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SepB^{CTF4} Is Required for the Formation of DNA-Damage-Induced UvsC^{RAD51} Foci in *Aspergillus nidulans*

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ABSTRACT

SepB is an essential, conserved protein required for chromosomal DNA metabolism in *Aspergillus nidulans*. Homologs of SepB include yeast Ctf4p and human hAnd-1. Molecular and bioinformatic characterization of these proteins suggests that they act as molecular scaffolds. Furthermore, recent observations implicate the yeast family members in lagging-strand replication and the establishment of sister-chromatid cohesion. Here, we demonstrate that SepB functions in the *A. nidulans* DNA damage response. In particular, analysis of double mutants reveals that SepB is a member of the UvsC^{RAD51} epistasis group. In accord with this prediction, we show that UvsC^{RAD51} forms DNA-damage-induced nuclear foci in a manner that requires SepB function. We also provide evidence that implicates SepB in sister-chromatid cohesion, thereby suggesting that cohesion may play a role in regulating the localization and/or assembly of UvsC^{RAD51} complexes.

IN eukaryotic cells, the repair of damaged DNA and the maintenance of genome integrity are often dependent on homologous recombination (reviewed by Jackson 2002). The RecA homolog Rad51 performs an essential role in this process by searching for homologous sequences and catalyzing strand exchange (reviewed by Symington 2002). *In vitro* studies show that Rad51 forms filaments on single-strand DNA coated with replication protein A (Sung and Robberson 1995) in a process that requires Rad52 and other associated proteins (Sung 1997; Shinohara and Ogawa 1998). In *Saccharomyces cerevisiae*, Rad51 forms subnuclear foci in mitotic cells that have suffered DNA damage (Gasior *et al.* 2001; Caspari *et al.* 2002). These foci are thought to represent sites of ongoing recombination, and, consistent with the *in vitro* observations, their formation requires replication protein A and Rad52 (Miyazaki *et al.* 2004; Wang and Haber 2004). Additional studies show that the repair of DNA strand breaks by homologous recombination is affected by chromatin organization and the establishment of sister-chromatid cohesion (Hartsuiker *et al.* 2001; Sjogren and Nasmyth 2001; Jaskelioff *et al.* 2003). However, the role of these functions in modulating Rad51 localization in response to DNA damage has not yet been investigated.

The SepB/And-1 protein family encompasses homologs in organisms ranging from fungi to humans (Williams and McIntosh 2002). Notable features of these proteins include the presence of WD40 repeats in the amino-terminus and a central conserved region termed the SepB domain (Kohler *et al.* 1997; Williams and McIntosh 2002). On the basis of the known ability of WD40 repeats to adopt a β-propeller conformation (Smith *et al.* 1999), SepB/And-1 proteins are likely to serve as scaffolds that interact with multiple partners. In support of this notion, the yeast homologs *S. cerevisiae* Ctf4p and *Schizosaccharomyces pombe* Mcl1 interact with multiple proteins involved in lagging-strand replication, including DNA polymerase α, Rad27p, and Dna2p (Formosa and Nittis 1999; Williams and Mcintosh 2002). In addition, Ctf4p and Mcl1 have been implicated in the establishment of sister-chromatid cohesion during S phase, perhaps by facilitating polymerase switching (Hanna *et al.* 2001; Williams and McIntosh 2002). Although little is known about the metazoan And-1 proteins, the Xenopus homolog xAnd-1 was found to associate with interphase chromatin (Kohler *et al.* 1997).

We have previously reported that SepB is an essential protein required for faithful chromosome segregation in *Aspergillus nidulans* (Harris and Hamer 1995). In particular, the temperature sensitive (Ts) lethal *sepB3* mutation causes several phenotypes suggestive of a defect in DNA metabolism, including increased mitotic recombination and chromosome nondisjunction and delayed progression through mitosis. In the process of characterizing genetic interactions between *sepB3* and mutations affecting the *A. nidulans* DNA damage response, we noted that *sepB3* also causes modest sensitiv-

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TABLE 1

Strains used in this study

Strain	Genotype	Source
A28	*pabaA6 biA1*	FGSC
ASH15	*sepB3; argB2; wA2*	Lab collection
ASH49	*sepB3; pyrG89; pyroA1; wA2*	Lab collection
ASH60	*sepB3; pabaA6 yA2*	Lab collection
ASH208	*sepB3; pabaA6; wA2*	Lab collection
ASH201	*uvsB110; chaA1*	Lab collection
ASH202	*uvsB110; sepB3; chaA1*	Lab collection
ASH215	*uvsC114; wA2*	Lab collection
ASH218	*uvsC114; sepB3; pabaA1; wA2*	Lab collection
ASH383	*musN227; chaA1*	Lab collection
ASH380	*musN227; sepB3; chaA1*	Lab collection
ASH568	*uvsC114*; *pyrG89*; *wA1*	Lab collection
ASG15	pSDW194-*sepB*::3xHA transformed into ASH15; integrated at *argB2*	Lab collection
ASG16	pSDW194-*sepB*::3xHA transformed into ASH15; integrated at *sepB3*	Lab collection
ASG17	pRG3-*uvsC*::FLAG transformed into ASH568; integrated at *uvsC*	Lab collection
ASG19	*uvsC*::FLAG; *sepB3*; *wA1*	Lab collection
ASG70	Δ*sldA*; *wA2*	Lab collection
ASG72	Δ*sldA*; *sepB3*; *wA2*	Lab collection
ASG76	Δ*sldA*; *wA2*	Lab collection
ASG78	Δ*sldA*; *sepB3*; *wA2*	Lab collection
ASG80	Δ*sldB*; *yA2*	Lab collection
ASG82	Δ*sldB*; *sepB3*; *wA2*	Lab collection
ASG84	Δ*sldB*; *yA2*	Lab collection
ASG86	Δ*sldB*; *sepB3*; *yA2*	Lab collection

FGSC, Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Lawrence, Kansas.

ity to DNA-damaging agents. Here, we provide evidence that SepB functions in a Rad51-mediated pathway for the repair of DNA damage by homologous recombination. Notably, we report that SepB is required for the formation of DNA-damage-induced UvsC^{RAD51} foci. We also present results implicating SepB in sister-chromatid cohesion. Our observations suggest that cohesion may play a role in Rad51 localization.

MATERIALS AND METHODS

Strains, media, and reagents: All strains used in this study are described in Table 1. Media used for the growth of *A. nidulans* include CM (1% dextrose, 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, nitrate salts, vitamins, and trace elements; pH 6.5), MAG (2% dextrose, 2% malt extract, 0.2% peptone, trace elements, and vitamins), YGV (2% dextrose, 0.5% yeast extract, and vitamins), and MNV (1% dextrose, 5% nitrate salts, trace elements, and vitamins; pH 6.5). Nitrate salts, trace elements, and vitamins were added as described in the appendix to Kafer (1977). Arginine (1 mm), uridine (5 mm), and uracil (10 mm) were added as needed. Media were solidified using 1.5% agar. When necessary, 0.01% Triton X-100 was added to restrict colony growth. Benomyl (BEN), methyl methanesulfonate (MMS; both Sigma-Aldrich Chemical, St. Louis), and phleomycin (PLM) D1 copper chelate chlorohydrate salt (CAYLA, Toulouse, France) were added to media at the appropriate concentration after autoclaving.

Growth conditions and genetic manipulations were as described previously (Harris *et al.* 1994). The DNA sequences of oligonucleotide primers are available from the authors upon request. The corrected sequence of SepB, which includes N-terminal and C-terminal extensions of 70 and 59 amino acids, respectively, is deposited at NCBI (accession no. X86399.1).

Cloning of *hAND-1* and complementation of *sepB3*: RT-PCR was performed on human placental RNA (gift from Andrew Arnold, University of Connecticut Health Center) using the Ambion (Austin, TX) RETROscript kit. The 3.4-kb *hAND-1* PCR product was cloned into pCR2.1-TOPO using the Invitrogen (San Diego) TOPO TA kit. The *hAND-1* gene was subsequently cloned into pSDW194 (gift from Steven W. James, Gettysburg College), such that its expression is regulated by the ethanol-inducible *alcA* promoter. pSDW194-*hAND1* was transformed into strain ASH15, and transformants in which the plasmid had integrated were identified by Southern blot analysis. Transformants were plated on MNV plates with 2% ethanol, 1% glycerol, or 1% dextrose as the inducing, nonrepressing, and repressing carbon source, respectively, or they were grown on MAG.

Cloning of the *sepB3* mutant allele: Genomic DNA was prepared from lyophilized mycelia obtained from the strains A28 and ASH60. The wild-type and *sepB3* alleles were amplified by PCR and cloned using the TOPO TA kit. Three independent clones derived from each allele were pooled and sequenced.

Viability assays: The viability of growing hyphae was measured as follows. For each strain tested, conidiospores were plated at ~10^6 conidia/plate on MAG and allowed to form a uniform mycelial mat. Mycelial agar plugs were made by using the large end of a 14.6 cm × 5 mm Pasteur pipette and were subsequently placed on CM media containing appropriate concentrations of MMS or PLM. One plug was made for each strain and placed on the same plate (*i.e.*, each plate contained wild-type, *sepB3*, single-mutant, and double-mutant plugs). Radial growth was measured at multiple time points up to 48 hr at 28° (permissive temperature) and 35° (semipermissive temperature). All growth was measured as percentage change in radial growth rate compared to the untreated CM control plate. Experiments were repeated three times.

The sensitivity of conidiospores to acute genotoxic stress was measured as follows. Dormant spores were harvested and diluted at ~10^6 conidia/ml of water. They were subsequently inoculated with a DNA-damaging agent (0.001–0.025% MMS or 5–40 μg/ml PLM) and incubated at 37° for 45 min with constant agitation. Spores were washed with water and plated on CM + Triton X-100 (0.01%) plates at a concentration of ~10^2 conidia or ~10^3 conidia/plate. Viability was measured as percentage survival compared to untreated controls.

The effect of microtubule depolymerization on viability was tested as follows. Dormant spores were incubated for 6 hr at 37° in YGV (supplemented with 0.2% Tween 20 to prevent clumping) containing 10 μg/ml BEN. Spores were diluted and plated on CM + Triton X-100 plates, and viability measured as described above.

Protein extraction and Western blot assays: Strain ASG16 was inoculated in YGV at ~10^6 conidia/ml and grown for 18 hr at 28°. The cultures were treated with 50 mm hydroxyurea (HU) for 2 hr, 5 μg/ml benomyl for 2 hr, 1 μg/ml nitrosoguanidine for 10 min, 10 μg/ml PLM for 10 min, 0.05% MMS for 10 min, or left untreated. Mycelia were harvested via filtration and rinsed with stop buffer (Moreno *et al.* 1989), pressed dry between paper towels, frozen in liquid nitrogen, and lyophilized overnight. Lyophilized mycelia were crushed to a fine powder using a spatula. Protein extraction and immunopre-

cipitation was performed following the protocol provided with the Roche anti-HA affinity matrix. For Western blots, proteins were separated by SDS-PAGE and transferred to Immobilon-P PVDF transfer membrane (Millipore, Bedford, MA) by electroblotting. Membranes were probed with a 0.4 mg/ml solution of 12CA5 anti-HA antibody (Roche, Indianapolis) at a 1:400 dilution, or with a 0.45 mg/ml solution of mouse monoclonal M2 anti-FLAG (Sigma, St. Louis) at a 1:400 dilution. A 1/10,000 dilution of anti-mouse IgG A3562 (whole cell) alkaline phosphatase conjugate (Sigma ImmunoChemicals, St. Louis) was used as a secondary antibody. Western analysis was performed by enhanced chemiluminescent detection (Roche, Indianapolis).

Localization of SepB and UvsC: A SepB-HA fusion protein was constructed by designing two complementary 117-bp oligonucleotides that contain the 3x-HA tag (YPYDVPDYAG) from pGTEPI (gift from Aaron Mitchell, Columbia University). The oligonucleotides were annealed using the protocol for adapter production from Life Technologies. The annealed product was subsequently cloned into the pCR2.1-TOPO vector. The HA tag was cloned in frame at the C terminus of *sepB* in pSDW194-SEPB. pSDW194-SEPB-HA was transformed into strain ASH15 to generate strains ASG15 and ASG16 (Table 1). In both cases, transformants displayed wild-type growth at 42°, demonstrating that the SepB-HA fusion protein was functional. For localization experiments, ASG16 was grown on coverslips in YGV for 12 hr.

A UvsC-FLAG fusion protein was constructed by PCR amplification using primers designed to amplify the entire *uvsC* gene with the FLAG epitope (DYKDDDDK; Hoffmann *et al.* 2001) added in frame to the C terminus. The *uvsC*-FLAG allele was cloned into the pRG3 (pUC19; *pyr-4*) vector, and the resulting pRG3-*uvsC*-FLAG plasmid was then transformed into strain ASH568 to generate ASG17 (Table 1). Transformants displayed wild-type resistance to ultraviolet (UV) irradiation and MMS, demonstrating that the UvsC-FLAG fusion protein was functional. ASG17 was crossed with ASH49 to generate ASG19, which introduces the *sepB3* mutation into the *uvsC*-FLAG background (Table 1). For localization experiments, ASG17 and ASG19 were grown on coverslips in YGV for 12 hr. The coverslips were treated with 50 mm HU for 1 hr, 10 μg/ml PLM for 1 hr, 0.02% MMS for 1 hr, or left untreated.

Immunofluorescence was performed following a standard protocol (Harris *et al.* 1999) using the following primary antibodies: 12CA5 anti-HA mouse monoclonal (Roche) as a 0.4 mg/ml solution in 50 mm PIPES, 25 mm EGTA, 5 mm $MgSo_4$ (PEM)-BSA or M2 anti-FLAG mouse monoclonal (Sigma) as a 0.45 mg/ml solution in PEM-BSA. A 1:200 dilution of FITC-conjugated anti-mouse IgG (Sigma) was used as the secondary antibody. Nuclei and cell wall were detected using Hoechst 33258 (Molecular Probes, Eugene, OR) and calcofluor white (Sigma), respectively, as previously described (Harris *et al.* 1994).

Analysis of Western blots revealed that the M2 anti-FLAG antibody detected additional bands in *A. nidulans* extracts (see Figure 3). Therefore, immunofluorescence experiments were also performed on a wild-type strain that does not possess the UvsC-FLAG construct (A28). These experiments show that the background cytoplasmic staining observed in *uvsC-FLAG* or *sepB3 uvsC-FLAG* hyphae is due to the additional FLAG-reactive proteins (supplemental Figure 1B at http://www.genetics.org/supplemental/). In no case was FLAG localization observed in nuclei of A28 hyphae (supplemental Figure 1B at http://www.genetics.org/supplemental/).

Slides were viewed using an Olympus BX51 fluorescent microscope. Images were captured with a Photometrics CoolSnap HQ CCD camera (Roper Scientific) and processed using IPLab software (Scanalytics) and Adobe PhotoShop 6.0. Confocal images were obtained with an Olympus FW500/BX61 confocal laser scanning microscope using the following laserlines: 405 nm for Hoechst 33258 and 563 nm for Cy3. Images were captured by direct acquisition with a Z step of 1–2 μm and were subsequently processed using ImageJ and Adobe PhotoShop 6.0.

RESULTS

Predicted organization of SepB/And-1 proteins: A schematic of the fungal and metazoan SepB/And-1 proteins is shown in Figure 1A. All homologs possess conserved WD40 and SepB domains. The Ts lethal *sepB3* mutation (P618S) affects a conserved residue within the SepB domain (Figure 1B), emphasizing the potential functional importance of this region. Differences between the fungal and metazoan homologs include the presence of different carboxy-terminal motifs, as well as a fungal-specific region similar to the amino-terminus of the catalytic subunit of DNA polymerase α (Figure 1, A and C). To determine the extent of functional conservation between family members, we expressed *hAND-1* in a *sepB3* mutant. However, instead of complementation, we found that expression of *hAND-1*, but not *sepB*, exacerbated the growth defects caused by the *sepB3* mutation (supplemental Table 1 at http://www.genetics.org/supplemental/). This may reflect the ability of hAnd-1 to interfere with the formation of stable SepB complexes or to cause the formation of nonfunctional complexes. This observation suggests that hAnd-1 and SepB may be able to form a heteropolymer, thereby implying that the two proteins might be functional homologs.

To examine SepB localization, a SepB-HA fusion protein was constructed and shown to complement the Ts growth defects caused by the *sepB3* mutation. Like the other homologs (Kohler *et al.* 1997; Hanna *et al.* 2001; Williams and McIntosh 2002), we found that SepB localizes to the nucleus and appears to be uniformly distributed throughout the nuclear compartment (Figure 1D). The localization pattern did not change when hyphae were exposed to DNA-damaging agents (PLM, MMS) or arrested in specific cell cycle phases using either HU (S-phase arrest) or Ben (mitotic arrest).

***sepB3* hyphae and conidiospores display sensitivity to agents that cause DSBs:** Ctf4p and Mcl1 are each required for an uncharacterized aspect of the DNA damage response (Kouprina *et al.* 1992; Williams and McIntosh 2002). To determine if SepB functions in the *A. nidulans* DNA damage response, we used a radial colony growth assay to measure the ability of *sepB3* hyphae to grow on media containing DNA-damaging agents at semipermissive temperature. These experiments revealed that growing *sepB3* hyphae are approximately twofold more sensitive to PLM (20–40 μg/ml) and to MMS (0.01–0.025%) than are wild type (Figure 2). Since hyphae are composed of a relatively asynchronous population of cells, we also tested *sepB3* sensitivity

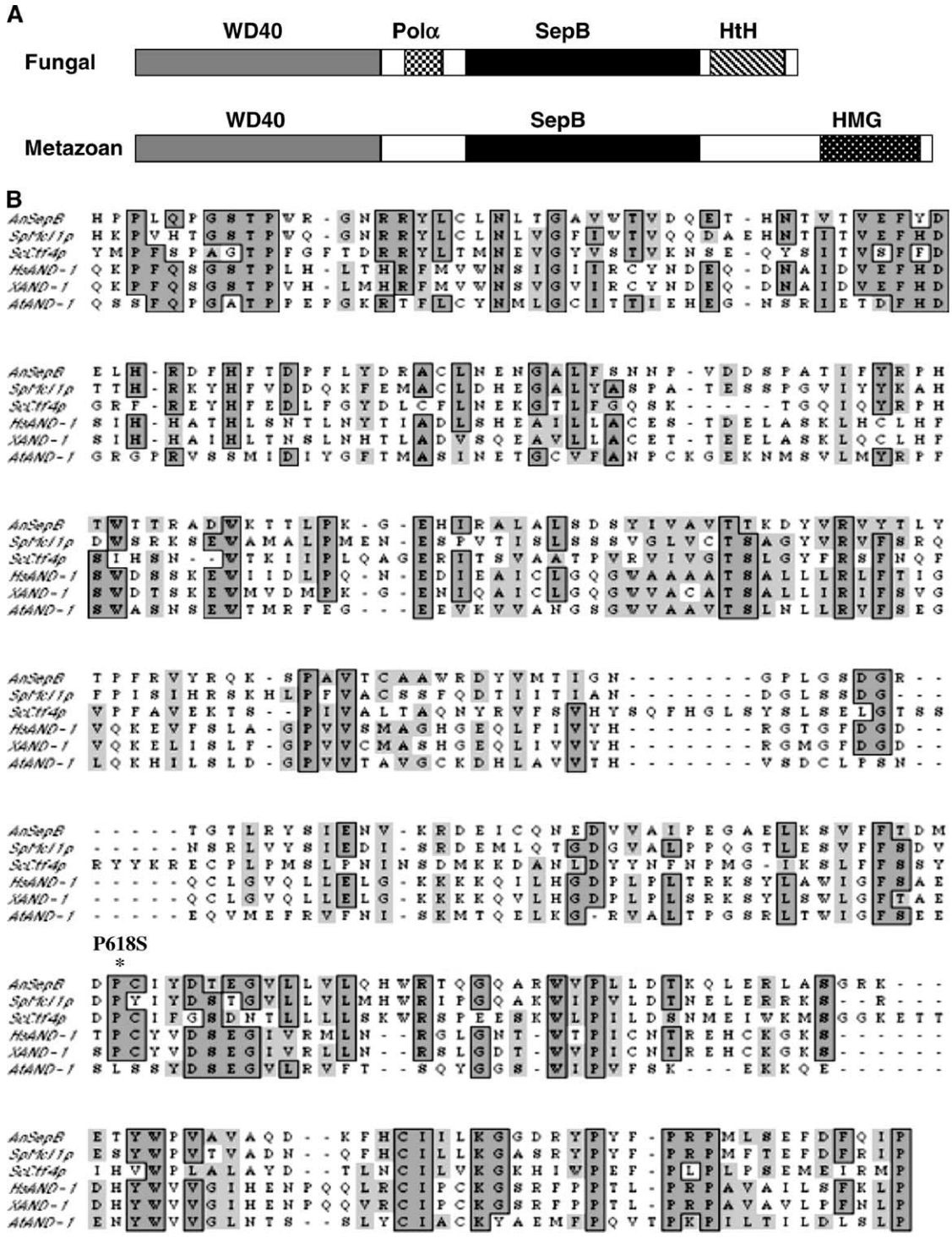

FIGURE 1.—Predicted organization of the SepB/And-1 protein family. (A) A schematic of the fungal and metazoan members of the SepB/AND-1 protein family. The WD40 (shaded) and the SepB (solid) domains are conserved in all members. In contrast, the Pol α checkerboard) and the HtH (diagonal lines) domains are specific to the fungal homologs, whereas the HMG domain (stippled) is found only in the metazoan homologs. (B) The SepB Domain. The predicted protein sequence of the SepB domain from the SepB/And-1 family members AnSepB (*A. nidulans*; X86399.1), SpMcl1p (*S. pombe*; AL590605.1), ScCtf4p (*S. cerevisiae*; M94769.1), HsAND-1 (*Homo sapiens*; AJ006266.1), XAND-1 (*Xenopus laevis*; X98884.1), and AtAND-1 (*Arabidopsis thaliana*; AL138640.1) were aligned using the MacVector 7.0 software. Note that the site of the *sepB3* mutation (P618S; indicated by an asterisk) is highly conserved. (C) The Pol α domain. An alignment of the 18-amino-acid Pol α domain from the catalytic subunit of DNA polymerase α and the fungal SepB homologs. A consensus sequence for the domain is shown in boldface type below the line. (D) SepB is a nuclear protein. ASG16 hyphae were grown on coverslips in YGV for 12 hr. SepB::3xHA was detected by indirect immunofluorescence using the anti-HA monoclonal antibody 12CA5. Nuclei were visualized using Hoechst 33258. Bar, 4 μm.

C

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
DNA Polymerase α																		
H.sapiens	R	Q	D	D	D	W	I	V	D	D	D	G	I	G	Y	V	E	D
X.laevis	R	Q	D	D	D	W	I	V	D	D	D	G	T	G	Y	V	E	D
D.melanogaster	K	Y	G	D	D	W	I	-	E	E	D	G	T	G	Y	A	E	D
C.elegans	R	Q	K	D	N	F	I	V	D	D	D	G	M	G	Y	V	D	N
S.pombe	L	D	E	D	D	F	V	V	D	D	D	G	A	G	Y	V	D	N
S.cerevisae	L	L	H	D	D	R	V	V	D	D	N	G	V	G	Y	V	D	R
Consensus	#	Q	*	D	D	@	I	V	D	D	D	G	-	G	Y	V	*	D
Polα domain																		
AnSepB	Q	D	M	E	D	F	V	E	D	D	D	G	A	G	Y	V	E	D
ScCtf4	F	E	D	E	E	F	I	D	D	D	D	G	A	G	Y	I	S	G
Consensus	**-**	**D**	**D**	**E**	**D**	**F**	**V**	*****	**D**	**D**	**D**	**G**	**A**	**G**	**Y**	**-**	**-**	*****

* acidic
@ aromatic
# basic

D

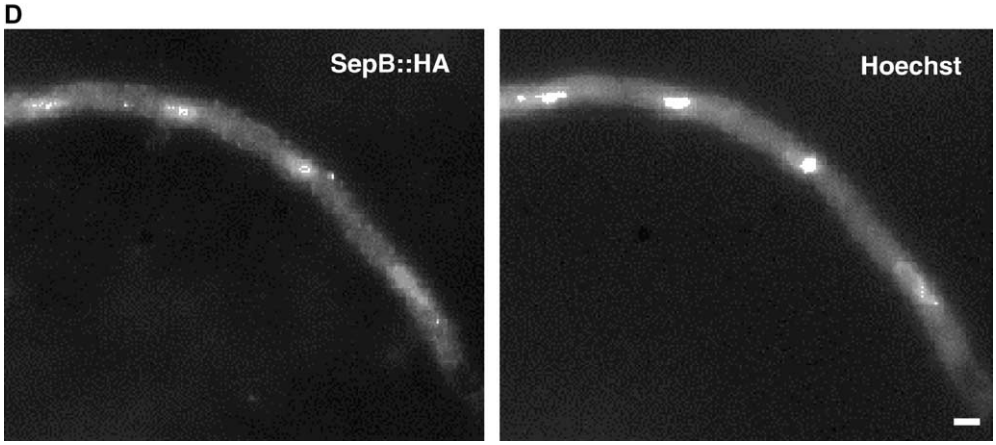

FIGURE 1.—*Continued.*

to DNA-damaging agents using dormant conidiospores, which are naturally synchronized in the G_1 phase of the cell cycle (BERGEN and MORRIS 1983). Spores were allowed to break dormancy at semipermissive temperature (37°) for 45 min in the presence or absence of DNA-damaging agents and were then plated at permissive temperature. Under these conditions, *sepB3* mutants were extremely sensitive to PLM (Figure 2), but not to MMS (data not shown). In addition, both the mycelial and dormant spore assays demonstrated that *sepB3* mutants display normal responses to UV irradiation and HU. Collectively, these observations suggest that SepB function is particularly important for the repair of PLM-induced double-strand breaks (DSBs) as cells progress through S phase. Analysis of a functional SepB-HA fusion protein expressed under control of its endogenous promoter indicated that neither its expression nor its apparent mobility is affected by exposure to PLM or other DNA-damaging agents (data not shown).

SepB is a member of the UvsC epistasis group: In *A. nidulans*, genetic analysis has revealed the existence of at least four distinct DNA repair pathways (GOLDMAN *et al.* 2002). To obtain additional insight into the potential role of SepB in DSB repair, we used the radial colony growth assay to test for epistatic interactions between *sepB3* and mutations affecting other repair pathways. These experiments showed that *sepB3 uvsC114* and *sepB3 musN227* double mutants are no more sensitive to MMS than either single mutant (Figure 2). In contrast, *sepB3* displayed additive interactions on MMS with other repair mutations such as *uvsB110* (Figure 2). Similarly, *sepB3 uvsC114* double mutants appear to display an epistatic interaction in response to PLM exposure (Figure 2). $UvsC^{RAD51}$ is the *A. nidulans* ortholog of Rad51 and is required for the repair of DNA strand breaks (VAN HEEMST *et al.* 1997). In addition, on the basis of a plasmid integration assay, *uvsC* mutants display severe defects in homologous recombination (ICHIOKA *et al.* 2001). $MusN^{RECQ}$ is the *A. nidulans* homolog of RecQ/Sgs1, and is also a member of the UvsC epistasis group (KAFER and CHAE 1994; HOFMANN and HARRIS 2001). By contrast, $UvsB^{ATR}$, which is the *A. nidulans* ATR/Mec1 homolog (HOFMANN and HARRIS 2000), does not function within this pathway (KAFER and MAYOR 1986). Accordingly, the epistatic interaction between the *sepB3*, *musN227*, and *uvsC114* mutations suggests that SepB participates in a $UvsC^{RAD51}$-mediated homologous recombination pathway required for the repair of DSBs.

SepB is required for the formation of DNA-damage-induced UvsC nuclear foci: In *S. cerevisiae*, lagging-strand replication is required after strand invasion for the repair of a DSB by homologous recombination

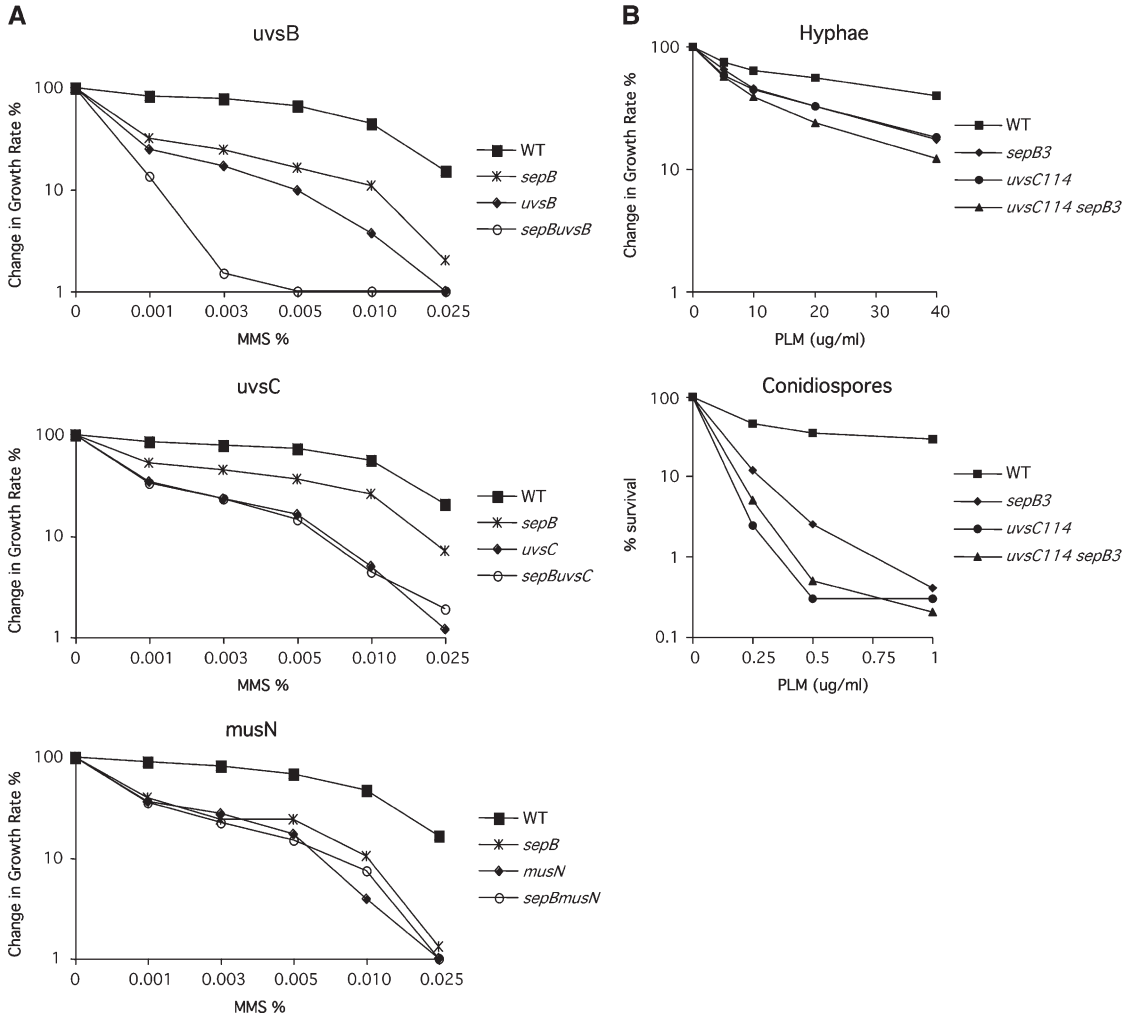

FIGURE 2.—SepB is involved in the DNA damage response. (A) Viability curves for wild-type, *sepB3*, *uvsB110*, *sepB3 uvsB110*, *musN227*, *sepB3 musN227*, *uvsC114*, and *sepB3 uvsC114* hyphae grown on media containing MMS at 35°. Growth was measured as percentage change in radial growth rate compared to an untreated CM control plate. (B) Viability curves for wild-type, *sepB3*, *uvsC114*, and *sepB3 uvsC114* hyphae (top) or conidiospores (bottom) exposed to PLM. Hyphae were grown on media containing PLM at 35° and growth was measured as percentage change in radial growth rate compared to an untreated CM control plate. Dormant conidiospores spores were suspended in sterile water and treated with the indicated doses of PLM for 45 min at 37° with constant agitation. Conidia were plated on CM + Triton X-100 plates and viability was measured as percentage survival compared to untreated controls.

(HOLMES and HABER 1999). Since Ctf4p, the yeast homolog of SepB, has been implicated in lagging-strand replication (FORMOSA and NITTIS 1999), SepB may be involved in an analogous process downstream of UvsC. Alternatively, since the yeast homologs also function in sister-chromatid cohesion (HANNA *et al.* 2001; WILLIAMS and MCINTOSH 2002), which is presumably a prerequisite for strand exchange (SJOGREN and NASMYTH 2001), SepB may function upstream of UvsC. We surmised that it might be possible to characterize the function of SepB in recombination-mediated repair by assessing its effect on $UvsC^{RAD51}$ localization. In yeast cells, Rad51 homologs form irradiation-induced subnuclear foci (GASIOR *et al.* 2001; CASPARI *et al.* 2002). To determine if $UvsC^{RAD51}$ localized to similar structures, we constructed a functional UvsC-FLAG fusion protein expressed under the control of *uvsC* promoter sequences. This protein fully complemented the UV and MMS sensitivity caused by the *uvsC114* mutation (data not shown). In untreated hyphae, UvsC-FLAG was expressed (Figure 3), but failed to localize to nuclei (Figure 4, A–D). By contrast, when hyphae were exposed to PLM (10 μg/ml for 1 hr), UvsC-FLAG localized to discrete subnuclear foci (average 1.8/nucleus; Figure 4, E, F, and I; supplemental Figure 1A at http://www.genetics.org/supplemental/) that may correspond to sites of recombination-mediated repair. When examined in a *sepB3* background, PLM-induced nuclear localization of UvsC-FLAG was severely reduced (average 0.3/nucleus, Figure 4, G–I; supplemental Figure 1A at http://www.genetics.org/supplemental/). Note that the background staining observed in the latter set of images appears to be due to interactions between

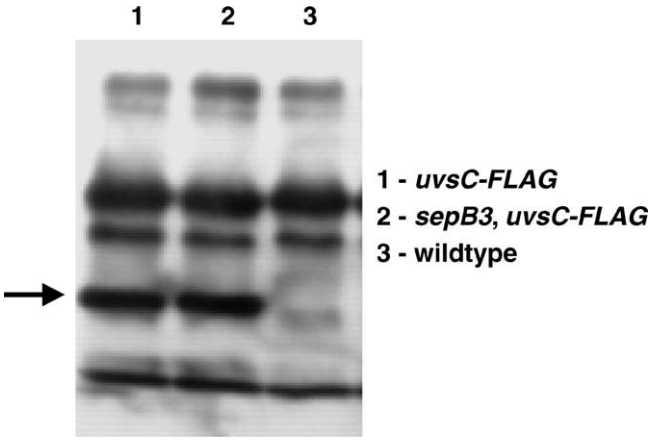

FIGURE 3.—Expression of UvsC-FLAG in wild-type and *sepB3* mutants. ASH17 (*uvsC-FLAG*), ASG19 (*uvsC-FLAG; sepB3*), and A28 (wild-type control) hyphae were incubated in YGV for 18 hr at 28°. Whole-cell extracts were precipitated with anti-FLAG agarose beads and detected by Western blot with anti-FLAG antibodies. Arrows depicts UvsC-FLAG (39 kD).

the anti-FLAG antibody and an additional epitope in *A. nidulans* (supplemental Figure 1B at http://www.genetics.org/supplemental/; also see MATERIALS AND METHODS) and likely does not reflect cytoplasmic localization of $UvsC^{RAD51}$. We also observed SepB-dependent formation of $UvsC^{RAD51}$ subnuclear foci in hyphae exposed to MMS or HU (C. SEMIGHINI, S. GYGAX and S. HARRIS, unpublished results). Since UvsC-FLAG expression was not affected by the *sepB3* mutation (Figure 3), these observations suggest that SepB is required for the localization of $UvsC^{RAD51}$ to sites of homologous recombination.

***sepB3* mutants display phenotypes consistent with defects in sister-chromatid cohesion:** In *S. cerevisiae*, mutations that affect sister-chromatid cohesion cause a characteristic set of phenotypes (*i.e.*, see HANNA *et al.* 2001). These include sensitivity to spindle-deploymerizing agents such as benomyl, which triggers prolonged metaphase arrest. In wild-type cells, sister chromatids remain aligned during the arrest and segregate properly upon recovery. By contrast, sister chromatids separate precociously in cohesion mutants, which leads to random segregation and loss of viability during recovery. In addition, cohesion mutants are typically sensitive to loss of the spindle assembly checkpoint. This presumably reflects the role of cohesins in generating the tension required to establish stable bipolar attachment of sister chromatids to microtubules (TANAKA *et al.* 2000). In the absence of this function, the spindle assembly checkpoint maintains viability by preventing premature segregation (reviewed in LEW and BURKE 2003). Because sister-chromatid cohesion defects could account for the failure to properly localize $UvsC^{RAD51}$, we screened for analogous phenotypes in *sepB3* mutants.

When incubated at semipermissive temperature (35°) on media containing BEN, *sepB3* mutants display a striking growth defect (Figure 5A). Although BEN triggers a normal metaphase arrest in *sepB3* mutants, which is reflected by the increased chromosome mitotic index (*i.e.*, the fraction of nuclei with condensed chromatin as determined by staining with Hoechst), the first mitosis during recovery appears slower relative to untreated controls (Table 2). Moreover, when *sepB3* conidiospores are treated with BEN for 6 hr and then plated at permissive temperature (28°), the fraction of viable colonies that display abnormal morphology is threefold higher compared to untreated controls (12.2% *vs.* 4.1%, Figure 5B; note that for wild-type conidiospores, the fraction is 0.2% for both control and treated samples). Because abnormal colony morphology is indicative of aneuploidy in *A. nidulans* (KAFER and UPSHALL 1973; HARRIS and HAMER 1995), this observation suggests that the *sepB3* mutation causes increased chromosome loss during recovery from spindle depolymerization.

The *sldA* and *sldB* genes encode the *A. nidulans* homologs of the spindle assembly checkpoint proteins Bub1p and Bub3p, respectively, and are required for metaphase arrest in response to spindle assembly defects (EFIMOV and MORRIS 1998). As shown in Figure 6, *sepB3* mutants exhibit a synthetic lethal interaction with deletion mutations in *sldA* and *sldB*. The double mutants fail to grow at 35° and display reduced colony formation at 32°. Notably, these interactions are more severe than those previously observed between *sepB3* and mutations that compromise the DNA damage checkpoint (HARRIS and KRAUS 1998). However, unlike the DNA damage checkpoint mutations, neither Δ*sldA* nor Δ*sldB* permits *sepB3* mutants to form septa at restrictive temperature (data not shown). Because *sepB3* mutants are sensitive to spindle-depolymerizing agents and cannot tolerate abrogation of the spindle assembly checkpoint, we propose that, like the yeast homologs Ctf4p and mcl1 (HANNA *et al.* 2001; WILLIAMS and MCINTOSH 2002), SepB may be required for sister-chromatid cohesion (see DISCUSSION for further comment).

DISCUSSION

Molecular characterization of SepB suggests that it is a conserved protein scaffold involved in multiple aspects of chromosomal DNA metabolism. We show here that SepB functions in the *A. nidulans* DNA damage response. In particular, SepB is a member of the $UvsC^{RAD51}$ epistasis group and is required for the formation of DNA-damage-induced $UvsC^{RAD51}$ foci. Because *sepB* mutants display phenotypes consistent with a defect in sister-chromatid cohesion, our results raise the interesting possibility that cohesion may have a role in Rad51 localization.

SepB family members possess multiple interaction domains: A characteristic feature of SepB/And-1 family

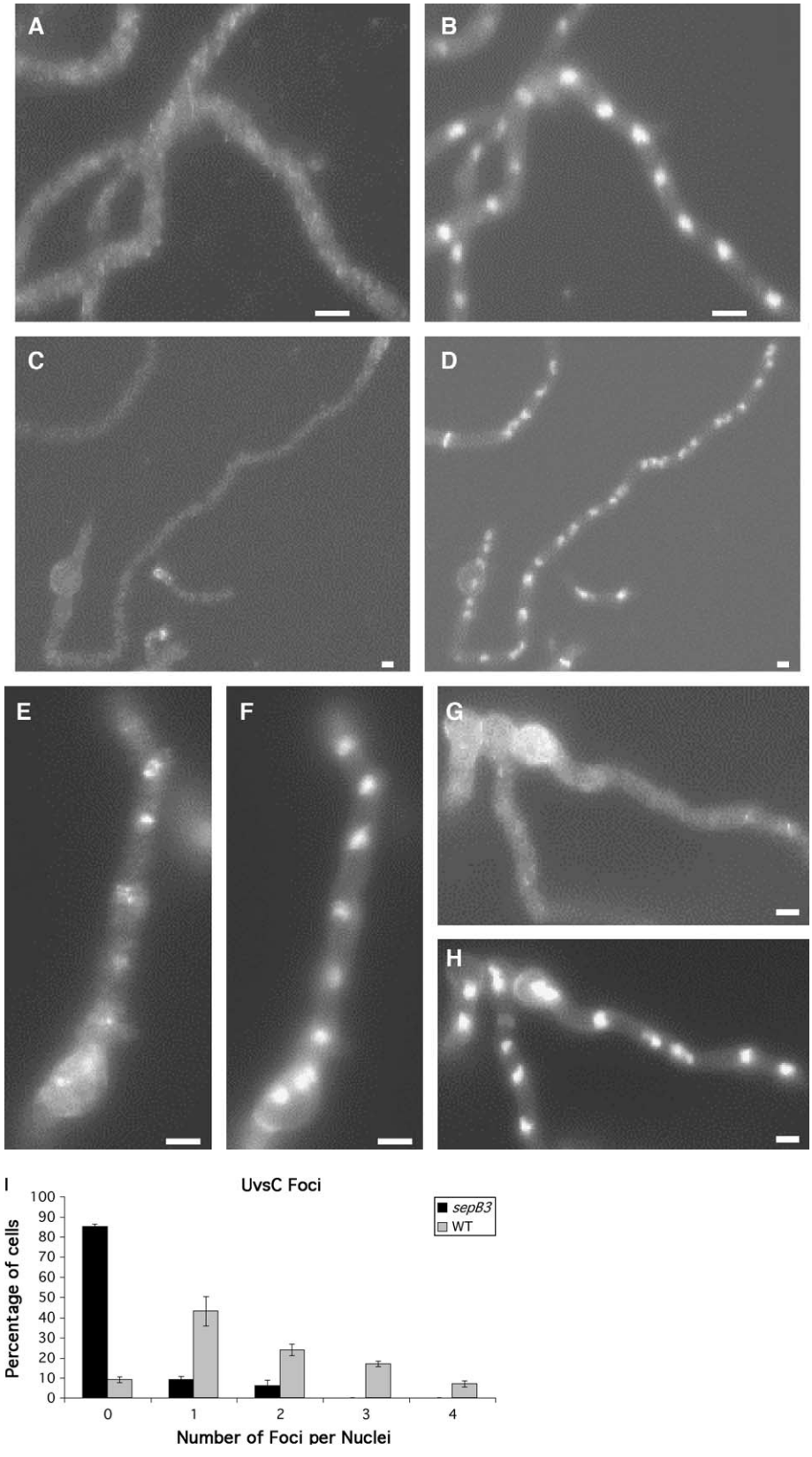

FIGURE 4.—UvsC localizes to DNA-damage-induced subnuclear foci. (A, B, E, and F) Wild-type (ASG17) hyphae. (C, D, G, and H) *sepB3* (ASG19) hyphae. Strains were grown in YGV for 14 hr at 28° and then left untreated (A–D) or exposed to 10 μg/ml PLM (E–H) and examined by immunofluorescence microscopy. UvsC-FLAG was detected using anti-FLAG antibodies (A, C, E, and G), and nuclei were stained using Hoechst 33258 (B, D, F, and H). (I) The number of UvsC-FLAG foci per nucleus was determined for wild-type (ASG-17) and *sepB3* (ASG19) hyphae treated with 10 μg/ml PLM. UvsC-FLAG was detected as described above, and hyphae were examined by immunofluorescence microscopy. For each sample, 100 nuclei were examined. Bars, 4 μm.

members is the presence of two distinctive β-propeller domains. SepB, Mcl1, and And-1, but not Ctf4p, possess five to seven degenerate WD40 repeats in their respective N termini (KOHLER *et al.* 1997; WILLIAMS and MCINTOSH 2002). In addition, all family members harbor a conserved ~300-amino-acid central region termed the

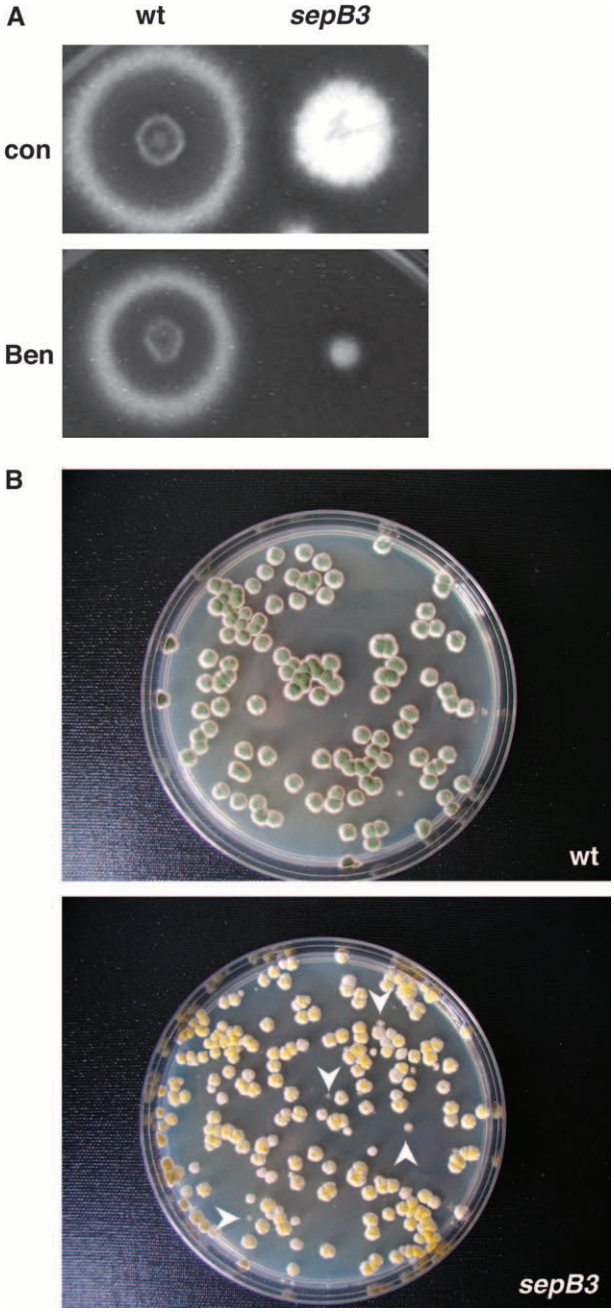

FIGURE 5.—*sepB3* mutants are sensitive to BEN. (A) Wild-type (A28) and *sepB3* (ASH60) conidiospores were patched onto MAG plates containing either 0.4 μg/ml BEN or no drug. Plates were incubated for 60 hr at 35°. (B) Increased appearance of abnormal colonies following exposure of *sepB3* mutants to Ben. Wild-type (A28) and *sepB3* (ASH60) conidiospores were germinated for 6 hr at 42° in YGV or YGV + 10 μg/ml BEN. Germlings were diluted and plated on CM + Triton X-100 and incubated at 28° for 3 days. Only plates containing BEN-treated conidiospores are shown. White arrowheads indicate colonies that display abnormal morphology.

SepB domain (WILLIAMS and MCINTOSH 2002). Analysis of SepB using two different algorithms that predict protein structure (available at http://bmerc-www.bu.edu/wdrepeat/ and http://www.bmm.icnet.uk/servers/3dpssm) revealed that the SepB domain is capable of forming a four-bladed β-propeller. Therefore, SepB/And-1 family members are largely composed of multiple β-propeller platforms that presumably mediate interactions with several different partner proteins. The extensive web of genetic interactions observed for both *ctf4* and *mcl1* mutants supports this notion (HANNA *et al.* 2001; WILLIAMS and MCINTOSH 2002; WARREN *et al.* 2004).

Unlike And-1, the fungal SepB homologs possess an acidic region that links the WD40 and SepB β-propeller platforms. This region displays striking homology to an N-terminal motif that is conserved in the catalytic subunit of DNA polymerase α from all eukaryotes (WANG *et al.* 1989). Although the role of this motif in polymerase function remains unknown, our preliminary observations show that mutations altering specific acidic residues within the motif (*i.e.*, D9 and D10 in Figure 1C) do not affect SepB function (K. CROWLEY, S. GYGAX and S. HARRIS, unpublished results). Accordingly, we propose that the crucial property of this motif is its general acidic nature. One attractive possibility is that SepB and Pol α compete for binding to a partner protein via this motif.

Role of SepB in the DNA damage response: Our observations suggest that SepB is involved in the UvsC^{RAD51}-mediated pathway that repairs DNA damage via homologous recombination. The yeast homologs of SepB appear to be particularly important for the repair of DSBs induced during S phase (MILES and FORMOSA 1992; HANNA *et al.* 2001; WILLIAMS and MCINTOSH 2002). The specific function(s) of these proteins that contribute to viability remain unclear, although involvement in lagging-strand replication or sister-chromatid cohesion are attractive possibilities (HOLMES and HABER 1999; WILLIAMS and MCINTOSH 2002). We propose that either of these functions may account for the role of SepB in homologous recombination. At this time, we have not yet determined whether, like yeast, SepB promotes repair during S phase (*i.e.*, attempts to construct double mutants that would permit synchronization in S phase have been unsuccessful). Nonetheless, several observations provide modest support for this conclusion. First, the characterization of *sepB3* mutants clearly implicates SepB in some aspect of S-phase DNA metabolism (HARRIS and HAMER 1995). Second, *sepB3* mutants are extremely sensitive to DSBs induced upon release from G_1 arrest, which, under normal conditions, are presumably repaired during the subsequent S phase. Finally, the accumulation of subnuclear UvsC^{RAD51} foci in wild-type hyphae arrested in S phase with HU is abolished by the *sepB3* mutation (S. GYGAX and S. HARRIS, unpublished results)

Links between S-phase functions and sister-chromatid cohesion: It has become increasingly apparent that sis-

TABLE 2

Effects of benomyl on nuclear division in *sepB3* mutants

Strain	Treatment	T = 6 hr (+BEN) Chromosome mitotic index	T = 6 hr (+BEN) % ≤2 nuclei	T = 10 hr (BEN rel): % ≤2 nuclei
Wild type	Con	7	62	0
	Ben	51	100	0
sepB3	Con	11	84	25
	Ben	36	100	49

Wild-type (A28) and *sepB3* (ASH60) conidiospores were germinated on coverslips at 37° in YGV or YGV + 10 μg/ml BEN. After 6 hr, one coverslip was removed (+BEN), and the remainder were washed and shifted to prewarmed YGV at 37° for an additional 4 hr (BEN release). Samples were fixed and stained with Hoechst 33258. At the 6-hr time point, the chromosome mitotic index was determined to verify that BEN triggered mitotic arrest. The experiment was performed twice, and representative results from one experiment are shown. Con, untreated control conidiospores, Ben, benomyl-treated conidiospores.

ter-chromatid cohesion is linked to functions associated with DNA replication (Carson and Christman 2001). For example, several proteins involved in replication or S-phase checkpoint activity promote sister-chromatid cohesion in *S. cerevisiae*, including Ctf4p, Mre11p, and Xrs2p (Hanna *et al.* 2001; Warren *et al.* 2004). It has been suggested that these proteins regulate the formation of a chromatin environment required for cohesion (Warren *et al.* 2004). Our observations provide preliminary support for the notion that SepB also mediates sister-chromatid cohesion, although definitive proof awaits the development of appropriate fluorescent *in situ* hybridization protocols. Nevertheless, if substantiated, this would be the first clue suggesting the existence of a previously unsuspected link between S-phase functions, sister-chromatid cohesion, and Rad51 localization. This link may be direct, whereby cohesion facilitates Rad51 localization by holding the ends of DSBs in proximity to each other (Hartsuiker *et al.* 2001; Sjogren and Nasmyth 2001; Kim *et al.* 2002). Alternatively, a common chromatin configuration generated during S phase may independently promote both cohesion and Rad51 localization (Alexiadis and Kadonaga 2002; Jaskelioff *et al.* 2003).

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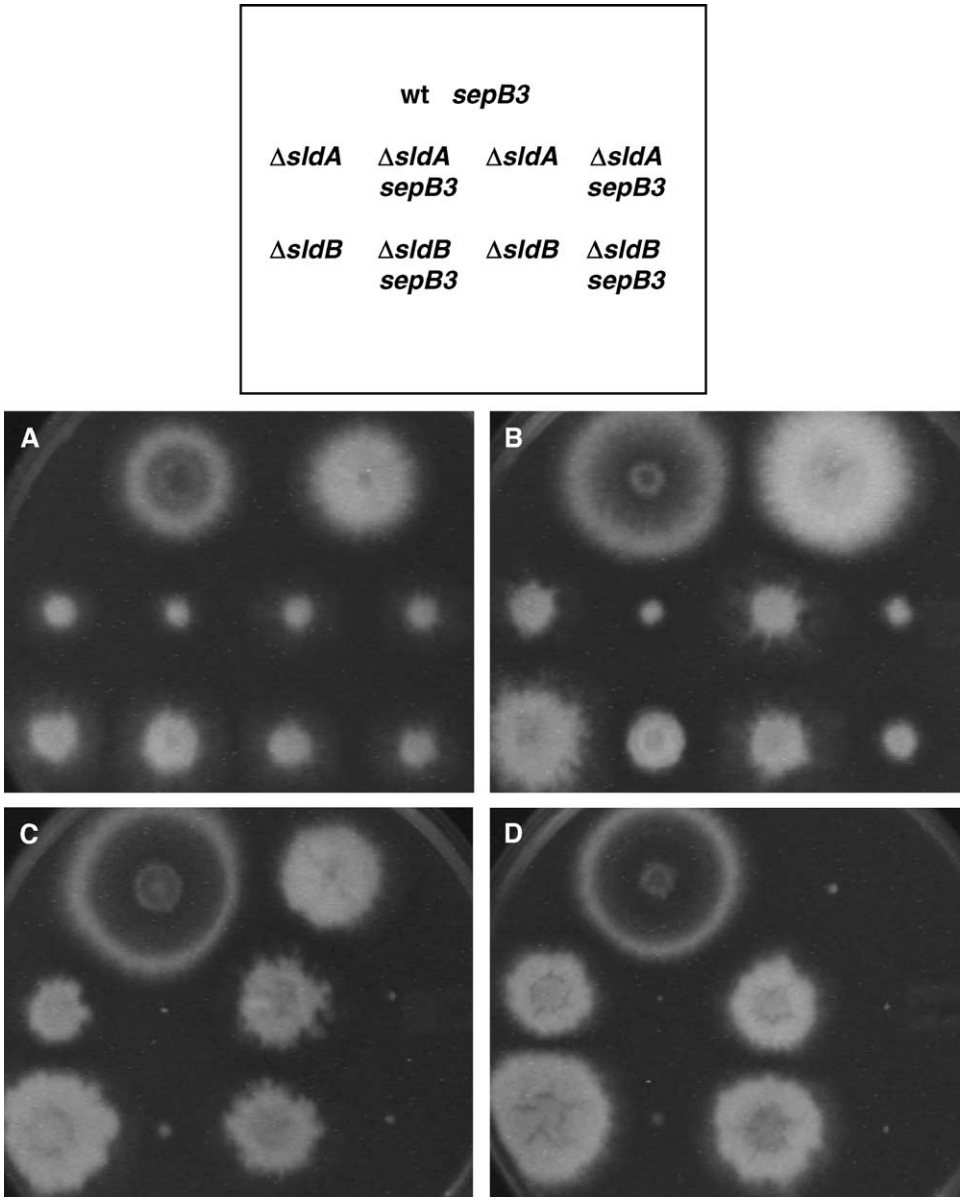

FIGURE 6.—*sepB3* displays synthetic lethal interactions with spindle assembly checkpoint mutations. Conidia were patched onto MAG plates in the order shown. The following strains were used: top row (left to right), A28 and ASH60; middle row (left to right), ASG70, ASG72, ASG76, and ASG-78; bottom row (left to right), ASG80, ASG82, ASG84, and ASG-86. Plates were incubated for 60 hr at 28° (A), 32° (B), 35° (C), or 42° (D).

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