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Resistance Caused by Decreased Penetration of β-Lactam Antibiotics into *Enterobacter cloacae*

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Strains of *Enterobacter cloacae* were selected on the basis of resistance to aztreonam, ceftazidime, moxalactam, or imipenem. All strains produced the same E2 β-lactamase, with an isoelectric point greater than 9.5 and with high hydrolytic activity in the presence of cephaloridine. Resistance to β-lactams could not be correlated with the amount of β-lactamase present in the various strains. β-Lactamase activity was induced strongly by moxalactam and imipenem in the wild-type and moxalactam-resistant strains, with β-lactamase representing as much as 4% of the total cellular protein after induction (2×10^5 molecules per cell). Ceftazidime and aztreonam were poor inducers. None of the antibiotics studied was readily hydrolyzed by the E2 β-lactamase; aztreonam and moxalactam inhibited the enzyme with apparent K_i values of 1.2 and 100 nM, respectively. Aztreonam, which bound covalently to the E2 β-lactamase with a half-life of 2.3 h at 25°C, was used to measure penetrability of β-lactam into the periplasmic space of the resistant *E. cloacae* strains. In all of the E2-producing organisms studied, a significant permeability barrier existed. A maximum concentration of 0.02 μg of aztreonam per ml should have saturated the periplasmic β-lactamase in the highest enzyme producers studied. However, fully active β-lactamase was observed in the periplasm of cells exposed to aztreonam at concentrations at least 1,000-fold higher than that theoretically necessary to inhibit the total enzyme within the cell. Thus, the major cause for resistance to β-lactam antibiotics in these *E. cloacae* strains was lack of penetration across the outer membrane.

Resistance to β-lactam antibiotics has been a problem throughout the history of the usage of these antimicrobial agents. A primary cause for the inactivity of penicillins and cephalosporins is hydrolysis of the β-lactam bond by an appropriate β-lactamase (15). Recently, compounds such as the monobactams and third-generation cephalosporins have appeared with a high degree of stability to many of the inactivating β-lactamases (2). As a result, bacterial resistance has developed due to mechanisms other than enzymatic hydrolysis.

Resistance to these new agents has been frequently observed in various strains of *Enterobacter cloacae* that possess the ability to produce elevated levels of β-lactamases (5, 16). These enzymes often exhibit high affinities for the β-lactam antibiotics but do not readily hydrolyze these molecules. Then and Angehrn have suggested that resistance may be due to "trapping" of these agents by the high concentrations of β-lactamase in the periplasm (16). Other explanations for resistance in *Enterobacter* strains have also been proposed, including the presence of a β-lactamase-related permeation barrier (12, 13).

In the present study, strains of *E. cloacae* were selected for resistance to aztreonam, moxalactam, ceftazidime, or imipenem. Although β-lactamase binding of these agents may be a contributing factor to the observed resistance patterns, the most significant characteristic of poorly active third-generation cephalosporins and monobactams was unfavorable penetration into the periplasmic space.

(A preliminary account of this work has been presented previously [R. B. Sykes, D. P. Bonner, K. Bush, N. Georgopapadakou, and K. Tanaka, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 23rd, Las Vegas, Nev., abstr. no. 428, 1983].)

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MATERIALS AND METHODS

Antibiotics. Aztreonam was prepared at E. R. Squibb & Sons, Princeton, N.J. Moxalactam and cephaloridine were gifts from Eli Lilly & Co., Indianapolis, Ind.; ceftazidime was from Glaxo Laboratories, Greenford, Middlesex, England; imipenem was from Merck & Co., Rahway, N.J.; cefoperazone was from Pfizer, Inc., New York, N.Y. SQ 24,902, a chromogenic cephalosporin (1), was synthesized at the Squibb Institute.

Bacterial strains. *E. cloacae* SC 12368 (wild type [WT]) was a clinical isolate known to exhibit cefoxitin-induced resistance to aztreonam and third-generation cephalosporins. Resistant mutants (Table 1) were isolated by direct selection on antibiotic-containing Diagnostic Sensitivity Test agar (DST; Oxoid Ltd., Columbia, Md.). All mutants were identified as *E. cloacae* by biochemical testing (API 20 E; Sherwood Medical, Plainview, N.Y.).

Susceptibility testing. Antibiotic susceptibilities were determined by agar dilution (14), using DST agar. Inocula were prepared by diluting overnight broth cultures to 10^7 CFU/ml (Antibiotic Assay Broth no. 3, BBL Microbiology Systems, Cockeysville, Md.). Plates were inoculated with a multipoint inoculating unit delivering 1 μl (Denley Instrument Ltd., Sussex, England).

Induction of resistance was determined in whole cells by the double-disk diffusion method described by Waterworth and Emmerson (18).

Purification of β-lactamases. E2 β-lactamase was purified from *E. cloacae* SC 12629 (strain DR) by a procedure modified from that of Ross and Boulton (11). SC 12629 was grown in shake flasks overnight in 0.5% yeast extract. A volume of 400 ml was centrifuged, and the pelleted cells were washed once with 0.1 M phosphate buffer (pH 7.0). The cells were then suspended in 20 ml of phosphate buffer, sonicated for 3 min at 4°C, and centrifuged for 45 min at 14,000 × *g*, and the resulting supernatant was frozen over-

TABLE 1. Bacterial strains producing E2 β-lactamase

Strain	SC No.	Parent	Selection (μg/ml)	Isolation frequency
WT	12368			
DR	12629	12368	Aztreonam[a]	
Az^r	12975	12368	Aztreonam (50)	3.9×10^{-7}
Cf^r	12976	12368	Ceftazidime (100)	1.2×10^{-6}
Mx^r	12977	12368	Moxalactam (50)	1.7×10^{-7}
Az^rMx^r	12979	12977	Aztreonam (100)	1.4×10^{-7}

[a] Spontaneous mutant isolated from disk diffusion zone.

night. An 18-ml sample was loaded onto Sephadex G-75 (5.0 by 52 cm) and eluted with 50 mM phosphate buffer (pH 7.0). Active β-lactamase fractions were combined and concentrated by bringing the solution to 95% saturation with ammonium sulfate. The precipitated β-lactamase was dissolved in a minimal volume of deionized water and dialyzed at 4°C for 2 h against 4 liters of water and for 12 h against 2 liters of 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer (pH 8.0).

Dialyzed β-lactamase was then loaded onto QAE-Sephadex and eluted with the 20 mM TES buffer. β-Lactamase activity appeared in the void volume of the column. The fractions with the highest specific activity were combined and utilized for kinetic and inhibition studies. On isoelectric focusing, no β-lactamase activity could be visualized in the pH range 3.5 to 9.2 (LKB Ampholine PAGplate; pH 3.5 to 9.5), using SQ 24,902 as a chromogenic substrate. All β-lactamase activity appeared as a single band at the anode, indicating a pI greater than 9.5. When the gels were stained for protein with Coomassie brilliant blue, more than 70% of the total protein appeared in a band corresponding to the β-lactamase activity. This purity was confirmed during titration studies with aztreonam as inhibitor (see below). At a purity of 70%, the E2 β-lactamase employed for all kinetic and inhibition studies had a catalytic activity of 650 molecules of substrate hydrolyzed per molecule of enzyme per s with cephaloridine at 25°C.

P99 β-lactamase was purified to homogeneity from *E. cloacae* SC10435 (11). The β-lactamase preparation used for kinetic determinations had a catalytic activity of 845 molecules of substrate hydrolyzed per molecule of enzyme per s with cephaloridine at 25°C and exhibited a pI of 8.2 on isoelectric focusing (LKB Multiphor, LKB Instruments Inc., Rockville, Md.).

β-Lactamase induction. Cultures were grown overnight in shake flasks at 37°C (0.5% yeast extract broth). A 5% transfer was made to Davis medium (6), and inducer (2% [vol/vol]) was added after 2 h of growth to give inducer levels of 0.01 to 100 μg/ml as indicated. Two cultures were grown as controls for each experiment, with buffer added in place of inducer. After another 2 h, cells were harvested by centrifugation (10 min, 14,000 × *g*) and washed with 30 ml of 0.05 M phosphate buffer (pH 7.0) at 5°C. The cells were centrifuged as above, weighed to give a "wet weight of cells," and suspended in 5.0 ml of cold 0.05 M phosphate buffer (pH 7.0). After sonication for 2× 1.0 min at 0°C (Heat Systems Ultrasonics Sonicator, model W350), 50 μl of 1.0% sodium azide was added to the tubes, and the resulting suspension was centrifuged for 30 min at 28,000 × *g*. The supernatants were decanted and used for β-lactamase analyses. β-Lactamase activity was monitored periodically by using SQ 24,902 (0.04 mg/ml) as substrate. When inhibitory β-lactams were used as inducers, the clarified sonicated cell preparations were incubated overnight at 25°C to allow for complete turnover of inhibited β-lactamase. β-Lactamase activity was monitored at intervals of at least 60 min until two consecutive assays yielded the same specific activity (micromoles of substrate hydrolyzed per minute per milligram [wet weight] of cells). Replicate experiments yielded enzyme activities that varied 10% or less.

Kinetic studies. Hydrolysis of β-lactams was monitored spectrophotometrically (model 250 spectrophotometer; Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 25°C in 0.1 M phosphate buffer (pH 7.0) as described by Bush et al. (2). Purified β-lactamase preparations were used to obtain all kinetic constants. Half-times for deacylation were determined by incubating excess β-lactam with enzyme at 25°C in a volume of 50 to 100 μl; the recovery of β-lactamase activity was then monitored as a function of time by diluting 2 μl of incubation mixture into 1.0 ml of cephaloridine (1.0 mM) and following the initial rate of cephaloridine hydrolysis at 295 nm (2).

Inhibition studies with isolated enzymes. Inhibition studies were performed by incubating 2 μl of purified β-lactamase with 50 μl of inhibitor in a cuvette for 5.0 min at 25°C. After the addition of 1.0 ml of cephaloridine (1.0 mM), reaction rates were monitored spectrophotometrically for at least 30 s. Inhibition constants were determined from Dixon plots (3). With the exception of imipenem inhibition of E2 β-lactamase, all inhibitors exhibited significantly lower K_i values if enzyme and inhibitor were preincubated before substrate addition.

Inhibition of β-lactamase in whole cells. Inhibition of periplasmic β-lactamase in intact cells was determined as follows. Cultures were grown as described above for the induction studies. After 2 h of growth in Davis medium (6), either moxalactam or aztreonam was added as inhibitor (external concentration, 0.01 to 500 μg/ml). Incubation was continued at 37°C for 2 h to permit equilibration of antibiotic into the periplasm. Cells were harvested, washed, and sonicated as in the induction studies. All samples were maintained at 0 to 4°C until initial assays were completed. Initial assays for β-lactamase activity in clarified sonic extracts were performed 60 to 90 min after sonication. An automated Gilford 202 spectrophotometer was used to monitor hydrolysis rates of SQ 24,902 (two samples per min). No more than eight cultures were handled in each set to minimize the time involved between sonication and initial assay. After initial activities were determined, the sonicated cell preparation supernatants were incubated at 25°C until all residual bound β-lactam was hydrolyzed and enzyme activity reached a plateau. The amount of free β-lactamase in the whole cell was expressed as % active β-lactamase = [(initial activity after sonication)/(final activity after incubation)] × 100.

Each strain was analyzed at least twice with aztreonam at two concentration ranges spanning the level at which 50% active β-lactamase was observed in the initial assay. Variations of less than 20% between experiments were calculated.

β-Lactamase concentration determinations. *E. cloacae* SC 10435 was grown in 0.5% yeast extract broth overnight. Immediately before harvest, broth was removed for determination of cell counts per milliliter. (For other studies, these cell counts could also be correlated with the weight of wet packed cells from the same cultures.) Total P99 β-lactamase activity in sonicated cell preparations of these cultures was measured in triplicate by determining V_{max} with cephaloridine, using Lineweaver-Burk plots. The number of molecules of P99 β-lactamase could then be determined by using the catalytic constant (k_{cat}) for cephaloridine hydro-

TABLE 2. β-Lactamase levels and antimicrobial susceptibilities of selected *E. cloacae* strains

Strain	β-Lactamase Type	Basal level of β-lactamase (U)[a]	MIC (μg/ml)[b]			
			Aztreonam	Ceftazidime	Moxalactam	Imipenem
WT	E2	10	0.5	1.0	0.5	2.0
DR	E2	1,160	125	125	62.5	1.0
Az^r	E2	970	250	250	250	2.0
Cf^r	E2	1,000	125	500	62.5	1.0
Mx^r	E2	11	4.0	7.8	1,000	31.3
Az^rMx^r	E2	1,250	500	1,000	>1,000	62.5
SC 10435	P99	1,600	25	50	12.5	0.4

[a] One unit = 1 nmol of cephaloridine hydrolyzed per min per mg (wet weight) at V_{max}.
[b] Agar dilution; inoculum = 10^4 CFU per spot.

lysis at 25°C. A k_{cat} value of 1,000 molecules of cephaloridine hydrolyzed per s per molecule of enzyme was measured with highly purified P99 β-lactamase. The total number of molecules of P99 enzyme in crude sonicated cell preparations could then be correlated with the total number of cells in the sample to obtain an estimate of β-lactamase molecules per cell.

A similar procedure was followed for estimating β-lactamase concentrations in SC 12368 and its progeny.

Permeability ratio. Quantitation of permeability was determined by using variations of the methods of Zimmermann and Rosselet (19) and Kojo et al. (8, 9). Because aztreonam and moxalactam bind rapidly, stoichiometrically, and pseudo-irreversibly to the β-lactamases studied, short-term inhibition of periplasmic β-lactamase would serve as an indication of the amount of inhibitor that had gained access to the periplasmic space. Using the data obtained from the whole-cell studies of inhibited β-lactamase, "% active β-lactamase" was plotted against "inhibitor concentration." As a reference point, the inhibitor concentration giving 50% active enzyme at the time of initial assay was determined graphically. A theoretical curve was then drawn to predict the range of inhibitor concentrations required to inhibit that enzyme preparation from 10 to 90%, based upon the calculation of the number of molecules of β-lactamase within the cell, assuming no interference with penetration. The point at which 50% of the total β-lactamase activity should be inhibited theoretically (IC_{50}) was determined. A permeability ratio was then calculated: permeability ratio = (IC_{50} observed)/(IC_{50} predicted), where IC_{50} (observed) was the inhibitor concentration observed to initially inhibit 50% of the final β-lactamase activity in that sonic extract, and IC_{50} (predicted) was the inhibitor concentration predicted to inhibit 50% of the calculated number of β-lactamase molecules in that sonic extract. A permeability ratio of 1.0 would indicate that inhibitor was distributed equally on either side of the outer membrane.

RESULTS

Antimicrobial susceptibilities of various *E. cloacae* strains are shown in Table 2, together with the basal level of β-lactamase in these organisms. Among the mutants derived from strain WT, resistance to moxalactam was observed in all isolates; resistance to imipenem was less marked than that found with aztreonam or ceftazidime. Susceptibility to chloramphenicol and tetracycline was unchanged from that of strain WT in the β-lactam-resistant mutants.

No correlation could be made between resistance and the basal level of β-lactamase in these strains. Strains DR and Az^rMx^r, which produced equivalent levels of β-lactamase, required MICs that were at least eightfold different for both ceftazidime and moxalactam. Strain Mx^r, selected on the basis of its resistance to moxalactam, exhibited a β-lactamase content equivalent to that of its parent, WT, yet was more than eightfold more resistant to the β-lactam antibiotics studied. SC 10435, which produces P99 β-lactamase, contained the highest level of β-lactamase of all the organisms studied; however, MICs for the β-lactam antibiotics were lower than those for the derepressed SC 12368 (DR) mutants with less β-lactamase activity.

Inducibility of β-lactamase activity was studied in the presence of 0.01 to 100 μg of β-lactam antibiotic per ml. Strains Az^r and Az^rMx^r did not exhibit inducible β-lactamase activity when treated with either moxalactam or aztreonam. Apparent β-lactamase levels could be increased twofold in DR after exposure to any of the four β-lactam antibiotics at concentrations of 10 μg/ml or greater. However, induction of β-lactamase activity was most impressive in WT and Mx^r (Fig. 1). Imipenem and moxalactam were potent inducers of the E2 β-lactamase, whereas aztreonam caused no significant induction at antibiotic levels less than 100 μg/ml. No strain differences in induction profiles for WT and Mx^r were evident for aztreonam, ceftazidime, or imipenem at all levels studied. However, induction characteristics of β-lactamase activity by moxalactam were different in these strains, in that WT β-lactamase was induced by lower concentrations of moxalactam than enzyme in the moxalactam-resistant mutant, Mx^r.

To determine whether multiple forms of β-lactamase might be produced in these mutants, isoelectric focusing was performed with sonicated cell preparations of all induced and noninduced cultures. These studies revealed only a single β-lactamase activity identical to that observed in the purified β-lactamase preparation from DR.

Interactions between the β-lactam antibiotics and the two *Enterobacter* β-lactamases were studied (Table 3). None of these antibiotics was a good substrate for either β-lactamase, although hydrolysis of ceftazidime and imipenem proceeded at measurable rates. Both enzymes exhibited strong affinities for aztreonam and moxalactam. Titration of the E2 and P99 β-lactamases with aztreonam indicated full inhibition at a stoichiometry of 1.0 mol of aztreonam per mol of enzyme.

Because aztreonam remained covalently bound to the E2 and P99 β-lactamases with half-times for turnover exceeding 2 h at 25°C, this monobactam was used to determine how much external β-lactam was required to saturate the periplasmic β-lactamase in the various *E. cloacae* strains. The results from these experiments would then indicate how easily aztreonam could penetrate the outer membrane of these organisms. Aztreonam also was an excellent choice for this study because of its poor induction capability.

For these penetration studies, cultures were grown to a

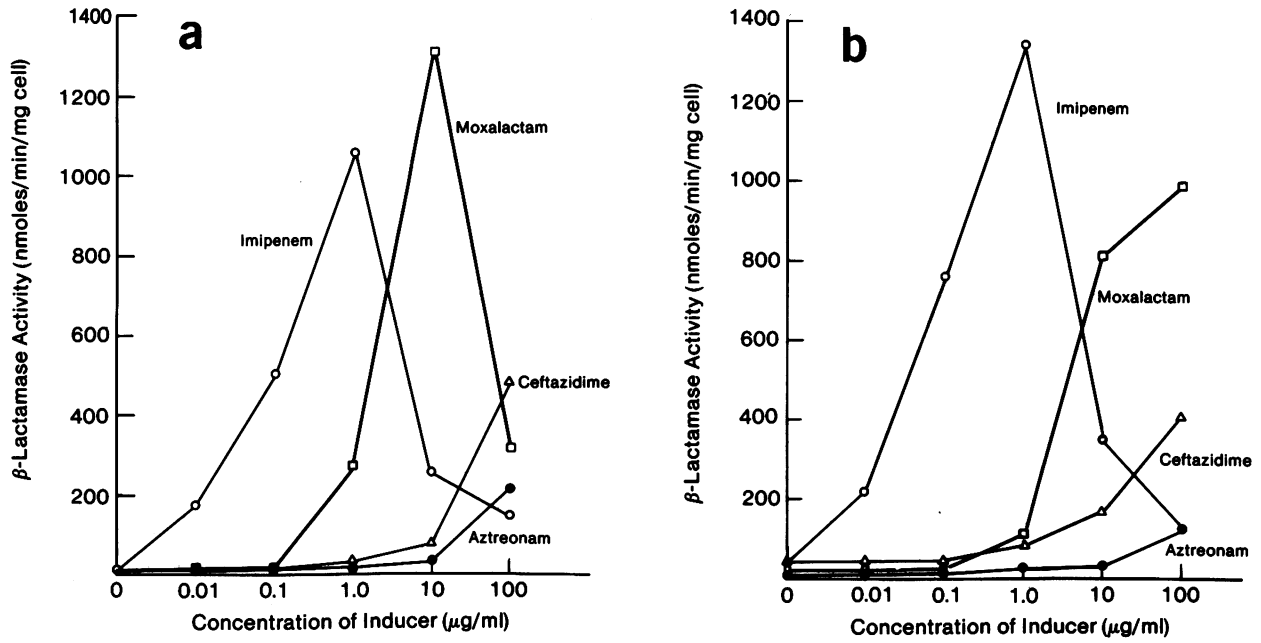

FIG. 1. (a) Induction of β-lactamase in strain WT. (b) Induction of β-lactamase in strain Mx^r. Inducers: aztreonam (●), ceftazidime (△), moxalactam (□), imipenem (○).

density of ca. 10^8 CFU/ml. By relating cell counts, total β-lactamase activity in sonicated cell preparations, and specific activity of purified β-lactamase, the number of enzyme molecules in a single cell could be estimated. SC 10435 produced 2.3×10^5 molecules of P99 β-lactamase per cell. For the wild-type strain WT, the basal level of E2 β-lactamase was 1,300 molecules per cell. (This number represents an upper limit for β-lactamase concentration in the WT strain. After maximal induction, β-lactamase would constitute as much as 4% of the total protein in WT sonicated cell preparations, with 1.8×10^5 molecules of β-lactamase per cell. These maximal values of β-lactamase concentrations agree well with the calculations of Vu and Nikaido [17], who reported 1.9×10^5 β-lactamase molecules per *E. cloacae* cell [3.7% total protein], using different methodology.) The total enzyme population in uninduced WT was thus ca. 10^{11} molecules per ml. Because aztreonam bound stoichiometrically to the E2 β-lactamase, under ideal conditions enzyme should have been fully inhibited when 10^{11} molecules of aztreonam per ml (0.08 ng/ml) were present—assuming unrestricted penetrability through the outer cell membrane and negligible binding to other cellular components.

Results of the penetrability experiments are shown in Fig. 2 for the two strains of *E. cloacae* that produced inducible E2 β-lactamase, for two of the mutants that possessed derepressed E2 enzyme, and for a strain of *E. cloacae* that produced derepressed P99 β-lactamase. In all organisms, a significant permeability barrier existed, as evidenced by the appearance of fully active β-lactamase at aztreonam concentrations at least 100 times that predicted for inhibitory activity. Although differences in permeability were observed between WT and Mx^r, strains producing comparable levels of β-lactamase, the greatest permeability barriers were present in Az^r and Az^{r}Mx^r, derepressed mutants of WT. The permeability factors for the latter mutants were much larger than for those strains containing inducible β-lactamases (Table 4). Moreover, all of the *Enterobacter* strains containing E2 β-lactamase exhibited permeability factors significantly higher than that for the P99-bearing SC 10435.

Similar experiments were conducted in which moxalactam was used as the inhibitor of E2 β-lactamase in whole cells. The results obtained were quite comparable to those observed with aztreonam, in that IC_{50} (observed) values were at least 1,000 times higher than predicted for strains containing inducible β-lactamase, and IC_{50} (observed) values were

TABLE 3. Kinetic parameters for *E. cloacae* β-lactamase with selected β-lactam antibiotics

Antibiotic	E2 β-lactamase				P99 β-lactamase			
	Relative V_{max}	$t_{1/2}$[a] (min)	K_m[b] (μM)	K_i[c] (μM)	Relative V_{max}	$t_{1/2}$ (min)	K_m (μM)	K_i (μM)
Cephaloridine	100	ND[d]	810	ND	100	ND	580	ND
Aztreonam	<0.0001	140	ND	0.0012	<0.0001	410	ND	0.0024
Ceftazidime	0.012	0.15	98	1.0	0.002	<0.1	20	3.0
Moxalactam	<0.0001	66	ND	0.10	<0.0001	210	ND	0.0051
Imipenem	0.002	0.8	2.3	2.7	0.0002	9	6.6	0.020

[a] Half-time for hydrolysis of β-lactam.
[b] K_m values were determined by using initial rates of hydrolysis.
[c] K_i values were measured after enzyme and inhibitor were preincubated for 5.0 min.
[d] ND, Not determined.

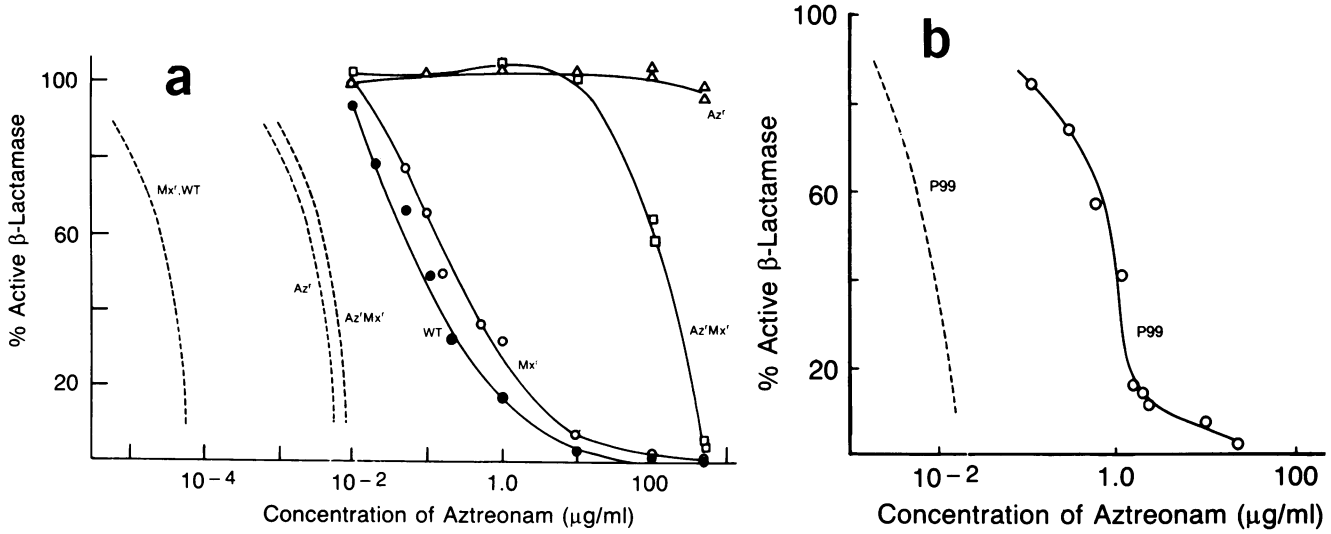

FIG. 2. Penetration of aztreonam into *E. cloacae*. After treatment of whole cells with aztreonam, β-lactamase activity was determined in fresh sonic extracts. Initial activity was then compared with the final activity observed after incubation of the sonic extract at 25°C. Dashed lines indicate theoretical values that were calculated assuming stoichiometric addition of aztreonam to β-lactamase in each strain, assuming no permeability barrier. (a) Symbols: ●, WT; ○, Mx^r; □, Az^rMx^r; △, Az^r. (b) Symbols: ○, SC 10435 (P99).

greater than 25,000 times those predicted for strains Az^r and Az^rMx^r producing derepressed E2 β-lactamase. Although these studies were complicated by the induction of enzyme in WT and Mx^r, the permeability factors calculated were within the same order of magnitude for both aztreonam and moxalactam.

DISCUSSION

If β-lactamase involvement is to be considered an important aspect in *Enterobacter* resistance, kinetic parameters for the various β-lactams must be evaluated. In the presence of E2 β-lactamase, the hydrolysis of ceftazidime and imipenem proceeded at slow but measurable rates. Thus, hydrolysis of these antibiotics may be responsible for the observed resistance of strain Az^rMx^r. Vu and Nikaido have also indicated that low hydrolysis rates may be important factors in the appearance of resistance of *E. cloacae* (17). However, the mutants Az^r and Cf^r with equivalent high levels of β-lactamase were both resistant to ceftazidime but susceptible to imipenem. Factors other than hydrolytic destruction must therefore be considered.

Aztreonam and moxalactam both bind tightly to the E2 and P99 β-lactamases. If a "trapping mechanism" were the only factor contributing to resistance, those strains containing essentially the same high concentrations of β-lactamase should bind the same amount of β-lactam and thus require the same increases in MIC. For the derepressed strains DR, Az^r, Cf^r, and Az^rMx^r with similarly high amounts of β-lactamase, it was calculated that antibiotic levels of 0.02 μg/ml or less would be sufficient to inhibit the periplasmic β-lactamase completely. However, these strains exhibited MICs ranging from 62.5 to greater than 1,000 μg/ml for moxalactam (increased from 0.5 μg/ml for WT).

Another argument against "trapping" was furnished by the inhibition studies with the aztreonam-resistant mutant Az^r. This strain attained its MIC of 250 μg/ml with no detectable inhibition of periplasmic β-lactamase by aztreonam. Therefore, resistance to aztreonam was not necessarily related to covalent binding of β-lactamase.

Poor correlation was observed between β-lactam susceptibility and amount of β-lactamase. In the *Enterobacter* strains studied above, SC 10435, with the highest β-lactamase level of all the organisms, exhibited MICs of an intermediate range, whereas strain Mx^r, containing an inducible low level of β-lactamase, exhibited an exceedingly high MIC of moxalactam. Similar results were reported by Farrar and Krause (4), who observed no relationship between β-lactamase activity and resistance of *Enterobacter* strains to cephalothin. In their strains, permeability barriers to cephalothin were described.

Permeability barriers were also implicated in this work as significant factors in the observed resistance patterns. Permeability differences were first suggested by the differential in induction potential of moxalactam between wild-type WT and the mutant Mx^r.

However, the most significant evidence for diminished permeability was provided in the inhibition studies in whole cells. In these studies, large excesses of β-lactam were required outside the cell before β-lactamase inside the cell could be inhibited. Based on the "permeability ratios" calculated, a series of permeability mutants could be identified, using both aztreonam and moxalactam as probes. Despite structural differences between these two molecules, penetrability into the periplasm was restricted in a similar manner.

It is tempting to suggest that this penetration defect may be related to a mutation that affects porin properties. At least two kinds of porins with different selectivities have been identified in *E. cloacae* (7). In *Pseudomonas aeruginosa*, loss of porin protein F resulted in a significant decrease in outer membrane permeability (10). However, further studies must be performed to determine whether a similar defect in *Enterobacter* would cause the observed resistance patterns described above.

Once β-lactam penetrated the outer membrane, the assumption was made that tight-binding β-lactamase inhibitors such as aztreonam and moxalactam would saturate the periplasmic β-lactamase before the antibiotics could reach the killing sites of the cell. However, in strain Az^r, killing levels of β-lactams were attained before the β-lactamase was inhibited. It is therefore possible that rates of penetration or rates of diffusion through the periplasm may differ for the

TABLE 4. Permeability barrier to aztreonam entry into *E. cloacae*

Strain	Aztreonam concn (ng/ml) required to inhibit 50% of β-lactamase		Permeability ratio (O/T)
	Theoretical (T)	Observed (O)	
WT	0.04	70	1,800
Mx^r	0.04	350	8,800
Az^r	3.9	>500,000	>125,000
Az^rMx^r	5.0	200,000	40,000
SC 10435	8.0	830	104

various mutants. Other possible contributions to resistance may include alterations in penicillin binding proteins, either in number or in affinities for the β-lactam in question. A smaller number of target sites or altered affinity for β-lactam by penicillin binding proteins may also be involved in resistance. Although the situation leading to bacterial death is complex, the primary event in the process is entry of the β-lactam into the cell.

In conclusion, development of resistance in selected *E. cloacae* strains could not be directly correlated with β-lactamase concentrations, with induction potential, or with affinity of binding of antibiotic to β-lactamase. Resistance to ceftazidime and imipenem may be related to slow hydrolysis of these molecules. However, decreased penetrability of the β-lactams into resistant strains was shown to be the major factor in decreased susceptibility.

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