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Modes of Action and Inhibitory Activities of New Siderophore–β-Lactam Conjugates That Use Specific Iron Uptake Pathways for Entry into Bacteria

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We describe here the mechanism of inhibition of two new siderophore–β-lactam conjugates against *Escherichia coli* X580. One conjugate is a spermidine-based catechol siderophore-carbacephalosporin (JAM-2-263), and the other is an N^5-acetyl-N^5-hydroxy-L-ornithine tripeptide hydroxamate siderophore-carbacephalosporin (EKD-3-88). In an agar diffusion test, both conjugates produced large inhibitory zones against strain X580. Resistant strains (i.e., JAM^R and EKD^R) could be isolated after exposure of X580 to the conjugates JAM-2-263 and EKD-3-88, respectively. No cross-resistance was observed in these individual isolates. JAM^R and EKD^R were studied further to elucidate the mechanism of inhibition of each conjugated drug. The affinities of JAM-2-263 and EKD-3-88 for penicillin-binding proteins (PBPs) of isolated inner membranes were determined by a competition assay with ^{125}I-penicillin V. JAM-2-263 targeted primarily PBPs 1A/B and 5/6, while EKD-3-88 targeted PBPs 1A/B and 3. Strains X580, JAM^R, and EKD^R showed similar PBP affinities for the conjugates. However, marked changes were observed in the iron-regulated outer membrane proteins of resistant isolates grown on agar plates depleted of iron. EKD^R lost the expression of FhuA (78 kDa) and its sensitivity to phages T1 and T5, whereas JAM^R lost the expression of Cir (74 kDa) and its sensitivity to colicin Ia. These results revealed the requirement of FhuA and Cir for the inhibitory activities of EKD-3-88 and JAM-2-263, respectively. In an antibiotic diffusion assay, ferrichrome (1 μM) strongly antagonized the activities of both conjugates against X580 and JAM^R, including the residual activity of JAM-2-263 against JAM^R. However, the susceptibility of strain EKD^R lacking the ferrichrome receptor (FhuA$^-$) to the two conjugates remained the same in the presence of ferrichrome. The antagonistic effect of ferrichrome on the activity of JAM-2-263 may also indicate a role for FhuA in the activity of this β-lactam conjugate. A FhuA$^-$ Cir$^-$ double mutant confirmed this hypothesis, since it showed a higher level of resistance to JAM-2-263. To reproduce iron-restricted in vivo growth conditions, we grew X580 and EKD^R cells in diffusion chambers implanted in the peritoneal cavities of rats. Strain EKD^R showed impaired growth in such a cultivation system. This is the first report of β-lactam drug transport into *E. coli* cells that involves the FhuA outer membrane protein.

Several authors have reviewed the role of iron in infections (1, 15, 49, 61). Iron is necessary for all microorganisms, plants, and animals. Most aerobic, facultative anaerobic, and saphrophytic microorganisms have the ability to produce high-affinity iron-binding compounds, termed siderophores, that are capable of chelating ferric iron and that allow its assimilation through cell surface receptors. It is thought that many pathogenic microorganisms acquire their essential iron from their hosts by this means (7, 31, 41).

The nature of the bacterial receptors and mechanisms of transport of siderophores has been best studied in *Escherichia coli*. These receptor proteins are multifunctional and also serve as receptors for bacteriophages and colicins (8, 15, 31, 41).

E. coli is able to produce a phenolate-catecholate siderophore, enterochelin, as well as a hydroxamate siderophore, aerobactin. It is also able to utilize ferrichrome, a siderophore produced by some fungi (40). The synthesis of enterobactin is governed by several chromosomal genes (8). Ferric enterobactin uptake is mediated by a transport system including the genes *fepA*, encoding the 81-kDa outer membrane receptor, *fepB*, encoding a permease, and four other genes (*fepCDEG*) (8). The gene *fes* codes for an intracellular esterase that allows the removal of iron from ferric enterobactin (41, 43). The Fur protein interacts thereafter with Fe^{2+} and acts as a repressor of the transcriptional expression of all components of the iron transport system (8).

The uptake of ferrichrome by *E. coli* requires two genes, designated *fhuA* and *fhuB*. The *fhuA* gene product is a 78-kDa outer membrane protein, and the *fhuB* gene product is a cytoplasmic membrane constituent. The ExbB, FhuC, and FhuD products are also implicated in the utilization of hydroxamate siderophores (6, 13, 19, 22). The FhuE product is the 76-kDa outer membrane receptor of coprogen and ferrirhodotorulate (17). Another gene, *tonB*, is necessary for the uptake of iron by all high-affinity outer membrane receptor-mediated iron transport systems (8). *exbB* mutants are also unable to take up iron, suggesting a function for *exbB* similar to that for *tonB*.

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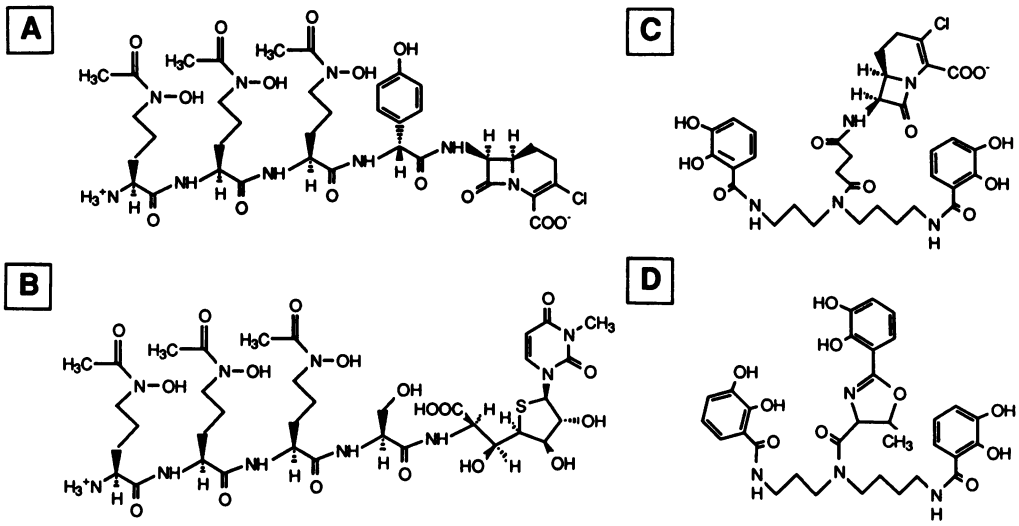

FIG. 1. Chemical structures of two new siderophore-carbacephalosporin conjugates (JAM-2-263 and EKD-3-88) in comparison with those of known iron-chelating molecules. (A) N^5-acetyl-N^5-hydroxy-L-ornithine tripeptide hydroxamate siderophore-carbacephalosporin β-lactam conjugate (EKD-3-88). (B) Albomycin. (C) Spermidine-based catechol siderophore-carbacephalosporin conjugate (JAM-2-263). (D) Agrobactin.

The ferric citrate uptake system is also dependent on *tonB* and *exbB* and involves two other gene products: the FecA protein (80-kDa outer membrane receptor) and the FecB, FecC, FecD, and FecE cytoplasmic membrane proteins (58). The 83-kDa Fiu and 74-kDa Cir outer membrane proteins are also inducible in response to iron limitation, but their functions are still unknown. These proteins seem to be implicated in a TonB-dependent transport of ferri-monocatechols, a function that could be associated with the recapture of the hydrolytic products of enterobactin (42).

The aerobactin iron transport genes are encoded by certain ColV plasmids but can also be found in the chromosome of invasive *E. coli* (60). The *iucABC* genes are responsible for aerobactin synthesis, while *iutA* and *iutB* code for a 74-kDa outer membrane receptor and a transport protein, respectively. Again, this transport system relies on TonB, ExbB, and FhuCDB functions (8).

Mammalian hosts limit the availability of essential nutrients, such as iron, to limit the growth of pathogens. One of the mammalian iron-withholding mechanisms thought to be very important is the contraction of iron in the plasma compartment and the expansion of iron in the storage compartment (1, 63). In plasma, iron is contained as complexes with iron-binding proteins. There are three major iron-binding proteins in humans: transferrin, lactoferrin, and ferritin. Nevertheless, pathogenic bacteria and fungi are able to acquire iron. Iron uptake systems may be turned against some pathogens by taking advantage of these pathways for drug delivery. The concept of siderophore-mediated drug delivery is the focus of this paper.

The hypothesis that we have tested in the present paper is that new hydroxamate (EKD-3-88) or spermidine-based catechol (JAM-2-263) siderophore-carbacephalosporin conjugates (Fig. 1) can use microbial iron transport processes to actively carry the β-lactam antibiotic into *E. coli* cells. The tripeptide portion of EKD-3-88 is similar to that of the previously described iron-chelating antibiotic albomycin (4, 20). The iron-chelating portion of JAM-2-263 is similar to the microbial siderophore agrobactin (47).

The mechanism of action of these conjugates was investigated through studies of resistant bacterial isolates. Our data indicate that the new antibiotic conjugates inhibit growth through binding to penicillin-binding proteins (PBPs), the cellular targets of traditional β-lactams (62), and that their mode of entry into bacterial cells is clearly dependent on the iron uptake system of *E. coli*.

MATERIALS AND METHODS

Antibiotics. We describe here the inhibitory mechanisms of a recently synthesized N^5-acetyl-N^5-hydroxy-L-ornithine tripeptide hydroxamate siderophore-carbacephalosporin conjugate (EKD-3-88) (10–12) and of a new spermidine-based catechol siderophore-carbacephalosporin conjugate (JAM-2-263) (33). The structures of these β-lactam conjugates are illustrated in Fig. 1 and compared with those of some naturally found siderophore molecules. Cephaloridine (Sigma Chemical Co., St. Louis, Mo.) and the chromogenic cephalosporin nitrocefin (Oxoid Canada Ltd., Nepean, Ontario, Canada) were also used in this study.

Bacterial strains. β-Lactam-hypersensitive *E. coli* X580 was used in this work and was from the strain collection of Lilly Research Laboratories (Eli Lilly and Co., Indianapolis, Ind.). The feature of this strain responsible for this hypersensitivity is unknown. Strains resistant to JAM-2-263 (JAM^R) and EKD-3-88 (EKD^R) were selected and isolated after exposure of strain X580 to the siderophore–β-lactam conjugates at 10 μg/ml (37). Strain JAM^R/EKD^R and reciprocal strain EKD^R/JAM^R are double mutants exhibiting cross-resistance to both conjugates and were isolated after exposure of JAM^R to the conjugate EKD-3-88 and EKD^R to the conjugate JAM-2-263, respectively.

Strains H455 [*aroB malT tsx thi* Δ(*pro lac*)] and its derivatives H1196 (*fhuA*::Mu dI), H1187 (*fepA*::Mu dI), H1300 (*cir*::Mu dI), H1252 (*tonB*::Mu dI), and ZI17 (*exbB*::Mu dI), strain H1443 (*E. coli* MC4100 *aroB*), strain H1594 (H1443 *fiu*::Mu dIX), strain H1619 (H1443 *fhuE*::Mu dIX), and strain ZI379 (*aroB tsx malT lac*::Tn*10 fecA*::Mu dIX) were used for the identification of specific outer mem-

brane proteins and were kindly provided by K. Hantke, Universität Tübingen, Tübingen, Germany (16–18, 53, 64).

For use in an antibiotic diffusion test and an outer membrane permeability assay, *E. coli* cells were transformed with pBR325 plasmid DNA encoding a TEM-1 β-lactamase by a calcium chloride procedure (30).

Media. Bacterial strains were cultivated with Miller's Luria broth or agar (30) or on Iso-Sensitest (IST) agar (Oxoid Canada Ltd.). Supplemental EDDA at 100 μg/ml and human apotransferrin (Sigma) at 1 mg/ml were added to produce iron-deficient agar plates when needed.

Antibiotic susceptibility testing. (i) Broth microdilution test. Antibiotic MICs were determined by a broth dilution technique (14) with 96-well plates. Bacteria were grown overnight in Luria broth and diluted to 10^6 CFU/ml for use as inocula.

(ii) Disk diffusion test. Inhibitory zones were measured around disks containing the β-lactam conjugates placed on a lawn of bacteria on Luria agar plates. Iron-deficient IST agar plates (see above) were also used for this test, with or without the addition of ferrichrome at 2 μM.

Frequency of isolation of resistant cells. The frequency of isolation of *E. coli* X580 cells resistant to the siderophore–β-lactam conjugates was estimated as follows. Cells grown overnight in Luria broth were diluted to 10^8 CFU/ml in saline, and a volume of 100 μl was spread on the surface (60.8 cm^2) of Luria agar plates. Thereafter, a disk containing 30 μg of the β-lactam conjugate was applied to the agar plates. The plates were incubated at 37°C for 24 h. The number of colonies found in a 2-cm^2 surface within the inhibitory zone caused by the β-lactam conjugate was measured and served as an estimate of the frequency of isolation of resistant cells.

Whole-cell permeability assays. The rate of diffusion of β-lactams across the outer membrane of whole bacterial cells was measured by the spectrophotometric method of Zimmermann and Rosselet (65). The rate of β-lactam hydrolysis by the periplasmic β-lactamase of intact cells compared with that of broken cells was determined as described before (28) by monitoring the decrease in the A_{482} and A_{256} over time in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.0) at 37°C for nitrocefin and cephaloridine, respectively.

Inner membrane preparation. Membranes were isolated as previously described (28). Log-phase cells from 1-liter cultures were harvested by centrifugation. The cells were suspended in 15 ml of 20% sucrose–50 mM Tris-HCl (pH 7.9)–0.2 mM dithiothreitol and disrupted by three passages through a French pressure cell (18,000 lb/in^2). Debris was removed by low-speed centrifugation (500 × *g* for 10 min). The supernatant was loaded onto a discontinuous sucrose gradient (52, 58, 64, and 70%) and centrifuged at 100,000 × *g* for 16 h. The low-density material (inner membranes) was collected, suspended in 50 mM Tris-HCl (pH 7.9), and stored frozen (−20°C). The protein concentration was determined by the method of Lowry et al. (26). The membranes were used in PBP assays (see below).

PBP assays. Radiolabeling of PBPs from *E. coli* membranes was performed by a modification of the method of Spratt (57) as previously described (28) with ^{125}I-penicillin V (3) as the labeled β-lactam (51). In a 20-μl reaction mixture, inner membranes (100 μg) were incubated with 10 μg of ^{125}I-penicillin V per ml (37.3 Ci/mmol) for 10 min at room temperature. The membrane samples were suspended in electrophoresis sample buffer containing 1% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. The samples were heated to 100°C for 5 min before being loaded for electrophoresis in discontinuous 0.1% SDS–10% polyacrylamide gels (25). Gels were stained with 0.1% Coomassie brilliant blue and dried for autoradiography (1 to 7 days) on Kodak X-Omat AR5 film.

In PBP competition experiments, the membrane samples were prelabeled with various concentrations of the siderophore–β-lactam conjugates for 10 min prior to the addition of ^{125}I-penicillin V. The concentration of the competing β-lactam needed to block at least 50% (I_{50}) of the subsequent binding of the radiolabeled penicillin to a particular PBP was determined by scanning of the PBP profiles obtained on the X-ray film with a Bio-Rad model 620 video densitometer.

Outer membrane preparation. Membranes were isolated by the method of Carlone et al. (5). Cells from iron-deficient IST agar plates were suspended in M9 minimal mineral medium (30) with 100 μg of EDDA per ml, washed by centrifugation, and finally suspended in 10 ml of M9 minimal mineral medium. After 2 h of incubation at 37°C, the cells were disrupted by three passages through a French pressure cell (18,000 lb/in^2). Unbroken cells were removed by low-speed centrifugation (500 × *g* for 10 min), and all membranes were harvested by ultracentrifugation at 100,000 × *g* for 45 min and suspended in 35 μl of 10 mM HEPES (pH 7.4). Inner membrane proteins were solubilized by the addition of 1% Sarkosyl (*N*-lauroylsarcosine; Sigma) and 30 min of incubation. Insoluble outer membrane proteins were washed with 0.5 ml of 10 mM HEPES (pH 7.4) and stored frozen (−20°C). The protein concentration was determined by the method of Lowry et al. (26). The outer membrane proteins were loaded for electrophoresis in discontinuous 0.1% SDS–8% polyacrylamide gels as described above.

Colicin sensitivity tests. The sensitivities of *E. coli* X580, JAM^R, and EKD^R to some group B colicins were determined by the deferred antagonism test as previously described (48), except that arginine was omitted from the overlay agar containing the tested strain. Colicin B and colicin Ia need to bind specifically to the *E. coli* outer membrane receptors FepA and Cir for activity, respectively. The sensitive control strain *E. coli* HfrH180, the colicinogenic strains (AG097, K-12 W3110, K-12 167, and CA53), and the specific colicin-resistant mutants (R-B, R-D, and R-Ia) were as previously described (59).

Phage sensitivity tests. The sensitivities of *E. coli* strains to bacteriophages T1 and T5, which are known to bind specifically to the outer membrane protein receptor (FhuA) of ferrichrome and albomycin, were tested. *E. coli* cells were spread on Luria agar plates and incubated for 2 h at 37°C before 3-μl quantities of laboratory phage stocks of T1 and T5 at 10^8 PFU/ml were spotted on the bacterial lawn. The plates were incubated at 37°C for an additional 15 to 18 h before zones of bacterial lysis were examined. Phages were kindly provided by H. W. Ackermann (Université Laval, Québec, Québec, Canada).

Diffusion chambers and in vivo growth conditions. Diffusion chambers (15 by 10 mm) were constructed from polypropylene tubing (Nalgene Co., Rochester, N.Y.) as described before (27). Millipore (Bedford, Mass.) MF 0.22-μm-pore-size filters were cut to the diameter of the barrels and attached by melting the chambers. The chambers were autoclaved. Overnight cultures of bacteria diluted to ~10^5 CFU/ml in saline were injected through a needle hole melted through the side of the barrels. The hole was subsequently closed with a hot glass rod. The chamber volume was approximately 500 μl. Sprague-Dawley female rats, weighing approximately 250 g, were anesthetized with ketamine

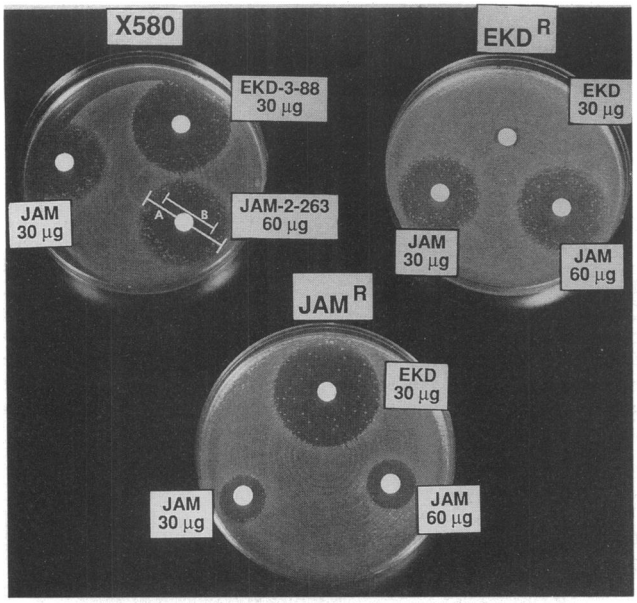

FIG. 2. Zones of inhibition produced by JAM-2-263 (JAM) and EKD-3-88 (EKD) in 30- or 60-μg disks for *E. coli* X580, EKD^R, and JAM^R. JAM-2-263 produced two zones of inhibition for strain X580, 29 mm (A) and 15 mm (B).

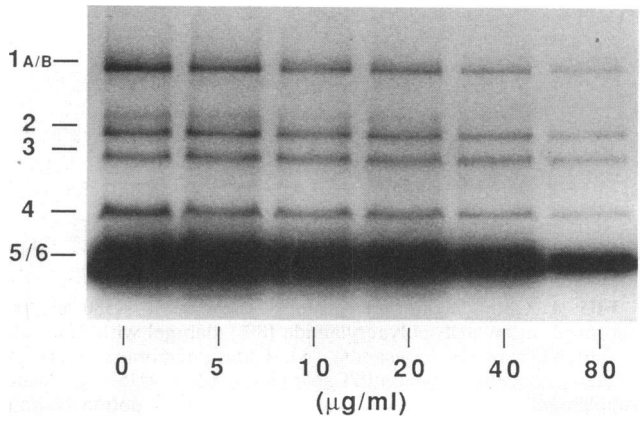

FIG. 3. Autoradiograph of JAM^R in a PBP competition experiment with the spermidine-based siderophore-carbacephalosporin conjugate JAM-2-263. Inner membranes (100 μg) were preincubated for 10 min with the competing β-lactam at the indicated concentration for each lane. Inner membrane PBPs were then labeled with 10 μg of ^{125}I-penicillin V per ml for 20 min and electrophoresed on an SDS-polyacrylamide (10%) slab gel. The PBPs of *E. coli* are indicated on the left side of the panel.

HCl (87 mg/kg) and xylazine (13 mg/kg) intraperitoneally. Four chambers were placed in the peritoneal cavity through a small longitudinal incision in the abdomen. Animals were sacrificed with carbon dioxide before the chambers were removed. Cells were removed from the chambers and collected for various tests.

RESULTS

Antibacterial activities. The antibacterial activities of the new siderophore-carabacephalosporin conjugates were evaluated by an antibiotic diffusion test. The antibacterial activities of 30 μg of the conjugates JAM-2-263 and EKD-3-88 on disks were demonstrated by the large inhibition zones (35 and 29 mm, respectively) obtained for *E. coli* X580 (Fig. 2). Alternatively, in a Luria broth antibiotic microdilution test, both conjugates demonstrated low inhibitory activities against *E. coli* X580, with MICs of 32 and 512 μg/ml for EKD-3-88 and JAM-2-263, respectively. The large number of resistant colonies seen in inhibition zones for susceptible strains (Fig. 2) suggests an explanation for the high conjugate MICs recorded in broth.

The compounds were also tested in disk diffusion tests against resistant strains selected and isolated after exposure to the conjugates: EKD^R was resistant to EKD-3-88, and JAM^R was resistant to JAM-2-263. As shown in Fig. 2, JAM-2-263 and EKD-3-88 demonstrated reduced activities against strains JAM^R and EKD^R, respectively, but no cross-resistance was observed. Interestingly, the 29-mm inhibition zone produced by JAM-2-263 for susceptible strain X580 included a 15-mm zone cleared of any resistant colony and was equivalent to the single 15-mm zone obtained for resistant strain JAM^R (Fig. 2). This two-zone phenomenon observed for JAM-2-263 may indicate two modes of entry into cells for this conjugate, and JAM^R would be resistant to only one of those modes. In contrast, almost complete resistance to EKD-3-88 was acquired in EKD^R, a result suggesting that a single mutation may neutralize its mode of action. The high frequency of isolation of X580 cells resistant to EKD-3-88 was estimated to be 3.1×10^{-5}.

The addition of a plasmid-encoded β-lactamase to strain X580 generated resistance to both siderophore–β-lactam compounds (no inhibition zone), indicating the requirement of the β-lactam portion of the conjugates for activity. The binding of the siderophore–β-lactam conjugates to PBPs of isolated inner membranes also indicated that the mechanism of inhibition of these new antibiotics may be dependent on the β-lactam portion of the molecule. An autoradiograph resulting from a PBP competition experiment with JAM-2-263 is shown in Fig. 3. JAM-2-263 bound primarily to PBPs 1A/B and 5/6 (i.e., with which it competed most for the binding of ^{125}I-penicillin V). The I_{50}s of JAM-2-263 for PBPs 1A/B and 5/6 were 17.5 and 24.7 μg/ml (strain X580) and 17.5 and 19.4 μg/ml (strain JAM^R). In contrast, EKD-3-88 bound primarily to PBPs 1A/B and 3, but again, there was no marked difference in the I_{50}s of the antibiotic for susceptible strain X580 and resistant strain EKD^R. The I_{50}s of EKD-3-88 for PBPs 1A/B and 3 were 16 and 18 μg/ml (strain X580) and 12 and 12 μg/ml (strain EKD^R). These data suggest that the resistance in JAM^R or EKD^R is not likely to be mediated by alterations in PBPs.

Cell surface of resistant bacteria. The outer membrane protein profiles of strains X580, EKD^R, and JAM^R obtained on SDS-polyacrylamide gels after electrophoresis were examined and compared. There was no apparent change in the electrophoretic mobility or the relative expression of OmpA and the porins OmpC and OmpF in the different strains, and reduced outer membrane permeability to β-lactams such as nitrocefin and cephaloridine through the nonspecific porin pathway could not be detected in the Zimmermann-Rosselet permeability assay (data not shown). The low sensitivity of this permeability assay did not allow adequate calculation of the outer membrane diffusion rate for the siderophore–β-lactam conjugates because of the slow hydrolysis of the carbacephalosporin nucleus by the plasmid-encoded β-lactamase used in this study and the interference of the siderophore portion during spectrophotometric measurements.

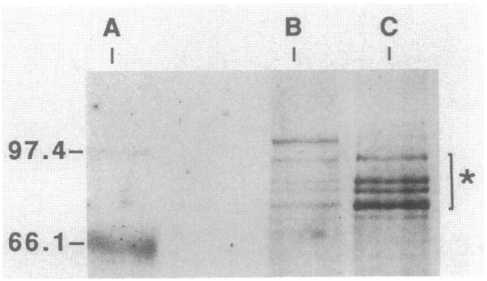

FIG. 4. Outer membrane proteins of *E. coli* X580 electrophoresed on an SDS-polyacrylamide (8%) slab gel with molecular weight markers (in thousands) (A). Outer membranes were extracted from cells grown on IST agar plates (B) or on IST agar plates supplemented with EDDA (100 μg/ml) and human apotransferrin (1 mg/ml) (C). The asterisk shows the iron-regulated outer membrane proteins of strain X580.

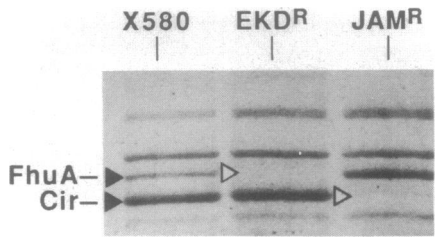

FIG. 5. Outer membrane proteins of *E. coli* X580, EKD^R, and JAM^R electrophoresed on an SDS-polyacrylamide (8%) slab gel. Outer membranes were extracted from cells grown on IST agar plates supplemented with EDDA (100 μg/ml) and human apotransferrin (1 mg/ml). The black arrowheads indicate the positions of the iron-regulated outer membrane proteins FhuA and Cir, and the white arrowheads indicate the positions of the missing proteins in the resistant strains.

As expected, several outer membrane proteins were expressed when strains were grown under iron-restricted conditions. Figure 4 shows the induction of iron-regulated proteins in the isolated outer membrane of strain X580 grown on IST agar plates and on IST agar plates supplemented with EDDA and human apotransferrin. Major differences were observed in these iron-regulated outer membrane proteins when strains X580, EKD^R, and JAM^R were compared (Fig. 5). The outer membrane of resistant strain JAM^R showed a striking decrease in the expression of the protein Cir, and the outer membrane of resistant strain EKD^R showed a decrease in the expression of the protein FhuA. The identities of these proteins were confirmed by comparison with strains having well-characterized mutations in the iron transport system (obtained from K. Hantke; see Materials and Methods). In addition, Table 1 suggests that JAM^R lacks the outer membrane protein Cir, which normally acts as the receptor for colicin Ia. Finally, phages T1 and T5 infected and lysed strains X580 and JAM^R but not strain EKD^R. This bacteriophage sensitivity test confirmed that only EKD^R lacks the phage receptor FhuA. These results suggest that the inhibitory activity of JAM-2-263 and EKD-3-88 on *E. coli* cells requires the presence of Cir and FhuA, respectively, two proteins induced by growth in iron-deficient media.

Antagonists of antibacterial activity. To further investigate the mode of entry of JAM-2-263 and EKD-3-88 into *E. coli* cells, we attempted to antagonize the inhibitory activity of these siderophore–β-lactam conjugates by the addition of various iron-related compounds in antibiotic inhibition tests with the hope that they would interfere or compete with cell surface receptors for the conjugated drugs. In an antibiotic diffusion assay on agar plates, a striking antagonistic effect of ferrichrome on the activity of the conjugates was observed (Fig. 6). Ferrichrome antagonized the inhibitory activity of the two conjugates against strain X580 (Fig. 6A) and against strain JAM^R (Fig. 6B). It is noteworthy that even the residual activity of JAM-2-263 against JAM^R was also antagonized by ferrichrome. The susceptibility of EKD^R to the two conjugates remained the same on plates with or without ferrichrome (Fig. 6C), suggesting that the antagonistic effect of ferrichrome on the inhibitory activities of both β-lactam conjugates against strains X580 and JAM^R was dependent on the presence of the outer membrane protein FhuA. These data also suggested that the small difference in the susceptibilities of strains X580 and EKD^R to JAM-2-263

TABLE 1. Sensitivity of selected bacterial strains to specific colicins

Strain	Relevant property	Sensitivity (S) or resistance (R) to the following colicinogenic strain (colicin produced):			
		AG097 (B)	K-12 W3110 (D)	K-12 167 (Ia)	CA53 (Ia)
X580	Control	S	S	S	S
EKD^R	Spontaneous mutant resistant to EKD-3-88	S	S	S	S
JAM^R	Spontaneous mutant resistant to JAM-2-263	S	S	R	R
EKD^R/JAM^R	Double mutant	S	S	R	R
JAM^R/EKD^R	Double mutant	S	S	R	R
H455	Control	S	S	S	S
H1196	FhuA$^-$	S	S	S	S
H1187	FepA$^-$	R	R	S	S
H1300	Cir$^-$	S	S	R	R
H1443	Control	S	S	S	S
H1594	Fiu$^-$	S	S	S	S
HfrH180	Control	S	S	S	S
R-B	Resistant to colicin B	R	R	S	S
R-D	Resistant to colicin D	R	R	S	S
R-Ia	Resistant to colicin Ia	S	S	R	R

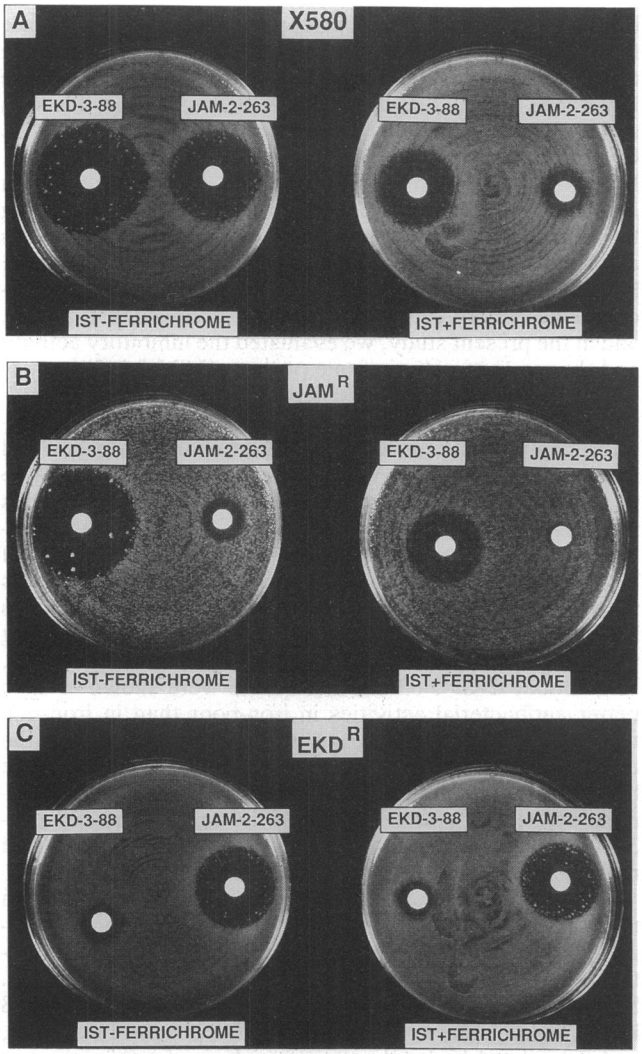

FIG. 6. Zones of inhibition produced by JAM-2-263 and EKD-3-88 for X580 (A), JAM^R (B), and EKD^R (C) grown on supplemented IST agar plates further enriched with the antagonist ferrichrome (2 μM) as indicated.

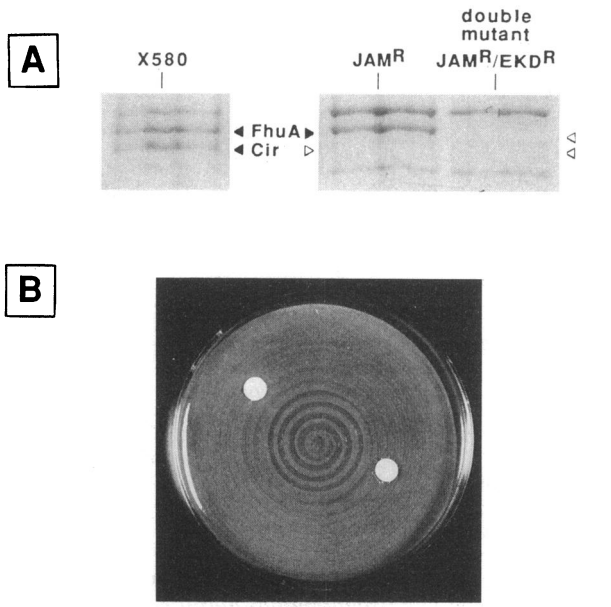

FIG. 7. (A) Outer membrane proteins of *E. coli* X580, JAM^R, and JAM^R/EKD^R electrophoresed on an SDS-polyacrylamide (8%) slab gel. Outer membranes were extracted from cells grown on IST agar plates with EDDA (100 μg/ml) and human apotransferrin (1 mg/ml). The black arrowheads indicated the positions of the iron-regulated outer membrane proteins FhuA and Cir, and the white arrowheads indicate the positions of the missing proteins in the resistant strains. (B) Disk diffusion susceptibility test of strain JAM^R/EKD^R with JAM-2-263 (disk on the right) and EKD-3-88 (disk on the left).

(29- and 22-mm inhibition zones in Fig. 6A and C, respectively) may also have been due to the presence and absence of FhuA in these strains, respectively. Control experiments showed that the addition of ferrichrome to IST agar plates did not modify the expression of the iron-regulated outer membrane proteins that were already induced by the presence of EDDA and human apotransferrin in the medium (data not shown). In contrast, the addition of an excess of ferric chloride to IST agar plates did antagonize the antibacterial activities of the siderophore conjugates by reducing the expression of the iron-regulated outer membrane proteins.

Double mutant studies. Strains that were already resistant to JAM-2-263 (JAM^R) and EKD-3-88 (EKD^R) were used to produce double mutants resistant to both antibiotics. The iron-regulated outer membrane proteins of one of the double mutants (JAM^R/EKD^R) are shown in Fig. 7. This double mutant and the reciprocal double mutant EKD^R/JAM^R expressed neither of the outer membrane proteins FhuA and Cir. The double mutants were not sensitive to phages T1 and T5 or to colicin Ia (Table 1). Furthermore, the double mutant JAM^R/EKD^R showed a higher level of resistance to JAM-2-263 than JAM^R, an observation that strongly supports the idea of a dual mode of entry for JAM-2-263 (Fig. 7). In other words, the residual inhibitory activity of JAM-2-263 against resistant strain JAM^R (Cir$^-$) was abolished in the double mutant JAM^R/EKD^R (Cir$^-$ FhuA$^-$), which had lost the second uptake route. These results confirmed the role of FhuA and of FhuA and Cir in the inhibitory activity of EKD-3-88 and JAM-2-263, respectively.

In vivo growth of resistant bacteria. The in vivo growth of strains X580 and EKD^R was monitored by viable cell plate counting at intervals after the implantation of diffusion chambers in rats (Fig. 8). Strain EKD^R showed impaired growth in diffusion chambers implanted in rats. It appears that EKD^R cells were dying for a period of 3 h before being able to multiply in vivo. A phage sensitivity test was also performed on bacteria isolated at each time point (Fig. 9). Bacteriophages T1 and T5 infected and lysed strain X580 grown in vitro and in vivo, but strain EKD^R, which was initially not sensitive to these phages, became sensitive to phages T1 and T5 after incubation for at least 4 h in rats. In addition, bacterial isolates recovered from the diffusion chambers in which strain EKD^R had been placed became susceptible to the siderophore–β-lactam conjugate EKD-3-88 after growth in rats. These data suggested that the full expression of iron-regulated outer membrane proteins is important for in vivo growth.

DISCUSSION

Recently, ureidopenicillins having a terminal catechol moiety were synthesized and found to have 30- to 60-fold

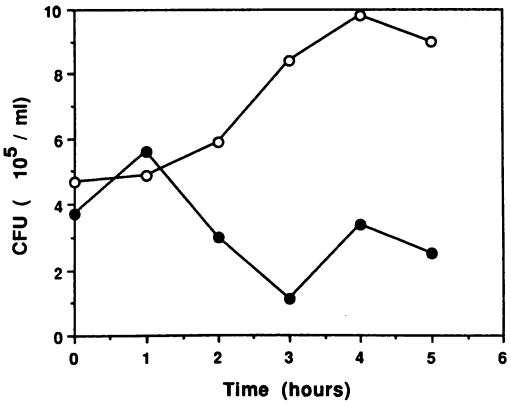

FIG. 8. In vivo growth of strains X580 (○) and EKD^R (●) monitored by viable cell plate counting at intervals after the implantation of diffusion chambers in rats.

increase in activity relative to piperacillin against various microbes, including *Pseudomonas aeruginosa* strains, which are generally less susceptible to β-lactam antibiotics (44, 46). Related catechol derivatives of ureidocephalosporins and ureidocephamycins also displayed enhanced activity against *P. aeruginosa* (45). It was speculated that all of these compounds owed their enhanced antimicrobial activity to their ability to bind iron and be actively carried into cells by iron transport mechanisms. Similarly, the antimicrobial activity of the catechol-containing cephalosporin M14659 was found to be enhanced in iron-poor environments, suggesting that this drug may be assimilated by microbial iron transport systems (38). E-0702 (61) and GR69153 (56) are also iron-chelating catechol-containing cephalosporin derivatives that have been shown to be incorporated into *E. coli* cells by the *tonB*-dependent iron transport system. All of these cephalosporin derivatives have the catechol moiety attached to the α-acyl group of the β-lactam. Other antibacterial compounds in which catechols are incorporated at the C-3 position of the cephalosporin nucleus have also been prepared (9, 39, 42). The activity of some of the catechol-containing cephalosporin derivatives has also been correlated with *tonB*-dependent illicit transport across the outer membranes of bacteria. When investigated, the process involved jointly and specifically the Fiu and Cir iron-regulated outer membrane proteins (9, 42, 56).

For some older antibiotics, microbial iron uptake systems were also implicated in inhibitory activity. The antibiotic albomycin has been shown to be a linear peptide attached to a toxic thioribosyl unit. As in the siderophore ferrichrome, the iron-binding section of albomycin is a tri-N^5-hydroxy-N^5-acetyl-ornithine peptide. While ferrichrome is a potent growth factor for many microbes, albomycin is a potent antibiotic. Evidence now indicates that albomycin is actively carried into microbial cells by normal iron transport processes via the FhuA outer membrane receptor and, once in the cells, the toxic thioribosyl moiety is enzymatically released, perhaps by peptidase-mediated cleavage (4, 20).

Albomycin analogs with attached antibiotics that display less mammalian toxicity than the thioribosyl unit of albomycin have long been sought. The primary limitation to exploring this interesting approach to the development of novel antimicrobial agents has been the inability to synthesize large amounts of the essential N^5-hydroxy-N^5-acetyl-ornithine residues and the corresponding peptides. We have been able to synthesize these components on a small scale for use in preliminary work, and we are currently exploring alternate, more practical large-scale approaches (35, 36). We have been able to attach a carbacephalosporin (a β-lactam antibiotic) to a number of albomycinlike peptides (10–12). One of these siderophore-carbacephalosporin conjugates, EKD-3-88, was further studied here. We have also been interested in another type of siderophore for illicit drug transport. Spermidine-based catechol siderophores such as agrobactin and parabactin have been isolated (40, 47). We have been able to synthesize some spermidine-based catechol siderophore-carbacephalosporin conjugates (32, 33), and in the present study, we evaluated the inhibitory activity and the mode of action of one of them (JAM-2-263).

The new siderophore-carbacephalosporin conjugates studied here are structurally related to the iron-chelating antibiotic albomycin and the microbial siderophore agrobactin and may similarly use the iron uptake systems of bacteria to penetrate the outer membrane. Supportive evidence for this view was provided in our recent structure-activity relationship study (37), in which the isolated siderophore portions of EKD-3-88 and JAM-2-263 were shown to form complexes with ferric iron and to have good growth-promoting activities for *E. coli* X580 under iron-restricted conditions, indicating the ability of *E. coli* to use these molecules for iron uptake. In addition, EKD-3-88 and JAM-2-263 were shown to have better antibacterial activities in iron-poor than in iron-rich media (12, 29, 37), presumably because of the increase in the expression of the siderophore receptors at the surface of the bacteria. All these observations already implied the involvement of the iron uptake system in the penetration of EKD-3-88 and JAM-2-263 into *E. coli* cells. We characterized resistant mutants to test this hypothesis.

Examination of iron-regulated outer membrane proteins in the resistant strains EKD^R and JAM^R indicated the requirement of the proteins FhuA and Cir for the inhibitory activities of EKD-3-88 and JAM-2-263, respectively (Fig. 5). Strain EKD^R lacked the FhuA outer membrane protein (78 kDa), which is the specific receptor for phages T1 and T5, and the related hydroxamate siderophores ferrichrome and albomycin. To our knowledge, this is the first report showing evidence of illicit transport of a β-lactam antibiotic via the

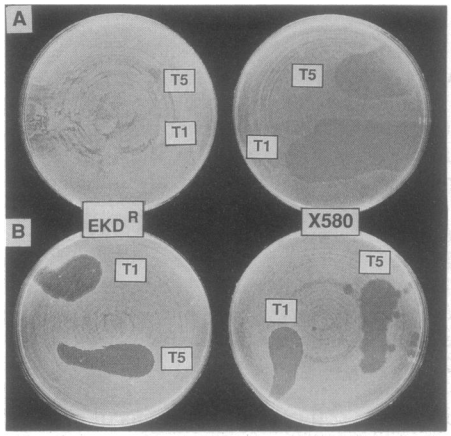

FIG. 9. Sensitivity of *E. coli* X580 and EKD^R to bacteriophages T1 and T5. (A) Sensitivity tests performed on *E. coli* strains maintained and cultivated on Luria agar plates. (B) Sensitivity tests performed on the same strains but reisolated after 5 h of incubation in diffusion chambers implanted in rats.

FhuA protein, although a semisynthetic rifamycin derivative has been shown to use the FhuA-TonB iron uptake pathway to penetrate across the outer membrane and exert its intracellular inhibitory activity on *E. coli* (52).

Strain JAM^R lacked the outer membrane protein Cir (74 kDa), inducible under iron-poor conditions, and was accordingly resistant to colicin 1a. Thus far, the physiological role of the Cir protein is still unclear. Cir and Fiu have been implicated in the transport of monomeric catechols (42). Interestingly, it was the use of β-lactam antibiotics containing iron-chelating catechol groups and resistant bacterial mutants that provided this information (9, 42, 56). Resistance was observed in *cir* or *fiu* mutants, and higher levels of resistance to the β-lactam tested were achieved in *cir fiu* double mutants, indicating two routes for the uptake of the β-lactam–catechol compounds (9, 42, 56). For compound JAM-2-263, we similarly suspected two modes of entry into *E. coli* cells, because the antibiotic disk diffusion assay revealed a two-zone phenomenon related to the inhibitory activity of the siderophore–β-lactam conjugate against sensitive strain X580 (Fig. 2). However, the natures of the two outer membrane proteins that would be involved in the two routes seem to differ from those suggested for the uptake of β-lactam–monomeric catechol conjugates. The antagonistic effect of ferrichrome on the activity of JAM-2-263 against strains X580 and JAM^R but not strain EKD^R indicated a role of the FhuA protein in addition to the Cir protein in the activity of JAM-2-263 (Fig. 6). The route of entry for JAM-2-263 into *E. coli* cells would be primarily via protein Cir and secondly via protein FhuA. Double mutant studies confirmed this hypothesis. A JAM^R isolate selected for its resistance to EKD-3-88 (strain JAM^R/EKD^R) did not express the iron-regulated outer membrane proteins Cir and FhuA and showed a higher level of resistance to JAM-2-263 than JAM^R (Fig. 7). This is the first evidence of a spermidine-based catechol siderophore–β-lactam conjugate (JAM-2-263) that may use two distinct receptor proteins for transport, one known to be specific for hydroxamate siderophores (FhuA) and the other known to be specific for monomeric catechols (Cir). These results also suggest that Cir and Fiu, two receptor proteins that are generally associated (9, 42, 56), have different affinities and selectivities for catechol iron chelators and perhaps different physiological functions in *E. coli*.

Unfortunately, the genetically defined *E. coli* mutants that have known defects in various aspects of the iron uptake system (provided by K. Hantke) could not be used in susceptibility tests to corroborate our results obtained with EKD^R (FhuA$^-$) and JAM^R (Cir$^-$). The reason for this is that all of K. Hantke's strains (including control strains) were resistant to both of our compounds in the antibiotic disk diffusion test. For this study, we had to use β-lactam-hypersensitive *E. coli* X580, which was susceptible to our experimental siderophore–β-lactam conjugates. The use of insertion or deletion mutants would have been preferable.

The experimental siderophore–β-lactam conjugate EKD-3-88 was shown to bind to *E. coli* PBPs 1 and 3, whereas JAM-2-263 bound primarily to PBPs 1A/B and 5/6. These results indicate that despite their large molecular size, these new antibiotics may exert their inhibitory activity through the inhibition of these peptidoglycan-synthesizing enzymes, like other traditional β-lactams (62). In addition, the presence of a β-lactamase abolished their inhibitory activity.

One of our resistant strains was tested for its ability to grow in animals, an environment in which iron is restricted. The resistant mutant EKD^R showed impaired growth in diffusion chambers implanted in rats, and the populations of bacteria isolated from such a cultivation system were thereafter sensitive to phages T1 and T5 (Fig. 8 and 9); i.e., these results suggest that EKD^R reverted to the wild type. These results also indicate that mutant strains lacking the FhuA receptor, such as strain EKD^R, are less likely to survive in vivo. Under our experimental conditions, however, the revertant strain was not submitted to antibiotic pressure during growth in vivo. Such environmental pressure could have favored the maintenance of resistant isolates. Besides, as pointed out by Nikaido and Rosenberg (42) for β-lactam–catechol conjugates that use two protein receptors for entry into cells, a high level of resistance to these drugs would require either a spontaneous double mutation or a *tonB* mutation, which would eliminate all high-affinity transport and internalization routes of iron complexes, a condition not likely to allow the growth of pathogenic bacteria in vivo. This observation, in addition to our observed high frequency of resistance to EKD-3-88, which penetrates *E. coli* via a single FhuA-mediated pathway, certainly supports the idea that the identification of an iron complex receptor that would be absolutely essential for growth in vivo may be the key element for future attempts at illicit drug transport. Also, a siderophore moiety that uses more than one essential iron transport route would certainly be desirable. Although our compounds showed low inhibitory activities and pharmacological data on such large molecules need to be provided, the study of these new experimental compounds certainly helped to rationalize the design of future conjugated drugs. Current work in our laboratories include the synthesis of siderophore-drug conjugates that are active against pseudomonads (23, 24).

Finally, it is important to specify here that some important pathogenic bacteria that do not produce siderophores acquire iron by other means. *Yersinia* species (50) and members of the family *Neisseriaceae* (2, 55) have been shown to use iron from hemin, transferrin, or lactoferrin. The same phenomenon has been observed for *Haemophilus influenzae* (21, 54) and *Bordetella* species (34). The use of siderophore-antibiotic conjugates for the control of infections caused by such microorganisms would certainly not be efficient. More studies on the interactions of bacterial siderophores, mammalian iron-binding molecules, and new antibiotic conjugates with receptors for outer membrane proteins from all pathogenic organisms will be necessary.

In the present study, we evaluated the inhibitory activity and the mode of action of new carbacephalorosporins conjugated to a hydroxamate siderophore (such as albomycin) and to a spermidine-based catechol siderophore (such as agrobactin) against *E. coli* cells. Our data indicate that the new antibiotic conjugates inhibit growth through binding to PBPs, the cellular targets of traditional β-lactams, and that their mode of entry into bacterial cells is clearly dependent on the iron uptake system of *E. coli*.

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