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Mutational Enzymatic Resistance of *Enterobacter* Species to Beta-Lactam Antibiotics

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Mutants with enhanced β-lactam resistance were selected from strains of *Enterobacter cloacae* and *E. aerogenes* by using three antibiotics. High-level β-lactamase-producing mutants had similar degrees of increased resistance, enzyme substrate profiles, and isoelectric (pI) values irrespective of the selective agent. Reverse mutants from a resistant *E. cloacae* mutant regained the susceptibility pattern originally exhibited by the wild type, or were of enhanced susceptibility, and no longer expressed increased β-lactamase production. β-Lactamases of the mutants were similar in pI values to the wild-type enzyme. The increased resistance of the mutants therefore appeared to be accounted for by increased β-lactamase production.

Previous studies from this laboratory (4, 10) and others (18) have shown that cefamandole- and ampicillin-resistant mutants of *Enterobacter* species occur at a frequency as great as 10^{-5}. Two classes of resistant mutants were selected by the antibiotics, one of which had markedly enhanced ability to inactivate the antibiotic in vitro. In our studies, the proportions of these two classes of mutants were approximately equal. We suggested that cefamandole resistance of the mutants with increased β-lactamase production might be explained by a mutational change involving the enzyme which extended its spectrum of activity to include cefamandole. We further demonstrated cross-resistance between ampicillin and cefamandole in strains with enhanced enzyme production. Ott et al. (18) reported a lack of correlation between β-lactamase production and susceptibility to cefamandole, found no qualitative differences between the substrate specificities of enzymes from a wild-type *Enterobacter* strain and its enhanced enzyme-producing mutant, and concluded that these mutants may have owed their resistance to genetic determinants other than those coding for β-lactamase production.

This study focused exclusively on mutants with enhanced β-lactamase production and was designed to resolve these differences in interpretation. It involved determining whether enhanced enzyme-producing mutants selected with different β-lactam antibiotics were similar, whether the β-lactamases of mutants differed from each other and from those of the wild type, and whether the enhanced β-lactamase production explained the increased resistance. The results support all of these hypotheses.

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MATERIALS AND METHODS

Bacterial strains. *E. aerogenes* 2046 and *E. cloacae* 2016 used in this study were previously described (10) and were originally derived from clinical material. Stocks of these strains and of their resistant mutants were held in equal parts of fetal calf serum and Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at −70°C.

Antibiotics. Cefamandole and cephaloridine were kindly provided by the Eli Lilly & Co. (Indianapolis, Ind.). Other antibiotics (ampicillin, carbenicillin, cephalothin, and penicillin G) were purchased from the University Hospital pharmacy.

Antibiotic susceptibility tests. Disk diffusion tests were performed according to the standardized single-disk test (3). Agar dilution susceptibility tests were performed according to the International Collaborative Study protocol (2), except that Isosensitest (Oxoid, Ltd., Basingstoke, Hampshire, England) medium solidified with 1.5% agarose (Calbiochem, La Jolla, Calif) was used.

Selection of resistant mutants. Between 10^7 and 10^9 bacteria were added to approximately 25 ml of melted nutrient agar containing antibiotic. With ampicillin and cefamandole, the antibiotic concentration was 128 μg/ml. With carbenicillin, it was 32 μg/ml. Isosensitest medium solidified with 1.7% agarose was used with ampicillin, and Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) was used with carbenicillin and cefamandole. Colonies that developed within 48 h were picked and streaked on antibiotic-free medium, and single-colony isolates from this source were tested for resistance to the selecting antibiotic and for β-lactamase production.

Selection of reverse mutants. Overnight cultures of high-level β-lactamase-producing mutants grown in

the absence of antibiotic were centrifuged at 1,350 × *g* for 15 min. The pellet was suspended in T2 buffer (13), an isotonic balanced salt phosphate buffer, to a density equivalent to a 0.5 McFarland turbidity standard (3), which corresponded to approximately 10^8 bacteria per ml. A 3-ml volume was transferred to a glass petri dish bottom and, while being gently agitated, was irradiated by a UV lamp (General Electric G8T5, 8 W) at a distance of 50 cm for 40 to 75 s. This resulted in approximately 99% loss of viability. Samples were then diluted 2 × 10^{-4}, and 0.1-ml amounts of this dilution were spread over the surface of a Trypticase soy agar plate. These procedures, and subsequent incubation, were performed in a darkened room. After overnight incubation at 35°C, a mixture of 4 ml of molten nutrient agar, 1 ml of a solution of 10^6 Units of potassium penicillin G per ml, and 1 ml of 4% Andrades indicator (11) was poured over the colonies. Colonies producing high levels of β-lactamase turned bright pink immediately. Colonies that did not were subcultured immediately to Trypticase soy agar for susceptibility testing and determination of β-lactamase production.

Preparation of cell-free extracts. Cultures were grown in 50 ml of brain heart infusion broth in a shaking water bath at 37°C. For some experiments involving possible β-lactamase induction, benzylpenicillin (50 μg/ml) was added after 1 and 4 h of incubation according to the protocol recommended for *Enterobacter* species (19). Cultures thus treated were described as induced. After overnight incubation, the culture was centrifuged for 15 min at 5,000 × *g*, and the supernatant fluid was discarded. The sediment was washed once in 0.01 M phosphate buffer, pH 7, and finally resuspended in 7 ml of the same buffer. The cells were disrupted in an ice bath with a Sonifier (Branson Sonic Power Co., Long Island, N.Y.) for five 45-s bursts. Cellular debris was removed by centrifugation at 18,000 × *g* for 30 min. Protein content was determined by the procedure of Lowry et al. (12). This material will be referred to as crude enzyme preparation.

Determination of β-lactamase activity (i) Qualitative tests. Antibiotic inactivation screening was performed by the Kjellander and Myrback "cloverleaf" modification of the Gotts test (9). β-Lactamase activity was tested for directly with the chromogenic cephalosporin nitrocefin (Glaxco Research Ltd., Greenford, Middlesex, England) (16).

(ii) Assays for hydrolysis of β-lactam antibiotics. The spectrophotometric assay of O'Callaghan et al. (17) was used to determine the rate of hydrolysis of cephaloridine and cefamandole by β-lactamases. Antibiotic stock solutions were diluted to give final concentrations of 100 μM in 1.25 ml of 0.1 M phosphate buffer (pH 7.0). Reactions were started by the addition of 50 μl of crude enzyme preparation, and the decrease in absorbance was measured at 37°C in a recording spectrophotometer (Gilford Instruments, Oberlin, Ohio). Wavelengths used were 255 nm for cephaloridine and 270 nm for cefamandole. Substrate and buffer blanks were included in each experiment. Units of β-lactamase activity, expressed as moles of antibiotic hydrolyzed per minute, were calculated according to the formula given by Ross and O'Callaghan (19).

Inhibition of β-lactamase hydrolysis of cephaloridine by *p*-chloromercuribenzoate (PCMB) or β-lactam antibiotics was determined by a modification of the method described by Jack and Richmond (6). Equal volumes of 1.0 mM PCMB and enzyme were preincubated 10 min at 0°C. Then 20 μl of the mixture was added to 1.25 ml of the cephaloridine substrate and assayed as described above. A control experiment was carried out with the enzyme in the absence of PCMB.

In antibiotic inhibition studies with ampicillin, carbenicillin, dicloxacillin, methicillin, or oxacillin, equal volumes of cephaloridine (200 μM) and inhibitor (200 μM) in 0.01 M phosphate buffer, pH 7.0, were incubated for 5 min to record any spontaneous hydrolysis, and the reaction was started by the addition of 50 μl of crude enzyme preparation. Cephaloridine hydrolysis was measured as described above.

Isoelectric focusing of cell-free extracts. Isoelectric focusing was carried out at 4°C in an LKB Multiphor 2117 (LKB Instruments, Inc., Rockville, Md.) equipped with an LKB 2103 power supply and cooled with a Lauda K-2/RD circulator as previously described (1). Gels of pH 7.8 to 10 were prepared as recommended by the manufacturer (LKB application note no. 250) and used immediately after the gels had set. Samples (25 to 50 μl) of the crude enzyme preparations, each containing 250 μg of protein, were applied to the gel surface on Whatman GF/A wicks (0.5 by 1.0 cm) and were centered at 3.5 cm from the edge of the anode and 6.1 cm from the cathode. The samples were focused for 3 h at constant power (25 W). Upon completion of the run, gels were marked at 1.0-cm intervals with a punch. The pH was measured at 4°C with a Beckman phase 1 digital pH meter equipped with an Ingold surface electrode (model 6122). The instrument had been standardized at 4°C with pH 7.0 and 10.0 buffers.

β-Lactamase bands were visualized with a 2% agarose overlay containing 250 or 500 μg of nitrocefin per ml. Bands of β-lactamase activity were easily detected, although some diffusion of color change occurred as the reaction developed. Parallel gels were stained for protein with Coomassie brilliant blue R-250 as previously described (1).

RESULTS

Resistant mutants with enhanced β-lactamase activity and reverse mutants. Stable resistant mutants were selected from two *Enterobacter* strains with ampicillin, carbenicillin, or cefamandole as described in Materials and Methods. Under the conditions used, mutant frequencies ranged from 1.5 × 10^{-6} to 5.5 × 10^{-8}. Approximately half of the mutants showed enhanced β-lactamase activity by the nitrocefin and modified Gotts procedures, and several were selected for study. Reverse mutants with little or no β-lactamase activity were selected from M2016 Am4, a resistant mutant of *E. cloacae* 2016 selected with ampicillin. The frequency of their detection in relation to the original irradiated suspension was approximately 10^{-8}, but, because of the technical difficulty of selecting them, this figure cannot be equated to the frequency of their occurrence in a culture. Enzyme-producing mutants were reisolated from

TABLE 1. Agar dilution minimal inhibitory concentrations (MICs) of parent strains and resistant mutants

Bacterial strains and mutants	Antibiotic used for mutant selection	MIC (μg/ml) of:			
		Ampicillin	Carbenicillin	Cephalothin	Cefamandole
E. cloacae 2016		16	≤4	≤32	≤2
M2016 Am4	Ampicillin	512	128	≥2,048	128
M2016 Cb1	Carbenicillin	1,024	128	≥2,048	128
M2016 Cfm8	Cefamandole	512	64	≥2,048	256
E. aerogenes 2046		64	≤4	≤32	≤2
M2046 Am2	Ampicillin	1,024	16	≥2,048	32
M2046 Cb1	Carbenicillin	1,024	32	≥2,048	64
M2046 Cfm29	Cefamandole	1,024	32	≥2,048	64

these susceptible clones by incubation in the presence of antibiotic.

Antibiotic susceptibilities of wild-type strains of *Enterobacter* and of their mutants. The original wild-type strains of *Enterobacter* were susceptible to a variety of non-β-lactam antibiotics when tested by the standardized disk diffusion method on Mueller-Hinton agar, but were resistant to ampicillin and cephalothin. Mutants selected with ampicillin, carbenicillin, or cefamandole showed diffusion test susceptibility patterns identical to those of the wild types with the non-β-lactam antibiotics, but all had become resistant to carbenicillin and cefamandole and showed greater resistance than the wild types to ampicillin and cephalothin when measured by quantitative susceptibility tests (Table 1). The degree of resistance of the mutants to each of the four antibiotics appeared to be comparable irrespective of the selecting agent. All resistant mutants showed evidence of enhanced inactivation of cephalothin and of inactivation of ampicillin, carbenicillin, and cefamandole by the cloverleaf screening procedure (9).

A non-β-lactamase-producing reverse mutant (RM2016) selected from a resistant mutant of *E. cloacae* (M2016 Am4) showed no antibiotic inactivation. Table 2 shows disk diffusion susceptibilities of the sequentially derived mutants. The wild-type strain was resistant to ampicillin and cephalothin. The resistant mutant was, in addition, resistant to carbenicillin and cefamandole. The reverse mutant was susceptible to all four β-lactam antibiotics, but a resistant mutant derived from it regained resistance to all four antibiotics.

Activity of β-lactamases from *E. cloacae* 2016 and its resistant mutants. Cell-free extracts of uninduced cultures and extracts of cultures induced with penicillin were prepared. β-Lactamase activities, as units per milligram of protein, were determined from rates of hydrolysis of cephaloridine and cefamandole by the spectrophotometric assay (Table 3).

Resistant mutants showed greatly enhanced enzyme activity when compared with the wild types. Relative activities against the two antibiotics were comparable irrespective of the antibiotic used for mutant selection. The susceptible reverse mutant showed evidence of enzyme activity in only one assay with cephaloridine (Table 3). There was no evidence of enzyme induction by penicillin in the mutants.

Inhibition of β-lactamase activity. To further characterize the β-lactamases of the mutants, their susceptibility to inhibition by PCMB and certain β-lactam antibiotics was determined spectrophotometrically with cephaloridine as the assay substrate. Cell-free extracts of the three resistant mutants of *E. cloacae* 2016 were inhib-

TABLE 2. Disk diffusion test results and interpretation for *E. cloacae* 2016 and three sequentially selected mutants

Strain	Derivation	Disk test result (zone diam in mm)[a]			
		Amp	Carb	Cph	Cfm
2016	Wild type	6(R)	26(S)	6(R)	28(S)
M2016 Am4	Resistant mutant	6(R)	16(R)	6(R)	7(R)
RM2016	Susceptible reverse mutant	24(S)	26(S)	24(S)	27(S)
MRM2016	Resistant mutant from reverse mutant	6(R)	12(R)	6(R)	9(R)

[a] Abbreviations: Amp, ampicillin; Carb, carbenicillin; Cph, cephalothin; Cfm, cefamandole; R, resistant; S, susceptible by the standardized procedure (3).

TABLE 3. Specific β-lactamase activity of cell-free extracts of *E. cloacae* 2016 and its mutants

Source of cell-free extracts	Penicillin induced	Antibiotic used for mutant selection	Sp act (U/mg of protein)	
			Cephaloridine	Cefamandole
2016	−		0.01	0[a]
M2016 Am4	−	Ampicillin	26.8	0.21
	+		23.2	0.23
M2016 Cb1	−	Carbenicillin	20.9	0.22
	+		21.6	0.25
M2016 Cfm8	−	Cefamandole	21.5	0.22
	+		11.2	0.24
RM2016[b]	−		0.2	0[a]
	+		ND[c]	ND

[a] 0, No activity detected.
[b] Susceptible reverse mutant from M2016 Am4.
[c] ND, Not done.

ited by 100 μM concentrations of ampicillin, carbenicillin, dicloxacillin, oxacillin, or methicillin, but not by 100 μM PCMB. These findings were compatible with those described by others for *Enterobacter* chromosomal β-lactamases (21). A similar experiment with a cell-free extract of an ampicillin-selected mutant of *E. aerogenes* 2046 showed complete inhibition of cephaloridine hydrolysis by the antibiotics that inhibited the *E. cloacae* enzyme activity. There was, however, an 88% inhibition of β-lactamase activity by PCMB. In this regard, it differed from the *E. cloacae* enzyme.

Isoelectric focusing of cell-free extracts. Analytical isoelectric focusing was carried out on cell-free extracts of induced cultures of the two *Enterobacter* strains and on resistant mutants derived from them. A reverse, susceptible mutant derived from a resistant mutant of *E. cloacae* 2016 and the resistant mutant reselected from the susceptible derivative were also tested.

Initial preparations from the resistant mutants showed the presence of dark-staining protein bands with an isoelectric point above pH 8.0. Gels were therefore prepared and run in a pH range of 7.8 to 10 (Fig. 1 and 2). *E. cloacae* 2016 gave a faint protein band having β-lactamase activity with an isoelectric point of 8.95 (Fig. 1a). A resistant mutant from this strain showed new dense protein bands with isoelectric points of 8.86 and 9.06 (Fig. 1b), corresponding to areas of most marked β-lactamase activity.

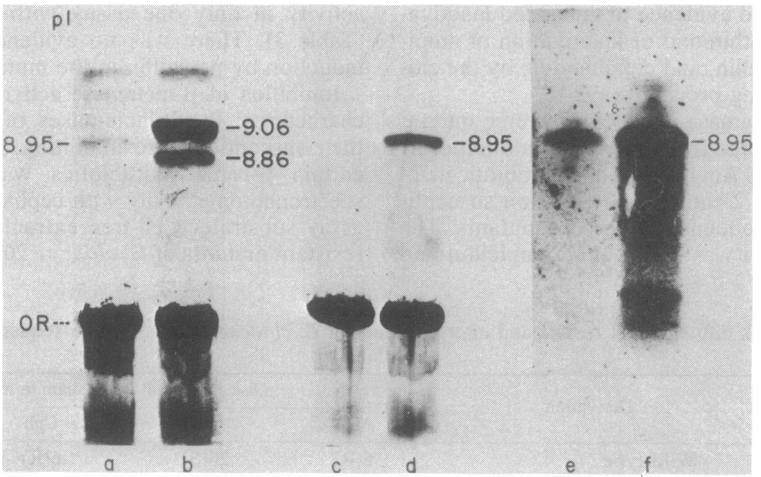

FIG. 1. Isoelectric focusing protein patterns of crude enzyme preparations from wild-type *Enterobacter* strains and their representative β-lactam antibiotic-resistant mutants. The acrylamide gel, pH range 7.8 to 10, was stained for proteins with Coomassie brilliant blue R-250 (lanes a through d) or nitrocefin (lanes e and f). The isoelectric points (pI) of the major bands demonstrating β-lactamase activity are indicated (————). Samples containing 250 μg of protein were applied at the location indicated (OR -----). Analyses are shown for strains (a) wild-type 2016, (b) M2016 Am4, (c, e) wild-type 2046, (d, f) M2046 Am2. Their antibiotic susceptibilities are given in Table 1.

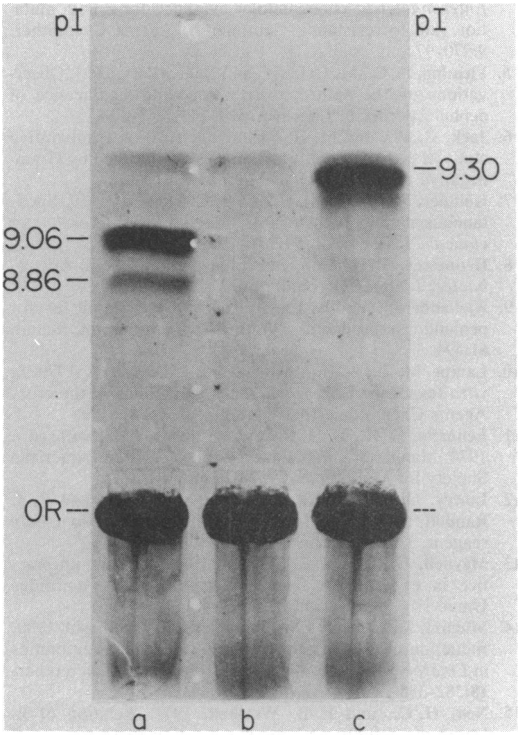

FIG. 2. Isoelectric focusing patterns of crude enzyme preparations from (a) *Enterobacter* mutant M2016 Am4, (b) its β-lactam antibiotic-susceptible reverse mutant (RM2016), and (c) a resistant mutant (MRM2016) selected from RM2016. The pH 7.8 to 10 gel was prepared as described in Materials and Methods and stained with Coomassie blue. The antibiotic susceptibilities of the strains are given in Table 2.

The *E. aerogenes* 2046 mutant showed a striking protein band having a pI of 8.95 (Fig. 1d) with marked β-lactamase activity (Fig. 1f). The wild type showed no protein band at a pI of 8.95 when the gel was stained by Coomassie blue (Fig. 1c), but a band at this position was demonstrated by the more sensitive nitrocefin method (Fig. 1e).

The *E. cloacae*-susceptible reverse mutant (RM2016), selected from the resistant mutant M2016 Am4, showed no β-lactamase activity or protein bands in locations corresponding to those of the mutant from which it was derived (Fig. 2a and b). A resistant mutant, MRM2016, selected from the reverse mutant by direct antibiotic selection, showed a major new protein band (Fig. 2c) having a pI of 9.30, with strong β-lactamase activity.

DISCUSSION

Previous reports from our laboratory have shown that mutants resistant to cefamandole and with increased resistance to ampicillin occur at a relatively high frequency in cultures of *Enterobacter* species. About half of the mutants exhibited enhanced β-lactamase activity (4, 10). The results reported here show that mutants producing apparently identical β-lactamases can be selected with carbenicillin, ampicillin, and cefamandole.

The isoelectric points of the β-lactamase from the wild-type *E. aerogenes* 2046 and of its mutants were similar, and thus the increased enzymatic activity of the mutants was probably due to enhanced production of the same enzyme. In the case of the *E. cloacae* strain, two dense new bands of enzyme activity with pI different from that of the wild-type enzyme were present in the mutants. These bands probably reflect increased amounts of altered forms of the same enzyme. A more pronounced difference in the physical behavior of the β-lactamase from a resistant mutant was shown in the case of the mutant derived from a susceptible reverse mutant of the *E. cloacae* strain. This enzyme differed in pI value from the β-lactamases of the wild type and of the resistant mutants directly selected from it (Fig. 2).

The evidence is good that the increase in resistance of the mutants from these strains to β-lactam antibiotics was due primarily to their increased production of β-lactamase. There was close correlation between the degree of resistance to the different antibiotics and the activity of the β-lactamase. Reverse mutants selected for their loss of β-lactamase activity became fully susceptible to all of the β-lactam antibiotics tested, in contrast to the wild type, which showed low-level β-lactamase activity with associated resistance to cephalothin and ampicillin. Similar findings were reported earlier by Goldner et al. (7). Resistant mutants selected from our reverse mutant regained enhanced β-lactamase production and the degree of resistance of the mutants which were selected directly from the wild type.

The substrate profiles of the β-lactamases of the resistant mutants corresponded quite closely with those previously reported for wild-type *Enterobacter* chromosomally determined β-lactamases (5, 8, 15, 21). In a biological assay, cephalothin and cephaloridine were the most actively hydrolyzed, followed by benzylpenicillin, cefamandole, ampicillin, and carbenicillin, in that order (data not shown). They were primarily cephalosporinases, and there was no or only minimal activity against ampicillin and carbenicillin.

There was no evidence of penicillin inducibility of the β-lactamases of the mutants. In the case of the susceptible reverse mutant, the induction procedure resulted in the selection of a

resistant mutant of enhanced β-lactamase activity which accounted for apparent "induction." It is important to exclude this possibility in studies of inducibility of *Enterobacter* β-lactamases, although clear evidence of inducibility of wild-type *Enterobacter* β-lactamases by low concentrations of cefoxitin and other cephamycin derivatives has been presented by Waterworth and Emmerson (22), Sanders and Sanders (20), and Minami et al. (14) and in earlier studies with penicillin induction (8).

Our findings do not settle the issue of the mechanism of increased β-lactamase production by the mutants. In the case of *E. aerogenes* 2046, the mutation could have involved a regulatory gene controlling β-lactamase production in the wild type leading to permanent derepression of enzyme production. With *E. cloacae* 2016, increased production of certain forms of β-lactamase seemed to have occurred in the resistant mutants, although enzymes of the same pI value could have been below the level of detection in the wild type. With the resistant mutant derived from the reverse susceptible mutant of *E. cloacae* 2016, an enzyme of altered pI value from that of the original mutant was seen.

ADDENDUM IN PROOF

Shortly before this paper was submitted, an abstract by Gootz and Sanders was published (Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, Atlantic City, N.J., abstr. no. 675, 1981). They also found that *Enterobacter* mutants were derepressed for β-lactamase production.

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