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Cefoxitin, a Semisynthetic Cephamycin Antibiotic: Resistance to Beta-Lactamase Inactivation

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Cefoxitin is a new, cephalosporin-like antibiotic which is highly resistant to hydrolysis by β-lactamase. Ninety-one cultures were selected either for their general resistance to cephalosporin antibiotics or for their ability to produce β-lactamase. Some of these cultures were resistant to cefoxitin. The capacity of each of the 91 strains to hydrolyze cefoxitin with β-lactamase was determined. Only seven of the cultures degraded the antibiotic as determined by a general assay for β-lactamase. Several others were able to hydrolyze cefoxitin after enzyme was induced by low concentrations of the antibiotic. The role of the constitutive and inducible enzyme in bacterial resistance to the antibiotic was investigated. Enzymatic destruction of cefoxitin was found to be an important factor contributing to bacterial resistance. However, the complete and rapid degradation of cefoxitin is not essential to resistance since one strain, *Enterobacter cloacae* 1316, hydrolyzed the antibiotic very slowly but was able to grow unaffected in the presence of cefoxitin. The presence of the enzyme is not necessarily sufficient to confer resistance since another culture, *Klebsiella* D535, readily hydrolyzed the antibiotic but was susceptible to it.

Cefoxitin (Fig. 1) is a semisynthetic cephamycin analog (12), a new cephalosporin-like antibiotic, with activity against both gram-positive and gram-negative bacteria (21). One of its important properties is its uniquely high resistance to hydrolysis by β-lactamase (EC 3.5.2.6 penicillin [cephalosporin] amido-β-lactam hydrolase). The capacity of a given bacterial strain to produce β-lactamase is often an important factor in its resistance to the penicillin- and cephalosporin-like antibiotics. The gram-negative bacteria have been shown to produce several β-lactamases with different substrate profiles (11). We have investigated the resistance of cefoxitin to β-lactamase hydrolysis in relation to its activity on 91 strains of gram-negative bacteria selected either for their representative resistance patterns to cephalosporin-like antibiotics or for their capacity to produce β-lactamase. This paper reports the results obtained from such a study.

MATERIALS AND METHODS

Cultures. The cultures used in this study were described by us in a recent publication (3). Briefly, 458 clinical isolates were obtained from five metropolitan hospitals and tested for their in vitro susceptibility to the various cephalosporin antibiotics. Fifty-four cultures were then selected as representative of the cephalosporin-resistant patterns for the various genera included in the original clinical isolates. An additional 38 gram-negative cultures were obtained from M. H. Richmond (Department of Bacteriology, The Medical School, University of Bristol, England), who used these cultures in a study on the classification of β-lactamases produced by gram-negative bacteria (11).

Enterobacter cloacae HSC 18410/62, which produces a potent cephalosporinase constitutively (5, 6), and *E. cloacae* HSC 18410-M66, which is a spontaneous mutant of HSC 18410/62 that does not produce β-lactamase (9), were obtained from M. Goldner (The Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada).

The cultures were maintained on brain heart infusion (BHI; Difco) slants at 3 to 5 C and transferred regularly for short periods of storage or frozen at −80 C for longer periods of storage.

Antibiotic susceptibility. The susceptibility of the microorganisms to cefoxitin was determined by the Bauer-Kirby method (2). Organisms which gave a zone of inhibition of 18-mm diameter or greater with less than five colonies in the zone of inhibition, and obtained with 0.25-inch (about 0.07 cm) disks containing 30 μg of cefoxitin, were designated suscepti-

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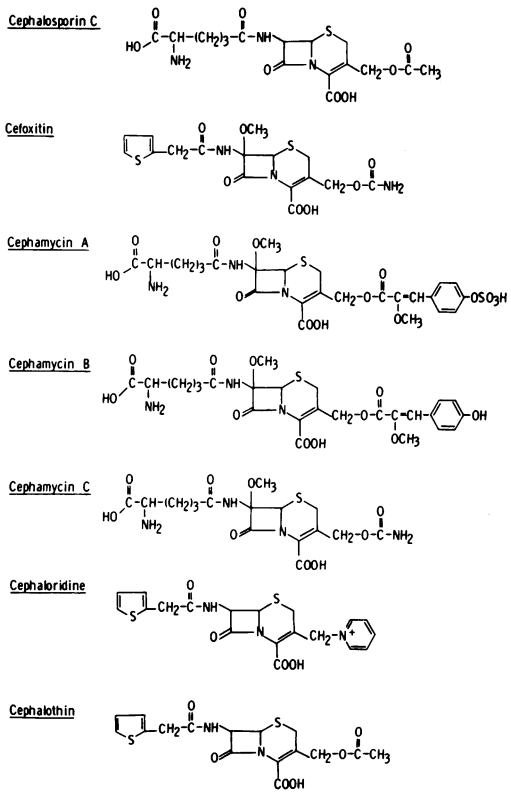

FIG. 1. *Structure of several cephalosporin-like antibiotics including the cephamycin family and cefoxitin.*

ble. The basis for the selection of an 18-mm zone diameter as the criterion for susceptibility is explained elsewhere (21).

Microbiological assay for antibiotic activity. The bioassay was performed by the standard agar diffusion-disk method using BHI plates seeded with *Staphylococcus aureus* MB-2786.

Assay for β-lactamase activity. The general assay used to detect β-lactamase activity was described previously (3). The bacteria were grown overnight at 37 C on BHI slants. The cells were washed off the slants into 0.067 M phosphate buffer, pH 7.0, and diluted to a standard optical density at a wavelength of 550 nm (Spectronic 20, Bausch and Lomb, Rochester, N.Y.). The cell suspension and the antibiotic were allowed to react for 1 h at 37 C. The concentration of hydrolyzed antibiotic was determined by a modification of the methods described by Novick (17) and by Goldner et al. (8). A starch-iodine indicator was prepared in 0.5 M acetate buffer, pH 4.0. This mixture was pipetted into the reaction tubes after the 1-h incubation period. Exactly 10 min after the reaction mixture and the indicator were mixed, the reduction of the starch-iodine indicator was determined spectrophotometrically at a wavelength of 620 nm. This value was corrected for the nonspecific loss of color with appropriate controls. The amount of hydrolysis was determined from a standard curve. This technique was used throughout the study in order to determine β-lactamase activity.

β-Lactamase preparations. Crude β-lactamase was prepared from *E. cloacae* HSC 18410/62 by a method which has been described previously (3). A cell-free preparation of β-lactamase was prepared from *Klebsiella* D535 in a similar manner, but it was not processed beyond the high-speed centrifugation step.

The rates of reaction were measured by the microiodometric method described above. The reactions were carried out in 0.067 M phosphate buffer, pH 7.0, at 37 C. Protein was determined by the Lowry method (14).

Induction of β-lactamase. Bacterial cultures were induced for β-lactamase production with cefoxitin. The cultures were grown to log phase in BHI broth at 37 C on a rotary shaker (220 rpm). The bacteria were diluted 1:10 into both a fresh BHI broth control and into fresh BHI broth containing cefoxitin at a final concentration of 25 μg/ml. Incubation was continued at 37 C for 3 h on the shaker. At the end of the induction period, the cells were centrifuged at 10,000 × *g* for 10 min, washed once in 0.067 M phosphate buffer, pH 7.0, and resuspended to give an optical density of 1.0 at a wavelength of 550 nm when diluted 1:10.

The control and induced cultures were assayed for β-lactamase activity both as whole cells and after sonication (M.S.E., Inc., Westlake, Ohio) at full amplitude for 5 min in an ice-water bath.

Effect of chloramphenicol on β-lactamase induction in Escherichia coli L-105 and Klebsiella Burpee. Two bacterial strains inducible for β-lactamase production, *E. coli* L-105 and *Klebsiella* Burpee, were grown to log phase in BHI broth. The cells were centrifuged and resuspended to a concentration of 3×10^9 to 5×10^9 bacteria/ml. These cultures were diluted 1:10 into BHI broth containing a final cefoxitin concentration of 25 and 50 μg/ml for *E. coli* L-105 and *Klebsiella* Burpee, respectively. The induction mixtures were incubated at 37 C in a shaking water bath (model G76, New Brunswick Scientific, New Brunswick, N.J.). At various times over a 2-h period, dry chloramphenicol (CM) was added such that the final CM concentration would be 250 and 500 μg/ml for *E. coli* L-105 and *Klebsiella* Burpee, respectively. Thus, all of the nine separate samples for each strain were exposed to cefoxitin for 2 h, but each separate sample was exposed to CM for a different period of time. The CM concentrations were sufficiently high to prevent proliferation but low enough to maintain viability. At the end of the 2-h induction period, the cells were centrifuged, washed once in 0.067 M phosphate buffer, pH 7.0, and sonicated. Each sample was assayed for β-lactamase activity and for protein concentration. Cephaloridine was used as the substrate to measure β-lactamase activity since this antibiotic is much more enzyme labile than is cefoxitin.

Effect of the induced β-lactamase on the susceptibility of E. coli L-105 and Klebsiella Burpee to cefoxitin. The two inducible cultures were grown and

prepared as described above and exposed to inducing concentrations of cefoxitin. At various times during the induction period, a 10-fold serial dilution was made from the cefoxitin-exposed culture. A viable count was determined, and 0.1 ml from each dilution tube was used to inoculate 0.9 ml of BHI broth containing 200 and 400 μg of cefoxitin per ml for *E. coli* L-105 and *Klebsiella* Burpee, respectively. The plates, dilution blanks, and cefoxitin tubes were incubated for approximately 18 h at 37 C. Since we knew the number of viable cells at each time period, we were able to determine the number of viable cells used to inoculate the last tube containing cefoxitin which permitted visible growth. The growth in the dilution blanks was a control which indicated that the number of viable cells used to inoculate the first cefoxitin tube which inhibited growth was sufficient to permit growth in the absence of the antibiotic.

Growth rate as a function of bioactive cefoxitin. *Enterobacter cloacae* HSC 18410/62, *Enterobacter cloacae* 1316, *Escherichia coli* L-105, *Klebsiella* Burpee, and *Klebsiella* D535 were grown in BHI broth containing 50 μg of cefoxitin per ml. At various time intervals, the optical density (at a wavelength of 550 nm) of the cultures was recorded and a sample was removed, membrane-filtered (Millipore Corp., Bedford, Mass.; 45 μm pore size), and quickly frozen in dry ice for bioassay at a later time.

RESULTS

β-Lactamase degradation of cefoxitin by cephalosporin-resistant and β-lactamase-producing, gram-negative bacteria. The percentage of the initial cefoxitin concentration degraded in 1 h at 37 C by β-lactamase from intact cells and the in vitro susceptibility of these 91 gram-negative bacteria are presented in Table 1. Data from a similar survey of cephaloridine, cephalothin, and cephamycin C presented in a recent report (3) are included in this table for comparison. Cefoxitin was remarkably resistant to β-lactamase hydrolysis by these gram-negative bacteria which were selected either for their representative resistance patterns to cephalosporin-like antibiotics or for their capacity to produce β-lactamases. Enzymatic degradation of cefoxitin was detectable in only seven of the 91 cultures tested, whereas 42 cultures degraded cephalothin, 73 degraded cephaloridine, and 28 degraded cephamycin C (Table 2). The comparative advantage of cefoxitin with respect to β-lactamase resistance was not a function of species variation.

Species variation is an important factor in a comparison of the susceptibility of the 91 cephalosporin-resistant or β-lactamase-producing bacteria to the four antibiotics tested. Cefoxitin was slightly more active than cephamycin C against four of the nine species included in the group of gram-negative cultures tested. Cefoxitin was more effective than cephalothin or cephaloridine against the *Aerobacter-Enterobacter* species, the *Klebsiella* species, and especially against the *Proteus* species. In addition, *Escherichia coli* was more susceptible to cefoxitin than to cephalothin. Interestingly, cephaloridine was more readily degraded by β-lactamase than was cephalothin (Table 2), yet the advantage of β-lactamase-resistant cefoxitin with respect to antibacterial effect was greater when cefoxitin is compared with cephalothin than when cefoxitin is compared with cephaloridine. In a species-by-species comparison of susceptibility to the four cephalosporin-like antibiotics, the antibacterial effect of cefoxitin was at least equivalent to that of the other three antibiotics. With the cephalosporin-resistant or β-lactamase-producing representatives of several species, however, cefoxitin had a considerable advantage in antibacterial effect.

Kinetics of cefoxitin hydrolysis by Enterobacter cloacae β-lactamase. The resistance of cefoxitin to β-lactamase degradation was quantitated by determining the kinetics of this enzyme reaction with cefoxitin as the substrate. Fleming has demonstrated that the β-lactamase of *E. cloacae* HSC 18410/62 is a potent cephalosporinase (8). The maximal rate of reaction (V_{max}) and the dissociation constant (K_m) were derived from a Lineweaver-Burk plot (13) by standard procedures. Data presented by us in a similar study (3) with other cephalosporin-like antibiotics are presented in Table 3 for comparison. Cefoxitin, in agreement with the data in Table 1, was an extremely poor substrate for the *E. cloacae* β-lactamase. Cephamycin C, of which cefoxitin is an analog (Fig. 1), was degraded about 170 times more rapidly than cefoxitin. Of the six antibiotics compared with cefoxitin, cephamycin B (Fig. 1) was the second most stable to enzyme degradation. Yet even this compound was degraded about 50 times more rapidly than cefoxitin by the *E. cloacae* β-lactamase. The affinity of the enzyme for cefoxitin was similar to that of cephalothin. The relationships defined between these antibiotics to this particular enzyme are no doubt valid. However, the absolute values may not be valid because a crude enzyme preparation was used.

Induction of β-lactamase by cefoxitin. Preliminary experiments indicated that β-lactamase activity could be induced in some organisms which showed no detectable enzyme. Therefore, 20 representative cultures, which produced little or no β-lactamase, were induced with 25 μg of cefoxitin per ml in BHI at 37 C for

TABLE 1. *Antibiotic susceptibility of clinical isolates and their capacity to degrade the antibiotics enzymatically*[a]

Culture	Susceptibility[b]				Antibiotic degraded (%)			
	Cephalothin	Cephaloridine	Cephamycin C	Cefoxitin	Cephalothin	Cephaloridine	Cephamycin C	Cefoxitin
Aeromonas sp.								
18	S	S	S	S	0	20	0	0
2/37	S	S	S	S	0	24	0	0
241	S	S	S	S	12	84	0	0
1390	S	S	S	S	0	24	0	0
Enterobacter cloacae								
HSC18410/62	R	R	R	R	87	97	100	15
HSC18410M66	S	S	S	S	0	0	0	0
P99	R	R	R	R	78	100	100	19
53	R	S	S	S	0	100	0	0
214	R	R	R	R	16	42	0	0
177	R	R	R	R	5	92	0	0
1082E	R	R	S	S	74	100	0	0
1929	R	R	R	R	5	100	0	0
1316	R	R	R	R	0	54	0	0
Aerobacter sp. 2/46	R	S	S	S	12	54	0	0
Enterobacter sp.								
6	R	R	R	R	22	49	0	0
15	R	R	R	S	0	0	0	0
42	R	R	R	R	26	45	44	0
349	R	R	R	S	30	100	0	0
369	R	R	R	R	42	94	40	0
68	S	R	R	R	16	36	5	2
231	R	R	R	R	10	32	10	0
242	R	R	S	R	0	0	0	0
244	R	R	R	R	28	50	10	0
301	R	R	R	R	22	44	0	0
Alcaligenes sp.								
97	R	R	R	R	0	0	0	0
249	R	R	R	R	0	0	0	0
251	R	R	R	R	0	0	0	0
257	S	S	S	S	0	0	0	0
385	R	S	S	S	36	100	0	0
Paracolon								
53	R	R	R	R	0	18	0	0
111	R	R	R	R	12	50	10	0
237	R	R	R	R	0	50	20	0
256	R	R	R	R	16	38	0	0
260	R	R	R	R	10	36	0	0
309	R	R	R	R	10	32	0	0
Providencia								
2	R	R	R	R	0	20	0	0
21	R	R	R	R	0	0	0	0
Serratia sp.								
187	R	R	R	R	0	0	0	0
377	R	R	R	S	46	90	0	0
Escherichia coli								
35	R	S	S	S	0	46	44	0
65	R	R	R	R	26	100	52	0
105	R	R	R	R	12	38	10	0
61	R	R	R	R	12	50	0	0

[a] Isolates were selected either for their representative resistance patterns to cephalosporin-like antibiotics or for their known production of β-lactamase.

[b] R, resistant; S, susceptible.

TABLE 1—*Continued*

Culture	Susceptibility[b]				Antibiotic degraded (%)			
	Cephalothin	Cephaloridine	Cephamycin C	Cefoxitin	Cephalothin	Cephaloridine	Cephamycin C	Cefoxitin
120	R	R	R	R	53	100	61	26
Fink	R	R	R	R	54	89	88	0
McTee	R	S	S	S	32	87	64	0
Lovell	R	R	R	S	38	87	64	0
8	S	S	S	S	0	66	0	0
071	S	S	S	S	0	34	0	0
284	S	S	S	S	0	0	0	0
719	R	R	R	R	63	100	48	16
1758	R	R	R	R	0	0	5	0
2/40/1	R	R	R	R	34	94	14	0
Denston 5003	R	S	S	S	0	100	0	0
CJL	R	R	R	R	19	100	20	2
R$^+$TEM	R	S	S	S	12	52	0	0
NPL-3	R	R	R	R	26	100	48	0
Wickham	S	S	S	S	12	62	26	0
Klebsiella sp.								
17	R	R	S	R	0	100	0	0
31	R	R	S	S	0	87	0	0
36	R	R	R	R	20	87	0	0
39	R	R	S	R	0	88	0	0
202	R	R	R	S	0	50	0	0
239	R	R	R	R	12	34	20	0
266	R	R	R	R	0	0	0	0
Burpee	R	R	R	R	0	30	0	0
115	S	S	S	S	0	40	0	0
264	S	S	S	S	0	26	0	0
311	S	S	S	S	0	44	5	0
418	S	S	S	S	0	5	5	0
466	R	S	S	S	8	82	0	0
481	S	S	S	S	0	30	0	0
D535	R	R	R	S	82	100	95	6
1169	R	S	S	S	0	58	0	0
9527	S	S	S	S	0	50	0	0
McDonald	R	S	S	S	46	100	0	0
Robinson	S	S	S	S	0	42	0	0
Shine	R	S	S	S	34	100	5	0
Proteus morganii								
1266	S	S	S	S	0	0	0	0
Collier	R	R	R		0	10	0	
Detenly	R	R	R	R	0	0	0	0
Mauser	R	R	S	S	0	0	0	0
P. mirabilis								
23	R	R	S	S	0	3	0	0
46	R	R	S	S	0	0	0	0
58	S	R	S	S	0	24	0	0
P. vulgaris 3117	S	S	S	S	0	64	0	0
Proteus sp.								
11	R	R	R	R	12	41	0	0
16	R	R	R	S	0	15	0	0
20	R	R	S	S	0	5	0	0
26	R	R	R	R	0	3	0	0
63	R	R	R	S	0	0	0	0
92	R	R	R	R	12	40	10	0

TABLE 2. *Antibiotic susceptibility and β-lactamase activity according to genus*

Genus	No.	Cephalothin[a]			Cephaloridine			Cephamycin C			Cefoxitin		
		β-lac[b]	No. R	No. S	β-lac	No. R	No. S	β-lac	No. R	No. S	β-lac	No. R	No. S
Enterobacter	20	15	18	2	17	17	3	7	15	5	3	14	6
Aeromonas	4	1	0	4	4	0	4	0	0	4	0	0	4
Alcaligenes	5	1	4	1	1	3	2	0	3	2	0	3	2
Escherichia coli	19	13	15	4	17	11	8	13	11	8	3	10	9
Klebsiella	20	6	13	7	19	9	11	5	6	14	1	6	14
Paracolon	6	4	6	0	6	6	0	2	6	0	0	6	0
Proteus	13	2	10	3	7	11	2	1	6	7	0[c]	4	9
Providencia	2	0	2	0	1	2	0	0	2	0	0	2	0
Serratia	2	0	2	0	1	2	0	0	2	0	0	1	1
Total	91	42	70	21	73	61	30	28	51	40	7	46	45

[a] Number showing significant (>9%) β-lactamase degradation of the antibiotic.
[b] R, resistant; S, susceptible.
[c] No data for *Proteus* Collier.

TABLE 3. *Enzyme kinetics of Enterobacter cloacae HSC18410/62 β-lactamase with six cephalosporin-like antibiotic substrates*

Substrate	V_{max} ($\times 10^{-3}$)[a]	K_m ($\times 10^{-2}$) (μmol)
Cephamycin A	1.2	9.5
Cephamycin B	1.0	6.2
Cephamycin C	3.3	2.4
Cephalothin	9.1	0.6
Cephaloridine	100.0	9.2
Cephalosporin C	200.0	14.3
Cefoxitin	0.019	0.6

[a] Expressed as micromolar per minute per milligram of protein.

3 h. Both whole and disintegrated cells were tested for basal and induced β-lactamase activity. No β-lactamase activity was associated with uninduced whole cells in most cases (Table 4). Enzyme activity became evident when these strains were tested after sonication. This indicates the presence of the crypticity factor discussed by Hennessey (10). Substantial enzyme activity was associated with induced whole cells. The whole cells were tested in stationary phase. In many cases, cefoxitin had retarded growth, suggesting that these cultures are not completely resistant to the antibiotic. Thus, it is not clear whether the increased β-lactamase activity observed with induced whole cells was completely due to induction or partially due to the disruption of the crypticity factor by the lysis of stationary-phase cultures as suggested by Hennessey (10) or to leakiness caused by antibiotic activity as suggested by Sabath et al. (20).

Increases in β-lactamase activity of 10- to 15-fold were observed in the sonicates of cefoxitin-induced cultures (Table 4). The magnitude of induction was in the range observed by Ayliffe (1), who used various antibiotics to

TABLE 4. *Effect of β-lactamase induction on the capacity of gram-negative bacteria to hydrolyze cefoxitin*

Culture	Whole cells[a]		Sonic extracts[b]	
	Control	Induced	Control	Induced
Enterobacter cloacae HSC18410/62	>2.1	>2.1	7.8	13.9
Alcaligenes DC249	0	0.2	0.1	0.1
Enterobacter cloacae 214	0.6	>2.1	0.5	13
Enterobacter				
B6	0	1.3	0.2	2.8
B42	0.1	>2.1	0.5	3.5
DC68	0	>2.1	0.2	3.5
DC242	0	0.1	0	0.1
Escherichia coli				
L-105	0	1.5	0.2	2.4
1758R	0	1.2	0.3	1.0
2/40/1	0	0.3	0.5	0.4
CJL	0.8	1.6	1.7	2.1
Klebsiella				
B17	0	0	0.4	0.7
L 239	0	1.8	0.2	3.2
Burpee	0	0.8	0	1.6
Paracolon				
DC111	0	0.8	0.2	5.7
DC237	0	>2.1	0.2	2.9
DC256	0	>2.1	0.2	3.0
Providencia L21	0	0.2	0.2	2.4
Proteus				
B-11	0	0	0.2	0.6
DC92	0	1.7	0.4	2.4

[a] Expressed as micromolar degraded per minute per milliliter of culture.
[b] Expressed as micromolar degraded per minute per milligram of protein.

induce β-lactamase production. Of the 19 cultures tested (*E. cloacae* HSC 18410/62 was included as a control), 15 had more than three times the basal β-lactamase activity after treatment with cefoxitin, but only two cultures produce more than 25 times the basal level. These results may be a consequence of the lengthy induction period. In two experiments described in a later section, the induced β-lactamase activity in strains of *E. coli* and *Klebsiella* reached a peak between 60 to 80 min. After the inducer was destroyed, the enzyme activity apparently was diluted out by the growth of the organisms.

Effect of chloramphenicol on β-lactamase activity in Escherichia coli L-105 and Klebsiella Burpee. The effect of chloramphenicol on the induction of β-lactamase in *E. coli* L-105 and *Klebsiella* Burpee was investigated by the method detailed above. Both strains have very low basal levels of enzyme activity. Enzyme production was stimulated in both strains by low concentrations of cefoxitin. The susceptibility to CM of increasing enzyme activity (Fig. 2) indicates that enzyme production is related to protein synthesis. Interestingly, β-lactamase activity decreased in *Klebsiella* Burpee (Fig. 2) after reaching a peak at 60 min. This phenomenon was also observed with *E. coli* L-105 (Fig. 2) when the enzyme activity is plotted as a function of the number of viable cells (not shown). This decrease in activity was probably due to the hydrolysis of the cefoxitin inducer by the induced enzyme. As the cefoxitin concentration was decreased, the enzyme was apparently diluted out in the newly divided cells which were no longer producing β-lactamase.

Effect of β-lactamase induction on the ability of E. coli L-105 and Klebsiella Burpee to survive high concentrations of cefoxitin. The cell concentration of *E. coli* L-105 or *Klebsiella* Burpee required to initiate growth in BHI broth containing a high concentration of cefoxitin decreased as a function of the time of exposure to inducing concentrations of the antibiotic (Fig. 3). The decrease in enzyme activity observed above after reaching a maximum is corroborated by the increase in the number of cells required to initiate growth after reaching a minimum. No significant inoculum effect was observed with uninduced cells. These data indicate that the induced β-lactamase plays an important role in the resistance of these two strains to cefoxitin.

Growth of Enterobacter cloacae HSC 18410/62, Enterobacter cloacae 1316, Escherichia coli L-105, Klebsiella Burpee, and Klebsiella D535 as a function of bioactive cefoxitin. Rapid and complete destruction of cefoxitin was neither essential nor necessarily sufficient to confer resistance to the antibiotic. This was demonstrated by growing *Enterobacter cloacae* HSC 18410/62, *Enterobacter cloacae* 1316, *Escherichia coli* L-105, *Klebsiella* Burpee, and *Klebsiella* D535 in BHI broth containing cefoxitin. The growth was monitored by optical density, and the cefoxitin concentration was monitored by bioassay.

In the case of *Enterobacter cloacae* HSC 18410/62, a steady decline in the bioactive cefoxitin concentration was associated with a growth rate that was identical to that of the control culture growing in the absence of cefoxitin (Fig. 4A). On the other hand, the growth curve of *E. cloacae* 1316, a microorganism described by Jack and Richmond (11), in the presence of cefoxitin is identical to that of the control, although very little cefoxitin was degraded (Fig. 4B).

The two strains which were inducible for β-lactamase activity, *Escherichia coli* L-105 (Fig. 4C) and *Klebsiella* Burpee (Fig. 4D), reacted differently to cefoxitin. No growth occurred with *E. coli* L-105 in the presence of 50 μg

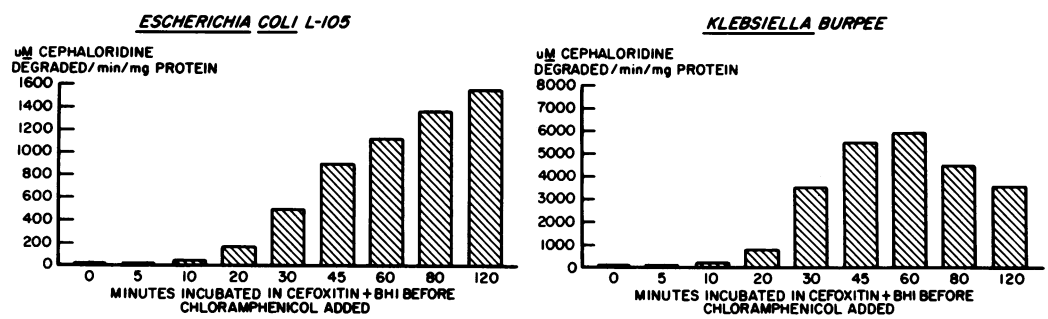

FIG. 2. *Effect of chloramphenicol on the induction of β-lactamase by cefoxitin in Escherichia coli L-105 and Klebsiella Burpee.*

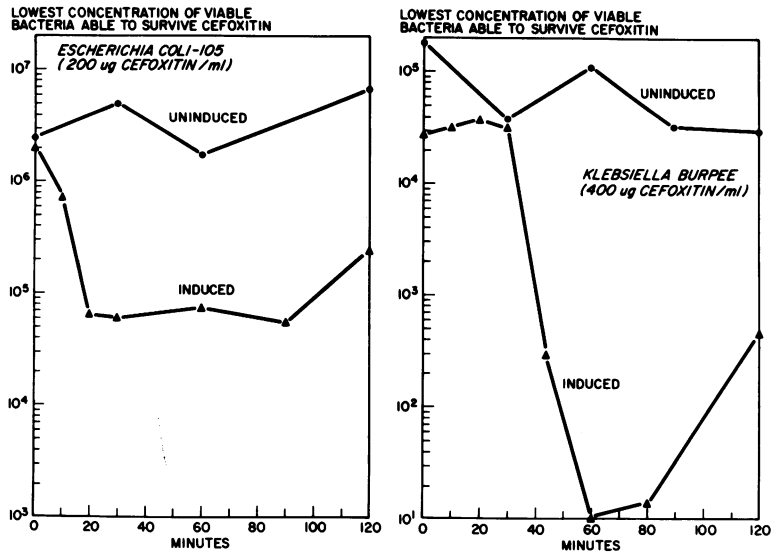

FIG. 3. *Capacity of uninduced bacteria and of bacteria induced for β-lactamase to survive cefoxitin.*

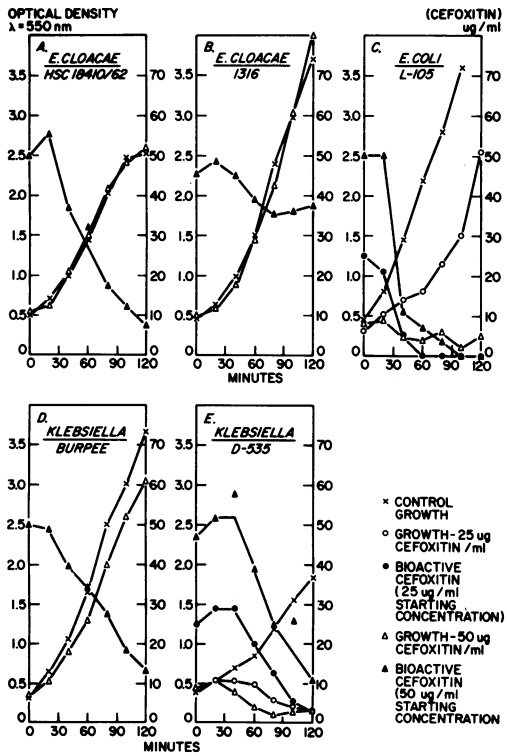

FIG. 4. *Relationship between bacterial growth and bioactive cefoxitin concentration in several bacterial strains capable of β-lactamase production.*

of cefoxitin per ml, although the antibiotic concentration declined steadily. In the presence of 25 μg of cefoxitin per ml, however, uninhibited growth began after 60 min, a time corresponding to the greatest activity of β-lactamase per viable cell. *Klebsiella* Burpee grew in a manner similar to that of the control culture in the presence of 50 μg of cefoxitin per ml without a perceptible lag for β-lactamase induction.

Klebsiella D535, also described by Jack and Richmond (11), provides an interesting contrast to the cases presented above. This organism actively degraded cefoxitin (Fig. 4E), yet it was susceptible to the antibiotic. That the decrease in cefoxitin bioactivity was due to hydrolysis by β-lactamase is indicated by the kinetics of a crude enzyme preparation in Table 5.

DISCUSSION

Cefoxitin is a semisynthetic, cephalosporin-like antibiotic which is a member of the cephamycin family. Specifically, cefoxitin is an analog of cephamycin C (12). The cephamycin family of antibiotics exhibits an increased resistance to hydrolysis by β-lactamase when compared with other cephalosporin-like antibiotics (3). This property of the cephamycins is no

TABLE 5. *Kinetics of a crude β-lactamase preparation from Klebsiella D535*

Substrate	V_{max} (× 10^{-3})[a]	K_m (× 10^{-2}) (μmol)
Cefoxitin	0.001	1.6
Cephalothin	2.2	1.0

[a] Expressed as micromolar per minute per milligram of protein.

doubt due to the presence of the α-methoxy group at the seven position of the cephalosporin nucleus (Fig. 1). This resistance to β-lactamase was enhanced in the new antibiotic, cefoxitin, by the substitution of the thienylacetamido group for the aminoadipoyl group of cephamycin C, also at the seven position (Fig. 1). Cefoxitin was degraded by the β-lactamase of *Enterobacter cloacae* HSC 18410/62 at a rate which is almost 200-fold less than that for cephamycin C. Of the 91 microorganisms tested for their capacity to hydrolyze cephaloridine, cephalothin, cephamycin C, and cefoxitin (Tables 1 and 2), 28 degraded cephamycin C but only seven degraded cefoxitin. In those instances where a microorganism could degrade both cephamycin C and cefoxitin (e.g., *Escherichia coli* 120 or *Klebsiella* D535, Table 1), a significantly greater amount of cephamycin C was degraded as compared with cefoxitin. However, in studying the relationship of this resistance to β-lactamase to the response of 91 gram-negative bacteria chosen either for their representative resistance patterns to cephalosporin-like antibiotics or for their capacity to produce β-lactamase, we have found that the activity of cefoxitin on a particular bacterial strain is the result of a complex combination of factors. Independent of the degradation of the antibiotic by β-lactamase, a basic tolerance is an important determinant in the response of a bacterial strain to cefoxitin. The basic tolerance is itself the consequence of several components, including the binding of the antibiotic to the bacteria and the susceptibility of the lethal target site or sites.

The importance of β-lactamase in the response of a bacterial strain to a particular antibiotic is based on the capacity of the enzyme to complement the basic tolerance or to compensate for the lack of it.

The β-lactamase of *Enterobacter cloacae* HSC 18410/62 completely compensated for a low basic tolerance to the cephalosporin-like antibiotics that we have tested. That the hydrolysis of cephalosporin-like antibiotics is the primary mode of resistance in this strain is corroborated by the sensitivity of a β-lactamase-deficient mutant (*E. cloacae* HSC 18410-M66) to the cephalosporin-like antibiotics. The fact that Miller et al. (15) have shown that cefoxitin protects mice against *E. cloacae* HSC 18410/62 at a concentration which is much lower than that required for cephaloridine or cephalothin is in agreement with the kinetics data for the β-lactamase of this strain (Table 3).

On the other hand, the basic tolerance of *Klebsiella* D535 is so low that the hydrolysis of cefoxitin by the β-lactamase of this strain is not sufficient to confer resistance. This phenomenon is not unique to the relationship of *Klebsiella* D535 to cefoxitin. Sabath and Finland (19) have made similar observations with *Proteus morganii*, *Escherichia coli*, and, most dramatically, with *Klebsiella* in relation to both the cephalosporin-like and penicillin-like antibiotics.

The complexity of the relationship between β-lactamase degradation and basic tolerance in the response of gram-negative bacteria to cefoxitin is exemplified in *Enterobacter cloacae* 1316, *Escherichia coli* L-105, and *Klebsiella* Burpee. In *Enterobacter cloacae* 1316, the resistant response is primarily due to basic tolerance. After 2 h, the bioactive cefoxitin concentration remained at 70% of the initial concentration, but the growth rate was totally unaffected by the antibiotic. In this case, the enzyme may play a less obvious role in the resistance of this strain to cefoxitin. Borrowing an idea from Neu (16), the strategic location of the β-lactamase on or in the cell could possibly compensate for the low reaction rate of the enzyme. In addition to its hydrolytic activity, this enzyme may offer protection to the cell by providing a binding site for cefoxitin which is highly competitive with the lethal target site of the antibiotic.

We have attempted to separate the function of the basic tolerance in the resistance to cefoxitin from that of the β-lactamase by studying the response of *Escherichia coli* L-105 and *Klebsiella* Burpee to cefoxitin both before and after the enzyme has been induced. The induction process followed approximately the same time-course in both strains (Fig. 2). The rate of decline in bioactive cefoxitin concentration associated with *E. coli* L-105 was slightly greater than that associated with *Klebsiella* Burpee (Fig. 4C and 4D). However, *E. coli* L-105 grew normally only when the bioactive cefoxitin concentration was considerably reduced, whereas the growth of *Klebsiella* Burpee was unaffected by the antibiotic. This difference in response to cefoxitin is the result of a greater basic tolerance of the antibiotic by *Klebsiella* Burpee than by *E. coli* L-105. The difference in the dependency upon the two separate functions, basic tolerance and β-lactamase, which resulted in the resistance of these two strains to cefoxitin was emphasized by the approximate minimal number of viable cells required to establish growth in a cefoxitin concentration which was eightfold greater than the inducing concentration (Fig. 3). This parameter followed

the time-course of β-lactamase induction in *E. coli* L-105 but remained constant in *Klebsiella* Burpee during the first 40 min of induction. Apparently the β-lactamase is an important factor in the capacity of *E. coli* L-105 to withstand cefoxitin, but, in *Klebsiella* Burpee, the basic tolerance is of such sufficiency that the induced capacity to degrade the antibiotic has little effect on the initial response of this strain to cefoxitin. We have also observed that when the enzyme activity is expressed as a function of the viable cell concentration rather than protein concentration (Fig. 2), it is 10 to 20 times greater at the five sampling times between 10 and 60 min in *E. coli* L-105 than in *Klebsiella* Burpee. We interpret this to mean that viability is closely related to the capacity to produce β-lactamase in *E. coli* L-105 but that enzyme production is not a strict requirement for viability in *Klebsiella* Burpee. The resistance of *E. coli* L-105 to cefoxitin is almost completely dependent on the enzymatic degradation of the antibiotic (Fig. 4C). The enzymatic hydrolysis of cefoxitin multiplies the resistance of *Klebsiella* Burpee to this antibiotic, but the enzyme serves more of an auxiliary than a primary function.

It is possible that, in some cases, the resistant response to cefoxitin is partially determined by a cooperative effect. A crypticity factor was evident when β-lactamase activity was assayed in whole cells and in the corresponding sonicated preparations (Table 4). The crypticity factor was less apparent when induced whole cells were assayed for the enzyme. It is possible that cefoxitin can reduce the crypticity barrier by causing leakiness in the cells as well as inducing the production of the enzyme. The released enzyme, in turn, may reduce the bioactive cefoxitin concentration to a level that the surviving cells can tolerate. The bioactive concentration to which the cefoxitin must be reduced would be ultimately dependent on the basic tolerance of the bacterial strain.

The concept of basic tolerance is not novel. Sabath and Finland (19) demonstrated that β-lactamase activity and bacterial resistance do not always correlate. Farrar and Krause (4) emphasized that β-lactamase plays an important, but not always a determinant, role in the resistance of many *Enterobacter* strains. Garber and Friedman (7) have suggested that the resistance of *Pseudomonas aeruginosa* to β-lactam antibiotics is primarily due to an intrinsic resistance rather than to β-lactamase. Richmond et al. (18) concluded that gram-negative bacteria which produce β-lactamase may be resistant to β-lactam antibiotics but that the loss of this capability does not always result in susceptible bacteria.

In a comparison of the antibiotic susceptibility of 91 gram-negative bacteria, selected either for their representative cephalosporin resistance patterns or for their ability to synthesize β-lactamase, the effectiveness of cefoxitin was at least equal to that of the other three antibiotics tested. With several species represented in this selected group of bacteria, there was a decided advantage for cefoxitin with respect to antibacterial effectiveness. A partial explanation for this superiority may lie in the extreme resistance of cefoxitin to hydrolysis by β-lactamase. When the resistant response of a particular bacterial strain to the cephalosporin family of antibiotics is primarily due to enzyme degradation, cefoxitin probably has an advantage in efficacy. However, Wallick and Hendlin (21) have shown that many of those gram-negative bacteria which are susceptible to cephalosporin-like antibiotics are most susceptible to cefoxitin. Therefore, the superiority of cefoxitin with respect to the susceptibility of many cephalosporin-resistant or β-lactamase-producing bacteria may be due to a requirement for a higher basic tolerance to withstand this antibiotic as well as to its property of resistance to β-lactamase hydrolysis.

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