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Antibiotic MIC/MBC analysis of *Bacillus*-based commercial insecticides: use of bioreduction and DNA-based assays

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Minimum inhibitory concentration (MIC) assays, monitored by colony counts, growth (turbidity) and bioreduction of non-toxic XTT [2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide, inner salt], were used to assess the performance of several types of antibiotics against: (1) commercial BT products made from scale-up sporulation phase cultures of *Bacillus thuringiensis* subsp *israelensis* (*Bti*) and subsp *kurstaki* (*Btk*); (2) vegetative cells derived from these BT products; and (3) Gram-positive and Gram-negative bacteria used as controls. The XTT-kinetic assay improved sensitivity and early reading of MIC breakpoints. The conventional colony count method for determining minimal bactericidal concentration (MBC) was used to validate a multi-sample dot-blot assay in which organisms in individual MIC assays are trapped free of residual antibiotic and their viability is estimated by *in situ* conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to insoluble formazan. Tolerance (MBC/MIC) for most antibiotics was low (≤4). Resistance to β-lactams was attributed to β-lactamase activity in both BT products and cultures derived from them. MIC and MBC breakpoints in spore-based assays were also approximated by changes in genome copy, using δ-endotoxin and β-lactamase genes as probes. The DNA assays are effective for monitoring and authenticating organisms in microbe-containing biotechnology products.

Keywords: β-lactamase; biopesticides; MIC/MBC; MTT; nitrocefin assay; PCR; tetrazolium; XTT

Introduction

Microbe-based biotechnology products (MBPs) have considerable potential for solving modern risk-induced environmental problems. However, because MBPs are themselves recent innovations, they are also considered modern risks, and require special criteria for evaluation [10,29,30]. The most extensively used environmental MBPs are BT products used to control blood-sucking and foliage-eating insects at their larval stages [8]. Most BT products on the market are derived from late-stage fermentations of Gram-positive *Bacillus thuringiensis* subsp *israelensis* (*Bti*) and subsp *kurstaki* (*Btk*); they are viscous concentrates consisting of spores (usually $>10^9$ ml^{-1}), variable amounts of crystal-like parasporal inclusion bodies (PIBs) and related byproducts such as cell wall debris, polypeptides and nucleic acids [29,30,34,35]. Determining the toxicity of these BT products is a complex process, involving solubilization and proteolytic activation of pro-δ-endotoxins, sequestered within PIBs and related amorphous aggregates, and byproducts from spore-generated vegetative cells [8,29,34,35]. *In vitro* bioassays of BT products demonstrate that the dominant toxic agents for both insect and human cells are exo-cytolytic products. These products are made *de novo* during vegetative cell production (optima: 37 ± 2°C; pH 7) which can be blocked by the antibiotic, gentamicin [29,35].

Currently, standardized automated methods for assessing antibiotic tolerances of microbes in commercial biopesticides are lacking. Some disc-diffusion and culture tests have been applied to certain *B. thuringiensis* strains and isolates in order to investigate mycotoxin sensitivity [7], plasmid content [27,28], effects of antibiotics on *in vivo* insecticidal activity and antibiotic sensitivity as a criterion in subspecies classification (for reviews of early papers see [5], Table 1 and [26]). However, there are few remedial data in reference to safety of BT products, considering the genetic relatedness of *B. thuringiensis* to *B.cereus–B.anthracis* pathogens, the animal cell-like growth preferences, environmental hardiness and infection-toxicity potential [1,2,9,12,17,19,28–30,35]. Here we report on testing of *Bti*- and *Btk*-based commercial products with 24 antibiotics distributed within groupings A to D suggested for routine testing [21,24]. In addition to determining the minimal inhibitory (MIC) and bactericidal (MBC) concentrations for estimation of BT products' spore tolerance (MBC/MIC), we also derived comparative data for reference strains and vegetative cells produced from BT products. In addition, conventional growth/viability assays for MIC and MBC were used to validate methods involving bioreduction and genome copy determinations. These relatively novel assays extend efforts to improve output and

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Abbreviations: BT, commercial insecticides derived from scale-up, sporulation phase cultures of *Bacillus thuringiensis* subsp *israelensis* (*Bti*) and subsp *kurstaki* (*Btk*); CFU, colony forming units; IU, international unit of BT potency or active ingredient; MBC, minimal bactericidal concentration of an antibiotic expressed in μg ml^{-1} resulting in <0.1% survivors; MIC, minimal inhibitory concentration, the lowest antibiotic concentration in μg ml^{-1} at which no visible *in vitro* growth of test organism occurs; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, thiazoyl blue; MH, Mueller–Hinton broth; PCR, polymerase chain reaction; XTT, sodium [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide, inner salt].

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Table 1 Reports on *B. thuringiensis* subspecies antibiotic susceptibilities

Subspecies	Antibiotic	Code	Medium[a]	Amount (μg ml^{-1})	R/S[b]	Reference
Btk	*Ampicillin	AMP	S-BHI	100	R	[38]
Bti			S-PM	100	R	[16]
Btk	Bacitracin		S-BHI	1000	R	[38]
Bti	Carbencillin		S-PM	50	R	[16]
	Cephalexin		S-PM	30	R	[16]
	*Chloramphenicol	CHL	S-PM	30	R	[16]
	Cloxacillin		S-PM	1	S	[16]
	Colistin		S-PM	10	R	[16]
	Co-trimoxazole		S-PM	25	R	[16]
Btk	*Erythromycin		S-BHI	50	R	[38]
Bti			S-PM	15	S	[16]
Btt				1000	S	[27]
Btk,*Bti*	*Gentamicin	GEN	LB,GM	50	S	[34,35]
Bti			S-PM	10	S	[16]
	(Sisomycin)	(GEN)	S-PM	30	S	[16]
Btk	*Kanamycin	KAN	S-BHI	100	R	[38]
Bti			S-PM	30	S	[16]
Btt				1000	S	[27]
Bti	Nalidixic acid		S-PM	30	R	[16]
	Nitrofurantoin		S-PM	300	R	[16]
	Novobiocin		S-BHI	100	R	[27]
Btk,*Bti*	Penicillin G	P-G	S-PM	10	I	[22]
Bti			S-PM	10	I	[16]
Btt			S-PM	15	S	[7]
Bti			S-PM	100 units	R	[16]
Bti	Polymyxin B		S-BHI	100	R	[38]
Btk	*Streptomycin	STR	S-PM	30	S	[16]
Btt				1000	S	[27]
Bti	*Tetracycline	TET	S-PM	10	S	[16]

*Tested individually and/or as part of PASCO® Gram-positive panel in this study. See additional drugs tested in Table 2.
[a]S-BHI, Bauer-Kirby testing on brain heart infusion agar medium; S-PM, Solid polymedium (peptone, 5 g L^{-1}; meat extract, 10 g L^{-1}; yeast extract, 10 g L^{-1}; sodium chloride, 3 g L^{-1}; glycerol 10 ml L^{-1}; agar, 20 g L^{-1}; pH 7.2).
[b]Classification of susceptibility: R, resistant; I, intermediate and S, sensitive.

accuracy of antibiotic tolerance determinations for all types of microbes, particularly spore producers. They also provide needed methodology for quality control (QC) and safety evaluation of MBPs.

Materials and methods

Antibiotics and bacterial strains tested

Antibiotics listed in Tables 1 and 2 were from Difco, Detroit, MI, USA (the Pasco® MIC Gram-Positive Panel, Lot 118150PA), Gibco/BRL (Rockville, MD, USA) and Sigma Chemical Co (St Louis, MO, USA). Clavamox® (CLV) (Pfizer Animal Health, Canada Inc, London, Ont, Canada) is a broad spectrum formulation for pet and livestock use. CLV tablets contain 50 mg amoxicillin trihydrate and 12.5 mg potassium clavulanate. Working stocks of antibiotics were 1% (w/v) in filter-sterilized water with storage at –20°C for up to 6 weeks.

Reference bacterial strains and their acquisition sources are listed in Table 2; they are the same as used previously [30]. The BT products were derived from either *Btk* (BT-1, Foray 48B®; BT-2, Foray 76B® and BT-3, Thuricide® C.I.L.) or *Bti* (BT-4, Vectobac® 12AS). BT-1-,BT-2 and BT-4 were provided by Abbott Industries (North Chicago, IL, USA). Dose characterizations of these BT products have been made using conventional and bioreduction methods [29]. All dilutions were mixed vigorously immediately before use to ensure uniform sampling of particulate matter.

Reference bacteria and vegetative cells derived from spores of BT products were cultured in LB broth or Mueller-Hinton (MH) broth at 37°C (220 rpm). Multiwell, turbidometric growth assays were conducted at 37°C using a scanning spectrophotometer (Spectramax 250, Molecular Devices Corp, Sunnyvale, CA, USA). Replica 96-well plates were mass-produced with 90 or 180 μl well^{-1} of MH or LB broth and stored frozen (–20°C) with protective wrapping [3]; in this case Parafilm 'M' (American National Can, Greenwich, CT, USA). Wells (other than along the perimeter) were inoculated with 10 or 20 μl of cells or medium before incubating the plates. Cell counts (most probable cell number) were determined by spreading aliquots (50–100 μl) of BT product dilutions or their cultures on LB or MH agar plates. Colony forming units (CFU) were scored after 12–24 h at 37°C or at 22°C if reduced colony size was required.

MIC and MBC assays

Antibiotics were added to broth in serial dilution steps. Based on National Committee for Clinical Laboratory Standards [24], culture stocks (5×10^7 CFU ml^{-1}) were made by adjusting their optical density (OD) to equal that of 0.5 units of McFarland reagent (a 0.24 mM $BaCl_2$, 0.18 M H_2SO_4 solution that yields an OD of 0.123 at 600 nm or

Table 2 MIC and tolerance (MBC/MIC) values derived from cell growth and XTT bioreduction assays

Test strain[a]	MIC (μg ml^{-1}) and corresponding tolerance values (MBC/MIC)									
	AMP	CHL	CLV	GEN	KAN	NEO	PEN	P-ST	STR	TET
BT-1, spore	≥1000 R	0.78 (1.0)	500 (2.0)R	0.19 (2.0)	1.56 (4.0)	0.39 (1.0)	≥1000 R	6.25 (1.0)	3.12 (2.0)	0.19 (1.0)
BT-1, veg.	≥1000 R	1.56 (1.0)	650 (2.0)R	1.56 (0.4)	6.25 (2.0)	3.12 (1.0)	≥1000 R	3.12 (1.0)	6.25 (1.0)	0.19 (1.0)
BT-2 spore	≥1000 R	1.56 (1.0)	≥1000 R	0.78 (0.5)	1.56 (1.0)	1.56 (1.0)	≥1000 R	3.12 (2.0)	1.56 (4.0)	0.19 (1.0)
BT-2, veg.	≥1000 R	1.56 (1.0)	≥1000 R	3.12 (1.0)	12.5 (2.0)	3.12 (1.0)	≥1000 R	6.25 (1.0)	25.0 (2.0)	0.39 (2.0)
BT-3, spore	≥1000 R	0.78 (2.0)	500(2.0)R	0.39 (1.0)	1.56 (1.0)	1.56 (1.0)	≥1000 R	6.25 (1.0)	1.56 (2.0)	0.39 (1.0)
BT-3, veg.	≥1000 R	0.39 (2.0)	650 (2.0)R	1.56 (1.0)	6.25 (2.0)	3.12 (1.0)	≥1000 R	3.12 (4.0)	25.0 (1.0)	0.19 (1.0)
BT-4, spore	≥1000 R	1.56 (1.0)	125 (1.0)R	0.39 (1.0)	1.56 (1.0)	0.78 (1.0)	≥1000 R	3.12 (1.0)	1.56 (2.0)	0.19 (1.0)
BT-4, veg.	≥1000 R	0.39 (4.0)	125 (1.0)R	1.56 (1.0)	6.25 (2.0)	1.56 (2.0)	≥1000 R	6.25 (1.0)	12.5 (1.0)	0.10 (2.0)
B. subtilis, spore	1.0 (1.0)	0.78 (1.0)	3.12 (2.0)	0.39 (1.0)	0.39 (1.0)	0.78 (1.0)	6.25 (1.0)	<0.1 (1.0)	50 (1.0)R	3.12 (1.0)
B. subtilis, veg.	250 (1.0)R	3.12 (4.0)	3.12 (4.0)	0.78 (1.0)	6.25 (1.0)	0.39 (1.0)	500 (2.0)R	50 (2.0)R	125 (10.0)R	3.12 (1.0)
E. coli	62.5 (1.0) (2–8)*	3.12 (1.0) (2–8)*	6.25 (1.0) (2–8)*	6.25 (1.0) (0.3–1)*	6.25 (1.0) (1–4)*	12.5 (1.0)	500 (2.0)R	500 (2.0)R	31.2 (2.0)R	0.78 (2.0) (1–4)*
P. aeruginosa	>1000R	250 (1.0)R	–	3.9 (1.0) (1–4)*	>1000R	125 (2.0)R	>1000R	125 (2.0)R	31.2 (4.0)R	31.2 (8.0)R
S. typhimurium	15.6 (1.0)	1.56 (2.0)	–	6.25 (2.0)	12.5 (1.0)	12.5 (1.0)	7.81 (1.0)	12.5 (2.0)	125 (2.0)R	1.56 (16.0)
S. aureus	0.39 (4.0) (0.25–1)*	6.25 (1.0) (2–8)*	–	0.39 (1.0) (0.12–1)*	1.56 (2.0) (1–4)*	6.25 (2.0)	1.95 (2.0) (0.25–1)*	1.0 (1.0)	62.5 (4.0)R	0.19 (1.0) (8–32)*

[a]BTk- 1 to 3 and BTi-1 refer to commercial strains of *kurstaki* and *israelensis* (see Methods). Species and ATCC strain accession numbers: *Bacillus subtilis* 6051, *Escherichia coli* 25922, *Pseudomonas aeruginosa* 27853, *Salmonella typhimurium* 14028, *Staphylococcus aureus* 25923. R- considered resistant when M1C is >32 μg ml^{-1}.
()*Range of tolerance values from NCCLS 1996. Drugs also tested from PASCO® panel giving MIC values ≤GEN (cefixime, ciprofloxacin, clarithromycin, erythromycin, lomefloxacin, ofloxacin, ripampicin, tobramycin, vancomycin) and ≥STR (cefazolin, cefotaxime, ceftraxone, cefuroxime, oxacillin).

0.104 at 625 nm) and then mixing the suspension with one volume of fresh culture medium. Since this method was inaccurate for BT products, actual spore (cell) contents were determined and adjusted to 5×10^7 CFU ml^{-1}.

Multi-sample liquid and solid (nylon membrane) MIC bioreduction assays were similar to those recently described [29]. Mass production of microwell plates with XTT (2,3-bis [2-methoxy-4-nitro-5-sulphophenyl]2H-tetrazolium-5-carboxanilide, inner salt), (Sigma Chemical Co) was done by dissolving XTT (0.05%, w/v final) in the broth of choice at 60°C for 2 min, followed by filter-sterilization and dispensing aliquots (either 90 or 180 μl well^{-1}). Plates were wrapped and stored at –20°C until use. Data on XTT-formazan production were collected ($\lambda = 480$ nm; 15-min intervals, 37°C) and processed with appropriate software (Softmax Pro, Molecular Devices and Excel 5.0, Microsoft). Formazan-based antibiograms are completed assays, inoculated with either spores or vegetative cells and terminated at or beyond the 16-h endpoint.

Conventional MBC testing was done by spread-plating each 16-h MIC dilution series and scoring CFU after 12–18 h [21]. For filter-based MBC assays, replica samples (10 μl) from each MIC multiwell assay were dispersed in 100 μl sterile water and trapped on sterile nylon membranes (10×6.5 cm, Zeta-Probe, Bio-Rad Lab, Hercules, CA, USA), using a 96-well dot-blot filtration manifold (Gibco BRL). Alternatively, larger samples (50–100 μl in 10 ml water) were trapped on 47-mm diameter filters (0.45-μm pore size, Sartorius) using a 6-tower filtration manifold. Following a water rinse (100 μl for microwells, 10 ml for single samples), the filters were incubated at room temperature for 16–24 h on sterile, LB-soaked absorbent paper or agar in sterile trays. Colonies arising from entrapped cells were detected at the micro stage ($\leq$ 1 mm dia.) by infusing membranes for $\leq$ 30 min with LB and 1 μg ml^{-1} of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), (Sigma Chemical Co) and then air-drying them [29].

DNA assays

Relative *Bt* genomic DNA copy number was determined in microwell samples containing BT product (330 IU or *ca* 1×10^6 CFU ml^{-1}) alone or with antibiotic dilutions. Immediately after a 3-h incubation at 37°C, a viability count (CFU) of each sample was determined and additional aliquots (50 μl each) were frozen or subjected to heat disruption using a thermal cycler (Model 9600 Perkin-Elmer: three cycles of 95°C/30°C with 2-min dwell). The heat-treated samples were either stored (–80°C) or used directly in PCR assays and/or in preparation of DNA dot-blots. PCR assays contained: 5 μl of heated sample and 45 μl of reaction mix (50 mM Tris-HCl, pH 7.4, 4.0 mM $MgCl_2$, 200 μM deoxyribonucleotide triphosphates or DIG DNA substrate) (Boehringer Mannheim Biochemica, Montreal, Canada), 20 pM each of forward and reverse primers, and 1.25 units Taq polymerase (Perkin-Elmer). After an initial step (95°C, 2-min dwell), reaction tubes were subjected to 25 cycles (55°C/72°C/95°C, 30-s dwell each step) terminated by steps at 55°C (30 s), 72°C (10 min) and 4°C (20 min) and storage (–20 to –80°C). The amplified DNA was sized by electrophoresis (1.6% agarose [w/v] in Tris-acetate-EDTA) using 100-bp increment DNA ladders (Gibco) in the presence of ethidium bromide [23], and quantified with a camera digitization-gel documentation/analysis system (UN-SCAN-IT, Silk Scientific, Utah, USA). Diagnostic primer sequences for detecting *cry*1A(c) δ-endotoxin gene were TB-1 (RB-19 of *Btk* HD-73, nucleotides 1779–1795, 5′-GGGACTGCAGGAGTGAT-3′) and TB-2 (US-15c of *Btk* HD-1, nucleotides 2408–2424, 5′-CAGGATTCCATTCAAGG-3′) [6]. Diagnostic primers for β-lactamase gene sequence were: LAC-1 (nucleotides 349–369: 5′-CAGGAGGGGCACTGCAAGTT-3′) and LAC-2 (nucleotides 989–1008: 5′-CCAGTCAG TTGGTACGCCTG-3′) [39].

β-Lactamase activity

The assay for β-lactamase activity used nitrocefin (3-[2,4-dinitrostyryl]-[6R,7R]-7-[2-thienylacetamido]-ceph-3-em-4-carboxylic acid, E-isomer) (Oxoid, Hampshire, UK) [25,26]. This substrate was made to 0.05% (w/v) in 10% DMSO and 0.1 M H_2NaPO_4 and stored without light at 4°C. Nitrocefin hydrolysis results in a change in coloration (light yellow to red), which can be measured at 480 nm. Optimization of assays with BT products and reference strains (10^5 CFU well^{-1}) were carried out using 96-well plates with various dilutions (0 and 0.3–5 μg ml^{-1}) of nitrocefin in LB, and time (up to 24 h) at 37°C. β-Lactamase activity in supernatant and pellet fractions of whole BT products ($12\,500 \times g$; 30 min) were compared with that from 'pure' spores. The latter were obtained by repeated washes and protease digestions of BT product pellets [29] and adjusted to CFU ml^{-1} content of BT products. Control β-lactamase activity was filtrate from an 18-h culture of *E. coli* C600 (ATCC 47048) in LB at 37°C.

Results and discussion

MIC and MBC determinations

Standard MIC assay methods [3,4,21,24] were used to obtain comparative MIC estimates for spores in BT products, vegetative cells derived from them and reference bacteria. These assays were also used to validate potentially superior, semi-automated quantification approaches based on XTT and MTT redox reporter dyes [29]. A summary of MIC, MBC and tolerance values determined for all antibiotics tested is given in Table 2. Typical examples of MIC and MBC antibiograms derived from the redox dyes reporting on spores in BT products and *E. coli* control are shown in Figure 1 a–c. Numerical values can be deduced for each MBC using tolerance data given in Table 2 (see MBC/MIC).

For most MBC determinations, results were similar to those of corresponding MIC assays. The single values reported for each antibiotic of Table 2 reflect that there was no discrepancy in multi-tests using cell proliferation-induced turbidity or cell proliferation-induced bioreduction of XTT-formazan. The values obtained for reference bacterial strains under identical testing regimes agree within one-half of a dilution unit of the published antibiotic concentrations [21,24]. The antibiotic tolerances of the spore-based BT products were usually low (1–4) for most antibiotics tested. The most potent antibiotic was tetracycline

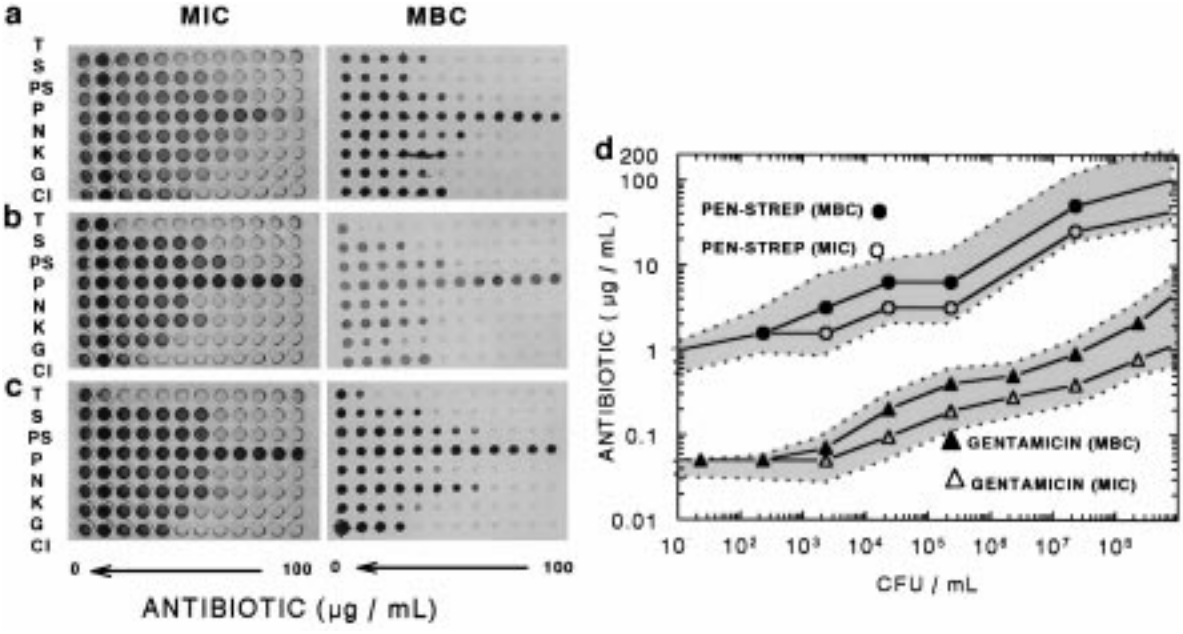

Figure 1 Comparison of MIC and MBC for various antibiotics. (a,b,c) Photographs of antibiotograms of MIC (left panels, 16-h endpoint liquid XTT microwell assay) and corresponding MBCs (right panels) derived by trapping aliquots of MIC assays on nylon filters, incubation for 12 h on antibiotic-free LB and infusion with MTT for *in situ* production of insoluble formazan (see Methods). Antibiotics were tested as a dilution series: 0, 0.1, 0.20, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 24, 50 and 100 μg ml^{-1}. (d) Effect of BT-1 product (*Btk* spore) concentration (CFU or IU) on MIC and MBC endpoints of antibiotic examples. Shaded areas in (d) signify experimental error. (a,b,c) Correspond to results from *E. coli*, BT-1 (*Btk*) and BT-4 (*Bti*), respectively (see Methods). Abbreviations: CL, chloramphenicol; G, gentamicin; K, kanamycin; N, neomycin; P, penicillin; PS, penicillin/streptomycin; S, streptomycin; T, tetracycline.

(Table 2, Figure 2). However, the MIC and MBC assays of ampicillin and penicillin showed that they were not effective, requiring more than the upper dose limit investigated (1000 μg ml^{-1}). These observations are in significant contrast to other reports using various *Bt* strains (Table 1). The attentuation of streptomycin effects in PEN-STR tests (Figure 1c and Table 2) is a result of dilution of STR and possibly interference by the large quantity of free-nucleic acid in BT products [30]. The resistance to β-lactam antibiotics, even in combination with a β-lactamase inhibitor such as clavulanic acid (Table 2 and Methods) is consistent with the finding of lactamase activity associated with the BT products and vegetative cells derived from the spores (see Figure 3).

Comparisons between spores and vegetative cells

When dealing with spore-rich BT products, the MIC and MBC of an antibiotic may be measurements of interference of spore activation and germination as well as vegetative cell propagation. At the standard cell concentration, comparisons of MIC and MBC results for spores and vegetative cells of *B. subtilis* and *Bti* and *Btk* from BT commercial products showed that metabolically active vegetative cells were twice as resistant to antibiotic as spores, but the tolerance values observed for the two subspecies (*Bti* and *Btk*) were in good agreement. These observations are consistent with the remarkable subspecies similarities in growth requirements [29]. In other experiments (Figure 1d) it was found that these tolerance values changed as BT product dose (the spores) or vegetative cell equivalents (data not shown) were increased in concentration.

In terms of bioreducing capacity, *Btk* and *Bti* spores are essentially dormant and have 40 times less activity than vegetative cells [29]. As the concentration of any antibiotic is increased, growth and XTT bioreduction curves of either *Bti* or *Btk* typically exhibited a lengthening in lag time before onset of exponential growth. This observation is similar to that seen when BT products (spores) are serially diluted (Figure 2c) and is consistent with the number of viable spores or vegetative cells that are present [29]. Similar studies with reference strains of *B. subtilis*, *E. coli* and *P. aeruginosa* (data not shown) revealed that their growth curves, as detected by XTT reduction, were less co-operative and easily distinguished from those of *Bti* and *Btk* cells. The capacity to reduce XTT by *B. subtilis* and the two species of Gram-negative bacteria could be significantly enhanced by using an electron coupler (eg, phenazine methosulfate), which is not required by either *Bti* or *Btk* cells and is toxic to them at levels >5 μg ml^{-1} [29]. At sub-MIC concentrations with most antibiotics and with β-lactam antibiotics at high concentrations, XTT-formazan production does not fully correlate with cell number. It appears that rapidly growing and dividing cells may be preferentially killed, resulting in either direct loss of XTT reduction or release of extracellular XTT reductase activity. This would preferentially leave late-log cells and possibly spores to reduce XTT, but at lower rates than seen with exponentially growing vegetative cells.

Performance of MIC/MBC bioreduction assays

MTT and also a related dye, INT (2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride), have been used to improve a microdilution ('autobac') MIC assay [3] and colony detection in disk-diffusion and broth assays

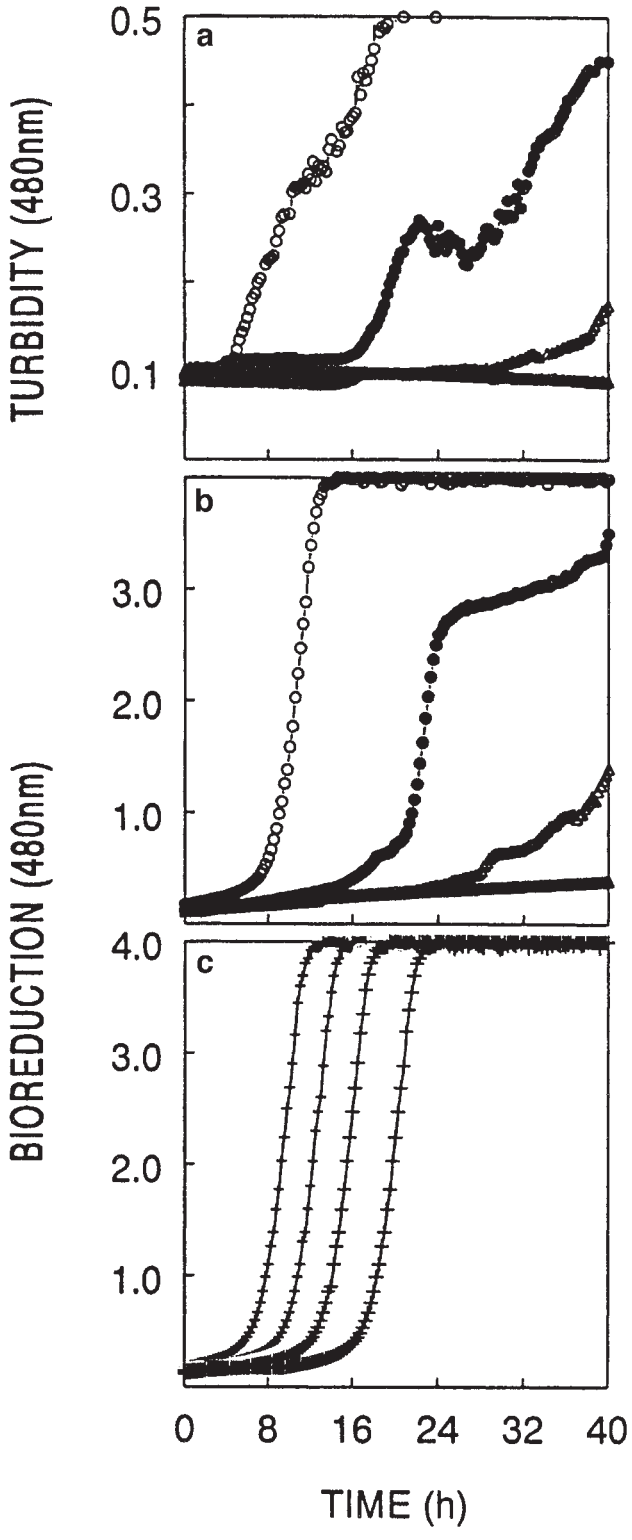

Figure 2 Kinetic derivations of tetracycline MIC for BT product. (a) Microwell turbidity measurement of 41.7 IU of BT-1 (~10^5 spores) in LB broth, without (○) and with tetracycline (●, 0.20; △, 0.39; ▲, 0.78 μg ml^{-1}). (b) Identical assays with XTT added (note scale change). (c) XTT bioreduction assay using BT-1 dilution series (10^{-4}, 10^{-6}, 10^{-8} and 10^{-10}, extreme right), where 1× BT-1 = 12.7 million IU ml^{-1} or ~3.15×10^{10} spores.

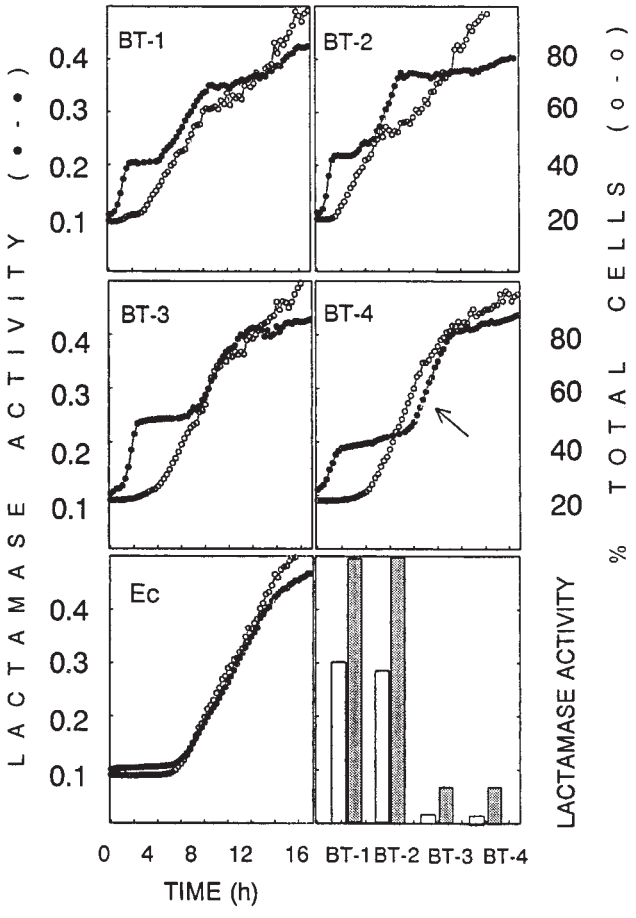

Figure 3 Nitrocefin-reducing activity of BT products and *E. coli*. β-lactamase of an *E. coli* (Ec) culture was used to develop a miniwell spectrophotometric assay with nitrocefin as substrate. Graphs illustrate vegetative cell production (○) and corresponding coloration (480 nm) due to nitrocefin hydrolysis (●) by BT products and Ec in LB broth. All experiments used 5 μg ml^{-1} nitrocephin, except BT-4 (2.5 μg ml^{-1}) to minimize inhibition of vegetative cell production. Bottom right panel shows histogram representations of lactamase activity intrinsic to BT products without (open) and with (filled) exogenously added *E. coli* β-lactamase (60 min assay at 37°C).

[11,18,31,33]. The assays described in the present work are significant improvements in quantification because of improved dye properties (XTT) and equipment (eg, use of a programmable microwell scanning system and end-point data capture in solid support assays by quantification of cell-bound reporter dye). The XTT bioreduction MIC assay resembles that of an earlier version using a fluorogenic dye [32], but builds on previous studies of *Bti* and *Btk* spores of BT products [29]. Extensive side-by-side performance comparisons of broth dilution tests, based on turbidity measurement and XTT-bioreduction, demonstrated that the latter test method significantly improves assay attributes (sensitivity and throughput time). This is illustrated using tetracycline MIC data (Figure 2a,b) with product BT-1 (spores). Compared to turbidity measurements taken at the 24-h endpoint, the same assay with XTT-formazan as reporter would be 10 times more sensitive after reaching a 16-h endpoint. The formazan (coloration) generated by XTT or MTT in liquid assays was stable for at least 2 weeks at room temperature and dry-filter versions have

retained color quality and intensity for over 5 years. LB-based and MH-based assays gave the same results (data not shown); intrinsic bioreducing activity of LB at the 24-h endpoint was <5% of total potential formazan conversion and is three times less than that of either MH or BHI broth [29]. MH broth-associated reducing activity was linear and decreased assay capability only after 30 h.

To improve on conventional MBC sample processing (volume and handling time) and to link it directly with MIC testing, replica aliquots derived from 96-well MIC assays were trapped on nylon membranes, using either single well or multi-dot blot filtration manifolds. This permitted removal of residual antibiotic by washing and incubation without antibiotic to test for cell viability. In comparing data from direct plating of MIC samples and trapped (washed) MIC samples, the former were the most variable and often under-estimated the MBC due to localized deposit of carryover antibiotic (data not shown). The membrane blot MTT detection method was superior as it used less material, more sample throughput and continuity with MIC assays. Also, since the MTT-formazan reporter kills vegetative *Bt* cells within 10 min of exposure, it is ideal for quantifying cell number and metabolic state [29]. Such assays, when allowed to progress for at least 16 h before MTT was added, easily detected the colony produced from one spore or vegetative cell. The developed membrane (Figure 1a–c right panel) with its standard $\log_{10}$ dilution series, once air-dried, provides a lab record for at least 5 yrs.

β-Lactamase production

Ampicillin, and particularly penicillin concentrations required for *Btk* and *Bti* resistance reported by others (Table 1) are low in comparison to values obtained in the present study (Table 2), which indicate both spores and vegetative cells derived from either *Bti*- or *Btk*-based BT products are resistant to these β-lactam antibiotics. Since evidence exists for the presence of at least a group-I β-lactamase gene in all commercial strains tested by us (see next section), we investigated the possibility that the vegetative cells derived from spores of BT products produce an active β-lactamase and that this enzyme activity may even be present in the BT products themselves. For these investigations we used nitrocefin [25,36], a chromogenic cephalosporin. At high concentrations (usually >5 μg ml^{-1}), nitrocefin can transiently inhibit growth from *Btk* and *Bti* spores. As shown in Figure 3, significant levels of *de novo* lactamase activity appeared in all BT-product cultures as early as 2 h post-inoculation at 37°C. This interval includes time for spore germination, which occurs 30–45 min after inoculation in liquid medium at temperatures >12 and <55°C [29]. As expected, the β-lactamase-positive *E. coli* control produced a similar level of hydrolytic activity. The lack of overlap of hydrolytic activity and cell production (turbidity) from BT-4 (*Bti*) commercial product (Figure 3) is consistent with a slight growth inhibition by the 5 μg ml^{-1} of nitrocefin used in these assays. Similar assays using BT product supernatants (spore- and PIB-free fraction) indicated that only BT-1 and BT-2 products were β-lactamase-positive (Figure 3). The pellet fractions of all BT products which contain the spores and other particulate matter (PIB and subcomponents) were only weakly positive in the presence of gentamicin, used to inhibit spores from producing vegetative cells. However, the low-level β-lactamase activity of BT-3 (also a *Btk*-based product), but not BT-4 (*Bti*) increased by the third wash cycle, suggesting removal of an inhibitor or release of activity from disaggregated subproducts in the pellet fraction such as PIBs. Since results from incubating either BT-3 or BT-4 product supernatants with *E. coli* β-lactamase indicated a 5-fold reduction of nitrocefin over control measurements, the presence of inhibitor(s) or proteases in the BT-product formulations is also likely.

DNA-Assays related to MIC-MBC

PCR has been used to identify specific target microorganisms in drug susceptibility studies [36]. We examined the use of PCR and DNA hybridization to independently predict antibiotic breakpoints by measuring changes in genome copy level rather than cell number in spore and vegetative cell MIC and MBC assays. As relevant probes, we used gene sequences diagnostic for *Btk*-δ-endotoxin (*cry*lAc) [6,29] and β-lactamase, which is highly homologous to the group-I gene of *B. cereus* [39]. Lactamase activity of some non-commercial *Bt* strains may be encoded by gene(s) carried on the host genome rather than on an extrachromosomal element [38]. Optimization of the DNA assays revealed a four to six-fold amplification of these target genes when spores from BT-1 and BT-2 formulas were subjected to a 3-h pre-culture step. This step allowed spores to germinate and three to four cell generations to occur. When vegetative cells were used instead of spores (BT products), differences in PCR amplicon levels were usually <two-fold. This change is likely due to the increased efficiency of template DNA released from vegetative cells compared to spores (V Seligy, unpublished). As shown in the penicillin-streptomycin study (Figure 4a–c), both cell count and amplified DNA product drop significantly at the transition established by other types of MIC assays. The final reduction in amplicon yields of β-lactamase (0.75 kbp) and *cry*1Ac (0.65 kbp) shown in agarose gels (Figure 4a and b), when quantified (Figure 4c), was similar to that generated by non-germinated spores. This reduction in PCR product formation was not caused by direct effects of antibiotics on DNA *in vitro* synthesis in the amplification reactions, as control reactions with λ-DNA template and increasing antibiotic concentrations showed no effect (data not shown). In the case of *Bti*, the PCR assay gave similar results with β-lactamase primers, but nothing with *cry*1Ac primers, as expected.

Detection of the *cry*1Ac amplicon using excised samples from MBC membrane assays of MIC experiments, demonstrated in Figure 4d, further establishes the utility of the PCR assay to detect breakpoint changes even in the presence of MTT-formazan. Data on dot-blot DNA hybridizations (not shown) improve on PCR detection using vegetative cells, but the hybridization method required considerably more time and effort. Both DNA assay methods could be used in heterogenous mixtures of organisms, but probably would not be cost effective for large-scale screening of antibiotics.

Detection of a β-lactamase amplicon, when only *Btk* or

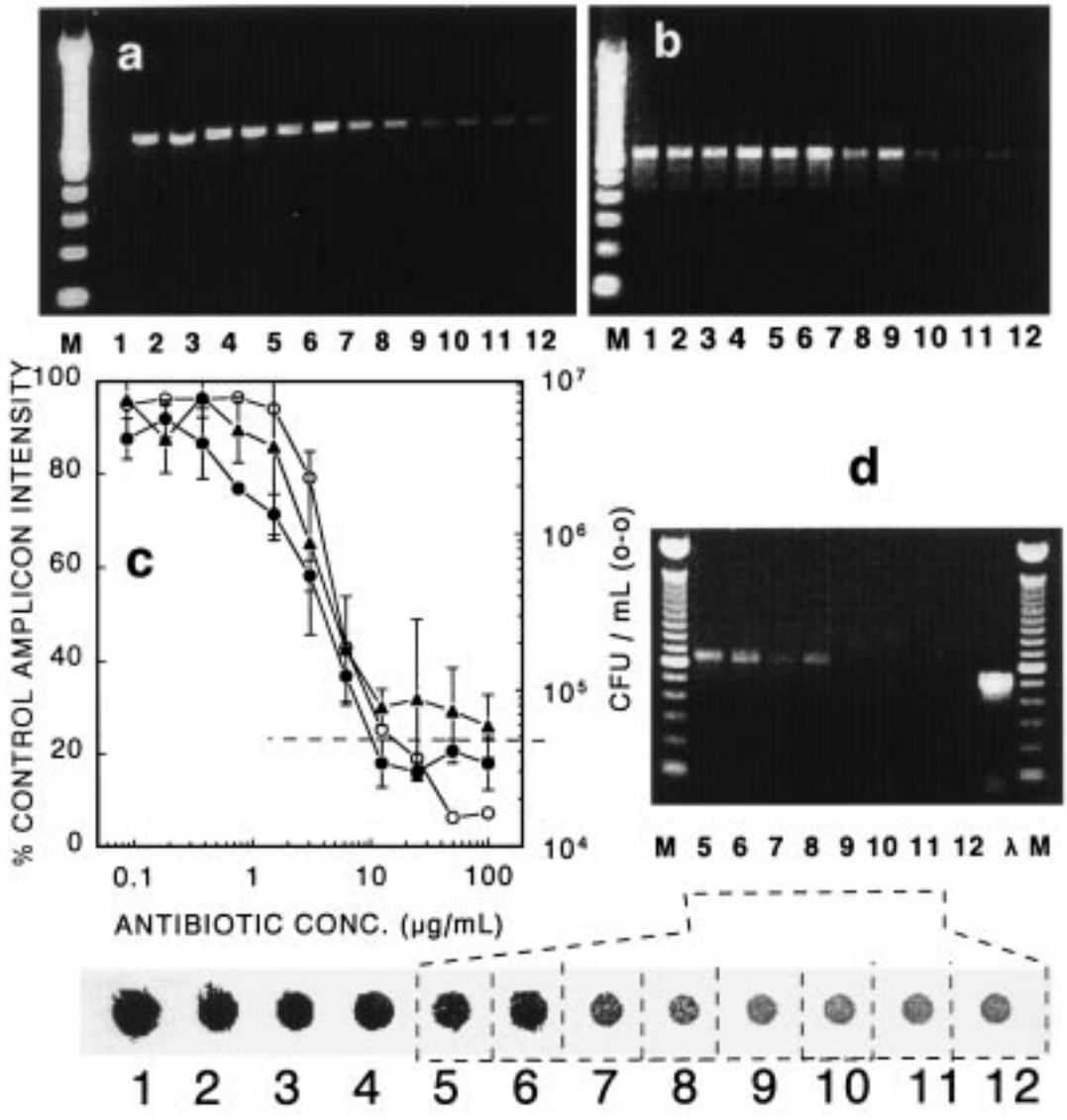

Figure 4 PCR use in analysis of MIC and MBC assays. (a and b) PCR amplicons produced by primers identifying diagnostic gene segments of β-*lac* (0.75 kbp, □) and *cry*1Ac (0.65 kbp, ●) with template DNA (5 μl) released from heat-lyzed samples of BT-1 (10^5 spores microwell^{-1}) incubated for 3 h at 37°C in LB, containing zero (lane 1) or 0.1–100 μg ml^{-1} (lanes 2–12) penicillin/streptomycin (1:1). (c) Shows relative amplicon yields from three experiments as in (a) and (b) and corresponding CFU (○) content. (d) PCR analysis (agarose gel) of DNA in excised membrane-trapped MBC assay samples (indicated by dotted lines) using spores of the MIC penicillin/streptomycin assay (as in a and b). Viable micro-colonies on the MBC membrane were stained by MTT-formazan reaction (see Methods). Ethidium bromide-stained agarose gels contained a 100-bp increment marker DNA (m).

Bti colonies or respective commercial BT products (spores) were used, supports the observations on β-lactamase activity and β-lactam resistance of BT products (Figure 3 and Table 2), and suggests that these organisms have very similar β-lactamase genes. In contrast, no PCR products were obtained with *B. subtilis* or *E. coli*, and/or primer-reaction mix alone, which rules out potential contamination by β-lactamase sequences [36].

Antibiotic testing of microbe-based biotechnology products

General information on antibiotic susceptibility of *B. sphaericus, Bti* (H-14) and *Bt* subsp *neolensis*, collected by zone-inhibition, disc method [16], and liquid assays [20] using *Bt* subsp *darmstadiensis* and serovars, *dakota* (H-15) and *indiana* (H-16) [13], fit within the determinations presented here. The earlier antibiotic sensitivity studies provided no detailed data on tolerance (MBC/MIC) of cell phases (see references cited in Introduction and Table 1). The need for such data becomes important as BT products and other organisms for bioremediation are used in high concentration in populated areas [14,15,17,30]. Our viability tests with BT products at pH 0.8 suggest that *Btk* and *Bti* spores would survive acidic conditions of the human stomach [29]. It has been suggested that outbreaks of food poisoning attributed to the enteropathogen *B. cereus* may have been caused by *B. thuringiensis* [12,17]. In most instances penicillin treatment would control *B. anthracis*

isolates, unlike strains of *B. cereus* and *Btk* and *Bti* tested here [12,17,22]. Knowledge about effective antibiotic use against such environmental contaminants would benefit all individuals, particularly those in high risk groups such as infants, those with cystic fibrosis and those with severe immune disorders [14]. Accidental infections attributed to vegetative cells arising from BT product use [28,37] have been treated with benzylpen, cephazolin, erythromycin and gentamicin, but details of dosage are lacking. In hindsight, the present MIC/MBC data would have supported a variety of treatment strategies. Also, use of gene-diagnostic probes as illustrated here would have determined if the infectious bacteria harboured *cry* genes, the only reliable diagnostic for distinguishing *B. cereus* from *B.t*-based commercial products.

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