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Effect of β-Lactamase Location in *Escherichia coli* on Penicillin Synergy

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Resistance to ampicillin in *Escherichia coli* is due generally to the presence of a β-lactamase (penicillinase). Resistant strains have been found to fall into two groups: those with high-level resistance (1,000 μg/ml or greater) and those with low-level resistance (8 to 250 μg/ml). Most of the high-level resistant organisms possess β-lactamases whose synthesis is episomally mediated. These strains release penicillinase from the cell when they are subjected to osmotic shock. Low-level resistant strains do not release the enzyme with osmotic shock. High-level resistant strains are not susceptible to the synergistic action of a penicillinase-resistant penicillin with ampicillin. Seventy eight per cent of low-level resistant strains are susceptible to the synergistic action of ampicillin and oxacillin. The two types of β-lactamases are similar in regard to most properties; both enzymes are subject to competitive inhibition by penicillinase-resistant penicillins. The difference in location in the cell might explain why only some strains of *E. coli* are susceptible to the synergistic action of penicillin combinations.

The proliferation of semisynthetic penicillins and cephalosporins, a number of which show activity against gram-negative organisms, has stimulated interest in the role of β-lactamases in the resistance of these organisms. β-Lactamases have been studied thoroughly in gram-positive species such as *Staphylococcus aureus* (10) and *Bacillus licheniformis* (12). However, the β-lactamases produced by members of the *Enterobacteriaceae* have only recently been given attention (1-3, 5, 7, 11, 13). The finding that synthesis of penicillinase could be episomally mediated was reported by Datta and Kontomichalou (2). Subsequently, Datta and Richmond (3) purified a penicillinase of *Escherichia coli*. They noted that the enzyme could be partially released from the cell by an osmotic shock technique developed by Neu and Heppel (9). I (7) showed that episomally mediated penicillinases are surface enzymes in many strains of *E. coli* and *S. typhimurium*, and suggested that the penicillinase whose synthesis is chromosomally mediated is more firmly bound to the cell.

The synergy between certain penicillins, such as ampicillin and oxacillin, has been thought to be due to the competitive inhibition of the β-lactamase by the penicillinase-resistant analogue, permitting the more easily hydrolyzed penicillin to exert its antibacterial action for a greater period. However, not all strains of *E. coli* possessing penicillinases have been susceptible to this synergistic effect (6, 14). It was felt that knowledge of the location of the penicillinase in the cell, as well as of the characteristics of the organisms, might explain the differences among the strains.

This paper suggests an explanation for the resistance of certain strains of *E. coli* to the ampicillin-oxacillin combination.

MATERIALS AND METHODS

E. coli strains were obtained from the diagnostic laboratory of the Presbyterian Hospital, New York City. Organisms were grown in Penassay Broth (Difco) or in the phosphate medium of Neu and Chou (8). Ampicillin resistance was determined by tube dilution in Trypticase Soy Broth (BBL) by use of a 10^5 inoculum from an overnight culture.

Osmotic shock was performed in the following manner. Organisms were grown to early stationary phase. They were harvested and washed in 0.85% NaCl. Cells were suspended in 0.5 M sucrose-0.03 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.5) at 21 C at a ratio of 10^{10} cells to 80 ml of sucrose-Tris. Ethylenediaminetetraacetic acid was added to a concentration of 1 mM, and, after 5 min of mixing, the cells were removed by centrifugation. The pellet of the cells was resuspended in water at 3 C and mixed for 5 min; the cells were again removed by centrifugation. Penicillinase was assayed in the osmotic shock fluid. In those organisms that failed to release the enzyme when subjected to osmotic shock, a sonic extract was prepared with a Branson Sonifier.

The supernatant fluid of a 20-min, 30,000 × *g* centrifugation of the extract was used to determine enzyme activity.

The presence of R factors was determined by the method of Watanabe (17). Penicillinase activity was determined by a modification of Novick's (10) iodometric assay. Standard preparations of ampicillin, oxacillin, methicillin, and dicloxacillin were provided through the courtesy of Bristol Laboratories.

RESULTS

Table 1 illustrates the results of subjecting seven representative strains of *E. coli* to osmotic shock. Strains such as DB103 and DB117, which contain an R factor for resistance to ampicillin, released essentially all of their penicillinase when subjected to osmotic shock. The same was true for strains such as FW/R26 and FW/R108, into which an episome had been transferred from a wild strain. Strains that lack an episome mediating resistance to ampicillin, but which are nonetheless penicillinase producers, did not release the enzyme when subjected to osmotic shock. However, such strains do have normal surface characteristics since they release other "surface enzymes," such as 5'-nucleotidase and cyclic phosphodiesterase (8).

Comparison of the level of ampicillin resistance of these two types of penicillinase strains is seen in Table 2. Those strains which contain episomes mediating ampicillin resistance and in which the penicillinase was released by osmotic shock showed high-level resistance. Of 23 strains tested, 22 were resistant to greater than 1,000 μg. Eighty-seven per cent were resistant to more than 2,000 μg of ampicillin. Those strains which failed to release the penicillinase when subjected to osmotic shock showed low to medium resistance. Of these, 63% were resistant to 63 μg or less.

Table 3 shows a comparison of the synergistic action of oxacillin plus ampicillin on two strains of *E. coli*. DB103 released its penicillinase when subjected to osmotic shock. DB116 lacks an episome for ampicillin resistance and did not release its penicillinase when it was osmotically shocked. Synergism is seen when the penicillinase is of a type that is not released by osmotic shock.

By use of a turbidimetric assay system with DB103, 1,000 μg of ampicillin and 1,000 μg of oxacillin caused no delay in growth of an exponential-phase culture. Strains DB108 and

TABLE 1. *Release of penicillinase by use of osmotic shock*

Organism	Per cent penicillinase released	R factor[a]
DB103	95	A, T, Sm
DB117	95	A, C
DB108	5	None
DB21	0	C, T, Su
DB120	0	None
FW/R26	85	A, C, T, Su
FW/R108	88	A, C, Sm

[a] A = ampicillin; C = chloramphenicol; T = tetracycline; Su = sulfonamide; Sm = streptomycin.

TABLE 2. *Comparison of the level of ampicillin resistance in strains that release penicillinase by osmotic shock with those strains that do not*

Ampicillin (μg/ml)[a]	No. of sensitive strains	
	Release by osmotic shock	Do not release by osmotic shock
0		
2		
4		
8		2
16		3
31		6
63		3
125		6
250	1	2
500		
1,000	2	
2,000	20	

[a] Final concentrations are represented. Each tube contained 1.0 ml of which 0.1 was a 10^{-5} dilution of culture, 0.1 was ampicillin, and 0.8 was Trypticase Soy Broth.

TABLE 3. *Comparison of the synergistic action of oxacillin on a strain that releases penicillinase by osmotic shock (DB103) with a strain that does not (DB116)*

Concn of ampicillin (μg/ml)	DB103		DB116	
	Ampicillin	Ampicillin + oxacillin[a]	Ampicillin	Ampicillin + oxacillin
0	+[b]	+	+	+
4	+	+	+	+
8	+	+	+	0
16	+	+	+	0
32	+	+	+	0
63	+	+	+	0
125	+	+	0	0
250	+	+	0	0
500	+	+	0	0
1000	+	+	0	0

[a] Oxacillin at 250 μg/ml.
[b] Symbols: +, growth; 0, no growth.

DB116, which do not release penicillinase, were grown in 30 μg of ampicillin per ml and 250 μg of oxacillin per ml and showed a profound lag in growth, compared with growth in ampicillin alone.

Table 4 summarizes the data obtained with all of the strains of *E. coli* tested for synergy between oxacillin and ampicillin. Of 22 strains that released the enzyme when subjected to osmotic shock, only 1 showed synergy, but 14 of 18 strains that failed to release penicillinase by means of osmotic shock showed synergy. In none of the strains did the penicillinase-resistant penicillin show antibacterial activity against the strains being tested. A two-dimensional tube dilution was performed on selected strains to obtain an isobol demonstrating synergy and to rule out an additive effect. Similar results were obtained with methicillin and dicloxacillin.

Identical results were obtained with 30 strains of *Salmonella typhimurium*. *Salmonella* strains that released β-lactamase when subjected to osmotic shock and in which β-lactamase production is episomally mediated were not susceptible to the synergistic action of ampicillin and a penicillinase-resistant penicillin.

Previously I described a method for obtaining penicillinase from strains in which the synthesis of the penicillinase is episomally directed (7). Penicillinase was obtained by subjecting the cells to osmotic shock. The enzyme was purified by diethylaminoethyl (DEAE)-cellulose chromatography followed by hydroxylapatite chromatography. The penicillinase from cells that did not release the enzyme when subjected to osmotic shock was obtained by disrupting the cells with sonic treatment. The sonic extract was dialyzed and then subjected to DEAE-cellulose chromatography by use of a linear, 0 to 0.2 M NaCl gradient in 0.01 M Tris-hydrochloride buffer. Peak tubes were compared for *p*H optimum, inhibition by iodine, competitive inhibition by penicillinase-resistant penicillins, and absorption to surfaces such as glass. No differences were noted. Similarly, neither type of penicillinase was inducible in vivo.

Neither enzyme was excreted during the growth of the cells. The surface penicillinase of all *E. coli* strains, unlike the staphylococcal penicillinase, is entirely cell-bound during growth.

TABLE 4. *Summary of the effect of the penicillinase inhibitor combination with ampicillin*[a]

Determination	No. showing synergism/no. tested	Per cent
Enzyme released by osmotic shock	1/22	4.5
Enzyme not released by osmotic shock	14/18	78

[a] Oxacillin was used as the penicillinase-resistant penicillin. Synergy is defined as a fourfold or greater reduction in minimal inhibitory concentration.

DISCUSSION

These data suggest that there may be two types of β-lactamases in *E. coli*. One is surface located and hence is subject to release by means of the osmotic shock technique used to demonstrate surface enzymes in the *Enterobacteriaceae* (8, 9). The other type of penicillinase is not released by osmotic shock. Essentially all of those strains that release the penicillinase show high-level ampicillin resistance (1,000 μg/ml or greater). In most of these organisms, the synthesis of the β-lactamase is episomally mediated. Strains with low-level resistance (8 to 250 μg/ml) are of the type that do not release the enzyme when subjected to osmotic shock.

The synergistic action of a penicillinase-resistant, semisynthetic penicillin and ampicillin is seen primarily with those strains that have a low level of ampicillin resistance. This might explain the fact that only some *E. coli* strains are susceptible to the synergistic action of two penicillins. Most strains in which the synthesis of penicillinase is episomally mediated will be resistant to the combined action of a nonhydrolyzable penicillin, such as methicillin or oxacillin, and ampicillin. Episomally mediated penicillinase has a high affinity for penicillins such as oxacillin. Indeed, there do not seem to be significant differences between the β-lactamases from strains that release the enzyme during osmotic shock and β-lactamases obtained from strains that do not.

A possible explanation of in vivo differences of the strains in regard to synergistic penicillin action is that the surface enzyme is so advantageously situated that it hydrolyzes the penicillin before it can enter the cell and be recognized by the transpeptidase enzymes concerned with cell wall synthesis. No enzyme is excreted in these organisms, so secretion of the β-lactamase cannot be used to explain the higher resistance. Differences in the enzymes are under study and can only be determined by K_m and K_I data for the purified enzymes. However, it has been difficult to obtain a homogeneous preparation of the β-lactamase that is not released from *E. coli* by osmotic shock.

The technique of osmotic shock provides a rapid means of screening organisms for synergistic penicillin action in the case of *E. coli*. The process can be performed in 45 min on an overnight culture. If the β-lactamase is released there is little

chance that the organism will be susceptible to the synergistic action of two penicillins.

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LITERATURE CITED

1. Ayliffe, G. A. J. 1965. Cephalosporinase and penicillinase activity of gram-negative bacteria. J. Gen. Microbiol. 40:119–126.
2. Datta, N., and P. Kontomichalou. 1965. Penicillinase synthesis controlled by infectious R-factors in *Enterobacteriaceae*. Nature 208:234–241.
3. Datta, N., and M. H. Richmond. 1966. The purification and properties of a penicillinase whose synthesis is mediated by an R-factor in *Escherichia coli*. Biochem. J. 98:204–209.
4. Farrar, W. E., Jr., N. M. O'Dell, and J. M. Krause. 1967. Modification of antibiotic resistance in gram-negative bacteria by penicillinase inhibition. Antimicrob. Agents Chemother.—1966, p. 316–320.
5. Goldner, M., D. G. Glass, and P. C. Fleming. 1968. Characteristics of *Aerobacter* beta-lactamase. Can. J. Microbiol. 14:139–145.
6. Hamilton-Miller, J. M. T., and J. T. Smith. 1964. Inhibition of penicillinase from gram-positive and gram-negative bacteria by substrate analogues. Nature 201:999–1011.
7. Neu, H. C. 1968. The surface localization of penicillinases in *Escherichia coli* and *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. 32:258–263.
8. Neu, H. C., and J. Chou. 1967. Release of surface enzymes in *Enterobacteriaceae* by osmotic shock. J. Bacteriol. 94: 1934–1945.
9. Neu, H. C., and L. A. Heppel. 1964. On the surface localization of enzymes in *E. coli*. Biochem. Biophys. Res. Commun. 17:215–219.
10. Novick, R. P. 1962. Staphylococcal penicillinase and the new penicillins. Biochem. J. 83:229–235.
11. Percival, A., W. Brumfitt, and J. de Louvois. 1963. The role of penicillinase in determining natural and acquired resistance of gram-negative bacteria to penicillins. J. Gen. Microbiol. 32:77–89.
12. Pollock, M. R. 1965. Purification and properties of penicillinases from two strains of *Bacillus licheniformis*: a chemical, physiochemical and physiological comparison. Biochem. J. 94:666–673.
13. Sabath, L. D., M. Jago, and E. P. Abraham. 1965. Cephalosporinase and penicillinase activities of a beta-lactamase from *Pseudomonas pyocyanea*. Biochem. J. 96:739–752.
14. Sabath, L. D., C. E. McCall, N. H. Steigbigel, and M. Finland. 1967. Synergistic penicillin combination for treatment of human urinary-tract infections. Antimicrob. Agents Chemother.—1966, p. 149–155.
15. Shirley, R. L., and J. W. Moore. 1965. *Pseudomonas aeruginosa* urinary tract infection. New Engl. J. Med. 273:283–288.
16. Sutherland, R., and F. R. Batchelor. 1964. Synergistic activity of penicillins against penicillinase from gram-positive and gram-negative bacteria by substrate analogues. Nature 201:868–869.
17. Watanabe, T. 1964. Selected methods of genetic study of episome mediated drug resistance in bacteria. Methods Med. Res. 10:202–220.