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Simple Screening Method for Isolation of Penicillin Acylase-Producing Bacteria

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A new screening method for bacteria capable of producing penicillin acylase is described. The method is based on the use of *Serratia marcescens* sensitive to 6-aminopenicillanic acid but comparatively resistant to benzylpenicillin. It is simple, quite specific, and requires no special equipment. It can also be used to screen for phenoxymethylpenicillin acylase activity. We also suggest an acidimetric method for rapid detection of cloned genes in genetic engineering studies of penicillin acylase.

Microorganisms are an important source of penicillin acylase (EC 3.5.1.11), which hydrolyzes penicillins to 6-aminopenicillanic acid (6-APA), the starting material for the manufacture of several semisynthetic antibiotics. Consequently, many methods have been developed to screen for microorganisms that produce this enzyme. They include the use of benzylpenicillin as the sole source of carbon in the screening medium in which the 6-APA formed is measured by bioassay after treatment with phenylacetyl chloride (5). Walton (9) has modified this method by incorporating colorless phenylacetyl-*p*-nitroaniline into the screening medium. Upon hydrolysis, the substrate gives yellow *p*-nitroaniline, the appearance of which is the basis for selecting acylase producers. However, this method is not very sensitive, owing to the low solubility of the substrate. A comparable screening method was suggested by Baker (1), using ampicillin as the substrate for enzyme hydrolysis. His method is based on the observation that ampicillin, but not 6-APA, gives a characteristic green color with the biuret reagent. This test, however, is not very practical either since the color fades rapidly. Recently, Szewczuk et al. (8) have suggested a method which uses paper disks saturated with phenylacetyl-4-aminobenzoic acid as a substrate for such a screen. After contact with bacterial colonies, the paper disk is treated with specific reagents; red spots develop only where the paper disk has previously contacted colonies of penicillin acylase-producing bacteria. The intensity of the red spots, measured densitometrically, is reported to correlate quantitatively with the acylase activity in the bacterial colonies. The disadvantage of this method is that it involves several steps and that some solutions for color development have to be freshly prepared. Moreover, it generally works well with bacteria capable of producing only cell-bound but not extracellular acylases.

We were interested in developing a simple and rapid method for screening bacteria producing either cell-bound or extracellular penicillin acylase. We modified a microbiological method for detection of 6-APA (6) and an acidimetric method for the detection of β-lactamase (4). Both methods are simple and require no special reagents or equipment. The microbiological method is quite sensitive and specific, and the acidimetric method is rapid and sensitive but nonspecific. Consequently, the latter method is suitable for the detection of acylase-producing colonies in specific cases, such as a search for the transfer of the penicillin acylase gene to recipient bacteria.

The following reagents were used: 6-APA, benzylpenicillin, phenoxymethylpenicillin, ampicillin, phenylacetic acid (PAA), and bromcresol green (Sigma Chemical Co., St. Louis, Mo.); methyl red (Difco Laboratories, Detroit, Mich.); resorcin blue and litmus (BDH Chemicals Ltd., Poole, England); chlorphenol red, sodium alizarin sulfonate, and ready-made silica gel thin-layer chromatography plates (article no. 5554; E. Merck AG, Darmstadt, West Germany); and methylene blue (BBL Microbiology Systems, Cockeysville, Md.).

Only isolates of *Escherichia coli* and *Bacillus* spp. were examined. To obtain isolates of *E. coli*, fecal specimens were streaked onto eosin methylene blue agar (Difco) plates. Those which developed a characteristic metallic sheen were selected and confirmed to be *E. coli* by biochemical tests (7). Only 1 isolate was selected from each fecal sample, and a total of 411 isolates were collected. *E. coli* ATCC 9637, a known penicillin acylase producer, was used as a reference strain. The organisms were maintained on

nutrient agar (Difco) slants at 4°C and transferred biweekly until they were used.

To obtain *Bacillus* strains, a total of 176 soil samples were suspended in sterile distilled water (ca. 0.5 g/5 ml of water), heated in a water bath at 60°C for 20 min, diluted, and plated on nutrient agar. Only one colony that resembled *Bacillus* spp. was selected from each soil sample. The isolates were maintained on nutrient agar slants at 4°C along with a stock culture of *Bacillus megaterium* ATCC 14945, a known penicillin acylase producer.

For the microbiological screening method, isolates of *E. coli* were point inoculated on nutrient agar plates containing 0.15% PAA as an inducer. In the case of *Bacillus* spp., PAA was omitted since it is toxic to *Bacillus* strains. After overnight incubation (*E. coli*, 37°C; *Bacillus* spp., 28°C), the plates were overlaid with 5 ml of nutrient agar containing 5 mg of benzylpenicillin per ml. Then 0.2 ml of an 18-h culture of *Serratia marcescens* ATCC 27117 (ca. 4.1×10^9 cells per ml), grown in liquid broth containing 1% peptone and 0.5% sodium chloride, was spread over the hardened upper layer. The test plates were incubated overnight at 28°C. Failure of growth of *S. marcescens* resulted in clear inhibition zones around colonies of test isolates and provided presumptive evidence for penicillin acylase activity. (With this strain of *S. marcescens*, inhibition zones around the wells in agar plates could be seen with 4.5 μg of 6-APA delivered in 30 μl of distilled water.)

To confirm presumptive production of acylase, the isolates of *E. coli* were grown at 28°C for 18 h in liquid medium containing 0.5% Bacto-Peptone (Difco), 0.3% beef extract (Difco), and 0.15% PAA, pH 7.0. The culture was centrifuged, and the sediment was suspended and readjusted to 30 mg (cell dry weight) per ml with 0.05 M phosphate buffer, pH 7.5. The reaction mixture contained 5 mg of benzylpenicillin per ml, phenoxymethylpenicillin or ampicillin, and 1 ml of bacterial suspension (30 mg [cell dry weight]) in 5 ml of phosphate buffer (0.05 M, pH 7.5). The mixture was incubated at 40°C for up to 1 h, and 0.5 ml of the reaction mixture was then assayed for 6-APA by the *p*-dimethylaminobenzaldehyde method (2).

As a further check for acylase activity, a small amount of the cell-free supernatant was examined by silica gel thin-layer chromatography with butyl acetate–1-butanol–acetic acid–water (80:15:40:24) as the running solvent for 1.5 h. For the detection of benzylpenicillin and 6-APA, the chromatograms were air dried, and one of three methods was used: (i) incubating in an iodine chamber, (ii) assaying microbiologically with *S. marcescens* (6), or (iii) spraying with methanol-sulfuric acid (50:50) and heating.

Penicillinase (β-lactamase) was assayed by the method of Catlin (3).

Only confirmed acylase-producing *E. coli* isolates were further tested by the acidimetric method. They were grown overnight at 28°C on nutrient agar with 0.15% PAA. Then each colony was transferred as a spot on a filter paper (Whatman no. 1; diameter, 7.0 cm) saturated with benzylpenicillin (20 mg/ml) and 0.04% chlorphenol red. The filter paper was then incubated at 40°C for 1 h. The appearance of a yellow zone around the spot indicated the formation of penicillin acylase by the corresponding colony, whereas a violet color indicated its absence.

Of 411 isolates of *E. coli* and 176 of *Bacillus* spp., only 20 and 5 isolates, respectively, caused clear inhibition zones of *S. marcescens*. The diameters of the clear zones varied from 1.0 to 2.0 cm and depended on the sizes of the colonies and the thickness of growth of *S. marcescens*. Comparison of the zone size with the results of the acylase assay (*E. coli* only) showed no quantitative correlation, and hence zone size was considered unreliable as a quantitative indication of enzyme production. Each of the 20 isolates of *E. coli* and the stock culture (ATCC 9637) gave positive results by the acidimetric method and negative results by the penicillinase assay. As expected, the acidimetric test was not specific since it also gave positive results with isolates which were acylase negative but penicillinase positive.

To eliminate the possibility that inhibition of *S. marcescens* was caused by a substance(s) other than 6-APA, a total of 27 isolates of *E. coli* and *Bacillus* spp. were tested by the usual protocol except that benzylpenicillin was removed from the agar overlayer. With all of the 20 *E. coli* isolates and with *E. coli* ATCC 9637, no clear zones were observed. On the other hand, of the five isolates of *Bacillus* and the reference *B. megaterium*, four showed inhibition zones in the absence of benzylpenicillin, indicating the production of some inhibitory substance(s) other than or in addition to 6-APA.

Chromatography of the reaction mixtures of *E. coli* strains 194 and ATCC 9637 showed the presence of benzylpenicillin and 6-APA (R_f, 0.7 and 0.25, respectively) as detected both chemically and microbiologically.

Furthermore, a substrate specificity test of the enzyme from strain 194 showed that its activity was of the benzylpenicillin acylase type, as it was about 10 times more active with benzylpenicillin and about 7 times more active with ampicillin than with phenoxymethylpenicillin.

These results have shown that our procedure is a simple and reliable way of detecting bacteria which produce both cell-bound and extracellular penicillin acylase. It is equally applicable to

TABLE 1. Final pH values for nutrient broth with various additions of benzylpenicillin, 6-APA, or PAA

Substance (mg/ml) added	Final pH
None	6.8
Benzylpenicillin (5)	6.4
6-APA (2)	4.7
PAA (1)	4.4
6-APA (2) + PAA (1)	4.4

benzyl- and phenoxymethylpenicillin, and it is easier to use than methods previously described (8, 9).

The acidimetric method described here for detecting acylase activity is based on the acidity produced after the enzymatic cleavage of benzylpenicillin to 6-APA and PAA. Assuming 60% conversion and starting with 5 mg of benzylpenicillin, ca. 2 mg of 6-APA and 1 mg of PAA would be obtained (Table 1). These products lower the pH of nutrient broth to 4.4. We have tested various indicators that change color within this range (pH 4.4 to 6.4): methyl red (0.04% in water), resorcin blue (0.5% in ethanol), litmus (0.5% in water), chlorphenol red (0.04% in water), sodium alizarin sulfonate (1% in water), bromcresol green (0.04% in water), and mixtures of bromcresol green (0.075%) with methyl red (0.05%) or methyl red (0.1%) with methylene blue (0.05%). Only chlorphenol red was a reliable indicator for the purpose. Although the test was not specific, it provided a rapid selection method for detecting penicillin acylase in specific cases, e.g., to screen for successful gene transfer to penicillinase-deficient recipients.

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