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Differences in the Resistant Variants of *Enterobacter cloacae* Selected by Extended-Spectrum Cephalosporins

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The rates of development of resistance to ceftriaxone, ceftazidime, cefepime, and cefpirome in 10 strains of *Enterobacter cloacae* were determined by daily transfer for 7 days to fresh medium containing twofold serial dilutions of the antibiotics. Development of resistance to ceftriaxone was the most rapid; this was followed by ceftazidime, cefpirome, and cefepime. Resistant variants selected by ceftriaxone and ceftazidime were cross-resistant and produced very high levels of β-lactamase. On the other hand, resistant variants selected by cefepime and cefpirome often had moderately high levels of β-lactamase and diminished levels of the 39- to 40-kDa porin protein.

For *Enterobacter cloacae*, therapy with some extended-spectrum cephalosporins may result in a rapid development of resistance to the drug (6–8, 27). The newer cephalosporins cefepime and cefpirome are often active against strains of *Enterobacter* that are resistant to ceftazidime and cefotaxime (9, 21). The increased levels of activity of cefepime and cefpirome against gram-negative bacteria is the result of their markedly reduced affinity for *E. cloacae* chromosomal β-lactamase and their enhanced permeation across the outer membrane compared with the levels of permeation of other broad-spectrum cephalosporins (13, 22).

Spontaneous mutants of *E. cloacae* with high-level resistance to older, extended-spectrum cephalosporins occur at frequencies of 10^{-6} to 10^{-7} (11, 17), compared with the lower frequencies of 10^{-7} to $<10^{-9}$ for the isolation of mutants that are less susceptible to cefepime (21, 25, 30). Likewise, in a murine peritonitis model, resistance to cefepime emerged less frequently than resistance to ceftazidime or ceftriaxone during therapy (19, 24).

In the study described here, we compared the rate of stepwise resistance development of 10 clinical isolates of *E. cloacae* (Table 1) to the extended-spectrum cephalosporins ceftazidime, ceftriaxone, cefepime, and cefpirome. Stepwise resistance was developed by the broth macrodilution method as described before (12). Briefly, on each day, growth from half the MIC tube was used as the inoculum, after adjustment to 1×10^5 to 5×10^5 CFU/ml, for a further series of tubes containing doubling concentrations of the cephalosporin. This procedure was continued for 7 days. When there was a notable increase in the MIC during the course of the 7-day study, less susceptible variants were isolated and characterized for their outer membrane protein patterns, their β-lactamase levels, and their susceptibilities to the cephalosporins. Since reduced susceptibilities to chemically unrelated agents have been reported for some cephalosporin-resistant mutants (23), we determined the MICs of carbapenems, ciprofloxacin, tetracycline, and chloramphenicol for our cephalosporin-selected variants.

MICs were determined by an agar dilution method in accordance with the procedures outlined by the National Committee for Clinical Laboratory Standards (20). The detection of chloramphenicol acetyltransferase (CAT) was performed by the method of Azemun et al. (1). The β-lactamase assay was performed spectrophotometrically with sonicated cell extracts (9). One unit of β-lactamase activity was defined as the amount of enzyme that hydrolyzed 1 μmol of nitrocefin per min per ml of protein. Isoelectric focusing of the supernatants from centrifuged sonicated cell extracts was performed as reported previously (9) by using *E. cloacae* SC 12629 (E2 chromosomal β-lactamase; pI = 8.9) and SC 10435 (P99 chromosomal β-lactamase; pI = 7.8) as standards. Western blot (immunoblot) analysis (14) of a TEM β-lactamase was performed by using rabbit antiampicillinase to the β-lactamase of pBR322 (5 Prime→3 Prime, Inc., Boulder, Colo.) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Jackson Laboratories, West Grove, Pa.) for detection. Outer membrane proteins were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (10).

Prior to serial passage, the MICs of the four selecting cephalosporins for the 10 *E. cloacae* strains were within the susceptible range (Table 1), with modal MICs of cefepime of 0.03 μg/ml and modal MICs of cefpirome and ceftriaxone of 0.06 μg/ml; a bimodal distribution was found for ceftazidime, with modal MICs of 0.25 and 2 μg/ml. Seven strains produced low basal levels of β-lactamase (<150 mU/mg of protein), but most were inducible for the production of the enzyme. The remaining three strains with higher β-lactamase levels (>1,300 mU/mg of protein) contained both the group 1 chromosomal β-lactamase (pI = 7.9 to 8.9) and a TEM β-lactamase, as confirmed by Western blot analysis, with pIs of 5.4 to 5.7 (4). The eight *E. cloacae* strains analyzed had both major porin proteins, with molecular masses of 37 to 38 kDa and 39 to 40 kDa. The 39- to 40-kDa protein (Omp39-40) is believed to be involved in the permeation of tetracycline and chloramphenicol as well as those of cephalosporins (16). In this report, porins are referred to by their molecular sizes; for example, Omp39 stands for the 39-kDa outer membrane protein.

For the 10 *E. cloacae* strains, passage in the presence of ceftriaxone led to the most rapid increase in the MIC (Fig. 1).

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TABLE 1. Characteristics of clinical *E. cloacae* strains

E. cloacae strain	MIC (μg/ml)[a]										β-Lactamase activity (mU/mg of protein)[b]			Size of outer membrane proteins (kDa)[c]	pI(s) of β-lactamase(s)
	FEP	CPO	CRO	CAZ	CTX	Imip	Merop	Cipro	TET	CAM	Basal	After induction with imipenem at:			
												0.1 μg/ml	1 μg/ml		
A20957	0.06	0.06	0.13	2	0.06	1	0.06	0.03	4	8	78	77	1,790	40/39/37	8.1/8.4
A20650	0.015	0.03	0.06	0.25	0.06	0.25	0.03	0.015	2	2	39	6,700	ND[d]	39/37	8.9
A15156	0.03	0.03	0.06	0.13	0.06	1	0.03	0.015	1	4	23	253	1,441	40/38/37	8.4/8.7
A26261	0.13	0.25	0.06	0.5	0.25	0.25	0.03	0.007	2	>128	5,600	6,500	ND	39/37	5.5/7.9
A20960	0.03	0.06	0.13	1	0.25	0.25	0.06	0.015	2	>128	122	121	339	39/37.5/37	7.85/8.1
A20476	0.06	0.13	0.5	0.5	0.5	0.25	0.03	0.03	16	8	37	3,100	ND	39/38	7.85
A26259	0.25	0.5	0.5	2	0.5	0.25	0.06	0.015	>128	>128	1,300	6,200	ND	39/37	5.4/8.3
A21405	0.13	0.5	0.25	2	0.25	0.25	0.03	0.015	128	>128	5,100	6,300	ND	39/37	5.7/7.5/8.6
A26883	0.03	0.06	0.25	0.25	0.25	0.13	0.03	0.015	2	8	67	1,200	ND	ND	8.6
A26884	0.03	0.06	0.13	0.25	0.25	0.13	0.03	0.015	2	8	59	242	3,999	ND	7.6

[a] FEP, cefepime; CPO, cefpirome; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; Imip, imipenem; Merop, meropenem; Cipro, ciprofloxacin; TET, tetracycline; CAM, chloramphenicol.
[b] β-Lactamase activity following 2 h of induction with imipenem.
[c] Major outer membrane proteins in the range of 37 to 40 kDa, as determined by SDS-PAGE.
[d] ND, not determined.

By the second day, seven strains developed resistance (MIC, ≥64 μg/ml) to ceftriaxone, and by the fifth day, only strain A20650 remained susceptible to the drug. Rapid development of ceftazidime resistance (MIC, ≥32 μg/ml) was also observed. By the second day, four strains developed resistance to ceftazidime, and by day 5, four more strains became resistant. Only strains A26884 and A26883 remained susceptible to ceftazidime after the 7-day passage. Resistance to cefpirome (MIC, ≥32 μg/ml) developed less quickly than resistance to ceftriaxone and ceftazidime. By days 2, 5, and 7, resistance to cefpirome was observed in three, five, and six strains, respectively. Resistance to cefepime (MIC, ≥32 μg/ml) was the least likely to develop, with zero, one, and two strains becoming resistant to the drug on days 2, 5, and 7, respectively.

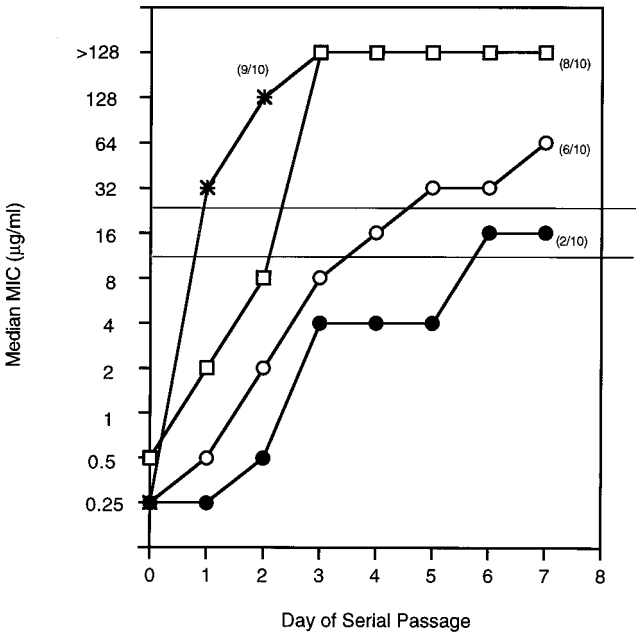

FIG. 1. Median MICs for 10 *E. cloacae* strains during 7-day serial passage with a cephalosporin. The median MIC represents the sixth MIC observation when the MICs for the 10 strains on each day of testing are listed from the lowest to the highest value. The values in parentheses are the number of strains among the 10 strains tested for which the MIC was in the resistant range (≥32 μg/ml for cefepime, cefpirome, and ceftazidime; ≥64 μg/ml for ceftriaxone) at the end of the 7-day serial passage. ∗, ceftriaxone; □, ceftazidime; ○, cefpirome; ●, cefepime. The upper and lower lines in the figure are cutoffs for resistance and susceptibility, respectively.

For all seven *E. cloacae* strains lacking the TEM-like β-lactamases within the ceftriaxone MIC series on days 0 and 1, growth was not found to occur in all tubes (skipped tube phenomenon). In five cases, a single tube or two consecutive tubes containing concentrations of ceftriaxone lower than the MIC had no growth, and in the other two cases, a single tube distal to the tube containing the drug at the MIC exhibited growth. For four of these strains, the skipped tube phenomenon was also shown with ceftazidime. The skipped tube phenomenon did not occur with cefepime or cefpirome. With ceftriaxone, resistance development more rapid than that depicted in Fig. 1 would have occurred had the inoculum used for passage been prepared from growth in skipped tubes containing drug at concentrations higher than the MIC (data not shown).

Twenty variants, selected from serial passage in ceftriaxone (11 variants) or ceftazidime (9 variants), were examined. Representative variants are listed in Table 2, since most of the unlisted variants are phenotypically similar to A26261 d 1-CRO 1. For 18 of these variants, the MICs of the selecting cephalosporin were ≥32 μg/ml, and most of the variants were cross-resistant to the other cephalosporin and produced high levels of β-lactamase (6,000 to 77,000 mU/mg of protein). Although for the variants selected by ceftriaxone and ceftazidime the MICs of the newer extended-spectrum cephalosporins were higher, all variants were susceptible to cefepime and for four variants cefpirome MICs were ≥16 μg/ml. The susceptibilities of these variants to other drug classes were, for the most part, unchanged from those of their parents. Two ceftriaxone- and ceftazidime-selected variants had diminished levels or no Omp39-40 (Fig. 2), and two others had diminished levels of Omp37 (data not shown).

Of the 27 variants selected by cefepime (13 variants) or cefpirome (14 variants), cefpirome and ceftazidime MICs were ≥16 μg/ml for 19 and 14 variants, respectively, whereas cefepime MICs were ≥16 μg/ml for only 8 variants. Cross-

TABLE 2. Characteristics of representative *E. cloacae* variants selected by the extended-spectrum cephalosporins

E. cloacae strain	Variant[a]	MIC (μg/ml)[b]										β-Lactamase activity (mU/mg of protein)[c]	Outer membrane proteins[d]
		FEP	CPO	CRO	CAZ	CTX	Imip	Merop	Cipro	TET	CAM		
A20957	d 7-FEP 1	1	2	1	4	2	1	0.5	0.25	32	16	79	$Omp39^{dim}$
	d 3-CAZ 128	2	4	>128	128	>128	1	1	0.06	8	4	25,000	$Omp39^{dim}$
A20650	d 7-FEP 0.25	0.5	1	4	4	4	2	2	0.06	2	1	25	$Omp39^{dim}/37^{-}$
	d 7-CPO 1	1	2	0.5	2	2	0.25	0.13	0.5	32	128	51	$Omp39^{dim}/37^{dim}$
	d 0-CRO 1	0.06	0.13	8	16	8	1	0.06	0.015	2	8	1,700	Parent
	d 5-CRO 1	0.06	0.06	0.25	0.5	0.25	1	0.06	0.06	4	32	119	Parent
	d 5-CAZ 16	0.25	0.5	0.5	32	1	0.25	0.03	0.015	2	>128	48	Parent
A15156	d 7-FEP 0.13	0.13	0.25	0.5	2	0.5	2	0.13	0.06	2	8	36	Parent
	d 4-CPO 1	4	4	2	8	8	1	0.25	0.25	16	32	23	$Omp40^{dim}$
	d 3-CRO 128	0.5	1	64	64	128	1	0.5	0.06	2	2	20,000	Parent
	d 5-CAZ 128	8	32	>128	>128	>128	0.25	0.25	0.03	2	>128	45,000	$Omp40^{-}$
A26261	d 1-FEP 1	2	4	0.5	2	1	0.25	0.06	0.03	2	>128	8,600	$Omp^{dim}/37^{dim}$
	d 2-CPO 16	32	64	4	16	4	4	8	0.03	2	>128	11,000	$Omp39^{-}$
	d 1-CRO 1	2	4	32	128	128	0.25	0.13	0.015	2	>128	55,000	Parent
A20960	d 7-FEP 8	16	32	>128	>128	>128	1	1	0.06	2	>128	53,000	$Omp39^{dim}$
	d 2-CPO 32	4	16	2	16	1	0.25	0.13	0.03	>128	>128	11,000	$Omp39^{dim}$
	d 1-CRO 8	0.5	1	64	32	32	0.25	0.06	0.015	2	>128	307	Parent
A20476	d 7-FEP 8	8	64	>128	>128	>128	1	1	0.25	64	32	56,000	$Omp39^{dim}$
	d 7-CPO 64	32	128	>128	>128	>128	1	2	0.25	128	64	69,000	$Omp39^{dim}$
	d 1-CRO 128	2	16	128	128	>128	0.25	0.06	0.03	16	8	57,000	Parent
	d 1-CAZ 16	2	16	>128	128	>128	0.25	0.06	0.13	64	32	54,000	Parent
A26259	d 3-FEP 2	4	4	2	4	2	0.25	0.25	0.03	>128	>128	2,300	$Omp39^{dim}$
	d 3-CPO 4	8	16	4	8	4	0.25	0.25	0.06	>128	>128	3,400	Parent
A21405	d 1-CPO 8	8	32	2	16	1	1	0.5	0.03	>128	>128	6,500	$Omp39^{dim}$

[a] The variants are designated by their day of isolation and the concentration of the selecting drug. For example, A20957 d 3-CAZ 128 was a variant of A20957 selected from the third day of passage in Mueller-Hinton broth containing 128 μg of ceftazidime per ml. The variant A20650 d 0-CRO 1 was obtained from determination of the MIC for A20650 in the tube containing 1 μg of ceftriaxone per ml prior to the first serial passage.

[b] MICs of noncephalosporins are underlined for variants for which MICs are eightfold or more higher than those for the parental strain listed in Table 1. See footnote *a* of Table 1 for definitions of drug name abbreviations.

[c] Uninduced level of β-lactamase.

[d] Only outer membrane protein (Omp) changes are indicated: Omp^{dim} or Omp^{-} means a diminished or absent Omp band of the designated molecular mass, respectively; parent means that the variant had the same Omp level as the parental strain.

resistance to ceftriaxone and cefotaxime was observed in six β-lactamase-hyperproducing variants (>50,000 mU/mg of protein). While 75% of the variants selected by ceftriaxone and ceftazidime produced β-lactamase at levels of >20,000 mU/mg of protein, only 25% of the variants selected by cefepime and cefpirome produced this amount of the enzyme.

The variants selected by ceftriaxone and ceftazidime also differed from those selected by cefepime and cefpirome in their levels of Omp39-40 (Table 2; Fig. 2). While only 10% of the variants selected by ceftriaxone and ceftazidime lacked or had diminished levels of Omp39-40, 80% of the variants selected by the newer cephalosporins had this phenotype. The absence or diminished level of Omp39-40 correlated with increased meropenem, ciprofloxacin, tetracycline, and chloramphenicol MICs. No CAT activity was detected in variants that developed resistance to chloramphenicol. Moreover, if multidrug resistance is defined as greater than fourfold increases in the MICs of three or more drug classes, none of the variants selected by ceftriaxone or cefotaxime was multidrug resistant. In contrast, six cefpirome-selected variants and one cefepime-selected variant exhibited decreased susceptibilities to multiple drug classes; all seven variants had diminished levels of Omp39-40. The multidrug resistance and diminished levels of

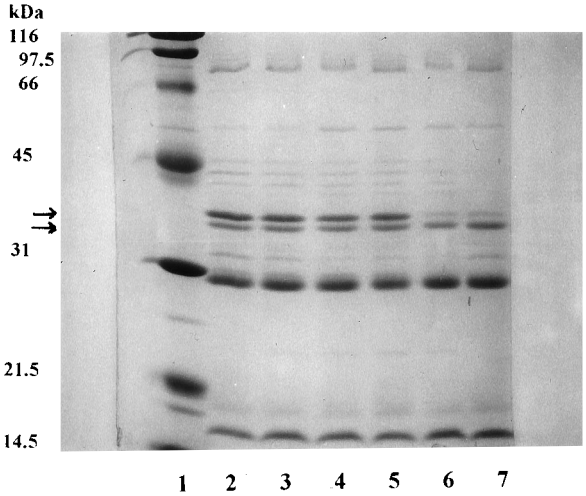

FIG. 2. SDS-PAGE of outer membrane proteins of *E. cloacae* A20476 and its cephalosporin-selected variants. The migration positions of standard proteins are shown on the left. Omp39-40 and Omp37 are indicated by the two arrows. Lanes: 1, molecular size standards (see text for details); 2, A20476; 3, A20476 d 1-CRO 128; 4, A20476 d 1-CAZ 16; 5, A20476 d 3-CAZ 128; 6, A20476 d 7-FEP 8; 7, A20476 d 7-CPO 64.

Omp39-40 are similar to the Mar phenotypes described for *Pseudomonas aeruginosa* (31). Four variants lacked or had diminished levels of Omp37.

These results indicate that in vitro, as in the clinical setting (2, 29), variants resistant to ceftriaxone and ceftazidime can readily be isolated. These variants are cross-resistant to the older extended-spectrum cephalosporins, primarily because of the hyperproduction of the chromosomal β-lactamase. In contrast, resistance to the newer cephalosporins, in particular, to cefepime, developed less rapidly. This has been observed in a murine peritonitis model (19, 23, 24) and, more importantly, in clinical trials. While none of the 58 evaluable, cefepime-treated patients infected with an *Enterobacter* sp. acquired a variant less susceptible to cefepime during or after therapy (greater than a twofold increase in the cefepime MIC or a ≤5-mm decrease in the zone diameter around the 30-μg disk compared with the results for the initial isolate), 2 (or 9.1%) of the 22 evaluable patients treated with ceftazidime developed resistance to ceftazidime and failed therapy (2a, 28).

Our finding that cefepime- and cefpirome-selected variants of *E. cloacae* often had diminished levels of Omp39-40 confirms the report by Piddock and Traynor (26), but is contrary to that of Büscher et al. (3). Possible explanations for this discrepancy could be strain differences, and the cefpirome MICs for the variants examined by Büscher et al. (3) were lower (≤10 μg/ml).

Although it appears that the absence or diminished amount of Omp39-40 is associated with decreased susceptibility to cefepime and cefpirome, high-level resistance (i.e., MICs ≥32 μg/ml) to these drugs seems to require both a diminished amount of this porin protein and high levels of β-lactamase. For instance, for the variants of strain A15156 (Table 2), the cefpirome MICs were 4, 1, and 32 μg/ml for A15156 d 4-CPO ($Omp40^{dim}$; low basal level of β-lactamase), A15156 d 3-CRO 128 (same level of Omp as that in the parent strain; β-lactamase level of 20,000 mU/mg of protein), and A15156 d 5-CAZ 128 ($Omp40^{-}$; β-lactamase level of 45,000 mU/mg of protein), respectively. The four cefepime-resistant variants in the present study had moderate to high β-lactamase levels (11,000 to 69,000 mU/mg of protein) and diminished levels of Omp39-40. Likewise, our results indicate that the lack of Omp39-40 often resulted in decreased susceptibility to ceftazidime, but concurrent β-lactamase hyperproduction is responsible for high-level resistance to this drug, as has been reported by Bush et al. (5).

The reduced levels of Omp39-40 and the high levels of cephalosporinase activity in the cefpirome- and cefepime-resistant variants of *E. cloacae* are analogous to those reported for imipenem-resistant mutants of *Enterobacter* spp. (18). Cefepime, cefpirome, and imipenem are zwitterionic, which may allow for their more rapid penetration through the porin channels of *E. cloacae* isolates (21), and they are more stable than older extended-spectrum cephalosporins to hydrolysis by the enterobacterial β-lactamase (5, 15, 21).

In summary, in *E. cloacae*, the rates of development of resistance to cefpirome and, in particular, cefepime are less rapid than those to cefriaxone and ceftazidime. High-level resistance to cefpirome and cefepime is often the result of moderately high levels of β-lactamase and diminished levels of Omp39-40.

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