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Species Selectivity of New Siderophore-Drug Conjugates That Use Specific Iron Uptake for Entry into Bacteria

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Siderophores selectively bind ferric iron and are involved in receptor-specific iron transport into bacteria. Several types of siderophores were synthesized, and growth-promoting or inhibitory activities when they were conjugated to carbacephalosporin, erythromycylamine, or nalidixic acid were investigated. Overall, 11 types of siderophores and 21 drug conjugates were tested against seven different bacterial species: *Escherichia coli*, *Bordetella bronchiseptica*, *Pasteurella multocida*, *Pasteurella haemolytica*, *Streptococcus suis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*. In some species, the inhibitory activities of the drug conjugates were associated with the ability of the bacteria to use the siderophore portion of the molecules for growth promotion in disc diffusion tests (0.04 μmol of conjugate or siderophore per disc). *E. coli* used catechol-based siderophore portions as well as hydroxamate-based tri-δ-OH-*N*-OH-δ-*N*-acetyl-L-ornithine ferric iron ligands for growth under iron-restricted conditions achieved by supplemental ethylenediamine di(O-hydroxyphenylacetic acid) (100 μg/ml) and was sensitive to carbacephalosporin conjugated to these siderophore types (up to a 34-mm-diameter inhibition zone). *B. bronchiseptica* used desferrioxamine B and an isocyanurate-based or trihydroxamate in addition to catechol-based siderophore portions for promotion but was not inhibited by β-lactam conjugates partly because of the presence of β-lactamase. *P. multocida* and *P. haemolytica* did not use any of the synthetic siderophores for growth promotion, and the inhibitory activities of some conjugates seemed partly linked to their ability to withhold iron from these bacteria, since individual siderophore portions showed some antibacterial effects. Individual siderophores did not promote *S. suis* growth in restrictive conditions, but the type of ferric iron ligands attached to β-lactams affected inhibitory activities. The antibacterial activities of the intracellular-acting agents erythromycylamine and nalidixic acid were reduced or lost, even against *S. aureus* and *S. epidermidis*, when the agents were conjugated to siderophores. Conjugate-resistant *E. coli* mutants showed the absence of some iron-regulated outer membrane proteins in gel electrophoresis profiles and in specific phage or colicin sensitivity tests, implying that the drugs used outer membrane receptors of ferric complexes to get into cells.

Pathogenic bacteria have a strict nutritional requirement for iron, and in vivo they must contend with the natural ability of the host to withhold free iron in the form of iron-protein complexes. Most aerobic, facultative anaerobic, and saprophytic 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 (27, 47, 57). Siderophores commonly occur in two broad chemical classes, hydroxamates and catechols, one of which occurs with almost every group of bacteria (40).

Several iron(III) transport systems have been characterized for *Escherichia coli*. This bacterium uses and synthesizes the hydroxamate siderophore, aerobactin, citrate, and the phenolate-catecholate siderophore, enterobactin (enterochelin). *E. coli* also uses the siderophore ferrichrome (and the closely related ferrichrocin and ferrichrysin), coprogen, and rhodotorulic acid synthesized by various fungi (5). Five transport systems are defined by five specific outer membrane receptors whose production is regulated by the availability of ferric iron in the environment and the consequent intracellular iron pool (41). The receptors are FepA (81 kDa) for ferric enterobactin, FecA (80 kDa) for ferric citrate, FhuA (78 kDa) for ferric ferrichrome, FhuE (76 kDa) common for ferric coprogen and rhodotorulic acid, and IutA (74 kDa) for ferric aerobactin (7). Fiu (83 kDa) and Cir (74 kDa) are also outer membrane proteins that are inducible in response to iron limitation. These proteins seem to be implicated in TonB-dependent transport of ferric monocatechols (42). Iron(III) ligated by hydroxamate siderophore (aerobactin, ferrichrome, coprogen, and rhodotorulic acid) is transported across the cytoplasmic membrane by some common internal transport components, while iron(III)-citrate and iron(III)-enterobactin use different transport proteins (52). The product of the *tonB* gene is necessary for the uptake of iron by all high-affinity outer membrane receptor-mediated iron transport systems, and *exbB* mutants are also unable to take up iron, suggesting an interactive function for *exbB* and *tonB* gene products (48).

Recently we have shown that a new hydroxamate siderophore-carbacephalosporin conjugate with a tripeptide portion

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similar to that of iron-chelating antibiotic albomycin, or a spermidine-based catechol siderophore-carbacephalosporin conjugate with a chelating portion similar to the microbial siderophore agrobactin, can use microbial iron transport processes to actively carry the β-lactam antibiotic into *E. coli* cells (3). In this article, we have performed a systematic study of several siderophore-antibiotic conjugates that possess a wide variety of synthetic siderophore portions that include mixed ligands. The hypothesis to be tested is that the newly synthesized siderophore-antibiotic conjugates can use one or several microbial iron transport pathways to carry drugs into *E. coli* cells. We have also explored the antibacterial activities of erythromycylamine and nalidixic acid conjugates in addition to siderophore–β-lactam conjugates to provide information on the effect of siderophore conjugation on such drugs acting in the cytoplasm. For comparison with *E. coli*, we also wanted to determine which of the synthetic siderophore analogs and drug conjugates could be specifically or commonly recognized by other pathogenic species and which of the conjugates would show antimicrobial properties improved compared with those of the drug alone. The drug conjugates were specifically tested against some swine and bovine pathogens for which iron transport systems have not been fully characterized.

Pasteurella multocida, *Bordetella bronchiseptica*, and *Streptococcus suis* are pathogens causing infections of the respiratory tract of pigs and are responsible for important economic losses in the swine industry (2, 22, 23, 56). Little is known about iron acquisition by these pathogens, although previous studies showed that iron acquisition may be important in the disease process (9, 12, 16, 24, 28, 29, 32). *Pasteurella haemolytica* A1 is the principal microorganism associated with bovine pneumonic pasteurellosis, a major cause of sickness, death, and economic loss in the feedlot cattle industry in North America (1, 43, 58), and iron acquisition may also be important in pathogenesis (15). In this study, a siderophore portion test and the use of siderophore antibiotic conjugates have provided new insights on the iron transport systems of these bacterial species.

MATERIALS AND METHODS

Antibiotics, siderophores, and conjugates. The chemical structures of siderophore–β-lactam (carbacephalosporin), siderophore-macrolide (erythromycylamine), and siderophore-nalidixic acid conjugates are shown in Table 1. The list also includes some unconjugated siderophores, or drugs, used in growth promotion (Table 2) or inhibition (Table 3) tests, respectively. The siderophore analogs to be tested are of both types (hydroxamate and catechol) or have mixed ligands. The iron chelating portions of hydroxamate siderophores are similar to the microbial siderophore ferrichrome (containing bis or tri-δ-*N*-OH-δ-*N*-acetyl-L-ornithine) or arthrobactin [containing 1 amino-5-(*N*-acetyl-*N*-hydroxy)amino pentane]. The catechol siderophores are derived from hydroxybenzoyl-based spermidine or lysine [containing N^1,N^{10}-bis or tri(2,3-dihydroxybenzoyl)-N^5-succinoylspermidine or lysine]. Their iron chelating groups are similar to those of agrobactin, enterobactin, or parabactin. The siderophores and antibiotic conjugates were stored as 10-mM solutions at −20°C in *N*,*N*-dimethylsulfoxide or in methanol. Under these conditions, the compounds were stable for at least 18 months (37). The inhibitory effects of each antibiotic conjugate also were compared to those of the unconjugated drugs throughout the study. Loracarbef and erythromycylamine were obtained from Eli Lilly and Co. (Indianapolis, Ind.), and nalidixic acid was obtained from Sigma (St. Louis, Mo.). Ferrichrome was obtained from Porphyrin Products (Logan, Utah), and desferrioxamine B (Desferal) was obtained from Ciba Geigy.

Bacterial strains. The β-lactam-hypersensitive strain *E. coli* X580 was from the strain collection of Lilly Research Laboratories (Eli Lilly and Co.). Representative strains of *P. multocida* (strain 1703, capsular type D), *B. bronchiseptica* (strain 1804), and *S. suis* 1591 (type 2) were field isolates from our strain collection. *P. haemolytica* (strain B122) was from Veterinary Infectious Disease Organization. *Staphylococcus aureus* 25923 and *Staphylococcus epidermidis* 31432 were from the American Type Culture Collection and were used as erythromycylamine-susceptible control strains in some experiments. Only *B. bronchiseptica* produced a β-lactamase as detected by a nitrocefin test (Becton Dickinson). Various resistant *E. coli* strains selected and isolated from zones of inhibition after exposure to different drug conjugates in disc diffusion tests are listed in Table 4. Some resistant strains isolated after exposure to the first drug also were exposed to a second drug for selection of double mutants exhibiting cross-resistance to two drug conjugates. Strain H455 [*aroB malT tsx thi* Δ(*pro lac*)] and the genetically defined iron transport derivatives H1196 (*fhuA*::Mu dI), H1187 (*fepA*::Mu dI), H1300 (*cir*::Mu dI), H1252 (*tonB*::Mu dI), ZI17 (*exbB*::Mu dI), H1594 (*fiu*::Mu dIX), H1619 (*fhuE*::Mu dIX), and strain ZI379 (*aroB tsx malT lac*::Tn*10 fecA*::Mu dIX) were used for the identification of specific outer membrane proteins involved in uptake of siderophore-drug conjugates and were kindly provided by K. Hantke Universität Tübingen, Tübingen, Germany.

Growth conditions and media. Strains were cultivated onto Mueller-Hinton agar (MHA) plates or in Mueller-Hinton broth (MHB). Iron-restricted conditions (45) were obtained after addition of 100 μg of EDDHA [ethylenediamine di(O-hydroxyphenylacetic acid); Sigma] per ml of MHA plates or MHB (Difco Laboratories, Detroit, Mich.). Iron-rich media were obtained by adding $FeCl_3$ to the media at 5 μM. For large-scale cultures used in membrane preparation, 50 ml of overnight cell cultures in MHB was used to inoculate 1 liter of MHB containing EDDHA at the concentration indicated above, and growth was continued at 37°C (200 rpm) until the A_{600} reached 0.5.

Disc diffusion tests. The ability of bacteria to use individual unconjugated siderophores or the susceptibility of bacteria to siderophore drug conjugates was tested by antibiotic disc diffusion tests under both iron-restricted (45) and iron-sufficient conditions. Freshly prepared agar plates containing exactly 20 ml of medium were inoculated with a sterile cotton swab dipped in a bacterial suspension in saline (approximately 10^8 CFU/ml) and used to streak the surface of the plates. Discs (6.0 mm diameter) containing 0.04 μmol of each siderophore or siderophore-antibiotic conjugate were placed on the surface of agar plates to allow growth inhibition or promotion. Plates were incubated at 37°C for exactly 24 h. *E. coli* X580 was used repetitively in each series of plate assays to essentially validate test reproducibility. Closely related compounds (i.e., D and L isomers) were always tested on the same plate. Growth inhibition or promotion zones were measured around the discs, and when possible, isolation of resistant bacteria derived from the parental *E. coli* X580 was performed by picking colonies present in the observed inhibition zones and then cultivating them into iron-rich broth before they were frozen and preserved in a liquid nitrogen storage system.

Outer membrane preparation. The method of Carlone et al. (6) was followed for outer membrane preparation isolation. Briefly 1 liter of culture was adjusted to an A_{600} of 0.5. After centrifugation, cells were suspended and washed in 10 mM HEPES *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, (Sigma) buffer (pH 7.4). Cells were then disrupted by three passages through a French pressure cell (18,000 lb/in^2). Unbroken cells were removed by low-speed centrifugation (3,000 × *g* for 10 min), and all membranes were harvested by ultracentrifugation at 100,000 × *g* for 1 h and suspended in 10 mM HEPES buffer (pH 7.4). Cytoplasmic membrane proteins were solubilized by the addition of 1% Sarkosyl (*N*-lauroylsarcosine; Sigma) and a 30-min incubation period. Insoluble outer membrane proteins were then collected by centrifugation and washed with 10 mM HEPES buffer (pH 7.4) and stored frozen (−20°C). 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 on a discontinuous 0.1% SDS–8% polyacrylamide gel system (26). Gels were stained with 0.1% Coomassie brilliant blue.

Colicin sensitivity tests. The sensitivities of *E. coli* X580 and resistant *E. coli* mutants to group B colicins were determined by the deferred antagonism test as previously described (46) except that arginine was omitted from the overlay agar containing the test strain. Colicin B and D and colicin Ia need to bind specifically to *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 previously described (54).

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 by the method previously described by Brochu et al. (3). Agar plates were inoculated with a sterile cotton swab dipped in a overnight bacterial suspension adjusted to 10^8 CFU/ml in saline and used to streak the surface of the plates. Plates were incubated for 2 h at 37°C before 2-μl aliquots of laboratory phage stock 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.

RESULTS

Growth promotion activities of hydroxamate peptides and catechol siderophores. The abilities of hydroxamate peptides and catechols to reverse the growth inhibition effect of EDDHA were determined in growth promotion assays (Table 2). Figure 1A illustrates a typical example of a zone of growth promotion provided by a siderophore in iron-restricted conditions (i.e., a positive value in Table 2) and, in Fig. 1B, a zone of inhibition of growth caused by an antibiotic conjugate in iron-rich conditions (i.e., a negative value in Table 3). In some cases, zones

TABLE 1. Structures of antibiotics, siderophores, and siderophore-antibiotic conjugates used in this study

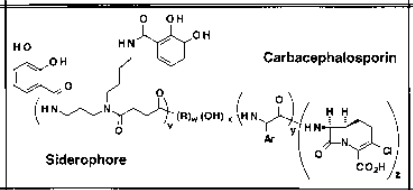
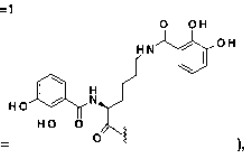
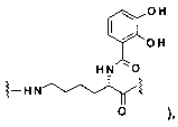
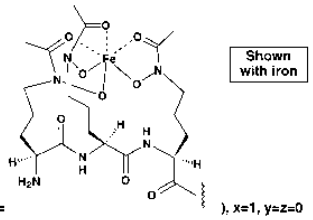
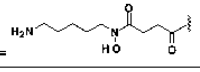
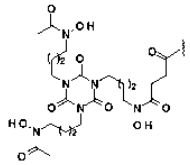
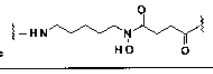
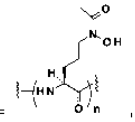
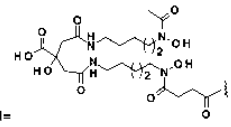
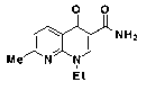
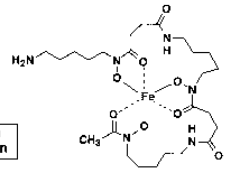
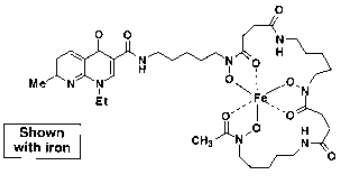
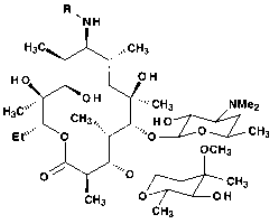
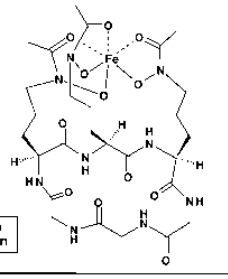
no.	Identification	Chemical Structure	Reference
	Generalized structure		
1	Loracarbef	v=0, w=1 (R=H), x=0, y=1 (Ar=Ph, D-isomer), z=1	Gift from Eli Lilly and Co.
2a	Siderophore	v=1, w=0, x=1, y=z=0	3, 31, 33, 34, 36, 37
2b	Conjugate	v=1, w=0, x=0, y=0, z=1	3, 31, 33, 34, 36, 37
2c	Conjugate	v=1, w=0, x=0, y=1 (Ar=Ph, D-isomer), z=1	33, 34, 36
2d	Conjugate	v=1, w=0, x=0, y=1 (Ar=Ph, L-isomer), z=1	35
2e	Conjugate	v=1, w=0, x=0, y=1 (Ar=*p*-HO-Ph, D-isomer), z=1	34
2f	Conjugate	v=1, w=0, x=0, y=1 (Ar=*p*-HO-Ph, L-isomer), z=1	34
3a	Siderophore	v=0, w=1  (R=), x=1, y=z=0	31, 33, 35
3b	Conjugate	v=0, w=1 (R= of 3a), x=0, y=0, z=1	31, 33, 35
4a	Siderophore	v=1, w=1  (R=), x=1, y=z=0	Not tested
4b	Conjugate	v=1, w=1 (R= of 4a), x=0, y=0, z=1	30, 33, 35
5a	Siderophore	v=0, w=1  (R=), x=1, y=z=0	10, 11, 33, 34, 35, 36
5b	Conjugate	v=0, w=1 (R= of 5a), x=0, y=1 (Ar=Ph, D-isomer), z=1	10, 11, 33, 34, 35, 36
5c	Conjugate	v=0, w=1 (R= of 5a), x=0, y=1 (Ar=*p*-HO-Ph, D-isomer), z=1	3
6a	Siderophore	v=0, w=1  (R=), x=1, y=z=0	Not tested
6b	Conjugate	v=0, w=1 (R= of 6a), x=0, y=0, z=1	18
6c	Conjugate	v=0, w=1 (R= of 6a), x=0, y=1 (Ar=Ph, D-isomer), z=1	18
7a	Siderophore	v=0, w=1  (R=), x=1, y=z=0	20
7b	Conjugate	v=0, w=1 (R= of 7a), x=0, y=1 (Ar=Ph, D-isomer), z=1	20
7c	Conjugate	v=0, w=1 (R= of 7a), x=0, y=0, z=1	20
8a	Siderophore	v=1, w=1  (R=), x=1, y=z=0	Not tested
8b	Conjugate	v=1, w=1 (R= of 8a), x=0, y=0, z=1	17
8c	Conjugate	v=1, w=1 (R= of 8a), x=0, y=1 (Ar=Ph, D-isomer), z=1	17
9a	Siderophore	v=1, w=1  (R= , n=1), x=1, y=z=0	Not tested
9b	Conjugate	v=1, w=1 (R= of 9a, n=1), x=0, y=1 (Ar=Ph, D-isomer), z=1	17
10a	Siderophore	v=1, w=1 (R= of 9a, n=3), x=1, y=z=0	Not tested
10b	Conjugate	v=1, w=1 (R= of 9a, n=3), x=0, y=1 (Ar=Ph, D-isomer), z=1	17
11a	Arthrobactin	v=0, w=1  (R=), x=1, y=z=0	18
11b	Conjugate	v=0, w=1 (R= of 11a), x=0, y=0, z=1	18
12	Nalidixic acid		Sigma Chemical Co.
13a	Desferrioxamine B		Gift from Ciba Geigy
13b	Conjugate		19
14	Erythromycylamine		Gift from Eli Lilly and Co.
15b	Conjugate	14 (R= of 5a)	33, 34, 35
15c	Conjugate	14 (R= of 7a)	21
16a	Ferrichrome		Porphyrin Products, Inc.

TABLE 2. Stimulation or inhibition of growth by various siderophore portions in disc diffusion tests[a]

Siderophore[b]	Structural type	Zone size (mm)[c]					
		ECX580	ECH455	BB	PM	PH	SS
2a	Spermidine-based bis-catechol	+22	+25	+24	−15	−16	0
3a	Lysine-based bis-catechol	+32	+25	+29	−14	−13	0
5a	Tri-δ-*N*-OH-δ-*N*-acetyl-L-ornithine (trihydroxamate)	+27	+18	+11	−15	−16	0
7a	Isocyanurate-based trihydroxamate	0	0	+12	−14	−16	0
11a	Arthrobactin (citrate-based hydroxamate)	0	0	0	0	−12	0
13a	Desferrioxamine B (trihydroxamate)	0	0	+26	0	0	0
16a	Ferrichrome (trihydroxamate)	+36	+22	+33	0	0	0

[a] Disc diffusion assays were performed on MHA plates supplemented with EDDHA (100 μg/ml) to evaluate the growth promotion abilities of siderophore portions or on regular unsupplemented agar to evaluate their inhibitory activities. All siderophores were used at 0.04 μmol per disc.
[b] Test compounds are numbered as in Table 1.
[c] EC, *E. coli*; BB, *B. bronchiseptica*; PM, *P. multocida*; PH, *P. haemolytica*; SS, *S. suis*; +, stimulation of growth; −, inhibition of growth.

of inhibition were caused by unconjugated siderophores on iron-rich media and zone sizes were thus attributed a negative value in Table 2. Inversely, inactive conjugated drugs that promoted growth in iron-restricted conditions were attributed a positive value in Table 3. Tests showed that *E. coli* X580 and H455 had their growth promoted in the presence of all catechol siderophore types when these compounds were used as the sole iron source in plates containing 100 μg of EDDHA per ml. The two strains of *E. coli* were also able to use some hydroxamate-based siderophores such as ferrichrome, as well as a synthetic portion of this natural siderophore (tri-δ-*N*-hydroxy-δ-*N*-acetyl-L-ornithine) for growth promotion. In general, strain X580 was more responsive to these siderophore-promoting activities. *B. bronchiseptica* used desferrioxamine B, and isocyanurate-based or tri-δ-*N*-hydroxy-δ-*N*-acetyl-L-ornithine hydroxamates in addition to catechol-based siderophore portions for promotion. *P. multocida* and *P. haemolytica* did not use any of the synthetic siderophores for growth promotion, and consequently some observed inhibitory activities may be linked to the ability of some siderophore types to withhold iron from these bacteria when tests were performed in iron-rich media. None of the siderophores tested promoted *S. suis* growth in iron-restrictive conditions. Additional but limited promotion tests were also done for some other gram-positive organisms (data not tabulated). Growth of *S. epidermidis* was promoted by the two siderophores tested: the spermidine-based bis-catechol and the isocyanurate-based trihydroxamate (18- and 20-mm zones of promotion, respectively). Neither of these individual siderophores promoted growth of *S. aureus.*

Antibacterial activities. With *E. coli*, the inhibitory activities of the drug conjugates were associated with the ability of the bacteria to use the siderophore portion of the molecules for growth promotion. *E. coli* used catechol-based and hydroxamate-based ferric iron ligands for growth under iron-restricted conditions and was sensitive to carbacephalosporin conjugated to these siderophore portions (Table 3). However, both the type of siderophore and the carbacephalosporin derivative determined the overall potency of the drug conjugate. For example, with the spermidine-based bis-catechol as the siderophore portion (no. 2a in Table 1), a relation for the best antibacterial activity may tentatively be outlined as follows after measurements of three separate disc diffusion tests for which standard deviations never exceeded 1.5 mm in zone diameter are averaged: D-phenylglycyl (no. 2c, zone of 33.0 mm) > D-hydroxyphenylglycyl (no. 2e, zone of 30.7 mm) > L-phenylglycyl (no. 2d, zone of 28.7 mm) > L-hydroxyphenylglycyl-carbacephalosporin (no. 2f, zone of 27.7 mm). Interestingly, the spermidine-based tricatechol conjugate (no. 4b in Table 1) which possesses appropriate hexadentate ligands for iron chelation abolished the inhibitory property of the carbacephalosporin. Most importantly, it was also observed that a mixed ligand (no. 8c in Table 1) having both hydroxamate and catechols in the same siderophore construct could provide one of the best antibacterial activities. Nevertheless, none of the conjugated drugs surpassed the inhibitory potential of the drug alone, and this was particularly true for siderophore-erythromycylamine or -nalidixic acid conjugates for all the bacterial species tested.

None of the β-lactam conjugates had an inhibitory effect on *B. bronchiseptica*, probably because of the presence of the β-lactamase produced by this strain and the minimal inhibitory potential of the unconjugated carbacephalosporin against that species (Table 3). Remarkably, *B. bronchiseptica*, which showed a great ability for using siderophore portions as growth-promoting agents (Table 2), was also able to use many of the drug conjugates as a source of iron. *P. multocida* and *P. haemolytica* did not use any of the synthetic siderophores for growth promotion, and accordingly, the inhibitory activity of some conjugates seemed partly linked to their ability to withhold iron from these bacteria. No significant antibacterial activity was obtained for the gram-positive organism *S. suis*. Erythromycylamine also lost or partly lost its antibacterial activity against other gram-positive species (*S. aureus* and *S. epidermidis*) when conjugated to the isocyanurate-based trihydroxamate siderophore, even though this siderophore could be used as growth stimulant by *S. epidermidis* (see above).

Susceptibility of resistant mutants. Some *E. coli*-resistant strains were selected and isolated from zones of inhibition after exposure to different drug conjugates in the disc diffusion test. Susceptibility of the isolated mutants to some siderophore-carbacephalosporin conjugates is reported in Table 4. Single-step mutants always showed a reduced susceptibility to the drug conjugate against which they were selected. However, two interesting observations were noted: (i) resistance in single-step mutants is not absolute; a persistent antibacterial activity remains, and (ii) cross-resistance is seen for conjugates having

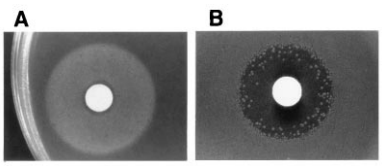


FIG. 1. (A) Zone of growth promotion provided by a siderophore placed on a disc in diffusion assays performed in iron-restricted conditions; (B) zone of inhibition caused by a siderophore-drug conjugate in assays performed in iron-rich media. Note the resistant colonies that may be isolated from such an inhibition zone and that can be used for subsequent mode-of-action studies.

TABLE 3. Inhibition or stimulation of growth by various siderophore-drug conjugates in disc diffusion tests[a]

Conjugate[b]	Siderophore portion	Drug portion	Zone size (mm)[c]				
			ECX580	BB	PM	PH	SS
1	None	Phenylglycyl-carbacephalosporin	−38	−12	−21	−25	−19
2b	Spermidine-based bis-catechol	Carbacephalosporin	−24	0	−15	−12	ND
2c	Spermidine-based bis-catechol	D-Phenylglycyl-carbacephalosporin	−33	0	−18	−16	−13
2d	Spermidine-based bis-catechol	L-Phenylglycyl-carbacephalosporin	−29	0	−12	−14	ND
2e	Spermidine-based bis-catechol	D-4-OH-Phenylglycyl-carbacephalosporin	−31	0	−17	−16	ND
2f	Spermidine-based bis-catechol	L-4-OH-Phenylglycyl-carbacephalosporin	−29	0	−13	−15	ND
3b	Lysine-based bis-catechol	Carbacephalosporin	−23	+24	−15	−12	ND
4b	Spermidine-based tricatechol	Carbacephalosporin	0	+18	−11	−10	ND
5b	Tri-δ-*N*-OH-δ-*N*-acetyl-L-ornithine	D-Phenylglycyl-carbacephalosporin	−33	+20	−19	−18	−12
6b	Amino-ω-(hydroxyamino)alkane	Carbacephalosporin	−13	0	0	0	0
6c	Amino-ω-(hydroxyamino)alkane	D-Phenylglycyl-carbacephalosporin	−31	0	−19	0	−18
7b	Isocyanurate-based trihydroxamate	D-Phenylglycyl-carbacephalosporin	−29	+12	−18	−23	−15
7c	Isocyanurate-based trihydroxamate	Carbacephalosporin	−26	+12	−15	−21	−15
8b	Mixed-ligand-based siderophore	Carbacephalosporin	−11	+16	0	0	−11
8c	Mixed-ligand-based siderophore	D-Phenylglycyl-carbacephalosporin	−34	+16	−24	−20	−15
9b	Mixed-ligand-based siderophore	D-Phenylglycyl-carbacephalosporin	−13	ND	ND	ND	ND
10b	Mixed-ligand-based siderophore	D-Phenylglycyl-carbacephalosporin	−25	ND	ND	ND	ND
11b	Citrate-based arthrobactin	Carbacephalosporin	−9	0	0	0	0
12	None	Nalidixic acid	−26	−20	−26	−20	ND
13b	Desferrioxamine B	Nalidixic acid	0	+18	0	0	ND
14	None	Erythromycylamine	−26	−23	−18	−17	0
15b	Tri-δ-*N*-OH-δ-*N*-acetyl-L-ornithine	Erythromycylamine	0	0	−12	−17	0
15c	Isocyanurate-based trihydroxamate	Erythromycylamine	0	0	0	0	0

[a] Disc diffusion assays were performed on MHA plates to evaluate the inhibitory activities of siderophore-drug conjugates or on plates supplemented with EDDHA (100 μg/ml) to evaluate growth promotion effects. All drug conjugates were used at 0.04 μmol per disc.
[b] Test compounds are numbered as in Table 1.
[c] EC, *E. coli*; BB, *B. bronchiseptica*; PM, *P. multocida*; PH, *P. haemolytica*; SS, *S. suis*; ND, not determined; +, stimulation of growth; −, inhibition of growth.

either similar or unrelated siderophore portions. Both of these observations may indicate that conjugates can profit from more than one iron transport system for entry into cells. Tests with double mutants support this view, since full resistance was observed only after a second selective pressure with a different type of siderophore conjugate (Table 4).

Cell surface of resistant bacteria. The outer membrane protein profiles of the *E. coli* strains X580 and conjugate-resistant mutants obtained on SDS-polyacrylamide gels after electrophoresis were examined. As expected, several outer membrane proteins were expressed when strains were grown under iron-restricted conditions, and major differences were observed in these iron-regulated proteins when strains were compared. Although we could not clearly identify all of the modifications observed in the outer membrane protein profiles, some of the mutant strains were lacking a specific siderophore receptor(s) as revealed by colicin sensitivity tests and their susceptibilities to phages T1 and T5 (Table 5). The bacteriophage sensitivity tests confirmed that mutant 5c-R and the 5c-R double mutant (2b-R/5c-R), which are resistant to the carabacephalosporin conjugated to a siderophore portion similar to that of ferrichrome, lack the 78-kDa protein FhuA, the outer membrane receptor for ferrichrome and phages T1 and T5 (Table 5 and Figure 2). Besides, resistance to the spermidine-based bis-catechol carbacephalosporin conjugate observed in 2b-R and 2b-R double mutants (2b-R/5c-R and 2b-R/8c-R) was partly explained by the lack of the 74-kDa Cir protein in the outer membrane (Fig. 2), as concluded by the resistance to colicin Ia

TABLE 4. Inhibition zones obtained with some siderophore-carbacephalosporin conjugates against conjugate-resistant *E. coli* mutants in disc diffusion tests

Strain	Resistance selected on the following agent:		Siderophore portion of selecting agent	Inhibition zone of test compound[a] (mm)				
	First	Second		1 (loracarbef)	5b (5c analog)	2c (2b analog)	8c	6c
X580	None	None	None	38	30	33	31	28
6c-R	6c	None	Amino-ω-(hydroxyamino)alkane	33	19	17	16	14
5c-R	5c	None	Tri-δ-*N*-OH-δ-*N*-acetyl-L-ornithine	33	13	30	28	26
8c-R	8c	None	Mixed-ligand-based siderophore	32	26	17	18	9
2b-R	2b	None	Spermidine-based bis-catechol	30	26	15	26	26
2b-R/5c-R	2b	5c	Spermidine-based bis-catechol and Tri-δ-*N*-OH-δ-*N*-acetyl-L-ornithine	32	19	15	26	28
2b-R/8c-R	2b	8c	Spermidine-based bis-catechol and mixed-ligand-based siderophore	38	29	0	0	14
6c-R/8c-R	6c	8c	Amino-ω-(hydroxyamino)alkane and mixed-ligand-based siderophore	34	27	17	ND	12

[a] Disc diffusion assays were performed on MHA. All siderophore-drug conjugates were used at 0.04 μmol per disc. ND, not determined.

TABLE 5. Sensitivities of conjugate-resistant mutants and selected bacterial strains to specific colicins or phages

Strain	Siderophore portion of selecting agent or colicin to which strain shows resistance	Result[a] for the following colicinogenic *E. coli* strain (colicin produced/colicin receptor) or phage (receptor):				
		AG097 (B/FepA)	K-12 W3110 (D/FepA)	K-12 167 (Ia/Cir)	CA53 (Ia/Cir)	ΦT1, T5 (FhuA)
X580	None	S	S	S	S	S
6c-R	Amino-ω-(hydroxyamino)alkane	S	S	S	S	S
5c-R	Tri-δ-*N*-OH-δ-*N*-acetyl-L-ornithine	S	S	S	S	R
8c-R	Mixed-ligand-based siderophore	S	S	S	S	S
2b-R	Spermidine-based bis-catechol	S	S	R	R	S
2b-R/5c-R	Spermidine-based bis-catechol and Tri-δ-*N*-OH-δ-*N*-acetyl-L-ornithine	S	S	R	R	R
2b-R/8c-R	Spermidine-based bis-catechol and mixed-ligand-based siderophore	S	S	R	R	S
6c-R/8c-R	Amino-ω-(hydroxyamino)alkane and mixed-ligand-based siderophore	S	S	R	R	S
HfrH180	None	S	S	S	S	ND
B-R	Colicin B	R	R	S	S	ND
D-R	Colicin D	R	R	S	S	ND
Ia-R	Colicin Ia	S	S	R	R	ND

[a] Abbreviations: S, sensitive to the colicin or phage; R, resistant to the colicin or phage; ND, not determined.

(Table 5). Mutant 6c-R showed the presence of FhuA and was sensitive to all the colicins tested, and these tests could not explain the modifications observed in the outer membrane protein profile (Fig. 2). The outer membrane protein profile of the double mutant 6c-R/8c-R was similar to that of 2b-R (Fig. 2), as was its colicin sensitivity profile (Table 5), while mutant 8c-R was not different from the parent strain in its outer membrane protein profile or in phage and colicin tests, although its drug susceptibility profile was different (Table 4). Modification in the cytoplasmic membrane proteins of these mutants was not investigated.

Defined mutant studies. The use of *E. coli* strains having well-characterized mutations in iron transport systems also helped in the identification of specific outer membrane proteins involved in both siderophore-mediated iron transport and siderophore-antibiotic conjugates. Susceptibility tests for these mutants revealed that the absence of outer membrane proteins FhuA, FecA, Cir, Fiu, and FhuE and in the periplasmic proteins TonB and ExbB affects the inhibitory activities of the carbacephalosporin conjugates tested (Table 6). The limited susceptibility of the parental strain *E. coli* H455 to the carbacephalosporin used in this study and the limited preference of that strain for hydroxamate-based siderophores in growth promotion tests (Table 2) did not allow for extensive collection of data, and observations were limited. However, Table 6 clearly shows that some conjugated drugs require FecA, Ton B, or ExbB to exert their antibacterial activity.

DISCUSSION

Several natural iron-chelating antibiotics have been described (49, 51). The antibiotic albomycin has been shown to be a linear peptide attached to a toxic thioribosyl unit (4). Like the siderophore ferrichrome, the iron-binding section of albomycin is a tri-δ-*N*-hydroxy-δ-*N*-acyl-L-ornithine peptide. Evidence now indicates that albomycin is actively carried into microbial cells by normal iron transport process via the FhuA outer membrane protein which also is the receptor of ferrichrome-Fe(III) (4). Several workers showed that it was possible to use outer membrane iron-regulated proteins to transport drugs into gram-negative bacteria. Some investigators have shown that semisynthetic β-lactams having a terminal catechol moiety have increased activity relative to other derivatives against various microbes, including *P. aeruginosa* strains (14, 33, 44, 53). Curtis et al. (8) showed that activity of a dihydroxybenzoyl derivative of a cephalosporin was dependent on a TonB-mediated transport through the bacterial outer membrane. More specifically, this compound was actively transported into *E. coli* cells via the outer membrane proteins Fiu and Cir (42).

In this study, we have evaluated several new siderophore-antibiotic conjugates, but we first studied the ability of various bacterial species to use siderophore portions as growth-promoting agents. Along with *E. coli* strains, *B. bronchiseptica* could utilize many sources of synthetic and natural siderophores. Foster and Dyer (13) previously showed that *B. bronchiseptica* could use its own siderophores for removal of iron from lactoferrin and transferrin rather than relying upon a receptor for these host ferri-binding proteins, and this scenario may also explain the large number of growth-promoting siderophores identified for *B. bronchiseptica* in the present study. Reissebrodt et al. (50) showed that *P. multocida* and *P. haemolytica* could used the iron-loaded siderophore ferrioxamine B diffusing from discs containing 2 nmol of the siderophore when grown in a candle jar on media containing 10 to 30 μM of EDDHA. In our study, EDDHA was used at 100 μg/ml (i.e., 280 μM) and desferrioxamine B was used as the iron-depleted siderophore placed on discs in promotion tests performed aerobically. In such experimental conditions, we were able to show the strong ability of *B. bronchiseptica* to use desferrioxamine B (as well as many other siderophores) but were unable to observe growth promotion of *Pasteurella* spp. by such a siderophore. In this respect, the discrepancy between the results of

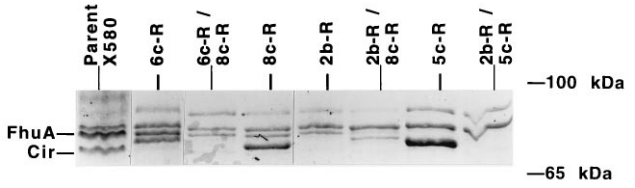


FIG. 2. OMP profiles of some conjugate-resistant mutants isolated in this study. The relevant portion of the Coomassie-stained gel is shown (molecular masses ranging from 65 to 100 kDa).

TABLE 6. Inhibition zones obtained with some siderophore-carbacephalosporin conjugates against genetically defined *E. coli* mutants in disc diffusion tests

Strain	Relevant phenotype (element lacking)	Inhibition zone (mm) caused by test compound (structural type of ligand in siderophore portion of conjugate)[a]				
		1 (loracarbef)	5b (hydroxamate)	2c (catechol)	8c (mixed ligands)	6c (OH-aminoalkane)
H455	Parental strain	16	0	17	16	0
H1594	Fiu (mono-catechol receptor)	ND	0	12	11	0
H1187	FepA (enterochelin receptor)	ND	0	16	16	0
Z1379	FecA (ferric dicitrate receptor)	ND	0	0	0	0
H1196	FhuA (ferrichrome receptor)	ND	0	13	15	0
H1619	FhuE (rhodotorulate receptor)	ND	0	13	11	0
H1300N	Cir (mono-catechol receptor)	ND	0	14	14	0
H1252	TonB (receptor-mediated transport of iron-III)	ND	0	0	0	0
ZI17	ExbB (receptor-mediated transport of iron-III)	ND	0	0	0	0

[a] Disc diffusion assays were performed on MHA. All drugs were used at 0.04 μmol per disc. ND, not determined.

Reissebrodt et al. (50) and our findings may be explained by the higher stringency of our test. In any case, these species were not affected by our desferal-nalidixic acid conjugate.

Even though the synthetic bis-catechols and trihydroxamates were very good growth-promoting agents for *E. coli*, the conjugation to drugs could not increase their antibacterial activity. Several studies showed that the addition of a catechol moiety to the acyl group of β-lactams may enhance the antimicrobial activities of these drugs in iron-restricted conditions (38, 39, 55) and that totally synthetic trihydroxamates and bis-catechols could deliver β-lactam antibiotics to the periplasmic targets (the penicillin-binding proteins) through the outer membrane of gram-negative bacteria via iron transport pathways (3). Besides, with a limited number of siderophores conjugated to antibiotics acting intracellularly, such as nalidixic acid and erythromycylamine, we have not been able to improve or sustain the activity of the parent drug. For gram-positive organisms and drugs acting in the cytoplasm, more studies are needed to identify adequate linkages and species-selective siderophore portions for drug delivery to cellular targets.

One of the most intriguing results encountered in this study was that examination of conjugate-resistant bacteria implied nonfunction or the absence of the specific outer membrane receptor for the ferric siderophore used in the preparation of the conjugate. Tests with double mutants also showed that full resistance may only be achieved after a second selective pressure with a different type of siderophore conjugate. These results indicated that at least two mutations are needed to achieve greater resistance levels. As was expected, we have also shown that a disabled TonB protein in the periplasmic space which is normally essential for the assimilation of both catechol and hydroxamate siderophore by *E. coli* (25, 48) would allow resistance to antibiotic conjugates being transported by either way (37).

We now know that a lower frequency of resistance is a very good indicator of multiple receptor-mediated entry occurring simultaneously (3). This observation is very important and validated the feasibility of using mixed siderophore ligands in a conjugate or using a mixture of conjugates to achieved greater inhibitory activity concomitantly with a low frequency of resistance. Also in the case of siderophore conjugates, the development of resistance should be viewed with much more latitude and vision than in the case of traditional antibiotic evaluation. For example, it is possible that some resistant mutants will not display cross-resistance, that with some conjugates some mutants will occur at a very low frequency because more than one or two mutations are needed to achieve greater resistance, and most importantly, that even one single type of mutation selects strains that are not necessarily able to survive or grow in an in vivo environment where competition for iron availability is crucial for pathogenesis. Indeed, a previous study has shown that some conjugate-resistant mutants could not grow efficiently in an animal model (3).

Our results demonstrate that siderophore-antibiotic conjugates can be used against pathogenic bacteria which produce and use siderophores to acquire iron. The ideal siderophore and drug combination remains to be determined, but this study showed that a multitude of species-directed or broadly active conjugates may be envisioned.

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