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Mechanism of *tonB*-Dependent Transport of KP-736, a 1,5-Dihydroxy-4-Pyridone-Substituted Cephalosporin, into *Escherichia coli* K-12 Cells

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The mechanism of transport of KP-736, a novel cephalosporin with a 1,5-dihydroxy-4-pyridone moiety at the C-7 position, into the *Escherichia coli* K-12 cell was investigated by determining the susceptibilities of iron transport mutants to KP-736. The *tonB* mutant showed a higher degree of resistance to KP-736, indicating that KP-736 was incorporated into *E. coli* cells via the *tonB*-dependent iron transport system. The product of the *exbB* gene was also necessary for the maximal antibacterial potency of KP-736. Cir-lacking and Fiu-lacking mutants showed a moderate level of resistance to KP-736. However, mutants lacking any one of the proteins FepA, FecA, FhuA, and FhuE did not show any increased resistance to KP-736. Two types of spontaneous mutants (e.g., KT1004 and KT1011) could be isolated from *cir* and *fiu* mutants by selection for KP-736 resistance and showed the same level of resistance to KP-736 as a *tonB* mutant. KT1004 showed *tonB* phenotypes, resistance to phage ϕ80, and loss of FecA, whereas KT1011 did not. KT1011 lost the ability to express both Cir and Fiu proteins. These results indicate that the Cir and Fiu outer membrane proteins are involved specifically in the *tonB*-dependent transport process of KP-736. Against OmpF- and OmpC-deficient transformants producing various groups of β-lactamases, KP-736 was more effective than the other cephalosporins tested.

Many bacteria require a free iron concentration of 0.1 μM for growth (for a review, see reference 3). Under aerobic conditions, most iron is found in an extremely insoluble ferric hydroxide polymer at pH 7, with the free ferric iron concentration being on the order of 10^{-12} M. To obtain iron under such iron-depleted conditions, many bacteria release high-affinity iron chelators known as siderophores. For example, *Escherichia coli* produces the phenolate-type siderophore enterochelin (3, 27, 44) and the hydroxamate-type siderophore aerobactin (2, 3). Furthermore, *E. coli* can utilize siderophores that *E. coli* strains do not synthesize (3). After siderophores chelate and solubilize ferric iron, ferric siderophores are transported into cells through iron-regulated outer membrane proteins (IROMPs).

In *E. coli*, five ferric iron transport systems that use enterochelin, aerobactin, citrate, and the fungal hydroxamates ferrichrome and coprogen have been identified (6). Each system requires IROMPs (81-kDa FepA, ferric enterochelin; 80.5-kDa FecA, ferric citrate; 78-kDa FhuA, ferrichrome; 76-kDa FhuE, coprogen and rhodotorulic acid) and several cytoplasmic membrane proteins including TonB (3). Furthermore, some of these IROMPs serve as binding sites for B-group colicin and certain phages (FepA, colicins B and D; FhuA, colicin M and phages T1, T5, and ϕ80; and 74-kDa Cir, colicins Ia and Ib) (3).

The TonB protein transmits cytoplasmic energy to the outer membrane receptors and allows the release of these substrates bound on IROMPs into the periplasm (11, 12, 43, 45, 46). TonB-dependent energy transmission is modulated by the ExbB protein, which stabilizes TonB, and also is possibly modulated by several other proteins including ExbC (19, 43). The *tonB* mutant is defective in all known high-affinity iron transport systems mediated by siderophores, is tolerant to B-group colicin, and is resistant to phages T1 and ϕ80 but not to T5 (1, 3, 43, 44, 56). All ferric iron transport systems are regulated by the iron supply and by the *fur* locus (14, 15, 17). Under iron-depleted conditions, the outer membrane proteins and enterochelin are synthesized in high amounts, and the activities of the iron transport systems are increased.

Mammalian hosts limit the availability of iron to restrict the growth of pathogens in body fluids. In human plasma, most iron is complexed with several iron-binding proteins including transferrin, lactoferrin, and ferritin, resulting in a low-iron environment. Nevertheless, pathogenic bacteria are able to acquire iron. The iron uptake systems of bacteria may be used as a transport pathway of drugs into bacterial cells. The concept of siderophore-mediated drug delivery is the focus of this report.

KP-736 is a novel cephem antibiotic with a 1,5-dihydroxy-4-pyridone moiety at the C-7 position of the cephem nucleus (Fig. 1), and the in vitro activities of KP-736 were reported previously by Maejima et al. (35). KP-736 showed more potent antibacterial activity than other cephalosporins against most members of the family *Enterobacteriaceae* and glucose nonfermenters including *Pseudomonas aeruginosa*. In addition, Yokota et al. (50) suggested that KP-736 may be incorporated into *P. aeruginosa* cells not only through the porin but also via the iron transport channels. They also reported that KP-736 was effective against clinical isolates of *P. aeruginosa* resistant to several antibiotics and with altered outer membranes.

In the study described in this report we investigated whether KP-736 is transported into *E. coli* cells by any of the iron transport systems and whether it is mainly transported through either a iron transport pathway or a porin pathway. We also examined whether KP-736 is effective in *E. coli* strains lacking porin and producing β-lactamase.

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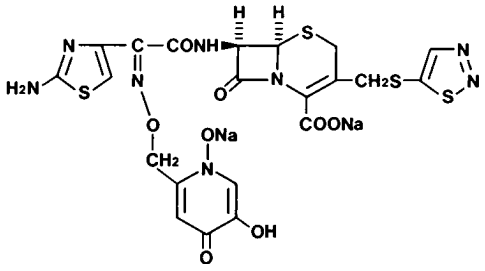

FIG. 1. Structure of KP-736.

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MATERIALS AND METHODS

Antibiotics and reagents. KP-736 was synthesized at the Central Research Laboratories, Kaken Pharmaceutical Co., Ltd., Tokyo, Japan, and the structure of KP-736 is illustrated in Fig. 1. The other antibiotics were obtained from the indicated companies: cefclidin, Eisai Co., Ltd., Tokyo, Japan; cefoxitin, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; cefepime, Bristol-Myers Squibb Research Institute, Tokyo, Japan; cefuzonam, Lederle Japan Co., Ltd., Tokyo, Japan. α,α′-Dipyridyl and Sarkosyl (*N*-lauroylsarcosine sodium salt) were purchased from Sigma Chemical Co. (St. Louis, Mo.). $FeCl_3 \cdot 6H_2O$ and citrate were also purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Bacterial strains and bacteriophages. All strains used in the study were *E. coli* K-12 derivatives and are listed in Table 1. The iron transport mutants were provided by K. Hantke, Universität Tübingen, Tübingen, Germany (13–19, 26, 49, 60). KT1002 and KT1013 were isolated from H1300 by selection for KP-736 resistance. KT1011 and KT1004 were isolated from H1594 by selection for KP-736 resistance. KT1015 was isolated from KT1011 by selection for cefoxitin resistance. *E. coli* MC4100 and MH1160 were provided by S. Mizushima, Nagoya University, Nagoya, Japan (27, 28). MH1160 is an *ompR1* mutant of MC4100 and lacks the outer membrane proteins OmpF and OmpC (25), while MC4100 has an osmoregulated production of OmpF and OmpC (25). Transformants of MH1160 and MC4100 with pMS510, pMS509, pMS185-2, or pMS182-5 plasmid DNA (25), which contain cloned β-lactamase genes of the types penicillinase group 2b (TEM-1) of Rms212 (57), penicillinase (PCase) group 2d (OXA-1) of Rms213 (57), cephalosporinase (CSase) group 1 of *Citrobacter freundii* GN346 (52), and oxyimino-cephalosporinase (CXase) group 2e of *Proteus vulgaris* GN7919 (36), respectively, were maintained as stock cultures in our laboratory (25). pMS363 plasmid DNA (30), which contains the cloned β-lactamase gene of CXase group 3 of *P. aeruginosa* GN17203, was provided by S. Iyobe, Gunma University, Gunma, Japan (30). The pMS363 plasmid was introduced into *E. coli* MC4100 and MH1160 by transformation with calcium chloride (9). Bacteriophage ϕ80 was provided by K. Mizobuchi and H. Uchida, Tokyo University, Tokyo, Japan. Bacteriophage T5 was also provided by A. Nishimura, National Institute of Genetics, Shizuoka, Japan.

TABLE 1. *E. coli* K-12 strains used in the study

Strain	Relevant genotype	Reference
AB2847	*aroB malT tsx thi*	13
H455	Same as AB2847, but Δ(*pro lac*)	14
H1196	Same as H455, but *fhuA*::Mu d1	14
H1187	Same as H455, but *fepA*::Mu d1	14
H1300	Same as H455, but *cir*::Mu d1	14
ZI379	Same as AB2847, but *fecA*::Mu d1 *lac*::Tn*10*	60
H1252	Same as H455, but *tonB*::Mu d1	14
ZI17	Same as H455, but *exbB*::Mu d1	19
H1608	Same as H455, but *exbC*::Mu d1X	14
H1274	Same as H1196, but *fur cys*	14
H1443	*araD* Δ(*lac*) *aroB rpsL relA flbB deoC ptsF rbsR*	16
H1594	Same as H1443, but *fiu*::Mu d1X	16
H1619	Same as H1443, but *fhuE*::Mu d1X	49
KT1013[a]	Same as H1300, but *tonB*	This study
KT1002[a]	Same as H1300, but *fiu*	This study
KT1004[a]	Same as H1594, but *tonB*	This study
KT1011[a]	Same as H1594, but *cir*	This study
KT1015[b]	Same as KT1011, but loss of OmpF and OmpC	This study
MC4100	F^- Δ*lacU169 araD139 rpsL relA thiA flbB*	7
MH1160	Same as MC4100, but *ompR1* (*ompB101*)	48

[a] Spontaneous mutants isolated by selection for KP-736 resistance (see text).
[b] Spontaneous mutants isolated by selection for cefoxitin resistance (see text).

Determination of MICs. MICs were determined by the twofold agar dilution method with Mueller-Hinton agar (MHA; Difco Laboratories, Detroit, Mich.). Overnight cultures of the bacterial strains in Mueller-Hinton broth (MHB; Difco) were diluted with saline to a final concentration of about 2×10^7 CFU/ml. Then, 5 μl of each bacterial suspension, corresponding to about 10^5 CFU, was spotted with an inoculator (Microplanter; Sakuma Seisakusho, Tokyo, Japan) onto MHA plates containing twofold serial dilutions of the antibiotics. The inoculated plates were incubated for 18 h at 30°C. The MIC was defined as the lowest concentration of antibiotic inhibiting visible growth of the bacteria on the agar plate.

Bactericidal activity test. The bacterial cells in the mid-logarithmic phase (about 10^6 CFU/ml) were exposed to antibiotics at the MIC in MHB only, iron-replete MHB (to which 200 μM $FeCl_3$ was added), or iron-depleted MHB (to which 100 μM α,α′-dipyridyl was added). Two 50-μl portions of each culture were taken at fixed times, and several 10-fold dilutions were prepared in saline and were mixed with 10 ml of drug-free MHA. The number of colonies was counted after 48 h of incubation at 30°C. The antibiotic carryover did not affect colony formation. The MICs of the antibiotics for the test strains were examined in MHB containing twofold serial dilutions of the antibiotics before the bactericidal activity test.

Isolation of spontaneous mutants resistant to KP-736. Spontaneous mutants resistant to KP-736 were isolated as follows. Overnight cultures of the strains in MHB were spread onto MHA plates containing a serial dilution of the antimicrobial agent at concentrations of between 10^7 and 10^8 cells per plate. Resistant colonies appeared after 36 h of incubation at 30°C and were randomly picked from plates containing 0.78 μg of KP-736 per ml and were purified on drug-free MHA. Similarly, spontaneous mutants lacking the two major outer membrane proteins, OmpF and OmpC, were isolated on MHA plates containing 12.5 μg of cefoxitin per ml (31, 50).

Preparation of outer membrane proteins and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Bacteria were grown at 30°C with shaking to the mid-exponential phase (cell optical density at 650 nm, 0.5) in Luria broth (34) supplemented with α,α′-dipyridyl and citrate at 100 μM and 1 mM, respectively. The amounts of protein in the outer membrane preparations were determined by the method of Lowry et al. (33), with bovine serum albumin used as the standard. The outer membranes were separated as a Sarkosyl-insoluble fraction by the method of Inokuchi et al. (28). The composition of the polyacrylamide gel and the conditions of electrophoresis were the same as those specified by Wagegg and Braun (54).

Phage susceptibility test. The FhuA protein is the receptor for phages ϕ80 and T5 (3). The susceptibilities of the cells to phage ϕ80 were *tonB* dependent, whereas those of the cells to phage T5 were *tonB* independent (3). Therefore, the presence of the functional *tonB* gene was examined preliminarily on the basis of the susceptibilities of the cells to phage ϕ80 and T5. Susceptibility to phage was tested as follows. Bacterial strains were spread onto Luria agar (34) plates at a concentration of about 10^7 cells per plate, and the plates were incubated for 2 h at 30°C. Then, 5 μl of phage suspension (10^9 phage per ml) in Luria broth containing 2.5 mM $CaCl_2$ was spotted onto the bacterial lawn. The plates were incubated for 18 h at 30°C, and zones of bacterial lysis were observed.

RESULTS

Susceptibilities of *E. coli* mutants iron transport to KP-736. The susceptibilities of *E. coli* iron transport mutants to KP-736, cefuzonam, and cefoxitin are given in Table 2. The *tonB* mutant (H1252) was 256-fold less susceptible to KP-736 than its parents (AB2847 and H455). The *exbB* mutant (ZI17) was fourfold less susceptible to KP-736 than its parents. Cir-lacking (H1300) and Fiu-lacking (H1594) mutants were 16- and 4-fold less susceptible to KP-736, respectively, than their parents. However, mutants lacking any one of FepA, FhuA, and FhuE did not show any increased level of resistance to KP-736. Conversely, the FecA-lacking mutant (ZI379) and the *fur* mutant (H1274) showed significantly increased susceptibilities to KP-736. In contrast, the MICs of cefuzonam and cefoxitin for these mutants were the same as those for their parents. Thus, these

TABLE 2. Susceptibilities of *E. coli* mutants to KP-736, cefuzonam, and cefoxitin

E. coli K-12 strain[a]	Salient characteristic[a]	MIC (μg/ml)[b]		
		KP-736	Cefuzonam	Cefoxitin
AB2847	Control	0.024	0.10	3.13
H455	Control	0.024	0.10	3.13
H1187	*fepA*	0.024	0.10	3.13
H1196	*fhuA*	0.024	0.10	3.13
H1300	*cir*	0.39	0.10	3.13
ZI379	*fecA*	0.006	0.10	3.13
H1252	*tonB*	6.25	0.10	3.13
ZI17	*exbB*	0.10	0.10	3.13
H1608	*exbC*	0.05	0.10	3.13
H1274	*fhuA fur*	0.001	0.10	3.13
KT1013	*cir tonB*	6.25	0.10	3.13
KT1002	*cir fiu*	3.13	0.10	3.13
H1443	Control	0.05	0.10	3.13
H1594	*fiu*	0.20	0.10	3.13
H1619	*fhuE*	0.05	0.10	3.13
KT1004	*fiu tonB*	6.25	0.10	3.13
KT1011	*fiu cir*	6.25	0.10	3.13
KT1015	*fiu cir* $OmpF^-$ $OmpC^-$	12.5	0.78	50
MC4100	Control	0.006	0.024	1.56
MH1160	*ompR1*	0.012	0.20	25

[a] See Table 1 for full genotypes and strain derivations.
[b] The MICs were determined by the agar dilution method with an inoculum of 10^5 CFU per spot.

mutants did not show cross-resistance to cefuzonam and cefoxitin.

Isolation and characterization of spontaneous mutants resistant to KP-736. On MHA plates containing 0.78 μg of KP-736 per ml spontaneous mutants could be isolated from *E. coli* AB2847, H455, and H854 (controls), H1187 (*fepA*), H1196 (*fhuA*), ZI379 (*fecA*), H1619 (*fhuE*), and H1608 (*exbC*) at a frequency of approximately 10^{-7} to 10^{-8} and from H1594 (*fiu*), H1300 (*cir*), and ZI17 (*exbB*) at a frequency of approximately 10^{-6}. The susceptibilities of spontaneous mutants to KP-736, cefuzonam, and cefoxitin are given in Table 2. Spontaneous mutants (e.g., strains KT1002, KT1013, KT1011, and KT1004) showed the same level of resistance to KP-736 as the *tonB* mutant H1252 and acquired a higher level of resistance to KP-736. In contrast, the MICs of cefuzonam and cefoxitin for spontaneous mutants were the same as those for their parents. Thus, spontaneous mutants did not show cross-resistance to cefuzonam and cefoxitin.

Table 3 shows the susceptibilities of spontaneous mutants and their parents to phages ϕ80 and T5. All parent strains were susceptible to ϕ80 and T5. Two distinct types of phage ϕ80

TABLE 3. Susceptibilities of KP-736-resistant spontaneous mutants to phages

E. coli K-12 strain	Susceptibility to phage	
	ϕ80	T5
AB2847	+	+
H455	+	+
H1196	−	−
H1252	−	+
KT1013	−	+
KT1002	+	+
H1443	+	+
KT1004	−	+
KT1011	+	+

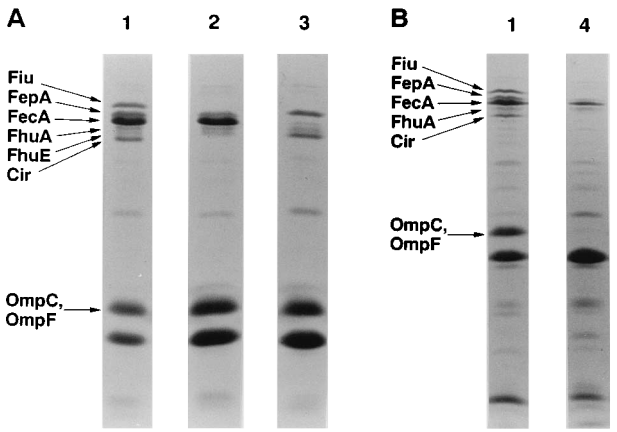

FIG. 2. Outer membrane protein profiles of *E. coli* AB2847, KT1011, KT1004, and KT1015. Lanes: 1, AB2847; 2, KT1011; 3, KT1004; 4, KT1015. Outer membranes were extracted from cells grown in Luria broth supplemented with α,α′-dipyridyl (100 μM) and citrate (1 mM). A total of 65 μg of each protein was analyzed on an SDS-polyacrylamide slab gel: (A) 10% polyacrylamide; (B) 12.5% polyacrylamide.

susceptibility were observed with spontaneous mutants (e.g., KT1004 and KT1011) derived from *cir* (H1300) and *fiu* (H1594) mutants. KT1004 showed a *tonB* phenotype, resistance to phage ϕ80. However, KT1011 was susceptible to phage ϕ80, which indicates that it has a functional *tonB* gene. Both of the mutants were susceptible to phage T5, which indicates that they have the receptor for phages ϕ80 and T5, the FhuA protein.

The outer membrane protein profiles of spontaneous mutants and that of the parent (AB2847) are compared (Fig. 2). Several IROMPs were expressed when wild-type strain AB2847 was grown in the presence of α,α′-dipyridyl and citrate (Fig. 2A, lane 1). In contrast, KT1011 lost the ability to express both Cir and Fiu proteins (Fig. 2A, lane 2), so that the mutant was identified as a *fiu cir* mutant. It should also be noted that FecA was not induced in the *tonB* mutant (KT1004), as has been reported by other researchers (4, 26, 60) (Fig. 2A, lane 3). The positions of these IROMPs were confirmed by the absence of designated IROMPs of iron transport mutants. We observed no dramatic reduction in the levels of the OmpF and OmpC proteins between these spontaneous mutants and AB2847 (Fig. 2A, lanes 2 and 3).

tonB mutants were isolated from all strains, whereas *cir fiu* double mutants were isolated from the only *cir* and *fiu* mutants.

When H1594 and H1300 were selected with 0.78 μg of KP-736 per ml, *tonB* mutants were obtained in about the same proportion that *cir fiu* double mutants were obtained. With selection of H1594 and H1300 at concentrations greater than 0.78 μg/ml, more *tonB* mutants than *fiu cir* double mutants were obtained. Selection of all *E. coli* strains at concentrations greater than 3.13 μg/ml was unsuccessful. Apart from the two types of mutants described above, no other types were found among 50 independent KP-736-resistant spontaneous mutants on the basis of an analysis of their outer membrane protein profiles.

Susceptibilities of OmpF- and OmpC-lacking *E. coli* mutants derived from wild-type strain and the *fiu cir* mutant to KP-736. The susceptibilities of OmpF- and OmpC-lacking *E. coli* mutants (MH1160 and KT1015) derived from wild-type (MC4100) and the *fiu cir* mutant (KT1011) to KP-736 and other cephalosporins, respectively, are given in Table 2. MH1160 was 8- and 16-fold less susceptible to cefuzonam and

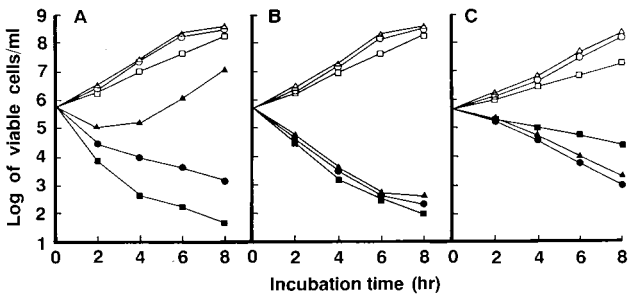

FIG. 3. Effect of iron on the bactericidal activities of KP-736 against *E. coli* AB2847 (A), KT1011 (B), and H1252 (C). The MICs of KP-736 for *E. coli* AB2847, KT1011, and H1252 in MHB were 0.05, 6.25, and 6.25 μg/ml, respectively. Symbols: ○, MHB only; △, MHB plus $FeCl_3$ (200 μM); □, MHB plus α,α′-dipyridyl (100 μM); ●, MHB plus KP-736 (at the MIC); ▲, MHB plus $FeCl_3$ (200 μM) plus KP-736 (at the MIC); ■, MHB plus α,α′-dipyridyl (100 μM) plus KP-736 (at the MIC).

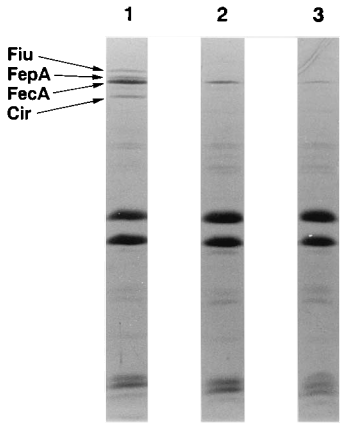

FIG. 4. Outer membrane protein profiles of *E. coli* AB2847 in relation to concentrations of iron. Outer membranes were extracted from cells grown in MHB supplemented with α,α′-dipyridyl (100 μM) (lane 1), in MHB only (Lane 2), or in MHB supplemented with $FeCl_3$ (200 μM) (lane 3). A total of 65 μg of each protein was analyzed on an SDS-polyacrylamide (12.5%) slab gel.

cefoxitin, respectively, than its parent (MC4100), but it was only 2-fold less susceptible to KP-736. KT1015 was isolated from KT1011 by selection for cefoxitin resistance and lost the ability to express OmpF and OmpC (Fig. 2B, lane 4), presumably because of the mutation in the *ompB* gene locus (31). KT1015 showed the same levels of resistance to cefuzonam and cefoxitin as MH1160, but it was only twofold less susceptible to KP-736 than its parent (KT1011).

Effect of iron on bactericidal activity of KP-736. To confirm whether KP-736 was transported across the iron-regulated outer membrane proteins, the effect of iron on the bactericidal activity of KP-736 was investigated (Fig. 3). In iron-depleted MHB, KP-736 showed an enhanced bactericidal activity against *E. coli* AB2847 at a concentration of 0.05 μg/ml compared with that in MHB only, and the number of viable cells decreased by 3 log units in 4 h (Fig. 3A). In contrast, the addition of 200 μM $FeCl_3$ to MHB completely inhibited the bactericidal activity of KP-736. This effect of iron was not observed with *fiu cir* (KT1011) and *tonB* (H1252) mutants (Fig. 3B and C). On the other hand, the bactericidal activity of cefuzonam against these strains was not affected by the addition of α,α′-dipyridyl or $FeCl_3$ to MHB (data not shown).

As the concentration of iron in MHB decreased, the outer membrane of AB2847 showed increased levels of expression of Fiu, FepA, FhuA, and Cir proteins (Fig. 4).

Antibacterial activity of KP-736 against the wild type and its OmpF- and OmpC-lacking transformants producing various groups of β-lactamases. We examined whether KP-736 was effective against *E. coli* strains lacking porin proteins and producing β-lactamase (Table 4). Against MC4100 transformants producing various groups of β-lactamases, KP-736 showed higher levels of activity than cefuzonam. The MICs of cefuzonam for MH1160 transformants were 16-fold greater than those for MC4100 transformants, when CSase group 1 of *Citrobacter freundii* or CXase group 2e of *Proteus vulgaris* was produced. An even greater synergistic effect of porin deficiency and β-lactamase production was observed with MH1160 transformants, for which the MICs of cefclidin and cefepime were 16- to 64-fold higher than those for MC4100 when PCase group 2d (OXA-1), CXase group 2e of *P. vulgaris*, or CXase group 3 of *P. aeruginosa* was produced. However, the MICs of KP-736 for MH1160 transformants were similar to those of KP-736 for MC4100 transformants. Thus, KP-736 showed higher levels of activity than the other cephalosporins against

TABLE 4. Antibacterial activities of KP-736 and other cephalosporins against the wild-type strain and its OmpF- and OmpC-lacking transformants producing various groups of β-lactamases

E. coli K-12 strain	Plasmid	β-Lactamase[a]	MIC (μg/ml)[b]			
			KP-736	Cefuzonam	Cefclidin	Cefepime
MC4100 ($OmpF^+$ $OmpC^+$)	Host	None	0.006	0.024	0.05	0.012
	pMS510	PCase 2b	0.006	0.024	0.10	0.024
	pMS509	PCase 2d	0.024	3.13	3.13	1.56
	pMS185-2	CSase 1	0.10	0.20	0.10	0.024
	pMS182-5	CXase 2e	1.56	12.5	0.78	0.39
	pMS363	CXase 3	1.56	25	3.13	1.56
MH1160 ($OmpF^-$ $OmpC^-$)	Host	None	0.012	0.20	0.20	0.10
	pMS510	PCase 2b	0.012	0.39	1.56	0.39
	pMS509	PCase 2d	0.05	12.5	50	25
	pMS185-2	CSase 1	0.20	3.13	0.39	0.39
	pMS182-5	CXase 2e	1.56	200	50	6.25
	pMS363	CXase 3	3.13	50	100	25

[a] The groups of β-lactamases are based on the classification of Bush (4, 5).
[b] The MICs were determined by the agar dilution method with an inoculum of 10^5 CFU per spot.

OmpF- and OmpC-lacking mutants producing various groups of β-lactamases.

DISCUSSION

In recent years, new β-lactam antibiotics that possess a catechol or pyridone moiety have been synthesized and reported to show potent antibacterial activities against most gram-negative bacteria, including *P. aeruginosa*, which have low levels of outer membrane permeability (29, 32, 35, 38, 39, 41, 42). This enhanced activity was due to the ability to bind iron and to undergo active transport into cells by iron transport mechanisms. The catechol-substituted cephalosporins, such as BO-1236 (22), BO-1341 (22, 47), E-0702 (53), M14659 (37), Compound A (8), and GR69153 (51), have been shown to be actively incorporated into *E. coli* cells via the *tonB*-dependent iron transport system. Curtis et al. (8) found that outer membrane receptors specific to catechol-substituted cephalosporins were Cir and Fiu proteins. Nikaido and Rosenberg (40) found that pirazmonam, a monobactam that possesses a 3-hydroxy-4-pyridone moiety at the C-3 position, was also incorporated into *E. coli* cells via the same transport system by which catechol-substituted cephalosporins are transported.

The new cephalosporin, KP-736, studied here possesses a novel 1,5-dihydroxy-4-pyridone moiety at the C-7 position. The vicinal carboxylate and hydroxyl groups in the pyridone moiety seem to actively chelate the ferric iron, as reported by Harris et al. (21) for those in aerobactin. KP-736 enters into *E. coli* cells through the iron transport pathway, as do siderophores. To demonstrate this hypothesis, we determined the susceptibilities of iron transport mutants to KP-736 and characterized spontaneous mutants resistant to KP-736.

In the present study, the *tonB* mutant showed a higher level of resistance to KP-736, indicating that KP-736 was incorporated into *E. coli* cells via the *tonB*-dependent iron transport system. The *exbB* mutant showed a moderate level of resistance to KP-736. This result indicates that the product of the *exbB* gene is also required for the full expression of the antibacterial potency of KP-736. For Cir-lacking and Fiu-lacking mutants there was a moderate decrease in activity of KP-736, whereas mutants lacking any one of the FepA, FecA, FhuA, and FhuE proteins did not show any increased resistance to KP-736. The *fur* mutant showed an increased susceptibility to KP-736 because it may express IROMPs constitutively (14). These observations agreed with those of Nikaido and Rosenberg (40) obtained with pirazmonam and extended their findings. In contrast, none of the iron transport mutants showed any cross-resistance to cefuzonam (23, 24), which has the same structure as KP-736 except for the 1,5-dihydroxy-4-pyridone group substituted at the C-7 position in the KP-736 structure. This indicates that the 1,5-dihydroxy-4-pyridone moiety in the KP-736 structure is necessary for the *tonB*-dependent transport of KP-736.

We thought that KP-736 enters into *E. coli* cells via two routes, Cir and Fiu proteins, because *cir* and *fiu* mutants acquired low levels of resistance to KP-736. This hypothesis was confirmed by an analysis of spontaneous mutants (e.g., KT1011) selected from *cir* and *fiu* mutants. KT1011 showed the same high level of resistance to KP-736 as the *tonB* mutant but had a functional *tonB* gene. KT1011 lost the ability to express both Cir and Fiu proteins. These results indicate that the Fiu and Cir proteins are involved jointly and specifically in the *tonB*-dependent transport of KP-736.

The expression of IROMPs is regulated by the levels of iron in the medium (14, 15, 17). The bactericidal activity of KP-736 against *E. coli* was remarkably enhanced under iron-depleted conditions. This was presumably due to the increase in the level of expression of Cir and Fiu proteins and the high degree of affinity of *E. coli* for the penicillin-binding proteins 1A, 1B(s), and 3 (35). In contrast, the bactericidal activity of KP-736 was completely inhibited under iron-replete conditions, presumably because of the repressed expression of Cir and Fiu receptors. KP-736 seems to kill the bacteria effectively under a low-iron environment in vivo when the iron transport systems are derepressed.

We also showed that the main route of entry of KP-736 into bacteria is not the porin channels but IROMPs. This hypothesis is supported by three facts: that the increase in KP-736 resistance by the loss of OmpF and OmpC from a *fiu cir* mutant was only 2-fold, that *cir fiu* double mutants showed a 64-fold higher level of resistance to KP-736 than the *ompR1* mutant, and that the molecular size of KP-736 (molecular weight, 683) is larger than that (<600 Da) of a compound able to diffuse into the periplasm through the porin channels (10). Perhaps the route of entry for KP-736 into *E. coli* cells will be primarily via the protein Cir, secondly via the protein Fiu, and thirdly through porin channels, because the KP-736 MIC for a *cir* mutant was four- and eightfold higher than those for *fiu* and *ompR1* mutants, respectively.

In general, small and hydrophilic β-lactams predominantly utilize the OmpF and OmpC porin channels to cross the outer membrane of *E. coli* (31, 50, 59). However, pathogenic bacteria can easily develop high levels of resistance to β-lactams by both the loss of porin proteins and the high-level production of β-lactamases (6, 20). Indeed, Hiraoka et al. (25) demonstrated that porin-deficient mutants showed high levels of resistance to β-lactams compared with the resistance of the wild-type strain when a large amount of active β-lactamase existed in the periplasm. However, the activity of KP-736 was less affected by the combined effect of porin loss and β-lactamase production. This is presumably because KP-736 largely depends on the *tonB*-dependent iron transport systems for its penetration route. Against wild-type transformants producing β-lactamases, KP-736 showed higher levels of activity than cefuzonam. However, the β-lactamase stability and the penicillin-binding protein affinity of KP-736 may be similar to those of cefuzonam (23, 24). This suggests that hydrolysis of KP-736 by β-lactamases is overcome by the effective penetration of the drug into the periplasm via the iron transport system.

A high level of resistance to KP-736 would require either a double mutation or a *tonB* mutation. Indeed, the isolation frequency of *tonB* mutants from the wild-type strain by selection of KP-736 resistance was high. However, it is doubtful that the *tonB* mutants, which are deficient in all high-affinity iron transport systems, are able to grow under an iron-depleted environment in vivo. Furthermore, the exclusive double mutant will not be isolated, possibly because of the extremely low frequency of double mutations. Indeed, we could not isolate spontaneous double mutants from the wild-type strain. If some clinical *E. coli* isolates naturally lack either Fiu or Cir, then *cir fiu* double mutants might be spontaneously selected. However, we found that a *cir* or a *fiu* mutant with lower levels of resistance was even more susceptible to KP-736 in iron-depleted medium (data not shown).

In conclusion, KP-736, an aminothiazolyl-oxyiminocephalosporin with a novel 1,5-dihydroxy-4-pyridone group at the C-7 position, was recognized by the Cir and Fiu iron-regulated outer membrane receptor proteins and was predominantly transported into *E. coli* cells via the *tonB*-dependent iron transport system rather than through the OmpF and OmpC porin channels. KP-736 may be an effective agent against infections caused by gram-negative bacteria, including those strains re-

sistant to several β-lactams as a result of porin deficiency and β-lactamase production.

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