Control of Cell Shape in Bacteria: Helical, Actin-like Filaments in *Bacillus subtilis*

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Summary

In the absence of an overt cytoskeleton, the external cell wall of bacteria has traditionally been assumed to be the primary determinant of cell shape. In the Grampositive bacterium *Bacillus subtilis*, two related genes, *mreB* and *mbl*, were shown to be required for different aspects of cell morphogenesis. Subcellular localization of the MreB and Mbl proteins revealed that each forms a distinct kind of filamentous helical structure lying close to the cell surface. The distribution of the proteins in different species of bacteria, and the similarity of their sequence to eukaryotic actins, suggest that the MreB-like proteins have a cytoskeletal, actinlike role in bacterial cell morphogenesis.

Introduction

Cell shape in eukaryotes is determined primarily by the action of various filamentous structures, particularly actin filaments. These are formed by polymerization of the 42 kDa actin monomer into long linear or branched structures. In vivo, actin polymerization is a highly regulated process controlled both by ATP binding and hydrolysis, and by the action of a number of actin binding proteins that initiate, cleave, cross-link, stabilize, or destabilize the filaments (Korn et al., 1987; Schmidt and Hall, 1998).

Bacteria exhibit a wide diversity of shapes, and shape has commonly been used as an important taxonomic criterion. In the well studied rod-shaped bacteria, Escherichia coli and Bacillus subtilis, a number of mutants that have a spherical morphology have been isolated and characterized. Most of the mutations affect processes associated with cell envelope synthesis, either peptidoglycan (e.g., pbpA and rodA; Spratt, 1975; Tamaki et al., 1980; Henriques et al., 1998) or teichoic acid synthesis (rodC/tagF in B. subtilis; Honeyman and Stewart, 1989). This is consistent with the perceived importance of the cell envelope in the determination of cell shape in bacteria. Moreover, the isolated peptidoglycan sacculus retains some elements of the shape associated with the cell from which it is obtained (Höltje, 1998). On the other hand, another group of genes, designated mreB, mreC, and mreD, are not clearly associated with cell envelope synthesis, and how they participate

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or intervene in cell shape determination is unknown (Wachi et al., 1987; Doi et al., 1988; Levin et al., 1992; Varley and Stewart, 1992). *B. subtilis* has two additional *mreB*-like genes, and disruption of one of these, *mbl*, also has an effect on cell shape (Abhayawardhane and Stewart, 1995). At the primary sequence level, MreB and its relatives show weak similarity to the actin superfamily, and to another bacterial morphogenic protein, FtsA, which is required for cell division. However, the superfamily also contains a number of proteins of noncytoskeletal function, such as hexokinases and the chaperone Hsp70/DnaK (Bork et al., 1992).

Here, we show that MreB and Mbl have distinct, complementary roles in cell shape determination in *B. subtilis* and that they form helical filamentous structures that are probably homologous to actin filaments of eukaryotic cells.

Results and Discussion

MreB and Mbl Have Contrasting and Complementary Roles in Cell Shape Determination

Previous work on mreB of B. subtilis indicated that the gene was probably essential but the phenotypic consequences of mreB disruption were not described (Varley and Stewart, 1992). In preliminary experiments, we also were unable to obtain stable disruptants of B. subtilis mreB, by insertion either of an antibiotic resistance cassette or an integrating plasmid. We therefore attempted to construct a merodiploid strain with a repressible copy of mreB located at an ectopic locus (amyE). For reasons that we do not understand, expression of mreB only at the amyE locus did not complement a disruption of mreB at its natural position, but we were able to generate an inducible strain when the whole mreBCD cluster of genes was placed at amyE under the control of the repressible P_{xy} promoter. We were then able to disrupt the natural copy of mreB with either a kanamycin resistance cassette or an insertion of plasmid pMUTIN4, both of which provide read-through trancription to maintain the expression of downstream genes (Daniel et al., 1996; Vagner et al., 1998). The resultant strain (2060) was viable in the presence of the inducer, xylose, but failed to grow when plated in the absence of xylose. Control experiments showed that these effects were unlikely to be due to a polar effect on *mreC* or *mreD*, because each of these genes can be disrupted individually by insertion of an integrating plasmid (to give strains 2066 and 2059, respectively; see Experimental Procedures) with only mild phenotypic consequences (data not shown).

To examine the phenotypic effects of depletion of MreB protein, a culture of strain 2060 (grown in the presence of inducer) was divided into two portions, from one of which inducer was withheld. Depletion was slow to take effect, but after 5 culture doublings in the absence of inducer, the unsupplemented culture showed a marked reduction in growth rate and eventually the culture optical density fell as the cells lysed. This confirmed that the *mreB* gene is essential in *B. subtilis* (in



Figure 1. Contrasting Effects of Mutations in the *mreB* and *mbl* Genes on Cell Shape, as Determined by Phase Contrast Microscopy (A) Wild-type *B. subtilis* grown in S medium, showing the typical rod shape.

(B–D) Effect of MreB depletion on culture growth rate and cell morphology. Strain 2060, bearing a xylose-inducible copy of *mreB*, was grown in S medium containing inducer (0.25% xylose), then washed twice in inducer-free medium and resuspended in fresh S medium with (B) or without (C and D) inducer. After about 5 (B and C) or 6 (D) culture doubling times, samples were examined by phase contrast microscopy. (E and F) Loss of control over the linear axis of growth in *mbl* mutant cells. Strain 2505 with a disruption of the *mbl* gene was grown to mid-exponential phase in S medium (E) and CH medium (F) (doubling times: 73 and 40 min respectively; Sharpe et al., 1998).

(G and H) Immunostaining of the cell division protein FtsZ in wild-type cells (G) and in an mbl mutant (H) (strain 2505) grown in CH medium.

contrast to *mreB* of *E. coli*, which is not essential; Doi et al., 1988). Microscopic examination of cells at this time revealed that the arrest in growth was associated with a change in cell morphology from the normal rod shape (Figures 1A and 1B) to an inflated, rounded morphology, typical of the general class of Rod mutants (Figure 1C). In later samples, many lysed cells were evident (Figure 1D; arrows), confirming that the shape defect is ultimately lethal. In contrast, cells grown in the presence of inducer had a normal rod-shaped morphoplogy (Figure 1B), indistiguishable from that of wild-type *B. subtilis* cells (Figure 1A). We conclude that disruption of the *mreB* gene of *B. subtilis*, as in *E. coli*, results in loss of cell width control, though in *B. subtilis* the effect is ultimately lethal.

We then examined cells with a disruption of the related mbl gene. As previously described, this gene is nonessential, though the mutant cells grow slowly and have a highly abnormal cell shape (Abhayawardhane and Stewart, 1995). As shown in Figure 1 (E and F), cells of the mbl mutant were bent and twisted at irregular angles. The morphological distortion was more obvious with rapidly growing cells (Figure 1F), under which conditions, B. subtilis cells remain associated in chains (Holmes et al., 1980). A proportion of the mbl mutant cells were also affected in cell width, with some bulges, again particularly at the higher growth rate (arrows in Figure 1F). However, the main effect with this mutant seemed to lie in control of the longitudinal axis of cell growth. Consistent with this notion, we discovered that FtsZ (a tubulin-like protein that directs septation by formation of a cytokinetic ring in prokaryotes; Lutkenhaus and Addinall, 1997) forms spiral and irregular circular structures in the distorted mbl mutant cells (Figure 1H) instead of the normal perpendicular rings (seen typically as bands in the wild-type cells illustrated in Figure 1G). We suggest that propagation of an FtsZ structure around the regular cylinder of the wild-type cell envelope allows formation of a closed, circular FtsZ ring, but when the side walls are irregular, the curving Z structure sometimes fails to close upon itself, instead forming a spiral or helix. Note also that circular Z rings were also frequently seen (lower panels of Figure 1H), where a closed Z ring had formed but did not lie in the normal perpendicular plane. An alternative view of the significance of the distorted FtsZ rings would be that they are responsible for the shape effects and that the MbI phenotype arises because this protein is involved in orientating the FtsZ rings. However, we think this less likely because cells with a distorted shape and an essentially normal FtsZ ring were not infrequent.

In contrast to *mbl* mutants, distorted cell divisions were not observed with the *mreB*-depleted cells (data not shown). Thus, it appears that *mreB* and *mbl* are both involved in cell shape control in *B. subtilis*, but their two proteins appear to be functionally specialized for different aspects of shape determination.

MreB Localizes in the Form of Helical Filaments that Encircle the Cell

To investigate how MreB participates in cell morphogenesis, we wanted to determine its subcellular localization. GFP fusions to either the N or C terminus of the protein were nonfunctional and nonlocalized (results not shown). We therefore raised antiserum against overexpressed, affinity-tagged protein so as to determine its location



Figure 2. Subcellular Localization of MreB Protein in *B. subtilis*

(A) Western blot showing specificities of the anti-MreB antiserum (lanes 1–3) and the anti-c-Myc monoclonal antibody (lanes 4–6). MW markers are shown to the left. Lanes 1 and 4 contain extracts of wild-type cells (strain 168). The other lanes contain extracts of strains with a xylose-inducible copy of the *c-myc-mreB* gene (strain 2060) grown in the presence (lanes 2 and 5) or absence (lanes 3 and 6) of 0.25% xylose.

(B–D) Localization of c-Myc-MreB detected with a monoclonal antibody against c-Myc. Strain 2060 was grown in S medium at 42°C. (B) Field of cells (unprocessed images) showing typical localization of MreB. (C) Optical sections through cell marked "a" in (B), at three different levels in the z axis after deconvolution. (D) 3D reconstruction of the same cell shown from above and during rotation through to 45°.

(E) Cells prepared as for (B), but stained with polyclonal anti-MreB antiserum.

(F and G) Wild-type (168) cell stained with polyclonal anti-MreB antiserum shown as an unprocessed image (F) and at three sections at different levels in the z axis after deconvolution (G).

(H) Correlation between the number of bands of MreB and cell cycle progression. The cells were divided into three age classes on the basis of their nucleoid morphology (DAPI stain): "new" cells had a single monolobed

nucleoid; "mid" cells had a single bilobed nucleoid; and "old" cells had two separate nucleoids. Black shading shows the frequency of cells (% of total cells measured) with a single MreB band; gray shading represents 2 bands and white represents >2 bands. Cells of strain 2060 were prepared and stained as for (E).

by immunofluorescence microscopy. We also made an epitope-tagged derivative of MreB, with an N-terminal c-Myc tag. Unlike GFP, addition of this short tag had no discernible effect on MreB function. The specificity of both antisera was verified as shown in Figure 2A. The polyclonal serum reacted strongly with a single protein of expected mobility in extracts of wild-type B. subtilis cells (Figure 2A, lane 1) and in extracts of strain 2060 grown in the presence of inducer (lane 2). The antiserum was specific for MreB, and did not cross-react with MbI protein, for example (which is related in sequence and has a similar MW), because no band was detected in cells from which the MreB protein was depleted (lane The monoclonal anti-c-Myc antibody reacted strongly with a single band of similar mobility to MreB in cells expressing the tagged protein (lane 5), but no significant signal was obtained with uninduced cells (lane 6) or the wild type (lane 4). Control immunofluorescence experiments also confirmed the antibody specificities (not shown).

Cells of several different genotypes were examined by immunofluorescence microscopy, using both antisera, under a range of different growth conditions. The clearest and most reproducible staining patterns were obtained when c-Myc-MreB was expressed from the P_{xyl} promoter, the cells were incubated at higher temperatures, and stained with the monoclonal antibody (Figure 2B). Typically, a range of fluorescent structures were seen, the most prominent being transverse bands, often with adjacent partial bands and dots. The bands were reminiscent of those seen with various cell division proteins, which assemble into a ring structure at the site of incipient division (Lutkenhaus and Addinall, 1997; see above), except that they were usually slanted rather than being perpendicular to the long axis of the cell, and they were generally less precisely demarcated. By focusing up and down through the cell, it appeared that at least some of the structures were helical. Such structures are intrinsically difficult to reproduce as flat images. In the case of cell division rings, for example, the ring is perpendicular to the plane of focus, so a well demarcated band appears, with all of the fluorescence, both in focus and from above and below the plane of focus, contributing to the band. With a helical structure, however, fluorescence from above and below the plane of focus would be displaced laterally from the main in-focus signal, giving a much less distinct image. To try to clarify the 3D form of these structures, we collected stacks of optical sections and used a deconvolution process to reduce out-of-focus light. Figure 2C shows a set of optical sections corresponding to planes at three different levels in the cell marked "a" in Figure 2B. This cell is one of a pair of newborn sister cells that have not yet separated from each other. This set of images and the rotation of the 3D reconstruction of this cell (Figure 2D) clearly showed that the transverse band corresponded to part of a helix.

Inspection of a range of cells analyzed in this way (or



Figure 3. Subcellular Localization of Mbl

(A) Western blot showing specificity of the anti-Mbl antiserum. MW markers are shown to the left. Lane 1 contains extract of wild-type cells (strain 168), and lane 2 from a null mutant for the *mbl* gene (strain 2505).

(B–E) Wild-type strain 168 was grown in S medium and processed for immunofluorescence detection of MbI. (B) shows unprocessed images of 3 typical cells of different lengths. The same cells are shown after deconvolution of an image stack, as a "max projection" (C), and as three separate optical sections taken at low, medium, and high positions in the z axis of the cell (D). (E) shows a stereo pair for cell "a". (F) Localization of an MbI-GFP fusion protein in cells of strain 2521 growing in CH medium (unprocessed image).

by direct examination through the microscope) established that most of the fluorescent structures comprised arcs or helices, the latter frequently extending to about 1 to 1.25 turns around the periphery of the cell. These reconstructions also confirmed that the helices were right handed. The helical pitch was estimated to be about 0.73 \pm 0.12 μm (based on measurements of 85 clearly defined structures). The most prominent bands tended to be located some distance in from the poles of the cell. However, it was not possible to measure this distance precisely, because the observed end point of the helix was dependent on the position of the focal plane in the cell. Measurements of the number of bands per cell (Figure 2H) revealed that they tended to increase during cell-cycle progression, suggesting that the structures undergo some kind of replicative process during cell growth. However, much more work is needed to define the precise pathway through which new structures are generated. The dimensions of the structures observed and superimposition of immunofluorescence and phase contrast images (not shown) showed that the helical structures are located close to the cell surface. Since MreB is likely to be a cytosolic protein, the helices presumably lie just under the surface of the cytoplasmic membrane.

Similar fluorescent structures were also seen with the polyclonal antiserum, detecting either the c-Myc-MreB fusion protein (Figure 2E) or in wild-type cells (Figures 2F and 2G), though the results were less clear, and

under the staining conditions used, fewer cells stained uniformly. Nevertheless, these images established that the results described above are not an artifact arising from ectopic expression of MreB or use of the c-Myc tag. Moreover, we have not seen structures like these with antibodies against a wide range of other *B. subtilis* proteins, suggesting that they are not a fixation artifact. We conclude that MreB protein monomers polymerize into, or associate with, helical filamentous structures that encircle the cell cytoplasm just under the membrane.

To determine whether there was sufficient MreB protein in the cell to account for the filamentous structures it appeared to form, we determined the cellular content of MreB protein by quantitative immunoblotting. For cells growing vegetatively in CH medium, we found that there were approximately 8000 molecules of MreB per cell (data not shown). This is certainly sufficient to form a linear polymer that could encircle the cell several times and is slightly higher than the amount estimated for the FtsZ protein (Feucht et al., 2001) that forms a cytokinetic ring involved in cell division (Lutkenhaus and Addinall, 1997).

Mbl Protein Forms Helical Filamentous Structures of Longer Pitch, which Run the Length of the Cell

Since Mbl protein is related in sequence to MreB and also implicated in control of cell shape, albeit a different



aspect of shape determination, we wondered whether it too would form filamentous structures visible by fluorescence microscopy. Again, we used affinity tagged purifed Mbl protein to raise a polyclonal antiserum. This antiserum was also specific, as demonstrated in Figure 3A. A single band of expected mobility was present in wild-type cells (lane 1) and absent from extracts of mbl mutant cells (lane 2). No cross reaction with MreB was detected. When wild-type cells were examined by immunofluorescence microscopy using this antiserum, a complex pattern of bands and dots was seen, again suggesting the existence of filamentous helical structures running around the periphery of the cell. These experiments were more straightforward because wildtype cells readily gave uniform staining patterns with the polyclonal antiserum. Unprocessed images of several typical cells of different lengths are shown in Figure 3B. The patterns were again clearer when a stack of images were deconvolved (Figure 3C). The detailed threedimensional arrangements of the structures seen were resolved by viewing individual optical slices from different depths (Figure 3D) and by viewing 3D reconstructions at different rotational angles (not shown). These, and stereo pairs (example shown in Figure 3E) established that the Mbl structures were again helical and right handed, but they differed in several ways from those of the MreB structures. First the helical pitch was more than twice that of MreB (1.7 \pm 0.28 μ m; n = 66). Second, the pattern often took on a "figure of 8" appearance. The simplest interpretation of this would be that each cell contains a double helix, with the two helices running perfectly out of phase with respect to each other; this arrangement is quite clear in the deconvolved images shown in Figure 3D. Finally, the patterns were rather simpler to interpret, with each helical strand running apparently the length of the cell, and usually making more than one complete turn around the cell periphery. Generally, in the smallest cells (e.g., "a" in Figure 3B), the filaments appeared to follow one full helical turn,

Figure 4. Effects of Missense Mutations in *mreB* on Cell Shape

Strains 168 (wild type, [A]), 2080 (*mreB42*, [B]), 2079 (*mreB18*, [C]) were grown in PAB medium at 37°C to midexponential phase. Samples of the cells were fixed with ethanol and viewed by phase contrast microscopy. (D) Images from >100 cells of each strain were analyzed for cell length and width using MetaMorph v4.1. Symbols: wild type, closed circles; *mreB42*, gray triangles; and *mreB18*, open circles.

(E) Western blot analysis of MreB protein in extracts of cells of the wild-type (lane 1), *mreB42* (lane 2), and *mreB18* (lane 3) strains.

whereas in longer cells (e.g., cells "b" and "c"), one and a half or two full turns were evident. Whatever the cell length, the filaments appeared to extend right into both cell poles. A simple interpretation of this would be that the structures grow continuously in parallel with cell elongation.

To confirm that the pattern of fluorescence seen by immunofluorescence was correct, we constructed a GFP fusion to the C terminus of Mbl. Unlike MreB, this fusion gave a distinct pattern of fluorescence distinctly similar to that of the immunofluorescent images. As shown in Figure 3F, helical filamentous structures were clearly visible in live cells containing this fusion as the only source of Mbl protein. Unlike MreB, this fusion was almost completely functional, with the cells showing only slight perturbations in shape. The similarity of these images to those of the immunfluorescence experiments lent strong support to the notion that the MreB and Mbl proteins form or associate with filamentous helical structures in living cells. Since we have now seen two distinct patterns of filamentous structures, it is most likely that the 3D structure of these filaments is a function of the MreB and Mbl proteins themselves rather than association with different kinds of as yet undetected filamentous structures in the cells. Consistent with this, quantitative immunoblotting revealed that Mbl is present in about 12,000 to 14,000 molecules per cell, compared to 8000 molecules per cell of MreB (data not shown).

Isolation of Nonlethal Point Mutations in *mreB* that Alter the Dimensions of the Cells

Though the above experiments strongly suggested that MreB and Mbl have a direct role in cell shape determination, it was also possible that they have a more passive role, for example in the maintenance of cell shape. If the role was a direct one, it should be possible to isolate alleles of *mreB* or *mbl* with an altered cell shape. To isolate a range of missense alleles of *mreB*, we designed a mutagenesis strategy that took advantage of the lethal

	Phosphate 1
actshum	~~~~MCDEDETTALVCDNGSGLVKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIITNWDDMEKIWHH
actyeast	~~~~MDSEVAALVIDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGIMVGMCQKDSYVGDEAQSKRGILTLRYEIEHGIVTNWDDMEKIWHH
mrebbs	~~~~~MFGIGARDLGIDLGTANTLVFVKGKGI.VVREPSVVALQT.DTKSIVAVGNDAKNMIGRTPGNVVALR.PMKDGVIADYETTATMMKY
stcoel	~MGNSMSFIG.RDMAVDLGTANTLVYVRGRGI.VLNEPSVVAINT.NTGGILAVGAEAKKMIGRTPGNIVAVR.PLKDGVIADFEITERMLRY
mblbs	~~~~~~MFARDIGIDLGTANVLIHVKGKGI.VLNEPSVVALDKNSGKVLAVGEEARRMVGRTPGNIVAIR.ELKDGVIADFEVTEAMLKH
mrebec	~MLKKFRGMFSNDLSIDLGTANTLIYVKGQGI.VLNEPSVVAIRQDR.AGSPKSVAAVGHDAKQMLGRTPGNIAAIR.PMKDGVIADFFVTEKMLQH
chl	~~~~RVFNFFSGNVGIDLGTANTLVYVRGRGI.VLSEPSVVAVDAQTHAVLAVGHKAKAMLGKTPRKIVAVR.PMKDGVIADFEIAEGMLKA
heli	MIFSKLIGLFSHDIAIDLGTANTIVLVKGQGI.IINEPSIVAVRMGLFDSKAYDILAVGSEAKEMLGKTPNSIRAIR.EMKDGVIADYDITAKMIRY
trep	~~~MFFLRRFSADVGIDLGTCNTIIYVEGRGI.VVNEPSVVAVERGTKSVVAVGSDAKRMLWKTPGN.IVAIR.BLKDGVIADMDTTEKMIRY
	Connect 1 Phosphate 2
actshum	TFYNELRVAPEEHPTLLTEAPLNPKANREKMTQIMFETFNVPAMYVAIQAVLSLYASGRTTGIVLDSGDGVTHNVPIYEGYALPHAIMRLDL
actyeast	TFYNELRVAPEEHPVLLTEAPMNPKSNREKMTQIMFETFNVPAFYVSIQAVLSLYSSGRTTGIVLDSGDGVTHVVPIYAGFSLPHAILRIDL
mrebbs	YINQAIKNKGMFTRKEYYMVCVESGITAVEERAVIDATRQAGARDAYPIEEPFAAAIGANLPVWEPTGSMVVDIGGGTTEVAIISLGGIVTSQSIRV
stcoel	FILKIHKRRYLARPRVVVCVPSGITGVERRAVIEASSQAGARQVHIIEEPMAAAIGSGLPVHEATGNMVVDIGGGTTEVAVISLGGIVTAQSIRV
mblbs	FINKLNVKG., LFSKERMLICCETNITSVEQKAIKEAAEKSGCKHVYLEEEPKVAAIGAGMEIFQPSGNMVVDIGGTTDIAVISMGDIVTSSSIKM
mrebec	FIKQVHSNS.FMRPSPRVLVCVPVGATQVERRAIRESAQGAGAREVFLIEEPMAAAIGAGLPVSEATGSMVVDIGGGTTEVAVISLNGVVYSSSVRI
chl	LIKRVTPARSMFREKILIAVESGITGVEKRAVEDSALHAGAQEVILIEEPMAAAIGVDLPVHEPAASMIIDIGGUTTEIAIISLGGIVESRSLRI
heli	FIEKAHKRKTWIRDRIMVCVDYGLTSVERNAVKESALSAGAREVFLIEEPMAAAIGAGLPVKEPQGSLIVDIGGGTTEIGVISLGGLVISKSIRV
trep	FISKILPRHRLIKERMVIGIESCITDVECRAVHESASKAGAGEVEVLEESLAAAIGANIPIEEPAGNMVCDIGGETTEVSVESLGMVVTNAIRV
actshum	AGROLTDYLKKILTERGYSFVTTAEREIVRDIKEKLCYVALDFENEMATAASSSSLEKSYELPDGOVITTONERFRCPETLFOPSFIGMESAGHETTY
actyeast	A GROLTDYLMKILSERGYSFSTTAEREIVRDIKEKLCYVALDFEGEMQTAAQSSSIEKSYELPDGQVITIENEKRAPEALFHPSVLGLESAGDQTTY
mrebbs	AG. DEMDDAILNYIRKTYNLMIGDR
stcoel	AG. DEFDMEITOHVKKEYSLLLGER
mblbs	AG, DKFDEAILNYIKKEYKLLIGER
mrebec	GG. DRI DECTI NY VRENYGSLIGEA
Chi	AG. DELDQSTINYMRKTYNLMIGPR
nerr	AG. DER DUATVELIKKENTULLIGER
crep	G. DEP WATKING VINLING
actehum	
actvoast	NSTMECTORNEL VOLTANT WSCOMMENDED A DEMONSTRATA A DESMEVICITA DE DEVISION AL ADDRATICO TA ADDRATI
mrehbs	STIERTOPIALATINDERIVIT CONTINUEVISET KMPVILAEDDIDCUATORCALEHTHIEKKCKTE*
stcoel	TTLDKCPPELSCDTMDRGTVLTGGATLRGLDERLRRET GMPTHTAEDPLDSVALGSGKCVEEFEALOOVLDASPRR*
mblbs	OVLERPPEELSADTIDEGVITTGGGALINGLOOLLAEEL KVPVLVAENPMOCVATGTGVMLDNMDKLPKRLS*
mrebec	VALEOCPPELASDISERGMVLTGGGALLENLDRLLMEETGIPVVVAEDPLTCVARGGGKALEMIDHHGGDLFSEE*
chl	LTLEKCPPELSADLVERGNVLASGGALIKGLDKALSKNTGLSVITAPHPLLAVCLGTGKALEHLDOLKK*
heli	SVLEEVKPDLVKDIVONGVVLT CGALIKGLDKYLSDMVKLPVYVGDEPLLAVAKGTGEAIODLDLLSRVGFSE*
trep	RTLARTPPELAADIVERGIVMTSGGSLLKGLPKLISKETHVPVILAENPMNCVAISGAGRYHEVYKDISGDRSLYAGLNS*

Figure 5. Sequence Alignment of MreB Proteins from Various Bacteria with Eukaryotic Actins

Sequences were as follows: human ActS (actshum), *Schizosaccharomyces pombe* Act1 (actyeast), *B. subtilis* MreB (mrebbs), and Mbl (mblbs), and MreB-like proteins from *Streptomyces coelicor* (stcoel), *E. coli* (mrebcc), *Chlymadia trachomaist* (chl), *Helicobacter pylori* (heli), and *Treponema pallidum* (trep). The black shading highlights areas of complete sequence identity, and the light gray shading areas of sequence similarity in all 8 proteins. The dark gray boxes surround the conserved regions previously described by Bork et al. (1992) and are labeled according to the functions assigned to them on the basis of the crystal structure of actin. The sequences were aligned by fixing the boxes defined by Bork and using Clustal W to align the intervening regions. The sequence data for the proteins was obtained from the SwissProt database. Amino acids are represented by the following single-letter abbreviations: A, ala; C, cys; D, asp; E, glu; F, phe; G, gly; H, his; I, ile; K, lys; L, leu; M, met; N, asn; P, pro; Q, gln; R, arg; S, ser; T, thr; V, val; W, trp; and Y, tyr. The black dots lie under the two residues altered in the mreB18 and mreB42 mutants.

phenotype of null mutants. Strain 2063 has its mreB gene inactivated with a kanamycin-resistance cassette, its viability being maintained by a xylose-inducible $mreB^+$ gene at the *amyE* locus. We mutagenized a plasmid-borne copy of mreB (incapable of replication in B. subtilis) and transformed strain 2063, plating in the absence of xylose. The end points of the insert in the plasmid were designed such that integration of the plasmid by any single crossover mechanism would also be lethal. Approximately 50% of the transformants arose by a double-crossover mechanism in which the kanamycinresistance cassette in mreB was replaced with the mutagenized mreB DNA. Individual transformants were screened microscopically for morphological abnormalities. A range of mutants were obtained, including several with severely abnormal shape and reduced growth rates, not dissimilar from the null mutants. However, among the collection were several that grew with normal growth rates and had stable alterations in shape. Two examples are shown in Figure 4 (B and C). Mutant 2079 (mreB18) had an increased cell width that was accompanied by a substantial decrease in cell length (Figures 4C and 4D). The cells also tended to bulge at the poles, becoming rather kidney shaped. The mreB gene of this mutant was sequenced and found to contain a single base substitution (ttg-tcg) that would produce the substitution L296S in MreB protein (see Figure 5). The residue affected is a highly conserved hydrophobic residue in one of the sequence motifs thought to be important for the folding of actin-like proteins (see Bork et al., 1992). Mutant 2080 (mreB42) had a less obvious phenotype but its average cell width was again increased and rather variable but with no significant decrease in cell length (Figures 4B and 4D). This mutant again had a single base substitution in mreB (atg-acg), giving rise to an M104R substitution affecting a different region of MreB, and again a fairly conserved residue. By contrast, in wild-type cells, as reported previously (Sharpe et al.,

Table 1. Distribution of <i>mreB</i> -like Genes in the Bacterial Subkingdoms			
Organism/group	Shape		
mreB present			
Bacillus subtilis (3 genes); B. cereus, Escherichia coli, Rickettsia prowazekii, Pseudomonas fluorescens, Haemophilus influenzae, Aquifex aeolicus, Thermotoga maritima (2 genes), Methanobac- terium thermoautotrophicum*	Rod		
Vibrio cholera, Caulobacter crescentus, Pasteurella multocida	Curved rod		
Streptomyces coelicolor (3 genes)	Filamentous		
Campylobacter jejuni, Helicobacter pylori (3 isolates), Wolinella succinogenes, Treponema pallidum, Borrelia burgdorferi	Helical		
Chlamydia (2 species)	Pleiomorphic		
mreB absent	-		
Streptococcus pneumoniae, Enterococcus faecalis, Staphylococcus aureus, Deinococcus radiodurans, Methanococcus jannaschii*, Neisseria (2 species), Archaeoglobus fulgidus*, Pyrococcus horikoshii*, Synechocystis sp	Round		
Mycoplasma (2 species), Mycobacterium tuberculosus	Pleiomorphic		

BLAST2 searches were run on EMBnet-CH or The Institute for Genomic Research databases using the predicted amino acid sequence of *B. subtilis* MreB protein. Scores (E value) of $<1 \times 10^{-49}$ were considered to represent MreB homologs. This family formed a homogeneous group distinct from the DnaK-like proteins (also members of the actin superfamily; Bork et al., 1992) that had E values of $>1 \times 10^{-16}$. Archaebacterial organisms have asterisks.

1998), the cell width is maintained within a very tight range (Figures 4A and 4D). On the basis of Western blot analysis (Figure 4E), neither of the new mutations had a detectable effect on the accumulation of MreB protein.

The isolation of *mreB* missense mutants with stable alterations in the dimensions of the cell provides strong support for the notion that this protein has a direct role in cell shape determination.

Widespread Presence of MreB-like Proteins in Bacteria with Complex Shapes

Given the apparent role for MreB and MbI in cell shape determination, it was interesting to investigate the distribution of MreB-like proteins around the bacterial subkingdom and its possible correlation with cell shape. Sequence database searches revealed the existence of clear homologs of mreB/mbl in the genomes of a wide range of eubacteria and at least one archaebacterium (Table 1). All of these organisms turned out to be related in having a nonspherical, and therefore probably an actively detemined cell shape. Many of the organisms are rod shaped, similar to B. subtilis and E. coli. Organisms with more complex shapes, including curved, filamentous, and helical bacteria, were all abundantly represented. Missing from the collection, however, were bacteria with a coccoid (spherical) morphology, both Gram-positive and Gram-negative. To confirm the absence of mreB-like genes from these bacteria, we searched the complete or near complete genome sequences of a number of organisms. None of the sequences examined (Table 1) showed a significant match to MreB. We therefore suggest that a sphere is the default shape taken by cells in the absence of an active, mreB-dependent shape determining system, and that more complex shapes are usually determined in part by mreB systems.

Conservation of Key Structural Residues between MreB-like Proteins and Actins

How might MreB and Mbl mediate an effect on cell shape? As pointed out previously (Bork et al., 1992),

many of the highly conserved residues in the MreB-like proteins are also conserved in the actin superfamily. However, several other proteins that resemble actin have functions that appear to have little to do with the cytoskeleton, particularly hexokinases, heat shock chaperones (Hsp70/DnaK), and the bacterial cell division protein FtsA. This superfamily of proteins share conserved amino acid residues that tend to cluster around the ATP binding pocket of actin, corresponding to positions that are critical for the overall folded structure of actin (Ayscough and Drubin, 1996). MreB pro-



Figure 6. Model for Shape Control by MreB and Mbl

The shape of the wild-type cell (center) is maintained by the combined action of the MreB (black) and Mbl (gray) helical filaments. The two systems are mainly responsible for width control and linear axis control, respectively, as indicated above. In the absence of one of the genes (below), the remaining structure exerts partial control over shape giving a characteristic aberrant morphology. The nucleoid (oval in the middle of the cell) is shown only to provide perspective. teins, however, are closer than any of the other proteins to actins in overall size. As shown in Figure 5, alignment of MreB proteins and actins requires relatively few gaps, and a large number of residues in each of the four major subdomains of actin are conserved in 7 diverse MreB proteins. Most of the gaps lie in the MreB alignment, accounting for this protein's slightly smaller size. Two small gaps (5 and 1 residue, only) needed to be inserted in the actin sequence. The 8 gaps in the MreB sequence range from 1 to 13 residues. Examination of the structure of actin (complexed with DNAase I; Kabsch et al., 1990) revealed that most of the residues missing in the MreB sequences lie in surface exposed loops or turns. It therefore seems likely that these proteins will have very similar folds. In contrast, the other members of the superfamily all tend to have large insertions relative to actin, consistent with their distinct functions. Thus, the sugar kinases all have large (>50 aa) insertions in their N-terminal regions, either the extreme N terminus or between the "phosphate 1" and "connect 1" domains (Figure 5; see Bork et al., 1992). The Hsp70/DnaK proteins have a major additional domain of >250 aa near their C terminus. FtsA is more closely related in overall structure to actin/MreB, but it too has a major insertion or replacement (about 50 aa) relative to domain Ib of actin (between the "phosphate 1" and "connect 1" sequences), which perhaps contributes to its specialized role in cell division. These observations are consistent with the notion that actins and MreB proteins have a particularly close functional relationship within the superfamily.

Clearly structural and biochemical studies would be required to confirm the equivalence of the functions of MreB and actin, but this seems a distinct possibility on the basis of the phenotype of the *mreB* and *mbl* mutants, and filamentous nature of the structures they are associated with in the cell. By analogy with actin, we propose that the filamentous structures we have observed are formed by assembly of MreB or Mbl monomers into linear polymers, and that ATP binding and hydrolysis is involved in regulation of filament dynamics.

An Actin-like Cytoskeletal Function for MreB and Mbl

Figure 6 summarizes the distinct morphologies of mreB and mbl mutants, an interpretation of the configurations of MreB and Mbl cytoskeletal structures, and our view of how they could contribute to the determination of cell shape in *B. subtilis*. We suggest that the different architectures of the filamentous structures the two proteins form is a reflection of their specialized roles in different aspects of cell shape control. On the basis of the *mreB* mutant phenotype, it appears that MreB is mainly concerned with cell width control. In vivo, the protein forms structures of short pitch mainly located at about mid-cell. By running almost perpendicular to the long axis of the cell, these structures could influence cell diameter, helping to fix the width at the midpoint of the rod. In contrast, MbI seems to be more important in maintaining the linearity of the longitudinal axis of the cell. Correspondingly, its helical structures have a much longer pitch, running virtually the length of the cell. Such structures would seem to be intrinsically less able to exert an effect on cell diameter because their path lies

so far from the perpendicular. However, they could be suited to fixing the relative orientation of the two poles of the cell, which, as pointed out for fission yeast (Mata and Nurse, 1998), must remain parallel if the cell is to grow in a straight line.

It is not yet clear whether the MreB and Mbl structures act directly to control cell shape in the sense of acting as a structural brace. More likely, by analogy with the postulated role of actin in yeast (Pruyne and Bretscher, 2000), they operate by exerting spatial control over the cell-wall-synthesizing machinery. At the moment, it is not clear how such control might be exerted. Whatever the mechanism, on the basis of our new data, bacterial cells appear to contain structures that have at least one of the functions of the eukaryotic actin cytoskeleton. The proteins themselves show weak similarity to actin, particularly in key structural residues. The level of sequence conservation would not be dissimilar from the relationship between tubulin and its likely prokaryotic homolog FtsZ (Mukherjee and Lutkenhaus, 1994). We suggest that our observations are best explained by supposing that MreB and actin are homologous, having evolved in a common ancestor before the divergence of prokaryotes and eukaryotes. If so, elucidation of the precise roles of MreB and Mbl in cell shape determination may have important and widespread implications for the still largely unresolved questions of cell shape determination in yeasts and higher organisms.

Experimental Procedures

General Methods

Methods for growth of *B. subtilis*, transformation, selection of transformants, etc., have been described extensively elsewhere (Marston and Errington, 1999).

Disruption of *mreB* and Complementation by an Engineered Inducible Allele

The B. subtilis mreBCD genes were amplified from strain 168 by PCR. A BamH1 site was introduced between the RBS and the start codon of mreB and an EcoRI site 6 bp downstream of the mreD stop codon. The BamHI- and EcoRI-digested fragment was then ligated to BamHI- and EcoRI-digested pSG1168 (bla amyE::spc Pxv/c-myc; P. J. Lewis, personal communication) to generate pSG1469. pSG1469 was transformed into strain 168 with selection for spectinomycin (50 $\mu\text{g/ml})$ to generate strain 2056. To disrupt the wildtype copy of mreB, the gene was amplified from 168, generating a BamHI-EcoRI fragment. This fragment was ligated to BamHI- and EcoRI-digested pSG1301 (Stevens et al., 1992) to generate pSG1446. A neo resistance cassette (Itaya et al., 1989) was excised with PstI and inserted into the unique PstI site in pSG1446 to generate pSG1447. Strain 2060 was generated by transforming 2056 with pSG1447 with selection for kanamycin (5 μ g/ml) in the presence of 1% xvlose. Strain 2060 was xvlose dependent and a c-Mvc fusion protein of the correct size could be immunodetected.

Isolation of Point Mutations in the mreB Gene

A C-terminal truncated copy of the *mreB* gene was amplified by PCR from strain 168. The BamHI- and HindIII-digested fragment was ligated to BamHI- and HindIII-digested pMUTIN4 (Vagner et al., 1998) to give plasmid pSG1485. Mutations were introduced into the plasmid by amplification in the *E. coli* strain XL1-Red (Novagene). Strain 2063 (*trpC2* Ω (*amyE*::P_{xyf}-c-*myc-mreBCD spec*) Ω (*mreB*::neo)) was transformed to xylose independence with the mutagenized plasmid library. The resulting transformants were streaked to single colonies and stored at -70° C. To avoid recombination between the mutant allele and the wild-type gene under *P*_{xyf} control at the *amyE* locus, each mutant was immediately transformed to chloramphenicol resistance with pJPR1 (*amyE*::P_{xyf} cat, J. Rawlins, personal communication). This resulted in replacement of the wild-type *mreB* gene with *cat*. The *mreB* gene and 0.5 kbp of upstream DNA were amplified from the mutants by PCR and sequenced directly using a departmental DNA sequencing service.

Disruption of mreC and mreD

For the disruption of the *mreC* gene, a 1184 bp DNA segment internal to the *mreB* and *mreC* genes was amplified from strain 168 by PCR using the oligonucleotides MREB1 and MREC2. The fragment was digested with BamHI (at a site in one of the primers) and at the unique HindIII in the *mreC* gene. The 242 bp fragment internal to the *mreC* gene was cloned into BamHI- and HindIII-digested pMUTIN4 (Vagner et al., 1998) to give pSG1464. Strain 168 was transformed to erythromycin resistance with plasmid pSG1464 to give an *mreC*-disrupted strain, 2066. For the *mreD* disruption, approximately the middle third of the *mreD* gene was amplified by PCR and cloned into BamHI- and HindIII-digested pMUTIN4, resulting in plasmid pSG1465. Strain 168 was transformed to erythromycin resistance with plasmide strain 2059.

Disruption of mbl

The full-length *mbl* gene was amplified by PCR with *Pfu* DNA polymerase and cloned into the Hindlll/BamHI-digested pSG1301 (Stevens et al., 1992) to give plasmid pSG4501. The *spc* cassette from plC156 (Steinmetz and Richter, 1994) was released by Smal digestion, gel purified, and inserted into the unique EcoRV site of pSG4501 to produce pSG4502. The *spc* cassette disrupts *mbl* after the 160th codon, and is flanked by two segments of *B. subtilis* DNA, each of approximately 500 bp. *B. subtilis* 168 was transformed with pSG4502, with selection for spectomycin resistance. Transformants arising by the desired double crossover mechanism were detected by the absence of the vector-encoded chloramphenicol resistance. Disruption of *mbl* was confirmed in the resulting strain (2505) by PCR analysis of chromosomal DNA.

Overproduction and Purification of Affinity-Tagged Derivates of MreB and Mbl

The MreB and Mbl-coding sequences were amplified by PCR from strain 168 chromosomal DNA, using flanking oligonucleotides that introduced the STREPII affinity tag (WSHPQFER) with an Ncol site at the N terminus, and a HindIII site at the C ternimus. The Ncoland HindIII-digested fragments were then cloned into the pET21d and pET3a vectors (Stratagene) respectively, and restricted with Ncol and HindIII, to generate plasmids pSG1478 and pSG4600.

To overproduce StrepII-MreB, plasmid pSG1478 was transformed into the E. coli expression host strain BL21 (pLysS). A 500 ml culture was inoculated from a fresh overnight plate and grown at 37°C in 2YT medium (Sambrook et al., 1989) containing ampicillin (100 $\mu\text{g}/$ ml). When the culture reached an $\mathsf{OD}_{\scriptscriptstyle 600}$ of 0.5, the cells were induced by the addition of 25 μ M isopropyI- β -D-thiogalactopyranoside (IPTG) and then grown for a further 3 hr. For StrepII-Mbl overproduction, pSG4600 was transformed BL21 (DH3) and the resultant strain was grown at 30°C to an OD₅₅₀ of 0.2–0.3, induced with 100 μ M IPTG and then grown overnight at 16°C. Cells were harvested, washed, resuspended in 5 ml of buffer (50mM Tris-HCI [pH 8.0], 10% sucrose, 1 mM EDTA, and 1 mM PMSF) and disrupted by sonication. The fusion proteins, in inclusion bodies, were recovered from the insoluble fraction and solubilized with Sarkosyl (Nguyen et al., 1993). The solubilized proteins were then purified on a StrepTactin column (Institut für Bioanalytik GmBH, Göttingen, Germany). For MreB, an aliquot of the solubilized inclusion bodies was also purified on the BioRad Model 4691 Prep Cell using an 8% SDS-PAGE. The affinitypurified and Prep Cell samples were pooled, concentrated by dialysis against PEG8000, and used by a commercial supplier (Cymbus Biotechnology, UK) to raise rabbit polyclonal antibodies by standard procedures.

Confirmation of Antibody Specificity by Western Blotting

Samples from xylose-induced and xylose-depleted cultures of strain 2060, and from cultures of the disrupted *mbl* strain 2505 and the wild-type strain 168, were taken and suspended to give an OD₆₀₀ of 1.0 in a final volume of 100 μ l. Cell pellets were resuspended in 1 × SDS-PAGE loading buffer and lysed by sonciation. Fifteen μ l were

loaded onto a 12% SDS gel and separated by electrophoresis before transferal to nitrocellulose membranes (Hybond-C extra, Amersham) by electroblotting. Western blots were carried out essentially as described by Wu and Errington (1994). Blots were dried for 10 min and blocked for 1 hr with PBS, 0.1% Tween (v/v), and 5% milk powder (w/v) at room temperature, with shaking. Antibodies were added in blocking solution and incubated shaking at room temperature for one hour at the following dilutions: anti-MreB, 1:10,000; anti-chabit-HRP conjugate, 3:10,000 and anti-mouse-HRP conjugate, 3:10,000. Blots were washed in PBS and 0.1% Tween (v/v). Detection was performed with an ECL kit (Amersham).

Growth and Microscopy on mreB Missense Mutants

Strains were grown to mid exponential stage from a fresh overnight plate at 37°C in PAB medium, then diluted to an OD₆₀₀ of 0.5 in warm PAB medium. When an OD₆₀₀ of between 0.7 and 0.8 was reached, 200 μ l cell samples were taken and fixed in 70% ethanol (Hauser and Errington, 1995). Cell pellets were resuspended in 50 μ l PBS, of which 5 μ l was examined microscopically on agarose-coated slides as described by Glaser et al. (1997).

Immunofluorescence Conditions

Cells were prepared for immunofluorescence microscopy as described previously (Glaser et al., 1997) with the following exceptions: for MreB immunolocalisation, all washes and blockings were performed in the 5% BSA, 0.01% Tween, and PBS; anti-FtsZ, anti-MreB, and anti-Mbl antisera were used at 1:5000, 1:7500, and 1:10,000 (vol/vol) dilutions, respectively, and the secondary FITC-anti-rabbit antibodies (Sigma) were used at dilutions of 1:1000, 3:1000 and 1:1000, respectively. For c-Myc detection, the anti-c-Myc antibody (Zymogen) was used at a 1:100 dilution and all washes and blockings were done as above, but in the presence of 5% milk powder.

Construction and Analysis of an mbl-gfp Fusion

A fusion of GFP to the C terminus of Mbl was constructed by cloning an Xhol-HindIII C-terminal coding region of the *mbl* gene (622bp) between these sites in plasmid pSG1151 (Lewis and Marston, 1999), to generate plasmid pSG4521. This plasmid was transformed into wild-type *B. subtilis* cells with selection for chloramphenicol resistance. After integration into the chromosome by Campbell-type mechanism, the only intact copy of *mbl* was the one fused to *gfp*. Cells of the resulting strain (2521) had minor alterations in cell size and shape.

To study the localization of MbI-GFP, strain 2521 was grown to exponential phase in hydrolyzed casein (CH) medium at 37°C. Samples for fluorescence microscopy were taken at an OD_{600} of 0.7–0.9 and immobilized on 1.2% agarose-coated microscope slides as described by Glaser et al. (1997).

Image Acquisition and Processing

Image acquisition, image stack acquisition, and deconvolution was performed essentially as described previously (Lewis et al., 2000). Image stacks were taken at 50 nm intervals. Stereo pair images were generated using MetaMorph v. 4.0 software from sequential view angles of 3D reconstructions, separated by 6° of rotation. "Max projections" of the deconvolved stacks of serial optical sections were generated (AutoDeBlur v. 5.1 software) by selecting the highest intensity values along the z axis and displaying them as an XY (2D, plane) projection.

Quantitation of MreB and Mbl Proteins

The total amounts of MreB and Mbl per cell were determined by quantitative immunoblot analysis. A 10 ml culture of cells grown to an OD₆₀₀ of 0.7 in CH medium were harvested and resuspended in a final volume of 600 µl in 1× SDS-PAGE loading buffer. The cells were lysed by sonication (2 times 30 s). Samples (2 to 25 µl) of cell extract were run in tandem with dilution series of StrepII-MreB or StrepII-Mbl (purified as described above) on 12% and 10% SDS-PAGE, respectively, and transferred to nitrocellulose membranes (Hybond C-extra) by electroblotting. The membranes were subjected to immunoblotting as described above, except that the secondary antibody was an alkaline phosphate-conjugate and the blots

were developed by exposure to ECF substrate (Amersham). Each blot was scanned using a Storm System (Molecular Dynamics). ImageQuant was used to calculate the quantity of protein in the cell extract relative to the purified protein. The cell number per OD unit was determined previously (Feucht et al., 2001).

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