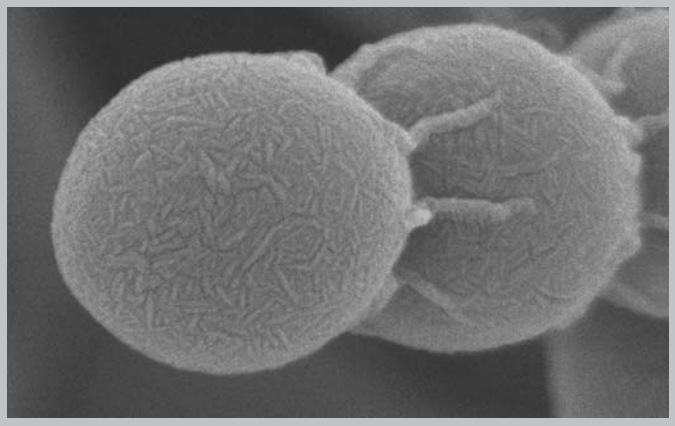
Control of sporulation-specific cell division in Streptomyces coelicolor

Noens, E.

FtsX and FtsE import autolytically produced peptidoglycan subunits during sporulation-specific cell division in *Streptomyces coelicolor*

Elke E. E. Noens, Henk K. Koerten and Gilles P. van Wezel



ABSTRACT

Cell division results in the formation of a septum that divides the mother cell into daughter cells. This is initiated by the formation of a ring of the tubulin-like protein FtsZ (the Z-ring), which functions as a scaffold for the construction of the septum. Subsequently, other cell division proteins are recruited to the Z-ring, forming the divisome or septosome. The cell division proteins FtsE and FtsX together form an ABC transporter, with FtsE functioning as the ATP-binding protein and FtsX as the membrane transporter. In *E. coli*, both proteins localise to division sites during the later stages of cell growth. *E. coli ftsE* null mutants are only viable on high salt medium and have a filamentous phenotype. We constructed an *ftsX* null mutant of *S. coelicolor*, but despite many efforts were unable to create an *ftsE* mutant. The fact that *ftsX* mutants are viable but not *ftsE* mutants suggests that FtsE may interact with at least one other ATP-requiring and essential transport protein. The *ftsX* mutant produces branches close to the base of a spore chain and accumulates peptidoglycan subunits between adjacent maturing spores. We show that FtsE localises to sporulation septa, at the same sites where peptidoglycan subunits accumulate and that FtsEX are not required for Z-ring formation. Our experiments demonstrate that FtsEX participate during the last stages of cell division, during autolytic spore separation, and most likely function by re-importing peptidoglycan subunits for recycling.

INTRODUCTION

Cell division involves the formation of a septum at the mid-cell position in most of bacteria. In *E. coli*, a collection of genes is required for septum formation, with two main features: the proteins encoded by these genes localise to the site of septum synthesis and mutants fail to septate at the non-permissive temperature (*fts*, filamentation temperature sensitive) (Errington *et al.*, 2003). The name *fts* has been extended to other cell division genes even if they are not known temperature-sensitive alleles. The first event of septation is the polymerisation of FtsZ, the prokaryotic homologue of tubulin, into a Z-ring functioning as a template for the construction of the septum. The other proteins are recruited to the Z-ring in a hierarchical order, which is almost completely linear in *E. coli* (FtsA, ZipA, (ZapA) → FtsEX → FtsK → FtsQ → FtsL/YgbQ → FtsW → FtsI → FtsN → AmiC) (Schmidt *et al.*, 2004) although recent work suggests that assembly of the divisome in *E. coli* involves the formation of subcomplexes, which are assembled into the divisome in a concerted mode (Vicente and Rico, 2006). Conversely, in *B. subtilis* similar division proteins (DivIB, DiVIC, FtsL, PBP-2B and FtsW) are cooperatively recruited to the division site and the division proteins are all completely interdependent for assembly (Errington *et al.*, 2003). Hence, the mode of division ring assembly appears to be quite similar in these two bacteria.

While in most bacteria a single septum is formed, dividing the mother cell into two daughter cells, two separate and morphologically distinct cell division events occur in the Gram-positive mycelium bacterium *Streptomyces*; cross-walls are laid down in vegetative hyphae to subdivide these into large multinucleoid compartments, while during development aerial hyphae erect from the lysing vegetative mycelium and many septa are simultaneously produced to form long chains of spores (Chater, 2001). There are some major and very interesting differences between cell division in streptomycetes and that in other eubacteria. For example, multiple septa are simultaneously produced in a ladder-like fashion (Schwedock *et al.*, 1997), and cell division is not essential for reproduction. The latter was shown by the viability of an *ftsZ* or *ftsQ* null mutant in *S. coelicolor*, while both proteins are essential in several kinds of bacteria (Bennett and McCormick, 2001; McCormick *et al.*, 1994; McCormick and Losick, 1996). Furthermore, assembly of the Z-ring is probably differently organised, as streptomycetes lack FtsA and ZipA, which anchor the Z-ring to the membrane in *E. coli* and *B. subtilis* (Errington *et al.*, 2003; Lowe *et al.*, 2004), while of the MinCDE control system for septum-site localisation (Autret and Errington, 2001; Marston *et al.*, 1998),

only homologues of MinD are present. These homologues do not seem to play a role in septum-site localisation (J. McCormick and G.P. van Wezel, unpublished data). Hence, a lot of information on septum formation in *S. coelicolor* is still missing, and we focus in particular on the differences between the 'non-physical division' by cross-wall formation in vegetative hyphae and the 'separative division' during sporulation.

In this chapter, we investigate the function of the cell division proteins FtsE and FtsX in *S. coelicolor*. *ftsEX* form an operon and the proteins they encode show similarity to ABC transporters, with FtsE resembling the ATP-binding component interacting with the membrane component FtsX. Typically, ABC transporters are anchored in the membrane by two separate regions, each containing six transmembrane domains. These residues, most likely, form the channel through which the substrates cross the membrane (Higgins, 1992). In bacteria, ABC transporters are mainly involved in nutrient uptake, although they also participate in the export of toxins, antibiotics and other undesired compounds, among others contributing to bacterial multidrug resistance (Locher, 2004). FtsX harbours only four hydrophobic segments, which are potential transmembrane helices (de Leeuw *et al.*, 1999). In most bacteria, *ftsEX* form an operon with *ftsY*, encoding the receptor of the signal recognition particle (SRP), which is essential for the correct insertion of FtsX, FtsE and other proteins into the plasma membrane (de Leeuw *et al.*, 1999; Du and Arvidson, 2003; Gill and Salmond, 1990). In actinomycetes, *ftsY* is located elsewhere in the genome, which does not necessarily mean that its role in the membrane topology of FtsEX – if any - is less important. In *E. coli*, FtsEX participate directly in cell division. An *ftsE* null mutation, with polar effects on *ftsX*, is viable on high salt medium only, where it shows moderate filamentation without being temperature sensitive. A decrease in salt concentration results in extreme filamentation and in cell death. Therefore, *ftsE* is a conditional salt-dependent essential gene in *E. coli*. Both proteins localise to the septal ring during late stages of cell growth and are important for the stability of the septal ring, especially in salt-free media (de Leeuw *et al.*, 1999; Schmidt *et al.*, 2004).

Here we describe the characteristics of an *ftsX* deletion mutant in *S. coelicolor*. By using an FtsZ-EGFP translational fusion in this mutant, we were able to draw conclusions about the order proteins are recruited to the divosome. We also show that FtsE localises at spore septa in maturing spore chains. All these data show that FtsEX are important in the later stages of cell division.

MATERIALS AND METHODS

Bacterial strains and media

The bacterial strains described in this work are listed in Table 1. *E. coli* K-12 strains JM109 (Sambrook *et al.*, 1989) and ET12567 (MacNeil *et al.*, 1992) were used for routine cloning and plasmid propagation and were grown and transformed by standard procedures (Sambrook *et al.*, 1989). *E. coli* ET12567 containing pUZ8002 was used for conjugation to *S. coelicolor* (Kieser *et al.*, 2000). *E. coli* transformants were selected in L-broth containing the appropriate antibiotics. *Streptomyces coelicolor* A3(2) M145 was obtained from the John Innes Centre strain collection and was the parent of the *ftsX* mutant described in this work. All media and routine *Streptomyces* techniques used are described in the *Streptomyces* manual (Kieser *et al.*, 2000). SFM agar plates were used for making spore suspensions and R2YE agar plates for regeneration of protoplasts and, after the addition of the appropriate antibiotic, for selecting recombinants. For standard cultivation and for plasmid isolation, YEME or TSBS (tryptone soy broth (Difco) containing 10% (w/v) sucrose) were used. For microscopical analysis, streptomycetes were grown on SFM agar plates.

Table 1: Bacterial strains.

Bacterial strain	Genotype	Reference
S. coelicolor A3(2) M145	SCP1$^-$ SCP2$^-$	(Kieser *et al.*, 2000)
S. coelicolor A3(2) MT1110	SCP1$^-$ SCP2$^-$	(Kieser *et al.*, 2000)
GSX1	M145 *ftsX*::Tn*5062*	This chapter
E. coli JM109	See reference	(Sambrook *et al.*, 1989)
E. coli ET12567	See reference	(MacNeil *et al.*, 1992)
E. coli ET 12567/pUZ8002	See reference	(Gust *et al.*, 2003)

Plasmids, constructs and oligonucleotides

All plasmids and constructs described in this chapter are summarised in Table 2. PCRs were done with *Pfu* polymerase (Stratagene), in the presence of 10% (v/v) DMSO, with an annealing temperature of 58°C. All used oligonucleotides are listed in Table 3.

General cloning vectors

pIJ2925 is a pUC19-derived plasmid used for routine subcloning (Janssen and Bibb, 1993). The shuttle vectors pHJL401 (Larson and Hershberger, 1986) and pSET152 (Bierman *et al.*, 1992) were used for cloning in *Streptomyces*, which both have the pUC *ori* for high-copy number replication in *E. coli* and the SCP2* *ori* on pHJL401 (around five copies per

chromosome) and the *attP* sequence, allowing integration at the attachment site of bacteriophage φC31, on pSET152 for maintenance in *S. coelicolor.* The suicide vector pSET151 contains the pUC *ori* for replication in *E. coli* and *oriT* RK2 for conjugation from *E. coli* to *Streptomyces* (Bierman *et al.*, 1992) and was used for recombination experiments.

Table 2: Plasmids and constructs.

Plasmid/ Cosmid	Description	Reference
pHJL401	*Streptomyces/E. coli* shuttle vector (5-10 and around 100 copies per genome, respectively)	(Larson and Hershberger, 1986)
pSET151	*Streptomyces* integrating plasmid with *E. coli* ori	(Bierman *et al.*, 1992)
KF41	pSET152-derived integrative vector expressing FtsZ-EGFP	(Grantcharova *et al.*, 2005)
pGWS113	pIJ2925 with 2,5 kb fragment harbouring a translational fusion of *ssgA* and *egfp*	(Noens *et. al.,* 2007)
E59	Cosmid clone containing *ftsX* and *ftsE*	(Bentley *et al.*, 2002)
E59(*ftsX*::Tn*5062*)	Cosmid E59 carrying a Tn*5062* insertion in *ftsX*	(Bishop *et al.,* 2004)
E59(*ftsE*::Tn*5062*)	Cosmid E59 carrying a Tn*5062* insertion in *ftsE*	(Bishop *et al.,* 2004)
pGWS136	pSET152 with 1,6 kb fragment harbouring an in frame fusion of *ftsE* and *gfp*	This chapter
pGWS137	pSET152 with 1,6 kb fragment harbouring an in frame fusion of *ftsX* and *gfp*	This chapter
pGWS144	pSET151 with 2 kb fragment harbouring the putative *ftsEX* promoter region and *ftsX* (-1512/+15 and +677/+2173, relative to *ftsE*)	This chapter
pGWS145	pHJL401 with 2 kb fragment harbouring the putative *ftsEX* promoter region and *ftsX* (-1512/+15 and +677/+2173, relative to *ftsE*)	This chapter
pGWS146	PGWS144 harbouring an apramycin resistance cassette replacing *ftsE*	This chapter

Homologous recombination experiments and construction of the ftsX mutant

Derivatives of cosmid E59 carrying a Tn*5062* insertion in *ftsX* and *ftsE*, respectively, were generated by *in vitro* transposition (Bishop *et al.*, 2004). These recombinant cosmids were transferred to *E. coli* ET12567 containing the conjugative plasmid pUZ8002, allowing direct conjugational transfer of the mutant cosmid to *S. coelicolor*. Simultaneous screening for loss of the cosmid sequences (Kan^S) and presence of the resistance cassette ($Apra^R$) is indicative of the desired mutant.

The *ftsE* disruption constructs were made as follows. Primers were designed in such a way as to allow amplification of the -1512/+15 (using FtsE-F1 + FtsE-R1) and +677/+2173 (using FtsE-F2 and FtsE-R2) sections relative to *ftsE* from the *S. coelicolor* genome. After digestion with *Eco*RI-*Xba*I and *Xba*I-*Hin*dIII, respectively, these fragments were ligated simultaneously into *Eco*RI-*Hin*dIII-digested pSET151, resulting in pGWS144 containing an in frame deletion of *ftsE*. Subsequently, the *aac(3)IV* gene, conferring apramycin resistance was inserted into the *Xba*I site of pGWS144, creating pGWS146, which has the +16/+676 region of *ftsE* replaced by *aac(3)IV*. Thiostrepton resistance was a selectable marker for the vector sequences for both pGWS144 and pGWS146.

Construct for the complementation of the ftsX mutant

For the complementation of the *ftsX* mutant, the insert of pGWS144 was cloned as an *Eco*RI-*Hin*dIII fragment into pHJL401 generating the low-copy vector pGWS145 that contains wild type *ftsX* expressed from its own promoter.

Constructs for FtsX-EGFP and FtsE-EGFP

A 978 bp fragment harbouring the putative *ftsEX* promoter region and *ftsE* and a 1893 bp fragment harbouring the putative *ftsEX* promoter region and *ftsEX* were amplified from genomic DNA from *S. coelicolor* using oligonucleotides E59_27_28_F + E59_27 (-STOP) and E59_27_28_F + E59_28 (-STOP), respectively. In this way, the stop codons of *ftsE* and *ftsX* were replaced by a *Kpn*I site. The two fragments were inserted into *Eco*RI-*Kpn*I-digested pGWS113 (Chapter 4), replacing *ssgA* and thereby creating an in frame fusion of *ftsE* and *ftsX* with *egfp,* respectively. The inserts of the two constructs were inserted as an *Eco*RI-*Bgl*II fragment into an *Eco*RI-*Bam*HI digested pSET152, creating pGWS136 and pGWS137, respectively.

Table 3: Oligonucleotides.

Primer	Sequence (5' → 3')	Location 5' end	Relative to
FtsE-F1	gctg**gaattc**gctttgaacattcggaatggtgagg	-1512	*ftsE*
FtsE-R1	gctg**tctaga**gtcgaatcggatcacggatgc	+15	*ftsE*
FtsE-F2	gctg**tctaga**taccagcactgacgagccacag	+677	*ftsE*
FtsE-R2	gctg**aagctt**cactgcgcagccggtcgccctcc	+2173	*ftsE*
E59_27_28_F	gctg**gaattc**cagttcgcgcacaccaaccggtc	-248	*ftsE*
E59_27 (-STOP)	gctgcg**ggtacc**ggcggcggcatcatcggcggcggccaccttcaggtacttgcgcaacgc	+690	*ftsE*
E59_28 (-STOP)	gctgcg**ggtacc**ggcggcggcatcatcggcggcggcgtgctggtagccgtagacaccgc	+918	*ftsX*

Restriction sites are presented in bold face.

Microscopy

Electron microscopy

Morphological studies of surface-grown aerial hyphae and spores of *S. coelicolor* M145 and GSX1 by cryo-scanning electron microscopy (cryo-SEM) were performed as described previously, using a JEOL JSM6700F scanning electron microscope (Keijser *et al.*, 2003). Transmission electron microscopy (TEM) for the analysis of cross-sections of hyphae and spores was performed with a Philips EM410 transmission electron microscope as described previously (van Wezel *et al.*, 2000).

Fluorescence microscopy

For the visualisation of strains containing proteins translationally fused to EGFP, sterile coverslips were inserted at a 45° angle into SFM plates and spores were inoculated in the acute angle. After 2 days (for FtsZ-EGFP) and 4-5 days (for FtsE-EGFP and FtsX-EGFP) of incubation at 30°C, coverslips were removed and samples positioned in a drop of water/1% agarose on a microscope slide.

Visualisation of DNA (propidium iodide (PI), Sigma) and cell wall material (FITC-WGA, Biomedica) by confocal fluorescence microscopy was performed as described previously (Chapter2). For these experiments, strains were grown on SFM for 5 days at 30°C.

Immuno-fluorescence microscopy of FtsE was carried out as described previously (Schwedock *et al.*, 1997). For this, *S. coelicolor* M145 was grown on SFM for 3 days at 30°C. Antibodies against FtsE were used in a dilution of 1:1000 and visualised using an Alexa Fluor® 488-labelled goat anti-rabbit secondary antibody (Molecular Probes) at a concentration of 7,5 µg/ml. Antibodies against FtsE were a kind gift of Joen Luirink (VU Amsterdam) and were directed against *E. coli* FtsE monomers and dimers.

Computer analysis

The TMHMM2 program (http://www.ch.embnet.org/software/TMPRED_form.html) was used for the prediction of transmembrane domains in proteins and T-Coffee (http://www.ch.embnet.org/software/TCoffee.html) and Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html) for multiple protein alignment.

RESULTS

Alignment of FtsE and FtsX homologues

FtsEX belong to the family of ABC transporters; FtsE resembles the ATP-binding protein and interacts with the membrane component FtsX (de Leeuw *et al.*, 1999; Schmidt *et al.*, 2004; Ukai *et al.*, 1998). Surprisingly, the predicted amino acid sequence of *S. coelicolor* FtsE (ScFtsE) shares only 49% identical and 70% similar amino acid residues with *E. coli* FtsE and 55% and 62% aa identity with FtsE from *B. subtilis* and *M. tuberculosis*, respectively (Fig. 1A).

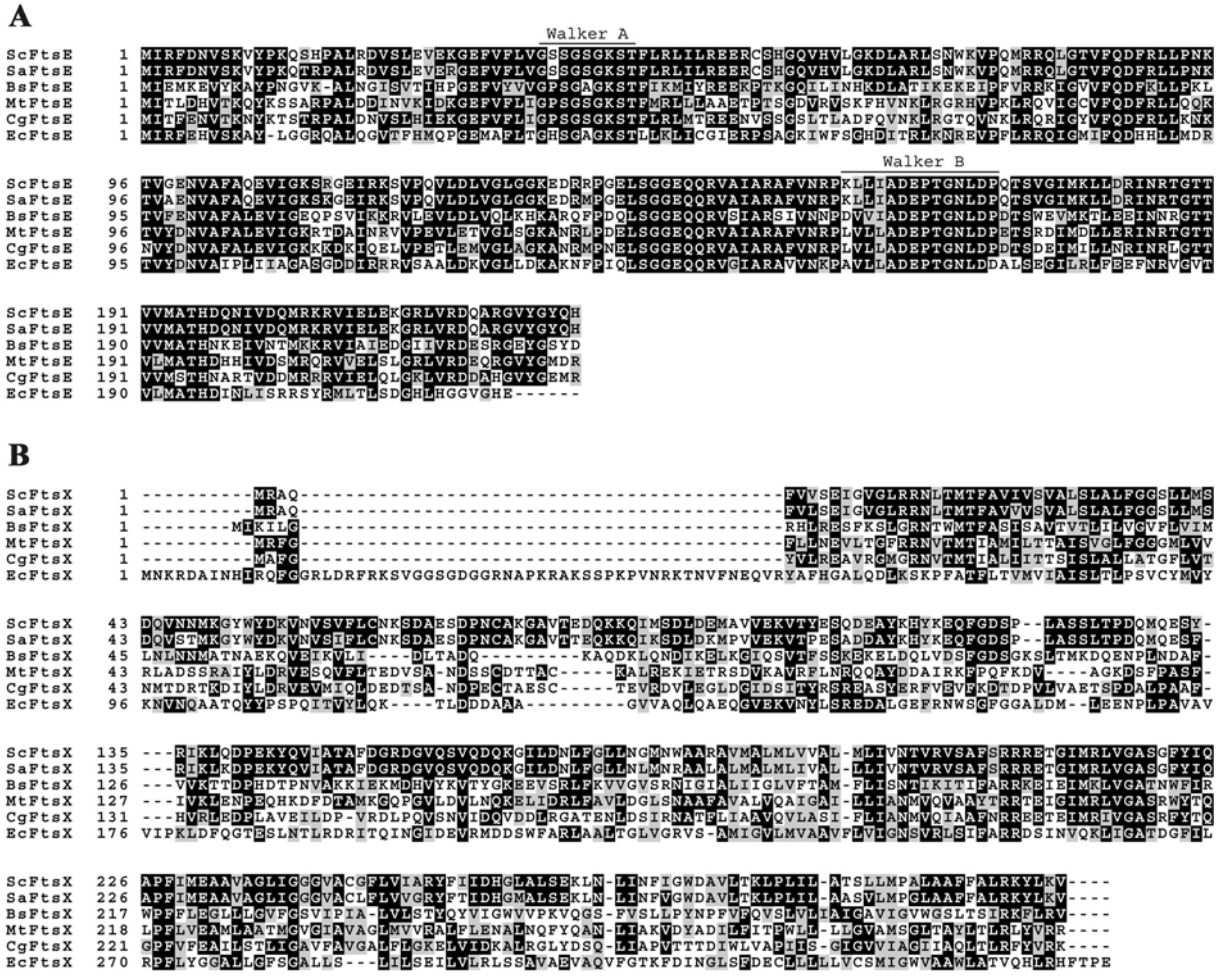


Figure 1: Sequence alignment of FtsE (A) and FtsX (B). Multiple alignments from various bacterial genera were carried out using T-Coffee (http://www.ch.embnet.org/software/TCoffee.html). Amino acids marked with black or grey boxes indicate sequence identity or similarity, respectively. The dashes indicate the gaps introduced to optimise the alignment. This was done using Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). ScFtsE/X: FtsE and FtsX of *S. coelicolor*, SaFtsE/X: FtsE and FtsX of *S. avermitilis*, BsFtsE/X: FtsE and FtsX of *B. subtilis*, MtFtsE/X: FtsE and FtsX of *Mycobacterium tuberculosis*, CgFtsE/X: FtsE and FtsX of *Corynebacterium glutamicum* and EcFtsE/X: FtsE and FtsX of *E. coli*. Walker A and B consensus sequences in FtsE are marked.

ScFtsE contains the Walker A and B consensus motifs that make up the ATP-binding site in proteins of the ABC family. The Walker A motif (GXXGXGKT/S) of both ATP and GTP-binding proteins is located between aa residues 36-43 of ScFtsE, which also contains the P-loop lysine residue that is in direct contact with the β- and γ-phosphate of the bound NTP (Saraste *et al.*, 1990). Even more surprisingly, the homology between FtsX proteins of different species is lower than that of FtsE proteins (Fig. 1B). For example, *S. coelicolor* FtsX (ScFtsX) shows only very limited similarity to the homologue from *E. coli* (20% identical and 45% similar residues). Similarly to FtsX of *E. coli*, ScFtsX is predicted to have four transmembrane domains, instead of the six typical of membrane components of other ABC transporters.

Construction of an ftsX mutant

ftsE (SCO2969) and *ftsX* (SCO2968) form an operon and lie relatively close to the origin of replication on cosmid E59 of the ordered cosmid library of *S. coelicolor* (Redenbach *et al.*, 1996). Upstream of *ftsE* lies SCO2970, encoding a membrane protein, while downstream of *ftsX* a cluster of genes is found that is the tmRNA-mediated trans-translation (Withey and Friedman, 2002), including ssrA for tmRNA, smpB (SCO2966) for small protein B (Braud *et al.*, 2006) and ctpA (SCO2967) a carboxy-terminal processing protease, most likely involved in proteolytic degradation of proteins tagged by the tmRNA protein tagging system (Fig. 2).

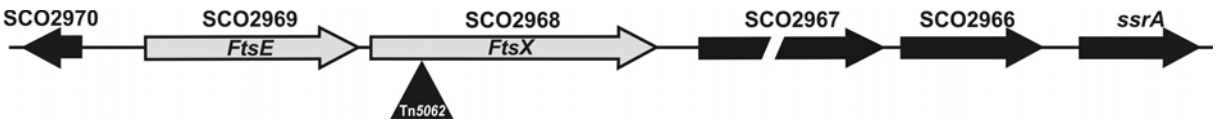


Figure 2: Genomic organisation of *ftsEX*. *ftsEX* are shown as grey arrows while adjacent genes are shown as black arrows. The corresponding SCO-numbers are shown above the arrows. SCO2967 encodes for a carboxy-terminal processing protease, most likely involved in proteolytic degradation of proteins tagged by the tmRNA tagging system. SCO2970 encodes for a membrane protein. The triangle indicates the position of TN*5062* insertion inactivating *ftsX*.

E59(*ftsX*::Tn*5062*) and E59(*ftsE*::Tn*5062*) were introduced into *S. coelicolor* M145 by conjugation from *E. coli* ET12567/pUZ8002. For E59(*ftsX*::Tn*5062*), simultaneous screening for loss of the cosmid sequences (Kan^S) and presence of the resistance cassette ($Apra^R$) gave several independent mutants. Southern hybridisation confirmed that the correct recombination event had occurred (not shown). This mutant was designated GSX1 (*ftsX*::Tn*5062*). Surprisingly, despite many attempts we were unable to obtain mutant double recombinants for

E59(*ftsE*::Tn*5062*). Using the plasmids pGWS144, harbouring an in frame deletion of *ftsE*, and pGWS146, where the +16/+676 region of *ftsE* was replaced by an apramycin cassette, we tried to create an *ftsE* deletion strain, in a different manner. Since *ftsE* is preceding the co-transcribed *ftsX*, introduction of pGWS146 into the genome of *S. coelicolor* will have a polar effect on the transcription of *ftsX*. Both plasmids were introduced into *S. coelicolor* M145. Double recombination was checked for both constructs, giving loss of thiostrepton resistance for pGWS144 and additional apramycin resistance for pGWS146. After introduction of pGWS146 and during screening for double recombination, only 10% of the cells were single cross-overs and apramycin resistant. In other words, 90% of the cells had lost the plasmid and recombination invariably led to the wild type genotype. Also after introduction of pGWS144, all double recombinants checked harboured the wild type *ftsE*. Since in *E. coli* an *ftsE* null mutant is only viable in the presence of more than 0.5% NaCl (de Leeuw *et al.*, 1999), the knock-out experiments were repeated with and without the addition of different concentrations of NaCl, but in either case double recombinants were never obtained. Our inability to create an *ftsE* deletion mutant using three different techniques may be an indication that (in contrast to *ftsX*) *ftsE* is an essential gene in *S. coelicolor*.

Characterisation of the *ftsX* mutant

When plated on SFM agar plates for 5 days, the *ftsX* mutant GSX1, had a lighter grey appearance than the parental strain M145, suggesting it produced significantly fewer spores (Fig. 3). To characterise the *ftsX* mutant in more detail, the mutant was grown together with the parental strain on SFM for 5 days at 30°C and analysed by electron and fluorescence microscopy. Cryo-scanning electron microscopy revealed relatively regular spore chains.

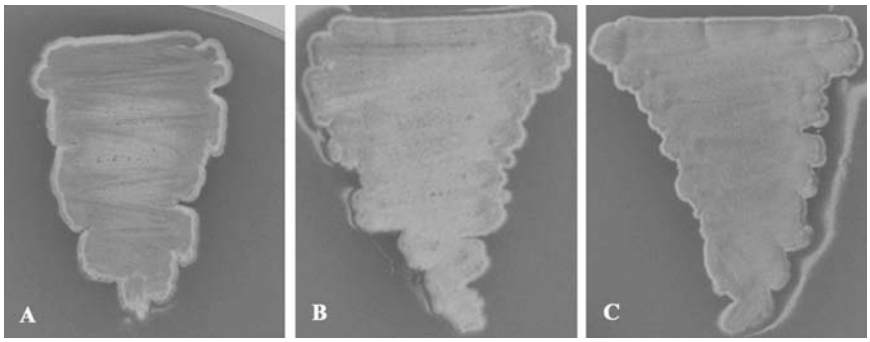
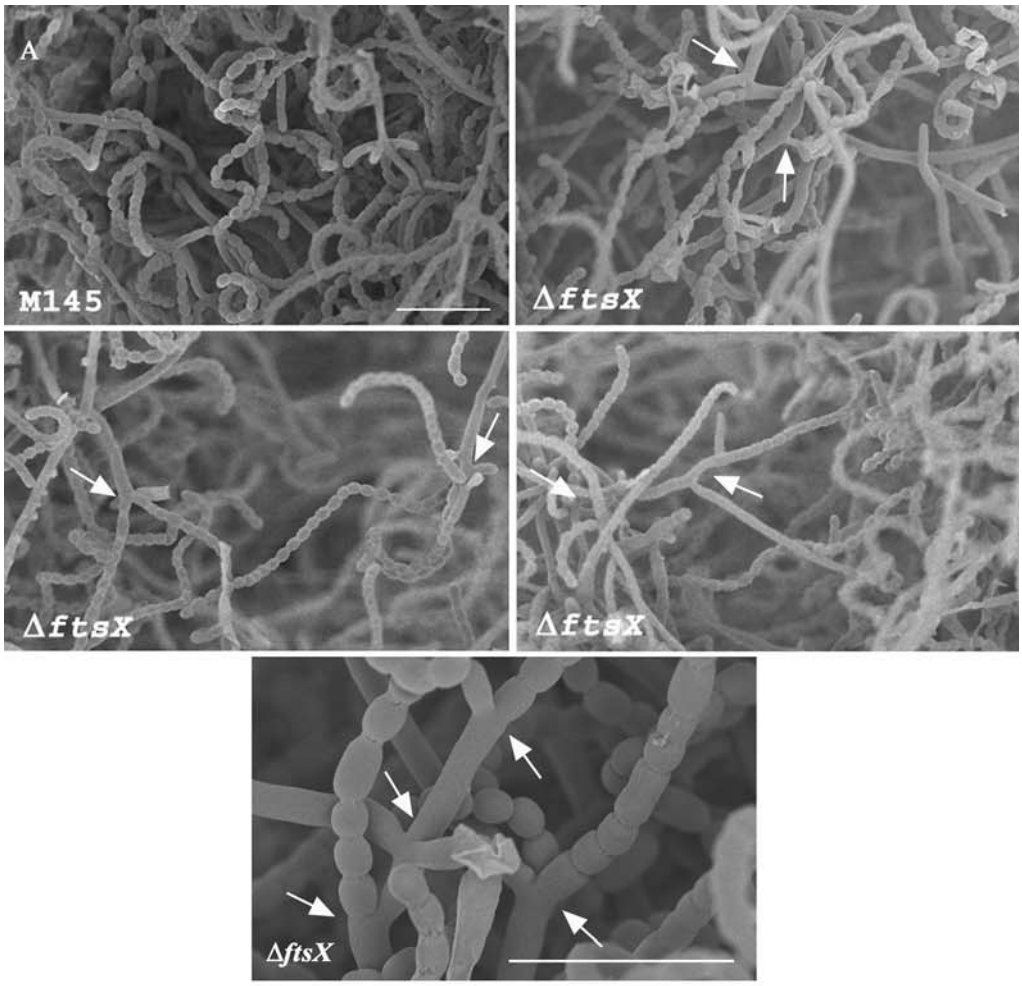


Figure 3: Phenotypes of the *ftsX* mutant (B), its genetically complemented derivative (C) and its congenic parent *S. coelicolor* M145 (A) on solid media. Strains were grown on SFM at 30°C for 5 days.


A
M145
ΔftsX
ΔftsX
ΔftsX
ΔftsX

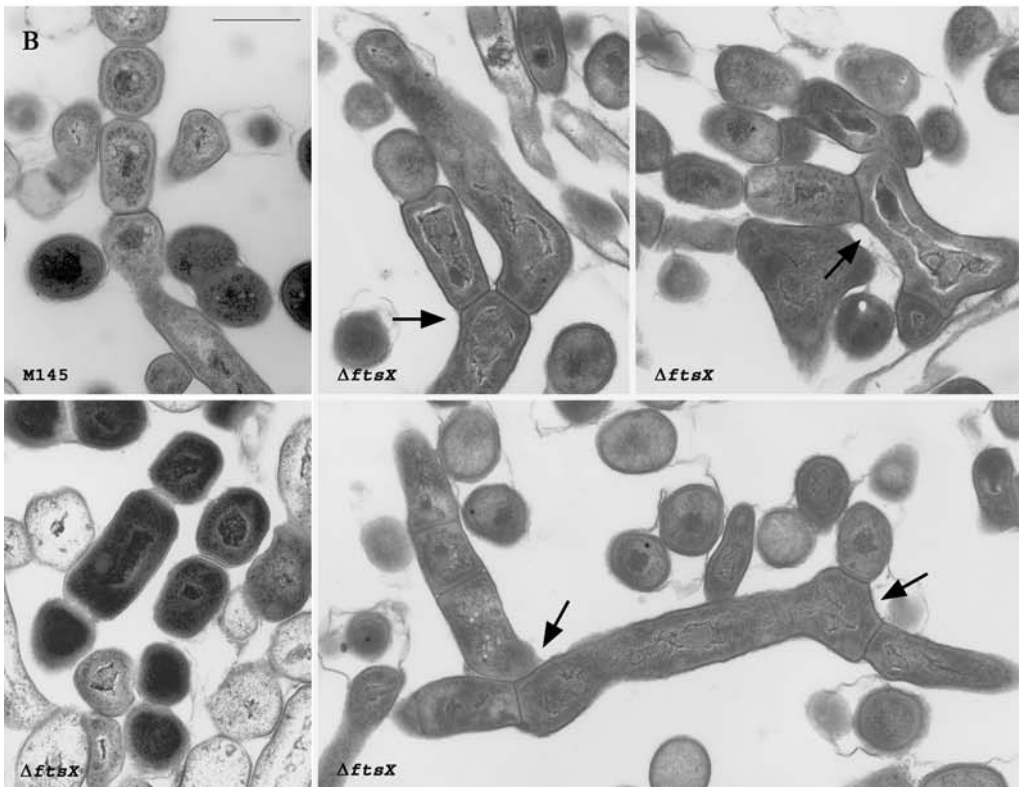

B
M145
ΔftsX
ΔftsX
ΔftsX
ΔftsX

Figure 4: Phenotypic characterisation of an *ftsX* mutant by cryo-scanning electron microscopy and transmission electron microscopy. Samples were taken from 5-day old cultures grown on SFM at 30°C. **A.** Cryo-SEM of spores and aerial hyphae of M145 and the *ftsX* mutant. Bar = 5 μm. **B.** TEM of aerial hyphae and spore chains of M145 and the *ftsX* mutant. Bar = 1 μm. **A-B:** Branches of aerial hyphae close to the base of the spore chain were detected in the *ftsX* mutant (arrows).

Surprisingly however, the mutant aerial hyphae frequently showed branching close to the base of the spore chain, which was never observed in the wild type strain (Fig. 4A). The same branches were also identified by high-resolution transmission electron microscopy, which also revealed irregular spore sizes (Fig. 4B).

Nucleic acid distribution in the *ftsX* mutant appeared to be very similar as in the wild type. However, around 70% of the Δ*ftsX* spores showed WGA-stained foci at the spore poles in comparison with the staining at the spore poles in the wild type (Fig. 5, for a full colour version, see p184).

GSX1 was restored to the phenotype of the parental strain by the introduction of a low copy-number vector harbouring the relevant *ftsX* gene and its promoter sequence (pGWS145). This complemented Δ*ftsX* strain was included in the microscopical analysis, verifying its re-established phenotype (not shown).

Localisation of FtsZ-EGFP in the *ftsX* mutant

In *E. coli*, studies were performed to reveal an order of assembly for Fts proteins into a multiprotein complex, called the divisome. Localisation of FtsX in *E. coli* appears to require FtsZ, FtsA and ZipA, but not the downstream division proteins FtsK, FtsQ, FtsL and FtsI (Schmidt *et al.*, 2004). To determine if FtsZ is dependent on FtsX for localisation at the septum site, we constructed an *ftsX* mutant harbouring FtsZ fused to EGFP. Regularly spaced FtsZ rings were detected in sporogenic aerial hyphae (Fig. 6A, for a full colour version of Fig. 6, see p185). This suggests that the correct localisation of FtsZ is not dependent on FtsX.

Localisation of FtsX and FtsE

In *E. coli*, FtsE and FtsX were localised at the division site in cells, which were on average longer, indicating that these proteins are functional during later stages of cell growth and remain at the division site until division is complete. Therefore, it has been suggested that FtsEX might be involved in the constriction of the septal ring or alternatively, may assist in the insertion of one or more division proteins into the cytoplasmic membrane (Schmidt *et al.*, 2004).

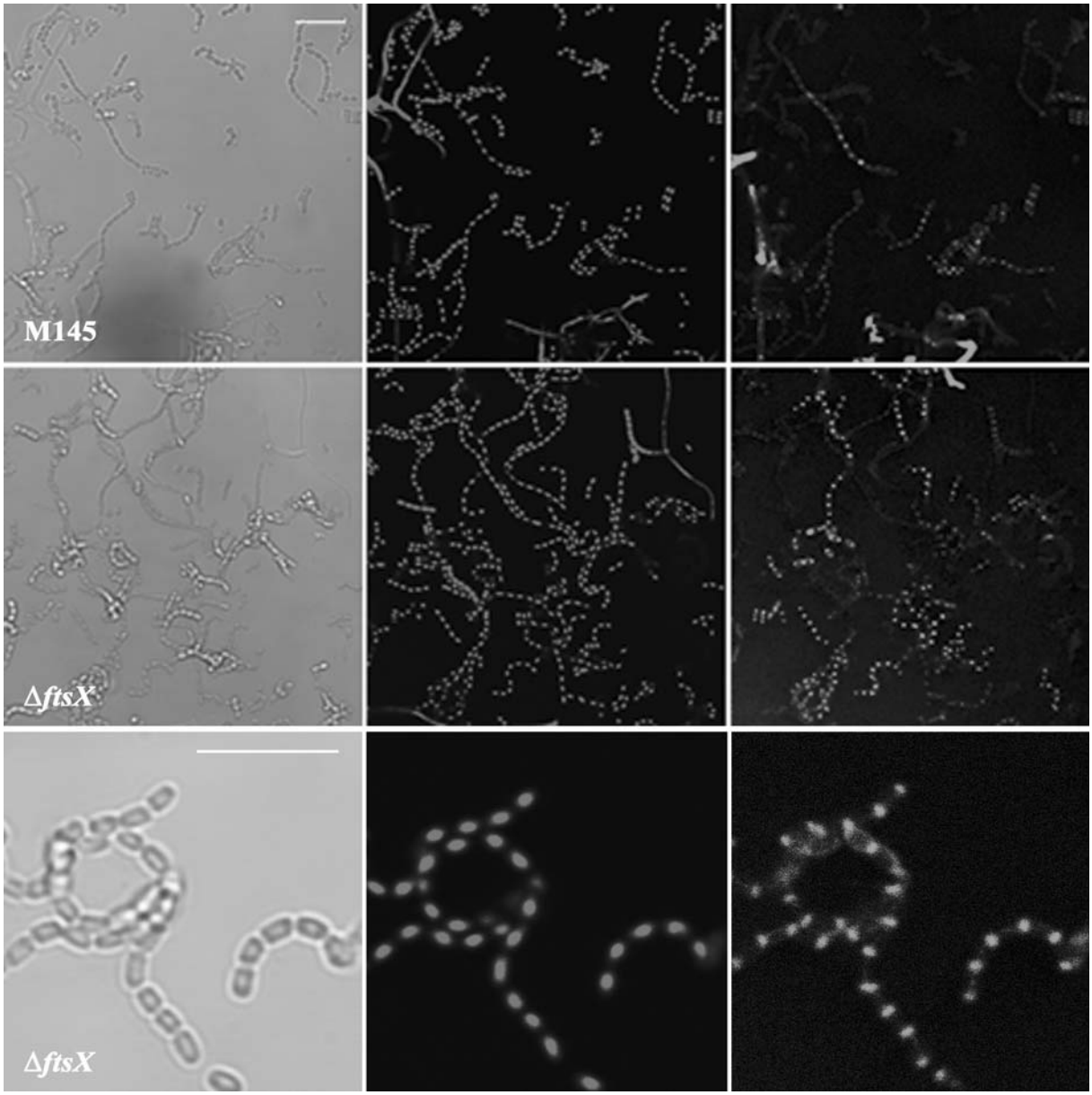


Figure 5: Analysis of an *ftsX* mutant by confocal fluorescence microscopy. Samples were prepared from 5-day old surface-grown cultures at 30°C of the parental strain M145 and the *ftsX* mutant. DNA and peptidoglycan subunits were visualised with PI (middle column) and fluorescein-WGA (right column). The left column shows light microscopy images. Bar = 5 μm. (Full colour version, see p184).

In order to localise FtsE and FtsX, we constructed pGWS136 and pGWS137, expressing in frame fusions of *ftsE-egfp* and *ftsX-egfp*, respectively, from their natural promoters (See Materials and Methods section). However, we could not detect any specific localisation of the GFP-tagged proteins in *S. coelicolor* transformants harbouring either pGWS136 or pGWS137, which could mean that the fusion with EGFP interferes with the

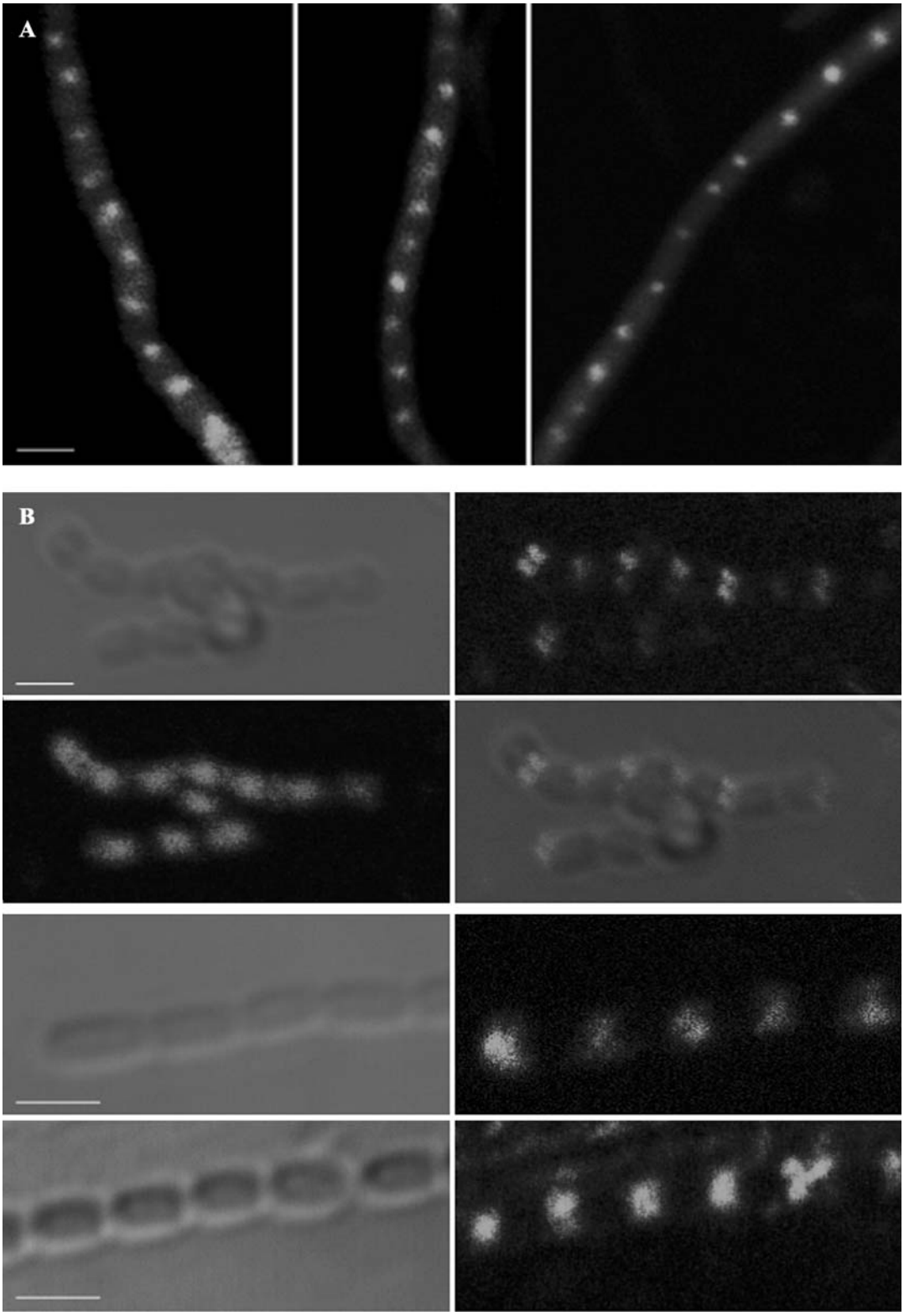


Figure 6: A. FtsZ-rings in an *ftsX* mutant. Strains were grown on SFM for 2 days at 30°C. **B. Cellular localisation of FtsE using anti-FtsE antibodies.** Strains were grown on SFM for 5 days at 30°C. Propidium iodide was used to visualise DNA. The left column shows light microscopy images and the right column represents FtsE localisation (Row 1-3-4) or the left column shows DNA and the right column shows an overlay of the images of row 1 (Row 2). Bar = 1 μm. (Full colour version, see p185).

proper function of the protein. In *E. coli*, researchers also could not identify specific foci for FtsE-EGFP (Schmidt *et al.*, 2004). Considering our failure to identify the GFP-tagged proteins, we attempted localisation of the proteins with immuno-fluorescence microscopy using *E. coli* antibodies. Cells were stained after treatment with lysozyme. Propidium iodide was used to visualise the DNA. FtsE localised at a time after the completion of DNA segregation, at the spore septa of maturing spore chains, which was visualised as a ladder. The fluorescent signal was brighter at either side of the septum (close to the hyphal walls) with less bright fluorescence in the middle of the septum (Fig. 6B). The localisation pattern of FtsE in the *ftsX* mutant was similar as in the parental strain (not shown). This localisation pattern of FtsE is similar to that of SsgB (Chapter 3), which may indicate that these proteins are recruited to the divisome at the same time, and may interact directly (see also Chapter 3).

DISCUSSION

In this chapter, we have investigated the function of FtsE and FtsX in *S. coelicolor*. These two proteins are homologous to an ABC transporter complex in *E. coli* that is since long known to play a role in cell division, although its precise function is still unknown. Sequence comparison indicates that FtsEX can be placed with importers rather than exporters (Schmidt *et al.*, 2004). An *ftsX* mutant of *S. coelicolor* was not obviously defective in cell division. The strain produced regular spore chains and normal vegetative septa. However, frequent branching was observed in the aerial hyphae, at the base of the spore chain. In comparison to the wild type, a significantly larger proportion of *ftsX* mutant mature spores showed accumulation of peptidoglycan (PG) precursors during autolytic separation. PG subunits released during septal constriction might be transported back into the spores, perhaps mediated by FtsEX, to enter a recycling pathway. Recycling of PG was already reported to happen during PG biosynthesis, while a significant proportion of the turnover is related to cell division (Goodell, 1985; Park, 1993). Subsequently, these recycled subunits could participate in spore wall synthesis and/or cell wall synthesis during spore germination and hyphal growth. Cells lacking FtsX may be disturbed in this PG import and the remaining subunits are then stained with Fluo-WGA. This staining of PG subunits at the spore poles was also seen in spores lacking SsgF (Chapter 2), although the typical 90° rotation of loosely attached spores, due to incomplete breakdown of PG subunits, was not seen in the *ftsX* mutant. This could be another indication that the stained subunits accumulate because of a disturbance in transport

rather than a deficiency in breakdown. FtsEX could also function in recruiting cell division proteins functional in this process. The phenotype of an *ftsX* mutant could be the result of the lack of *ftsX* and/or of free FtsE, which might interact with another protein and exerts its function as an ATP-binding protein in another process. Attempts to create an *ftsE* knock out strain failed, even with the addition of extra salt in the medium, suggesting that FtsE might be an essential protein interacting with at least one other ATP-requiring and essential transport protein. There is at least one other example reported of an ATP-binding protein assisting more than one ABC transport system. For example, MsiK was originally identified as the ATPase for the maltose transport system but apparently provides energy to no less than 30 other processes (Schlosser *et al.*, 1997) and S. Rigali, personal communication). ScFtsE has a homology of about 50% with EcFtsE, and working with constructs containing the full sequence of *ScftsEX* in *E. coli*, was difficult and appeared to be toxic for the *E. coli* cells.

Localisation studies of FtsE and FtsX, using a fusion with EGFP failed to work, possibly because these proteins are not able to function properly as GFP fusions. The cellular localisation of FtsE was discovered using peptide antibodies directed against FtsE. As in *E. coli*, where both FtsX and FtsE localise at the septum in a later stage of cell growth, *S. coelicolor* FtsE was localised to the septum and this was after DNA segregation in maturing spores.

Fluorescence was brighter at the sides of the septum, indicating that FtsE might be forming an open ring-like structure. Assuming that, as in *E. coli*, FtsX and FtsE form a complex together, it is very tempting to speculate that FtsX localises, with FtsE, at septum sites. The localisation of FtsE at the periphery of the septum in maturing spores is again suggesting that FtsEX have a function during autolytic spore separation. This observation supports the hypothesis that FtsEX are active during later stages of sporulation. It was also suggested that in *E. coli* FtsEX are involved in constriction of the septal ring or in insertion of a division protein into the membrane (Schmidt *et al.*, 2004). Our data strongly suggest that the latter is not the case, but rather that FtsX allows transport of autolytically produced PG subunits back into the cells.

FtsZ is capable of producing the normal Z-ring in an *ftsX* mutant. Therefore, FtsZ does not need FtsX for its function. During FtsE localisation, DNA segregation was finished and septa appeared to be closed in the maturing spores, leading us to speculate that FtsK, involved in DNA segregation during sporulation, and FtsI, taking part in septum synthesis, will have the ability to localise and function without FtsX. It appears that in *S. coelicolor* the order of

recruiting proteins to the septal ring is different from that in *E. coli*. This could be the result of the different order of events taking place in cell division in *S. coelicolor*, where DNA segregation happens after septum initiation and constriction occurs after septum closure, while in *E. coli* segregation of the chromosome happens before the start of septum synthesis and constriction happens during septum synthesis.

We anticipate that *S. coelicolor* FtsE and FtsX, interact with each other and constitute an ABC transporter. From the localisation at septum sites in maturing spores and the accumulation of PG subunits during constriction, we hypothesise that FtsEX function during spore separation by autolysis, most likely as a recycling component. Still, the exact function of these proteins needs further investigation. Two crucial question that remain include are they really transporters and if so, what do they import.

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