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MreB of *Streptomyces coelicolor* is not essential for vegetative growth but is required for the integrity of aerial hyphae and spores

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Summary

MreB forms a cytoskeleton in many rod-shaped bacteria which is involved in cell shape determination and chromosome segregation. PCR-based and Southern analysis of various actinomycetes, supported by analysis of genome sequences, revealed *mreB* homologues only in genera that form an aerial mycelium and sporulate. We analysed MreB in one such organism, *Streptomyces coelicolor*. Ectopic overexpression of *mreB* impaired growth, and caused swellings and lysis of hyphae. A null mutant with apparently normal vegetative growth was generated. However, aerial hyphae of this mutant were swelling and lysing; spores doubled their volume and lost their characteristic resistance to stress conditions. Loss of cell wall consistency was observed in MreB-depleted spores by transmission electron microscopy. An MreB–EGFP fusion was constructed to localize MreB in the mycelium. No clearly localized signal was seen in vegetative mycelium. However, strong fluorescence was observed at the septa of sporulating aerial hyphae, then as bipolar foci in young spores, and finally in a ring- or shell-like pattern inside the spores. Immunogold electron microscopy using MreB-specific antibodies revealed that MreB is located immediately underneath the internal spore wall. Thus, MreB is not essential for vegetative growth of *S. coelicolor*, but exerts its function in the formation of environmentally stable spores, and appears to primarily influence the assembly of the spore cell wall.

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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 (Young, 2003; Cabeen and Jacobs-Wagner, 2005). However, not only the enzymes that are directly involved in the synthesis and assembly of peptidoglycan can affect the morphology. It is becoming clear that cytoskeletal elements exist in the bacterial cytoplasm (Löwe *et al*., 2004), and that they are determining the architecture of the cell wall and have strong impacts on cell shape. These include homologues of all three major types of eukaryotic cytoskeletons. Thus, MreB proteins are actin homologues that produce microfilament-like fibres and determine rod shape in many bacteria (van den Ent *et al*., 2001; Jones *et al*., 2001); crescentin (CreS) produces intermediate filament-like elements in *Caulobacter crescentus* and give rise to the curved shape of those cells (Ausmees *et al*., 2003); and 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* polymerizes *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 homologues have been studied primarily in *Escherichia coli* (*mreB*), *Bacillus subtilis* (*mreB*, *mbl*, *mreBH*) and *C. crescentus* (*mreB*), and been found with immunofluorescence microscopy and GFP tagging to form helical-like structures underneath the cell envelope (Jones *et al*., 2001; Shih *et al*., 2003; Figge *et al*., 2004; Soufo and Graumann, 2003). Deletion of *mreB* in *E. coli* resulted in loss of the rod shape and in the production of spherical cells (Doi *et al*., 1988). In *B. subtilis*, *mreB* is an essential gene, and depletion of MreB resulted in increased cell width, loss of cell shape and eventually lysis (Jones *et al*., 2001; Lee and Stewart, 2003; Formstone and Errington, 2005). Furthermore, 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). Despite these observations that suggest that MreB proteins form actin-like fibres that define the bacterial cell shape, the specific function of MreB proteins remain still unclear. Recent studies link MreB to peptidoglycan synthesis (Daniel and Errington, 2003), correct chromosome segregation (Kruse *et al*., 2003; Soufo and Graumann, 2003; Gitai *et al*., 2005) and establishment of cell polarity (Gitai *et al*., 2004). Interestingly, in the rod- and coccobacillus-shaped *Rhodobacter sphaeroides* MreB failed to produce the typical helical structures observed in *Bacillus* but rather formed a ring at the mid-cell position of elongating cells, suggesting a role for MreB both in septation and in peptidoglycan synthesis in this organism (Slovak *et al*., 2005). Thus, it appears that MreB may have different functions in different organisms.

All three genes in the highly conserved *mre* gene cluster, encompassing *mreB*, *mreC* and *mreD*, are required for cell shape determination in the rod-shaped *E. coli* and *B. subtilis* (Wachi *et al*., 1987; Jones *et al*., 2001; Kruse *et al*., 2005; Leaver and Errington, 2005). In both organisms, *mreC* and *mreD* are essential genes, and depletion of the gene products resulted in lost control of cell shape, spheroid morphology and eventually lysis (Lee and Stewart, 2003; Kruse *et al*., 2005; Leaver and Errington, 2005). It has been suggested that the membrane proteins MreC and MreD may connect the cytoplasmic MreB and Mbl filaments to the cell wall synthetic machinery that is located on the outer face of the membrane (Kruse *et al*., 2005; Leaver and Errington, 2005).

The observation that the *mre* gene cluster is absent from coccoid species such as *Streptococcus*, *Staphylococcus* and *Lactococcus* suggested that a spherical shape may be correlated with the absence of an *mre*-dependent system, and that MreB proteins are particularly important in rod-shaped cells (Jones *et al*., 2001). However, in contrast to most other rod-shaped bacteria, the actinomycete *Corynebacterium glutamicum* lacks *mreB*-like genes in its genome. In accordance with this, it apparently establishes its rod shape in a different and MreB-independent way, through growth of the cell wall only at the cell poles (Daniel and Errington, 2003). Most other actinomycetes also lack MreBs, but the *Streptomyces* genus is a notable exception as they encode well conserved MreB homologues (see below). This raises the question of what the roles may be for the *Streptomyces* MreBs. Streptomycetes are Gram-positive filamentous actinobacteria known for their complex morphology and their ability to produce a wide range of secondary metabolites. Their morphological development resembles in many ways that of filamentous fungi. They produce a vegetative mycelium consisting of a network of branching, multinucleoidal, hyphal filaments, which grow in a highly polarized way by tip extension. This polarized growth differs from that of *C. glutamicum* by being independent of cell division and occurring at hyphal tips generated by branching or germ tube emergence, rather than at the poles created by cell division (Flärdh, 2003). Upon starvation colonies enter the reproductive phase by forming aerial hyphae that eventually produce chains of spores (Chater and Hopwood, 1993). This involves a specialized form of cell division that divides long multigenomic aerial hyphae into a large number of spore compartments, which then mature into thick-walled dormant spores (Chater and Losick, 1997; Flärdh, 2003). The genome of *Streptomyces coelicolor* contains two homologues of *mreB*, but the functions of them during growth and development are poorly understood. One of these genes, *mreB*, is located in a cluster with *mreC* and *mreD*, while the other one is located elsewhere. We have previously identified and studied the *mreBCD* homologues in *S. coelicolor*, and gene disruption experiments resulted in a viable *mreC* knockout mutant that showed growth retardation and reduced sporulation, while an *mreB* mutant could not be created by conventional gene replacement (Burger *et al*., 2000). Transcriptional analysis of the *mreBCD* gene cluster by promoter probing and S1 nuclease mapping suggested that the three genes are regulated as an operon from three promoters upstream of *mreB* (Burger *et al*., 2000). In this article, we provide evidence that MreB is not essential for vegetative growth in *S. coelicolor* but plays a vital role in formation of spores resistant to various forms of environmental stress.

Results

MreB *is found only in actinomycetes that convert aerial hyphae into spore chains*

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 the partial sequence of *S. scabies* (http://www.sanger.ac.uk/Projects/S_scabies) revealed almost complete conservation of MreB, and between 85% and 90% amino acid identity for MreC and for MreD. In addition, a second *mreB* homologue was detected in all these organisms. So far no *mreB* homologue was identified in non-sporulating actinomycetes, including *Corynebacterium*, *Kineococcus*, *Mycobacterium*, *Nocardia* and *Rhodococcus* (Jones *et al*., 2001; http://nocardia.nih.go.j/; http://Rhodococcus.ca; http://genome.jgi-psf.org/mic_home.html).

In *S. coelicolor*, *S. avermitilis* and *S. scabies*, the *mreBCD* operon lies between *ndk*, encoding a nucleoside diphosphate kinase, and *pbp83* (= *pbp2*), encoding a penicillin-binding protein (PBP) (Burger *et al*., 1998). In order to test how widespread the *mreB* genes are among rare actinomycetes, genomic DNA was isolated from various actinomycetes and PCR was performed using primers PM13 and PM14 (see Table 4) that were designed at two highly conserved regions of *mreB*. The primers did not distinguish between the two homologues of *mreB* found in the streptomycete genomes, and should detect both genes, although the products did not differ in size. Following the example from *B. subtilis*, we designate the second homologue that is located at a position distant from the *mre* cluster, *mbl*. We obtained a band of the expected size in all tested *Streptomyces* strains, in *Streptoverticillum mobaraense* and in *Streptosporangium roseum* (Table 1). In contrast, we failed to detect such a band with actinomycetes that do not produce aerial hyphae (Miyadoh, 1997), such as *Actinoplanes* (*A. friuliensis* and *Actinoplanes* sp.), *Micromonospora* sp., *C. 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 1). MreB proteins therefore seem to be present only in strains that produce both aerial mycelium and spores (see *Discussion*).

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 overexpressed *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 MS plates containing 10 μg ml^{-1} thiostrepton, the strain failed to grow (Fig. 1B), while the same strain harbouring the control plasmid pGM190 (without insert) showed normal growth (Fig. 1D); 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 S-medium and grown at 30°C. After 8–12 h

Table 1. Presence of *mreB* in different actinomycetes and the relationship of the ability to form aerial mycelium and spores.

Strain	Reference	Formation of aerial mycelium[1]	Production of spores[2]	Amplified product[3]	Detection by Southern blot[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 (http://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 hybridizing to the *S. coelicolor mreB* probe (**4**).

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

n.t., not tested.

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*.

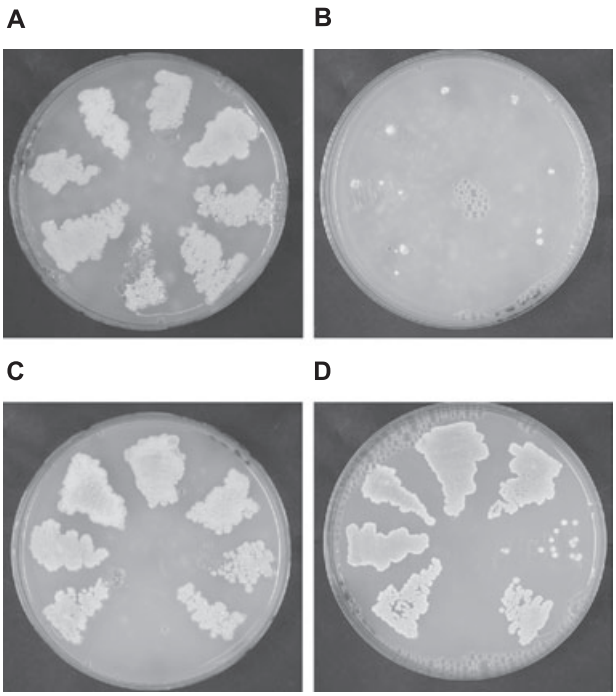

Fig. 1. Lethal effect of MreB overexpression on solid growth.
S. coelicolor M145 strains were plated in the absence (A and C) and presence (B and D) of 10 μg ml^{-1} of the inducer thiostrepton.
S. coelicolor M145 carrying pPM1 [*tipAp-mreB*] (A and B) and the vector pGM190 (C and D) were streaked onto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

of growth, MreB expression was induced by the addition of 10 μg ml^{-1} 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. 2). Swelling of the extremities of the hyphae and extensive lysis of the mycelium of pPM1 transformants were observed already 2 h after induction (Fig. 2C), while the pGM190 transformants remained unaffected. Overexpression of MreB in the pPM1 strain was confirmed by Western blot analysis with anti-MreB antibodies (data not shown).

To analyse whether MreB overexpression affects spore germination, spores of *S. coelicolor* M145 transformed with pPM1 were inoculated in liquid culture containing thiostrepton (Fig. 2D–F). In this case the spores were able to germinate, but the elongation of germ tubes was inhibited (Fig. 2F) and the hyphae failed to elongate properly.

A mutant deficient in mreB *was constructed by PCR targeting*

Previous attempts to inactivate *mreB* by gene disruption using the temperature-sensitive *Streptomyces* vector pGM9 were unsuccessful (Burger *et al.*, 2000). However, the use of REDIRECT technology (see *Experimental procedures*) allowed the isolation of an insertion mutant

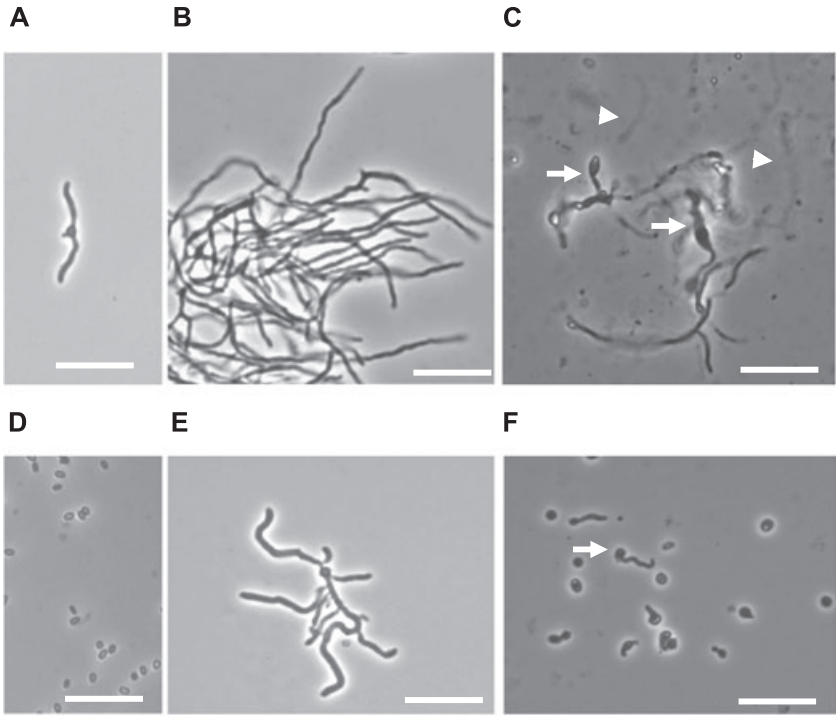

Fig. 2. Effect of the MreB overexpression on vegetative growth (A and C) in liquid culture and on spore germination (D and E).
A–C. Spores of *S. coelicolor* M145 carrying pPM1 [tipAp-*mreB*] were inoculated in S-medium. After 12 h of growth (A) the cultures were supplemented (C) or not (B) with 10 μg ml^{-1} of the inducer thiostrepton. Images were taken 8 h after induction. Scale bar: 10 μm. (C) Arrow heads show hyphal lysis and arrows swelling of hyphae induced by MreB overexpression.
D–F. To analyse the effects on spore germination, spores of the strain carrying pPM1 [*tipAp-mreB*] were inoculated in S-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.
Scale bar: 10 μm.

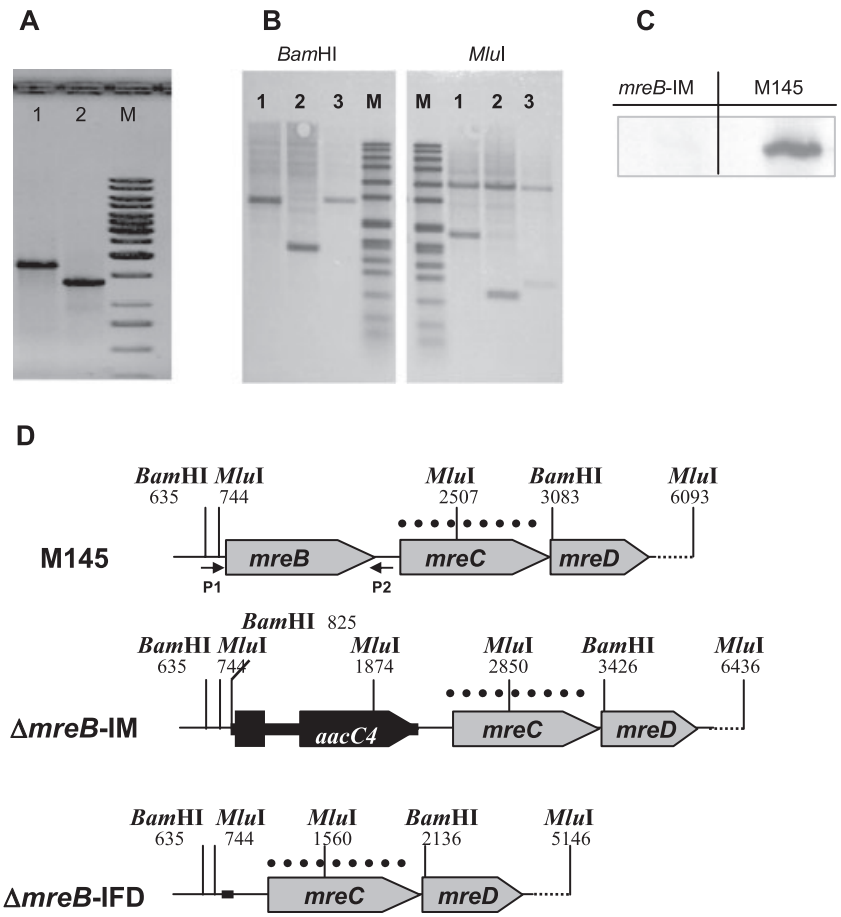

Fig. 3. Confirmation of the *mreB* mutants by PCR (A), Southern (B) and immunoblot (C) analysis.
A. PCR was conducted on DNA of M145 (lane 2) and *mreB*-IM (lane 1) using a pair of primers that anneals 200 bases upstream the start codon (P1 in D) and 20 bases downstream the stop codon (P2 in D). If the gene has been replaced by the *aacC4* cassette the PCR product obtained with these primers has to be 356 bases bigger than the wild type. M: 1 kb ladder, Fermentas.
B. Southern blot analysis was performed with genomic DNA of M145 (1), *mreB*-IFD (2) and *mreB*-IM (3) digested with BamHI or MluI using the *mreC* gene as a dig-labelled probe (dotted line in D). M, DNA marker VII, DIG-labelled (Roche), with fragment sizes (bp) of 8576, 7427, 6106, 4899, 3639, 2799, 1953, 1882, 1515, 1482, 1164, 992, 710.
C. For Immunoblot analysis protein extracts of M145 and *mreB*-IM were separated by SDS-PAGE and blotted on a nitrocellulose membrane. Immunoblotting performed with MreB-specific antiserum detected a band of the expected size (37 kDa) in the wild type but not in the *mreB*-IM mutant.
D. The schematic drawing shows maps of the chromosomal *mreBCD* regions of *S. coelicolor* M145, and the *mreB* mutants *mreB*-IM and *mreB*-IFD. Restriction sites used in Southern blot analysis are indicated.

(*mreB*-IM) in which *mreB* was replaced with the apramycin resistance cassette *aacC4* (1384 bp). The replacement of *mreB* with the apramycin cassette was verified with PCR and Southern blot analysis (Fig. 3A and B), and the loss of expression of MreB was confirmed with Western blot analysis using anti-MreB antiserum (Fig. 3C). The *mreB*-IM mutation is expected to have polar effects to *mreC* and *mreD*, as these 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 also constructed removing the apramycin cassette from the chromosome of the *mreB*-IM mutant (see *Experimental procedures*). The loss of the resistance marker and the exact location of the deletion were confirmed by PCR (data not shown) and Southern blot analysis (Fig. 3B).

The mreB *deletion mutant produces swollen aerial hyphae and swollen spores*

Unexpectedly, in the case of the *mreB*-IM mutant, colony morphology and vegetative growth of the substrate mycelium was normal on plates and in liquid-grown cultures (data not shown). However, differentiation was significantly affected: many aerial hyphae had a swollen appearance, were irregular in shape (Fig. 4B) and showed a large degree of lysis. Spore dimensions were also abnormal. Measurement of spore sizes (Table 2) using the ANALYSIS® program (SIS, Soft Imaging System GmbH) showed that the *mreB*-IM mutant spores had an average width of 1.31 μm while the spores of the parental strain *S. coelicolor* M145 had an average width of only 0.75 μm. Mutant spores were also longer (Table 2), as the average length of *mreB*-IM mutant spores was 1.65 μm while the average length of parental spores measured 1.09 μm. In addition, both light (Fig. 4C) and scanning electron microscopy (data not shown) frequently showed premature germination of spores already in the spore chains. The phenotype of the in-frame *mreB* deletion mutant (*mreB*-IFD) strongly resembled that of the *mreB*-IM replacement mutant, again with swollen, kinky and lysing aerial hyphae (data not shown). Spores were also significantly larger and wider than the parental strain showing average values of width (1.16 μm) and length (1.64 μm) comparable to the insertional mutant (Table 2).

To confirm that the absence of an intact *mreB* was responsible for the observed phenotype, a single copy of the *mreB* gene under its own promoter (construct pPM6; see *Experimental procedures*) was inserted at the ϕC31

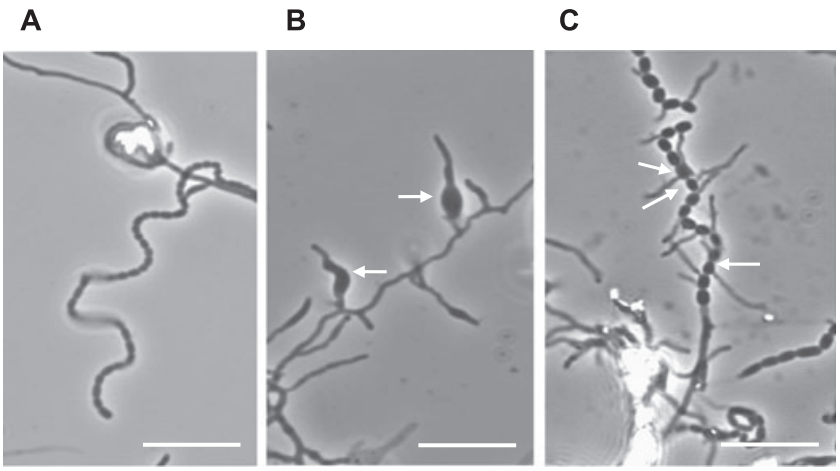

Fig. 4. Effect of *mreB* deletion on morphological differentiation. *S. coelicolor* M145 (A) and *mreB*-IM (B and C) were grown on MS agar for 2–3 days before being prepared for light microscopy. White arrows indicate swollen aerial hyphae (B) and prematurely germinating spores (C) caused by the deletion of *mreB*. Bar = 10 μm.

attachment site in the chromosome of the *mreB*-IFD mutant, generating the strain *mreB*-IFDc. Insertion of *mreB* complemented the phenotype of the *mreB* mutant. Aerial hyphae of the strain *mreB*-IFDc did not swell and lyse (data not shown). Length (1.23 μm) and width (0.84 μm) of the spores of *mreB*-IFDc were almost completely restored to parental values (Table 2), suggesting that indeed the altered spore morphology was due to deletion of *mreB*.

Integrity of the envelope is severely compromised in mreB *mutant spores*

To analyse the defects of the *mreB* mutant of *S. coelicolor* in more detail, thin sections of sporulating aerial mycelia of the mutant and its parental strain M145 were analysed by high-resolution transmission electron microscopy (TEM). Typical examples of dividing aerial hyphae, prespore chains and mature spores are presented in Fig. 5. In line with the observations with light microscopy, the *mreB* mutant showed extensive lysis of the aerial hyphae, producing bloated structures (data not shown). Especially remarkable was the altered appearance of the nucleoids, invariably surrounded by electron-lucent (white) material (Fig. 5B). Spores had highly heteromorphous shapes, and collapsed, dented and broken spores evidenced loss of spore wall consistency (Fig. 5B), when compared with the parental M145 (Fig. 5A). Apparently, both nucleoid organization and integrity of the spore envelope were severely compromised in the *mreB* mutant.

The spores of the mreB *mutants are sensitive to heat and detergent*

Streptomyces spores are dormant cells which are relatively resistant to desiccation, sonic vibration, enzymatic digestion and exposure to moderately high temperature (McBride and Ensign, 1987). To test whether the mutant spores were as heat resistant as those of the parental strain, spore suspensions (10^5 spores ml^{-1}) were incubated at 60°C for different intervals and then plated on SFM and further cultivated for 2 days at 30°C. After 5 min of heat treatment, 84% of the spores of the parental M145 survived, while of the *mreB* mutant only 7.5% (*mreB*-IFD) had survived (Fig. 6). Extended heat treatment (60 min) was required to decrease the survival rate of M145 spores to 30%, and during those conditions only 0.01% of the *mreB*-IFD mutant could survive (Fig. 6).

It is known that *Streptomyces* spores are resistant to treatment with SDS and this detergent has also been reported to activate spore germination (Grund and Ensign, 1982). The effect of SDS on *mre*-depleted *S. coelicolor* spores was therefore tested. SDS (final concentration 5%) or water (as a control) was added to spore suspensions of M145 and of the *mreB*-IFD mutant and incubated at room temperature for 1 h. Spores were then diluted, plated on MS agar and incubated at 30°C for

Table 2. Average values of spore length and width of spores in chains of M145, MreB-depleted strains, complemented mutants and *mre*-IM complemented with the *mreB–egfp* fusion gene.

	M145	*mreB*-IM	*mreB*-IMgfp	*mreB*-IFD	*mreB*-IFDc
Spores length (μm)[a]	1.09 ± 0.29	1.65 ± 0.33	1.26 ± 0.31	1.64 ± 0.24	1.23 ± 0.27
Spores width (μm)[a]	0.75 ± 0.12	1.31 ± 0.25	0.82 ± 0.11	1.16 ± 0.21	0.84 ± 0.14

a. Average values for 60 spores measured by phase contrast microscopy.

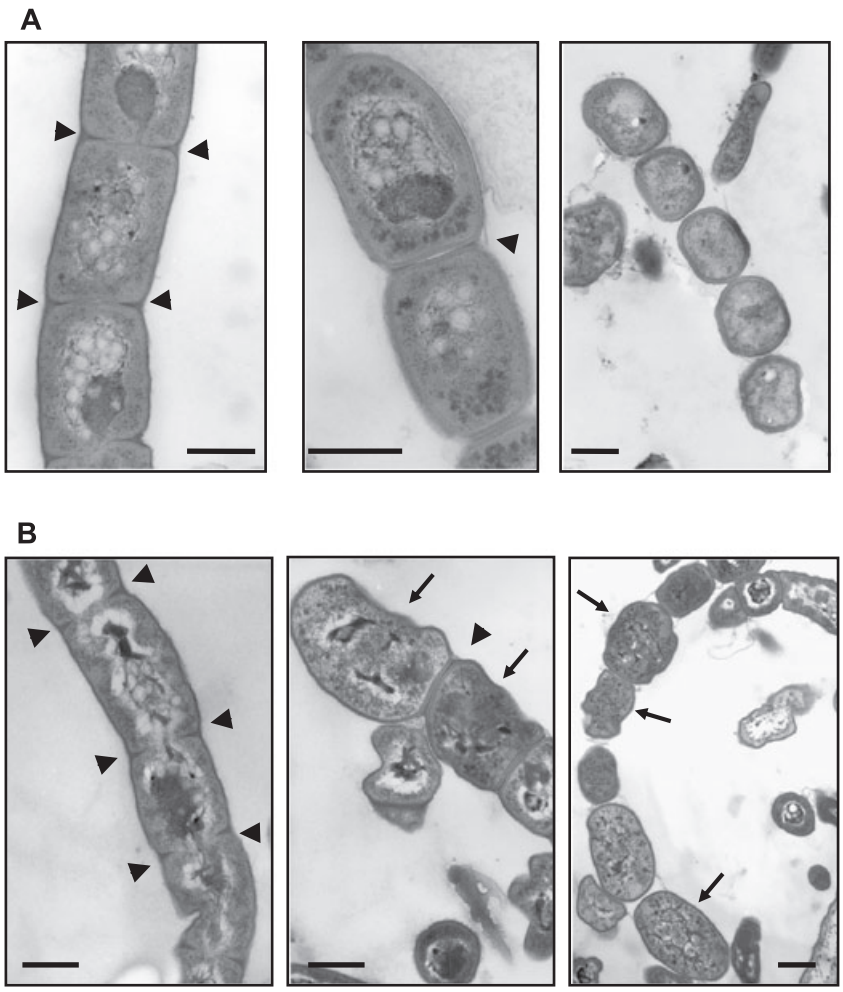

Fig. 5. Transmission electron micrographs of the *mreB* mutant. Thin sections of sporogenic aerial hyphae of *S. coelicolor* M145 (A) and its *mreB*-IM gene replacement mutant (B) were analysed at high resolution by transmission electron microscopy. Left, middle and right panels show prespores, almost mature spores and mature spores respectively. While septation (arrowheads) is not affected by the deletion of *mreB*, integrity of cell walls is highly compromised, resulting in strongly deformed spores (arrows). Bar = 500 nm.

2 days. Viable count of wild-type spores did not decrease after incubation either in water or in SDS. However, the viable count of the *mreB* mutant was reduced to 0.1% after SDS treatment. As the spores of the *mreB* mutant strains are, unlike the wild type, sensitive to some physiological stresses, the response of these spores to mechanical stress was also analysed. Spore suspensions were treated with ultrasound as described in *Experimental procedures*. The wild type and the mutants were equally resistant to this treatment, and even after five cycles of ultrasonication the survival rate was more than 50% in both cases (data not shown).

MreB is a membrane-associated protein that localizes 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 (Jones *et al*., 2001; Kruse *et al*., 2003; Shih *et al*., 2003; Formstone and Errington, 2005) 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 localize MreB in *S. coelicolor* M145, the entire *mreB* gene and its three promoters were positioned upstream of the *egfp* gene, to generate a fusion between the two genes, encod-

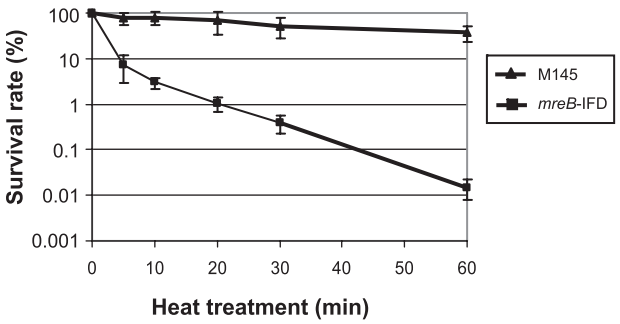

Fig. 6. Effect of the *mreB* mutation on resistance of the spores to heat treatment. Spore suspensions of *S. coelicolor* M145 and the *mreB* mutant *mreB*-IFD were incubated at 60°C for different intervals (indicated on the *x*-axis) and plated on MS agar. Survival rate was calculated as described in *Experimental procedures*. After 60 min heat treatment the survival rate of *mreB*-IFD was about 1000 times lower than that of the parental strain *S. coelicolor* M145. Each value is the mean of three to five replicates.

ing an MreB–EGFP fusion protein under the control of the natural *mreB* promoters (see *Experimental procedures*). The fusion gene was cloned in pSET152, allowing integration at the ϕC31 attachment site of the *S. coelicolor* chromosome generating the strain SCPM6. The same plasmid (pPM4) was also integrated into the chromosome of the *mreB*-IM mutant to analyse whether the fusion protein is functional and able to complement the MreB deficiency. The presence of a single copy of *mreB–egfp* complemented the defect causing the pre-germination of spores (data not shown) and reduced the swollen spore size to almost parental values (Table 2), suggesting that the MreB–EGFP fusion protein was active.

During vegetative growth of SCPM6, only diffuse fluorescence was observed in the hyphae although expression of MreB–EGFP was confirmed by Western blot analysis with both anti-MreB and anti-GFP antibodies (data not shown). In contrast, study of surface-grown cultures revealed very clear fluorescence signals that were specifically localized (Fig. 7) at the septa of sporogenic aerial hyphae. As shown in Fig. 7B, bands of fluorescence coincided with the constrictions caused by sporulation septation. Simultaneous labelling of septa with fluorescent conjugates of wheat germ agglutinin confirmed that MreB–EGFP overlapped with the septa (data not shown). However, it is not clear whether MreB–EGFP also localized to the basal septa sometimes seen at the bottom of sporogenic hyphal cells (Kwak *et al*., 2001). In prespore chains, foci were localized at the cell poles generated by sporulation septation (white arrows in Fig. 7D). Successively, in more mature spores, the MreB–EGFP signal completely surrounded the spores, giving rise to ring-like appearance of the fluorescence (Fig. 7F). However, in fully mature spores most of the fluorescence from

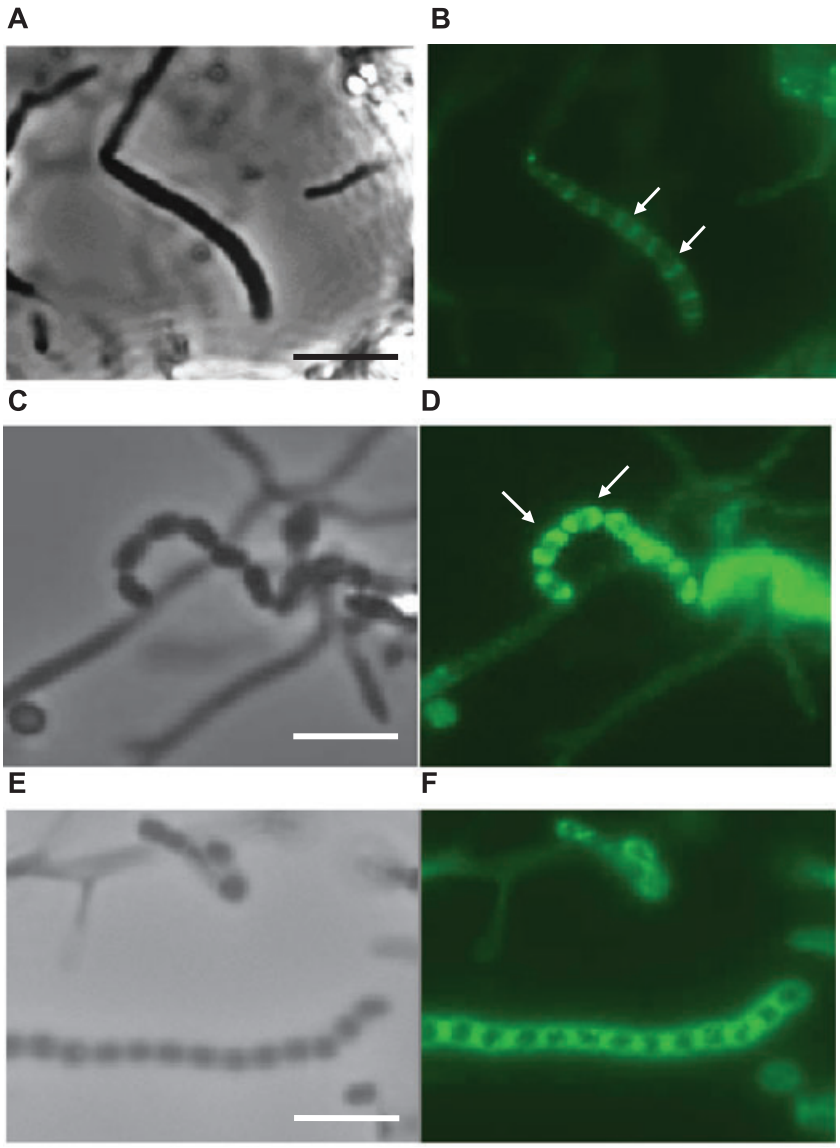

Fig. 7. Localization of the MreB–EGFP fusion protein during morphological differentiation. MreB–EGFP was detected at sporulation septa in aerial hyphae (B), at the poles of prespores (D) and subsequently covering the whole spore wall in spore chains (F). A, C, E: phase contrast; B, D, F: fluorescence microscopy. Bar = 5 μm.

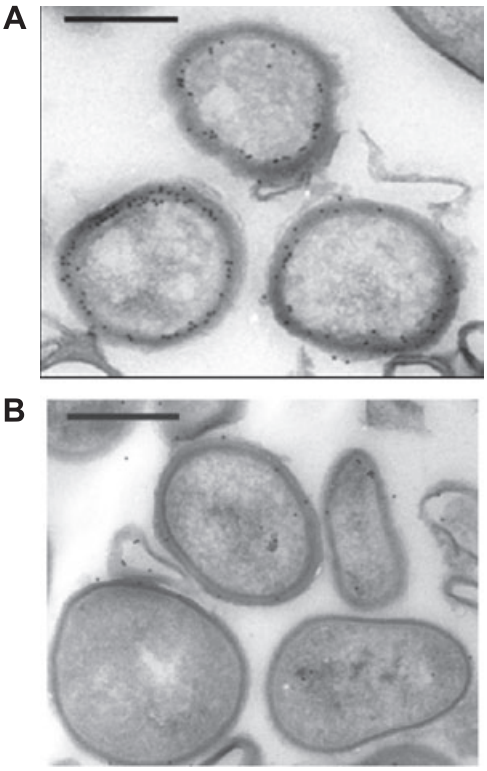

Fig. 8. High-resolution localization of MreB in *S. coelicolor* spores. Specific localization of MreB in spores by *in situ* hybridization of thin sections (immunoelectron microscopy) of spore preparations of *S. coelicolor* M145 (A), using gold-labelled anti-MreB antibodies. The MreB-specific antibodies localized almost exclusively around the inner side of the spore envelope. Occasional particles found outside the spores were attached to cell debris. Expectedly, 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 MreB–EGFP fusion had disappeared (data not shown).

Closer inspection of the localization of MreB was performed by immunogold electron microscopy of spore preparations from *S. coelicolor* M145 (Fig. 8). As a negative control we used the *mreB* insertion mutant (*mreB*-IM). While in the mutant only background gold labelling was obtained, similar to when anti-MreB antibody was omitted (not shown), in the parental strain clear and specific labelling, particularly close to the inside of the spore walls was detected.

The cellular localization of MreB was further examined by cell fractionation and Western analysis using anti-MreB antibodies. For this, the mycelium and spores grown on MS agar were disrupted by French press treatment and the cytoplasmic, the membrane-associated and the membrane fractions were individually isolated. MreB was detected both in the soluble fraction and mostly in the membrane-associated fraction, while we failed to detect MreB in the membrane fraction (data not shown). Cellular localization of the MreB–EGFP fusion protein was also conducted. Indeed, the protein was localized in the same fractions as the native MreB, namely in the soluble fraction and in the membrane associated fractions (data not shown), indicating that the fusion protein was localized equivalent to the parent protein.

Discussion

In this study, an *mreB* deletion mutant was obtained in the filamentous Gram-positive bacterium *S. coelicolor*, and it was demonstrated that MreB is dispensable for vegetative growth, but essential for spore formation and assembly of the spore cell wall. Previous attempts to inactivate *mreB* in *S. coelicolor* by gene disruption using the temperature-sensitive *Streptomyces* vector pGM9 (Muth *et al*., 1989) were unsuccessful (Burger *et al*., 2000). However, using a PCR-targeting method (REDIRECT method; Gust *et al*., 2003) we were able to generate deletion mutants in *mreB*. 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. As several *mreB* mutants were isolated in independent experiments with frequencies that are typical of 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 *mreB* in *S. coelicolor* gave no obvious phenotype during vegetative growth in liquid culture or on solid plates. However, transcriptional analyses by S1 nuclease mapping (Burger *et al*., 2000) and by microarray and reverse transcription polymerase chain reaction (RT-PCR) studies (E.E. Noens and G.P. van Wezel, unpublished) revealed that *S. coelicolor mreB* is transcribed during vegetative growth, and expression was confirmed by Western blot analysis of MreB protein in liquid-grown cultures (data not shown). Thus, the possible role of MreB in young mycelium is unclear and needs further analysis, and it can not be excluded that there could be some degree of redundancy between MreB and the second MreB homologue Mbl. However, based on the lack of a clear phenotype of the *mreB* mutant during vegetative growth, and the lack of MreB–EGFP signals at hyphal tips, it seems unlikely that MreB would have a direct role in the elongation of the hyphal cell wall, which occurs primarily at the tips. Sequence analysis of genomic DNA of different actinomycetes showed that species forming only substrate mycelium, such as *Rhodococcus* sp. and *Nocardia* sp. (http://Rhodococcus.ca; http://nocardia.nih.go.jp), do not have an *mreBCD* cluster, supporting the idea that the Mre

proteins may not be required for vegetative growth in actinomycetes.

While inactivation of *mreB* did not compromise vegetative growth of *S. coelicolor*, ectopic 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 at the wrong spatial compartment severely interferes with normal growth, perhaps by recruiting PBPs and preventing them from their function in cell wall synthesis.

Mutational analysis clearly demonstrated that MreB in *S. coelicolor* is required for correct sporulation, and appears to affect spore wall formation. The aerial hyphae and spores of the *mreB* deletion mutant were swollen (Fig. 4; Table 2), spores were sensitive to heat and treatment with SDS (Fig. 6), and irregularities in the spore cell walls were observed using TEM (Fig. 5B). This is consistent with the observation made in *B. subtilis* that deletion of *mreB* causes increased cell width and cell lysis (Formstone and Errington, 2005). Formstone and Errington (2005) proposed that loss of MreB lead 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 (Formstone and Errington, 2005). In analogy, the depletion of MreB in *S. coelicolor* appears to impair assembly of the cell wall, but only 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 (Glauert and Hopwood, 1961; Ensign, 1978). 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.

The involvement of MreB in spore formation is consistent with the PCR and Southern blot analysis of different actinomycetes. Using primers corresponding to a highly conserved region of *mreB*, genomic DNA of sporulating actinomycetes gave rise to an amplified fragment of the expected size, while genomic DNA of non-sporulating actinomycetes did not (Table 1). Surprisingly two sporulating genera, *Actinoplanes* and *Micromonospora*, did not give an amplified product (Table 1). These two genera share an important characteristic: they sporulate, but they do not form aerial mycelium. The genus *Actinoplanes* produces motile spores enveloped in structures called sporangia (Waksman, 1961). The genus *Micromonospora* grows as straight or curved branching substrate hyphae without cross-walls (Waksman, 1961). Like in *Actinoplanes*, aerial mycelium is not formed and multiplication occurs by means of fragments of mycelium and special spores formed singularly. The genus *Actinoplanes* is a member of the family of the Actinoplanaceae together with the genus *Streptosporangium* which in contrast produces an aerial mycelium which resembles that of most species of *Streptomyces* (Waksman, 1961). Unlike *Actinoplanes*, *Streptosporangium* DNA was shown to encode an *mreB* homologue (Table 1). Apparently, the differentiation process in the two genera is highly different, suggesting that the *mre* genes are needed only in *Streptosporangium* sporulation that indeed resembles that of *Streptomyces*. From these observations, MreB seems not to be required in sporulation processes of actinomycetes in general, but only in a sporulation process which involves septation of aerial mycelium.

In *B. subtilis* and *E. coli*, which both are rod-shaped bacteria, MreB forms helical filaments lying beneath the cell surface (Jones *et al.*, 2001; Shih *et al.*, 2003). In *C. crescentus*, MreB undergoes two distinct patterns of localizations: during the cell elongation phase it appears like spirals that traverse along the longitudinal axis of the cell, while during cell division it forms an FtsZ-dependent transverse band at the mid-cell (Figge *et al.*, 2004). Therefore, it was speculated that MreB has a role in coordinating the switch between longitudinal and septal peptidoglycan synthesis. In *S. coelicolor*, we failed to detect the typical helical-like structures described for *E. coli*, *B. subtilis* and *C. crescentus*. The signal generated from the MreB–EGFP fusion during vegetative growth resulted in diffused fluorescence in the hyphae, indicating that MreB monomers are randomly distributed in the vegetative mycelium without a specific localization. In contrast, during differentiation the MreB–EGFP localized at the septa of aerial hyphae and successively, when spores were formed, in the spores (Fig. 7) probably underneath the cytoplasmic membrane, as indicated by immunogold electron microscopy (Fig. 8). Considering that MreB is organized in helical structures in rod-shaped bacteria, it is possible that in *S. coelicolor* MreB forms dense spirals just underneath the cytoplasmic membrane, but no such patterns could be discerned in our analyses. As it is possible that fusions of GFP to the C-terminus of MreB can interfere with its function, we can not exclude that our C-terminal MreB–EGFP fusion did not fully reproduce the localization patterns of MreB itself. However, we could demonstrate that MreB–EGFP localized to the same subcellular fractions of *S. coelicolor* as the MreB protein did. Furthermore, MreB–EGFP was able to complement the mutant phenotype of the *mreB*-IM mutant by restoring the spore size to almost parental values (Table 2) as well as by preventing pre-germination of spore chains.

In *S. coelicolor*, septation occurs during both vegetative growth and sporulation, but MreB localization 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 (Fig. 5B) and produced

viable spores. On the other hand, it should be tested whether MreB localization could be FtsZ dependent, as already seen in *C. crescentus* (Figge *et al*., 2004). It will be therefore interesting to test in future experiments MreB localization in different *S. coelicolor ftsZ* mutants; for example, an *ftsZ* mutant impaired in Z-ring formation in sporogenic aerial hyphae, but not in vegetative mycelium (Grantcharova *et al*., 2003). In *R. sphaeroides*, MreB localizes predominantly at the mid-cell position (Slovak *et al*., 2005). The authors speculate that this mid-cell localization 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 localize 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. Thus, MreB in *S. coelicolor* has a novel localization pattern as well as a somewhat unique mode of action that has not been observed in the rod-shaped bacteria and may be typical of actinomycetes that form an aerial mycelium and spores.

Based on the localization of MreB at the spore wall and its important role in maintaining the integrity of the spore wall, we anticipate that MreB is involved in thickening of the spore wall and may be recruiting PBPs and other peptidoglycan-related proteins during the sporulation process. The *mre* genes in *S. coelicolor* and *S. avermitilis* are physically linked with *pbp83* and *sfr* (Burger *et al*., 2000). Pbp83 (Pbp2; Bentley *et al*., 2002), encodes a PBP with high similarity to *E. coli* PBP2, which is involved in cell elongation in *E. coli* (Vinella *et al*., 1993), and with SpoVD, a PBP important for sporulation of *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 *pbp83* by gene disruption lead to an impairment in differentiation (A. Burger and W. Wohlleben, unpublished), a phenotype that resembles that of the *mreB* mutant that is reported in this article. A protein interaction model of the MreBCD, RodA and PBPs in *B. subtilis* has been proposed by Errington (2003). According to this model a multi-PBP complex interacts with RodA, MreC and MreD and this assembly is spatially controlled by the MreB cables in the cytosol. These proposed interactions between the Mre proteins were partially confirmed in *E. coli* (Kruse *et al*., 2005). We observed that in *S. coelicolor*, deletion of *mreB*, *mreC*, *mreD* individually, or of the whole *mreBCD* cluster, leads to a similar phenotype (K. Schirner, P. Mazza, G. Muth, and W. Wohlleben, unpublished) suggesting that also in this organism the three proteins may participate in the same process. A complex that includes PBPs, a RodA homologue, and the Mre proteins, and which primarily is acting during spore wall assembly, is therefore imaginable in *S. coelicolor*. This leads to the following hypothesis on the mode of action of MreB in *S. coelicolor*: it is expressed at a basal level during vegetative growth and it is localized rather dispersely in the cytosol. When sporulation septation occurs, MreB condenses at the sporulation septa, possibly in an FtsZ-dependent manner. An increased expression of *mreB* before sporulation that was detected by S1-mapping experiments (Burger *et al*., 2000) may allow the cells to accumulate a sufficient number of MreB units to form a ring- or a shell-like structure underneath the spore wall. 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 localize proteins responsible for spore wall formation, perhaps including Pbp83 and the product of *sfr*. Further studies to localize Pbp83 and Sfr in *mre* mutants and interaction studies between the Mre proteins and the products of *pbp83* and *sfr* are underway to understand the unique role of MreB in *S. 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.

Experimental procedures

Bacterial strains and media

The *E. coli* and *S. coelicolor* A3(2) strains used are listed in Table 3. *S. coelicolor* strains were cultivated on MS agar plates or in S-medium (Kieser *et al*., 2000). Cultivation of strains and procedure for DNA manipulation were performed as previously described for *E. coli* (Sambrook *et al*., 1989) and *S. coelicolor* (Kieser *et al*., 2000).

Construction of plasmids

Plasmid pPM1 was obtained by amplifying the *mreB* gene from the *S. coelicolor* M145 genome using the primers PM1 and PM2 (Table 4), digesting the PCR product with NdeI and HindIII and cloning the fragment under the control of the thiostrepton-inducible promoter *PtipA* into the *Streptomyces* multicopy expression plasmid pGM190 (G. Muth, unpubl. data). To construct a C-terminal fusion with the EGFP protein, the *mreB* gene was amplified using the primers PM5 and PM6 (Table 4), cut with NdeI and BglII and cloned in front of the *egfp* gene in the plasmid pTST101 (J. Altenbuchner, pers. comm.) cut with NdeI and BamHI. To transform *S. coelicolor* M145 the resulting *mreB–egfp* fusion was cut out from pTST101 and cloned in the integrative *Streptomyces* vector pSET152 (Bierman *et al*., 1992) generating pPM4. Plasmid pPM6 was constructed by amplifying the *mreB* gene with its promoter region from *S. coelicolor* M145 with the primers PM11 and PM12 (Table 4) and cloning the PCR product as BglII–EcoRI fragment in pSET152 (Bierman *et al*., 1992).

Table 3. Strains of *E. coli* and *S. coelicolor* used in this work.

Strain	Characteristics	Reference
E. coli BL21(DE3)pLysS	F$^-$*ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm araB*::T7RNAP-*tetA*	Invitrogen
E. coli ET 12567	GM2929 *hsdM*$^-$*hsdR*$^-$*zjj*-202	MacNeil *et al*. (1992)
E. coli DH5α	*supE44* Δ*lacU169* (*80 lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Woodcock *et al*. (1989)
E. coli BW 25113/pIJ790	Chromosome: Δ*(araD-araB)567* Δ*lacZ4787 (::rrnB-4) lacIp-4000 (lacI^Q)* λ^{-1}*rpoS369(Am) rph-1* Δ*(rhaD-rhaB)568 hsd514*; Plasmid: [*oriR101*] [*repA101*(ts)] *araBp-gam-beta-exo*	Gust *et al*. (2003)
S. coelicolor M145	Prototrophic, SCP1$^-$ SCP2$^-$	Kieser *et al*. (2000)
mreB-IM	M145 Δ*mreB::ΩaacC4*	This work
mreB-IFD	M145 Δ*mreB*	This work
mreB-IFDc	M145 Δ*mreB attB*$_{\Phi C31}$ *::mreB*	This work
SCPM6	M145 *attB*$_{\Phi C31}$ *::mreB–egfp*	This work

Protein expression, purification and antibody production

mreB from *S. coelicolor* M145 was amplified by PCR with the primers PM7 and PM2 that include BamHI and HindIII site, respectively, and cloned in pRSETB (Invitrogen) so that it forms an in-frame fusion with the His-tag (pPM5). His-tagged MreB was expressed in *E. coli* BL21 cells containing pPM5 and purified with Ni-Nta spin columns (Qiagen) under denaturing condition as described in the Ni-Nta manual (Qiagen). Rabbit polyclonal antibodies were raised against the purified protein (Eurogentec).

Overexpression of MreB

Protoplasts of *S. coelicolor* M145 were transformed with the plasmids pPM1 or pGM190 as described previously (Kieser *et al*., 2000). Spores of transformants were inoculated in S-medium containing 50 μg ml^{-1} kanamycin and incubated with shaking at 30°C. To overexpress MreB, thiostrepton was included at a concentration of 10 μg ml^{-1}.

Overexpression of MreB was confirmed by Western blot analysis with anti-MreB serum. Mycelium was collected after 18 h of growth with or without thiostrepton and broken with French press. Crude extracts were mixed with an equal volume of 2× sample buffer (125 mM Tris-HCl pH 6.8; 4% SDS; 20% glycerol; 2.0 mM EDTA; 0.02% bromophenol blue; 3% dithiothreitol), boiled for 5 min and loaded on SDS-polyacrylamide gels (12.5%). Proteins were capillary transferred to a pure nitrocellulose blotting membrane (Pall Corporation) and MreB was detected using a 1:2500 dilution of polyclonal anti-MreB serum.

Cell fractionation

Streptomyces coelicolor M145 and the strain SCPM6 carrying the *mreB–egfp* fusion were grown for 2–3 days in 150 ml of S-medium with or without antibiotics at 30°C and harvested by centrifugation. Mycelium was resuspended in 6 ml of 25 mM Tris-HCl (pH 7.5), 100 mM NaCl and 1 mM protease inhibitor (Complete EDTA-free tablets, Roche), and the cells were broken using the French press. The cell extract was centrifuged at 90 000 *g* for 30 min at 4°C and the supernatant, containing the cytoplasmic fraction, was saved and stored at –20°C. Pellet was resuspended in 4 ml of 25 mM Tris-HCl (pH 7.5), 1 M NaCl and 20% glycerol, stirred for 2 h at 4°C and centrifuged at 90 000 *g* for 30 min at 4°C. The supernatant containing membrane-associated proteins was saved and stored at –20°C. To obtain the membrane fraction, pellet was resuspended in 1.5 ml of 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 *g*. The samples obtained from cell fractionation were loaded on SDS-gels and Western blot was performed as described above.

Creation of an mreB *null allele*

To create an *mreB* mutant, the PCR-targeting procedure described by Gust *et al*. (2003) was used. The *ΩaacC4* cassette (which confers apramycin resistance) from the plasmid pIJ773 was amplified using the oligonucleotides PM9 and PM10 (Table 4) designed so that the 3′-ends can anneal to

Table 4. Primers used in this work.

Primer	Sequence[a]
PM1	cg*catatg*gggaactcaatgtcgttc
PM2	ga*aagctt*acgtcatctacggggcg
PM5	cg*catatg*tgatccttcttcgggac
PM6	gg*agatct*gatgaacgacattga
PM7	aa*ggatcc*ggggaactcaatgtcg
PM9	ccctcaaaagctcctgggaaggccagtcgaatcctgatggatatcattccgg*ggatcc*gcgtacc
PM10	ggagatcgtctcgtacggcggaaccgaagtgttacgtcagatatctgtaggctggagctgcttc
PM11	g*cgagatc*tgacgccatgtcagtcga
PM12	gc*gaattc*ggcggaaccgaagtgtta
PM13	gccgtgcgcccgctgaaggacgg
PM14	agcagcgcgcgccgccgccggt

a. Restriction sites are written in italics.

the resistance cassette gene, whereas the 5′-ends are homologous to the sequences flanking *S. coelicolor mreB*. The PCR products was transferred into *E. coli* BW 25113/pIJ790 carrying the cosmid C88 and the λ RED system induced as described (Gust *et al*., 2003). The correct gene replacement on the cosmid C88 isolated from an apramycin-resistant clone was confirmed with PCR using a set of oligonucleotides priming outside the region of recombination. This cosmid was designated pPM11. pPM11 was introduced into *E. coli* ET12567 containing the plasmid pUZ8002 and then transferred to *S. coelicolor* M145 by conjugation as previously described (Kieser *et al*., 2000). Exconjugants were selected for kanamycin and apramycin resistance and then replicated on plates with apramycin and with or without kanamycin. DNA of kanamycin-sensitive and apramycin-resistant clones were isolated and tested with PCR and Southern blot to confirm the absence of the chromosomal *mreB*.

In order to remove the disruption cassette, the cosmid pPM11 was introduced in the *E. coli* strain DH5α/pCP20 that expresses the FLP recombinase (Gust *et al*., 2003). The resulting cosmid carrying the in-frame deletion was named pPM12 and used to transform the *S. coelicolor* mutant strain containing the marked deletion (Kieser *et al*., 2000). Transformants were first selected for insertion of the cosmid by single cross-over (kanamycin resistant) and then screened for the double-cross-over event (kanamycin sensitive and apramycin sensitive). The loss of the disruption cassette was confirmed with PCR and Southern blot analysis.

Heat and detergent treatment

To test whether the mutant spores were as heat resistant as the ones of the parental strain, spore suspensions of different strains (10^5 spores ml^{-1}) were incubated at 60°C for different time. After incubation spore suspensions were diluted 1:10 or 1:100 and 100 μl of the dilution was plated on MS agar. Spore suspension of the *mreB* mutant incubated longer than 20 min at 60°C were plated without being diluted. Plates were further cultivated for 2 days at 30°C. Survival rate was calculated as number of colonies grown on plates after treatment at 60°C divided by the number of colonies grown on plates without treatment at 60°C in percentage.

To test the effect of the detergent SDS on *mreB*-depleted spores, 50 μl of spore suspensions (10^9 ml^{-1}) of M145 and of the *mreB* mutant (*mreB*-IFD) was incubated in 5% SDS, 1% SDS or water for 1 h at room temperature. Appropriate spore dilutions were plated on MS agar and incubated at 30°C for 2 days. Titre of colonies surviving SDS treatment were determined in relation to those incubated in water.

Ultrasonic treatment

To test spore resistance to ultrasonication, spore suspension (10^5 spores ml^{-1}) was treated with ultrasounds (SONOPULS GM2070 digital ultrasonic homogenizers, 20 kHz; BANDELIN electronic GmbH) five cycles of 10 s and after each cycles 100 μl of spore suspension was collected, diluted, plated on MS agar and further incubated at 30°C for 2 days.

Microscopy

Phase contrast microscopy. For light microscopy, sterile coverslips were inserted at a 45° angle into MS agar and spores were inoculated in the acute angle along the glass surface. Coverslips were removed after 3- to 4-day incubation at 30°C and mounted in PBS containing 50% glycerol on poly L-lysine-coated slides. Alternatively liquid culture was spotted directly on microscope slides covered with 1% agarose and covered with coverslips. Samples were observed with an Olympus System Microscope BX60 and pictures taken with an Olympus F-view II camera.

Electron microscopy. TEM for the analysis of ultrathin cross-sections of hyphae and spores was performed as described previously (van Wezel *et al*., 2000).

For immunoelectron microscopy spores were fixed in 2% paraformaldehyde with 0.2% glutaraldehyde in PHEM buffer for 2 h 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, cryoprotected in 2.3 M sucrose and snapfrozen in liquid nitrogen. Ultrathin cryosections were labelled with rabbit anti-MreB (1:2000) followed by 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).

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