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E-0702, a New Cephalosporin, Is Incorporated into *Escherichia coli* Cells via the *tonB*-Dependent Iron Transport System

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E-0702, a new cephalosporin with a potent antipseudomonal action, was synthesized. In the study of the mode of action of this antibiotic in *Escherichia coli*, it was found that mutants which acquired resistance to E-0702 were isolated spontaneously and could be shown to be susceptible to its closely related derivatives, E-0702-060 and E-0702-061, and other representative β-lactam antibiotics. In these mutants, no increased production of β-lactamase was detectable. No apparent differences between the resistant mutants and the parental strains were observed in the affinity of E-0702 for penicillin-binding proteins. Furthermore, no significant reduction in or loss of both OmpF and OmpC porin proteins in the outer membrane was observed. The mutation was mapped to the *tonB* gene, which is known to be essential for the iron transport system of bacteria. The bactericidal action of E-0702 was rapidly expressed against iron-starved cells in which the iron transport system was induced, whereas the bactericidal action against iron-supplemented cells was ineffective. It is suggested that E-0702 is incorporated into bacterial cells as a chelator of iron via the *tonB*-dependent iron transport system, after which its strong and rapid bactericidal action is manifested.

Many new cephalosporins with broad spectra of antibacterial activity have been developed over the past few years. Several of these antibiotics are in clinical use for the treatment of a wide range of bacterial infections.

In our studies directed toward the development of new cephalosporins, E-0702 {(6*R*,7*R*)-3-[(1-carboxymethyl-1*H*-tetrazol-5-yl)-thiomethyl]-7-[(2*R*)-2-(6,7-dihydroxy-4-oxo-4*H*-1-benzopyran-3-carboxamido)-2-(4-hydroxyphenyl)-acetamido]-8-oxo-5-thia-1-azabicyclo-[4,2,0]-oct-2-ene-2-carboxylic acid disodium salt} was synthesized (Fig. 1). This compound showed a broad antibacterial spectrum and a potent antipseudomonal activity.

The characteristics of E-0702 were reported previously (24). The in vitro antibacterial activity of E-0702 is strongly affected by the size of inoculum, which is not the case with other β-lactam antibiotics. In addition, E-0702 exhibited more potent bactericidal activity, especially against enteric bacteria, than other β-lactam antibiotics did.

To investigate the mode of action of E-0702 on bacteria, spontaneous mutants which were shown to be highly resistant to E-0702 were isolated from *Escherichia coli* strains.

Generally, the mechanisms of resistance to β-lactams in bacteria are divided into three categories: inactivation of the antibiotics by β-lactamase; alteration of the targets; and reduced penetration of the antibiotics through the outer membrane as a result of alteration of porin proteins (20). In this report, we analyze spontaneous E-0702-resistant mutants and demonstrate that the resistance to E-0702 may be the result of the reduced penetration of E-0702 through the outer membrane. However, these resistant mutants showed no reduction in the porin proteins, such as OmpF and OmpC, which form the pores in the outer membrane that allow most cephalosporins to diffuse rapidly into the periplasmic space (16, 25, 33, 34). It is suggested that the pathway of permeation of E-0702 through the outer membrane of *E. coli* may be different from those of other β-lactams and that E-0702 is incorporated into bacterial cells via the *tonB*-dependent iron transport system. The possibility of antimicrobial treatment via such an active transport system is also discussed.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. The strains of *E. coli* K-12 used in this study are shown in Table 1. Other bacterial strains are maintained as stock cultures in our laboratory. *E. coli* ER31 was isolated from *E. coli* MIHJ JC-2 by selection for E-0702 resistance; *E. coli* ER36 was from *E. coli* No. 23; *Salmonella typhimurium* ER42 was from *S. typhimurium* 1406; *Klebsiella pneumoniae* ER47 was from *K. pneumoniae* IID875; *Serratia marcescens* ER52 was from *S. marcescens* IID620; *Proteus mirabilis* ER59 was from *P. mirabilis* OM-1; and *Pseudomonas aeruginosa* ER64 was from *P. aeruginosa* PAO1. Bacteriophages T5, BF23, φ80, and P1 *vir* were kindly provided by K. Mizobuchi and H. Uchida.

Culture media. The iron-deficient minimal medium contained the following: 100 mM Tris hydrochloride buffer (pH 7.4), 0.64 mM KH_2PO_4, 20 mM NaCl, 3 mM Na_2SO_4, 2 mM NH_4Cl, 1 mM $MgCl_2$, 1 μM $CaCl_2$, 100 μM α,α′-dipyridyl, 0.5% glucose, and 0.05% Casamino Acids (Difco Laboratories, Detroit, Mich.). The iron-supplemented medium was prepared in the same way as the iron-deficient medium was, except for the addition of 200 μM $FeCl_3$ in place of α,α′-dipyridyl. The minimal medium for Hfr mating and P1 transduction consisted of 100 mM Tris hydrochloride buffer (pH 7.4), 0.64 mM KH_2PO_4, 20 mM NaCl, 3 mM Na_2SO_4, 2 mM NH_4Cl, 1 mM $MgCl_2$, 1 μM $CaCl_2$, 2 μM $FeCl_3$, and 0.5% glucose. When necessary, the following supplements were added: thiamine (1 μg/ml), L-amino acids (30 μg/ml), and streptomycin (final concentration, 10 μg/ml). The glassware for the manipulations with iron-deficient medium was rinsed with 0.1 N HCl followed by rinses with copious quantities of deionized, distilled water before being autoclaved. The glucose and Casamino Acids were sterilized separately by autoclaving. $MgCl_2$, $CaCl_2$, $FeCl_3$, thiamine, L-amino acids, and streptomycin were sterilized separately by filtration through membranes (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). Mueller-Hinton broth and Mueller-Hinton agar medium (BBL Microbiology Systems,

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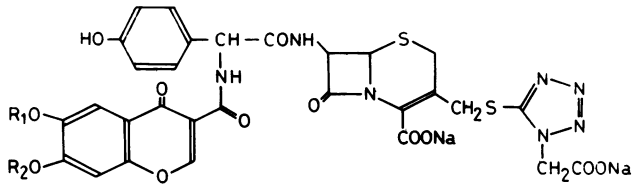

Antibiotics	R_1	R_2
E-0702	H	H
E-0702-060	CH_3	H
E-0702-061	H	CH_3

FIG. 1. Chemical structures of E-0702 and its derivatives.

Cockeysville, Md.) were used for determining the inhibitory concentrations of the antibiotics. L broth consisted of 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose at pH 7.4 adjusted by the addition of 1 N NaOH. Antibiotic medium 3 (Difco) and nutrient agar that contained bromothymol blue and lactose were also used. All solidified media contained 1.5% (wt/vol) agar.

Antibiotics. E-0702 and its derivatives were synthesized in the research laboratories of Eisai Co. The following antibiotics were obtained commercially: cefazolin (Fujisawa Pharmaceutical Co., Osaka, Japan); cephaloridine, moxalactam, and gentamicin (Shionogi Pharmaceutical Co., Osaka, Japan); cefoxitin (Daiichi Seiyaku Co., Tokyo, Japan); cefoperazone (Toyama Chemical Co., Toyama, Japan); ampicillin, benzylpenicillin, and streptomycin (Meiji Seika Co., Tokyo, Japan); erythromycin (Dainippon Pharmaceutical Co., Osaka, Japan); chloramphenicol (Sankyo Co., Tokyo, Japan); and tetracycline (Sigma Chemical Co., St. Louis, Mo.).

Determination of MICs. MICs were determined by an agar dilution method. Overnight cultures of the bacterial strains in Mueller-Hinton broth were diluted to a final concentration of about 10^6 CFU/ml, and 5 μl of each bacterial suspension was spotted onto Mueller-Hinton agar plates that contained twofold serial dilutions of antibiotics. MICs were measured after incubation for 18 h at 37°C.

Detection of β-lactamase activity. Two methods were used to detect β-lactamase activity. The ability of the strains to produce β-lactamase was assayed by dropping 50 μl of the overnight cultures onto a Cefinase disk (BBL). The β-lactamase activity of crude cell extracts was detected by the spectrophotometric method (41, 42) by using E-0702, cephaloridine, and benzylpenicillin as the substrates.

Isolation of E-0702-resistant mutants. Spontaneous mutants resistant to E-0702 were isolated as follows. Overnight cultures of strains in antibiotic medium 3 were spread on bromothymol-blue-containing nutrient agar plates that contained 1.56 or 3.13 μg of E-0702 per ml at a concentration of between 10^7 and 10^8 cells per plate. After incubation at 37°C for 18 to 24 h, resistant colonies on the plates were randomly picked and purified. Similarly, spontaneous mutants lacking the two major outer membrane proteins, OmpF and OmpC, were isolated by selection for cefoxitin resistance (20, 43).

Membrane preparation. Fractions enriched for the outer membrane were prepared from the whole envelope, which was treated with Triton X-100 to solubilize the cytoplasmic membrane by the method of Schnaitman (44). The protein concentrations were estimated by the method of Lowry et al. (28) by using bovine serum albumin as the standard.

Affinity of E-0702 for PBPs. The affinity of E-0702 for the penicillin-binding proteins (PBPs) of *E. coli* was determined by the conventional competition assay with benzyl-[^{14}C]-penicillin (Amersham Corp., Arlington Heights, Ill.), as described by Spratt and others (10, 45).

Bactericidal activity. Overnight cultures of test strains in liquid medium were harvested by centrifugation at 6,000 × *g* for 20 min at 4°C. Cells were suspended in the appropriate fresh medium at about 10^5/ml and were incubated at 37°C. E-0702 was added after 1 h of incubation, and the numbers of surviving cells, at appropriate times, were counted on bromothymol-blue-containing nutrient agar plates.

Genetic techniques. Mating analysis to locate the mutation and control genetic markers on the chromosomal map of *E. coli* was conducted as described by Miller (31). Streptomycin was used for counterselection. The transduction experiment was performed with phage lysates prepared from cultures infected with the P1 *vir* bacteriophage from strain ER16 (27). Cotransduction frequencies were used to determine the distance between markers by the method of Wu (48).

Phage sensitivity. The sensitivity of strains to various phages was measured qualitatively by spot tests on agar plates (2). Bacterial strains from overnight cultures in antibiotic medium 3 were spread on bromothymol-blue-containing nutrient agar plates, and 1 drop (10 μl) of phage suspension (10^9 phage per ml) in L broth that contained 2.5 mM $CaCl_2$ was added. The extent of lysis was determined after incubation overnight at 37°C.

Absorption spectra of E-0702. The visible and UV spectra of E-0702 and its metal complex were obtained on a Hitachi model 220A recording spectrophotometer.

Measurement of cell growth. The cells grown in minimal medium without $FeCl_3$ and α,α′-dipyridyl for 18 h at 37°C were inoculated, at about 10^6/ml into 10 ml of minimal medium that contained 200 μM $FeCl_3$ or 100 μM α,α′-dipyridyl and incubated at 37°C. Transmittance was measured continuously on a JASCO model Bio-LogII photometer.

RESULTS

Isolation of E-0702-resistant mutants. Spontaneous E-0702-resistant mutants were isolated from *E. coli* ML1410, 3000, NIHJ JC-2, and No. 23 at a frequency of 10^{-6} to 10^{-7}. The mutants were selected on agar medium that contained 1.56 μg of E-0702 per ml. Five to ten colonies were picked randomly for each strain; after purification, the susceptibility to a series of antibiotics was tested. Since all the selected mutants from a given strain showed the same spectrum of

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

Strain	Relevant characteristics	Origin
ML1410	F⁻ *met nalA*	Laboratory collection
ER10	E-0702 resistant, from ML1410	This study
ER24	Cefoxitin resistant, from ML1410	This study
AB1133	F⁻ *thr leu thi-1 supE44 lacY1 galK2 ara-14 xyl-6 mtl-1 proA2 his-4 argE3 str33* λ	K. Mizobuchi
3000	HfrH *thi rel* λ⁻ P01	K. Mizobuchi
ER16	E-0702 resistant, from 3000	This study
CS101	HfrC *metB1 rel-1 tonA22* T_2^r P02A	K. Mizobuchi
ER21	E-0702 resistant, from CS101	This study
KE110	*lac-85*/F_{13} *lac*⁺ *trp*	Laboratory collection
Ymel	*mel-1 supE57 supF58*	K. Mizobuchi
FRE101	*tonB* derivative of Ymel	K. Mizobuchi

TABLE 2. Susceptibility of *E. coli* strains to antibiotics

Strain	MIC (μg/ml)[a]												
	E-0702	E-0702-060	E-0702-061	CEZ	CER	CFX	CPZ	LMOX	ABPC	EM	CP	TC	GM
ML1410	0.012	3.13	1.56	1.56	3.13	6.25	0.2	0.2	6.25	100	6.25	3.13	0.4
ER10	6.25	3.13	1.56	1.56	3.13	6.25	0.2	0.2	6.25	100	6.25	3.13	0.2
ER24	0.012	3.13	1.56	25	25	50	0.8	3.13	6.25	200	12.5	3.13	0.4
3000	0.006	0.8	0.4	1.56	3.13	3.13	0.05	0.1	3.13	50	6.25	1.56	0.1
ER16	3.13	0.8	0.4	1.56	3.13	3.13	0.05	0.1	3.13	50	6.25	1.56	0.1
NIHJ JC-2	0.05	1.56	0.4	1.56	3.13	0.8	0.1	0.2	3.13	6.25	0.8	1.56	0.4
ER31	3.13	1.56	0.4	1.56	3.13	0.8	0.05	0.2	3.13	6.25	0.8	1.56	0.4
No. 23	0.003	1.56	0.8	NT[b]	3.13	NT	0.1	0.2	6.25	50	6.25	NT	0.1
ER36	6.25	1.56	0.8	NT	3.13	NT	0.1	0.2	6.25	50	6.25	NT	0.1
Ymel	0.003	0.8	0.4	0.8	1.56	3.13	0.1	0.1	3.13	50	6.25	3.13	0.1
FRE101	3.13	0.8	0.4	0.8	1.56	3.13	0.1	0.2	3.13	25	3.13	3.13	0.05

[a] Abbreviations: CEZ, cefazolin; CER, cephaloridine; CFX, cefoxitin; CPZ, cefoperazone; LMOX, moxalactam; ABPC, ampicillin; EM, erythromycin; CP, chloramphenicol; TC, tetracycline; GM, gentamicin.
[b] NT, Not tested.

susceptibility, one mutant from each strain was chosen; they were designated ER10, ER16, ER31, and ER36.

The MICs of E-0702 for these mutants were between 3.13 and 6.25 μg/ml, and these values were 100- to 1,000-fold higher than those for the parental strains. In contrast, the MICs of derivatives of E-0702 (E-0702-060 and E-0702-061), of other β-lactam antibiotics, and of other antibiotics for the mutants were almost the same as those for the parental strains (Table 2). Thus, no cross resistance was observed for E-0702 and the other antibiotics tested in the mutants resistant to E-0702.

E-0702 is a cephalosporin containing vicinal hydroxyl groups at the 6,7-positions of the chromone carboxylate in the 7β-side chain of the cephalosporin nucleus, whereas its derivatives, E-0702-060 and E-0702-061, have the same structure as E-0702 does, except for the substitution by one methoxyl group for one hydroxyl group on the chromone carboxylate (Fig. 1).

These facts suggest that the vicinal hydroxyls on the chromone moiety of E-0702 play an important role in the antibacterial activity of E-0702.

Properties of mutants. The β-lactamase activity of the E-0702-resistant mutants was not detected by either the spectrophotometric assay or the Cefinase-disk test. Since these mutants were shown to be susceptible to cephaloridine, cefoperazone, and ampicillin (Table 2), which are substrates for β-lactamase, it can be concluded that resistance to E-0702 did not result from production of β-lactamase.

The affinity of E-0702 for PBPs was investigated by the method of Spratt (45). No differences in the pattern of the PBPs and in the affinity of E-0702 for the PBPs were detected between the mutants and the parental strains. Considering that the other β-lactams, all of which are known to act on the same targets as E-0702 does, are active against the mutants, it is quite unlikely that the resistance to E-0702 is the result of any alteration in PBPs.

In the outer membrane fractions of the E-0702-resistant mutant, we observed no drastic reduction in the levels of OmpF and OmpC proteins (Fig. 2), which are known to be responsible for the permeation of hydrophilic cephalosporins through the outer membrane (16, 25, 33, 34). Also, a slight difference in the relative amounts of porin proteins between the mutant and parental strains and additional protein bands above the OmpF band were observed. However, these differences are not important for the porin channels, which are sufficient to reveal cefoxitin susceptibility in the mutant strain. In the outer membrane of the cefoxitin-resistant mutant, the levels of both OmpF and OmpC proteins were extremely reduced (Fig. 2), presumably because of the mutation in the *ompB* gene locus (20). This mutant, ER24, was resistant to all tested cephalosporins, except for E-0702 and its derivatives (E-0702-060 and E-0702-061), but it was susceptible to penicillins and other antibiotics (Table 2). This result is consistent with that of Sawai et al. (43). Thus, it is likely that E-0702 penetrates the outer membrane not via the porin channels but via a nonporin and, probably, E-0702-specific pathway. E-0702 resistance appears to be the result of a defect in this E-0702-specific pathway.

Location of the E-0702-resistant mutation. Strains ER16 and ER21 were isolated from strains 3000 (HfrH) and CS101 (HfrC), respectively, by selection on agar medium that contained 1.56 μg of E-0702 per ml and were crossed with the multiauxotrophic recipient strain AB1133. The loci for resistance to E-0702 were transferred not by HfrC but by HfrH when streptomycin was used for counterselection. In a

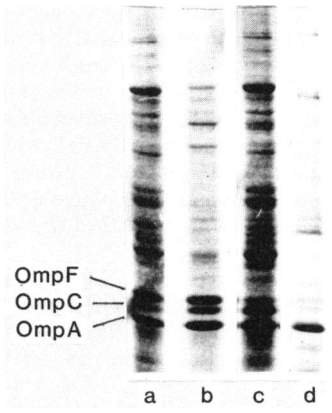

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins. The proteins were prepared from Triton-treated cell envelopes as described in Materials and Methods and analyzed by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis as described by Laemmli (26). Lanes: a, strain ML1410 grown in iron-supplemented medium; b, strain ML1410 grown in iron-deficient medium; c, strain ER10 grown in iron-supplemented medium; d, strain ER24 grown in iron-supplemented medium.

TABLE 3. Sensitivity of *E. coli* strains to phages

Strain	Sensitivity to phage		
	ϕ80	T5	BF23
ML1410	+	+	+
ER10[a]	−	+	+
3000	+	+	+
ER16[a]	−	+	+
Ymel	+	+	+
FRE101[b]	−	+	+

[a] Strains ER10 and ER16 are the E-0702-resistant mutants isolated from ML1410 and 3000, respectively.
[b] *tonB* mutant derived from Ymel.

cross of ER16 with AB1133, the transfer frequencies of lac^+, gal^+, and his^+ recombinants were 2.1×10^{-4}, 9.9×10^{-6}, and 1.6×10^{-7}, respectively. Under these conditions, the E-0702-resistant phenotype was observed at a frequency of 1.1×10^{-6}, indicating that the mutation was located at approximately 28 min on the *E. coli* chromosome between *gal* (17 min) and *his* (44 min) (1).

Transduction of the E-0702 resistance gene by the P1 *vir* phage was performed to determine the linkage with *trp* at 28 min. A P1 lysate was prepared from strain ER16, and the *trp* marker was transduced to strain KE110, which was susceptible to E-0702. Of the *trp* transductants, 69% were found to be resistant to E-0702. The distance between the resistance gene and the *trp* gene was calculated to be 0.23 min (map unit) from this frequency of cotransduction by the equation of Wu (48).

Since E-0702 resistance is considered to result from the decreased ability of E-0702 to penetrate the outer membrane as described above, it is likely that the E-0702 resistance mutation is located in the *tonB* region (28 min), which is required for the active transport of many substances.

Mutations at the level of the cell envelope sometimes result in phage resistance. Sensitivity to ϕ80, T5, and BF23 phages was tested qualitatively in the E-0702-resistant mutants and their parental strains. The mutants were found to be resistant to phage ϕ80 and sensitive to phages T5 and BF23. With respect to phage sensitivity, the *tonB* mutant and the E-0702-resistant mutant were phenotypically identical (Table 3). This result confirmed that the mutation to E-0702 resistance is located in the *tonB* gene.

To determine whether a defect in the *tonB* gene caused E-0702 resistance, we tested the susceptibility to various antibiotics of the *tonB* mutant (FRE101), which was isolated from Ymel for resistance to phage T1. Strain FRE101, which has been shown to be resistant to phage ϕ80 (Table 3), was found to be resistant only to E-0702 of the antibiotics tested (Table 2).

Therefore, we concluded that E-0702 resistance results from a defect in the functioning of the *tonB* gene. Furthermore, it seemed likely that the *tonB* gene or its product plays an important role in E-0702 permeation of the outer membrane of *E. coli*.

Growth of E-0702-resistant mutants. The *tonB* function is required not only for the killing action of phages and colicins but also for the energy-dependent uptake of ferric enterochelin (12, 19, 38, 39), ferric citrate (11, 14, 18), and ferrichrome (6, 15). It is known that *tonB* mutants require a high level of iron for normal growth (46). When the growth of strains in iron-supplemented (200 μM $FeCl_3$ added) or iron-deficient (100 μM α,α'-dipyridyl added) medium was investigated, strain ML1410 grew normally in both media, but strain ER10 did not grow at all in iron-deficient medium, as expected (Fig. 3). This result supports the hypothesis that the E-0702-resistant mutant is deficient in the high-affinity system for the transport of iron.

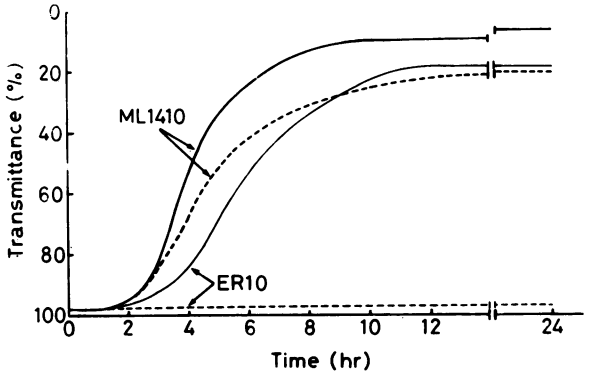

FIG. 3. Iron requirement of E-0702-resistant mutant. Growth was monitored as a decrease in transmittance at 550 nm. ——, Growth of the strains in minimal medium that contained 200 μM $FeCl_3$; -----, growth in minimal medium that contained 100 μM α,α'-dipyridyl.

Chelation of metals by E-0702. The absorption spectrum of iron-supplemented E-0702 in phosphate buffer (pH 7.0) is shown in Fig. 4. When $FeCl_3$ was added to E-0702 in phosphate buffer (pH 7.0), the solution turned yellow-brown. A new absorption band appeared in the visible region, the E-0702 absorption peaks at 352 and 234 nm shifted toward higher wavelengths, and the peak at 264 nm disappeared upon the addition of $FeCl_3$. In contrast, no spectral changes were observed in similar experiments with E-0702 and $MgCl_2$ or $CaCl_2$ or with E-0702-060 and $FeCl_3$. Thus, E-0702 seems to be very specific for the chelation of ferric ion, possibly by the vicinal hydroxyls on the chromone moiety.

Bactericidal activity of E-0702. E-0702 exhibited extremely

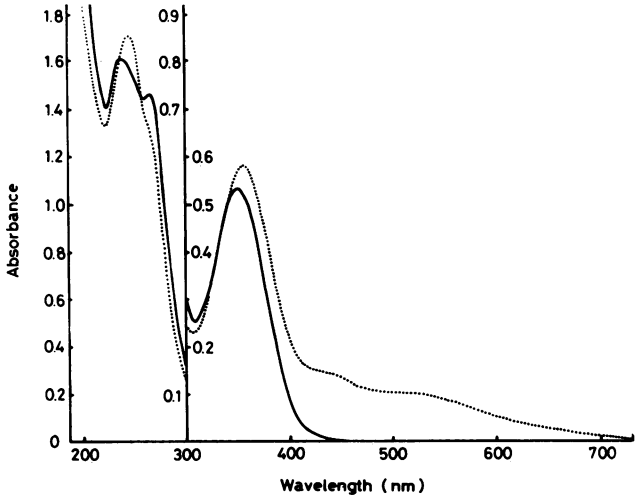

FIG. 4. Absorption spectra of E-0702. A solution of 40 μM E-0702 in 30 mM phosphate buffer (pH 7.0) was used for the measurement. ——, No supplement or addition of $MgCl_2$ or $CaCl_2$; -----, supplementation with $FeCl_3$. In each case, the final concentration of metal ions was 40 μM.

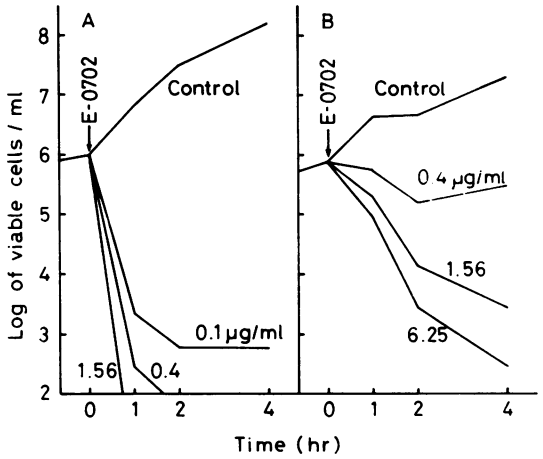

FIG. 5. Bactericidal activity of E-0702 against strains ML1410 (A) and ER10 (B). Cells were grown in antibiotic medium 3.

rapid bactericidal activity against strain ML1410 of *E. coli*. A killing effect of 99.9% was observed, even at 0.1 μg of E-0702 per ml, within 1 h of the addition of E-0702 (Fig. 5). None of the other cephalosporins has exhibited such a rapid killing action at low concentrations so close to their MICs. Against the resistant strain, however, the bactericidal activity of E-0702 was poor, and a killing effect of only 90% within 1 h was observed even at 6.25 μg of E-0702 per ml.

Since E-0702 is a chelator for iron and the E-0702-resistant mutant is phenotypically identical to the *tonB* mutant, it is probable that iron plays an important role in the action of E-0702.

It is known that *E. coli* cells are able to take up iron by the action of iron carriers, which are called siderophores, under conditions of iron starvation (32, 40). These iron transport systems are regulated by the level of iron in media, and the critical concentration of iron for derepression of these systems is about 10^{-6} M (6, 19).

The effect of iron on the bactericidal activity of E-0702 was investigated to study the permeation pathway of E-0702. When 0.1 μg of E-0702 per ml was added to cells grown in iron-deficient medium that contained 100 μM α,α'-dipyridyl, the number of viable cells decreased immediately, and the killing rate of E-0702 was shown to be 99.9% after 2 h (Fig. 6). On the other hand, E-0702 showed hardly any bactericidal activity at 0.1 μg/ml within 2 h against cells grown in iron-supplemented medium that contained 200 μM $FeCl_3$ (Fig. 6). At 0.01 μg/ml, the bactericidal activity of E-0702 against iron-starved cells was also higher than that against iron-supplemented cells.

Isolation of E-0702-resistant mutants from other bacteria. Mutants resistant to E-0702 were also isolated from strains of bacteria other than *E. coli* at a frequency of 10^{-6} to 10^{-7}. However, spontaneous E-0702-resistant mutants could not be isolated from *Staphylococcus aureus* strains.

The resistant mutants from the family *Enterobacteriaceae*, *K. pneumoniae*, *S. typhimurium*, *S. marcescens*, and *P. mirabilis* showed a high degree of resistance only to E-0702 and revealed the same spectrum of susceptibility to other antibiotics as did the mutants from *E. coli* (Table 4). On the other hand, the level of resistance to E-0702 of *P. aeruginosa* mutants was low in comparison with the levels of resistance of mutants from the *Enterobacteriaceae*.

DISCUSSION

It is important for the elucidation of the mode of action of antibiotics and the design of new antibiotics to understand the mechanism of resistance of bacteria to antibiotics. The mechanisms of resistance of bacteria to β-lactam antibiotics are generally categorized into the following three types: (i) production of β-lactamase; (ii) alteration of PBPs; and (iii) decrease of permeability of β-lactam antibiotics through the outer membrane, especially by reduced levels of porin proteins.

In this study, we showed that the first two types could be excluded in the E-0702-resistant mutants isolated from *E. coli* strains. We also showed that the porin channels are not the main pathway of entry of E-0702. This hypothesis is also supported by the fact that the antibacterial activity of E-0702 against a double mutant, isolated from strain ER10 by selection for cefoxitin resistance, was the same as that against ER10 (data not shown) and by the fact that the molecular size of E-0702 (molecular weight, 770) is larger than that of a compound able to diffuse into the periplasmic space through the porin channels (9).

Since E-0702 resistance could not be explained by the three usual types of resistance mechanisms described above and since it was expected to result from a new and E-0702-specific-mechanism of resistance, the genetic characterization of the resistance mutation was investigated. It was shown that resistance to E-0702 results from a chromosomal mutation localized in the *tonB* gene.

Although analysis of the *tonB* gene, using genetic techniques, has recently allowed the identification of the gene product as a 36,000-M_r protein and the determination of its nucleotide sequence (36, 37), the cellular location of the *tonB* gene product remains uncertain because of its rapid turnover (4, 21). However, studies of the *tonB* mutation have proved that the *tonB* product is required for the proper function of the receptors involved in the specific transport systems in the outer membrane of *E. coli* (15, 39, 46, 47).

The *tonB* mutants are insensitive to bacteriophages T1 and φ80, tolerant to many colicins (6, 38, 47), and defective in the transport of vitamin B_{12} (3). In addition, the *tonB* mutants are defective in all known high-affinity iron transport systems that are mediated by enterochelin (12, 19, 38, 39), ferrichrome (6, 15), citrate (11, 14, 18), and aerobactin (5). Enterochelin is produced by cells and is a catechol-

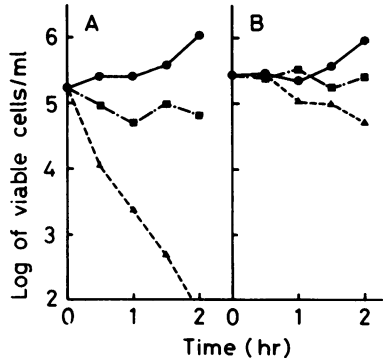

FIG. 6. Effect of iron on bactericidal activity of E-0702. (A) Strain ML1410 grown in iron-deficient medium that contained 100 μM α,α'-dipyridyl; (B) strain ML1410 grown in iron-supplemented medium that contained 200 μM $FeCl_3$. Symbols: ●, control; ■, 0.01 μg of E-0702 per ml; ▲, 0.1 μg of E-0702 per ml.

TABLE 4. Susceptibility of bacterial strains to antibiotics

Strain	MIC (μg/ml)[a]												
	E-0702	E-0702-060	E-0702-061	CEZ	CER	CFX	CPZ	LMOX	ABPC	EM	CP	TC	GM
Klebsiella pneumoniae													
IID875	0.006	1.56	0.8	1.56	0.8	3.13	0.2	0.1	12.5	200	6.25	3.13	0.4
ER47	3.13	1.56	0.8	1.56	0.8	3.13	0.2	0.1	12.5	200	6.25	3.13	0.4
Salmonella typhimurium													
1406	0.0015	1.56	0.8	1.56	1.56	0.8	0.4	0.05	1.56	200	6.25	3.13	0.4
ER42	12.5	3.13	1.56	1.56	1.56	1.56	0.4	0.05	1.56	200	6.25	3.13	0.4
Serratia marcescens													
IID620	0.05	3.13	1.56	>100	100	3.13	0.4	0.1	12.5	100	6.25	3.13	0.2
ER52	6.25	3.13	0.8	>100	100	3.13	0.4	0.1	12.5	100	6.25	3.13	0.2
Proteus mirabilis													
OM-1	0.05	1.56	0.8	3.13	6.25	3.13	0.025	0.05	6.25	200	3.13	25	0.8
ER59	6.25	1.56	0.4	3.13	6.25	1.56	0.025	0.05	6.25	200	3.13	25	0.8
Pseudomonas aeruginosa													
PAO1	0.1	12.5	25	>100	>100	>100	6.25	12.5	>100	400	25	12.5	1.56
ER64	1.56	12.5	25	>100	>100	>100	6.25	12.5	>100	400	25	12.5	0.8

[a] For abbreviations, see Table 2, footnote *a*.

containing siderophore. Its free phenolic hydroxyls act as ligands for iron (35, 40). We showed that E-0702 also coordinates to iron via the vicinal hydroxyls and that the killing action of E-0702 is affected by the level of iron in the medium. These findings indicate that iron-chelated E-0702 is incorporated into *E. coli* cells through the outer membrane via the *tonB*-dependent transport system for iron with subsequent rapid expression of the killing action of E-0702, which exhibits high affinity for the essential PBPs (23).

The transport systems for phages, colicins, iron, and vitamin B require specific receptors for proper function, in addition to the functional product of the *tonB* gene (47). When mutants were isolated by selection for resistance to phages and colicins, most of them were defective in receptor proteins in the outer membrane. However, all the E-0702-resistant mutants tested turned out to be defective in the *tonB* gene function, and no defects in the receptor proteins in the outer membrane were observed. This result could be explained as follows. (i) Receptors are not necessary for E-0702 transport. (ii) Various receptors for E-0702 are present in the outer membrane. If this assumption were true, receptor-defective mutants would not be isolated, since E-0702 could penetrate the outer membrane as a result of the proper functioning of other receptors and could act on PBPs, even if one type of receptor was defective. (iii) The receptor for E-0702 is essential for cell viability. The loss of this receptor would lead to cell death. (iv) The mutation frequency of the gene for the receptor protein is much lower than that of the *tonB* gene.

Since iron transport systems are dependent on an energized membrane state (39), it is expected that uncouplers should affect the killing action of E-0702. However, cells treated with uncouplers are unable to grow sufficiently for the action of β-lactams to be tested. Thus, experiments to observe the effect of uncouplers on the bactericidal activity of β-lactams cannot be performed, and it remains uncertain whether the transport system of E-0702 is dependent on an energized membrane state.

E-0702-resistant mutants were also isolated from other enteric bacteria, and such mutants showed a high level of resistance only to E-0702. Members of the family *Enterobacteriaceae* very probably carry a *tonB*-like gene on their chromosomes, so that iron-chelated E-0702 can be incorporated into these cells via the iron transport systems that are dependent on a *tonB*-like function under iron-limiting conditions. In glucose-nonfermentative rods, such as *P. aeruginosa*, mutants that acquired resistance to E-0702 showed a lower level of resistance to E-0702 than the *Enterobacteriaceae*. However, some *Pseudomonas* variants are known to produce hydroxamate-type iron siderophores, such as pyochelin (8), and probably incorporate iron via an active transport system that is dependent on a *tonB*-like function. E-0702 may, therefore, be incorporated through an E-0702-specific pathway that is a part of the iron transport system. The fact that E-0702 showed potent activity against *P. aeruginosa* also suggests the presence of an E-0702-specific pathway in *P. aeruginosa*.

Albomycin (17), tetracyclines (29, 30), aminoglycosides (13), and fosfomycin (22) are antibiotics which are incorporated into bacterial cells by active transport systems. However, no β-lactam antibiotics are known to penetrate the outer membrane via the active transport systems, and E-0702 is the first β-lactam antibiotic which may be incorporated into cells by the active transport system that is dependent on the *tonB* function. Recently, new β-lactam antibiotics which show high levels of activity against the *Enterobacteriaceae* and *P. aeruginosa* and which contain the catechol group in their molecules have been developed. We previously designated the term catechol effect for the relationship between the spectra of antibacterial activity and the chemical structures of these β-lactam antibiotics (Y. Machida, S. Nomoto, S. Negi, H. Ikuta, I. Sugiyama, H. Yamauchi, K. Kitoh, and I. Saito, Program Abstr. 22nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 618, 1982). We expect that these new β-lactams are also incorporated into bacterial cells via the *tonB*-dependent iron transport system.

The use of microbial iron ligands as the carriers for antibiotics has been attempted. There are inhibitory compounds, called sideromycins, which are structurally analogous to the hydroxamate-type siderophores (49). However, no inhibitory compound analogous to the enterochelin-type

siderophore has been described. E-0702 is the first compound in this class. Sideromycins, as antimicrobial agents, serve as vehicles to bring the antibiotically active part of drugs into the cell and to facilitate uptake, but their use is mainly limited by the high frequency of spontaneous resistant mutants with defects in their transport systems (7). Indeed, the isolation frequency of spontaneous E-0702-resistant mutants was high. However, it is doubtful that these mutants, which require a high level of iron for normal growth, are able to grow in their hosts. Therefore, antimicrobial treatment via the iron transport system remains an extremely fascinating possibility.

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