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SmeA, a small membrane protein with multiple functions in *Streptomyces* sporulation including targeting of a SpoIIIE/FtsK-like protein to cell division septa

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Summary

Sporulation in aerial hyphae of *Streptomyces coelicolor* involves profound changes in regulation of fundamental morphogenetic and cell cycle processes to convert the filamentous and multinucleoid cells to small unigenomic spores. Here, a novel sporulation locus consisting of *smeA* (encoding a small putative membrane protein) and *sffA* (encoding a SpoIIIE/FtsK-family protein) is characterized. Deletion of *smeA-sffA* gave rise to pleiotropic effects on spore maturation, and influenced the segregation of chromosomes and placement of septa during sporulation. Both *smeA* and *sffA* were expressed specifically in apical cells of sporogenic aerial hyphae simultaneously with or slightly after Z-ring assembly. The presence of *smeA*-like genes in streptomycete chromosomes, plasmids and transposons, often paired with a gene for a SpoIIIE/FtsK- or Tra-like protein, indicates that SmeA and SffA functions might be related to DNA transfer. During spore development SffA accumulated specifically at sporulation septa where it colocalized with FtsK. However, *sffA* did not show redundancy with *ftsK*, and SffA function appeared distinct from the DNA translocase activity displayed by FtsK during closure of sporulation septa. The septal localization of SffA was dependent on SmeA, suggesting that SmeA may act as an assembly factor for SffA and possibly other proteins required during spore maturation.

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Introduction

The life cycle of streptomycetes is one of the most complex developmental processes among prokaryotes. A germinating spore grows out to form a vegetative mycelium of branched hyphae. Nutrient limitation and possibly other stimuli then trigger a complex multicellular development, which includes the emergence of a specialized aerial mycelium on the surface of the colony and the production of antibiotics and other secondary metabolites (Chater and Losick, 1997; Elliot *et al.*, 2007). Subsequently, a profound reprogramming of regulation and modification of fundamental morphogenetic and cell cycle processes take place in the aerial hyphae, leading to the differentiation of these filamentous, multinucleoid cells into chains of unigenomic spores that have thick cell walls and low metabolic activity, permitting dispersal and long-term survival.

A key morphogenetic event in the sporulation of aerial hyphae is the switch in the mode of cell division from the widely spaced thin crosswalls, or vegetative septa, that delimit the multinucleoid hyphal cells in the substrate mycelium to sporulation septation, which partitions the sporogenic aerial hyphal cell into prespores (Chater and Losick, 1997; Chater, 2000). Sporulation septa are thicker, regularly spaced between every nucleoid, and lead to deep cell wall constriction and eventually separation of daughter cells (Wildermuth and Hopwood, 1970; Flärdh and Van Wezel, 2003). Sporulation requires a developmentally induced upregulation of *ftsZ* (Flärdh *et al.*, 2000), encoding the tubulin homologue that directs bacterial cell division (Margolin, 2005). FtsZ then polymerizes into helical filaments that are remodelled to form a series of regularly spaced FtsZ rings along the sporulating hyphal cell, and subsequently organize the formation of sporulation septa (Schwedock *et al.*, 1997; Grantcharova *et al.*, 2005). Simultaneously, mechanisms for segregation of chromosomes have to be activated in order to ensure that each spore inherits one copy of the genome.

The ParA and ParB proteins, which are similar to proteins involved in chromosome and plasmid partitioning in many other bacteria, have a role in developmentally controlled nucleoid partitioning in *Streptomyces coelicolor* (Kim *et al*., 2000; Jakimowicz *et al*., 2006). However, the relatively mild phenotype caused by the lack of ParA and ParB (only about 13% of the spores contained aberrant amounts of DNA) implies that additional proteins are involved (Kim *et al*., 2000). It has been previously reported that the constriction of sporulation septa in streptomycetes initiates before nucleoid partitioning is complete (Schwedock *et al*., 1997; Miguelez *et al*., 1998; Flärdh, 2003; Noens *et al*., 2005), and thus, some mechanism for transport or movement of DNA through the closing septa must exist. One candidate likely to be involved in this process is the *S. coelicolor* FtsK protein (SCO5750), which is homologous to the SpoIIIE/FtsK DNA translocases (Errington *et al*., 2001). Indeed, FtsK was recently reported to localize at sporulation septa and to affect the integrity of chromosomes after sporulation in *S. coelicolor*, suggesting that it may be involved in sporulation-specific genome segregation (Wang *et al.*, 2007). However, as the majority of spores of the *ftsK* null mutant contained intact chromosomes, other mechanisms are likely to be involved as well.

Some of the key regulators of the development of aerial hyphae into spores in *S. coelicolor* are known, but our overall understanding of the pathways and mechanisms is still fragmentary. Commitment to sporulation requires the RNA polymerase sigma factor WhiG (Chater *et al*., 1989). In the absence of *whiG*, the aerial hyphae remain straight and form widely spaced vegetative-type hyphal crosswalls (Flärdh *et al*., 1999). Two target genes under the control of *whiG* have been characterized, *whiH* encoding a GntR-family repressor and *whiI* encoding a response regulator (Ryding *et al*., 1998; Ainsa *et al*., 1999). *whiH* and *whiI* are both required for efficient sporulation septation, but the respective mutants have different phenotypes (Ainsa *et al*., 1999; Flärdh *et al*., 1999; Tian *et al*., 2007). The genes *whiA* and *whiB* constitute a second converging pathway in the control of sporulation in aerial hyphae and are both required for sporulation septation in the aerial hyphae (Chater, 1972; Flärdh *et al*., 1999; Ainsa *et al*., 2000). Orthologues of WhiA are ubiquitous among Gram-positive bacterial genomes, and WhiB is the founding member of a large family of *Actinobacteria*-specific proteins (Soliveri *et al*., 2000; Gao *et al*., 2006). Both *whiA* and *whiB* are upregulated during sporulation in a *whiG*-independent fashion (Soliveri *et al*., 1992; Ainsa *et al*., 1999), but their exact functions are unclear. These five early *whi* genes are all required for full sporulation septation, most spore maturation processes, and the developmental upregulation of a number of genes including *ftsZ*, *parAB* and *sigF* (encoding a σ factor that affects spore maturation), as well as *ssgB* (which encodes a member of the SsgA-like family of proteins that appear to modify peptidoglycan/cell wall during sporulation) and the genes of the *whiE* cluster (which encode the biosynthetic genes for the grey spore pigment) (Kelemen *et al*., 1996; 1998; Flärdh *et al*., 2000; Chater, 2001; Sevcikova and Kormanec, 2003; Noens *et al*., 2005; Jakimowicz *et al*., 2006). Apart from some σ^{WhiG} regulon members, no direct targets for any of the sporulation regulatory proteins have been identified, and it is still an open question as to how these early regulatory genes can trigger the profound morphogenetic changes taking place during the differentiation of aerial hyphae into spores.

In this article, we characterize a novel locus whose expression depends on both *whiA* and the *whiG*/*whiI*/*whiH* pathway, and is constrained to the sporogenic aerial hyphae at a time coinciding with the assembly of multiple FtsZ rings. The locus consists of two genes with new roles in sporulation: one encodes a small putative membrane protein of unknown function (SmeA) and the other a member of the SpoIIIE/FtsK family (SffA). We show that deletion of these genes has a pleiotropic effect on spore development, and that *smeA* is required for the specific targeting of SffA to the sporulation septa. SffA influences the accuracy of nucleoid partitioning, but has a different function from that of FtsK.

Results

smeA-sffA *(SCO1415-16) constitutes a novel sporulation locus*

In order to identify new genes involved in spore development, and to further elucidate the regulatory networks controlling spore formation in *S. coelicolor*, we have analysed gene expression profiles of the wild-type strain M145 and sporulation-deficient mutant strains throughout development using a whole-genome microarray approach (comprehensive results will be published elsewhere, P. Salerno *et al.*, in preparation). In a pilot microarray experiment we compared the developmental gene expression profile of the wild-type strain with those of its congenic mutants lacking *whiG*, *whiA*, *whiH* and *whiI* to identify genes that were upregulated during development in the parent strain, but failed to be upregulated in at least one of the mutants. This initial analysis detected activation of several known sporulation genes (Fig. S1), including open reading frames (ORFs) in the *whiE* cluster and *ssgB* (Kelemen *et al*., 1998; Kormanec and Sevcikova, 2002). In addition, it revealed a number of previously unrecognized genes which appeared to have sporulation-specific expression patterns (to be described elsewhere), including a putative operon consisting of genes SCO1415 and SCO1416, whose expression was upregulated in the later

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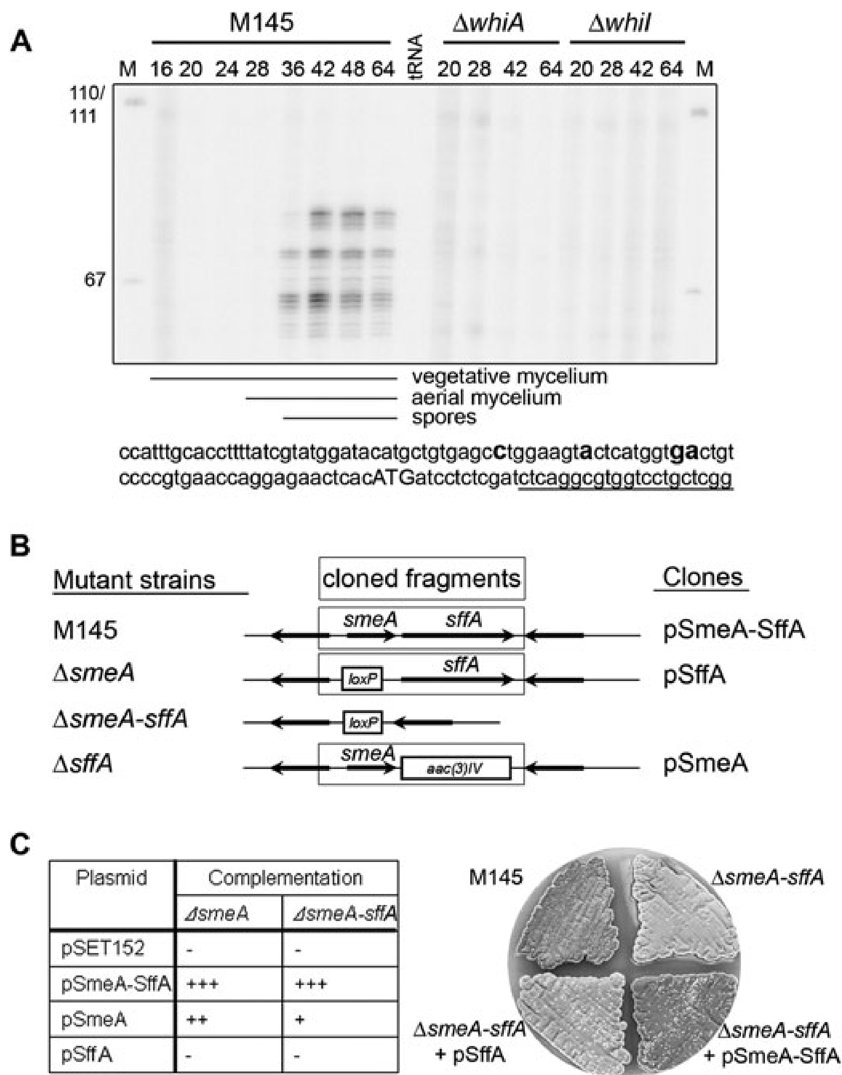

Plasmid	Complementation	
	ΔsmeA	ΔsmeA-sffA
pSET152	-	-
pSmeA-SffA	+++	+++
pSmeA	++	+
pSffA	-	-

Fig. 1. A. S1 nuclease protection analysis of transcription of *smeA* during development on SM agar plates in the wild-type strain M145 and in the non-sporulating *whiA* and *whiI* mutants. Numbers above indicate time in hours of development. M designates a lane containing a DNA size marker, and tRNA indicates a control reaction with an equal amount of yeast tRNA. Below is shown the beginning of *smeA* open reading frame with upstream region. Capital letters indicate the predicted *smeA* start codon, bold letters indicate the first 5′ nucleotides of the mRNA transcripts identified by 5′RACE, and the primer sequence used for S1 assays is underlined. The start point of the shortest transcript could not be exactly assigned to the G or A as polyC tailing of the cDNA was used. A terminal G on the mRNA could therefore not be distinguished from the added stretch of C on the complementary strand.
B. Schematic presentation of the *smeA-sffA* locus, deletion mutants and clones used in phenotypic studies and for complementation. LoxP indicates in-frame deletion of *smeA* or *smeA-sffA*, and *aac(3)IV* indicates replacement of *sffA* by an apramycin resistance cassette. Plasmids pSmeA-SffA, pSffA and pSmeA correspond to plasmids pNA276, pNA277 and pNA485 respectively.
C. Left: complementation of the light grey spore phenotype of the Δ*smeA* and the Δ*smeA-sffA* strains by *smeA*, *sffA* and *smeA-sffA in trans*. Triple plus symbol (+++) designates restoration of spore colour to the wild-type level; single plus (+) and double plus (++) symbols indicate spore colour that is darker than that of the mutant but lighter than wild-type colour. Right: photograph of a 6-day-old MS plate containing the following strains: wild-type M145, Δ*smeA-sffA*, Δ*smeA-sffA* with *smeA-sffA in trans* and Δ*smeA-sffA* with only *sffA in trans*.

stages of development in the wild-type strain but not in any of the *whi* mutant strains examined (Fig. S1). SCO1415 encoded a small protein (63 amino acids) and was designated *smeA* (small membrane protein). *In silico* analysis of SmeA revealed a centrally located putative transmembrane domain and indicated a putative N-terminal signal peptide, but did not reveal any motifs to infer a possible cellular function (see *Discussion* and Fig. 6) (Cserzo *et al.*, 1997; Nielsen *et al.*, 1997). Nevertheless, BLAST and PSI-BLAST searches revealed that similar proteins are widespread among *Streptomyces* and *Frankia* (see *Discussion*). SCO1416 encoded a protein with an N-terminal transmembrane region and a C-terminal domain with similarity to ATPase domains of SpoIIIE/FtsK-family proteins, and as such, was termed *sffA* (Figs S2 and S3).

To confirm the expression pattern revealed by the microarray experiment, transcription of *smeA* was investigated using S1 nuclease protection assays on independent RNA preparations. The largest protected fragments corresponded to a transcription start site ~40–50 bp upstream of the *smeA* start codon, and transcripts were seen only in samples from time points at which visible signs of sporulation were detected (Fig. 1A), confirming that transcription of *smeA* is activated during sporulation in *S. coelicolor*. No *smeA* transcripts were detected in samples from the *whiG*, *whiA* and *whiI* mutants, and only very faint bands were seen in the *whiH* mutant (Fig. 1A and data not shown), indicating that *smeA* expression was completely dependent on the products of *whiG*, *whiA* and *whiI*, and strongly affected by the *whiH* gene product. The *smeA* mRNA 5′ ends were also amplified and cloned using the rapid amplification of 5′ cDNA ends (5′RACE) method, from RNA prepared independently of a sporulating wild-type culture. Of 10 sequenced inserts, six showed an mRNA start point at 28 or 29 bp upstream of the predicted start codon for *smeA*, two started at 38 bp and two at 46 bp from the start codon (Fig. 1A, bottom). This

Table 1. Strains and plasmids.

Strain/plasmid	Description	Source
Strain		
S. coelicolor		
J1984	M145 *sigF::tsr*	Kelemen *et al.* (1998)
J2400	M145 *whiG::hyg*	Flärdh *et al.* (1999)
J2401	M145 *whiA::hyg*	Flärdh *et al.* (1999)
J2408	M145 Δ*whiH::ermE*	Flärdh *et al.* (1999)
J2450	M145 *whiI::hyg*	Ainsa *et al.* (1999)
K102	M145 Δ*glk-esp119 ftsZ17*(Spo)	Grantcharova *et al.* (2003)
K127	M145 *ftsZ::*pKF40[Φ(*ftsZ–egfp*)Hyb], merodiploid strain, contains both *ftsZ* and *ftsZ–egfp*	K. Flärdh Grantcharova *et al.* (2005)
K150	M145 Δ*smeA::loxP*	This study
K151	M145 Δ*smeA-sffA::loxP*	This study
K152	M145 Δ*sffA::[aac(3)IV oriT]*	This study
K158	M145 *sffA::*pNA303[Φ(*sffA–egfp*)Hyb], *sffA* is replaced by *sffA–egfp*	This study
K159	M145 Δ*smeA::loxP sffA::*pNA303[ϕ(*sffA–egfp*)Hyb]	This study
K161	M145 Δ*ftsK::[aac(3)IV oriT]*	This study
K169	M145 Δ*smeA-sffA::loxP* Δ*ftsK::[aac(3)IV oriT]*	This study
K170	M145 *ftsK*::pNA585 [Φ(*ftsK–mCherry*)Hyb]	This study
K171	M145 Δ*smeA::loxP ftsK*::pNA585 [F(*ftsK–mCherry*)Hyb]	This study
M145	Plasmid-free prototroph	Kieser *et al.* (2000)
E. coli		
DH5a	Cloning strain	
DY380	Δ(*mrr-hsdRMS-mcrBC*) *mcrA recA1* λ *cI857* Δ(*cro-bio*)<>*tet*, for PCR-targeted mutagenesis	Lee *et al.* (2001)
GM2929	*dam-13*::Tn*9 dcm-6 hsdR2 recF143 galK2 galT22 ara-14 lacY1 xyl-5 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtl-1 glnV44 leuB6 rfbD*	M. Marinus
EKF104	LE392 *lacZ::(cI857 λp-cre)*, expressing Cre recombinase for excision of *loxP* flanked cassettes	This study
ET12567/pUZ8002	*dam-13::*Tn*9 dcm-6 hsdM*, carries RK2 derivative with defective *oriT* for plasmid mobilization	Kieser *et al.* (2000)
LE392	*supF supE hsdR galK trpR metB lacY tonA*	
TOP10	Cloning strain	Invitrogen
Plasmid		
pIJ82	Hygromycin-resistant derivative of pSET152	Helen Kieser, JIC, Norwich, UK
pNA276 (pSmeA-SffA)	pSET152 containing *smeA* and *sffA* with upstream region	This study
pNA277 (pSffA)	pSET152 containing *sffA* with upstream region where *smeA* is replaced by *loxP*	This study
pNA303 (pSffA–GFP)	Plasmid pEGFP-N2 encoding the C-terminal part of SffA fused to EGFP	This study
pNA473 (pPsmeA–mCh)	pSET152 containing the promoter region of *smeA* translationally fused to *mCherry*	This study
pNA485 (pSmeA)	pIJ82 containing *smeA* with upstream region and *aac(3)IV* which replaces *sffA*	This study
pNA540 (pPsigF–mCh)	pSET152 containing the promoter region of *sigF* translationally fused to *mCherry*	This study
pNA547	pSET152 containing *smeA* with internally inserted *mCherry* and *sffA*	This study
pNA585	Plasmid pEGFP-N2 encoding the C-terminal part of FtsK fused to mCherry and with *egfp* deleted	This study
pSET152	*E. coli–S. coelicolor* shuttle vector, apramycin resistance	Bierman *et al.* (1992)

corresponded very well with the sizes of the fragments detected in the S1 nuclease protection assay. However, it is unclear whether the multiple 5′ ends represent multiple transcription start sites or could be generated by mRNA processing.

To further study the putative sporulation-related roles of *smeA* and *sffA*, we deleted both genes from the genome of the wild-type strain M145, which normally produces dark grey spores on MS agar plates. A Δ*smeA-sffA* strain (K151, Table 1) produced uniformly light grey colonies, indicating that indeed, *smeA* and *sffA* were important for normal sporulation (Fig. 1B and C). Introduction of *smeA-sffA* (pNA276, Table 1) *in trans* restored the spore pigmentation of the mutant to the wild-type level, confirming that the observed developmental defect was indeed caused by *smeA-sffA* deletion (Fig. 1B and C). In order to clarify the individual contributions of *smeA* and *sffA* to the defective sporulation phenotype, we constructed an in-frame Δ*smeA* deletion mutant (K150, Table 1; Fig. 1B) and a Δ*sffA* mutant (K152, Table 1; Fig. 1B). The *smeA* deletion strain had a light grey spore phenotype, similar to that of the Δ*smeA-sffA* strain, while deletion of *sffA* alone resulted in a very slight reduction in spore pigmentation, suggesting that the lack of SmeA was primarily responsible for the light grey phenotypes of both the Δ*smeA* and Δ*smeA-sffA* mutants. However, *smeA* alone *in trans* was only able to partially complement the Δ*smeA* strain (where *sffA* was still present) and not the Δ*smeA-sffA* strain,

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indicating that both SmeA and SffA are needed for optimal sporulation (Fig. 1C). Full complementation was only obtained when *smeA* and *sffA* were expressed *in cis*, suggesting that coexpression was necessary for the optimal function of both SmeA and SffA. Together, these results strongly suggest that *smeA-sffA* constitutes a novel sporulation locus in *S. coelicolor*.

SmeA and SffA influence multiple developmental processes during later stages of sporulation

Because reduced spore pigmentation is often indicative of defects in underlying developmental processes leading to spore maturation, we studied the Δ*smeA-sffA* strain by phase-contrast, fluorescence and electron microscopy to gain better understanding of the developmental roles of SmeA and SffA. Growth of the vegetative mycelium was not affected by deletion of *smeA-sffA*; however, several differences between the wild-type and the Δ*smeA-sffA* strains were observed in sporulating aerial hyphae. First, fluorescence microscopy of fixed and DAPI-stained sporulating hyphae revealed that the chromosomes in Δ*smeA-sff* spores displayed a more diffuse staining pattern than chromosomes in wild-type spores (Fig. 2A). The lower panel in Fig. 2A shows the distribution of fluorescence signal intensity along a line through the middle of five consecutive spore compartments. Peaks representing wild-type chromosomes were sharp and declined almost to a background level at positions corresponding to constrictions between spore compartments, whereas peaks of the mutant strain were wider and flatter, indicating the inability of the Δ*smeA-sffA* strain to condense its chromosomal DNA to the wild-type level during sporulation. Furthermore, DAPI staining also revealed that *smeA-sffA* deletion caused a slight defect in chromosome segregation. Anucleate spore compartments within spore chains occurred with a frequency of 0.6% in the Δ*smeA-sffA* strain, compared with the wild-type frequency of < 0.1% (> 10 000 spore compartments were counted for both wild-type and Δ*smeA-sffA* strains).

Transmission electron microscopy (TEM) revealed architectural defects in the spore envelope of the Δ*smeA-sffA* strain (Fig. 2B). The cell walls of the mutant spores were thinner and showed less defined layers than those of the wild-type spores, which had clearly discernible layers of light and dense material, as described previously (McVittie, 1974). In particular, the pronounced electron-dense outer layer of the wild-type spores appeared considerably thinner and 'paler' in the mutant (Fig. 2B). Reduced spore wall thickness is often correlated with decreased spore resistance to detergents and high temperatures (Potuckova *et al*., 1995; Molle *et al*., 2000; Mazza *et al*., 2006), and correspondingly, we observed that mutant spores were more susceptible to heat than wild-type spores. For example, 10.3% of the wild-type spores survived treatment at 65°C for 20 min, whereas the corresponding figure for mutant spores was 2.4% (after 30 min the corresponding values were 3.3% and 0.4% respectively). However, mutant and wild-type spores appeared to be equally resistant to detergents (up to 5% SDS, data not shown). Intriguingly, SmeA and Sff also appeared to be required for spore separation, as scanning electron microscopy (SEM) images of 8-day-old colonies grown on MS agar revealed that, while free spores were readily apparent on the surface of the wild-type strain, spores from the Δ*smeA-sff* strain appeared almost exclusively in chains (Fig. 2C). Furthermore, the uneven compartment size of developing spore chains of the Δ*smeA-sffA* strain, seen clearly in Fig. 2C, was also observed by phase-contrast microscopy and by TEM (data not shown).

The phenotype of the Δ*smeA* strain was indistinguishable from that of the Δ*smeA-sffA* double mutant strain at the level of microscopic observation. In contrast, the *sffA* deletion strain showed apparently normal spore maturation and spore wall structure, except that phase-contrast and electron microscopy revealed an uneven septation defect similar to what is characteristic of the Δ*smeA-sffA* strain (example in a young Δ*sffA* spore chain is shown in Fig. 2B). Taken together, these observations show that the products of the *smeA-sffA* operon influence several processes of normal formation of prespore compartments and spore maturation, including division septum placement, chromosome segregation and condensation, spore separation, spore pigmentation, maturation of the spore envelope and development of heat resistance.

The smeAp *promoter is induced specifically in sporogenic aerial hyphal cells independently of the sporulation sigma factor gene* sigF

Interestingly, most features of the pleiotropic *smeA-sffA* phenotype overlap with the phenotype associated with the deletion of the RNA polymerase sigma factor gene *sigF*, which is also known to affect late stages of sporulation (Potuckova *et al*., 1995). *sigF* is specifically induced in sporulating hyphae (Sun *et al*., 1999) and like *smeA-sffA*, its transcription depends on the early *whi* genes (*whiG*, *whiA*, *whiB*, *whiH*, *whiI* and *whiJ*) (Kelemen *et al*., 1996). Thus it was possible that *smeA-sffA* transcription was σ^F-dependent. To test this hypothesis, we fused the promoter region, ribosome binding site and start codon of *smeA* to the gene for the red fluorescent protein mCherry (*smeAp–mCherry*, pNA473, Table 1), and used this translational fusion to monitor *smeA* expression. In the wild-type, a strong fluorescence signal was observed in sporulating aerial hyphae with constrictions or sporulation septa, and a weaker signal was seen in some unconstricted aerial hyphae, but no signal was detected in the

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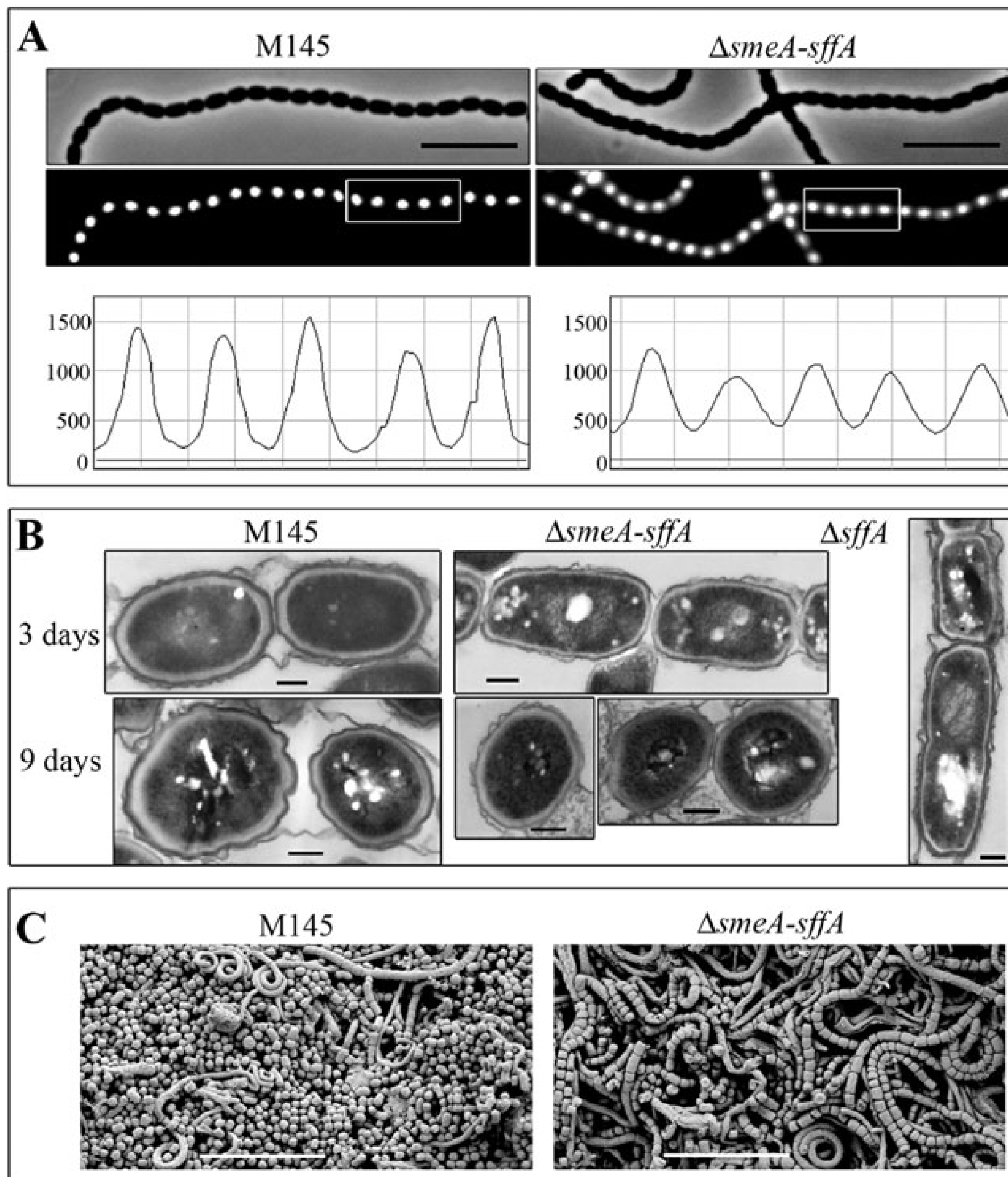

Fig. 2. Deletion of *smeA-sffA* causes multiple defects in spore development.
A. Phase-contrast (top) and DAPI fluorescence images (bottom) of fixed sporulating aerial hyphae of the wild-type strain M145 and the Δ*smeA-sffA* deletion strain K151 after growth on MS agar for 48 h. Size bar corresponds to 5 μm. White rectangles mark the regions for fluorescence intensity measurements shown below. The graphs represent fluorescence signal intensity along a line drawn through the middle of five consecutive spore compartments. The straight line indicates the level of background fluorescence. Numbers on *y*-axis represent arbitrary units of fluorescence intensity.
B. Transmission electron micrographs of thin sections of spores of M145, Δ*smeA-sffA* and Δ*sffA* strains (K151 and K152 respectively) grown on MS agar for 3 and 9 days (M145 and K151) or just 3 days (K152). Size bars correspond to 200 nm.
C. Scanning electron microscopic images of 9-day-old colony surfaces of MS agar-grown M145 and Δ*smeA-sffA* (K151) strains. Size bars correspond to 10 μm.

vegetative mycelium (Fig. 3A). This showed that there was a specific compartmentalized activation of the *smeA* promoter in the apical cell of aerial hyphae undergoing sporulation. A similar pattern of expression of *smeAp–mCherry* was also seen in the Δ*smeA-sffA* strain, indicating that SmeA and SffA are not involved in regulating their own expression (data not shown). A wild-type developmental pattern of *smeAp–mCherry* expression was also observed in the *sigF* deletion strain (J1984, Table 1), showing that σ^F is not required for the sporulation-specific expression of *smeA-sffA* (Fig. 3A). Conversely, we also tested whether *sigF* expression depended on *smeA-sffA*. A *sigFp–mCherry* translational reporter fusion (pNA540, Table 1) was introduced into the wild-type (M145), Δ*sigF*

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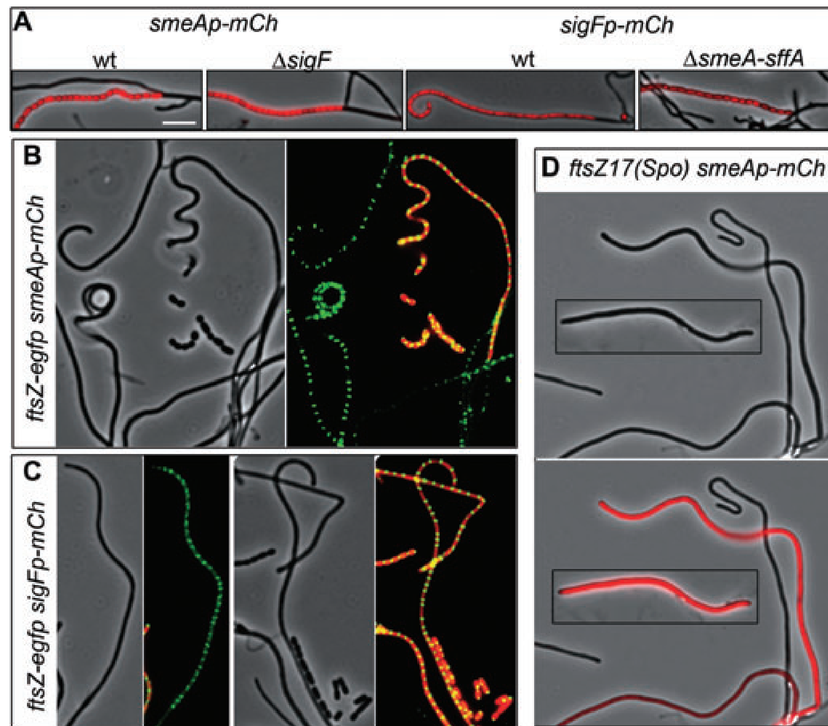

Fig. 3. Developmental timing of *smeA* and *sigF* expression.
A. Translational fusions of the *smeA* and *sigF* promoters to *mCherry* (*smeAp–mCh* and *sigFp–mCh*) were monitored in the wild-type M145, the Δ*sigF* strain J1984 and the Δ*smeA-sffA* strain K151. Overlays of phase-contrast and fluorescence images (red) show compartmentalized promoter activity only in sporulating aerial hyphae.
B and C. Phase-contrast images and fluorescence overlay images of FtsZ–EGFP (green) and mCherry (red) of live aerial hyphae in different developmental stages of the KF127 strain containing the *smeAp–mCherry* fusion (B) and the KF127 strain containing the *sigFp–mCherry* fusion (C) are shown. Hyphae in an earlier developmental stage display only FtsZ–EGFP structures, while those in later stages show both FtsZ–EGFP and *smeAp–mCherry* (B) or *sigFp–mCherry* (C) fluorescence, showing similar timing of the firing of both promoters.
D. Phase-contrast image of aerial hyphae of the *ftsZ17*(Spo) strain carrying the *smeAp–mCherry* fusion (top) and the same image overlaid with the mCherry fluorescence image (red, bottom) are shown. Insets in both panels depict separated phase-dark hyphae showing a high level of *smeAp*-driven mCherry production.

and Δ*smeA-sffA* strains. Strong sporulation-specific expression of mCherry fluorescence was observed in all three strains, showing that *sigF* expression did not depend on SmeA-SffA or σ^F itself (Fig. 3A and data not shown). Thus, *sigF* and *smeA-sffA* are expressed independently of each other in sporulating aerial hyphae.

Activation of smeA-sffA *expression occurs after Z-ring formation, but is not dependent on regular septation*

Expression of both *sigF* and *smeA-sffA* depends on the early *whi* genes. As the early *whi* genes are also needed for the formation of sporulation septa, and *ftsZ* mutants that are specifically defective in the formation of sporulation septa have pleiotropic sporulation defects including a strong reduction in the production of the grey spore pigment (Flärdh *et al*., 2000; Grantcharova *et al*., 2003), it has been speculated that septum formation might represent a morphological checkpoint that triggers the expression of late sporulation genes whose products are needed after compartmentalization of the aerial hyphae (Flärdh *et al*., 2000). To test whether *smeA-sffA* was subject to this putative cell-division checkpoint, we first needed to determine the timing of *smeA-sffA* expression in relation to Z-ring formation. For this purpose, the *smeAp–mCherry* reporter was introduced into strain K127, which produces both wild-type FtsZ and FtsZ–EGFP. Most sporulating hyphae displayed both FtsZ–EGFP and *smeAp*–mCherry fluorescence. However, some unconstricted aerial hyphae had clearly visible Z-rings but no detectable mCherry fluorescence (Fig. 3B), but we never detected *smeAp–mCherry* expression without an FtsZ–EGFP signal. These results suggested that *smeA* expression occurred no earlier than, and possibly after, FtsZ-ring formation. The same experiment was conducted with the *sigFp–mCherry* reporter, which allowed comparison with this previously known late sporulation gene (Kelemen *et al*., 1996). The timing of *sigF* expression was indistinguishable from that of *smeA-sffA* (Fig. 3C), indicating that both *smeA-sffA* and *sigF* gene expression could in prin-

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ciple be subject to septum checkpoint regulation. We next introduced the *smeAp–mCherry* promoter probe into a strain carrying the mutant allele *ftsZ17*(Spo) (K102, Table 1), which is severely impaired in the formation of regular Z-rings and sporulation septa in the aerial mycelium (Grantcharova *et al*., 2003). If expression of *smeA-sffA* required normal FtsZ-ring formation and septation, we would expect to see reduced *smeAp–mCherry* fluorescence in the aerial hyphae in this mutant background. Interestingly, many aerial hyphae showed *smeAp–mCherry* fluorescence with intensity comparable to the wild-type situation (Fig. 3D). Furthermore, it has been shown that sporulation septa form with low frequency in the *ftsZ17*(Spo) strain, causing separation of some aerial hyphal fragments (Grantcharova *et al*., 2003). Such separated fragments (inset in Fig. 3D) showed not only intense mCherry fluorescence, but were also thick and phase-dark, reminiscent of the spore-like aerial hyphal fragments formed by *whiH* and *ftsZ*Δ*2p* mutants (Flärdh *et al*., 1999; 2000; Grantcharova *et al*., 2003). Thus, there does not seem to be a quantitative dependence of *smeA* expression on the formation of multiple Z-rings and sporulation septa in aerial hyphae. However, it is still possible that the dependence was qualitative, and formation of one, or a few, sporulation septa per aerial hypha suffices to trigger *smeA-sffA* expression.

SffA localizes to late sporulation septa in a SmeA-dependent manner

The homology of SffA to FtsK-like DNA translocases and the increased frequency of anucleate spores in the Δ*smeA-sffA* mutant suggested a possible involvement of this locus in segregation of chromosomes. It was therefore important to determine whether SmeA and SffA were targeted to the sporulation septa or to other specific sites. Unfortunately, all attempts to create a fully functional tagged derivative of SmeA were unsuccessful. Both N-terminal and C-terminal fusions to mCherry or EGFP, respectively, were non-functional, as was SmeA carrying a short C-terminal FLAG tag. However, insertion of mCherry internally, immediately following a putative signal sequence cleavage site, produced a partially functional fusion protein as indicated by a slight restoration of spore pigmentation on MS agar plates (pNA547, Table 1). This fusion protein (mCh–SmeA) displayed a developmental expression pattern similar to the *smeAp–mCherry* reporter (Fig. 3A) and the SffA–EGFP protein (see below and Fig. 4) and was evenly distributed in the cell periphery (Fig. 4A), thus supporting the predicted membrane localization of SmeA. However, as the fusion protein was only weakly active, we cannot exclude that the native SmeA protein might have a more distinct cellular localization pattern, such as, for example, colocalization with SffA to the sporulation septa.

Replacement of *sffA* by *sffA–egfp* in the chromosome of the wild-type strain M145 (K158, Table 1) did not cause the characteristic uneven septation phenotype of the Δ*sffA* strain, indicating that the SffA–EGFP fusion protein was at least partially functional. In agreement with the results seen with the *smeAp–mCherry* reporter, no SffA–EGFP fluorescence was detected during vegetative growth. Weak SffA–EGFP fluorescence appeared in some early non-constricted sporogenic hyphae (Fig. 4B). In later stages, when visible constrictions had appeared between spore compartments, SffA–EGFP had re-localized and accumulated into strongly fluorescent foci in the middle of the constricting septa (Fig. 4C and D). Thus, SffA has a cell type-specific expression and septal localization in *S. coelicolor*, which would be consistent with a hypothetical role as a translocase protein suggested by its homology to FtsK/SpoIIIE proteins. In addition, SffA–EGFP was also seen at some vegetative-type septa in the subapical segment of aerial hyphae (data not shown), which otherwise showed no SffA–GFP upregulation.

To investigate the cellular role of SmeA and the putative functional relationship with SffA, we studied the localization of SffA–EGFP in a Δ*smeA* background. *sffA* was replaced by *sffA–egfp* in the chromosome of the Δ*smeA* strain (K159, Table 1), and as expected, the Δ*smeA sffA–egfp* strain was phenotypically similar to the Δ*smeA* strain and showed normal activation of *sffA–egfp* expression in sporogenic hyphae (Fig. 4E). Strikingly, however, SffA–EGFP did not relocalize to the constricting sporulation septa of the Δ*smeA* deletion strain (Fig. 4F and G). In contrast, SffA–EGFP localization to the vegetative-type septa (arrows in Fig. 4E and F) in the non-sporulating part of the hyphae occurred both in the *smeA* and *smeA*$^+$ strains. Introduction of *smeA-sffA in trans* fully restored the dynamic localization pattern of SffA–EGFP (Fig. 4H) and the dark grey spore colour to the *smeA sffA–egfp* strain. Thus, SmeA was specifically required for SffA accumulation at the constricting sporulation septa, but the localization of SffA–EGFP to vegetative-type septa in basal parts of sporulating aerial hyphae was SmeA-independent. It is important to note that while both the Δ*smeA* (SffA mislocalized) and the Δ*smeA-sffA* (SffA missing) strains had very similar phenotypes, the Δ*sffA* strain had a less severe sporulation defect, suggesting that the mislocalization of SffA was not solely responsible for the developmental phenotype of the *smeA* strains. Consequently, SmeA must fulfil other functions in addition to its role as a localization determinant for the DNA-translocase-domain protein SffA.

Sporulation septa contain two SpoIIIE/FtsK-family proteins with distinct functions

The septal localization of SffA resembled that of FtsK and SpoIIIE proteins from other bacteria, which localize to the

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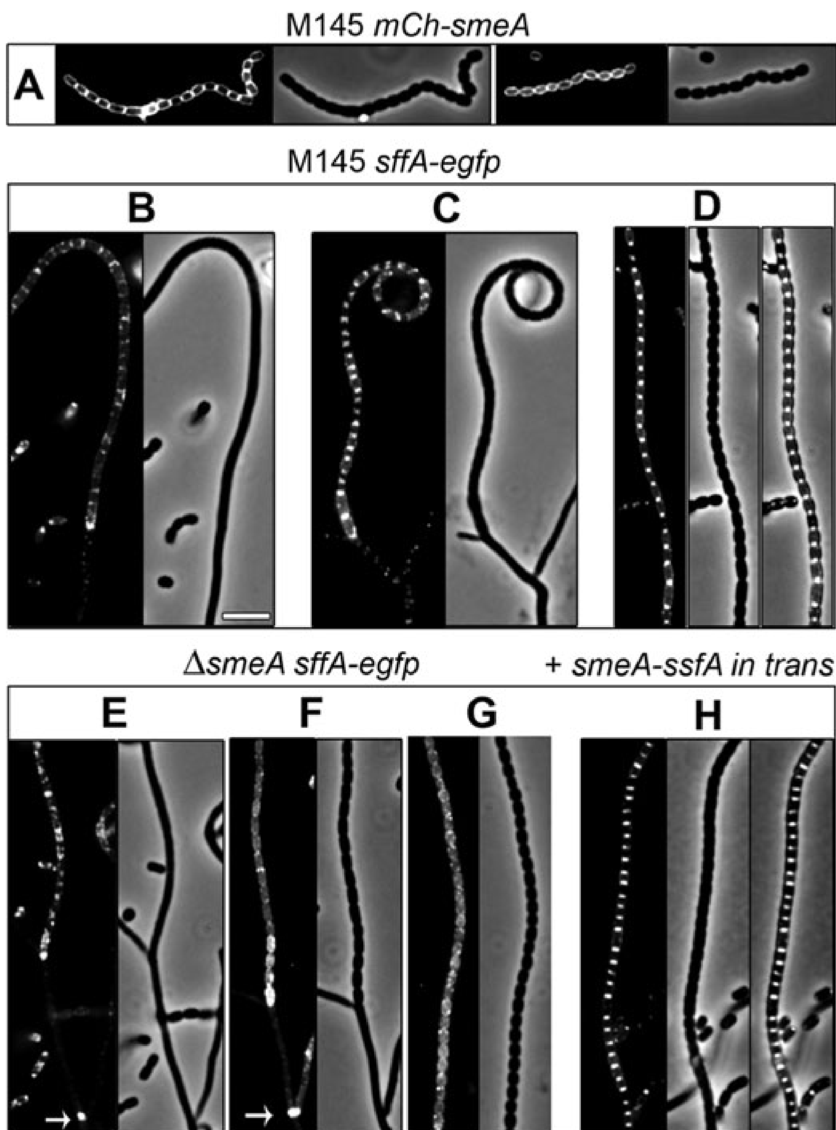

Fig. 4. mCherry–SmeA localizes to the membrane of sporulating hyphae and SffA–GFP localizes to sporulation septa in a SmeA-dependent manner.
A. Phase-contrast (right) and fluorescence (left) images of spore chains of strain M145 containing plasmid pNA547 expressing SmeA with internally inserted mCherry. Peripherally localized fluorescence indicates a close association of mCherry–SmeA with the cell membrane.
B–D. Different sporulating aerial hyphae of the *sffA–gfp* strain in developmental succession from smooth to strongly constricted state showing accumulation and localization of SffA–GFP. Overlay of fluorescence and phase-contrast images in (D) shows that the localization of SffA–GFP foci coincides with cell division sites.
E–G. Images of smooth and constricted aerial hyphae of the Δ*smeA sffA–gfp* strain showing that SffA–GFP is upregulated but delocalized in the sporulating part. Arrows indicate vegetative-type septa to where SffA–GFP is localized also in the absence of SmeA.
H. A spore chain of the Δ*smeA sffA–gfp* strain complemented by *smeA-sffA in trans*, showing full restoration of SffA–GFP localization to constricting sporulation septa. GFP fluorescence (left) and corresponding phase-contrast images (right or middle) of live cells from MS agar cultures are shown in all panels.
Size bar corresponds to 5 μm.

middle of cell division or sporulation septa and are involved in active translocation of DNA across the closing septum as well as in resolution of chromosomes (Bath *et al*., 2000; Aussel *et al*., 2002). A recent report established that the SCO5750 protein has an FtsK-like function in *S. coelicolor*, affecting segregation and/or stability of chromosomes (Wang *et al*., 2007). Therefore, it was important to establish whether SffA and FtsK have overlapping or perhaps redundant functions. To this end, we constructed deletion strains where *ftsK* was replaced by an apramycin resistance cassette both in the wild-type (M145) and in the Δ*smeA-sffA* backgrounds, yielding Δ*ftsK* and Δ*smeA-sffA* Δ*ftsK* mutants (K161 and K169, Table 1). In contrast to *Escherichia coli* and *Bacillus subtilis*, in which FtsK and SpoIIIE have essential functions for viability and sporulation, respectively, the *S. coelicolor* Δ*ftsK* strain grew as well as the wild-type parent and sporulated abundantly, but showed an increased occurrence of aberrant colonies upon restreaking, confirming previous observations (Wang *et al*., 2007). However, fluorescence microscopy of DAPI-stained spore chains showed a subtle but revealing difference between strains with and without *ftsK*. In the wild-type strain, 88% (14 out of 16) of the occasional anucleate spore compartments were flanked by a spore compartment containing approximately twice as much DNA as an average spore. Similarly, in the Δ*smeA-sffA* strain, which has a higher occurrence of anucleate spores, 87% of those (27 out of 31) were flanked by a spore with two chromosomes (example shown in Fig. 5A). Only situations where an anucleate spore was flanked by another spore on both sides were considered. In the Δ*ftsK* and Δ*smeA-sffA* Δ*ftsK* strains, however, spores with severely reduced DNA content were readily detected, but spores that were completely devoid

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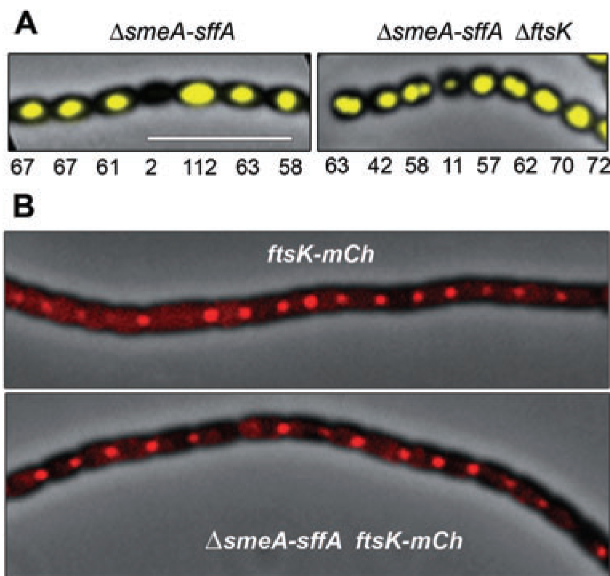

Fig. 5. FtsK is targeted to sporulation septa independently of SmeA and its activity as a cross-septum translocase is not redundant with the function of SffA.
A. Overlays of phase-contrast and DAPI fluorescence images (yellow) of spore chain segments of the Δ*smeA-sffA* and the double mutant Δ*smeA-sffA* Δ*ftsK* strains, containing anucleate spores. Numbers below the images indicate the mean intensity of fluorescence signal (reflecting the DNA content) in a corresponding spore compartment. The anucleate spore compartment of the Δ*smeA-sffA* strain, but not the partially DNA-free spore of the Δ*smeA-sffA* Δ*ftsK* strain, is flanked by a spore with two chromosomes.
B. Overlays of phase-contrast and FtsK–mCherry fluorescence (red) images of live aerial hyphae of the *ftsK–mCherry* and the Δ*smeA-sffA ftsK–mCherry* strains. In both strains FtsK–mCherry, which is replacing the wild-type FtsK, shows similar localization in the middle of sporulation septa.
Size bar corresponds to 5 μm.

of DNA were not observed, even though more than 10 000 spores were examined for each strain. In clear contrast to the situation in the *ftsK*$^{+}$ counterparts, in either the Δ*ftsK* strain or the Δ*smeA-sffA* Δ*ftsK* strain none of 43 observed spores with strongly reduced DNA content were flanked by spores with double DNA content (Fig. 5A). Our interpretation of these results is that the cross-septum DNA translocase activity of FtsK normally contributes to the formation of unigenomic spores by removing chromosomal DNA that becomes trapped under a closing septum to the proper adjacent spore compartment. However, on occasions when septum placement or chromosome positioning is impaired, the trapped chromosome may be pumped in the 'wrong' direction resulting in an empty spore flanked by a spore with two chromosomes. These occasions are rare in the wild type, but become more frequent when chromosome positioning is disturbed. In support of this hypothesis, the Δ*smeA-sffA* strain, which showed chromosome segregation and septum placement defects, showed proportionally more occasions of zero/double chromosome pairs. This resembles the function of *B. subtilis* SpoIIIE in moving trapped chromosomes out of closing cell division septa (Sharpe and Errington, 1995). As most empty spores of the Δ*smeA-sffA* strain were also flanked by spores with double chromosomes, it seems likely that the clearing of DNA from closing septa is dependent on FtsK activity and not that of SffA.

We also replaced *ftsK* by *ftsK–mCherry* in the wild-type and Δ*smeA-sffA* background. Like SffA–EGFP, FtsK–mCherry localized to the sporulation septa in the wild-type background (Fig. 5B), confirming the FtsK localization reported by Wang *et al.* (2007). The same localization pattern was observed in the Δ*smeA-sffA* background (Fig. 5B), indicating that, in contrast to SffA, the localization of FtsK was independent of SmeA. Thus, we have shown that the SffA and FtsK putative translocase proteins both localize to sporulation septa, but their localization mechanisms and their cellular functions are different. FtsK appears to be involved in DNA translocation between spore compartments, but is not directly involved in regulating sporulation. SffA, on the other hand, appears to be functionally related to SmeA, which has a pleiotropic role in spore maturation.

Discussion

In this article we have identified and characterized two dedicated sporulation genes whose expression is specifically activated in sporogenic aerial hyphae. In contrast to several previously characterized sporulation-specific genes (*whi* genes) which encode regulators of gene expression, the gene products of *smeA* and *sffA* are not likely to be directly involved in transcriptional control. Nevertheless, the small membrane protein SmeA was shown to influence several processes during spore development, including septum placement, DNA segregation and condensation, spore wall thickening and spore separation. Although the deletion of *sffA* caused a subtle phenotype, its specific activation in sporulating aerial hyphae, as well as its genetic and functional coupling to SmeA, suggested a role for SffA in sporulation as well. Understanding the functions of SmeA and SffA in more detail will help to shed new light on the molecular mechanisms underlying sporulation.

smeA-sffA organization is conserved

BLAST and PSI-BLAST searches revealed that the sequenced *Streptomyces* species *S. coelicolor*, *S. avermitilis* and *S. scabies* contain four, seven and six *smeA*-like genes, respectively, and several self-transmissible plasmids from different *Streptomyces* species harboured single copies of *smeA* homologues (Fig. 6). Furthermore, two sequenced *Frankia* strains also encoded one SmeA-

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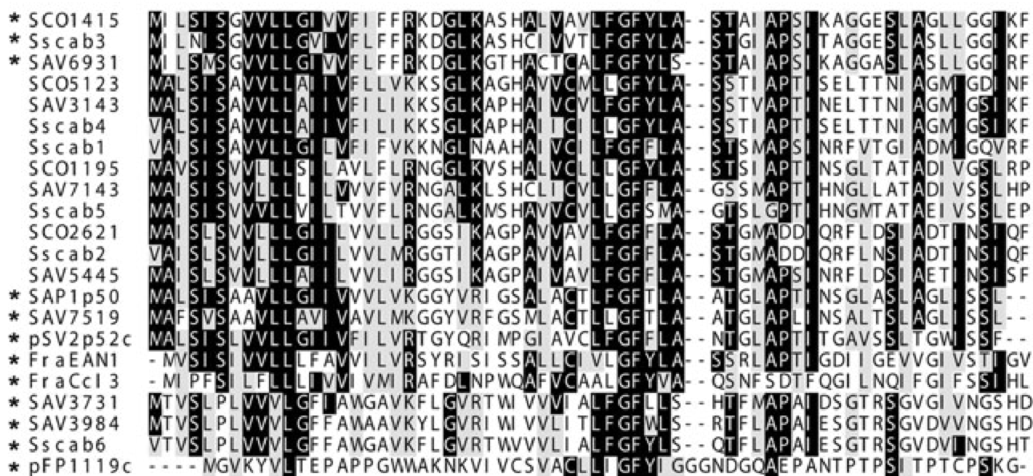

Fig. 6. The SmeA family of small *Actinomycete* membrane proteins. MULTALIN (Corpet, 1988) alignment of the SmeA-like proteins from *Streptomyces coelicolor* (designated by their respective sequence tags SCO), *S. avermitilis* (sequence tags SAV), *S. scabies* (arbitrarily named Sscab1–6), two proteins with locus tags Francci3_0137 and Franean1DRAFT_3434 from *Frankia* sp. strains CcI3 and EAN1pec respectively. pFP1119c, pSV2p52c and SAP1p50 represent plasmid-borne SmeA homologues from *Streptomyces* sp. circular plasmid pFP11, *Streptomyces violaceoruber* linear plasmid pSV2 and *S. avermitilis* linear plasmid SAP1 respectively. The predicted secretion signal sequence and the internal transmembrane domain of SmeA are shown by grey bars above the alignment. A star designates close genetic linkage of the respective gene to a gene encoding a SpoIIIE/FtsK-domain protein. Multiple alignment was performed with default settings and the output order reflects the relatedness of the input sequences.

like protein each, but we did not find homologoues in other currently available actinomycete genomes. In total, we have identified 22 members of the Sme family, but we suspect that more distantly related proteins may not have been detected using BLAST, due to the short length of the query sequence (Fig. 6). All members of the SmeA family contain a transmembrane domain predicted with high probability by most algorithms. Furthermore, the SignalP algorithm recognized a putative N-terminal secretion signal in several, but not in all SmeA homologues (Nielsen *et al*., 1997). For example, the probabilities for the four *S. coelicolor* SmeA proteins to possess an N-terminal signal sequence vary between 0.684 and 0.995 according to the hidden Markov model prediction (Nielsen and Krogh, 1998). Each of the three sequenced *Streptomyces* species encodes one canonical SmeA-SffA pair in a conserved genetic environment. Strikingly, 12 of the 22 members of the *sme* family are adjacent to genes encoding SpoIIIE/FtsK-family proteins, including the single *sme* family genes of both *Frankia* species and all *sme* genes harboured on plasmids (indicated by asterisks in Fig. 6). Given that conservation of genetic linkage often indicates a functional relationship, and as the plasmid-encoded SpoIIIE/FtsK-family proteins (called Tra proteins) function as DNA translocases during conjugal plasmid transfer (Grohmann *et al*., 2003), the plasmid-encoded SmeA-like proteins may have a function associated with DNA transfer. Several of the chromosomally encoded Sme-Sff-like pairs appear to be part of integrated plasmids, based on neighbouring genes encoding typical plasmid functions; however, the chromosomal neighbourhood of *smeA-sffA* in *S. coelicolor* does not contain plasmid-related genes. Thus, an intriguing possibility is that SmeA and SffA function in some aspects of moving or organizing chromosomal DNA during sporulation.

What could be the cellular role of SmeA?

One consequence of SmeA deletion is the inability to correctly localize SffA to the sporulation septa. However, the complex phenotype of *smeA* deletion could not be explained solely by mislocalization of SffA. Perhaps SmeA is needed for proper localization or membrane assembly of sporulation-specific proteins in addition to SffA. Such a function would be reminiscent of the roles of several small membrane proteins in other bacteria. In *E. coli*, for example, CcmD (69 aa) has been proposed to function as an assembly factor for the formation of a membrane-bound multiprotein complex functioning in the haem delivery process (Ahuja and Thony-Meyer, 2005), while in *B. subtilis*, the membrane-associated protein SpoVM (29 aa) tethers the spore coat to the outer spore membrane (Ramamurthi *et al*., 2006).

Are SmeA and SffA involved in DNA translocation?

In addition to the effects of Δ*smeA-sffA* and Δ*sffA* mutations on the accuracy of chromosome segregation in developing spore chains, several observations support a role for SffA as a DNA translocase. SffA shares the

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typical domain architecture of other FtsK-like proteins, with an N-terminal membrane-spanning region, and a C-terminal P-loop ATPase domain that is clearly, although distantly, related to the translocase domains of canonical FtsK proteins (Figs S2 and S3) (Rost *et al.*, 2004). When the sequence of SffA was compared with the consensus sequence of the large FtsK-HerA superfamily of pumping ATPases, SffA contained all conserved, distinguishing motifs of this family (Iyer *et al.*, 2004). Most of the characterized proteins of this family are involved in pumping or packaging DNA (Iyer *et al.*, 2004), with the only known exception being the YukA-like proteins containing three tandem ATPase domains, which are implicated in the secretion of extracellular peptides (Pallen, 2002). Another feature shared between SffA and the FtsK proteins is its specific localization to cell division septa. While we have shown that both SffA and FtsK localize to sporulation septa in *S. coelicolor*, experimental evidence only supported the role of FtsK as a DNA pump. Furthermore, the SffA amino acid sequence showed some curious deviations from the FtsK-HerA superfamily. For example, the P-loop sequence of the Walker A motif of SffA (consensus Gx4GK[TS]) contains a threonine in place of the conserved lysine residue, and a conserved glutamic acid residue in the Walker B motif is replaced by an arginine in SffA. The family of SpoIIIE/FtsK-domain proteins is large and its members exist in most bacteria and archaea, and yet the exact function is known only for a handful of members. Therefore, the similarities and differences in the sequence and function of the distantly related SffA and FtsK proteins make SffA an attractive target for further functional and structural studies.

Compartmentalized expression of smeA-sffA *does not require multiple sporulation septa and occurs also in poorly septated* whiH *and* ftsZ *mutants*

A central but so far unresolved question is what mechanisms are involved in triggering developmentally induced gene expression specifically in the sporulating aerial hyphal cells. In analogy to the role of the asymmetric septum in *B. subtilis* sporulation (Barak and Wilkinson, 2005), it has been suggested that sporulation septation may act as a morphological checkpoint to which the progression of the developmental programme would be linked (Chater, 2000; Flärdh *et al.*, 2000). In support of such a model, developmental activation of several late sporulation promoters (*sigFp*, *whiE1p*, *whiE2p* and *parAB1p*) is abolished in mutants that completely lack sporulation septa (such as *whiG*, *whiA* and *whiB*), but can be detected, although at significantly reduced levels, in *whiH* mutants that are still capable of forming some sporulation septa (in addition, *parAB* was upregulated in an *ftsZ∆2p* mutant that also forms only occasional sporulation septa) (Kelemen *et al.*, 1996; 1998; Flärdh *et al.*, 1999; Jakimowicz *et al.*, 2006). We found that *smeA-sffA* expression also follows the same pattern, suggesting a quantitative dependence of its expression on the number of sporulation septa. Using *smeAp–mCherry* and *sigFp–mCherry* constructs in a strain also containing FtsZ–EGFP, we showed that the developmental activation of both late sporulation genes was restricted to the apical sporogenic cell of aerial hyphae, and most probably happened after the assembly of Z-rings (Fig. 3B and C). Furthermore, by monitoring the *smeAp–mCherry* fluorescence in the *ftsZ17*(Spo) mutant (which produces only a few sporulation septa and is similar to the *whiH* and *ftsZ∆2p* mutants), we were able to show that *smeA* upregulation in fact did not require the formation of multiple Z-rings/septa per aerial hyphal cell. Based on these results, we propose that in the *ftsZ17*(Spo) and *whiH* mutants expression of late sporulation genes is activated in these mostly unseptated hyphal fragments, triggering spore maturation processes that give rise to their already documented spore-like appearance (Flärdh *et al.*, 1999; 2000; Grantcharova *et al.*, 2003). The strongly reduced abundance of transcripts corresponding to *smeA* and possibly other late sporulation genes in the *whiH* mutant may then be explained by the lower abundance of such spore-like hyphal compartments in this mutant compared with the number of developing spore chains in wild-type strains (K. Flärdh, unpubl. obs.).

It is obvious from these and other recent studies that the improvement of cytological tools for analysis of cell type-specific gene expression will be essential for further progress in understanding the control of morphological differentiation in *S. coelicolor*. The use of the red fluorescent protein mCherry (Shaner *et al.*, 2004) enabled us to avoid the significant problem of the strong autofluorescence of *Streptomyces* hyphae in the spectral region used to study GFP. This often appears as relatively bright fluorescent spots within the hyphae and has limited the use of GFP fusions to cases with a strong signal or very specific pattern of subcellular localization. Thanks to the low and uniform autofluorescence in the red spectral region, we were able to reliably detect even relatively weak mCherry expression. The use of such cytological reporters and markers provides a productive avenue ahead for detailed dissection of the development and sporulation of the aerial mycelium in streptomycetes.

Experimental procedures

Bacterial strains and media

The *S. coelicolor* A3(2) and *E. coli* strains used in this work are listed in Table 1. *E. coli* strains DH5α (Hanahan, 1983)

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and TOP10 (Invitrogen) were used for cloning, and strain ET12567/pUZ8002 was used to drive conjugative transfer of non-methylated DNA from *E. coli* to *S. coelicolor* as described previously (Kieser *et al*., 2000). Strain GM2929 was used to prepare non-methylated plasmid DNA for direct transformation of *S. coelicolor* protoplasts. Strain EKF104 was constructed by integrating the *cre* expression cassette from p705-Cre (Zhang *et al*., 1998) into *E. coli* strain LE392. Cultivation of *E. coli* strains was performed as described in Sambrook *et al*. (1989). *S. coelicolor* strains were grown on mannitol soy flour agar plates (MS agar), in yeast extract-malt extract medium (YEME), in tryptone soy broth (TSB) or on R2YE agar (Kieser *et al*., 2000).

Construction of S. coelicolor *mutants*

The PCR-targeting procedure was used for generation of *Streptomyces* gene knockout mutants essentially as described in Gust *et al*. (2003). The primers used are listed in Table S1. The apramycin resistance cassette was obtained from plasmid pIJ773 (for replacement of *sffA* and *ftsK*) and the chloramphenicol resistance cassette from plasmid pLox-Cat2 (for the in-frame deletions of *smeA* and *smeA-sffA*) (Palmeros *et al*., 2000). Target genes were first replaced by resistance cassettes on cosmids 6D7 (*smeA*, *sffA*) and 7C7 (*ftsK*) (Redenbach *et al*., 1996) in the *E. coli* strain DY380, which contains an inducible λ RED system (Yu *et al*., 2000). The LoxCat2 cassette that replaced *smeA* or *smeA-sffA* was subsequently excised by inducing the Cre recombinase in strain EKF104, leaving only a short sequence consisting of the *loxP* sequence and flanking restriction sites. Mutated cosmids were introduced into *S. coelicolor* wild-type or mutant strains by conjugation or protoplast transformation according to established protocols (Kieser *et al*., 2000) and screened for clones where a double recombination event had replaced the target gene by a respective mutant allele present on the cosmid. Mutants were verified by diagnostic PCR.

Plasmid construction

The plasmids used are listed in Table 1. DNA manipulation and cloning were carried out according to standard protocols (Sambrook *et al*., 1989). All primers used for cloning are listed in Table S1. Plasmid constructs were verified by DNA sequencing. Inserts for construction of plasmids pNA276 (pSmeA-SffA), pNA485 (pSmeA) and pNA277 (pSffA) were obtained by PCR using chromosomal DNA of strain M145, or mutated derivatives of cosmid 6D7 as templates, as shown in Fig. 1. Four hundred and thirty-five base pires of *smeA* upstream sequence and 317 bp of *sffA* downstream sequence were included in the clones. For EGFP and mCherry fusion constructs 996 bp of *sffA* and 1042 bp of *ftsK* encoding the C-terminal halves of the proteins were fused in frame to *egfp* or *mCherry*, respectively, on plasmid pEGFP-N2, creating plasmids pNA303 and pNA585, which were then used to transform *S. coelicolor* protoplasts. Transformants were selected where homologous recombination via a single crossing-over event had created a full-length recombinant fusion allele in the wild-type locus under control of the native promoter, as well as a second disrupted copy of gene. For insertion of *mCherry* (Shaner *et al*., 2004) internally into *smeA*, a KpnI site was introduced into *smeA* between regions encoding the putative signal sequence and the putative transmembrane helix by PCR-based mutagenesis of plasmid pNA276 (pSmeA-SffA). This site was then used for in-frame insertion of PCR-amplified *mCherry* lacking its start and stop codons, resulting in plasmid pNA547. Red fluorescent promoter probe constructs were constructed by cloning PCR-amplified promoter regions (660 bp for *smeA* and 494 bp for *sigF*) and including respective ribosome binding sites and start codons of *smeA* and *sigF* in frame with the *mCherry* ORF in plasmid pSET152, resulting plasmids pNA473 and pNA540.

Light microscopy

Samples for light and fluorescence microscopy were obtained either by pressing a coverslip on developing colonies on MS agar or by growing the strains in the angle between an inserted coverslip and the MS agar surface (Kieser *et al*., 2000). GFP and mCherry fluorescence was observed directly after mounting these coverslips on a glass slide with 50% glycerol in phosphate-buffered saline. For visualization of nucleoids, samples were first methanol-fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI) as described previously (Flärdh *et al*., 1999). All fluorescence and phase-contrast microscopy was performed using an Axioplan II imaging fluorescence microscope equipped with appropriate filter sets, an Axiocam charge-coupled device camera and Axiovision software (Carl Zeiss Light Microscopy). Digital images were processed using Adobe Photoshop CS version 8.0 software.

Electron microscopy

For SEM the samples were pre-fixed with 2.5% glutaraldehyde, washed three times in phosphate-buffered saline and fixed in 1% osmium tetraoxide. After dehydration in ethanol the samples were injected through nucleopore filters with 0.2 μm pore size, critical point-dried, mounted on Cambridge alloy stubs, silver sputtered and examined in a Zeiss Supra 35-VP field emission SEM equipped with a STEM detector, EDAX Genesis 4000 EDS.

For TEM the samples were pre-fixed with 2.5% glutaraldehyde, washed in phosphate-buffered saline and fixed in 1% osmium tetraoxide. After dehydration in ethanol (20–100%) and infiltration in acetone-resin 1:1, the samples were infiltrated in pure resin and then embedded in silicon plates. Sections (60 nm thick) were made on a LKB-ultramicrotome with a diamond knife from Dupont, stained with lead citrate and uranyl acetate and examined in a Zeiss Supra 35-VP field emission SEM equipped with a STEM detector, EDAX Genesis 4000 EDS.

RNA preparation, S1 nuclease protection assay and 5′ RACE

S1 nuclease protection assays were carried out on the same set of RNA preparations and with the same procedure as

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described previously (Flärdh *et al*., 2000). The probe was prepared by PCR using primers KF129 (aggcggcgagcatgacgagg) and 5′ radiolabelled KF130 (cgagcaggaccacgcctgag).

5′RACE was carried out using the 5′RACE kit, version 2.0 (Invitrogen), according to instructions from the manufacturer. The template RNA for this analysis was prepared from *S. coelicolor* strain M145 grown on a cellophane membrane on MS agar for 48 h, using RNeasy Protect Bacteria kit (Qiagen) according the protocol from The *S. coelicolor* Microarray Resource, University of Surrey (http://www.surrey.ac.uk/SBMS/Fgenomics/Microarrays/html_code/ExperimentalDesign.html). The amplified 5′RACE products were cloned using the TOPO TA cloning kit (Invitrogen) and 10 randomly chosen plasmids were investigated by DNA sequencing to determine the 5′ ends of the *smeA* transcript.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. DNA microarray analysis of transcription of selected genes during development of the *S. coelicolor* strain M145 and its non-sporulating mutants on MS agar.

Fig. S2. Schematic representation of the domain architectures of the SffA and FtsK proteins.

Fig. S3. MULTALIN alignment of the translocase domains of SffA and of some selected FtsK/SpoIIIE-family proteins.

Table S1. Primers used in this work.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2007.05877.x
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