Localization and Expression of MreB in *Vibrio parahaemolyticus* under Different Stresses

Shen-Wen Chiu, Shau-Yan Chen, and Hin-chung Wong*

*Department of Microbiology, Soochow University, Taipei, Taiwan 111, Republic of China*

Received 6 May 2008/Accepted 16 September 2008

MreB, the homolog of eukaryotic actin, may play a vital role when prokaryotes cope with stress by altering their spatial organization, including their morphology, subcellular architecture, and localization of macromolecules. This study investigates the behavior of MreB in *Vibrio parahaemolyticus* under various stresses. The behavior of MreB was probed using a yellow fluorescent protein-MreB conjugate in merodiploid strain SC9. Under normal growth conditions, MreB formed helical filaments in exponential-phase cells. The shape of starved or stationary-phase cells changed from rods to small spheroids. The cells differentiated into the viable but nonculturable (VBN) state with small spherical cells via a “swelling-waning” process. In all cases, drastic remodeling of the MreB cytoskeleton was observed. MreB helices typically were loosened and fragmented into short filaments, arcs, and spots in bacteria under these stresses. The disintegrated MreB exhibited a strong tendency to attach to the cytoplasmic membrane. The expression of *mreB* generally declined in bacteria in the stationary phase and under starvation but was upregulated during the initial periods of cold shock and VBN state differentiation and decreased afterwards. Our findings demonstrated the behavior of MreB in the morphological changes of *V. parahaemolyticus* under intrinsic or extrinsic stresses and may have important implications for studying the cellular stress response and aging.

**Materials and Methods**

**Bacterial strains and growth conditions.** *V. parahaemolyticus* strain 1137 (Kanagawa phenomenon positive, serotype O3:K6) was isolated in Taiwan from a clinical specimen (38). The strain 1137 that contained low-copys-number plasmid pSC10 (**P** 6.0, spc::mreB, lacP, MBL ori, MobRP4, Ap′, Cm′) was designated strain SC9 (6). The yellow fluorescent protein-conjugated protein (YFP-MreB) generated in strain SC9 was used to monitor the behavior of MreB (6). *V. parahaemolyticus* strains were grown in tryptic soy broth (Difco, Becton-Dickinson Diagnostic Systems, Sparks, MD) supplemented with 3% sodium chloride (TSB-3% NaCl) at 37°C, except where stated otherwise. Chloramphenicol was added at 5 μg/ml to the medium to culture SC9.

**Stress conditions.** *V. parahaemolyticus* was grown in TSB-3% NaCl at 37°C for 3 h (mid-exponential phase), 12 h (early stationary phase), or 36 h (prolonged stationary phase). Other stresses were imposed on the cultures in the mid-exponential phase. For carbon starvation, the cells were cultured in modified Morita mineral salt (MMS) medium supplemented with 0.4% glucose at 25°C, harvested by centrifugation at 12,000 *g* for 5 min, and washed twice with equal volumes of MMS medium without glucose (39). The cells were resuspended in an equal volume of MMS medium that was devoid of glucose and incubated at 25°C for different intervals. For cold shock, *V. parahaemolyticus* cells in the mid-exponential phase were cooled to 10°C and incubated for different intervals. The VBN state was induced by incubating at 4°C in MMS medium as previously described (39). Culture samples were taken for microscopic and mRNA analyses.
IPTG (isopropyl-β-D-thiogalactopyranoside) was used at a final concentration of 10 μM to induce the expression of YFP-MreB in *V. parahaemolyticus*. For pulse induction, the cells were grown in TSB-3% NaCl in the presence of IPTG for 1 to 1.5 h. IPTG was removed before the application of stresses. For constant induction, IPTG was reintroduced into the MMS-washed cultures.

**Microscopy and image analysis.** FM4-64 (Molecular Probes, Carlsbad, CA) was added to the sample at a final concentration of 1 μg/ml and incubated at 25°C in the dark for 10 min for membrane staining (27). To examine the integrity of the membrane, the cells were incubated with propidium iodide (Molecular Probes) at a final concentration of 30 μM at 25°C in the dark for 20 min. The cells were immobilized on agarose pads or poly-L-lysine-coated slides (Sigma Co., St. Louis, MO) before microscopic examination. Agarose pads were made by adding 1.5% of low-melt agarose to the TSB-3% NaCl, LB, or MMS medium. Antibiotics or IPTG were added to the culture medium when needed. Three microliters of the sample was placed on top of the agarose pads or coated slides.

The cells were examined with a Nikon Eclipse E800 fluorescence microscope equipped with a Nikon Plan Fluor 100 × 1.30 N.A. oil-immersion objective at room temperature (6). Images were obtained using an Evolution VF charge-coupled device camera (Media Cybernetics, Inc., Silver Spring, MD) and Image-Pro Express software (Media Cybernetics, Inc.), Five to seven images of optical sections of fluorescence images were obtained for a cell at spacings of about 140 nm.

**RNA preparation and RT-PCR analysis.** The expression of mreB was determined by reverse transcription (RT)-PCR (19). Approximately 10⁶ cells were taken from each sample to extract RNA using an RNApure kit (Genesis Biotech, Burlington, Harrington Court, Canada), as described by the manufacturer. For PCR analysis, two pairs of primers were used to amplify the cDNA of 16S rRNA

**RESULTS**

**Localization of YFP-MreB in stationary-phase *V. parahaemolyticus* cells.** To investigate MreB localization in living *V. parahaemolyticus* cells, a merodiploid strain (*V. parahaemolyticus* SC9) that expresses the YFP-MreB fusion protein was constructed (6). The expression of YFP-MreB, induced by IPTG at the level of 10 to 50 μM, resulted in the formation of rod-shaped cells, which were indistinguishable from the wild-type cells. The 10 μM concentration was used herein. YFP-MreB and native MreB assembled into the same cytoskeletal structure and formed hybrid filaments (6).

In exponential phase, the cells were under rapid growth, and newborn, elongated, and dividing cells were frequently observed (Fig. 1A, left). Fluorescence microscopy revealed that most cells in this phase contained MreB helical structures that extended along the long axis, and two newborn cells (Fig. 1Bi) and an elongated cell (Fig. 1Bii) were shown. Detail patterns of MreB structures in the exponential-phase *V. parahaemolyticus* were reported in another paper (6).

Cells of strain SC9 changed from bacillus to small-coccoid form when they entered the stationary phase (Fig. 1A, right). The helical structure of YFP-MreB in the cells in the exponential phase disappeared when the cells entered the early stationary phase (Fig. 1C). About 12% of the cells contained several short arcs or limp filaments (Fig. 1Ci and Cii). In around 88% of the cells, a single straight (Fig. 1Civ) or slightly curved filament (Fig. 1Ciii) was found. These observations revealed that the helical MreB cytoskeleton became loose and disassembled in the stationary phase. The localization of YFP-MreB in cells was examined after prolonged incubation in the stationary phase. The mean cell length in this prolonged stationary phase (1.02 ± 0.05 μm) was shorter than that in the early stationary phase (1.46 ± 0.67 μm). The MreB filament disappeared in 83% of the prolonged stationary-phase cells, and the YFP-MreB localized peripherally with some diffused fluorescence signals in the cytoplasm (Fig. 1Diii and Dii). In about 11% of the cells, YFP-MreB was in the form of a straight and thick filament that crossed the whole cell (Fig. 1Di). In around 6% of the cells, YFP-MreB also was in peripheral localization with multiple short cytoplasmic filaments (Fig. 1Dv). The viability of these cells was demonstrated by propidium iodide staining, and only viable cells exhibited the YFP signal. This observation reveals that viable cells lost the helical
geometry of the MreB cytoskeleton when they entered the stationary phase.

Localization of YFP-MreB in starved V. parahaemolyticus cells. Starvation altered the morphology of V. parahaemolyticus cells. The cells of V. parahaemolyticus strain SC9 grown in TSB medium exhibited cell lengths of 3.31 ± 0.33 μm in the exponential phase. The lengths of cells that underwent carbon starvation for 5 h decreased to 1.20 ± 0.08 μm, while the cell widths remained constant. The cell lengths further decreased to 0.68 ± 0.05 μm after 20 h of starvation. The expression of YFP-MreB was induced in two modes, constant induction or pulse induction. However, no apparent difference was observed between the localization patterns of YFP-MreB in these different modes. Most cells (78%) that were starved for 20 h exhibited a peripheral localization of YFP-MreB (Fig. 2A). Some cells (17%) contained loose MreB filaments that crossed the cytoplasm or were attached to the cytoplasmic membrane (Fig. 2B). However, the MreB filaments that crossed the cytoplasm may also have been located underneath the cell membrane but could not be distinguished by the microscopy adopted herein. YFP-MreB also diffused throughout the cytoplasm in 5% of the cells (Fig. 2C).

Localization of YFP-MreB in cold-shocked V. parahaemolyticus cells. V. parahaemolyticus cells ceased growing at temperatures below 10°C. Incubation in TSB-3% NaCl at 10°C for 48 h did not change the morphology of V. parahaemolyticus. However, the localization of YFP-MreB changed drastically in cells that were cold-shocked for 1 h. YFP-MreB was distributed randomly near the cell surface (Fig. 3Ai). Some of the cells exhibited peripheral spots (Fig. 3Aii to Aiv), indicating the presence of freely diffused YFP-MreB. After cold-shocking for 4 h, YFP-MreB dispersed peripherally in about 92% of the cells (Fig. 3B).

Localization of YFP-MreB in VBNC V. parahaemolyticus cells. Previous works have demonstrated that V. parahaemolyticus cells enter the VBNC state when they encounter a combination of starvation and low-temperature treatment and that the cells become small spheroids (39). A time course study with short intervals was conducted to follow changes in the morphology and localization of YFP-MreB during the early stage of VBNC state differentiation. An intriguing swelling-waning process was observed in this early stage (Fig. 4A). Briefly, about 62% of the cells swelled at 18 h and waned after 36 h (Fig. 4A). Swelling seemed to occur at different cell positions to produce rabbit head-like cells (Fig.4Aiii) or drumstick-like cells (Fig.4Ai). The proportion of spheroid cells changed from about 14% at time zero to 92% at 36 h (Fig.4Aii). Jiang and Chai (16) also reported that during the first week in the differentiation of the VBNC state, V. parahaemolyticus cells swell slightly and change from rod-shaped to irregularly shaped cells, finally becoming spherical. The sizes of the cells further declined, presumably via the waning process when the cells starved at a low temperature for at least a week. In the waning process, part of the cytoplasmic membrane peeled off from the cell walls of the swollen cells, with the cytoplasmic compartments shrunk to leave empty space in these cells. Finally, the sizes of these cells further reduced, and the empty space disappeared (Fig. 4A). The cytoplasmic membrane remained intact during this process, as monitored by propidium iodide staining (data not shown).

The localization of YFP-MreB in strain SC9 cells during this VBNC state differentiation was examined. Constant and pulse induction both produced similar localization patterns. YFP-MreB localized evenly or with distinct foci around the cytoplasmic membrane during swelling (Fig. 4B). In the rabbit head-like and drumstick-like cells, YFP-MreB was distributed mainly in the nonswelling parts (Fig.4Bi, Biib, and Biv). In the bulges, YFP-MreB was principally diffused in the cytoplasm (Fig.4Bi, Biib, and Biv). YFP-MreB localized asymmetrically at the cytoplasmic membrane of spherical cells (Fig.4Bii), which might differentiate to the waning, gibbous stage (Fig. 4A). When cells entered the waning process, YFP-MreB appeared only in the cytoplasm-containing parts, like the “last quarter"
The YFP signals localized as different short fragments beneath the cytoplasmic membrane and diffused in the cytoplasm (Fig. 4C). In the small spheroids, YFP-MreB formed aggregates and also diffused in the cytoplasm (Fig. 4Civ). Following 30 days of starvation at a low temperature, more than 99% of the strain SC9 cells became VBNC (39). YFP-MreB diffused in the cytoplasm of these VBNC cells, but a few membrane-associated fluorescent spots also appeared in some cells (Fig. 4D). Our previous investigation has demonstrated that VBNC *V. parahaemolyticus* cells can resuscitate after a temperature-upshift treatment at 25°C for 1 to 3 days (39). The YFP-MreB recovered its membrane localization and participated in filamentous structures when VBNC cells were transferred to 25°C for one day (Fig. 4E).

**Analysis of mreB expression in stressed *V. parahaemolyticus* cells.** To further clarify the function and the regulation of MreB in cells under various stresses, the expression kinetics of *mreB* were examined by RT-PCR. RNA was extracted from wild-type *V. parahaemolyticus* strain 1137 cells in different growth phases or under various environmental stresses. The *mreB* level was downregulated during transition from the exponential phase into the stationary phase and remained almost constant throughout the late stationary phase (Fig. 5A). Consistent with this fact, the level of *mreB* transcripts declines when *B. subtilis* (22) and *Helicobacter pylori* (33) cells enter the stationary phase. *mbl* expression is also downregulated in stationary-phase *B. subtilis* cells (1).

To study the effect of starvation, *V. parahaemolyticus* cells were starved for 24 h, and then transferred to TSB-3% NaCl for 30 min, 1 h, 1.5 h, and 2 h. *mreB* was downregulated during starvation (Fig. 5B), continuously upregulated during the first hour when the cells were shifted to rich medium, and gradually approached a constant level thereafter (Fig. 5B). In *B. subtilis*, *mbl* expression is also repressed by the stringent response (13).
The presence of different structures of the MreB cytoskeleton in *V. parahaemolyticus* in the exponential phase (Fig. 1B) or under various stresses (Fig. 1 to 5) suggests that MreB was highly dynamic. When the cells of *V. parahaemolyticus* entered the exponential phase (Fig. 1) or underwent carbon starvation (Fig. 2), cold shock (Fig. 3), or the VBNC state (Fig. 4), the MreB helices generally loosened and fragmented into short filaments, arcs, and spots. Then, the MreB filaments presumably depolymerized. When bacterial cells encounter stresses, the MreB cytoskeleton may collapse in the following steps: (i) relaxation and fragmentation of the MreB helical bundles, (ii) disassembly of the bundled protofilaments, and (iii) depolymerization of MreB from protofilaments into oligomers and monomers. Consistent with this notion, the depolymerization of MreB by A22 yields a variety of MreB structures, like patches and bands, which later depolymerize to form a diffused pattern (17).

Soufo and Graumann indicated that starvation destroys MreB helices in *B. subtilis* cells in the exponential phase in only a few minutes (32). In *B. subtilis* cells in the stationary phase, green fluorescent protein-MreB localized as bright foci that are randomly located within the cells (32). These large foci may represent the aggregation of MreB. YFP-MreB foci were also observed in cold-shocked *V. parahaemolyticus* (Fig. 3). The YFP-MreB foci in *V. parahaemolyticus* were smaller than those found in stationary-phase *B. subtilis* cells and, unlike them, localized peripherally; they probably also exhibited a strong tendency to attach to the cytoplasmic membrane (Fig. 3).

An intriguing observation in our study is that MreB has a great propensity for membrane attachment. The disintegrated MreB cytoskeleton, whether in the form of loose filament, short arc, spot, or oligomer/monomer, principally localized beneath the cytoplasmic membrane. Although sequence analysis predicts that MreB is probably a cytoplasmic protein, several pieces of evidence show that MreB is associated with the membrane of the cell. Subcellular fractionation reveals that MreB is primarily associated with the membrane in *Escherichia coli* (36), *Caulobacter crescentus* (14), and *Streptomyces coelicolor* (24). This binding may proceed via the direct interaction of MreB with the membrane or indirectly via other membrane proteins. Two long-considered candidate proteins are MreC and MreD (20).

How do different environmental stresses influence the MreB cytoskeleton? Alterations of membrane composition in bacteria under stress may also account for the redistribution of MreB. Subtle changes in the lipid composition of the cell membrane under different environmental and physicochemical factors alter lipid-protein interactions (10). As demonstrated in another cytoskeleton, an oscillating MinD helix disintegrates into compact spots which randomly migrate around the mutant cells without phosphatidylethanolamine, an anionic phospholipid in the cell membrane (25).

Both stationary phase and carbon starvation represent composite stresses in bacterial cells, which then experience two major intrinsic difficulties, the deprivation of energy (especially ATP and adenylate energy charge) and oxidative damage. The ATP level declines as bacteria enter the stationary phase or are under starvation conditions (18), and the change of ATP level influences the dynamics of the MreB filaments. The actin cytoskeletal network of the budding yeast *Saccharomyces cerevisiae* becomes disorganized and shifts toward less-dynamic, ADP-actin-rich aggregates following ATP depletion in the sta-
tionary phase (15). ATP depletion may produce ADP-MreB-rich aggregates as in bacteria. In prokaryotic cells, MreB requires a bound purine nucleotide for polymerization and rapidly hydrolyzes it following assembly; moderate differences between the ATP and ADP critical concentrations influence the treadmilling of MreB (4).

In eukaryotic cells, oxidative stress also severely disrupts the actin cytoskeleton, mainly by oxidative modification of the sulfhydryl group of the conserved Cys374 residue of actin (8). The formation of the intramolecular Cys285-Cys374 disulfide bond or intermolecular disulfide linkages reduces the disassembly rate of actin filaments (8). The sequences of MreB homologs were examined herein, and four cysteine residues (Cys113, Cys116, Cys278, and Cys324) were identified in the MreB of V. parahaemolyticus. Cys113 is conserved in all prokaryotic MreB homologs (MreB, Mbl, and MreBH). Cys116 is only present in vibrios. Cys278 is conserved in MreB of gammaproteobacteria and MreBH. Cys324 is also highly conserved except for the MreB of Thermotoga maritima (34). A comparison with the structure of MreB revealed that the Cys116 and Cys278 residues of the MreB homolog of V. parahaemolyticus are probably assessable under oxidation. Stress usually causes intrinsic oxidation, which injures or kills the bacteria (7). Therefore, oxidation can mediate the change in the configuration of the MreB cytoskeleton in bacteria under stress.

As well as being a victim of stress, MreB may also play an active role in morphological differentiation in bacteria under stress. MreB participates in spatially coordinating cell morphogenesis in conjunction with Mrc, a protein that wraps around the outside of the cell and becomes physically associated with penicillin-binding proteins. Mrc is required for the spatial organization of components of the peptidoglycan-synthesizing holoenzyme, while MreB directs the localization of a peptidoglycan precursor synthesis protein (11). Microarray analysis reveals that MreBCD and Mbl in B. subtilis are upregulated in the “K-state,” which is a growth-arrested and genetically competent state that is distinct from sporulation and probably a unique adaptation to stress (5). The similar stress-resistant state is also observed in other nonsporulating bacteria such as E. coli and V. parahaemolyticus. Cells typically become small and rounded during the differentiation of this state (35). It is believed that VBNC cells become small coci in a manner similar to that of starved or stationary-phase cells (28). However, carefully examining the early course of the differentiation process revealed a heretofore undiscovered swelling-waning process. Cells swelled and became irregular before being miniatu- rized (Fig. 4). Concomitant changes in the expression and localization of MreB in V. parahaemolyticus reported here may constitute a mechanism by which the cells can develop an adaptive morphology. Some bacterial species, at least under certain circumstances, also differentiate into swollen dormant cells. B. subtilis cells change from rod-shaped to spherically or irregularly shaped cells under particular microculturing conditions (31), resembling the V. parahaemolyticus cells in the early stage of VBNC state differentiation, including bulges at the poles (Fig. 4).

MreB is upregulated in the shape-changed cells of B. subtilis (31). The relevant literature was thoroughly reviewed and original data from various transcriptomic and proteomic studies compiled. In summary, the production of MreB homologs is downregulated by Fur in the stationary phase or in the stringent response of bacteria, while such factors as acid stress, chromosome exposure, heat shock, nitrogen limitation, osmotic stress, salinity shock, sporulation, the VBNC state, c-diGMP, σ45 (RpoN), σ2, the FlhD/FlhC transcriptional regulatory complex, YveQ-YveP (two-component regulatory systems), ComK (associated with the “K-state”), and others upregulate the production of MreB homologs. In this work, the expression of mreB was downregulated in stationary-phase and starved V. parahaemolyticus cells but upregulated in the initial periods of differentiation of cold-shocked and VBNC-state cells (Fig. 5). The upregulation of mreB expression or the enhanced production of MreB in bacteria under environmental stresses or stringent physiological states is probably limited to the initial periods of the responses. The level of mreB transcript was increased only in the first few days of VBNC-state differentiation (Fig. 5D), as in the formation of transient morphological abnormality and intensive reconfiguration of the MreB cytoskeleton (Fig. 4). Following this initial period, the cells became spheroid and the mreB level was downregulated, remaining low throughout the VBNC state. The amounts of mreB transcripts in V. parahaemolyticus strain 1137 were also determined under starvation and VBNC state at different intervals by quantitative real-time RT-PCR (3), and similar patterns were observed (unpublished data).

What other functions does the MreB cytoskeleton have in cells under stress? In S. cerevisiae, the depolarization of the actin cytoskeleton activates cell-wall-integrity signaling pathways (21). The integrity of the MreB cytoskeleton per se, and its associations with other molecules, may be used by the cell to monitor its physiological state and cellular functions. Moreover, a bacterial cell must coordinate the dynamics of the MreB cytoskeleton with ATP availability for homeostasis during stresses. The hypothetic ADP-MreB-rich aggregates may slow down the dynamics of the MreB cytoskeleton and save the available ATP for essential housekeeping tasks in the stressed cells. Actin aggregates that resemble the stress-induced MreB structures reported herein are also found in dormant eukaryotic cells (29). Since MreB is an abundant protein, the oxidation of this protein may also act as a “radical sink” to neutralize reactive species generated in the cell under stress, as opposed to actin (23).

Conclusions. This work elucidates the patterns of expression and localization of MreB in V. parahaemolyticus under normal and stressed conditions. The changes in the expression level of mreB and the subcellular localization of MreB were related to changes in subcellular architecture and cell morphology. This information will improve our understanding of the mediation by the MreB cytoskeleton of cellular responses to both internal and external signals. The striking similarities between the behaviors of prokaryotic MreB and eukaryotic actin cytoskeletons under stress suggest some universal mechanisms that are used by cells to cope with environmental fluctuations.

ACKNOWLEDGMENTS

We thank the National Science Council of Taiwan, Republic of China, for financially supporting this research under contract no. NSC 94-2313-B-031-003, NSC 95-2313-B-031-002, and NSC96-2313-B-031-001. Ted Knoy is appreciated for his editorial assistance.
REFERENCES