THE SURFACE LOCALIZATION OF PENICILLINASES IN ESCHERICHIA COLI AND SALMONELLA TYPHIMURIUM[1]

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The penicillinases (beta-lactamases) of Gram-negative bacteria have been minimally studied in contrast to the penicillinases of Gram-positive species. Datta and Richmond (1966) related that 35 per cent of the penicillinase of E. coli strain TEM was released by the osmotic shock technique of Neu and Heppel (1964). This small release of penicillinase was in contrast to the total enzyme release of a number of degradative enzymes described by Neu and Heppel (1965). Neu and Chou (1967) showed that the technique of osmotic shock for release of "surface" enzymes was applicable to most Enterobacteriaceae. Using the technique of osmotic shock we have been able to achieve 100 per cent release of penicillinase from E. coli and S. typhimurium strains in which synthesis of the penicillinase is episomally mediated. Penicillinases whose synthesis appears to be chromosomally mediated and the penicillinases of several Klebsiella and Citrobacter strains are not released by the osmotic shock technique.

Utilizing the penicillinase released into the osmotic shock fluid we have purified several penicillinases to homogeneity as judged by acrylamide gel and sucrose density gradient centrifugation.

Materials and Methods

E. coli, Salmonella, Enterobacter and Citrobacter strains were obtained from the diagnostic laboratory of the Presbyterian Hospital. Organisms were

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grown in Penassay broth (Difco) or the previously described phosphate medium (Neu and Chou, 1967). The techniques for osmotic shock are those described by Neu and Chou (1967). Presence of R-factors was determined by the method of Watanabe (1964). Penicillinase activity was determined by Novick's (1962) iodometric method or microbiological assay using Sarcina lutea (Citri, 1964). The assay for 5'-nucleotidase was described (Neu, 1967).

Results and Discussion

E. coli and Salmonella typhimurium strains that posses an R-factor which mediates the synthesis of penicillinase totally release the penicillinase when subjected to a sudden osmotic transition (Table I). None of the penicillinase is released when the cells are growing; nor is there excretion of the enzyme by

TABLE I

Organism	Per cent enzyme released		R-factor
	Penicillinase	5'-nucleotidase	
E. coli			
E1	88	93	A, T, S
FW/R126	85	92	A, C, T, Su
DB103	100	95	A, T, Sm
DB11	87	97	C, A, T, Su, K
DB117	95	96	A, C
K12 A100	78	90	A, T, S
S. typhimurium			
100-T	82	--	A, C, T
Sa-100	97	--	A
E. aerogenes			
DB27	52	59	A, T, C, Sm

Organisms were grown to early stationary phase in Penassay broth. They were harvested and washed in 0.85% NaCl. Cells were suspended in 0.5 M sucrose-0.03 M Tris-HCl, pH 7.5 at 21° at a ratio of 1 g (wet weight) to 80 ml of sucrose-Tris. EDTA was added to a concentration of 1 mM and after 5 minutes of mixing the cells were removed by centrifugation. The pellet of cells was resuspended in water at 3°, mixed for 5 minutes and the cells again removed by centrifugation. Penicillinase and 5'-nucleotidase were assayed in the osmotic shock fluid. The cells were sonically disrupted, and the enzymes remaining in the cells after osmotic shock determined. R-factors were determined previously by passage of the ampicillin marker into a sensitive test strain by the method of Watanabe. T = Tetracycline, A = Ampicillin, C = Chloramphenicol, Su = Sulphonamide, K = Kanamycin, Sm = Streptomycin.

late stationary phase cells. This release of penicillinase is identical with that seen for the 5'-nucleotidase and cyclic phosphodiesterase as originally noted by Neu and Heppel (1964). Cells that have released the penicillinase are able to grow normally but are susceptible to penicillin immediately after they have released their penicillinase. This is not due to alteration of permeability caused either by the EDTA-Tris or the osmotic shock, since shocked cells that do not release their penicillinase grow normally in the presence of ampicillin (Figure 1). The antibiotic resistant pattern conferred by the R-factor did not seem to influence the location of the penicillinase.

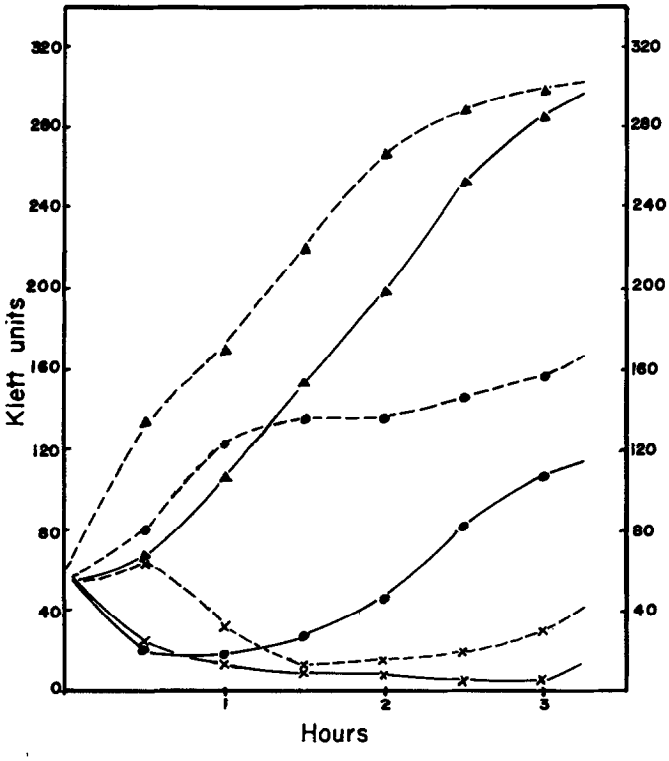

Figure 1. Regrowth of osmotically shocked exponential phase E. coli in ampicillin. E. coli were grown to mid-exponential phase in the high phosphate medium (Neu and Chou, 1967). They were suspended in 0.5 M sucrose-0.03 M Tris, pH 7.5, 0.1 mM EDTA for 5 minutes and then the cells were removed by centrifugation. The cells were resuspended in 5 x 10^{-4} M $MgCl_2$ at 3° for 5 minutes and then removed by centrifugation. The cells were finally resuspended in high phosphate medium to a volume equal to the original sucrose-Tris and diluted 10-fold in medium. Growth was followed with the Klett. FW/R126 in this medium reaches a level of only 200 Klett units as compared with DB100 at 300. Solid line denotes ampicillin 30 μg/ml. Dash line indicates no ampicillin. ▲ DB100 (chromosomal penicillinase); ● FW/R126 (episomal penicillinase); ✕ K12 (sensitive).

TABLE II

Organism	Per cent enzyme released		R-factor
	Penicillinase	5'-nucleotidase	
E. coli			
DB5	10	97	None
DB21	0	94	C, T, Su
DB7	0	88	S, C, Su
DB30	0	95	None
DB120	0	80	None
DB25	5	92	C, Su
Enterobacter			
DB33	7	58	C, T
DB38	5	47	C, T, K
Citrobacter			
DB115	3	48	None

Organisms were grown to early stationary phase in Penassay broth. They were subjected to osmotic shock as detailed in Table I. Penicillinase was assayed in the osmotic shock fluid and in sonically disrupted cells that had been shocked. In addition to the iodometric assay, a microbiological assay was used to test the shock fluid. Sonically prepared extracts were tested with the iodometric assay using 6-aminopenicillanic acid as substrate to obviate the chance that resistance was due to amidase formation. R-factor transfer was by Watanabe's method (1964). Cells grown to mid-exponential phase or grown in phosphate medium gave identical results.

As Table II illustrates, the penicillinase was not released by osmotic shock from organisms in which ampicillin resistance was not R-factor mediated and apparently chromosomally directed. It is not valid, however, to state that the penicillinase whose synthesis is chromosomally mediated is not a "surface" enzyme. Failure to show episome transfer does not exclude a defective episome.

Several strains of *Klebsiella* which produced penicillinases were subjected to osmotic shock. Only one strain of *Enterobacter aerogenes* released a penicillinase. In this organism there was an R-factor for ampicillin resistance. However, as noted in Table I, penicillinase activity was still present in the cells. *Enterobacter* species consistently release only 50 per cent of 5'-nucleotidase. The penicillinase that remains in the cells could be

unreleased penicillinase whose synthesis is episomally mediated or a chromosomally directed enzyme.

Using the osmotic shock technique we have purified the penicillinases of two E. coli and one S. typhimurium strains. The osmotic shock fluid was applied to a DEAE cellulose column (Whatman 32) and the penicillinase eluted with a linear 0 to 0.25 M NaCl gradient in 0.01 M Tris, pH 7.5. The enzyme was concentrated by negative pressure in a collodion bag and applied to a hydroxylapatite column. Penicillinase fails to adsorb and passes through the column leaving other enzymes bound to the column. Material at this stage is pure as judged by molecular seive chromatography and sucrose-density gradient centrifugation. A single protein and enzymatic band is found on acrylamide gel electrophoresis (Figure 2).

Both E. coli and S. typhimurium penicillinases have pH optima of 7.2 to 7.5. Only 30 per cent of activity is lost after 10 minutes at 60°. The enzymes do not adsorb to glass nor to cell membranes. The enzymes hydrolyze

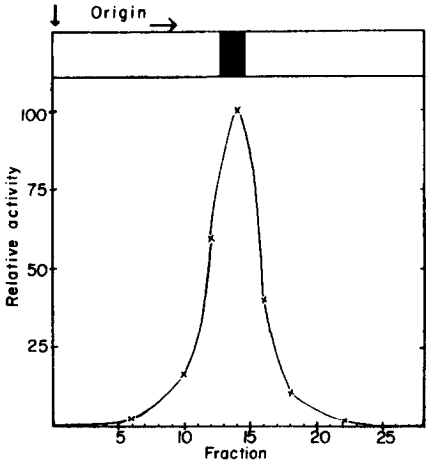

Figure 2. Acrylamide gel electrophoresis of E. coli DB103 using 7% polyacrylamide in Tris-glycine buffer, pH 8.9 for 1 hour at 15°. The fraction, 50 µg, was applied in 0.01 Tris, pH 7.5 to two gels. One gel was stained with Amido schwarz dye. The other gel was sliced into 28 equal fractions. The enzyme was eluted by shaking in 0.2 ml of 0.01 M potassium phosphate, pH 7.2 at 3°. The iodometric assay was used for penicillinase.

benzylpenicillin G and ampicillin with a Km of 10^{-5} M. Cephaloridine is hydrolyzed almost as well as benzylpenicillin. The enzymes have a high affinity for oxacillin and cephalosporin but hydrolyze these substrates poorly. EDTA and urea had no effect on the enzymes. In contrast with the TEM penicillinase of Datta and Richmond (1966) iodine did not inhibit these penicillinases. These enzymes do not seem to be induced by benzylpenicillin or the semisynthetic penicillins such as oxacillin. Phosphate content of the growth medium does not affect the enzyme synthesis.

These data show that penicillinases of E. coli and S. typhimurium whose synthesis is episomally mediated are totally released by osmotic shock. They can thus be grouped with a number of other enzymes which are felt to be "surface" or "periplasmic" enzymes. Preliminary studies suggest that the penicillinases whose synthesis is chromosomally directed are bound to the cell more firmly and possess different enzymatic properties.

Studies of the amino acid composition and enzymatic properties of several E. coli, Salmonella and Enterobacter penicillinases are in progress to compare the enzymes.

References

Citri, N., Methods Med. Res. 10, 221 (1964).
Datta, N. and Richmond, M. H., Biochem. J. 98, 204 (1966).
Neu, H. C., J. Biol. Chem. 242, 3896 (1967).
Neu, H. C. and Chou, J., J. Bacteriol. 94, 1934 (1967).
Novick, R. P., Biochem. J. 83, 236 (1962).
Watanabe, T., Methods Med. Res. 10, 202 (1964).