p-inducing conditions. The explanation for this class of transformants is that recombination between the plasmid-borne *sepA5*[figA] sequences and the chromosomal *sepA* gene has led to: (i) repair of the *sepA1* mutation; (ii) transfer of the Ts mutation into the *sepA5*[figA] sequence such that it can no longer inhibit growth when over-expressed. These observations also indicate that the *sepA1* mutation resides within the 3′-end of the *sepA* gene in the region spanned by the *sepA5*[figA] sequence.

The finding that *sepA5*[figA] is a dominant negative allele of *sepA* prompted us to examine the phenotype of *sepA5*[figA] transformants grown under inducing conditions. However, microscopic observations revealed that the transformants possess no defects in septum formation.

sepA is essential for growth at high temperature
The *sepA* gene was disrupted to determine if it is essential for growth. Plasmid pLH9, in which a 2.2 kb *Bam*HI fragment internal to the *sepA* gene was replaced with the *argB* gene, was introduced into a haploid strain (ATW17) and Arg+ transformants were selected. DNA blot analysis indicated that two transformants (ALH1 and ALH2) had undergone a homologous integration event which resulted in disruption of the *sepA* gene (Figure 4B). No wild-type segregants were obtained when ALH1 (*sepA4ΔBm*) was crossed to ASH34 (*sepA1*), indicating that we had indeed cloned and disrupted the bona fide *sepA* gene.

Surprisingly, *sepA4ΔBm* mutants were able to grow at 28°C, although they exhibited a highly restricted colonial phenotype (Figure 4C). This result indicates that the *sepA* gene is not essential for growth. However, *sepA4ΔBm* mutants failed to grow at 42°C (Figure 4C), conditions under which Ts *sepA* mutants form small, tightly restricted colonies. At 42°C, *sepA4ΔBm* spores germinate to form aberrantly branched hyphae devoid of septa. These results demonstrate that *sepA* function is essential for growth at higher temperature and also indicate that the Ts mutants retain partial activity at 42°C.

sepA mutants fail to make an actin ring
We have previously shown that Ts *sepA* mutants fail to make septa, display an increase in hyphal width and exhibit an aberrant pattern of apical branching when incubated at the restrictive temperature (Harris *et al.*, 1994). Examination of *sepA4ΔBm* hyphae by fluorescent microscopy using Calcofluor and Hoechst 33258 revealed that they display similar phenotypes. Specifically, the disruptants form broad hyphae which display the aberrant branching pattern that is characteristic of *sepA* mutants (Figure 5A and C). Although the hyphae are initially aseptate, scattered septa do begin to appear following prolonged incubation (i.e. >20 h) at 28°C (Table II). Following 40 h incubation at 28°C, all hyphae formed by the disruptant contained septa (Table II), but the number of septa/hyphae was much lower than wild-type levels. These observations indicate that a second gene product, perhaps another FH1/2 protein, can functionally substitute for the loss of *sepA* (see below).

Previous attempts to assess the status of both the actin and tubulin cytoskeletons in the Ts *sepA* mutants were confounded by the inability to effectively remove the cell wall from hyphae incubated at higher temperatures. We were able to circumvent these difficulties by performing immunofluorescent microscopy on *sepA4ΔBm* hyphae grown at 28°C for 12 h and stained with either anti-actin or anti-α-tubulin antibodies. In wild-type cells, an intense patch of actin staining is observed at the hyphal tip (Harris *et al.*, 1994). Even though *sepA4ΔBm* mutants exhibit defects consistent with an inability to maintain hyphal polarity, each hyphal tip does contain a dense patch of actin staining (Figure 5D). Wild-type cells also form an actin ring in association with the developing septum (Figure 5B, arrows). In contrast, actin rings are not observed in *sepA4ΔBm* hyphae (Figure 5D). Staining with anti-α-tubulin antibodies revealed that inactivation of *sepA* has no effect upon the structure of cytoplasmic or spindle microtubules (Figure 5E and F).

sepA functions after mitosis to promote septum formation
The septation defects observed in Ts *sepA* mutants were previously shown to be readily reversible (Trinci and Morris, 1979; Harris *et al.*, 1994). Specifically, following a shift from restrictive to permissive temperature, properly spaced septa were formed throughout the previously aseptate hyphae within an interval of 90–120 min. These results suggested that septation sites were marked by a mechanism which may involve the *sepA* gene product. Conceivably, SepA could function as a positional marker which is activated by adjacent mitotic nuclei. Alternatively, SepA could be required for septum formation at marked
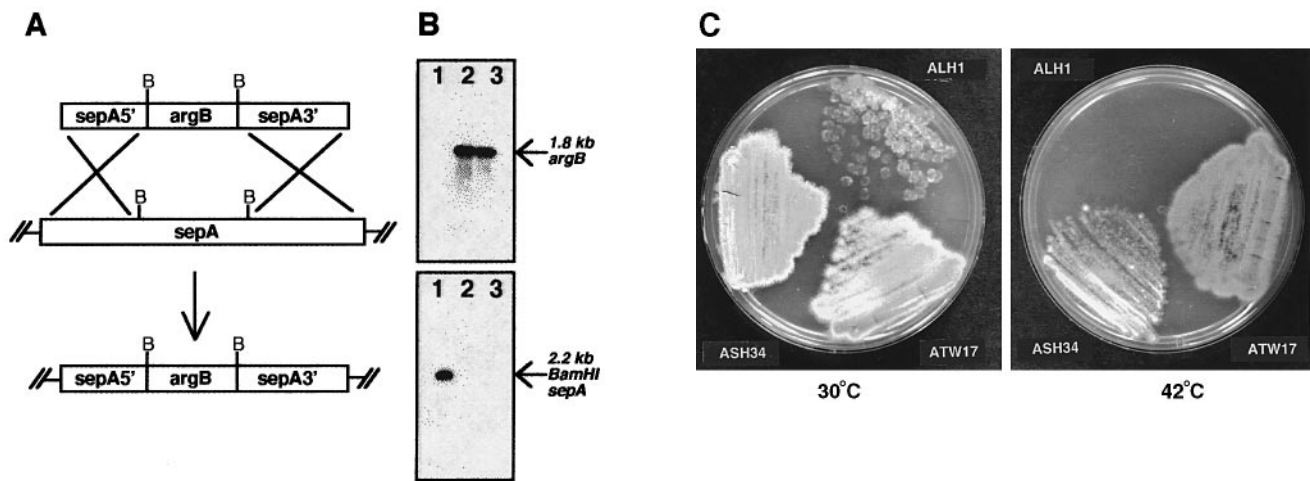
Fig. 4. *sepA* is essential for growth at high temperature. (**A**) A schematic diagram showing how the *sepA* disruption was constructed. The structure of the disruption allele (*sepAΔBm*) is shown. (**B**) Confirmation that the *sepA* gene was disrupted. A 1.8 kb *Bam*HI fragment from pSalArgB containing *argB* (upper panel) and the 2.2 kb *Bam*HI fragment from pLH14 containing part of *sepA* (lower panel) were hybridized to *Bam*HI-digested genomic DNA from the parent strain (ATW17) and two transformants. ATW17 should contain only the 2.2 kb *Bam*HI fragment from *sepA* and the disruptants should contain only the 1.8 kb *Bam*HI fragment from *argB*. (**C**) Growth of a *sepA* disruptant (ALH1) compared with other genotypes [wild-type (ATW17) and *sepA1* (ASH34)]. Plates were incubated for 2 days at either 30 or 42°C. See text for details.

sites in a manner which is independent of mitosis. To distinguish between these possibilities, we determined if blocking progression through mitosis with either hydroxyurea (HU) or benomyl (BEN) could prevent formation of septa when a *sepA1* mutant is returned to the permissive temperature.

Growth of a *sepA1* strain (ASH34) at semi-permissive temperature (37°C) for 12 h led to the formation of aseptate hyphae (Table I). Within 2 h following a return to the permissive temperature >70% of the hyphae had formed at least one septum (Table I). However, <20% of *sepA1* hyphae could form septa when returned to the permissive temperature in the presence of either HU or BEN (Table I). The residual septation observed in the presence of these drugs is likely due to hyphae completing mitosis at 28°C prior to imposition of the block. These results show that *sepA* function is tightly coupled to the completion of mitosis.

sepA is required for maintenance of cellular polarity during conidiation

Although hyphae possessing a disruption of the *sepA* gene display the same spectrum of morphogenetic defects (i.e. failure to form septa, aberrant branching, increased hyphal width) when incubated at 28°C as do Ts *sepA* mutants incubated at restrictive temperature, they are still able to grow and produce a conidiating colony. This observation suggested that the *sepA* gene product is not required for conidiophore development. To test this idea further, we examined wild-type and *sepA4ΔBm* hyphae by DIC microscopy (Figure 6). Conidiophores produced by *sepA4ΔBm* mutants do not exhibit gross developmental defects and form abundant conidia. However, they do exhibit striking defects in the maintenance of cellular polarity, leading to formation of branched stalks (Figure 6B) and split vesicles (Figure 6C, arrowhead). These observations show that the *sepA* gene product is required for maintenance of cellular polarity in additional *A.nidulans* cell types other than growing hyphae.

Examination of conidiophores formed by *sepA4ΔBm*

mutants revealed that they are capable of undergoing cell division and possess abundant septa (Figure 6; K.Sharpless and S.Harris, unpublished results). This observation lends additional support to the notion that a second gene product may be able to functionally substitute for *sepA* during conidiation. Since the initiation of conidiophore development is known to be suppressed when hyphae are grown in submerged culture (Timberlake, 1991), the delayed appearance of septa in *sepAΔBm* cultures (Table II) indicates that expression of the other gene(s) cannot be subject to developmental control. However, the unfavorable growth conditions found in older cultures and associated with initiation of conidiation may contribute to expression of this gene(s). If a functional homolog of *sepA* does exist, it is not detectable in hybridization experiments using DNA probes derived from the *sepA* gene.

Discussion

A characteristic feature of cell division is that it requires dramatic re-organization of the actin cytoskeleton. In fungal cells, septum formation proceeds via formation of an actin ring at a precise cortical location with proper timing relative to the cell cycle. Amongst the growing list of proteins shown to be required for septum formation are those whose known function is to control the dynamics of actin polymerization (Balasubramanian *et al.*, 1992, 1994). We have found that the *A.nidulans* *sepA* gene, which is required for septum formation, encodes a protein whose predicted properties suggest that it may interact with the actin cytoskeleton.

sepA is required for septum formation and maintenance of hyphal polarity

Temperature-sensitive mutations of the *sepA* gene cause a failure to form septa, an increase in hyphal width and aberrant branching from hyphal tips (Trinci and Morris, 1979; Harris *et al.*, 1994). We have found that disruption of the *sepA* gene results in the same pleiotropic effects. A number of observations support the notion that the
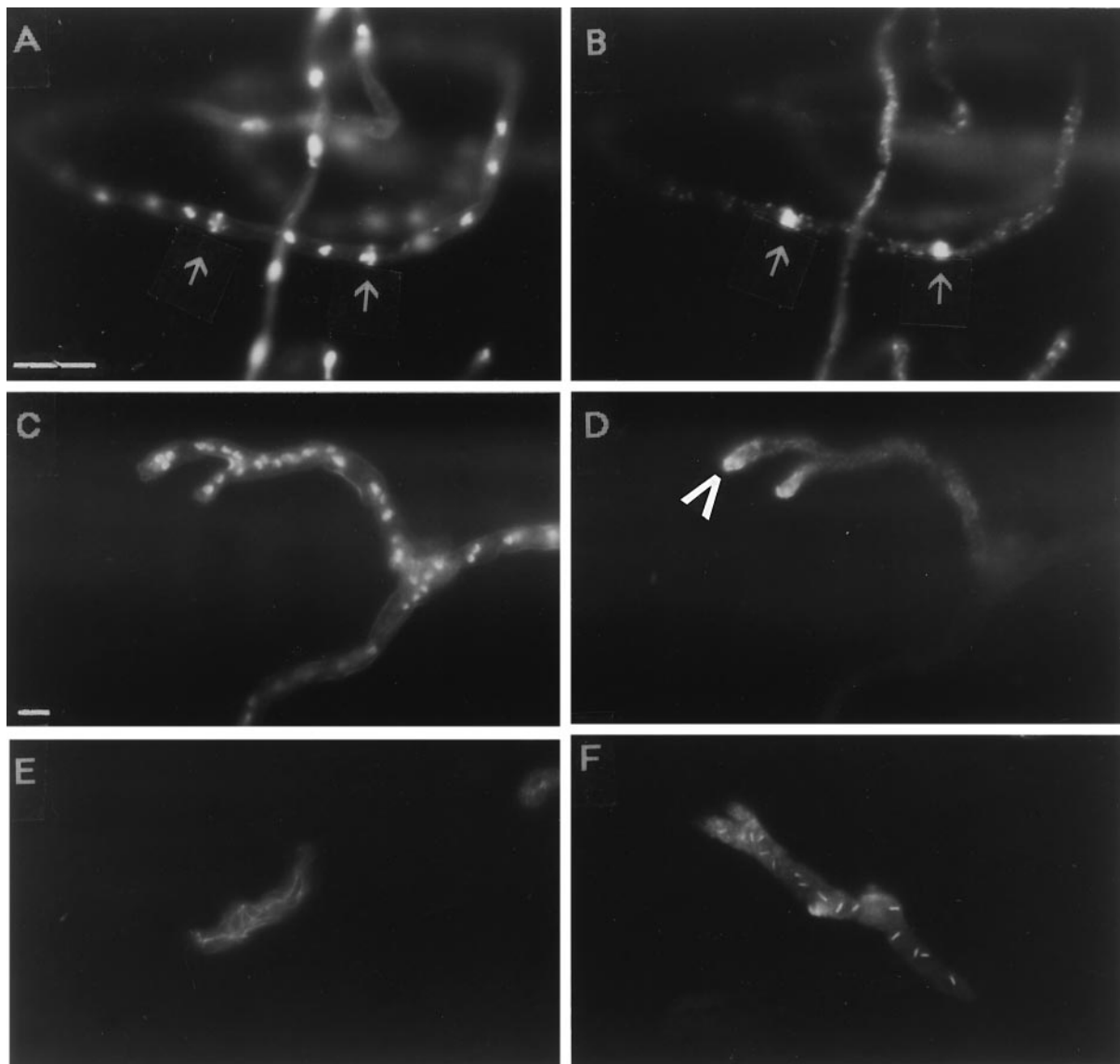
Fig. 5. *sepA* mutants fail to form an actin ring. Wild-type (**A** and **B**) and *sep4ΔBm* hyphae (**C–F**) were examined by immunofluorescence microscopy following incubation at 28°C for 12 h. Hyphae were stained with either Calcofluor and Hoechst 33258 (A and C), a mouse anti-actin monoclonal antibody (B and D) or a mouse anti-α-tubulin monoclonal antibody (E and F). The arrows in (A) and (B) show septal staining with either Calcofluor (A) or the anti-actin antibody (B). *sepAΔBm* mutants fail to form septa (C) and do not contain actin rings (D). They do display normal arrays of cytoplasmic (E) and spindle (F) microtubules. Scale bars in (A) and (C) represent 2 μm.

Table I. *sepA* functions after mitosis to promote septum formation

Treatment[a]	Time following return to permissive temperature (h)	No. of septa			
		0	1	2	>2
Control	0	200	0	0	0
	1	107	41	25	27
	2	55	42	34	69
HU	0	200	0	0	0
	1	152	30	13	5
	2	171	26	2	1
BEN	0	200	0	0	0
	1	163	20	10	7
	2	159	19	11	11

[a]ASH34 hyphae were incubated at 37°C (semi-permissive temperature) for 12 h, after which they were transferred to media containing either 20 mM HU, 2.5 mg/ml BEN or no addition. Samples were taken at 1 and 2 hours following the temperature shift. Calcofluor was used to visualize septa.

defects in septation caused by *sepA* mutations are due to an inability to promote formation of actin rings at incipient division sites. First, septum formation in *A.nidulans* is known to require formation of contractile actin rings (Harris *et al.*, 1994; M.Momany and J.Hamer, unpublished results). Second, we have not been able to detect actin rings by indirect immunofluorescence microscopy in *sepA* mutants. In contrast, *sepA* mutations have no effect upon the appearance of the actin patch located at hyphal tips. Finally, molecular analysis of the *sepA* gene shows that it encodes a protein which may be capable of promoting formation of actin filaments.

Loss of *sepA* function, while not preventing accumulation of actin at hyphal tips, does lead to defects in polarized growth. Preliminary observations indicate that the increased hyphal width observed in *sepA* mutants is due to a severe delay in establishment of spore polarity (S.Harris, unpublished observations). Difficulties in properly organizing the actin cytoskeleton at the site of
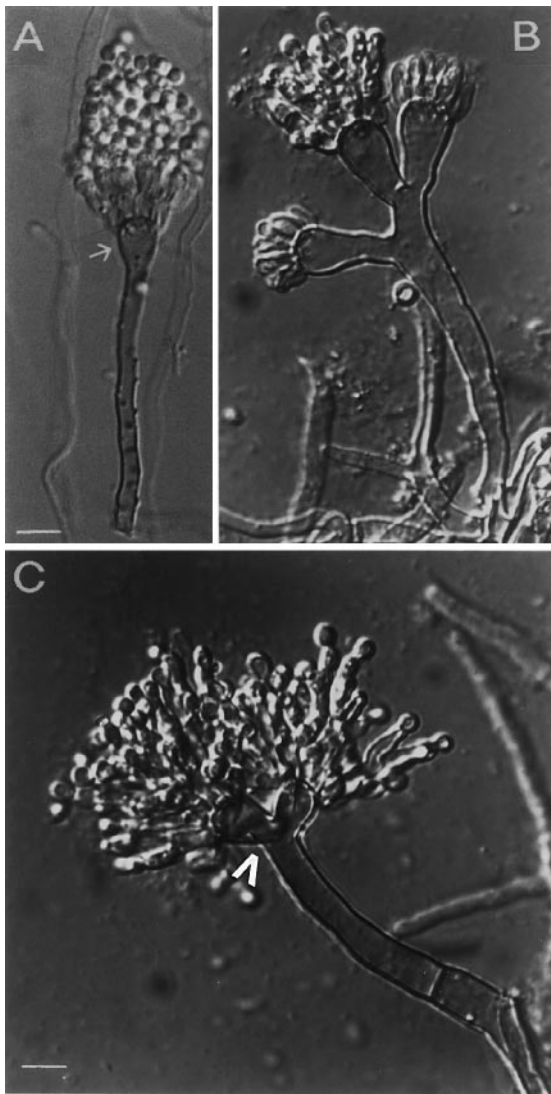
Fig. 6. The maintenance of cellular polarity during conidiation requires a functional *sepA* gene. Conidiophores formed by wild-type (**A**) and *sepA4ΔBm* (**B** and **C**) cultures grown at 28°C were harvested and examined by DIC microscopy. Normally, a single conidiophore vesicle (arrow in A) is formed at the apex of the stalk. In *sepA* mutants the aerial conidiophore stalk frequently appears to generate multiple growing tips, resulting in formation of branched stalks (B) or multiple vesicles upon a single stalk (C, arrowhead). Note the septum at the base of the stalk formed by the *sepA* mutant in (C). Scale bar, 5 μm.

germ tube emergence could presumably cause such a delay. Similar problems in maintaining actin organization at hyphal tips could also lead to the aberrant branching pattern observed in *sepA* hyphae and conidiophores. The ability of *sepA* mutants to form actin-based structures at hyphal tips but not at septation sites may reflect intrinsic differences in the nature of these structures.

Surprisingly, *sepA* is essential for growth and colony formation only at 42°C. Hyphae formed by *sepA* null mutants grow slowly at 28°C and exhibit severe delays in septum formation. We attribute the eventual appearance of septa in hyphae formed by *sepAΔBm* mutants to the presence of a functionally redundant gene product (perhaps a second *A.nidulans* FH1/2 protein) which may be expressed under adverse growth conditions. In support of this idea, both *S.pombe* and *Drosophila* possess at least

two genes encoding FH1/2 proteins (see below). However, since all *sepA* mutants are defective in septum formation at 42°C, the redundant activity must be inherently temperature sensitive.

sepA is a member of the FH1/2 class of proteins

Molecular analysis of the *sepA* gene product demonstrates that it is a member of the FH1/2 class of proteins. This expanding family includes members in species ranging from yeast to mice. The level of similarity between *sepA* and the other FH1/2 proteins ranges from 28 to 65% and is mainly confined to the FH1 and FH2 domains. An additional feature shared by *sepA* and most of the other FH1/2 proteins is the presence of regions capable of adopting a coiled coil conformation. This suggests that these proteins may function as dimers or oligomers.

The participation of FH1/2 proteins in cellular processes which require formation of microfilaments suggests that they may be functionally related. The most striking conservation of function occurs between *sepA* and both *cdc12*[+] and *diaphanous*. We have shown that mutations in the *A.nidulans sepA* gene lead to defects in septum formation, presumably due to a failure to form actin rings. The *cdc12*[+] gene is required for assembly of the contractile actin ring during septum formation in *S.pombe* (Chang *et al.*, 1996). Mutations in *cdc12*[+] cause delocalized clusters of actin patches to accumulate in the middle of the cell, suggesting that it functions early during actin ring assembly. The *Drosophila diaphanous* gene is required for cytokinesis in all tissues which have been examined (Castrillon and Wasserman, 1994). Furthermore, the absence of cleavage furrows in mutant larval neuroblasts suggests that *diaphanous* may also act early during contractile ring formation. Functional similarities also exist between *sepA* and the *S.cerevisiae BNI1* gene product. *bni1* mutants exhibit partial defects in cytokinesis (forming a broader mother–bud junction) and fail to display a bipolar pattern of bud site selection (Jansen *et al.*, 1996; Zahner *et al.*, 1996). The increase in hyphal width and the aberrant branching of both hyphae and conidiophores caused by *sepA* mutations may represent defects in polarized growth which are analogous to those caused by *bni1* mutations. Collectively, these observations suggest that the re-organization of the actin cytoskeleton which precedes cell division in animal and fungal cells occurs via an evolutionarily conserved mechanism involving FH1/2 proteins. These proteins may stimulate assembly of actin rings or contractile rings by nucleating formation of actin filaments.

The presence of multiple prolines within the FH1 domain suggests at least two possible mechanisms by which FH1/2 proteins could stimulate formation of actin filaments. One mechanism is through interactions with proteins containing either SH3 or WW domains. These proteins bind to ligands containing a proline-rich motif (Sudol, 1996) and, in the case of SH3 proteins, have been implicated in mediating signal transduction to the actin cytoskeleton (Pawson, 1995). Consistent with this possibility, the mouse formins have recently been shown to interact with SH3 and WW proteins (Chan *et al.*, 1996). The polyproline tracts may also mediate interactions with the actin binding protein profilin. Profilin is known to modulate the kinetics of actin filament assembly (Pantaloni

Table II. Septation defects caused by the *sepAΔBm* mutation

Time (h)[a]	Septation	Strain[b]					
		ATW17		ASH34		ALH1	
		28°C	42°C	28°C	42°C	28°C	42°C
0	−	100	100	100	100	100	100
	+	0	0	0	0	0	0
10	−	100	18	97	100	100	100
	+	0	82	3	0	0	0
12	−	75	0	74	100	100	100
	+	25	100	26	0	0	0
15	−	0	0	0	100	97	100
	+	100	100	100	0	3	0
20	−	0	0	0	100	73	100
	+	100	100	100	0	27	0
40	−	0	0	0	100	0	100
	+	100	100	100	0	100	0

[a]ATW17 (wild-type), ASH (*sepA1*) and ALH1 (*sepAΔBm*) hyphae were incubated at 28 or 42°C for the indicated period of time. Samples were removed and stained with Calcofluor to visualize septa.
[b]The percentage of cells containing no (–) or >1 (+) septa. $n = 100$.

and Carlier, 1993) and avidly binds to polyproline (Janmey, 1991). Furthermore, genetic and/or two-hybrid interactions have been observed between profilin and FH1/2 proteins in both flies and yeast (Manseau *et al.*, 1996; Chang *et al.*, 1997; Evangelista *et al.*, 1997). It is tempting to speculate that the inhibition of growth caused by over-expression of the FH1/2 region in the *sepA5*[figA] allele is due to titration of an interacting protein(s) which is required for organization of the actin cytoskeleton.

Roles for FH1/2 proteins in cytokinesis

Our results, together with other studies on proteins possessing FH1 and FH2 domains, suggest that these gene products may stimulate assembly of actin filaments at specified cortical sites through interactions with actin-associated proteins. In this manner, *sepA* may play a critical role in directing formation of the actin ring at the division site in *A.nidulans*. The *sepA* gene product may be recruited to the incipient division site by interacting with a class of proteins termed septins. Septins are a novel class of cytoskeletal proteins required for cell division in numerous organisms (Longtine *et al.*, 1996). One of their primary functions may be to serve as a scaffold for assembly of the division apparatus. The observation of genetic interactions between a septin and an FH1/2 protein in *S.cerevisiae* (Longtine *et al.*, 1996) indicates that similar interactions between *sepA* and septins may also occur in *A.nidulans*. At least three septin-encoding genes have been identified in *A.nidulans* and their function is currently being assessed (Momany *et al.*, 1995). The observation that mitotic progression is required for *sepA* to promote septum formation indicates that the *sepA* gene product may localize to the incipient division site in response to mitotic signals. Alternatively, mitotic signals may 'activate' *sepA* and trigger actin filament formation. In either case, these signals could be transduced to the division apparatus by small GTP binding proteins of the *rho* class, which are known to be required for cytokinesis in a number of cell types (Kishi *et al.*, 1993; Mabuchi *et al.*, 1993; Larochelle *et al.*, 1996).

Materials and methods

Strains, media and growth conditions
The following strains were used in this study: A28 (*pabaA6 biA1*), A376 (*suA1adE20 lysF88 pabaA1 yA2 adE20*), ASH20 (*sepA1 argB2 yA2*), ASH34 (*sepA1 argB2 yA2*), ASH51 (*sepA1 pyrG89 wA2*), ATW17 (*ΔargB::trpCΔB*), ALH1 (*sepA4ΔBm*) and TTAP22 (*biA1 ΔargB::trpCΔB methG1 trpC801 alcA(p)::figA*). Strains were either obtained from the Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center, Kansas City, KS) or constructed specifically for this study.

Media used [complete medium (CM), minimal medium (MN) and yeast extract glucose (YEG)] and general growth conditions were as described previously (Harris *et al.*, 1994). YGV is yeast extract glucose + 0.1% vitamins (Kafer, 1977). For induction of the *alcA* promoter, MN containing 100 mM threonine and no glucose was used.

Aspergillus nidulans was grown on coverslips in liquid media as described previously (Harris *et al.*, 1994). Temperature down-shift experiments were initiated by growing strain ASH34 on coverslips at 37°C in YGV for 12 h. Coverslips with adherent cells were then transferred to YGV containing either no additions, 20 mM HU or 2.5 mg/ml BEN and shifted to 28°C. Coverslips were removed at 1 and 2 h following the temperature shift and processed for microscopy. To examine the phenotype of strains overexpressing *sepA5*[figA], strain TTAP22 was grown on coverslips at 28°C in MN for 12 h. Coverslips with adherent hyphae were then transferred to either MN or MN+Thr medium and incubated for an additional 6 h at 30°C before being processed for microscopy. Septation time course experiments were initiated by growing strains ATW17, ASH34 and ALH1 on coverslips at either 28 or 42°C in CM supplemented with arginine. Coverslips were removed at 0, 10, 12, 15, 20 and 40 h post-inoculation and processed for microscopy. Samples removed at 0 and 10 h were inoculated at a density of 10^5 conidia/ml, samples removed at 12, 15 and 20 h were inoculated at a density of 10^4 conidia/ml and samples removed at 40 h were inoculated at a density of 2.5×10^3 conidia/ml.

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

All recombinant DNA manipulations followed standard protocols (Sambrook *et al.*, 1989). Previously described procedures were employed for isolation of nucleic acids from *A.nidulans* mycelia (Timberlake, 1980; Dobinson *et al.*, 1993) and for transformation of *A.nidulans* (Oakley and Osmani, 1993).

Plasmid constructs
The plasmids pSalArgB, pDHG25 and pTAP22 have been previously described (Gems *et al.*, 1991; Marhoul and Adams, 1995). Plasmid pON48 contains the ~13 kb *Hin*dIII fragment from cosmid L15G11

(Brody *et al.*, 1991) subcloned into pBS SK+ (J.Marhoul and T.Adams, personal communication). Plasmid pKES1 contains the 6.1 kb *Hin*dIII–*Sph*I fragment from pON48 subcloned into pUC119. Plasmid pKES2 contains the partially digested 4.1 kb *Sph*I fragment from pON48 subcloned into pUC119. Plasmids for sequencing of the 5′ *sepA* region were obtained by subcloning the following fragments from pON48 into pBK KS⁻: ~2.2 kb *Bam*HI (pLH14); ~1.6 kb *Xba*I (pLH15); ~0.9 kb *Xba*I–*Spe*I (pLH19); ~1.7 kb *Xba*I–*Bam*HI (pLH20); ~1.0 kb *Eco*RI (pLH21). An ~2.0 kb cDNA fragment from a λgt10 library was cloned into pBS SK⁺ to form pLH22. pLH22 and pLH23 (containing an ~1.4 kb *Cla*I fragment from pLH22) was used for sequencing of the 3′ *sepA* region. The *sepA* disruption plasmid pLH9 was constructed in three steps. First, the 6.1 kb *Hin*dIII–*Sph*I fragment from pON48 was subcloned into pBR322 (pLH5). Second, pLH5 was digested with *Bam*HI and re-ligated (pLH7). Third, the 1.8 kb *Bam*HI fragment containing the *argB* gene was ligated to *Bam*HI-digested, dephosporylated pLH7.

Sequencing and sequence analysis

Sequencing of the plasmids pLH14, pLH15, pLH19, pLH20, pLH22 and pLH23 was performed on a Pharmacia Alf sequencer using the T7, T3, Universal and Reverse primers. The sequence was analysed using XREFdB database search tools (Bassett *et al.*, 1995) and the Coils (Lupas *et al.*, 1991) and Paircoil (Berger *et al.*, 1995) software.

Microscopy

Coverslips with adherent cells were processed for microscopy and stained with Hoechst 33258 and Calcofluor as described previously (Harris *et al.*, 1994). Immunofluorescence microscopy for detection of actin and tubulin cytoskeletons was performed using standard protocols (Oakley and Osmani, 1993; Harris *et al.*, 1994). Primary antibodies used were the mouse anti-actin N.350 monoclonal (Amersham) at 1:500 and the mouse anti-α-tubulin DM1A monoclonal (Sigma Immunochemicals) at 1:200. Secondary antibodies used were FITC-conjugated anti-mouse (Sigma Immunochemical) at 1:200.

Acknowledgements

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