The Evolution in Food Production:
The Relevance of *Bacillus* Species to Agriculture and IPM

Copa Cogeca / IBMA workshop.
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Jacob Eyal, Ph.D.
Executive Vice President, Certis USA
The Relevance of *Bacillus* Species to Agriculture

Topics to Cover:

- *Bacillus* genus: history, background, and use
- *Bacillus* spp. (other than Bt) as biocontrol agents
- *Bacillus thuringiensis* (Bt) spp. as biocontrol agents.
- Advantages and limitations of using Bt in agriculture
- Commercial Bt ‘s Genomic sequence, markers and survey
- Use of *Bacillus* spp in European and global markets
Bacillus: History, Background, and Use

• 1835: Christian Ehrenberg first to isolate bacteria named *Vibrio subtilis*

• 1872: Ferdinand Cohn creates the genus *Bacillus* and renames *Vibrio subtilis* as *Bacillus subtilis*

• 16rRNA gene sequencing, reveal the genus is divided into different genera: *Bacillus, Brevibacillus, Paenibacillus* and others

• *Bacillus cereus* falls in the *Bacillus subtilis* group and is closely related to *B. thuringiensis*, *B. anthracis*, *B. mycodies*, etc.

• Gram-positive spore-forming bacteria, rod-shaped and aerobic

• Diverse and commercially useful in many industrial applications: human and animal health, food, chemicals, bioremediation, and agriculture
• The spore-forming ability makes *Bacillus* spp. ideal candidates for developing biocontrol agents

• *Bacillus* spores have a high tolerance for drying

• *Bacillus* bacteria function like a “microbial factory” for manufacturing a vast array of enzyme and biologically-active molecules

• *Bacillus*-based biopesticides are used as fungicides, nematicides, bactericides, and insecticides
**Bacillus amyloliquefaciens (Ba)**

- *Bacillus amyloliquefaciens (Ba)* is an common soil bacterium described in 1943 (Fukumoto)
- Name: producer (*faciens*), of Amylase (*amylo*), and liquefying of starch (*lique*)
- *Ba* spp and *Bacillus subtilus* spp are very closely related both produce the restriction enzyme *BamHI* (CGSTCC)
- Confusion in identification and exact taxonomic position of *B. amyloliquefaciens* spp resulted in different names *Bacillus subtilis, Bacillus subtilis var amyloliquefaciens*, etc
- Whole sequence genome analysis, DNA-DNA hybridization, 16sRNA and phylogenetic analysis, and other genomic methods may reveal the differences between *Bacillus amyloliquefaciens sb spp*
Bacillus amyloliquefaciens (Ba) - continue

- B. *amyloliquefaciens* spp produce metabolites with antimicrobial properties with several classes:
  
  a. Peptide and Polyketides: *Bacilysin, rhizocticin, subtilisin, bacillaene, difficidin, macrolaotin*

  b. Lipopeptides families: *surfactin, iturin, and fungycin*

- The association between the production of these metabolites and sporulation is not fully understood

- *Ba* spp are commercialized in agriculture to control pathogens, improve tolerance in salty soil, solubilize phosphate, promote growth of rhizobacteria, quickly colonize roots, produce biofilm, etc
<table>
<thead>
<tr>
<th>Bacillus spp.</th>
<th>Strain Name</th>
<th>Commercial Name</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. amyloliquefaciens</strong></td>
<td>D747</td>
<td>Amylo-X®, Double Nickel®, Ecoshot®</td>
<td>Broad spectrum biofungicides for preventative control or suppression of fungal and bacterial plant diseases (<em>These strains were formerly identified as B. subtilis</em>)</td>
</tr>
<tr>
<td></td>
<td>MBI 600*</td>
<td>Serifel®, Botokiller®</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FZB24</td>
<td>Taegro®</td>
<td></td>
</tr>
<tr>
<td></td>
<td>QST 713*</td>
<td>Serenade®, Impression®</td>
<td></td>
</tr>
<tr>
<td><strong>B. firmus</strong></td>
<td>I-1582</td>
<td>Nortica®, Votivo®</td>
<td>Plant parasitic nematodes applied as seed treatment or WP (lawn and turf uses)</td>
</tr>
<tr>
<td><strong>B. pumilus</strong></td>
<td>QST 2808</td>
<td>Sonata®</td>
<td>Biofungicide for control of fungal plant diseases</td>
</tr>
<tr>
<td><strong>B. sphaericus</strong></td>
<td>ABT 1743</td>
<td>Vectolex®, Spheratax®</td>
<td>Control of larva of Culex spp.; effective in turbid water</td>
</tr>
<tr>
<td><strong>B. mycoides</strong></td>
<td>Isolate J</td>
<td>LifeGard™</td>
<td>New class of biological disease control; used to manage early and late potato blight as well as white mold</td>
</tr>
<tr>
<td><strong>B. licheniformis</strong></td>
<td>SB3086</td>
<td>Ecoguard™</td>
<td>Fungal disease control on ornamentals, lawn and turf</td>
</tr>
<tr>
<td><strong>B. subtilis</strong> spp.</td>
<td>B. Megaterium B. Mojavensis</td>
<td>Various names</td>
<td>Broad spectrum biopesticide, phosphate enhancing agent, biostimulate and plant growth promoter</td>
</tr>
</tbody>
</table>
Mode of Action of *Bacillus*: Plant diseases biocontrol

**Competition for Space**
- Better adapted to various environmental and nutritional conditions than plant pathogens
- Ability to survive under conditions that are unfavourable to the plant pathogens
- Ability to grow more rapidly than the pathogenic strain

**Competition for Nutrients**
- Prevents spore germination and growth of pathogens
- *Bacillus* spp. take nutrients away from the pathogens more rapidly
  - Initial concentration of *Bacillus* should be high
  - Most effective concentration ranges between $10^7$ to $10^8$ CFU/mL
Mode of Action of *Bacillus*: Elicit pores on fungal cell membranes

Stained fungal cells (*Rhizoctonia solani*) after treatment with 100 g/mL of fengycin for 2 hr; the leaked cytoplasm from hyphae is labeled by arrows (Gaofu et al, 2010)
Mode of Action of *Bacillus*: Root colonization (persistence in the rhizosphere)

Colonization of tomato root hairs 2 weeks after seed treatment with *Bacillus amyloliquefaciens*

- Spores germinate & grow with the seedling root
- Colonization of root surfaces (biofilm formation)
- Competitive exclusion of pathogenic fungi
- Enhancement of nodulation (legumes)
- Induced systemic resistance (ISR)

Life *Bacillus mycodies* triggers Systemic Acquire Resistance (SAR) by directly activating NRR1 Proteins (SAR protein) downstream of salicylic acid

Activated NRR1 translocated into Nucleus activates Transcription of pathogenesis–related (PR) genes

Similar to Acibenzolar-S-methyl (Actigard, Bion)

No phytotoxicity detected

Downy mildew in Chardonnay grapes

References:
Overview of *Bacillus* lipopeptide interaction for control of plant diseases
Surfactin

Fengycin

Iturin

Modified from Trend in Microbiology Vol.16 No.3 2007

Biofilm formation Spreading Anti-bacterial Anti-fungal Signal for plant cells

Leaf disease reduction

Pathogen

Benfical rhizobacteria

Root colonization

Rhizosphere competence

Antagonism

Direct inhibition (antibiosis) of phytopathogens

Induced resistance

Host plant immunization
Summary - Mode of action of **Bacillus spp.**

Pathogen (fungi or bacteria)

Bacillus spp.

**Competition**

Bacillus colonize

**Induced resistance**

- Lipopeptides
- Growth promotion
- Yield increase
- Plant strength

**Competition**
Typical Manufacturing & Quality Control Process of *Bacillus spp.*

### Strain selection
- **Starting culture**
- **Shaker flasks**
- **Stock culture collection**

### Quality Control (stock cultures)
- Growth & morphology
- Cry gene & toxin analysis
- Insect bioassay

### Scale-up and production
- **Main fermenter**
- **Spray drying**
- **Inoculate**
- **Harvest**

### Finished product
- **Formulation**
- Technical powder

**Quality Control:** bioassay, microbial purity, mammalian safety, physical characteristics
Bacillus thuringiensis (Bt) Species as Biocontrol Agents
Background: *Bacillus thuringiensis*

- Common soil bacterium
- Discovered in 1901 in Japan by Shigetane Ishiwata
- Rediscovered in 1911 in Germany by Ernest Berliner
- Many subspecies, strains, and isolates
- Many are pathogens of insects
- Used as a microbial insecticide since the 1930s in France
- Entered commercial use in the United States in the 1950s
- Characterized by presence of crystalline protein toxins (Cry toxins)
## Economically important *Bt* subspecies

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>Target insects</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.t. kurstaki (Btk)</td>
<td>Lepidoptera larvae (caterpillars)</td>
<td>Delfin®, Costar®, Dipel®, Biobit®, Foray®, Rapax®</td>
</tr>
<tr>
<td>B.t. aizawai (Bta)</td>
<td></td>
<td>Agree®, Xentari®, Certan®</td>
</tr>
<tr>
<td>B.t. israelensis (Bti)</td>
<td>Diptera larvae (mosquitoes, blackflies)</td>
<td>Teknar®, VectoBac®, Aquabac®</td>
</tr>
<tr>
<td>B.t. tenebrionis (Btt)</td>
<td>Coleoptera larvae &amp; adults (beetles)</td>
<td>Trident®, Novodor®</td>
</tr>
</tbody>
</table>
Bt Life Cycle: Production of Spores and Crystals

Sporulated Bt

Lysis

Crystal

Spore

Free spores & crystals in technical powder

3-D view of crystal

Copa Cogeca/IBMA workshop: Regulatory policy on Bacillus cereus and the risk of a future without microbial pesticides in Europe. 28th March 2017. Brussels, BE.
Mode of Action of *Bacillus thuringiensis* subsp. *kurstaki*

1. Larva ingests Bt.
2. Toxin crystals dissociate in alkaline midgut.
3. Protoxin molecules are released from dissociated crystal.
4. Protoxin molecules “activated” by the insect’s digestive enzymes.
5. Activated δ-endotoxin molecules bind to receptors on midgut cells.
6. Toxin destroys the cellular lining of the midgut.
7. Gut is paralyzed, insect stops feeding.
8. Death from osmotic shock, septicemia, or starvation.
The Mode of Action of Cry toxins

Modified from de Maagd et al., Trends in Genetics (2001)

Bt crystals & spores ingested by larva

Crystal dissolves in alkaline (pH10) midgut

Protoxin

“Activation” by digestive enzymes

Activated δ-endotoxin (Cry toxin) binds to midgut

Toxin inserts into cell membrane, opening a pore

Midgut cell membrane
Cry toxin profiles and target pest susceptibility

<table>
<thead>
<tr>
<th>Cry toxin group:</th>
<th>1Aa</th>
<th>1Ab</th>
<th>1Ac</th>
<th>1C</th>
<th>1D</th>
<th>2A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cry toxin content of product technical powders</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bt kurstaki</em> strains (natural)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Bt aizawai</em> strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural strain</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Transconjugant</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Cry toxin susceptibility of representative target insects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cabbage looper (<em>Trichoplusi</em>)</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Diamondback moth (<em>Plutella</em>)</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Corn earworm (<em>Helicoverpa</em>)</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Armyworms (<em>Spodoptera</em>)</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
</tr>
</tbody>
</table>
Important Cry Toxins vs. Lepidoptera in Agriculture

- Nearly 300 unique Cry toxins identified!
  - Some are toxic to insects, ticks, nematodes, other invertebrates
  - Many have no known toxicity
  - Some have been engineered into plants, algae, or other bacteria (GMO)
- Cry1 and Cry2 groups are most important in Bt products for Lepidoptera
- Broadest spectrum & most active on Lepidoptera: Cry1Ac
- Best Spodoptera activity: Cry1C
Potency of Bt products: International Units (IU)

• Determined by standard bioassay against cabbage looper

\[
\text{IU/mg} = \frac{\text{LC}_{50} \text{ of standard}}{\text{LC}_{50} \text{ of sample}} \times \text{Assigned potency of standard}
\]

(Potency standard for US-HD1-1980: 16,000 IU/mg)
Other markets for Bt products

Forestry

Consumer

Public Health

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Advantages of *Bt* Bioinsecticides

- Highly-specific compared to many synthetic insecticides
  Btk, Bta: *Lepidoptera* larvae only

- Safe to users, livestock, wildlife
  Mammals have acidic gut and different proteases than insects

- Highly compatible with Integrated Risk Management (IPM)
  No effect on beneficial insects
  No toxic residues
  Lowest available entry period after Spray
  Lowest available PHI (spray and harvest on the same day)

- Unique mode of action
  No cross-resistance with synthetic chemical insecticides
Limitations of *Bt* Bioinsecticides

• High specificity = narrow spectrum  
Ineffective against many key pests (i.e., aphids, mites, thrips, etc)

• Most effect against young larvae  
Proper timing of sprays is critical  
L1-L2 best; L3—maybe; L4-L5—too late  
No effect on eggs, pupae, adults

• Sensitive to environmental conditions  
Solar UV radiation (spray late in day, use sunscreen)  
Alkaline water (high pH)

• No contact activity  
Must be ingested to be effective  
Good spray coverage is critical to success
Use of *Bt* and *B. Spp* in European and global markets
Market share for *Bacillus* spp
Global Biofungicide Treated Hectares

By country:
- China (2015) 38%
- Russian Fed. (2015) 15%
- USA (2015) 11%
- Others 3%
- Canada (2015) 3%
- Costa-Rica (2012) 3%
- Chile (2015) 1%
- Brazil (2015) 3%
- Mexico (2015) 4%
- Guatemala (2014) 8%
- India (2015) 4%
- Colombia (2015) 4%
- France (2015) 3%
- Costa Rica (2012) 3%
- Chile (2015) 1%
- Others 3%

By crop:
- Vegetable Crops 22%
- Rice 18%
- Cotton 11%
- Bananas 9%
- Potatoes 8%
- Tobacco 6%
- Oilseed Rape 6%
- Peas/beans 3%
- Vines/grapes 4%
- Tropical Fruit 2%
- Flowers+ornamentals 2%
- Pome/Stone Fruit 2%
- Others 4%

Bacillus amyloliquefaciens / subtilis / pumilis

Total treated Ha – 2.0 MM

Source: Sigma CP database- Kynetec Ltd.2016.
Copa Cogeca/IBMA workshop: Regulatory policy on Bacillus cereus and the risk of a future without microbial pesticides in Europe. 28th March 2017. Brussels, BE.

Bt treated hectares in major EU countries

**By country**
- **Italy (2015)**: 39%
- **Spain (2015)**: 32%
- **France (2015)**: 8%
- **Holland (2014)**: 8%
- **Portugal (2014)**: 3%
- **Germany (2015)**: 1%
- **Austria (2013)**: 1%
- **Belgium (2013)**: 1%
- **Others**: 1%
- **Greece (2015)**: 3%
- **Turkey (2015)**: 1%
- **Belgium (2013)**: 1%
- **Austria (2013)**: 1%

**By crop**
- **Vegetable Crops**: 30%
- **Pome/Stone Fruit**: 24%
- **Tomatoes**: 10%
- **Vines/grapes**: 17%
- **Undefined Crops**: 2%
- **Soft Fruit**: 2%
- **Citrus**: 1%
- **Others**: 2%
- **Flowers+ornamentals**: 3%
- **Forestry**: 6%
- **Olives**: 3%

Total treated Ha – 459,000

Source: Sigma CP database- Kynetec Ltd.2016.
Global *Bt* treated hectares (except China)

**By countries**
- Brazil (2015) 62%
- India (2015) 6%
- Australia (2015) 4%
- USA (2015) 3%
- Morocco (2013) 2%
- Mexico (2015) 2%
- Indonesia (2014) 2%
- Italy (2015) 2%
- Malaysia (2015) 2%
- Spain (2015) 2%
- Vietnam (2014) 2%
- Others 11%

**By crop**
- Soybeans 28%
- Vegetable Crops 16%
- Cotton 15%
- Others 15%
- Cereals 6%
- Tomatoes 6%
- Pome/Stone Fruit 3%
- Oil Palm 3%
- Corn 3%
- Vines/grapes 3%
- Forestry 2%

**Total treated Ha – 8.8 MM**

Source: Sigma CP database- Kynetec Ltd.2016.
Thank You.

Contact:
Jacob Eyal, Executive Vice President
Certis USA LLC
9145 Guilford Road, Suite 175
Columbia MD 21046 USA
jeyal@certisusa.com