

## SMC Protein-Dependent Chromosome Condensation during Aerial Hyphal Development in *Streptomyces*<sup>∇†</sup>

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**Members of the SMC (structural maintenance of chromosomes) protein family play a central role in higher-order chromosome dynamics from bacteria to humans. So far, studies of bacterial SMC proteins have focused only on unicellular rod-shaped organisms that divide by binary fission. The conversion of multi-genomic aerial hyphae of the mycelial organism *Streptomyces coelicolor* into chains of unigenomic spores requires the synchronous segregation of multiple chromosomes. Here we focus on the contribution of SMC proteins to sporulation-associated chromosome segregation in *S. coelicolor*. Deletion of the *smc* gene causes aberrant DNA condensation and missegregation of chromosomes (7.5% anucleate spores). In vegetative mycelium, immunostained SMC proteins were observed sporadically, while in aerial hyphae about to undergo sporulation they appeared as irregularly spaced foci which accompanied but did not colocalize with ParB complexes. Our data demonstrate that efficient chromosome segregation requires the joint action of SMC and ParB proteins. SMC proteins, similarly to ParAB and FtsZ, presumably belong to a larger group of proteins whose expression is highly induced in response to the requirement of aerial hyphal maturation.**

Higher-order chromosome structure is particularly critical in the segregation of replicated chromosomes during cell division in eubacteria, eukaryotes, and archaea. Recent studies have shown that members of the SMC (structural maintenance of chromosomes) protein family play a central role in higher-order chromosome dynamics, acting either as condensins or cohesins (15–17, 30, 32, 36, 40). SMC are large proteins (110 to 170 kDa) that form dimers. Their N- and C-terminal parts form a single head domain (see references 13, 15–17, and 38 for reviews). SMC proteins exhibit ATPase activity that is required for their dynamic interaction with DNA (17). The results of recent studies demonstrated that bacterial SMC protein functions in complexes with other proteins, i.e., ScpA and ScpB (Scp's are segregation and condensation proteins) (6, 29, 37).

In contrast to eukaryotes, in eubacteria the cell-cycle events, including replication, condensation, and segregation, take place simultaneously (16, 26). Thus, during the bacterial cell cycle, chromosomes must undergo dynamic changes. So far, studies regarding chromosome dynamics have focused on *Bacillus subtilis*, *Caulobacter crescentus*, and *Escherichia coli*. In these organisms, SMC proteins (or MukB, a functional homologue in *E. coli*) appear to play similar roles; they are involved in chromosome organization and segregation. The genes encoding these proteins are not essential, although their deletion results in temperature-sensitive growth (2, 9, 22, 31, 32, 38). In *B. subtilis*, *smc* deletion mutants exhibit complex phenotypes under permissive conditions, including poorly compacted nucleoids and a relatively large amount of anucleate cells (up to 15% of the cells were anucleate) (10). Interestingly, the

inactivation of *smc* enhances the chromosome segregation defect of *spo0J*- or *soj*-null mutants (25). The proteins encoded by these genes, Soj (ParA, an ATPase) and Spo0J (ParB, a DNA-binding protein), form a nucleoprotein complex at the *oriC* region that ensures its proper segregation. Many attempts have been made to localize SMC proteins in bacterial cells. Despite some differences in SMC protein localization, comprehensive microscopic observations in *B. subtilis* revealed that SMC proteins form foci or a punctate pattern associated with the nucleoid (42). SMC protein colocalizes with its interacting partners ScpA and ScpB, and the specific localization of SMC protein depends on both Scp proteins, showing that all three components of the SMC complex are required for proper localization (41).

Here we extend the studies of SMC proteins, which have previously focused only on unicellular rod-shaped bacteria that divide by binary fission, to the more-complex bacterium *Streptomyces coelicolor*. Streptomycetes are gram-positive multicellular sporulating bacteria that grow by tip extension and hyphal branching to form a dense mycelial network of vegetative hyphae in which septation occurs at some distance from the tips (7). Compartments of the vegetative hyphae contain several copies of the large (8 to 9 Mbp), linear uncondensed chromosome (4). Older *Streptomyces* colonies differentiate to form aerial hyphae, which subsequently develop into chains of spores (3, 4). The rapid growth of the aerial hyphae is accompanied by intensive replication of the chromosomes, such that 50 or more uncondensed nonsegregated chromosomes may be present in one long tip compartment (33). After an aerial hypha has stopped growing, a large number of FtsZ rings form a regular ladder in the long tip compartment, giving rise to sporulation septa that delimit the prespore compartments (8). At this stage, dozens of chromosomes are condensed and uniformly aligned along the hyphal tip, ensuring that each prespore compartment receives a single copy (21).

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TABLE 1. Strains used in this study

Strain	Relevant genotype or phenotype	Source or reference
<i>E. coli</i>		
DH5 $\alpha$	<i>supE44</i> $\Delta$ <i>lacU169</i> ( $\phi$ 80 <i>lacZ</i> $\Delta$ <i>M15</i> ) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Laboratory stock
BW25113/pIJ790	K-12 derivative; $\Delta$ <i>araBAD</i> $\Delta$ <i>rrhBAD</i> $\lambda^-$ Red( <i>gam bet exo</i> ) <i>cat</i> <i>araC</i> <i>rep101</i> (Ts)	12
ET12567/pUZ8002	<i>dam-13::Tn9</i> <i>dcm</i> <i>cat</i> <i>tet</i> <i>hsd zjj-201::Tn10</i> <i>tra neo</i> RP4	32a
BL21	B F $^-$ <i>dcm ompT</i> <i>hsdS</i> (r $_B^-$ m $_B^-$ ) <i>gal</i>	Stratagene
<i>S. coelicolor</i> M145		
	SCP1 $^-$ SCP2 $^-$	1
	$\Delta$ <i>parB</i> (J3305)	D. Jakimowicz, unpublished data
	$\Delta$ <i>parA</i> (J3306)	21
	<i>parB-egfp</i> (J3310)	19
	<i>dnaN-egfp</i> (J3337)	33
	<i>attBfC31::KF41</i> ( <i>ftsZ-egfp</i> ) (K202)	8
	<i>smc-egfp acc(3)IV</i> (RMD2)	5a
	pIJ8641	39
	$\Delta$ <i>smc::apra</i>	This study
	$\Delta$ <i>scpAB</i>	This study
	$\Delta$ <i>parB</i> $\Delta$ <i>smc::apra</i>	This study
	$\Delta$ <i>parA</i> $\Delta$ <i>smc::apra</i>	This study
	<i>parB-egfp</i> $\Delta$ <i>smc::apra</i>	This study

Very little is known about the orchestration of major cell cycle processes, particularly the synchronization of condensation and the segregation of several dozen chromosomes during the formation of unigenomic spores. Here we focus on the contribution of SMC proteins to sporulation-associated chromosome segregation in *S. coelicolor* A3(2), the model organism for the *Streptomyces* species.

#### MATERIALS AND METHODS

**DNA manipulation and bacterial strain growth conditions.** DNA manipulations were carried out according to standard protocols (34). Enzymes were supplied by Fermentas, New England BioLabs, or Roche; fluorescent reagents were from Invitrogen (Molecular Probes); and oligonucleotides were from The Institute of Biochemistry and Biophysics (Poland). The *S. coelicolor* and *E. coli* strains are listed in Table 1. Culture conditions, media, antibiotic concentrations, and transformation and conjugation methods followed the general procedures for *E. coli* (34) and *Streptomyces* (23). *S. coelicolor* was cultivated in Trypticase soy broth-yeast extract-malt extract (1:1) complex liquid medium or on mannitol soy flour agar plates unless otherwise stated.

**Construction of strains carrying a deletion of *smc* or *scpAB*.** A PCR-targeted gene replacement strategy (11, 12) was used for the construction of deletions of the *smc* and *scpAB* genes. Deletion of *smc* was created by replacing the *smc* gene within cosmid 7A1 with *apra-oriT*, an apramycin resistance (*Apra* $^r$ ) cassette flanked by FLP recombinase recognition target sites. The cassette was amplified from pIJ773 (12) with the pair of primers p $_{\Delta$ smc $_f$  and p $_{\Delta$ smc $_r$  (Table 2). The resulting construct, 7A1 $\Delta$ *smc::apra*, was used to transform ET12567/pUZ8002, from which it was mobilized into *S. coelicolor* M145 or other *S. coelicolor* derivatives (Table 1). The *Apra* $^r$  exconjugants were screened for the loss of Kan $^r$  (the *kan* gene is a part of cosmid 7A1 $\Delta$ *smc::apra*), indicating that there was double-crossover allelic exchange of the *smc* locus.

An in-frame *scpAB* deletion was created by using the appropriate pair of primers, p $_{\Delta$ scp $_{AB}}$  $_f$  and p $_{\Delta$ scp $_{AB}}$  $_r$ . Cosmid StI51 $\Delta$ *scpAB::apra* was digested with *Swa*I and religated. The *amp* gene in the SuperCos part of cosmid StI51 $\Delta$ *scpAB* was subsequently exchanged for an *apra-oriT* cassette, yielding StI51 $\Delta$ *scpAB* *amp::apra-oriT*. This construct was used to transform ET12567/pUZ8002 and for conjugation into *S. coelicolor* M145. The *Apra* $^r$  exconjugants were screened for the loss of both *Apra* $^r$  and Kan $^r$ , indicating a double-crossover allelic exchange of the  $\Delta$ *scpAB* locus of M145.

**Purification of SMC, ScpA, and ScpB proteins.** The vectors pET-21a and pGEX-6P-2 were chosen to overexpress the recombinant SMC protein and the fusion proteins glutathione-S-transferase (GST)-ScpA and GST-ScpB. Cosmid 7A1, which contains the entire coding region of the *S. coelicolor* *smc* gene, was used for subcloning the *smc* gene into pET-21a. The 5' end (1,135 bp) of the *smc*

gene was PCR amplified using the appropriate pair of primers, p $_{smc_5\_fw}$  and p $_{smc_5\_rv}$  (Table 2). The resulting DNA fragment was digested with *Nde*I and *Xho*I and inserted into the *Nde*I- and *Xho*I-restricted pET-21a. Afterward, the rest of the *smc* gene was subcloned into the expression vector as follows. An *Xho*I restriction site was created directly before the stop codon of *smc* on cosmid 7A1 by using PCR targeting directly, and then a 2,456-bp fragment of *smc* was cut out and subcloned into *Nco*I- and *Xho*I-restricted pET-21a carrying *smc* (1,135 bp). The *S. coelicolor* His $_6$ -SMC protein was isolated as described previously (43).

The *scpA* and *scpB* genes were PCR amplified with the appropriate pairs of primers, p $_{scpA\_fw}$  and p $_{scpA\_rv}$  and p $_{scpB\_fw}$  and p $_{scpB\_rv}$  (Table 2). The PCR products were cloned into pGEM-T Easy (Promega) and later recloned as *Bam*HI-*Xho*I fragments into pGEX-6P-2. All the PCR-derived clones were analyzed by DNA sequencing to check their fidelity. The fusion proteins GST-ScpA and GST-ScpB were purified on glutathione-Sepharose 4B as described earlier (27). For removal of the GST part, the bound fusion proteins were treated with PreScission protease (Amersham-Pharmacia-Biotech) and ScpA or ScpB protein was released from the beads. The purified SMC, ScpA, and ScpB proteins were more than 95% pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (see Fig. S1A in the supplemental material).

**Preparation of antisera.** Antisera were obtained by immunization of rabbits with the purified SMC and ScpA proteins mixed with the adjuvant monophosphoryl lipid A (a derivative of lipid A from *Hafnia alvei* PCM 1200) (5). Serum samples were taken 10 days after the second booster injection. Cellular particles were removed by centrifugation, and the serum was stored at  $-20^\circ\text{C}$ . Anti-SMC and anti-ScpA antibodies were purified by solid-phase chromatography (14).

**SDS-PAGE and Western blotting.** Proteins were separated by SDS-PAGE (10% or 12% acrylamide) as described by Laemmli (24) and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature with TBST (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% Tween 20) containing 3% bovine serum albumin (BSA) and subsequently incubated with polyclonal anti-SMC antibody. Afterwards the membrane was incubated with a goat anti-rabbit secondary antibody conjugated with alkaline phosphatase. The membrane was stained with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium.

**Dot blot overlay assay.** SMC or ScpB protein was spotted onto the nitrocellulose membrane strips. After drying, the strips were placed in a blocking solution (0.5% BSA, 0.25% gelatin, 0.5% Nonident P-40, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5) and subsequently incubated with ScpA protein. The strips were exhaustively washed with TBST buffer containing 3% BSA. Afterwards, ScpA protein was detected by a polyclonal anti-ScpA antibody as described above.

**Microscopy.** Strains used for fluorescence microscopic observations were inoculated into the acute-angle junction of coverslips inserted at an angle of  $45^\circ$

TABLE 2. Oligonucleotides used in this study

Name	Sequence <sup>a</sup>	Application
PScpA_fw PScpA_rv	<b>GGATCC</b> GTGCGGCTCGCCAAC <b>TT</b> CGAGG (BamHI) <b>CTCGAG</b> CGCCTTCCGCTCCTC <b>TT</b> CGG (XhoI)	<i>scpA</i> gene amplification
PScpB_fw PScpB_rv	<b>GGATCC</b> GTGAGTGAGCGGATCACGGAGG (BamHI) <b>CTCGAG</b> AAATCCGCTCTGTGCTGCGTC (XhoI)	<i>scpB</i> gene amplification
PDelspn_f PDelspn_r	GACGACGGTGTCTTCAAGGTGCGGCTCGCCAAC <b>T</b> TCGAGAT <b>TTAAATATTCCGGGGATCCGTCGACC</b> (SwaI) TTCCGCTCTTGTGCTGCTGCGTCCCCGGCATCCGGG GCGTCA <b>TTAAATGTAGGCTGGAGCTGCTTC</b> (SwaI)	<i>scpA</i> and <i>scpB</i> (SCO1769 and SCO1770) deletion in cosmid St151
PSmc_5_fw PSmc_5_rv	<b>CATATG</b> CACCTGAAGGCC <b>CTGACC</b> CTC (NdeI) <b>CTCGAG</b> GGCGGGCG <b>TTCC</b> TCATGGCC (XhoI, NcoI)	<i>smc</i> gene fragment amplification
PSmc_ap_fw PSmc_ap_rv	CGGTGTGTCGAAGGTCATCAGCCAGCGGTTGCGT CAGCC <b>CTCGAGTGTAGGCTGGAGCTGCTTC</b> (XhoI) AGTCAAGAGGGCGAGAGCTGTGACT <b>TT</b> CAGGGG AGCTGAAATCCGGGGATCCGTCGACC	XhoI sites before codon stop <i>smc</i> gene in cosmid 7AI
PDelsmc_f PDelsmc_r	TCGTGGAGTGAGACAGCCGAAAGGTAGAGTC CGACGGC <b>ATTCCGGGGATCCGTCGACC</b> GCTGAAGTGGTTATGTGTTCAAGTCTTGAAGAAC GAGGG <b>TTGTAGGCTGGAGCTGCTTC</b>	<i>smc</i> (SCO5577) deletion in cosmid 7AI

<sup>a</sup> Boldface indicates restriction sites, and italics indicate *apra* cassette complementary fragment.

into minimal medium containing 1% mannitol (23) and cultured for 24 to 72 h. The staining procedures were described previously (21, 35). Briefly, mycelium was fixed for 10 min with a paraformaldehyde-glutaraldehyde mixture, digested for 2 min with 2 mg ml<sup>-1</sup> lysozyme, washed with phosphate-buffered saline (PBS), and blocked with 2% BSA. For immunostaining, samples were incubated with purified antibody against SMC (1:2,500 dilution) overnight, washed six times with PBS, and then incubated for 1 h with secondary antibody (antirabbit) conjugated with Alexa Fluor 546. For DNA staining, samples were incubated with 0.1 to 1 µg ml<sup>-1</sup> 4',6'-diamidino-2-phenylindole (DAPI; Molecular Probes), and for cell wall visualization with 10 µg ml<sup>-1</sup> wheat germ agglutinin (WGA)-Texas Red or Alexa Fluor 350 conjugate (Molecular Probes). After five washes with PBS, the coverslips were mounted in Vectashield (Vector Laboratories) antifade reagent. Fluorescence microscopy was carried out using a Zeiss Axio Imager Z1 microscope equipped with a 100× objective. The relative intensities of the ParB foci (maximal pixel value with the background signal subtracted) in images of aerial hyphae of the M145 *Δsmc parB-egfp* and M145 *parB-egfp* (expressing enhanced green fluorescent protein) strains taken with the same camera settings were measured.

## RESULTS

**Identification of the *Streptomyces coelicolor smc*, *scpA*, and *scpB* homologues.** Sequence analysis identified *smc*, *scpA*, and *scpB* genes in all the *Streptomyces* chromosomes sequenced so far (<http://www.genomesonline.org/>). The *smc*, *scpA*, and *scpB* genes encode putative proteins with calculated molecular masses of 128, 30, and 24 kDa, respectively. Sequence analysis of the *S. coelicolor* chromosome indicates that the *smc* gene is presumably independently transcribed, while *scpA* and *scpB* may be cotranscribed as part of a larger cluster of genes (*scpA* being the first gene of the operon, with *scpB* and four other genes, encoding putative pseudouridine synthase, putative DNA hydrolase, putative glycohydrolase, and a hypothetical protein, downstream of this gene) (1). The SMC, ScpA, and ScpB proteins from *S. coelicolor* share extensive homology with chromosomally encoded orthologues from other bacteria (se-

quence similarities to those of *B. subtilis* are 47%, 47%, and 44%, respectively). All the SMC signature motifs (Walker A and B, ABC signature) and domain organization (coiled-coil domains, hinge) are conserved in the predicted *Streptomyces* SMC protein.

In order to test whether ScpA interacts with SMC/ScpB, as the corresponding proteins do in *B. subtilis*, a dot blot overlay experiment was performed (see Fig. S1B in the supplemental material). Purified SMC and ScpB proteins (see Materials and Methods and see Fig. S1A in the supplemental material) were spotted onto a nitrocellulose membrane, followed by incubation with ScpA. The membrane was then washed extensively and probed with anti-ScpA antibodies. Immunoreactive spots were observed for the two proteins but not for the negative controls BSA or binding buffer alone, indicating that ScpA interacts with SMC and ScpB. These interactions presumably exist in vivo as well.

**Deletion of *smc* or *scpAB* genes causes aberrant condensation and missegregation of chromosomes.** In *B. subtilis* and *C. crescentus*, deletion of the *smc* gene is not lethal, but it does cause defects in DNA compaction and chromosome segregation. In order to evaluate the importance of the *smc* and *scpAB* genes for viability, growth, and chromosome segregation in *Streptomyces*, deletion mutants were constructed.

Since the *smc* gene is supposed to be independently expressed, the insertion-deletion mutant was constructed by replacing the entire gene with the apramycin resistance cassette (see Materials and Methods). The deletion was confirmed by Southern hybridization (data not shown) and Western blotting (Fig. 1B). The resulting *Δsmc* mutant strain, *S. coelicolor*, showed no obvious defect in growth or morphology when cultured in liquid or on solid medium. However, visualization of

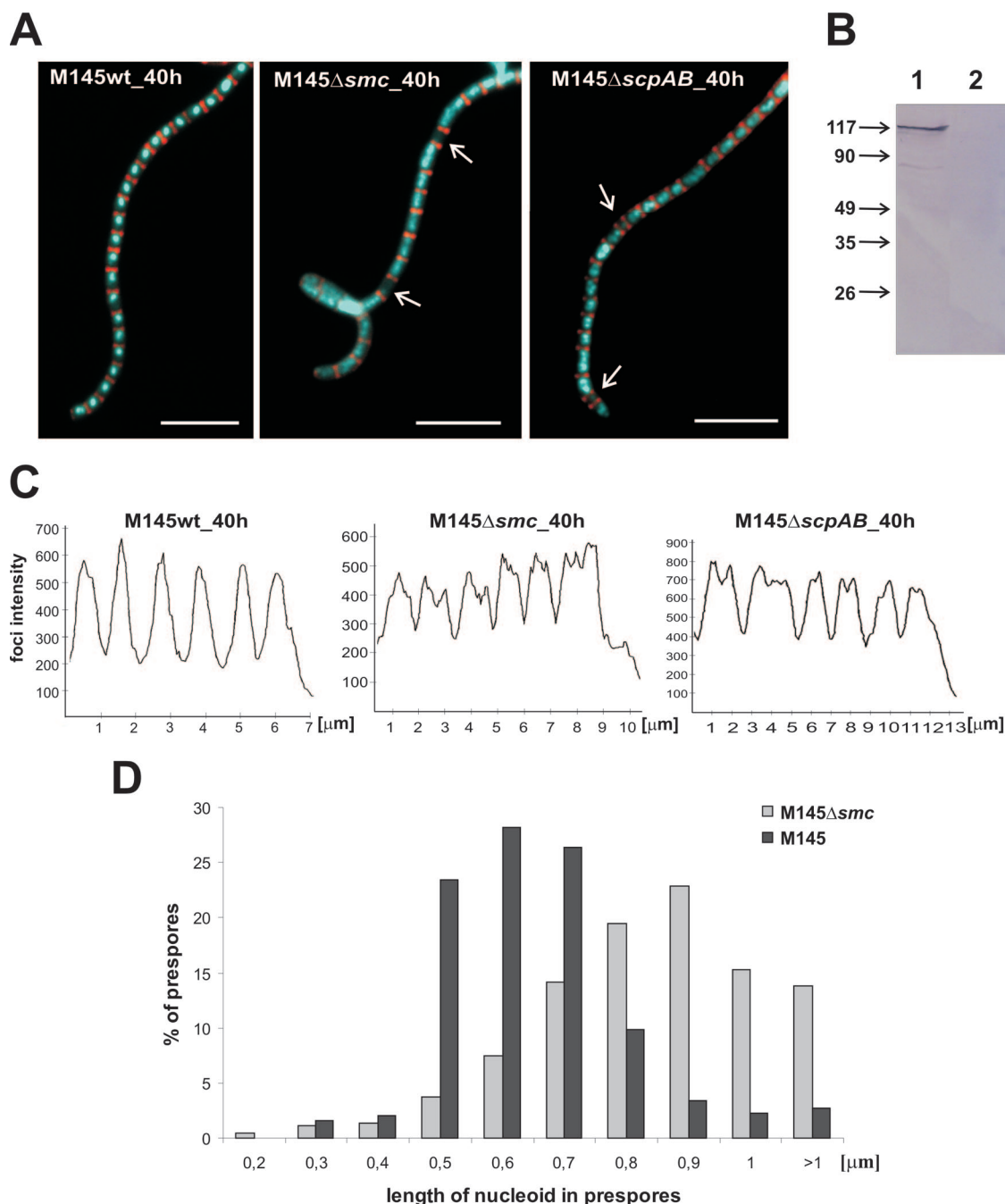


FIG. 1. Influence of *smc* or *scpAB* deletion on chromosome condensation and segregation. (A) Examples of images showing hyphae with cell walls stained with WGA (red) and DNA stained with DAPI (blue). Anucleate compartments are indicated by white arrows. Scale bar, 5  $\mu$ m. (B) Western blotting. Total proteins (20  $\mu$ g) were separated by SDS-PAGE, and then SMC protein was identified by using antibodies raised against the recombinant SMC protein. Lanes: 1, wild type; 2,  $\Delta$ *smc* mutant. Molecular sizes in kDa are shown on the left. (C) DNA signal intensity in spore compartments: fluorescent intensity of DNA stained with DAPI, presented in arbitrary units, was measured along a line drawn through the middle region of the spore compartments. (D) Percentage of prespores exhibiting different degrees of DNA compaction (length of nucleoid in prespores). wt, wild type; h, hour.

the DNA (DAPI stained) and cell walls (stained with WGA conjugated with Texas red) of sporulating hyphae revealed missegregation of chromosomes; approximately 8% of prespore compartments were anucleate. Since both the *smc* and partitioning gene (*parA* and *parB*) deletion mutants have defects in chromosome segregation, we tested the phenotypes of

double mutants  $\Delta$ *smc*  $\Delta$ *parA* and  $\Delta$ *smc*  $\Delta$ *parB*. The double mutants showed no obvious defects in morphology. The number of anucleate prespores resulting from  $\Delta$ *smc*  $\Delta$ *parB* mutations was approximately equal to the sum of the anucleate prespores observed with the two single mutations (Table 3). Interestingly, the total amount of anucleate prespores resulting

TABLE 3. Segregation defects in  $\Delta smc$  and  $\Delta scpAB$  strains

Strain or genotype	Anucleate prespores (%) <sup>a</sup>
Wild-type M145	1.4
$\Delta smc$	7.5
$\Delta scpAB$	6.3
$\Delta parA$	24.1
$\Delta parB$	19.5
$\Delta smc \Delta parB$	24.5
$\Delta smc \Delta parA$	24.7

<sup>a</sup> Calculated for 400 to 700 prespores of each mutant and the wild type.

from the double  $\Delta smc \Delta parA$  mutation was approximately the same as in the  $\Delta parA$  mutant strain (Table 3).

Deletion of *smc* also perturbed chromosome condensation in comparison with the chromosome condensation of the wild type; the  $\Delta smc$  mutant's nucleoids filled almost all of a prespore's volume and did not stain very brightly with DAPI (Fig. 1A, C, and D), whereas the nucleoids of the wild type became smaller and stained very brightly during the formation of prespores (Fig. 1A, C, and D). This suggests that the nucleoids of the  $\Delta smc$  mutant are less condensed during the early stages of sporulation (the smaller volume of mature spores made it difficult to assess chromosome condensation at later stages).

The *scpA* and *scpB* genes are supposed to be part of a larger operon. Indeed, disruption of the *scpAB* genes with the insertion of the Apra cassette caused markedly delayed growth (data not shown), suggesting perturbation in the transcription of genes that are located downstream of *scpAB* (including *scs1768*, encoding a putative pseudouridine synthase). To avoid polar effects of *scpAB* deletion on these genes, a strain carrying a nonpolar, unmarked, in-frame deletion of *scpAB* was constructed (see Materials and Methods). The *scpAB* unmarked deletion was verified by Southern hybridization. The *S. coelicolor*  $\Delta scpAB$  mutant showed a phenotype similar to that of the *S. coelicolor*  $\Delta smc$  mutant, including aberrations in chromosome condensation and segregation (Fig. 1A and C), although slightly fewer anucleate prespores were observed (6.3%).

In conclusion, these observations show that the SMC, ScpA, and ScpB proteins are involved in DNA condensation and influence chromosome segregation during sporulation.

**In aerial hyphae, SMC forms irregularly spaced foci before septation.** In *Streptomyces*, dozens of linear chromosomes have to condense and segregate accurately and synchronously during sporulation to ensure that each spore receives a single copy of the chromosome.

To localize SMC protein in the hyphae of a sporulating colony, rabbit polyclonal antibodies raised against the purified SMC protein (see Materials and Methods) were used in immunofluorescence microscopy. The affinity-purified anti-SMC antibodies reacted specifically with a protein of approximately 130 kDa that was present in a whole-cell extract of wild-type *S. coelicolor*, but they did not detect any protein when the *S. coelicolor*  $\Delta smc$  mutant strain was tested (Fig. 1B). In agreement with the Western blot analysis, immunofluorescent signals (foci) were observed in the wild-type strain but not in the *smc* deletion mutant (Fig. 2). In the vegetative mycelium, sporadic immunostaining of SMC was observed (Fig. 2), while in

the aerial hyphae, the number of foci increased as hyphae became close to full-length, reaching a maximum level before septation. At this stage, SMC protein was visualized as clusters of irregularly spaced foci. The abundance of foci was approximately seven foci/10  $\mu\text{m}$  (Fig. 2). They were observed over uncondensed DNA; however, when the DNA was condensed in prespores, SMC foci were no longer detectable (Fig. 2). The foci disappeared after septum formation (see Fig. 4 and 6).

**Correlation of SMC localization with replication and segregation.** In rod-shaped bacteria dividing by binary fission, chromosome replication, condensation, and segregation take place simultaneously; the nascent origin regions are condensed and segregated toward the cell poles soon after replication starts (26, 36, 40). In *Streptomyces*, several dozen chromosomes need to be condensed and uniformly segregated along the hyphal tip, ensuring that each prespore compartment receives a single copy (21). In order to correlate SMC localization with particular cell-cycle events (replication and segregation) and stages of aerial hyphal development, SMC protein was immunolocalized in strains which allow the monitoring of replication (DnaN-EGFP) (33) and formation of partitioning complexes (ParB-EGFP) (19).

**SMC and DnaN-EGFP foci formations are presumably spatially restricted.** Rapid growth of aerial hyphae is accompanied by intensive replication of the chromosomes, such that 50 or more nonsegregated chromosomes may be present in one long apical compartment (33). To correlate the appearance of SMC foci with ongoing replication and aerial hyphal maturation, the SMC protein was immunostained. The examination of more than 100 apical compartments ( $n = 112$ ) of the strain expressing DnaN-EGFP revealed that SMC foci did not generally colocalize with replisomes (Fig. 3). Instead, replisomes and/or SMC protein were frequently detected as separated arrays of foci representing, respectively, zones of intensive replication and/or condensation (Fig. 3). Thus, intensive replication and the formation of SMC foci appear to be mutually exclusive, presumably due to temporal separation of both processes, with the former preceding the latter.

**ParB and SMC cooperate at the stage of chromosome segregation.** In young aerial hyphae, ParB-EGFP is visible as irregularly spaced and rather weak foci. However, during subsequent steps of aerial hyphal development, ParB forms bright and regularly distributed foci (19). They appear before DNA condensation and disassemble after septum formation. To define the timing of SMC foci formation with respect to ParB complexes, a substantial number of presporulation aerial hyphae were examined ( $n = 102$ ). Apical compartments containing ParB-EGFP foci were classified as described earlier (Fig. 4B) (21). It was found that SMC foci appeared before the assembly of regular ParB complexes (Fig. 4; B1S1, 21%). During later developmental stages, the number of SMC foci increased (B1S2, 26%), reaching a "maximum" level when the regular ParB complexes were formed (B2S2, 14%). Interestingly, SMC foci disappeared after septum formation, while ParB foci were still visible (Fig. 4; B3S0 14%). Taken together, SMC is observed as irregularly spaced foci which accompany but do not colocalize with ParB complexes in aerial hyphae about to undergo sporulation. SMC foci disappeared earlier than ParB complexes.

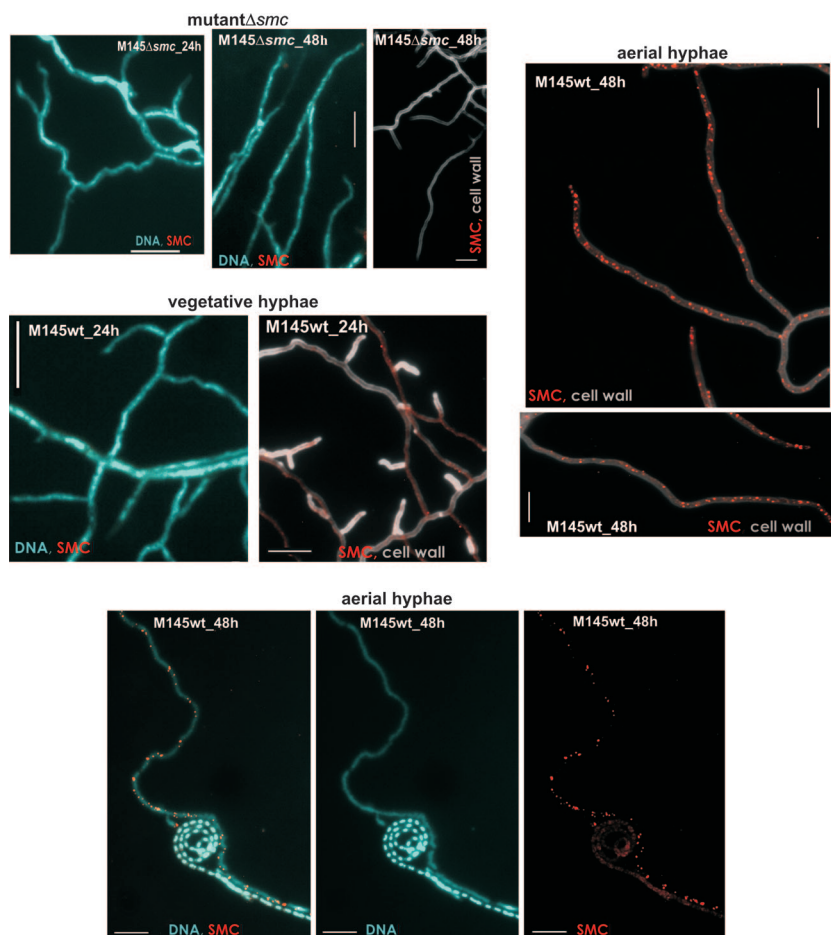


FIG. 2. Immunolocalization of SMC protein in the vegetative and aerial mycelium of the wild-type strain and in the  $\Delta smc$  mutant. Immunolabeled SMC (red), cell wall stained with WGA (gray), and DNA stained with DAPI (blue) in the mycelium of strain grown for 24 to 48 h on solid minimal medium. Scale bar, 5  $\mu\text{m}$ . wt, wild type; h, hour.

**Deletion of *smc* influences the formation of ParB segregation complexes.** To determine whether SMC protein affects the positioning and/or formation of segregation complexes, we analyzed ParB localization in an *smc* deletion strain. The *smc* deletion was introduced into a strain which expressed ParB-EGFP protein instead of the wild-type ParB. The resulting strain, *S. coelicolor parB-egfp*  $\Delta smc$ , was used to monitor microscopically the formation of ParB-EGFP complexes. As in the wild type, we observed arrays of regularly spaced ParB complexes, but they appeared smaller and less intensely fluorescent; the average signal intensity of the ParB foci in the  $\Delta smc$  mutant was about 70% of that in the wild type (see Materials and Methods and Fig. 5). The results indicate that SMC protein may facilitate the formation of large segregation complexes.

**SMC foci appear shortly before FtsZ spiral formation.** During sporulation, the key cell division protein FtsZ forms spiral-shaped filaments along hyphae that are gradually replaced by regularly spaced rings (8). In order to correlate SMC protein localization more precisely in relation to particular stages of cytokinesis, we immunostained SMC protein in strain K202, in which, in addition to FtsZ encoded by the normal chromosomal copy, FtsZ-EGFP fusion protein is expressed from an

integrated plasmid (8). Several compartments had SMC signals but no FtsZ, implying that SMC aggregation marginally preceded FtsZ aggregation (Fig. 6B; Z0S1 7.0%, and Z0S2, 5.8%). Compartments exhibiting FtsZ signals were grouped into three subclasses as described previously (21), and the abundance of SMC foci in each subclass was estimated (Fig. 6B). A substantial number of compartments contained both SMC foci and FtsZ spirals (Z1S2, 26.7%). SMC protein was also observed in compartments with some spirals and some irregular Z-rings (Z2S2, 17.4%) and in compartments where regular Z-rings were already present (Z3S2, 10.5%). The foci and the Z-rings disappeared rapidly after septum formation (sS0, 13.9%).

## DISCUSSION

In eukaryotes, major events of the chromosome cycle, i.e., replication, cohesion, condensation, and segregation, are temporally separated and occur at discrete stages of the cell cycle. In contrast, replication, condensation, and chromosome segregation take place simultaneously in rod-shaped bacteria. Not much is known about the coupling of replication, condensation, and segregation in multinucleoid compartments of filamentous multicellular bacteria, such as *Streptomyces*. Here we

M145*dnaN-egfp\_66h*

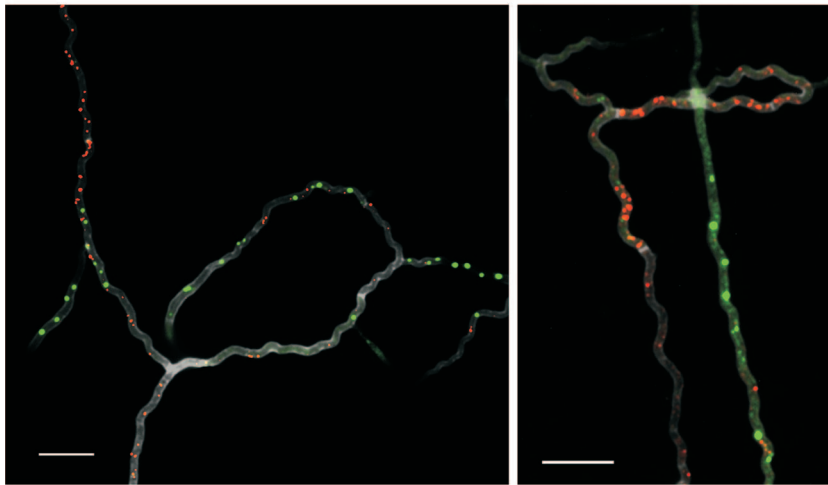
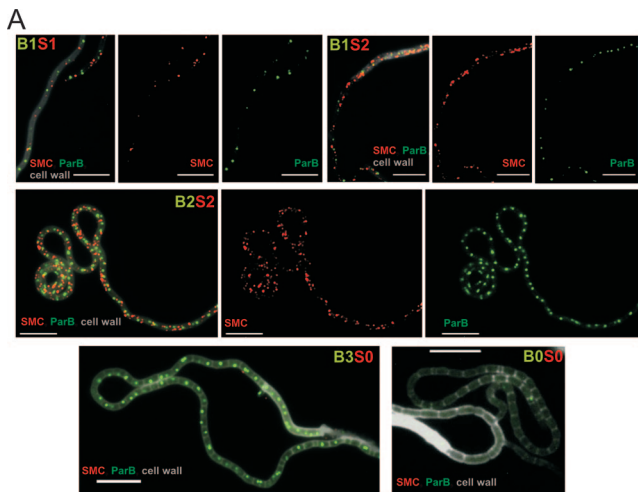


FIG. 3. Immunolocalization of SMC protein in the aerial hyphae of a strain expressing DnaN-EGFP fusion protein. The strain was grown for 66 h on solid minimal medium. SMC immunofluorescence is visible as red, DnaN-EGFP as green, and cell wall in grey scale. Scale bar, 5  $\mu$ m. h, hour.



**B**

ParB septa	SMC		
	SMC foci < ParB foci <b>S1</b>	SMC foci $\geq$ ParB foci <b>S2</b>	No signal <b>S0</b>
No regular ParB foci, no septa <b>B1</b>	21% ( <b>B1S1</b> )	26% ( <b>B1S2</b> )	2%
Regular strong ParB foci, no septa <b>B2</b>	7% ( <b>B2S1</b> )	14% ( <b>B2S2</b> )	0%
Regular ParB foci, septa <b>B3</b>	3%	0%	14% ( <b>B3S0</b> )
No ParB signal, septa <b>B0</b>	0%	0%	13% ( <b>B0S0</b> )

FIG. 4. Localization of SMC and ParB proteins in the aerial hyphae. (A) Immunolocalization of SMC protein in a strain expressing ParB-EGFP fusion protein. The strain was grown for 44 h on solid minimal medium. SMC immunofluorescence is visible as red, ParB-EGFP as green, and cell walls in grey scale. The alphanumerical abbreviations indicate developmental stages as shown in panel B. Scale bar, 5  $\mu$ m. (B) Developmental stages. Table shows percentages of aerial hyphae falling into specific developmental subclasses (100% = 102 apical compartments). Boldface indicates the most frequently observed stages.

studied the influence of *smc*, *scpA*, and *scpB* deletions on the condensation of *Streptomyces* chromosomes and attempted to relate this process to their replication and segregation during the differentiation of sporulating aerial hyphae.

Genes encoding the SMC, ScpA, and ScpB proteins are

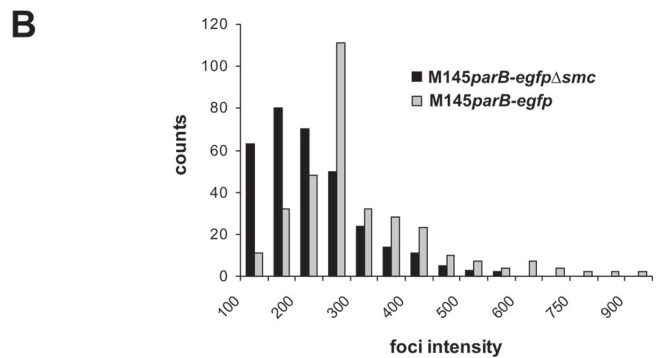
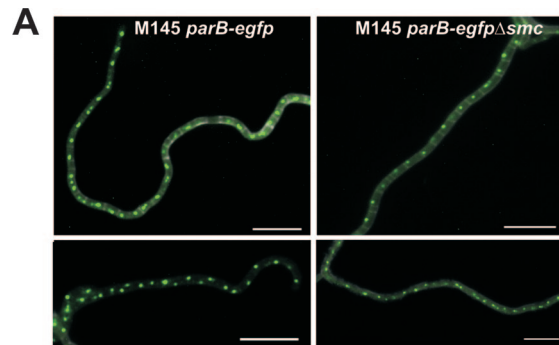


FIG. 5. Influence of *smc* deletion on ParB complex formation. (A) Examples of merged images showing ParB-EGFP (green) and cell walls stained with WGA (gray). Scale bar, 5  $\mu$ m. (B) Frequency of occurrence of ParB foci with indicated signal intensity (in arbitrary units) in aerial hyphae of strain M145 *parB-egfp* and *parB-egfp*  $\Delta$ *smc* mutants.

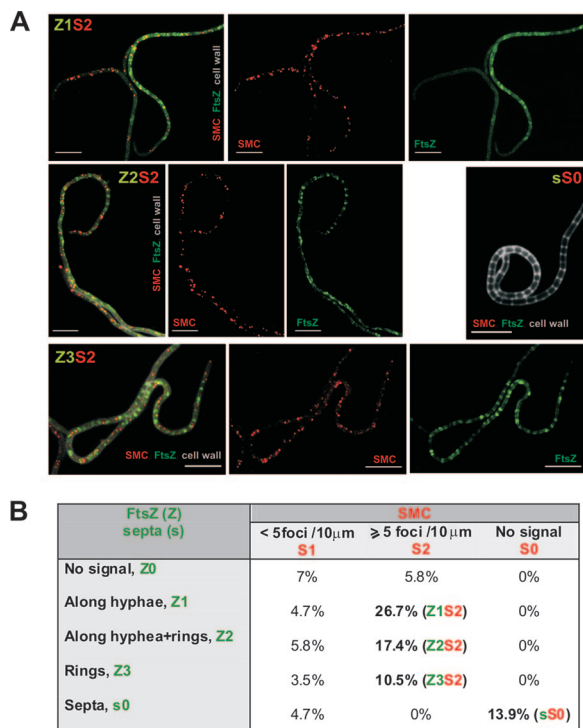


FIG. 6. Localization of SMC and FtsZ in the aerial hyphae. (A) Immunolocalization of SMC protein in a strain expressing FtsZ-EGFP fusion protein. The strain was grown for 44 h on solid minimal medium. SMC immunofluorescence is visible as red, FtsZ-EGFP as green, and cell walls in grey scale. The alphanumerical abbreviations indicate developmental stages as shown in panel B of the figure. Scale bar, 5 µm. (B) Developmental stages. Table showing percentages of aerial hyphae falling into specific developmental subclasses. (100% = 105 apical compartments). Boldface indicates the most frequently observed stages.

present in the sequenced *Streptomyces* species *S. coelicolor*, *S. avermitilis*, and *S. scabies*. As for other bacteria, the deletion of *smc* revealed that the product of this gene is not essential to *S. coelicolor*. However, *smc* deletion affects DNA condensation (Fig. 1) and correct chromosome segregation during sporulation (Table 3). The *scpAB* deletion mutant displays a phenotype similar to that of  $\Delta smc$  (Table 3). In addition, we demonstrated in vitro that the ScpA protein interacts with the ScpB and SMC proteins. Thus, the ScpA, ScpB, and SMC proteins presumably act in concert in vivo. Probably, SMC/ScpA/ScpB protein complexes interact randomly with DNA, with subsequent interactions between complexes bringing about condensation.

**Developmental-stage-specific assembly of irregularly spaced SMC complexes in aerial mycelium.** In *Streptomyces* hyphae, linear chromosomes are uncondensed during both vegetative and aerial growth and remain so until sporulation septation is completed. Using in situ immunodetection, we demonstrated that SMC foci occur sporadically in vegetative hyphae. In contrast, irregularly spaced foci are detectable over uncondensed DNA in aerial hyphae; their number increases during aerial hyphal development, reaching a maximum before septation. At this stage, the number of SMC foci was about equal to or slightly exceeded the number of ParB complexes (Fig. 4). Since

each ParB complex is bound to a single chromosome (21), we can assume that statistically there is one SMC focus per nucleoid. In contrast to ParB complexes, the SMC foci are not regularly positioned. Like other SMC proteins, *S. coelicolor* SMC protein presumably does not exhibit any sequence specificity. Thus, we assume that SMC molecules bind randomly to a few distinct regions and, by bringing them into close proximity, form a condensation center, i.e., a condensome. The foci presumably reflect ongoing DNA condensation. Indeed, the lack of SMC protein causes inefficient condensation of chromosomes in prespores (Fig. 1).

The presence of SMC foci is presumably a direct consequence of increased SMC protein production (see Fig. S2 in the supplemental material) in aerial hyphae; *smc* transcription is probably developmentally controlled. This is consistent with the idea that there is a need for a greater amount of SMC protein at the time of sporulation; several dozen chromosomes have to be condensed simultaneously in order to fit into tiny prespore compartments. SMC complexes are transiently localized at the time of sporulation, when DNA has to be compacted and segregated. Thus, SMC, like FtsZ (8) and ParAB (19, 20), seems to belong to the larger group of proteins whose expression is highly induced in response to the requirement of aerial hyphal maturation. The SMC foci, like FtsZ and ParAB, disappear after septation. In *B. subtilis*, when the cells enter the stationary phase, SMC protein is subject to induced proteolysis (28). A future question will be to identify the agents of this presumed proteolysis during spore maturation, as well as their mechanisms of activation.

**Efficient chromosome segregation requires the joint action of ParB and SMC.** In addition to the effect of *smc* and *scpAB* deletion on DNA condensation, our observations suggest a role for SMC/ScpA/ScpB complexes in accurate chromosome segregation: the elimination of any of these genes resulted in the relatively frequent formation of anucleate prespores (about 7.5%) compared with their rate of formation in the wild-type strain (1.4%). Moreover, in the  $\Delta smc$  mutant, the ParB foci were smaller and less intensive, although the ParB-EGFP protein was produced at about wild-type levels (data not shown). In *S. coelicolor*, ParB binds to numerous *parS* sites scattered over a 400-kbp chromosomal segment containing *oriC* and assembles into a large nucleoprotein complex that is necessary for proper chromosome segregation (18, 19). Probably, condensation brought about by SMC/ScpA/ScpB protein complexes is needed for the effective formation of segregosomes, perhaps by bringing *parS* sequences closer together by condensing the DNA around the *oriC* region. The resulting nucleoprotein complexes may enhance ParB oligomerization. Condensomes and segregosomes do not colocalize, but they appear at nearly the same time, at the beginning of aerial hyphal maturation. In contrast to *B. subtilis* (31), deletion of the *smc* gene does not influence the positioning of the regularly spaced ParB complexes. Interestingly, in *S. coelicolor* the Z-rings and, consequently, septa are also not mispositioned in the  $\Delta smc$  mutant—measurements did not reveal a statistically significant difference in spacing between Z-rings and septa compared with that in the wild type (data not shown). In *S. coelicolor*, ParA provides a scaffolding for the proper distribution of ParB complexes and controls the synchronized segregation of several dozen chromosomes, possibly mediating a segregation



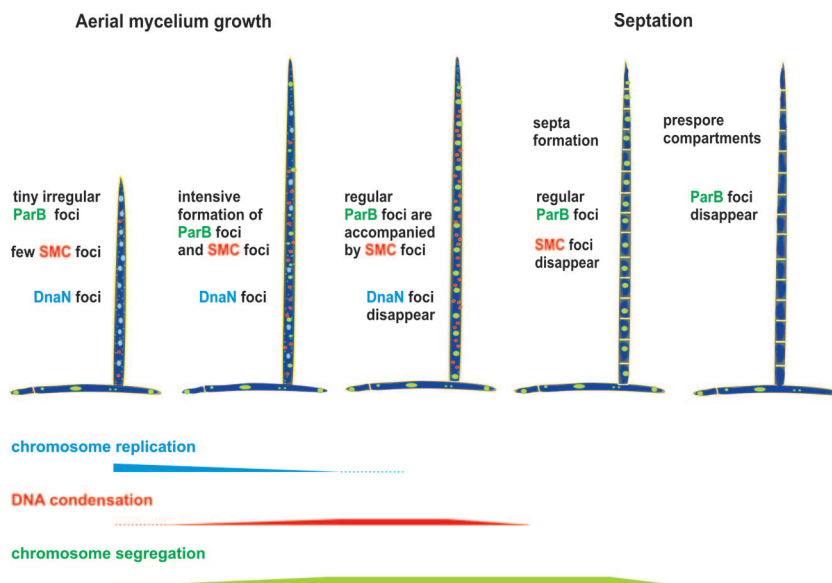


FIG. 7. Sequential stages of aerial hypha development with respect to replication, condensation, and segregation of chromosomes.

and septation checkpoint. On the basis of our current and previous results (21), we suggest that the formation of the ParA scaffolding precedes chromosome condensation and segregation events, and therefore, deletion of the *smc* gene in the  $\Delta parA$  mutant does not significantly increase the number of anucleate prespores. In contrast to *Streptomyces*, the deletion of *B. subtilis smc* unmasked a role for Soj in chromosome segregation; a *soj*-null mutation dramatically enhanced the production of anucleate cells in an *smc*-null mutant (25). In *S. coelicolor*, ParA probably promotes ParB binding to *parS* sites, possibly by enhancing oligomerization (21), while SMC-dependent chromosome condensation may help in clustering ParB binding sites.

**Sequential stages of aerial hyphal development with respect to the replication, condensation, and segregation of chromosomes.** In rod-shaped bacteria, such as *B. subtilis* and *E. coli*, SMC protein (MukB in *E. coli*) is present within defined bipolar foci, one in each cell half, while the replication machinery is located mid-cell. Newly replicated DNA, which moves from the central part of the cell toward opposite poles, is probably condensed within SMC centers. In contrast, in vegetative as well as in young and short aerial hyphae of *Streptomyces*, chromosome replication is not accompanied by condensation: the chromosomes remain uncondensed until the aerial hyphae are converted into prespores. The rapid growth of the aerial hyphae requires intensive chromosome replication, and our results suggest that at this stage of growth there is no chromosome condensation machinery (we could observe separate zones of intensive replication and condensomes). A decrease in replication intensity and an increase in the formation of condensomes was accompanied by the formation of segregation complexes and could be observed before Z-ring and septum formation (Fig. 6). Thus, in *Streptomyces*, all three processes, chromosome replication, condensation, and segregation, only partially overlap during aerial hyphal maturation, and they all precede Z-ring formation (Fig. 7).

**Addendum.** In related work, Dedrick et al. (5a) have obtained similar results for the segregation phenotype of an *smc* mutant and SMC localization.

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