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CHANGE OF THE QUANTITY OF PENICILLIN-BINDING PROTEINS AND OTHER CYTOPLASMIC AND MEMBRANE PROTEINS BY MUTATIONS OF THE CELL SHAPE-DETERMINATION GENES *mreB*, *mreC*, AND *mreD* OF *ESCHERICHIA COLI*

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Single and multiple mutations in the *mreB*, *mreC*, and *mreD* genes of *Escherichia coli*, which are involved in rod shape determination and the accompanied change in the cell's mecillinam resistance, caused an increase in the quantity of septum-peptidoglycan synthetase penicillin-binding protein (PBP)-3, and more general peptidoglycan synthetase PBP-1B. The *mreD* mutant, but not the *mreB* and *mreC* mutants, showed a decreased growth which could be suppressed by deletion of the total *mreBCD-orfEF* area on the chromosome. The *mre* mutations additionally caused a complex nature increase/decrease of several other cytoplasmic and membrane protein quantities. These results suggest that the *mreB*, *mreC*, and *mreD* genes are involved in a complex regulatory mechanism in the process of cell growth, division and shape determination.

Many genes involved in cell growth and cell division mechanisms are located in several distinct regions of the chromosome of *Escherichia coli*, thereby forming clusters of genes (*5*). Among these, genes located in two regions on the chromosome map are thought to be responsible for cell shape determination, i.e., *mrd*

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(*mu*rein synthesis gene cluster *d*) at 15 min (*9*) and *mre* (*mu*rein synthesis gene cluster *e*) at 71 min (*10*). It was previously reported that the *mre* region encompassed three genes *mreB*, *mreC* and *mreD* (*11*), with its base sequence also being determined (*1*, *11*). A mutation in one of these three *mre* genes caused the formation of a spherical like cell which showed resistance to amidinopenicillin mecillinam (*11*). Originally a mutant strain *⊿mre678* was isolated which had a deletion of a 5 kb chromosomal region encompassing *mreB*, *mreC* and *mreD* genes (*10*), and also two open reading frames *orfE* and *orfF*, coding for 22 kD and 51 kD proteins, respectively (*13*). Single mutants of *mreB*, *mreC*, *mreD*, *orfE*, and *orfF* were constructed from the *⊿mre678* mutant by introducing plasmid that contained a 6.5 kb chromosomal fragment encompassing *mreB*, *C*, *D* and *orfE*, *F*, with each fragment having an appropriate frame-shift mutation (*11*). By using these constructed mutants, all three *mre* mutations were shown to cause rounding of the cell, although *orfE* and *orfF* mutations did not cause any change of cell morphology. In the present study functions of the *mreB*, *mreC*, and *mreD* genes were studied further, by examining the quantity of various proteins contained in the cytoplasm and membranes.

MATERIALS AND METHODS

The bacterial strains and plasmids used are shown in Table 1.

Cells were grown at 30°C in a modified Lennox broth (see the reference 11) supplemented with 20 mg of thymine and 100 μg/*l* of lipoic acid (L′-lip broth). Kanamycin was added to the broth at a concentration of 25 mg/*l* in order to maintain the plasmids. Materials used were [^{14}C]-benzylpenicillin potassium salt (59 Ci/mol, Amersham Int. plc., Buckinghamshire, England), Mecillinam (Leo Pharmaceutical Products, Copenhagen, Denmark), sodium dodecyl sulfate (SDS, Sigma Chemical Co., St. Louis, Mo.), with the other reagents being commercial products. Penicillin-binding proteins (PBPs) were observed by binding [^{14}C]-benzylpenicillin, followed by performing electrophoretic separation of the proteins on a SDS-polyacrylamide gel, and fluorography as previously described (*10*). For membrane preparation, cells grown at 30°C were collected and disrupted by sonication in a sodium phosphate buffer (50 mM, pH 7.5) under ice water cooling. Crude membranes were collected by centrifuging cell extracts for 30 min at 4°C (100,000 × *g*), were washed twice with the above buffer, and then suspended in the sodium phosphate buffer containing 0.5% Sarkosyl (pH 7.5). The suspension was kept for 20 min at 20°C and then centrifuged for 30 min at 20°C (7,000 × *g*). The supernatant was used as the inner membrane fraction, and the sediment, after washing once and suspending in the above sodium phosphate-Sarkosyl buffer, was used as the outer-membrane fraction. SDS-PAGE was performed according to Laemmli and Favre (*3*).

Table 1. *E. coli* strains and plasmids used.

Strain	Genotype	Source
PA340	F⁻ *argH1 thr-1 leuB6 ghd-1 gltB31 thi-1 lacY1 gal-6 xyl-7 ara-14 mtl-2 malA1 rpsL9 tonA2*	M. A. Berkerich
PA340-678	As PA340 except *gltB*⁺ *Δmre-678* (*ΔmreBCDorfEF*)	Sexual cross: AT2472-678 × PA340 (*10*)
Plasmid	**Source and Construction**	
pLG339	Obtained from B. G. Spratt (*8*), a low-copy-number cloning vector derived from pSC101 containing unique *Bam*HI, *Sph*I, *Sal*I, and *Hin*cII sites within the Tc^R gene and unique *Sma*I and *Xho*I sites within the Km^R gene.	
pMEL1	Constructed by ligation of a chromosomal 6.5-kb *Sal*I fragment containing the *mreB*, *mreC*, and *mreD* genes and two other coding frames with *Sal*I-digested pLG339 (*10*)	
pMEL1K	Same as pMEL1 except a −4 bp frameshift at the *Kpn*I site in the *mreB* gene (*11*)	
pMEL1C	Same as pMEL1 except a +2 bp frameshift at the *Cla*I site in the *mreC* gene (*11*)	
pMEL1Ba	Same as pMEL1 except a +4 bp frameshift at the *Bam*HI site in the *mreD* gene (*11*)	
pMEL1S	Same as pMEL1 except a −4 bp frameshift at the *Sph*I site in the coding frame of the 22-kDa protein (*11*)	
pMEL1Bg	Same as pMEL1 except a +4 bp frameshift at the *Bgl*II site in the coding frame of the 55-kDa protein (*11,13*)	

RESULTS AND DISCUSSIONS

Over-production of PBP-1B and PBP-3 in the mreB, C and D mutants

As shown in Fig. 1, simultaneous over-production of PBP-3 (septum peptidoglycan synthetase, references *2* and *4*) and PBP-1B (peptidoglycan synthetase, *7*) was caused by a single mutation in any one of the three *mre* genes. The multiple deletion mutation *Δmre678* also caused over-production of the PBPs (lane b) as previously reported (*10*), which was mitigated and returned to normal, i.e., "rescued," by introducing the plasmid pMEL1 which covered the total deletion of *Δmre678* (lane c), whereas not by pMEL1K (frameshift in *mreB*, lane d), pMEL 1C (frameshift in *mreC*, lane e), or pMEL1Ba (frameshift in *mreD*, lane f). Over-production of PBP-3 and PBP-1B in *Δmre678* was similarly rescued by the plasmid pMEL1S (lane g) and pMEL1Bg (lane h), with a respective frameshift in *orfE* and *F*, thereby indicating that *orfE* and *F* are not involved in the regulation of PBP-1Bs and PBP-3. Wachi and Matsuhashi (*12*) used a plasmid that contained the fusion gene *ftsI-lacZ*, and suggested the *mreB* gene's negative regulatory function in expression of *ftsI*, the structural gene of septum peptidoglycan synthetase PBP-3. It is believed that *mreC* and *mreD* also function in a similar manner.

Decrease of the growth rate caused by the mreD mutation and its suppression

The modes of action of MreB, MreC and MreD proteins, however, are not

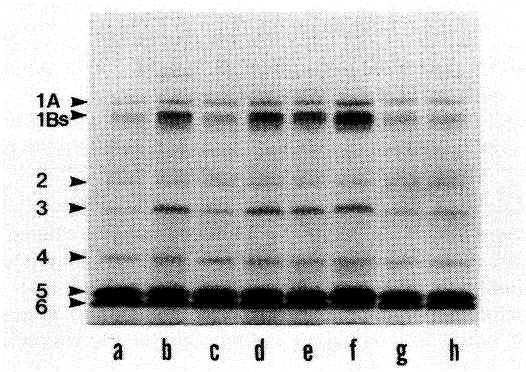

Fig. 1. Overproduction of PBP-1 Bs and PBP-3 in *mreB*, *mreC*, and *mreD* mutants. A fluorogram of a SDS-PAGE electrophoregram is shown. a: *E. coli* strain PA340/pLG339 (wild type); b: strain PA340-678/pLG339 (*ΔmreBCDorfEF*); c: PA340-678/pMEL1 (wild type); d: PA340-678/pMEL1K (*mreB*); e: PA340-678/pMEL1C (*mreC*); f: PA340-678/pMEL1Ba (*mreD*); g: PA340-678/pMEL1S(*orfE*); h: PA340-678/pMEL1Bg (*orfF*). Numbers show PBPs.

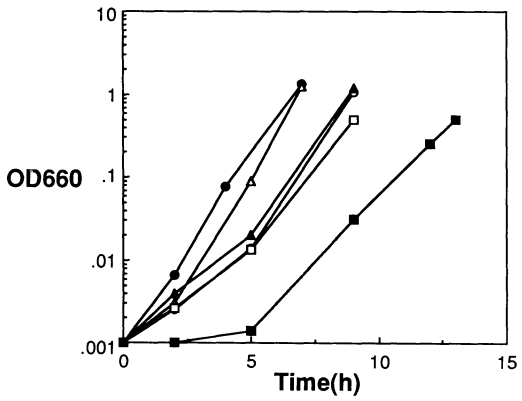

Fig. 2. Growth curves of *mre* mutants. Cells were grown in an exponential phase at 30°C in L′-lip broth. Cells were then inoculated at 10^6 cells/ml in fresh L′-lip broth and grown at 30°C. Growth was followed by measuring the absorption at 660 nm (10 mm light path). ●: *E. coli* strain PA340/pLG339 (wild type); ○: strain PA340-678/pLG339 (*ΔmreBCDorfEF*); △: PA340-678/pMEL1 (wild type); ▲: PA340-678/pMEL1K (*mreB*); □: PA340-678/pMEL1C (*mreC*); ■: PA340-678/pMEL1Ba (*mreD*).

exactly the same, as was observed from the phenotypical changes of the cell's shape in the respective mutations (*11*). The *mreB* and *Δmre678* mutations caused rounding of the cell, without significant effect in its growth rate, whereas the *mreC*, and especially the *mreD* mutations, caused formation of swollen, round shaped cells which indicated spontaneous cell lysis. The *mreD* mutant also showed a decreased

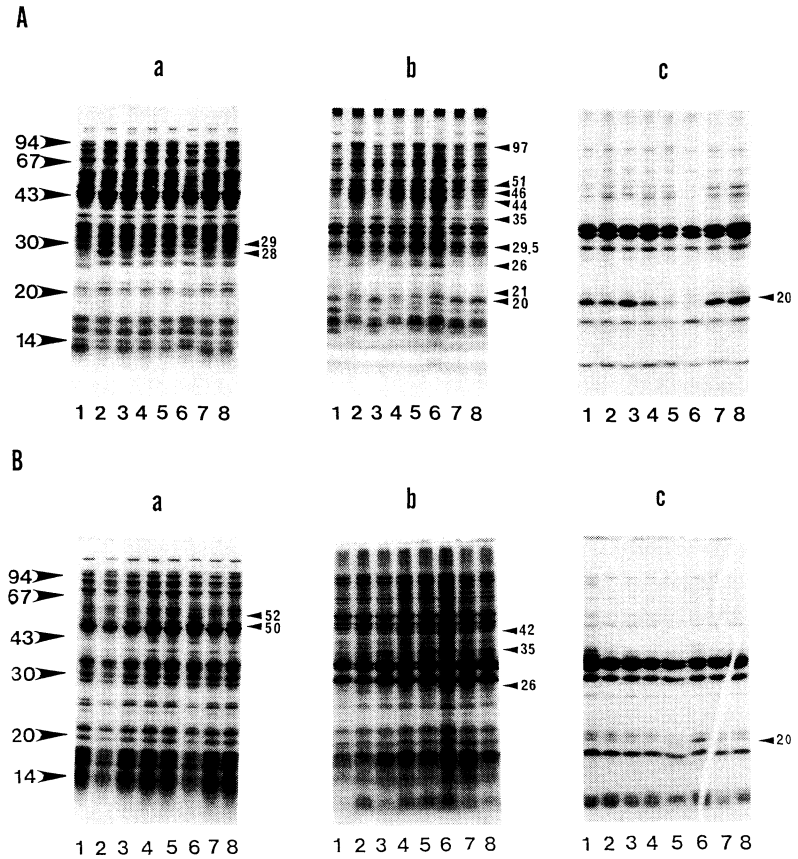

Fig. 3. Protein pattern of *mre* mutant cells. Coomassie brilliant blue R-stained gel (10 cm × 10 cm × 1 mm, containing 12.5% monoacrylamide and 0.33% bis-acrylamide) was used. Preparation methods for the cellular fraction and SDS-PAGE are described in the text. (A), fractions from stationary cells; (B), fractions from exponentially growing cells. a: soluble fraction; b: cytoplasmic membrane; c: outer membrane. Lanes 1–8 are proteins from *E. coli* strains a–h in the legend to Fig. 1, respectively. Arrowheads at the left of electrophoregrams show molecular weight standards and those at the right show obvious changes in the protein pattern among the wild type and *mre* mutant cells.

cell growth. Figure 2 shows the growth curves of the *mre* mutant cells, and indicates that the apparent cell growth, as measured by an increase in absorbance at 660 nm, was most significantly inhibited in the *mreD* mutant cells. Total deletion of chromosome from *mreB* to *orfF* suppressed the decrease of the growth of the *mreD* mutant (see the *Δmre678* mutant, Fig. 2).

Change of other protein quantities in the mreB, C and D mutants

As shown in Fig. 3(Aa), in the stationary cells the quantity of the cytoplasmic

29 kD protein increased in all the multiple and single *mre* mutants (lanes 2 and 4–6), yet in the cytoplasmic 28 kD protein it increased only in the *⊿mre678* (lane 2), *mreB* (lane 4) and *mreC* (lane 5) mutants, whereas not in the *mreD* mutant (lane 6). In the exponentially growing cells (Fig. 3Ba), both the cytoplasmic 52 kD and 50 kD proteins increased in only the *mreD* mutant (lane 6), with these increases not being seen in the *⊿mre678* mutant (lane 2).

Pattern changes of the cytoplasmic membrane proteins were more frequent, with obvious increases in the proteins having a Mr of 97, 51, 46, 44, 29.5, 26 and 21, and decreases of the 20 kD protein in all the stationary phase *mre* mutants (Fig. 3 Ab, lanes 2 and 4–6). An increase in the 35 kD cytoplasmic membrane protein occurred only in the stationary phase *mreD* mutant (lane 6). There was a significant decrease of the outer-membrane 20 kD protein from the stationary phase *mre* mutants (Fig. 3Ac, lanes 2 and 4–6) which may be identical to the peptidoglycan associated lipoprotein (PAL) having a calculated molecular weight of 16,600 (*6*). In the exponentially growing *mreD* mutant cells, the 20 K protein increased in the outer membrane (Fig. 3Bb, lane 6). Experiments were repeated showing similar results.

These changes in the cell's protein patterns can either be a cause or a result of a failure in the shape determination process. Presented results nonetheless suggest a complexed action of *mreB*, *mreC*, and *mreD* gene products in the cell-cycle control process. The *mreB* product protein was reported to show several similarities in its amino acid sequence to animal heat-shock proteins and the *E. coli* DnaK protein, which are both supposed to be a protein kinase (*5*). The MreB protein is suggested to be involved in the regulatory mechanisms where protein-phosphorylation and dephosphorylation are involved (unpublished experiments). However, since no similarities were found between the amino acid sequences of the MreC and MreD proteins and those of the MreB protein, heat-shock proteins, or DnaK protein, a hypothesis concerning the action mechanism of the MreC and MreD proteins cannot yet be proposed.

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