AN AGAR PLATE METHOD FOR THE SCREENING OF ANTIBIOTICS TRIGGERING AUTOLYTIC ENZYMES

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The strategy of destroying a pathogen with its own autolytic enzymes is very appealing. Recently KITANO and TOMASZ[1] showed that the relative effectiveness of β-lactams in autolysin triggering was parallel to the effectiveness of the same drugs in causing rapid loss of viability, culture lysis and spheroplast formation. The first direct evidence of the role of autolytic enzymes in killing due to the inhibition of cell wall synthesis was provided by TOMASZ *et al.*[2].

Though cell-wall-active antibiotics are known to cause bacterial lysis no systematic attempts have been made to specifically search for agents inducing autolytic enzymes in bacteria. The lack of development of an appropriate screening test is largely responsible for this situation. This in turn is due to the fact that antibiotic-induced bacterial lysis has remained a relatively poorly understood phenomenon[3].

Two conventional methods used for the detection of lysis are: (1) Drop in the optical density of the antibiotic-treated growing culture and (2) microscopic examination of antibiotic treated bacteria to observe spheroplast formation due to autolytic cell wall degradation[1]. Both these methods are inherently slow and not suitable for testing the lytic activity of hundreds of broth samples.

Recently, KITANO and TOMASZ[1] have developed a sensitive test system which measures autolytic cell wall degradation by measuring the radioactivity liberated from the drug-treated bacteria containing radioactive diaminopimelic acid in its cell wall. However, in the opinion of TOMASZ, this method is too complicated to be suitable as a primary stage screen[4].

We have studied the β-lactam and other cell-wall-active antibiotics mediated induction of lysis in resting cells of *Escherichia coli* and developed a simple rapid screening method for detecting agents capable of triggering autolysin. In this test a mutant *E. coli* M6, supersensitive to β-lactams is employed with the intention of increasing the sensitivity of the method. The method is as follows: 4 ml of 6 hours old broth culture of *E. coli* M6 is inoculated into 40 ml of nutrient medium containing 0.25% glucose and 0.5% each of peptone (Oxoid, U. K.), beef extract (Oxoid, U. K.) and sodium chloride, pH 7.0. The culture is grown for 4 hours at 37°C in a New Brunswick water bath shaker operating at a speed of 100 rpm. Cells are harvested by centrifuging at $10,000 \times g$ for 10 minutes at 10°C and washed by suspending them in one half the culture volume of 0.2 M Tris-maleate buffer of pH 7.0. Cell suspension is recentrifuged under similar conditions, supernatant discarded and cells are resuspended uniformly in the same buffer to give an O. D. of 0.65~0.7 at 430 nm. The cell suspension is then mixed with an equal volume of 2.4% Difco Bacto Agar. 40 ml of this heavily seeded agar is poured into a Petri dish of 15 cm diameter and allowed to set. Wells (6.5 mm diameter) are punched and 20 to 50 μl of test broths or solutions of antibiotics are dispensed in each. Plates are incubated at 37°C for 16 hours. Zones of clearance due to autolysis are visible around the wells for appropriate compounds.

Thus, the most useful feature of this method is that triggering of lysis is carried out with a non-growing culture and it permits visual observation of lysis. Hence it does not involve the use of radioactive chemicals and other sophisticated equipment.

It is also found that amino acids added individually to the buffered agar (10~15 μg/ml) stimulate the lytic activity of cell-wall-active antibiotics. Zones of lysis formed in the presence of amino acids are clearer, larger and sharper, thus enabling in some cases, the detection of 0.1 to 0.5 μg of compound. The effect of amino acids is non-specific in nature and among various amino acids tested asparagine and lysine caused marked increase in the lysis triggering activity of various antibiotics (Table 1). The exact reasons for the stimulatory effect of amino acids is not known. Perhaps it involves some aspect of the stringent control system. GOODELL and TOMASZ[5] have demonstrated that in stringently controlled bacteria, amino acid starvation has protective effect against penicillin-induced lysis. Another very interesting observation made was that triggering of lysis in 'resting cells' is inhibited by

Table 1. Lytic activity of various antibiotics.

Antibiotic	Concentration (μg/well)	Lysis zone diameter (mm)			
		Additions to 0.1 M Tris-maleate buffer agar (μg/ml)			
		Control (0)	Asn* (12.5)	Lys (12.5)	CP (20)
Benzylpenicillin	10	11**	14	14	—
Ampicillin	10	11**	14	13	—
Mecillinam	200	—	—	—	—
Clavulanic acid	50	11**	13	13	—
Cephalexin	25	12	18	17	—
Cephalothin	5	12	18	19	—
Cephaloridine	5	14	19	19	—
Cefoxitin	5	15	19	19	—
Cefotaxime	5	12	17	16	—
HR-221	5	10**	14	14	—
HR-810	5	13	19	18	—
Cycloserine	2.5	20	30	28	—
Fosfomycin	25	12	15	15	—
Vancomycin	50	11	14	13	—
Moenomycin	50	11	15	14	—
Polymyxin	25	20**	20**	19**	23
Chloramphenicol	25	—	—	—	—
Bacitracin	200	—	—	—	—

* Asn: Asparagine, Lys: lysine, CP: chloramphenicol.
** Hazy zone of lysis.
HR-221 and HR-810 are semi-synthetic cephalosporins related to cefotaxime.

chloramphenicol, indicating that the triggering event requires protein biosynthesis. A similar effect of chloramphenicol on lysis of a growing culture is reported by a number of other investigators[1,6,7].

This effect of chloramphenicol can be used to differentiate lysis caused by compounds acting on cell membrane, *e.g.* polymyxin and those triggering autolysins. Lysis caused by the former is not inhibited by chloramphenicol. Table 1 shows results of these experiments.

Results obtained with various β-lactams were similar to those reported by KITANO and TOMASZ[1]. β-Lactams like cephaloridine and cephalothin which are known to bind penicillin binding protein-1b (PBP-1b) preferentially were the most powerful triggering agents. On the other hand, cephalexin which binds selectively to PBP-3 is a relatively weak inducer of lysis and mecillinam which binds exclusively to PBP-2 did not cause any lysis even at the high concentration of 200 μg/well. Results for a number of other β-lactams tested but not shown here confirm the method.

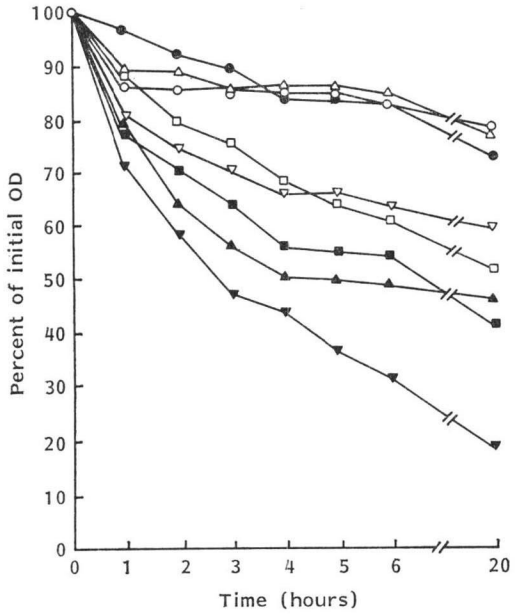
Among the non β-lactam cell-wall inhibitors tested, cycloserine exhibited strong lytic activity. Fosfomycin also showed good lytic activity whereas vancomycin and moenomycin showed moderate lysis triggering activity. Bacitracin even at a high concentration (200 μg/well) failed to induce lysis. Lysis triggered by all the cell-wall-active antibiotics is inhibited by chloramphenicol incorporated at a concentration of 20 μg/ml.

Initial studies with the broths of soil isolates producing cell-wall-active antibiotics such as cycloserine and β-lactams of various kinds (penicillins, cephalosporins, cephamycins, carbapenems and monobactams) gave positive results.

To elucidate the mechanism of formation of the zone of lysis, the following experiment was designed. Cells of the late log phase culture of *E. coli* M6 were harvested, washed thoroughly to remove media constituents and then resuspended in 0.1 M Tris-maleate buffer, pH 7.0 to give an OD of 0.3~0.35 at 430 nm. Four ml of this suspension were dispensed in tubes containing different antibiotics. Tubes are incubated at 37°C and their ODs recorded for 6 hours at hourly intervals and finally at the end

Fig. 1. Lytic effect of antibiotics on suspension of nongrowing cells of *E. coli* M6.

All additions were done at 0 hour. No addition (○); mecillinam 50 μg/ml (△); chloramphenicol 25 μg/ml (●); cephalexin 50 μg/ml (▽); polymyxin 50 μg/ml (□); ampicillin 50 μg/ml (▲); cycloserine 25 μg/ml (■); cephaloridine 25 μg/ml (▼).



of the twentieth hour. Results are shown in Fig. 1.

The formation of zones of lysis can be explained on the basis of different rates of lysis of untreated cells and cells treated with an inducer of autolytic enzymes. This results in the dissolution of the cells in the vicinity of wells and formation of a clear zone of lysis.

The simple nature of the method makes feasible large scale screening of autolysin-triggering agents of microbial origin. It may be possible to find compounds which deregulate the autolytic enzymes and hence cause lysis of cultures treated with powerful yet non-lytic antibiotics such as mecillinam, cephalexin and cefaclor. Such substances may considerably improve the *in vivo* efficacy of these antibiotics. It may be possible to find 'super' triggers or inducers of lytic enzymes. Such 'super' inducers might be able to provide the strong stimulus requisite for eliciting autolytic cell wall degradation in bacteria whose lysis is suppressed phenotypically or genotypically. All these substantiate the promising nature of the method.

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