Control of sporulation-specific cell division in Streptomyces coelicolor

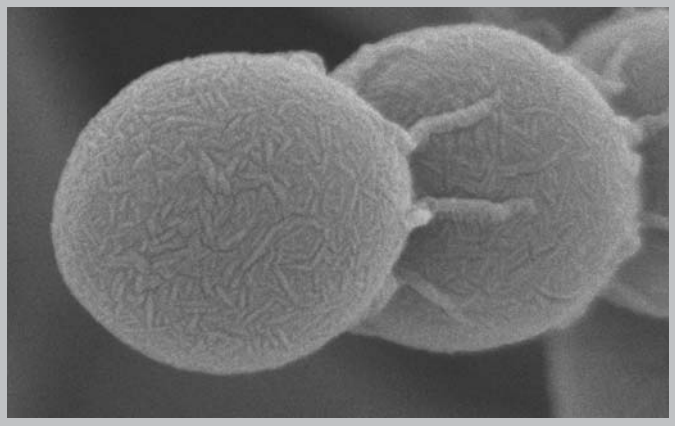
Noens, E.

5

MreBCD and Mbl of *Streptomyces coelicolor* are required for the integrity of aerial hyphae and spores

Elke E. E. Noens, Paola Mazza, Kathrin Shirner,
Nina Grantcharova, Mieke Mommaas, Henk K. Koerten,
Gunther Muth, Klas Flärdh, Wolfgang Wohlleben
and Gilles P. van Wezel

Parts of this chapter have been published in
Mol Microbiol (2006) 60: 838-852



ABSTRACT

The cytoskeletal protein MreB is involved in cell shape determination and chromosome segregation in many rod-shaped bacteria. PCR-based and Southern analysis of various actinomycetes, supported by analysis of genome sequences, revealed *mreB* orthologues only in genera that form an aerial mycelium and spores, although a distant relative was found in *Rhodococcus*. We analysed MreBCD and Mbl in *Streptomyces coelicolor*. Ectopic overexpression of *mreB* impaired growth, and caused swellings and lysis of hyphae. *mreB* and *mbl* null mutants were created, which showed normal vegetative growth but aberrant development. Analysis of null mutants deleted for either *mreB, mreC, mreD, mreBCD*, *mbl* or *pbp2* were subjected to an intensive study using electron microsocpy. All mutants had similar anomalies, with swelling and lysing aerial hyphae and spores of aberrant dimensions, indicative of disruption of the cell wall integrity. An MreB-EGFP fusion protein localised at the septa of sporulating aerial hyphae, as bipolar foci in young spores, and as ring- or shell-like pattern inside mature spores. No specific localisation was observed in vegetative hyphae, although transcription of *mreB* was at least as high as in aerial hyphae. Immunogold electron microscopy using MreB-specific antibodies revealed that MreB is located immediately underneath the spore wall. Thus, MreBCD and Mbl are not essential for vegetative growth of *S. coelicolor*, but exert their function in the formation of environmentally stable spores, thereby primarily influencing the assembly of the spore cell wall.

INTRODUCTION

Bacterial morphologies range from spherical and rod-shaped to curved, helical and filamentous. A major determinant of cell shape is the bacterial cell wall, which consists of glycan strands cross-linked by short peptides. Isolated peptidoglycan sacculi can retain the shape of the bacterial cell, and mutants defective in peptidoglycan synthesis typically show an altered morphology (Cabeen and Jacobs-Wagner, 2005; Young, 2003). However, not only the enzymes that are directly involved in the synthesis and assembly of peptidoglycan affect the morphology. It is becoming increasingly clear that cytoskeletal elements exist in the bacterial cytoplasm (Lowe *et al.*, 2004) and that these determine the architecture of the cell wall, with a strong impact on cell shape. These include homologues of all three major types of eukaryotic cytoskeletons. MreB-proteins are actin homologues that produce microfilament-like fibers and determine rod-shape in many bacteria (Jones *et al.*, 2001; van den Ent *et al.*, 2001); crescentin (CreS) produces intermediate filament-like elements in *Caulobacter crescentus* that give rise to a curved cell shape (Ausmees *et al.*, 2003); FtsZ is a tubulin-homologue that assembles into a cytokinetic ring at the site of cell division and directs cell division and formation of the septal peptidoglycan (Margolin, 2005).

MreB is a member of the HSP70-actin-sugar kinase (ASHKA) superfamily of proteins (Bork *et al.*, 1992), and its crystal structure is strikingly similar to the structure of actin (van den Ent *et al.*, 2001). Purified MreB of *Thermotoga maritima* polymerises *in vitro* to form filaments with a spacing between the MreB monomers of 51 Å, which is reminiscent of the spacing between the subunits (55 Å) in actin filaments (van den Ent *et al.*, 2001). MreB-like proteins have been studied primarily in *E. coli* (MreB), *B. subtilis* (MreB, Mbl, MreBH) and *C. crescentus* (MreB), and immuno-fluorescence microscopy and GFP-tagging showed that they form helical-like structures underneath the cell envelope (Jones *et al.*, 2001; Shih *et al.*, 2003; Soufo and Graumann, 2003). MreB is essential in *E. coli* and *B. subtilis*, and its depletion resulted in increased cell width, loss of cell shape and eventually lysis (Formstone and Errington, 2005; Jones *et al.*, 2001; Kruse *et al.*, 2005; Lee and Cohen, 2003). The MreB-like Mbl protein is required for determination of the rod-shape of *B. subtilis*, and forms helical cytoskeletal structures that are needed for incorporation of new peptidoglycan along the lateral wall during cell elongation (Daniel and Errington, 2003). While these observations suggest that MreB-like proteins form actin-like fibers defining the bacterial cell shape, the precise cellular function of MreB proteins remains unclear. Recent studies link MreB to

peptidoglycan synthesis (Daniel and Errington, 2003), correct chromosome segregation (Gitai *et al.*, 2005; Kruse *et al.*, 2003; Soufo and Graumann, 2003), and cell polarity (Gitai *et al.*, 2004). Interestingly, the MreB homologue of the rod- and coccoid-shaped *Rhodobacter sphaeroides* failed to produce helical structures and rather formed a ring at the mid-cell position of elongating cells, suggesting it also plays a role in septal peptidoglycan synthesis in this organism (Slovak *et al.*, 2005). Thus, MreB may well have different functions in different organisms.

mreB is almost invariably part of an operon with *mreC* and *mreD*, and typically followed by *pbp2*, which is transcribed from its own promoter. Like *mreB*, the *mreC* and *mreD* genes are essential in *E. coli* and *B. subtilis,* and their depletion results in lost control of cell shape, spheroid morphology and lysis (Kruse and Gerdes, 2005; Leaver and Errington, 2005; Lee and Stewart, 2003). A link between the actin-like cytoskeleton formed by the MreB family and the lateral cell wall synthesis machinery is suggested (Daniel and Errington, 2003; Errington, 2003; Kruse and Gerdes, 2005). *pbp2* from *S. coelicolor*, encoding a penicillin-binding protein (PBP) has high similarity with *E. coli* PBP2, which is involved in elongation of the lateral cell wall (Vinella *et al.*, 1993) and with SpoVD, a PBP important for sporulation in *B. subtilis* (Daniel *et al.*, 1994). Absence of the elongation system in *E. coli* and *B. subtilis* leads to the formation of rounded cells (Wei *et al.*, 2003).

The absence of an *mre* gene cluster in coccoid species such as *Streptococcus*, *Staphylococcus* and *Lactococcus* suggests that a spherical shape may relate to the absence of an *mre*-dependent system and that MreB is particularly important in rod-shaped cells (Jones *et al.*, 2001). However, the rod-shaped actinomycete *Corynebacterium glutamicum* also lacks *mreB*-like genes in its genome and establishes its rod-shape in an MreB-independent way, through growth of the cell wall only at the cell poles (Daniel and Errington, 2003). Most other actinomycetes also lack *mreB*, but streptomycetes are a notable exception as their genomes contain well-conserved *mreB*-homologues (see below). The genome of *S. coelicolor* contains an *mreBCD-pbp2* gene cluster and a separately located *mbl* gene. In this chapter, we describe the creation and study of cytoskeletal mutants, which are studied by advanced electron microscopy, confocal fluorescence microscopy and transcriptional analysis. We provide evidence that all components play an important role in the control of the shape of aerial hyphae and spores.

MATERIALS AND METHODS

Bacterial strains and culturing conditions

The *E. coli* and *S. coelicolor* A3(2) strains are listed in Table 1. *S. coelicolor* strains were cultivated on SFM or R2YE agar plates or in TSB medium, with or without the appropriate antibiotics as indicated (Kieser *et al.*, 2000). Cultivation of strains and procedure for DNA manipulation was performed as previously described for *E. coli* (Sambrook *et al.*, 1989) and *S. coelicolor* (Kieser *et al.*, 2000). Protoplasts of *S. coelicolor* M145 were transformed with the plasmids pPM1 or pGM190 as described previously (Kieser *et al.*, 2000).

Table 1: Strains of *E. coli* and *S. coelicolor*.

Strain	Characteristics	Reference
E. coli BL21(DE3)pLysS	See reference	Invitrogen
E. coli ET 12567	See reference	(MacNeil *et al.*, 1992)
E. coli DH5α	See reference	(Woodcock *et al.*, 1989)
E. coli BW 25311/pIJ790	See reference	(Gust *et al.*, 2003)
S. coelicolor M145	SCP1$^-$ SCP2$^-$	(Kieser *et al.*, 2000)
mreB-IM	M145 *ΔmreB::aac(3)IV*	This chapter
mreB-IFD	M145 *ΔmreB*	This chapter
mreB-IFDc	M145 *ΔmreB attB$_{\Phi C31}$::mreB*	This chapter
Δ*mreC*	M145 Δ*mreC::aac(3)IV*	(Burger *et al.*, 2000)
Δ*mreD*	M145 Δ*mreD::aac(3)IV*	W. Wollheben
Δ*mreBCD*	M145 Δ*mreBCD::aac(3)IV*	W. Wollheben
Δ*mbl*	M145 *Δmbl::aac(3)IV*	This chapter
Δ*pbp2*	M145 Δ*pbp2::aac(3)IV*	G. Hobbs
SCPM6	M145 *attB$_{\Phi C31}$:: mreB-egfp*	This chapter

Plasmids

All plasmids and constructs are listed in table 2. The oligonucleotides are listed in table 3.

To obtain a construct for the expression of MreB in *S. coelicolor*, the *mreB* gene was amplified from *S. coelicolor* M145 genomic DNA by PCR using primers PM1 and PM2, the PCR product was digested with *Nde*I-*Hin*dIII and cloned behind the thiostrepton-inducible promoter *PtipA* by inserting it in the *Streptomyces* multi-copy plasmid pGM190 (G. Muth, unpublished data) digested with the same enzymes. The resulting plasmid was designated pPM1. To construct a C-terminal fusion with the EGFP protein, *mreB* was amplified from *S. coelicolor* M145 genomic DNA using primers PM5 and PM6, and cloned as an *Nde*I-*Bgl*II fragment in front of the *egfp* gene in plasmid pTST101 (J. Altenbuchner, personal communication). The resulting *mreB-egfp* fusion was removed from pTST101 with *Nde*I and *Bam*HI and cloned in the integrative *Streptomyces* vector pSET152 (Bierman *et al.*, 1992)

digested with the same enzymes, generating pPM4. Plasmid pPM6 was constructed to complement the *mreB* deletion mutant by amplifying the *mreB* gene with its promoter region from *S. coelicolor* M145 genomic DNA with primers PM11 and PM12 and cloning the PCR product as a *Bgl*II-*Eco*RI fragment in pSET152 (Bierman *et al.*, 1992). Finally, to allow high-level production of MreB in *E. coli* to use for preparation of antibodies, *mreB* was amplified by PCR from *S. coelicolor* genomic DNA using primers PM7 and PM2, and the resulting DNA fragment was digested with *Bam*HI and *Hin*dIII and cloned in pRSETB (Invitrogen), so as to form an in frame fusion with the His-tag (pPM5).

Table 2: Plasmids and constructs.

Plasmid/ Cosmid	Description	Reference
pGM190	*Streptomyces* multi-copy plasmid with thiostrepton-inducible promoter *PtipA*	G. Muth; unpublished data
pTST101	*E. coli* plasmid containing *malE-egfp*	J. Altenburgher
pSET152	*Streptomyces/ E. coli* shuttle vector (integrative in *Streptomyces*, high copy number in *E. coli*)	(Bierman *et al.*, 1992)
pRSETB	*E. coli* plasmid with high level expression from the bacteriophage T7 promoter and N-terminal polyhistidine (6xHis) tag	Invitrogen
C88	Cosmid clone containing *mreB*	(Bentley *et al.*, 2002)
C24	Cosmid clone containing *mbl*	(Bentley *et al.*, 2002)
PM1	pGM190 with a DNA fragment harbouring *mreB*	This chapter
PM4	pSET152 with a DNA fragment harbouring *mreB* and *egfp*	This chapter
PM5	pRSETB with a DNA fragment harbouring *mreB*	This chapter
PM6	pSET152 with a DNA fragment harbouring *mreB* with its promoter region	This chapter
PM11	Mutant cosmid C88 with coding region of *S. coelicolor mreB* replaced by *aacC4*	This chapter
PM12	Mutant cosmid C88 with an in frame deletion of *S. coelicolor mreB*	This chapter
C24/Δ*mbl*	As PM11, but for *S. coelicolor mbl*	This chapter

Protein purification and Western analysis

His_6-tagged MreB was expressed in *E. coli* BL21 containing pPM5 and purified with Ni-NTA spin columns (Qiagen) under denaturing conditions, as described in the supplier's manual. Rabbit polyclonal antibodies were raised against the purified protein (Eurogentec). For expression of MreB in *S. coelicolor*, transformants were inoculated in TSB containing kanamycin (50 μg ml^{-1}), and thiostrepton was added to 10 μg ml^{-1} (final concentration) for induction of expression. After 18h of growth, mycelium was collected and lysed with a French Press. Crude extracts were mixed with an equal volume of 2X sample buffer (125 mM Tris-HCl pH 6.8; 4% SDS; 20% glycerol; 2.0 mM EDTA; 0.02% bromophenol blue; 3% dithiothreitol), heated at 100°C for 5 min and loaded on SDS-polyacrylamide gels (12.5%).

Proteins were capillary transferred to a nitrocellulose membrane (Pall Corporation). MreB was detected using a 1:250 dilution of polyclonal anti-MreB serum.

Table 3: Oligonucleotides.

Primer	Sequence (5'→3')
PM1	cg**catatg**gggaactcaatgtcgttc
PM2	ga**aagctt**acgtcatctacggggcg
PM5	cg**catatg**tgatccttcttcgggac
PM6	gg**agatct**gatgaacgacattga
PM7	aa**ggatcc**ggggaactcaatgtcg
PM9	ccctcaaaagctcctgggaaggccagtcgaatcctgatggatatcattccgg**ggatcc**gtcgacc
PM10	ggagatcgtctcgtacggcggaaccgaagtgttacgtcagatatctgtaggctggagctgcttc
PM11	g**cagatc**tgacgccatgtcagtcga
PM12	gc**gaattc**ggcggaaccgaagtgtta
PM13	gccgtgcgcccgctgaaggacgg
PM14	agcagcgcgcgccgccgccggt
mbl-F	ggccccgcacggtccgcgctctcgggaggattcgccatgattccggggatccgtcgacc
mbl-R	tgcgtcaggcttccccggtcgccgcccggcggcggatcatgtaggctggagctgcttc
mreB-Fli	cgtccgtcgtcgcgatcaacacc
mreB-Rli	gtgatctcgaagtcggcgatgacg
mreC-Fli	cctcaaggcgaagctcggcagc
mreC-Rli	cgctcctatggcgatgacctgg
mreD-Fli	ctggtgatccaggtgagcgtcc
mreD-Rli	ccgacgtggccgtagaccagg

Restriction sites are written in bold.

Cell Fractionation

S. coelicolor M145 and its derivative SCPM6 (containing *mreB-egfp*) were grown for 2-3 days in 150 ml TSB with appropriate antibiotics at 30°C and harvested by centrifugation. Mycelium was resuspended in 6 ml 25 mM Tris-HCl (pH 7.5), 100 mM NaCl and 1 mM protease inhibitor (Complete EDTA-free tablets, Roche), and the cells were lysed using the French Press. The cell extract was centrifuged at 90,000 x g for 30 min at 4°C; the cytosolic fraction was stored, while the pellet fraction was resuspended in a buffer of 25 mM Tris-HCl (pH 7.5), 1 M NaCl and 20% glycerol, stirred for 2h at 4°C and centrifuged at 90,000 x g for 30 min at 4°C. The supernatant containing membrane-associated proteins was stored at –20°C, and the membrane-containing pellet fraction was solubilised in 25 mM Tris-HCl (pH 7.5), 1 M NaCl, 20% glycerol and 2% Triton X-100, stirred overnight at 4°C and again centrifuged for 30 min at 90,000 xg.

Creation of *mreB* and *mbl* null mutants

To create *mreB* and *mbl* mutants, the PCR-targeting procedure described by (Gust *et al.*, 2003) was used. The *ΩaacC4* cassette (which confers apramycin resistance) was PCR-

amplified from pIJ773 with oligonucleotides PM9 and PM10 for *mreB* and mbl-F and mbl-R for *mbl*. Cosmids C88 and C24 were used for *mreB* (C88.22c, SCO2611) and *mbl* (C24.22, SCO2451), respectively. The subsequent steps were described in detail in (Noens *et al.*, 2005). DNA of mutant candidates was isolated and tested with PCRs and Southern hybridisations to confirm the absence of the chromosomal *mreB* or *mbl*, respectively. The resulting mutants were designated *mreB*-IM (for the *mreB* insertional mutant) and Δ*mbl* (for the *mbl* insertional mutant).

In order to remove the disruption cassette still present in *mreB*-IM, the knock-out cosmid pPM11 was introduced in *E. coli* DH5α/pCP20 that expresses the FLP recombinase for removal of the *aacC4* cassette (Gust *et al.*, 2003). The resulting cosmid pPM12 carrying the in frame deletion of *mreB* was transformed to *S. coelicolor mreB*-IM (Kieser *et al.*, 2000). Transformants were first selected for insertion of the cosmid by single cross over (Km^R) and then screened for the double-cross over event (loss of both kanamycin and apramycin resistance). The loss of the disruption cassette was confirmed with PCR and Southern blot analysis. This mutant was designated *mreB*-IFD.

RNA isolation and RT-PCR

mreB-IFD and its parent *S. coelicolor* M145 were grown on cellophane disks on MM containing 1.5% Hispan agar and 0.5% mannitol as the carbon source. Mycelium was harvested at times corresponding to vegetative growth (20h-24h), aerial growth (36h-40h-48h) or sporulation (48h-60h-72h-96h). *mreB*-IM and M145 were grown in TSBS medium and mycelium was collected during exponential growth at a OD of around 0.4. RNA was purified using a modified version of the Kirby protocol (Kieser *et al.*, 2000). RNA purification columns (RNeasy, Qiagen) and DNaseI treatment were used as well as salt precipitation (final concentration 3M NaAc pH 4.8) to purify the RNA and remove any traces of DNA. RT-PCR analysis was carried out using the Superscript III one step RT-PCR System with Platinum® *Taq* DNA polymerase (Invitrogen). For each RT-PCR reaction 100 ng of RNA was used together with 1μM (final concentration) of each primer. Reactions without reversed transcription were used as a control for the absence of genomic DNA in the samples. The program was as follows: 45 min cDNA synthesis (reversed transcription) at 48°C, followed by 2 min at 95°C and 25, 29, 31 or 35 cycles of: 45 s at 94°C (denaturation), 30 s at 68°C (annealing) and 30 s at 68°C (elongation). The reaction was completed by 5 min incubation at 68°C. 5μl of each sample was tested by electrophoresis on a 2% agarose gel in

1xTAE buffer and EtBr-stained DNA visualised on a UV transilluminator. The oligonucleotides are listed in Table 2.

Microscopy

Phase contrast microscopy.

For light microscopy, sterile coverslips were inserted at a 45° angle into SFM agar and spores were inoculated in the acute angle along the glass surface. Coverslips were removed after 3-4 days of incubation at 30°C and mounted in PBS containing 50% glycerol on poly L-lysine-coated slides. Alternatively, 10 µl of liquid-grown culture was spotted directly on microscope slides covered with 1% agarose under a coverslip. Samples were analysed with an Olympus System Microscope BX60 with F-view II camera.

Electron Microscopy.

Morphological studies of surface-grown aerial hyphae and spores of *S. coelicolor* M145 and its mutants by cryo-scanning electron microscopy (cryo-SEM) was performed as described previously, using a JEOL JSM6700 SEM (Keijser *et al.*, 2003). Strains were grown for 5 days at 30°C on SFM agar plates. Transmission electron microscopy (TEM) for the analysis of ultra thin cross-sections of hyphae and spores was performed as described previously (van Wezel *et al.*, 2000). Samples were taken after 5 days of growth at 30°C on SFM. For immuno-electron microscopy spores were fixed in 2% paraformaldehyde with 0.2% glutaraldehyde in PHEM buffer for 2 hours at room temperature. After washing in PBS the spores were pelleted and embedded in 12% gelatine. The pellet was cut into 1 mm^3 cubes, cryo-protected in 2.3 M sucrose and snap frozen in liquid nitrogen. Ultra thin cryo-sections were labelled with rabbit anti-MreB (1:2000) and 15 nm protein A-gold particles. The labelled sections were embedded and contrasted in methylcellulose with uranyl acetate. All samples were viewed with a Philips EM 410 electron microscope (Eindhoven, The Netherlands).

RESULTS

The *mre* genes are highly conserved among streptomycetes and analysis of the sequenced genomes of *S. coelicolor* (Bentley *et al.*, 2002) and *S. avermitilis* (Ikeda *et al.*, 2003) and of the partial sequence of *S. scabies* (http://www.sanger.ac.uk/Projects/S_scabies) revealed

almost complete conservation of the predicted gene products MreB (SCO2611; 343 aa) and between 85-90% amino acid identity for MreC (SCO2610; 341 aa) and for MreD (SCO2609; 223 aa). In addition, a gene encoding the mreB-like protein Mbl (SCO2451; 360 aa) was detected in all sequenced streptomycetes, which shares 43% amino acid identity with MreB. The genomic organisation around *mreBCD* and *mbl* is presented in Fig. 1.

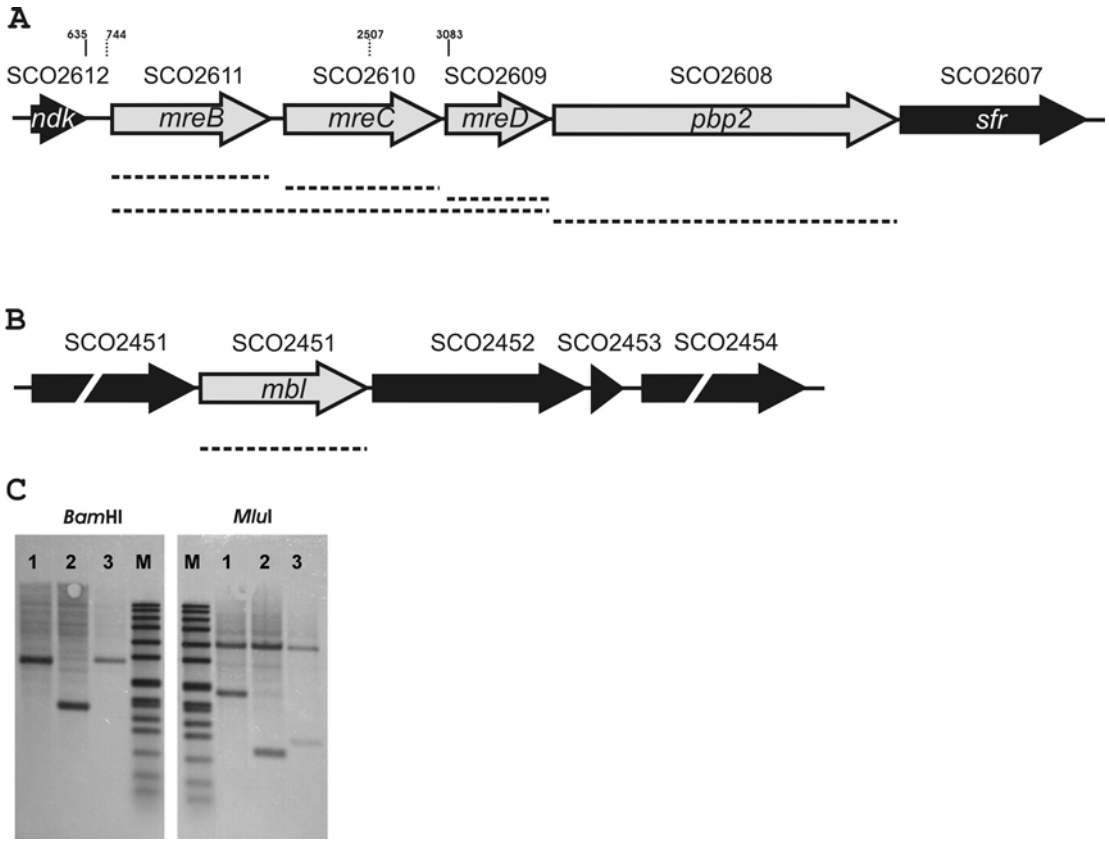


Figure 1: Genomic organisation of *mreBCD, pbp2* (A) and *mbl* (B) in *S. coelicolor.* *mreBCD, pbp2* and *mbl* are shown as grey arrows, while adjacent genes are shown as black arrows. Above the arrows are the corresponding SCO numbers. The dotted line under the genes represents the codon sequence, which is replaced by the apramycin cassette *aac*C4 in *mreB*-IM, Δ*mreC*, Δ*mreD*, Δ*mreBCD* and Δ*pbp2*, respectively (**A**) and in Δ*mbl* (**B**). For *mreB*-IFD, the same frame is deleted as *mreB*-IM. *Bam*HI and *Mlu*I restriction sites are presented as a full and dotted vertical line in A, respectively. **C.** Southern blot analysis performed with genomic DNA of M145 (1), *mreB*-IFD (2) and *mreB*-IM (3). The genomic DNA was digested with *Bam*HI or *Mlu*I and the *mreC* gene was used as probe. (M) DNA marker VII, DIG labelled (Roche).

In the sequenced genomes of *S. coelicolor*, *S. avermitilis* and *S. scabies,* the *mreBCD* operon is preceded by *ndk* (SCO2612), encoding a nucleoside diphosphate kinase, and followed by *pbp2* (SCO2608), encoding a penicillin-binding protein (Burger *et al.*, 1998) and *sfr* (SCO2607), coding for an FtsW/SpoVE/RodA family protein. MreC, MreD, PBP2 and Sfr are all predicted membrane proteins, with MreC and PBP2 (769 aa) harbouring a single N-terminal transmembrane (TM) domain at the immediate N-terminus (TM corresponding to aa

9-29 for MreC and to aa 14-32 for PBP2), so that the bulk of each protein faces the exterior, while MreD is a highly hydrophobic protein with five predicted TM domains, probably with the N-terminus facing the cytoplasm and the C-terminal part outside the cell. Sfr (372 aa) contains 10 predicted TM domains.

Table 4: Presence of *mreB* in different actinomycetes and the relationship of the ability to form aerial mycelium and spores.

Strain	Reference	Aerial mycelium[1]	Production of spores [2]	Amplified product [3]	Southern blot detection[4]
Microbacterium testaceum	DSM 20166	-	-	-	-
Nocardioides simplex	DSM 20130	-	-	-	-
*Corynebacterium glutamicum**	DSM 20300	-	-	-	-
Rhodococcus rhodochrous	DSM 43241	-	-	-	-
Tsukamurella paurometabola	DSM 20262	-	-	-	-
Gordonia sp. ACTA 2262	lab collection	-	-	-	-
Actinoplanes friuliensis	Aretz *et al*., 2000	-	+	-	-
Actinoplanes sp.	ATCC 31042	-	+	-	n.t
Micromonospora sp. Tü53	lab collection	-	+	-	-
Streptomyces olivaceus Tü8	lab collection	+	+	+	+
Streptomyces reticuli Tü45	lab collection	+	+	+	+
Streptomyces rimosus Tü58	lab collection	+	+	+	+
Streptosporangium roseum Tü74	lab collection	+	+	+	+
Streptoverticillum mobaraense Tü1063	lab collection	+	+	+	+

*: *mreB* not found after BLAST search in the sequenced genome (www.expasy.org/tools/blast/?CORGL.)

-: absence of an amplified product of the expected size ([3]), absence of aerial mycelium formation ([1]), absence of spores ([2]), absence of a band hybridysing to a *S. coelicolor mreB* probe.

+: presence of an amplified product of the expected size ([3]) a band hybridysing to a *S. coelicolor mreB* probe ([4]), formation of aerial mycelium ([1]), formation of spores ([2]).

[3] The presence of an *mreB* homologue in the tested strains deduced by the amplified PCR product obtained using primers designed in highly conserved regions of *mreB*.

n.t.: not tested

mreB is found only in actinomycetes that produce an aerial mycelium and spores

In order to assess how widespread the *mreB* genes are among actinomycetes, genomic DNA was isolated from various actinomycetes and PCR was performed using primers PM13 and PM14 (Table 3) designed to match two highly conserved regions of *mreB* and, therefore, did not distinguish between the two *mreB*-like genes on the *Streptomyces* genomes, and the PCR reactions should amplify both *mreB* and *mbl*, producing products of the same size. We obtained a band of the expected size in all tested *Streptomyces* strains, as well as in *Streptoverticillum mobaraense* and in *Streptosporangium roseum*. In contrast, we failed to detect such a band with actinomycetes that do not produce aerial hyphae, including *Actinoplanes* (*A. friuliensis* and *Actinoplanes sp*.), *Micromonospora sp*., *Corynebacterium glutamicum*, *Microbacterium testaceum*, *Nocardioides simplex, Gordonia sp.* and *Rhodococcus rhodochrous*, suggesting the lack of an *mreB* homologue in these organisms.

These findings were confirmed by Southern blot analyses using *mreB* of *S. coelicolor* as a probe (Table 4). MreB proteins therefore seem to be present only in strains that produce both aerial mycelium and spores (see Discussion). Interestingly, a single *mreB* homologue was identified in the genome sequence of the non-sporulating actinomycete *Rhodococcus* sp. RHA1 (www.Rhodococcus.ca). While a blastP search with the predicted gene product showed only significant homology to MreB, the overall similarity was significantly lower than expected for a true MreB homologue (around 40% amino acid identity to many proven or expected MreB proteins). The function of this interesting protein awaits further analysis.

Expression of MreB in S. coelicolor leads to growth impairment and lysis

Overexpression of *E. coli* MreB in *E. coli* leads to inhibition of cell division (filamentous phenotype), probably due to a reduction of FtsI activity, which is enhanced in *mreB* mutants (Wachi and Matsuhashi, 1989). To analyse the effects of overexpression of *S. coelicolor* MreB in *S. coelicolor*, the gene was cloned under the control of the thiostrepton-inducible *tipA* promoter in the *E. coli-Streptomyces* shuttle vector pGM190; the construct was designated pPM1. When *S. coelicolor* M145 transformed with pPM1 was plated on SFM plates containing 10 μg/ml thiostrepton, the strain failed to grow (Fig. 2B), while the same strain harbouring the control plasmid pGM190 (without insert) showed normal growth (Fig. 2D); this indicated that overexpression of MreB on solid medium is lethal.

To study the effect of *S. coelicolor* MreB overexpression on the growth of substrate mycelium, spores of *S. coelicolor* carrying either pPM1 or the control plasmid were inoculated in TSB medium and grown at 30°C. After 8-12 hours of growth, MreB expression was induced by the addition of 10 μg/ml thiostrepton. Interestingly, it was possible to overexpress MreB in liquid-grown cultures, although these cultures grew significantly slower than those of the parental strain harbouring the empty vector pGM190 (Fig. 3). Swelling of the extremities of the hyphae and extensive lysis of the mycelium of pPM1 transformants was observed already two hours after induction (Fig. 3C), while the pGM190 transformants remained unaffected. Overexpression of MreB in *S. coelicolor* containing pPM1 was confirmed by Western blot analysis with anti-MreB antibodies (data not shown). To analyse whether MreB overexpression affects spore germination, spores of pPM1 transformants were inoculated in liquid culture containing thiostrepton (Fig. 3D-E-F). The spores were able to germinate, but the elongation of germ tubes was inhibited (Fig. 3F) and the hyphae failed to elongate properly.

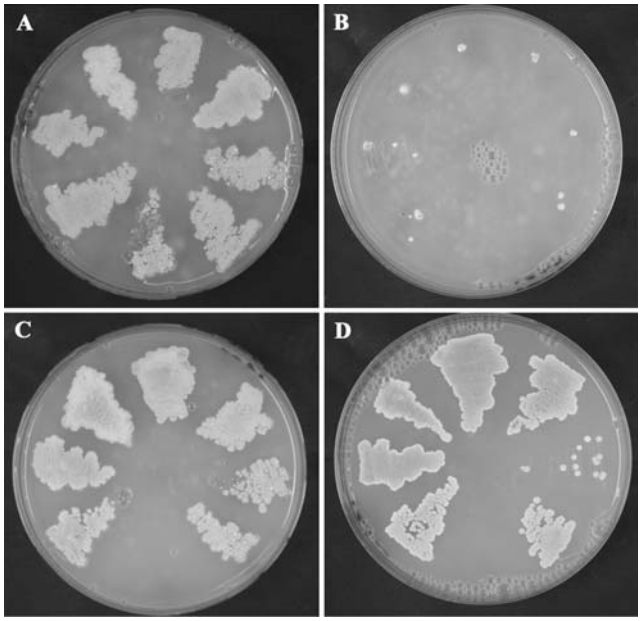


Figure 2: Lethal effect of MreB overexpression on solid growth. *S. coelicolor* M145 strains were plated in absence (**A-C**) and presence (**B-D**) of 10 μg ml^{-1} of the inducer thiostrepton. *S. coelicolor* M145 carrying pPM1 [*tipAp-mreB*] (**A-B**) and the vector pGM190 (**C-D**) were streaked onto the plates indicated. Each patch is an independent isolate of the strains.

Mutational analysis of *mreB* and *mbl*

Previous attempts to inactivate *mreB* by gene disruption using the temperature-sensitive *Streptomyces* vector pGM9 were unsuccessful (Burger *et al.*, 2000). Despite this failure, a renewed attempt using the Redirect technology allowed us to construct an insertion mutant of *mreB* (*mreB*-IM) in which *mreB* was replaced by the apramycin resistance cassette *aac(3)IV* (1384 bp) (Fig. 1A). The gene replacement was verified with PCR and Southern analysis (Fig. 1C), and the lack of MreB was confirmed with Western blot analysis using anti-MreB antiserum (data not shown) (Mazza *et al.*, 2006). The *mreB*-IM mutation is expected to have polar effects to *mreC* and *mreD*, as these genes appear to be transcribed as an operon (Burger *et al.*, 2000). In order to avoid such polar effects, an *mreB* in frame deletion mutant (*mreB*-IFD) was constructed removing the apramycin cassette from the chromosome of the *mreB*-IM mutant (see Materials and Methods section). The loss of the resistance marker and the exact location of the deletion were confirmed by PCR and Southern analysis (Fig. 1C). To confirm that the absence of an intact *mreB* was responsible for the observed phenotype, pPM6, containing a single copy of the *mreB* gene under its own promoter was introduced in *mreB*-IFD, generating the *mreB*-complemented strain *mreB*-IFDc. We simultaneously analysed the *mreB* mutants with *mreB*-IFDc to verify that the observed mutant phenotype was solely due to the gene replacement and not to a second-site mutation.

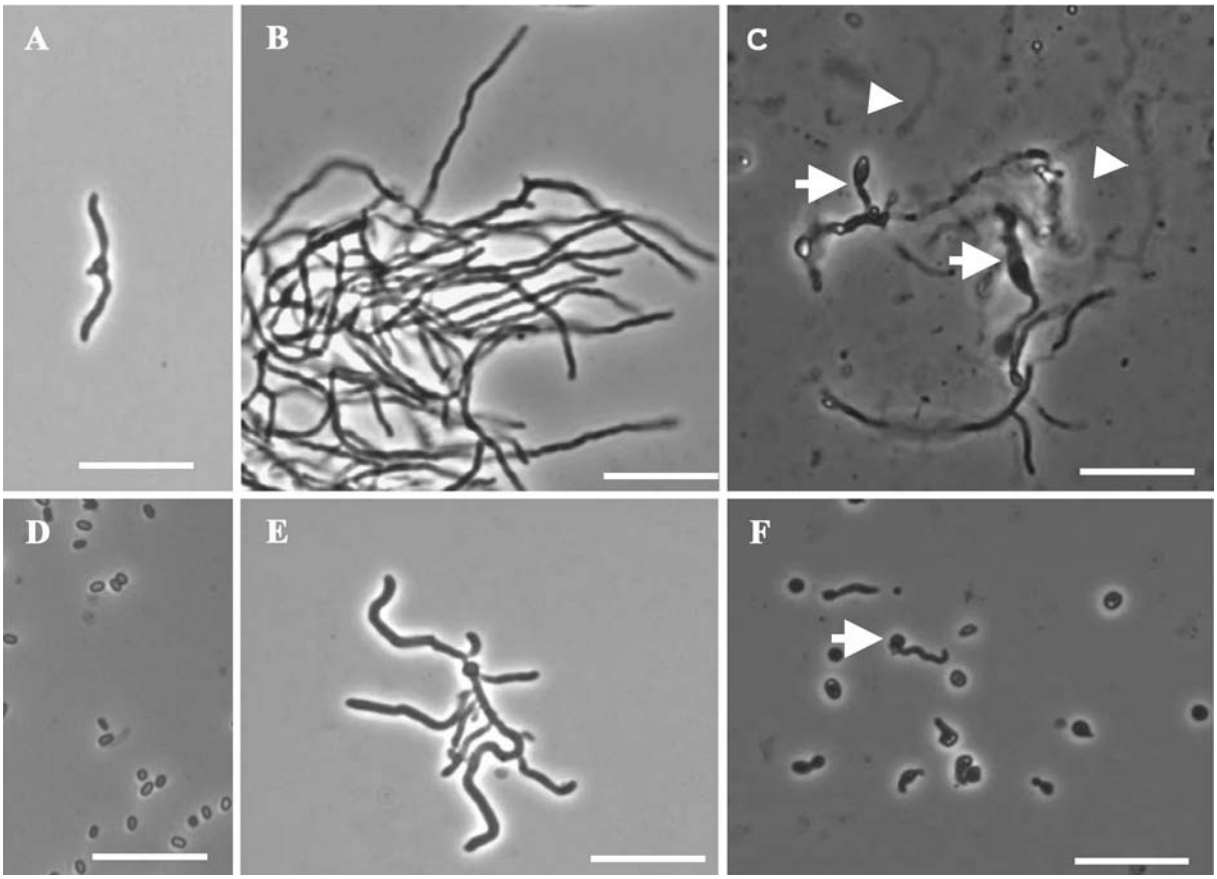


Figure 3: Effect of MreB overexpression on vegetative growth (A-B-C) in liquid culture and on spore germination (D-F-E). Spores of *S. coelicolor* M145 carrying pPM1 [tipAp-*mreB*] were inoculated in TSB medium. After 12 h of growth (**A**) the cultures were supplemented (**C**) or not (**B**) with 10 μg $ml^{-1}$of the inducer thiostrepton. Images were taken 8 hours after induction. **C**: Arrowheads show hyphal lysis and arrows swelling of hyphae induced by MreB overexpression. Bar = 10 μm. Spores of the strain carrying pPM1 [*tipAp-mreB*] were inoculated in TSB medium (**D**) and grown in the absence (**E**) and presence (**F**) of 25 μg $ml^{-1}$of the inducer thiostrepton. Under inducing conditions spores were able to germinate (arrow), but elongation of germ tubes was inhibited. Bar = 10 μm.

Using a similar approach as described for *mreB*-IM, we created an *mbl* mutant (Δ*mbl*; Fig. 1B). In this mutant *mbl* was replaced with the apramycin cassette *aacC4*, which was verified by PCR and Southern blot analysis (data not shown). An in frame deletion mutant has not yet been made for *mbl*. Considering the short distance between the genes, it is not unlikely that *mbl* forms an operon with the downstream-located genes SCO2452 (for a two-component sensor histidine kinase) and SCO2453 (for a protein with unknown function). Therefore, polar effects due to the insertion of the apramycin cassette have to be taken into consideration.

Transcriptional analyis of *mreBCD*

mreBCD appear to be transcribed as an operon. To establish if indeed transcription of *mreCD* was not affected by the deletion of *mreB* in the *mreB-IFD* mutant or by the substitution of *mreB* by *aacC4* in the *mreB*-IM mutant, we analysed the transcription of *mreBCD* using RT-PCR. Total RNA was isolated from M145 and *mreB*-IFD grown on SFM at different time points corresponding to vegetative growth (20h-24h), aerial growth (36h-40h) and sporulation (48h-60h-72h-96h) and from two independently grown liquid cultures of both M145 and *mreB*-IM, exponentially grown until an OD of around 0.4. Analysis of the RNA samples by RT-PCR with oligonucleotide pairs for *mreB*, *mreC* or *mreD* showed that indeed *S. coelicolor mreB*, *mreC* and *mreD* were transcribed throughout the *S. coelicolor* life-cycle and with a similar expression pattern (Fig. 4A). Transcription of *mreC* and *mreD* were similar in M145 and in *mreB*-IFD, underlining that developmental defects observed for *mreB*-IFD would be solely due to the deletion of *mreB* and not to polar effects on *mreCD* (Fig. 4B). Finally, the transcription of *mreC* appeared to be higher in *mreB*-IM than in M145, while *mreD* transcription seemed to be similar or higher in *mreB*-IM than in M145 (Fig. 4C). Therefore, certain anomalies of the *mreB*-IM mutant may be the result of a different expression of mreC*D*.

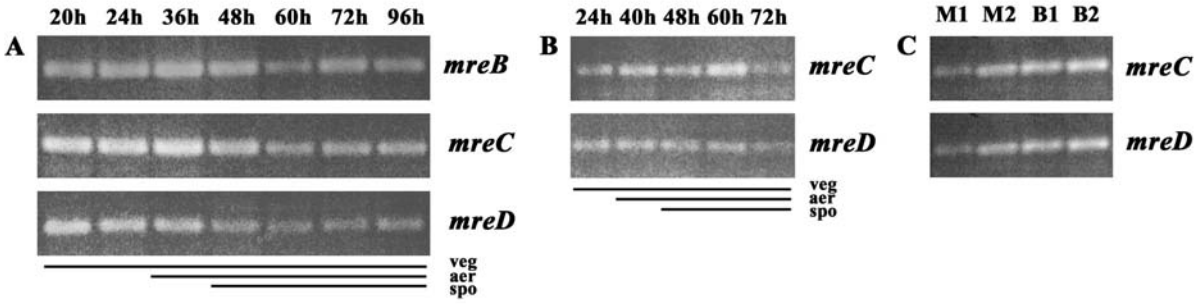


Figure 4: Transcriptional analysis of *mreB, mreC* and *mreD* in the parental strain M145 (A), in *mreB*-IFD (B) and in *mreB*-IM (C). RNA from M145 and *mreB*-IFD was isolated at different time points from cultures grown on cellophane disk on SFM. The corresponding developmental stages are indicated by the bars under the lanes (**A-B**). RNA was isolated from two liquid cultures of both M145 (M1-M2) and mreB-IM (B1-B2), independently grown until an OD of around 0.4 (**C**).

mre, pbp2 and *mbl* mutants are compromised in development

S. coelicolor M145 and its mutant derivatives *mreB*-IM, *mreB*-IFD, Δ*mreC,* Δ*mreD,* Δ*mreBCD,* Δ*mbl* and Δ*pbp2* were plated on R2YE (Fig. 5A) and SFM agar plates (Fig. 5B), and the ability of the strains to produce grey-pigmented spores was first assessed visually. The *mreB*-IM and *pbp2* mutants produced spores after 5 days of growth on R2YE agar plates, although they had a lighter pigmentation than the parent M145. In contrast, *mreB*-IFD, Δ*mreC,* Δ*mreD,* Δ*mreBCD* and Δ*mbl* all failed to produce aerial hyphae or spores (bald phenotype) within 5-6 days. After prolonged incubation (7 days for Δ*mreD* and Δ*mbl*; 10 days for *mreB*-IFD and Δ*mreC*) these five mutants produced a small amount of aerial hyphae as well as some spores on R2YE, except Δ*mreBCD*, which never entered morphological differentiation and thus had a strictly bald phenotype.

On SFM agar plates, all mutants produced significantly fewer spores than the wild type, as indicated by their lighter grey appearance, although this effect was significantly less pronounced in Δ*mreD* and Δ*mreB*-IM (Fig. 5). The ability of Δ*mreBCD* to produce abundant aerial hyphae and spores underlined that like many *bld* mutants, the *mreBCD* mutant had a conditionally non-sporulating phenotype (Nodwell and Losick, 1998; Rigali *et al.*, 2006), with sporulation on mannitol-containing media (such as SFM), but a non-sporulating, bald phenotype on glucose-containing media (such as R2YE).

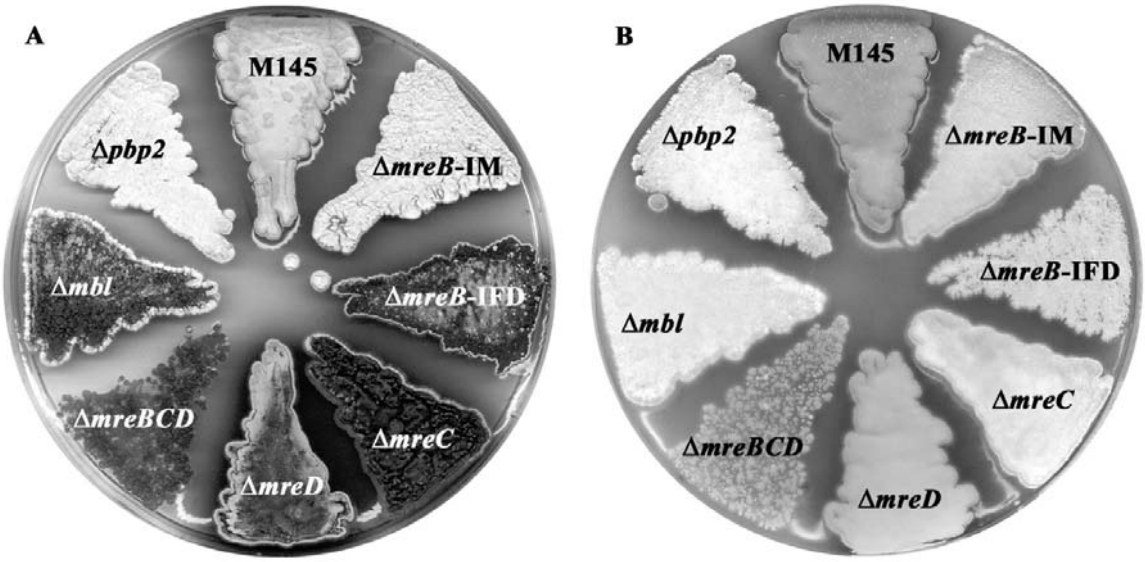


Figure 5: Phenotype of the mutants and their congenic parent *S. coelicolor* M145 on solid media. Strains were grown for 5 days at 30°C on R2YE (**A**) and SFM (**B**).

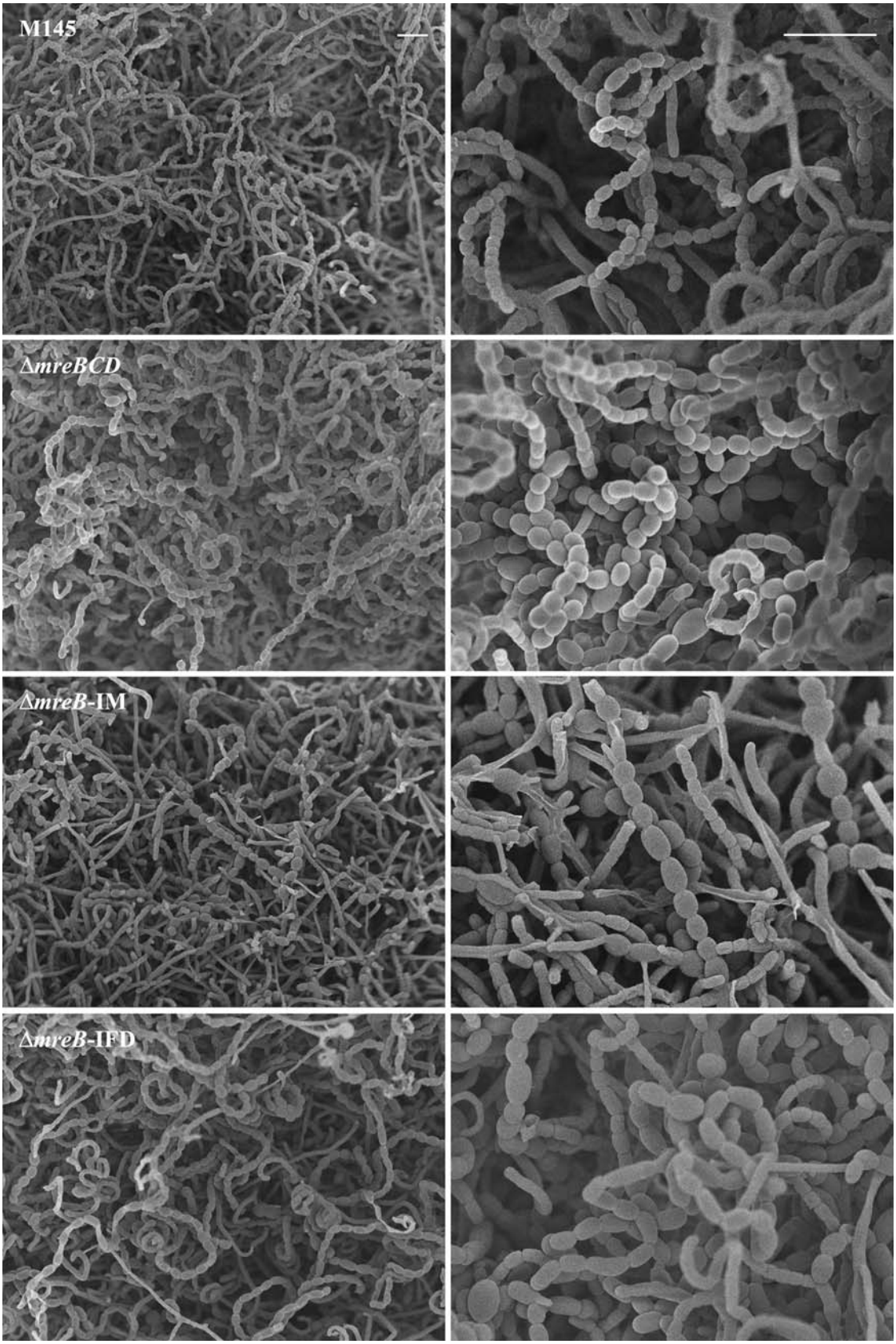
Analysis of the mutants by electron microscopy

To analyse the phenotypes of the mutants in more detail, all mutants were grown on SFM agar plates for 5 days and analysed in detail by cryo-scanning electron microscopy (Fig. 6) and transmission electron microscopy (Fig. 7). The results are summarised in Table 5. After 5 days of growth on solid medium, M145 showed typical examples of sporogenic aerial hyphae, prespore chains and mature spores (Fig. 6A-7A). The *mreBCD* mutant occasionally produced spores with irregular lengths and/or a swollen appearance (Fig. 6B-7B). The aerial hyphae were regularly swollen and more frequently lysed than in the parental strain. Around 25% of the vegetative cross walls of Δ*mreBCD* were thick and irregular.

All four *mre* single mutants (*mreB*-IM, *mreB*-IFD, Δ*mreC* and Δ*mreD* (Fig. 6C-D-E-F; 7C-D-E-F) produced spores with irregular sizes and swollen spores/hyphae, as did Δ*mreBCD* (Fig. 6B-7B). Many spores were found with a thin spore wall typical of that of vegetative or young aerial hyphae (Fig. 7C-D-E-F; arrowheads). Aerial hyphae were also more frequently lysed in these mutants. This defective phenotype was most prominent in *mreB*-IM, which produced many more misshapen spores than the other *mre* mutants, often with extremely heteromorphous shapes (Fig. 6C-7C). As in Δ*mreBCD*, around 25% of the vegetative cross walls of *mreB*-IM were thick and irregular, around 6% of which were unfinished, suggestive of a thick, asymmetric ring. Mutant *mreB*-IM accumulated electron-dense granules in its hyphae and spores (Fig. 7C), similar to those observed in *ssgB* mutants, which are likely to contain unincorporated cell wall precursor material (Keijser *et al.,* 2003). Spores of both *mreB* single mutants showed premature germination. Additionally, *mreB*-IM harboured completely segregated chromosomes, as visualised by propidium iodide (not shown), while nucleoids of *mreB*-IM were invariably surrounded by electron-lucent (white) material. This was also observed in *mreB*-IFD, Δ*mreC* and Δ*mreD* (Fig. 7D-E-F; arrows). The nature and origin of this material is unknown.

The defects observed in Δ*mbl* corresponded to those previously described for the *mre* mutants, with a high proportion of spores showing aberrant shapes and different sizes, and premature germination occurring regularly (Fig. 6G-7G). However, spore formation appeared to be more severely compromised in this mutant.

pbp2 mutants produced significantly fewer spores than the parental strain. Few spores appeared to be swollen although frequently, spores with irregular shapes were observed (Fig.


M145
ΔmreBCD
ΔmreB-IM
ΔmreB-IFD

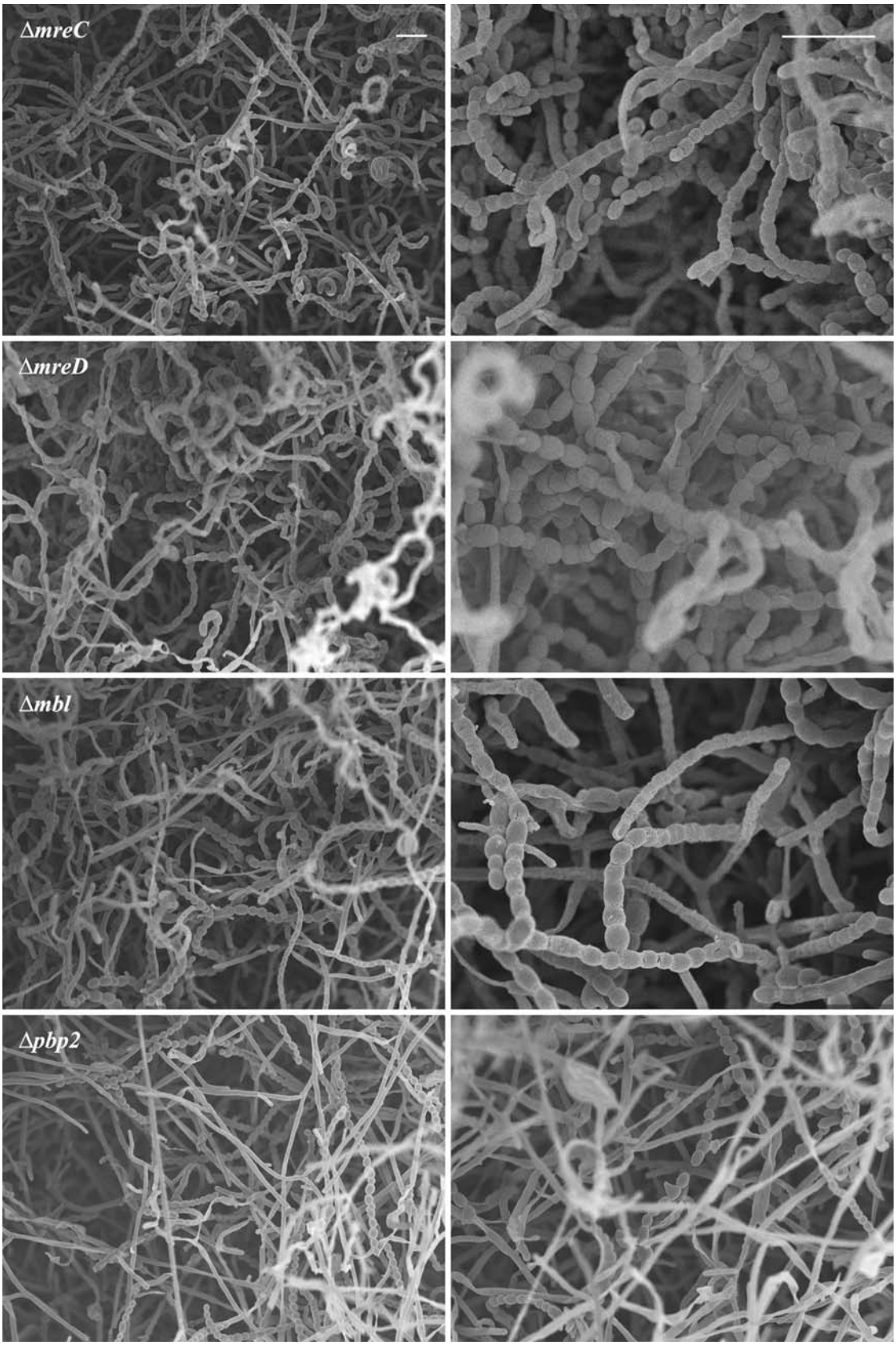


Figure 6: Phenotypic characterisation of the parental strain M145 and the mutants by cryo-scanning electron microscope. Samples were taken after 5 days of growth on SFM. Bar = 5 µm.

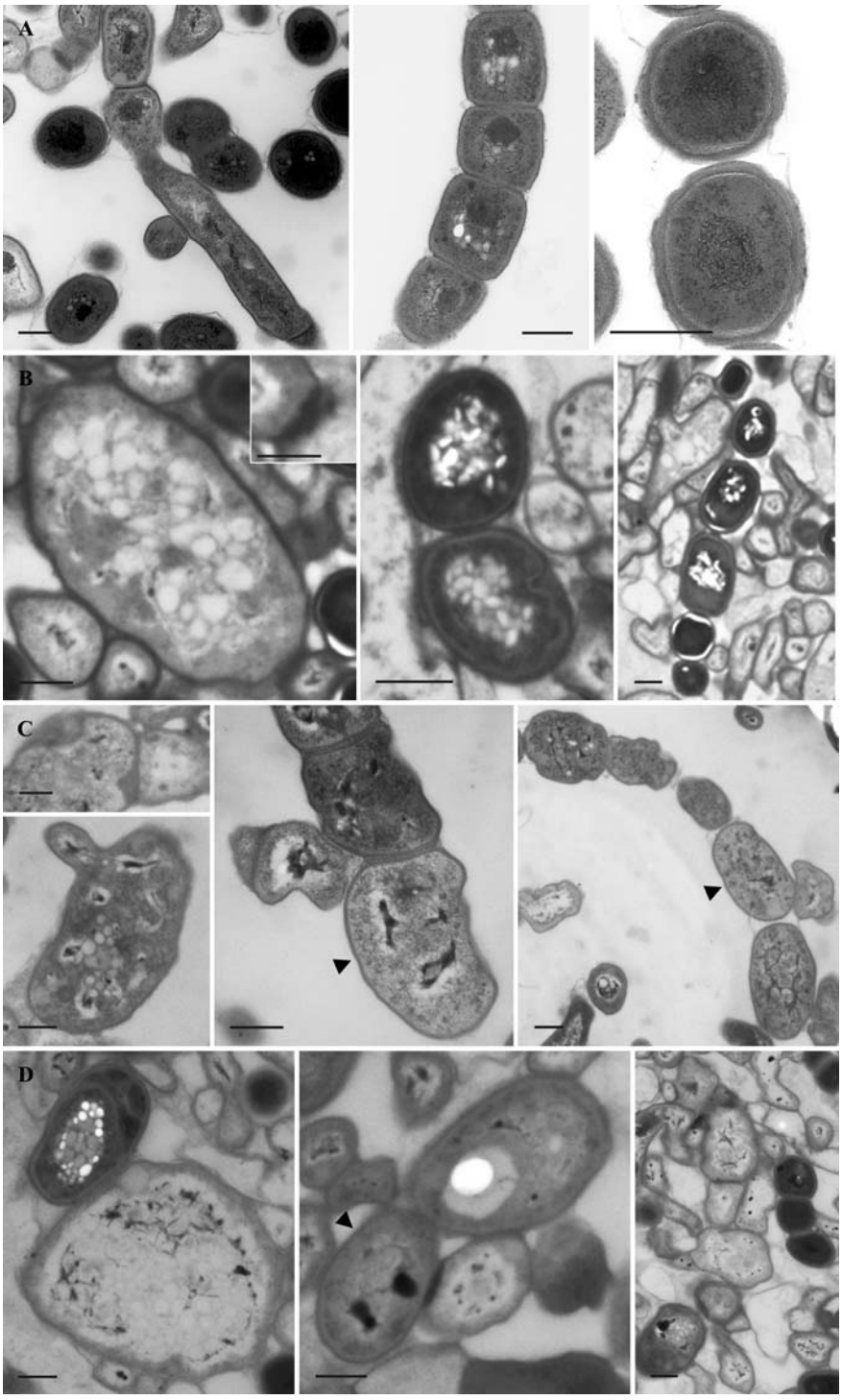
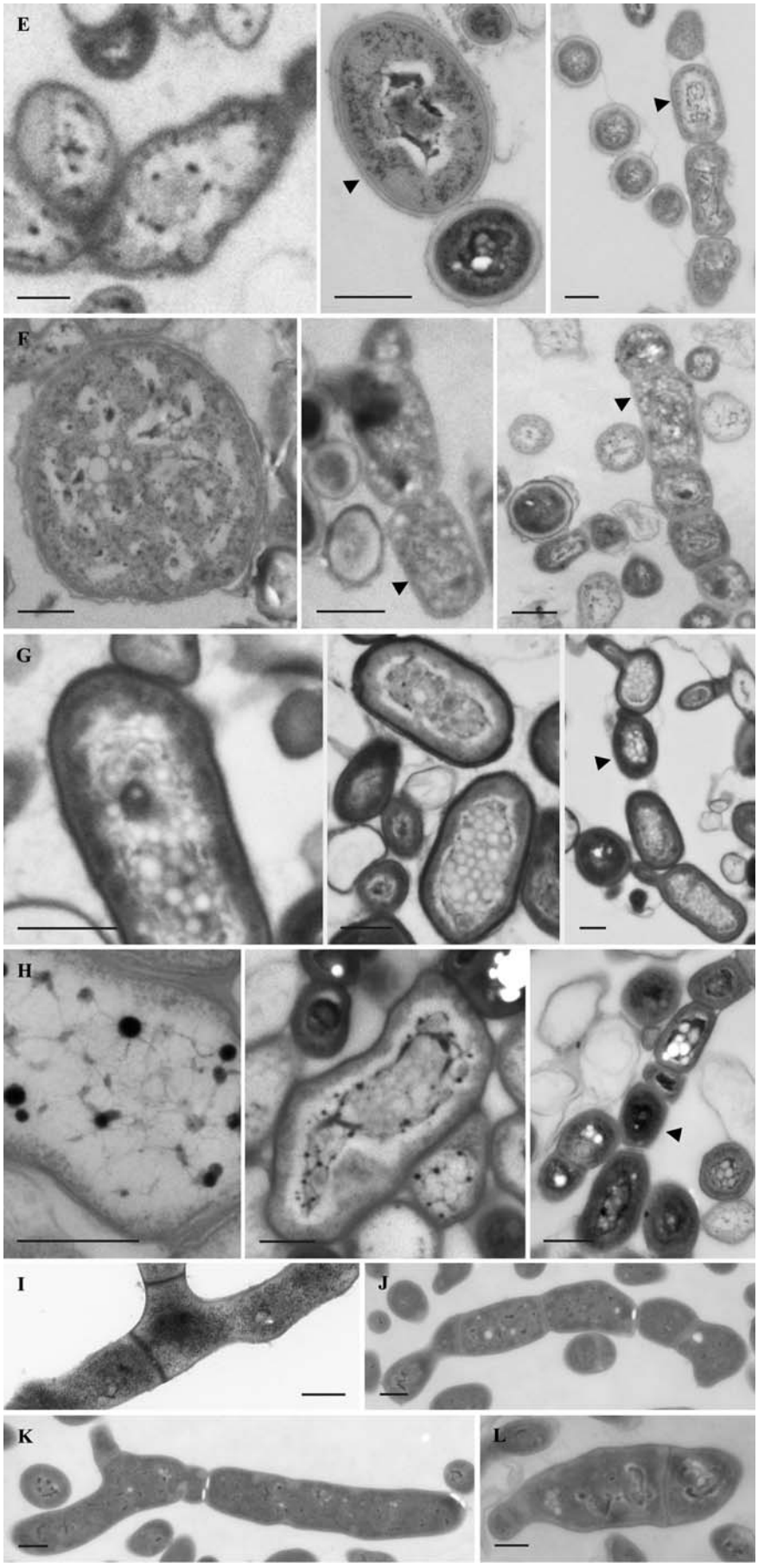


Figure 7: Transmission electron microscope images of hyphae and spores of the parental strain M145 and the mutants. A-H: Samples were taken after 5 days of growth on SFM. **A**: M145; **B**: Δ*mreBCD*; **C**: *mreB*-IM; **D**: *mreB*-IFD; **E**: Δ*mreC*; **F**: Δ*mreD*; **G**: Δ*mbl* and **H**: Δ*pbp2*. **A**: aerial hyphae (left), immature spores (middle) and mature spore chains (right). **B-G**: bloated hyphae (left), swollen spores (middle) and spores with irregular dimensions (right). **H**: Electrodense granules accumulated in hyphae (left), swollen hyphae (middle) and irregular spores (right). Aberrant cross walls are observed in B and C (insert). Arrowheads point to the cell wall of spores, lacking the thick peptidoglycan layer, typical for mature spores. **I-L**: Images of vegetative hyphae were taken after 2 days of liquid growth. **I**: M145; **J**: *mreB*-IM; **K-L**: Δ*mbl*. Bar = 0.5 μm.


E
F
G
H
I
J
K
L

6H-7H). Aerial hyphae were frequently lysed. As in *mreB*-IM, electron-dense granules accumulated in the hyphae and spores of the *pbp2* mutant.

To investigate if *mreB* and *mbl* play a role during vegetative growth, cross sections of two days old solid-grown cultures of Δ*mbl* and *mreB*-IM were analysed by TEM. The morphology of vegetative hyphae and cross-walls was very similar to that of the wild type strain. However, the distance between cross-walls of liquid-grown hyphae of *mreB*-IM and Δ*mbl* was often less than 2 μm, while the cross-walls of wild type hyphae were laid down with a frequency of approximately one per 8 μm. Analysis of liquid-grown mycelium of both mutants revealed swollen hyphae (Fig. 7I-J-K-L), providing supportive evidence that Mbl and MreB are important for the stability of both vegetative and aerial growth.

Table 5: Characteristics of different mutants.

	Spores	Swollen spores	Premature germina-tion	Irregular spore length	Irregular, thin spore wall	Lysed aerial hyphae	Swollen hyphae
M145	++	-	-	-	-	+/-	-
***mreB*-IM**	+	+++	+	+	+	+	+
***mreB*-IFD**	+	+	+	+	+	+	+
ΔmreC	+	+	-	+	+	+	+
ΔmreD	++	+	-	+	+	+	+
ΔmreBCD	+++	+	-	+	-	+	+
Δmbl	+	++	++	+	+	+	+
Δpbp2	+	+	-	+	+/-	+	+

+++: very high amounts. ++: high amounts. +: average amounts. +/-: rarely but seen. -: absent.

MreB is a membrane-associated protein that localises at the septa of aerial hyphae and at the spore wall

Recent results showed that MreB in *B. subtilis* and *E. coli* forms helical structures located on the inner surface of the cytoplasmic membrane (Formstone and Errington, 2005; Jones *et al.*, 2001; Kruse *et al.*, 2003; Shih *et al.*, 2003) and that it is only weakly associated with the membrane (Slovak *et al.*, 2005), while MreC and MreD are membrane proteins (Kruse *et al.*, 2005). In order to localise MreB in *S. coelicolor* M145, an MreB-EGFP fusion protein was created under the control of the natural *mreB* promoters, and cloned in pSET152, generating pPM4 (see Materials and Methods). This plasmid almost fully complemented *mreB*-IM, indicating that the MreB-EGFP fusion protein was functional.

During vegetative growth, only diffuse fluorescence was observed in the hyphae, although expression of MreB-EGFP was confirmed by Western blot analysis with both anti-

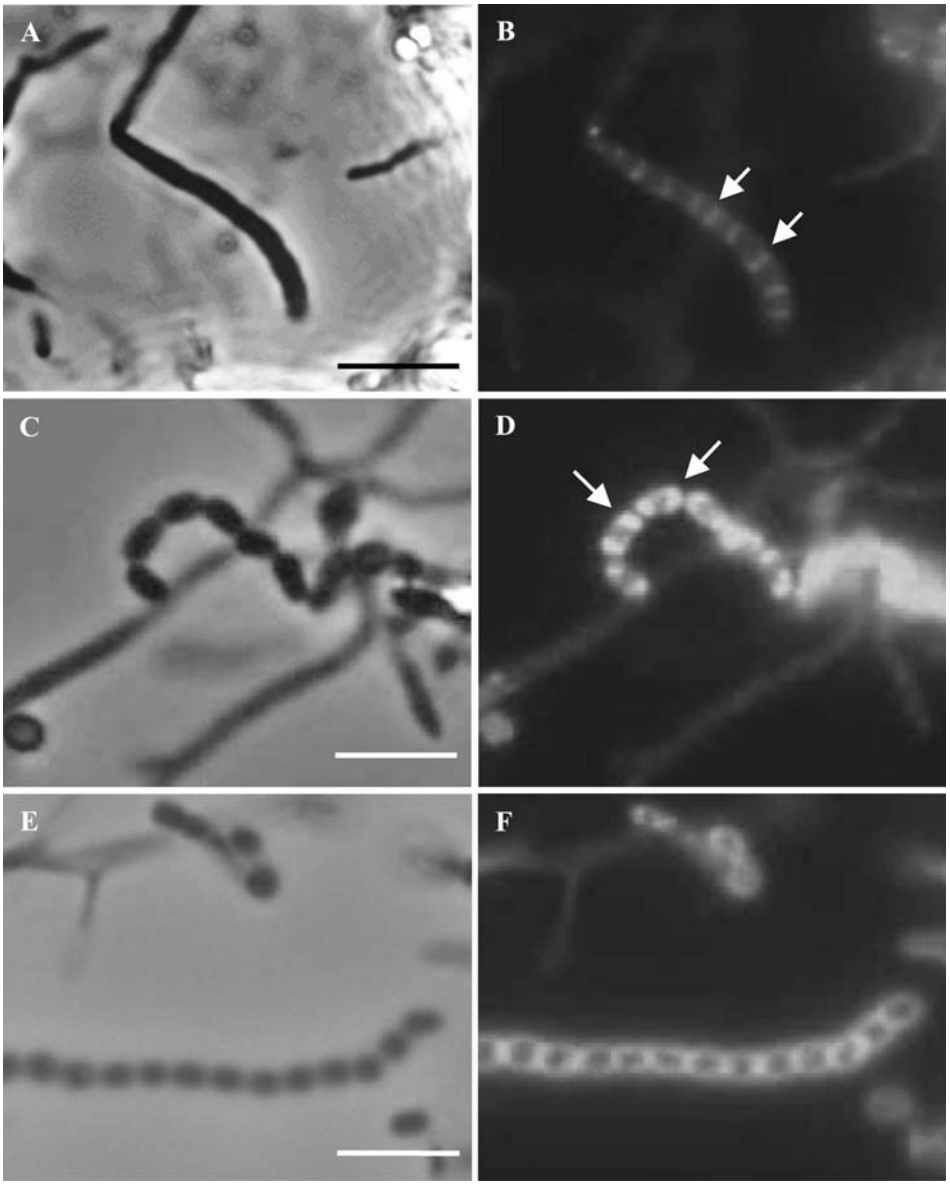


Figure 8: Localisation of the MreB-EGFP fusion protein during morphological differentiation. MreB-EGFP was detected at sporulation septa in aerial hyphae (**B**), at the poles of prespores (**D**) and subsequently covering the whole spore wall in spore chains (**F**). **A-C-E**: phase contrast; **B-D-F**: fluorescence microscopy. Bar = 5 μm.

MreB and anti-GFP antibodies (data not shown). However, very clear fluorescence signals were specifically localised at the septa of sporogenic aerial hyphae (Fig. 8). As shown in Fig. 8B, bands of fluorescence coincided with the constrictions caused by sporulation septation. Simultaneous labeling of septa with fluorescent conjugates of wheat germ agglutinin confirmed that MreB-EGFP overlapped with the septa (data not shown). MreB-GFP was also localised at the tip of the sporogenic aerial hyphae. However, it is not clear whether MreB-EGFP also localised to the basal septa sometimes seen at the bottom of sporogenic hyphal

cells (Kwak *et al.*, 2001). In prespore chains, foci were localised at the cell poles generated by sporulation septation (Fig. 8D; arrows), including the tip of last spore in the chain, which is not preceded by a septum. Successively, in more mature spores, the MreB-EGFP signal completely surrounded the spores, giving rise to ring-like appearance of the fluorescence (Fig. 8F). However, in fully mature spores most of the fluorescence from the MreB-EGFP fusion had disappeared (data not shown). Closer inspection of the localisation of MreB by immunogold electron microscopy revealed abundant and specific labelling, particularly close to the inside of the spore walls of wild type spores (Fig. 9). In the control experiment where hyphae and spores from the *mreB* insertion mutant (*mreB*-IM) were imaged, only background labeling was obtained.

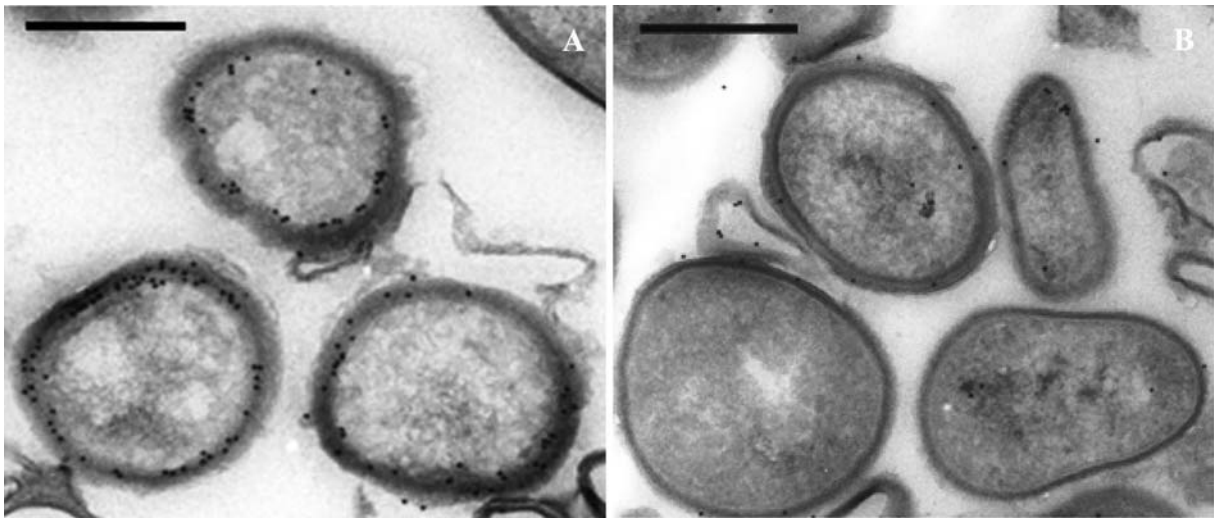


Figure 9: High-resolution localisation of MreB in *S. coelicolor* spores. Specific localisation of MreB in spores by *in situ* hybridisation of thin sections (immuno-electron microscopy) of spore preparations of *S. coelicolor* M145 (**A**), using gold-labelled anti-MreB antibodies. No specific labelling was obtained with any of the independent *mreB* mutants (**B**). Notice the highly variable spore sizes and less dense spore wall of the mreB null mutant. Bar = 500 nm.

The cellular localisation of MreB was further examined by cell fractionation and Western analysis using anti-MreB antibodies. Mycelium and spores grown on SFM agar plates were disrupted in a French-Press and the cytoplasmic, the membrane-associated and the membrane fractions were individually isolated. MreB was detected in the cytoplasmic fraction and in the membrane-associated fraction, while we failed to detect MreB in the membrane fraction (data not shown). The same result was obtained when localisation of the MreB-EGFP fusion protein was examined, indicating that the localisation of MreB-EGFP was the same as for the wild type protein.

DISCUSSION

In this study, we present the mutational and functional characterisation of the *mreBCD* and *mbl* genes, which encode actin-like cytoskeletal proteins, and of *pbp2*. The creation of the deletion of *mreB* is surprising in itself, as earlier failed attempts suggested that in contrast to *mreC* and *mreD*, *mreB* was an essential gene (Burger *et al.*, 2000). The discrepancy may be explained by a higher frequency of allelic exchange in the REDIRECT method, due to the much larger regions flanking the *mreB* locus that were available for recombination. Since several *mreB* mutants were isolated in independent experiments with frequencies that are typical for gene inactivation experiments using the REDIRECT procedure, it appears unlikely that *mreB* is essential in *Streptomyces* and that lethality of the *mreB* mutations was masked by (a) suppressor mutation(s), as has been described for other bacteria (Kruse et al., 2005). This is further supported by the observation that *mreB* is absent from many non-sporulating actinomycetes.

Deletion of either *mreB* or *mbl* gave no obvious phenotype during vegetative growth in early solid samples, but a significantly detrimental effect was observed on liquid-grown mycelia, where hyphae of *mreB* mutants and to lesser extent of *mbl* mutants had a swollen appearance, with a significantly shorter spacing between the cross walls as in the parent. Furthermore, in *mreB*-IM and Δ*mreBCD* approximately 30% of the cross wall in the vegetative hyphae are thick and irregular, which could be due to an enhancement of FtsI, also observed in *mreB* mutants in *E. coli* (Wachi and Matsuhashi, 1989).

Transcriptional analysis by S1 nuclease mapping (Burger *et al.*, 2000) and RT-PCR studies (this work) revealed that *S. coelicolor mreB* and *mbl* are transcribed at similar levels throughout the *S. coelicolor* life-cycle, and expression of MreB was confirmed by Western blot analysis in liquid-grown cultures (data not shown). Thus, MreB and Mbl play an important role in the stability of the young hyphae under certain growth conditions, although the exact function needs further analysis. It cannot be excluded that there could be some degree of redundancy between MreB and Mbl, and only a double mutant can rule this out. However, based on the lack of MreB-EGFP signals at hyphal tips, it seems unlikely that MreB would play a direct role in the elongation of the hyphae, which occurs primarily at the tips. Sequence analysis of genomic DNA of different actinomycetes showed that only strains forming both an aerial mycelium and spores possess an *mreBCD* cluster, supporting the idea that the Mre proteins may not be required for vegetative growth in actinomycetes. One

apparent exception was *Rhodococcus* sp. RHA1, an actinomycete that only grows vegetatively, and has an mreB gene, although the low similarity of its predicted gene product to other MreBs (at best 40% amino acid identity) suggests that it may have a different function. While deletion of *mreB* affected hyphal stability but otherwise allowed normal vegetative growth, enhanced expression of *mreB* was highly toxic. After germination of spores the elongation of germ tubes was inhibited and hyphae lysed. Obviously, the presence of too much MreB severely interferes with normal growth, perhaps by recruiting penicillin-binding proteins, thereby inhibiting their function in cell wall synthesis.

From our data we conclude that MreBCD and Mbl are required for correct sporulation and play an important role in spore wall synthesis. The aerial hyphae and spores of the *mreB, mreC, mreD* and *mreBCD* deletion mutants were swollen, and irregularities in the spore cell walls were observed using TEM and spores of *mreB*-IFD were sensitive to heat and treatment with SDS. This is consistent with the observation that deletion of *mreBCD* in *B. subtilis* causes increased cell width and cell lysis (Formstone and Errington, 2005; Leaver and Errington, 2005). Loss of MreB leads to a change in resistance of the cell wall to osmotic or mechanical stress, due to the absence or incorrect assembly of cell wall components. In analogy, the absence of MreBCD in *S. coelicolor* impairs cell wall assembly, primarily during sporulation. The spore wall composition of streptomycetes is not well studied. However, it is different in thickness from vegetative hyphal walls and has often two layers, although qualitative differences in peptidoglycan components have not been reported (Ensign, 1978; Glauert and Hopwood, 1961). The importance of the spore wall may be indicated by the fact that spores acquire resistance to different physiological and mechanical stresses, which the substrate mycelium does not have. Consistently, the *mreB* mutants failed to mount resistance against two types of stress, namely heat and incubation with SDS.

The fact that deletion of *mreB, mreC* or *mreD* or the entire *mreBCD* gene cluster leads to a similar phenotype, strongly suggests that MreB, MreC and MreD act together and do not have any obvious independent role in the control of *Streptomyces* morphology and development. For example, in *mreBCD* mutants there is no expression of *mreB, mreC* or *mreD* and the phenotype of this mutant is highly similar to that of *mreB*-IFD. However, while *mreB*-IM and *mreB*-IFD have similar phenotypes, *mreB*-IM shows more ‘swollen’ spores, and many aberrant cross-walls, and this may be due to the polar effect of the insertional mutation on *mreC* and *mreD*. It was published previously that the apramycin resistance cassette has strong promoter activity (van Wezel *et al.*, 2005), and it is, therefore, unclear how

the expression of *mreCD* and flanking genes (*ndk, pbp2, sfr*) is affected in *mreB*-IM. Conceivably, deregulated expression of MreC and MreD might lead to recruitment of PBPs and thus prevent them for correct functioning.

Both *mbl* and *mreB* mutants produced spores with heteromorphous shapes that often germinate prematurely, and this even more frequently in the *mbl* mutant. The altered appearance of the nucleoids in *mreB* mutants was not observed in the *mbl* mutant, suggesting that only genes in the *mreBCD* cluster affect DNA segregation. Despite differences in the frequency and the degree of the observed anomalies, most of the defects were of similar nature, suggesting that the functions of Mbl and MreB may be similar, and that functional redundancy may exist between the two actin-like proteins.

To learn more about the function of MreB we studied the localisation of MreB-EGFP. In the rod-shaped bacteria *B. subtilis* and *E. coli* MreB forms helical filaments just underneath the cell surface (Jones *et al.*, 2001; Shih *et al.*, 2003), while in *Caulobacter crescentus* MreB undergoes two distinct localisation patterns, namely during the cell elongation phase as spirals that traverse along the longitudinal axis of the cell, and during cell division as an FtsZ-dependent transverse band at the mid-cell position (Figge *et al.*, 2004). In *S. coelicolor,* we failed to detect the typical helical-like structures described for *E. coli*, *B. subtilis* and *C. crescentus*. MreB-EGFP was observed as diffuse fluorescence in vegetative hyphae, indicating that MreB was randomly distributed in the vegetative mycelium, perhaps suggesting it occurred primarily in its monomeric form. Conversely, during development MreB-EGFP localised at the septa of sporogenic aerial hyphae and subsequently underneath the cytoplasmic membrane of the spores, as shown at high resolution by immunogold electron microscopy. While it is possible that the C-terminal fusion of GFP to MreB interferes to some extent with its function, and hence we cannot ascertain that MreB-EGFP precisely reproduced the localisation patterns for the natural MreB, the fusion protein was able to complement the *mreB* mutant phenotype and MreB-EGFP localised to the same subcellular fractions of *S. coelicolor* as MreB itself.

In *S. coelicolor,* septation occurs both during vegetative growth and sporulation, but MreB localisation was specific for sporulation septa. It is clear that MreB is not required for the formation of sporulation septa *per se*, as *mreB* mutants were still able to septate and produced viable spores. However, it should be tested whether MreB-localisation could be FtsZ-dependent, as seen in *C. crescentus* (Figge *et al.*, 2004), for example by localising MreB in different *S. coelicolor ftsZ* mutants, such as in an *ftsZ* mutant impaired in Z-ring formation

in sporogenic aerial hyphae, but not in vegetative mycelium (Grantcharova *et al.*, 2003). In *Rhodobacter sphaeroides*, MreB localises predominantly at the mid-cell position (Slovak *et al.*, 2005). The authors speculate that this mid-cell localisation for MreB might reflect the fact that this region is the main site of peptidoglycan synthesis rather than that MreB plays a direct role in septation. In an analogous manner, MreB in *S. coelicolor,* which was shown to localise at the sporulation septa, could be needed in subsequent steps for spore formation, rather than playing a crucial role in the sporulation-specific cell division.

Based on the localisation of MreB at the spore wall and the important role of MreB, MreC, MreD and Mbl in maintaining the integrity of the spore wall, we anticipate that these proteins are involved in thickening of the spore wall and may be recruiting PBPs and other peptidoglycan-related proteins during the sporulation process. The *mre* genes of *S. coelicolor* and *S. avermitilis* are linked genetically to *pbp2* and *sfr* (Burger *et al.*, 2000). PBP2 is a penicillin-binding protein with high similarity to *E. coli* PBP2, which is involved in cell elongation (Vinella *et al.*, 1993), and with SpoVD, a PBP important for synthesis of the spore cortex in *B. subtilis* (Daniel *et al.*, 1994). The product of *sfr* shows similarity to RodA, a protein involved in peptidoglycan synthesis during cell elongation in *E. coli*, and SpoVE, a protein required for the synthesis of the spore cortex peptidoglycan in *B. subtilis* (Henriques *et al.*, 1998). Inactivation of *pbp2* leads to a phenotype that resembles that of the *mreB* mutant. In *E. coli,* MreB, MreC and MreD were proposed to function as a membrane-bound complex directing the PBP2-dependent longitudinal cell wall synthesis (Kruse *et al.*, 2005), while in *B. subtilis*, it was suggested that MreC and MreD couple the helical Mbl 'cables' to the extracellular cell wall synthesising machinery (Leaver and Errington, 2005). We suggest that also in *S. coelicolor* these proteins participate in the same process. A complex may be formed including PBPs and the Mre proteins, which acts primarily during spore wall assembly. This leads to the following hypothesis on the mode of action of MreB in *S. coelicolor*: MreB is expressed at similar levels during the life cycle and it is localised rather dispersely in the cytosol of vegetative hyphae. At this stage, it contributes to the stability and stress resistance of the cell wall. When sporulation septation occurs, MreB condenses at the sporulation septa, possibly in an FtsZ-dependent manner. As MreB most likely is a membrane-associated protein, this structure may be anchored to the cell membrane through the two membrane proteins MreC and MreD and used to recruit and localise proteins responsible for spore wall formation, perhaps including PBP2 and the product of *sfr*. Further studies to localise PBP2 and Sfr in *mre* mutants and interaction studies between the Mre

proteins and the products of *pbp2* and *sfr* are necessary to understand the unique role of MreB in *Streptomyces coelicolor* A3(2). It will also be important to clarify the role of Mbl in this organism, and to determine whether there could be some functional redundancy between this protein and MreB. A mutant with combined deletions of *mbl* and *mreB* is the first obvious step to better out understanding of the role of these important morpho-proteins in the life cycle of streptomycetes.

ACKNOWLEDGEMENTS

We thank Wolfgang Wohlleben for Δ*mreC*, Δ*mreD* and Δ*mreBCD* and Glyn Hobbs for Δ*pbp2*.