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High and Selective Resistance to Mecillinam in Adenylate Cyclase-Deficient or Cyclic Adenosine 3',5'-Monophosphate Receptor Protein-Deficient Mutants of *Escherichia coli*

RIKIZO AONO†*, MAKARI YAMASAKI, AND GAKUZO TAMURA

Department of Agricultural Chemistry, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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Adenylate cyclase-deficient (*cya*) mutants of *Escherichia coli* K-12 were selectively and highly resistant to mecillinam (FL1060) among several β-lactam antibiotics in the absence of cyclic adenosine 3',5'-monophosphate (cAMP). They became sensitive to the drug in the presence of cAMP. Also, cAMP receptor protein-negative (*crp*) mutants, with the exception of strain 5333, were highly resistant to mecillinam in the presence and in the absence of cAMP. Mecillinam exerted two distinct and sequential effects in both cya^+ strains and *cya* strains supplemented with cAMP: (i) rounding of cells and (ii) cessation of cell division. The first effect was accompanied by a decrease in growth rate, whereas the second effect was accompanied by enlargement and lysis of the rounded cells. The second effect of mecillinam was dependent on inoculum size and cAMP. When the cell density was above about 10^6 cells per ml, the rounded cells stopped dividing but did not lyse. In the absence of cAMP, *cya* strains neither stopped dividing nor lysed; they were resistant to the second, lethal effect of mecillinam.

We have been interested in the basis of the spherical or short rod morphology of *cya* or *crp* mutants of *Escherichia coli* K-12. This morphological abnormality may be due to a lack or a defect in protein(s) which is able to synthesize a rodlike peptidoglycan or constitute a rod frame of an envelope on which a peptidoglycan should be synthesized as a rod layer. We could not detect such envelope protein(s) determining the shape of cell by a method in which the envelope proteins were fractionated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue (2).

It is well known that β-lactam antibiotics interfere with the synthesis of peptidoglycan and can cause changes in the cell shape of *E. coli*. Particularly mecillinam, which is 6β-[(hexahydro-1H-azepin-1-yl)-methyleneamino]penicillanic acid and otherwise called FL1060, has been known to cause the formation of large ovoid cells (6, 8, 9, 12).

Mecillinam-resistant mutants take various morphologies in the absence of the agent: normal rod, short rod, spherical, or mixtures of these shapes (9). Mecillinam binds exclusively to one of the six benzylpenicillin-binding proteins in the inner membrane of *E. coli*. This protein is designated penicillin-binding protein 2 (PBP2), and 20 molecules of PBP2 are supposed to exist per bacterium (17, 18). A mecillinam-resistant mutant grew slowly as rounded cells, even in the absence of mecillinam. The mutant had no PBP2 or a defective PBP2 failing to bind penicillin (15).

The ovoid shape and low growth rate of the mecillinam-resistant mutant and the rounding of mecillinam-treated wild strains resemble the phenomena shown by *cya* or *crp* strains: slow growth rate and spherical or short rod morphology. We assumed that the synthesis or physiological activity of PBP2 may be regulated by cyclic AMP (cAMP) and cAMP receptor protein and that the morphological changes in *cya* or *crp* strains might have been due to a defect of PBP2. In fact, as we reported earlier, *cya* strains are selectively resistant to mecillinam (21). In the present paper, we describe in detail the mecillinam resistance of *cya* and *crp* strains.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the bacterial strains employed and their genetic properties. We always cultured the *cya* or *crp* mutants on MacConkey lactose agar at 37°C overnight and employed cells from small Lac^- colonies in order to exclude revertants.

Sensitivities to β-lactam antibiotics. Benzylpenicillin, 6-aminopenicillanic acid, ampicillin, mecillinam, and cephaloridine sensitivities of each strain

† Present address: The institute of Physical and Chemical Research, Wako-shi, Saitama-ken, 351, Japan.

TABLE 1. *Strains of E. coli K-12*

Strain	Genotype	Reference
W2252*thy*	HfrC *metB thy rel*	1
AO10	*crp* mutant from W2252 *thy*	
AO33	*cya* mutant from W2252 *thy*	1, 21
AO60	*cya* mutant from W2252 *thy*	1
AO33R1	Spontaneous *cya*$^+$ revertant from AO33	21
AO33R2	Spontaneous *cya*$^+$ revertant from AO33	21
CA8000	HfrH *thi relA*	14
CA7902	*cya* mutant from CA8000	14
CA7902R1	Spontaneous *cya*$^+$ revertant from CA7902	1, 2, 21
CA7902R2	Spontaneous *cya*$^+$ revertant from CA7902	21
CA8306	*cya* deletion mutant from CA8000	3
CA8445	*cya* and *crp* deletion mutant from CA8000	13
5333	HfrH *thi crp*	4
5333R1	Spontaneous *crp*$^+$ revertants from 5333	2
5333R2	Spontaneous *crp*$^+$ revertant from 5333	

were determined by the conventional serial dilution technique in liquid medium. Each drug was serially diluted twofold with an inoculum of about 10^4 cells per ml of nutrient broth in the presence or absence of 1 mM cAMP. The growth response in each culture was judged after 24 h of incubation at 37°C without shaking. The minimal inhibitory concentration (MIC) was the minimum concentration of each drug at which no growth occurred.

Colony-forming abilities of some strains were also examined. Cultures which were exponentially growing in nutrient broth in the absence of cAMP were diluted with 0.8% sodium chloride and plated in nutrient soft agar together with each drug over a range of concentrations in the presence or absence of 1 mM cAMP. After 2 days of incubation at 37°C, the number of colonies was counted.

Effect of mecillinam on the growth. AO33 and other strains were inoculated in nutrient broth in the presence or absence of 1 mM cAMP and cultured at 30°C with shaking. Cultures in logarithmic phase of growth (about 10^8 cells per ml) were diluted to 10^6 cells per ml with fresh medium prewarmed at 30°C. Mecillinam was added and cell morphology was followed microscopically. Samples were also diluted with 0.8% sodium chloride and plated in nutrient soft agar in the presence or absence of 1 mM cAMP. After 2 days of incubation at 30°C, the number of colonies was counted.

To examine the effect of mecillinam at high cellular density, mecillinam was added directly to log phase cultures at 10^8 cells per ml.

Chemicals. Mecillinam was the product of Leo Laboratories, Ballerup, Denmark. The other β-lactam antibiotics were purchased from the following sources: benzylpenicillin, Meiji Seika Co., Japan; ampicillin, Takeda Pharmaceutical Inc., Japan; 6-aminopenicillanic acid, Sigma Chemical Co., St. Louis, Mo.; and cephaloridine, Eli Lily & Co., Indianapolis, Ind. The sodium salt of cAMP was the generous gift of Yamasa Shoyu Co., Japan. The composition of media and other chemicals were described previously (1, 2, 21).

RESULTS

Selective and high resistance of *cya* or *crp* strains to mecillinam. MICs to five β-lactam antibiotics were examined with various strains of *E. coli* K-12. The *cya* strains in the absence of cAMP were highly resistant to mecillinam (21) (Table 2). There were very large differences between the MICs in the presence of 1 mM cAMP and those in the absence of cAMP. The differences were 800-fold for strains AO33 and CA7902 and 400-fold for strain CA8306. Their parental and revertant strains were no longer resistant to mecillinam, but had sensitivities about the same as those of the respective *cya* strains in the presence of 1 mM cAMP.

The *crp* strains AO10 and CA8445 were also highly resistant to mecillinam in the presence and absence of 1 mM cAMP (Table 2). The *crp* strain 5333, however, was essentially as sensitive as its spontaneous *crp*$^+$ revertants 5333R1 and

TABLE 2. *MICs of β-lactam antibiotics in the presence or absence of cAMP*[a]

Strain	Presence of:			MIC (μg/ml)	
	cya	*crp*	cAMP	Ampicillin	Mecillinam
W2252*thy*	+	+	−	NT	0.24
			+	NT	0.12
AO33	−	+	−	0.63	63
			+	0.31	0.078
AO33R1	+	+	−	1.3	0.039
			+	0.63	0.039
AO33R2	+	+	−	0.63	0.078
			+	0.31	0.039
AO10	+	−	−	NT	31
			+	NT	16
CA8000	+	+	−	NT	0.078
			+	NT	0.078
CA7902	−	+	−	13	125
			+	3.1	0.16
CA7902R1	+	+	−	6.3	0.16
			+	1.6	0.16
CA7902R2	+	+	−	3.1	0.31
			+	1.6	0.16
CA8306	Δ	+	−	NT	63
			+	NT	0.16
CA8445	Δ	Δ	−	NT	31
			+	NT	31
5333	+	−	−	13	0.31
			+	13	0.31
5333R1	+	+	−	6.3	0.16
			+	3.1	0.31
5333R2	+	+	−	3.1	0.31
			+	3.1	0.16

[a] MICs were determined by the broth dilution method by using nutrient broth supplemented with or without 1 mM cAMP. R in the strain number represents a spontaneous revertant of corresponding strain. Δ designates a deletion mutation. NT, Not tested.

5333R2. This may be due to its leaky mutation of *crp* gene (4).

AO33 and CA7902 were two- to fourfold more sensitive to ampicillin in the presence of cAMP than in its absence. However, resistance to ampicillin was much less than that observed in the case of mecillinam (Table 2). The sensitivities of AO33, CA7902, 5333, and their parental and revertant strains to benzylpenicillin, 6-aminopenicillanic acid, and cephaloridine were not affected by the addition of 1 mM cAMP (21; data not shown).

The colony-forming ability of strain AO33 was examined in nutrient soft agar containing each of the above-mentioned drugs.

Figure 1 demonstrates the very high mecillinam resistance of AO33 cultured in the absence of cAMP. From such results, we determined the concentration of each drug at which 50% of the cells of AO33 were capable of forming colonies (Table 3). Although AO33 was more resistant to all of the β-lactam antibiotics in the absence of cAMP than in its presence, the ratios of resistance levels (without cAMP versus with cAMP)

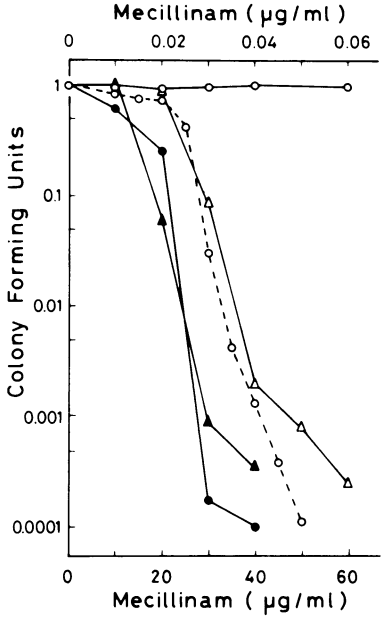

FIG. 1. *Effect of mecillinam on the colony-forming abilities of AO33 and AO33R1. The cells of AO33* (●, ○) *and its spontaneous revertant AO33R1* (▲, △) *growing exponentially in nutrient broth in the absence of cAMP were plated in nutrient soft agar containing mecillinam and supplemented with* (●, ▲) *or without* (○, △) *1 mM cAMP. After 2 days of incubation at 37°C, the number of colonies was counted. Solid lines correspond to the upper scale of abscissa, and the broken line corresponds to the lower.*

TABLE 3. *Concentrations of β-lactam antibiotics at which 50% of AO33 cells formed colonies*

Drug	LD_{50} (μg/ml)[a]		Ratio of LD_{50} with cAMP to LD_{50} without cAMP
	without cAMP	with cAMP	
Benzylpenicillin	5.2	3.8	1.4
Ampicillin	0.51	0.33	1.5
6-Aminopenicillanic acid	15	12	1.3
Mecillinam	24	0.013	1,800
Cephaloridine	1.5	1.3	1.2

[a] LD_{50} is the concentration at which 50% of the cells of AO33 formed colonies. It was determined as described in the legend to Fig. 1.

for the other antibiotics were very low in comparison with the 1,800-fold ratio in the case of mecillinam. The increase in sensitivity to mecillinam in the *cya* strain was dependent on the concentration of cAMP in the medium (Fig. 2A). The addition of 1 mM cAMP made strain AO33 as sensitive to mecillinam as the *cya*$^+$ revertant AO33R1. AO33 was no longer sensitive to mecillinam at cAMP concentrations of 0.01 mM or less. Strains A060 (*cya*) and A010 (*crp*) were also highly resistant to mecillinam (Fig. 2B).

Suppression of a lytic effect of mecillinam. The growth of strain AO33 in the presence of 1 mM cAMP was completely inhibited by the addition of 0.1 or 1 μg of mecillinam per ml (Fig. 3A). The increase in the viable cell number ceased and began to decrease 2 to 2.5 h after the addition of mecillinam. In the absence of cAMP, AO33 grew exponentially, although the growth rate decreased in higher concentrations of mecillinam (Fig. 3B).

Progressive morphological change in AO33 treated with mecillinam depended on cAMP in the medium (Fig. 4). At 1 h after the addition of 1 μg of mecillinam per ml, most of the cells grown in the presence of cAMP had converted to an oval shape (Fig. 4B). At 2 h, the center of the cells expanded further. The cells became large and more rounded in shape. At this stage, in the absence of cAMP, AO33 cells also became oval, but these cells were smaller than those in the presence of cAMP. Both the shape and the diameter of the cells grown in the absence of cAMP were maintained thereafter (Fig. 4F). In the presence of cAMP, the diameter of the rounded cells continued to increase. By 3 h, a small fraction of the cells incubated in the presence of cAMP formed large ghosts that maintained rounded shapes but contained no intracellular substance (Fig. 4C). By 4 h, half of the cells were converted to ghosts (Fig. 4D). By 6 h,

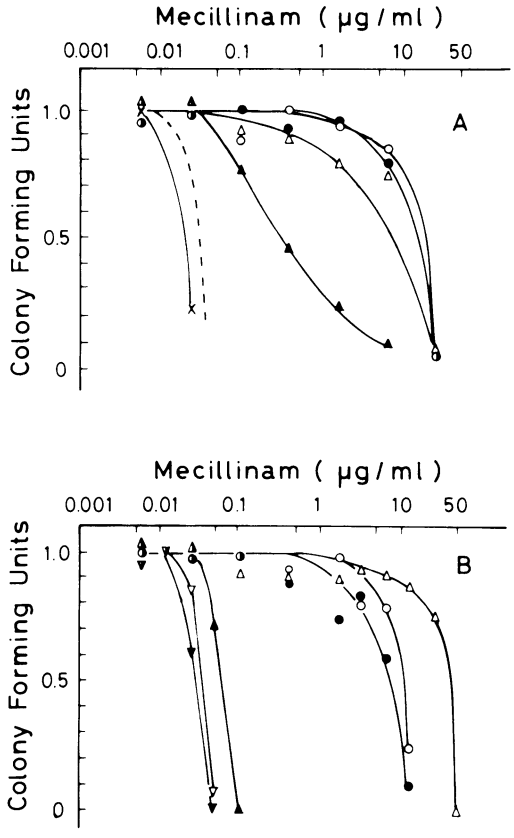

FIG. 2. *Regulation of mecillinam sensitivity by cAMP and cAMP receptor protein. (A) Effect of cAMP concentration in media on the sensitivity of AO33 to mecillinam. The cells of AO33 were plated in mecillinam-containing nutrient soft agar supplemented with 0 (○), 0.001 (●), 0.01 (△), 0.1 (▲), or 1 mM (×) cAMP. The dotted line shows colony-forming units of AO33R1 without the addition of cAMP. (B) Colony-forming abilities of crp mutant AO10 (●, ○), cya mutant AO60 (▲, △), and their parental strain, W2252thy (▼, ▽). Solid symbols represent the results in the presence of 1 mM cAMP, and open symbols represent those in the absence of cAMP.*

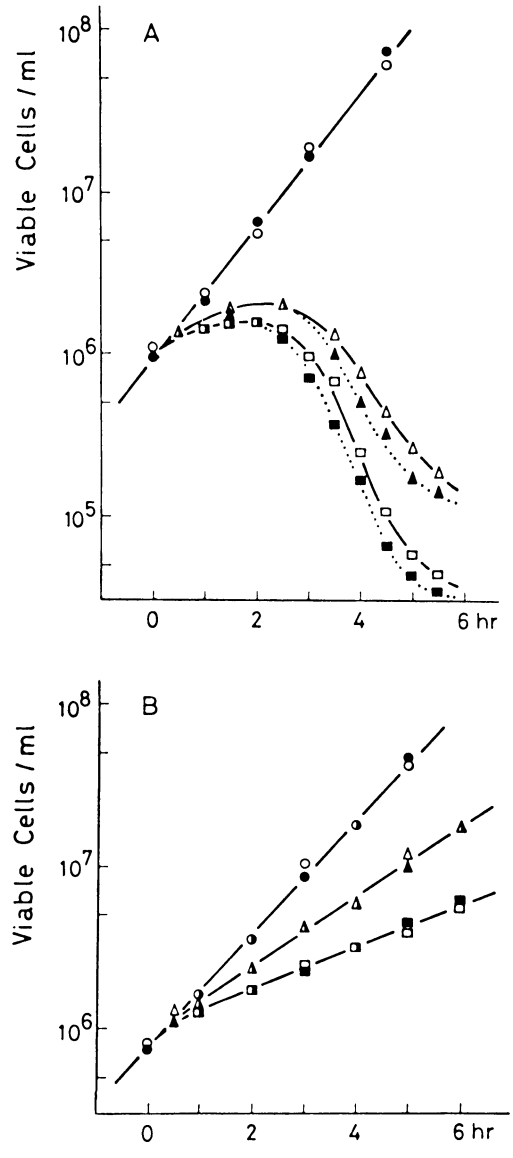

FIG. 3. *Effect of mecillinam on growth of AO33. (A) AO33 was cultured at 30°C in the presence of 1 mM cAMP. When an exponentially growing culture reached about 10^8 cells per ml, it was diluted 100-fold with prewarmed medium with 1mM cAMP and divided into three parts. Each culture was supplemented with 0 (●, ○), 0.1 (▲, △), or 1 μg (■, □) of mecillinam per ml at zero time and incubated at 30°C with shaking. Periodically a part of each culture was diluted with 0.8% NaCl and plated in nutrient soft agar in the presence (solid symbols) or absence (open symbols) of 1 mM cAMP. The number of colonies was counted after 2 days of incubation at 30°C. (B) All experimental procedures and symbols were the same as in (A), except that cAMP was not added to the cultures.*

most of the ghosts had ruptured, and the few surviving cells were huge and irregular rather than rounded in shape. The time of first appearance of ghosts (Fig. 4) corresponded to the time when viable cell number first began to decrease (Fig. 3A).

The morphological changes and the lysis caused by mecillinam in strain CA7902 were also dependent on the addition of cAMP. With strain AO33R1, the rupture of the rounded cells did not require the addition of cAMP, although this was more distinct in the presence of cAMP (data not shown). However, when mecillinam was administered to growing cultures at high density

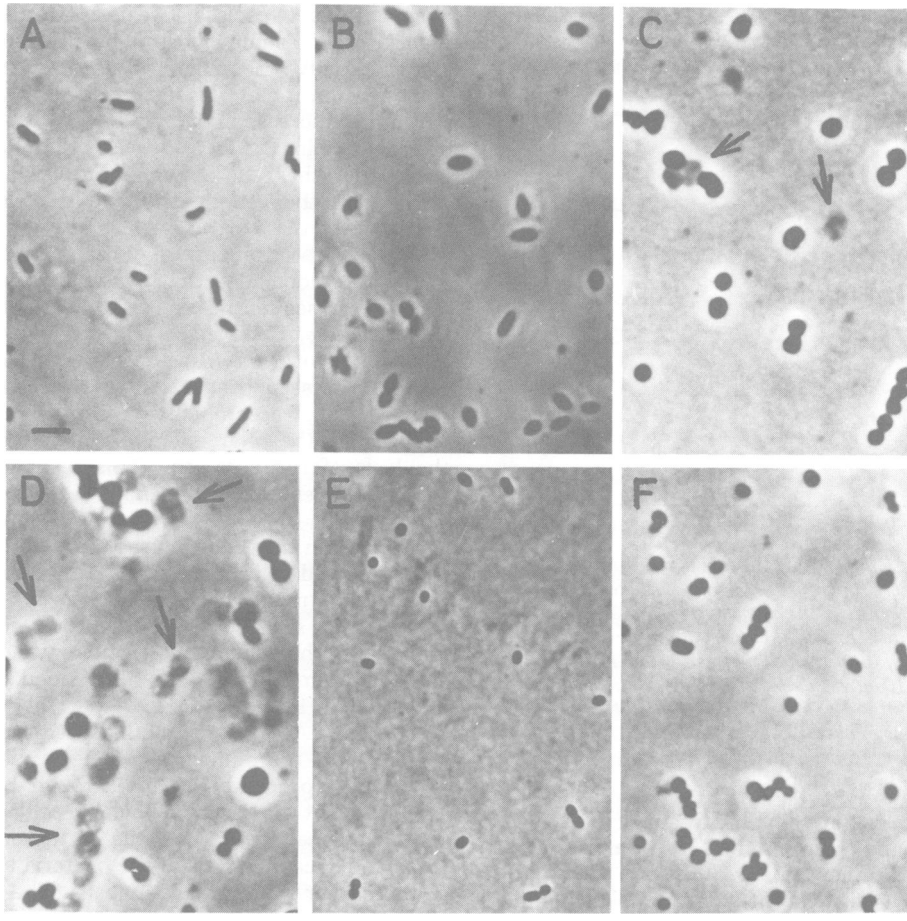

FIG. 4. *Effect of mecillinam on the morphology of strain AO33 cultured in the presence (A through D) or absence (E and F) of 1 mM cAMP at 30°C. To the cultures (about 10^8 cells per ml) 1 μg of mecillinam per ml was added. A part of each culture was withdrawn at zero time (A and E), and at 1 (B), 3 (C), 4 h (D), and 6 h (F) after the addition of mecillinam. The cells were immobilized on a thin layer of agar and phase-contrast photomicrographs were taken. All of the figures are at the same magnification. Bar, 5 μm. The arrows indicate ghost cells.*

even in the presence of cAMP, the morphological changes ceased at the stage of the rounded cells. The number of viable cells stopped increasing but did not decrease. The optical density at 550 nm increased continuously in the culture treated with mecillinam (Fig. 5). The mecillinam per cell was probably sufficient in the experiment shown in Fig. 5, since the number of the cells was 60 times higher and the concentration of mecillinam was 10 or 100 times higher in the experiment shown in Fig. 5 than those in the experiment shown in Fig. 3.

DISCUSSION

Morphological abnormality in *cya* or *crp* strains might have resulted from a defect or a low activity of enzyme(s) that determine the shape of the peptidoglycan layer, for example, a transpeptidase. We examined the sensitivities of these strains to β-lactam antibiotics which are well known to interfere with the synthesis of peptidoglycan. These strains, with the exception of 5333, were highly resistant to mecillinam but not to four other β-lactam antibiotics (Tables 2 and 3). The addition of cAMP made *cya* strains as sensitive to mecillinam as their respective revertants and parental strains. These strains were not as resistant to ampicillin as were those reported by Kumar (7).

Mecillinam did not completely inhibit the growth of *E. coli* (i.e., the increase in the turbidity of the cultures), and it did not induce lysis under some conditions (5, 6, 9–12, 16, 20; Fig. 5). Moreover, mecillinam-resistant mutants occur

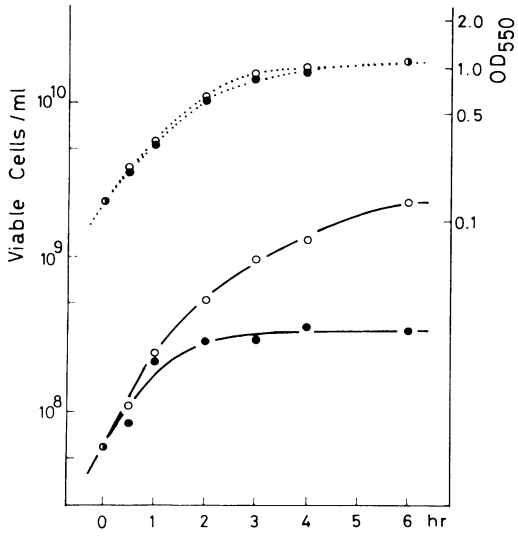

FIG. 5. *Effect of mecillinam on the growth of AO33 at high cell density. A033 was cultured at 37°C in nutrient broth in the presence of 1 mM cAMP. The exponentially growing culture (optical density at 550 nm [OD_{550}],0.15) was divided into two halves. Mecillinam at 10 μg/ml was added to one half at zero time (●) and not to the other (○). OD_{550} (dotted lines) and the number of viable cells (solid lines) of each culture were measured periodically.*

with a comparatively high frequency (12, 19, 20). In order to determine the MIC of a drug having such a complex action, it is not sufficient to observe merely whether a culture becomes more turbid in the presence of the drug.

It is very interesting to note that cAMP and cAMP receptor protein controlled the sensitivity to only one drug of a group of drugs with similar chemical structures and similar biochemical targets in bacteria. On the other hand, it has already been established that mecillinam has a unique capacity to interfere with the maintenance of rod shape in *E. coli*.

Many investigators have reported that *E. coli* treated with mecillinam gradually became rounded and formed greatly enlarged osmotically stable spheres (6, 8, 9, 12, 16, 17). We suppose that "the osmotically stable rounded cells" in these reports refer to the rounded cells, which we obtained by treatment with mecillinam at a comparatively high cell density. The results in these papers are from experiments in which mecillinam was added to cultures containing more than 10^7 bacteria per ml, and, in some cases (5, 6, 9, 16), the cultures were incubated for relatively short periods after the addition of the drug. Moreover, growth was measured either by the total cell number (Coulter counter) or by the total cell mass (turbidometry) (6, 9, 16). The comparison between Fig. 3A and 5 indicates that mecillinam does not kill at high cell concentration. Actually, mecillinam caused *E. coli* to convert from rods to rounded cells, but did not inhibit the increase in optical density at 550 nm or kill the cells (Fig. 5). Some early investigators noted that the lytic action of mecillinam depended on the cell density and that *E. coli* treated with the drug at low cell density would eventually lyse after several divisions (8, 10, 20).

We have concluded that mecillinam has two distinct and sequential effects. The first effect is the rounding of cell shape and the lowering of growth rate of *E. coli*. It is not influenced with cAMP. The second effect is the halting of cell division (6), accompanied with successive changes, i.e. the enlargement of cell volume and the lysis of the organism. This second effect needs endogenous or exogenous cAMP (Fig. 3 and 4).

Mecillinam treatment caused the formation of clear rounded cells from spherical or short rod cells of *cya* strains when grown in the absence of cAMP (Fig. 4E and F). These rounded cells maintained their rounded morphology and characteristic dimensions but still divided in the presence of 1 μg of the drug per ml. In the presence of cAMP, mecillinam caused the same sequence of morphological changes as found with wild-type cells (Fig. 4A through D). We conclude that the decrease in viable cell number caused by mecillinam was due to the enlargement and sucessive lysis of the bacteria but not to the earlier rounding of the cells. The resistance of *cya* strains to mecillinam is due to the insensitivity of these cells to the secondary effect of mecillinam (i.e., interference with cell division).

Mecillinam binds specifically to PBP2 in the inner membrane of *E. coli* (15–18) and apparently inhibits the unknown physiological function of PBP2 (9, 12). Even in the absence of cAMP, the growth rate of strain AO33 was lowered by the addition of mecillinam (Fig. 3B). Therefore, we think that PBP2 exists in the inner membrane of these cells and that mecillinam can bind to PBP2 in the absence of cAMP. Mecillinam may exert its physiological activity after dissociation from PBP2 involving a cAMP-dependent process. The relationship between penicillin-binding proteins and resistance of *cya* and *crp* mutants of *E. coli* to mecillinam will be published elsewhere.

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