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Characterization of β-Lactamases In Situ on Polyacrylamide Gels

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An inhibitor-based characterization system which allowed the identification of beta-lactamases after isoelectric focusing on polyacrylamide gels was developed. This system, using potassium clavulanate and oxacillin, distinguished type I chromosomally mediated enzymes from other beta-lactamases of gram-negative bacteria.

The use of analytical isoelectric focusing to characterize beta-lactamases was first described by Matthew et al. in 1975 (2). Since then, several modifications of the original method of Matthew et al. have been made to enhance the utility of the procedure. During studies of the type I chromosomal beta-lactamases of gram-negative bacteria, we encountered problems in distinguishing minor bands on gels that also contained major bands. Also, it became necessary to distinguish between plasmid- and type I chromosomally mediated beta-lactamases in strains possessing both types. This report describes a method that provides an easy solution to both of these problems.

Beta-lactamases were focused in 7% polyacrylamide gels (165 by 215 by 2 mm) containing pH 3 to 10 ampholytes by the method of Vecoli et al. (5) with the following modifications. The gels were focused across the width at 4°C and 1,600 V for 90 min by using an LKB Multiphor unit (LKB Instruments, Inc., Rockville, Md.). Crude preparations of beta-lactamases were obtained by sonication of cell pellets harvested from overnight cultures in Mueller-Hinton broth. The organisms used were (i) various derepressed mutants of *Enterobacter cloacae* and *Pseudomonas aeruginosa* producing type I beta-lactamases constitutively, which were isolated in this laboratory, (ii) *Escherichia coli* A20343 (kindly provided by F. Leitner of Bristol Laboratories) and *E. cloacae* P99 (kindly provided by L. Koupal of Merck Sharp & Dohme), which also produce type I beta-lactamases constitutively, (iii) *Proteus mirabilis* A20342 and *Klebsiella pneumoniae* A20346 (kindly provided by F. Leitner), which produce chromosomal beta-lactamases of Richmond and Sykes types II and IV, respectively, and (iv) various *E. coli* and *P. aeruginosa* strains possessing well-characterized plasmid-mediated beta-lactamases of Richmond and Sykes types III and V (kindly provided by A. A. Medeiros of Brown University).

The usual method for visualizing beta-lactamase bands after isoelectric focusing involves the placement of filter paper moistened with nitrocephin on the focused gel (2). As noted by Matthew et al., major bands visualized by this method appear rapidly and may diffuse before the appearance of some weakly reacting minor bands (2). Thus, serial photographs are needed to obtain a complete record of gels containing both major and minor bands. Because a number of beta-lactamases have both major and minor bands, a modified detection system that did not require serial photographs to be taken was developed. After focusing at 4°C, the gel was overlaid with molten agar (3.8%; Difco Laboratories, Detroit, Mich.) containing 50 μg of nitrocephin per ml. Once the agar had hardened, beta-lactamases appeared as pink bands in a yellow background. The agar diminished diffusion of the major bands while minor bands were developing and allowed a single picture to be taken as long as 1 h after the application of the nitrocephin agar. The developed gel was then photographed with a Polaroid MP-4 camera using a Tiffen 58 dark green filter and type 51 high-contrast film. The visualization in a single photograph of distinct minor satellite bands surrounding a single major band is shown in Fig. 1, lanes 1, 2, 3, and 5.

A second problem encountered was the need to distinguish rapidly between chromosome- and plasmid-mediated beta-lactamases. Many strains can possess both types of enzyme, and pIs of the enzymes can overlap (1, 3, 4). This problem was solved by developing an inhibitor-based characterization system which could be applied directly to focused gels. In this system, filter paper is moistened with solutions containing various beta-lactamase inhibitors, and the paper is applied to the focused gel surface for 10 to 20 s. The paper is then removed, and the molten nitrocephin agar is overlaid on the surface of the gel. If the beta-lactamase is susceptible to the inhibitor at the concentration applied, bands will not develop in the nitrocephin agar in the first 10 min. Preliminary studies are required to determine the necessary concentration of inhibitor to distinguish between the enzymes likely to be encountered.

This inhibitor-based characterization system was used to distinguish type I chromosomally mediated beta-lactamases from other beta-lactamases of gram-negative bacteria. In this particular study, filter paper moistened with 1,000 μM cloxacillin or 1,000 μM potassium clavulanate was used. Type I beta-lactamases which are highly susceptible to cloxacillin did not develop after treatment with this inhibitor, whereas the other beta-lactamases, which are less susceptible, did develop (Fig. 1, lanes 8 to 13). Conversely, type I beta-lactamases are relatively resistant to potassium clavulanate, so only these developed after treatment of the gel with this inhibitor (Fig. 1, lanes 15 to 20). Crude enzyme preparations were applied to the gel in 10-μl samples. Preparations can be applied undiluted if 50 μl does not completely hydrolyze 150 μl of a 50-μg/ml nitrocephin sample faster than 5 s. More active preparations must be diluted. This system was used to successfully distinguish between type I chromosomally mediated beta-lactamases

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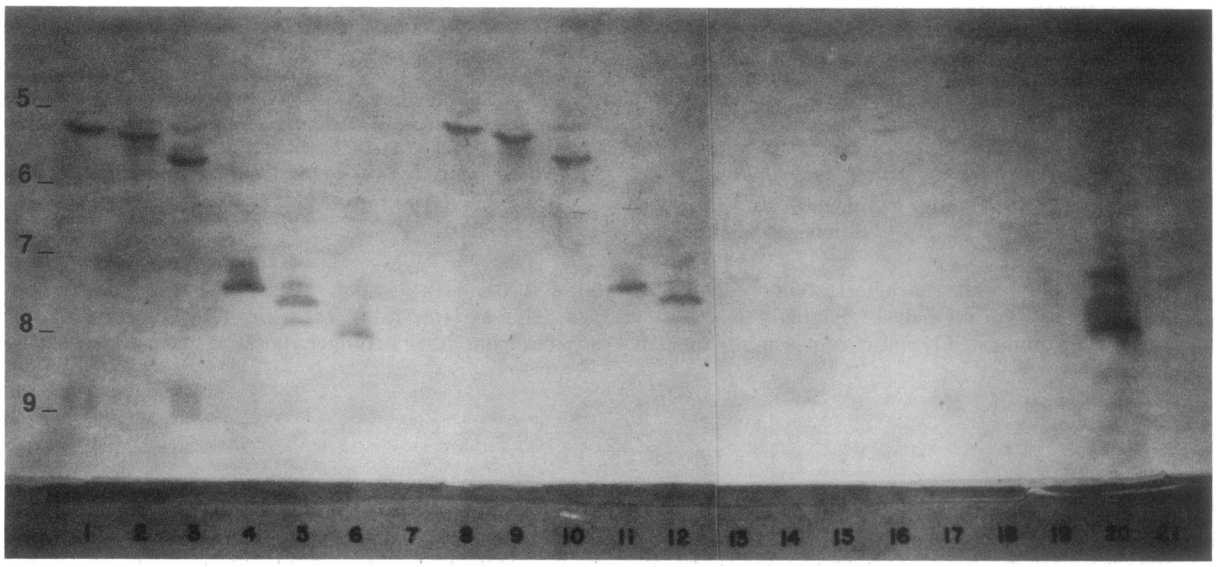

FIG. 1. Isoelectric focusing of beta-lactamases using nitrocephin agar for detection and inhibitor-based characterization system. Lanes 1 to 6 were untreated before being overlaid with nitrocephin agar. Lanes 8 to 13 were treated with 1,000 μM cloxacillin, and lanes 15 to 20 were treated with 1,000 μM potassium clavulanate before being overlaid with nitrocephin agar. The enzymes in each lane were as follows: *P. aeruginosa* Pu 21(pMG 19), PSE-4 in lanes 1, 8, and 15; *E. coli* RTEM(R6K), TEM-1 in lanes 2, 9, and 16; *P. aeruginosa* Pu 21(RPL-11), PSE-1 in lanes 3, 10, and 17; *E. coli* J53(R1010), SHV-1 in lanes 4, 11, and 18; *K. pneumoniae* A20346, type IV in lanes 5, 12, and 19; and *E. cloacae* P99, type I in lanes 6, 13, and 20. Numbers at left indicate pH.

and type III plasmid-mediated enzymes (TEM-1, TEM-2, SHV-1, HMS-1, PSE-1, PSE-3, and PSE-4), type V plasmid-mediated enzymes (OXA-1, OXA-2, OXA-3, and PSE-2), and type IV chromosomally mediated enzymes. Type II penicillinases did not visualize in the nitrocephin agar system. Thus, this inhibitor-based characterization system using cloxacillin and potassium clavulanate was used to distinguish type I beta-lactamases from all other major Richmond and Sykes types. It also distinguished between these enzymes when they were applied to the gel as mixtures.

It should be possible to extend this inhibitor-based characterization to other beta-lactamase inhibitors, which would allow further differentiation of the enzymes in situ on polyacrylamide gels. The development of the nitrocephin agar overlay was a necessary first step which allowed the subsequent development of the inhibitor-based characterization system.

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