Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

21-Dec-2010

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

ENV/JM/MONO(2010)43
Unclassified

Report of the 1st OECD BioPesticides Steering Group Seminar on Identity and Characterisation of Micro-Organisms

Series on Pesticides
No. 53

JT03294666

English - Or. English

OECD Environment, Health and Safety Publications

Series on Pesticides

No. 53

REPORT OF THE 1st OECD BIOPESTICIDES STEERING GROUP SEMINAR ON IDENTITY AND CHARACTERISATION OF MICRO-ORGANISMS

1 July 2009, OECD, Paris

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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Paris 2010

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No. 49 *OECD Guidance Document on Defining Minor Uses of Pesticides* (2009)

No. 50 *Report of the OECD Seminar on Pesticide Risk Reduction through Better National Risk Management Strategies for Aerial Application* (2010)

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No. 52 *OECD Survey of Pollinator Testing, Research, Mitigation and Information Management: Survey Results* (2010)

No.53 *Report of the 1st OECD BioPesticides Steering Group Seminar on Identity and Characterisation of Micro-organisms* (2010)

Published separately

OECD Guidance for Country Data Review Reports on Plant Protection Products and their Active Substances-Monograph Guidance (1998, revised 2001, 2005, 2006)

OECD Guidance for Industry Data Submissions on Plant Protection Products and their Active Substances-Dossier Guidance (1998, revised 2001, 2005)

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Report of the OECD Pesticide Aquatic Risk Indicators Expert Group (2000)

Report of the 2nd OECD Workshop on Pesticide Risk Indicators (1999)

Guidelines for the Collection of Pesticide Usage Statistics Within Agriculture and Horticulture (1999)

Report of the [1st] OECD Workshop on Pesticide Risk Indicators (1997)

Report of the OECD/FAO Workshop on Pesticide Risk Reduction (1995)

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FOREWORD

This report presents the outcomes of a biopesticides Seminar on issues related to the identity and characterisation of micro-organisms used for pest control, which took place on 1st July 2009 at OECD, in Paris, France. This Seminar was held back-to-back with the annual meeting of the BioPesticides Steering Group (BPSG), a sub-group of the OECD Working Group on Pesticides (WGP). The Seminar was the first one of a series of BPSG seminars that focus on biopesticide-related issues of interest to OECD member countries' governments.

The objectives of the Seminar were to:

- identify key issues related to the identification and characterisation of micro-organisms used for pest control;
- exchange information on national and international activities in the area concerned; and
- make recommendations for further actions and/or possible activities.

The Seminar was chaired by Jeroen Meeussen (the Netherlands), the Chairman of the BPSG. Thirty five experts from 16 countries and IBMA (International Biocontrol Manufacturers Association) participated in the Seminar. The list of Participants is in Annex 2.

The Seminar consisted of two main sessions with first, presentations addressing the following topics: *Government and Experience Perspectives* and *Stakeholder Experience and Perspectives*, and second, a round-table discussion session. The Seminar participants' conclusions, observations and recommendations are included in the first part of this report. The Seminar Programme is presented in Annex 1. The abstracts of presentations are compiled in Annex 3, while presentations are provided in Annex 4.

The draft Seminar report was approved out-of-session by the Working Group on Pesticides by written procedure was finished on 1st September 2010.

This document is being published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology, which has agreed that it be unclassified and made available to the public.

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INTRODUCTION

1. This report presents the results and recommendations of an OECD Seminar of the BioPesticides Steering Group (BPSG) on issues related to the identity and characterisation of micro-organisms. This one-day Seminar, held on 1 July 2009, was chaired by Jeroen Meeussen (the Netherlands), Chairman of the OECD BPSG, and took place at OECD, in Paris, France.

2. This Seminar was the first in a series of Seminars on biopesticides to be organised by the OECD BPSG, a sub-group of the OECD Working Group on Pesticides (WGP). The BPSG Seminars will focus on key issues on biopesticides of interest to OECD governments. "Identity and characterisation of micro-organisms" was selected as the topic of this Seminar considering its significance for the registration of biopesticides.

3. The importance of identification, taxonomy, characterisation, manufacturing process and -acceptable level of- contaminants is already stressed in the OECD Working Document on the Evaluation of Microbials for Pest Control (Series on Pesticides No. 43, 2008) and will be a topic of future Working Documents. These issues were also highlighted in the Workshop on the Regulation of Biopesticides: Registration and Communication issues (Series on Pesticides No. 44, 2009).

PARTICIPANTS

4. People attending the OECD Seminar included representatives of the pesticide regulatory authorities of OECD countries, representatives from industry, IBMA (International Biocontrol Manufacturers Association), and international experts familiar with issues related to identity and characterisation of micro-organisms. A participant list is provided in Annex 2.

PURPOSE AND SCOPE OF THE SEMINAR

5. The main objectives of the Seminar included:

- to identify key issues and challenges in the area of the identification and characterisation of micro-organisms including issues related to taxonomy, the manufacturing process and -acceptable- level of contaminants;
- to provide updates of national and international activities and initiatives in the area of identification and characterisation of micro-organisms;
- to exchange information on OECD countries' current activities in the area of the identification and characterisation of micro-organisms;
- to suggest and discuss options of further steps for OECD countries and key stakeholders in OECD and non-OECD countries to address the identified issues; and,
- to recommend possible further steps for OECD.

6. In particular the following issues were presented during the Seminar:

- what methods should be used for identification of micro-organisms;
- at what taxonomic level is verification required;
- what identification data package should be submitted to conclude if strains are similar;
- standard operational procedures, including quality control measures regarding the manufacturing process; and
- maximum acceptable amount of contaminants.

STRUCTURE OF THE SEMINAR

7. The Seminar intended to follow the format developed for the OECD-Risk Reduction Steering Group Seminar series, with presentations in the morning and roundtable discussions in the afternoon. However during this Seminar it was decided to have a short discussion after each presentation due to the diversity of topics discussed within groups of micro-organisms. The Seminar programme is provided in Annex 1.

GOVERNMENT AND STAKEHOLDER EXPERIENCE & PERSPECTIVES AND ROUND TABLE DISCUSSIONS WITH REGARD TO IDENTITY AND CHARACTERISATION OF MICRO-ORGANISMS

8. Below are listed the main topics covered in the presentations and discussions. More detailed information can be found in the abstracts (Annex 3) and presentations (Annex 4).

- From a regulatory point of view, it is agreed that identification needs to be done at strain level. In some cases a number or collection of methods (e.g. AFLP, MLST) is needed for identification. From a registration point of view, it is important to know, within species strains that might be present, which ones are human pathogens.
- It is not always necessary to identify the strain at gene-level (although this might be challenging from a scientific point of view), but it is important that the method can distinguish between strains which are human pathogens and strains which are not. In this respect it is also important to realise that genes can be expressed differently depending on the host.
- There was also a discussion as to whether there are any benefits from having detailed taxonomy details over just a unique collection identification. It was suggested that taxonomy was useful in identifying whether an organism is related to other organisms e.g. whether it is related to a plant pathogen or if there is a likelihood of producing metabolites/toxins.
- Because taxonomy advances it will become more and more difficult to base a dossier on public literature. In particular because open literature does not always make it clear which species

and/or strain has been used in the tests. Therefore, it is very difficult to identify which data is relevant.

- The European Commission has agreed in one specific case that the protoplast fusion of two *Trichoderma* strains did not result in a GMO

- Regarding contaminants and secondary metabolites it was suggested that while there are no contaminants in the fermentor, the problem is that they can be introduced into the formulation. Contaminants can come from workers, machinery and co-formulants (e.g. clay is very difficult to sterilise). Therefore, the Seminar wondered whether it was really feasible to meet contaminant levels, particularly when there will be exposure from such contaminants coming from sources other than the product.

- Data on toxins will not be asked for if they are only produced in target organisms. It was highlighted that Canada usually only asks for information on those of concern from open literature.

- The difficult faced in the EU with pre-submission meetings was highlighted. The difficulty being that the meeting only provides one Member State's view, so other issues can still come out during peer review.

- The issue of clinical strains and the difficulties caused by strains used in clinical papers not being added to culture collections was highlighted. There was a general plea that deposition in culture collections should be encouraged.

- Regarding characterisation issues it was recommended that a tiered approach be followed. Industry also highlighted that it does not help to have a comparison of numerous other strains to other strains in the same species. As stated before it is important to distinguish between strains which are human pathogens and strains which are not.

- It was also suggested that consideration should be given to incorporating new techniques into the guidelines and data requirements for micro-organisms.

SEMINAR CONCLUSIONS AND RECOMMENDATIONS

9. This Seminar was a good opportunity to exchange information of what OECD countries, industry and researchers were actually doing in the area of identification and characterisation of micro-organisms. There was a valuable exchange of information between regulators, scientists and industry.

- It was highlighted that there were still a number of issues to consider further and unfortunately general guidance was often difficulty, the case-by-case approach often being the only answer for different groups of micro-organisms.

- There was also a proposal to set up a discussion group to take forward some issues and for further questions for discussion to be raised. This could be web-based and it will be explored whether OECD can facilitate this.

ANNEX 1

SEMINAR PROGRAMME

OECD BPSG Seminar on Identity and Characterisation of Micro-organisms 1st July 2009, OECD, Paris, France

9.00 - 9.30 [PPT 0]	**Introduction** • Purpose and structure of the Seminar • Tour de table: Introduction of participants • Presentation on the OECD and the work of OECD-BPSG *by Jeroen Meeussen, The Netherlands*
9.30 - 11.30 [PPT 1] [PPT 2] [PPT 3] [PPT 4]	**Government Experience and Perspectives** • OECD-countries will present their view: - Taxonomy, characterisation and identification of *Bacillus thuringiensis*: experience in preparing Draft Assessment Reports. ***Niels Bohse Hendriksen*** (Department of Environmental Chemistry and Microbiology, Aarhus University; Denmark) - Taxonomy, characterisation and identification of *Trichoderma*: experience in preparing Draft Assessment Reports. ***Kersti Gustafsson***(Swedish Chemicals Inspectorate – KEMI; Sweden) - Contaminants (secondary metabolites) ***Claude Alabouvette*** (Laboratoire de Recherches sur la Flore Pathogène dans le Sol, I.N.R.A., Dijon; France) - US-regulatory system and perspective on identity and characterisation issues for micro-organisms ***William Schneider*** (BioPesticides Division, EPA, Arlington; USA)

11.30 - 12.15 [PPT 5] [PPT 6] 12.15 - 13.45 13.45 - 15.00 [PPT 7] [PPT 8] [PPT 9] [PPT 10]	**Stakeholder Experience and Perspectives** • Industry, IBMA and Research Institutes will present their view: - Baculoviruses ***Rüdiger Hauschild*** (GAB Consulting GmbH, Lamstedt; Germany) - Identity and molecular characterization of baculoviruses ***Johannes Jehle*** (DLR Rheinpfaltz, Neustadt/ Weinstrasse; Germany) **Lunch** - Joint presentation: • Contamination in bacterial and fungal plant protection products ***Willem Ravensberg*** (Koppert, Berkel en Rodenrijs; The Netherlands) • Contamination with regard to baculovirus products ***Philip Kessler*** (Andermatt Biocontrol; Grossdietwil; Switzerland) - Genotypic/Phenotypic characterization of biocontrol and clinical strains of *Pantoea agglomerans* ***Brion Duffy*** *(*Agroscope, Wädenswil ; Switzerland) - Characterisation issues on bacteria ***Denise Munday, Maria Herrero*** (Valent Biosciences; Switzerland) and ***Sherry Heins*** (AgraQuest)
15.00 – 16.45	**Round-table Discussion** • What methods should be used for identification of micro-organisms; • At what taxonomic level is verification required; • What identification data package should be submitted to conclude if strains are similar; • Standard operational procedures, incl. quality control measures regarding the manufacturing process; and • Maximum acceptable amount of contaminants.
16.45 - 17.00	**Summary of discussions, ideas for follow-up, recommendations for possible further OECD work**
17.00	**End of the Seminar**

ANNEX 2

List of Participants

OECD BPSG Seminar on Identity and Characterisation of Micro-organisms
1st July 2009, OECD, Paris

Australia/Australie	Alan NORDEN Manager, Minor Use Australian Pesticides and Veterinary Medicines Authority (APVMA)
Austria/Autriche	Wolfgang BERGERMAYER Institute for Plant Protection Products Evaluation Authorization Austrian Agency for Health and Food Safety Ltd.
	Britta MÖBES-HANSEN AGES - Austrian Federal Agency for Health & Food Safety Institute for Plant Protection Products Evaluation & Authorization
Belgium/Belgique	Jérémy DENIS Bioingénieur FPS service Health, Food Chain Safety and Environment
Canada/Canada	Esther SETO Senior Evaluation Officer Pest Managment Regulatory Agency Health Canada
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Germany/Allemagne	Bilgin KARAOGLAN Environmental Risk Assessment and Management of Plant Protection Products, EU Active Substances Program Federal Environmental Office (UBA)
	Johannes JEHLE DLR Rheinpfalz
	Eckhard KOCH Julius Kuehn-Institut, Bundesinstitut fuer Kulturpflanzen, Institut fuer Biologischen Pflanzenschutz
	Vera RITZ Safety of Substances and Preparations Federal Institute for Risk Assessment (BfR)
Italy / Italie	Marco NUTI University of Pisa
Netherlands/Pays bas	Jeroen MEEUSSEN (CHAIR) EU Co-ordinator Board for the Authorisation of Plant Protection Products and Biocides
Slovak Republic/République slovaque	Nadezda ONDEJKOVA Department of Diagnostics Central Controlling and Testing Institute in Agriculture
Sweden/Suède	Kersti GUSTAFSSON Principal Scientific Adviser Pesticides and Biotechnical Products Swedish Chemicals Agency

Switzerland/Suisse	Brion DUFFY Plant Protection Division Agroscope Changins-Wädenswil ACW
Turkey/Turquie	Alev BURCAK Head of Department General Directorate of Agriculture Research Plant Protection Research Department Ministry of Agriculture and Rural Affairs
United Kingdom/Royaume-Uni	John DALE Approval Committee Branch Pesticides Safety Directorate
United States/États-Unis	William SCHNEIDER Biopesticides and Pollution Prevention Division (7511) US Environmental Protection Agency Office of Pesticide Programs
International Biocontrol Manufacturers Association (IBMA)	Bernard BLUM Head International Affairs International Biocontrol Manufacturers Association (IBMA) Agrometrix Integrated Crop Management
	Jacob EYAL Exective Vice President Certis USA LLC
	Rüdiger HAUSCHILD Head of Microbials Department GAB Consulting GmbH
	Sherry HEINS Production Registration Manager AgraQuest, Inc.
	Maria HERRERO Regulatory Affairs Manager Valent BioSciences Corp

IBMA (continued)	Denise MUNDAY President IBMA SCAE-Valent Biosciences Sàrl.
	Willem RAVENSBERG R & D Microbials Manager Koppert Biological Systems
	Eda REINOT Head R & D Becker Underwood
	Azzurra ABELLI Regulatory Affairs Agrifutur srl
	Philip KESSLER Regulatory Affairs Andermatt Biocontrol AG
	Sergio FRANCESCHINI Regulatory Affairs Director Intrachem Production S.r.l.
	Ulf HEILIG International Relations – Regulatory Affairs International Biocontrol Manufacturers Association (IBMA)
	Peter LUETH Managing Director Prophyta Biologischer Pflanzenschutz GmbH
	Roma GWYNN Biocontrol Consultant Rationale Biopesticide Consultants
OECD/OCDE	Marie-Chantal HUET Administrator ENV/EHS OECD

Davina TILL
Assistant
ENV/EHS
OECD

ANNEX 3

Abstracts of Presentations

Presentation on the OECD and the work of OECD-BPSG
By Jeroen Meeussen, The Netherlands

Taxonomy, characterisation and identification of *Bacillus thuringiensis*: experience in preparing Draft Assessment Reports
By Niels Bohse Hendriksen (Department of Environmental Chemistry and Microbiology, Aarhus University; Denmark)

Taxonomy, characterisation and identification of *Trichoderma*: experience in preparing Draft Assessment Reports
By Kersti Gustafsson (Swedish Chemicals Inspectorate – KEMI; Sweden)

Contaminants (secondary metabolites) and residues
By Claude Alabouvette (Laboratoire de Recherches sur la Flore Pathogène dans le Sol, I.N.R.A., UMR Microbiologie du Sol et de l'Environnement, Dijon; France)

US-regulatory system and perspective on identity and characterisation issues for micro-organisms
By William Schneider (BioPesticides Division, EPA, Arlington; USA)

Baculoviruses
By Rüdiger Hauschild (GAB Consulting GmbH, Lamstedt; Germany)

Identity and molecular characterization of baculoviruses
By Johannes Jehle (DLR Rheinpfaltz, Neustadt/ Weinstrasse; Germany)

The Biopesticide Industry view on microbial contaminants and limits in microbial pest control product based on baculoviruses
By Willem Ravensberg (Koppert, Berkel en Rodenrijs; The Netherlands)

Contamination with regard to baculovirus products
By Philip Kessler (Andermatt Biocontrol; Grossdietwil; Switzerland)

Genotypic/Phenotypic characterization of biocontrol and clinical strains of *Pantoea agglomerans*
By Brion Duffy (Agroscope, Wädenswil; Switzerland)

Characterisation issues on bacteria
By Denise Munday, Maria Herrero (Valent Biosciences; Switzerland) and Sherry Heins (AgraQuest)

Presentation on the OECD and the work of OECD-BPSG

By Jeroen Meeussen (Board for the Authorisation of Pesticides, The Netherlands) [PPT 0]

In 1961 the Organisation for Economic Co-operation and Development (OECD) was established with a trans-Atlantic and then global reach. Today the OECD has 33 member countries. More than 70 developing and transition economies are engaged in working relationships with the OECD. OECD is a forum in which governments work together to address the economic, social and environmental challenges of interdependence and globalisation. OECD is also a provider of comparative data, analysis and forecasts to underpin multilateral co-operation.

The OECD work on agricultural pesticides (i.e. chemical and biological pesticides) aims to help member countries improve the efficiency of pesticide control, share the work of pesticide registration and re-registration, minimise non-tariff trade barriers and reduce risks to human health and the environment resulting from their use. In support of these goals, the Pesticides Programme has undertaken work to:

i. identify and overcome obstacles to work-sharing;

ii. harmonise data requirements and test guidelines; and

iii. harmonise hazard/risk assessment approaches.

The BioPesticides Steering Group (BPSG) was established by the WGP in 1999 to help member countries harmonise the biological pesticides assessment and improve the efficiency of control procedures. Biological pesticides involve: microbials, pheromones and other semiochemicals, plant extracts (botanicals) and invertebrates as biological control agents. The BPSG has been chaired by Canada since its inception and by The Netherlands from mid 2005 onward. The first tasks of the BPSG consisted of:

i. reviewing regulatory data requirements for three categories of biopesticides (microbials, pheromones and invertebrates); and

ii. developing formats for dossiers and monographs for microbials, and pheromones and other semio-chemicals.

This was achieved in 2004 and resulted in several OECD-publications in the Series of Pesticides (No. 12, 2001; No. 18, 2003 and No. 21, 2004).

The BPSG then decided to concentrate its efforts on science issues that remain as barriers to harmonisation and work-sharing. This resulted in the preparation of a “working document” which does not provide 'mandatory' guidance but being essentially a set of examples/case studies aimed at helping the regulatory authorities. The document is titled: *“Working Document on the Evaluation of Microbials for Pest Control”* and has been published in OECD Series on Pesticides No. 43, 2008.

The report of the *Workshop on the Regulation of Biopesticides: Registration and Communication issues, 15 – 17 April 2008, EPA, Arlington, USA*, is the most recent publication of the work of the BPSG in the OECD Series on Pesticides (No. 44, 2009).

This seminar on *Identity and Characterisation of micro-organisms* is the 1st Seminar in -hopefully- a series of seminars on biopesticides to be organised by the BPSG. "Identity and characterisation of micro-organisms" was selected as the topic of this Seminar considering its significance for the registration of biopesticides.

The importance of identification, taxonomy, characterisation, manufacturing process and -acceptable level of- contaminants is already stressed in previous mentioned OECD-publications. The objectives, scope and structure of the Seminar are described in detail in the 'Seminar outline'.

Taxonomy, characterisation and identification of *Bacillus thuringiensis*: experience in preparing Draft Assessment Reports

By Niels Bohse Hendriksen (Department of Environmental Chemistry and Microbiology, Aarhus University; Denmark)
[PPT 1]

Niels Bohse Hendriksen, National Environmental Research Institute, Århus University, Frederiksborgvej 399, P.O. Box 358, DK-4000 Roskilde.

Denmark has been the responsible EU-country for the preparation of three Draft Assessment Reports (DARs) on strains of *Bacillus thuringiensis* subsp. *kurstaki* (Btk) a bacterium notably active against larvae of pests from the insect-order Lepidoptera (Butterflies). The three DARs cover in total five different Btk strains from four companies (Valent, Certis, Intrachem and Probelte).

From the data provided by the companies it appeared that the strains have different origins, that they are member of the *Bacillus cereus* group producing parasporal crystals consisting of delta-endotoxins (cry1 and cry2) and with the 3a3b(3c) serotype– confirming their affiliation to *B. thuringiensis* subsp. *kurstaki.* Thus the classification of the five strains to Btk is unequivocal based on the provided data. Further it appears that there exist small differences between the five strains e.g. at the level of the relative concentrations of the cry-toxins and the presence and size of plasmids.

The relationship between Btk and other species within the *B. cereus* group (notably *B. cereus* and *B. anthracis*) could be established on the basis data provided by some of the applicants and from the open peer-reviewed literature. The data exists of Amplified Fragment Length Polymorphism typing (AFLP) and Multi Locus Sequence Typing (MLST) of numerous strains from the group. It appeared from these analyses that: Btk is a homogenous group most likely of monophyletic origin, that Btk is not closely related to *B. anthracis*, emetic *B. cereus* or exotoxin producing *B. thuringiensis* strains. However, in addition to this the results also show that Btk is related to some pathogenic, mainly entero-toxic, *B. cereus* strains. It is therefore necessary to take the relationship to entero-toxic *B. cereus* into consideration when assessing risks associated to Btk.

It has been concluded that unequivocal identification at strain level is needed for control-activities of authorities. One of the companies have established a system for unequivocal identification of one Btk strain, such systems need still to be established for the four remaining strains.

Taxonomy, characterisation and identification of *Trichoderma*: experience in preparing Draft Assessment Reports

By Kersti Gustafsson (Swedish Chemicals Inspectorate – KEMI; Sweden)
[PPT 2]

Within the EU review program for plant protection products, which is formulated in Commission Regulation (EC) No 2229/2004 of 3 December 2004 laying down further detailed rules for the implementation of the fourth stage of the programme of work referred to in Article 8(2) of Council Directive 91/414/EEC, Sweden has acted as Rapporteur Member State for *Trichoderma harzianum* and *T. polysporum,* that were on the so called List 4. As there were several companies involved Sweden got three dossiers, one of which was a compiled dossier from a Task Force. Taxonomy in general is in a revolutionizing period with molecular biology and development of DNA techniques rendering some difficulties with the assessment.

For *T. harzianum* Rifai proposed in 1969 nine species aggregates and this classification was still valid when the Council Directive 91/414/EEC of July 15, 1991, became effective in July 1993. As taxonomy evolved the four *T. harzianum* in the task Force dossier ended up to be two *T. harzianum*, one *Trichoderma asperellum* and one *Trichoderma atroviride*. Sweden chose to assess all these four strains in one draft assessment report as they were originally seen as one species and the important point for risk assessment must be rather the phenotype than the genotype. Strain specific information was compared however no specific differences were found yet.

One of the *Trichoderma* strains was developed via protoplast fusion from two mutants, one with biocontrol capacities and the other with rhizosphere colonisation capacities. Protoplast fusion might be compared with anastomosis which means approximately that two hyphae grow together. In the Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC - Commission Declaration techniques of genetic modification are defined and protoplast fusion is mentioned. In consultation with the EU Commission it was assessed that the organism is formed by protoplast fusion of organisms, which are capable of exchanging genetic material by traditional breeding methods; it should therefore on this basis be exempt from Directive 2001/18/EC.

Sweden also drafted assessment reports for another strain of *T. harzianum* and *T. polysporum*. The main difference between those two strains was the range of temperature activity, which was both the reason for having both species in one product and also for producing two draft assessment reports.

All these *Trichoderma* strains have been included in Annex 1 to Directive 91/414/EEC via Commission Directive 2008/113/EC of 8 December 2008 amending Council Directive 91/414/EEC to include several micro-organisms as active substances. There was a condition for the Annex 1 inclusion, *i.e.* the Commission is to request the EFSA to deliver its view on the draft review reports by 31 December 2010 at the latest.

Contaminants (secondary metabolites)

By Claude Alabouvette (Laboratoire de Recherches sur la Flore Pathogène dans le Sol, I.N.R.A., UMR Microbiologie du Sol et de l'Environnement, Dijon; France)
[PPT 3]

The directive 2001/36, being the transposition to microorganisms of the directive 91/414 prepared for chemicals, requirements concerning secondary metabolites, contaminants and residues are difficult to fulfil.

It is required to characterize "metabolites", but it seems difficult to detect and identify all the secondary metabolites which could be produced by microbial biocontrol agents. In fact, both bacteria and fungi are producing, usually at low concentration, a large variety of secondary metabolites, including antibiotics, toxins, hormone-like substances etc. The secondary metabolites produced during the fermentation process might be present in the technical product, and the same or other metabolites might be produced after release of the MBCA in the environment. It is therefore impossible to characterize all the metabolites produced by the MBCA at the different stages of its life cycle. Only those present at a quantifiable level in the technical product, and known to be dangerous, might be of concern for risk assessment.

It is then required to identify and characterize contaminants. In the case of MBCAS, the presence of microbial contaminants should be avoided. Therefore, the MBCAs should always be produced in pure culture. But this is not always possible, for example in the case of viruses, and even when the fermented product is clean, microbes from the environment can pollute the product during the formulation process. Obviously the microbial contaminants should be kept at a low concentration in the commercial product; otherwise the batch will have to be destroyed. It seems difficult to set a maximum level of contaminants, since the hazards depends greatly depends on the nature of the contaminants. Therefore a case by case approach has to be followed.

Finally, as for chemical, residues on food and feed have to be characterized. But in the case of MBCAs the residues are not exclusively of chemical nature. According to the directive, the residues are divided in viable residues, and non viable residues. The biocontrol agent itself constitutes the major part of the viable residues which also include the microbial contaminants. In contrast to chemical pesticides the MBCAS can establish and sometimes proliferate on food and feed. The non viable residues are the metabolites which can be produced by the MBCA on or in food and feed. As already stated, it is quite impossible to characterize all the secondary metabolites present as traces at the surface of, for example, vegetables or fruits treated several weeks before harvest. Thus again, only a case by case approach can be recommended, taking into account that agricultural products do not have to be sterile to be put on the market!

US-regulatory system and perspective on identity and characterisation issues for micro-organisms

By William Schneider (BioPesticides Division, EPA, Arlington; USA)
[PPT 4]

The US Microbial Pesticide Data Requirements were recently revised to better reflect the actual regulatory practices that had evolved since they were previously published in 1984. They were published in Title 40, Part 158.2100, of the Code of Federal Regulations with an effective date of December 26, 2007. They are available on the EPA Biopesticides website at www.epa.gov/pesticides/biopesticides. The data requirements relevant to this topic are in 40CFR158.2120, Product Analysis, i.e. Product Identity, Manufacturing Process, Deposition of a Sample, Discussion of Formation of Unintended Ingredients, and Analysis of Samples. In addition, the US has guidelines for these data requirements. The guidelines are our recommendations on how to best satisfy the data requirements and are available on the EPA pesticides website at www.epa.gov/opptsfrs/home/guidelin.htm

The microbial identity is very important to allow comparison to related microorganisms in the scientific and clinical literature for a pre-submission evaluation of what data might be needed or, possibly, not needed, to adequately assess the potential risks. A proper taxonomic description also is used to identify the strain of the microorganism that is used as a pesticide. However, microbial taxonomy alone is not sufficient to adequately identify a microbial pesticide active ingredient since the nomenclature can change and often is not specific enough to separate toxicant-producing variants. The US has required companies to add a unique isolate identification designation to the taxonomic name and also to maintain their registered microbial pesticide in a nationally recognized culture collection. The company is responsible to ensure that the sample remains on deposit to allow, if needed, subsequent comparison of the microbial pesticide being sold and the deposited strain that was registered. Genetic analysis of an isolate may also be useful, particularly for biocontrol viruses, whose taxonomy is often more uncertain.

The US also does an analysis of the potential for contamination with pathogens in the microbial growth process and/or in the end use product. This requires a case-by-case analysis of the growth media, production process, and the final formulation. Batch monitoring may be required as part of the manufacturing process. The microbial pesticides that have to be produced in living organisms, such as the baculoviruses which are often grown in living insects, require close attention to the potential for contamination.

Baculoviruses
Experience and Perspectives

By Rüdiger Hauschild (GAB Consulting GmbH, Lamstedt; Germany)
[PPT 5]

What is a baculovirus?

Baculoviruses are rod-shaped enveloped viruses with a circular double-stranded DNA. The family consists of two morphological groups, Granulovirus (GV) and Nucleopolyhedrovirus (NPV). Baculovirus species are named according to the morphology of the occlusion body and the host where the virus was isolated from. However, this system alone is not appropriate to distinguish between viruses with different origins and the traditional names do not contain sufficient information for species distinction or a phylogenetic assignment. DNA sequence analyses revealed that phylogenetics of Baculoviruses follows the phylogeny of the host insects and is not directly linked to occlusion body morphology. A phylogenetic species criterion was proposed based on the similarity of three partial sequences of conserved genes (Lange et al., 2004; Jehle et al., 2006).

Regulatory situation

Baculoviruses are used since many years in Plant Protection Products in different countries. From a regulatory point of view, baculoviruses are "micro-organisms" in all systems. As for other micro-organisms, regulation is accomplished at strain level. However, baculovirus isolates are genetically heterogenous and "strains" do not exist for species that are used in plant protection products. In the EU, two species are currently included in Annex I of Directive 91/414: *Spodoptera exigua* Nucleopoly-hedrovirus (SeMNPV) was included as a "new active substance" in August 2007. *Cydia pomonella* Granulovirus (CpGV) is listed as "existing active substance" since 2008, and the peer review is expected in 2010/2011. Three further species are currently under evaluation as new active substances: *Adoxophyes orana* Granulovirus (AdorGV / AoGV), *Helicoverpa armigera* Nucleopolyhedrovirus (HearNPV), and *Spodoptera littoralis* Nucleopolyhedrovirus (SpliNPV). The completeness of these dossiers was published in 2007.

The "OECD Consensus document"

In 2002 the OECD published the "Consensus Document on Information used in the Assessment of Environmental Applications Involving Baculoviruses". This document summarizes all information on potential risks of baculoviruses to humans or the environment. The following properties are summarized: For most species, the host range is restricted to a single species, or few related species within the same family. Baculoviruses are not infective, pathogenic, genotoxic, mutagenic, or carcinogenic for mammals and do not replicate in mammalian cells. No effects on sensitisation of humans were observed for products containing different baculoviruses. Metabolites are not produced and effects on non-target species can be excluded. Finally, data from different species can be used for the risk assessment of a given species or isolate. The OECD Consensus document concludes that **"the use of baculoviruses is safe"**.

Regulatory development

Based on the conclusion from the OECD Consensus document and the outcome of the EU evaluations for SeMNPV and CpGV, the general regulatory principle for micro-organisms that each strain/isolate has to be regarded as a new active ingredient can be abandoned for baculoviruses. A proposal for a simplified

regulatory procedure for other species or isolates was developed during the EU policy support action REBECA and implemented as "SANCO Guidance Document 0253/2008 rev.2 to facilitate the Annex I inclusion of new isolates of a species already included in Annex I". According to this document, a molecular identification and characterisation (by RFLP) and data on the host range have to be provided. The application for Annex I inclusion of a new isolate is submitted to a member state together with an application for national authorisation of a product containing this isolate. Data requirements for the products, especially for efficacy, depend on national requirements. After evaluation, the MS reports to the Commission with a proposal to amend Annex I inclusion.

Experiences

In the dossiers for Annex I inclusion of baculovirus isolates submitted so far, the numbers of studies were not considerably reduced when compared to other micro-organisms. Data and studies from other baculovirus species were used together with scientific justifications. This strategy was accepted so far, but the peer review for CpGV and for the "new active substances" AoGV, HearNPV, and SpliNPV is still pending. Annex I inclusion in the EU is still difficult and time-consuming for new baculoviruses. Applications were made in 2005 and 2006, respectively, and timelines for Annex I inclusion can still not be predicted, even while no additional data are requested. Furthermore, the possibility to apply for national registrations after the completeness decision as foreseen in Directive 91/414 is refused by some member states that wait for the DAR or even the Annex I inclusion before they assess dossiers for national registrations. National registrations are in many cases not issued before Annex I inclusion.

The Sanco Guidance Document 0253/2008 facilitates Annex I inclusion of new isolates of a species listed in Annex I and the placing on the market of products containing new isolates. This procedure is urgently required to obtain access to the market for CpGV isolates that are able to break the resistance towards the "Mexican" isolate CpGV-M.

In the future, further experience with baculovirus isolates and products might accelerate Annex I inclusion for further species

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Identity and molecular characterization of baculoviruses

By Johannes A. Jehle (Dienstleistungszentrum Ländlicher Raum Rheinpfaltz, Neustadt/ Weinstrasse; Germany)
[PPT 6]

Viruses are not living biological entities and lack important features which define life. They do not have a cellular organization, they do not propagate by cell division, and they lack a compartmented metabolism as it is typical for all procaryotic and eucaryotic life forms. The unifying features of all viruses are their protein capsid and a genome of nucleic acid in form of DNA or RNA, as well as a one-step growth curve. Though viruses differ from living organisms, similar rules and taxonomic levels, such as species, genus, family and order are currently used. According to the current concept, a virus species is defined as a polythetic class of viruses which constitute a replicating lineage and occupy a particular niche. The responsible organization of virus classification and taxonomy is the International Committee on Taxonomy of Viruses (ICTV) within the International Union of Microbiological Societies (IUMS).

The only viruses commercially used as microbial pest control agent belong to the Family *Baculoviridae*. More than 600 different baculoviruses have been described in the literature. Baculoviruses are mainly specific for the insect orders Lepidoptera, Diptera and Hymenoptera. Based on phylogenetic studies and genome comparisons four genera have been established. The Alphabaculoviruses comprise lepidopteran-specific nucleopolyhedroviruses, the Betabaculoviruses consist of lepidopteran-specific granuloviruses, whereas Gammabaculoviruses and Deltabaculoviruses include nucleopolyhedroviruses specific for Hymenoptera and Diptera, respectively.

Different methods are applied for identification and classification of baculoviruses.

1) DNA endonuclease restriction (REN) analysis allows a fast and reliable identification of different virus isolates. By using different restriction endonucleases, the DNA of a given virus can be cut in specific fragments and separated on agarose gels. The obtained REN profiles are typical for a given isolate or species. However, for phylogenetically based taxonomy the power of REN analysis is very limited. Here, concepts of molecular evolution need to be applied. Therefore,

2) Genome sequencing became more and more useful. With second generation sequencing technologies genome sequencing of a baculovirus is also economically feasible.

3) A molecular approach using Polymerase Chain Reaction (PCR) to amplify and sequence highly conserved gene fragments (e.g. polyhedrin, lef-8 and lef-9) was successfully developed for more than 100 baculoviruses and allows a very fast and reliable classification.

In conclusion, **REN analyses** are state-of-the-art for baculovirus identification; they allow identification of isolates as well as genome heterogeneities of a given isolate. REN analyses are fast, reliable and cost effective. In general, 4-6 digests using different enzymes are suitable to distinguish different genotypes. **Genome sequencing** and **PCR** based studies of marker genes are useful for initial characterization and screening of isolates as well as for phylogentic studies and classification.

The Biopesticide Industry view on microbial contaminants and limits in microbial pest control product based on baculoviruses

By Willem Ravensberg (Koppert, Berkel en Rodenrijs; The Netherlands)
[PPT 7]

For the registration of microbial pesticides information is required on the presence of microbial contaminants in the final product. There are, however, no clear criteria provided in the regulatory guidelines, nor are any guidance documents available on acceptable levels of microbial contaminants. The current regulations in the EU, in the USA (EPA) and in Canada (PMRA) are rather vague and ambiguous regarding this topic, which leads to unknown criteria and various interpretations and risk assessment conclusions. To overcome the lack of criteria, a draft OECD Issue Paper has been written by PMRA ("Discussion on microbial contaminants limits for microbial pest control products", Version 2, 23 March 2009).

In this presentation the biopesticide industry gives its comments on this draft paper. Bacterial and fungal biopesticides are addressed in this paper. Baculovirus-based products are reported on by Phillip Kessler of Andermatt Biocontrol, Switzerland, in another presentation/paper. The introductory part in this paper refers to all kind of biopesticides. Whitin the development of a biopesticide, purity is an important topic. Producers try to limit the number of contaminants, since they may adversely affect the product's stability and field performance, and they may pose a risk to the applicator, consumer and the environment. Plant pathogens obviously should be absent. The industry agrees with the need to set standard criteria and limits, as discussed in the draft paper, but emphasizes that biopesticides are very different products compared to food, supplements and probiotics.

The production technology of the various types of micro-organisms has a great impact on the probability of presence of contaminants. Bacteria, fungi and yeasts are produced under sterile conditions; baculoviruses and protozoa are produced in vivo, and therefore control of contaminants is limited. Production of bacteria, fungi and yeasts is started with pure inoculum and is performed in sterilized equipment on sterilized medium. This production process must be sterile in order to have an efficient yield. In the down stream process and during formulation, and packaging, equipment and formulants are used that are not sterile and may contain low numbers of micro-organisms. Formulations, however, are typically dry (< 10% moisture), oil-based, or prepared with preservatives (flowables) and packed under low oxygen conditions, and stored at low temperatures which all makes growth of micro-organism almost impossible. During the production process, many Q.C. parameters generally are checked including sampling on contaminants in a number of phases of the production. Product quality control includes checking contaminants and products are only released for sale once Q.C specifications are met.

Microbial pesticides are only a small part of input in agriculture and microbial contaminants from this source should be considered in the broad context of agricultural activities. Other inputs are manure, fertilizers, compost, growing medium, water, plants, equipments and people, which all are potentially a source of microorganisms. Therefore we suggest to consider microbial contaminants exposure via biopesticides against natural background levels. Studies show that natural occurrence of micro-organisms reach high numbers of fungi and bacteria. Levels of 10^4 for fungi and 10^7 for bacteria were found per gram of egg plant (see slide nr. 13; source DAR *Trichoderma harzianum* (EFSA)). Similar occurrence is found in compost used in agriculture and Quality Standard criteria allow for up to 10^6 - 10^8 cfu/gdw of various types of microorganisms. HACCP criteria, based on EC/2005/2073 (microbiological criteria for foodstuffs), have set maximum levels of only two indicator species on ready-to-eat fruit and vegetables: 10^3 for *E. coli* and 10^3 for *Salmonella* per gram of produce.

The draft paper recommends testing for indicator species for three areas of concern: human pathogens, microbial activity, and human, fecal and environmental contamination.

For each area several indicator species are recommended. The industry agrees with some indicator species and levels, but considers others not relevant.

The recommendations from industry are provided in the table below.

Table: PMRA proposal of indicator species and limits and the industry's proposal

Indicator	**OECD Draft Paper March 2009**	**Industry proposal July 2009**
Salmonella	Absence in 25 g or 25 mL	Absence in 25 g
Listeria monocytogenes	Absence in 25 g or 25 mL	not relevant; omit
Vibrio	Absence in 25 g or 25 mL	not relevant in EU; omit
Shigella	Absence in 25 g or 25 mL	not relevant in EU; omit
Aerobic Plate Count	< 1 × 105 CFU/g or mL	< 0.1% if a.i. level, with a maximum of 10E7 CFU/g
Anaerobic spore-formers	<105 CFU/g	not relevant, cannot develop; omit
Yeast and Mould Count	< 1000 CFU/g or mL	not relevant; omit
Escherichia coli or Thermophilic (fecal) coliforms	Absence in 1 g or mL<10 CFU/g or mL	Coliforms: < 1000 CFU/g
Staphylococci	Absence in 1 g or mL	S. aureus < 1000 cfu/g
Pseudomonas aeruginosa	Monitoring*	Not relevant
Mouse IP/SC assay	No evidence of infection or injury in test animals	only in some cases when need has been proven

Industry recommends to have four indicator tests with the given limits for fungal and bacterial products. This is doable and affordable as a Q.C. test for each batch of final product. With these recommendations harmonization can be reached for the EU, US and Canada.

Justification for proposed amendments of Draft paper of PMRA.

- Aerobic plate count: the acceptable level should be raised since natural background levels, e.g. on fruit, reach similar levels. Moreover, biopesticides are generally diluted strongly for application. The maximum of 10^7 is set so that biopesticide formulations with a very high spore content such as 10^{11} sp/g are not allowed to have 10^8 microbial contaminants. This seems higher than natural levels.

- Anaerobic spore-formers: these organisms cannot grow in aerated production systems, nor in the formulated products. Not relevant to test.

- Yeast and mould counts: not relevant; cannot be distinguished in products based on fungi and yeast. Are covered by the aerobic plate count. Natural background levels are much higher than

the limit suggested by PMRA.

- Coliforms: we recommend to test on the whole group because the group is a good indicator for sanitary conditions in the production. The level of 10^3 is set since E coli levels are set on 10^3 as the maximum on fruit and vegetables in EC/2005/2073

- Staphylococci: we suggest to test only on *S. aureus* and to set a maximum of <1000 cfu/g as in EC/2005/2073 where it is only tested in fish and seafood, and not in fruit and vegetable.

- *Pseudomonas aeruginosa*; not relevant since it is ubiquitous in nature, and only clinical isolates pose a risk.

- In case production takes place in tropical areas, criteria may be adapted.

Still some items remain to be discussed and considered when the requirements for biopestices are definitely established. These are:

- Criteria for BVs should not be different than for other MBCPs since the use of the products is identical

- Are chemical and other non-microbial pesticides free of microbial contaminants? Why are they not tested?

- Do microbial contaminants pose a risk compared to other agricultural inputs?

- And compared to natural background levels?

- Do microbial contaminants pose a risk to applicators with protective clothing, gloves and eye/face protection?

Contamination with regard to baculovirus products

By Philip Kessler (Andermatt Biocontrol; Grossdietwil; Switzerland)
[PPT 8]

The draft OECD issue paper on *Microbial Contaminants Limits for Microbial Pest Control* product is discussing possible regulatory guidelines for acceptable limits of presences of various microbial contaminants. A comprehensive overview and comments for limits for microbial pest control products is given in the previous abstract of Willem Ravensberg (Koppert Biological Systems, NL), without discussing the limits for baculovirus products.

This abstract addresses the limits for baculovirus products, as separate limits for baculoviruses were proposed in the OECD issue paper.

In-vivo production of baculoviruses

The mass production of active baculoviruses can only be done *in-vivo*, on living host insects. Host insects need to be infected, and the viruses will be harvested from dead larvae. As a consequence some parts of larval bodies and insect diet will be integrated in the formulation, which leads to some degree of contamination by micro-organisms. A mass production under complete sterile conditions is not possible. Firstly, the insects used for the mass production are living organisms and are not sterile. A mass production under 100% sterile conditions is not practicable and a considerable microbial flora in the midgut of larvae cannot be avoided. Secondly, the inoculum with active baculoviruses itself for the infection of the insects is not sterile. Any sterilisation processes will have negative impact on the viability of the active ingredient.

Contamination by micro-organisms is influencing the quality of the end-product (e.g, activity, stability, physical and chemical properties). Due to this fact, the industry is already investing considerable resources to bring the contamination on an acceptable level in order to guarantee baculovirus products at a high and reliable quality.

Proposed limits for baculoviruses

The proposed limits for baculoviruses are listed in the recent draft paper from March 2009. It is based on proposed limits on a previous draft, but also includes recommendations proposed by the REBECCA virus group 2007 (working group of EU regulators, science and industry). The proposals of the industry are fully in accordance with the proposed limits by the REBECCA virus group.

Microbial contaminant	**Limits (recommended in OECD 2nd draft, 2009)**	**Limits (recommended by REBECCA (2007) Industry view**	**Comments**
Total Aerobic Bacteria (Mesophilic)*	< 1 x 10^8 CFU/g or mL	< 1 x 10^8 CFU/g or mL	
Bacillus cereus	< 1 x 10^7 CFU/g or mL	< 1 x 10^7 CFU/g or mL	Not possible to produce at < 1 x 10^6 CFU/g or mL regularly

Total coliforms Fecal coliforms/ Escherichia coli*	< 100 CFU/g or mL ***or*** Absence in 1 g or mL	Absence in 1 g or mL	Testing for E.coli as suitable indicator of fecal contaminants
Staphlococcus aureus*	Absence in 1 g or mL	Absence in 1 g or mL	
Salmonella*	Absence in 25 g or mL	Absence in 25 g or mL	
Shigella	Absence in 25 g or mL	Not recommended	Test is not sensitive, quantification rarely performed
Vibrio	Absence in 25 g or mL	Not recommended	Routine testing for Vibrio cholera is not practical and are too insensitive and time-consuming. V. cholerae not in Europe
Yeast and Mould	Visually monitored; evaluation based on levels that occur	Visually monitored; evaluation based on levels that occur	Routine screening for considerable yeast and mould contamination during production process
Mouse IP/SC tests (case-by-case)	No evidence of infection or injury in test animals	Not recommended	Mouse testing too expensive, time-consuming. Animal testing not tolerable. Food is also not tested by mouse IP/SC tests

Bacillus cereus

Bacillus cereus is ubiquitous in the environment, and can be isolated from the soil, plants and as mentioned is also a part of the natural microbial gut-flora of *Cydia pomonella* larvae. *B. cereus* is also frequently isolated as a contaminant of various foods. The consumption of foods that contain more than 10^5 CFU/g may result in food poisoning. The contamination limits for *B. cereus* for food items are 10^3 for baby food to 10^5 CFU/g (Germany (DGHM)). *Bacillus cereus* is a substantial part of the microbial contamination in baculovirus products containing *Cydia pomonella* granuloviruses (CpGV). *B. cereus* is known to be a part of the microbial flora in larval guts of *Cydia pomonella*, which can lead to an end contamination of $1x10^7$ CFU/g in the baculovirus product. It is not possible to reduce the contamination of *B. cereus* without reducing the viability of the active ingredient in the same time.

A baculovirus product is applied highly diluted. Following worst case scenario for MADEX (CpGV product of Andermatt Biocontrol) shows the maximal degree of contamination with *B. cereus* after application over an entire season. The application of 1 hectare with 9 times (3 applications on 3 generation of Cydia pomonella) a standard dosage of 100ml of MADEX containing a maximal contamination of B. cereus of 10^7 CFU per ml, will result in an overall contamination over the entire season of 9×10^9 CFU/per ha/per season. Assuming that the surface of a standard apple is 200 cm^2 and the apple has a weight of 150 g, the contamination with B. cereus would be 1.2×10^2 CFU/g apples. Respecting that that the UV radiation reduces the *B. cereus* viability over time, and respecting that due to the leaf index the realistic exposition of the apple surface is lower, the realistic contamination with B. cereus on the apple is about 10 to 100 lower. A realistic contamination of *B. cereus* after using a CpGV product over an entire season would be 1-10 CFU/g apple at harvest and is therefore 100 to 1000 times lower than the accepted level for baby food.

Furthermore, the production of fruits like apples and pears is not a sterile process. A natural contamination of the apple surface with *B. cereus* can already be expected, as *B. cereus* is a ubiquitous organism in the environment. The surface (and even the inside) of an apple is already covered with a large number of naturally occurring fungi and bacteria. An uncontrolled multiplication of *B. cereus* will not be possible under such a competitive environment. Furthermore, the ingestion of parts of an apple, infested by a *Cydia pomonella* larva and its faeces, would provoke a far higher intake of *B. cereus* due to the natural presence of *B. cereus* in the midgut of the larvae, than an application with a CpGV product would do.

Conclusively, there is no realistic risk for consumers caused by contamination with *B. cereus* on fruits, which were treated with baculovirus products containing a contamination of *B. cereus* of $1x10^7$ CFU/g. The realistic contamination with *B. cereus* would be far lower than accepted thresholds for food items such as baby food. The EU specified an upper limit for *B. cereus* of 1×10^6 CFU/g after the inclusion of CpGV on Annex 1 of 91/414/EEC. Due to the postponed peer-review, the industry had not the possibility to comment this specification. The specified limit by the EU of $1x10^6$ CFU/g would be an unreasonable obstacle for the industry.

A risk for applicators is minimal as the products are sprayed highly diluted. Furthermore applicators are required to protect themselves with protective clothing, gloves and eye/face protection.

Test for human pathogens such as *Shigella* and *Vibrio* spp.

These pathogens are not endemic in many countries of the world. A permanent screening for these pathogens is not justified. Furthermore, available test are not sensitive and its quantification is rarely performed. Therefore it should be proposed to omit this specification, at least as long as the production is based outside of countries, where these human pathogens are regularly present.

Mouse IP/SC assay

The use of mouse IP/SC tests would not give further relevant data for human pathogens in a baculovirus product, as the product is already tested for three indicators of human pathogens (*Salmonella*, *Escherichia coli* and *Staphylococcus aureus*). Furthermore the mouse testing usually need to be performed by external laboratories, and are therefore too expensive and time-consuming, and would be an unreasonable obstacle for the industry. Furthermore, there are world-wide efforts to reduce the number of animal testing, which would not justify a permanent screening of baculovirus products for microbial contamination by a mouse IP/SC test. Also food items are also not tested by mouse IP/SC tests to screen for microbial contamination.

Therefore, the mouse IP/SC assays would be an unreasonable obstacle for the industry, without giving more relevant information.

Overall conclusions and proposals for limits for microbial contaminants for baculovirus products by the industry:

- Agree that screening for microbial contaminants/ pathogen is necessary
- Microbiological pest control agents are not intended for consumption by humans or other animals.
- The limits of microbial contamination chosen for food item are not applicable for microbiological pest control products such as baculovirus products.

- The dietary exposure needs to be assessed at the realistic level of contamination after application.
- A certain contamination in the product can be accepted as the baculovirus products are highly diluted before applying. The end-dietary contamination after application is not over passing limits as tolerated in food items.
- Screening for pathogens should be limited to a reasonable number of indicators/pathogens
- No testing for pathogens those are obviously unlikely.
- No animal testing should be accepted.

Genotypic/Phenotypic characterization of biocontrol and clinical strains of *Pantoea agglomerans*

By Brion Duffy *(*Agroscope, Wädenswil; Switzerland)
[PPT 9]

Pantoea agglomerans **biocontrol agent biosafety: strain-level identification and genotypic comparison of biocontrol and clinical isolates**

Fabio Rezzonico[1], Esther Badosa[2], Theo H.M. Smits[1], Jürg E. Frey[1], Emilio Montesinos[2], **Brion Duffy[1]**

[1] Agroscope Changins-Wädenswil ACW, Plant Protection Division, CH-8820 Wädenswil, Switzerland; [2] Institute of Food and Agricultural Technology-CIDSAV-XaRTA, University of Girona, E-17071 Girona, Spain

Pantoea agglomerans strains are common plant epiphytes with promising applications for biocontrol of bacterial and fungal plant diseases. Commercial strains in the US/Canada/New Zealand are among the most promising alternatives to antibiotic use against fire blight. European registration has been hampered by the Jekyll-Hyde nature of *P. agglomerans* with clinical as well as beneficial strains reported.

Comparative genetic and phenotypic analysis however, raises serious questions regarding the validity of current classification as a biosafety-level 2 organism. A major problem rests on inaccurate identification using standard clinical diagnostic methods (e.g., biochemical, 16S rDNA), and use of outdated systematics for the genus. A majority of clinical isolates obtained from international strain collections were found to be erroneously identified when more accurate methods were applied (i.e., MLST). Total genomic analysis (fAFLP) identified a biocontrol strain-specific band to differentiate from clinical isolates. Comparative analysis of complete genomes of biocontrol and clinical strains found no evidence of typical virulence elements. Secondary metabolites involved in biocontrol activity were also found that are primarily absent in clinical isolates and can be used in genetic screening for biosafety. Phenotypic assays for plant disease suppression found that biocontrol strains could be distinguished from clinical strains based on potential beneficial efficacy. However, the fundamental problem is that clinical isolates show no evidence of being pathogenic, and most often are polymicrobial isolations. Koch's postulates have never been fulfiled. We found no evidence for pathogenicity of biocontrol (or clinical isolates) in standardised hemolytic, mouse, and nematode models. Regulatory assumptions based on poorly detailed clinical reports appear to be unjustified by scientific analysis.

Characterisation issues on bacteria

Denise Munday, Maria Herrero (Valent Biosciences; Switzerland)
and ***Sherry Heins*** (AgraQuest)
[PPT 10]

The registrant, AgraQuest, Inc., presented historical data characterizing the MPCA QST 713 strain of *Bacillus subtilis*, utilizing both classical characterization physiological methods, Analytical Profile Index 50 CHB identification system (bioMerieux Vitek), 16s rRNA gene sequencing and Riboprinter Analysis using the Qualicon System.

The results of these methods were deemed insufficient to the EU regulators and a comparative analysis utilizing Ribotyping, which involves Southern blotting of digested chromosomal DNA of the organism of interest, probing with the *E. coli* rRNA operon, and computer analysis of the resulting patterns, was conducted.

These resulting patterns may be compared to a database for identification or to other strains for strain differentiation. A custom method was developed distinguish the MPCA from other 13 B. subtilis in the database. The findings supported the registrant's initial identification.

The registrant sought to clarify that the regulators should consider practical methods for strain identification. Furthermore, once the identification is supported the burden should fall to the registrant to ensure the integrity of the production strain.

As technology changes it becomes a burden to registrants to analyze their MPCA by every method available. Strict adherence to strain preservation, storage and propagation ensures a robust master lots of production strains.

ANNEX 4

TITLES OF PRESENTATIONS

Presentation on the OECD and the work of OECD-BPSG
By Jeroen Meeussen, The Netherlands

Taxonomy, characterisation and identification of *Bacillus thuringiensis*: experience in preparing Draft Assessment Reports
By Niels Bohse Hendriksen (Department of Environmental Chemistry and Microbiology, Aarhus University; Denmark)

Taxonomy, characterisation and identification of *Trichoderma*: experience in preparing Draft Assessment Reports
By Kersti Gustaffson (Swedish Chemicals Inspectorate – KEMI; Sweden)

Contaminants (secondary metabolites) and residues
By Claude Alabouvette (Laboratoire de Recherches sur la Flore Pathogène dans le Sol, I.N.R.A., UMR Microbiologie du Sol et de l'Environnement, Dijon; France)

US-regulatory system and perspective on identity and characterisation issues for micro-organisms
By William Schneider (BioPesticides Division, EPA, Arlington; USA)

Baculoviruses
By Rüdiger Hauschild (GAB Consulting GmbH, Lamstedt; Germany)

Identity and molecular characterization of baculoviruses
By Johannes Jehle (DLR Rheinpfaltz, Neustadt/ Weinstrasse; Germany)

The Biopesticide Industry view on microbial contaminants and limits in microbial pest control product based on baculoviruses
By Willem Ravensberg (Koppert, Berkel en Rodenrijs; The Netherlands)

Contamination with regard to baculovirus products
By Philip Kessler (Andermatt Biocontrol; Grossdietwil; Switzerland)

Genotypic/Phenotypic characterization of biocontrol and clinical strains of *Pantoea agglomerans*
By Brion Duffy (Agroscope, Wädenswil; Switzerland)

Characterisation issues on bacteria
By Denise Munday, Maria Herrero (Valent Biosciences; Switzerland) and Sherry Heins (AgraQuest)

Presentation on the OECD and the work of OECD-BPSG
By Jeroen Meeussen, The Netherlands

Seminar on Identity and Characterisation of Micro-organisms

Biopesticides Steering Group

1 July 2009, Paris, France

OECD 1 OCDE

Seminar on Identification and Characterisation of Micro-organisms

- A few words about OECD
- OECD Work on (Bio)Pesticides
- Today's seminar: purpose, scope and structure

OECD 2 OCDE

OECD

- OECD: The Organisation for Economic Co-operation and Development

OECD

What is OECD?

- A forum in which governments work together to address the economic, social and environmental challenges of interdependence and globalisation.
- A provider of comparative data, analysis and forecasts to underpin multilateral co-operation

OECD's Mission

OECD brings together the governments of countries committed to democracy and the market economy from around the world to:

- Support sustainable economic growth;
- Boost employment;
- Raise living standards;
- Maintain financial stability;
- Assist other countries' economic development;
- Contribute to growth in world trade.

OECD 5 OCDE

A tool for governments

- Started after World War II;
- Transformed in 1961 into the Organisation for Economic Co-operation and Development with trans-Atlantic and then global reach;
- Today the OECD has 30 member countries;
- More than 70 developing and transition economies are engaged in working relationships with the OECD.

OECD 6 OCDE

OECD - Working Group on Pesticides

The OECD work on **agricultural pesticides** aims to help member countries:

- improve the efficiency of pesticide control;
- share the work of pesticide registration and re-registration;
- minimise non-tariff trade barriers;
- reduce risks to human health and the environment.

OECD 7 OCDE

OECD - Working Group on Pesticides

- Registration Steering Group
- Risk Reduction Steering Group
- BioPesticides Steering Group

OECD 8 OCDE

OECD-BPSG

The BioPesticides Steering Group (BPSG) was established by the WGP in 1999:

- to help member countries harmonise the methods and approaches used to assess biological pesticides and

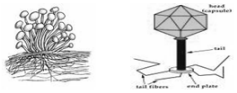
- improve the efficiency of control procedures.

OECD 9 OCDE

OECD-BPSG

Biological Pesticides:

- Macro-organisms
- Microbial Biopesticides
- Semiochemicals
- Plant extracts/Botanicals



OECD 10 OCDE

OECD-BPSG

The first tasks of the BPSG consisted of:

(i) reviewing regulatory data requirements for three categories of biopesticides; and
(ii) developing formats for dossiers and monographs for microbials, and pheromones and other semio-chemicals.

OECD 11 OCDE

OECD-Publications

Registration requirements:

- for pheromones (Series on Pesticides, No. 12 , 2001);
- for microbial pesticides (Series on Pesticides, No. 18 , 2003);
- for invertebrate biocontrol agents/IBCAs (Series on Pesticides, No. 21, 2004).

OECD 12 OCDE

OECD-Publications

- *OECD Guidance for **Industry Data Submissions** for Microbial Pest Control Products and their Microbial Pest Control Agents (Dossier Guidance for Microbials), August 2006.*
- *OECD Guidance for **Country Data Review Reports** on Microbial Pest Control Products and their Microbial Pest Control Agents (Monograph Guidance for Microbials), August 2006.*

OECD 13 OCDE

OECD-BPSG

The BPSG then decided to concentrate its efforts on **science issues** that remain as barriers to harmonisation and work-sharing.

OECD 14 OCDE

Working Document

"Working Document on the Evaluation of Microbials for Pest Control"

This document is essentially a set of examples/case studies aimed at helping the regulatory authorities to deal with these issues in the assessment of (microbial) biopesticides.

OECD 15 OCDE

Working Document - chapters

- Taxonomic identification of micro organisms in MPCP
- Genetic toxicity assessment of microbial pesticides
- Exposure (operators, bystanders, consumers)
- Microbial metabolite residues in food
- Efficacy evaluation of microbials

OECD 16 OCDE

Workshop on the Regulation of Biopesticides

- *"Workshop on the Regulation of Biopesticides: registration and Communication issues"*

- *15-17 April 2008, EPA, Arlington, USA*

OECD 17 OCDE

Workshop on the Regulation of Biopesticides - Results

The objectives of the workshop are met:

- To collect input to resolve science issues;
- To improve communication and information exchange;
- To take forward some of the conclusions from REBECA.

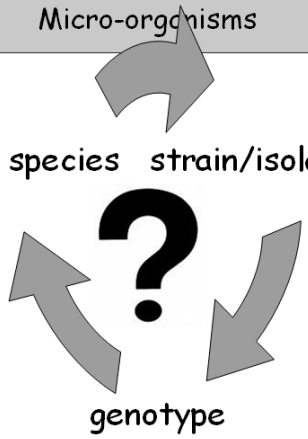
OECD 18 OCDE

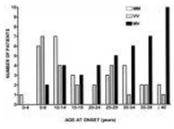
OECD-Publications

- OECD, *Working Document on the Evaluation of Microbials for Pest Control*, OECD Environment, Health and Safety Publications, Series on Pesticides No. 43, 2008.
- OECD, *Report of Workshop on the Regulation of Biopesticides: Registration and Communication issues, 15 – 17 April 2008, EPA, Arlington, USA*, OECD Environment, Health and Safety Publications, Series on Pesticides No. 44, 2009

OECD 19 OCDE

Seminar on Identification and Characterisation of Micro-organisms





OECD 20 OCDE

Seminar on Identification and Characterisation of Micro-organisms

List 4 micro-organisms	**formerly**
Lecanicillium muscarium	*Verticilium lecanii*
Metarhizium anisopliae var. *anisopliae*	*Metarhizium anisopliae*
Trichoderma atroviride	*T. harzianum*
Trichoderma asperellum	*T. harzianum*
Trichoderma gamsii	*T. viride*
Verticillium albo-atrum	*Verticillium dahliae*

OECD 21 OCDE

Seminar on Identification and Characterisation of Micro-organisms
Objectives

- To identify key issues and challenges in the area of identification and characterisation of micro-organisms including issues related to taxonomy, manufacturing process and -acceptable- level of contaminants;
- To provide updates of national activities and initiatives and exchange information;
- To suggest further steps for OECD-countries, industry and OECD.

OECD 22 OCDE

Seminar on Identification and Characterisation of Micro-organisms

Scope

- What methods should be used for identification of micro-organisms;
- *At what taxonomic level is verification required;*
- *What identification data package should be submitted to conclude if strains are similar;*
- *Standard operational procedures, incl. quality control measures regarding the manufacturing process;*
- *Maximum acceptable amount of contaminants.*

OECD 23 OCDE

Seminar on Identification and Characterisation of Micro-organisms

Structure

Presentations on:

- governement experience and perspectives,
- stakeholder experience and perspectives,

followed by a round table discussion.

OECD 24 OCDE

Seminar on Identification and Characterisation of Micro-organisms

I wish you an interesting and useful seminar!

Thank you very much for your attention.

OECD 25 OCDE

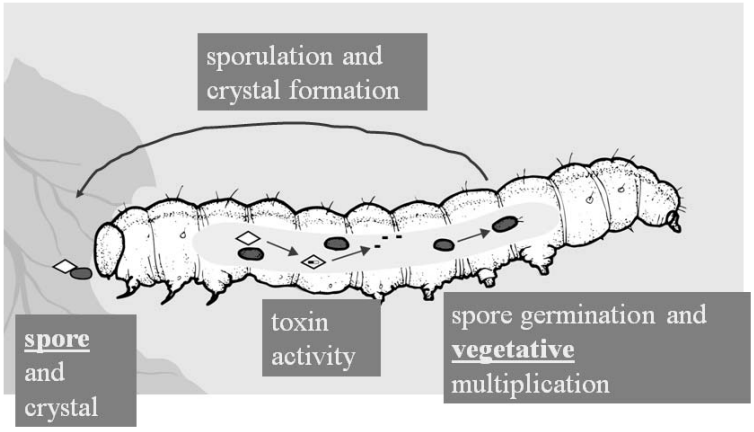
Taxonomy, characterisation and identification of *Bacillus thuringiensis*: experience in preparing Draft Assessment Reports

By Niels Bohse Hendriksen (Department of Environmental Chemistry and Microbiology, Aarhus University; Denmark)

Taxonomy, characterisation and identification of *Bacillus thuringiensis*: experience in preparing Draft Assessment Reports

Niels Bohse Hendriksen, National Environmental Research Institute, Aarhus University, Denmark

B. thuringiensis spores and crystalline toxins

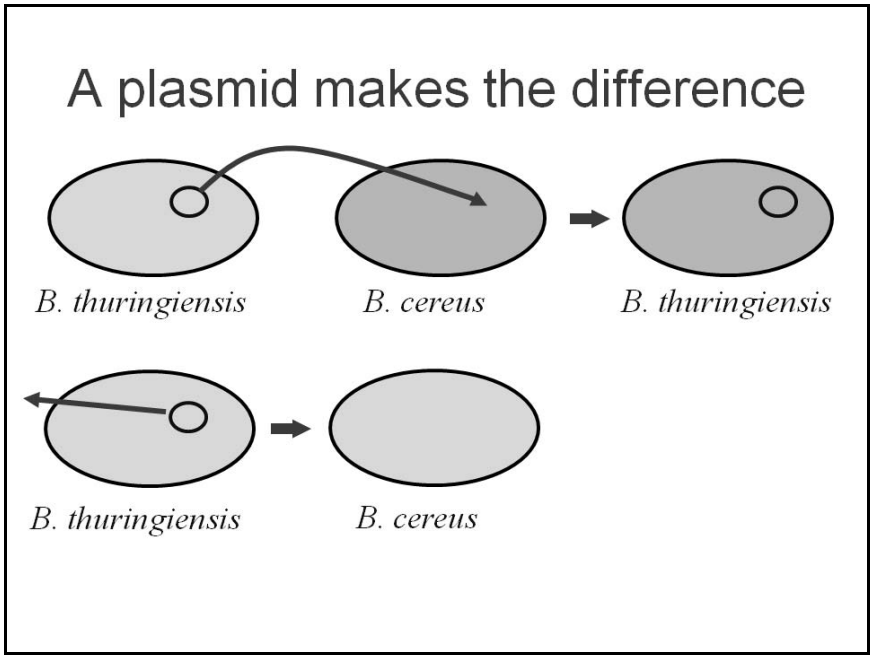


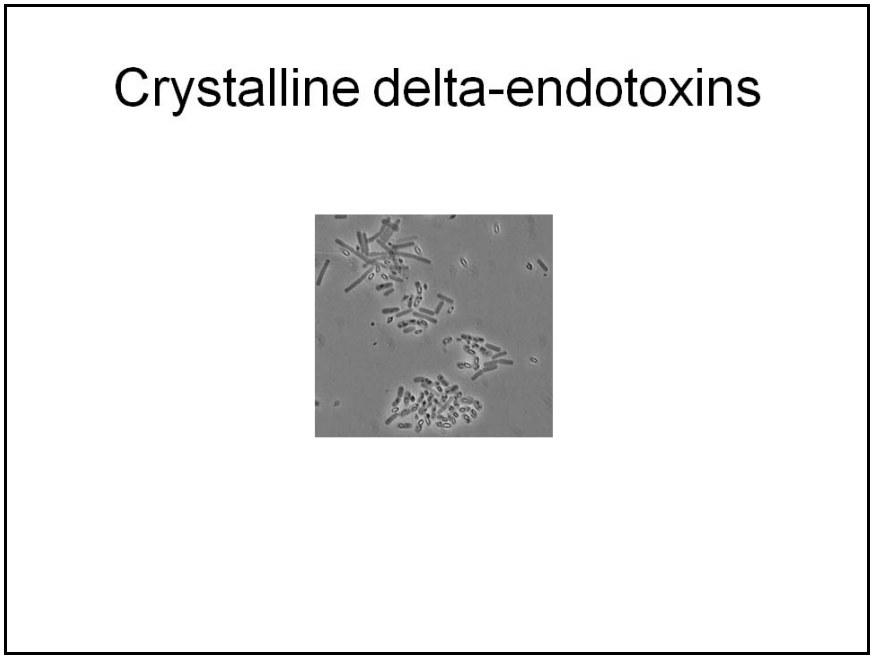
B. thuringiensis indistinguisable from *B. cereus*

- morphology
- utilisation of many organic compounds
- whole cell composition (fatty acids; sugars)
- serological typing
- phage typing
- multilocus enzyme electrophoresis

Bacillus thuringiensis

- Produces parasporal crystalline proteins
- consisting of δ-endotoxins
- Pathogenecity to invertebrates, notably insects
- Genes coding for δ-endotoxins located on large plasmids


A plasmid makes the difference
B. thuringiensis
B. cereus
B. thuringiensis
B. thuringiensis
B. cereus


Crystalline delta-endotoxins

Draft Assessment Reports

- *B. thuringiensis kurstaki* ABTS351 (Sumitomo, Valent)
- *B. thuringiensis kurstaki* SA-11, SA-12, EG2348 (Mitsui (Certis), Intrachem)
- *B. thuringiensis kurstaki* PB54 (Probelte)

Data provided

- Origin of strains
- Overall classification (Biochemical properties)
- Specific classification (crystals)
- Serotype
- Delta-endotoxins
- Plasmid-profiles
- Relationsships to other strains

Conclusions from that

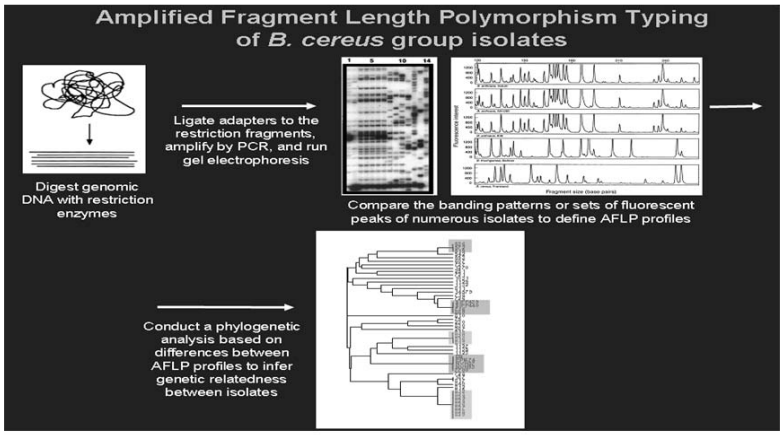
- The strains have different origins
- Members of the B. cereus group
- Produce parasporal crystals consisting of delta-endotoxins – B. thuringiensis
- Serotype 3a3b(3c) kurstaki
- Delta-endotoxins restricted to cry1 and cry2
- Small differences between strains at the level of toxins and plasmids

Relations within the B.cereus group

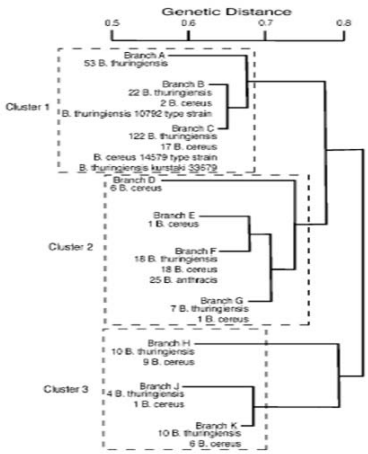
- AFLP data provided by some applicants
- MLST data from open litterature

- What to learn from this

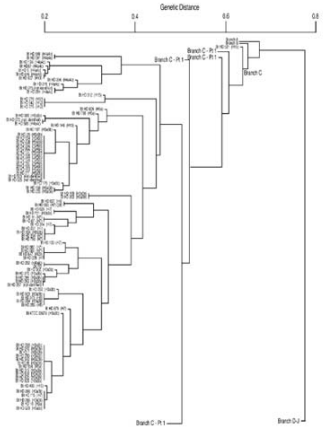
AFLP



AFLP B.cereus group



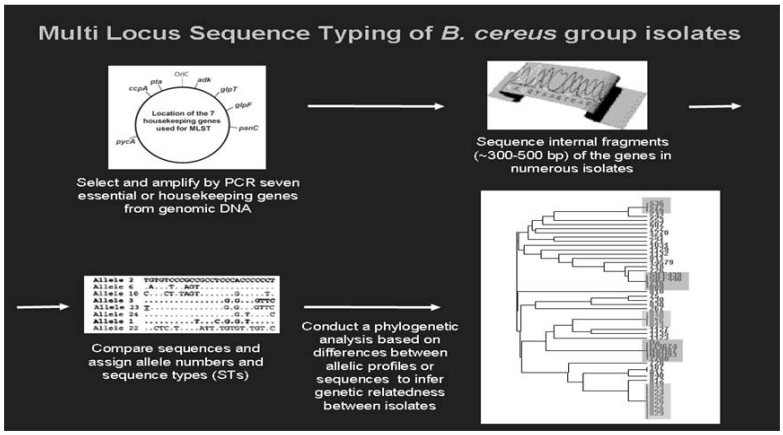
AFLP Cluster 1 Branch C



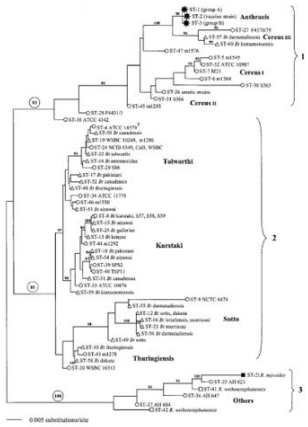
Conclusions from AFLP

- B. thuringiensis kurstaki a homogenous group – monophyletic origin
- Not closely related to B. anthracis
- Not closely related to emetic B. cereus
- Not closely related to exotoxin producing B. thuringiensis
- Closely related to some B. cereus strains

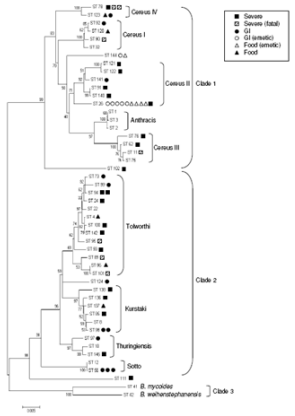
MLST



MLST – B.cereus group



MLST – including pathogenic B. cereus strains



MLST – including other pathogenic Bc strains

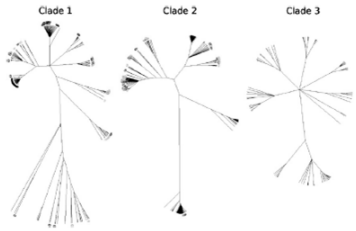


Fig. 1. Majority-rule consensus trees built from the output of ClonalFrame for each of the three clades, with pathogenic isolates colour-coded according to the disease they caused: anthrax in black, blood infection in red, diarrhoea in green, vomiting in blue, lung infection in mauve and wound infection in cyan. Trees drawn using FigTree [51].

Conclusions from MLST

- B. thuringiensis kurstaki a very homogenous group – monophyletic origin
- Not closely related to B. anthracis
- Not closely related to emetic B. cereus
- Not closely related to exotoxin producing B. thuringiensis strains
- Closely related to some pathogenic B. cereus strains (mainly entero-toxic)

Identification

- Methods for unequivocal identification at strain level needed
- Data from open litterature shows that it is possible
- One applicant has provided a method

Overall conclusions

- Classification clear
- Relationships to B. cereus need to be taken into considerations in the assessment
- Identification methods at strain level need to be established for all strains

”Species” in the *B.cereus* group

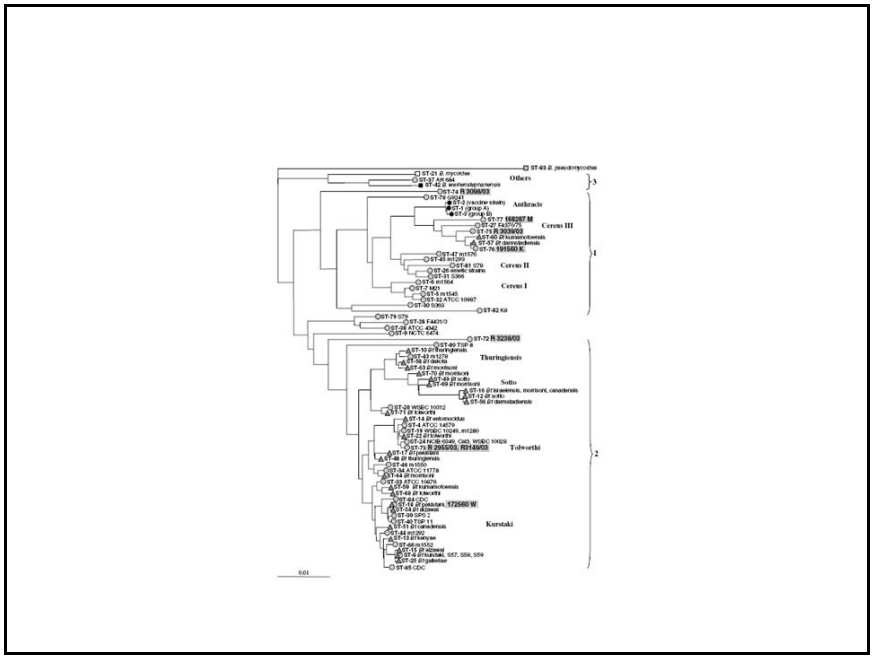
- *B. cereus (Bc)*
- *B. antracis (Ba)*
- *B. thuringiensis (Bt)*
- *B. mycoides (Bm)*
- *B. weihenstephanensis (Bw)*
- *B. pseudomycoides (Bps)*

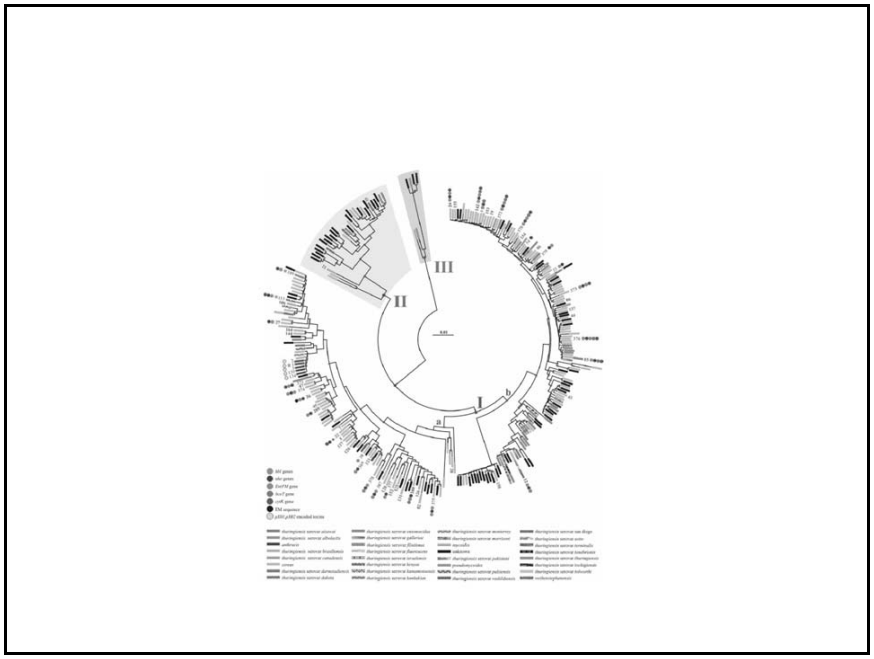
"Species" in the *B.cereus* group

- *B. cereus (Bc)*
- *B. antracis (Ba)*
- *B. thuringiensis (Bt)*
- *B. mycoides (Bm)*
- *B. weihenstephanensis (Bw)*
- *B. pseudomycoides (Bps)*

Serotyping the classical way to divide *B. thuringiensis* into subspecies

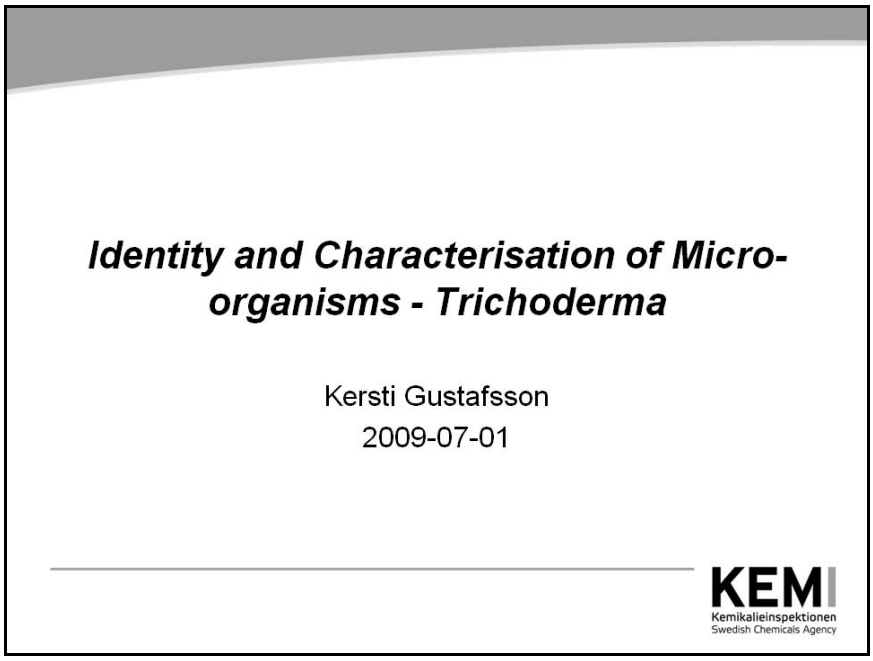
- H-serotyping – reaction to flagellar antigens
- At least 69 H-serotypes recognised
- The methodology not maintained
- Attempts to to find alternatives – based on sequencing (Flaggelin genes)
- Not always related to phylogeny

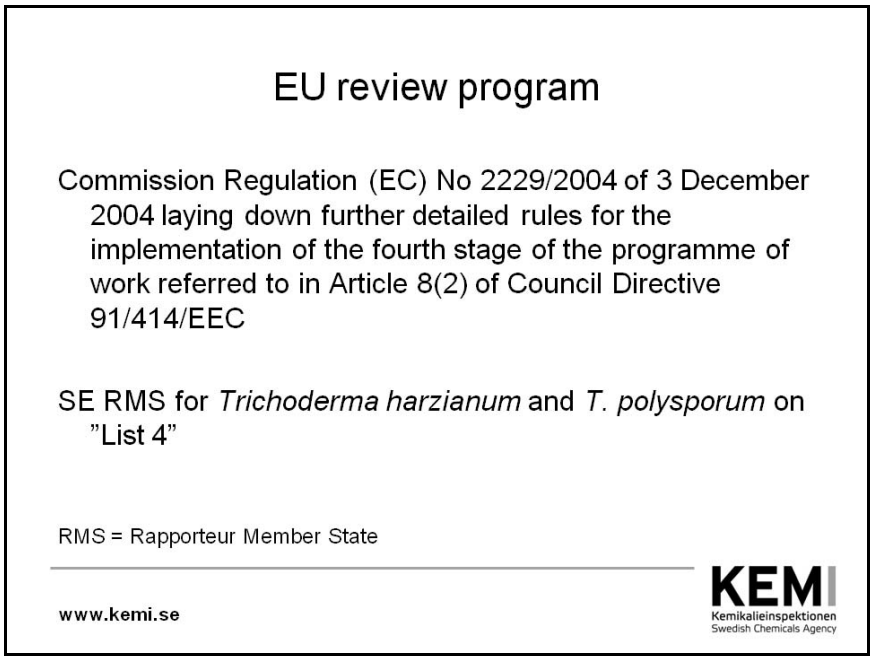

Others
Anthracis
Cereus III
Cereus II
Cereus I
Thuringiensis
Sotto
Tolworthi
Kurstaki
0.01


III
II
I

Taxonomy, characterisation and identification of *Trichoderma*: experience in preparing Draft Assessment Reports

By Kersti Gustaffson (Swedish Chemicals Inspectorate – KEMI; Sweden)





3 Dossiers and 3 DARs

- *Trichoderma harzianum* Rifai
  - *T. harzianum* T-11, T-22, ICC012, ITEM 908
- *Trichoderma harzianum*
  - *T. harzianum* IMI 206040
- *Trichoderma polysporum*
  - *T. polysporum* IMI 206039

www.kemi.se

KEMI Kemikalieinspektionen Swedish Chemicals Agency

T. harzianum Rifai

- Rifai (1969) proposed nine species aggregates (*T. piluliferum*, *T. polysporum*, *T. hamatum*, *T. koningii*, *T. aureoviride*, *T. harzianum*, *T. longibrachiatum*, *T. pseudokoningii* and *T. viride*)
- This classification still valid when the Council Directive 91/414/EEC of July 15, 1991, became effective in July 1993.

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T. harzianum Rifai cont.

- 2000 comparison of ITS1 sequences from different biocontrol isolates of *Trichoderma spp.* → phylogenetic tree with five groups

- *T. harzianum* / *T. inhamatum*; biocontrol strains
- Mushroom competitors;
- *T. paraceramosum* / *T. longibrachiatum*;
- *T. hamatum* / *T. asperellum*; biocontrol strains
- *T. viride*, *T. atroviride*, and *T. koningii*. biocontrol strains

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Swedish Chemicals Agency

T. harzianum Rifai cont.

- 2004 phylogenetic analyses with 53 *Trichoderma* isolates using the ITS1 sequence and a part of the translation elongation factor (*tef*) gene.
- The analyses lead to a finer resolution of the phylogeny within the genus *Trichoderma*. Phylogenetic trees for both genes showed biocontrol isolates in all groups except *T. aggressivum*.

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Swedish Chemicals Agency

RMS SE comments

- Taxonomy in general in a revolutionizing period with molecular biology and DNA techniques
- *Trichoderma harzianum* on List 4 - all four strains in the compiled dossier originally belonged to *Trichoderma harzianum* Rifai
- Development of oligonucleotide barcodes for species identification (2005) - possible to strictly identify the four strains to species level
- Checking the gene sequences with a gene bank, NCBI (National Centre for Biotech Information), the following species identification was the result:
- **T-22** and **ITEM 908** are *Trichoderma harzianum* = *Hypocrea lixii*;
- **ICC012** is *Trichoderma asperellum sensu* Hermosa *et al* 2000 (there is 100 % equivalence in ITS1 and 2 with strains IMI 296237 and IMI20268 in their article); and
  T-11 is *Trichoderma atroviride sensu* Hermosa *et al* 2000 (they sequenced this isolate).

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Genotype *versus* phenotype

- DAR - these four strains treated as one species;
- Strain specific information compared even though there are no specific differences found yet.

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Strain specific data

T-11	T-22	ITEM 908	ICC012
Indigenous wild type	Protoplast fusion between T12 - T95; sensitive to benomyl, fully prototrophic; combines biocontrol abilities in T12 with rhizosphere competence in T95.	Indigenous wild type	Indigenous wild type
Identification by amplification and sequence analysis of the internal transcribed spacer (ITS1-2) region and the translation elongation factor 1 (*tef1*) gene.			
SCAR marker can clearly distinguish strain 11 from other closely related *Trichoderma* strains.	Distinguished from parent strain T-95 and most other *T. harzianum* strains by isoenzyme electrophoresis, but not from parent T-12.		Can be characterised by RAPD-PCR.

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Strain specific data cont.

T-11	T-22	ITEM 908	ICC012
Conidiogenous cells > 10 µm length; subglobose conidia > 2.4 µm Ø; terminal chlamydo-spores < 10 µm Ø.	Morphology determined microscopically of asexual spore-bearing structures on malt extract agar; appearance of colonies grown from spores can be strongly influenced by physiological conditions and media. Colony appearance on PDA. Typical well-isolated colonies ~ 2 cm Ø. Hyphal growth dense both on agar surface and submerged. Aerial hyphae profuse, especially at the edge of well isolated colonies, giving a white, cottony appearance. Spores green, dense sporulation in centre of the colony, gives a pale to dark green colour. Crowded colonies do not expand radially resulting in reduction or absence of the white, cottony aerial mycelium around the colony edge and the spore mass covering the colony appears denser with a darker shade of green. PDA = potato dextrose agar	Colonies on PDA at 26°C moderately fast-growing (30 mm Ø 2 d, 66 mm 3 d), richly sporulating. Conidiation effuse, appearing granular in young cultures, crusty with age to dense conidiation; rapidly turning olive-green. Reverse beige. Odour distinct. Conidiophores verticillate, forming a pyramidal structure. Phialides ampulliform, usually 3-4 verticillate. Conidia are globose 2.9 × 2.7 µm, smooth walled, pale green.	*Solid medium:* Irregularly spread colonies after 36-48 h, circular, almost regular colonies after 5 d; white aerial mycelium; dark green, greyish spores. *Liquid medium:* Confluent not homogenous growth; dark green, greyish spores.

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T-22

- **T-22** protoplast fusion 1988 from mutants of T-12 and T-95
- **T-22** have a combination of biocontrol capacities of T12 originally isolated from USA and of rhizosphere colonisation capacities of T-95 isolated from Colombia.

- Protoplast fusion ~ anastomosis

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T-22 cont.

- Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC - Commission Declaration
- Annex 1A: Techniques of genetic modification
  - (3) cell fusion (including protoplast fusion) or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally
- Annex 1B: Techniques/methods of genetic modification yielding organisms to be excluded
  - (2) cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods

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T-22 cont.

- EU Commission response
  - The organism is formed by protoplast fusion of organisms, which are capable of exchanging genetic material by traditional breeding methods; it should therefore on this basis be exempt from Directive 2001/18/EC

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***T. harzianum* IMI 206040 and *T. polysporum* IMI 206039**

Main difference is range of temperature activity - reason for having both species in one product

Reason for producing two DARs

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Inclusion in Annex 1 to Directive 91/414/EEC

Commission Directive 2008/113/EC of 8 December 2008 amending Council Directive 91/414/EEC to include several micro-organisms as active substances

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Annex 1 inclusion cont.

Trichoderma atroviride (formerly *T. harzianum*)
STRAIN: IMI 206040; Culture collection No IMI 206040, ATCC 20476;
STRAIN: T11; Culture collection: No Spanish type culture collection CECT 20498, identical with IMI 352941

Trichoderma asperellum (formerly *T. harzianum*)
STRAIN: ICC012; Culture collection No CABI CC IMI 392716

Trichoderma harzianum Rifai
STRAIN: *Trichoderma harzianum* T-22; Culture collection No ATCC 20847
STRAIN: *Trichoderma harzianum* ITEM 908; Culture collection No CBS 118749

Trichoderma polysporum
STRAIN: *Trichoderma polysporum* IMI 206039; Culture collection No IMI 206039, ATCC 20475

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Condition for Annex 1 inclusion

Commission Directive 2008/113/EC:

In accordance with Article 25a of Regulation (EC) No 2229/2004 the Commission is to request the EFSA to deliver its view on the draft review reports by 31 December 2010 at the latest.

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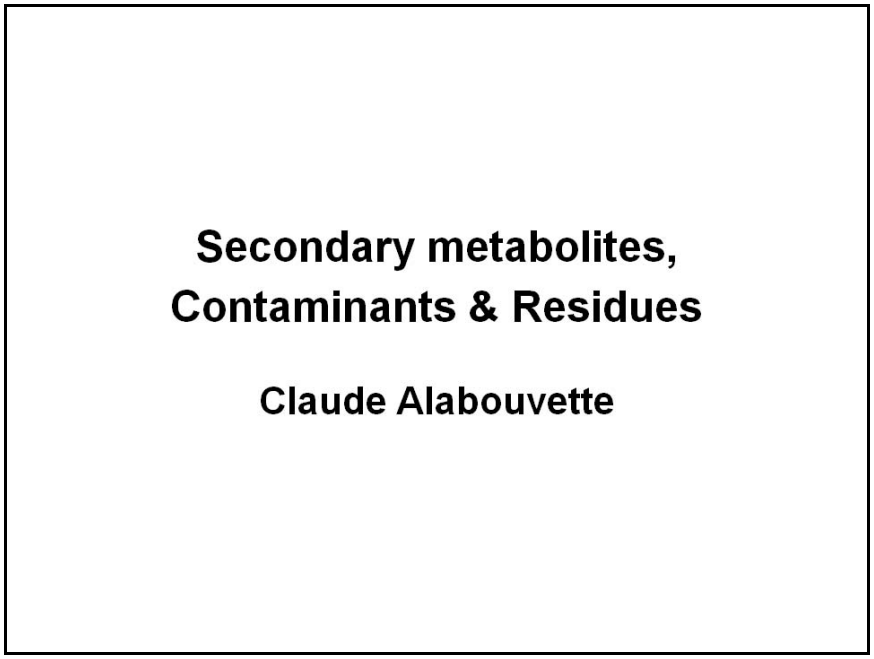
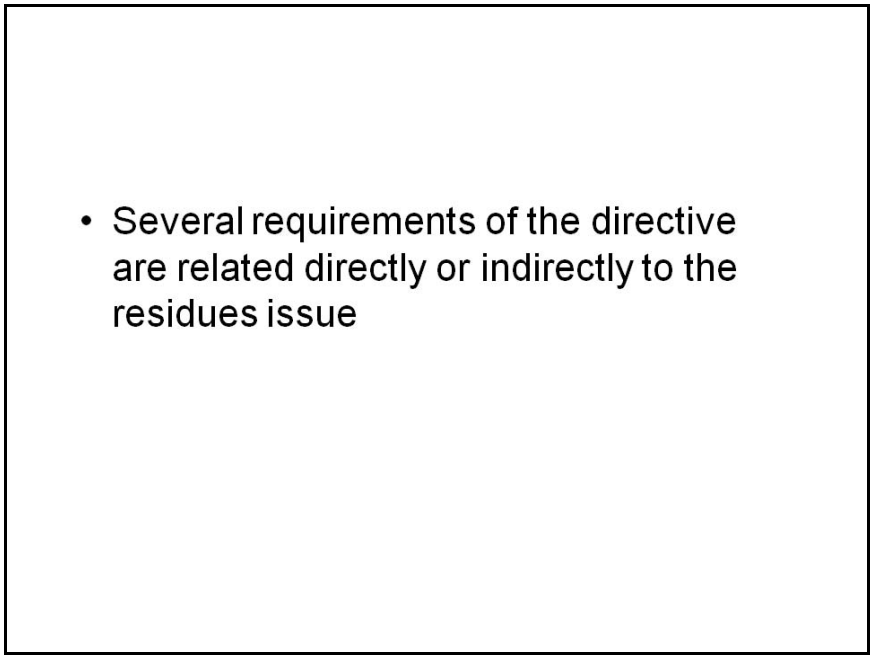
Contaminants (secondary metabolites) and Residues

By Claude Alabouvette (Laboratoire de Recherches sur la Flore Pathogène dans le Sol, I.N.R.A., Dijon, France)





6/ Residues (microorganism) in or on treated products, food and feed

- It is considered that the main residue is the microorganism itself
- But there is some concern regarding accumulation of secondary metabolites and of contaminants

There is a lack of guidelines to address these questions

1/ Identity of the active substance

- The first requirement is a perfect identification of the biological control agent in order to utilize all the data from the literature

2/ Biological properties of the microorganisms

- **2.2.2 modes of action** :
  - there are always several modes of action
  - the modes of action described in vitro are not necessary the same as in nature
  - when secondary metabolites are produced, their concentration is always very low

Many secondary metabolites

- **Strains of *Trichoderma* spp are producing many different secondary metabolites**
- **The same strain is producing different secondary metabolites depending on**
  - **the plant on which it is applied**
  - **The pathogen to which it is confronted**

Many secondary metabolites

- Strains of fluorescent *Pseudomonas* spp are producing many different secondary metabolites
- The same strain is producing different secondary metabolites depending on
  - the soil environmental conditions
- It has been recently demonstrated that the strain CHA0 has the capacity to produce toxins with insecticidal properties

Questions related to secondary metabolites

How to characterize the modes of action

in situ ?

How to characterize the secondary metabolites produced in situ ?

3/ Further information on the active substance

- 3.4/ method of production and quality control

  Methods must be described, but nothing is said about a maximum threshold for contaminants

Contaminants

- The BCAS must be produced in pure culture
- Contaminants should be absent from the technical product

Origine of the contaminants?

- Pollution from the atmosphere of the plant
- Contamination from the co-formulants

Is the identification of these contaminants needed?

Contaminants

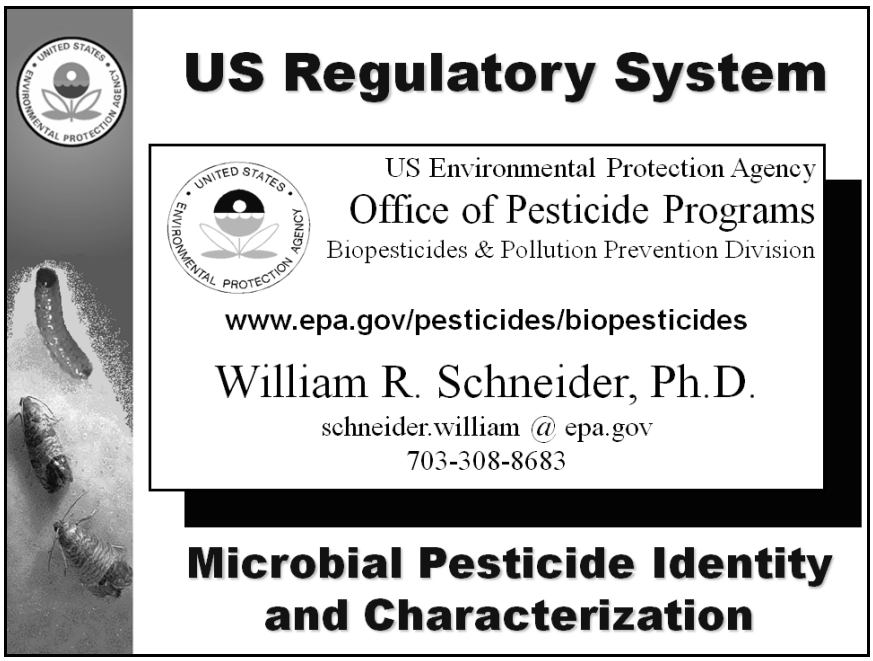
How to prevent contamination?

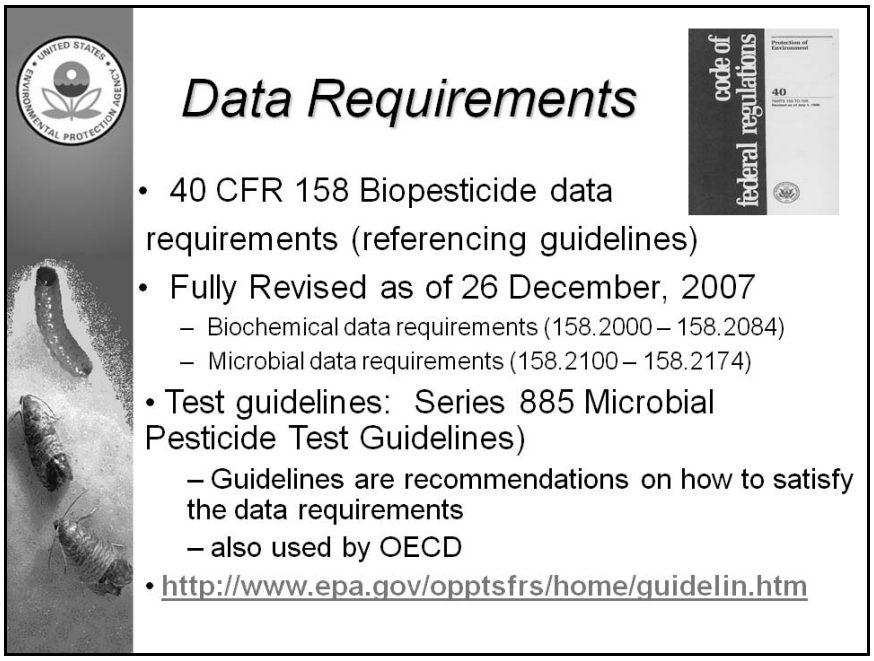
Should the contaminants be identified?

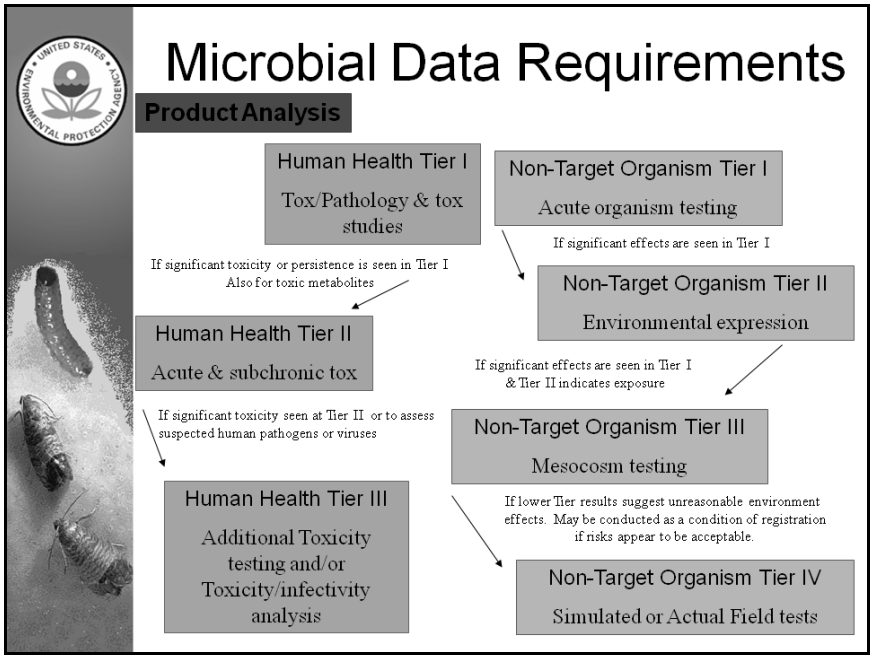
What is the maximum content of contaminants to be tolerated?

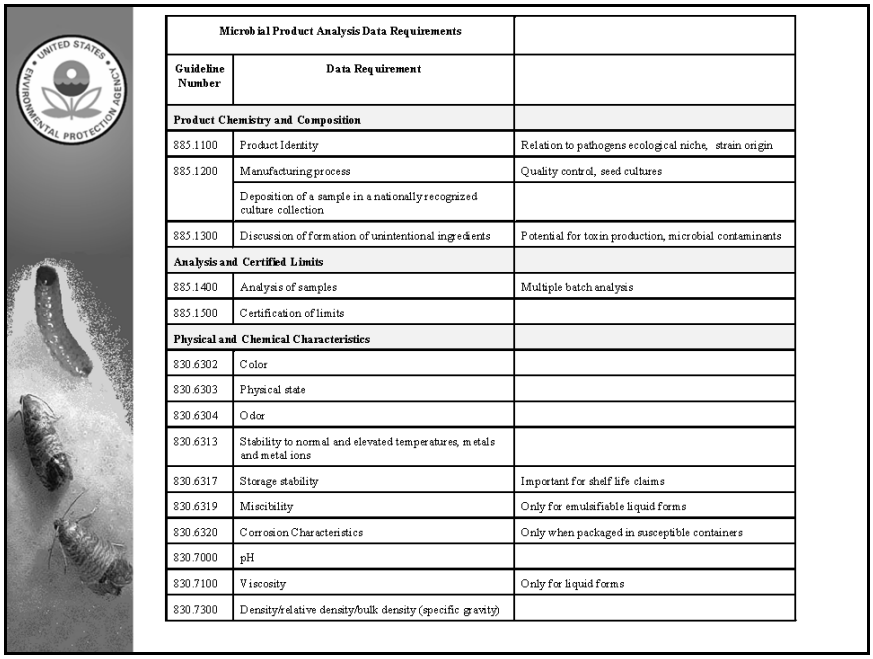
US-regulatory system and perspective on identity and characterisation issues for micro-organisms

By William Schneider (BioPesticides Division, EPA, Arlington; USA)

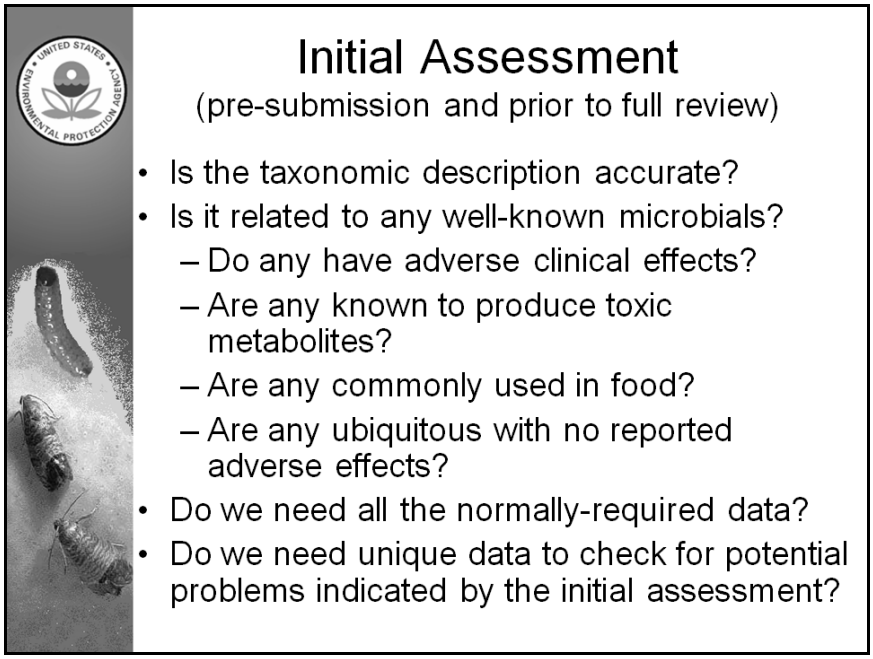


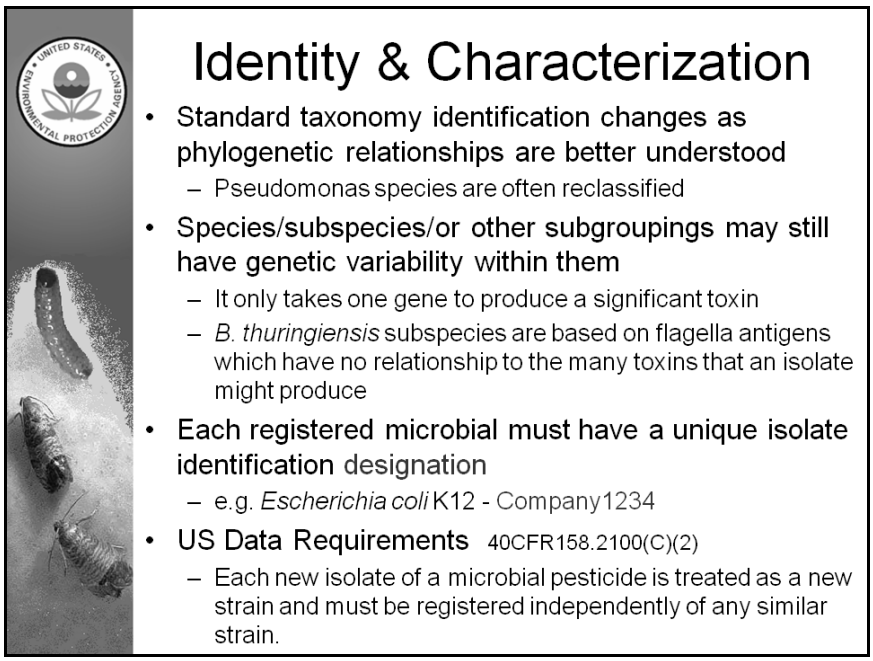


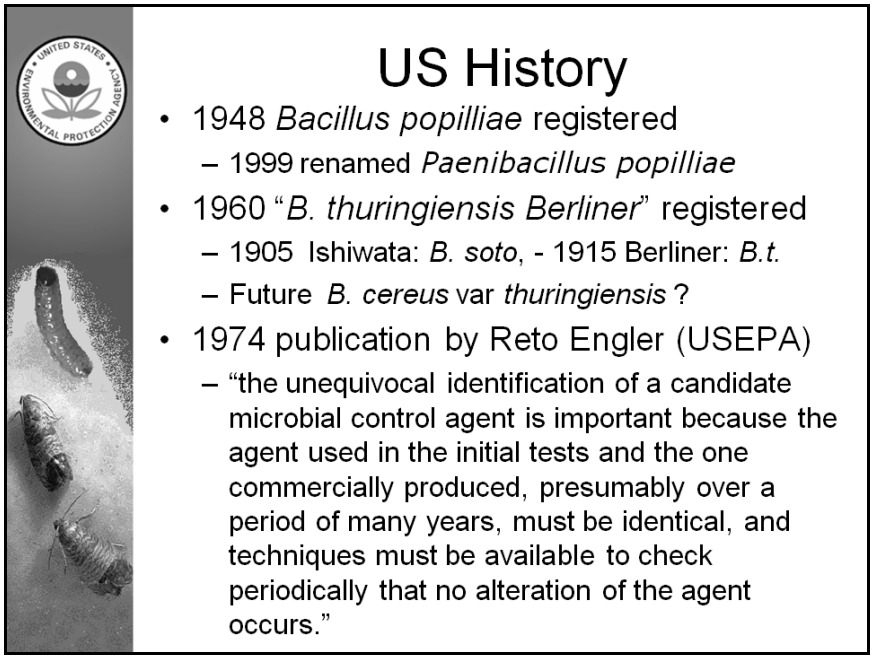


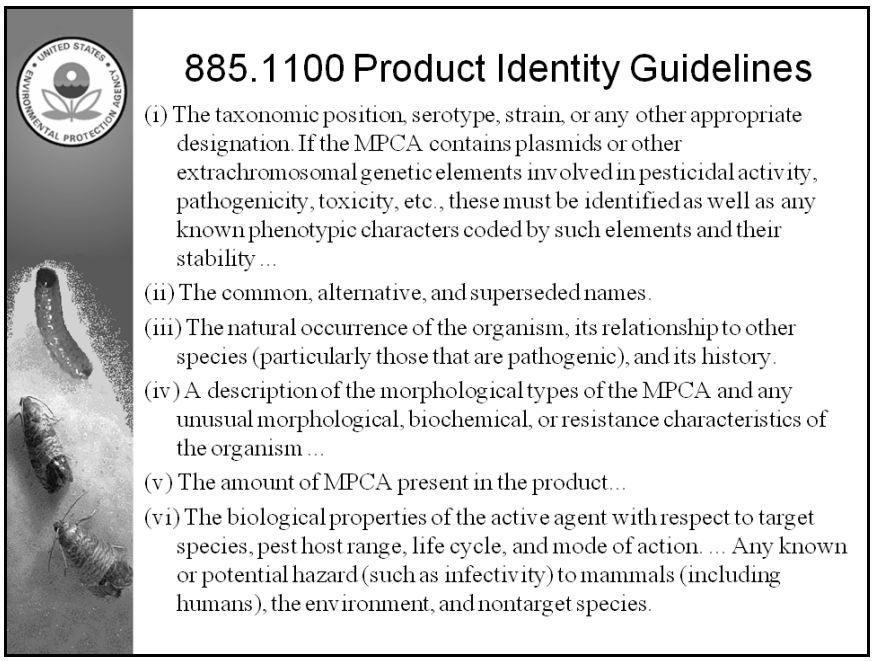


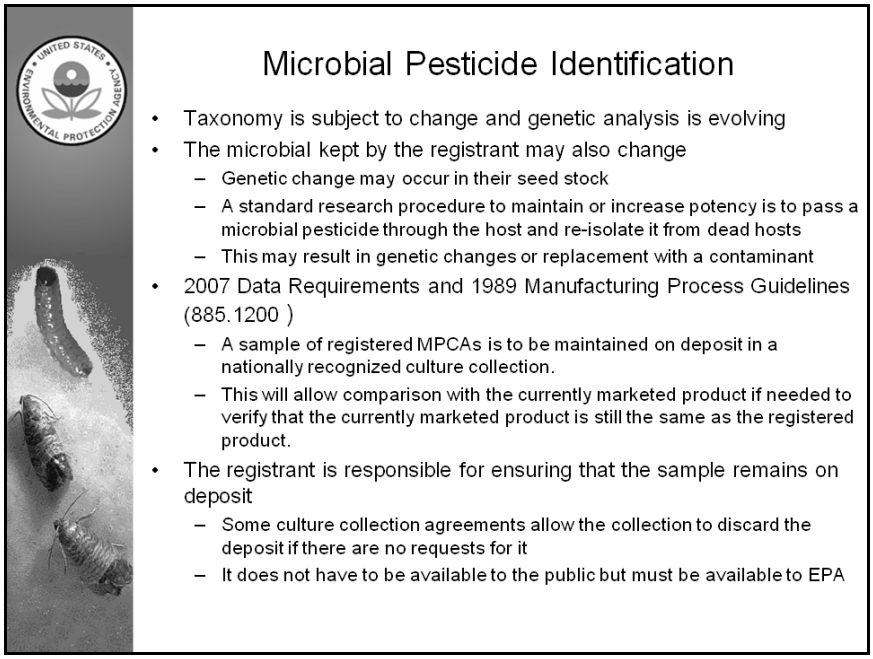
Microbial Product Analysis Data Requirements		
Guideline Number	**Data Requirement**	
Product Chemistry and Composition		
885.1100	Product Identity	Relation to pathogens ecological niche, strain origin
885.1200	Manufacturing process	Quality control, seed cultures
	Deposition of a sample in a nationally recognized culture collection	
885.1300	Discussion of formation of unintentional ingredients	Potential for toxin production, microbial contaminants
Analysis and Certified Limits		
885.1400	Analysis of samples	Multiple batch analysis
885.1500	Certification of limits	
Physical and Chemical Characteristics		
830.6302	Color	
830.6303	Physical state	
830.6304	Odor	
830.6313	Stability to normal and elevated temperatures, metals and metal ions	
830.6317	Storage stability	Important for shelf life claims
830.6319	Miscibility	Only for emulsifiable liquid forms
830.6320	Corrosion Characteristics	Only when packaged in susceptible containers
830.7000	pH	
830.7100	Viscosity	Only for liquid forms
830.7300	Density/relative density/bulk density (specific gravity)	

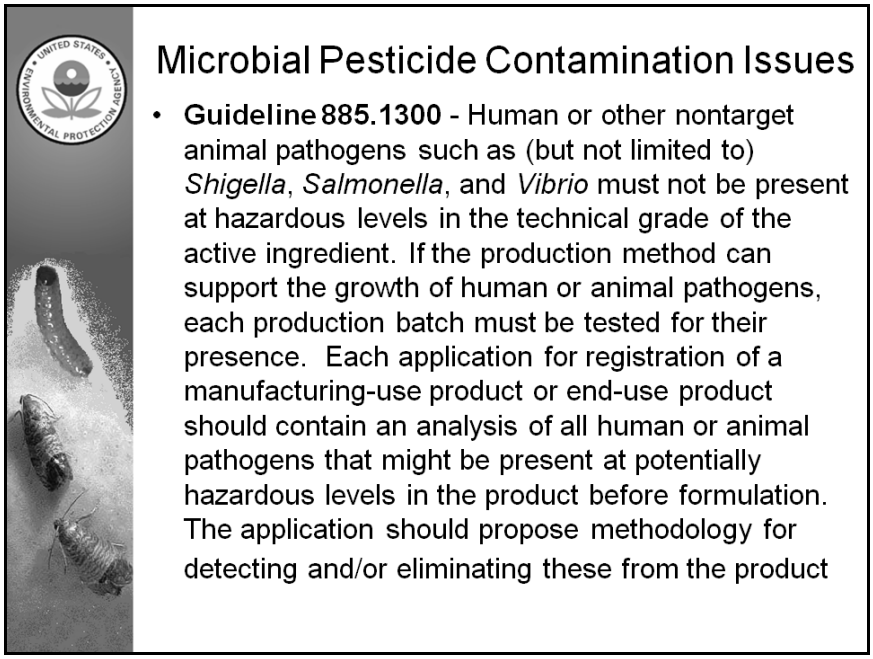

Initial Assessment
(pre-submission and prior to full review)
• Is the taxonomic description accurate?
• Is it related to any well-known microbials?
– Do any have adverse clinical effects?
– Are any known to produce toxic metabolites?
– Are any commonly used in food?
– Are any ubiquitous with no reported adverse effects?
• Do we need all the normally-required data?
• Do we need unique data to check for potential problems indicated by the initial assessment?

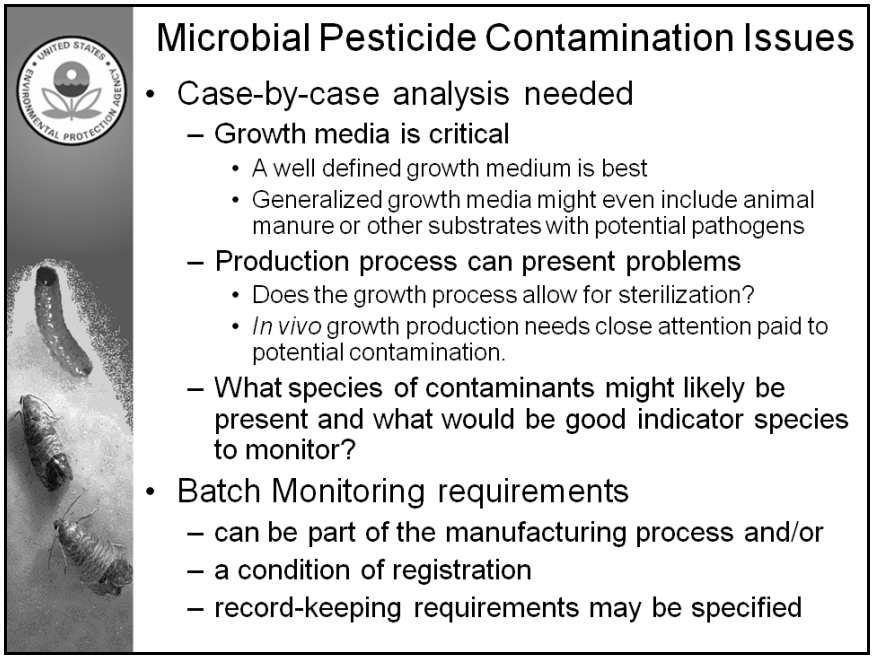

Identity & Characterization
• Standard taxonomy identification changes as phylogenetic relationships are better understood
– Pseudomonas species are often reclassified
• Species/subspecies/or other subgroupings may still have genetic variability within them
– It only takes one gene to produce a significant toxin
– B. thuringiensis subspecies are based on flagella antigens which have no relationship to the many toxins that an isolate might produce
• Each registered microbial must have a unique isolate identification designation
– e.g. Escherichia coli K12 - Company1234
• US Data Requirements 40CFR158.2100(C)(2)
– Each new isolate of a microbial pesticide is treated as a new strain and must be registered independently of any similar strain.

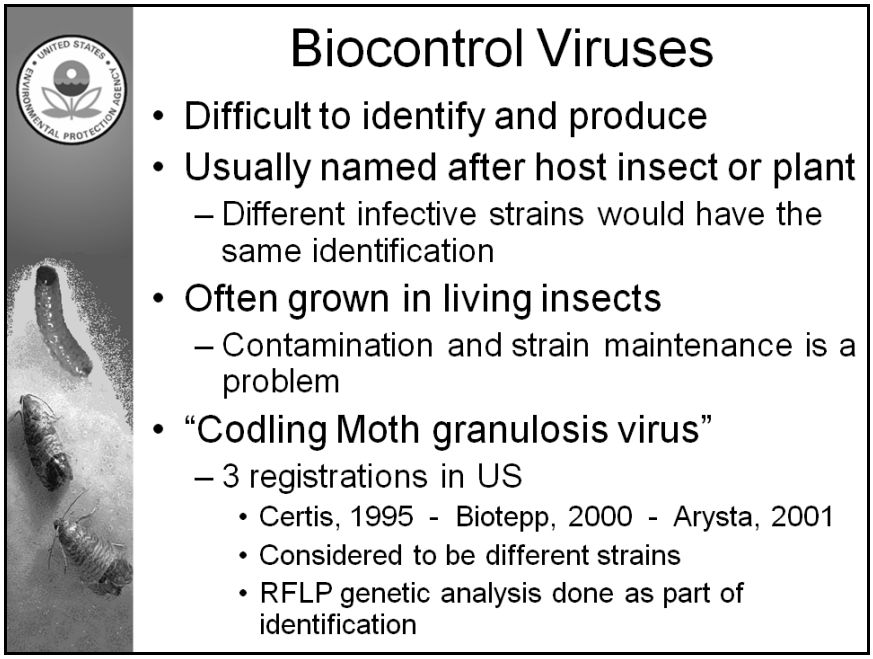

US History
• 1948 *Bacillus popilliae* registered
– 1999 renamed *Paenibacillus popilliae*
• 1960 "*B. thuringiensis Berliner*" registered
– 1905 Ishiwata: *B. soto*, - 1915 Berliner: *B.t.*
– Future *B. cereus* var *thuringiensis* ?
• 1974 publication by Reto Engler (USEPA)
– "the unequivocal identification of a candidate microbial control agent is important because the agent used in the initial tests and the one commercially produced, presumably over a period of many years, must be identical, and techniques must be available to check periodically that no alteration of the agent occurs."


885.1100 Product Identity Guidelines
(i) The taxonomic position, serotype, strain, or any other appropriate designation. If the MPCA contains plasmids or other extrachromosomal genetic elements involved in pesticidal activity, pathogenicity, toxicity, etc., these must be identified as well as any known phenotypic characters coded by such elements and their stability ...
(ii) The common, alternative, and superseded names.
(iii) The natural occurrence of the organism, its relationship to other species (particularly those that are pathogenic), and its history.
(iv) A description of the morphological types of the MPCA and any unusual morphological, biochemical, or resistance characteristics of the organism ...
(v) The amount of MPCA present in the product...
(vi) The biological properties of the active agent with respect to target species, pest host range, life cycle, and mode of action. ... Any known or potential hazard (such as infectivity) to mammals (including humans), the environment, and nontarget species.


Microbial Pesticide Identification
• Taxonomy is subject to change and genetic analysis is evolving
• The microbial kept by the registrant may also change
– Genetic change may occur in their seed stock
– A standard research procedure to maintain or increase potency is to pass a microbial pesticide through the host and re-isolate it from dead hosts
– This may result in genetic changes or replacement with a contaminant
• 2007 Data Requirements and 1989 Manufacturing Process Guidelines (885.1200)
– A sample of registered MPCAs is to be maintained on deposit in a nationally recognized culture collection.
– This will allow comparison with the currently marketed product if needed to verify that the currently marketed product is still the same as the registered product.
• The registrant is responsible for ensuring that the sample remains on deposit
– Some culture collection agreements allow the collection to discard the deposit if there are no requests for it
– It does not have to be available to the public but must be available to EPA

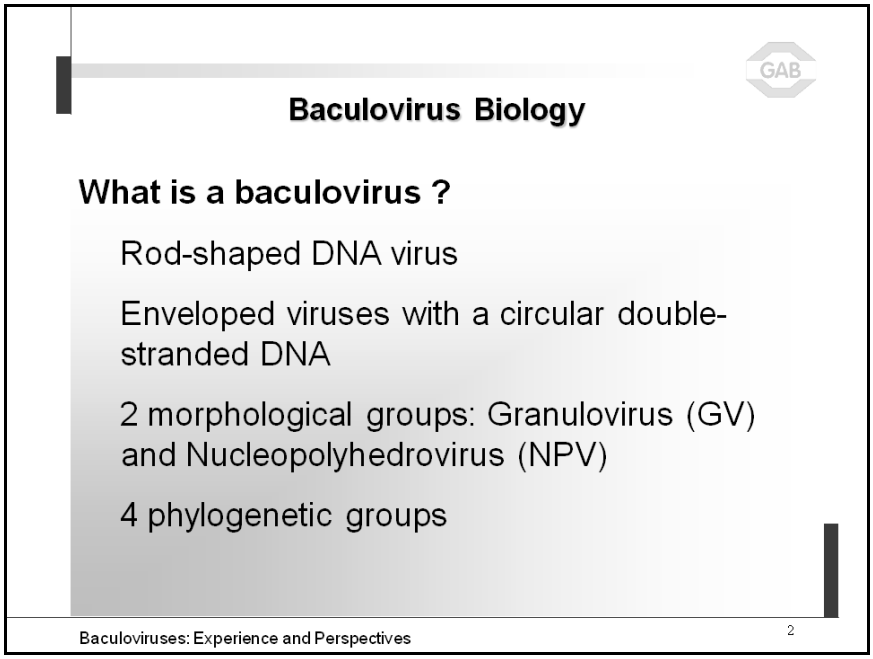

Microbial Pesticide Contamination Issues
• Guideline 885.1300 - Human or other nontarget animal pathogens such as (but not limited to) Shigella, Salmonella, and Vibrio must not be present at hazardous levels in the technical grade of the active ingredient. If the production method can support the growth of human or animal pathogens, each production batch must be tested for their presence. Each application for registration of a manufacturing-use product or end-use product should contain an analysis of all human or animal pathogens that might be present at potentially hazardous levels in the product before formulation. The application should propose methodology for detecting and/or eliminating these from the product

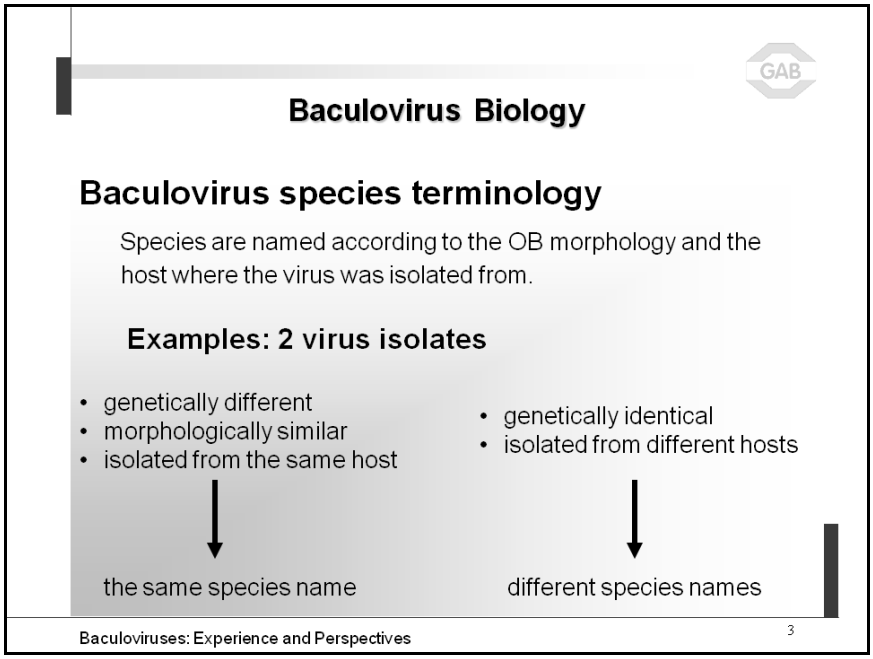

Microbial Pesticide Contamination Issues
• Case-by-case analysis needed
– Growth media is critical
• A well defined growth medium is best
• Generalized growth media might even include animal manure or other substrates with potential pathogens
– Production process can present problems
• Does the growth process allow for sterilization?
• *In vivo* growth production needs close attention paid to potential contamination.
– What species of contaminants might likely be present and what would be good indicator species to monitor?
• Batch Monitoring requirements
– can be part of the manufacturing process and/or
– a condition of registration
– record-keeping requirements may be specified

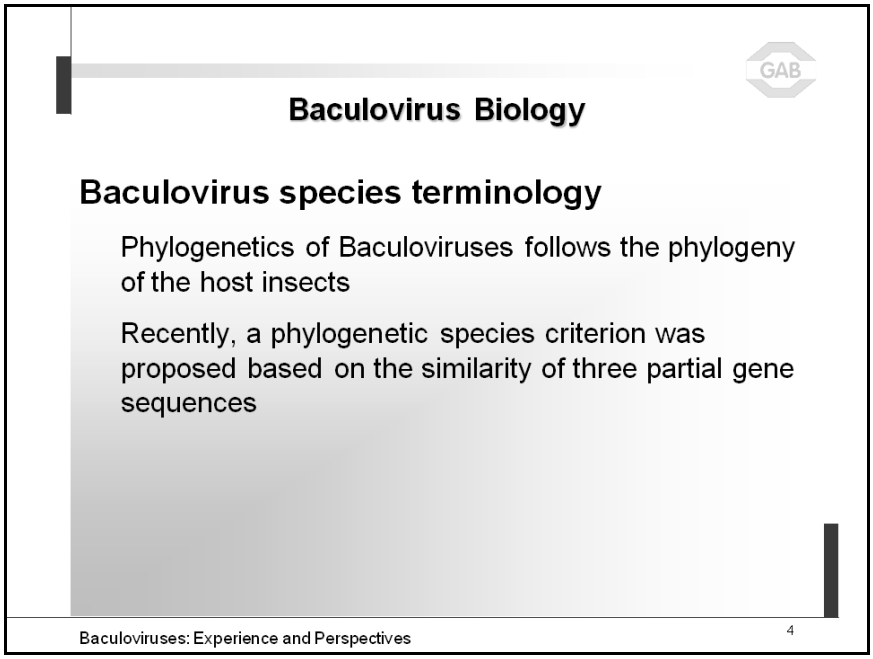

Biocontrol Viruses
• Difficult to identify and produce
• Usually named after host insect or plant
– Different infective strains would have the same identification
• Often grown in living insects
– Contamination and strain maintenance is a problem
• "Codling Moth granulosis virus"
– 3 registrations in US
• Certis, 1995 - Biotepp, 2000 - Arysta, 2001
• Considered to be different strains
• RFLP genetic analysis done as part of identification

Baculoviruses

By Rüdiger Hauschild (GAB Consulting GmbH, Lamstedt; Germany)




Baculovirus Biology
Baculovirus species terminology
Species are named according to the OB morphology and the host where the virus was isolated from.
Examples: 2 virus isolates
• genetically different
• morphologically similar
• isolated from the same host
the same species name
• genetically identical
• isolated from different hosts
different species names
Baculoviruses: Experience and Perspectives
3


Baculovirus Biology
Baculovirus species terminology
Phylogenetics of Baculoviruses follows the phylogeny of the host insects
Recently, a phylogenetic species criterion was proposed based on the similarity of three partial gene sequences
Baculoviruses: Experience and Perspectives
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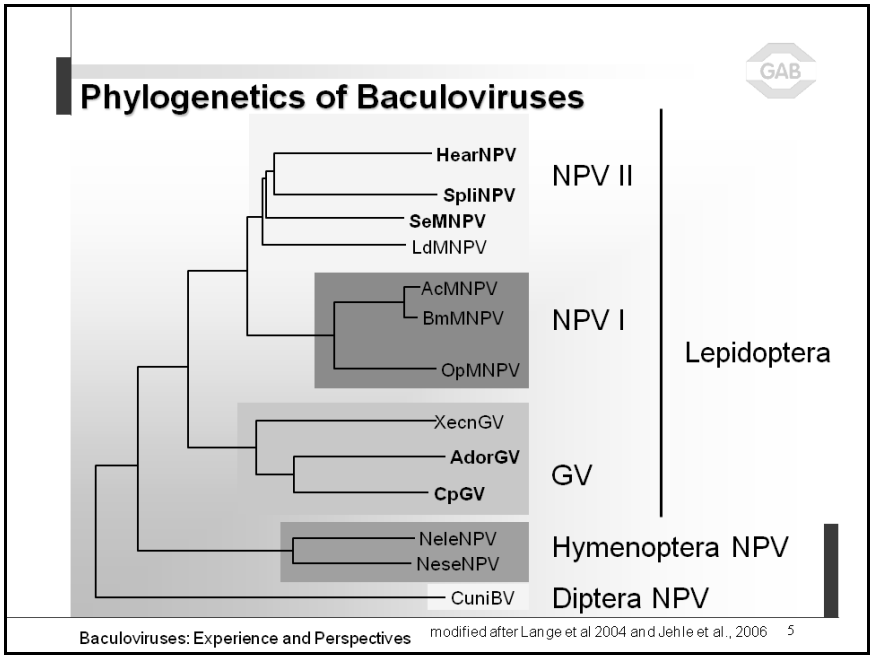
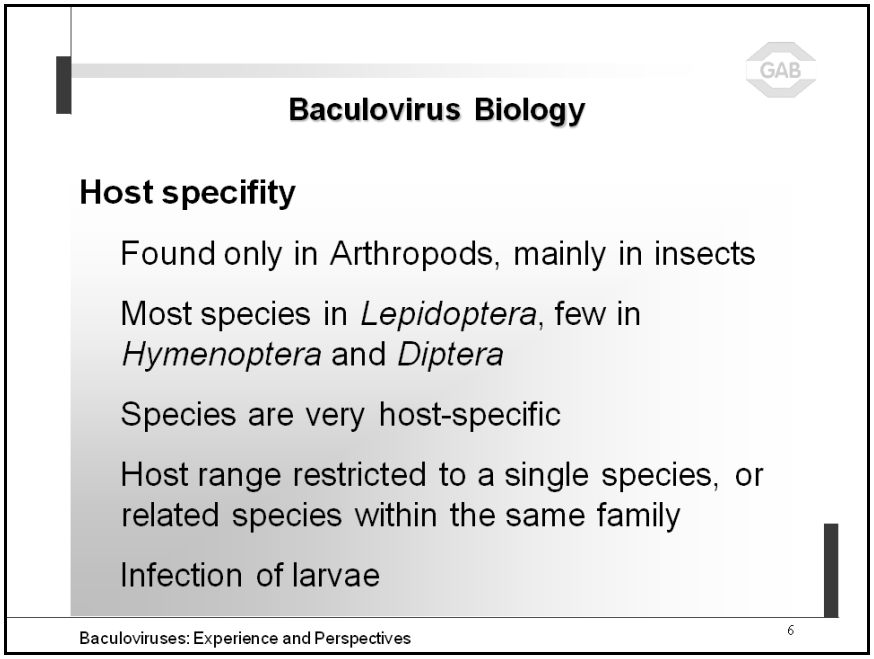

Phylogenetics of Baculoviruses
HearNPV
SpliNPV
SeMNPV
LdMNPV
AcMNPV
BmMNPV
OpMNPV
XecnGV
AdorGV
CpGV
NeleNPV
NeseNPV
CuniBV
NPV II
NPV I
GV
Lepidoptera
Hymenoptera NPV
Diptera NPV
Baculoviruses: Experience and Perspectives
modified after Lange et al 2004 and Jehle et al., 2006
5


Baculovirus Biology
Host specifity
Found only in Arthropods, mainly in insects
Most species in Lepidoptera, few in Hymenoptera and Diptera
Species are very host-specific
Host range restricted to a single species, or related species within the same family
Infection of larvae
Baculoviruses: Experience and Perspectives
6

Baculoviruses in Regulation

Regulatory situation

- Baculoviruses are "micro-organisms" in all regulatory systems
- Regulation is accomplished at strain level
- Baculovirus isolates are genetically heterogenous
- Genetic heterogeneity required to account for variations among host populations
- Differences to bacteria and fungi not always formally considered

Baculoviruses: Experience and Perspectives 7

Baculoviruses in Regulation

Regulatory situation in the EU

Two species included in Annex I:

- *Cydia pomonella* Granulovirus (CpGV) as "existing active substance", peer review soon
- *Spodoptera exigua* Nucleopolyhedrovirus SeMNPV (SeNPV) as "new active substance"

Three species under evaluation as new active substances, completeness of dossiers published

- *Adoxophyes orana* Granulovirus (AdorGV / AoGV)
- *Helicoverpa armigera* Nucleopolyhedrovirus (HearNPV)
- *Spodoptera littoralis* Nucleopolyhedrovirus (SpliNPV)

Baculoviruses: Experience and Perspectives 8

Baculoviruses in Regulation

"OECD Consensus document"

Series on Harmonization of Regulatory Oversight in Biotechnology, No.20, 2002:

"CONSENSUS DOCUMENT ON INFORMATION USED IN THE ASSESSMENT OF ENVIRONMENTAL APPLICATIONS INVOLVING BACULOVIRUSES"

Summary of available literature

Baculoviruses: Experience and Perspectives 9

Baculoviruses in Regulation

Properties of Baculoviruses (OECD 20)

- Extremely host-specific
- Not infective for mammals
- No replication in mammalian cells
- No pathogenic, genotoxic, mutagenic, or carcinogenic effect in mammals
- No metabolite production
- Effects on non-target species can be excluded

"The use of baculoviruses is safe"

Baculoviruses: Experience and Perspectives 10

Baculoviruses in Regulation

Potential risks from plant protection products containing baculoviruses

- No risks from the baculovirus itself.
- Potential risks from products might occur through other product components.
- No effects on sensitisation for products containing CpGV, SpliNPV, and LdMNPV.
- Microbial contaminants cannot be excluded.

Baculoviruses: Experience and Perspectives 11

Baculoviruses in Regulation

Regulatory strategy

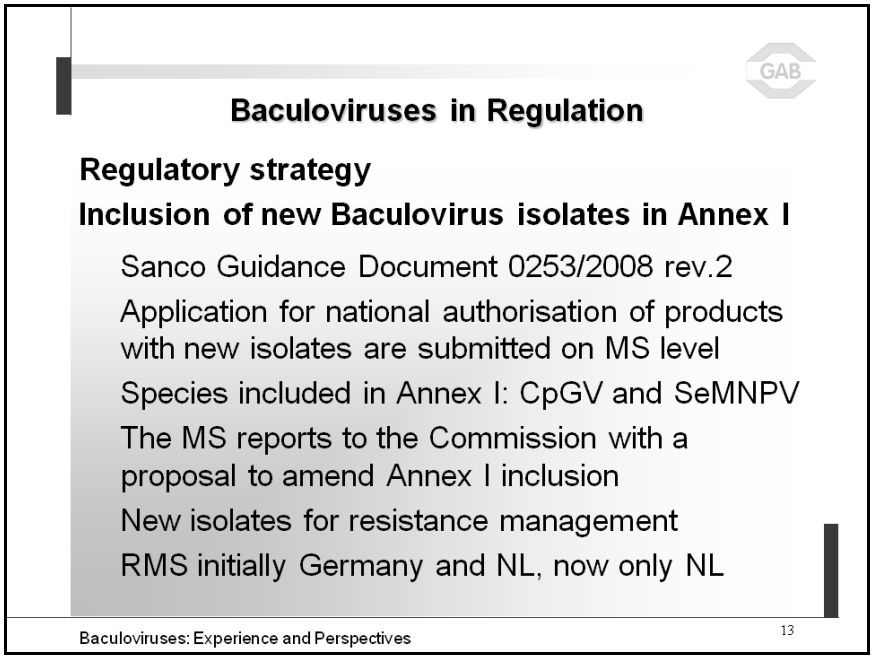
Molecular characterisation of the isolate (by RFLP)

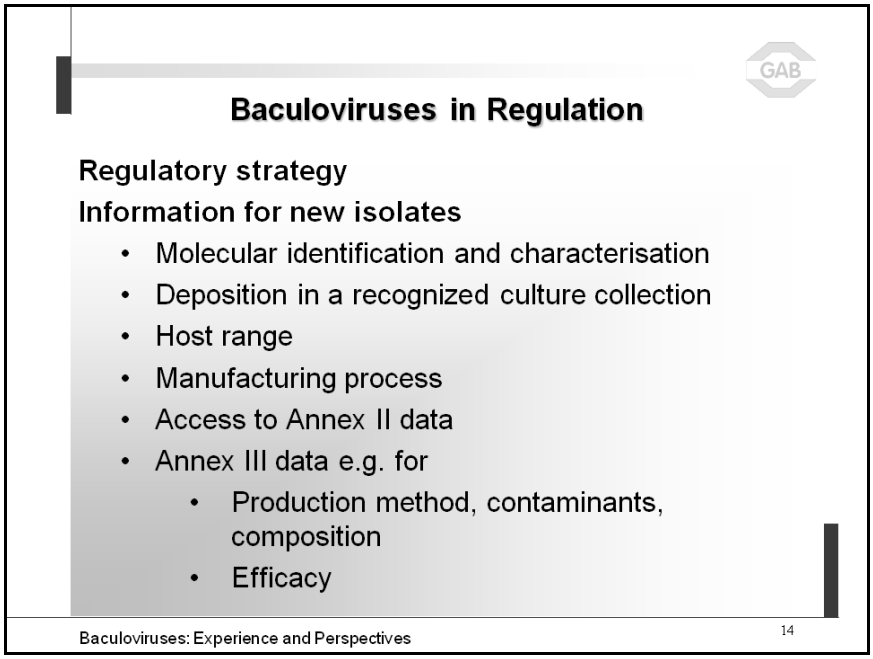
Production data for the isolate / product

Data from different species can be used for the risk assessment of a given species / isolate

The principle that each strain/isolate has to be regarded as a new active ingredient can be abandoned for baculoviruses.

Baculoviruses: Experience and Perspectives 12


Baculoviruses in Regulation
Regulatory strategy
Inclusion of new Baculovirus isolates in Annex I
Sanco Guidance Document 0253/2008 rev.2
Application for national authorisation of products with new isolates are submitted on MS level
Species included in Annex I: CpGV and SeMNPV
The MS reports to the Commission with a proposal to amend Annex I inclusion
New isolates for resistance management
RMS initially Germany and NL, now only NL
Baculoviruses: Experience and Perspectives
13


Baculoviruses in Regulation
Regulatory strategy
Information for new isolates
Molecular identification and characterisation
Deposition in a recognized culture collection
Host range
Manufacturing process
Access to Annex II data
Annex III data e.g. for
Production method, contaminants, composition
Efficacy
Baculoviruses: Experience and Perspectives
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Baculoviruses: Experience and Perspectives

Experiences

Numbers of studies in the dossiers submitted so far were not considerably reduced when compared to other microbials

Literature information was used extensively

Annex I inclusion in the EU is still difficult and time-consuming for new baculoviruses

National registrations are in many cases not issued before Annex I inclusion

No experience available until now for the procedure according to Sanco Guidance Document 0253/2008.

Baculoviruses: Experience and Perspectives 15

Baculoviruses: Experience and Perspectives

Perspectives

Sanco Guidance Document 0253/2008 will facilitate the placing on the market of products containing new isolates.

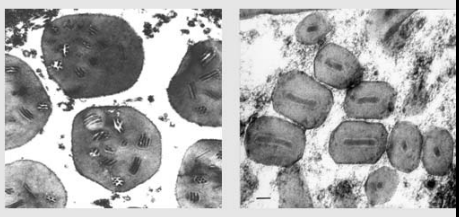
Further experience with baculovirus isolates and products might accelerate Annex I inclusion for further species

Baculoviruses: Experience and Perspectives 16


GAB
Baculoviruses: Experience and Perspectives
Many Thanks !
Rüdiger Hauschild
GAB Consulting GmbH
ruediger.hauschild@gab-consult.de
17

Identity and molecular characterization of baculoviruses

By Johannes Jehle (DLR Rheinpfaltz, Neustadt/ Weinstrasse; Germany)



Identity and molecular characterization of baculoviruses

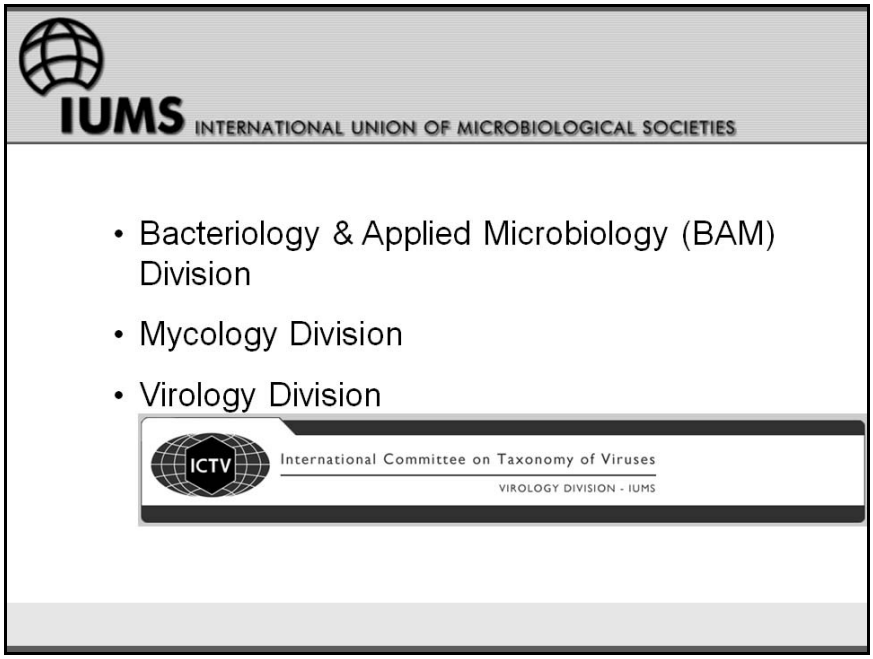
Johannes A. Jehle

Laboratory of Biotechnological Crop Protection,
Agricultural Service Center (DLR Rheinpfalz), Neustadt Wstr., Germany

Paris, July 1, 2009

Outline

- International Committee on Taxonomy of Viruses
- Classification
- Methods of identification
  - Restriction analyses
  - Genome Sequencing
  - PCR based methods
- Conclusions



Invertebrate Virus Subgroup (2008-2011)

Name	Study Group	Country
Krell, Peter J.	Chair	Canada
Bigot, Yves	Chair - Ascoviridae Study Group	France
Jehle, Johannes A.	Chair - Baculoviridae Study Group	Germany
Nakashima, Nobuhiko	Chair - Dicistroviridae Study Group	Japan
Hyatt, Alex	Chair - Iridoviridae Study Group	Australia
Lo, Grace	Chair - Nimaviridae Study Group	Taiwan
Thiery, Richard	Chair - Nodaviridae Study Group	France
Strand, Michael	Chair - Polydnaviridae Study Group	USA
Walker, Peter	Chair - Roniviridae Study Group	Australia
Gordon, Karl	Chair - Tetraviridae Study Group	Australia

ICTV
International Committee on Taxonomy of Viruses
VIROLOGY DIVISION - IUMS

Baculovirus Study Group (2008-2011)

Name	Study Group	Country
Jehle, J. A.	Chair	Germany
Arif, B.	Member	Canada
Blissard, G. W.	Member	USA
Bonning, B.	Member	USA
Becnel, J.	Member	USA
Harrison, W.	Member	USA
Herniou, E. A.	Member	France
Theilmann, D. A	Member	Canada
Vlak, J. M.	Member	The Netherlands

ICTV International Committee on Taxonomy of Viruses
VIROLOGY DIVISION - IUMS

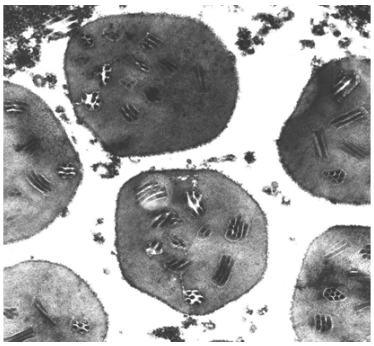
Baculovirus Classification (ICTV)

Taxonomic Level	Example
Order	-
Family	*Baculoviridae*
Genus	*Alphabaculovirus*
Species	*Autographa californica nucleopolyedrovirus* (AcMNPV)

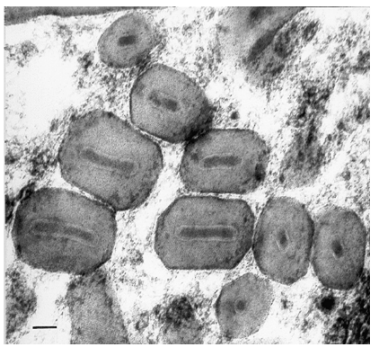
Classification of Baculoviruses

- Morphology
- Nucleic Acid
- Proteins
- Host Range
- Molecular Phylogeny

Occlusion Body Types

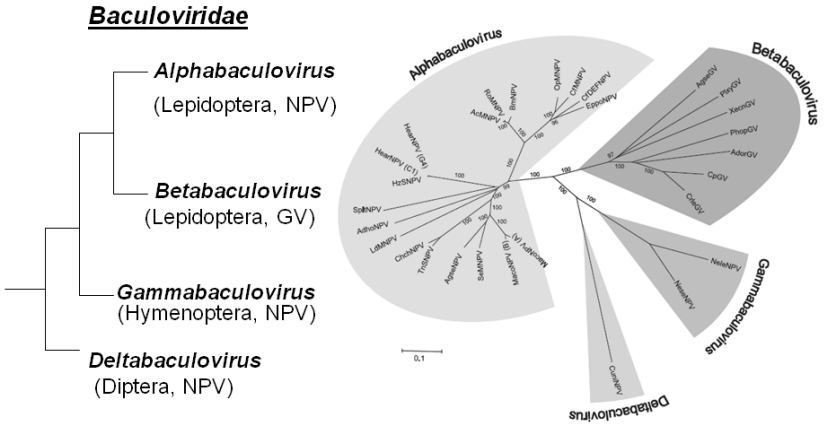


Nucleopolyhedrovirus (= NPV)



Granuloviruses (= GV)

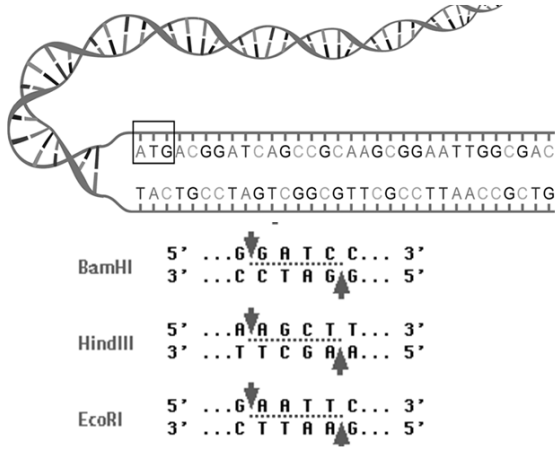
Classification of Baculoviruses



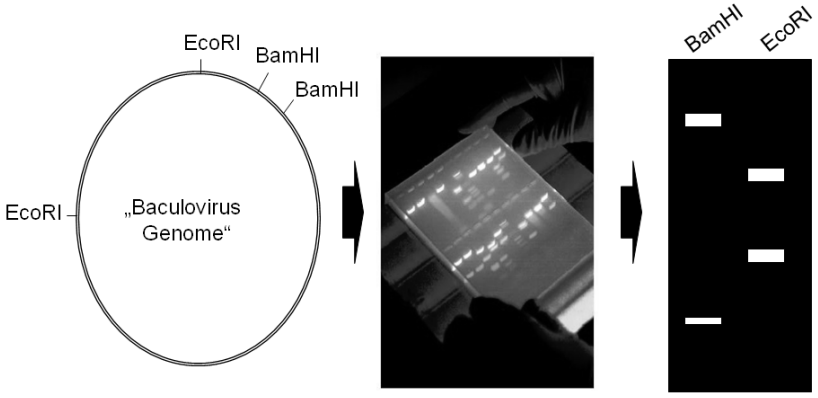
Methods for identification

- DNA restriction endonuclease digestion
- Genome sequencing
- PCR based methods

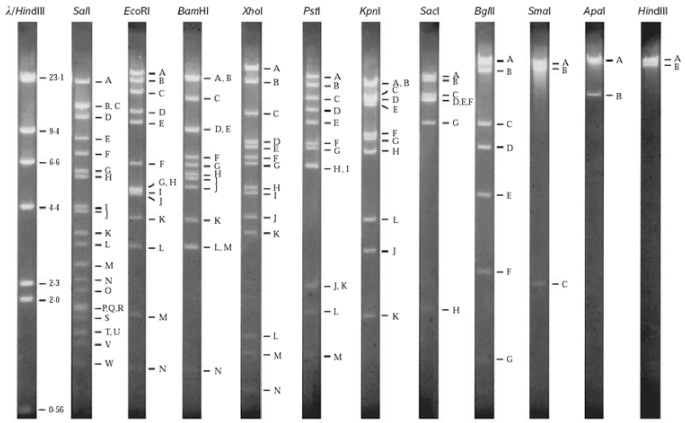
Restriction Endonuclease (REN) analysis



REN Analysis

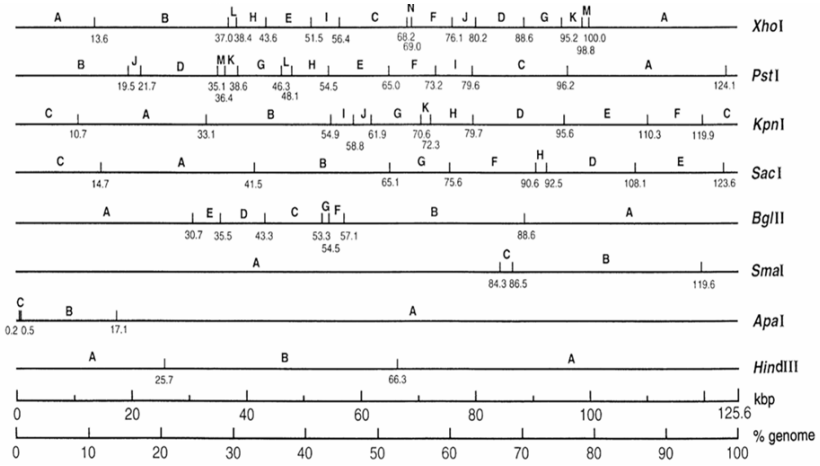


CpGV REN-Analysis



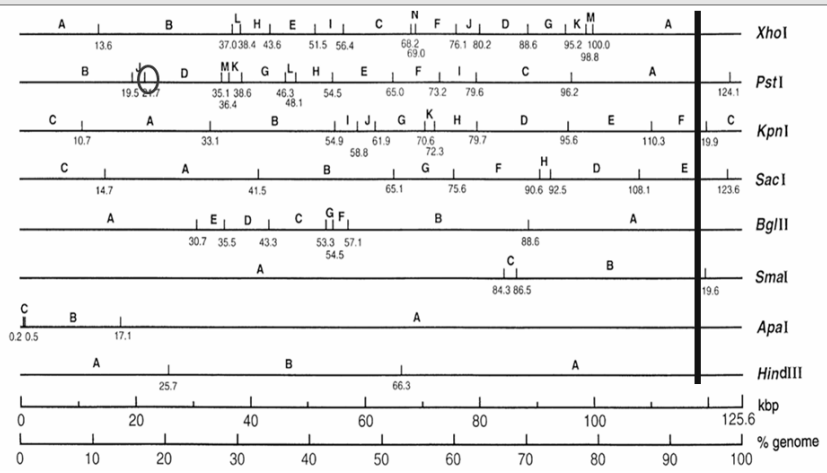
Crook et al., 1997

Restriction map of CpGV



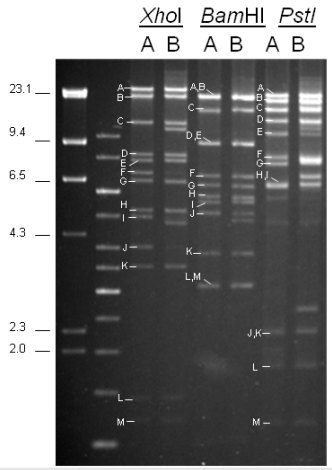
Crook et al., 1997

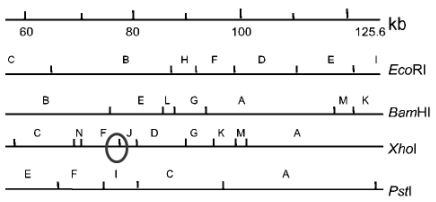
Restriction map of CpGV



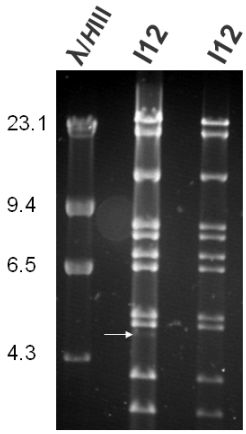
Crook et al., 1997

Genetic Heterogeneity in an Isolate





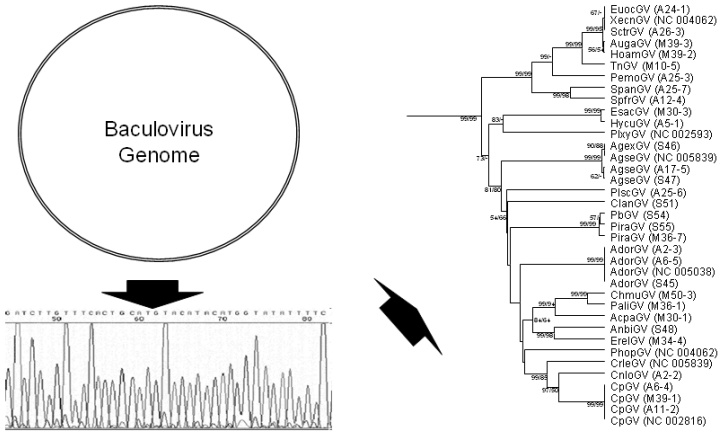
Pitfall: Incomplete Digestion of DNA



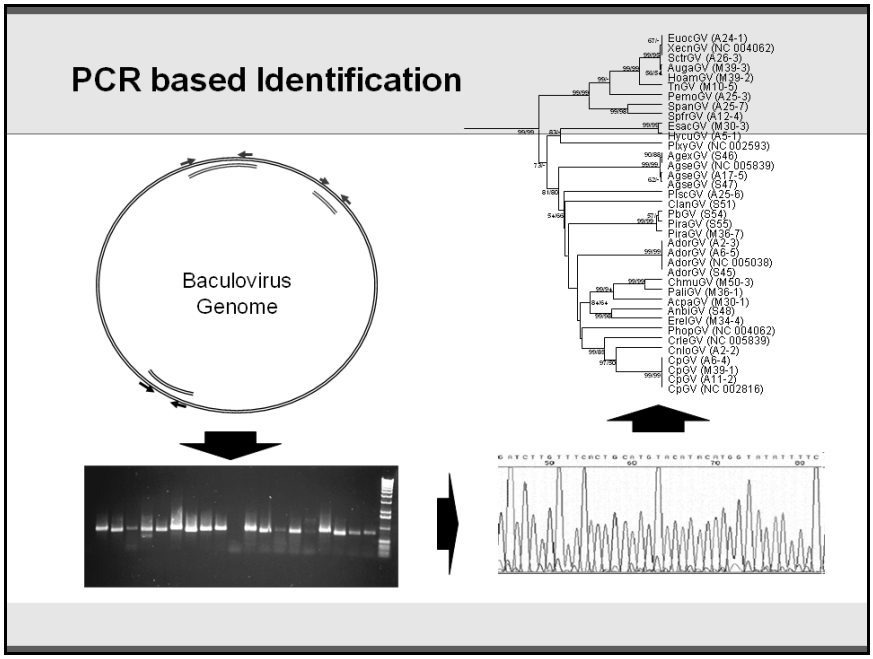
Incomplete digestion with XhoI:

- Sometimes difficult to interpret
- Some expert knowledge necessary

Genome Sequencing



PCR based Identification



PCR based Identification

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Virology 346 (2006) 180 – 193
VIROLOGY
www.elsevier.com/locate/yviro

Molecular identification and phylogenetic analysis of baculoviruses from Lepidoptera

Johannes A. Jehle [a,*], Martin Lange [a], Hualin Wang [a,b], Zhihong Hu [b], Yongjie Wang [a], Rüdiger Hauschild [a]

[a] *Laboratory for Biotechnological Crop Protection, Department of Phytopathology, Agricultural Service Center Palatinate (DLR Rheinpfalz), Breitenweg 71, 67435 Neustadt an der Weinstraße, Germany*
[b] *State Key Laboratory of Virology and Joint Laboratory of Invertebrate Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, People's Republic of China*

Received 30 August 2005; returned to author for revision 27 September 2005; accepted 25 October 2005
Available online 28 November 2005

Conclusions

	REN	Genome Sequence	PCR analyses
Complexity of information	+	+/-	-
Amount of information	+/-	+	-
Simplicity of methodology	+	-	+/-
Time needed	4 weeks	4 months	2 weeks
Simplicity of concepts	+	-	-
Cost effectiveness	+	-	+/-
Information for identification	+	+	+/-
Information for classification	-	+	+/-
Usefulness for science	+/-	+	+
Usefulness for producers	+	+	+
Usefulness for regulators	+	-	-

Conclusions

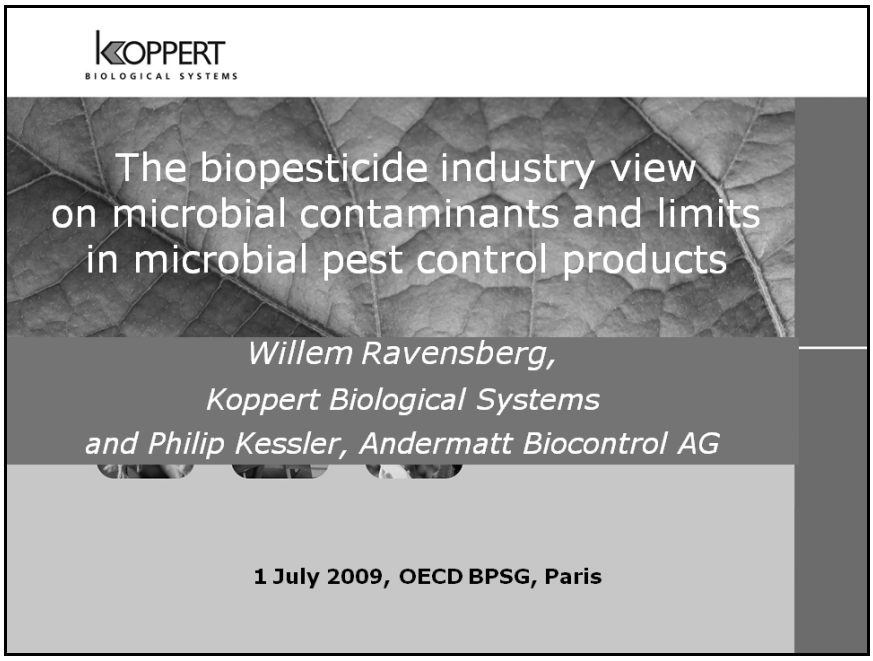
REN analysis

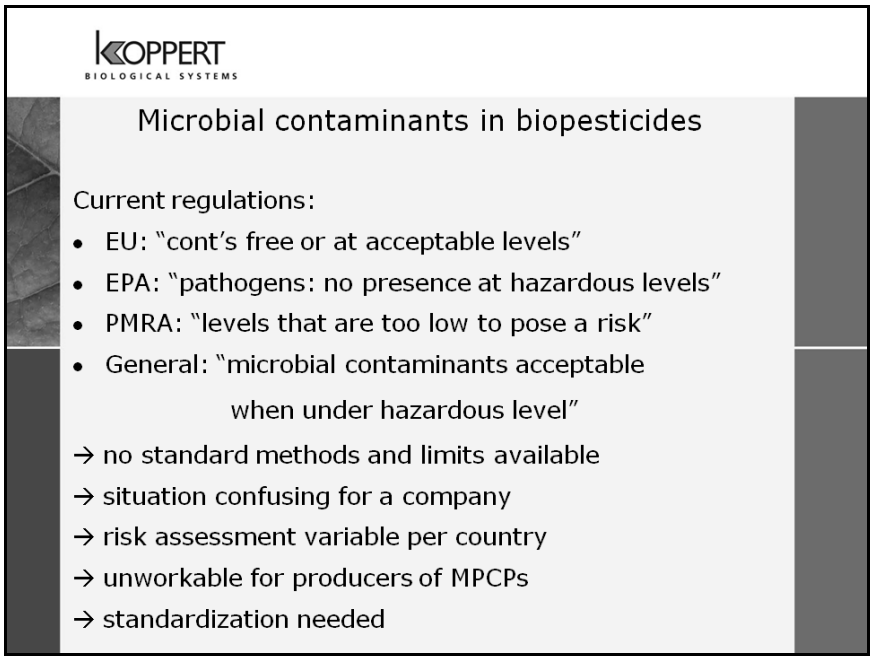
- is state-of-the-art for baculovirus identification
- allows identification of isolates, genome heterogeneity
- corresponds with the genome sequence
- is fast, reliable and cost effective
- 4-6 digests using different enzymes are suitable to distinguish different genotypes

Genome sequencing and **PCR based studies** of marker genes are useful for initial characterization and screening

Thank you!

The Biopesticide Industry view
on microbial contaminants and limits in microbial pest control products
By Willem Ravensberg (Koppert, Berkel en Rodenrijs; The Netherlands)





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Reasons to limit microbial contaminants in MPCPs

- Product stability
- Product efficacy
- Exposure to humans and animals
- Exposure to the environment
- Plant pathogens: should not be present

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Draft OECD discussion paper on "microbial contaminants and limits" from PMRA, Canada

- Cf. food and drinking water criteria → use methods and criteria from food, supplements, probiotics
- MPCPs may end up on food and in food or feed products
- Concern: direct and indirect exposure

- Industry agrees with necessity of standardization of methods and criteria
- But MPCPs are very different products compared to food, etc.!!
- Establish criteria relevant to MPCPs an their use

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Microbial contaminants in MPCP production

Production methods

- Bacteria → *in vitro* → sterile
- Fungi → *in vitro*→ sterile/non-sterile
- Yeasts → *in vitro* → sterile
- Baculoviruses → *in vivo* → non-sterile
- Protozoa → *in vivo* → non-sterile

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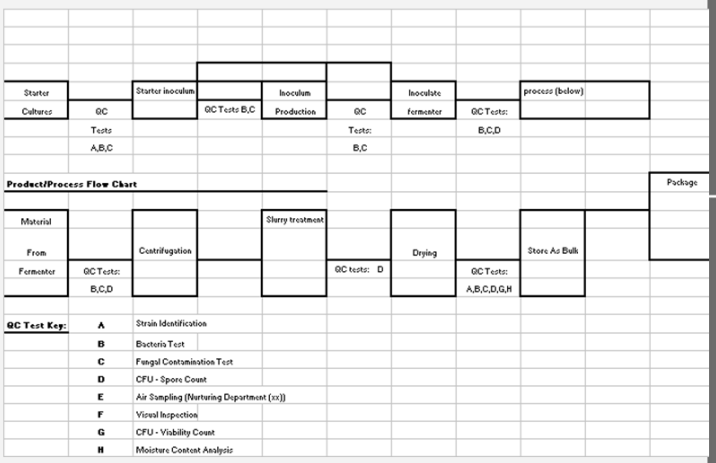
Flow chart of typical production of a MPCP



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Microbial contaminants in MPCP production

- *In vitro* production methods (bacteria, fungi, yeasts)
- Must be sterile
- Sterilized equipment and medium
- Selective and optimized medium for a.i.
- Pure inoculum
- Process parameters and QC checks
- Yield: QC

- Non-sterile fungi production: some cases where fungus overgrows medium very quickly (2-3 days)

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Down-stream process and formulation

- Non-sterile phase in production process
  - equipment (filtration, centrifugation, mixing, etc.)
  - co-formulants (food approved products, chemical and natural substances)
  - air, water
  - personnel

- Packaging
  - equipment
  - materials
  - personnel

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Formulations

- Dry formulations (WP, WG)
  - -Most formulations are dried: Aw< 0.5
- Oil formulations
  - -No water available
- Flowables, emulsions
  - -Often with preservatives, or high osmatic value
- MPCPs
  - -Formulation conditions do not allow growth of m.c.'s
  - -Often stored at < 10°C
  - -Often low O_2 conditions in package
- Lilelyhood of activity of m.c.'s in MPCPs very low

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Product Quality Control

- Content (% a.i.)
- Phys.-chem. properties
- Efficacy
- Microbial contaminants

- Only when results meet the product's specifications, the MPCPs are released for sale

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Perspective of biopesticides in agriculture

- (Bio)pesticides are inputs in agriculture, possibly with m.c.'s
- Other inputs, possibly with m.c.'s:
  - fertilizers / manure
  - compost / growing medium
  - water for irrigation, spraying, and rain
  - plants
  - equipment
  - people
  - air

--> Exposure to m.c.'s from all these inputs

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Example of biopesticide use and exposure of microbial contaminants

- Mycotal: *Lecanicillium muscarium* 10^{e}10 sp/gr
- Dose: 1 g/l, 1000 l/ha → 10^{e}13 spores/ha
- LAI = 3, two-sided→ LAI=6 → 30,000 sp/cm^2 of leaf
- On one tomato (ø 4 cm)→ 750.000 *L.muscarium* spores

- Suppose contaminants level: 10^{e}7 mesophiles
  - On one tomato: 750 spores
- Cont's level (coliforms): 100
  - Probability: one coliform on ≈ 1330 tomatoes
- Cont's level: 1 Salmonella per 25 gr
  - Probability: one Salmonella on ≈ 332,500 tomatoes

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Natural occurence of m.o.'s on fruit

Table B.7.2.2 Average number of micro-organisms (CFU/g fresh weight ± standard deviation) in/on eggplant fruits after treatment with Remedier

	Treated		Untreated	
	Surface	Flesh	Surface	Flesh
Trichoderma spp.	None	None	None	None
Penicillium spp.	$5.7 \pm 3.6 \times 10^{3}$	$3.3 \pm 1.8 \times 10^{3}$	$3.1 \pm 2.0 \times 10^{3}$	$2.5 \pm 1.5 \times 10^{3}$
Fusarium spp.	$4.5 \pm 1.9 \times 10^{3}$	$4.8 \pm 3.1 \times 10^{3}$	$4.5 \pm 2.2 \times 10^{3}$	$3.0 \pm 0.9 \times 10^{3}$
Other fungi (*Rhizopus*, *Geotrichum*, etc.)	$1.9 \pm 0.7 \times 10^{4}$	$5.0 \pm 3.3 \times 10^{3}$	$2.4 \pm 1.0 \times 10^{4}$	$3.3 \pm 1.8 \times 10^{3}$
Total fungi	2.9×10^{4}	1.3×10^{4}	3.2×10^{4}	8.8×10^{3}
Yeasts	$3.4 \pm 1.7 \times 10^{3}$	$3.0 \pm 2.3 \times 10^{3}$	$5.0 \pm 3.8 \times 10^{4}$	$2.2 \pm 1.3 \times 10^{3}$
Bacillus-like bacteria	$7.5 \pm 3.9 \times 10^{7}$	$1.4 \pm 0.9 \times 10^{7}$	$1.0 \pm 0.4 \times 10^{8}$	$1.1 \pm 0.5 \times 10^{7}$
Pseudomonas-like bacteria	$1.1 \pm 0.8 \times 10^{7}$	$\sim 10^{4}$	$5.0 \pm 4.4 \times 10^{8}$	$\sim 10^{4}$
Total bacteria	8.6×10^{7}	1.4×10^{7}	1.1×10^{8}	1.1×10^{7}

Conclusions: Following soil applications of Remedier at a rate of 2 x 2.5 kg product/ha (corresponding to a total 3.33×10^{3} CFU *T. harzianum* /g soil), no viable residues of *Trichoderma* spp. could be isolated from the surface or flesh of mature eggplant fruits.

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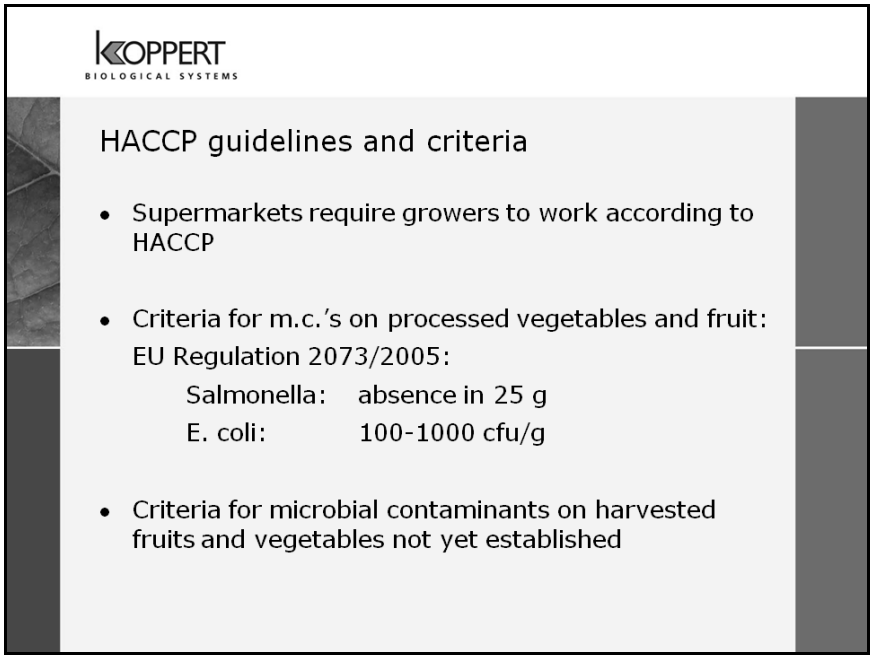
Quality Standard for Finished Compost

Heterotrophic Plate Count	1 x 108 - 1 x 1010 CFU/gdw
Anaerobic Plate Count	Aerobes: Anaerobes at 10:1 or greater
Yeasts and Molds	1 x 103 - 1 x 105 CFU/gdw
Actinomycetes	1 x 106 - 1 x 108 CFU/gdw
Pseudomonads	1 x 103 - 1 x 106 CFU/gdw
Nitrogen-Fixing Bacteria	1 x 103 - 1 x 106 CFU/gdw
Compost Maturity	>50% on Maturity Index at dilution rate appropriate for compost application.
Compost Stability	<100 mg O2/Kg compost dry solids-hour
E. coli	< 3 E. coli/g
Fecal Coliforms	<1000 MPN/g of dry solids
Salmonella	< 3 MPN/4g total solids

http://www.ciwmb.ca.gov/organics/products/Quality/CQStandards.htm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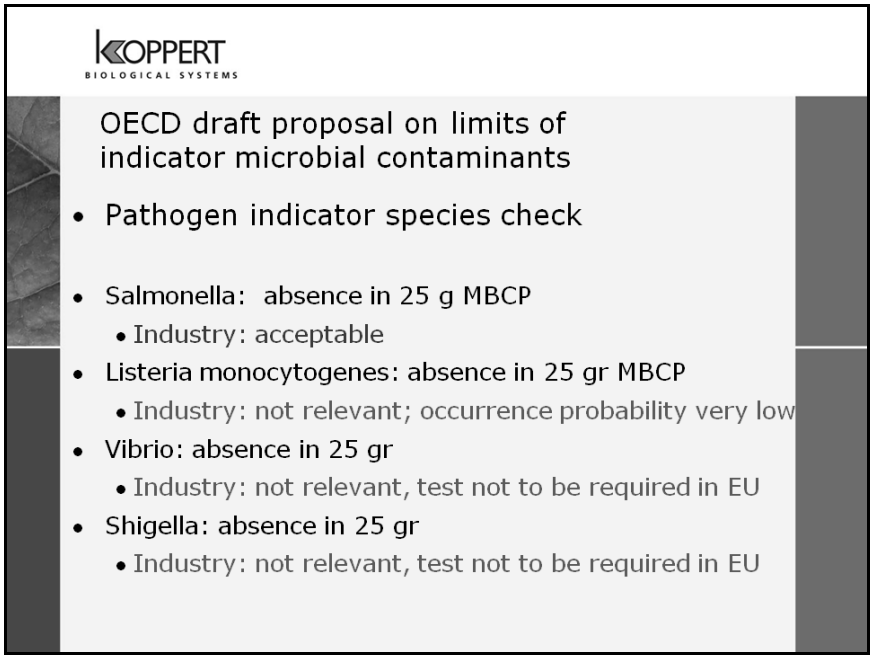
HACCP guidelines and criteria
• Supermarkets require growers to work according to HACCP
• Criteria for m.c.'s on processed vegetables and fruit:
EU Regulation 2073/2005:
Salmonella: absence in 25 g
E. coli: 100-1000 cfu/g
• Criteria for microbial contaminants on harvested fruits and vegetables not yet established


KOPPERT
BIOLOGICAL SYSTEMS
OECD draft proposal on limits of indicator microbial contaminants
• Pathogen indicator species check
• Salmonella: absence in 25 g MBCP
• Industry: acceptable
• Listeria monocytogenes: absence in 25 gr MBCP
• Industry: not relevant; occurrence probability very low
• Vibrio: absence in 25 gr
• Industry: not relevant, test not to be required in EU
• Shigella: absence in 25 gr
• Industry: not relevant, test not to be required in EU

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OECD draft proposal on limits of indicator microbial contaminants

- Microbial activity indicator check

- Aerobic plate count: < 10^e5 cfu/g (10^e8 for BVs)
  - Industry: 0.1% of a.i. level, with a maximum of 10^e7
- Anaerobic spore formers: < 10^e5 cfu/g
  - Industry: not relevant; cannot grow → not to be tested
- Yeasts and moulds: < 1000 cfu/g
  - Industry: not relevant; compare background levels, and no discriminating tests available

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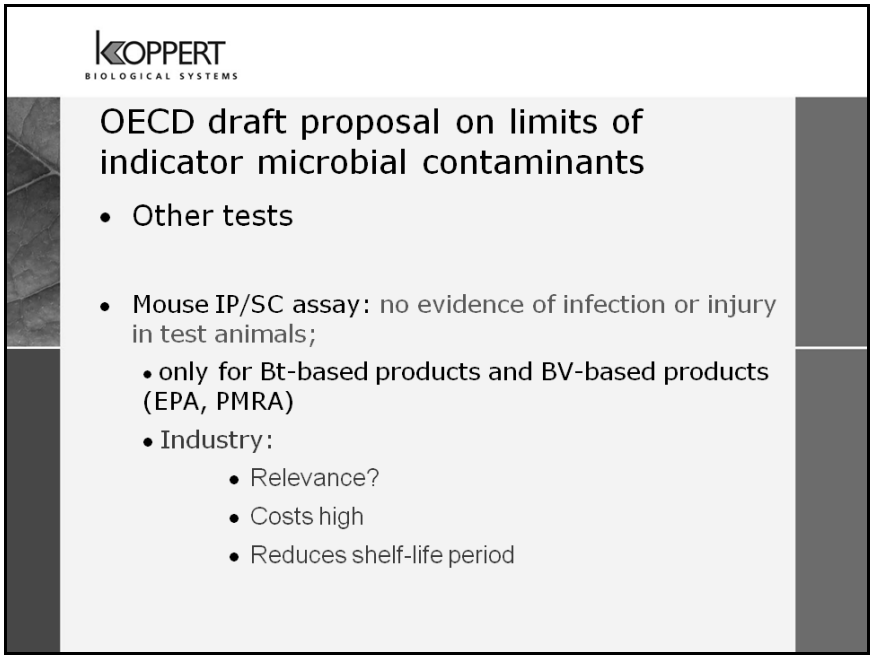
OECD draft proposal on limits of indicator microbial contaminants

- Human, feacal and environmental contamination indicator check

- E. coli or thermophilic coliforms: absence in 1 g or < 10 cfu/g
  - Industry: total coliforms:< 1000 cfu/g
- Staphylococci: absence in 1 g
  - Industry: acceptable
- Pseudomonas aeruginosa: monitoring
  - Industry: not relevant; not a pathogen; compare background levels, and no discriminating tests available in case of Pseudomonas-based products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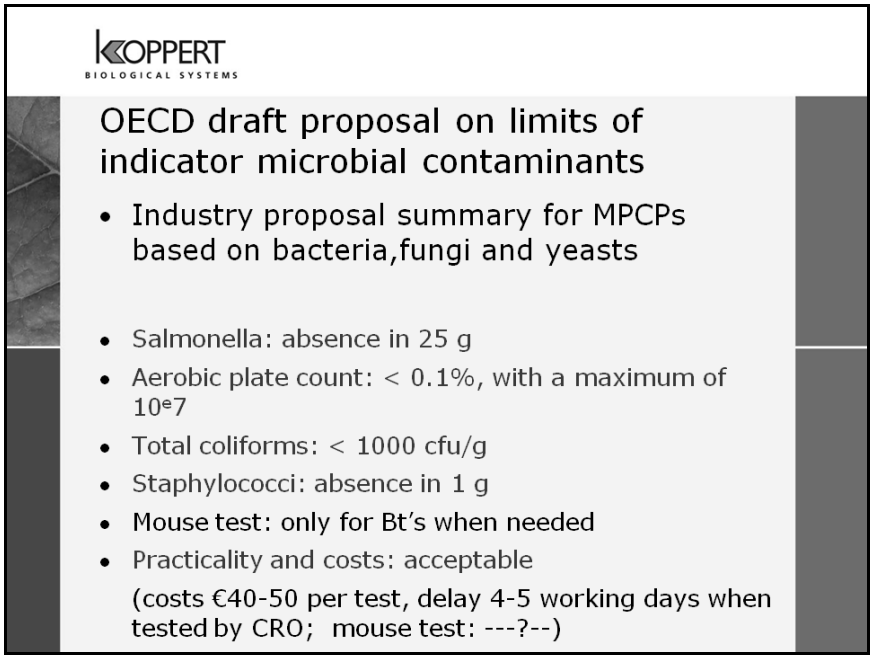
OECD draft proposal on limits of indicator microbial contaminants
• Other tests
• Mouse IP/SC assay: no evidence of infection or injury in test animals;
• only for Bt-based products and BV-based products (EPA, PMRA)
• Industry:
• Relevance?
• Costs high
• Reduces shelf-life period


KOPPERT
BIOLOGICAL SYSTEMS
OECD draft proposal on limits of indicator microbial contaminants
• Industry proposal summary for MPCPs based on bacteria,fungi and yeasts
• Salmonella: absence in 25 g
• Aerobic plate count: < 0.1%, with a maximum of 10e7
• Total coliforms: < 1000 cfu/g
• Staphylococci: absence in 1 g
• Mouse test: only for Bt's when needed
• Practicality and costs: acceptable
(costs €40-50 per test, delay 4-5 working days when tested by CRO; mouse test: ---?--)

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Summary of microbial contaminant limits

Indicator	OECD Draft Paper March 2009	Industry proposal July 2009
Salmonella	Absence in 25 g or 25 mL	Absence in 25 g
Listeria monocytogenes	Absence in 25 g or 25 mL	not relevant; omit
Vibrio	Absence in 25 g or 25 mL	not relevant in EU; omit
Shigella	Absence in 25 g or 25 mL	not relevant in EU; omit
Aerobic Plate Count	$< 1 \times 10^5$ CFU/g or mL	< 0.1% if a.i. level, with a maximum of 10E7 CFU/g
Anaerobic spore-formers	$<10^5$ CFU/g	not relevant, cannot develop; omit
Yeast and Mould Count	< 1000 CFU/g or mL	not relevant; omit
Escherichia coli or Thermophilic (fecal) coliforms	Absence in 1 g or mL<10 CFU/g or mL	Coliforms: < 1000 CFU/g
Staphylococci	Absence in 1 g or mL	Absence in 1 g or mL
Pseudomonas aeruginosa	Monitoring*	Not relevant
Mouse IP/SC assay	No evidence of infection or injury in test animals	only in some cases when need has been proven

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OECD draft proposal: discussion items

- Standardization needed (EU, USA, Canada)
- Differentiation needed for OECD countries (tropical regions)
- Criteria for BVs different than for other MBCPs??
- Are chemical and other non-microbial pesticides free of microbial contaminants? Why are they not tested?
- Do m.c.'s pose a risk compared to other agricultural inputs?
- And compared to natural background levels?
- Do m.c.'s pose a risk to applicators with protective clothing, gloves and eye/face protection?


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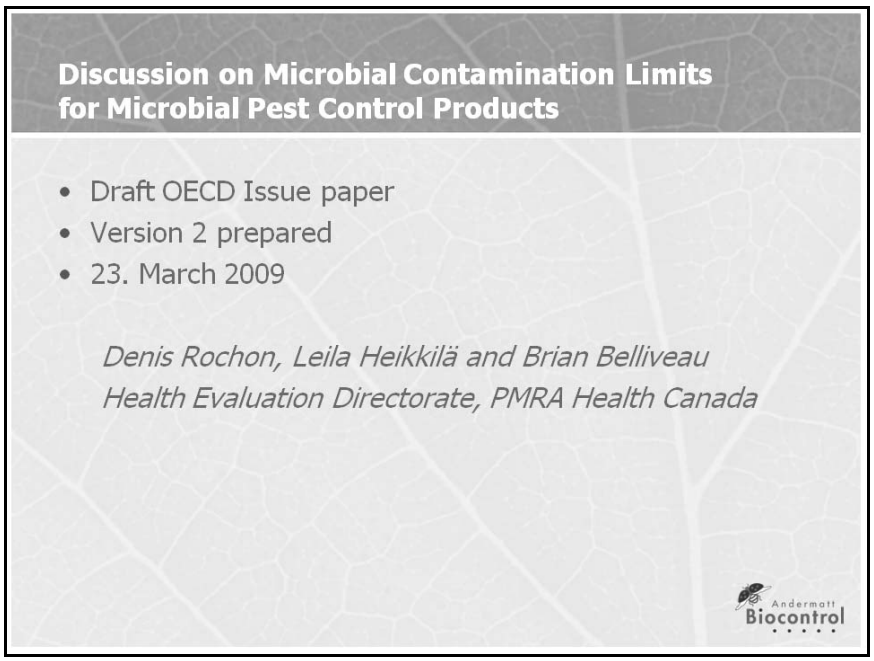
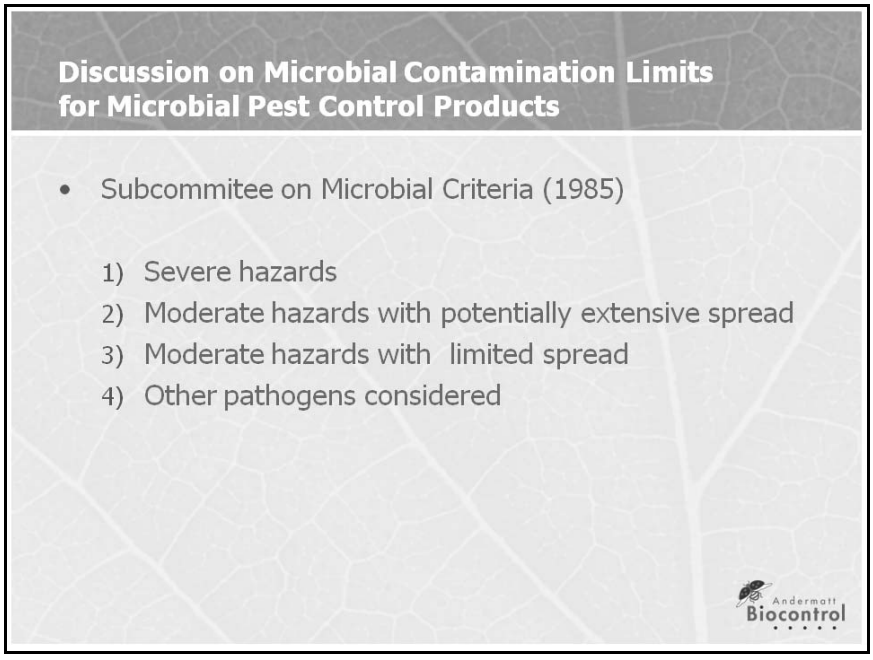
Contamination with regard to baculovirus products

By Philip Kessler (Andermatt Biocontrol; Grossdietwil; Switzerland)




Discussion on Microbial Contamination Limits for Microbial Pest Control Products
• Regulatory authorities have recognized the risks by contaminating micro-organisms
• Canada
• PMRA
• Regulatory Directive DIR2001-02
• Unites States
• U.S.EPA
• 40 CFR 158.2120
• European Union
• 91/414/EEC
• 1998/8/EC
Andermatt Biocontrol


Discussion on Microbial Contamination Limits for Microbial Pest Control Products
• Subcommitee on Microbial Criteria (1985)
1) Severe hazards
2) Moderate hazards with potentially extensive spread
3) Moderate hazards with limited spread
4) Other pathogens considered
Andermatt Biocontrol


Discussion on Microbial Contamination Limits for Microbial Pest Control Products
1) Severe hazards
• Brucella spp
• Clostridium botulinum
• Salmonella typhii, paratyphii A, B, C, sendai chlerae-suis
• Shigella dysenteriae
• Vibrio cholerae
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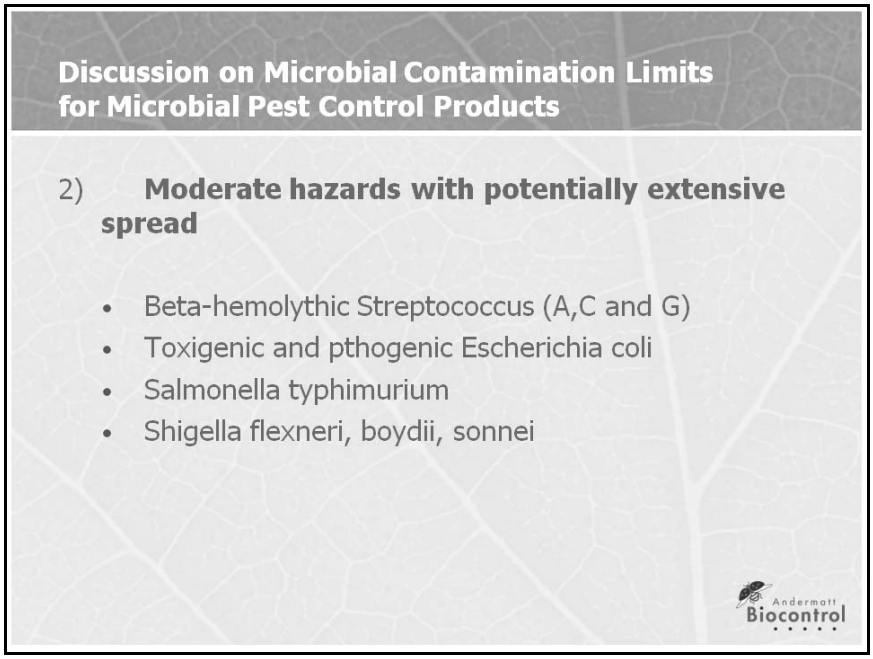
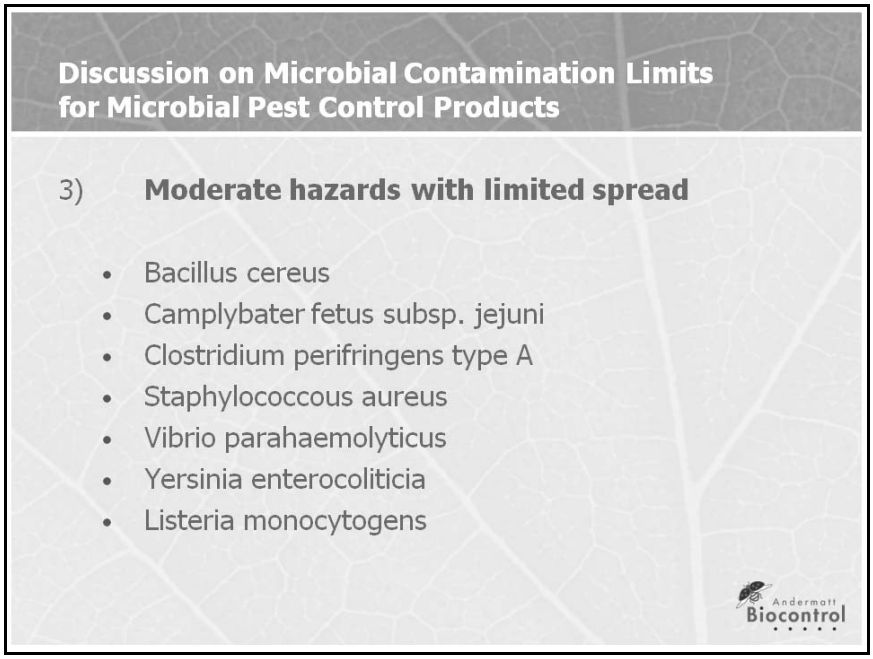

Discussion on Microbial Contamination Limits for Microbial Pest Control Products
2) Moderate hazards with potentially extensive spread
• Beta-hemolythic Streptococcus (A,C and G)
• Toxigenic and pthogenic Escherichia coli
• Salmonella typhimurium
• Shigella flexneri, boydii, sonnei
Andermatt Biocontrol


Discussion on Microbial Contamination Limits for Microbial Pest Control Products
3) Moderate hazards with limited spread
• Bacillus cereus
• Camplybater fetus subsp. jejuni
• Clostridium perifringens type A
• Staphylococcous aureus
• Vibrio parahaemolyticus
• Yersinia enterocoliticia
• Listeria monocytogens
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Discussion on Microbial Contamination Limits for Microbial Pest Control Products
4) Other pathogens considered in drinking water
• Aeromonas
• Legionella
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Discussion on Microbial Contamination Limits for Microbial Pest Control Products

- Indicator Micro-organisms
- Many microbiological specifications in water and food make use of indicator organisms instead of screening for the pathogens themselves
  1) Indicators that assess microbial numbers or activity
  2) Indicators of potential human pathogens or faecal contamination
  3) Indicators of post-heat processing contamination
  4) Metabolic products of pathogens that indicate the presence of a pathogen

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Discussion on Microbial Contamination Limits for Microbial Pest Control Products

1) **Indicators that assess microbial numbers or activity**
   - Aerobic plate count
   - Anaerobic plate count
   - Thermoduric, psychrotrophioc, thermophilic proteoloytic and lipolytic counts
   - Direct microscopic counts
   - Microscopic mould counts
   - Yeast and mould counts
   - Heat-resistant moulds
   - Thermopylic spore counts

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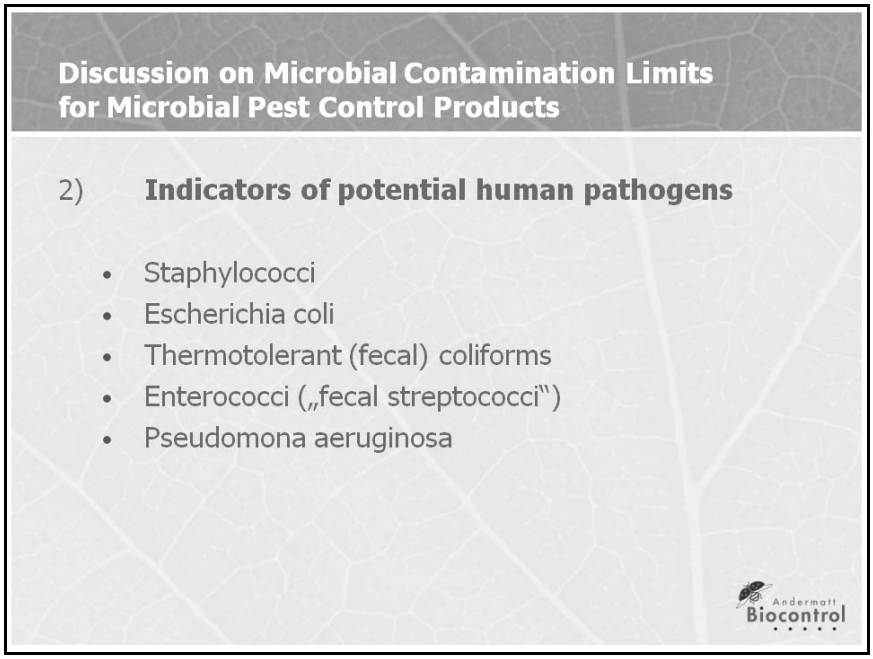
Discussion on Microbial Contamination Limits for Microbial Pest Control Products
2) Indicators of potential human pathogens
• Staphylococci
• Escherichia coli
• Thermotolerant (fecal) coliforms
• Enterococci („fecal streptococci")
• Pseudomona aeruginosa
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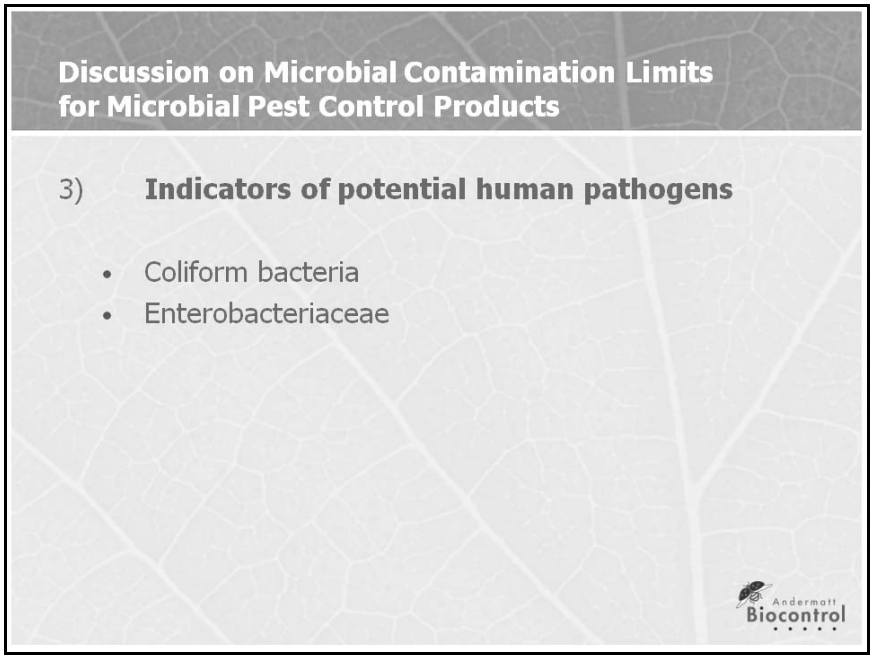
Discussion on Microbial Contamination Limits for Microbial Pest Control Products
3) Indicators of potential human pathogens
• Coliform bacteria
• Enterobacteriaceae
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Discussion on Microbial Contamination Limits for Microbial Pest Control Products

- OECD draft:
  - Chosen limits on microbial specifications and screening requirements must satisfy the data requirements of all OECD member countries
  - The list of contaminants has to be practical and feasible for registrants to monitor in their products
  - Appropriate standard screening methods have to be available for each of the selected indicators and/or pathogens
  - The chosen indicators and/or pathogens and corresponding screening methods must provide meaningful data to assess the overall acceptability and risk of microbial pest control products in order to avoid unnecessary testing and placing an unreasonable burden on applicants/notifiers
  - In the vent that the chosen screening requirements may put laboratory technicians at risk of infection, or outbreak, authorities must be willing to dictate applicants/notifiers without accredited laboratories employ the service of contract laboratories to satisfy requirements

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Discussion on Microbial Contamination Limits for Microbial Pest Control Products

- Proposed microbial specifications
  - in microbial pest control products (excl. baculoviruses)
  - Baculovirus products (manufactured in vivo)

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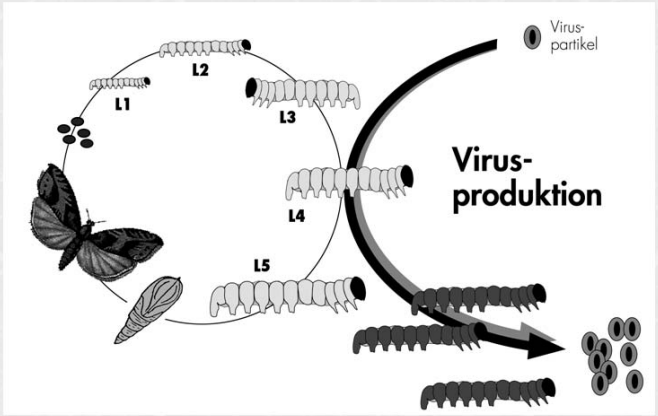
Discussion on Microbial Contamination Limits for Microbial Pest Control Products

- Baculovirus products (manufactured in vivo)



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Proposed microbial specifications for baculovirus products

Microbial contaminant	Limits (recommended in OECD 1st draft, 2006 (recommendations PMRA)	REBECCA (2007)	Limits (recommended in OECD 2nd draft, 2009)
Total Aerobic Bacteria (Mesophilic)*	< 1 x 10^7 CFU/g or mL	< 1 x 10^8 CFU/g or mL	< 1 x 10^8 CFU/g or mL
Bacillus cereus	< 1 x 10^6 CFU/g or mL	< 1 x 10^7 CFU/g or mL	< 1 x 10^7 CFU/g or mL
Other Aerobic Sporeformes	< 1 x 10^7 CFU/g or mL	Not recommended	Not recommended
Fecal Streptococci – Enterococci	Absence in 1 g or mL	Not recommended	Not recommended
Total coliforms Fecal coliforms/ Escherichia coli*	< 100 CFU/g or mL or Absence in 1 g or mL	Absence in 1 g or mL	< 100 CFU/g or mL or Absence in 1 g or mL
Staphlococcus aureus*	Absence in 1 g or mL	Absence in 1 g or mL	Absence in 1 g or mL
Salmonella*	Absence in 25 g or mL	Absence in 25 g or mL	Absence in 25 g or mL
Shigella	Absence in 25 g or mL	Not recommended	Absence in 25 g or mL
Vibrio	Absence in 25 g or mL	Not recommended	Absence in 25 g or mL
Yeast and Mould	< 1000 CFU/ g or mL	Visually monitored	Visually monitored
Mouse IP/SC tests (case-by-case)	No evidence of infection or injury in test animals	Not recommended	No evidence of infection or injury in test animals

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Proposed microbial specifications for baculovirus products

- *Bacillus cereus*
  - *CpGV DAR : Specification for CpGV a.i. < 10^6 CFU/g*
    - *Not acceptable for industry*
  - De-contamination methods not applicable so far for baculovirus products
  - Content of 10^7 CFU/g does not pose higher risk
    - *Bacillus cereus* is ubiquitous in the environment
    - Limits for food categories 10^3 to 10^5 CFU/g
    - Intoxication in food starting from 10^5 CFU/g
    - Baculovirus products gets highly diluted and applied
    - Realistic contamination on the fruit 1 to 10 CFU per g if initial contamination of the product is 10^7 CFU/g
    - No problem during 20 years of baculovirus production and application

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Proposed microbial specifications for baculovirus products

- *Bacillus cereus*
- *REBECCA 2007*
- Application of 2.7 Liter/ha per season (GAP Granupom)
- Contamination of the CpGV of the CpGV product: $1x10^7$ CFU/g or $1x10^{10}$ CFU/L
- Average yield of apples per season: 28t
  - 2.7×10^{10} CFU / 28t = 9.64×10^2 CFU/g apple
- Other factors to be considered
  - Deactivation by UV (factor 10)
  - Major part applied on leaves and soil (factor 5)
  - Growth of apple surface during the season (factor 2)
- **Realistic contamination by B. cereus 1-10 CFU/g apple** after intensive spraying of CpGV product containing B. cereus contamination of $1x10^7$ CFU/g

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Proposed microbial specifications for baculovirus products

- *Bacillus cereus*
- *Andermatt Biocontrol 2009*
- Application of 1.2 Liter/ha per season (GAP MADEX)
- Contamination of the CpGV of the CpGV product: $1x10^7$ CFU/g or $1x10^{10}$ CFU/L
- Application on 1 ha: => $1.2 x10^0$ CFU / mm^2
- Contamination per apple (20'000 mm^2) 2.4×10^4 cfu/apple
- Contamination per g apple (150g/apple) 1.6×10^2 cfu/g apple
- Other factors to be considered
  - Deactivation by UV (factor 10)
  - Leaf index (factor 5)
  - Growth of apple surface during the season (factor 2)
- **Realistic contamination by B. cereus 1-10 CFU/g apple** after intensive spraying of CpGV product containing B.cereus contamination of $1x10^7$ CFU/g

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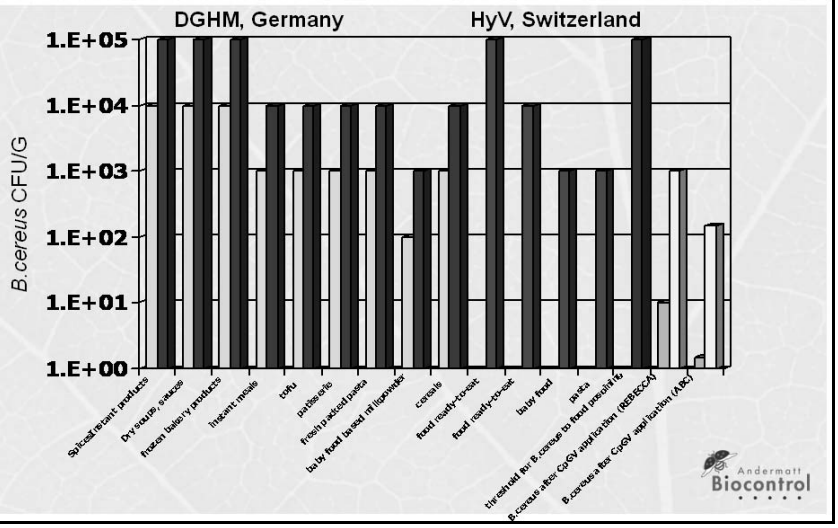
Proposed microbial specifications for baculovirus products



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Proposed microbial specifications for baculovirus products

- *Shigella*
  - The method for its detection is not sensitive and quantification is rarely performed
- *Vibrio*
  - Routine testing for *Vibrio cholerae* is not practical as detection methods are too insensitive and time consuming
  - Makes not sense if product is manufactured in region free from the pathogens

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Proposed microbial specifications for baculovirus products

- *Yeast and moulds*
  - During the production process visual screening for mould and yeast contamination is routinely performed.
  - Contaminated units are not used for further formulation

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Proposed microbial specifications for baculovirus products

- *Mouse IP/SC testing*
  - Only required by PMRA, and not mandatory by EPA
  - The EU does not specify the requirements for animal testing
  - It is commonly agreed that animal testing should be reduced at a minimum
  - The probability to get new information by Mouse IP/SC test is low.
  - Mouse IP/SC testing are expensive and can not be accepted as a routine testing methods to asses contamination of baculovirus production batches

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Discussion on Microbial Contamination Limits for Microbial Pest Control Products

- OECD draft:
- data required for microbial contamination assessments
  - Detailed description of manufacturing methods
  - Products for which sufficient information is available, an analysis of five bathes for microbial contaminants should be adequate.
  - Authorities may, however, request additional action be taken

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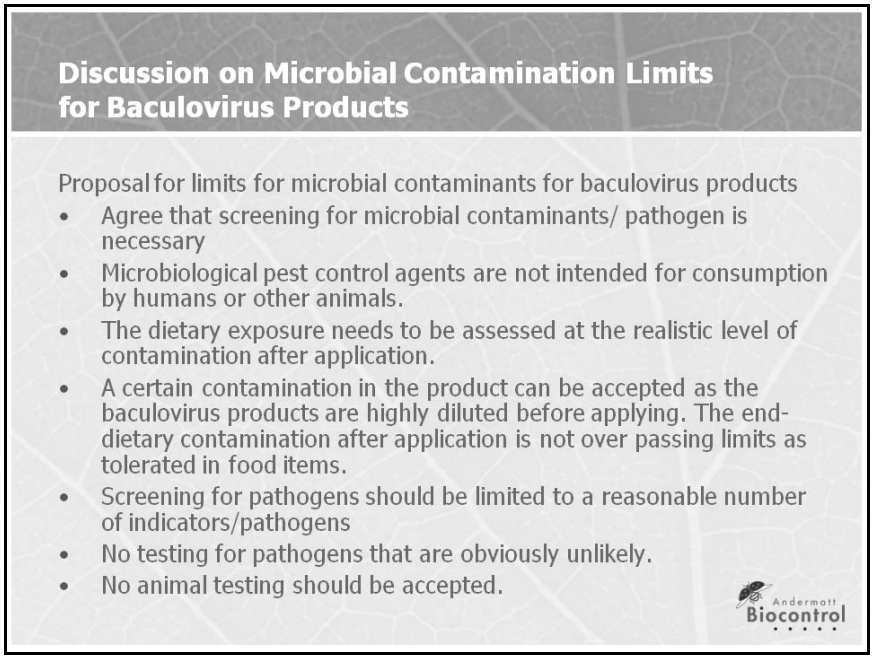
Discussion on Microbial Contamination Limits for Baculovirus Products
Proposal for limits for microbial contaminants for baculovirus products
• Agree that screening for microbial contaminants/ pathogen is necessary
• Microbiological pest control agents are not intended for consumption by humans or other animals.
• The dietary exposure needs to be assessed at the realistic level of contamination after application.
• A certain contamination in the product can be accepted as the baculovirus products are highly diluted before applying. The end-dietary contamination after application is not over passing limits as tolerated in food items.
• Screening for pathogens should be limited to a reasonable number of indicators/pathogens
• No testing for pathogens that are obviously unlikely.
• No animal testing should be accepted.
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Genotypic/Phenotypic characterization of biocontrol and clinical strains of *Pantoea agglomerans*

By Brion Duffy (Agroscope, Wädenswil; Switzerland)

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Federal Department of Economic Affairs DEA
Agroscope Changins-Wädenswil Research Station ACW

Pantoea agglomerans Biocontrol Agent Biosafety

Strain-level identification and genotypic comparison of biocontrol and clinical Isolates

Brion Duffy
Fabio Rezzonico, Theo H.M. Smits, Esther Badosa,
Mauro Tonolla, Guido Vogel, Emilio Montesinos, Jürg E. Frey

cost
European Cooperation in the Field of Scientific and Technical Research

COST 873
StoneFruitNutHealth

Pantoea agglomerans: A biocontrol agent with an identity crisis.

Commercial *P. agglomerans* products are already registered and sold in USA, Canada & New Zealand.

BlightBan C9-1™ (NuFarms, USA/CAN)

BlossomBless ™ (NZ)

Bloomtime Biological™ (Northwest Agricultural Products, USA/CAN)

Fire Blight biocontrol – *E. amylovora* (BL-2)

EU market disadvantage: European pome fruit growers face economic extinction due to fire blight; EU distributors/producers of biocontrol blocked from a promising solution.

2

Genotyping of *Pantoea agglomerans* isolates — Fabio Rezzonico – IOBC Interlaken 09.09.2008

Jekyll-Hyde Problem with *Pantoea agglomerans*

Many beneficial species used in biocontrol include isolates with clinical history – but are they really pathogens, really dangerous?

- Synonyms: *Erwinia herbicola*, *Enterobacter agglomerans*
- **Ubiquitous plant epiphyte -** Enterobacteriaceae

Reported as clinical isolate, thus automatic BL-2 status – usually polymicrobial infections though!

Why take this species seriously?

- Diversity and source of clinical infections
  - **Thorn wounds – *link to plant isolates ?***
  - **Blood, septicemia**
  - **Knee, arm, scalp, buttock, neck, heel, foot wounds**
  - **Urine, catheter, chest, peritonitis – *nosocomial origins***

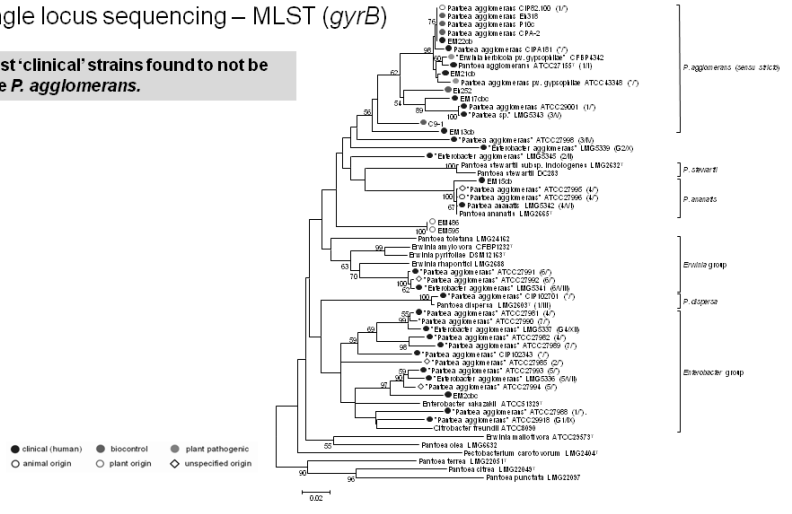
3

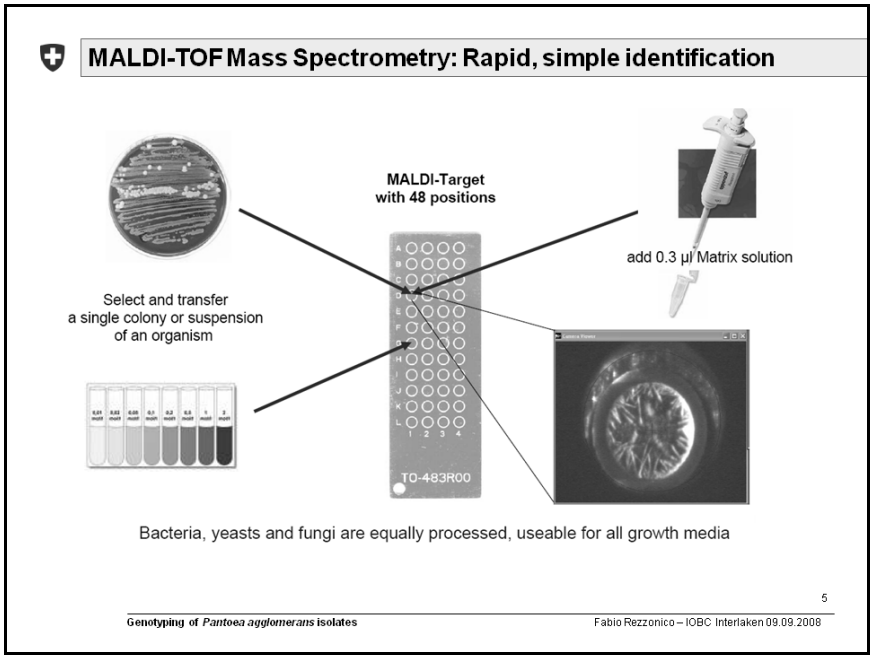
Genotyping of *Pantoea agglomerans* isolates Fabio Rezzonico – IOBC Interlaken 09.09.2008

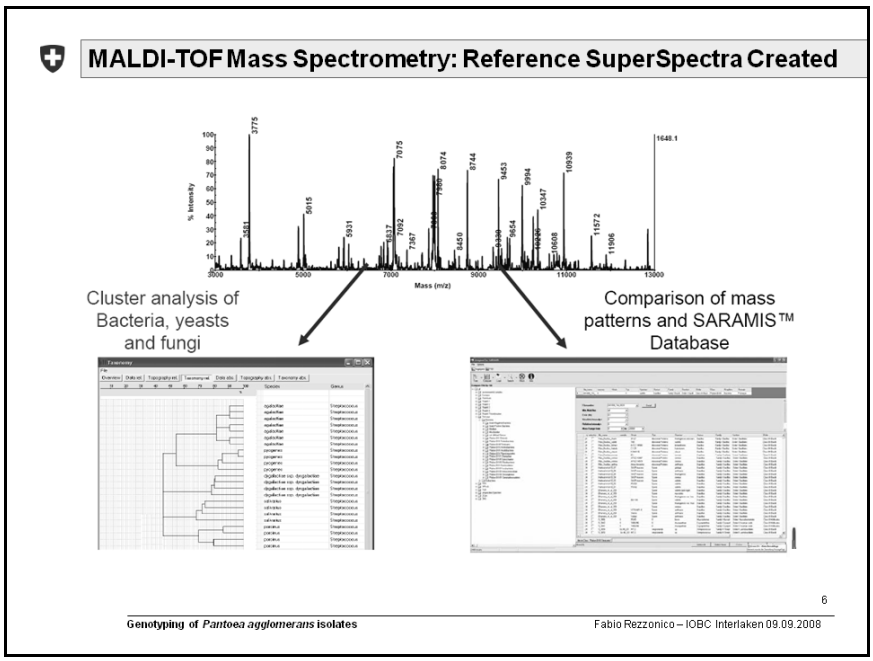
Basic research aim: Resolve biosafety issues critical to facilitate cautious but confident registration process in Europe

Single locus sequencing – MLST (*gyrB*)

Most 'clinical' strains found to not be true *P. agglomerans.*

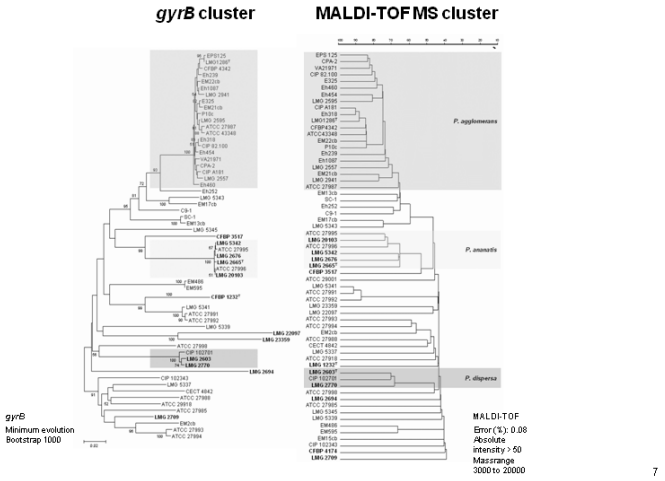



MALDI-TOF Mass Spectrometry: Rapid, simple identification
MALDI-Target
with 48 positions
add 0.3 µl Matrix solution
Select and transfer
a single colony or suspension
of an organism
TO-483R00
Bacteria, yeasts and fungi are equally processed, useable for all growth media
5
Genotyping of Pantoea agglomerans isolates
Fabio Rezzonico – IOBC Interlaken 09.09.2008


MALDI-TOF Mass Spectrometry: Reference SuperSpectra Created
% Intensity
Mass (m/z)
Cluster analysis of
Bacteria, yeasts
and fungi
Comparison of mass
patterns and SARAMIS™
Database
6
Genotyping of Pantoea agglomerans isolates
Fabio Rezzonico – IOBC Interlaken 09.09.2008

MALDI-TOF MS: Reliable identification in just a few minutes

Comparison of dendrograms derived from *gyrB* sequencing and MALDI-TOF MS spectra



Clinical diagnostics improved: reduced incidence of *P. agglomerans*

Data from ICM Bellinzona

Year	Wounds	Blood	Sputum	Urine	Biopsy	Eye	Catheter	**Total**
2005	1	2	0	1	0	1	1	**6**
2006	2	0	1	0	1	0	0	**4**
2007	4	2	2	1	0	0	0	**9**
2008	2	0	0	0	0	0	0	**2**

Biochemical identification

MALDI-TOF

- **Twenty-one cases of *P. agglomerans* reported in the last 4 years**

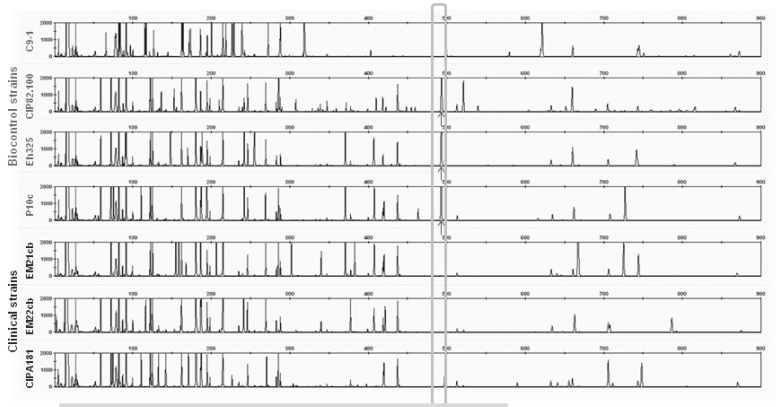
→ Almost the half in wounds (potentially contaminated by environmental germs)

→ No data about number of pure cultures

→ Integration of MALDI-TOF as additional method for identification has led to drop in the number of *P. agglomerans* cases

Biosafety markers in *P. agglomerans* (*sensu stricto*)

Random genomic markers search: AFLP (Amplified Fragment Length Polymorphisms)
→ Total DNA is digested with restriction enzymes and fragments are selectively amplified



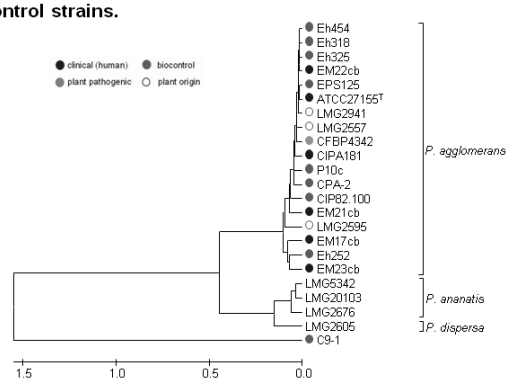
Biomarker 'unique' to biocontrol strains found.

9

Biosafety markers in *P. agglomerans* (*sensu stricto*)

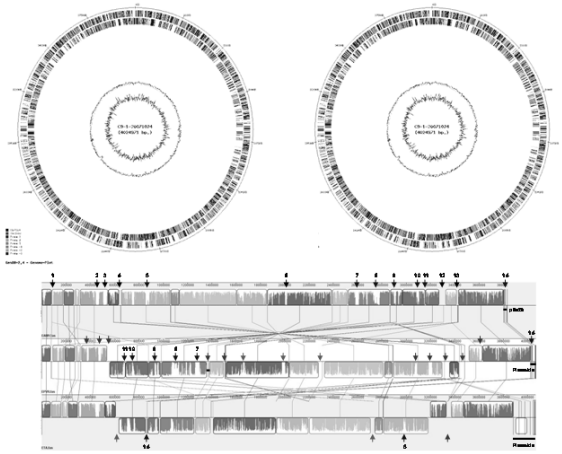
UPGMA analysis of AFLP-data gives phylogeny based on binary matrix (+/-peak)

**Congruent with MLST / MALDI-TOF MS data –
problem is that *some* true *P. agglomerans*
clinical strains cluster with biocontrol strains.**



10

Complete genome sequencing: C9-1 finished, E325 and clinical strain being polished: aim to ID subtle discriminatory biomarkers.



11

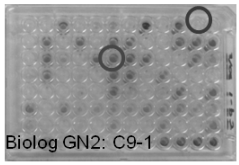
Genotyping of *Pantoea agglomerans* isolates — Fabio Rezzonico – IOBC Interlaken 09.09.2008

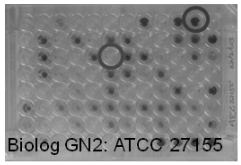
Comparative genomics: Typical virulence genes found to be absent in biocontrol strains.

Type 3 secretion system genes found in some *P. agglomerans* strains – BUT these are like those in biocontrol *Pseudomonas* – and not like in pathogenic bacteria.

Strain	*hrcN*	Strain	*hrcN*
■ ATCC 27987	-	○ LMG 2557	+
● C9-1	-	○ LMG 2595	-
○ CIP 82.100	+	○ LMG 2941	-
■ CIP A181	-	● P10c	-
● CPA-2	+	● P1SA	-
● E325	-	○ P2SA	-
● Eh1087	-	○ P3SA	+
● Eh239	+	○ P4SA	-
● Eh252	-	○ P5WA	-
● Eh318	-	○ P6WA	-
● Eh454	-	○ P7NSW	+
● Eh460	-	○ P9Qld	-
■ EM21cb	-	● USJ-C91	-
■ EM22cb	-	■ VA21971	+
● EPS125	-	□ ATCC 43348	+
■ LMG 1286^T	-	□ CFBP 4342	+

Differential metabolics of biocontrol vs. clinical strains



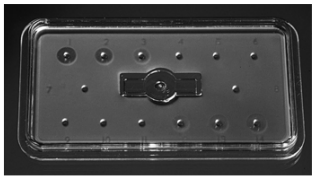


12

Genotyping of *Pantoea agglomerans* isolates — Fabio Rezzonico – IOBC Interlaken 09.09.2008

Phenotypic comparison: Genes may be differentially expressed in plant or animal habitats.

Hemolysis assay for animal virulence: ALL *P. agglomerans* strains negative.

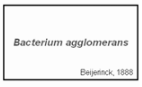


Strain	Broth	Agar	Supernatant
Control	-	-	-
CFBP 4342	-	-	-
CPA-2	-	-	-
EM 13 cb	-	-	-
C9-1	-	-	-
Eh 1087	-	-	-
Eh 318	-	-	-
EPS-125	-	-	-
P	-	-	-
CIPA 1811	-	-	-
VA 21971	-	-	-
EM 17 cb	-	-	-
ATCC27155	-	-	-
Eh 252	-	-	-
EM 22 cb	-	-	-
LM 45393	-	-	-
Melittin (control)	+	+	+

13

Taxonomic history of *Enterobacter* and *Erwinia* spp.

Countless taxonomical rearrangements in the genus *Enterobacter* probably resulted in the misassignment of many isolates to *P. agglomerans*



14

Koch's postulates never fulfilled for clinical isolates

Four criteria designed to establish a causal relationship between a causative microbe and a disease

- ? The microorganism must be found in abundance in all organisms suffering from the disease.
- ☑ The microorganism must be isolated from a diseased organism and grown in pure culture.
- ☒ The cultured microorganism should cause disease when introduced into a healthy organism.
- ☒ Finally, it must be reisolated from the inoculated, diseased host and identified as the original agent.

Clinical isolates rarely saved for confirming identity.

P. agglomerans biocontrol strains are ~safe~

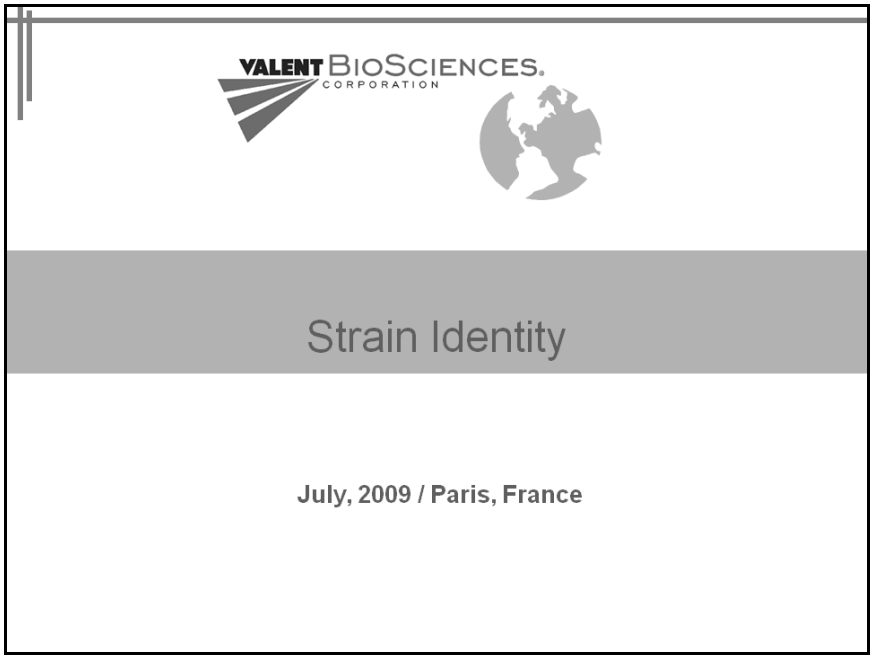
- Most clinical strains misclassified.
- Unique biocontrol biomarker found with AFLP.
- Lack of virulence genes in genome of biocontrol strains.
- Lack of virulence phenotype.

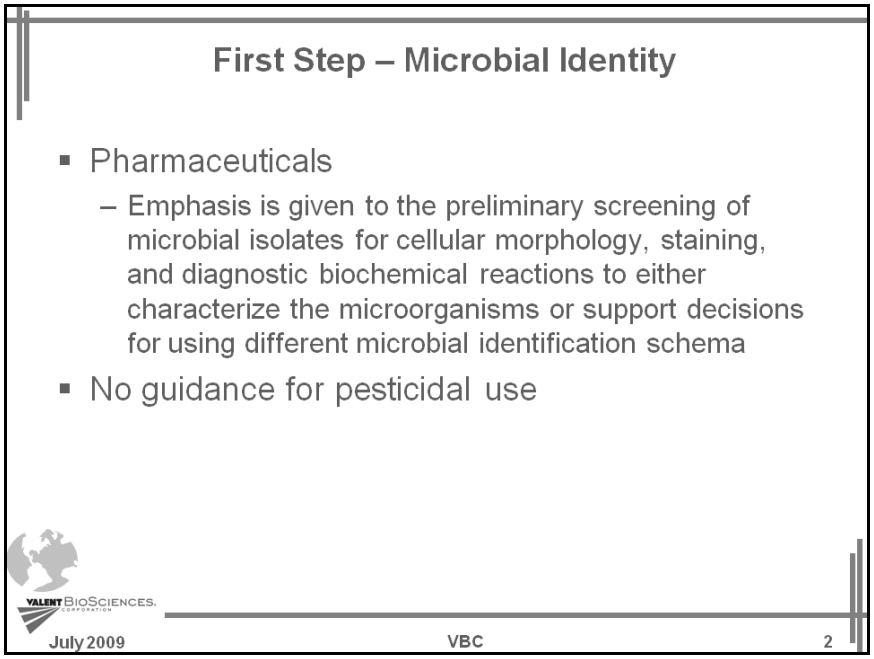
- Fundamental problem with clinical diagnostics; confirmation of role in clinical condition.

- Identification at species level using MLST, AFLP, MALDI-TOF MS.

Characterisation issues on bacteria

By Denise Munday and Maria Herrero (Valent Biosciences; Switzerland)





Categories / Characteristics

- Cultural
  - Colony morphology colony color and size, and pigment production
- Morphological
  - Cellular morphology, cell size, flagella type, reserve material, gram reaction and spore, and acid-fast staining.
- Physiological
  - Oxygen tolerance, pH range, temperature optimum and range, and salinity tolerance
- Biochemical
  - Carbon utilization, carbohydrate oxidation or fermentation, and enzyme patterns
- Inhibition
  - Bile salt-tolerance, antibiotic susceptibility, and dye tolerance
- Serological
  - Agglutination
- Chemo-Taxonomic
  - Fatty acid profile, microbial toxins and whole cell composition

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Bts belonging to VBC

- All categories listed previously were carried out

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Point of Departure

- Members of the Bacillus cereus group of organisms include Bacillus cereus, Bacillus anthracis and Bacillus thuringiensis. Collectively, these organisms represent microbes of high economic, medical and biodefense importance. Given this significance, this group contains the highest number of closely related fully sequenced genomes.

GROUND RULES

- Much of the disease and host specificity of members of this group can be attributed to their plasmids, which vary in size and number.
- Chromosomes exhibit a high level of synteny and protein similarity, with limited differences in gene content, questioning the speciation of the group members.

Rasko et al. 2005

Step I

- **Separation of the groups**
  - For Bt a large part of the separation was already accomplished through the traditional microbiological identification methods
  - H-serotyping has been used for the identification and classification of Bacillus thuringiensis strains. Côté et al. have recently shown a direct correlation between the flagellin (Hag protein) amino acid sequences and H-serotypes. H-serotyping, however, cannot distinguish closely related strains.

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Genotypic/phylogenic characteristics that may be employed in microbial taxonomy

- Genotypic
  - DNA base ratio (G + C content), restriction fragment patterns, and DNA probes
- Phylogenic
  - DNA-DNA hybridization, and 16s and 23s rRNA codon sequences
- Full sequence
  - 21 B. cereus group strains have been completely sequenced. These 21 isolates cover all three clusters, although the majority of them are B. anthracis strains or clinical strains closely related to B. anthracis due to the focus of genome sequencing projects. Tourasse and Kolstø (2008, Norway)

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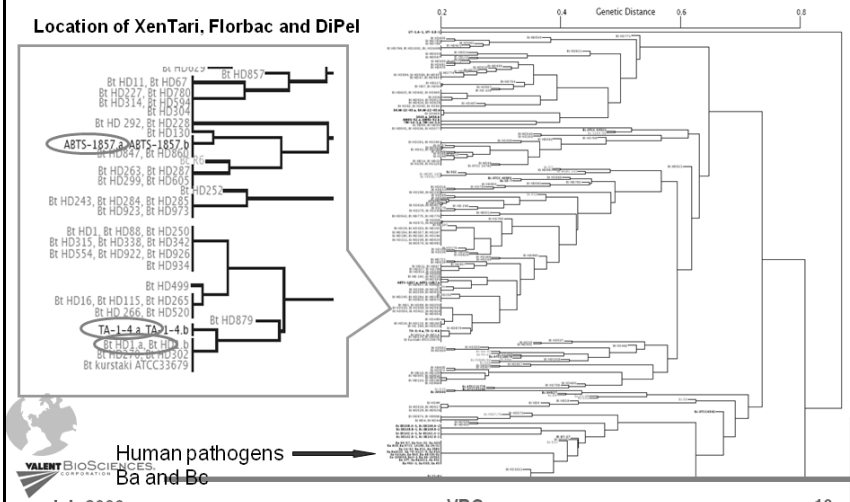
Step II

- Genetic Separation – comparative process
- Carried out AFLP profiles in comparison with 300 other isolates
  - Isolates shown in red are *B. anthracis*, *B. cereus* or *B. thuringiensis* isolates known to be pathogenic in humans or other mammals.
  - Isolates shown in green are *B. thuringiensis* isolates not know to have any pathogenic or toxigenic properties in vertebrates.
    - » **Hill, *et al.* 2004.** Fluorescent Amplified Fragment Length Polymorphism ...Appl. & Envir. Microbiol. 70(2): 1068-1080

How similar? DNA analysis.

Location of XenTari, Florbac and DiPel



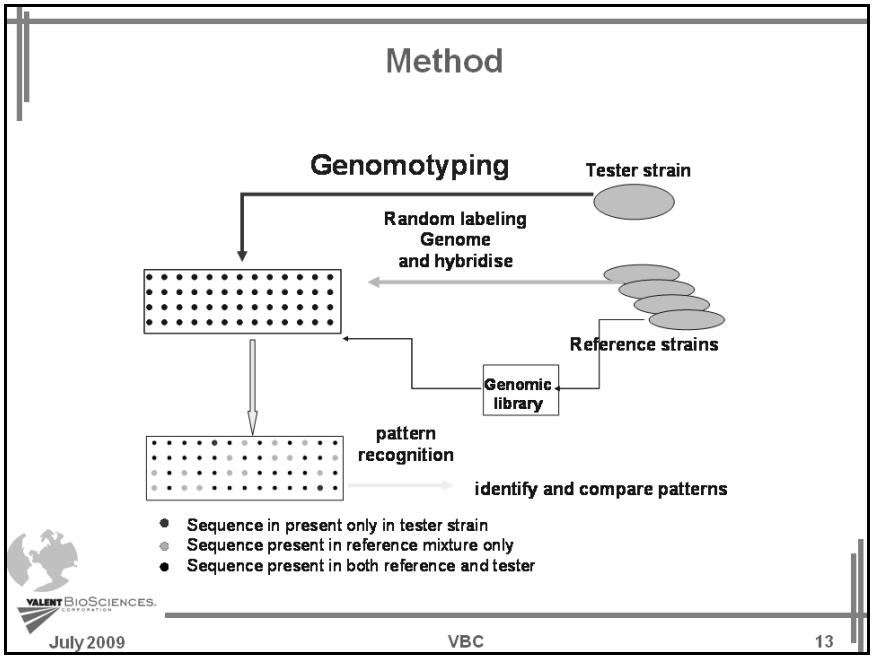
Human pathogens → Ba and Bc

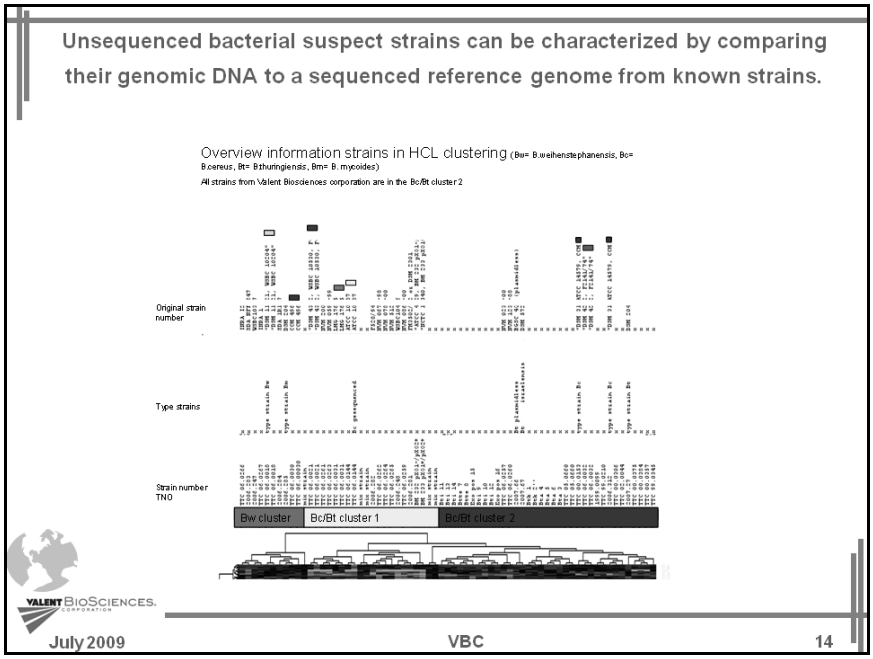
Annex I Inclusion

- Indication that Unequivocal Identification had not been accomplished to strain level. Monitoring methodology mandated.
- Contacted several Bt experts as to possible methodologies to try
  - A. Bishop (U. of Greenwich) tried MLST (multilocus sequence typing analysis). This had been used by Tourasse and Kolstø (2007, Norway) to separate out B.c., B.a., and B.t.
    - This methodology was not able to separate out separate Btk strains.
    - Within subspecies level, the genetic sequence are over 85% homologous. Need to identify which portion of the sequence is consistently variable to use MLST

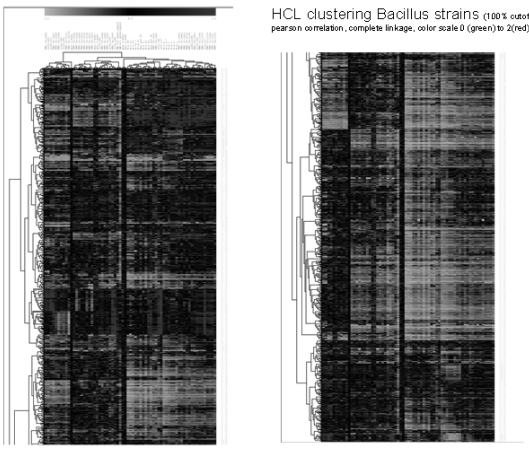
Genomotyping

- Describes the analysis of bacteria by comparison of their genomes using microarrays. DNA microarray technology has become an important research tool for microbiology and biotechnology as it allows for comprehensive DNA and RNA analyses to characterize genetic diversity and gene expression in a genome-wide manner.
  - This is important because of the clonal nature of Bt subspecies


Method
Genomotyping
Tester strain
Random labeling
Genome
and hybridise
Reference strains
Genomic
library
pattern
recognition
identify and compare patterns
Sequence in present only in tester strain
Sequence present in reference mixture only
Sequence present in both reference and tester
VALENT BIOSCIENCES CORPORATION
July 2009
VBC
13


Unsequenced bacterial suspect strains can be characterized by comparing their genomic DNA to a sequenced reference genome from known strains.
Overview information strains in HCL clustering (Bw= B.weihenstephanensis, Bc= B.cereus, Bt= B.thuringiensis, Bm= B. mycoides)
All strains from Valent Biosciences corporation are in the Bc/Bt cluster 2
Original strain number
Type strains
Strain number TNO
Bw cluster
Bc/Bt cluster 1
Bc/Bt cluster 2
VALENT BIOSCIENCES CORPORATION
July 2009
VBC
14

Gene complement differences can be observed even between strains of the same subspecies.



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Accomplishment.

- Identification to strain level has been accomplished.
  - limitation of the number of markers will affect strain specificity
- Statistical analysis can be used to show the amount of genetic separation.
- Unsequenced bacterial suspect strains can be characterized by comparing their genomic DNA to a sequenced reference genome from known strains.
- By definition of a set of specific markers (probes), a commercial hybridization platform can be arranged to have an analysis system operational for a field lab.

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Registrant's Perspective

- Agree that microbial identification must be done correctly as this provides information to regulators on points of concern that need to be addressed.
- Level to which this identification must occur, because it must be comparative, adds huge technical challenges and costs for questionable benefit and utilization.

Proposal

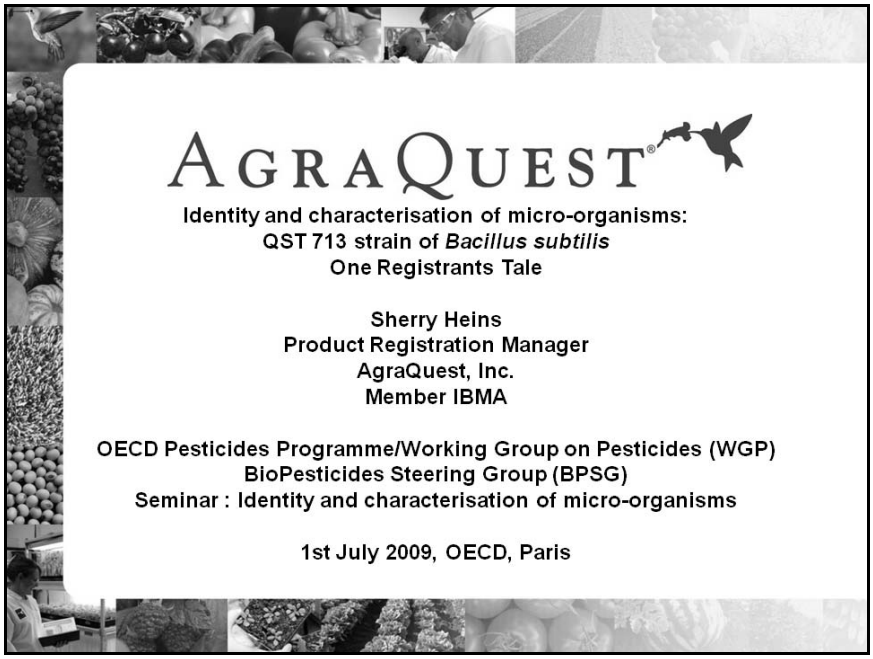
- Identification level takes on a Tiered Process, much as toxicology, etc.
- The level of concern raised by the micro-organism establishes level of methodology which must be established.
  - When is monitoring needed?
  - Are residue levels needed?
- Importance is on the *safety* of the strain and not its genetic characterization

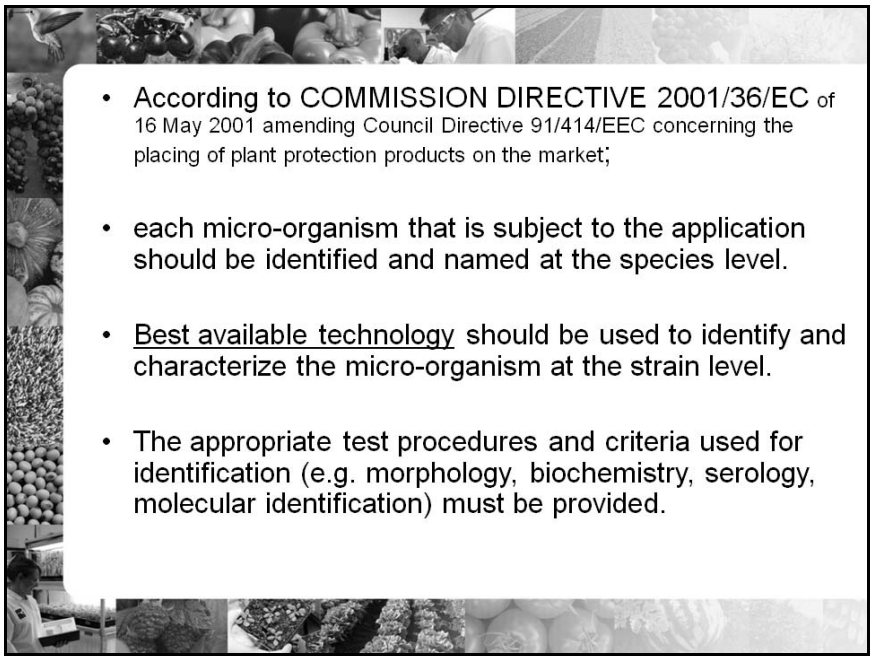
Data Requirements for New Strains

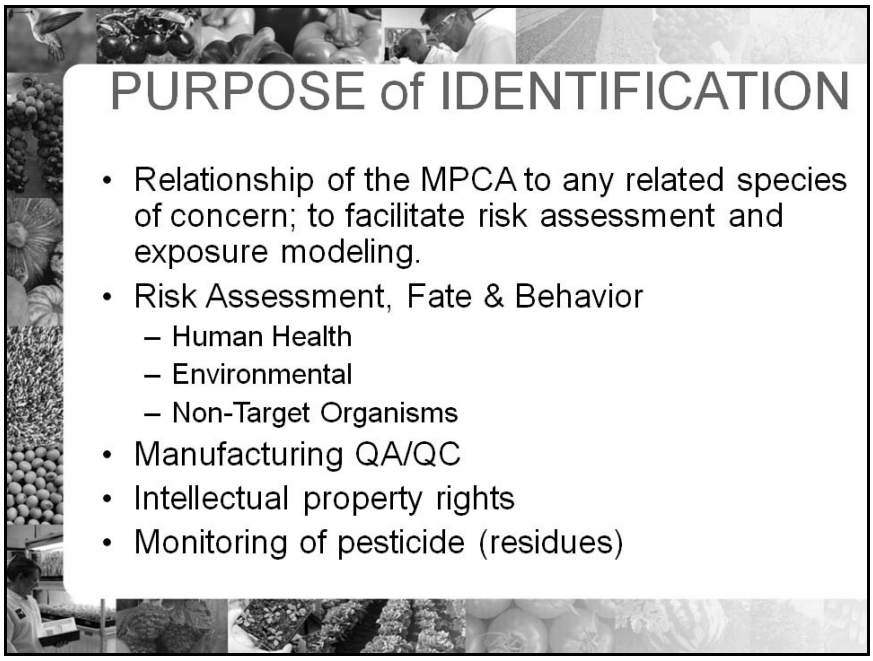
- Identification to subspecies level must be complete
- Toxins of concern should be addressed
- Toxicology (Mammalian and Eco.) data should not should not be read across as that establishes the safety of the strain isolate.
- Registration is at strain level, but strain designation pertains to the data showing its safety and efficacy.
  - Efficacy different from traditional subspecies should be questioned

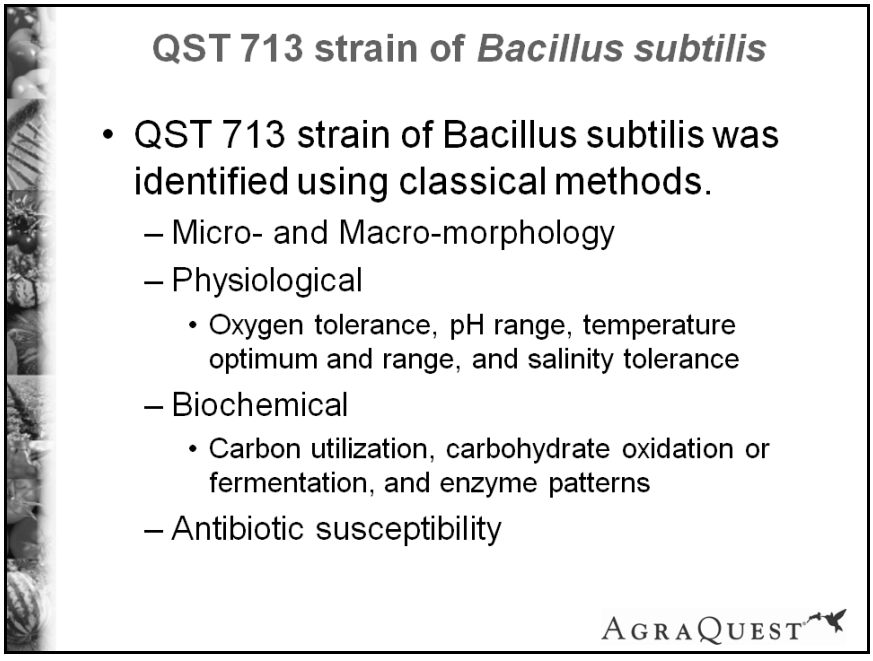
Thank you for your consideration.

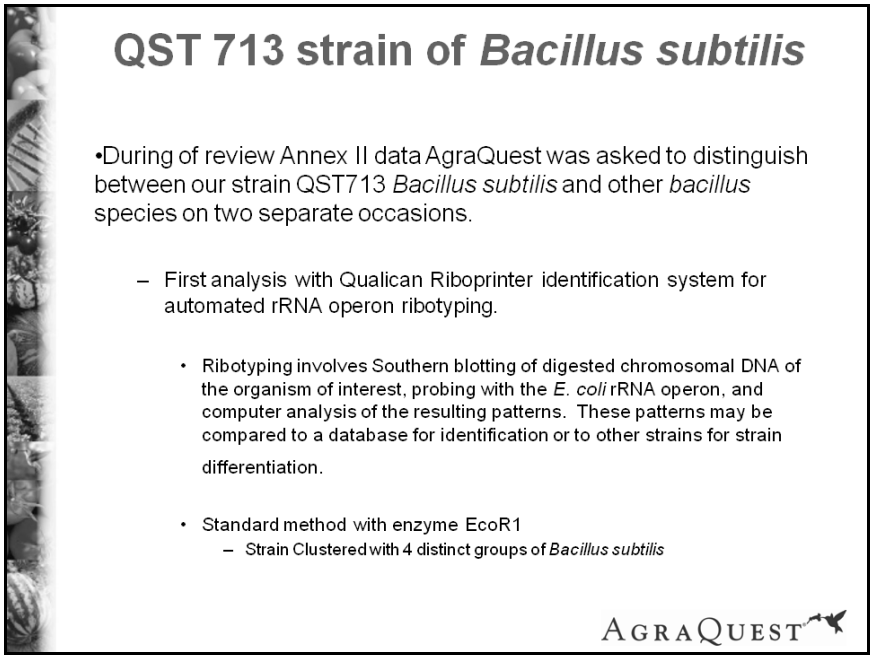
Characterisation issues on bacteria
By Sherry Heins (AgraQuest)

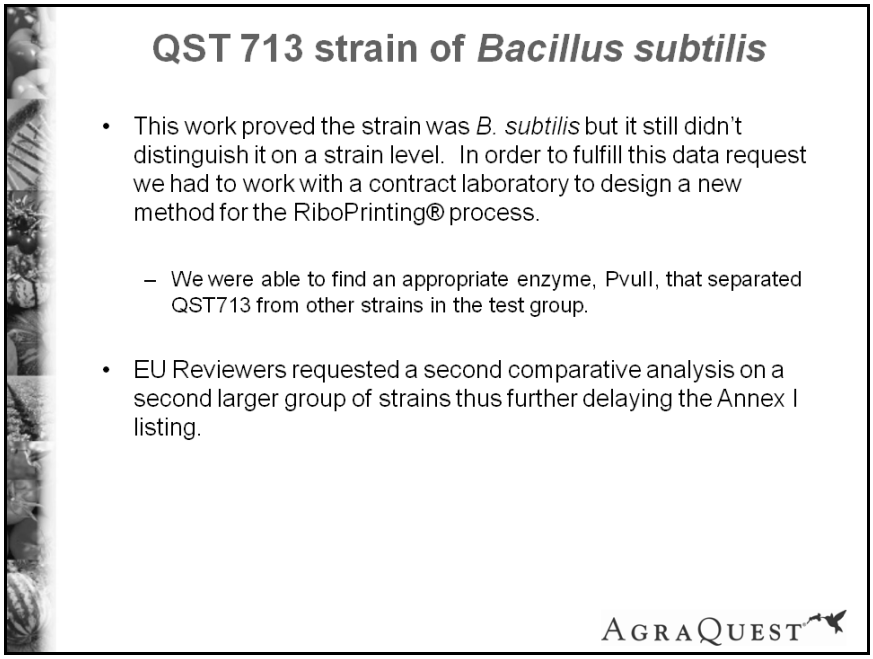


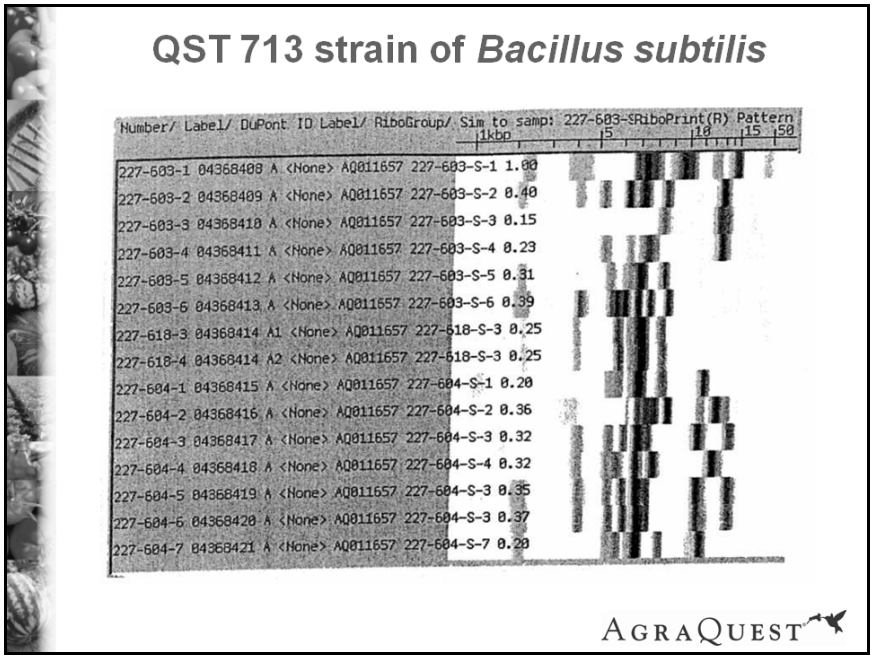


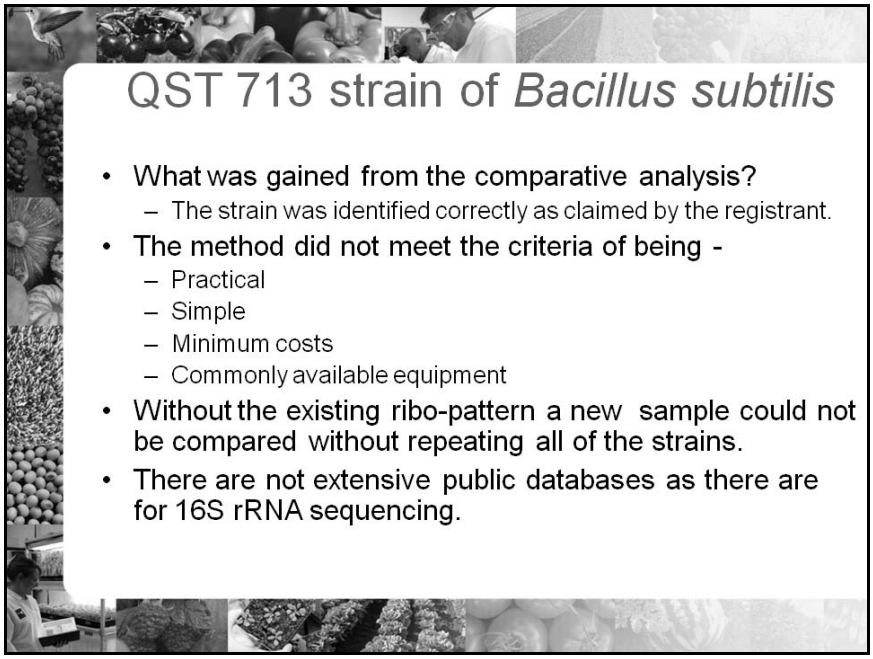

PURPOSE of IDENTIFICATION
• Relationship of the MPCA to any related species of concern; to facilitate risk assessment and exposure modeling.
• Risk Assessment, Fate & Behavior
– Human Health
– Environmental
– Non-Target Organisms
• Manufacturing QA/QC
• Intellectual property rights
• Monitoring of pesticide (residues)

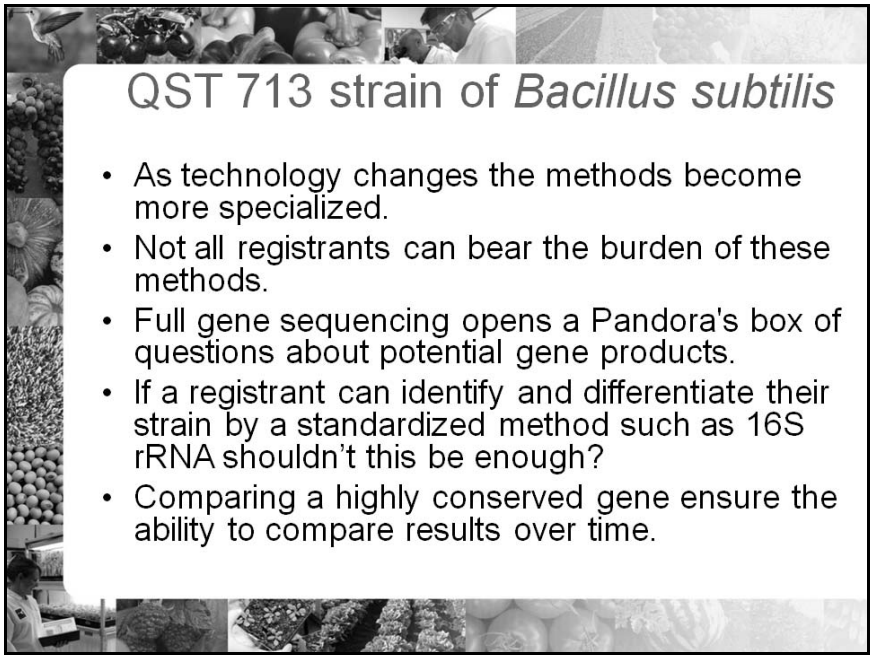

QST 713 strain of Bacillus subtilis
• QST 713 strain of Bacillus subtilis was identified using classical methods.
– Micro- and Macro-morphology
– Physiological
• Oxygen tolerance, pH range, temperature optimum and range, and salinity tolerance
– Biochemical
• Carbon utilization, carbohydrate oxidation or fermentation, and enzyme patterns
– Antibiotic susceptibility
AGRAQUEST

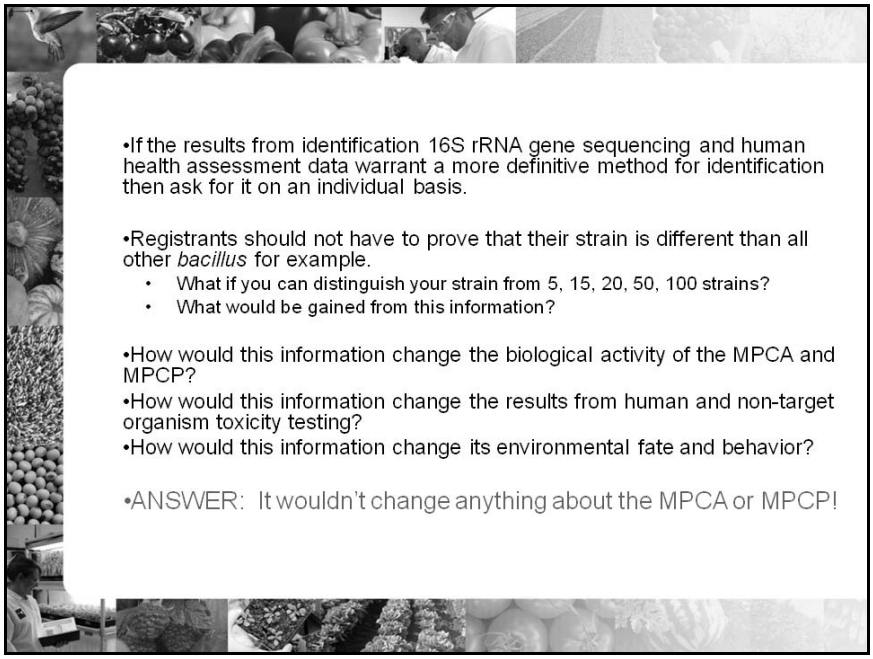

QST 713 strain of *Bacillus subtilis*
•During of review Annex II data AgraQuest was asked to distinguish between our strain QST713 *Bacillus subtilis* and other *bacillus* species on two separate occasions.
– First analysis with Qualican Riboprinter identification system for automated rRNA operon ribotyping.
• Ribotyping involves Southern blotting of digested chromosomal DNA of the organism of interest, probing with the *E. coli* rRNA operon, and computer analysis of the resulting patterns. These patterns may be compared to a database for identification or to other strains for strain differentiation.
• Standard method with enzyme EcoR1
– Strain Clustered with 4 distinct groups of *Bacillus subtilis*
AGRAQUEST

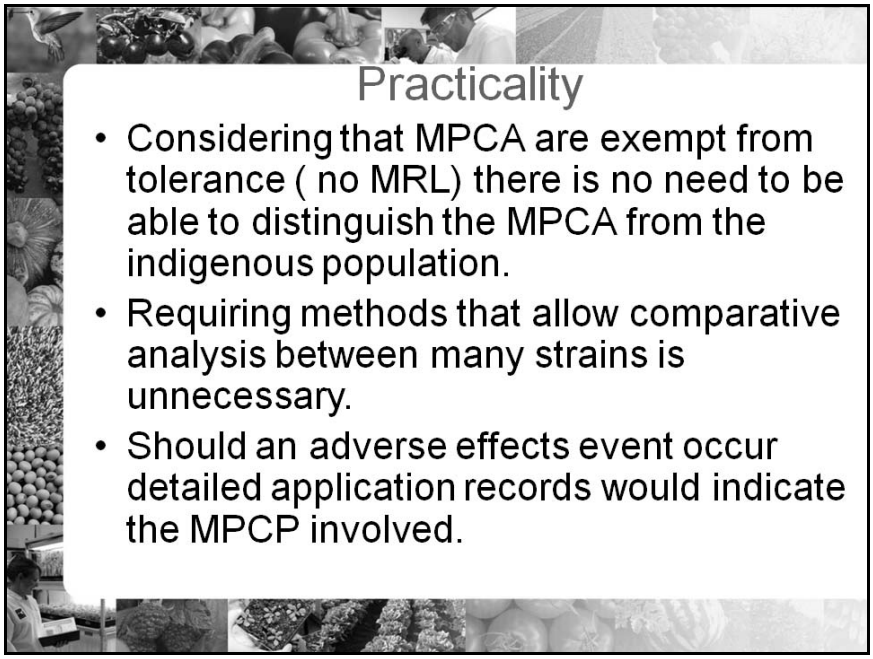

QST 713 strain of *Bacillus subtilis*
• This work proved the strain was *B. subtilis* but it still didn't distinguish it on a strain level. In order to fulfill this data request we had to work with a contract laboratory to design a new method for the RiboPrinting® process.
– We were able to find an appropriate enzyme, PvuII, that separated QST713 from other strains in the test group.
• EU Reviewers requested a second comparative analysis on a second larger group of strains thus further delaying the Annex I listing.
AGRAQUEST


QST 713 strain of Bacillus subtilis
Number/ Label/ DuPont ID Label/ RiboGroup/ Sim to samp: 227-603-S
RiboPrint(R) Pattern
1kbp
5
10
15
50
227-603-1 04368408 A <None> AQ011657 227-603-S-1 1.00
227-603-2 04368409 A <None> AQ011657 227-603-S-2 0.40
227-603-3 04368410 A <None> AQ011657 227-603-S-3 0.15
227-603-4 04368411 A <None> AQ011657 227-603-S-4 0.23
227-603-5 04368412 A <None> AQ011657 227-603-S-5 0.31
227-603-6 04368413 A <None> AQ011657 227-603-S-6 0.39
227-618-3 04368414 A1 <None> AQ011657 227-618-S-3 0.25
227-618-4 04368414 A2 <None> AQ011657 227-618-S-3 0.25
227-604-1 04368415 A <None> AQ011657 227-604-S-1 0.20
227-604-2 04368416 A <None> AQ011657 227-604-S-2 0.36
227-604-3 04368417 A <None> AQ011657 227-604-S-3 0.32
227-604-4 04368418 A <None> AQ011657 227-604-S-4 0.32
227-604-5 04368419 A <None> AQ011657 227-604-S-3 0.35
227-604-6 04368420 A <None> AQ011657 227-604-S-3 0.37
227-604-7 04368421 A <None> AQ011657 227-604-S-7 0.20
AGRAQUEST

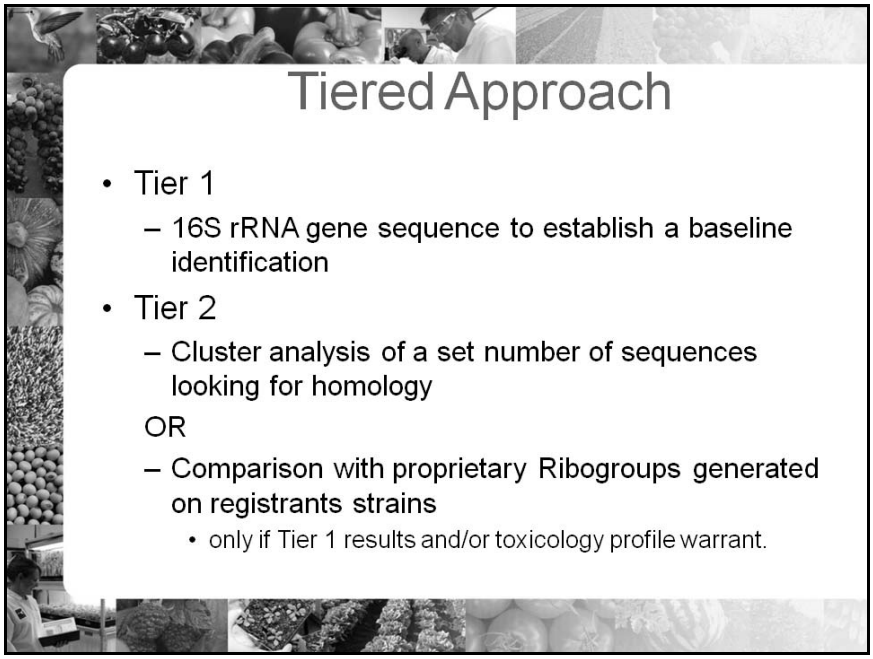

QST 713 strain of Bacillus subtilis
• What was gained from the comparative analysis?
– The strain was identified correctly as claimed by the registrant.
• The method did not meet the criteria of being -
– Practical
– Simple
– Minimum costs
– Commonly available equipment
• Without the existing ribo-pattern a new sample could not be compared without repeating all of the strains.
• There are not extensive public databases as there are for 16S rRNA sequencing.


QST 713 strain of *Bacillus subtilis*
• As technology changes the methods become more specialized.
• Not all registrants can bear the burden of these methods.
• Full gene sequencing opens a Pandora's box of questions about potential gene products.
• If a registrant can identify and differentiate their strain by a standardized method such as 16S rRNA shouldn't this be enough?
• Comparing a highly conserved gene ensure the ability to compare results over time.


•If the results from identification 16S rRNA gene sequencing and human health assessment data warrant a more definitive method for identification then ask for it on an individual basis.
•Registrants should not have to prove that their strain is different than all other *bacillus* for example.
• What if you can distinguish your strain from 5, 15, 20, 50, 100 strains?
• What would be gained from this information?
•How would this information change the biological activity of the MPCA and MPCP?
•How would this information change the results from human and non-target organism toxicity testing?
•How would this information change its environmental fate and behavior?
•ANSWER: It wouldn't change anything about the MPCA or MPCP!


Practicality
• Considering that MPCA are exempt from tolerance (no MRL) there is no need to be able to distinguish the MPCA from the indigenous population.
• Requiring methods that allow comparative analysis between many strains is unnecessary.
• Should an adverse effects event occur detailed application records would indicate the MPCP involved.


Quality Control/ Assurance
• Strict adherence to manufacturing process
• Product release specifications
– Viable unit (cfu/g etc.)
– Physical properties
– Unintentional microorganisms
Manufacturers ensure an uniform product that is equivalent the MPCA and MPCP batches that where testing and analyzed to obtain Annex I inclusion.


Tiered Approach
• Tier 1
– 16S rRNA gene sequence to establish a baseline identification
• Tier 2
– Cluster analysis of a set number of sequences looking for homology
OR
– Comparison with proprietary Ribogroups generated on registrants strains
• only if Tier 1 results and/or toxicology profile warrant.


Thank You For The Invitation
• Jeroen J. Meeussen
– EU-coordinator plant protection products
• Ulf HEILIG
– IBMA - International Relations Regulatory Affairs