ORIGINAL ARTICLE

Characterization of the biocontrol activity of three bacterial isolates against the phytopathogen *Erwinia amylovora*

Fadi Dagher[1] | Arvin Nickzad[1] | Jie Zheng[2] | Maria Hoffmann[2] | Eric Déziel[1]

[1]Centre Armand-Frappier Santé Biotechnologie, Institut national de la recherche scientifique (INRS), Laval, Québec H7V 1B7, Canada

[2]US Food and Drug Administration Regulatory Science Center for Food Safety and Applied Nutrition, College Park, Maryland, USA

Correspondence
Eric Déziel, Centre Armand-Frappier Santé Biotechnologie Institut national de la recherche scientifique (INRS), 531, boul. des Prairies, Laval, Qc H7V 1B7, Canada.
Email: eric.deziel@inrs.ca

Abstract

Antibiotics are sprayed on apple and pear orchards to control, among other pathogens, the bacterium *Erwinia amylovora*, the causative agent of fire blight. As with many other pathogens, we observe the emergence of antibiotic-resistant strains of *E. amylovora*. Consequently, growers are looking for alternative solutions to combat fire blight. To find alternatives to antibiotics against this pathogen, we have previously isolated three bacterial strains with antagonistic and extracellular activity against *E. amylovora*, both in vitro and in planta, corresponding to three different bacterial genera: Here, we identified the inhibitory mode of action of each of the three isolates against *E. amylovora*. Isolate *Bacillus amyloliquefaciens* subsp. *plantarum* (now *B. velezensis*) FL50S produces several secondary metabolites including surfactins, iturins, and fengycins. Specifically, we identified oxydifficidin as the most active against *E. amylovora* S435. *Pseudomonas poae* FL10F produces an active extracellular compound against *E. amylovora* S435 that can be attributed to white-line-inducing principle (WLIP), a cyclic lipopeptide belonging to the viscosin subfamily (massetolide E, F, L, or viscosin). *Pantoea agglomerans* NY60 has a direct cell-to-cell antagonistic effect against *E. amylovora* S435. By screening mutants of this strain generated by random transposon insertion with decreased antagonist activity against strain S435, we identified several defective transposants. Of particular interest was a mutant in a gene coding for a Major Facilitator Superfamily (MFS) transporter corresponding to a transmembrane protein predicted to be involved in the extracytoplasmic localization of griseoluteic acid, an intermediate in the biosynthesis of the broad-spectrum phenazine antibiotic D-alanylgriseoluteic acid.

KEYWORDS
apple, antagonism, Fire blight, lipopeptide, oxydifficidin

1 | INTRODUCTION

The bacterial plant pathogen responsible for fire blight is *Erwinia amylovora* (Holtappels et al., 2018; Singh & Khan, 2019). It is a rod-shaped Gram-negative bacterium capable of infecting different host plants in the *Rosaceae* family, including apple and pear trees (Philion, 2018). Annual losses due to fire blight can be significant in many countries (Acimovic et al., 2015). In the United States alone, apple and pear

producers' losses were estimated in millions of dollars during the year 2000 in Michigan (Norelli, Jones, et al., 2003, Norelli, Holleran, et al., 2003) and during 1998 in Washington and northern Oregon due to fire blight (Stockwell, Johnson, Sugar, et al., 2002). Growers have been spraying antibiotics on apple and pear orchards, but are looking for alternatives due to resistance development (Acimovic et al., 2015; McManus & Jones, 1994; McManus et al., 2002). There are several published articles exploring fire blight control and providing insights into the infection and propagation of *E. amylovora* on flowers and leaves; these form a platform for the development of solutions to control this disease. For example, Wright et al. (2001) experimented on a *Pantoea agglomerans* isolate (strain Eh318), which acts through antibiosis by inhibiting the growth of *E. amylovora*. This activity was attributed to the production of two antibiotics, pantocin A and B (Wright et al., 2001). Giddens et al. (2002) characterized a novel phenazine antibiotic gene cluster in *P. agglomerans* Eh1087 effective in the suppression of the fire blight pathogen (Giddens et al., 2002). Smits et al. (2011) studied a *Pantoea vagans* and found two main factors contributing to the biological activity against *E. amylovora*: (a) competition for limiting substrates and (b) production of antibacterial metabolites (Pusey et al., 2011). Niu et al. (2013) showed that polymyxin P is the main active ingredient in the suppression of *Erwinia* species by *Paenibacillus polymyxa* M-1 (Niu et al., 2013). Lee *et al.* (1987) found that culture filtrates of a strain of *Pseudomonas aeruginosa* strongly inhibited the growth of *E. amylovora* (Lee et al., 2013). Zhao et al. (2013) isolated a strain of *Bacillus amyloliquefaciens* subsp. *plantarum* with high activity against *E. carotovora* which infects vegetables in post-harvest (Zhao et al., 2013). *Aureobasidium pullulans* is also used as a biocontrol agent for fire blight protection in organic apple and pear production. They are highly efficient microorganisms that block *Erwinia amylovora* from colonizing the apple blossom. It works through natural competition for space and nutrients between pathogens and antagonists on the blossom (Temple et al., 2020). Bacteriophages, bacteria-infecting viruses, have been recently reconsidered as a biological control tool for preventing bacterial pathogens. A bacteriophage phiEaP-8 was isolated from apple orchard soil and was shown to control efficiently and specifically *E. amylovora* (Park et al., 2018).

Several species belonging to the genera *Pseudomonas*, *Pantoea*, *Bacillus*, and *Paenibacillus* are used to control several plant infectious diseases (El-Hendawy et al., 2005; Stockwell, Johnson, Loper, et al., 2002). They have been tested against phytopathogenic bacteria and have shown antagonistic activity against most phytopathogens (Allard et al., 2014; Mansfield et al., 2012; Pusey et al., 2009; Yin et al., 2011). Several effective inhibitory metabolites produced by the above active bacteria have been described, especially in *Bacillus* and *Paenibacillus* (Zimmerman et al., 1987). Polyketides are one important class of microbial secondary metabolites with a wide spectrum of antibacterial and antifungal activities in an agricultural context (Chen et al., 2006; Olishevska et al., 2019). They include many active ingredients such as antibiotics. Species belonging to the genera *Bacillus* and *Paenibacillus* also synthesize non-ribosomal peptides (NRPs). *Bacillus subtilis*, for example, synthesizes surfactin

belonging to the class of lipopeptides (Fan et al., 2017). Several species of fluorescent *Pseudomonas* are recognized for their ability to produce NRP-type secondary metabolites with antibacterial and antifungal characteristics (Omoboye et al., 2019). Several species of *Pseudomonas* and *Bacillus* produce lipopeptides that function not only in antagonism against pathogens but also in surface spreading motility, leading to the colonization of new habitats and the development of highly structured biofilms (Hennessy et al., 2017; Nielsen et al., 2002).

We have previously reported the isolation of three bacterial strains with antagonist activity against *E. amylovora* both *in vitro* and *in planta*: (1) *Pantoea agglomerans* NY60, (2) *Pseudomonas poae* FL10F and (3) *Bacillus amyloliquefaciens* subsp. *plantarum* FL50S (Dagher et al., 2020). Here, we characterized the inhibitory mode of action of each of these strains against *E. amylovora*.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and culture conditions

The bacterial strains used in this study are as follows: *B. amyloliquefaciens* subsp. *plantarum* FL50S was isolated from agricultural field soil in Wimauma, Florida, USA, *P. poae* FL10F was isolated from strawberry leaves collected in Dover, Florida, USA, *P. agglomerans* NY60 was isolated from Rome apple leaves collected in Geneva, New York, USA and *E. amylovora* S435 was originally isolated from an infected apple tree and supplied by the Institut de recherche et développement en agroenvironnement (IRDA), Québec, Canada). We have recently reported the characterization of the three antagonistic strains (Dagher et al., 2020). Unless otherwise specified, the bacteria were routinely grown from frozen glycerol stocks by culturing in 3 ml tryptic soy broth (TSB) (BD) overnight in test tubes incubated at 30°C and shaking in a TC-7 roller drum (New Brunswick Scientific Co., New Brunswick, NJ) at 240 rpm. For inoculation, appropriate aliquots of each overnight culture were used to inoculate culture media by adjusting the optical density at 600 nm (OD_{600}) to 0.01, corresponding to about 8×10^6 CFU/ml.

2.2 | Whole-genome sequencing

Genomic DNA was isolated from an overnight culture of each strain using a Qiagen DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA). Genome sequencing was performed using the Illumina MiSeq sequencing system (Illumina, San Diego, CA), achieving >50X average genome coverage. De novo assembly was achieved for each genome using SPAdes 3.0.0 (St. Petersburg genome assembler), and annotated with the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html). The taxonomy of each isolate was assigned using Kraken (Wood & Salzberg, 2014).

2.3 | PacBio sequencing and assembly

Single-molecule, real-time sequencing offers longer read lengths making it well-suited for unsolved problems in genome, transcriptome, and epigenetics research. The highly contiguous sequencing can close gaps in current reference assemblies and characterize structural variation (SV) in genomes (Ee et al., 2014; Rhoads & Au, 2015).

The resulting assembled sequences were polished with Illumina reads using a combination of BWA version 0.7.17-r1188 (Chen et al., 2009), SAMtools version 1.9 (Chen et al., 2009), and Pilon version 1.22 (Walker et al., 2014).

2.4 | Secondary metabolite gene cluster prediction and analysis

Genome mining of biosynthetic gene clusters included non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), hybrid PKS/NRPS, siderophores, and bacteriocins were predicted with antibiotics & Secondary Metabolite Analysis SHell (antiSMASH) (Weber et al., 2015) webserver version 5 (http://antismash.secondarymetabolites.org/).

2.5 | Production of secondary metabolites

The production of metabolites by bacterial isolates was carried out in 2 L conical flasks with 500 ml of the respective medium. *P. agglomerans* NY60 was cultivated in Difco Nutrient Broth (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). King's B medium was used as a production medium for *P. poae* FL10F isolate (King et al., 1954), while Landy medium was used for cultivation of *B. amyloliquefaciens* subsp. *plantarum* FL50S (Ben Ayed et al., 2015; Gordillo et al., 2009). Landy medium contained: 20 g/L dextrose, 5.0 g/L L-glutamic acid, 1.0 g/L yeast extract, 1.0 g/L K_2HPO_4, 0.5 g/L $MgSO_4$ ($7H_2O$), 0.5 g/L KCl, 1.6 mg/L $CuSO_4$, 1.2 mg/L $Fe_2(SO_4)_3$, 0.4 mg/L $MnSO_4$. All cultures were shaken at 30°C for 48 h at 200–250 rpm.

To obtain cell-free supernatants, cultures of all three strains were centrifuged at 17,700 × *g* for 1 h. The pellets were discarded and the supernatants were filtered using the Stericup vacuum filtration system (0.2 μm). Fresh cell-free supernatants were used for the present research, but they could also be stored at +4°C for 1 week, or at −20°C for 6 months while keeping their activity.

2.6 | Bioassay-guided fractionation and isolation of active metabolites

The cell-free medium supernatant was applied to 40 g of pre-equilibrated Amberlite XAD-16 resin packed in a column. The XAD-16 column was subsequently washed with 1 L of H_2O and then eluted with 1 L of 100% methanol. The methanolic elution was evaporated to dryness by rotary evaporation, and the brown residue was redissolved in 5 ml of purified water (MilliQ system; Millipore). The concentrated solution was then applied onto a 12 gram Biotage® SNAP Ultra C18 column for fractionation using reverse-phase flash chromatography on a Biotage Isorela One instrument (Stockholm, Sweden). The separation was performed using a linear gradient of acetonitrile from 5% to 100% over 50 min at 15 ml/min. To identify column fractions containing active compounds, each fraction was evaporated to dryness by rotary evaporation and redissolved in 2 ml of MilliQ water. The bioactivity of these fractions was further determined by performing agar disk diffusion assays.

2.7 | The activity of cell-free supernatants and fractions against *Erwinia amylovora* S435

To estimate the activity of bacterial metabolites and fractions against *E. amylovora* S435, an agar disk diffusion assay was performed. Blank paper disks (d = 6 mm) were saturated by cell-free supernatants or fractions (20 μl), then air-dried in a biosafety cabinet for 30 min. The antimicrobial activity was then tested by placing the disk on a lawn of freshly inoculated *E. amylovora* S435 TSB agar plate and measuring the growth inhibition area (mm²) after 48 h of cultivation at room temperature (~21°C). This assay was performed in triplicate.

2.8 | LC-ESI-MS/MS analyses

The cell-free supernatants and active fractions were further analyzed by high-performance liquid chromatography (HPLC; Waters 2795) equipped with a 250 × 4.6 mm i.d. Luna Omega Polar C18 reversed-phase column (particle size 3 μm) using a 1% acetic acid–acetonitrile gradient at a flow rate of 500 μl/min. The detector was a quadrupole mass spectrometer (Quattro Premier XE, Waters). Analyses were carried out in the positive electrospray ionization (ESI) mode with a mass-to-charge ratio (m/z) window ranging from 130–1930. Collision-induced dissociation (CID) MS/MS experiments were performed using argon gas at various collision energies.

2.9 | Random transposon mutagenesis and colony selection

Random transposon insertions in the *P. agglomerans* NY60 chromosome were generated by mating NY60 with DAP-auxotroph *E. coli* strain χ7213 carrying the pUT/mini-Tn5 Sm/Sp plasmid on Lysogeny broth (LB) agar plates supplemented with DAP at 37°C (de Lorenzo et al., 1990). Transposants were selected on TSA plates containing spectinomycin (15 μg/ml). The plates were incubated at room temperature (~21°C) for 2 days. Thereafter, a lawn of lab-derived streptomycin-resistant *E. amylovora* S435 was spread onto TSA

TABLE 1 Identified biosynthetic gene cluster regions in the genome of *B. velezensis* FL50S

Region on genome	Biosynthetic gene cluster type	Most similar known cluster from the MiBIG database	Similarity[a]	MIBiG accession number
302440–377131	NRPS	Surfactin	82%	BGC0000433
4851028–4956922	NRPS, transAT-PKS	Fengycin	80%	BGC0001095
4851028–4956922	NRPS, transAT-PKS	Iturin	88%	BGC0001098
2290903–2384088	transAT-PKS	Difficidin	100%	BGC0000176
1390407–1478530	transAT-PKS	Macrolactin	100%	BGC0000181
1698501–2006499	transAT-PKS, NRPS	Bacillaene	85%	BGC0001089
4554904–4605413	NRPS	Bacillibactin	100%	BGC0000309
3598088–3639506	other	Bacilysin	100%	BGC0001184

Abbreviations: MIBiG, The Minimum Information about a Biosynthetic Gene cluster https://mibig.secondarymetabolites.org/; NRPS, Nonribosomal peptides synthetases.

transAT-PKS, Polyketide synthase.

[a]Similarity shows the % of sequence similarity of the region on the genome to the entries in the MIBiG database using the ClusterBlast algorithm described by Medema et al. (2011)

TABLE 2 Identified biosynthetic gene cluster regions in the genome of *P. poae* FL10F

Region on genome	Biosynthetic gene cluster type	Most similar known cluster from the MiBIG database	Similarity[a]	MIBiG accession number
1706151–1809187	NRPS	Viscosin	43%	BGC0001312
1981700–2032473	NRPS	Pyochelin	100%	BGC0000412
3613987–3659622	NRPS	Bananamide 1	50%	BGC0001346
4133067–4179775	NRPS	Safracin A / Safracin B	100%	BGC0000421
6051250–6104146	NRPS	Pyoverdin	10%	BGC0000413

Abbreviations: MIBiG, The Minimum Information about a Biosynthetic Gene cluster https://mibig.secondarymetabolites.org/; NRPS, Nonribosomal peptides synthetases.

[a]Similarity shows the % of sequence similarity of the region on the genome to the entries in the MIBiG database using the ClusterBlast algorithm described by Medema et al. (2011)

plates using sterile cotton-tipped applicators. After incubation for another 2 days at 21°C, transposants forming smaller or no inhibition zones on the *E. amylovora* S435 lawns were selected for the determination of the transposon insertion site.

2.10 | Identification transposon insertions sites using transposon insertion sequencing (Tn-seq)

Genomic DNA of eight candidate transposants was extracted from cultures using a DNA extraction kit and pooled together. DNA concentrations were determined using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies). DNA was sequenced at the Génome Québec Innovation Centre, McGill University. DNA samples were generated using the NEB Next Ultra II DNA Library Prep Kit for Illumina (New England BioLabs) as per the manufacture protocol. TruSeq adapters and PCR primers were purchased from IDT. Size selection of libraries containing the desired insert size was obtained using SPRI select beads (Beckman Coulter). Briefly, genomic DNA was fragmented and tagged with adapter sequence via one enzymatic reaction (tagmentation). Thereafter, PCR was used to amplify the region between the end of the insertion (primer TnErwinia-CS1:

(5′ ACACTGACGACATGGTTCTACA**GCGGCCGCACTTGTGTATAA** 3′ [transposon-specific sequence is in Bold])), and the Illumina adapter with primer 2 (5′ TACGGTAGCAGAGACTTGG TCTCTAGCATAGAGTGCGTAGCTCTGCT 3′) to enrich for transposon insertion sites and allow multiplex sequencing. The thermocycler program was 94°C for 2 min, 94°C for 30 s, 55°C for 30 s 72°C for 30 s for 33 cycles, and 72°C for 7 min. This region was reamplified to add the Illumina adapters for MiSeq sequencing: PE1-CS1 (AGATCGGAAGAGCACACGTCTGAACTCCAGTCACAC ACTGACGACATGGTTCTACA) and primer 2. Thereafter, sequencing was performed on an Illumina MiSeq using the MiSeq Reagent Kit v2 Kit (500-cycles). To find the transposon insertion sites, 288,889 reads were mapped to *P. agglomerans* NY60 genome (CP034469.1).

3 | RESULTS

3.1 | Whole-genome sequencing

We have previously reported the identification of three bacterial isolates presenting an effective inhibitory activity against the phytopathogen *E. amylovora* (Dagher et al., 2020). We deposited

these bacterial isolates at the Center for Food Safety and Applied Nutrition (CFSAN), United States Food and Drug Administration. *Bacillus amyloliquefaciens* subsp. *plantarum* FL50S was reclassified as *Bacillus velezensis* FL50S, a strain corresponding to CFSAN strain CFSAN034340. Isolate FL10F was classified as *Pseudomonas poae* FL10F, a strain corresponding to CFSAN strain CFSAN034337. Isolate NY60 was classified as *Pantoea agglomerans* NY60, a strain corresponding to CFSAN strain CFSAN047153 (Dagher et al., 2020).

3.2 | Genome mining studies

We next wanted to identify the mode of action of these potential biocontrol agents.

Genome mining for biosynthetic gene clusters (BGC) was performed, including for NRPSs and other secondary metabolites, using the antiSMASH 5.0 prediction tool. The summary of the predicted secondary metabolites for strains *B. velezensis* FL50S and *P. poae* FL10F are shown in Tables 1 and 2, respectively.

For strain FL50S, a total of 15 putative BGCs were predicted, of which 8 correspond to known functions, including non-ribosomal peptide synthetases (NRPSs) attributed to the biosynthesis of surfactin, iturin, and fengycin, and polyketide synthases (PKSs) encoding for difficidin, macrolactin, and bacillaene (Table 1). For *Pseudomonas poae* FL10F, a total of 5 NRPSs gene clusters were predicted. Among them, two unlinked NRPS gene clusters with 100% similarity to viscosin and bananamide 1 gene clusters coding for NRPS responsible for the biosynthesis of cyclic lipopeptides (CLP) were identified (Table 2).

Since the activity of *Pantoea agglomerans* NY60 was found to be attributed to a direct cell-to-cell antagonistic effect against *E. amylovora* S435 and not to its cell-free supernatant (Dagher et al., 2020), the antiSMASH analysis did not reveal many secondary metabolite genes with antibacterial properties.

3.3 | Identification of metabolites and their inhibitory activity

The antiSMASH analysis suggests that *B. velezensis* FL50S has the biosynthetic machinery for the production of several CLPs and polyketides. To verify the antiSMASH results and see if these gene clusters are functional, cell-free supernatant of *B. velezensis* FL50S grown in TSB was analyzed by HPLC-ESI MS/MS. In accordance with antiSMASH results, MS spectra of *B. velezensis* FL50S indicate that it produces three families of CLP: surfactins, iturins, and fengycins. CLPs were identified by comparing the detected peaks with those from literature and the actual mass of CLP (Deng et al., 2017; Rokni-Zadeh et al., 2012). Pseudomolecular ion peaks of surfactin were observed at m/z 994.6, 1008.6, 1022.7, and 1036.8, while those of iturin were observed at m/z 1043.6, 1057.6, and 1071.6, and doubly charged pseudomolecular ion peak of fengycins were observed at 718.4, 725.4, 732.4, 739.4, 746.6, 753.6 (Figure 1). Regarding polyketides, only the oxidized form of difficidin, oxydifficidin, could be identified (Figure 2). Chen *et al.* (2006) reported that difficidin and oxydifficidin can only be detected in their deprotonated forms ([M-H]$^-$ = 543.4 and 559.3) in the negative ionization mode (Chen et al., 2006); in positive mode, we detected oxydifficidin in its dephosphorylated (m/z 463.4) and dimer (m/z 1121.3) species (Figure 2) (Wilson et al., 1987).

We performed activity-guided fractionation of the crude extracellular extract of FL50S to identify the *E. amylovora* -inhibiting metabolites. Out of 30 fractions of *B. velezensis* FL50S, four displayed activity against *E. amylovora* S435 (Table A1), with fraction #21 causing the largest inhibition zone. Performing the HPLC-ESI MS analyses on active fractions revealed that the polyketide oxydifficidin is the main inhibitory metabolite active against *E. amylovora* S435.

Three out of 30 fractions of *P. poae* FL10F displayed activity against *E. amylovora* S435 (Table A2) with fraction #20 causing the largest inhibition zone. Analyzing the MS spectra of the fractions of *P. poae* FL10F
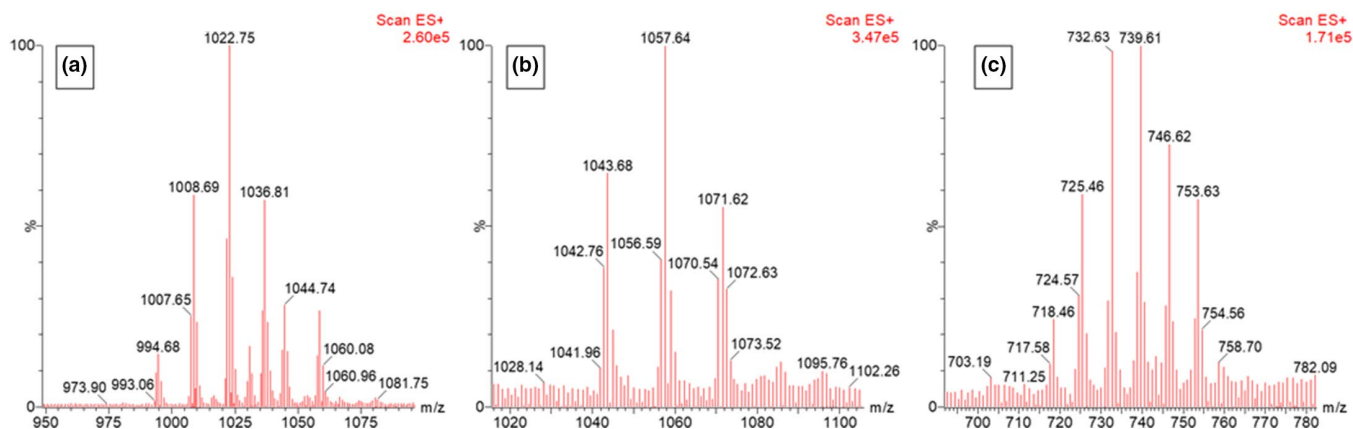

FIGURE 1 MS spectra of three families of cyclic lipopeptides produced by *B. velezensis* FL50S. (a) surfactins (b) iturins and (c) fengycins detected in positive ionization mode. In the case of fengycins, peaks correspond to the doubly charged ions

active against *E. amylovora* S435 showed the presence of a major pseudomolecular ion peak at m/z 1127.1 and some minor peaks including 1113.1 [M + H]⁺ with 14 unit mass difference with the major peak (Figure 3). Collision-induced dissociation tandem MS/MS of the major pseudomolecular ion peak at m/z 1127.1, combined with antiSMASH amino acid sequence prediction of a putative product of two unlinked NRPS gene clusters involved in the biosynthesis of cyclic lipopeptides, suggest that the 1127.1 peak corresponds to the white-line-inducing principle (WLIP) (Coraiola et al., 2006), a cyclic lipodepsipeptide belonging to the viscosin subfamily (Nguyen et al., 2016) (Figure 4).

3.4 | Screening for transposon mutants of *P. agglomerans* NY60 with decreased antagonist activity against *E. amylovora* S435

Regarding the *P. agglomerans* NY60 strain, while its cell-free supernatant from nutrient broth culture shows no activity against *E. amylovora* S435, it is still able to produce an inhibition zone on the lawn of *E. amylovora* S435 on TSA agar plates (Dagher et al., 2020). The co-culturing of *P. agglomerans* NY60 strain in the presence of *E. amylovora* S435 did not induce the production of free-cell supernatant active against *E. amylovora* S435, suggesting that the *P. agglomerans* NY60 inhibitory activity requires direct contact with live cells (Dagher et al., 2020).

To identify the functions responsible for the antagonistic activity of this strain, random mutagenesis of *P. agglomerans* NY60 was performed via insertion of a mini-Tn*5* transposon. Five transposants with reduced antagonistic activity were identified (Table A3). Using transposon insertion sequencing (Tn-seq), the transposon insertion sites of these mutants were mapped, leading to the identification of few genes that altered the production of antagonistic functions (Table 3).

Interestingly, one of the transposants has an insertion in a gene predicted to code for an MFS transporter located on the 211 kbp plasmid (CP034471) carried by strain NY60. BLASTing the sequence indicated that it matches the sequence for the EhpJ gene in *Pantoea agglomerans* (*Erwinia herbicola*) Eh1087 which is a transmembrane protein predicted to be involved in the extracytoplasmic localization of griseoluteic acid, an intermediate in the biosynthesis of the potent broad-spectrum phenazine antibiotic D-alanylgriseoluteic acid (AGA) (Giddens et al., 2002).
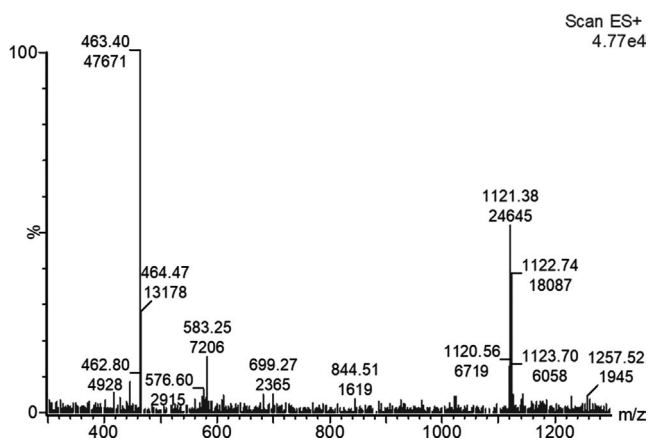
4 | DISCUSSION

The biocontrol activity of three bacterial isolates presenting an effective inhibitory activity against the phytopathogen *E. amylovora* was characterized.

For strain *B. velezensis* FL50S, through the HPLC-ESI MS analyses on fractions obtained from activity-guided fractionation of the crude extracellular metabolite, we found that the polyketide oxydifficidin is the major active metabolite against *E. amylovora* S435.
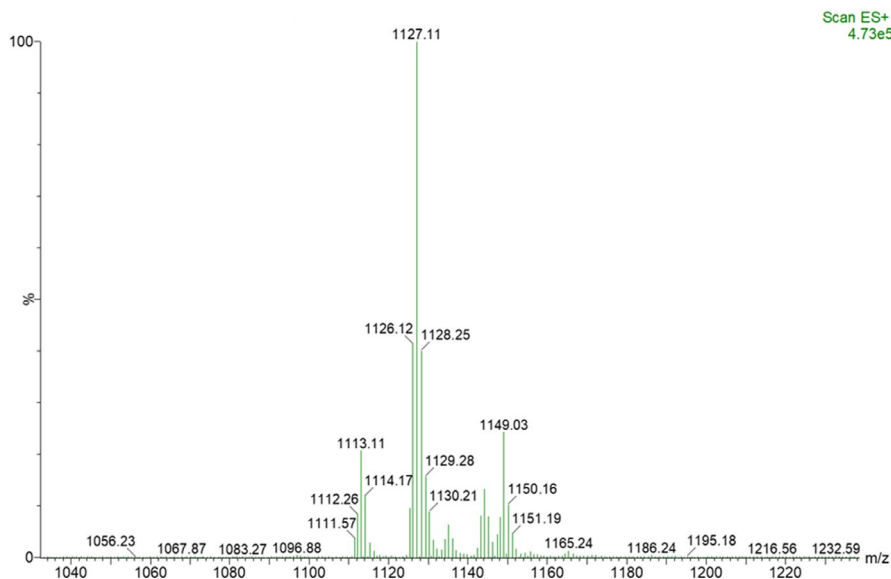

FIGURE 2 MS spectra of produced Oxydifficidin in the supernatant of *B. velezensis* FL50S culture detected in positive ionization mode

FIGURE 3 MS spectra of a fraction of *P. poae* FL10F culture extract active against *E. amylovora* S435
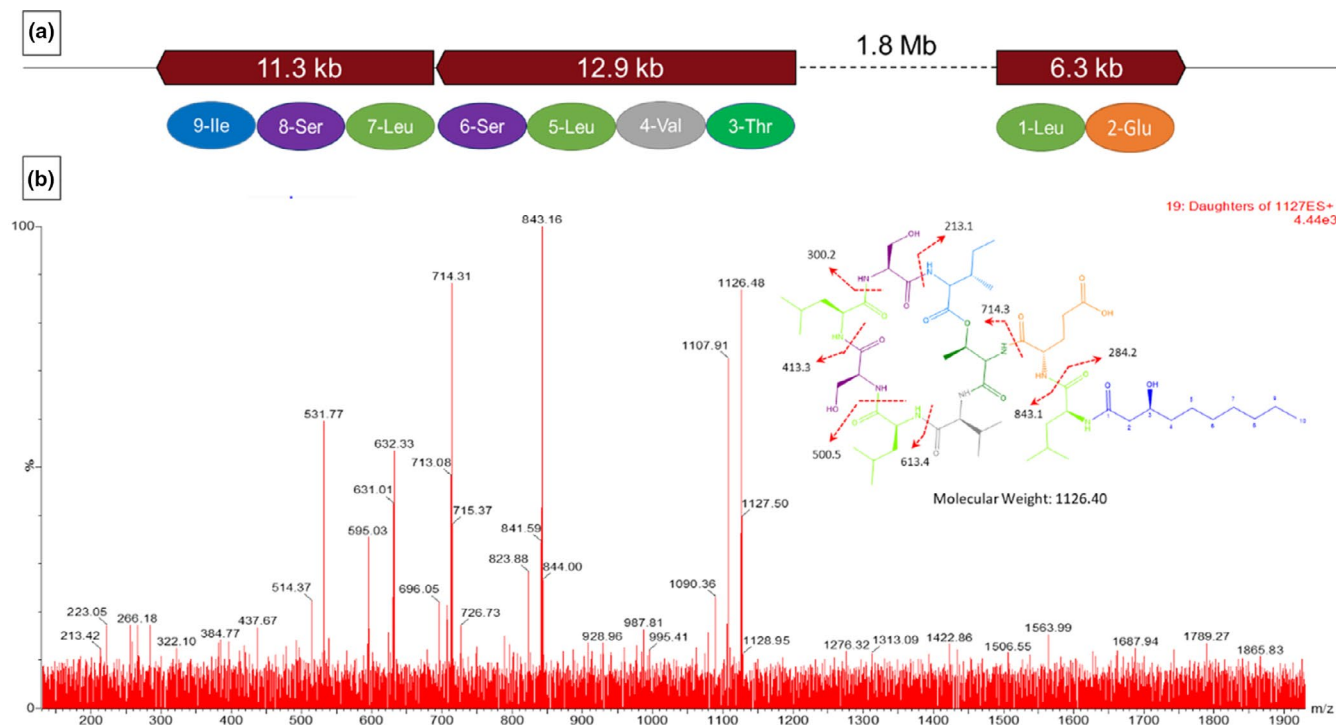
FIGURE 4 Biosynthetic gene clusters of *Pseudomonas poae* FL10F corresponding to the viscosin and bananamide 1 by antiSMASH. (a) Organization of CLP biosynthetic gene clusters of *Pseudomonas poae* FL10F corresponding to the viscosin and bananamide 1 gene clusters (BGC0001312 and BGC0001346 respectively) as identified by antiSMASH. (b) MS/MS fragmentation spectrum of the parent ion peak 1127.3 (m/z) at different collision energies in positive ionization mode. The daughter ion peaks are indicated on the proposed structure

TABLE 3 Identified transposon insertion sites in the genome of *P. agglomerans* NY60

Genome	Transposon insertion site	Locus tag	Putative *P. agglomerans* ortholog
CP034469 Chromosome			
	474915	D1628_02140	TonB-dependent siderophore receptor
	570285	D1628_02530	DNA sulfur modification protein *DndD*
	1416458	D1628_06515	Glycosyltransferase
	1526741	D1628_07110	Flagellar hook-filament junction protein FlgL
CP034471 Plasmid			
	97491	D1628_22515	MFS transporter

Accordingly, the corresponding biosynthetic genes were identified on the genome of FL50S. Oxydifficidin was previously known to be effective against plant phytopathogens such as *Ralstonia solanacearum*, a causal agent of tomato bacterial wilt (Im et al., 2020); however, to the best of our knowledge, this study is the first to show the specific activity of oxydifficidin on *E. amylovora*.

Our results demonstrate that *P. poae* FL10F produces an extracellular compound active against *E. amylovora* S435 that can be attributed to white-line-inducing principle (WLIP), a lipodepsipeptide belonging to the viscosin subfamily, which includes massetolides E, F, L, and viscosin. WLIP a member of the viscosin group of cyclic liponondepsipeptides featuring a Glu2 amino acid with both

antifungal as well as antibacterial activity (against gram-positive bacteria) (De Vleeschouwer et al., 2016; Lo Cantore et al., 2006). In addition to linking the known phenotypes of white line production and hemolytic activity of a WLIP producer with WLIP biosynthesis, additional properties of ecological relevance conferred by WLIP production were published, namely, antagonism against *Xanthomonas* as well as involvement in swarming and biofilm formation (Rokni-Zadeh et al., 2012). Pseudomonads produce molecules that scavenge nutrients, sense population density, and enhance or inhibit the growth of competing microorganisms (Nguyen et al., 2016). They produce several surface-active lipopeptides showing antimicrobial properties but that also facilitate surface motility and influence biofilm formation.

In contrast with the other two bacteria, culture supernatants of *P. agglomerans* NY60 do not inhibit the growth of *E. amylovora* (Dagher et al., 2020). Therefore, we could not use an approach based on activity-guided fractionation of culture fluids to identify the mode of action of this isolate. Instead, we performed random mutagenesis coupled with screening to identify mutants with defective inhibitory activities. Among the five transposants with reduced activity against *E. amylovora* we identified, one mutant has its transposon inserted in the middle of an operon encoding the biosynthesis of broad-spectrum phenazine antibiotic D-alanylgriseoluteic acid (AGA).

In phenazine-producing species like *P. agglomerans* Eh1087, the *phz* genes are on a plasmid. Its main phenazine product, AGA, acts in *P. agglomerans* like a typical antibiotic and is employed in competition with closely related bacterial species in its ecological niche. Interestingly, AGA itself is toxic to *P. agglomerans* Eh1087, and the *phz* pathway encodes a special phenazine-binding protein, EhpR, that prevents self-poisoning of the producer (Giddens et al., 2003; Mavrodi et al., 2010). Very recently, Mechan et al. (2020) also have pointed to, using a UV-mutagenesis screen, the AGA synthesis gene cluster as being at the base of the antimicrobial activity of the *P. agglomerans* isolate against *E. amylovora* (Mechan Llontop et al., 2020). Our data are also consistent with the literature and the emerging view of the antimicrobial activity of AGA against the phytopathogen *E. amylovora* (Giddens & Bean, 2007).

More research is needed to better understand the mode of action of our strains against *E. amylovora*, as well as the factors contributing to the expression of these inhibitory functions.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Fadi Dagher: Conceptualization (lead); Formal analysis (lead); Investigation (lead); Writing-original draft (lead); Writing-review & editing (lead). **Arvin Nickzad:** Formal analysis (equal); Investigation (equal); Methodology (equal); Validation (equal); Visualization (equal); Writing-original draft (supporting); Writing-review & editing (supporting). **Jie Zheng:** Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Resources (supporting); Writing-review & editing (supporting). **Maria Hoffmann:** Formal analysis (supporting); Methodology (supporting); Resources (supporting). **Eric Déziel:** Conceptualization (equal); Funding acquisition (lead); Methodology (supporting); Project administration (lead); Supervision (lead); Writing-review & editing (equal).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

The sequences of strain *P. agglomerans* NY60 and *B. velezensis* FL50S are available in GenBank under the accession numbers: CP034469 (https://www.ncbi.nlm.nih.gov/nuccore/CP034469) and LYNC00000000 (https://www.ncbi.nlm.nih.gov/nuccore/LYNC00000000), respectively.

ORCID

Eric Déziel https://orcid.org/0000-0002-4609-0115

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APPENDIX

TABLE A1 HPLC-ESI MS analyses- Activity of fractions of *B. velezensis* FL50S against *E. amylovora* S435

Fraction number	Diameter of inhibition zone of *E. amylovora* S435 strain (including disk diameter)*, mm
#1	0.00 ± 0.00
#2	0.00 ± 0.00
#3	0.00 ± 0.00
#4	0.00 ± 0.00
#5	0.00 ± 0.00
#6	0.00 ± 0.00
#7	0.00 ± 0.00
#8	0.00 ± 0.00
#9	0.00 ± 0.00
#10	**8.00 ± 0.50**
#11	0.00 ± 0.00
#12	0.00 ± 0.00
#13	0.00 ± 0.00
#14	0.00 ± 0.00
#15	0.00 ± 0.00
#16	0.00 ± 0.00
#17	**9.00 ± 0.50**
#18	0.00 ± 0.00
#19	0.00 ± 0.00
#20	0.00 ± 0.00
#21	**19.00 ± 0.50**
#22	**9.00 ± 0.50**
#23	0.00 ± 0.00
#24	0.00 ± 0.00
#25	0.00 ± 0.00
#26	0.00 ± 0.00
#27	0.00 ± 0.00
#28	0.00 ± 0.00
#29	0.00 ± 0.00
#30	0.00 ± 0.00

Note: ±Standard error of the mean (SEM) of three replicates, *Disk diameter = 6 mm (Joseph et al., 2011; Justesen et al., 2013)

TABLE A2 HPLC-ESI MS analyses- Activity of fractions of *Pseudomonas poae* FL10F against *E. amylovora* S435

Fraction number	Diameter of inhibition zone of *E. amylovora* S435 strain (including disk diameter)*, mm
#1	0.00 ± 0.00
#2	**8.00 ± 0.50**
#3	0.00 ± 0.00
#4	0.00 ± 0.00
#5	**9.00 ± 0.50**
#6	0.00 ± 0.00
#7	0.00 ± 0.00
#8	0.00 ± 0.00
#9	0.00 ± 0.00
#10	0.00 ± 0.00
#11	0.00 ± 0.00
#12	0.00 ± 0.00
#13	0.00 ± 0.00
#14	0.00 ± 0.00
#15	0.00 ± 0.00
#16	0.00 ± 0.00
#17	0.00 ± 0.00
#18	0.00 ± 0.00
#19	0.00 ± 0.00
#20	**22.00 ± 0.50**
#21	0.00 ± 0.00
#22	0.00 ± 0.00
#23	0.00 ± 0.00
#24	0.00 ± 0.00
#25	0.00 ± 0.00
#26	0.00 ± 0.00
#27	0.00 ± 0.00
#28	0.00 ± 0.00
#29	0.00 ± 0.00
#30	0.00 ± 0.00

Note: ±Standard error of the mean (SEM) of three replicates, *Disk diameter = 6 mm

TABLE A3 Direct antagonistic activity of 5 transposants with reduced antagonistic activity against *E. amylovora* S435 on TSA plates

Transposons	*Pantoea* colony diameter (mm) / inhibition zone on *E. amylovora* S435 lawn (including *Pantoea* diameter in mm)
Transposon #1	10.00 ± 0.50 / 20.0 ± 0.50
Transposon #2	11.00 ± 0.50 / 17.0 ± 0.50
Transposon #3	10.00 ± 0.50 / 24.0 ± 0.50
Transposon #4	9.00 ± 0.50 / 16.0 ± 0.50
Transposon #5	12.00 ± 0.50 / 18.0 ± 0.50
P. agglomerans NY60	10.00 ± 0.50 / 30.0 ± 0.50

Note: Tryptic Soy Agar (TSA) Plate, ±Standard error of the mean (SEM) of three replicates.