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Mapping of the Mecillinam-Resistant, Round Morphological Mutants of *Escherichia coli*

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Genes responsible for round morphology in mecillinam-resistant, round morphological mutants of *Escherichia coli* have been mapped. Three mutants, called *rodX*, mapped at around 14 min, and two, called *rodY*, mapped at around 70 min by P1 transduction. These are either the same or very close to the loci reported, respectively, for the *rodA* (H. Matsuzawa, K. Hayakawa, T. Sato, and K. Imahori, J. Bacteriol. **115**:436-442, 1973) and *envB* genes (B. Westling-Häggström and S. Normark, J. Bacteriol. **123**:75-82, 1975). This suggests that mecillinam can be used very efficiently to select for round morphological mutants of *rodA* and *envB* after nitrosoguanidine treatment.

A new penicillin, mecillinam (6-β-amidinopenicillanic acid; FL1060) (15), has a remarkably different phenotypic effect on *Escherichia coli* than other β-lactam antibiotics (19). At low concentrations of this antibiotic (10 μg/ml or less), *E. coli* becomes spherical and eventually dies. Matsuhashi et al. (17) isolated mutants of *E. coli* that were resistant to the amidino-penicillin after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment and reported a very high frequency of mutants with round morphology among resistant mutants (i.e., 5 out of 31 resistant mutants had a round morphology). Several morphological mutants of *E. coli* have been reported in the literature, with several of them having round morphology (2, 3, 9, 11-13, 16, 18, 21, 26). Since there are only four round *E. coli* mutants with a known mapping position, *rodA* (18), *envB* (21), *cya*, and *crp* (12), an attempt was made to map the mutations that resulted in round morphology of these mecillinam-resistant mutants. All the round morphology genes tested were cotransducible with either *lip* or *aroE*, which suggested that these round morphology mutants were similar or identical to *rodA* or *envB* mutants.

MATERIALS AND METHODS

Organisms. *E. coli* strains and their relevent properties are summarized in Table 1. Several strains in Table 1 were obtained from Coli Genetic Stock Center, Yale University School of Medicine, New Haven, Conn. Generalized transducing phage P1*vir*-1 was a gift from H. Ikeda.

Media and chemicals. Rich medium DYABT, is DYAB medium (17) plus 10 μg of thymine per ml. Minimal medium is M9 (1), with amino acids added to a concentration of 20 μg each, 10 μg of thymine, uracil, and adenine, and 1 μg of thiamine per ml. Lipoic acid (DL-6,8-thioctic acid) was purchased from Sigma Chemical Co., St. Louis, Mo. Shikimic acid was purchased from Calbiochem, La Jolla, Calif. Mecillinam was a gift from Leo Pharmaceutical Co., Copenhagen, Denmark.

Techniques in genetics. Transduction, conjugation, and other techniques in bacterial genetics were carried out by the method of Adams (1) and Miller (20).

Sensitivity to antibiotics. Bacterial strains were cultured overnight, and, after appropriate dilution, a 0.05-ml sample was spread on DYABT-rich agar plates supplemented with various concentrations of the drug to be tested. The plates were incubated for 2 to 3 days at 30°C, after which the colony numbers were counted.

RESULTS

Mapping of the round morphology gene. A conjugational cross between HL103 and PA3092 placed the round morphology gene of HL103 (called *rodX*) between 8 and 20 min (27). A *rodX* recombinant of PA3092 was mated to KL99, KL226, and several other Hfr strains. These crosses narrowed the *rodX* lesion to a region between 22 and 14 min, the origins of KL99 and KL226, respectively. Using P1 cotransductional analysis, the *rodX* lesions in HL3, HL29, and HL103 (or BF40) cotransduced with *lip* at similar high frequencies (Table 2). Thus, strains HL3, HL29, and HL103 have lesions in either the same gene or very closely linked genes. In addition, the high frequency of cotransduction with *lip* suggests that the *rodX* lesion is probably the *rodA* lesion previously described (18).

The round morphology gene of HL18 (designated *rodY*) was restricted by conjugation ex-

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TABLE 1. *Bacterial strains*

Strain	Sex	Envelope phenotype	Ampicillin sensitivity[a]	Genotype	Reference[b]
H2143	HfrH	Rod		*lys dap thi*	17
HL3	HfrH	Round (RodX)		Derivative of H2143	17
HL29	HfrH	Round (RodX)		Derivative of H2143	17
HL103	HfrH	Round (RodX)	*amp*^{ss}	Derivative of H2143	17
HL18	HfrH	Round (RodY)	*amp*^{ss}	Derivative of H2143	17
HL6	?	Round (RodY)		Derivative of H2143	17
KL96	Hfr	Rod		*thi-1 rel-1* λ^-	14
KL99	Hfr	Rod		*thi-1 rel-1 lac-42* λ^-	14
KL226	HfrC	Rod		*rel-1 tonA22* $T2^r$ λ^-	14
AB312	Hfr	Rod		*thr-1 leu-6 thi-1 lacZ4 str-8* λ^- *supE44*	27
AB313	Hfr	Rod		*thi-1 thr-1 leu-6 lacZ4 str-8 supE44*	28
PA3092	F$^-$	Rod		*thi thr leu argH his trp thy lacY mtl malA xyl str tonA* λ^r	23
AT2538	F$^-$	Rod	*amp*^{s}	*thi-1 pyrE60 argE3 his-4 proA2 thr-1 leu-6 mtl-1 xyl-5 ara-14 galK2 lacY1 str-31* λ^- *supE44*?	A. L. Taylor
CSH57	F$^-$	Rod	*amp*^{s}	*ara leu lacY purE gal trp his argG malA rpsL xyl mtl ilv metA or B thi*	20
AB2834	F$^-$	Rod		*aroE353 mal-352 tsx-352* $\lambda^r\lambda^-$ *supE42*?	22
AT1325 *lip-9*	F$^-$	Rod	*amp*^{s}	*thi-1 his-4 purB15 proA2 mtl-1 xyl-5 galK2 lacY1* λ^- *lip-9 str-35 supE44*?	10
BF40	F$^-$	Round (RodX)	*amp*^{s}	*proA*$^+$ derivative in a cross between HL103 and AT2538	This paper
BF9	F$^-$	Rod	*amp*^{s}	*proA*$^+$ derivative in a cross between HL103 and AT2538	This paper
BF11	F$^-$	Rod		*proA*$^+$ derivative in a cross between HL103 and AT2538	This paper
BF15	F$^-$	Round (RodX)	*amp*^{ss}	*proA*$^+$ derivative in a cross between HL103 and AT2538	This paper
WE13	F$^-$	Round (RodY)	*amp*^{ss}	*pyrE*$^+$ derivative in a cross between HL18 and AT2538	This paper
WE37	F$^-$	Rod	*amp*^{s}	*pyrE*$^+$ derivative in a cross between HL18 and AT2538	This paper
WE41	F$^-$	Rod	*amp*^{s}	*pyrE*$^+$ derivative in a cross between HL18 and AT2538	This paper
WE48	F$^-$	Round (RodY)	*amp*^{ss}	*pyrE*$^+$ derivative in a cross between HL18 and AT2538	This paper
HS24	F$^-$	Round (RodX)	*amp*^{ss}	P1 transductant of BF40 into AT1325 *lip-9*, selected for *lip*$^+$	This paper
CW9	F$^-$	Round (RodY)	*amp*^{ss}	P1 transductant of WE48 into CSH57, selected for *argG*$^+$	This paper
LS853	F$^-$			*cya-283 trpR55 trpA9605 his-29* λ^-	6
CA8306	HfrH			*cya854 thi*	6

[a] *amp*^{ss} was defined as absence of colony formation from 10^8 cells on plates supplemented with 1 μg of ampicillin per ml. At this concentration, the survival of wild type was about 50% (*amp*^{s}).

[b] Strains KL226, AB312, AB313, AT2538, KL96, AB2834, AT1325 *lip-9*, LS853, and KL99 were obtained from Coli Genetic Stock Center, Yale University School of Medicine, New Haven, Conn. CA8306 was obtained from E. Brickman and J. Beckwith.

periments with Hfr strains to the region between 66 and 81 min (origins of AB312 and AB313) by using AT2538 as the female recipient and various Hfr strains. The *envB* gene, another round morphology gene, was reported to be cotransducible with *aroE*, *rpsL*, and *argG* at 68 to 72 min (5, 29). A low frequency of cotransducibility between the round morphology gene of WE48 and *aroE*, *rpsL*, and *argG* was observed (Table 2). This implies that the round morphology genes of HL18 and *envB* are either the same gene or are very close to each other. This round morphology gene is closer to *aroE* (around 71.5 min) than to *argG* or *rpsL* (Table 2). The round

TABLE 2. *Cotransduction frequency between round morphology genes and various markers*

Recipient	Selection	No. of round cells among the total recipient cells selected				
		Donor				
		HL3	HL29	BF40	WE48	HL6
AT1325 *lip-9*	*lip*$^+$	118/130	91/100	106/112[a]	0/52	0/50
CSH57	*argG*$^+$			0/51	3/354	
KL96	*rpsL*				1/223[b]	
AB2834	*aroE*$^+$				7/100[c]	6/100

[a] In another experiment, HL103 was used as donor strain and the cotransduction frequency between *rodX* and *lip* was 80/96.

[b] In case of transduction of streptomycin-resistant marker, *rpsL*, the transductant was grown in rich medium for several hours for the phenotypic expression before it was plated on DYABT plate containing 100 μg of streptomycin per ml.

[c] In another experiment, HL18 was used as donor strain and the cotransduction frequency between *rodY* and *aroE* was 13/234.

morphology gene of HL6 was also found to be cotransducible with *aroE* (Table 2).

Relationship between mecillinam resistance and round morphology. Since all of these round HL mutants appeared among mecillinam-resistant mutants and with a high frequency (about 16%, reference 17; or 5%, M. Iwaya, B. Feingold, D. J. Tipper, J. L. Strominger, and B. G. Spratt, manuscript in preparation), the possibility that the mecillinam-resistance and round morphology determinants were the same was examined in conjugation and transduction experiments.

***rodX* and mecillinam resistance.** In conjugation experiments between HL103 (*rodX*) and AT2538 selecting for *proA*$^+$, four different phenotypes were observed (Fig. 1 and Table 3). BF40 was a round, mecillinam-resistant exconjugant, and BF9 was a mecillinam-sensitive rod. BF11 was also a mecillinam-sensitive rod (99% killing at 1 μg/ml), but the frequency of mecillinam-resistant cells in this exconjugant was extremely high (10^{-2}). The female recipient (AT2538) had a very high spontaneous frequency of mutation to resistance to mecillinam (about 10^{-4}). One (BF15) out of 12 *rodX* exconjugants appeared similar to BF11, but retained the round morphology. However, on subsequent testing, it was completely resistant to mecillinam (as BF40). In the P1 transduction experiments selecting *lip*$^+$ transductants, 2 out of 37 and 5 out of 45 *lip*$^+$ *rodX* transductants examined were similarly initially round and apparently mecillinam sensitive (as BF15), but on subsequent testing were mecillinam resistant. It is important that all mecillinam-resistant conjugants and transductants had the round morphology (Table 3).

***rodY* and mecillinam resistance.** In conjugation experiments between HL18 (*rodY*) and AT2538 selecting for *pyrE*$^+$, five different phenotypes were observed (Fig. 2 and Table 4).

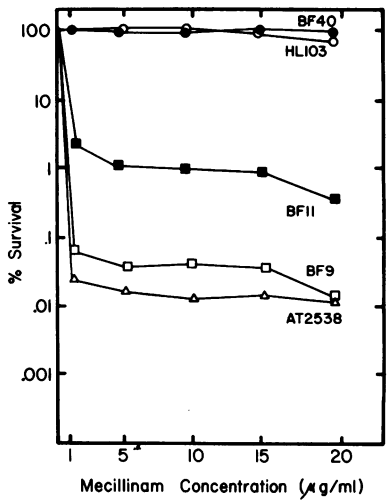

FIG. 1. *Sensitivity of HL103 (rodX) derivatives to mecillinam for colony formation. Dilution of overnight cultures were spread on DYABT agar plates supplemented with various concentrations of mecillinam. After incubation at 30 or 37°C for 1 or 2 days, the number of surviving colonies was counted and the percent survival was plotted on a semilog graph as a function of mecillinam concentration. In separate experiments, AT2538, BF9, and BF11 were shown to be as sensitive to 0.1 as to 1 μg of mecillinam per ml.*

WE48 was a round, mecillinam-resistant exconjugant, but WE37 was a mecillinam-resistant rod. The latter phenotype was never observed in studies of the *rodX* gene. WE13 was an example of a round strain with intermediate-level mecillinam sensitivity (Fig. 2). Strains with this intermediate level of sensitivity and rod morphology were also found. Both these strains exhibited sparse growth (0.1 to 10% survival) on plates containing 10 μg of mecillinam per ml. Finally, exconjugants were found, as WE41, that

TABLE 3. *Sensitivity of HL103 derivatives to mecillinam*

Determination	Selected marker	Donor strain	Envelope phenotype	No. tested[a]	*mec*[s][b]	*mec*[rev][b]	*mec*[r][b]
Conjugation (HL103 × AT2538)	*proA*$^+$		RodX$^-$	12	0	1	11
			RodX$^+$	85	83	2	0
	his$^+$		RodX$^-$	5	0	0	5
			RodX$^+$	43	43	0	0
	proA$^+$		RodX$^-$	6	0	0	6
P1 transduction (selection for *lip*$^+$)		BF40	RodX$^-$	45	0	5	40
			RodX$^+$	6	6	0	0
		HL103	RodX$^-$	37	0	2	35
			RodX$^+$	10	10	0	0

[a] Sensitivity to mecillinam for colony formation was carried out as described in the legend to Fig. 1.
[b] Abbreviations: s, sensitive; r, resistant; *mec*[rev], mecillinam sensitivity characteristic of BF11 (Fig. 1), with a high frequency of reversion to resistance.

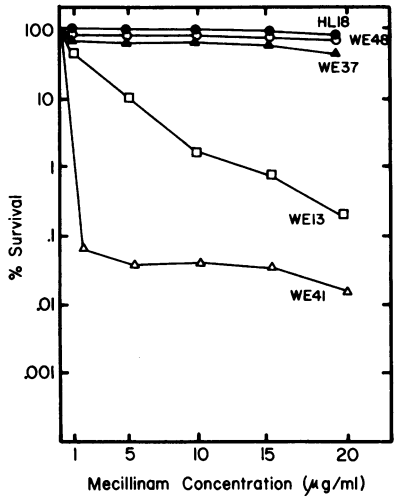

FIG. 2. *Sensitivity of HL18 (rodY) derivatives to mecillinam for colony formation. Experiments were carried out as described in the legend to Fig. 1.*

had the wild phenotype (mecillinam-sensitive rods). In P1 transduction experiments selecting *aroE*$^+$ transductants, only three of these phenotypes were observed (Table 4). Notably absent were the mecillinam- and intermediate-resistant rod phenotypes.

One other anomaly was observed. The streptomycin-resistant transductant of KL96 (Table 2) was originally as sensitive to mecillinam as strain AT2538 (Fig. 1), although it had round morphology. It was the only example of a round mecillinam-sensitive strain encountered in studies of the *rodX* and *rodY* mutants. However, this sensitivity was lost during storage, and it became completely resistant to mecillinam without changing the round morphology. It might be worth mentioning that all *rodX* and *rodY* conjugants were mucoid on DYABT plate, compared to wild type.

Spontaneous mecillinam-resistant mutants. The spontaneous mutation frequency from mecillinam-sensitive to -resistant (10 μg/ml) strains is quite high, that is, about 10^{-5} for strains AT2538, PA3092, or AT1325 *lip-9* and sometimes as high as 10^{-4}. About 200 spontaneous mecillinam-resistant colonies grown on mecillinam-containing plates (about 40 colonies each from 20-, 50-, 75-, 100-, and 150-μg/ml plates) were examined under the phase-contrast microscope after they were grown in DYABT medium without mecillinam. None had round morphology.

To see whether these spontaneous resistant colonies were due to adaptation and not to real mutation, 10 colonies of spontaneous resistant mutants on DYABT plate containing 10 μg of mecillinam per ml were selected, grown in DYABT medium without mecillinam, and plated on DYABT plates with or without mecillinam (10 μg/ml) at 30°C. All 10 strains formed 1/6 to 1/2 the number of colonies on mecillinam plates as on plates with no antibiotic. Thus, they were not adaptations. The extremely high spontaneous frequency of mutation to mecillinam resistance could be explained in either of two ways: that there are one or a few genes with a high spontaneous mutation frequency (7), or, alternatively, that there are many genes (100 to 1,000) with a normal, low spontaneous mutation frequency (10^{-6} to 10^{-8}). These could be distinguished by mapping the spontaneous mutations.

Transductional mapping of additional mecillinam-resistant, round morphology mutants. The frequency of round morphology mutants among mecillinam-resistant mutants selected after NTG treatment was about 5%. About 40 such mutants of AT2538 were selected (Iwaya, Feingold, Tipper, Strominger, and Spratt, manuscript in preparation). Four were randomly chosen and tested for cotransduction of their round morphology genes by P1 trans-

TABLE 4. *Sensitivity of HL18 derivatives to mecillinam*

Determination	Selected marker	Donor strain	Envelope phenotype	No. tested	mec^{ss}	mec^{int}	mec^{r}
Conjugation (HL18 × AT2538)	*pyrE*$^+$		RodY$^-$	24	0	2	22
			RodY$^+$[b]	24	22	0	2
	pyrE$^+$		RodY$^-$	4	0	0	4
			RodY$^+$	43	7	9	27
P1 transduction (selection for *aroE*$^+$)		WE48	RodY$^-$	7	0	2	5
			RodY$^+$	60	60	0	0
		HL18	RodY$^-$	5	0	0	5
			RodY$^+$	43	43	0	0

[a] Sensitivity to mecillinam for colony formation was carried out as described in the legend to Fig. 1. mec^{int} is the intermediate mecillinam sensitivity characteristics of WE13 (Fig. 2).

[b] The difference between the two conjugation crosses seems significant, although they were replicate experiments of the same design. The cause for this variation has not been investigated.

duction. One of these was cotransducible with *lip*, and three were cotransducible with *aroE*.

Sensitivity of *rodX* and *rodY* strains to ampicillin. The various strains used in this study were tested for ampicillin sensitivity (amp^s) and abnormal supersensitivity (amp^{ss}) (Table 1). amp^{ss} was cotransduced with both the *rodX* mutation of HL103 and the *rodY* mutation of HL18.

DISCUSSION

Relationship between round morphology genes of HL mutants and *rodA* and *envB* genes. One of the known round morphology genes, *rodA*, is cotransducible with *lip* at a frequency of 95% and also confers increased sensitivity to penicillin G (18). The cotransduction frequencies between the round morphology genes (*rodX*) of HL3, HL29, and HL103 and *lip* was 91, 91, and 95%, respectively (Table 2). HL103 or its round transductant were more sensitive to ampicillin on DYABT plates than were the parents H2143 and AT1325 *lip-9* (Table 1). These results strongly suggest that *rodA* gene and the round morphology genes (*rodX*) of HL3, HL29, and HL103 are the same or are similar genes situated very close to each other.

The round morphology gene (*rodY*) of HL18 was cotransducible with *argG*, *rpsL*, or *aroE* at a low frequency (Table 2), similar to the reported cotransduction frequencies between *envB* and *argG*, *rpsL*, or *aroE* (29). HL18, its round transductants (Table 2 and 4), or its round conjugant (Table 4) were also more sensitive to ampicillin on DYABT plate than was H2143, CSH57, or AT2538 (Table 1). *envB* mutants, like *rodA* mutants, were more sensitive to ampicillin and penicillin G than was the parent (21, 29). These results also suggest that *envB* and the round morphology gene (*rodY*) of HL18 are the same or are similar genes located close to each other.

Adler, Terry, and Hardigree (2) reported on a giant cell mutant (*mon*) of *E. coli*. All progeny in crosses with a variety of Hfr donors retained the unusual cell morphology of the female. This could be explained if one assumes that this unusual cell morphology gene is situated very close to *rpsL* (used for selection), as is the case for the round morphology gene of HL18 or *envB*; therefore, the segregation of these two genes is a rare event. The identity of *envB* and *mon* was found by S. Long and K. B. Low (personal communication; reference 4).

Relationship between mecillinam resistance and round morphology genes. (i) *rodX*. Is the mecillinam resistance gene identical to the round morphology gene (*rodX* or *rodA*) or are they closely linked? In conjugation and transduction experiments, no example was found in which these two genes were clearly separable (Table 3).

The reason why the frequency of *rodX* or *rodY* among mecillinam-resistant mutants increased 10-fold or more after NTG treatment is not clear at the present time. One possibility is that *rodX* (or *rodY*) itself confers a certain degree of mecillinam resistance that is not sufficient for mecillinam selection. However, the combination of *rodX* and other presumably closely linked mecillinam resistance genes that are also induced by NTG treatment (8) would make a cell more mecillinam resistant and therefore easier to select.

(ii) *rodY*. Mecillinam resistance of the *rodY* mutants can only be accounted for from the data of Table 4 by at least two mutations. The round morphology gene (*rodY*) may confer a variable degree of resistance since some of these strains have intermediate degrees of mecillinam resistance. In addition, it is either identical to or very closely linked to *envB*. The data require the existence of at least one other gene that confers mecillinam resistance without any morphological change, because in the conjugation

experiments a large number of mecillinam-resistant rods were obtained. All of the phenotypes found could be accounted for by two genes in the normal or mutated state.

Westling-Häggström and Normark (29) obtained a mecillinam-sensitive *envB* strain after conjugation. These workers also found a mecillinam resistance gene, called *sloB1*, which mapped near *rpsL*, a position definitely different from *envB*. The mutant in this gene is characterized by slow growth, which can confer variable degrees of mecillinam resistance of *E. coli*. Another possibility was suggested by the observation of Yamasaki et al. (30), who reported that *cya* (adenyl cyclase) mutant exhibited mecillinam resistance in the absence of cyclic AMP (cAMP) and was round shaped (even in the absence of mecillinam). However, it was mecillinam sensitive and rod shaped in the presence of cAMP. They suggested that the synthesis of the penicillin-binding protein 2 (25) was under the control of cAMP. Since the cAMP receptor protein gene (*crp*) maps at 73 min, close to *rodY* between *rpsL* and *aroB* (24), the possibility that *rodY* and *crp* might be the same was considered. Round morphology of *rodY* mutants (derivatives of HL18) was not converted to normal rod morphology in broth supplemented with 20% sucrose and 0.01 M $MgSO_4$ (unpublished results), under which condition *crp* mutant should have normal rod morphology (12). Also, the transductional mapping (Table 2) suggests the gene order as *argG*, *rodY*, *aroE*, and *rpsL*, which agrees with the data for *envB* (29). Thus *rodY* cannot be *crp* or *cya*, which lies between *ilv* and *metE* genes at 83 min (6) (although such mutants could conceivably be selected as mecillinam resistant with a round morphology). However, it remains possible that *rodY* controls the expression of the *rodX* gene in some unknown manner.

Recently we have examined a few *cya* mutants for their morphology in the absnece of cAMP. It seems that the morphological change of *cya* mutants depends on their genetic background, since LS853 (F^-, deletion for *cya* gene) showed normal short rods, whereas CA8306 (HfrH, deletion for *cya* gene) showed a mixture of short rods, oval cells, and round cells in the absence of cAMP. Also, the round cells of *rodX* and *rodY* are much larger than the oval or round cells of *cya* mutants.

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