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Role of β-Lactamases and Outer Membrane Proteins in Multiple β-Lactam Resistance of *Enterobacter cloacae*

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The chromosomal β-lactamase and outer membrane proteins of *Enterobacter cloacae* were examined to determine their relative contributions to multiple antibiotic resistance in this organism. Mutants altered in β-lactamase expression, whether derived in the laboratory or recovered from patients treated with one of the new β-lactam antibiotics, were found to have no detectable alterations in outer membrane proteins. Derepression of β-lactamase in these mutants was associated with high-level resistance to multiple β-lactam antibiotics, while loss of inducible β-lactamase (i.e., production of basal enzyme levels only) was associated with acquisition of susceptibility to many β-lactam antibiotics, including cephalothin. In contrast, alteration in outer membrane proteins was associated with only moderate-level resistance to β-lactam antibiotics. However, this included resistance to such drugs as amdinocillin and Sch 34343, which were unaffected by derepression of β-lactamase. Resistance to chloramphenicol and tetracycline also accompanied changes in outer membrane proteins. Although the outer membrane proteins of various strains of *E. cloacae* were similar, there did appear to be some major strain-to-strain variations. Thus, it appears that alterations in both β-lactamase and outer membrane proteins can affect the susceptibility of *E. cloacae* to many antibiotics. However, alterations in β-lactamase alone are sufficient to produce high-level multiple β-lactam resistance in this organism.

Mutants of *Enterobacter* spp. producing high levels of β-lactamase have been shown to be responsible for a number of clinical failures associated with emergence of resistance during therapy with the newer expanded-spectrum β-lactam antibiotics (16). These mutants occur spontaneously at a frequency of 10^{-6} to 10^{-7} and appear to have lost control of β-lactamase production (8, 10). Normally, production of the chromosomally mediated cephalosporinase of *Enterobacter* spp. is under repressor control (8). Loss of this normal control mechanism via mutation leads to high-level β-lactamase production. This event is associated with resistance to multiple β-lactam antibiotics (8, 10, 14, 16). This resistance has been difficult to explain solely on the basis of β-lactamase-mediated hydrolysis because it involves many drugs that appear to be poor substrates for the enzyme. Thus, it has been speculated that this resistance must also involve a change in permeability.

The permeability of gram-negative organisms to β-lactam antibiotics is determined primarily by outer membrane proteins (13). Studies by Sawai et al. (17) and Kaneko et al. (9) indicate that in *Enterobacter cloacae* two major outer membrane proteins appear to be involved in cephalosporin permeation. Alterations in these proteins have been shown to be associated with multiple β-lactam resistance in this organism (17). Since changes in β-lactamase expression and outer membrane proteins can be responsible for multiple β-lactam resistance, this investigation was designed to examine both these factors in *E. cloacae*.

MATERIALS AND METHODS

Strains. Four sets of wild-type/mutant (W/M) pairs of *E. cloacae* were evaluated in this study (Table 1). Two sets were laboratory derived (55W/55M and 84W/84M) and have been described in detail previously (8). The other two (91W/91M and 106W/106M) were pre- and posttherapy isolates from patients who were treated with one of the newer β-lactam antibiotics. Strain identity for these pairs was confirmed by determining their biotype in two systems (API 20E [Analytab Products, Plainview, N.Y.] and Enterotube II [Hoffman-La Roche, Inc., Nutley, N.J.]) and by plasmid profiles (5).

A mutant producing low levels of β-lactamase (55M-L) was derived from isolate 55M with nitrosoguanidine (4). Cells were treated with 500 μg of nitrosoguanidine per ml and then plated onto Mueller-Hinton agar. They were then replicate plated onto Mueller-Hinton agar containing 50 μg of cefotaxime per ml. Mutant 55M-L was detected as a colony unable to grow on the cefotaxime agar. A mutant altered in outer membrane proteins (55M-LP) was derived from 55M-L by exposing the strain to 32 μg of cefoxitin per ml in Mueller-Hinton broth for 24 h. A second mutant altered in outer membrane proteins (P99⁻-P) was derived from isolate P99⁻ (6), an *E. cloacae* strain possessing no detectable β-lactamase activity in whole-cell assays, by exposing the strain to 8 μg of cefoxitin per ml in Mueller-Hinton broth for 24 h.

Susceptibility tests. Antibiotic susceptibility tests were performed by serial twofold agar dilution tests in Mueller-Hinton agar. Inocula of 10^5 CFU per spot were applied with a Steers replicator (18). The MIC was defined as the lowest concentration preventing growth after incubation for 18 to 24 h at 35°C in air. The presence of three colonies or fewer was ignored.

β-Lactamase assays. Spectrophotometric assays utilizing whole cells or cell-free sonic extracts were done with 100 μM of cephalothin or cefamandole as the substrate (8). β-Lactamases were induced by growing the strains on Mueller-Hinton agar containing subinhibitory concentrations of cefoxitin (15).

Assay of outer membrane proteins. Outer membrane proteins were analyzed by vertical slab gel electrophoresis as described by Lugtenberg et al. (11). Cell envelopes were prepared from overnight cultures in antibiotic medium 3 (Difco Laboratories, Detroit, Mich.) by the method of Ames

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TABLE 1. Isolates of *E. cloacae* used

Isolate designation	Distinguishing feature	Source or reference
55W	Wild type, clinical isolate	8
55M	Mutant of 55W derepressed for β-lactamase	
84W	Wild type, clinical isolate	8
84M	Mutant of 84W derepressed for β-lactamase	
91W	Wild type, pre-cefamandole therapy	This study
91M	Mutant of 91W derepressed for β-lactamase, post-cefamandole therapy	
106W	Wild type, pre-aztreonam therapy	3
106M	Mutant of 106W derepressed for β-lactamase, post-aztreonam therapy	
55M-L	Mutant of 55M, low-level β-lactamase producer	This study
55M-LP	Mutant of 55M-L altered in outer membrane proteins	
P99$^-$	β-Lactamase-free mutant	6
P99$^-$-P	Mutant of P99$^-$ altered in outer membrane proteins	This study

TABLE 2. β-Lactamase activity in *E. cloacae* isolates

Isolate	Assay[a]	Cephalothin		Cefamandole	
		Uninduced	Induced	Uninduced	Induced
91W	Whole cell	68	85	10	51
91M		84	84	61	62
106W	Whole cell	100	100	0	60
106M		100	100	64	67
84W	Whole cell	100	100	16	72
84M		100	100	84	83
55W	Whole cell	39	67	0	67
55M		75	77	69	60
55M-L		30	38	0	0
55M-LP		7	14	0	0
55W	Cell free	46			
55M		6,140			
55M-L		51			
55M-LP		54			

[a] Results from whole-cell assays are expressed as nanomoles hydrolyzed per 10^9 cells. Whole-cell assays were incubated for 4 h except in tests with *E. cloacae* 55 isolates, in which assays with cephalothin were incubated for only 1 h. Results from cell-free assays are expressed as nanomoles hydrolyzed per 20 min per milligram of protein. Differences of more than 10 nmol are considered significant.

(1) as modified by Bavoil et al. (2). Purified outer membrane preparations were obtained by treatment of the cell envelopes with sodium N-lauroyl sarcosinate (17). A 25-μl sample (ca. 10 μg of protein) of each outer membrane preparation was applied to gels (0.7-mm thick; 15 by 15 mm) and electrophoresed at 30 mV until the dye front reached the bottom of the gel. Protein bands were stained with Coomassie blue.

RESULTS

The influence of derepression of β-lactamase upon antibiotic resistance was examined in four W/M pairs of *E. cloacae*. Each wild type possessed an inducible cephalosporinase, and its mutant was stably derepressed for β-lactamase production (Table 2). The contribution of outer membrane proteins to antibiotic resistance in *E. cloacae* was examined in a series of mutants possessing only low levels of β-lactamase. This approach was taken because inducible or high levels of β-lactamase were likely to mask susceptibility changes due to altered outer membrane proteins. Thus, *E. cloacae* 55M-L, a low-level β-lactamase producer, was obtained by treating *E. cloacae* 55M with nitrosoguanidine. *E. cloacae* 55M-LP was then obtained from *E. cloacae* 55M-L with cefoxitin.

The β-lactamase activity in *E. cloacae* 55W and its various mutants was examined initially in whole-cell assays (Table 2). When cephalothin was used as the substrate, the time of incubation was decreased to 1 h to allow examination of the substrate before complete hydrolysis. *E. cloacae* 55M-L produced uninducible, low levels of cephalosporinase (Table 2). This activity was similar to that observed for *E. cloacae* 55W before induction. β-Lactamase activity in *E. cloacae* 55M-LP appeared to be lower than in *E. cloacae* 55M-L. *E. cloacae* 55M-LP was ultimately found to possess altered outer membrane proteins (see below), and the decreased β-lactamase level could have been due to technical problems inherent in a whole-cell assay with such a mutant. β-Lactamase assays were therefore repeated with cell-free sonic extracts. Uninduced base-line levels of β-lactamase were similar for *E. cloacae* 55W, *E. cloacae* 55M-L, and *E. cloacae* 55M-LP in these assays (Table 2).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the outer membrane proteins of *E. cloacae* 91W and 91M (Fig. 1, lanes 1 and 2) and *E. cloacae* 106W and 106M (lanes 3 and 5) showed very little difference between the wild types and their derepressed mutants. Each isolate possessed two major proteins between 35,000 and 38,000 molecular weight and several minor proteins between 40,000 and 42,000 molecular weight. *E. cloacae* 84W differed from *E. cloacae* 84M in the intensity of several major protein bands: the 36,500-molecular-weight protein was more intense and the 38,000-molecular-weight protein was less intense in *E. cloacae* 84W. *E. cloacae* 55W, *E. cloacae* 55M, and *E. cloacae* 55M-L possessed three major outer membrane

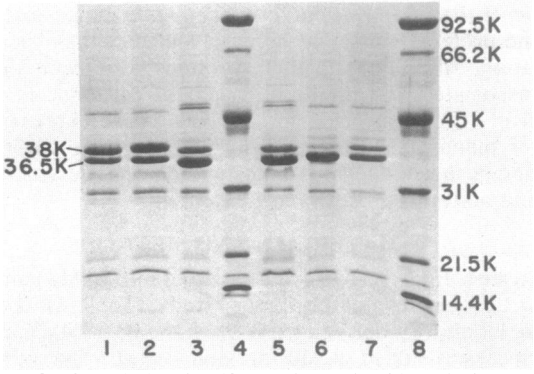

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins of *E. cloacae* 91W (lane 1), 91M (lane 2), 106W (lane 3), 106M (lane 5), 84W (lane 6), and 84M (lane 7). Molecular weight standards are in lanes 4 and 8. 38K, 38,000 molecular weight.

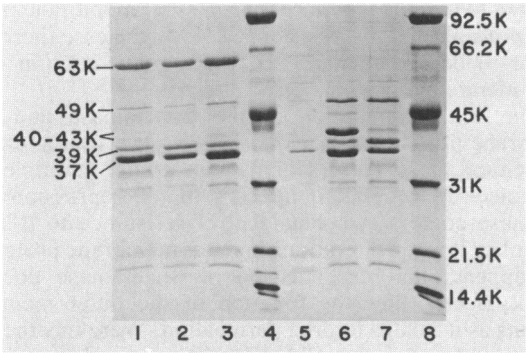

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins of *E. cloacae* 55W (lane 1), 55M (lane 2), 55M-L (lane 3), 55M-LP (lane 5), P99⁻ (lane 6), and P99⁻-P (lane 7). Molecular weight standards are in lanes 4 and 8. 63K, 63,000 molecular weight.

proteins of 63,000, 39,000, and 37,000 molecular weight (Fig. 2). All three proteins were significantly decreased in *E. cloacae* 55M-LP (lane 5). Minor proteins in the 40,000- to 43,000- and 49,000-molecular-weight regions were also diminished. The two major proteins in *E. cloacae* P99⁻ with molecular weights of 37,000 and 43,000 (Fig. 2, lane 6) were diminished in *E. cloacae* P99⁻-P, while the 39,000-molecular-weight protein was slightly increased (Fig. 2, lane 7).

The susceptibility of these *E. cloacae* strains to β-lactam antibiotics, aminoglycoside antibiotics, chloramphenicol, and tetracycline was determined in agar dilution tests. The mutants derepressed for β-lactamase were significantly less susceptible to the penicillins, cephalosporins, and aztreonam than their wild types (Table 3). The mutants showed no change in susceptibility to amdinocillin, Sch 34343, imipenem, chloramphenicol, tetracycline, or aminoglycoside antibiotics (data not shown). *E. cloacae* 55M-LP and *E. cloacae* P99⁻-P, two mutants with altered outer membrane proteins, were significantly less susceptible to penicillins, cephalosporins, aztreonam, amdinocillin, Sch 34343, chloramphenicol, and tetracycline than were their parental strains (*E. cloacae* 55M-L and *E. cloacae* P99⁻). They showed no change in susceptibility to imipenem or the aminoglycoside antibiotics (data not shown). The presence of basal levels only of uninducible β-lactamase in *E. cloacae* 55M-L was associated with an increased susceptibility to cephalothin and cefoxitin.

DISCUSSION

In *E. cloacae*, both β-lactamase and outer membrane proteins are significant determinants of the antibiotic susceptibility of the organism. Alterations in β-lactamase affect susceptibility to β-lactam antibiotics only, whereas alterations in outer membrane proteins affect susceptibility to a variety of unrelated antibiotics. As demonstrated in this study, the relative contribution of these two factors can be assessed only by analyzing them separately.

The role of β-lactamase in the antibiotic susceptibility of *E. cloacae* is most clearly illustrated by results obtained in tests with *E. cloacae* 55W, 55M, and 55M-L. In these isolates, outer membrane proteins were the same, but expression of β-lactamase differed. In comparison of *E. cloacae* 55W and 55M-L, it appeared that basal, uninducible levels of β-lactamase (55M-L) were not sufficient to confer resistance to most β-lactam antibiotics, although a moderate level of resistance to cephalothin could be demonstrated. The addition of an inducible enzyme system (55W) to basal enzyme levels produced high-level resistance to cephalothin and cefoxitin but not to other β-lactam antibiotics. The resistance to cephalothin probably results from the very high lability of this drug to the chromosomal cephalosporinase of *E. cloacae*. Thus, although cephalothin is a poor inducer of the enzyme (7, 12), even slightly elevated levels could confer high-level resistance to the drug. Cefoxitin, on the other hand, is an excellent inducer of the *E. cloacae* β-lactamase (7, 15). Thus, the high-level resistance to cefoxitin in *E. cloacae* 55W probably results from extensive induction of β-lactamase by the drug itself. The lack of an effect of this inducible β-lactamase system upon the activity of other β-lactam antibiotics most likely results either because they are poor enzyme inducers (12, 15), with β-lactamase remaining at basal levels, or because the concentration required for induction exceeds the concentration required for their antibacterial effect.

The major contribution of the *E. cloacae* β-lactamase to antibiotic resistance can be seen when the inducible system undergoes stable derepression (55M). Once this occurs,

TABLE 3. Antibiotic susceptibility of *E. cloacae* isolates

Isolate No.	MIC (μg/ml)												
	Cephalothin	Cefamandole	Cefoxitin	Cefotaxime	Moxalactam	Carbenicillin	Mezlocillin	Aztreonam	Amdinocillin	Sch 34343	Imipenem	Chloramphenicol	Tetracycline
91W	>128.0	4.0	>128.0	0.5	0.12	8.0	4.0	0.12	0.12	1.0	0.25	16.0	4.0
91M	>128.0	128.0	>128.0	32.0	4.0	128.0	16.0	16.0	0.25	1.0	0.12	16.0	4.0
106W	>128.0	1.0	>128.0	0.5	≤0.06	4.0	2.0	0.12	0.12	0.5	0.25	32.0	2.0
106M	>128.0	>128.0	>128.0	64.0	4.0	64.0	64.0	8.0	0.25	0.5	0.12	32.0	2.0
84W	>128.0	4.0	>128.0	0.12	0.12	16.0	4.0	4.0	0.25	2.0	0.25	8.0	>128.0
84M	>128.0	>128.0	>128.0	128.0	16.0	>128.0	128.0	128.0	0.25	2.0	0.25	8.0	>128.0
55W	>128.0	4.0	128.0	0.25	0.12	8.0	4.0	≤0.06	0.5	1.0	1.0	8.0	4.0
55M	>128.0	>128.0	>128.0	>128.0	16.0	>128.0	>128.0	16.0	0.25	0.5	0.5	4.0	8.0
55M-L	16.0	2.0	4.0	0.12	0.12	4.0	4.0	≤0.06	0.25	0.25	0.5	4.0	4.0
55M-LP	>128.0	64.0	>128.0	4.0	4.0	64.0	64.0	0.5	8.0	4.0	0.5	32.0	32.0
P99⁻	4.0	2.0	8.0	≤0.06	0.12	4.0	4.0	≤0.06	0.12	1.0	1.0	4.0	1.0
P99⁻-P	64.0	32.0	128.0	1.0	1.0	32.0	16.0	0.12	0.5	4.0	0.5	64.0	8.0

high-level resistance to many diverse β-lactam antibiotics results. The importance of derepression of β-lactamase to high-level, multiple β-lactam resistance was emphasized further in comparisons between *E. cloacae* 91W and *E. cloacae* 106W, two clinical isolates, and their stably derepressed mutants which arose during therapy with newer β-lactam antibiotics. These isolates and their mutants showed no differences in outer membrane proteins, indicating again that the resistance was mediated by the β-lactamase.

The results obtained with *E. cloacae* 55W, 55M, and 55M-L suggest that the genetic region governing the chromosomal β-lactamase of this organism is more complex than originally speculated (8). Rather than a single control region and single structural gene, there may in fact be two structural genes, only one of which is under repressor control. Such an arrangement would explain how mutagenesis of *E. cloacae* 55M, an isolate derepressed for β-lactamase, produced *E. cloacae* 55M-L, an isolate possessing only basal, uninducible β-lactamase. Alternatively, *E. cloacae* 55M-L could have resulted from mutations in the regulatory region and a single structural gene. Clearly, additional work will be required to fully determine the complexity of the genetic region governing β-lactamase production in *E. cloacae*.

The role of outer membrane proteins in the antibiotic susceptibility of *E. cloacae* is most clearly illustrated by results obtained in tests with *E. cloacae* 55M-L and 55M-LP and *E. cloacae* P99$^-$ and P99$^-$-P. In contrast to the β-lactamase-mediated resistance, alterations in outer membrane proteins produced a moderate level of resistance to diverse β-lactam antibiotics, including amdinocillin and Sch 34343, and to chloramphenicol and tetracycline. This cross-resistance between β-lactams, chloramphenicol, and tetracycline mediated by altered outer membrane proteins in *E. cloacae* was reported by Sawai et al. (17). However, the precise outer membrane proteins responsible for this resistance may vary from strain to strain.

Studies by Sawai et al. (17) with a single strain of *E. cloacae* (206) suggested that two outer membrane proteins of molecular weights 37,000 and 39,000 to 40,000 were involved in permeation of cephalosporins, tetracycline, and chloramphenicol. The 39,000- to 40,000-molecular-weight protein was of greater importance to cephalosporin permeation than the 37,000-molecular-weight protein. Subsequent studies by Kaneko et al. (9) with *E. cloacae* 206 confirmed these outer membrane proteins to be porins. However, a comparison of the outer membrane protein patterns of the five strains of *E. cloacae* studied here (Fig. 1 and 2) and of strain 206 studied by Sawai et al. (17) suggests that there may be discrete variations in outer membrane proteins of *E. cloacae* that may be involved in the permeation of β-lactams, chloramphenicol, and tetracycline. All five strains studied here (*E. cloacae* 91W, 106W, 84W, 55W, and P99$^-$) appear to possess outer membrane proteins of 35,000 to 37,000 and 37,000 to 39,000 molecular weight; apparent molecular weights for similar proteins from strain 206 were 35,000 and 37,000, respectively. In neither study were alterations in these outer membrane proteins alone associated with increased antibiotic resistance. Only *E. cloacae* P99$^-$ possessed a major protein (40,000 to 43,000 molecular weight) similar to that of *E. cloacae* 206 (39,000 to 40,000 molecular-weight). In both these strains, diminution of this protein was associated with increased antibiotic resistance. In addition, we recorded a 63,000-molecular-weight outer membrane protein that was not observed by Sawai et al. Since the antibiotic resistance of *E. cloacae* 55M-LP was associated with decreases in multiple outer membrane proteins, further studies will be required to identify which change in outer membrane protein is responsible for the resistance. Nevertheless, there does appear to be considerable strain-to-strain variation in the outer membrane proteins of *E. cloacae*.

From this examination of the β-lactamase and outer membrane proteins of *E. cloacae*, it is clear that alterations in each of these factors alone can confer multiple drug resistance. In general, it appears that derepression of β-lactamase confers a higher level of resistance to β-lactam antibiotics than do alterations in outer membrane proteins. It also appears that derepression of β-lactamase does not phenotypically alter the function of the outer membrane proteins as it relates to drug permeation. Were this the case, derepressed mutants should show an increased resistance to amdinocillin, Sch 34343, chloramphenicol, and tetracycline similar to those observed for mutants with altered outer membrane proteins. The interplay between alterations in β-lactamase and outer membrane proteins was not examined in this study. Whether alterations in both factors would amplify the resistance produced by alterations in one remains to be studied. However, it is clear that each factor alone significantly influences the antibiotic susceptibility of *E. cloacae*.

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