SCREENING FOR β-LACTAM ANTIBIOTICS IN NATURE

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The discovery and development of penicillin has been retold many times incorporating numerous personal and scientific perspectives. However, throughout the various interpretations one fact remains undisputed, the discovery of penicillin was a chance observation.

In 1945, GUISEPPE BROTZU designed his experiments to look specifically for antibiotic activity among the organisms of sewage effluent. The activity pursued by BROTZU was that elaborated by a strain of *Cephalosporium acremonium*, subsequently identified as penicillin N[1]. An additional antibiotic produced by BROTZU's *C. acremonium* strain and the origin of all true cephalosporins was discovered only by a kind of afterthought through careful experimentation and observation[2].

For the next 15 years following the discovery of cephalosporin C, naturally-produced β-lactam containing molecules lay undiscovered in the soil. No naturally-occurring β-lactam molecules of novelty were reported during this "golden era" of antibiotic research. By the 1970's this situation had changed dramatically with new and novel molecules being reported in relative abundance. This sudden change in fortune can be attributed to a number of factors foremost among which were the development of "mode of action" screens and advances in isolation technology.

In 1971, NAGARAJAN and his colleagues[3] reported their discovery of the cephamycins (7-α-methoxycephalosporins) as products of streptomycetes. These molecules were detected in screens developed to look for agents producing morphological changes in sensitive bacteria and thus represent the first naturally occurring β-lactams to emerge from directed screening procedures. Following the discovery of the cephamycins came clavulanic acid and the carbapenems, molecules detected on the basis of their ability to inhibit β-lactamases[4].

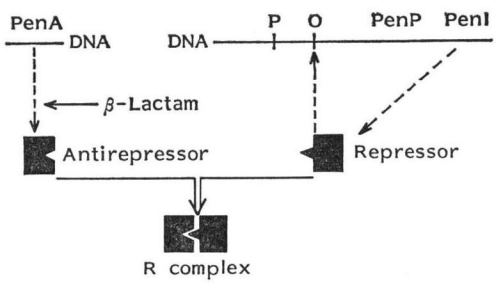
One of the most successful directed screening technologies for β-lactams has been the use of β-lactam antibiotic-supersensitive mutants. Using a supersensitive mutant of *Pseudomonas aeruginosa*, KITANO and his colleagues[5] reported on the production of penicillins and cephalosporins from fungal strains previously not associated with the production of these compounds. In a similar vein, workers at Fujisawa[6], discovered a new series of monocyclic β-lactams produced by *Nocardia* species, utilizing a supersensitive mutant of *Escherichia coli*.

The latest development in this ever broadening field has been the discovery of β-lactam antibiotics produced by bacteria. Within the last few years, bacteria have been shown to produce a series of novel monocyclic β-lactam antibiotics (monobactams), a carbapenem and a series of cephalosporins[7~10].

All of the above described bacterially produced molecules reported by workers at Squibb, were detected in screens developed to look for the induction of β-lactamases. Activated β-lactam containing molecules interact with two main groups of enzymes; the cell wall synthesizing enzymes *e.g.* transpeptidases, and the β-lactam hydrolyzing enzymes or β-lactamases. At the start of our studies, the only known substrates for β-lactamases were β-lactam antibiotics. In addition to acting as substrates, these molecules also possess the ability to induce β-lactamase production in certain strains of Gram-positive and Gram-negative bacteria. It is well recog-

Fig. 1. Model proposed for the regulation of penicillinase production.

P and O are the promoter and operator sequences for the penicillinase structural gene (PenP). PenI is a regulatory gene specifying the penicillinase repressor, and PenA is a regulatory region specifying a penicillinase antirepressor protein. Formation of the repressor/antirepressor complex (R complex) which takes place only when inducer molecules are present, inactivates the penicillinase repressor. Subsequently transcription occurs and penicillinase synthesis begins.



nized that penicillins and cephalosporins can increase the level of β-lactamase production in such strains as *Staphylococcus aureus, Bacillus* sp., enteric organisms and *P. aeruginosa*. A model for regulation of penicillinase production in Gram-positive bacteria was proposed by IMSANDE[11] the main features of which are shown in Fig. 1.

Many methods have been described for the detection of β-lactamases[12], one of the simplest being the use of chromogenic substrates.

In one of our screens we employed a strain of *Bacillus licheniformis* that in the absence of β-lactam antibiotics produces minimal levels of β-lactamase activity. However, in the presence of β-lactams, large amounts of enzyme are produced which can be detected in liquid or solid media by the use of a chromogenic cephalosporin substrate. This methodology has proved to be highly specific for β-lactam containing molecules. The only false positives to emerge so far are a group of bacterially produced β-lactones[7]. The methodology for such a screening system is described below.

Microorganism

B. licheniformis SC 9262 (ATCC 14580) was grown overnight in 500-ml Erlenmeyer flasks containing 300 ml of antibiotic assay broth (AAB) (Baltimore Biological Laboratory, Cockeysville, Maryland). The flasks were incubated at 28°C on a rotary shaker (150 rpm, G10 gyrotory shaker, New Brunswick Scientific Co., New Brunswick, New Jersey).

β-Lactamase Induction Assay

A 20% inoculum of the overnight *B. licheniformis* culture was added to BA2 agar (cooled to 50°C), consisting of (g/liter): BBL seed agar 30.5 and NaCl 5.0 in distilled water. Immediately following inoculation the agar was dispensed into Petri dishes. Paper discs containing fermentation broths or compounds to the tested were placed directly onto the agar surface. Inoculated plates were allowed to incubate 2~3 hours at 37°C and then overlayed briefly with a solution of chromogenic cephalosporin SQ 24,902 (500 μg/ml), dissolved in a small volume of dimethyl sulfoxide and then diluted in 0.05 M, of pH 7.0 phosphate buffer). β-Lactamase inducing compounds were detected by the rapid appearance of a red zone around the discs as a result of hydrolysis of the chromogenic cephalosporin.

The sensitivity of the induction assay was compared to a morphological screen and a screen using a β-lactam supersensitive strain of *E. coli*. As can be seen from the Table 1, the induction assay was orders of magnitude more sensitive than the morphological assay and 15-fold more sensitive than the β-lactam supersensitive *E. coli* strain.

Table 1. Detection limits of the various assay procedures for potassium benzylpenicillin.

Assay	Quantity detected (ng)
B. licheniformis SC 9262 β-Lactamase induction	1
E. coli[a] SC 12155 Inhibition	15
P. mirabilis[b] SC 3855	
a) elongated cells	200
b) spheroplasts	3,000

[a] *E. coli* SC 12155 is a β-lactam supersensitive mutant described by AOKI *et al.*[6].

[b] Observations were made following incubation of an overnight broth culture (diluted 1:50), in the presence of varying concentrations of penicillin for 3~4 hours in a 37°C water bath.

Characterization of β-Lactamase Inducers

Characterization of β-lactamase inducers from fermentation broths was carried out by the use of thin-layer chromatography (TLC) and high voltage electrophoresis (HVE). TLC of broth filtrates was carried out on either F1440 cellulose plastic sheets (Schleicher and Schuell, Keene, New Hampshire) or polysilicic acid gels impregnated glass fiber sheets (Gelman Sciences, Inc., Ann Arbor, Michigan). Various ratios of acetonitrile - H_2O were employed as the solvent system *e.g.* 3:2, 7:3, 4:1, or 5:1. The developed chromatograms were dried and then placed on assay plates containing *B. licheniformis*. Following a 30-minute incubation period at 37°C, the chromatograms were removed and the plates reincubated for an additional 1.5~2 hours. Inducing material was detected by overlaying the plates with chromogenic cephalosporin as described previously.

HVE was performed on a CAMAG high voltage paper electrophoresis system (CAMAG, Muttenz, Switzerland). Paper electrophoresis was run at 2,000 volts for 30 minutes at pH 2.4 (10% acetic acid), pH 7.0 (0.05 M phosphate buffer) or pH 9.0 (0.2 M borate buffer). On completion of the electrophoresis, the pherogram was removed, dried and layered onto a *B. licheni-*

formis assay plate as previously described.

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