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Determinations of the DNA Sequence of the *mreB* Gene and of the Gene Products of the *mre* Region That Function in Formation of the Rod Shape of *Escherichia coli* Cells

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The 6.5-kilobase *mre* region at 71 min in the *Escherichia coli* chromosome map, where genes involved in formation of a rod-shaped cell form a gene cluster, was analyzed by in vivo protein synthesis in a maxicell system and by base sequencing of DNA. An open reading frame that may code for a protein with an M_r of about 37,000 on sodium dodecyl sulfate-polyacrylamide gels was found and was correlated with the *mreB* gene. N-terminal amino acid sequencing of the hybrid *mreB-lacZ* protein confirmed the production by *mreB* of a protein of 347 amino acid residues with a molecular weight of 36,958. The amino acid sequence of this protein deduced from the DNA sequence showed close similarity with that of a protein of the *ftsA* gene which is involved in cell division of *E. coli*. Three other contiguous genes that formed three proteins with M_rs of about 40,000, 22,000, and 51,000, respectively, were detected downstream of the *mreB* gene by in vivo protein synthesis. The *mreB* protein and some of these three proteins may function together in determination of cell shape.

Two regions called *mrd* (15) and *mre* (11) on the *Escherichia coli* chromosome involve clusters of genes responsible for determination of the cell shape and the sensitivity of cell growth to an amidinopenicillin, mecillinam. The *mrd* region located at 15 min on the *E. coli* chromosome map involves two genes for formation of the rod shape of the cell, *mrdA* (= *pbpA*), which codes for penicillin-binding protein 2, a peptidoglycan synthetase, and *mrdB* (= *rodA*), which codes for the RodA protein which may also be necessary for functioning of the *mrdA* protein (5). The *mre* region located at 71 min on the chromosome map involves genes that probably function together in shape determination and mecillinam sensitivity of the cell (17). Previously, we reported preliminary results of gene analysis of the apparent 7-kilobase (kb) *mre* region neighboring the *fabE* gene (17). The gene we called *mreB* (11), which may be allelic to *envB* (18) and in which the mutation *mre-129* causing a round cell and supersensitivity to mecillinam is located, was cloned in a 2.8-kb DNA fragment. However, we found that another mutation, *mre-678*, causing a round cell, resistance to mecillinam, and overproduction of penicillin-binding proteins 1Bs and 3 was due to deletion of a 5.2-kb DNA fragment extending from the *mreB* gene and could not be complemented by the 2.8-kb fragment (17).

The present report describes the in vivo identification of the protein products of the genes located in the *mre* region (the size of the *Sal*I fragment is corrected to 6.5 kb from that in the previous report) by using a maxicell system (13) and DNA sequencing of a 2.1-kb *Hin*cII region involving the *mreB* gene.

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MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strain CSR603 (*uvrA6 recA1 phr-1 thr leu pro his thi arg lac gal ara xyl mtl rpsL*) used for protein synthesis in the maxicell system was obtained from B. J. Bachmann, Yale University School of Medicine. Strain JM109 (Δ*lac-proAB recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ⁻ F′ *traD36 proAB lacI*^{q} Δ*lacZM15*) used for DNA sequencing experiments was obtained from Takara Shuzo Co., Kyoto, Japan.

Culture media. Modified Lennox broth (9) supplemented with 20 mg of thymine per liter and 50 μg of lipoic acid per liter, called L′lip-broth (15), was used for growing cells unless otherwise indicated. For growing cells carrying plasmids, 25 mg of kanamycin, 25 mg of chloramphenicol, and 50 mg of aminobenzylpenicillin per liter were added to the broth.

[^{35}S]methionine labeling of proteins in the maxicell system. The maxicell system was used for labeling proteins with [^{35}S]methionine in in vivo synthesis of proteins coded by genes on plasmids (13). In a typical experiment, exponentially growing cells (2×10^8 cells per ml) in 7 ml of M9 medium plus 1% Casamino Acids (Difco Laboratories, Detroit, Mich.) were UV irradiated and then incubated at 30°C for 1 h with shaking. Then D-cycloserine (200 mg/liter) was added, and incubation was continued for 12 h. The cells were collected and washed twice with modified Hershey medium (19). They were then suspended in 1 ml of modified Hershey medium and incubated at 37°C for 1 h. Then [^{35}S]methionine (6.3 μCi) was added, and the reaction was carried out at 37°C for 1 h. The labeled proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (8), and radioactivity was detected by fluorography.

DNA sequencing. The dideoxynucleotide method (14) with the Klenow fragment of *E. coli* DNA polymerase I was applied directly to the denatured double-stranded DNA

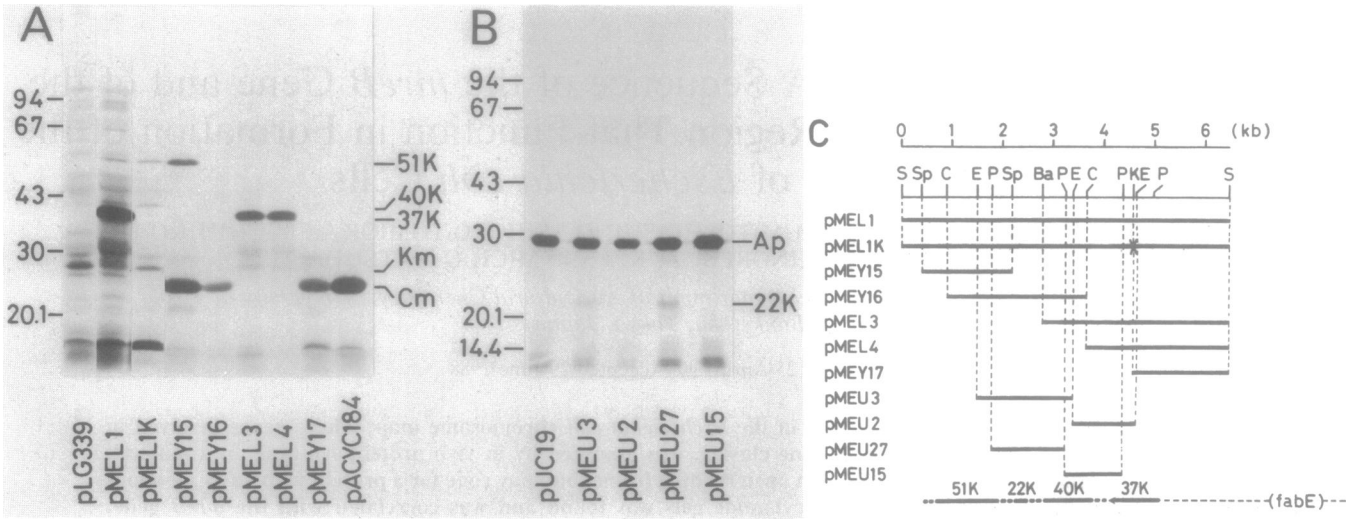

FIG. 1. Proteins encoded by plasmids in the maxicell system. Two gels, a 10% acrylamide gel (A) and a 12.5% acrylamide gel (B), were used. Numbers indicate molecular weight. The DNA fragments used for in vivo protein synthesis and a summary of the results (solid lines at the bottom indicating approximate location of the open reading frames) are shown in panel C. Abbreviations: Ap, β-lactamase; Km, kanamycin phosphotransferase; Cm, chloramphenicol acetyltransferase; Ba, *Bam*HI; C, *Cla*I; E, *Eco*RV; H, *Hin*cII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sp, *Sph*I.

plasmid pUC19 (3) carrying fragments of the *E. coli* chromosome.

Plasmid construction. Plasmid pMC1403 (1), used for construction of the *mreB-lacZ* fusion gene, was obtained from H. Matsuzawa, University of Tokyo. The isolations of low-copy plasmids pMEL1, pMEL1K, pMEL3, and pMEL4 have been reported (17). Plasmids pMEY15, pMEY16, and pMEY17 were constructed by ligation of high-copy plasmid pACYC184 with fragments of pMEL1 obtained by digestion with appropriate restriction endonucleases. Plasmids pME U2, pMEU3, pMEU15, and pMEU27 were constructed by ligation of appropriate endonuclease-digested DNA fragments of pMEL1 with high-copy plasmid pUC19.

Construction of *mreB-lacZ* fusion gene and N-terminal amino acid sequencing of fusion protein. The 1.1-kb *Hin*cII-*Eco*RV fragment (nucleotides 4 to 1107 in Fig. 2) of pMEL3 was inserted into the *Sma*I site of pMC1403, which could make an in-frame joint between the N-terminal part of *mreB* and the C-terminal part of *lacZ*. Strain JM109 carrying the constructed plasmid pEHB3 was grown in L'lip-broth, and the fusion protein was overproduced after amplification of the plasmid by treatment with 170 mg of chloramphenicol per liter. The cells were disrupted in a French press (16,000 lb/in^2), and the membrane fraction, in which the major part of the β-galactosidase activity was recovered, was collected by ultracentrifugation (100,000 × *g*) and subjected to SDS-polyacrylamide gel electrophoresis (7.5% gel). The band corresponding to an M_r of 150,000, which is the expected size of the *mreB-lacZ* fusion protein, was cut out from the gel. The protein was eluted and concentrated in a Max-Yield-GP protein concentrator (AE-3590; ATTO Co. Ltd., Tokyo, Japan) in *N*-ethylmorpholine acetate buffer (pH 8.5) containing 0.1% SDS. The protein was precipitated with acetone and introduced into a gas-phase amino acid sequencer (ABI 470A; Applied Biosystems, Inc., Foster City, Calif.).

Radioactive materials and reagents. [^{35}S]methionine (1,470 Ci/mmol) and [α-^{32}P]dCTP (410 Ci/mmol) were purchased from Amersham International plc, Amersham, Buckinghamshire, England. SDS and D-cycloserine were purchased from Sigma Chemical Co., St., Louis, Mo. Other reagents and enzymes for DNA recombination experiments were also commercial products.

RESULTS

Identification of gene products in *mre* region in the maxicell system. The clone of the 6.5-kb chromosomal DNA fragment containing the *mre* region (17) was separated into subclones for identification of gene products (Fig. 1). The proteins formed by genes located on the 6.5-kb DNA fragment were detected in the maxicell system. The SDS-polyacrylamide gel electrophoresis patterns of proteins labeled by [^{35}S] methionine are shown in Fig. 1A (10% acrylamide gel for higher-molecular-weight protein) and Fig. 1B (12.5% acrylamide gel for lower-molecular-weight protein). These plasmids formed proteins that could be attributed to the expression of the genes located on chromosomal fragments in the plasmids in addition to products of the drug resistance genes on the vector plasmids. The low-copy-number plasmid pMEL1, which contained the whole 6.5-kb fragment, formed three higher-molecular-weight proteins with M_rs of about 51,000, 40,000, and 37,000 on the 10% gel (Fig. 1A). A plasmid with a deletion of 4 base pairs at the unique *Kpn*I site, pMEL1K, which could not complement either the *mre-129* or *mre-678* mutation (17) did not form the 37-kilodalton (kDa) protein. These results established that the 37-kDa protein is formed from the *mreB* gene that contains the unique *Kpn*I site. This protein was also formed from the low-copy-number plasmids pMEL3 and pMEL4, which both contained a 2.8-kb *Cla*I-*Sal*I fragment, but was not formed from the high-copy-number plasmids pMEY15, pMEY16, and pMEY17, which did not have the entire fragment (Fig. 1A).

The two other proteins of 51 and 40 kDa were formed from pMEL1K. The 51-kDa protein was also formed from the high-copy-number plasmid pMEY15 containing the 1.8-kb *Sph*I fragment, but not from pMEY16, which did not contain the complete *Sph*I fragment (Fig. 1A). These results suggest that this protein was coded by the gene located on the 1.8-kb

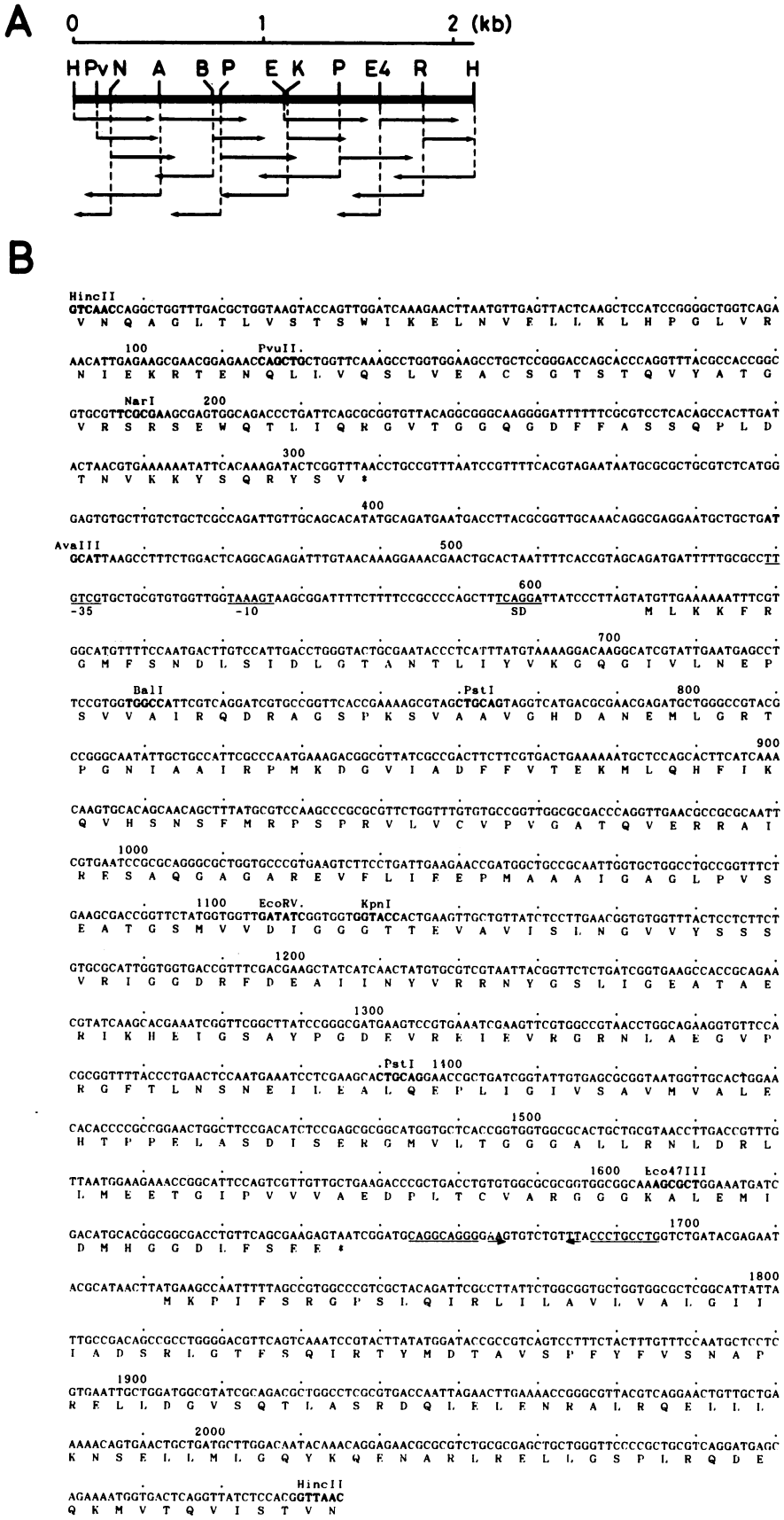

FIG. 2. Strategy and result of DNA sequencing (A) and deduced amino acid sequence (B). The possible promoter and Shine-Dalgarno (SD) sequences are underlined. The termination codon is shown by an asterisk. Arrows indicate a palindrome sequence. Abbreviations for restriction endonucleases are as described in the legend to Fig. 1; in addition, Pv, *Pvu*II; N, *Nru*I; A, *Ava*III; E4, *Eco*47III; R, *Rsa*I.

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mreB                                                    MLKKFRGMFSNDLSI 15

ftsA   MIKATDRKLVVGLEIGTAKVAALVGEVLPDGMVNIIGVGSCPSRGMDKGGVNDLESVVKC 60

mreB   DLGTANTLLYVKGQGIVLNEPSVVAIRQDRAGSPKSVAAVGHDANEMLGRTPGNIAAIRP 75

ftsA   VQRAIDQAELMADCQISSVYLALSGKHISCQNEIGMVPISEEEVTQEDVENVVHTAKSVR 120

mreB   MKDGVIASFFVTEKMLQHFIKQVHSNSFMRPSPRVLVCVPVGATQVERRAIRESAQGAGA 135

ftsA   VRDEHRVLHVIPQEYAIDYQEGIKNPVGLSGVRMQAKVHLITCHNDMAKNIVKAVERCGL 180

mreB   REVFLIEEPMAAAIGAGCRFLKATGSMVVDIGGGTTEVAVISLNGVVYSSSVRIGGDRFD 195
                                      ******** ::** : ::: ::: :: :*:  :
ftsA   KVDQLIFAGLASSYSVLTEDERELGVCVVDIGGGTMDIAVYTGGALRHTKVIPYAGNVVT 240

mreB   EAIINYVRRNYGSLIGEATAERIKHEIGSAYPGDEVREIEVRGRNLAEGVPRGFTLNS-N 254
       :: *:*   ::*:  ::*:* ::*  *:*  :   :: :*  ::::  **::  :: :
ftsA   SD-IAY---AFGTPPSDAEAIKVRH--GCALGSIVGKDESVEVPSVGGRPPRSLQRQTLA 294

mreB   EILEALQEPLIGIVSAVMVALEHT-PPELASDISERGMVLTGGGALLRNLDRLLMEETGI 313
       *::*:  : *:::*:: :::*::: ::: :::  : *:*****:* : :*
ftsA   EVIEPRYTELLNLVNEEILQLQEKLRQQGVKHHLAAGIVLTGGAAQIEGLAACAQRVFHT 354

mreB   PVVVAEDPLTCVARGGGKALEMIDMHGGDLFSEE 347

ftsA   QVRIGAPLNITGLTDYAQEPYYSTAVGLLHYGKESHLNGEAEVEKRVTASVGSWIKRLNS 414

ftsA   WLRKEF 420
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FIG. 3. Optimized alignment of *mreB* and *ftsA* proteins. The single-letter notation of amino acids is used. Identical residues are marked with an asterisk, and conservative replacements are shown by two dots. Insertions made during optimization are marked with a dash. The alignment is according to the program of Lipman and Person (10). Residue 338 of the FtsA protein given as A could be uncertain.

*Sph*I fragment. The 40-kDa protein was formed from pMEL1 or pMEL1K, but not from any of the subclones in the high-copy-number plasmid in Fig. 1C. Thus, the gene coding for this protein may encompass either the 0.9-kb *Bam*HI-*Cla*I region or a part of the terminal 0.4-kb *Sal*I-*Sph*I region of the total 6.5-kb *Sal*I fragment. However, we think the latter possibility is less likely.

Formation of the 22-kDa protein by the high-copy-number plasmids pMEU3 and pMEU27, which both involve the 1.5-kb *Pst*I fragment, could be detected in the 12.5% gel (Fig. 1B). Thus, the gene coding for the 22-kDa protein may be located between the genes coding for the 51- and 40-kDa proteins.

Determination of base sequence of *mreB* gene and N-terminal peptide sequence of *mreB* protein. The 2.1-kb *Hin*cII fragment, which could complement the *mre-129* mutation and involved the unique *Kpn*I site, was obtained from plasmid pMEL1 for sequencing the *mreB* gene. The strategy and result of DNA sequencing of the 2.1-kb *Hin*cII fragment containing the *mreB* gene are shown in Fig. 2. A large open reading frame encompassing the *Kpn*I site was detected as the sole candidate for the *mreB* gene. Three start codons were seen at GTG (544 to 546), GTG (553 to 555), and ATG (613 to 615). A termination codon for this frame was found at TAA (1654 to 1656).

To determine the N-terminal amino acid sequence of the *mreB* protein, we prepared a hybrid peptide of the N-terminal portion of the *mreB* protein and the C-terminal portion of the *lacZ* protein of *E. coli* as described in Materials and Methods. The N-terminal amino acid sequence NH_2-MLKKFRXMFXND- was obtained, consistent with that deduced from the base sequence starting at the start codon ATG (613 to 615).

From these results, the protein formed from *mreB* should have 347 amino acid residues with a molecular weight of 36,958. Provided the start codon is at ATG (613 to 615), candidates for promoter sequences may be TTGTCG (539 to 544, the −35 region) and TAAAGT (561 to 566, the −10 region). The possible Shine-Dalgarno sequence may be TCAGGA (595 to 600). The amino acid sequence upstream of the N-terminal methionine residue deduced from the DNA sequence, M (or MLRV) VGKVSGFSFPPQLSGLSLS, resembles a signal peptide sequence. If this sequence actually functions as a signal peptide, the starting codon would be GTG (544 to 546) or GTG (553 to 555). However, no promoterlike and Shine-Dalgarno-like sequences could be found in the close upstream region of these two GTG codons.

A palindrome sequence (1664 to 1695) that could construct an 11-base-pair stem and an 8-base loop was found shortly after the termination codon TAA (1654 to 1656). This sequence may function as a terminator, but this possibility requires further study.

The *mreB* gene is preceded by an apparent open reading frame on the same strand (1 to 306) continuing upstream of the *Hin*cII site, its possible protein product not having been detected. The termination codon of the unknown gene and the start codon ATG of the *mreB* protein are separated by a spacing sequence of 303 nucleotides. With a 65-base-pair spacing sequence downstream of the termination codon TAA of the *mreB* gene, a possible open reading frame on the same strand followed only the 384-base N-terminal sequence which is shown in Fig. 2. This probably codes for a 40-kDa protein. No reasonable promoterlike sequence could be found in this spacing region.

The hydropathy profile of the *mreB* protein sequence deduced from the base sequence was moderate (not shown). The codon usage indicated that this protein is formed in a large amount in the cells (4).

The homology of the base sequence of the *mreB* gene was surveyed in an EMBL gene library. Some homology was found with *ftsA* (12), which is a gene involved in cell

division. The homology of the protein products of the two genes was still higher. The C-terminal half of the *mreB* protein showed 24.5% amino acid identity in a 143-amino-acid overlap with a similar portion of the *ftsA* product (Fig. 3). The highest homologies were seen in two regions, eight consecutive amino acid residues from Val-163 through Thr-170 and five consecutive amino acid residues from Val-292 through Gly-296 of the *mreB* protein. The unique *Kpn*I site of the *mreB* gene was located in the homologous octapeptide region. Considering conservative replacement, the homology in the sequence of 143 amino acids was 71.3%.

DISCUSSION

The alignment of genes in the *mre* region is shown in Fig. 1C. The *mreB* gene coding for the 37-kDa protein was located close to the *fabE* gene. Based on the restriction map of the total *E. coli* chromosome (7), the *mreB* gene is transcribed counterclockwise, in the opposite direction of the *fabE* gene. Downstream of *mreB*, three genes were found that could code for a 40-kDa protein, a 22-kDa protein, and a 51-kDa protein in this order. The *mre-129* mutation was located in the *mreB* gene, but the *mre-678* mutation possessed a large deletion of the 5.2-kb DNA fragment extending over *mreB* and three other genes coding for the 22-, 40-, and 51-kDa proteins. The phenotyic changes caused by the *mre-678* mutation could only be recovered by plasmids which carried this chromosomal area, such as pMEL1, but not by those that carried only a part of this area, such as pMEL3 or pMEL4 (17). The *mreB* protein and at least one of the three other proteins are definitely involved in the formation of the rod shape, mecillinam sensitivity, and the regulation of formation of penicillin-binding proteins 1Bs and 3 of the *E. coli* cell.

The strong homology of the C-terminal half of the *mreB* gene product with the *ftsA* protein suggests some relationship between the functions of these two proteins. The *ftsA* protein is involved in a late step of cell division (2), and the *mreB* protein is involved in formation of the rod shape of the cell. These proteins might function similarly. They possibly antagonize each other.

Previously, a report from this laboratory (16) indicated the presence of a gene named *mreA* that may be responsible for regulation of the penicillin-binding proteins 1A and 2 and its mutation, which was recessive to the wild type. The position of the *mreA* mutation was mapped roughly by use of a lambda transducing bacteriophage close to *mreB* (11; S. Nakajima-Iijima, Ph.D. thesis, University of Tokyo, Tokyo, Japan, 1981). The exact location of this gene and identification of the functional gene product require further investigation.

Several Rod$^-$ mutations, named *rodY* (6) or *envB* (18), have been mapped close to this region. The fact that the *envB* mutation could be complemented by the plasmid pMEL4 (17) suggests that the *envB* gene could be identical to the *mreB* gene.

The mechanisms by which the *mreB* product and some other putative *mre* gene products function in formation of rod-shaped cells, induction of normal mecillinam sensitivity, and regulation of penicillin-binding proteins 1Bs and 3 need further investigation.

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