6th Pacific Rim Conference on the Biotechnology of Bacillus thuringiensis *and its Environmental Impact,* Victoria BC, 2005
Côté, J.-C., Otvos, I.S, Schwartz, J.-L. and Vincent, C. (eds)

Development and Application of Molecular Tools for Exposure, Toxicity and Pathogenicity Characterization of *Bacillus cereus* Group Organisms in Context of Biotechnology Use

Vern Seligy,[1*] Gordon Coleman,[1] Jennifer Crosthwait,[1] Kathy Nguyen,[1] Phil Shwed[1], Azam Tayabali,[1] George Arvanitakis, [2] Della Johnston, [2] Louis Bryden,[3] Michael Mulvey, [3] Brian Belliveau,[4] and Esther Seto[4]

[1]Environmental & Occupational Toxicology, Safe Environments Programme, Health Canada, Ottawa, Ontario, Canada, K1A 0K9
[2]Biotechnology, Product Safety Programme (PSP) of the Healthy Environments and Consumer Safety Branch (HECSB), Health Canada, Ottawa, Ontario, Canada, K1A 0k9.
[3]Antimicrobial Resistance & Nosocomial Infections, National Microbiology Laboratory, Public Health Agency, Health Canada, Winnipeg, Manitoba, Canada, R3E 3R2.
[4]Microbial & Pheromone Evaluation, Pest Management Regulatory Agency (PMRA), Health Canada, Ottawa, Ontario, Canada,K1A 0k9.

Keywords: antibiotics, cytokines, enterotoxins, fatty acids, genomics/microarrays, hemolysin, PCR

Both industry and regulators are interested in standardized methods, which can rapidly assess any MO or their subcomponents (or product formulation) for in vivo-relevant toxicological effects. In previous research, we focussed on developing in vitro approaches, which parallel assessment of dose effects of chemical contaminants (9,14,16), and allow quantification of cell/tissue specific multi-gene/protein indicators to inform on mode of toxification or immunologic effects. Here, we covered research approaches that make use of the latest advances in genomics of Bc group organisms and comparative murine and human immuno- and toxico- genomics/proteomics knowledge, and tools, which allows us to harmonize again with chemical testing, and earlier Bc group clinical tests and infection studies involving mouse models (6,7). The genotyping by microarray analysis validates the relatedness of Bt strains to Bc14579 (type strain), and also sharing of a number of factors that could be possibly engineered out of future biotech strains, if needed. This approach may be very important in addressing quorum-sensing factors (e.g., germination, chemotaxic signal factors, and pleiotropic regulators such as plcR and papR) which can coordinate expression/repression of genes (e.g., antibiotic resistance, and various hydrolytic enzymes and toxin-like activities) in mixed bacterial populations (3,4,7,9).

Introduction

The *Bacillus* genus contains taxa of interest to biotechnology (e.g., use in bioremediation and pest control), and also some of clinical concern. A key example is within the *B. cereus* (Bc) group, which includes Bc *sensu stricto*, *B. anthracis* (Ba) and *B. thuringiensis* (Bt). Interspecies comparisons indicate sharing of chromosomal features (e.g. cell cycle/ differentiation and adaptive functions including several virulence factors). Because of this sharing, there is need to qualify the intrinsic identity and safety status of the several dozen putative Bt subspecies which have commercial potential for scale-up applications and environmental release. Here, we survey some key criteria, tools and data relevant for use in assessment of Bc and Bt organisms covered by regulations under the Canadian Environmental Protection Act (1999) and the Pest Control Product Act. The tools featured here address species/strain identification, and scanning toxicity, and pathogenic / immunogenic potentials, using in vitro and in vivo strategies.

Background

Bacterial diversity and knowledge gaps. The *Bacillus* genus comprises 181 officially recognised species, and over two dozen taxa of biotech interest, including strains of *B. sphaericus, B. cereus* (Bc) (*sensu stricto*) and *B. thuringiensis* (Bt) which are relevant to this conference (1). The latter two species, along with *B. anthracis* (Ba) as well as three others comprise the *B. cereus* (Bc) group (2-4). This grouping is based on a number of shared biochemical, morphologic and physiologic (polyphasic) characteristics, and recent data indicating a high degree of relatedness at both gene and chromosome (synteny) levels (3-5). Currently, there are several hundred strains of each species in various collections. As well, there are several dozen candidate

* Corresponding author. Mailing address: Environmental & Occupational Toxicology, Safe Environments Programme, Health Canada, 0803A Tunney's Pasture, Ottawa, Ontario, Canada, K1A 0K9.. Tel: 613 952 5852. Fax: 613 941 4546. Email: vern_seligy@hc-sc.gc.ca

Bt subspecies, but none are officially recognised (1). Designation of strains to the Ba and Bt taxa, rather than Bc, relies on a few phenotypic features, which are mostly extra-chromosomally encoded (2-4). Less is known about the overall genomic organization of most Bc strains and Bt strains/subspecies compared to Ba strains, and how they might function in mammalian physiologic environments. Isolates are assigned to the Bt taxon based on presence of *cry* genes, and/or their encoded Cry endotoxins, and/or their associated parasporal inclusion body (PIB) structures, and/or flagellar serotype (H antigen) (5). However, these traits may be variably shared, transmitted and/or expressed. H-antigen serotyping is not always reliable because frequency of common flagellar antigens is very high between Bt and Bc strains (5). In addition to the many biotech-related strain collections under study within many countries, there are clinical collections of Bt strains exhibiting pathogenic effects (4,6-10). Genomic mapping will enable approaches to distinguish benign from pathogenic members of the Bc group.

Public safety and regulatory oversight. The session "Public Safety" as organized for this conference, includes this paper and two others, which address performance of past and recently used commercial formulations derived from Bt *kurstaki* (Btk) or *israelensis* (Bti). As a reference point, "Public Safety" (the prevention and protection of the general public from all manners of harm) is usually considered the domain of Emergency Services organisations. However, for biotechnology products such as those to be gleaned from use of Bc and Bt strains, there are overarching national and international authorities that prescribe extensive measures for authorization/registration and safe use. In the Canadian context (see Fig.1), only select strains of Bti, Btk and Bt *tenebrionis* (not addressed here) have been assessed for their relative safety and approved for use as biopesticides. Similar regulatory scrutiny is in place to assess strains of Bc and Bt members intended for other environmental applications (Fig.1; ref. 14). Hence, there is need to avoid lumping little known strains of Bc or Bt (as done in some literature) with the subset

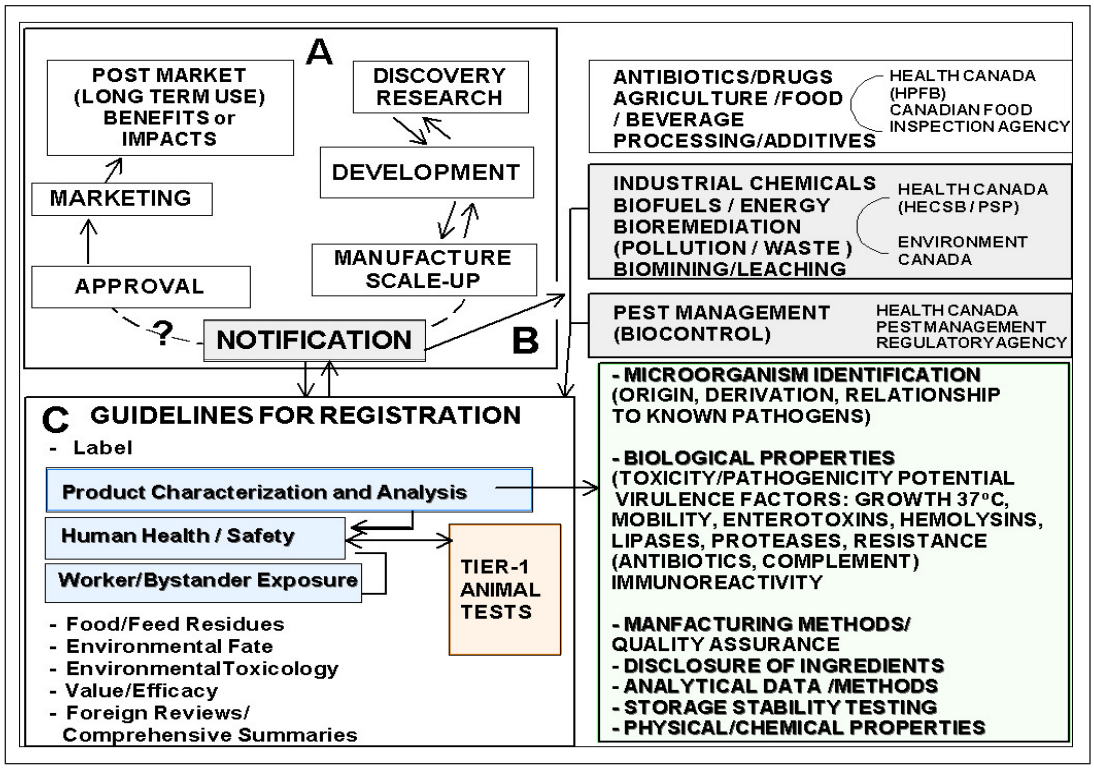

FIG.1. Environmental applications of microorganism (MO)-based products. (A) A successful path to market includes developing a safe product which requires working closely with regulatory authorities (B) to fulfil requirements (C) for import, manufacture and production of MOs. Canadian requirements for registration of MO use as pest control agents is governed by the Pest Control Products Act while other applications (e.g., bioenergy/fuels, bioremediation, biowaste recycling/composting) are stipulated by the Canadian Environmental Protection Act (CEPA (1999)). The latter overarching authority requires joint assessments by Environment Canada and Health Canada (Biotechnology Section, Product Safety Programme). A relevant example of this cooperation concerns MOs of the Domestic Substance List (DSL) such as Bc14579 and Bt13367, which are addressed in this paper and elsewhere (13).

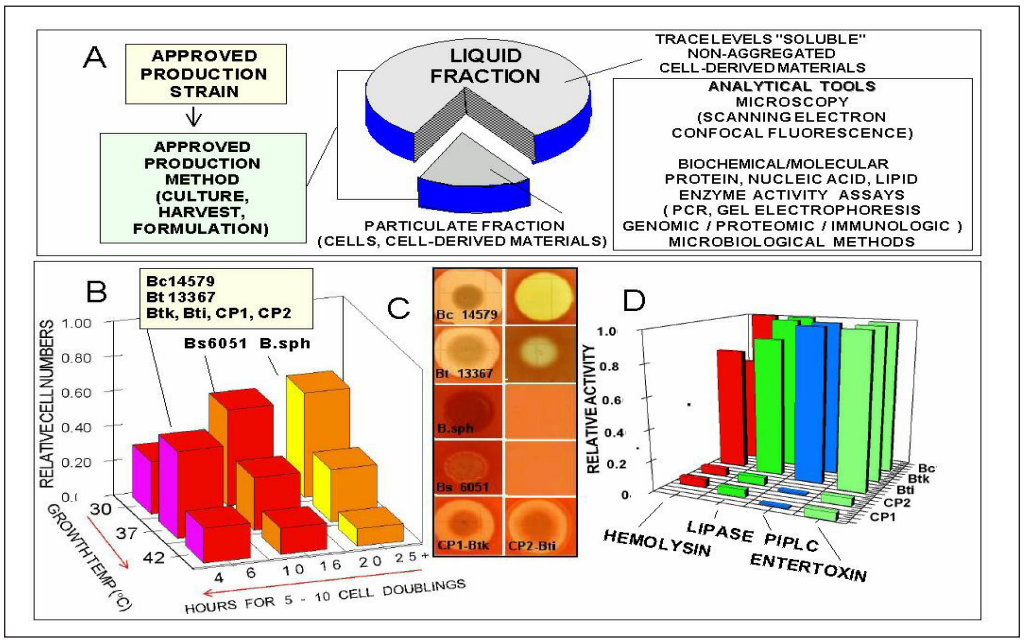

FIG.2. Characterization of micro-organism based biotech products and subcomponents. (A) Schematic of fractionation strategy and tools for principle component analysis of *Bacillus*-based products, modelled on commercial biopesticides (Btk, CP-1; Bti, CP-2). Screening potential for infectivity based on growth in mammalian physiologic conditions (B) and expression of virulence factors (C,D): Bc and all Bt strains exhibit peak growth at 34 to 39°C compared to other species. (C) Hemolytic activity assay using 5% sheep or human blood agar at 37°C: spores (5,000 per spot) after 24h, and culture filtrates (7uL) from (B) after 2h (right column). (D) Relative activity estimated from indicator plates (blood or egg yolk) for lipases (PIPLC and PLC) and assays of culture filtrates at 8h growth. Activity associated with Btk (CP1) and Bti (CP2) in presence of gentamicin to prevent spore outgrowth is < 1/20th of that from their outgrowths. Bc14579, Bt10792 and Bt13367 produce ~ 30% more haemolytic activity per cell than either Bti or Btk strains.

of strains that have been favourably evaluated. Given this context, our paper will briefly address the regulatory oversight for micro-organism (MO)-based products, and the ongoing need for data requirements, and the tools/methods, which may be used to characterize strains of the Bc group (other than Ba) showing promise for application.

Approaches used in characterization of MO and related biotech products

Criteria for MO risk screens. Requirements to assess MO risks to human health and safety, and to the environment, are fairly consistent between developed countries. The requirements outlined in Fig.1 to address microbial pest control agents (see PMRA Regulatory Directive DIR2001-03; www.hc-sc.gc.ca/pmra-arla) are similar to those prescribed for environmental applications (Fig.1; CEPA 1999, www.ec.gc.ca/substances/nsb/bioguide/eng/bi_s2_e.htm), and draw on comprehensive test guidelines of the U.S. Environmental Protection Agency (Series 885, www.epa.gov/opptsfrs/home/guidelin.htm) and the European Union. The generic format of these requirements provides flexibility for data presentation and accommodation of latest scientific and technological advances. However, the more extensive the detail and quality of information given in the characterization and analysis steps the better, as this information influences the nature, and possibly extent of data required to asses safety to human health (i.e., Tier 1 animal tests). Subsequent sections of this paper present some methods and results from select Bc and Bt strains, and a few other species which are relevant to both pest control and other environmental applications.

Bt-product formulations as models. Based on quantities released into the environment, Bti and Btk based biopesticides are the most used of all bacteria-based biotech products (11). Essentially, they are concentrates of scale-up cultures (fermentations) harvested at sporulation phase (Fig. 2A). Given the global extent of application, and their quality (consistency of ingredients), we have used these products as models for developing semi-quantitative methods to assess quality of liquid and powder (hydrated) formulations, and monitor product release and persistence (e.g., efficacy (12), and worker/bystander exposures (11)). Some key tools for product analysis are listed in Fig. 2A. Simple fractionation by centrifugation (> 5000 x g

10 min) or filtration (0.45 micron pore size) enabled us to assess formulation subcomponents (e.g., liquid or powder (hydrated). Analyses of several dozen Bti and Btk–based formulations showed that they contain ~30-35% particulate matter (v/v), comprising ≥ 10^9 spores per mL of liquid, and about an equal mass of other culture products, mostly PIB structures and amorphous aggregates, characteristic of each strain type, and their Cry products (10-13). Fieldwork with various organizations (11,12) indicated that quantitative detection of Bt formulations (delivered as sprays) was most accurate using the spore rather than the PIB or Cry components. In terms of active ingredient, an international unit would contain ~ 2400 spores, and as much as several million may be deposited as spray droplets per cm^2 of surface (10-13).

Identification by biochemical and microbiological methods. Additional assays for characterizing the bacterial cell component of products are described in Fig. 2B to D and Figs. 3-6. For this purpose we used type strains, Bc14579, and Bt Berliner 10792, commercial Bt-products, CP-1 (Btk) and CP-2 (Bti) (12), and others as indicated in Fig.3. Bc14579, Bt13367 and *B.subtilis* (Bs) 6051a are of interest for non-pesticide applications (14). The numerical designations for strains are mostly ATCC but also NRRL. A key part of our screening strategy was to make use of parameters that give an indication of potential for infectivity (growth in mammalian cell environment), and quorum sensing (triggering coordinated outgrowth and expression of virulence potentials) (9,10,15). Bc and Bt strains are separated from other *Bacillus* species by their growth optimum (37°C, Fig. 2B) exhibited in bacterial and mammalian cell medium, growth in the presence of mammalian cells (10,13,15; Figs. 2C, 5 and 6), and expression of animal cell damaging effects (Fig. 2C, D and Figs. 5 and 6). Also, Bc and Bt strains are separated from other bacterial species, but not themselves, in profiles of their growth with different classes of antibiotics (Fig. 3A) and cell-associated fatty acids (derived as methyl esters (FAME)) (Fig. 3B). To separate the Bt strains from Bc14579 a test for presence of PIB structures, *cry* genes and Cry products (by PCR and immunologic staining methods) would be required (10,12,13). Use of recently developed multivariate analytical software

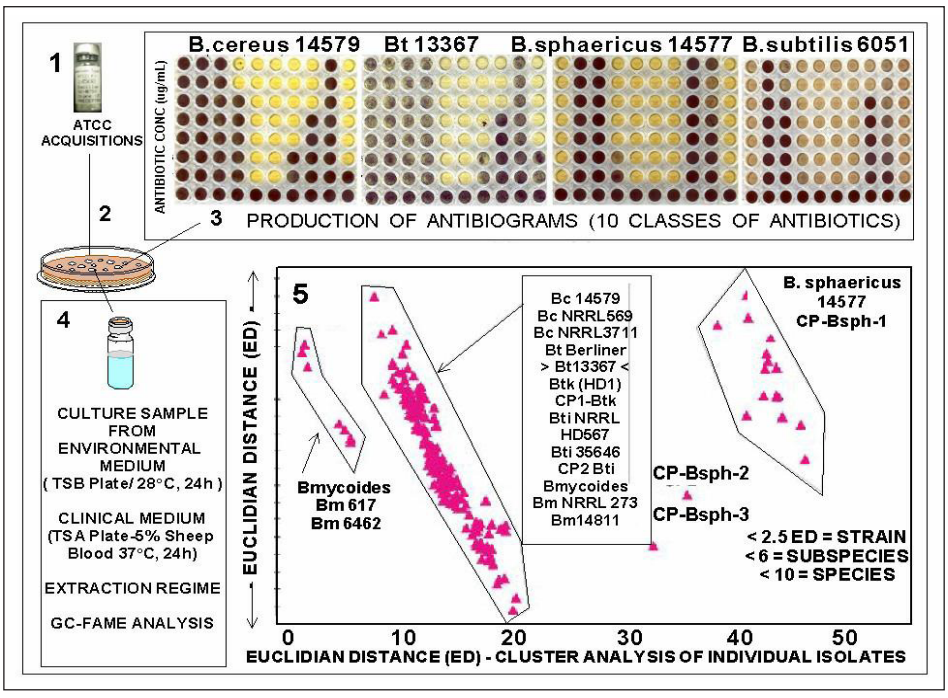

FIG. 3. Characterization of micro-organisms based on vegetative cell (spore outgrowth) properties. Step 3 - minimum inhibitory concentrations (MIC) ~20,000 cfu / mL, 37ºC incubated for 24h using half-fold dilutions of antibiotics (from 0.37 to 24 ug/mL), columns left to right: Amoxycillin; Amphotericin B; Aztreonam; Cephotaxime; Doxcycline; Erythromycin; Gentamicin; Nalidix acid; Trimethoprim; and Vancomycin. Bc and Bt strains as expected are resistant to penicillin and derivatives, but can be managed by others antibiotics. Steps 4,5 a method to obtain fatty acid (methyl esters/FAME) principle components using Agilent MIDI System and software and databases (Clinical and Environmental), also used by many labs. This methodology only partly discriminates species-level differences within the Bc group (e.g., some *B.mycoides* strains). The proprietary clinical database classes all Bt strains as Bc, but the bioterrorism database recognizes the possibility of misidentification of Bt.

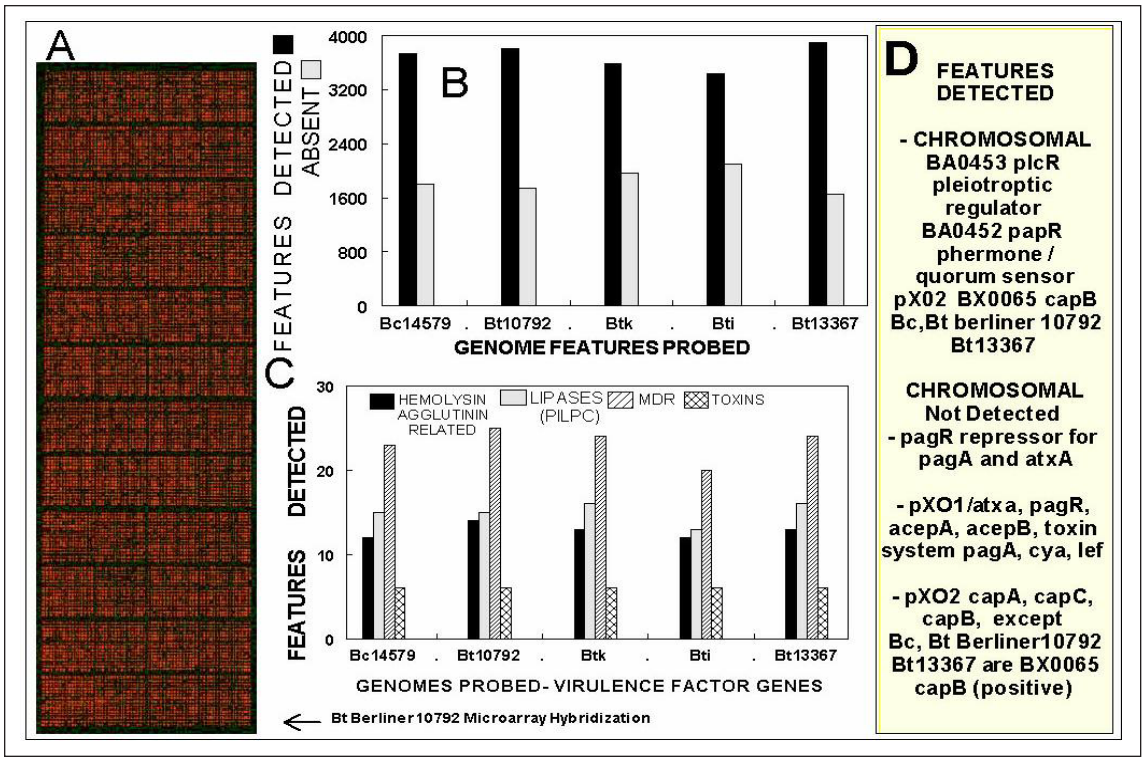

FIG. 4. Potential genome similarities and dissimilarities of Bc and Bt strains revealed by comparative genomic hybridisation arrays. (A) Example of hybridisation to glass slide microarray (manufactured by Qiagen and printed by DNA Core Facility NML/PHAC) composed of oligonucleotide features representing the BaA2012 (5.1 Mbp unfinished chromosome; 5444 features printed, 2486 functions unknown); pX01 (116 of 204 printed and denoted BXA…;156 unknown functions) and pX02 (56 of 104 denoted BXB..;78 unknown functions). (B) Summary of total features detected (triplicate hybridisations) using genomic DNA from Bc and Bt. (C) Common potential virulence functions by various categories. (D) Status of key functions indicating capacity of Bc and Bt strains to express and respond to quorum sensing signals (plcR/papR) but lacking pathogenic factors encoded by plasmids pXO1 and pXO2 of extreme virulent strains of Ba.

can incorporate all of the data collected for any given strain and give both a taxonomic assignment (against reference strains or published criteria (1)), and also a ranking of potential for infectivity and pathogenicity. We have used the latter hierarchy to prioritise in vivo testing. It is notable that 24h tests with commercial products (in presence of antibiotic) showed little or no damage to mammalian or insect cells (10,15; Fig.2C,D), albeit antigenic components (e.g., PI-P Lipase C) (15) are present, and consistent with their degradation by autolytic activity during sporulation phase.

Identification by genome/proteome mapping. Over the past decade there has been extensive use of PCR and DNA hybridisation methods to identify Bc group organisms, particularly in terms of commonality of their virulence factors (2-5,8,9). We have made use of medium to high-resolution sequence maps of several strains of Ba and Bc, and three Bt strains (3,4), to compare general genomic features of Bc14579 and Bt strains. Figure 4 summarizes intergenomic data using a custom microarray probe based on the BaA2012 chromosome and plasmids pXO1 and pXO2 (3,4). The Bt10792 x A2012 array hybridisation (Fig. 4A) illustrates the significant detection of features also seen using DNA from other genomes. Analysis (Imagene 6.0.0 Beta, BioDiscovery Inc., and Genespring 7.0, Silicon Genetics) indicate a high degree of commonality in features (~2800) compared to Bs168 or Bs6051a (data not shown, <700 features). Many of the shared features correspond to genes of hypothetical function, with potential virulence genes exhibiting >90% sharing (Fig.4 C), which are consistent with earlier analysis of genes and proteins (2-4). Given the small target size of the probe features, similarities between genomes may be underestimated. As genomic/proteomic detail increases, and major similarities between species and strains are understood, the novel aspects (new genes and allelic variations) can be used as strain level discriminators. A further layer of understanding is expected to come from multi-locus, expression analysis using probes from both bacterial and mammalian genomes to map cell-

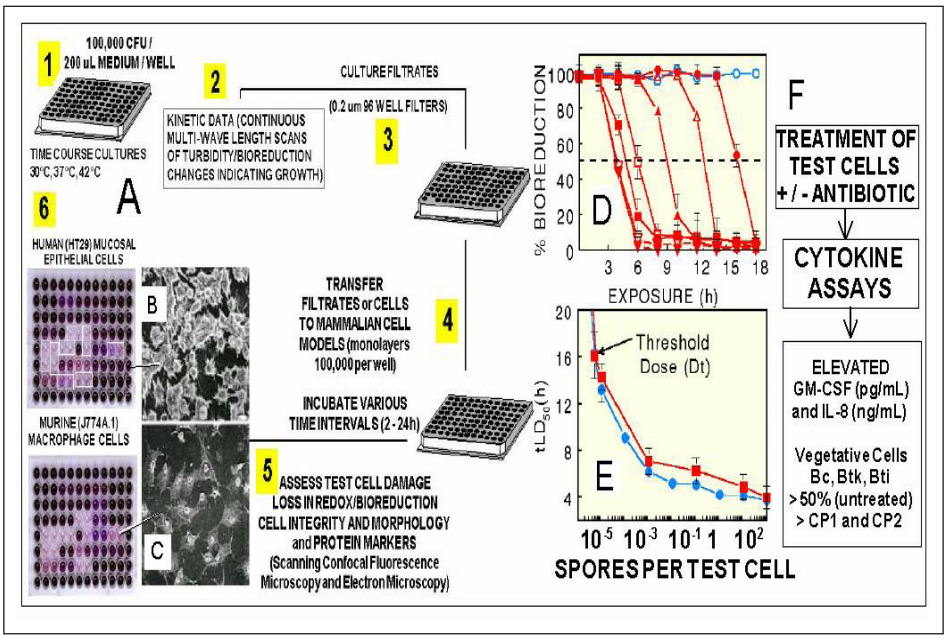

FIG. 5. In vitro toxicity screening protocol for quantifying exposure effects of microorganisms, their byproducts and possible formulation applications. (A) Steps 1 to 6 with end results (multiwell plates, step 6) after 6h exposure using of a number of *Bacillus* species/strains. Test cell toxicity is indicated by loss of bioreduction (MTT formazan) (clear versus dark wells) and structure (C) (scanning electron microscope image) versus control (B). Only strains of Bc, Bt and *B.mycoides* strains caused severe cytotoxic/lytic effects. The assay enables collection of Bc/Bt dose/time kinetic data (D and E). Also, both mucosal epithelial (HT29) and macrophage-like (J774A.1) can be assessed for cytokine production and release, prior to cell damage, which indicates potential for inflammatory reactions. With antibiotic (gentamicin 50ug/mL) effects of CP-1, CP-2 and purified Bc group spores were nil compared to their vegetative cells (VC) or debris from them (signifying autolytic activity), which induced high levels of cytokines.

cell and molecular interactions, and effects on immune defences (15) (see also next section).

In vitro animal cell models to test toxicity/ pathogenicity potentials. Summarized here are test systems using model cells of intestinal mucosa (HT29) (Fig. 5), and macrophage (J774A.1) (Fig. 6). J774A.1 along with RAW 264.7 are two of four cell lines that we have tested so far (15), and both have been used to study effects of *Acinetobacter* strains and Ba (16,17). In the presence of appropriate antibiotics, exposure effects from all types of bacterium are similar to untreated control cells. This includes treatments with PIB structures enriched from Bti and Btk commercial products (10,15). The latter exhibited low effects on immune systems of exposed migrant workers compared to spores (i.e., their vegetative cells) (11). However, in the absence of antibiotic, spores from all strains of Bc and Bt, germinate and grow in the presence of test cells, and produce exoproducts (as indicated in Fig. 2) which cause loss of metabolic activity and protein synthesis, and cell lysis (10). Dose-dependent effects begin with one spore or vegetative cell (Fig. 5B and C), and correlate with the time required for vegetative cells to increase to concentrations similar to that observed in in vivo infections (~ 10^5 per mL) (6,7,9,10). In early exposures (5 min to 1h), the macrophage model allows the assessment of innate immune defences, which include phagocytosis and cell signalling responses. The pathways outlined in Fig. 6 indicate potential exposure outcomes. Spores of Bc and Bt strains are engulfed at rates and amounts comparable to other *Bacillus* species spores, but after 3h, depending on spore dose, the macrophage exhibit internal infection and death. These effects have been seen in studies (9). Because both model systems have capacity to release cytokines, known to act in vivo as signals in innate immune responses (e.g., inflammatory reactions), these immune-mediating responses can be assessed as well. Results so far indicate that some cytokines (e.g., GM-CSF and IL-8 for mucosal cells and TNF-α, IL-1β, IL-6 for macrophage) are early markers for exposure to Bc and Bt vegetative cells and/or their cellular debris. A further layer of assays can be performed using multi-indicator arrays for specific proteins and genes (e.g., antibody and gene chip arrays) to reveal extent of effects in these exposures such as shown in one array hybridisation example Fig. 6B,C. (see ref. 16 application for Ba analysis).

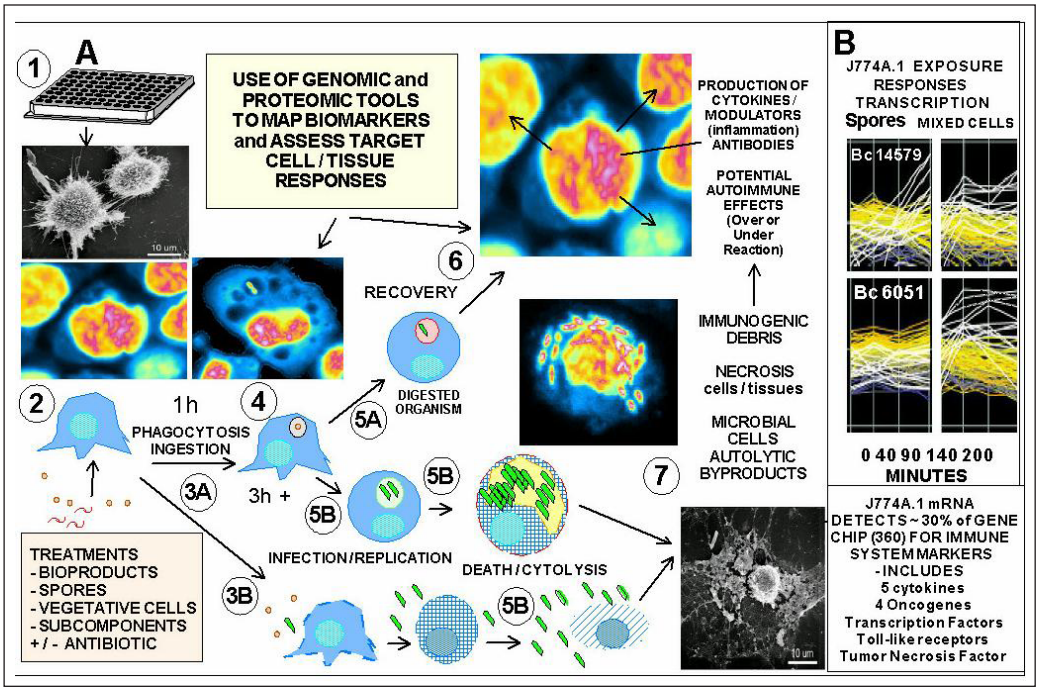

FIG. 6. Macrophage functional test to assess exposure effects of various types of micro-organisms and possible formulations. (A) in vitro assay based on Fig.5. uses J774A.1cells and known amounts of bacteria or subcomponents (step 2) equivalent to the bacteria from which they came. Uptake (phagocytosis) is completed by 45 to 60 min. Pathway 3A,4,5A,6 occurs with spores or vegetative (growing) cells (VC) of Bs6051 as well as either spores or VC derived from Bc14579 or Bt (either 13367, 10982 or CP1 (Btk) or CP2 (Bti) spores) + antibiotic (gentamicin 50ug/mL). Pathway 3A,4,5B,7 occurs with spores of Bc or Bt (all sources), beginning at 3h, and ending at ≥ 6h, and their VCs (with/without prewash to remove exoproteins). (B) Preliminary transcriptional assay using Mouse Autoimmune/Inflammatory Response GEArray: MM-602.3.

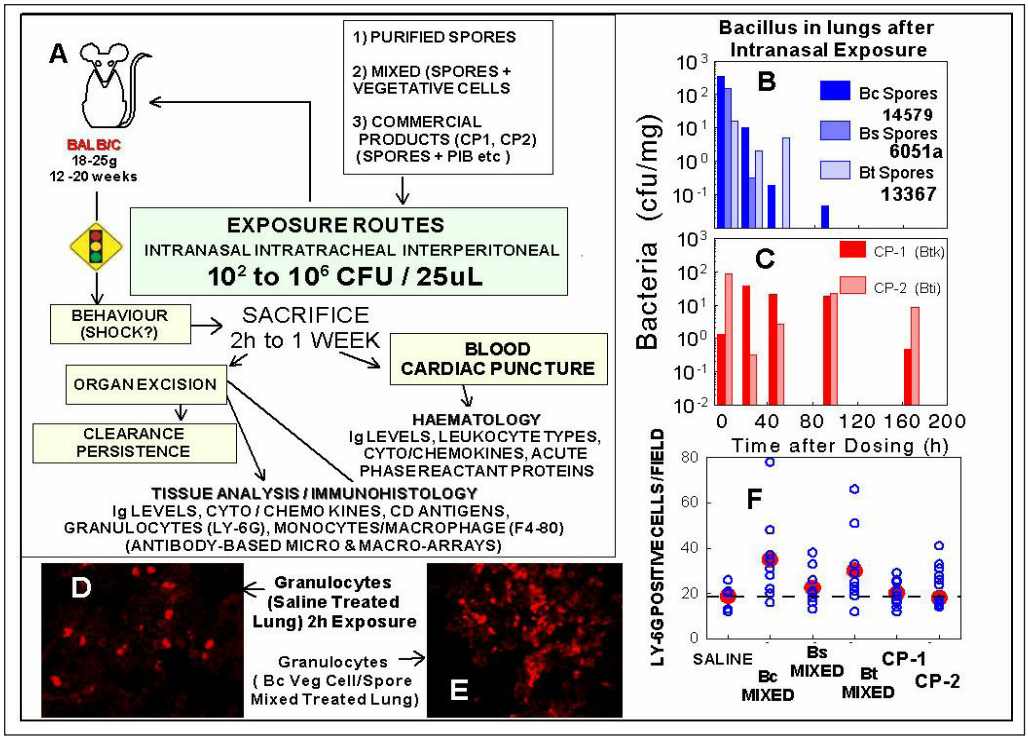

FIG.7. In vivo murine test model. (A) Health Canada Animal Care Committee approved protocol using behaviour (shock/stress symptoms) as endpoint followed with assays to address immunologic responses mostly. (B,C) Clearance times for purified (B) spores of Bc, Bt and Bs and equivalent amounts of spores as found in commercial products, CP-1 and Cp-2 (C). The latter contain PIB/CRY proteins (Fig.2). Migrant workers exposed to similar products exhibited relatively low immune reactivity to these components (10). Confocal images (D,E) of lung (thick sections) exhibiting accumulation of granulocytes which occur only in exposures to either Bc or Bt vegetative cell /spore preparations and not others (F).

In vivo exposure model. Tier-1 tests to assess human health and safety include regimes involving animals of different species, and test substances (e.g., technical grade active ingredient, microbial pest control agent, and/or end-use product), employing different routes of exposure/administration (e.g., oral, pulmonary, dermal). The test protocol shown in Fig.7A arose partly from joint work on in vivo testing of Bc and Bt strains assigned to the CEPA (1999) Domestic Substance List (see Fig1 and ref.13). This generic protocol is aimed at improving dose delivery and quantification and monitoring immune responses, as a follow up to studies of migrant workers exposed to Bt applications in the U.S.A. (10). The protocol makes use of early indicators for changes in behaviour signifying onset of stress/shock-like symptoms, and lower dose regimes (10^3 to 10^6 per animal), and accommodates a large number of assays involving multi-cellular/molecular markers for sub-lethal, largely immunologic effects. A brief scan of data from several tests demonstrates differences in clearance patterns of purified spores from sources indicated in Fig.7A, and commercial Bt-formulations (Fig.7B). However, effects from spores or Bt-formulations are relatively minor compared to immune responses in equivalent exposures with preparations involving mixtures of spores and vegetative cell (>60%) which triggered shock-like symptoms at ~ 2h and build up of various leukocyte populations such as shown here (see Fig.7D-E). These observations are consistent with earlier findings (6,7), and expected based on innate immune reactivity to presence of growing bacteria or their cellular debris.

References

1. **Euzéby, J.P.** 2005. List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.net).

2. **Pirttijarvi,T.S., M. Andersen, A.C. Scocing, and M.S. Salkinuja-Salonen.** 1989. Evaluation of methods for recognizing strains of the *B.cereus* group with food poisoning potential among Industrial and environmental contaminants. Syst.Appl Microbiol **22**: 133-144.

3. **Gohar, M., N. Gilois, R. Graveline, C. Garreau, V.Sanchis, and D. Leclercus.** 2005. A comparative study of *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* extracellular proteomes. Proteomics **5**:3696-3711.

4. **Rasko, D.A., M.R. Alther, C.S. Han, and J. Ravel.** 2005. Genomics of the *Bacillus cereus* group of organisms. FEMS Micriobiol. Reviews **29**: 303-329.

5. **Shisa, N., N. Wasano, A. Ohgushi, D-H. Lee, and M. Ohba.** 2002. Extremely high frequency of common flagellar antigens between *Bacillus thuringiensis* and *Bacillus cereus.* FEMS Microbiol. Letters **213**:93-96.

6. **Hernandez, E., F. Ramisse, T. Cruel, R. le Vagueresse, and JD. Cavallo.** 1999. *Bacillus thuringiensis* serotype H34 (subsp. *konkukian*) isolated from human and insecticidal strains serotypes 3a3b and H14 can lead to death of immunocompetent mice after pulmonary infection FEMS-Immunol-Med-Microbiol**.24**: 43-47

7. **Salamitou, S., F. Ramisse, M. Brehelin, D. Bourguet, N. Gilois, M. Gominet, E. Hernandez, and D. Lereclus.** 2000. The PlcR regulon is involved in the opportunistic properties of *Bacillus thuringiensis*, and *Bacillus cereus* in mice and insects. Microbiology **146**:2825-2832.

8. **Ehling-Schulz, M., B. Svensson, M.-H. Guinebretiere, T. Lindback, M. Andersson, A. Schulz, M. Fricker, A. Christiansson, P. E. Granum, E. Martlbauer, and C. Nguyen-The.** 2005. Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. Microbiol. **151**:183-197.

9. **Ramarao, N., and D. Lereclus.**2005. The InhA1 metalloprotease allows spores of the B.cereus group to escape macrophage. Cell. Microbiol.**7**:1357-1364.

10. **Tayabali A.F., and V.L. Seligy.** 2000. Human cell exposure assays of *B.thuringiensis* commercial insecticides:production of *Bacillus cereus*-like cytolytic effects from the outgrowth of spores. Env.Health Perspectives **108**: 919-930.

11. **Bernstein, I.L., J.A. Bernstein, M.E. Miller, S. Terzieva, D.I .Bernstein, Z. Lumus, M.J. Selgrade, D. Doerfler, and V.L. Seligy.** 1999. Health effects research on microbiologic insecticides in farm workers. Environ Health Perspectives **107**: 575-582.

12. **Seligy, V.L., G.R. Douglas, J.M. Rancourt, A.F. Tayabali, I. Otvos, K. van Frankenhuyzen, J. Dugal, and G. Rousseau**, 2000. Comparative performance of conventional and molecular dosimetry methods in environmental biomonitoring: assessment using *Bacillus*-based commercial biopesticides as models. *In* Rapid Methods for the Analysis of Biological Materials in the Environment. Eds P.J. Stopa and M.A.Bartoszcze, NATO ASI Series Kluwer Academic Publishers, Netherlands pp. 279-297.

13. **Seligy, V.L., and J.M. Rancourt.** 1999. Antibiotic MIC/MBC Analysis of *Bacillus*-based commercial insecticides: use of bioreduction and DNA-based assays. J Industrial Microbiol Biotechnol **21**: 1-10.

14. **El Ouakfaoui, S., L-A. Tsan, T. Pare, and K. Delgaty.** 2005. Environmental risk assessment of *Bacillus thuringiensis* ATCC 13367 under the Canadian Environmental Protection Act 1999 (CEPA 1999). Proc. 6th Pacific Rim Conference.

15. **Seligy, VL., PS. Shwed , and AF .Tayabali**. 2004. Comparison of Macrophage Cell Models exposed to spores of *Bacillus cereus* and *B.thuringiensis* sspp. Proc.12th Internat'l Immunol. Congress. Medimond S.r.l., Bologna Italy Vol.ISBN 88-7587-0769-1 pp 469-472.

16. **Bergman, NH., K.D. Passalacqua , R. Gaspard , L.M. Shetron-Rama, J. Quackenbush, and P.C. Hanna.** 2005**.** Murine Macrophage Transcriptional Responses to *Bacillus anthracis* Infection and Intoxication . Infection and Immunity **73**:1069-1080.

17. **Tayabali, AF., KC. Nguyen ,and VL. Seligy.** 2004. Immunotoxic Assays for Potential Bioremediation Bacteria: *Acinetobacter* and *Rhizobium radiobacter*. Proc.12th Internat'l Immunol.Cong. MedimondS.r.l.,Bologna Italy. Vol.88-7587-0769-1 pp269-273.