ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1972, p. 107-111
Vol. 1, No. 2

Printed in U.S.A.

Relation of β-Lactamase Activity and Cellular Location to Resistance of *Enterobacter* to Penicillins and Cephalosporins

HAROLD C. NEU[1] AND ELAINE B. WINSHELL
College of Physicians and Surgeons, Columbia University, New York, New York 10032

Received for publication 8 November 1971

The *Enterobacter* species *E. aerogenes*, *E. cloacae*, and *E. hafnia* were examined for resistance to penicillin and cephalosporin derivatives. All were resistant to benzyl penicillin, ampicillin, 6 [D(−)α-amino-*p*-hydroxyphenylacetamido] penicillanic acid, cephaloridine, cephalothin, and cephalexin. A significant number were sensitive to carbenicillin and 6 [D(−)α-carboxy-3-thienylacetamido] penicillanic acid. No differences among the three species were noted. The β-lactamase activity was cell-bound, and was not released by osmotic shock, toluene treatment, or diphenylamine treatment. It was rarely released into the growth medium. The β-lactamase activity was primarily directed against cephalosporin derivatives. Synthesis of β-lactamase was chromosomally mediated. Resistance to ampicillin seemed to be partly related to entry of the molecule into the bacteria since exposure to ethylenediaminetetraacetate lowered the minimal inhibitory concentration.

Members of the *Klebsielleae* have assumed greater importance as etiological agents of nosocomial infections in recent years. The observation that strains of *Enterobacter* were resistant to cephalothin was early noted by Kirby and coworkers (1). The resistance of *Enterobacter* strains to benzyl penicillin and ampicillin was not unexpected, because many of the *Klebsielleae* possess β-lactamases and many are intrinsically resistant to these compounds (4, 13). Fleming, Glass, and Goldner have demonstrated β-lactamase activity with predominantly cephalosporin specificity (4, 5). The observation by several groups (9, 14) that many *Enterobacter* strains are susceptible to carbenicillin concentrations that can be readily achieved in vivo prompted us to reexamine the resistance of *Enterobacter* species to penicillins and cephalosporins. We wished to evaluate particularly the role of β-lactamase activity, substrate specificity of *Enterobacter* β-lactamase, cellular location of the β-lactamase, alteration of cell wall permeability, and enzyme induction in the antibiotic resistance of these organisms.

MATERIALS AND METHODS

Bacteria. The strains of *Enterobacter* were obtained from cultures of patients admitted to the Presbyterian Hospital. Speciation was based on the criteria of Edwards and Ewing (2). Bacteria were maintained on Typticase Soy Agar slants (BBL).

Antibiotics. Solutions of antibiotics were prepared fresh for each experiment in phosphate buffer, *p*H 7.0. Sodium benzylpenicillin was a gift from The UpJohn Co. Ampicillin, carbenicillin, 6 [D(-)α-amino-*p*-hydroxyphenylacetamido] penicillanic acid (BRL-2333), and 6 [D(-)α-carboxy-3-thienylacetamido] penicillanic acid (BRL-2288) were gifts from Beecham Pharmaceuticals. Cephalothin, cephaloridine, and cephalexin were gifts from Eli Lilly & Co. Dicloxacillin was a gift of Bristol Laboratories.

Susceptibility tests. Broth dilutions in Trypticase Soy Broth (BBL) were performed with a 10^4 inoculum of an overnight culture. Incubation was for 18 hr at 35 C. The minimal inhibitory concentration (MIC) was that amount of antibiotic which inhibited the production of visible turbidity. Tests for synergy were performed with a checkerboard arrangement of test tubes as previously described (11). Assays for the amount of a compound hydrolyzed were performed on membrane filtrates of the culture by use of a previously described agar diffusion method (9). The test organism was *Bacillus subtilis*. In growth experiments, a Klett colorimeter was used, and optical density was recorded with a 520- to 580- nm filter.

Transfer of antibiotic resistance was determined by the technique of Watanabe (17) with *Escherichia coli* W1485 as recipient.

Penicillinase activity was determined by a modification of the microiodometric assay (12). Cephalosporinase activity was determined both by the

[1] Career Scientist, New York City Health Research Council.

TABLE 1. *Cumulative percentage of E. aerogenes, E. cloacae, and E. hafnia inhibited*[a]

MIC (μg/ml)	Penicillin	Ampicillin	BRL 2333[b]	Carbenicillin	BRL 2288[c]	Cephalothin	Cephaloridine
>1,000	100	100	100	100	100	100	100
1,000	0	84	88	100	100	30	30
500	0	32	12	100	100	10	10
250	0	0	0	100	100	5	5
100	0	0	0	100	100	0	0
50	0	0	0	72	52	0	0
25	0	0	0	20	12	0	0
10	0	0	0	8	4	0	0

[a] Broth dilutions on 30 strains.
[b] 6[D(−)α-amino-*p*-hydroxyphenylacetamido] penicillanic acid.
[c] [D(−)-α-carboxy-3-thienylacetamido] penicillanic acid.

microiodometric assay and by a spectrophotometric assay (8).

RESULTS

Distribution of species. In contrast to the experience at the Communicable Disease Center and the Boston City Hospital (14), *E. aerogenes* accounted for 52%, *E. cloacae* for 35%, and *E. hafnia* for 12% of the 60 strains tested during the period we sampled. We did not encounter *E. liquefaciens*. The majority of isolates came from sputum and urine specimens of hospitalized patients.

Susceptibility to antimicrobial agents. Table 1 shows the cumulative per cent susceptibility of various *Enterobacter* strains to penicillins and cephalosporins. The strains were susceptible only to carbenicillin and BRL-2288 in amounts that could be readily achieved in man (10). For example, for an individual strain, the MIC of ampicillin could be 500 μg/ml and that of carbenicillin could be 25 μg/ml. In most cases, the MIC of carbenicillin and BRL-2288 was 8- to 16-fold less than the MIC of other penicillins or cephalosporins.

Various *E. aerogenes* and *E. cloacae* strains were studied to determine the hydrolysis of penicillins and cephalosporins by intact cells. As Fig. 1 demonstrates, cepholoridine was rapidly hydrolyzed, but ampicillin and benzylpenicillin were destroyed to a lesser degree. Carbenicillin was not significantly hydrolyzed in 6 hr of incubation. Since β-lactamase activity in *Enterobacter* strains is inducible (6), it seemed that part of this effect might be due to induction of enzyme. However, the rate of hydrolysis of the compounds was identical in strains preliminarily induced with benzyl penicillin G or cephalothin. Preparation of sonic extracts resulted in relative rates of hydrolysis similar to that of intact cells. In disrupted cells, ampicillin was a less suitable substrate than cephaloridine or benzyl penicillin.

Table 2 shows that induction of β-lactamase

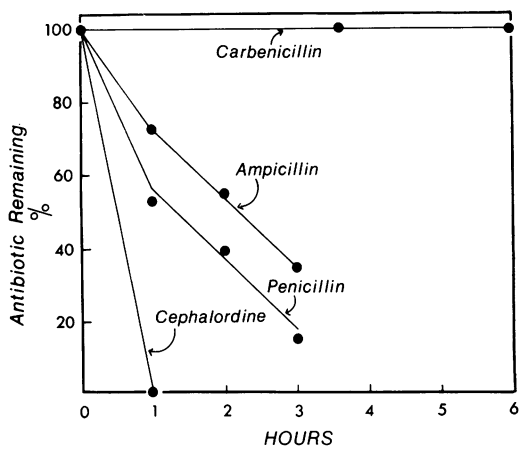

FIG. 1. *Hydrolysis of β-lactam antibiotics by E. aerogenes. A 1:10 dilution of logarithmic culture was prepared, and 100 μg of benzyl penicillin, ampicillin, or cephaloridine was added. The organisms were incubated on a shaker at 37 C, and samples were removed and membrane filtered. The filtrate was assayed for antibiotic remaining. The data illustrated refer to a single strain, but similar results were obtained with seven strains tested.*

activity with penicillin G, carbenicillin, and cephalothin resulted in β-lactamase of similar specific activity against penicillin G and cephaloridine. The basic activity of the enzyme was directed against cephalosporin compounds, and the induced enzyme was less specifically a β-lactamase with cephalosporinase activity. Strains which were susceptible to carbenicillin could be induced with cephalothin, penicillin G, or carbenicillin to produce a β-lactamase that hydrolyzed cephalosporins, pencillin G, and ampicillin, but not carbenicillin. At lower concentrations of ampicillin (Fig. 2), the antibiotic was not destroyed until late in the exponential phase, possibly as a function of enzyme induction. When cells at the same

TABLE 2. *Hydrolysis of penicillin G and cephaloridine by E. aerogenes induced with various substrates*

Inducer	Specific activity (units/mg)	
	Penicillin	Cephaloridine
Nothing	0	2.08
Penicillin G	7.5	8.24
Carbenicillin	7.46	6.62
Cephalothin	7.6	7.45

[a] *E. aerogenes* 604 was grown to exponential phase in Trypticase Soy Broth and divided into five portions to which penicillin G (500 μg/ml), cephalothin (500 μg/ml), or carbenicillin (30 μg/ml) was added. After 2 hr, the organisms were harvested and washed, and a sonic extract was prepared. Penicillinase and cephalosporinase activity was determined by the microiodometric method.

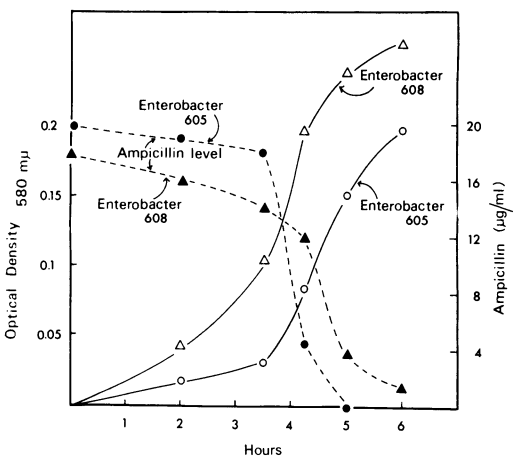

FIG. 2. *Hydrolysis of ampicillin by E. cloacae 605 and E. aerogenes 608. Cultures (10⁴ cells) were inoculated into Trypticase Soy Broth, and ampicillin (20 μg/ml) was added to the cultures. Growth was followed by change in optical density (△, ○). Hydrolysis of ampicillin was followed by agar plate assay of membrane filtrates of the culture (▲, ●).*

phase of growth were incubated with ampicillin and cepholoridine, they hydrolyzed the cephaloridine but failed to destroy the ampicillin.

To clarify further the cellular location of these enzymes, both uninduced and induced cells were exposed to a variety of treatments known to release surface enzymes (9, 15). Several strains of each species were subjected to cold osmotic shock (9). Table 3 demonstrates that no significant release of β-lactamase activity occurred. Preparation of spheroplasts by use of ethylenediaminetetra-

TABLE 3. *Attempt to release β-lactamase from E. cloacae 766*

Growth phase	β-Lactamase released (%)			
	Wash	Sucrose-Tris[b]-EDTA	Shock fluid	Sonic extract
Exponential	7.6	6.8	15	71
Stationary	0.5	8.3	1.2	90

[a] Organisms were grown in Trypticase Soy Broth to early exponential phase, and 100 μg of penicillin G/ml was added for 2 hr. Organisms were harvested, washed, and subjected to osmotic shock by standard procedures (9). Each fraction was assayed for penicillinase activity with benzyl penicillin as substrate. Fractions were of equal volume, and hence percentages were readily calculated.

[b] Tris(hydroxymethyl)aminomethane.

acetate (EDTA) and lysozyme also failed to release the enzyme. Exposure of cells to toluene or polymyxin B (15) was likewise unsuccessful. Diphenylamine (3) did not release β-lactamase activity. The finding of cephalosporinase activity in the growth medium of stationary-phase *E. cloacae* appears related to lysis of some cells and not to active excretion of the enzyme. Disrupted cells showed greater absolute rates of hydrolysis of cephaloridine than did intact cells, either induced or noninduced.

Attempts to transfer resistance to cephalosporins from *Enterobacter* strains to *E. coli* recipients were unsuccessful. Several strains which were resistant to 100 μg of carbenicillin/ml contained a β-lactamase which mediated resistance to penicillin G, ampicillin, and carbencillin. Such strains transferred penicillin resistance to *E. coli* W1485 but did not transfer resistance to cephalothin.

Previous studies from this laboratory had suggested that in various *Enterobacteriaceae* EDTA could cause a lowering of MIC of an antibiotic if resistance was not episomally mediated. Table 4 shows that EDTA caused a significant lowering of the MIC of cephaloridine, ampicillin, and carbenicillin. The MIC of cephaloridine could be reduced from 500 μg/ml to between 7.8 and 32 μg/ml by use of 2 mM EDTA. An EDTA concentration of 10 to 50 mM was required to lower the MIC of ampicillin from 500 μg/ml to between 16 and 62 μg/ml. When 20 mM EDTA was used, the MIC of carbenicillin was reduced from 100 to 12.5 μg/ml. The optimal amount of EDTA to achieve a significant lowering of the MIC varied from strain to strain, but was less for cephalosporin compounds than for ampicillin. Plotting the data

TABLE 4. *Effect of EDTA upon the MIC of cephaloridine, ampicillin, and carbenicillin*

Strain	MIC (μg/ml)					
	Carbenicillin		Ampicillin		Cephaloridine	
	−EDTA	+EDTA[a]	−EDTA	+EDTA[b]	−EDTA	+EDTA[c]
1	100	12.5	500	32	500	7.8
2	50	12.5	1,000	250	250	62.5
3	100	25	500	62.5	250	31.2
4	50	3.2	250	16	—	—

[a] EDTA, 20 mM.
[b] EDTA, 50 mM.
[c] EDTA, 2 mM.

TABLE 5. *Synergy of penicillinase-resistant penicillins with ampicillin and carbenicillin*

Organism	Minimal inhibitory concn (μg/ml)			
	Carbenicillin		Ampicillin	
	Above	With dicloxacillin[a]	Alone	With dicloxacillin
E. aerogenes..	50	3.2	500	125
E. aerogenes..	50	12.5	1,000	125
E. cloacae....	25	3.2	250	16
E. cloacae....	12.5	3.2	64	16

[a] Dicloxacillin, 62 μg/ml. The MIC of dicloxacillin is 1,000 μg/ml.

TABLE 6. *Effect of inoculum size upon the minimal inhibitory concentration*

Strain	Minimal inhibitory concn (μg/ml)			
	Carbenicillin		Ampicillin	
	10^{8}[a]	10^{4}	10^{8}	10^{4}
E. aerogenes.	50	25	500	125
E. aerogenes.	100	25	1,000	62
E. cloacae...	64	6	500	125
E. cloacae...	50	3	1,000	16
E. hafnia....	200	25	1,000	31

[a] Colony-forming units added to broth dilution.

as isobolograms produced hyperbolic curves suggesting a synergistic action.

β-Lactamase-resistant penicillins also showed a synergistic action when combined with ampicillin or carbencillin (Table 5). The MIC of ampicillin could be reduced from 250 to 16 μg/ml in the presence of 62 μg of dicloxacillin/ml. The carbenicillin MIC could be reduced from 50 to 3.2 μg/ml. The combination of dicloxacillin and cephaloridine produced a much less impressive reduction in the MIC of cephaloridine.

The effect of inoculum size upon the MIC of penicillins and cephalosporins has often been commented upon. Table 6 demonstrates this effect for ampicillin and carbenicillin. A 10^4 reduction in inoculum resulted in a two- to eightfold reduction in MIC. With all of the penicillin and cephalosporin derivatives tested, we encountered a marked effect of inoculum size. This was seen whether the cultures used were in the exponential or early stationary phase.

DISCUSSION

The results of this study on 60 *Enterobacter* strains confirm the resistance of these organisms to ampicillin (14) and cephaloridine (1) as well as their susceptibility to carbenicillin (10, 14) and a new similar α-carboxy penicillin derivative. The β-lactamase activity of *Enterobacter* strains is primarily directed toward cephalosporins and much less against penicillin derivatives, as has been suggested. The β-lactamase activity of almost all of the *Enterobacter* strains tested was internal and not surface-located as are β-lactamases which are episomal in origin (12). Changes in cell wall permeability by use of EDTA, which releases cell wall lipopolysaccharide (9), or of toluene (15), which has a similar effect, did not cause release of the enzyme from the bacteria. Diphenylamine, which affects cell wall phospholipid (3), did not effect a change in location of the β-lactamase activity. The fact that hydrolysis of a compound such as ampicillin occurred more readily after EDTA or toluene treatment does point to a "cryptic" nature of the β-lactamase as well as its poorer affinity for ampicillin.

EDTA treatment was able to potentiate the activity of cephaloridine, ampicillin, and carbenicillin. Since the EDTA effect on bacteria is primarily release of lipopolysaccharide from the cell wall and thus effects wall disorganization, it seems

unlikely that the resistance of these strains is due to poor affinity of the cell wall transpeptidase, but this does remain a possibility.

The synergistic action of penicillinase-resistant semisynthetic penicillins with ampicillin suggests a role of the β-lactamase in resistance, but is not conclusive evidence.

The marked effect of the size of the inoculum upon resistance of *Enterobacter* could be the result of poor entry of penicillins into the bacteria with slow hydrolysis of the compounds, resulting from an induction phenomenon.

In agreement with Medeiros and O'Brien (7), we found that resistance of *Enterobacter* to β-lactam antibiotics is rarely episomally mediated. The rare carbenicillin-resistant strain contained both a β-lactamase of primary cephalosporin affinity, whose synthesis was chromosmally mediated, and a resistance transfer factor-determined episomal β-lactamase of primary benzyl penicillin or ampicillin affinity.

Unfortunately, unlike *Staphylococcus aureus* and *E. coli*, in *Enterobacter* the mechanisms of resistance to penicillin and cephalosporin derivatives vary from strain to strain without a mechanism particular to all *Enterobacter* species. Resistance seems to be multifactorial, depending on entry of the antibiotic into the organism, β-lactamase activity of specific substrate affinity, location of the β-lactamase within the cell, induction of β-lactamase activity, and probably on cell wall synthesis factors not yet elucidated. Further study is necessary to clarify the interaction of these various mechanisms.

ACKNOWLEDGMENTS

This investigation was funded by grants from the New York Health Research Council, and by Public Health Service grant AI 06840 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Benner, E. J., J. S. Micklewait, J. L. Brodie, and W. M. M. Kirby. 1965. Natural and acquired resistance of Klebsiella-Aerobacter to cephalothin and cephaloridine. Proc. Soc. Exp. Biol. Med. 119:536–541.
2. Edwards, P. R., and W. H. Ewing. 1962. Identification of Enterobacteriaceae, 2nd ed. Burgess Publishing Co., Minneapolis.
3. Feingold, D. S. 1969. The serum bactericidal reaction. IV. Phenotypic conversion of *Escherichia coli* from serum-resistance to serum-sensitivity by diphenylamine. J. Infec. Dis. 120:437–444.
4. Fleming, P. C., M. Goldner, and D. C. Glass. 1963. Observation on the nature, distribution and significance of cephalosporinase. Lancet 1:1399–1401.
5. Goldner, M., D. G. Glass, and P. C. Fleming. 1968. Characteristics of *Aerobacter* beta-lactamase. Can. J. Microbiol. 14:139–145.
6. Hennessey, T. D. 1967. Inducible β-lactamase in Enterobacter. J. Gen. Microbiol. 49:277–285.
7. Medeiros, A. A., and T. F. O'Brien. 1968. R-factor mediated increments in levels of resistance to and enzymatic degradation of penicillins and cephalosporins. Antimicrob. Ag. Chemother. 1967, p. 271–278.
8. Neu, H. C. 1971. β-Lactamase production by *Pseudomonas aerugenosa*. Antimicrob. Ag. Chemother. 1970, p. 534–538.
9. Neu, H. C., and J. C. Chou. 1967. Release of surface enzymes in *Enterobacteriaceae* by osmotic shock. J. Bacteriol. 94:1934–1945.
10. Neu, H. C., and H. Swarz. 1969. Carbenicillin: clinical and laboratory experience with a parenterally administered penicillin for treatment of Pseudomonas infections. Ann. Intern. Med. 71:903–911.
11. Neu, H. C., and H. Swarz. 1970. The effect of carbenicillin on ampicillin resistant *Escherichia coli*. J. Gen. Microbial. 58:301–305.
12. Neu, H. C., and E. B. Winshell. 1970. Purification and characterization of penicillinases from *Salmonella typhimurium* and *Escherichia coli*. Arch. Biochem. Biophys. 139:278–290.
13. Seligman, S. J. 1968. Resistance of Klebsiella and Enterobacter to the penicillins. Proc. Soc. Exp. Biol. Med. 127: 915–919.
14. Slocombe, B., and R. Sutherland. 1970. β-Lactamase activity and resistance to ampicillin, carbenicillin, and cephaloridine of *Klebsiella*, *Enterobacter*, and *Citrobacter*. Antimicrob. Ag. Chemother. 1969, p. 78–85.
15. Teuber, M., and G. Cerney. 1970. Release of periplasmic ribonuclease I into the medium from *Escherichia coli* treated with membrane-active polypeptide antibiotic polymyxin B. Fed. Eur. Biochem. Soc. Letters 8:49–51.
16. Toala, P., Y. H. Lee, C. Wilcox, and M. Finland. 1970. Susceptibility of *Enterobacter aerogenes* and *Enterobacter cloacae* to 19 antimicrobial agents in vitro. Amer. J. Med. Sci. 260:41–55.
17. Watanabe, T. 1964. Selected methods of genetic study of episome-mediated drug resistance in bacteria. Methods Med. Res. 10:202–220.