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Cir and Fiu Proteins in the Outer Membrane of *Escherichia coli* Catalyze Transport of Monomeric Catechols: Study with β-Lactam Antibiotics Containing Catechol and Analogous Groups

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Recently, β-lactam agents containing iron-chelating moieties, such as E0702, which contains catechol, and pirazmonam and U-78,608, which contain 3-hydroxypyridone, have been developed. By determining the susceptibility to these agents of *Escherichia coli* mutants lacking various iron-repressible outer membrane proteins, we showed that two of these proteins with hitherto unknown functions, Fiu and Cir, were apparently involved in the transport of monomeric catechol and its analogs. These results confirm the conclusion of Curtis and co-workers, which was obtained by using a different set of catechol-containing antibiotics (N. A. C. Curtis, R. L. Eisenstadt, S. J. East, R. J. Cornford, L. A. Walker, and A. J. White, Antimicrob. Agents Chemother. 32:1879–1886, 1988). E0702 was shown to enhance the uptake of radioactive ferric iron into wild-type cells but not into *cir fiu* double mutants. By combining the influx of E0702 with its hydrolysis by a periplasmic β-lactamase, we showed that the wild-type cells transported unliganded E0702 at a rate comparable to or even higher than the rate of transport of the E0702-Fe^{3+} complex. We postulate that the main function of Cir and Fiu may be to recapture the hydrolytic products of enterobactin, such as 2,3-dihydroxybenzoic acid and 2,3-dihydroxybenzoylserine.

Most nutrients used by gram-negative bacteria move across the outer membrane through the nonspecific channels of porin (16), and a similar mechanism of diffusion is thought to be used by most antibiotics that are effective against these bacteria, including most β-lactams (13). Some nutrients, however, are too large for efficient diffusion through the porin channel, and these compounds, including iron-siderophore complexes, are often taken up by specific pathways present in the outer membrane. Thus, *Escherichia coli*, for example, is known to have at least seven outer membrane proteins whose production is regulated by the availability of ferric iron in the environment; four of them, the products of the *fepA*, *fecA*, *fhuA*, and *fhuE* genes, have been identified as the receptors for ferric enterobactin, ferric citrate, ferrichrome, and coprogen, respectively (12, 22). Recently, several β-lactam compounds with attached iron-chelating moieties have been developed (9, 18, 24). Moreover, evidence suggestive of the utilization of a siderophore receptor for outer membrane penetration was obtained for at least one of these compounds, E0702, because *E. coli* became resistant to this compound if it had *tonB* mutations, which abolish the transport of all known siderophores as well as vitamin B_{12} across the outer membrane (26). However, the identities of the outer membrane receptors used by these β-lactams were unknown, and this study was undertaken to identify these receptors. In the course of the present study, we learned that another laboratory studied the outer membrane permeation of a group of catechol-containing cephalosporins by using a similar approach (3). Our results, however, have been obtained by using antimicrobial agents different from those studied by Curtis et al. (3) and extend their data by the successful measurement of the influx rates of one of the antibiotics across the outer membrane.

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MATERIALS AND METHODS

Bacterial strains. All strains used were derivatives of *E. coli* K-12 and are listed in Table 1. HN593 was made by selecting colicin Ib-resistant mutants from H1594. It was sensitive to bacteriophage φ80, which indicates that it was still $TonB^+$, and the absence of the Fiu and Cir proteins in this strain was confirmed by sodium dodecyl sulfate-polyacrylamide gel analysis of the outer membrane proteins from cells grown in the presence of ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA) (21) (Fig. 1).

β-Lactam compounds. E0702, U-78,608, and pirazmonam (SQ-83,360) were obtained from Eisai Co., Tsukuba City, Japan; The Upjohn Co., Kalamazoo, Mich.; and E. R. Squibb & Sons, Princeton, N.J., respectively. Their structures are shown in Fig. 2.

Susceptibility experiments. The susceptibilities of various *E. coli* strains to β-lactams were determined by a broth dilution technique. Twofold successive dilutions of antibiotics in L broth (10 g of tryptone [Difco Laboratories, Detroit, Mich.], 10 g of yeast extract [Difco], and 5 g of NaCl per liter) were inoculated with overnight cultures of the *E. coli* strains so that the initial densities were about 10^4 CFU/ml. Since some of the strains were temperature sensitive because of the presence of Mu d(*lac*) fusions, the incubation was carried out at 30°C. The results were scored after 24 h of incubation.

Sodium dodecyl sulfate-polyacrylamide gel analysis of outer membrane proteins. For the analysis of outer membrane proteins, the outer membrane fraction was prepared by sucrose density centrifugation (23). The composition of the gel and the conditions of electrophoresis were as specified by Wagegg and Braun (25).

Uptake of ferric iron. For ferric iron uptake experiments, the bacteria were grown at 30°C with shaking in morpholinepropanesulfonic acid (MOPS) medium (11) modified by leaving out iron and *N*-(tris[hydroxymethyl]methyl)glycine (Tricine) (4), with appropriate additions of required growth

TABLE 1. Bacterial strains used for the study of β-lactam penetration

Strain	Relevant genotype	Other markers	Reference or source
MC4100	Wild type	*araD139 lacU169 rpsL thiA*	
AB2847	Wild type	*aroB351 malT354 tsx-354*	
W3100	Wild type		
RW193	*entA*	*thi proC leuB trpE lacY mtl xyl azi tsx rpsL*	27
RW1318	*fepA entA*	*thi proC leu trp*	J. Neilands
WA380	*fecA11*	*aroB tsx malT thi*	25
C600	*fhuA21*	*thr-1 leuB6 lacY1 supE44 thi-1*	Laboratory stock
JK114	*fhuA*	*argH ara lac gal ura trp his thi mal man xyl purC*	J. Konisky
H1619	*fhuE*::Mu d1X	*aroB araD139 lacU169 rpsL thiA*	22
H1594	*fiu*::Mu d1X	*aroB araD139 lacU169 rpsL thiA*	K. Hantke
JK2132	*cir*::Mu dII*lac*	*trpR55 trpA9605 his-85 metE lac*	J. Konisky[a]
JK116	*cir*	Same as JK114	2 (called JK114R3 therein)
BN3040	*cir entA*	*proC leuB trpE thi*	8
JK402	Φ(*cir-lac*)	Same as MC4100	28 (called PW402 therein)
HN593	*cir fiu*::Mu d1X	Same as H1594	This study
GUC6	*tonB50 fhuA21*	*thr-1 leuB6 thi-1 lacY1 supE44*	B. Bachmann
BN0203	*fepB*	*proC leu trp thi*	J. Neilands
WA27	*fecB34*	Same as WA380	25

[a] Derived from JK2131 by a method described in reference 6.

factors and with 0.2% glycerol as the carbon source. Cells in the exponential phase of growth were harvested when the Klett reading reached 100 (0.225 mg [dry weight]/ml) and were washed and suspended in 0.04 M MOPS-KOH buffer (pH 7.4) containing 1% glycerol. This suspension was shaken at 30°C in a water bath, ^{55}Fe-siderophore complexes were added, and 0.2-ml portions of the suspension were filtered at appropriate intervals through a type HA filter (diameter, 25 mm; Millipore Corp., Bedford, Mass.). The filter was washed immediately with 10 ml of 100 μM nitrilotriacetic acid (NTA) and dried, and the radioactivity was determined with a liquid scintillation counter. We subtracted from each value the radioactivity of the zero time sample, which presumably represented the nonspecific adsorption of ferric hydroxide to filters and cells; this amounted to about 20% of the total activity present in the 8-min sample with the wild-type cells. Similar uptake experiments were carried out simultaneously with a 2:1 complex of NTA-^{55}Fe, and the nonspecific transport rate thus obtained was subtracted from the rate obtained in the presence of E0702. This correction amounted to about 20% in the case of the wild type.

The solution of $^{55}FeCl_3$ in 0.1 M HCl (Amersham Corp., Arlington Heights, Ill.) was dried under a stream of nitrogen, and the ferric chloride was dissolved by adding either 2 mol of NTA or 3 mol of E0702 per mol of $FeCl_3$ in 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-NaOH buffer (pH 7.4).

Uptake of β-lactams. When the strain to be tested contained only the chromosomal *ampC* gene, which is not inducible in *E. coli*, R factor R1, with a gene coding for TEM β-lactamase, was transferred to the strain by conjugation. Other strains produced sufficient levels of TEM β-lactamase because they contained the *bla* gene as a part of the Mu d(*lac*) insertion sequence. The cells were grown in L broth and harvested by centrifugation when the culture reached a Klett reading of 100. They were washed once in 10 mM HEPES-NaOH buffer, pH 7.4, containing 50 μg of bovine serum albumin per ml and were resuspended in the same buffer at a density of 2 mg/ml. All centrifugations were

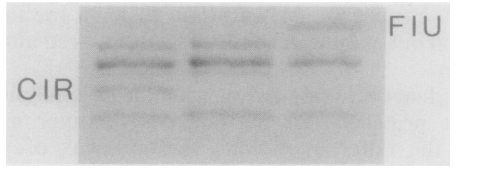

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel pattern of iron-repressible proteins. The strains were grown in L broth with shaking and were harvested 3 h after the addition of 1 mg of EDDA per ml. Outer membranes were prepared and analyzed as described in Materials and Methods. Only the portion of the gel containing iron-repressible proteins (about 70 to 90 kilodaltons) is shown. The strains shown are H1594 (*fiu*), HN593 (*fiu cir*), and BN3040 (*cir*) (left to right).

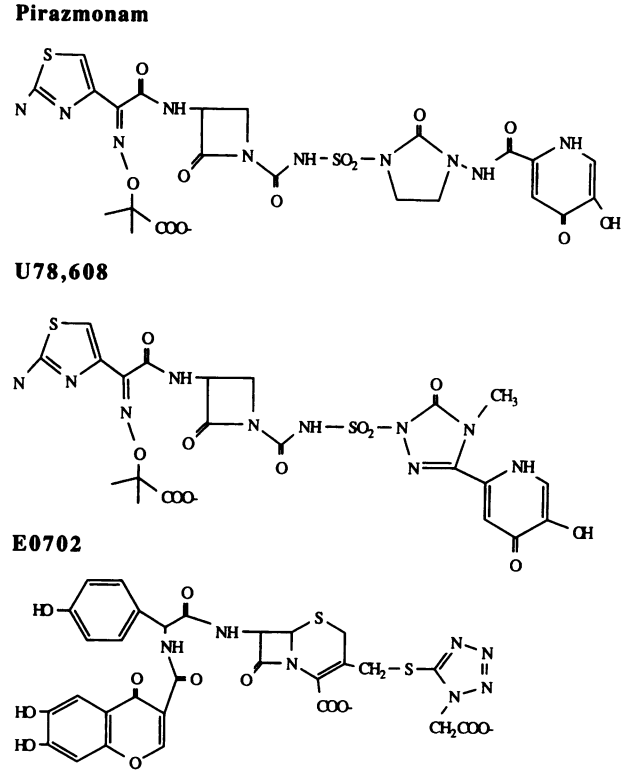

FIG. 2. Structures of the β-lactams tested.

TABLE 2. MICs of E0702, pirazmonam, and U-78,608 for various strains

Strain	Relevant genotype	MIC (μg/ml) of[a]:		
		E0702	Pirazmonam	U-78,608
MC4100	Wild type	0.05	0.1	0.1
W3100	Wild type	0.1	0.2	0.2
RW193	*entA*	0.2	0.2	0.2
RW1318	*fepA entA*	0.1	0.1	0.1
WA380	*fecA11*	0.01	0.002	0.001
C600	*fhuA21*	0.05	0.2	0.2
JK114	*fhuA*	0.05	0.1	0.2
H1619	*fhuE*::Mu d1X	0.2	0.1	0.04
H1594	*fiu*::Mu d1X	1.6	0.6	0.2
JK2132	*cir*::Mu dII*lac*	1.6	0.3	0.1
JK116	*cir*	3.2	2.0	0.6
BN3040	*cir entA*	6.4	2.3	1.0
JK402	Φ(*cir-lac*)	51.2	1.6	0.6
HN593	*cir fiu*	25.6	6.2	3.1
GUC6	*tonB50 fhuA21*	3.2	2.0	1.3
BN0203	*fepB*	0.025	0.05	0.025
WA27	*fecB34*	0.025	0.025	0.009

[a] Values are geometric means of at least three determinations.

carried out, and the final cell suspension was kept, at room temperature.

The hydrolysis of E0702 was measured spectrophotometrically. The reaction mixture contained 10 mM HEPES-NaOH buffer (pH 7.4), freshly dissolved E0702 at a final concentration of 10 μM, and cells at a final concentration of about 0.03 mg/ml. This was mixed in a cuvette (light path, 1 cm), and optical density (OD) changes were monitored for 15 min at two wavelengths, 274 and 246.8 nm, with a lambda 4B spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) controlled by a PC/XT personal computer (IBM Corp.) through the PECSS interface (Perkin-Elmer). The hydrolysis of E0702 produced maximum changes in OD at 274 nm (OD_{274}), whereas the $OD_{246.8}$ remained constant during hydrolysis. The changes in $OD_{246.8}$ were used to correct for changes in light scattering due to the swelling, shrinkage, or aggregation and sedimentation of the cells. Because we had to determine OD changes in the range of 0 to 0.0003/min (in the presence of ODs of 1 to 1.2 caused mainly by scattering by the cells) it was essential to read the ODs at two wavelengths and to use a spectrophotometer with a high degree of precision and an extremely low noise level (about 0.0001 absorbance unit peak-to-peak at zero absorbance with our instrument).

A portion of the cell suspension was centrifuged at the same time, and the supernatant was later used to determine the activity of leaked-out β-lactamase present in the extracellular medium. The hydrolysis rates obtained with the cell suspension were corrected for the contribution of the leaked-out enzyme. This correction was between 20 and 40% of the total activity with the wild-type cells.

RESULTS

MICs for various mutant strains. If the β-lactams tested mimic siderophores during their movement through the *E. coli* outer membrane, then mutants defective in the transport of siderophores are likely to be more resistant to these agents. A *tonB* mutant, which is deficient in all known siderophore transport processes across the outer membrane in spite of the presence of the receptors, was significantly more resistant to all three antimicrobial agents tested (Table 2). This extends the finding of Watanabe et al. (26) to pirazmonam and U-78,608 and is consistent with the assumption that all three agents indeed utilize the siderophore pathway(s). Among the six or seven *E. coli* outer membrane proteins that are derepressed under iron starvation conditions, FepA, FecA, FhuA, and FhuE are known to be receptors for ferric enterobactin, ferric citrate, ferrichrome, and coprogen, respectively (11, 22). Mutants lacking these proteins, however, did not show any increased resistance to the three β-lactam agents tested, and the *fecA* mutant unexpectedly showed a significantly increased susceptibility. In contrast, the MICs of the β-lactams tested were significantly higher for mutants lacking two iron-repressed outer membrane proteins of hitherto unknown functions, Cir and Fiu, than for the wild-type strains. For one of the four *cir* mutants used, JK2132, the increase in MIC was often much smaller, but this is probably due to the fact that the strain was thermolabile and showed significant lysis even when grown at 30°C (6); thus, the outer membrane barrier was likely to have been damaged in most members of the population.

TABLE 3. Influence of iron availability on MICs of E0702

Preculture medium	MIC (μg/ml) determined in[a]:		
	L broth + conalbumin	L broth	L broth + ferric citrate
L broth + conalbumin	<0.007	0.2	0.2
L broth	<0.007	0.2	0.6
L broth + iron citrate	<0.007	0.2	0.6

[a] Strain AB2847 was used, and MICs were determined by serial threefold dilutions in L broth (plus additions). The inoculum was about 10^4 cells per 0.5 ml. Conalbumin was added to a concentration of 4 mg/ml, and ferric citrate was added to a concentration of 10 μM.

Some strains contained the *bla* gene, which codes for TEM β-lactamase, as a part of the Mu d(*lac*) fusion, and this may have contributed to the resistance to E0702, which is easily hydrolyzed by the TEM enzyme (26). In fact, in one of the *cir* mutants, JK402, the TEM β-lactamase was expressed at a much higher level than in other strains (data not shown), and this strain also showed a higher resistance to E0702 (Table 2). However, the two monobactams with the oxyimino substituents are quite stable in the presence of this enzyme (24), and thus the increased MICs of these agents are not due to the presence of the *bla* gene. We note also that all three drugs tested had high MICs for BN3040, a *cir* mutant not containing the *bla* gene.

When the *cir* and *fiu* mutations were combined in a single strain (HN593), resistance was increased to a much higher level, suggesting that antibiotics tested can use the products of both of these genes for penetration of the outer membrane. We found identical high levels of resistance in six independent colicin-resistant isolates. We cannot explain why the resistance level in these mutants was higher than in the *tonB* strain. It seems possible that the *tonB* mutation used was functionally leaky.

If the β-lactams tested are transported across the outer membrane by siderophore receptors, the MICs of these compounds should be affected by conditions that alter the levels of expression of the receptor proteins. Indeed, the MICs of E0702 decreased greatly when the test medium contained conalbumin (Table 3), which binds iron and caused overproduction of the iron-repressible siderophore receptors, as detected by sodium dodecyl sulfate-polyacrylamide gel analysis of the outer membrane proteins (data not

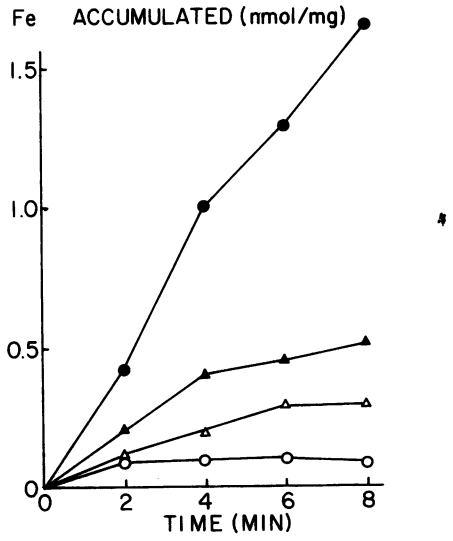

FIG. 3. Uptake of ^{55}Fe stimulated by the addition of E0702. A 3:1 complex of E0702 and ^{55}Fe (final concentration, 10 μM) was added to cells grown in modified MOPS medium, and the uptake was measured as described in Materials and Methods. Strains: wild-type (MC4100) (●); *cir* mutant (BN3040) (▲); *fiu* mutant (H1594) (△); *cir fiu* double mutant (HN593) (○).

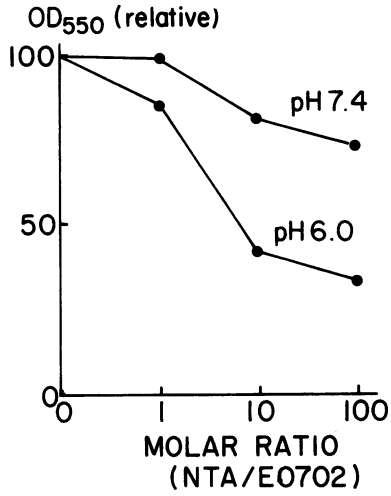

FIG. 4. Competition between E0702 and NTA for Fe^{3+}. A 10 mM solution of E0702 was added to an equal volume of freshly dissolved 10 mM $FeCl_3$ in the presence of various concentrations of NTA (pH 6). The mixture was diluted 50-fold in either 50 mM HEPES-NaOH (pH 7.4) or 50 mM potassium-phosphate (pH 6.0), and the absorption spectra between 400 and 700 nm were determined. The figure shows the OD_{550} of the NTA-containing samples as percentages of that of the sample that did not contain NTA.

shown). Interestingly, the nature of the medium in which the inoculum was precultured had only a very small effect on the MIC. It is also noteworthy that the inclusion of excess Fe^{3+} ions in the assay medium had little effect on MICs (Table 3).

The *fecA* strain WA380 was much more susceptible to the β-lactam agents tested. Although it contained an *aroB* mutation, which makes it impossible for this strain to synthesize its endogenous siderophore, enterobactin, iron deficiency caused by this mutation cannot entirely explain the hypersusceptibility of strain WA380, because another *aroB* strain, H1619, showed normal susceptibility. The mechanism of hypersusceptibility in WA380 remains a topic for future study.

Uptake of ferric ions. The repression of synthesis of Cir and Fiu proteins by high ferric ion concentrations in the environment (11) suggests that these proteins may function in the uptake of ferric ion-siderophore complexes. For this reason, the uptake of ^{55}Fe was studied. The most thorough study was done with the antibiotic E0702. Although the cells showed a significant rate of baseline uptake of Fe^{3+} when only the Fe-NTA (1:2) complex was added, there was a four- to fivefold accelerated uptake of Fe^{3+} into the wild-type cells when Fe^{3+} was supplied in the form of a 1:3 complex with E0702 (Fig. 3). This increased uptake was greatly reduced in the *cir* or *fiu* single mutants and was nearly absent in the *cir fiu* double-mutant strain (Fig. 3). Similar stimulation of Fe^{3+} uptake in the wild-type cells was observed with pirazmonam and U-78,608 as well as with compounds presumably corresponding to the iron-chelating moiety of the latter antibiotic, i.e., 3-hydroxypyridon-6-yl carboxylic acid and 3-hydroxypyridonyl-4-methyl-1,2,4-triazolone, although not all the mutant types were used with these compounds (data not shown).

It is customary to add a large excess of NTA to reaction mixtures for the assay of ferric iron uptake (see, for example, references 3 and 4) in order to suppress the low-affinity iron uptake systems. When such an approach was tried in our system, the addition of E0702 and other β-lactams did not produce a measurable stimulation of iron uptake (data not shown). Thus, our assay mixture contained no NTA, although it contained 1% glycerol in an effort to decrease the deposition of ferric hydroxide on the cell surface or on the filter.

We have performed preliminary experiments to examine the competition between E0702 and NTA. These compounds and $FeCl_3$ were mixed and then diluted in HEPES-NaOH buffer (pH 7.4) or potassium-phosphate buffer (pH 6.0), and the amount of E0702-Fe^{3+} complex present was estimated by A_{550}; at a wavelength of 550 nm, E0702, NTA-Fe complex, and $FeCl_3$ all show negligible absorption. The OD_{550} decreased sharply in the presence of NTA at a large molar excess, and this decrease seemed to occur more efficiently at pH 6.0; a 10-fold excess of NTA was enough to decrease the OD_{550} nearly maximally (Fig. 4). Although we do not completely understand the interaction between the three components, such as the persistence of a significant OD_{550} even at a very large molar excess of NTA (Fig. 4), it is clear that NTA, when present in excess, effectively competes against E0702 in the formation of Fe^{3+} complexes. This is not surprising when we consider that these monomeric catecholic compounds must form a 3:1, or perhaps 2:1, complex with ferric ions (26) and that the association constant for such complexes cannot be very large.

Preliminary data on antibiotic uptake. Because E0702 is hydrolyzed relatively rapidly by the TEM β-lactamase, it was possible to measure its rate of uptake by incubating whole cells in a buffer containing the antibiotic. In preliminary experiments, the kinetics of hydrolysis of E0702 by TEM β-lactamase was determined spectrophotometrically with a purified enzyme prepared as described earlier (14). Although the spectrum of E0702 changed significantly with minor changes of pH in the range of 6 to 8, presumably because of the protonation of phenolic hydroxyl groups, the difference spectrum between the unhydrolyzed and completely hydrolyzed compounds was not affected at all by pH and showed the maximum change and no change at A_{274} and $A_{246.8}$, respectively. Hydrolysis kinetics observed at 274 nm showed that the K_m for the TEM enzyme was 30 μM and the V_{max} was 21% of that for cephaloridine. Thus, at a 100 μM

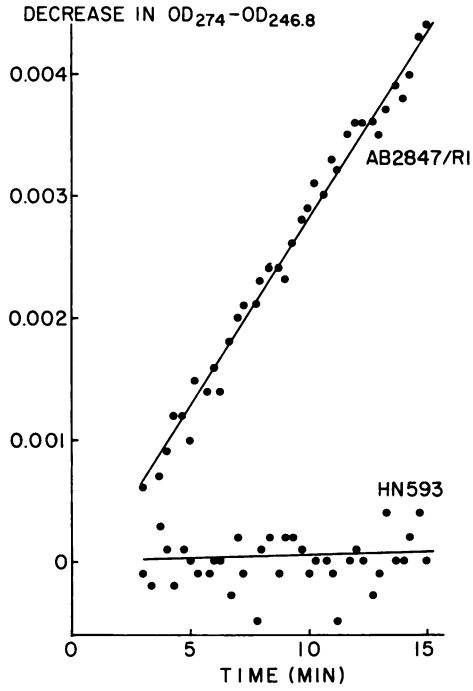

FIG. 5. Uptake of E0702 assayed by coupled hydrolysis by periplasmic β-lactamase. The strains were grown in L broth, and exponential-phase cells were used for the assay as described in Materials and Methods. Because there was a large fluctuation in the observed OD values during the first 2 to 3 min, presumably due to flow birefringence, only the data collected after this initial period are shown. For each datum point, $OD_{246.8}$ was subtracted from OD_{274}, and the analogous value from the supernatant, corresponding to hydrolysis by leaked-out enzymes, was further subtracted from the experimental value. Data were collected every 20 s, and individual datum points are shown to indicate the extent of scatter. The rate of decrease of OD_{274} with the wild-type strain corresponds to the hydrolysis (or transport) rate of 1.2 nmol/mg of cells per min. Although AB2847/R1 contained more β-lactamase (V_{max} for cephaloridine, 1.7 μmol/mg per min) than HN593 (V_{max}, 0.16 μmol/mg per min) did, the difference between the strains was not due to the content (or the leaking out) of the enzyme, since H1619 (*fhuE cir*$^+$ *fiu*$^+$), which contained even less β-lactamase (V_{max}, 0.12 μmol/mg per min), hydrolyzed E0702 at a rate about one-half of that of AB2847/R1 (not shown).

substrate concentration, the rate of hydrolysis of E0702 was about 68% of that of cephaloridine, which is fairly close to what has been reported elsewhere (9). In the assays of E0702 hydrolysis by intact cells (Fig. 5), the activity of the TEM β-lactamase present in the periplasmic space was always more than an order of magnitude higher than the rates observed. Thus, the intact cell rates were essentially determined by the slow uptake process across the outer membrane.

Because of the very low rates of hydrolysis, the currently available data are not very precise. Nevertheless, it was clear that a much more rapid influx of E0702 takes place in the wild-type cells than in a *cir fiu* double mutant strain (Fig. 5). This result is consistent with the idea that the products of these two genes are responsible for the uptake of catechol-containing β-lactams across the outer membrane. Since the concentration of the drug was fairly high (10 μM) and the cell concentration was low (0.03 mg/ml), it is difficult to imagine that E0702 combined with the Fe^{3+} leaking out of the cell and that the resulting complex was taken up. The data therefore suggest that E0702 could be transported efficiently without complexation with ferric iron. Unfortunately, it was not possible to study directly the rate of influx of E0702-Fe^{3+} (3:1) complex by measuring E0702 hydrolysis because the complex was hydrolyzed more slowly than the free compound, even by a purified enzyme.

DISCUSSION

We have shown in this study that β-lactam antibiotics containing catechol or 3-hydroxypyridone cross the outer membrane barrier mainly through siderophore transport channels Cir and Fiu. This conclusion is similar to that of Curtis and co-workers (3), which appeared during the course of our study. In fact, the observation that the MIC of pirazmonam is increased somewhat for *cir* mutants was first made by K. Bush and co-workers (K. Bush, S. K. Tanaka, S. Ohringer, and D. P. Bonner, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1218, 1987).

Because these proteins are induced by iron starvation, they have been presumed to function in the transport of ferric ion-siderophore complexes, but the identities of the siderophores remained unknown. Our results suggest that the proteins may be involved in the transport of complexes of ferric ions and siderophores resembling monomeric catechol. Although the natural ligand(s) for Cir and Fiu proteins remains to be identified, we note that such monomeric catecholic compounds are probably encountered rather frequently by *E. coli* cells; thus, 2,3-dihydroxybenzoic acid is a biosynthetic intermediate of the endogenous siderophore of *E. coli*, enterobactin (5), and ferric enterobactin is hydrolyzed to 2,3-dihydroxybenzoyl-L-serine by an esterase once it is transported into the cytoplasm (17). Although such monomeric catechol siderophores are expected to bind only rather weakly to ferric ions, many other bacteria are already known to utilize siderophores of this type (for a list, see reference 19). In fact, Hancock et al. (7) showed that *E. coli* can take up the ferric ion complex of 2,3-dihydroxybenzoic acid. Interestingly, this transport activity was decreased by 50% in a *cir* mutant (7).

We have been able to measure the rate of transport of E0702 by combining its rate of diffusion across the outer membrane with its rate of hydrolysis in the periplasmic space (Fig. 5). To our knowledge, this represents the first successful attempt to measure directly the rate of diffusion of any siderophore across the outer membrane. Earlier, all measurements of the transport of siderophores dealt with uptake into ill-defined compartments within the whole cell, and it was impossible to distinguish transport into periplasmic space from that into the cytoplasm. (One exception is the study by Matzanke et al. [10], which suggests, on the basis of results of osmotic shock treatment and the use of synthetic enterobactin analogs, the transient accumulation of ferric enterobactin in the periplasm.) Interestingly, we found that the Cir (and/or the Fiu) system transported unliganded siderophore as rapidly as or perhaps more rapidly than it transported the ferric ion-siderophore complex (compare the rates in Fig. 3 [0.2 nmol of Fe^{3+} per mg per min, and thus 0.6 nmol of E0702 per mg per min if the stoichiometry of the complex is 1:3] and Fig. 5 [1.2 nmol of E0702 per mg per min]). The cells used in the experiment whose results are shown in Fig. 5 had to be grown in L broth, because growth in modified MOPS medium (Fig. 3) produced excessive leakage of β-lactamase into the medium. Despite this complication, our conclusion is likely to remain

valid because the uptake of Fe^{3+} was much slower in cells grown in L broth (data not shown) than in those grown in MOPS medium (Fig. 3), presumably because of the repression of iron transporters such as Cir and Fiu. If these proteins can indeed transport unliganded catecholic compounds efficiently, this may be beneficial to the cell for the uptake of 2,3-dihydroxybenzoic acid and its derivatives. Thus, the system may also function in recovering the products of the hydrolysis of enterobactin so that they can be reutilized in the synthesis of enterobactin. We also note that free Fe^{3+} is essentially unavailable in the tissues of animals because of its binding with high-affinity iron-binding proteins; the catechol-containing β-lactams would not work well in such an environment if they had to be transported exclusively as Fe^{3+} complexes.

The extreme susceptibility of strain WA380 to the drugs tested is intriguing. Since the binding to targets, i.e., the penicillin-binding proteins, of compounds with similar structures requires concentrations in the range of 0.05 to 5 μg/ml, it is difficult to imagine that pirazmonam and U-78,608 bind to the target(s) at concentrations equal to the MICs observed, i.e., 0.001 to 0.002 μg/ml. A much more likely explanation is that the siderophore transport system(s) in this strain carries out a concentrative uptake of the compounds in the periplasmic space. Such uphill transport processes across the outer membrane have been suggested to occur with enterobactin (11) and with another ligand that is transported by a similar, TonB-dependent transport machinery, vitamin B_{12} (20).

The measured rates of E0702 uptake were much lower than the rates of diffusion of many other β-lactams through the porin channels of intact cells (15). However, we have to consider the fact that the latter rates were obtained at very high, pharmacologically irrelevant concentrations, typically 1 mM. In contrast, the initial rate of influx at a 10 μM external concentration is calculated by the first law of diffusion of Fick (15) to be 1.4 nmol/mg per min for a typical broad-spectrum cephalosporin, cefotaxime. Our measured rate of influx of E0702 at a 10 μM concentration was 1.2 nmol/mg per min (Fig. 5) and was thus comparable to the porin-mediated diffusion rates of more traditional compounds. Furthermore, with porin-mediated diffusion, the rate continues to decrease as more β-lactam molecules accumulate in the periplasm; however, with the siderophore analogs, the rate of influx stays constant if these compounds are indeed accumulated actively in the periplasm (see above).

The idea of utilizing specific transport machineries of the cell for the uptake of drugs is an old one (for example, see reference 1). However, it appears that very few useful antimicrobial agents have been developed along these lines, presumably because microorganisms can easily develop resistance by the mutational loss of the specific transport protein. The compounds containing catechol or its analogs appear to constitute an exception, apparently because they can utilize one of the two or more pathways present in the outer membrane; a high level of resistance to these compounds would require either an extremely rare double mutation or a mutation in the *tonB* locus, which would essentially abolish the ability of the organism to transport iron and thus make it nonpathogenic. However, it is not yet clear whether gram-negative bacteria other than *E. coli* also produce two pathways that can be used by these compounds.

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