

## Cluster of *mrdA* and *mrdB* Genes Responsible for the Rod Shape and Mecillinam Sensitivity of *Escherichia coli*

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Two closely linked genes, *mrdA* and *mrdB*, located at ca. 14.2 min on the *Escherichia coli* chromosomal linkage map, seem to be responsible for the normal rod shape and mecillinam sensitivity of *E. coli*. The product of *mrdA* was concluded to be penicillin-binding protein 2, because *mrdA* mutations caused formation of thermosensitive penicillin-binding protein 2. The product of the *mrdB* gene is unknown. At 42°C, mutation in either of these genes caused formation of spherical cells and mecillinam resistance. Both mutations were recessive, and complementation, as detected in +/−/+ meroheterodiploids having the wild-type phenotype, provided strong evidence that the two mutations are in different complementation groups. P1 transduction suggested that the most plausible gene order is *leuS-mrdA-mrdB-lip*. The *rodA* mutation reported previously seems to be similar to the *mrdB* mutations, but the identities of the two have not yet been proven.

Several *Escherichia coli* mutants that form osmotically stable spherical cells have been isolated (1, 3, 5, 6, 11, 12, 19, 20, 27). Some of these mutants are also resistant to mecillinam, an amidinopenicillin (10) that induces spherical cells of *E. coli* (10) and other gram-negative bacilli (18). A critical advance in studies on the mechanism of the sphere-rod conversion of cells and of the action of this  $\beta$ -lactam antibiotic was achieved by Spratt and Pardee (22) and Spratt (20), who observed that mecillinam specifically binds to penicillin-binding protein 2 (PBP-2) in *E. coli* and that mutation in this protein causes formation of spherical, mecillinam-resistant cells. The gene for PBP-2 has subsequently been mapped at 14.4 min (*rodA*, reference 23) on the *E. coli* chromosomal linkage map (100-min map [2]). The *rodA* mutant isolated previously, which has a mutation closely linked to the *lip* gene (12), is also spherical but has a normal PBP-2 (H. Matsuzawa, S. Asoh, and T. Ohta, unpublished data). This paper describes our recent finding: that at least two closely linked genes, *mrdA* and *mrdB*, that are responsible for formation of the rod shape and mecillinam sensitivity are present at about 14.2 min on the *E. coli* linkage map. The product of one of these genes seems to be PBP-2, but that of the other gene, which may be identical to gene *rodA*, is unknown.

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jima, H. Matsuzawa, S. Tamaki, and M. Matsuhashi, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, K(H)14, p. 173].)

### MATERIALS AND METHODS

**Strains.** The *E. coli* K-12 strains used in this study are listed in Table 1.

**Media.** Lennox broth (8) without NaCl but supplemented with 20  $\mu$ g of thymine and 50 ng of lipoic acid per ml (LLB minus NaCl) was used for isolation of spherical, mecillinam-resistant mutants. LLB was used for routine growth of most strains. Minimal medium M9 (4) was used for selection of recombinants by nutritional markers and for growth of F-prime strains. Final concentrations were as follows: sugars, 0.4%; amino acids, 50  $\mu$ g/ml; nucleosides, 20  $\mu$ g/ml; thiamine, 2  $\mu$ g/ml; lipoic acid, 50 ng/ml; streptomycin, 100  $\mu$ g/ml. For plates, broth was solidified with 1.5% agar (Wako Pure Chemical Industries, Osaka, Japan).

**Determination of sensitivity to mecillinam.** Stationary cultures of the strains in LLB were diluted to  $10^{-2}$  (ca.  $10^7$  cells per ml) and streaked on plates of LLB containing mecillinam in a series of threefold-increasing concentrations (0.03 to 100  $\mu$ g/ml). Incubations were carried out overnight at 30 and 42°C.

**Culture of cells for preparation of membrane fraction and assay of PBPs.** Cells were cultured as described previously (22, 24). Merodiploids and their control cells were cultured in appropriate media to the stationary phase of growth, centrifuged, suspended in an equal volume of LLB (for *lip<sup>+</sup> lac<sup>+</sup>/lip lac* merodiploids containing an F-prime factor covering the chromosomal region *lip-lac*, without lipoic acid), and cultured at 42°C for 1.5 h (ca.  $4 \times 10^8$  cells per ml). Cells were harvested by centrifugation, and the membrane fraction was prepared by sonicating the cells as described previously (22, 24). PBPs were assayed by

TABLE 1. *E. coli* K-12 strains used

Strain	Genotype	Source or derivation
AT1325lip9	F <sup>-</sup> <i>lip-9 thi-1 proA2 his-4 purB15 mtl-1 xyl-5 galK12 lacY1 str-35</i>	CGSC4286
TMM1	As AT1325lip9 but <i>mrda1</i>	Spontaneous <i>mrda</i> mutant of AT1325lip9
TMM2	As AT1325lip9 but <i>mrda2</i>	Spontaneous <i>mrda</i> mutant of AT1325lip9
TMM3	As AT1325lip9 but <i>mrda3</i>	Spontaneous <i>mrda</i> mutant of AT1325lip9
TMM4	As AT1325lip9 but <i>mrdb4</i>	Spontaneous <i>mrdb</i> mutant of AT1325lip9
TMM5	As AT1325lip9 but <i>mrda5</i>	Spontaneous <i>mrda</i> mutant of AT1325lip9
TMM6	As AT1325lip9 but <i>mrdb6</i>	Spontaneous <i>mrdb</i> mutant of AT1325lip9
TMM11	F <sup>-</sup> <i>lip-9 thi-1 proA2 purB15 mtl-1 xyl-5 galK12 lacY1 mrda1 recA str-35</i>	Recombinant from KL16-99 × TMM1
TMM13	F <sup>-</sup> <i>lip-9 thi-1 proA2 purB15 mtl-1 xyl-5 galK12 lacY1 mrda3 recA str-35</i>	Recombinant from KL16-99 × TMM3
TMM14	F <sup>-</sup> <i>lip-9 thi-1 proA2 purB15 mtl-1 xyl-5 galK12 lacY1 mrdb4 recA str-35</i>	Recombinant from KL16-99 × TMM4
TMM16	F <sup>-</sup> <i>lip-9 thi-1 proA2 purB15 mtl-1 xyl-5 galK12 lacY1 mrdb6 recA str-35</i>	Recombinant from KL16-99 × TMM6
SA51	F <sup>-</sup> <i>thi-1 proA2 his-4 purB15 mtl-1 xyl-5 galK12 lacY1 rodA51 str-35</i>	Lip <sup>+</sup> <i>rodA</i> (12) transductant of AT1325lip9
BW113	Hfr (P4X) <i>metB1</i> λ <sup>-</sup>	CGSC 4312
KL16-99	Hfr (KL16) <i>thi-1 rel-1</i> λ <sup>-</sup> <i>recA</i>	K. Yoda
TMM23	F <sup>-</sup> <i>thi-1 proA2 his-4 purB15 mtl-1 xyl-5 galK12 lacY1 mrda3 str-35</i>	Lip <sup>+</sup> transductant of TMM3
TMM24	F <sup>-</sup> <i>thi-1 proA2 his-4 purB15 mtl-1 xyl-5 galK2 lacY1 mrdb4 str-35</i>	Lip <sup>+</sup> transductant of TMM4
TMMP3	Hfr (P4X) <i>his-4 mrda3</i>	Recombinant from BW113 × TMM23
TMMP4	Hfr (P4X) <i>his-4 mrdb4</i>	Recombinant from BW113 × TMM24
SAP51	Hfr (P4X) <i>rodA51</i>	Recombinant from BW113 × SA51
ORF4/KL251	F254 ( <i>lac</i> <sup>+</sup> <i>lip</i> <sup>+</sup> )/KL251 ( <i>leuB proC pure trpE recA metE thi ara lacZ xyl mtl azi rpsL tonA tsx</i> λ <sup>-</sup> <i>supE</i> )	CGSC 4282
RL1	F <sup>-</sup> <i>leuS31 thi-1 proA2 his-4 purB15 mtl-1 xyl-5 galK12 lacY1 str-35</i>	D. Söll

sodium dodecyl sulfate-acrylamide slab gel electrophoresis of [<sup>14</sup>C]penicillin G-protein complexes and then by fluorography, as described previously (22, 24). For assay of the thermostability of PBPs, membrane fractions were heated at 43°C for 5 min in 0.05 M sodium phosphate buffer (pH 7.0) before binding of [<sup>14</sup>C]penicillin G at 43°C was measured. As controls, membranes were directly incubated with [<sup>14</sup>C]penicillin G at 30°C.

**Mating, transduction with bacteriophage P1, and F-duction.** The methods used for mating (25), P1 transduction (8), and F-duction (9, 15) were essentially as described previously.

**Reagents.** [<sup>14</sup>C]penicillin G (50 Ci/mol) was a product of the Radiochemical Centre, Amersham, England. Mecillinam was kindly supplied by Leo Pharmaceutical Products, Denmark, and Takeda Chemical Industries, Osaka, Japan. Other chemicals were standard commercial products.

## RESULTS

**Isolation of *E. coli* *mrda* and *mrdb* mutants forming spherical, mecillinam-resistant cells at 42°C.** About 5 × 10<sup>8</sup> cells of parent strain AT1325lip9 were spread on a plate of LLB minus NaCl containing 90 µg of mecillinam per ml. The plate was incubated at 42°C, and mecillinam-resistant colonies, which appeared at a frequency of ca. 10<sup>-7</sup>, were isolated. The morphology of the cells was observed microscopically after incubation at 30 and 42°C. From among 15 isolated mutants that showed mecillinam resistance and spherical morphology at 42°C, those with mutations close to the *lip* gene (six strains, TMM1 to TMM6) were selected after *lip*<sup>+</sup> transduction with phage P1 (90 to 95% cotransduction). The other nine strains had a

mutation linked to *aroE* (23% cotransduction). Two types of mutants with *lip*-linked mutations could be distinguished. Group A mutants (strains TMM1, TMM2, TMM3, and TMM5) had wild-type phenotypes at 30°C, i.e., were rod-shaped and mecillinam sensitive (minimal inhibitory concentration, about 0.1 µg/ml). Group B mutants (strains TMM4 and TMM6) at 30°C formed spherical cells and were mecillinam supersensitive (minimal inhibitory concentrations, 0.03 µg/ml for strain TMM4 and 0.01 µg/ml for strain TMM6). At 42°C all mutants of both groups formed spherical cells that were mecillinam resistant (minimal inhibitory concentration, ca. 100 µg/ml). The mecillinam supersensitivity caused by the *mrdB* mutations was confirmed subsequently with isogenic strains carrying the *mrdB*<sup>+</sup> and *mrdB*<sub>4</sub> genes.

Four group A mutant strains had thermosensitive PBP-2 when cultured at 30°C and no PBP-2 activity when cultured at 42°C. On the contrary, two group B mutant strains showed thermostable (wild-type) PBP-2 when cultured at either 30 or 42°C, although there seemed to be slightly less PBP-2 when the strain was cultured at 42°C. The *rodA* mutant previously isolated by H. Matsuzawa et al. (12) also had thermostable PBP-2.

In both group A and group B strains, the mutations seemed to be related to formation of the peptidoglycan (called murein by Weidel and Pelzer [26]) sacculus, and the genes involved seemed to be closely linked to each other. Therefore, these mutations are referred to as *mrD* (murein *D*) -A and *mrD*B, respectively. Clusters of genes *mra* (14) and *mrB* (14) and gene *mrc* for PBP-1Bs (24) have been reported previously.

The nine mutants in which the mutation was linked to *aroE* were also spherical and mecillinam resistant (minimal inhibitory concentration, about 100 µg/ml) at 42°C and had thermostable (wild-type) PBP-2. The mutation may be closely related to *rodY* (6), *envB* (27), or *slo* (27) mutations but details about these nine mutants will not be described here.

**Dominance studies of *mrDA* and *mrDB* mutations.** The purpose of the present study was to obtain proof that the mutations *mrDA* and *mrDB*, which cause formation of spherical cells and high resistance to mecillinam at 42°C, are different and are due to mutations of different genes located near *lip* on the chromosome. This proof could be obtained in studies of merodiploids carrying these mutations. Thus, dominance studies of these two mutations were made. F-prime strain ORF4/KL251, carrying the part of the chromosome covering *lac* to *lip*, was used as the donor of the F-prime factor. *recA* derivatives of the mutants were obtained by

crossing Hfr KL16-99 (*recA*) with each of the mutants TMM1, -3, -4, and -6 (F<sup>-</sup> *lip his lac rps mrDA* or *mrDB*) (His<sup>+</sup> Str<sup>r</sup> selection; Table 1). The five merodiploids were prepared from two *mrDA* strains (TMM11 and TMM13) and two *mrDB* strains (TMM14 and TMM16) by Lip<sup>+</sup> Lac<sup>+</sup> selection. They all showed the wild-type phenotypes of cell shape and mecillinam resistance. The putative *mrDA*<sup>+</sup> *mrDB*<sup>+</sup>/*mrDA* *mrDB*<sup>+</sup> meroheterodiploids contained a large amount of thermostable (wild-type phenotype) PBP-2. By eliminating the F-prime factor, spontaneously as well as in the presence of acridine orange, both *mrDA*<sup>+</sup> *mrDB*<sup>+</sup>/*mrDA* *mrDB*<sup>+</sup> and *mrDA*<sup>+</sup> *mrDB*<sup>+</sup>/*mrDA*<sup>+</sup> *mrDB* merodiploids recovered their respective mutant phenotypes. Thus, it can be concluded that the *mrDA* and *mrDB* mutations are both recessive in heterozygotes.

**Complementation studies of *mrDA* and *mrDB* mutations.** To demonstrate complementation of genes *mrDA* and *mrDB*, two series of meroheterodiploids carrying *mrDA* and *mrDB* mutations on their chromosome and episome were prepared. One series of strains carried *mrDA* mutations on the chromosome and *mrDB* or *rodA* mutations on the episome, and the other series of strains carried *mrDB* mutations on the chromosome and *mrDA* mutation on the episome. For preparing these meroheterodiploids, Hfr strains carrying the *mrDA* or *mrDB* (*rodA*) mutations were isolated by mating BW113 (Hfr P4X *metB1*) with TMM23, TMM24, or SA51 (F<sup>-</sup> *mrDA* *mrDB* or *rodA* *lip*<sup>+</sup> *lac*). Lac<sup>+</sup> and Met<sup>+</sup> recombinants were selected (strains TMMP3, TMMP4, and SAP51). About 80% of the selected recombinants were Hfr. Mating of Hfr strains carrying *mrDA* or *mrDB* (*rodA*) mutations (strain TMMP3, TMMP4, or SAP51) and F<sup>-</sup> strains carrying *mrDA* or *mrDB*, *lip*, *lac*, and *recA* (TMM11, TMM13, TMM14, and TMM16) on the selection plate for Lip<sup>+</sup> and Lac<sup>+</sup> resulted in isolation of meroheterodiploids (9, 15). Five strains were isolated independently that were supposed to have the following combinations (total, 30 strains): *mrDA*3 *mrDB*<sup>+</sup>/*mrDA*<sup>+</sup> *mrDB*4, *mrDA*3 *mrDB*<sup>+</sup>/*mrDA*<sup>+</sup> *mrDB*6, *mrDA*<sup>+</sup> *mrDB*4/*mrDA*1 *mrDB*<sup>+</sup>, *mrDA*<sup>+</sup> *mrDB*4/*mrDA*3 *mrDB*<sup>+</sup>, *mrDA*<sup>+</sup> *rodA*51/*mrDA*1 *mrDB*<sup>+</sup>, *mrDA*<sup>+</sup> *rodA*51/*mrDA*3 *mrDB*<sup>+</sup>.

All of the isolated meroheterodiploids showed wild-type phenotypes, i.e., rod-shaped morphology and mecillinam sensitivity, at both 30 and 42°C, and they also had thermostable (wild-type) PBP-2 (Fig. 1). After elimination of the F-prime factor spontaneously or after the addition of acridine orange, all of the strains regained the mutant phenotypes owing to mutations on their chromosome. As a control experiment, merodiploids that carried the following allelic muta-

## Penicillin Binding Protein

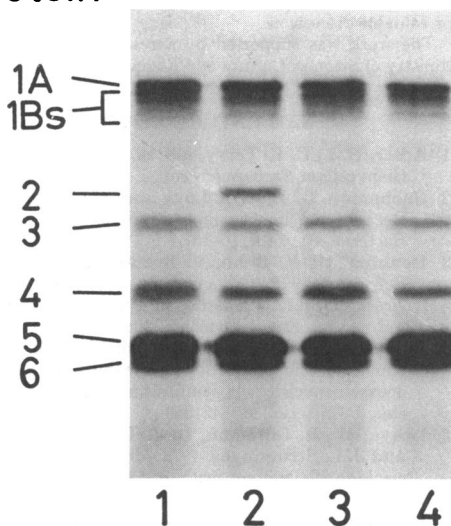


FIG. 1. PBPs of the *mrda mrdb*<sup>+</sup> strain and the *mrda*<sup>+</sup> *mrdb*/*mrda mrdb*<sup>+</sup> and *mrda mrdb*<sup>+</sup>/*mrda mrdb*<sup>+</sup> meroheterodiploids. Membranes were prepared from cells cultured at 42°C, and the binding experiment with [<sup>14</sup>C]penicillin G was performed at 43°C. A fluorograph is shown. For experimental procedures, see the text. 1, *mrda1 mrdb*<sup>+</sup> mutant strain TMM11; 2, *mrda*<sup>+</sup> *mrdb4*/*mrda1 mrdb*<sup>+</sup> meroheterodiploid; 3, *mrda1 mrdb*<sup>+</sup> mutant obtained by curing 2; 4, *mrda3 mrdb*<sup>+</sup>/*mrda1 mrdb*<sup>+</sup> meroheterodiploid.

tions were isolated in the same way: *mrda3 mrdb*<sup>+</sup>/*mrda1 mrdb*<sup>+</sup>, *mrda3 mrdb*<sup>+</sup>/*mrda3 mrdb*<sup>+</sup>, *mrda*<sup>+</sup> *mrdb4*/*mrda*<sup>+</sup> *mrdb4*, *mrda*<sup>+</sup> *mrdb4*/*mrda*<sup>+</sup> *mrdb6*, *mrda*<sup>+</sup> *rodA51*/*mrda*<sup>+</sup> *mrdb4*, and *mrda*<sup>+</sup> *rodA51*/*mrda*<sup>+</sup> *mrdb6*. Five strains for each combination were isolated independently. These strains showed no recovery of wild-type phenotypes (for PBPs, see Fig. 1).

It can be concluded, that genes *mrda* and *mrdb* are in different complementation groups.

We failed to obtain *rodA*/*mrdb* merodiploids with wild-type phenotypes, suggesting, but not proving, that these two genes are identical.

**Order of *leuS*, *mrda*, *mrdb*, and *lip*.** The map order of *leuS*, *mrda*, *mrdb*, and *lip* was determined by transduction with phage P1. Three-point crosses with the *lip mrda* or *lip mrdb* strain as a donor and the *leuS* strain as a recipient (*leuS*<sup>+</sup> selection) or the *leuS* strain as a donor and the *mrda lip* or *mrdb lip* strain as a recipient (*lip*<sup>+</sup> selection) indicated (Table 2) that the most probable gene order is *leuS*-*mrda* or *mrdb*-*lip*. Further mapping of the order of

TABLE 2. Cotransduction frequencies of *mrda* and *mrdb* with *leuS* and *lip*<sup>a</sup>

Expt	Genotype of transductants				No. of transductants
	<i>leuS</i>	<i>mrda</i>	<i>mrdb</i>	<i>lip</i>	
1	1	0		0	31
	1	0		1	3
	1	1		0	19
	1	1		1	52
2	1		0	0	20
	1		0	1	0
	1		1	0	3
	1		1	1	39
3	0		0	1	43
	0		1	1	53
	1		0	1	2
	1		1	1	89

<sup>a</sup> 1, Genetic markers derived from the donor; 0, those derived from the recipient. Selection was made for *LeuS*<sup>+</sup> or *Lip*<sup>+</sup>. The following strains were used. Experiment 1: Donor, TMM3 (*leuS*<sup>+</sup> *mrda lip*) (1 1 1); recipient, RL1 (*leuS mrda*<sup>+</sup> *lip*<sup>+</sup>) (0 0 0). Experiment 2: donor, TMM4 (*leuS*<sup>+</sup> *mrdb lip*) (1 1 1); recipient, RL1 (*leuS mrdb*<sup>+</sup> *lip*<sup>+</sup>) (0 0 0). Experiment 3: donor, RL1 (*leuS mrdb*<sup>+</sup> *lip*<sup>+</sup>) (1 1 1); recipient, TMM4 (*leuS*<sup>+</sup> *mrdb lip*). The *leuS*<sup>+</sup> genotype was estimated by the thermoresistant growth of the strain at 42°C in the absence of leucine, and *mrda* and *mrdb* were evaluated by strain morphology and mecillinam resistance at 30 and 42°C. An experiment carried out with RL1 (*leuS mrda*<sup>+</sup> *lip*<sup>+</sup>) as the donor and TMM3 (*leuS*<sup>+</sup> *mrda lip*) as the recipient (*Lip*<sup>+</sup> selection) gave similar results. The frequency of cotransduction of *mrdb* with *lip* (76%) in experiment 3 was low for unknown reasons.

*mrda* and *mrdb* with respect to *lip* was carried out by P1 transduction between *mrda* and *mrdb* strains, with selection for *Lip*<sup>+</sup>. The frequencies of recombinant cells with the wild-type phenotype should indicate the order of these two mutations with respect to *lip*. Two different recipient strains were used for each donor strain. Five wild-type recombinants were isolated from 200 *lip*<sup>+</sup> transductants when a *mrda3 lip*<sup>+</sup> mutant (strain TMM23) was used as a donor and a *mrdb4 lip* mutant (strain TMM4) was used as a recipient. In contrast, only one wild-type recombinant was isolated from 200 *lip*<sup>+</sup> transductants when a *mrdb4 lip*<sup>+</sup> mutant (strain TMM24) was used as a donor and *mrda3 lip* mutant (strain TMM3) was used as a recipient. Assuming that double mutants with the genotype *mrda mrdb* have mutant phenotypes, wild-type recombinants should have the genotype *mrda*<sup>+</sup> *mrdb*<sup>+</sup>. Thus, the above results favor the suggestion that wild-type recombinants were formed in the cross of the *mrda3*

*lip*<sup>+</sup> donor with the *mrdb4 lip* recipient from a double crossover and in the cross of the *mrdb4 lip*<sup>+</sup> donor with the *mrda3 lip* recipient from a quadruple crossover. The most plausible gene order may be *leuS-mrda3-mrdb4-lip*. The results became a little more inconclusive, however, when the *mrdb6 lip* strain (TMM6) was used as a recipient in the cross with strain TMM23. Only one wild-type recombinant was isolated from 200 *lip*<sup>+</sup> transductants. In the cross TMM24 × TMM1, no wild-type recombinants were isolated from 200 *lip*<sup>+</sup> transductants. Further investigation may be necessary to draw a conclusion.

### DISCUSSION

The exact function of PBP-2 is still unknown. Spratt (20, 21) isolated spherical mutants possessing thermosensitive PBP-2 and thus suggested that this protein acts transiently in the cell cycle to ensure that elongation at newly introduced growth sites occurs in the correct rod configuration (see also reference 7). It is tempting to consider that this protein functions as a transpeptidase cross-linking a specific site on the peptidoglycan sacculus. PBPs have been extracted from *E. coli* membranes with detergents (16), and recently peptidoglycan-polymerizing enzyme (transglycosylase) and cross-linking enzyme (transpeptidase) activities were demonstrated in a purified preparation of PBP-1Bs (17), but no enzymatic activities have so far been demonstrated in preparations of PBP-2. Assuming however that PBP-2, the *mrda* product, functions as a transpeptidase (and probably also as a transglycosylase), then what function could the *mrdb* product have? One possibility is that this protein functions as a peptidoglycan lytic enzyme that forms a nick in a specific position of the peptidoglycan sacculus to ensure that the transpeptidase (and probably also the transglycosylase) inserts new peptidoglycan fragments at a certain position in the sacculus. A defect in this specific lytic enzyme may cause formation of spherical, mecillinam-resistant cells. This lytic enzyme activity could, however, just as well be the product of another rod gene(s), such as that linked to *aroE*. It is also possible, that the *mrdb* product is involved in some regulatory mechanism concerning formation of a rod-shaped peptidoglycan sacculus, but no evidence for this has yet been obtained. Cyclic AMP may be involved in these processes, because its absence in *cya* or *crp* cells of *E. coli* causes formation of spherical cells and mecillinam resistance (28).

Although the product of *mrdb* is still unknown, the present work provides new evidence that the genes involved in formation of the peptidoglycan sacculus are at different positions in the same cluster on the chromosome (13, 14).

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