Influence of heterologous MreB proteins on cell morphology of *Bacillus subtilis*

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The prokaryotic cytoskeletal protein MreB is thought to govern cell shape by positioning the cell wall synthetic apparatus at growth sites in the cell. In rod-shaped bacteria it forms helical filaments that run around the periphery of the rod during elongation. Gram-positive bacteria often contain more than one *mreB* gene. *Bacillus subtilis* has three *mreB*-like genes, *mreB*, *mbl* and *mreBH*, the first two of which have been shown to be essential under normal growth conditions. Expression of an *mreB* homologue from the closely related organism *Bacillus licheniformis* did not have any effect on cell growth or morphology. In contrast, expression of *mreB* from the phylogenetically more distant bacterium *Clostridium perfringens* produced shape defects and ultimately cell death, due to disruption of the endogenous MreB cytoskeleton. However, expression of either *mreB^{B. licheniformis}* (*mreB^{Bl}*) or *mreB^{C. perfringens}* (*mreB^{Cp}*) was sufficient to confer a rod shape to *B. subtilis* deleted for the three *mreB* isologues, supporting the idea that the three proteins have largely redundant functions in cell morphogenesis. Expression of *mreBCD^{Bl}* could fully compensate for the loss of *mreBCD* in *B. subtilis* and led to the formation of rod-shaped cells. In contrast, expression of *mreBCD^{Cp}* was not sufficient to confer a rod shape to *B. subtilis* Δ *mreBCD*, indicating that a complex of these three cell shape determinants is not enough for cell morphogenesis of *B. subtilis*.

INTRODUCTION

For a long time, the presence of cytoskeletal elements has been considered a characteristic eukaryotic trait. However, in the last decade it has emerged that prokaryotes also have proteins with cytoskeletal function. One large group of cytoskeletal elements are the actin homologues, represented mainly by the MreB proteins in bacteria. Homologues of MreB have been found in most species with complex, i.e. non-spherical, cell morphology (Bork *et al.*, 1992; Carballido-López, 2006b; van den Ent *et al.*, 2001). Gram-negative organisms tend to have one *mreB* gene, usually in an operon with *mreC* and *mreD* genes, whilst Gram-positive bacteria often have multiple *mreB* isologues, but only one of these normally occurs in an operon with *mreCD*.

Bacillus subtilis encodes three actin-like proteins called MreB, MreBH and Mbl. The *mreB* gene is defined by its position in an operon with *mreC* and *mreD*, whilst the other two genes are organized in monocistronic units. The single mutants of any of the three *mreB* genes are viable if cultured in medium supplemented with high concentrations of Mg^{2+} : for *mreB* and *mbl* mutants, a concentration of approximately 3 mM is required, but the *mreBH* mutant

only needs 100 μ M Mg^{2+} to grow (Carballido-López *et al.*, 2006; Formstone & Errington, 2005; Schirner & Errington, 2009). All three single mutants have a defect in cell morphology. *mreB* mutant cells have an increased diameter and are straight, whereas *mbl* mutants are also wider but are characteristically twisted. *mreBH* mutant cells are straight but thinner than the wild-type. MreB, Mbl and MreBH co-localize, forming one helical structure along the long axis of the cell underneath the cytoplasmic membrane (Carballido-López *et al.*, 2006; Jones *et al.*, 2001). It has been suggested that they have a role in cell shape determination by organizing the peptidoglycan synthetic machinery at the cell surface (Carballido-López, 2006). This is consistent with observations that peptidoglycan synthesis occurs in a similar helical pattern (Daniel & Errington, 2003; Hayhurst *et al.*, 2008; Tiyanont *et al.*, 2006a; Kawai *et al.*, 2009a). MreC and MreD have a role in cell elongation, and interaction studies suggest that they are in the same morphogenic pathway as MreB (Figge *et al.*, 2004; Jones *et al.*, 2001; Kruse *et al.*, 2005; Leaver & Errington, 2005; Lee & Stewart, 2003; Rogers *et al.*, 1970; Wachi *et al.*, 1989). Viability of *mreC* and *mreD* deletion strains is restricted to conditions in which the growth medium is supplemented with high concentrations of Mg^{2+} , similar to *mreB* mutants, but additionally these strains require an osmoprotectant, such as sucrose, to prevent cell lysis; the mutant cells completely lose their rod shape and grow as

Abbreviations: NA, nutrient agar; PAB, Difco Antibiotic Medium 3; YFP, yellow fluorescent protein.

clusters of spheres (Leaver & Errington, 2005). A similar phenotype has been observed for a mutant deleted for the whole *mreBCD* operon (M. Leaver, unpublished data).

The current model suggests that the MreB helix is responsible for organizing the peptidoglycan synthetic apparatus, at least in part by interacting with membrane proteins, including MreC, MreD, RodA and one or more penicillin-binding proteins. MreBH has additionally been shown to be involved in the export of LytE, a cell wall hydrolase. By coordinating synthetic and lytic enzymes in one complex, the cell maintains the delicate balance between synthesis and hydrolysis of cell wall material (Carballido-Lopez, 2006a, b; Stewart, 2005).

Here we show that the three MreB-like proteins in *B. subtilis* have overlapping functions. Expression of one heterologous MreB protein from the closely related organism *Bacillus licheniformis* or the phylogenetically more distant organism *Clostridium perfringens* is enough for the formation of a long cell axis in a *B. subtilis* strain lacking all three endogenous MreB proteins. Interestingly, the presence of MreB^{*C. perfringens*} (MreB^{CP}) has a dominant-negative effect on *B. subtilis* wild-type cells, and leads to cell morphology defects and loss of viability by interfering with the localization of the endogenous MreB proteins.

METHODS

General methods. All strains were grown on nutrient agar (NA; Oxoid) plates or as liquid cultures whilst shaking in Difco Antibiotic Medium 3 (PAB) at 37 °C. Growth media were supplemented with 20 mM Mg²⁺ (added in the form of MgSO₄) and/or xylose at the concentrations indicated. Growth curves were obtained by measuring optical density at 600 nm at the time points indicated.

DNA manipulations and *Escherichia coli* DH5 α transformations were carried out using standard methods (Sambrook *et al.*, 1989). *B. subtilis* strains were transformed according to the method of Anagnostopoulos & Spizizen (1961) as modified by Jenkinson (1983). Selection for *B. subtilis* transformants was carried out on NA plates, supplemented with the following antibiotics, as required (μ g ml⁻¹): kanamycin (5), chloramphenicol (5), erythromycin (1), lincomycin (25) and/or spectinomycin (50).

Strain construction. All strains and plasmids used are listed in Table 1. To construct these strains, the *mreB* or *mreBCD* genes, respectively, were amplified from chromosomal DNA of *B. licheniformis* or *C. perfringens* with the oligonucleotides listed in Table 2. The PCR products were ligated into plasmid pJPR1, placing the gene(s) under control of the xylose inducible promoter between two segments of the *amyE* gene. For construction of strain 4630, primers amy1 and amy2 were used to amplify the region to be inserted in the chromosome, and *B. subtilis* 168 was transformed directly with the PCR product. Plasmids pSG5934, pSG5935 and pSG5936 were cloned using *E. coli* DH5 α . Transformation of *B. subtilis* with these plasmids or the linear PCR product and selection for chloramphenicol resistance resulted in strains carrying the heterologous gene(s) under control of the xylose inducible promoter inserted into the chromosome at the *amyE* locus. If required, deletions of endogenous genes were introduced afterwards.

Microscopic imaging. Cell morphology was assessed using light microscopy. Cells were grown on NA plates or in PAB with

supplements as indicated at 37 °C. Samples of exponentially grown cells were taken after diluting back once in the presence of inducer, and then mounted on microscope slides covered with a thin film of 1 % agarose in minimal medium (Glaser *et al.*, 1997). For fluorescence microscopy, overnight cultures were diluted into CH medium (Nicholson & Setlow, 1990) and grown for 2 h at 37 °C in the presence of 0.5 mM IPTG to induce expression of the fluorescent proteins. Expression of the heterologous MreB was induced by addition of 0.5 % xylose, and the strains were grown for 1 h before taking samples for microscopy. Images were acquired with a Sony CoolSnap HQ cooled CCD camera (Roper Scientific) camera attached to a Zeiss Axiovert M135 microscope or to a Zeiss Axiovert M200 microscope. ImageJ (<http://rsb.info.nih.gov/ij/>) was used to analyse the images. Manipulation was limited to altering brightness and contrast to obtain optimal prints.

RESULTS

Construction of strains expressing heterologous MreB proteins

Under typical bacterial culture conditions, the *mreB* gene is essential to *B. subtilis*, but viability can be restored by addition of high concentrations of Mg²⁺ to the growth medium. When Mg²⁺ is removed, the cells start to swell and lyse (Formstone & Errington, 2005). To investigate if expression of *mreB* from other bacteria could complement this defect, strains were constructed carrying the *mreB* genes amplified by PCR from *B. licheniformis* (*mreB^{Bl}*) or *C. perfringens* (*mreB^{CP}*), then placed under the control of a xylose-inducible promoter at the *amyE* locus. Additionally, constructs were made in which the whole *mreBCD* operon of *B. licheniformis* or *C. perfringens* was placed under the control of P_{xy1} at the *amyE* locus.

The heterologous *mreB* genes were identified by BLAST searches (Altschul *et al.*, 1990), and neighbourhood to paralogues of *mreCD* was used as a criterion to differentiate between *mreB* and endogenous orthologues. As in *B. subtilis*, the genome of *B. licheniformis* encodes three actin homologues (designated *mreB*, *mbl* and *mreBH*); in contrast, only two *mreB*-like genes were found in *C. perfringens* (designated *mreB* and *mbl*).

MreB^{Bl} is 92 % identical to MreB of *B. subtilis* (MreB^{Bs}), whilst MreB of the less closely related organism *C. perfringens* is 63 % identical to the *B. subtilis* protein. While MreC and MreD from *B. licheniformis* are 71 and 63 % identical, respectively, to the *B. subtilis* proteins, their homologues in *C. perfringens* share only 21 and 16 % identity, respectively. Table 3 summarizes the similarities of the MreB-like proteins of *B. licheniformis* and *C. perfringens* compared to their respective homologue in *B. subtilis* as determined by CLUSTAL W version 2.0 analysis (Larkin *et al.*, 2007).

Dominant-negative effect of *mreB^{CP}* expression

No effect was observed when expression of MreB^{Bl} was induced with 0.5 % xylose in a *B. subtilis* 168 wild-type

Table 1. Strains and plasmids

Strain/plasmid	Relevant genotype	Source/reference*
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
3728	<i>trpC2</i> Ω neo3427 Δ mreB	Formstone & Errington (2005)
2505	<i>trpC2</i> Ω (<i>mbl</i> :: <i>spc</i>)	Jones <i>et al.</i> (2001)
2537	<i>trpC2</i> <i>mreBH'</i> :: <i>lacZ lacI bla erm P_{spac}</i>	Carballido-López <i>et al.</i> (2006)
4334	<i>trpC2</i> Δ mreBCD::neo	M. Leaver, unpublished
4630	<i>trpC2</i> <i>amyE</i> :: <i>P_{xyI}-mreB^{Bl} cat</i>	This work
4631	<i>trpC2</i> <i>amyE</i> :: <i>P_{xyI}-mreB^{Cp} cat</i>	This work
4632	<i>trpC2</i> Ω neo3427 Δ mreB <i>amyE</i> :: <i>P_{xyI}-mreB^{Bl} cat</i>	This work
4633	<i>trpC2</i> Ω neo3427 Δ mreB <i>amyE</i> :: <i>P_{xyI}-mreB^{Cp} cat</i>	This work
4634	<i>trpC2</i> Ω (<i>mbl</i> :: <i>spc</i>) <i>amyE</i> :: <i>P_{xyI}-mreB^{Bl} cat</i>	This work
4635	<i>trpC2</i> Ω (<i>mbl</i> :: <i>spc</i>) <i>amyE</i> :: <i>P_{xyI}-mreB^{Cp} cat</i>	This work
4636	<i>trpC2</i> Ω neo3427 Δ mreB Ω (<i>mbl</i> :: <i>spc</i>) <i>mreBH'</i> ::pMUTIN4 (<i>lacZ lacI bla erm P_{spac}</i>) <i>amyE</i> :: <i>P_{xyI}-mreB^{Bl} cat</i>	This work
4637	<i>trpC2</i> Ω neo3427 Δ mreB Ω (<i>mbl</i> :: <i>spc</i>) <i>mreBH'</i> ::pMUTIN4 (<i>lacZ lacI bla erm P_{spac}</i>) <i>amyE</i> :: <i>P_{xyI}-mreB^{Cp} cat</i>	This work
4638	<i>trpC2</i> Δ mreBCD::neo <i>amyE</i> :: <i>P_{xyI}-mreBCD^{Bl}</i>	This work
4639	<i>trpC2</i> Δ mreBCD::neo <i>amyE</i> :: <i>P_{xyI}-mreBCD^{Cp}</i>	This work
4641	<i>trpC2</i> <i>amyE</i> :: <i>P_{xyI}-mreB^{Bl} cat aprE</i> :: <i>P_{spac}-yfp-mbl spc</i>	This work
4642	<i>trpC2</i> <i>amyE</i> :: <i>P_{xyI}-cat mreB^{Bl} aprE</i> :: <i>P_{spac}-yfp-mreB spc</i>	This work
4643	<i>trpC2</i> <i>amyE</i> :: <i>P_{xyI}-mreB^{Cp} cat aprE</i> :: <i>P_{spac}-yfp-mbl spc</i>	This work
4644	<i>trpC2</i> <i>amyE</i> :: <i>P_{xyI}-mreB^{Cp} cat aprE</i> :: <i>P_{spac}-yfp-mreB spc</i>	This work
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (<i>r_K⁻ m_K⁺</i>) <i>gal⁻</i> <i>phoA supE44 λ^- thi1⁻ gyrA96 relA1</i>	Invitrogen
Plasmid		
pJPR1	<i>bla amyE' cat P_{xyI} 'amyE</i>	J. Rowlings, unpublished
pSG5934	<i>bla cat P_{xyI}-mreB^{Cp}</i>	This work
pSG5935	<i>bla cat P_{xyI}-mreBCD^{Bl}</i>	This work
pSG5936	<i>bla cat P_{xyI}-mreBCD^{Cp}</i>	This work

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background strain (4630). Growth on NA plates or in liquid PAB was not affected in comparison to the behaviour of an uninduced strain (Fig. 1a). Also, cell morphology was not altered by expression of *mreB^{Bl}* (Fig. 1b). This result is consistent with observations made for overexpression of *gfp-mreB^{Bs}*, *gfp-mbl^{Bs}* or *gfp-mreBH^{Bs}* under the same conditions (Carballido-López *et al.*, 2006; Formstone & Errington, 2005; Jones *et al.*, 2001). Similarly, expression of *mreBCD^{Bl}* did not have obvious effects on viability or cell morphology (data not shown).

In contrast, induction of *mreB^{Cp}* expression had a deleterious effect on *B. subtilis*. Hardly any growth was observed on NA plates containing 0.5% xylose (strain 4631; Fig. 1a), and in liquid medium the growth rate decreased rapidly upon addition of inducer (data not shown). Microscopic examination revealed that induction of *mreB^{Cp}* expression led to severe morphological defects: twisted cells, formation of chains and lysis (Fig. 1b, c). This effect was correlated with the amount of xylose added, and we therefore concluded that it was due to *mreB^{Cp}*

expression levels (Fig. 1c). The cell morphology was reminiscent of an *mbl* mutant of *B. subtilis* (Abhayawardhane & Stewart, 1995; Jones *et al.*, 2001; Schirner & Errington, 2009), and similar to that strain, addition of Mg²⁺ restored viability (Fig. 1a) and ameliorated the cell shape defects (Fig. 1b). Comparable observations were made when the three genes *mreBCD^{Cp}* were expressed in a wild-type background (data not shown).

MreB^{Cp} disrupts the endogenous cytoskeleton

To test whether expression of *mreB^{Cp}* interfered with assembly of the MreB cytoskeleton of *B. subtilis*, we analysed the localization of YFP-Mbl and YFP-MreB in strains expressing heterologous *mreB* genes. In the absence of heterologous *mreB* genes, both YFP-Mbl and YFP-MreB localized along the periphery of the cells in a pattern that is thought to be based on an essentially helical structure [Fig. 2a(i), b(i), c(i), d(i)]. Expression of *mreB^{Bl}* did not change

Table 2. Oligonucleotides used in this study

Name	Restriction site	Sequence (5'–3')	Purpose
lichB-fwEco	EcoRI	ATCGTAGAATTCAATTTATGGGTTTTTGCTTAGG	Amplification of <i>mreB^{Bl}</i>
lichB-revBam	BamHI	TCACATGGATCCTGCGGCATGATTATACAC	Amplification of <i>mreB^B</i>
lichD-revBam	BamHI	GAGTTGGGATCCCTCTTTTCGACATCGGC	Amplification of <i>mreBCD^{Bl}</i>
ClospB-fw	Sall	ATCAGAGTCGACGAGACCCAAACACCTAGTAGAG	Amplification of <i>mreB^{Cp}</i>
ClospB-rev	BamHI	TAGTGAGGATCCGGCAACTGACAGTACTATAACAG	Amplification of <i>mreB^{Cp}</i>
ClospD-rev	BamHI	ACACTCGGATCCTTGAACCTTCTCATGTAACCACC	Amplification of <i>mreBCD^{Cp}</i>
amy1		CCGTCTAGACTAGTTAAACGGCGAACAATCGAAT	Amplification of insert from pJPR1
amy2		CCCTCTAGACTAGTTAAGCACTCCCgcgATCGCC	Amplification of insert from pJPR1

this localization pattern [Fig. 2a(ii), b(ii)]. In contrast, when *mreB^{Cp}* was expressed, the localization of both YFP-Mbl [Fig. 2c(ii)] and YFP-MreB [Fig. 2d(ii)] was severely disrupted. The pattern of tilted bands was completely disrupted and replaced with small numbers of bright foci that appear cytosolic rather than peripheral. This disruption of the endogenous MreB cytoskeleton provides a clear explanation for the dominant-negative phenotype on expression of *mreB^{Cp}*.

mreB^{Bl} but not *mreB^{Cp}* can compensate for the loss of *B. subtilis* *mreB*

To test if the heterologous MreB proteins could substitute for *B. subtilis* MreB, the *mreB* gene was deleted in strains harbouring the heterologous *mreB* genes (4632, 4633). When expression of *mreB^{Bl}* was induced, viability of the otherwise inviable *B. subtilis* Δ *mreB* strain was restored both on plates and in liquid culture without elevated Mg^{2+} concentrations (strain 4632; Fig. 3a, c). The formation of small colonies of strain 4632 on NA plates without supplements was probably due to incomplete repression of the xylose-inducible promoter. Although expression of *mreB^{Bl}* could partially restore growth, cell morphology was still impaired. The cells often appeared bent, but lysis was not apparent (Fig. 3b).

Table 3. Relative conservation of the MreB, Mbl and MreBH proteins

Source	Protein	Percentage identity relative to the <i>B. subtilis</i> protein		
		MreB	Mbl	MreBH
<i>B. subtilis</i>	MreB	100	53	56
	Mbl	53	100	51
	MreBH	56	51	100
<i>B. licheniformis</i>	MreB	92	54	56
	Mbl	53	97	51
	MreBH	56	51	90
<i>C. perfringens</i>	MreB	63	54	52
	Mbl	52	54	49

In contrast, expression of *mreB^{Cp}* did not restore viability of *B. subtilis* Δ *mreB* cells. No growth was observed on NA plates, nor in PAB medium containing 0.5 % xylose. The phenotype was ameliorated in medium containing both Mg^{2+} and xylose (strain 4633; Fig. 3a, d); here, twisting of the cells showed that the heterologous MreB protein was expressed, but cell lysis was not prevented (strain 4633; Fig. 3b).

mreBCD^{Bl} restores viability and rod shape of *B. subtilis* Δ *mreBCD*

B. subtilis cells deleted for the whole *mreBCD* operon are only viable when grown in the presence of high Mg^{2+} concentrations and the osmoprotectant sucrose. Such cells have a spherical morphology, similar to *mreC* or *mreD* single mutants (Leaver & Errington, 2005; M. Leaver, unpublished). We introduced the *mreBCD* operon from *B. licheniformis* into this strain background giving rise to strain 4638 and observed that expression of *mreBCD^{Bl}* was able to restore viability, in that growth occurred on NA plates containing xylose without further additives (Fig. 4a), and that the cells displayed a rod-shaped morphology. This was true with both solid and liquid media (Fig. 4b, c). Cell shape was slightly irregular, with occasionally bent cells and some variation in cell width. Addition of Mg^{2+} had little effect on cell viability or morphology (Fig. 4). In the absence of inducer, the strain grew as clumps of spherical cells, the expected morphology of an *mreBCD* deletion mutant (M. Leaver, unpublished).

In the same way, a strain carrying an inducible copy of the *mreBCD^{Cp}* operon was constructed (strain 4639). Just as for strain 4638, the cells had a spherical morphology in the absence of inducer and presence of Mg^{2+} (Fig. 4b, c). Interestingly, expression of *mreBCD^{Cp}* partially complemented a deletion of *mreBCD* in *B. subtilis*: in the presence of both Mg^{2+} and 0.5 % xylose, the strain was viable and grew better than without induction of *mreBCD^{Cp}* expression (Fig. 4a). In this strain, a high Mg^{2+} concentration was essential for viability: addition of xylose alone did not lead to colony formation. Unexpectedly, microscopic analysis showed that in contrast to *mreBCD^{Bl}*, expression of *mreBCD^{Cp}* did not restore the rod shape of *B. subtilis* either on plates or in liquid medium (Fig. 4b, c).

Fig. 1. Expression of *mreB^{CP}* has a dominant-negative effect on *B. subtilis*. (a) Growth of wild-type (wt), a strain with MreB^{Bl} (strain 4630) and a strain with MreB^{CP} (strain 4631) under control of a xylose inducible promoter on NA plates with additives as indicated. (b) Microscopic image of strains 4630 and 4631 grown in PAB with the supplements as indicated. (c) Phase-contrast microscopic images of strain 4631 grown in PAB with supplements as indicated. Bars, 5 μ m.

It should be noted that expression of either heterologous *mreBCD* operon at least partially supported sporulation, as spores were readily found after overnight culture on NA plates (Fig. 4b, arrows).

mreB^{Bl} can compensate for the loss of *mbl* in *B. subtilis*

Previous results suggested a partial redundancy in the function of actin homologues in *B. subtilis* (Defeu Soufo & Graumann, 2006; Schirner & Errington, 2009; Kawai *et al.*, 2009b). To test if *mreB^{Bl}* was able to compensate for the loss of *mbl*, we introduced the *mbl* deletion (Jones *et al.*, 2001) into a strain with *mreB^{Bl}* under the control of P_{xyb} creating strain 4634. Interestingly, expression of *mreB^{Bl}*

restored the viability of the *mbl* mutant on NA plates without supplemented Mg²⁺ (Fig. 5a). Also, cell shape was restored to an almost wild-type morphology, though cells were often slightly bent (Fig. 5b). In contrast, expression of *mreB^{CP}* did not enable growth of the *mbl* mutant strain (strain 4635) on NA plates with inducer. Indeed, expression of this heterologous gene had a detrimental effect on *B. subtilis* Δ *mbl* even in the presence of high Mg²⁺ concentrations, leading to the formation of smaller colonies than on plates containing Mg²⁺ only (strain 4635; Fig. 5a). In addition, cell morphology was not rescued by expression of *mreB^{CP}*: the cells still twisted, swelled and lysed (Fig. 5b), similar to the unsupplemented *mbl* mutant or to the effects of *mreB^{CP}* expression in a wild-type background (Fig. 1b, c).

Fig. 2. Expression of *mreB^{Cp}* disrupts the helical structures of Mbl^{Bl} and MreB^{Bl}. (a, b) Localization of YFP-Mbl (a) and YFP-MreB (b) in the absence [a(i), b(i)] and the presence [a(ii), b(ii)] of MreB^{Bl}. (c, d) Localization of YFP-Mbl (c) and YFP-MreB (d) in the absence of MreB^{Cp} [c(i), d(i)] and when MreB^{Cp} is expressed [c(ii), d(ii)]. Bar, 5 μ m.

The presence of one actin homologue is sufficient for determination of rod shape

Since *mreB^{Bl}* appeared to partially compensate for loss of either *mreB* or *mbl*, it was interesting to test whether it could restore growth to cells deleted for all three endogenous *mreB* isolagues. As shown in Fig. 6, it was possible to construct such a strain (4636). The cells were slow-growing and strictly dependent on supplementation of the growth medium with Mg^{2+} (Fig. 6a, c). In the presence of 0.5 % xylose, the cells were rod-shaped when growing on plates (Fig. 6b), but with a wide cell diameter and not uniformly shaped. In rich liquid medium such as PAB, however, the cells did not maintain their rod shape and slowly converted to a spherical morphology (Fig. 6c). Depletion of MreB^{Bl} under all conditions tested resulted in a more or less spherical morphology: cells tended to form clumps and lysis was frequent (Fig. 6b, c). The presence of small translucent colonies on NA plates with high Mg^{2+} concentrations might be due to the incomplete repression of the xylose-inducible promoter. Although *mreB^{Cp}* did not complement the *B. subtilis* *mreB* mutant, and even had a deleterious effect when expressed in a wild-type background, it was able to confer rod-like morphology to cells with a deletion of all three endogenous *mreB* homologues with the *P_{xyI}-mreB^{Cp}* construct (strain 4637). Although we cannot rule out additional suppressor mutations, strain 4637 was viable and the phenotype was similar to that of strain 4636, though colonies on NA plates appeared more transparent compared to those of the strain

expressing *mreB^{Bl}* (Fig. 6a). The cells were irregularly shaped, tended to form chains and were often twisted, but became clearly rod-shaped when *mreB^{Cp}* was expressed. In the absence of xylose, the cells became spherical and often lysed (Fig. 6b, c). Elevated Mg^{2+} concentrations were essential for viability (Fig. 6a, c). Nevertheless, these results showed that *B. subtilis* viability and cell elongation could be substantially rescued by expression of a single *mreB* gene only 63 % identical to that of the wild-type.

DISCUSSION

MreB^{Cp} interferes with the *B. subtilis* actin cytoskeleton

Surprisingly, the overexpression of *mreB^{Cp}* had a dominant-negative effect on viability of wild-type *B. subtilis*, whilst overexpression of *mreB^{Bl}* had no effect on cell shape or viability. The morphology of cells expressing *mreB^{Cp}* was highly reminiscent of that of *mbl* mutants: in both cases, the cells twist, swell and lyse, and they are not viable unless the medium is supplemented with high concentrations of Mg^{2+} (Abhayawardhane & Stewart, 1995; Jones *et al.*, 2001; Schirner & Errington, 2009). The delocalization of Mbl and MreB in a strain expressing *mreB^{Cp}* suggests that this heterologous MreB interferes with the polymerization of *B. subtilis* actin homologues, which have been shown to form a single helical structure and therefore probably interact with each other or a common target protein

Fig. 3. *mreB^{BI}* restores the viability of a *B. subtilis* *mreB* mutant. (a) Growth of wild-type, *mreB* mutant, an *mreB* mutant strain carrying *mreB^{BI}* (strain 4632) or *mreB^{CP}* (strain 4633) under the control of *P_{xyI}* on NA plates with supplements as indicated. (b) Phase-contrast microscopy images showing morphology of strains 4632 and 4633 when grown in PAB medium with supplements as indicated at 37 °C. Bars, 5 μ m. (c, d) Growth curves of strain 4632 (c) and strain 4633 (d) in PAB (♦), PAB supplemented with 20 mM Mg^{2+} (■), 0.5% xylose (✱) or both 20 mM Mg^{2+} and 0.5% xylose (▲) at 37 °C.

(Carballido-López *et al.*, 2006). Disruption of the helical structure leads to cell shape defects and ultimately loss of viability, possibly due to an imbalance of processes involved in cell wall synthesis and turnover which are regulated in subtly differentiated ways by the various isolates.

The MreBCD complex alone is not sufficient for determination of rod shape

MreC and MreD are important for rod-shape determination in *B. subtilis* and *E. coli*. They probably form a complex with MreB, and together they are involved in orchestrating lateral cell wall synthesis (Defeu Soufo &

Graumann, 2005; Kruse *et al.*, 2005; Leaver & Errington, 2005; Wachi *et al.*, 1989). The results presented here show that the MreBCD homologues of *B. licheniformis* are able to restore rod shape and viability in *B. subtilis* $\Delta mreBCD$, indicating that the similarity of MreC and MreD from *B. licheniformis* to the *B. subtilis* proteins (71 and 63 %, respectively) is high enough to substitute for the endogenous proteins. However, expression of *mreBCD^{CP}* failed to complement the *mreBCD* mutant, even though the presence of MreB^{CP} as the only MreB protein is enough for determination of rod shape. It is possible that the MreBCD^{CP} complex fails to interact with, or even inhibits, other proteins involved in synthesis of the cylindrical part of the cell wall, such as RodA, RodZ or penicillin-binding

Fig. 4. Viability of *B. subtilis* $\Delta mreBCD$ expressing *mreBCD^{BI}*. (a) Growth of *B. subtilis* $\Delta mreBCD$ carrying either *mreBCD^{BI}* (strain 4638) or *mreBCD^{CP}* (4639) on NA plates containing supplements as indicated. (b, c) Phase-contrast microscopy images showing morphology of strains 4638 and 4639 when grown on NA plates (b) or in liquid PAB medium (c) containing Mg²⁺ and xylose as indicated. Bars, 5 μ m.

Fig. 5. Expression of *mreB^{BI}* confers viability to *B. subtilis* Δmbl . (a) *B. subtilis* wild-type, *mbl* mutant, and *mbl* mutant strains carrying an inducible copy of *mreB^{BI}* (4634) or *mreB^{CP}* (4635) grown on NA plates with supplements as indicated. (b) Phase-contrast microscopic images showing cell morphology of strains 4634 and 4635 grown in PAB medium at 37 °C with additives as indicated. Bars, 5 μ m.

Fig. 6. Viability of a *B. subtilis* strain expressing only a single heterologous MreB protein. (a) Growth of *B. subtilis* $\Delta mreB \Delta mbl \Delta mreBH$ with an inducible copy of *mreB^{BI}* (4636) or *mreB^{CP}* (4637) on NA plates with additives as indicated. (b, c) Cell shape of strains 4636 and 4637 when grown on NA plates (b) or in PAB medium (c) at 37 °C under conditions as indicated. Bars, 5 μ m.

proteins (Alyahya *et al.*, 2009; Bendezu *et al.*, 2009; Rogers & Taylor, 1978; Shiomi *et al.*, 2008; Wei *et al.*, 2003), resulting in the formation of spherical cells. The membrane proteins MreC and MreD probably have a function in connecting the intracellular actin scaffold to the extracellular cell wall synthetic machinery (Leaver & Errington, 2005; van den Ent *et al.*, 2006). Since these proteins are only 21 and 16 % identical, respectively, to the *B. subtilis* homologues, MreC^{CP} and MreD^{CP} are probably

too divergent to fulfil their functions in *B. subtilis*. Because MreBCD^{CP} should be able to form a functional complex in *B. subtilis*, this result suggests that the formation of a functional MreBCD complex is not enough for determination of rod shape, but interactions with other morphogenic proteins are required. However, we cannot exclude the possibility that one or both of MreC^{CP} or MreD^{CP} do not accumulate sufficiently for reasons of gene expression levels, activity or stability in *B. subtilis*.

Overlapping functions of actin homologues

Although *B. subtilis* strains with single deletions of *mreB* homologues can be constructed and are viable in the presence of high Mg^{2+} concentrations, the only viable double mutant has deletions of *mbl* and *mreBH* (Defeu Soufo & Graumann, 2006; Kawai *et al.*, 2009b). Interestingly, overexpression of *mreB^{Bl}* not only restored viability of a *B. subtilis* *mreB* mutant, it also rescued an *mbl* deletion strain, supporting the idea of a functional overlap between *mreB* and *mbl*. Subsequently, strains carrying deletions of all three endogenous *mreB* homologues were constructed in the presence of *mreB^{Bl}* or *mreB^{CP}* under the control of *P_{xyt}*. Although these cells were by no means wild-type in phenotype, they clearly retained the ability to elongate and became distinctly rod-like. Thus, a single MreB homologue, only 63 % identical to the endogenous MreB, is sufficient for determination of longitudinal growth in *B. subtilis*. Therefore, it seems likely that there is significant functional redundancy between MreB iso-

logues. Given the apparent functional complementation of an *mreB* isologue triple mutant, it was surprising that *mreB^{CP}* had a deleterious effect when expressed in a wild-type background or in *mreB* or *mbl* single mutant strains (Figs 1, 3 and 5, respectively). Based on the results shown in Fig. 2, we suggest that this dominant-negative phenotype is due to interference in assembly of the endogenous MreB isologues into filaments, leading to disturbed cell growth. The interference effect can apparently be manifested through either MreB or Mbl. The partial complementation in a triple *mreB* null background suggests that the MreB^{CP} protein can nevertheless interact productively with the wall synthetic machinery. We hope to use *mreB^{CP}* as a tool to help identify and dissect the sites on MreB isologues involved in self-interaction, leading to filament assembly, versus those involved in interactions with the effector proteins of cell wall synthesis.

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