Development of a novel biological control agent targeting the phytopathogen *Erwinia amylovora*

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- Fire blight
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- Apple orchard
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**ABSTRACT**

Antibiotics are used extensively to control animal, plant, and human pathogens. They are sprayed on apple and pear orchards to control the bacterium *Erwinia amylovora*, the causative agent of fire blight. This phytopathogen is developing antibiotic resistance and alternatives either have less efficacy, are phytotoxic, or more management intensive. The objective of our study was to develop an effective biological control agent colonizing the host plant and competing with *Erwinia amylovora*. It must not be phytotoxic, have a wide spectrum of activity, and be unlikely to induce resistance in the pathogen. To this end, several bacterial isolates from various environmental samples were screened to identify suitable candidates that are antagonistic to *E. amylovora*. We sampled bacteria from the flowers, leaves, and soil from apple and pear orchards from the springtime bloom period until the summer. The most effective bacteria, including isolates of *Pseudomonas poae*, *Paenibacillus polymyxa*, *Bacillus amyloliquefaciens* and *Pantoaea agglomerans*, were tested in vitro and in vivo and formulated into products containing both the live strains and their metabolites that were stable for at least 9 months. Trees treated with the product based on *P. agglomerans* NY60 had significantly less fire blight than the untreated control and were statistically not different from streptomycin-treated control trees. With *P. agglomerans* NY60, fire blight never extended beyond the central vein of the inoculated leaf. The fire blight median disease severity score, 10 days after inoculation, was up to 70% less severe on trees treated with *P. agglomerans* NY60 as compared to untreated controls.

**Importance**

Increasing resistance to antibiotics represents a growing challenge. One factor contributing to global antibiotic resistance is the large-scale application of antibiotics in agricultural fields. The bacterial plant pathogen *Erwinia amylovora* is responsible for a disease called fire blight in different hosts, including apple and pear trees. Biological control agents against *E. amylovora* were developed through a screening approach specifically tailored to isolate bacteria effective against this phytopathogen. The cell-free supernatants of various bacteria demonstrated inhibitory activity against three different *E. amylovora* strains, including two streptomycin-resistant ones. A few isolates were even capable of completely killing the target phytopathogen by direct interaction. The developed products, based on both the live bacteria as well as their metabolites, were less phytotoxic than traditional treatment and also as effective as antibiotics in controlling the fire blight disease on apple trees.

**1. Introduction**

Resistance to antibiotics of human bacterial pathogens is a huge problem. The Center for Disease Control and Prevention (CDC) in the United States considers this as “one of the most pressing public health problems in the world” [1]. Many bacteria evolve resistance to the most commonly prescribed antibiotics, resulting in increasingly prolonged infections and higher medical costs.

Although the risk is small, a possible factor contributing to antibiotic resistance is the large-scale application of antibiotics in agricultural fields, for instance on fruit trees such as apple and pear orchards [2, 3]. The more that fields (trees and soil) are exposed to antibiotics, the higher is the likelihood that the environmental microbiota will develop resistance to antibiotics, which can lead to the transfer of resistance determinants to pathogens of animals, including humans [1].

The bacterial plant pathogen responsible for fire blight is *Erwinia amylovora* [4, 5, 6] a rod-shaped Gram-negative bacterium capable of...
and antibiotics in combating fire blight in apple and pear orchards. In addition, this biological product should possess a wider spectrum of activity and, ideally, multiple modes of actions against *E. amylovora* in order to reduce the likelihood of resistance development.

Using a screening approach tailored to isolate bacteria effective against *E. amylovora*, we present the development of a biological control to fire blight based on both bacterial active strains as well as their metabolites, which are less phytotoxic than copper but also as effective as antibiotics in controlling the disease caused by *E. amylovora*.

### 2. Materials and methods

#### 2.1. Media

Two nonselective media (Tryptic Soy Agar (TSA), and Plate Count Agar (PCA)) and three selective media (Benedict (for isolation of *Streptomyces* spp.) [23], BCSA (for isolation of *Burkholderia* spp.) [24] and Gould (for isolation of *Pseudomonas* spp.) [25] supplemented with 50 mg L⁻¹ cycloheximide (to limit the growth of fungi) were used to isolate bacteria from environmental samples.

#### 2.2. Isolation of microorganisms from plants

Ten seeds and three segments (0.5 cm²) randomly excised from each leaf, stem, root, and fruit were vortexed in 5 mL sterile 0.85% (w/v) NaCl. To isolate sporulating bacteria, the suspensions were preheated at 80 °C for 30 min. Aliquots (100 μL) of each suspension were spread onto the nonselective- and selective media plates (Table 1). To isolate bacteria, the plates were incubated in the dark for 2–5 days at room temperature (~21 °C) [26, 27, 28, 29, 30].

#### 2.3. Isolation of bacteria from soil

One gram of soil was added to 9 mL sterile phosphate-buffered saline (PBS) then agitated for 30 min. Sample suspensions were serially diluted (Table 1). One hundred microliters of each dilution (10⁻², 10⁻³, and 10⁻⁴) were spread onto nonselective- and selective media. Plates were incubated under the same conditions as above.

#### 2.4. Storage and culture of isolated bacteria

Colonies of bacteria were purified first and then colonies with different morphological characteristics were transferred to tubes containing 3 mL TSB and incubated overnight at 30 °C. Bacteria were stored at -80 °C in TSB amended with 20% v/v glycerol.

#### 2.5. Strains used to evaluate antimicrobial activity

The pathogenic strains used as indicators for antimicrobial activity were *E. amylovora* S43S, a strain isolated from an infected apple tree at the IRDA (Quebec, Canada), a research center conducting studies on fire blight; streptomycin-resistant *E. amylovora* S153 (Botany and Plant Pathology, Oregon State University, USA) and streptomycin-resistant *E. amylovora* S1605 (MAPAQ, Quebec, Canada).

#### 2.6. First screening step: antagonistic activity assays

The antagonistic activities of the bacterial isolates against *E. amylovora* were determined by an agar plate assay.

#### 2.6.1. Method

Aliquots of bacterial colonies were selected from each bacterial glycerol-frozen stock solution and incubated overnight at 30 °C in 3 mL TSB. Five microliters of each bacterial culture were deposited on lawns of *E. amylovora* S43S growing on TSA plates. These lawns were first made by incubating 50 μL of *E. amylovora* S43S overnight in 3 mL TSB at 30 °C and...
then resuspending them in sterile water to get an OD$_{620}$ of 0.2. Finally, 100 μL of the suspensions were spread onto TSA plates. The plates were incubated at room temperature (~21 °C) for 2 d. Bacterial isolates forming clear haloes (inhibition zones) on the _E. amylovora_ S435 lawns were selected for the second screening step.

### 2.7. Second screening step: antimicrobial activity assays

For evaluation of the extracellular antimicrobial activity, bacterial isolates produced clear inhibition zones in at least one of the previous assays were inoculated into 3 mL TSB at 30 °C and 150 rpm on a rotary shaker for 2 d. The cultures were then centrifuged at 18,000 x g for 10 min at 20 °C and the supernatants were passed through a 0.22-μm pore diameter filter to obtain sterile supernatant.

Antimicrobial activity against _E. amylovora_ S435 was assessed using a well-diffusion inhibition assay. First, lawns of the test bacteria were grown on agar plates. Fifty microliters of well-diffusion inhibition assay. First, lawns of the test bacteria were grown on agar plates. Fifty microliters of the cell-free bacterial culture supernatant. The plates were then incubated at room temperature (~21 °C) and the diameters of the inhibition zones around the wells were measured after 2 d. For the control, 200 μL TSB were added to one well. Plates were incubated under the same conditions described in previous sections. Each treatment was performed in triplicate.

### 2.8. In vitro growth and co-culture competition

The growth and competition of the active strains were measured by co-culturing them with _E. amylovora_ S435, S153 or S1605 in liquid media. Using TSB cultures incubated overnight, 50 μL of each test bacterium (diluted to OD$_{620}$ = 0.2) were mixed with 50 μL _E. amylovora_ strains (OD$_{620}$ = 0.02) and cultivated in 3 mL TSB at 30 °C with agitation at 150 rpm for 24 h. Serial dilutions up to 10$^{-3}$ were then prepared. One hundred microliters of each co-culture dilution were spread onto TSA plates and incubated at room temperature (~21 °C). After 2-3 d, the colony-forming units (CFUs) were counted (_Erwinia_ colonies were always distinct from the test isolates). For the control, 50 μL of pure _E. amylovora_ S435 (OD$_{620}$ = 0.02) was cultured.

### 2.9. Identification of bacterial isolates

#### 2.9.1. DNA extraction

DNA of active bacterial isolates were extracted according to Fastprep™ procedures and instruments (MP Biomedicals, Solon, OH, USA). The dry DNA pellet was resuspended in 50 μL sterile ddH$_2$O and maintained at -20 °C [31, 32, 33].

#### 2.9.2. 16S rRNA gene sequence analysis

To identify the isolates of interest, PCR amplification of the gene encoding the 16S rRNA was performed (Supplemental Table 1). PCR was carried out in a 50-μL reaction mixture consisting of 1X Taq buffer, 200 μM dNTPs mix, 0.4 μM pA-27f-YM, 0.4 μM pH, 1 unit Feland Taq DNA Polymerase (BioBasic Canada Inc., Markham, Ontario, Canada), and 50 ng extracted DNA. The amplifications were performed in a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) using an initial DNA denaturation step of 5 min at 95 °C followed by 29 cycles of 30 s at 95 °C, primer annealing for 40 s at 55 °C, primer elongation for 1.5 min at 72 °C, and a final extension step for 10 min at 72 °C. After DNA amplification, the PCR products were analyzed by agarose gel electrophoresis (1.0% w/v agarose, 100 V, 60 min). The DNA was stained with ethidium bromide (0.5 μg mL$^{-1}$) and visualized under UV illumination [34].

#### 2.9.3. 16S rRNA gene sequence analysis via Resphera Insight v 2.2

Resphera Insight v. 2.2 (Resphera Biosciences, Baltimore, MD, USA), which provides ultra-high-resolution taxonomic assignment of 16S rRNA sequences down to the species level, was used as illustrated by the manufacturer [35] to predict an accurate consensus lineage of the active isolates.

#### 2.9.4. Amplification of specific _Bacillus_ and _Paenibacillus_ genes

The _rpoB_, _gyrA_, and _gyrB_ gene fragments were used as molecular diagnostic markers to identify isolates within the _Bacillus subtilis_ group. To this end, specific primers for the amplification of each gene were used (Supplemental Table 1). PCR amplifications were carried out in a 25-μL reaction mixture as described above, using the appropriate forward- and reverse primers. The amplifications were performed using specific PCR temperature protocols. After DNA amplification, the _rpoB_, _gyrA_, and _gyrB_ genes were amplified and visualized under UV illumination.

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**Table 1. Environmental samples collected from various locations for bacterial isolation.**

| Sample | Location source and sampling date | Number of samples |
|--------|----------------------------------|-------------------|
| Agricultural field soil | [A]: Apple and pear orchards, Mont-Saint-Bruno (IRDA) Quebec, Canada, September 2014 | 4 |
| Agricultural field soil | [B]: Apple and pear orchards, Oka, Quebec, Canada, September, 2014 | 3 |
| Agricultural field soil | [C]: Sherrington, Quebec, Canada, September, 2014 | 4 |
| Agricultural field soil | [D]: Wimauma, Florida, USA, April, 2013 | 1 |
| Agricultural field soil | [E]: Sherrington, Quebec, Canada, November, 2011 | 4 |
| Agricultural field soil | [F]: Wimauma, Florida, USA, July, 2012 | 2 |
| Strawberry leaves | [G]: Dover, Florida, USA, April, 2013 | 1 |
| Tomato leaves and fruits | [H]: Wimauma, Florida, USA, July, 2012 | 3 |
| Apple (McIntosh and Honeycrisp cultivars) and pear (Beauté Flamande cultivar) leaves, stems, and fruits | [I]: Apple and pear orchard, Mont-Saint-Bruno (IRDA), Quebec, Canada, September, 2014 | 4 |
| Tomato leaves and fruits | [J]: Sherrington, Quebec, Canada, November, 2011 | 4 |
| Leaves, stems, and fruits of various plants | [K]: Orchard, Laval, Quebec, Canada, September, 2014 | 4 |
| Flowers, leaves, stems, and soil of Empire, Marshall McIntosh, Cortland, McIntosh, Paula Red, Honeycrisp, Royal Court and Lobo apple cultivars | [L]: Apple and pear orchards, Mont-Saint-Bruno (IRDA), Quebec, Canada, May–September, 2015 | 30 |
| Apple leaves, stems, and soil | [M]: Apple trees, Laval, Quebec, Canada, June, 2015 | 5 |
| Rome apple leaves and stems | [N]: Geneva, New York, USA, July, 2015 | 5 |
| Pear flowers, leaves, and stems | [O]: Laval, Quebec, Canada, May, 2015 | 5 |
| **Total** | | **79** |
fragments were analyzed by agarose gel electrophoresis (Supplemental Tables 1 and 2).

To refine the identification of the *Pseudomonas* spp. isolates, the *rpoB* gene was amplified (Supplemental Table 1) [36,37]. PCR amplifications were carried out in a 25-μL reaction mixture with 1X Taq buffer, 200 μM dNTPs mixture, 0.4 μM of each primer, 1 unit Felda Taq DNA Polymerase, and 50 ng bacterial DNA. The amplifications were performed as described above except that the primer was elongated for 35 s at 72 °C. After DNA amplification, the *rpoB* fragments (240 bp) were analyzed by agarose gel electrophoresis, All PCR products were excised and purified from the agarose gel using a gel extraction kit (Bio Basic Canada Inc., Markham, Ontario, Canada) and sequenced at Institut de recherches cliniques de Mont réal (IRCM). The same primers were used for the initial PCR reaction and the sequencing reactions with 16S rRNA and the *rpoB* gene from the *Pseudomonas* sp. isolates. The *rpoB* fragments from the *Pseudomonas* sp. isolates were cloned into a pGEM-T-Easy Vector [38] (pGEM-T Easy Kit, Promega, Madison, WI, USA) and sequenced using the universal primers Sp6 and T7. The *gyrA* fragments were sequenced with the same primers used in the initial PCR. The *gyrB* fragments were amplified with the universal primers UP-1 and UP-2r and sequenced with the UP-1S and the UP-2Sr primers.

The sequences obtained for each isolate were processed with the BioEdit™ sequence alignment editor (Ibis Therapeutics, Carlsbad, CA, USA) and analyzed with Ribosomal Database Project RDP [https://rdp. cme.msu.edu/seqmatch/seqmatch_intro.jsp] If the sequence identity was >99%, we concluded that the bacterial isolates belonged to the same species. If the sequence identity was >97%, then the strains were classified in the same genus or family.

### 2.10. Biological control products formulation

Six strains were selected from the antagonistic and antimicrobial activity assays based on their ability to control *E. amylovora* in vitro. Each of these products was formulated to suppress the pathogen via the active live strains and the secreted metabolites. The final formulation for each product was broth-based as illustrated below.

### 2.11. Formulation of spore-forming bacteria

To formulate two products based on strains of *P. polymyxa* 273 and *B. amyloliquefaciens* subsp. *plantarum* FL505, Schaeffer’s sporulation medium (SSM) was used [38]. After incubation for 72 h at 30 °C with agitation at 200 rpm, the cultures were heated for 10 min at 80 °C to kill any vegetative bacteria. These two products, each containing sporulated bacteria and supernatant extracellular metabolites, were prepared in triplicate and stored in polypropylene bottles at room temperature (~21 °C). The effect of heating on the activity of cell-free supernatants (CFS) was evaluated (Supplemental Table 1) [36,37]. PCR amplifiers were performed as described above except that the primer was elongated for 35 s at 72 °C. After DNA amplification, the *rpoB* fragments (240 bp) were analyzed by agarose gel electrophoresis, All PCR products were excised and purified from the agarose gel using a gel extraction kit (Bio Basic Canada Inc., Markham, Ontario, Canada) and sequenced at Institut de recherches cliniques de Montréal (IRCM). The same primers were used for the initial PCR reaction and the sequencing reactions with 16S rRNA and the *rpoB* gene from the *Pseudomonas* sp. isolates. The *rpoB* fragments from the *Pseudomonas* sp. isolates were cloned into a pGEM-T-Easy Vector [38] (pGEM-T Easy Kit, Promega, Madison, WI, USA) and sequenced using the universal primers Sp6 and T7. The *gyrA* fragments were sequenced with the same primers used in the initial PCR. The *gyrB* fragments were amplified with the universal primers UP-1 and UP-2r and sequenced with the UP-1S and the UP-2Sr primers.

The sequences obtained for each isolate were processed with the BioEdit™ sequence alignment editor (Ibis Therapeutics, Carlsbad, CA, USA) and analyzed with Ribosomal Database Project RDP [https://rdp. cme.msu.edu/seqmatch/seqmatch_intro.jsp] If the sequence identity was >99%, we concluded that the bacterial isolates belonged to the same species. If the sequence identity was >97%, then the strains were classified in the same genus or family.

### 2.12. Formulation of vegetative bacteria

For the formulation of products based on vegetative bacteria, *P. poae* FL10F, *P. fluorescens* IRDA4F, *P. agglomerans* NY160 and NY130 were cultivated in Difco Nutrient Broth (Becton, Dickinson and Co., Franklin Lanes, NJ, USA) supplemented with 0.5% v/v glycerol to promote the production of secondary metabolites [39]. Each culture was incubated for 2 d at 30 °C with agitation at 200 rpm. Then, NaCl was added at a final concentration of 4% v/v to preserve the bacterial products [40]. Each formulated product, consisting of both vegetative bacteria and their supernatant extracellular metabolites, was prepared in triplicate and stored in polypropylene bottles at room temperature (~21 °C). The same products were formulated as described above but without the addition of NaCl to assess the residual antibacterial action of the isolates (bacteria and metabolites) without preservative.

### 2.13. Stability and survival of stored isolates in vivo

Apple leaves (McIntosh) were disinfested by soaking them in 70% v/v ethanol for 1 min. They were then air-dried under a sterile hood for 30 min. Formulation of test bacteria that had been stored at room temperature (~21 °C) for 3 mo were diluted at a rate of one part product in twenty parts sterile water. One milliliter of this product was applied to each leaf with a trigger sprayer. The leaves were incubated on wet filters for 7 d at 25 °C and 40% RH. Three leaves were selected at t = 0 and another three were selected after 7 d to count CFUs. Each leaf was cut with sterile scissors, soaked in 5 mL NaCl (0.85% v/v), vortexed for 1 min, and incubated for 30 min with shaking at 200 rpm. Dilution series were prepared and used to inoculate TSA count plates. Three replicates were performed for each treatment described above.

### 2.14. Potted tree validation

A trial was conducted at the IRDA research station in Saint-Bruno-de-Montarville, Quebec to evaluate the efficacy of the best isolates previously screened. Inoculum was obtained by suspending a 5-d culture of the virulent strain *E. amylovora* 435 in potassium phosphate buffer (pH = 6.5) in King’s B agar which favors the growth of this phytopathogen [41, 42]. The bacterial density was adjusted to ~1 × 10⁸ CFU mL⁻¹. Actively growing shoots of potted trees (cv. McIntosh grafted onto M26) were inoculated by transversely bisecting the two youngest leaves with scissors dipped in the inoculum. Shoots without at least the last leaf unfurled were not used to ensure uniformity. One or two shoots were inoculated per tree. Two hours after inoculation, candidate biological control agent suspensions were sprayed to runoff onto five trees per treatment using a low-pressure sprayer. The experimental design was completely randomized. Nine treatments were applied in the potted-tree experiment and 5 trees per treatment. Thus, a total of 45 potted trees were used in this experiment. These treatments were sprayed with the following formulations respectively: 1) streptomycin (100 ppm); 2) water only; 3) sterile culture medium; 4) *P. polymyxa* 273; 5) *P. poae* FL10F; 6) *B. amyloliquefaciens* FL505; 7) *P. fluorescens* IRDA 4F; 8) *P. agglomerans* NY130 and 9) *P. agglomerans* NY60.

Inoculated controls were sprayed with the medium used to formulate the candidate biocontrol products. Uninoculated controls were sprayed only with water. The medium applied in Treatment 3 was the same one used to cultivate *P. poae* FL10F, *P. poae* FL10F, *P. agglomerans* NY60, *P. agglomerans* NY130, and *P. fluorescens* IRDA4F. It consisted of Nutrient Broth NB supplemented with 0.5% glycerol (w/w) and a 4% NaCl (w/w). The trees were incubated in a growth chamber at 25 °C and observed 2, 4, 7, and 10 d after treatment. Disease severity (DS) was rated as follows: 0 = absence of necrosis; 1 = necrosis limited to central vein of inoculated leaves; 2 = necrosis extending to petiole; 3 = necrosis reaching shoot; 4 = necrosis reaching other leaves on the inoculated shoot [43]. Fire blight severity scores were reported only 10 d after inoculation. No disease was found on the uninoculated controls. Severity scores were analyzed with a cumulative link mixed model (clmm) in the “ordinal” package of R [44]. Treatment and observation date were used as fixed effects. Individual trees were used as a random effect.

### 3. Results

#### 3.1. Characteristics of the most efficient bacterial strains isolated from the screening

#### 3.1.1. First screening for bacterial isolates

To isolate bacteria antagonistic against *E. amylovora*, the leaves, flowers, stems, and fruits of apples, pears, tomatoes, and strawberries, and agricultural field soil samples, were collected from various locations over a few years period (2011–2015) (Table 1).
3.2. Isolation of microorganisms from environmental samples

A total of 79 environmental samples were analysed. About 5,000 isolates were tested against *E. amylovora* S435 using a direct antagonistic activity assay as the first screening step. Of all the isolates assayed, 205 strains produced inhibition zones of varying diameters on a lawn of *E. amylovora* S435 (Figure 1A). These 205 selected isolates were stored at -80 °C, and the antagonistic screening process was repeated three times with similar results.

For the second screening step, the extracellular antimicrobial activities of the 205 isolates were determined and 32 isolates were retained for their cell-free supernatants (CFS) activities (Figure 1B and Table 2). The CFS of strains FL10F, 273, and FL50S displayed the strongest activity against *E. amylovora* S435. They also inhibited the growth of streptomycin-resistant *E. amylovora* S153 and *E. amylovora* S1605 (Table 3). Different strains of *E. amylovora* resistant and susceptible to streptomycin antibiotic were used in this screening in order to develop a biological control agent with a wide spectrum of activity against the phytopathogen regardless of the trait variations of each strain of *E. amylovora*. Cell-free supernatants from these three isolates formed inhibition halos 25.0–35.0 mm in diameter on *E. amylovora* S435 cultures and were kept for further analyses.

Interestingly, a subgroup of five isolates determined to be strongly inhibitory in the direct antagonism assay (step 1 of screening, Table 4) produced CFS with no visible inhibitory activity against *E. amylovora* S435 (Table 2, bottom).

3.3. Metabolite activity at various dilutions

To increase agar diffusion assay sensitivity and address relative differences in metabolite solubility, CFS of the strains most active against *E. amylovora* (with the largest inhibition zones, Table 2) were diluted by 2-fold and 10-fold and the *E. amylovora* S435 inhibition zone assays were repeated. Certain CFS displayed no inhibition against *E. amylovora* S435 when diluted by 10-fold. Nevertheless, the CFS from six of the isolates retained some inhibitory activity even at a 10-fold dilution (Table 2).

3.4. Identification of active isolates

3.4.1. 16S rRNA

A 1.6-kb 16S rRNA DNA fragment was amplified and sequenced for each bacterial isolate. Thirty-seven bacterial isolates were identified by gene sequencing (Table 2), including 21 isolates belonging to the *Bacillus subtilis* group and 2 isolates belonging to the *Paenibacillus* genus. Analysis of pairwise 16S rDNA sequence alignments were highly similar to each at the genus level but could not be used to distinguish different but closely related bacterial species such as *P. polymyxa*, *P. peoriae*, *P. jamilae*, and *P. kribbensis*. Nine isolates belonging to the fluorescent *Pseudomonas* group and five isolates belonging to the *Pantoea agglomerans* species were also identified.

3.4.2. Further identification of *Pseudomonas* and *Pantoea* strains using Resphera Insight™ (v2.2)

Resphera Insight™ (v2.2) which is a clinical-grade proprietary analysis protocol developed to provide ultra-high-resolution taxonomic assignment of 16S rRNA sequences to species-level membership was used to further identify *Pseudomonas* and *Pantoea* strains. This computationally intensive procedure maintains the capacity for 99.9% sensitivity and >99.5% species-level specificity for hundreds of bacterial strains, and in cases of ambiguous membership, predicts an accurate consensus lineage. The results Resphera Insight™ (v2.2) were supportive to the 16S rRNA findings (Table 5).

3.4.3. Identification of isolates via gyra, gyrB and rpoB gene sequences

The *gyrA* and *gyrB* sequences were used to discriminate the isolates belonging to the *Bacillus subtilis* group. Isolate 50S most closely resembled *Bacillus amyloliquefaciens*; their sequence similarity was 99%. Partial sequencing of the gene encoding the B protein subunit of DNA gyrase (*gyrB*) was performed on the FL3F, FL4F, FL9F, and FL10F isolates, all of which most nearly resemble a *Pseudomonas fluorescens* group strain with sequence similarities of 99–100%. Based on the *rpoB* gene sequence, isolate 273 was 99% similar to a *P. polymyxa* strain.

3.4.4. In vitro competition in co-cultures

The *in vitro* competition was assessed by co-culturing the active strains in liquid with *E. amylovora* S435, S153, and S1605 strains. Not all active strains completely killed each *E. amylovora* strain. However, all three of *E. amylovora* strains were below the detection limit of the enumeration technique following co-cultivation with either *Pantoea agglomerans* NY60 or NY130.

3.4.5. Formulations of products: stability and activity

The objective of this research was to formulate biological control products effective against fire blight on apple and pear trees. Based on the above data, six strains were selected based on their excellent activity against *E. amylovora* for the development of formulations. Each of these products was formulated to have a dual mode of action via 1) live strains and 2) metabolites present in extracellular CFS. When the product is sprayed on the trees, the active metabolites would control *E. amylovora*, while the live bacteria in the formula would grow, colonize, and outcompete any remaining or *forthcoming E. amylovora*.

Figure 1. Antagonistic activity of several bacterial isolates against *E. amylovora* S435. A: Isolates 90, 110, 130, 140, and 160 form clear haloes (inhibition zones) on the *E. amylovora* S435 lawn. B: Antimicrobial activity of two bacterial isolate supernatants CFS against *E. amylovora* S435. Left: isolate FL3F; Right: isolate FL4F.
The long-term survival of bacteria and stability of the antagonistic activities of the six biological control formulations were assessed over a period of 9 months. The products were stored at room temperature (~21 °C) in plastic bottles and were found to be relatively stable during the period. Based on CFU/ml counts, the densities of both fluorescent Pseudomonas spp. strains and both Pantoea strains decreased by ~2 log, and those of those of P. polymyxza 273 and B. amyloliquefaciens FL505 by ~1 log (Table 6). The mean results of three replicates are presented in Table 2. Activities of cell-free culture supernatants against Erwinia amylovora S435.

Table 2. Activities of cell-free culture supernatants against Erwinia amylovora S435.

| Bacterial isolates/16S rRNA gene sequence identification | Source | Medium of isolation | Diameter of growth inhibition zone (including well diameter)*, mm | 2-fold diluted CFS | 10-fold diluted CFS |
|--------------------------------------------------------|--------|---------------------|---------------------------------------------------------------|-------------------|--------------------|
| P. polymyxza 273                                       | [E]    | Benedict            | 26.66 ± 0.34                                                  | 21.00 ± 0.36      | 17.58 ± 0.50       |
| P. polymyxza 344                                       | [J]    | TSA                 | 23.67 ± 0.21                                                  | 17.08 ± 0.27      | no inhibition      |
| B. amyloliquefaciens subsp. plantarum FL505            | [F]    | PCA                 | 28.58 ± 0.33                                                  | 27.16 ± 0.21      | 19.08 ± 0.27       |
| B. amyloliquefaciens 304                               | [E]    | Benedict            | 27.25 ± 0.30                                                  | 20.16 ± 0.30      | no inhibition      |
| Bacillus sp. FD368                                     | [A]    | TSA                 | 27.42 ± 0.41                                                  | 25.16 ± 0.27      | no inhibition      |
| Bacillus sp. 331                                       | [J]    | TSA                 | 28.30 ± 0.42                                                  | 20.50 ± 0.43      | no inhibition      |
| Bacillus sp. FD402                                     | [B]    | TSA                 | 24.50 ± 0.43                                                  | 20.16 ± 0.47      | no inhibition      |
| B. amyloliquefaciens 417                               | [E]    | TSA                 | 27.33 ± 0.60                                                  | 20.16 ± 0.60      | no inhibition      |
| Bacillus sp. 418                                       | [E]    | TSA                 | 26.75 ± 0.31                                                  | 19.58 ± 0.25      | no inhibition      |
| B. subtilis 421                                        | [E]    | TSA                 | 25.16 ± 0.21                                                  | 22.66 ± 0.42      | no inhibition      |
| B. amyloliquefaciens 431                               | [E]    | TSA                 | 27.16 ± 0.40                                                  | 20.16 ± 0.30      | no inhibition      |
| Bacillus sp. FD604                                     | [A]    | TSA                 | 27.41 ± 0.20                                                  | 21.33 ± 0.49      | no inhibition      |
| Bacillus sp. IRDA27                                    | [L]    | TSA                 | 25.50 ± 0.34                                                  | 20.16 ± 0.30      | no inhibition      |
| Bacillus sp. IRDA63                                    | [L]    | TSA                 | 23.50 ± 0.34                                                  | 17.08 ± 0.27      | no inhibition      |
| Bacillus sp. IRDA618                                   | [L]    | TSA                 | 23.50 ± 0.50                                                  | 19.50 ± 0.18      | no inhibition      |
| Bacillus sp. IRDA619                                   | [L]    | TSA                 | 21.50 ± 0.50                                                  | 16.50 ± 0.22      | no inhibition      |
| Bacillus sp. IRDA627                                   | [L]    | TSA                 | 25.50 ± 0.34                                                  | 20.16 ± 0.30      | no inhibition      |
| Bacillus sp. IRDA672                                   | [L]    | TSA                 | 24.00 ± 0.50                                                  | 21.50 ± 0.34      | no inhibition      |
| Bacillus sp. IRDA675                                   | [L]    | TSA                 | 23.50 ± 0.50                                                  | 16.50 ± 0.22      | no inhibition      |
| Bacillus sp. IRDA683                                   | [L]    | TSA                 | 29.16 ± 0.30                                                  | 25.16 ± 0.27      | no inhibition      |
| Bacillus sp. IRDA684                                   | [L]    | TSA                 | 24.00 ± 0.50                                                  | 21.50 ± 0.34      | no inhibition      |
| Bacillus sp. IRDA685                                   | [L]    | TSA                 | 25.50 ± 0.34                                                  | 21.00 ± 0.36      | no inhibition      |
| Bacillus sp. IRDA687                                   | [L]    | TSA                 | 22.50 ± 0.50                                                  | 17.08 ± 0.27      | no inhibition      |
| Pseudomonas poae FL5F                                   | [G]    | PCA                 | 28.10 ± 0.27                                                  | 25.16 ± 0.30      | 19.50 ± 0.18       |
| P. poae FL4F                                           | [G]    | PCA                 | 29.92 ± 0.20                                                  | 23.75 ± 0.31      | 21.50 ± 0.34       |
| P. poae FL9F                                           | [G]    | PCA                 | 26.00 ± 0.26                                                  | 23.50 ± 0.34      | 16.50 ± 0.22       |
| P. poae FL10F                                          | [G]    | PCA                 | 35.08 ± 0.27                                                  | 30.41 ± 0.37      | 25.10 ± 0.27       |
| Pseudomonas fluorescens IRDA4F                         | [L]    | TSA                 | 29.16 ± 0.30                                                  | 19.50 ± 0.18      | no inhibition      |
| Pseudomonas sp. 41                                     | [L]    | TSA                 | 23.25 ± 0.36                                                  | 20.16 ± 0.30      | no inhibition      |
| Pseudomonas sp. 42                                     | [L]    | TSA                 | 23.33 ± 0.35                                                  | 17.08 ± 0.27      | no inhibition      |
| Pseudomonas sp. 43                                     | [L]    | TSA                 | 23.50 ± 0.34                                                  | 19.58 ± 0.25      | no inhibition      |
| Pseudomonas sp. NY1238                                 | [L]    | TSA                 | 21.17 ± 0.40                                                  | 16.50 ± 0.22      | no inhibition      |
| P. aeruginosa NY50                                     | [N]    | TSA                 | No activity                                                   | No activity       | No activity        |
| P. aeruginosa NY60                                     | [N]    | TSA                 | No activity                                                   | No activity       | No activity        |
| P. aeruginosa NY130                                    | [N]    | TSA                 | No activity                                                   | No activity       | No activity        |
| P. aeruginosa IRDA 36                                  | [L]    | TSA                 | No activity                                                   | No activity       | No activity        |
| P. aeruginosa IRDA 59                                  | [L]    | TSA                 | No activity                                                   | No activity       | No activity        |

Tryptic Soy Agar (TSA), Plate Count Agar (PCA). Benedict, ± Standard error of the mean (SEM) of three replicates. * Well diameter = 10 mm.

Table 3. Efficacies of the extracellular metabolites of P. polymyxza 273, B. amyloliquefaciens FL505 and Pseudomonas poae FL10F against E. amylovora S153 and S1605.

| N | Bacterial isolates | Diameter of growth inhibition zone of E. amylovora strains (including well diameter)*, mm |
|---|--------------------|------------------------------------------------------------------------------------------|
|   |                    | S153                                      | S1605                                      |
| 1 | Pseudomonas poae FL505 | 25.00 ± 0.25                             | 25.58 ± 0.37                              |
| 2 | Bacillus amyloliquefaciens subsp. plantarum FL505 | 27.30 ± 0.42                             | 27.83 ± 0.40                              |
| 3 | Pseudomonas poae FL10F  | 29.58 ± 0.52                             | 33.08 ± 0.27                              |

± Standard error of the mean (SEM) of three replicates. * Well diameter = 10 mm.
Table 4. Direct antagonistic activity of Pantoea isolates against E. amylovora S435.

| N  | Bacterial isolates | Pantoea colony diameter (mm) or inhibition zone on E. amylovora S435 lawn (including Pantoea diameter in mm) |
|----|--------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1  | P. agglomerans NY50 | 11.33 ± 0.33/28.33 ± 0.33                                                                                                           |
| 2  | P. agglomerans NY60 | 10.00 ± 0.57/30.66 ± 0.33                                                                                                           |
| 3  | P. agglomerans NY130| 10.66 ± 0.66/31.33 ± 0.66                                                                                                           |
| 4  | P. agglomerans IRDA 36| 10.66 ± 0.66/25.33 ± 0.66                                                                                                           |
| 5  | P. agglomerans IRDA 59| 10.33 ± 0.66/26.33 ± 0.88                                                                                                           |

All strains were isolated on TSA plates. ± Standard error of the mean (SEM) of three replicates.

Table 5. Summary of further Pseudomonas and Pantoea strains identification for isolates FL10F, FL9F, FL3F, IRDA 4F, NY60 and NY130.

| Isolates identification (%) similarity | Pseudomonas poae 99% | Pseudomonas poae 99% | Pseudomonas poae 98% | Fluorescent Pseudomonas sp. 99% |
|--------------------------------------|----------------------|----------------------|----------------------|---------------------------------|
| Resphera Insight                     | FL10F                | FL9F                 | FL3F                 | IRDA 4F                         |

Summary of further Pantoea strains identification for isolates NY60 and NY130

| Isolates identification (%) similarity | Pantoea agglomerans 99% | Pantoea agglomerans 99% |
|--------------------------------------|--------------------------|--------------------------|
| Resphera Insight                     | NY60                     | NY130                    |

Table 6. Stability of the biological control product formulations.

| N  | Biological control product | Concentration of bacteria (CFU/ml) |
|----|----------------------------|-----------------------------------|
|    |                            | Time:Zero 3 months 9 months       |
| 1  | PA. polymyxab 273          | 1.10 ± 0.02 x 10⁷ 1.01 ± 0.01 x 10⁷ 2.00 ± 0.11 x 10⁷ |
| 2  | Bacillus amyloliquefaciens subsp. plantarum FL50S | 2.10 ± 0.10 x 10⁷ 2.00 ± 0.10 x 10⁷ 1.03 ± 0.06 x 10⁷ |
| 3  | Pseudomonas poae FL10F | 1.96 ± 0.08 x 10⁷ 1.96 ± 0.14 x 10⁷ 1.96 ± 0.12 x 10⁷ |
| 4  | Pantoea agglomerans NY130 | 2.56 ± 0.03 x 10⁶ 1.96 ± 0.03 x 10⁶ 0.96 ± 0.13 x 10⁷ |
| 5  | Pantoea agglomerans NY130 | 2.56 ± 0.03 x 10⁶ 1.96 ± 0.03 x 10⁶ 0.96 ± 0.13 x 10⁷ |
| 6  | Pseudomonas fluorescens IRDA4F | 2.00 ± 0.10 x 10⁶ 1.96 ± 0.03 x 10⁶ 1.96 ± 0.12 x 10⁷ |

± Standard error of the mean (SEM) of three replicates.

Table 7. Antimicrobial activity of CFS of the biological control product formulations against E. amylovora S435.

| N  | Biological control product | Diameter of growth inhibition zone (including well diameter) *, mm |
|----|----------------------------|-------------------------------------------------------------------|
|    |                            | Time Zero 3 months 9 months                                      |
| 1  | PA. polymyxab 273          | 26.66 ± 0.34 23.33 ± 0.35 21.67 ± 0.21                          |
| 2  | Bacillus amyloliquefaciens subsp. plantarum FL50S | 28.58 ± 0.33 26.67 ± 0.16 25.30 ± 0.34                          |
| 3  | Pseudomonas poae FL10F | 35.08 ± 0.27 30.00 ± 0.50 29.16 ± 0.30                          |
| 4  | Pseudomonas fluorescens IRDA4F | 29.16 ± 0.30 27.66 ± 0.42 23.25 ± 0.36                          |

± Standard error of the mean (SEM) of three replicates, * Well diameter = 10 mm.
Pantoea strains were not tested for lack of CFS activity.

Table 8. Antagonistic activity of the biological control product formulations.

| N  | Biological control product | Time Zero* | 3 months* | 9 months* |
|----|----------------------------|------------|-----------|-----------|
| 1  | PA. polymyxab 273          | 10.00 ± 0.57/24.66 ± 0.33 11.00 ± 0.00/22.66 ± 0.33 11.33 ± 0.66/22.00 ± 0.00 |
| 2  | Bacillus amyloliquefaciens subsp. plantarum FL50S | 11.00 ± 0.57/26.00 ± 0.00 11.00 ± 0.57/26.66 ± 0.33 11.00 ± 0.00/24.30 ± 0.33 |
| 3  | Pseudomonas poae FL10F | 10.66 ± 0.33/29.66 ± 0.88 9.00 ± 0.00/28.33 ± 0.66 11.00 ± 0.57/28.00 ± 0.57 |
| 4  | Pseudomonas fluorescens IRDA4F | 11.00 ± 0.57/29.66 ± 0.33 10.00 ± 0.00/28.30 ± 0.66 9.00 ± 0.00/26.00 ± 0.57 |
| 5  | Pantoea agglomerans NY130 | 10.00 ± 0.57/30.66 ± 0.33 10.66 ± 0.33/29.50 ± 0.28 10.00 ± 0.57/29.30 ± 0.16 |
| 6  | Pantoea agglomerans NY130 | 10.66 ± 0.16/32.33 ± 0.15 10.00 ± 0.57/30.66 ± 0.33 10.00 ± 0.57/28.50 ± 0.28 ± |

*Biological control product colony diameter (mm)/inhibition zone on E. amylovora S435 lawn (mm). ± Standard error of the mean (SEM) of three replicates.
metabolites. They also demonstrated that the genes encoding that antibacterial pantocin A were found on a 28 kb chromosomal genomic island [49].

Among the 32 strains with active CFS, *P. polymyxa* 273, *B. amyloliquefaciens* subsp. *plantarum* FL505, and four *P. poae* strains (FL10F, FL3F, FL4F, FL9F) all retained their anti-*E. amylovora* inhibition activity even at 10-fold dilution indicating that either the anti-*Erwinia* metabolites are produced at high concentrations or are very effective at low concentrations.

The CFS of *P. poae* FL10F, *P. polymyxa* 273, and *B. amyloliquefaciens* subsp. *plantarum* FL505 demonstrated the strongest antimicrobial activity against three different *E. amylovora* strains, including two streptomycin-resistant strains. The activity of CFS is expected to be attributed to more than a single metabolite; thus, resistance is less likely to develop.

Although many strains produce extracellular inhibitory metabolites, the *Pantoea* isolates demonstrated the strongest antagonistic activity against *E. amylovora* S435. And only *Pantoea* strains NY60 and NY130 when they were co-cultured with *E. amylovora* S453, S153, and S1605 were capable of completely killing the target phytopathogens, presumably by direct interaction. Such effective inhibitory activity of *P. agglomerans* against *E. amylovora* has never been previously reported [41, 49, 50, 51].

Six strains were chosen based on their abilities to control *E. amylovora* in vitro and in vivo for the development of formulations. They were selected from different bacterial genera to increase variability in the formulated strains and to increase their efficacies on apple trees. Each of these formulations consisted of both whole cultures stabilized by heating (to prevent spore formation) and NaCl addition. Both the unidentified active metabolites present in the extracellular milieu maintained activity and a significant proportion of the bacteria remained alive after storage of 9 months at room temperature indicating that these strains can be probably formulated and stored at room temperature with a 9-month shelf life.

A trial was conducted to evaluate the effectiveness of the bacterial formulations against fire blight. Although the formulations were applied two hours after tree inoculation with *E. amylovora*, formulations based on *P. poae* FL10F, *P. agglomerans* NY60 or NY130 were effective and trees had significantly less fire blight than the controls. Importantly, trees treated with formulations based on *P. agglomerans* NY60 or NY130, strains isolated from an apple orchard, were similar to those treated with streptomycin, the standard treatment for fire blight. This suggests that *P. agglomerans* NY60 or NY130 formulations have a probable curative effect in controlling *E. amylovora* [52].

Our bacteria did not have to be exposed to phytopathogenic bacteria such as *E. amylovora* in order to produce cell-free supernatant (CFS) with efficient inhibitory activity. These active CFS are produced when our bacteria are cultivated in TSB even in the absence of a phytopathogen. The activity of the CFS is attributed to the presence of secondary metabolites, not hydrolytic enzymes (Dagher and Déziel, unpublished).

A single formulation of consortia based on the combination of the formulations contained CFS from several active bacteria could be
developed in future experiment. Here, we initially developed and compared the activity of separate formulations for two main reasons. First, we wanted to be closer to a real situation, so using bacterial formulations instead of only live bacteria was the best option. Second, and more importantly, we needed to identify which formulation/biocontrol strain was the most efficient. The results obtained will inform future experiments, including possible synergistic mixtures. However, this is a complex process, because biocontrol bacteria can also be antagonistic to each other.

Details on antimicrobial mechanisms will require further experimentation, ongoing investigations are being conducted. Although our active strains are natural isolates, more work is required on the safety of the biocontrol formulations, which will involve analyses of the whole genome sequences of the most active strains.

5. Conclusions

A major screening was conducted to select bacteria with strong inhibitory activity against *E. amylovora*. Bacterial control agents were isolated from various North American environmental samples and tested for their efficacy against this phytopathogen. Candidate bacteria were isolated from the flowers, leaves, and soil in apple- and pear orchards from springtime bloom to the summer season. In this way, the likelihood of isolating bacteria capable of colonizing the trees and competing against *E. amylovora* would be optimized. The most effective strains were tested in *vitro* and in *vivo*. They were formulated into stable products containing both living cells and their cell-free supernatants. A formulated biological control formulation based on *P. agglomerans* NY60 was not phytotoxic and was highly effective against fire blight relative to the antibiotic streptomycin. Further research is necessary to identify and isolate the gene products responsible for anti-*Erwinia* activity. Other studies are also required to elucidate the modes of action of the *Pantoea* strains whose intact cells are antagonistic against fire blight but whose CFS are not. Finally, field trials must be run to refine and perfect biological control agents which could successfully replace antibiotics and copper for fire blight control in commercial orchards.

Eric Déziel: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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