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Further Studies on *rodA* Mutant: a Round Morphological Mutant of *Escherichia coli* K-12 with Wild-Type Penicillin-Binding Protein 2

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Escherichia coli rodA mutant AOS151 grows as round cells at 30 and 42°C (H. Matsuzawa, K. Hayakawa, T. Sato, and K. Imahori, *J. Bacteriol.*, **115**, 436~442 (1973)). The mutant was found to be resistant to mecillinam at both temperatures. *lip*$^+$ transductants were prepared by P1 phage transduction *via* strain AOS151, the cotransduction frequency of round morphology (Rod$^-$) at 42°C with the *lip* gene being about 90%. At 42°C all 54 Rod$^-$ transductants tested were resistant to mecillinam. At 30°C all but two of these Rod$^-$ (at 42°C)-type transductants were rod-shaped, and all were sensitive to mecillinam; the two strains grew as ovoid cells. The original *rodA* mutant AOS151 probably involves an additional mutation(s), that expresses the round cell shape at lower temperature, whereas the *rodA51* mutation alone seems to result in temperature-sensitive expression of round cell morphology and mecillinam resistance. *rodA* mutant cells cultured at either 30 or 42°C had wild-type penicillin-binding protein 2, judging from penicillin-binding activity, electrophoretic mobility, and thermosensitivity.

Several mutants of *Escherichia coli* K-12 with round morphology have been isolated as radiation-resistant,[1] ampicillin-sensitive,[2] temperature-sensitive,[3] or mecillinam (6β-amidinopenicillanic acid derivative[4])-resistant mutants.[5~10] Moreover, *cya* and *crp* mutants[11] have also been found to have round cells in non-permissive conditions. Matsuzawa *et al.*[12] previously isolated a round-cell mutant of *E. coli* by chance among a group of acridine orange-sensitive mutants obtained by mutagenization with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and found that the gene responsible for the rod shape, designated as *rodA*, is closely located to the *lip* gene (14.2 min on the *E. coli* genetic map[13]).

Mecillinam is known to induce round cells of *E. coli*[4] and *Pseudomonas aeruginosa*.[14] In studies on the mechanism of action of this unique antibiotic, a number of mecillinam-resistant mutants of *E. coli* have been isolated and their biological and genetical character have been studied.[5~10] Critical findings in these studies were the observations of Spratt that mecillinam binds extensively to penicillin-binding protein (PBP) 2[15] and that in one mecillinam-resistant mutant, which had round cells, [^{14}C]benzylpenicillin and [^{14}C]mecillinam did not bind to PBP-2.[6]

From genetical studies on mecillinam-resistant, round-cell mutants of *E. coli*, Iwaya *et al.*[7] classified the mutants into *rodX* mutants linked to the *lip* gene and *rodY* mutants linked to *aroE* (71.7 min[13]). They concluded that their *rodX* mutation, which they sometimes call *rodA*, probably occurs in the gene for PBP-2.[8]

Tamaki, Matsuzawa, and Matsuhashi[10] analyzed mecillinam-resistant, morphological mutants of *E. coli* in more detail. They found that there are at least two closely linked genes, *mrdA* and *mrdB*, at about 14.5 min on the *E. coli* genetic map, which are responsible for

The following abbreviations are used: Rod$^-$, symbol for round morphology; PBP, penicillin-binding protein; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

the rod shape and mecillinam sensitivity. They identified the product of the *mrdA* gene as PBP-2, but that of *mrdB* is unknown. The *mrdB* gene is genetically indistinguishable from *rodA51* by complementation tests with *mrdB* and *rodA51* in meroheterodiploids. Complementation tests with *mrdA* and *rodA51* showed that the *rodA* gene is in a different complementation group from *mrdA*, the gene for PBP-2. The most plausible gene order was proposed to be *leuS-mrdA-mrdB(rodA)-lip*.[10] B.G.Spratt (personal communication) determined this gene order using λ phage carrying the respective parts of the chromosome.

This paper describes the penicillin-binding activity of PBP-2 and temperature-dependent changes of cell shape and mecillinam sensitivity of the original *rodA* mutant AOS151 and Rod$^-$ (at 42°C)-type transductants. The results suggest that strain AOS151 is not a mutant of PBP-2 and also that, in addition to *rodA51*, it may have some mutation(s) concerning cell morphology.

MATERIALS AND METHODS

Bacterial strains. The properties of the *E. coli* K-12 strains used are summarized in Table I. Transducing phage P1 was obtained from Y. Sugino.

Media. Bacteria were cultured at 30, 37, or 42°C with shaking in modified L-broth[12] supplemented with 20 mg thymine per liter. K10 medium[12] was used for transduction experiments. The amino acids and nucleotides required were added at final concentrations of 50 μg per ml, thiamine was added at 1 μg per ml, and lipoic acid was added at 50 ng per ml (also added to L-broth). Media were solidified with 1.5% agar.

Transduction technique. The procedure used[12] was based on that described by Lennox.[16] P1 phage lysate was prepared on the *rodA* or Rod$^-$ (at 42°C) strain and used to transduce *lip*$^+$ into strain AT1325 lip9. The shape of *lip*$^+$ transductant cells grown overnight at 30 or 42°C on L-agar plates was examined with a phase-contrast microscope.

Determination of sensitivity to mecillinam. Appropriate dilutions of overnight cultures at 37°C of each strain were mixed with 9 ml of L-agar (1%), and then poured into petri dishes with 1 ml of L-broth containing 100 μg of mecillinam. The plates were incubated at 30 or 42°C for 2 or 3 days, and then numbers of colonies formed (less than 300 per plate) were counted.

Detection of penicillin-binding proteins. The methods used[17] were essentially those of Spratt and Pardee.[15] Cells were cultured overnight at 37°C, and then 0.2 ml of the culture was inoculated into 200 ml of fresh medium. Bacteria were grown at 30 or 42°C for about 5 hr (*ca.* 3×10^8 cells per ml). Washed membranes were prepared from the cells as described previously.[15] For assay of PBPs, the membranes (*ca.* 600 μg of protein) from cells grown at 30°C were incubated at 30, 43, or 45°C for 10 min, and those from cells cultured at 42°C were incubated at 30 or 43°C for 10 min with [^{14}C]benzylpenicillin (50 mCi/mmol, 3 nmol). The [^{14}C]benzylpenicillin–protein complexes were separated by sodium dodecyl sulfate/acrylamide slab gel electrophoresis and located by fluorography as described previously.[15,17]

The penicillin-binding activity of PBP-2 in membranes of cells grown at 30°C was measured by counting the radioactivity in dried slices of PPO (2,5-diphenyloxazole)-impregnated slab gel in toluene–PPO–POPOP

TABLE I. STRAINS OF *Escherichia coli* K-12 USED

Strain	Genotype[a]	Source or derivative
JE1011	F$^-$ *thr leu trp his thy thi ara lac gal xyl mtl strA azi*	M. Ishibashi
AOS151	As JE1011 but *rodA51*, other *rod* mutation(s)	NTG-induced from JE1011 (12)
AT1325 lip9	F$^-$ *thi-1 his-4 purB15 proA2 mtl-1 xyl-5 galK2 lacY1 lip-9 str-35*	CGSC4286 (12)
R1	As AT1325 lip9 but *lip*$^+$	P1 transduction *via* AOS151 (12)
S1	As AT1325 lip9 but *lip*$^+$ *rodA51*	P1 transduction *via* AOS151 (12)
S2	As AT1325 lip9 but *lip*$^+$ *rodA51*, other *rod* mutation(s)	P1 transduction *via* AOS151 (12)
S2Revl	As AT1325 lip9 but *lip*$^+$ *rodA51*	Spontaneous revertant from S2, this paper
S4b	As AT1325 lip9 but *lip*$^+$ *rodA51*	P1 transduction *via* AOS151 (12)
SA51	As AT1325 lip9 but *lip*$^+$ *rodA51*	P1 transduction *via* AOS151 (10)

[a] The genetic symbols used are those described by Bachmann and Low.[18]

[2,2′-*p*-phenylene-bis-(5-phenyloxazole)] (1 liter: 4 g: 0.1 g) in a scintillation spectrometer at a counting efficiency of 78%, as described previously.[16]

Chemicals.. Mecillinam was a gift from F. Lund, Leo Pharmaceutical Products, Ballerup, Denmark. [^{14}C]Benzylpenicillin was a product of the Radiochemical Centre, Amersham, England. Other chemicals were standard commercial products.

RESULTS

Cell shape and mecillinam sensitivity of Rod⁻ strains

Rod⁻ transductants were prepared from strain AT1325 lip9 (recipient) by transduction with P1 phage grown on *rodA* mutant AOS151 (*lip*⁺ selection). The cotransduction frequency of the Rod⁻ phenotype at 42°C with *lip* was 86% (523 Rod⁻ at 42°C among 609 *lip*⁺ transductants). Table II shows the cell shapes and surviving fractions of the *rodA*⁺ parent, *rodA* mutant AOS151 and several transductants on plates containing 10 μg mecillinam per ml. The parental strain JE1011 and Rod⁺ transductant R1 were sensitive to mecillinam at 30 and 42°C. The original *rodA* mutant AOS151 grew as round cells at 30 and 42°C as described previously[12] and was resistant to 10 μg per ml of mecillinam at both temperatures. On the contrary, strain S1, S2, S4b, and SA51, the transductants with the Rod⁻ phenotype at 42°C, had round cells and were resistant to 10 μg per ml of mecillinam at 42°C. In contrast, at 30°C strains S1, S4b, and SA51 were rod-shaped and were sensitive to mecillinam to various degrees. The only exception was strain S2, which grew as ovoid cells and was sensitive to this antibiotic at 30°C. In another experiment, only one transductant with a phenotype similar to that of strain S2 was isolated from among 50 Rod⁻ (at 42°C)-type transductants, the other transductants having other phenotypes such as those of strains S1, S4b, and SA51 as mentioned above. This suggests that the *rodA51* mutation caused temperature-sensitive changes in cell shape and mecillinam sensitivity.

When the *rodA*-type transductant SA51 was used to transduce *lip*⁺ into strain AT1325 lip9, the Rod⁻ phenotype at 42°C was again cotransducible with the *lip* gene at a frequency of 87% (229 Rod⁻ at 42°C among 262 *lip*⁺ transductants). The changes of cell shape and mecillinam sensitivity of these Rod⁻ (at 42°C)-type transductants depending on the growth temperature were essentially the same as those of strain SA51, the *rodA* donor for transduction.

These results also suggest that the original *rodA* mutant AOS151 may have some additional mutation(s), which is responsible for the round morphology at 30°C. Probably when the *rodA51* mutation is present with another mutation(s), cells such as strains AOS151 and S2 are round at 30°C. These two strains could not grow on NaCl-free L-agar plates at 42°C.

TABLE II. CELL MORPHOLOGY AND MECILLINAM SENSITIVITY OF Rod⁺ AND Rod⁻ STRAINS

Strain	Growth temperature (°C)			
	30		42	
	Cell shape	Survival[a]	Cell shape	Survival[a]
JE1011	Rod	2.9×10^{-5}	Rod	3.0×10^{-5}
AOS151	Round	4.0×10^{-1}	Round	1.0
R1	Rod	1.7×10^{-5}	Rod	6.6×10^{-4}
S1	Rod	2.3×10^{-5}	Round	4.5×10^{-1}
S2	Ovoid	2.0×10^{-4}	Round	7.2×10^{-1}
S4b	Rod	3.6×10^{-4}	Round	7.1×10^{-1}
SA51	Rod	2.2×10^{-5}	Round	3.4×10^{-1}

[a] Sensitivity to mecillinam was examined in L-agar plates containing 10μg mecillinam per ml as described in the MATERIALS AND METHODS. Colony numbers were compared with those in plates containing no mecillinam.

Spontaneous revertant S2Rev1, which could grow in the same conditions, was obtained from strain S2. This revertant had the same phenotype as the Rod$^-$ (at 42°C)-type transductants S1, S4b, and SA51 (data not shown). When strain S2 was used to transduce *lip*$^+$, however, 64 Rod$^-$ (at 42°C)-type transductants examined were all strain S2-type transductants, which were ovoid at 30°C and round at 42°C, the cotransduction frequency with the *lip* gene being 88% (64 Rod$^-$ among 73 *lip*$^+$ transductants).

Penicillin-binding proteins of the rodA mutant

The penicillin-binding proteins of the membranes of *rodA* strains were studied. The original *rodA* mutant AOS151 and seven *rodA* transductants grown at 30°C appeared to have normal PBPs when compared with those of the parental strain JE1011 and isogenic *rodA*$^+$ strain R1. Figure 1 shows that PBP-2 of *rodA*$^-$ strains had similar heat-sensitivity to that of wild-type strains. When cells were grown at 42°C, six *rodA* transductants tested also had wild-type PBPs (data not shown).

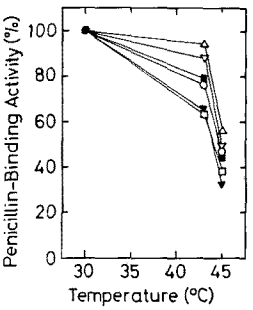

FIG. 1. Heat-Sensitivity of Penicillin-Binding Activity of PBP-2 in Membranes of *rodA*$^+$ and *rodA* Strains.

The membranes of cells grown at 30°C were prepared as described previously,[15,17] and incubated with [^{14}C]benzylpenicillin for 10 min at the indicated temperatures. The resulting [^{14}C]benzylpenicillin-PBP-2 complexes were obtained and their radioactivities were measured as described in the MATERIALS AND METHODS. The percentages of the radioactivity in PBP-2 in strains JE1011 (○), AOS151 (△), R1 (■), S1 (□), S2 (▼), and S4b (▽) were calculated.

DISCUSSION

The *E. coli rodA51* mutation described in this paper seemed to cause temperature-sensitive changes in cell morphology and mecillinam sensitivity.

Membranes of the *rodA* mutant grown at 30°C showed normal PBP-2 with respect to its electrophoretic mobility and penicillin-binding activity assayed at 30 and 43°C, and cells cultured at 42°C also had a normal amount of PBP-2. Tamaki *et al.*[10] recently reported that two genes, *mrdA* and *mrdB*, are responsible for the rod shape and mecillinam sensitivity and are located between genes *lip* and *leuS* on the *E. coli* chromosome. They demonstrated that the *mrdA* gene is the structural gene for PBP-2, which was referred to as *pbpA* by Spratt,[19] and that the *mrdA* gene is a different complementation group from *mrdB* and *rodA*.* Although the *rodA* and *mrdB* genes have so far been indistinguishable from each other, it is still uncertain whether they are identical, because their products are unknown.

We have not yet enough information to understand why the *rodA* mutant is resistant to mecillinam despite the fact that PBP-2 is the target protein of this antibiotic.[15] As discussed by Tamaki *et al.*,[10] however, one possible explanation is that the *rodA* product functions as a peptidoglycan lytic enzyme forming a nick at a specific position of the peptidoglycan sacculus, to ensure that transpeptidase or transglycosylase, which might be assumed for PBP-2, inserts new peptidoglycan fragments at a certain site in the sacculus. If so, the *rodA* mutant could not give the position where PBP-2 expresses the activity. Accordingly, *rodA* mutants seem to be resistant to mecillinam. This hypothetic role of the *rodA* product may, however, also be ascribed with equal probabilities to products of other *rod* genes such as *envB*[2] and *rodY*.[7]

Moreover, transduction experiments using the original *rodA* mutant AOS151 indicated

* The name *rodA* for the structural gene for PBP-2 is misleading,[8,9] because the *rodA* gene is distinctly different from the structural gene for PBP-2, *mrdA*.

the possibility that at least one other gene, which may be located close to *rodA* and *lip*, functions in expression of the rod-shaped morphology. However, on the contrary, results obtained using strain S2 as a donor for transduction suggest that the strain S2-type phenotype could be caused by a second mutation in the *rodA* gene. Nothing is known about this supposed mutation and further work on this problem is necessary.

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