NOTES

IS *BACILLUS SUBTILIS* ATCC 6633 A β-LACTAMASE PRODUCER ?

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(Received for publication September 22, 1978)

During a series of studies in which the presence and function of β-lactamase(s) and their inhibitions by clavulanic acid[4] were monitored by the color change (from yellow to red) which develops with nitrocefin (Glaxo chromogenic cephalosporin 87/312)[2] it was observed that *B. subtilis* ATCC 6633 gave this color reaction both in liquid and on solid media. This reagent detects very small amounts of β-lactamase(s) in strains which were considered previously as non-enzyme producers[2].

In an experiment, the results of which are shown in Table 1, it was found that the color-reaction starts to develop at room temperature after 30 minutes and the color continues to deepen with time. The development of color on solid media appears within a shorter time period.

Table 1. Detection of β-lactamase of *B. subtilis* ATCC 6633 with the color-reaction with nitrocefin and its influence by sodium clavulanate

B. subtilis ATCC 6633 in peptone-glucose-broth	Onset of color	Color with nitrocefin	
		Control	+20 μg/ml clavulanic acid
Spore suspension	None	None	None
Culture grown at room temperature	30 min. 60 min. 6 hrs.	Yellowish Pinkish Red	No Pinkish Red
Culture grown at 37°C	30 min. 60 min. 6 hrs.	Pinkish Pink Red	No Pink Red

Only the vegetative forms (grown culture) produce the characteristic color. This was never observed with the spore suspension of this *B. subtilis* strain. The development of color (pink-red) was somewhat faster when the culture was grown at 37°C than at room temperature. Sodium clavulanate (20 μg/ml) delayed but did not inhibit the development of the color.

The above observations obtained with the chromogenic cephalosporinase substrate were confirmed by biological assays. In these experiments, peptone-glucose-agar plates were separately seeded either with spore suspension or vegetative cells of *B. subtilis* ATCC 6633 and disced with susceptibility discs as shown in Table 2.

Table 2. Inhibition zones of β-lactam and non β-lactam antibiotic discs against *B. subtilis* ATCC 6633; spore or vegetative seeds

Antibiotic disc	Inhibition zone, diameter in mm	
	Spore seed	Vegetative seed
Benzylpenicillin 10 u	31	24 (ST)
Ampicillin 10 μg	29	27 (ST)
Carbenicillin 50 μg	32	30 (ST)
Methicillin 5 μg	27	27
Oxacillin 1 μg	21	21
Cephalothin 30 μg	39 (50)	38 (51)
Cefazolin 30 μg	35 (43)	34 (43)
Lincomycin 2 μg	13	12
Chlortetracycline 5 μg	26	26

(ST)=Satellite colonies in the inhibition zones
( )=Secondary zones

After overnight incubation at 37°C, the diameters of inhibition zones with vegetative inoculum tend to be smaller for the β-lactamase-sensitive antibiotics (benzylpenicillin, ampicillin and carbenicillin) than with the spore-seeded plates. There are no differences in zone sizes in the case of β-lactamase resistant β-lactams and other non-β-lactam antibiotics. In addition, it was observed that in the case of β-lactamase-labile antibiotics, there were numerous satellite colonies within the inhibition zones. Upon subculturing of these satellite colonies and comparing their sensitivities to benzylpenicillin discs with those of spore suspensions and original vegetative cells, it was found that these satellite cells destroyed more benzylpenicillin (smaller inhibition zone) than the original vegetative cells. Furthermore, it was also observed that on plates seeded with these satellite cultures, the red color with nitrocefin developed faster and became deeper than with the original vegetative cultures.

B. subtilis ATCC 6633 is a widely employed

strain in the biological assay of antibiotics using agar diffusion techniques. Our observation that the vegetative form (but never the spore form) of this organism possibly contains a unique class of β-lactamase(s), detected by the extremely sensitive[2] and highly specific[1] color-reaction with nitrocefin and verified by biological methods is of interest since this strain is employed in the assay of the β-lactamase sensitive antibiotics. However, it should be kept in mind that the presence of β-lactamase *per se* may not necessarily signify the expression of its activity. Nonetheless, it seems to support the view that β-lactamase may be an integral constituent of the growing cell in synthesizing cell materials and thus transitorily related to β-lactam structures[3]. Our finding that it is not present in the spores but is demonstrable in growing and developed cells provides data relating to this kind of role of β-lactamase(s) in the physiology of certain microbes.

Acknowledgment

The authors wish to express their thanks to Glaxo Research Ltd. (Dr. P. W. MUGGLETON) for the kind donation of a research sample of nitrocefin.

References

1) KAMMER, R. B.; D. A. PRESTON, J. A. TURNER & L. C. HANLEY: Rapid detection of ampicillin-resistant *Haemophilus influenzae* and their susceptibility to sixteen antibiotics. Antimicr. Agents & Chemoth. 8: 91～94, 1975
2) O'CALLAGHAN, C. H.; A. MORRIS, S. KIRBY & A. H. SHINGLER: Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicr. Agents & Chemoth. 1: 283～288, 1972
3) SYKES, R. B. & M. MATTHEW: The β-lactamases of gram-negative bacteria and their role in resistance to β-lactam antibiotics. J. Antimicr. Chemoth. 2: 115～157, 1976
4) URI, J. V.; P. ACTOR & J. A. WEISBACH: A rapid and simple method for detection of β-lactamase inhibitors. J. Antibiotics 31: 789～791, 1978