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Institut National de la Recherche Scientifique

DÉVELOPPEMENT DE PESTICIDES BIOLOGIQUES ACTIFS CONTRE DES BACTÉRIES PHYTOPATHOGÈNES

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Thèse présentée pour l'obtention

du grade de Philosophie Doctor (Ph.D.) en biologie

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RÉSUMÉ

Des antibiotiques sont pulvérisés dans les vergers de pommiers et de poiriers pour lutter, entre autres, contre la bactérie phytopathogène *Erwinia amylovora,* l'agent responsable de la maladie nommée «feu bactérien» ou «brûlure bactérienne». Comme pour de nombreux autres agents pathogènes, on observe l'émergence de souches d'*E. amylovora* résistantes aux antibiotiques. Par conséquent, les producteurs recherchent des solutions alternatives pour combattre le feu bactérien. Dans un effort pour trouver des alternatives aux antibiotiques contre ce pathogène, l'objectif de cette thèse était de développer un agent de contrôle biologique efficace contre *E. amylovora*, par exemple en colonisant la plante hôte et excluant ce pathogène. Idéalement, l'agent trouvé ne causerait pas de phytotoxicité, présenterait une activité à large spectre et serait peu susceptible d'induire une résistance chez le pathogène. À cette fin, plusieurs isolats bactériens provenant de divers échantillons environnementaux ont été criblés afin d'identifier des candidats appropriés antagonistes à *E. amylovora*. Nous avons prélevé des bactéries sur les fleurs, les feuilles et le sol de vergers de pommiers et de poiriers, de la période de floraison au printemps jusqu'à l'été. Les isolats les plus efficaces ont été identifiés comme appartenant aux espèces *Pseudomonas poae*, *Paenibacillus polymyxa*, *Bacillus velezensis* et *Pantoea agglomerans*. Ces bactéries ont été testées *in vitro* et *in vivo* et formulées en produits stables contenant à la fois les souches vivantes et leurs métabolites. Les arbres traités avec le produit à base de *P. agglomerans* NY60 avaient moins de brûlure bactérienne que le témoin non traité et n'étaient pas significativement différents des arbres témoins traités à la streptomycine. Avec *Pantoea agglomerans* NY60, le feu bactérien ne s'est jamais étendu au-delà de la veine centrale de la feuille inoculée. Aucun dommage chimique n'a été observé avec ce traitement. Le score médian de gravité de la maladie, 10 jours après l'inoculation, était jusqu'à 70 % moins élevé sur les arbres traités avec NY60 que sur les arbres non traités.

Parmi les trois souches bactériennes isolées ayant une activité antagoniste et extracellulaire contre *E. amylovora*, à la fois *in vitro* et *in planta*, correspondant à trois genres bactériens différents, nous avons identifié le mode d'action inhibiteur de chacun des trois isolats contre *E. amylovora*.

L'isolat *Bacillus amyloliquefaciens* subsp. *plantarum* (*B. velezensis*) FL50S produit plusieurs métabolites secondaires dont des surfactines, des iturines et des fengycines. Plus précisément, nous avons identifié l'oxydifficidine comme étant la plus active contre *E. amylovora*

S435. *Pseudomonas poae* FL10F produit un composé extracellulaire actif contre *E. amylovora* S435 qui peut être attribué au (white-line-inducing principle) (WLIP), un lipopeptide cyclique appartenant à la sous-famille des viscosines (massétolide E, F, L ou viscosine). *Pantoea agglomerans* NY60 a un effet antagoniste direct de cellule à cellule contre *E. amylovora* S435. En criblant des mutants de cette souche générés par insertion aléatoire d'un transposon démontrant une activité antagoniste réduite contre la souche S435, nous avons identifié plusieurs transposants défectueux. Un mutant d'un gène codant pour un transporteur de la Superfamille des Facilitateurs Majeurs (MFS), correspondant à une protéine transmembranaire supposée être impliquée dans la localisation extracytoplasmique de l'acide griséolutéique, un intermédiaire dans la production de l'antibiotique de type phénazine à large spectre, l'acide D-alanylgriséolutéique, présentait un intérêt particulier.

L'ensemble de notre approche et nos résultats a abouti à une plateforme de développement des solutions biologiques de contrôle d'agents phytopathogènes spécifiques tels que *E. amylovora.* Nous avons appliqué la même approche pour développer des solutions biologiques spécifiques contre un autre agent phytopathogène (*Xanthomonas* spp.). Nous avons déposé deux brevets décrivant la plateforme utilisée pour développer des solutions biologiques contre des agents phytopathogènes spécifiques.

REMERCIEMENTS

Je tiens tout d'abord à remercier les membres du jury, Dr. Richard Villemur, Dre. Isabelle Laforest-Lapointe et Dr. Martin Filion. Merci sincèrement d'avoir accepté d'évaluer mon travail de thèse.

Je remercie mon directeur de recherche, Dr. Éric Déziel, de m'accueillir dans son laboratoire malgré mon âge pour développer des solutions biologiques contre des agents phytopathogènes. Dr. Éric Déziel s'est montré toujours à l'écoute, et m'a aidé tout au long de ces années de thèse. Je le remercie pour sa patience envers moi et pour son encouragement à persévérer pour compléter ce projet tout en publiant deux articles et deux brevets.

Ce travail n'aurait pu se faire sans la participation de Dre. Snizhana Olishevska et de Dr. Arvin Nickzad qui m'ont aidé à exécuter les expériences scientifiques. Grâce à leur compétence scientifique, leur disponibilité, et leur patience, j'ai pu développer les solutions biologiques ainsi que comprendre le mode d'action des agents actifs contre des agents phytopathogènes.

Je remercie également les professeurs Philippe Constant, François Lépine et Claude Guertin qui m'ont beaucoup encouragé à persévérer dans ce chemin.

Un grand merci particulier à Marie-Christine, pour sa gentillesse, son aide ainsi que son sourire.

Enfin, un grand merci à tous les membres du laboratoire Déziel pour leur soutien.

Dédicace

Je dédie ce travail à :

Mon épouse Micheline qui était toujours compréhensive et disponible, m'écoutant pendant les périodes difficiles durant ces années. Son amour, sa gentillesse, sa motivation et sa patience m'ont toujours donné le courage de continuer à achever ce projet.

Ma fille Rebecca, source de ma joie, m'a donné le courage et le désir pour continuer dans ce trajet.

Mon père, mon frère, mes deux sœurs et surtout ma mère que j'ai perdu au début de la pandémie COVID-19. À toi Maman, je dédie aussi ce travail car tu étais toujours fière de mes accomplissements dans ma vie professionnelle.

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ARLA :	Agence de réglementation de la lutte antiparasitaire
NOSB :	Commission Nationale des Normes Biologiques
E. amylovora :	*Erwinia amylovora*
SST3 :	Système de sécrétion de type 3
EPS :	Extracellular Polymeric Substances (Polysaccharides extracellulaires)
UE :	Union européenne
NRP :	Non-ribsosomal Peptide (Peptides non ribosomiques)
AMB :	Acide L-2-amino-4-méthoxy-trans-3- buténoïque
EPA :	Environmental Protection Agency
SSC :	Surnageant sans cellules
MFS :	Major facilitator superfamily (Superfamille des facilitateurs majeurs)
WLIP :	White-line-inducing principle
AGA :	Acide D-alanylgriséolutéique

1. INTRODUCTION

1.1. Feu bactérien

Le feu bactérien, causé par *Erwinia amylovora*, est une maladie infectieuse très répandue chez les pommiers, les poiriers et autres plantes hôtes de la famille Rosacea qui peut tuer les jeunes arbres ou causer des dommages structurels permanents aux arbres adultes [1-3]. Elle peut même éliminer un verger entier en une saison de croissance [4]. L'étude du feu bactérien a commencé il y a plus de 200 ans [5]. C'est la première maladie des plantes à propos de laquelle il a été démontré qu'elle est d'origine bactérienne [6]. Après avoir été découverte pour la première fois en 1868 à New York, elle s'est étendue dans d'autres régions productrices de pommes et de poires du monde, notamment la Nouvelle-Zélande, le Royaume-Uni, l'Europe et le Moyen-Orient [4]. La bactérie infecte typiquement les plantes par les fleurs et les bourgeons en croissance active et peut être disséminée d'un arbre à l'autre par les insectes pollinisateurs. L'étude de l'*E. amylovora* et du feu bactérien a permis de mieux comprendre les interactions des plantes avec les bactéries pathogènes, notamment le rôle des pollinisateurs en tant que vecteurs de maladies [7].

Le feu bactérien constitue une menace grave pour la production de pommes et de poires dans le monde entier, entraînant des pertes économiques importantes dans de nombreux pays [4, 7, 8]. Aux États-Unis, les pertes et les coûts liés à la lutte d'*E. amylovora* sont chiffrés à plus de 100 millions de dollars par an [9]. Par exemple, en 2000 au Michigan, une épidémie de la brûlure bactérienne a entraîné des pertes économiques d'environ 42 millions de dollars, principalement associées à l'abattage et à l'élimination de 350 000 à 400 000 pommiers [10]. En 1998, les producteurs de pommes et de poires de l'état de Washington et du nord de l'Oregon ont subi une perte estimée à 68 millions de dollars en raison du feu bactérien [11]. Au Québec, la seule maladie bactérienne d'importance notable dans les vergers de pommiers est la brûlure bactérienne. Des cas isolés de feu bactérien sont rapportés chaque année au Québec, mais des épidémies majeures à l'échelle provinciale n'ont eu lieu qu'en 2002, 2012 et 2016 [12, 13]. Le feu bactérien reste une maladie difficile à contrôler, même en utilisant des pratiques culturales visant à réduire l'inoculum primaire et les pulvérisations de cuivre et d'antibiotiques pour limiter la multiplication de ces agents phytopathogènes [7].

En tant que membre de la famille Enterobacteriaceae, *E. amylovora* est génétiquement liée à de nombreuses bactéries pathogènes humaines et animales, telles qu'*Escherichia coli*,

Salmonella enterica, *Shigella flexneri*, et *Yersinia pestis* qui appartiennent à cette même famille. Cette bactérie à Gram négatif en forme de bâtonnet est capable d'infecter différents hôtes de la famille des Rosacées, dont toutes les espèces de la sous-famille des *Maloideae* dans laquelle on retrouve les pommiers et les poiriers [12, 14]. Exceptionnellement, certaines espèces appartenant à la sous-famille des *Spiraeoideae*, comme les spirées, cerisiers et pruniers, sont parfois touchées par le feu bactérien lorsqu'elles se trouvent à proximité de vergers infectés [12].

Chez les pommiers et les poiriers, les infections primaires causées par ce pathogène affectent d'abord les fleurs, qui constituent le point d'entrée principal de la bactérie dans l'arbre. La présence d'*E. amylovora* n'entraîne pas nécessairement une infection des pommiers et des poiriers. L'infection se produit seulement lorsque la population bactérienne dépasse les 10 000 bactéries par fleur, et que les conditions sont favorables à l'infection [12]. Ainsi, plus la température est élevée, plus la multiplication bactérienne sera favorisée. La température doit dépasser 18°C pendant la floraison afin que l'infection se manifeste en feu bactérien [15, 16]. D'autre part, l'infection de l'arbre par *E. amylovora* est favorisée si les fleurs sont mouillées par la pluie ou la rosée. L'humectation des fleurs est donc un vecteur permettant la formation d'un film aqueux qui conduit les bactéries des stigmates, leur site de multiplication, jusqu'aux glandes nectarifères. Ces nectaires sont le point d'entrée de l'infection dans l'arbre. Sans ce film d'eau, les infections seraient assez marginales et ne se traduiraient pas physiquement en feu bactérien [17].

Autres que les infections florales, certaines infections peuvent se manifester en absence de conditions favorables durant la floraison. Par exemple, l'infection peut se répandre directement des chancres, tâches brunes et concaves s'étendant sur le tronc ou sur les branches, jusqu'aux pousses environnantes via des blessures. Le feu bactérien peut aussi se propager aux autres pousses ou aux arbres situés à proximité par des insectes et par les filaments bactériens utilisant l'air comme vecteur de transport. Le tissu infecté meurt et change de couleur en été, devenant marron foncé à noir. Les fleurs et les feuilles infectées et contaminées se flétrissent, prenant une coloration brunâtre foncé à noire, après quoi elles dépérissent et dessèchent [18].

La Figure 1 montre le cycle d'*E. amylovora* dans les vergers. En général, la bactérie hiberne et se conserve dans les chancres. Elles commencent à se multiplier au printemps lorsque les températures favorables à la croissance (entre 12°C et 24°C) sont atteintes et que les autres conditions optimales sont présentes, telles qu'une humidité suffisante. En général, des

températures plus élevées favorisent une apparition plus rapide de la maladie [16].

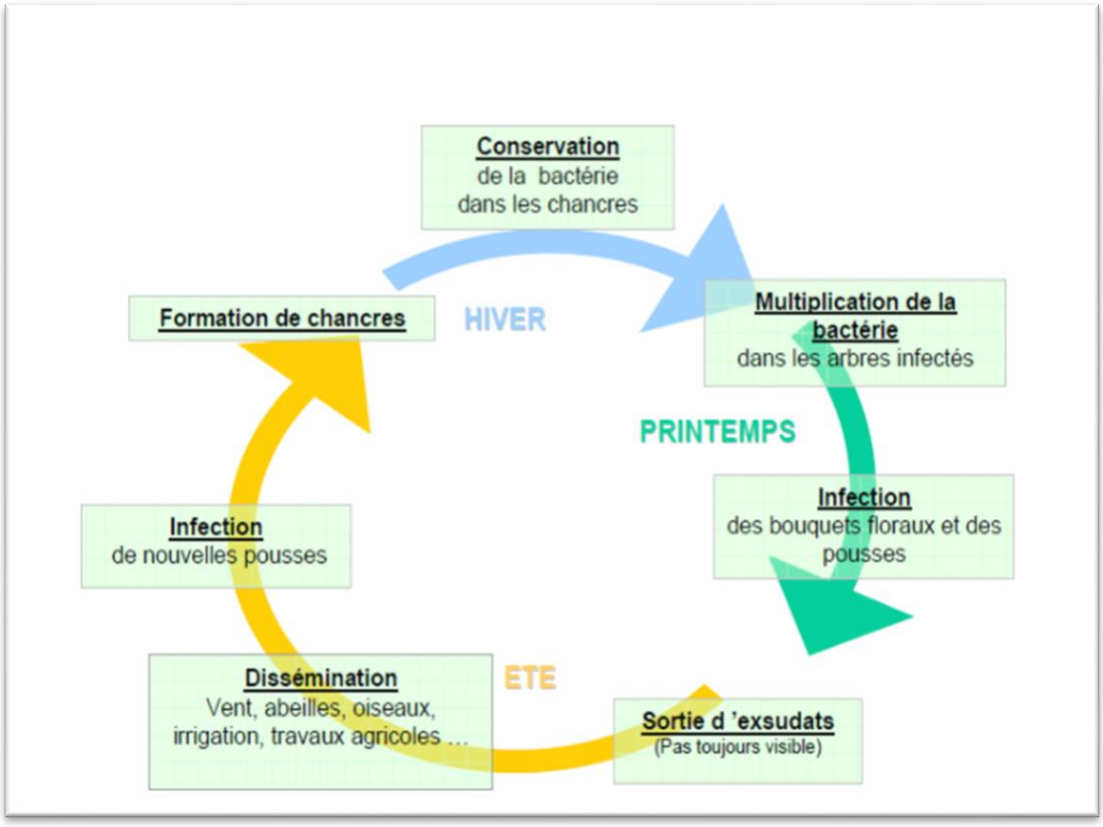

Figure 1: Cycle de la survie d'*Erwinia amylovora dans* les vergers. Image tirée de la fiche: « Le feu bactérien ». Fédération Régionale de Défense contre les Organismes Nuisibles de Provence Alpes Côte d'Azur (FREDON PACA) [19].

Visuellement, les symptômes associés au feu bactérien dans les vergers sont frappants. Les fleurs se flétrissent et se corrompent et les pousses herbacées se recourbent en une crosse caractéristique. Les branches, les pousses, et le feuillage prennent une coloration brune à noire qui donne l'impression visuelle de brûlure. Si les conditions sont favorables, l'infection se propagera rapidement des branches, vers les branches charpentières pour atteindre finalement le tronc, ce qui à terme, peut conduire à la mort de l'arbre. Sur les branches, la zone attaquée et contaminée se fissure formant à nouveau des chancres qui constituent un nouvel inoculum qui pourrait infecter les arbres l'année suivante [19]. Les figures 2 à 5 montrent les symptômes associés à la brûlure bactérienne sur différentes structures d'un pommier. De plus, les jeunes arbres sont particulièrement sensibles à la maladie, et les cultivars de poiriers sont généralement plus sensibles que les cultivars de pommiers. Alors, selon les cultivars et la gravité des cas, on observe un rougissement du feuillage ou encore un dessèchement généralisé, comme le montre

la figure 6.

Figure 2 : Symptômes de la brûlure bactérienne dans un champ. Image tirée de la fiche 105 : « Le feu bactérien : dépistage ». Institut de recherche et de développement en agroenvironnement, IRDA, Québec, Canada [20].

Figure 3 : Symptômes de la brûlure bactérienne sur une feuille. Image tirée de la fiche 105: « Le feu bactérien : dépistage ». Institut de recherche et de développement en agroenvironnement, IRDA, Québec, Canada [20].

Figure 4: Symptômes de la brûlure bactérienne sur les fruits. Image tirée de la fiche 105: « Le feu bactérien : dépistage ». Institut de recherche et de développement en agroenvironnement, IRDA, Québec, Canada [20].

Figure 5: Chancre d'un feu bactérien se développant sur les rameaux affectés et sa couleur brunâtre foncée visible à l'œil nu. Image tirée de la fiche 105: « Le feu bactérien : dépistage ». Institut de recherche et de développement en agroenvironnement, IRDA, Québec, Canada [20].

Figure 6: Pommiers gravement infectés par le feu bactérien donnant l'impression d'avoir été roussis par le feu. Photo tirée de l'extrait de la publication 310F « Lutte intégrée contre les ennemis du pommier ». Ministère de l'Agriculture, de l'Alimentation et des Affaires Rurales. Ontario. [16].

Il faut noter que certains cultivars sont plus sensibles au feu bactérien que d'autres comme indiqué dans le tableau 1. Par exemple, les cultivars tels que Fuji, Gala et Mutsu sont très sensibles. Par contre les cultivars tels que Cortland, Golden Delicious, McIntosh, Honeycrisp et Spartan sont considérés comme étant sensibles au feu bactérien. Enfin, les cultivars tels que Freedom, Liberty, et McFree sont les moins sensibles au feu bactérien [21].

Tableau 1**: Résistance relative au feu bactérien des cultivars et porte-greffes de pommiers. Tableau tiré de l'extrait de la publication 310F « Lutte intégrée contre les ennemis du pommier ». Ministère de l'Agriculture, de l'Alimentation et des Affaires Rurales. Ontario. [16].**

Cultivar	**Les moins sensibles**	**Moyennement sensibles**	**Très sensibles**
Pommier	Enterprise Freedom Jonafree Liberty Macfree Northern Spy Red Delicious Redfree	Ambrosia Cameo Cortland Creston Empire Golden Delicious Golden Supreme GoldRush Granny Smith Gravenstein Honeycrisp Jerseymac Macoun McIntosh Mutsu (Crispin) Nova Easygro Nova Mac Pioneer Mac Sansa Spartan Summered Sunrise Yataka	Braeburn Fuji Gala et types Gala Ginger Gold Golden Russet Idared Jonagold Jonathan Lodi Mutsu Paulared Pink Lady Rome Rome Beauty Spigold Tydeman Wealthy Winter Banana Yellow Transparent
Pommetier		Dolgo	Manchurian

			Snowdrift
Porte-greffe	M.7 B.95 Robusta 5 Cornell-Geneva (CG) série 5	MM.106 MM.111 M.41	M.9 M.26 M.27 Mark Ottawa 3

1.2. Les facteurs de virulence d'*Erwinia amylovora*

Les facteurs exprimés par *E. amylovora* favorisant l'infection et la dissémination sont bien connus. Ainsi, Molina et ses collaborateurs [22] ont démontré l'importance du « quorum sensing », un système de communication intercellulaire, dans la régulation de nombreux facteurs de virulence d'*E. amylovora* parmi lesquels figurent les polysaccharides de surface (l'amylovoran et le levan) ainsi que le système de sécrétion de type 3 (SST3) qui est un élément pathogène crucial pour plusieurs bactéries Gram-négatives (*E. coli*, *Shigella*, *Salmonella*, *Yersinia*, *Pseudomonas*) leur permettant de transférer directement les facteurs de virulence de la bactérie dans la cellule hôte cible. [23-25]. De plus, Koczan et ses collaborateurs [26] ont découvert que les polysaccharides extracellulaires (EPS) d'*E. amylovora* sont impliqués dans la formation du biofilm [27]. Ces EPS protègent *E. amylovora* vis-à-vis de pertes d'eau et de nutriments dans les conditions sèches. Ils sont impliqués dans l'apparition des symptômes tels que le flétrissement, par la diminution du flux vasculaire [28].

Le système de sécrétion de type 3 (SST3) d'*E. amylovora* sécrète des protéines effectrices de virulence parmi lesquelles figurent les protéines HrpA, HrpN, HrpW et DspE/A. Cette dernière constitue une protéine spécifique à la maladie [2, 28, 29]. Ces protéines effectrices sont injectées dans le cytosol, la zone à l'intérieur de la membrane cytoplasmique de la plante hôte. Elles manipulent ou modifient les activités cellulaires de la plante hôte et cela aide l'agent pathogène à coloniser l'arbre infecté. [28].

1.3. Contrôle des infections

Lorsque les symptômes de la maladie sont visibles, il n'existe pas de traitements curatifs efficaces pour traiter la plante du feu bactérien [30]. Pour sauver un arbre suite à une infection, les branches doivent être coupées d'au moins 0,3 m au-dessous des symptômes visibles. Si

l'infection se produit près du tronc d'un arbre, celui-ci doit être complètement abattu, puis brûlé [9]. Le contrôle efficace de la maladie est donc axé sur la prévention de la croissance de l'agent pathogène sur les surfaces de fleurs avant que l'infection ne se produise [31].

1.4. Antibiotiques utilisés contre le feu bactérien

La streptomycine est un antibiotique efficace contre le feu bactérien lorsque la bactérie se trouve à la surface de la fleur. L'oxytétracycline, qui inhibe la croissance de l'agent pathogène, mais ne le tue pas, est moins efficace [32]. Même si les producteurs peuvent utiliser des antibiotiques pour contrôler le feu bactérien, les épidémies de la maladie se produisent encore. Aux États-Unis, les épidémies du feu bactérien ont eu lieu lorsque les conditions météorologiques étaient favorables à la propagation de la maladie, et dans la plupart des cas, des populations d'*E. amylovora* résistantes à la streptomycine étaient détectées [32]. De plus, les souches d'*E. amylovora* résistantes aux antibiotiques se propagent mondialement dans les vergers. En 2002, et malgré l'utilisation des antibiotiques, l'épidémie de feu bactérien la plus virulente de ces 100 dernières années a frappé une grande région de la production de poires dans le sud de l'Oregon. Dans certains pays où les antibiotiques ne sont plus homologués pour l'agriculture, le feu bactérien a causé de graves dommages dans les vergers. Dans une tentative infructueuse d'éradiquer le feu bactérien en Italie à la fin des années 1990, environ 500 000 poiriers ont été détruits dans la plaine du Pô, une importante zone de la production de poires [33]. De même, près d'un million de poiriers, de pommiers et de cognassiers (arbre à coings) ont été détruits en Roumanie et en Croatie dans les années 1990 dans une tentative infructueuse d'enrayer la propagation du feu bactérien dans ces pays [34].

L'usage fréquent et abusif des antibiotiques surtout aux États-Unis contre les maladies infectieuses d'origine bactérienne est l'un des facteurs favorisant le développement et l'évolution de résistances microbiennes. Pour contrer l'action des antibiotiques, les bactéries utilisent plusieurs mécanismes parmi lesquels le brouillage (la production d'enzymes capables d'inactiver les antibiotiques), le blindage et l'efflux (capacité à se rendre imperméable à la pénétration de l'antibiotique ou encore capacité à le rejeter), le camouflage (modification de la structure des cellules cibles des antibiotiques), l'esquive ou la stratégie de contournement. Par conséquent, plusieurs souches d'*Erwinia amylovora* ont dévelopé une resistance à l'antibiotique vaporisé à plusieurs applications et concentrations dans certains États [35].

Face à la résistance contre les antibiotiques homologués contre le feu bactérien, Arysta LifeScience a obtenu l'homologation du produit Kasumin®2L au Canada en 2013. Il s'agit d'une formule liquide d'hydrate d'hydrochlorure de kasugamycine qui supprime la brûlure bactérienne des fruits à pépins. Cet antibiotique de la famille des aminoglycosides est appliqué à un taux de concentration de 100 ppm (par exemple, 5 L / ha dans 1000 L d'eau). Le volume de pulvérisation doit être suffisant pour permettre une bonne couverture du feuillage traité. Le produit est appliqué au stade de 20 ou 30% de la floraison lorsque les conditions sont favorables au développement de la maladie. Les traitements doivent se répéter à intervalles de 7 jours ou lorsque les conditions favorisent le développement de la maladie [36].

Toutefois, la résistance contre la kasugamycine également se développera inévitablement chez *E. amylovora*. Dans une expérience en laboratoire, des mutants ont été récupérés suite à l'exposition de souches d'*E. amylovora* à une faible concentration de kasugamycine (25 mg/L), et repiqués dans un milieu contenant des concentrations plus élevées de l'antibiotique. Les isolats résultants pouvaient ensuite croitre dans un milieu contenant jusqu'à 150 mg/mL de kasugamycine. Ils portaient tous des mutations dans le gène *ksgA*, qui code la protéine cible de la kasugamycine. Celle-ci est associée à la résistance à la kasugamycine spontanée chez *E. coli*. Ce travail suggère que les pratiques agricoles qui optent pour des traitements avec des concentrations plus faibles de Kasumin sur les fleurs de pommiers pourraient contribuer à une sélection plus rapide de la résistance chez *E. amylovora* [37].

1.5. Interdiction des antibiotiques

En 2016, la Commission Nationale des Normes Biologiques (NOSB) a bannie la streptomycine dans les vergers de culture biologique de pommes et de poires aux États-Unis [38]. De même, l'Union européenne et le Canada interdisent dorénavant l'utilisation des antibiotiques, incluant la kasugamycine, au sein des vergers de culture biologique. Des études menées en Allemagne ont montré que le miel était souvent contaminé par l'antibiotique utilisé contre le feu bactérien. Sur 183 échantillons, 21% contenaient des résidus de streptomycine. En raison de ce risque de contamination, la streptomycine n'est pas homologuée pour un tel usage dans la plupart des pays de l'Union européenne [39]. Concernant les normes en culture traditionnelle et conventionnelle au Québec, kasumin et streptomycine sont approuvés par ARLA; par contre, la tétracycline ne l'est pas pour combattre les maladies, telles le feu bacterien.

1.6. Alternatives au cuivre et aux antibiotiques

1.6.1. Phytotoxicité des bactéricides à base de cuivre

Les bactéricides à base de cuivre sont mondialement utilisés pour contrôler les bactéries pathogènes agricoles. Les ions de cuivre, toxiques pour les bactéries et les champignons, peuvent cependant causer des dommages aux feuilles et aux fruits. Pour réduire la phytotoxicité du cuivre, des formulations en cuivre « fixe » ont été développées. Le cuivre « fixe» est conçu pour laisser un résidu peu soluble à la surface des feuilles [40]. Lorsque les feuilles sont mouillées, les ions de cuivre s'échappent lentement de ces dépôts pour attaquer les bactéries. Cette libération graduelle du cuivre maintient une efficacité pendant une plus longue période et évite que la concentration d'ions en cuivre soit trop grande pour provoquer l'effet phytotoxique. Toutefois, le cuivre «fixe» n'élimine pas complètement les risques de la toxicité. Plus le cuivre est appliqué durant la saison, plus le risque est élevé sur les fleurs et peut causer une roussissure inacceptable sur les fruits de certains cultivars tels que les McIntosh et Empire ainsi qu'une baisse de la formation du fruit et du rendement [41]. Il est important de noter que l'efficacité du cuivre et la phytotoxicité sont intimement liés. Il est donc difficile de maximiser l'effet antibactérien tout en réduisant la phytotoxicité. Pour cette raison, les antibiotiques sont considérés comme les bactéricides standards utilisés par les cultivateurs pour traiter/prévenir la brûlure bactérienne [41].

1.6.2. Métabolites biologiques : une option intéressante pour remplacer le cuivre et les antibiotiques

Certaines souches bactériennes produisent des métabolites secondaires efficaces contre des microorganismes pathogènes. Par exemple, de nombreuses souches des espèces *Bacillus* et *Paenibacillus* sont connues pour produire des molécules inhibitrices efficaces contre plusieurs microorganismes, incluant des bactéries phytopathogènes [42]. Ces souches produisent les métabolites suivants: subtilosine, subtiline, ericine, sublancine, iturine, iturine A, surfactine, mycosubtiline, fengycine, plipastatine, bacillaene, macrolactine, bacilysine, bacillomycine, difficidine etc [43-48]. Ces métabolites secondaires sont des composés organiques qui ne sont pas directement impliqués dans le développement ou la reproduction microbienne. Leur absence ne conduit pas à la mort immédiate mais peut limiter leur survie. Un de leurs rôles principaux est d'augmenter la compétitivité de l'organisme qui les biosynthétise en leur procurant un avantage

sur les autres organismes. Les polycétides constituent l'une des classes importantes de métabolites secondaires microbiens possédant un spectre étendu d'activités antibactériennes et antifongiques dans un contexte agricole [49]. Ils incluent de nombreux composés actifs tels que les antibiotiques [50].

Les espèces appartenant aux genres *Bacillus* et *Paenibacillus* synthétisent aussi des peptides non ribosomiques (NRP) et peptides synthétases non ribosomiques (NRPS) qui sont une classe importante de métabolites secondaires peptidiques [50]. *Bacillus subtilis*, par exemple, synthétise la surfactine appartenant à la classe des lipopeptides [51-53]. Plusieurs espèces de ces genres produisent des lipopeptides qui ont des fonctions non seulement antagonistes contre les pathogènes mais aussi dans la motilité cellulaire, conduisant à la colonisation de nouveaux habitats et le développement de biofilms fortement structurés [54, 55]. Finalement, en plus de *Bacillus* et *Paenibacillus,* plusieurs espèces de *Pseudomonas*, dont *Pseudomonas fluorescens*, sont reconnus pour leurs capacités à produire des métabolites secondaires de type NRP ayant des caractéristiques antibactériennes et antifongiques [54, 56, 57].

Plusieurs espèces appartenant aux genres *Pseudomonas*, *Bacillus* et *Paenibacillus* ont été utilisées pour combattre plusieurs maladies des plantes [11, 58]. Certaines espèces ont montré une activité antagoniste contre l'un ou l'autre des dix principaux phytopathogènes bactériens qui sont, dans l'ordre, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Agrobacterium tumefaciens*, *Xanthomonas oryzae* pv. *Oryzae*, pathovars de *Xanthomonas campestris,* pathovars de *Xanthomonas axonopodis, Erwinia amylovora* , *Xylella fastidiosa*, *Dickeya (dadantii* et *solani), Pectobacterium carotovorum* et *Pectobacterium atrosepticum* [59-62].

1.7. Moyens alternatifs présentement disponibles et options possibles afin de lutter contre le feu bactérien

Les formulations à base de cuivre étant phytotoxiques et les antibiotiques étant de plus en plus décriés en raison des résistances qu'ils induisent dans les pommiers et les poiriers, et le danger qu'une telle utilisation à grande échelle peut avoir sur les phénomènes de multi-résistance des pathogènes infectant les humains et animaux, il existe un besoin criant exprimé par les cultivateurs et les organisations gouvernementales pour développer des biopesticides efficaces pour lutter contre le feu bactérien, particulièrement en agriculture biologique.

Il existe plusieurs études rapportant l'identification de microorganismes présentant une activité inhibitrice envers *Erwinia*. Niu et ses collaborateurs [63] ont montré que la polymyxine P est la molécule active permettant la suppression des espèces phytopathogènes *d'Erwinia* à partir de préparation à base de la rhizobactérie *Paenibacillus polymyxa* M-1 [64]. Zhao et ses collaborateurs [65] ont isolé une souche identifiée comme *Bacillus amyloliquefaciens* subsp*. plantarum* sur la base de la morphologie des colonies, la morphologie cellulaire, la sporulation et les séquences nucléotidiques partielles de l'ARNr 16S ainsi que le gène *gyrB*, et qui possède une activité contre *Erwinia carotovora* qui infecte les légumes en post-récolte [66]. Pour sa part, la souche de *Pantoea agglomerans* Eh318 produit, par antibiose, une zone complexe d'inhibition de croissance d'*E. amylovora* qui est attribuée aux deux antibiotiques sécrétés, nommés les pantocines A et B [67]. La microencapsulation de la souche *Pantoea* E325, une nouvelle technologie qui se base sur la libération contrôlée de l'agent actif formulé, a été utilisée comme antagoniste contre *E. amylovora*. Cette étude a fait la démonstration de l'efficacité de cette approche et pourrait servir de modèle pour d'autres études sur le développement de stratégies de gestion efficace contre d'autres maladies de plantes [68]. Smits et ses collaborateurs [69] ont étudié une souche de *Pantoea vagans* qui est aussi un agent de lutte biologique utilisé contre *E. amylovora*. L'analyse du génome a indiqué deux facteurs principaux contribuant à l'activité de lutte biologique: la compétition pour les substrats limitants et la production de métabolites antibactériens. Les gènes codant la pantocine A ont été trouvés sur un ilot chromosomique de 28 kb. En revanche, les gènes codant le dapdiamide E se trouvent sur le plasmide pPag2 [69]. Les bactériophages sont aussi utilisés dans le contrôle des populations bactériennes pathogènes sur les plantes comme l'oignon, la tomate et la pomme de terre. Muller et ses collaborateurs ont montré que leurs bactériophages lysent efficacement leurs hôtes et protègent les fleurs de pommiers du feu bactérien [70].

Non seulement les microorganismes mais aussi plusieurs composés chimiques et extraits de plantes ont été testés pour leur efficacité contre *E. amylovora.* Konecki et ses collaborateurs [71] a démontré une inhibition de la croissance d'*E. amylovora* et d'espèces apparentées à *Erwinia* en utilisant des acides gras à chaîne courte neutralisés, tels l'acide propionique. Yang et ses collaborateurs [72] ont expérimenté sur des petites molécules appartenant à salicylidène acylhydrazides qui ont inhibé l'expression de la sécrétion de type III ainsi que les gènes de biosynthèse de l'EPS amylovoran chez *E. amylovora*. Lee et ses collaborateurs [73] ont constaté que les filtrats de culture d'une souche *Pseudomonas aeruginosa* produisant du L-2-amino-4-

méthoxy-trans-3- buténoïque (AMB) inhibent fortement la croissance d'*E. amylovora*.

Fischer et ses collaborateurs [74] ont criblé les quinones, qui sont des extraits de plante, et ils ont trouvé que le composé le plus actif est le juglone, un extrait de noix. Il a un effet bactéricide puissant et spécifique contre *E. amylovora* et des concentrations minimales inhibitrices de seulement 2.5 à 10 µM. Marutescu et ses collaborateurs [75] ont testé une série de N-(1-méthyl-1H-pyrazole-4-carbonyl) et ces dérivés de thiourée pour leur activité contre vingt-deux souches d' *E. amylovora* isolées à partir de différentes régions de Roumanie. Leur étude a montré que six nouveaux composés de thiourée ont présenté une activité antibactérienne faible (valeurs MIC > 500 µg/mL), mais les concentrations sous-inhibitrices ont inhibé le développement d'un biofilm sur des substrats inertes. Ainsi, ces résultats pourraient suggérer l'utilité des composés testés comme agents de lutte pour empêcher la première étape (la colonisation) de l'infection par l'agent pathogène du feu bactérien.

Plusieurs études concernant la lutte biologique contre le feu bactérien ont été publiées, mais il existe peu de produits biologiques disponibles pour les cultivateurs afin de protéger leurs vergers contre *E. amylovora*. Les produits biologiques homologués les plus utilisés en Amérique Nord sont les suivants:

1. **Bloomtime Biological™ FD Biopesticide :** Un produit basé sur la souche *Pantoea agglomerans* E325 contenant un minimum de $1x10^{10}$ UFC/g. Il a été approuvé par l'« Environmental Protection Agency » (EPA) aux États-Unis avec le numéro d'enregistrement (EPA Reg. No. 71975-1).
2. **BlightBan®A506 :** Un produit à base des *Pseudomonas fluorescens* A506 contenant un minimum de $1x10^{10}$ UFC/g. Il a aussi été approuvé par l'EPA avec le numéro d'enregistrement suivant: EPA REG. NO. 228-710.
3. **Serenade®MAX™ :** Un produit à base de la souche QST 713 des cellules lyophilisées de *Bacillus subtilis* contenant un minimum de $7.3x10^{9}$ UFC/g (EPA No. 69592-11). Il a été développé pour contrôler plusieurs pathogènes agricoles.
4. **Blossom Protect :** Un produit à base d'*Aureobasidium pullulans* qui est homologué par Santé Canada pour la lutte contre le feu bactérien dans les pommiers et les poiriers. Ce produit contient deux souches vivantes de la levure *A. pullulans* dans un contenant, et un tampon à l'acide citrique dans un autre contenant. Les deux sont mélangés avant

l'application. Les souches de levures colonisent les mêmes parties des fleurs et utilisent les mêmes éléments nutritifs que la bactérie responsable du feu bactérien [76].

Ces produits ont été largement testés comme substituts à la streptomycine et l'oxytétracycline dans différentes régions des États-Unis. Ces produits ont été expérimentés durant plus de sept ans au Michigan, en Virginie et dans l'état de New York où ils ont montré une faible efficacité et de la variabilité dans les résultats d'une géographie à une autre et d'une année à une autre [77].

Au Québec, les produits approuvés commercialement disponibles tels: Blossom Protect, Double Nickel, BMJ WG, QST 713 et Serenade Opti sont utilisés surtout dans les cultures biologiques; toutefois, ces produits sont moins efficaces que les antibiotiques en général.

2. PROBLÉMATIQUE

L'antibiorésistance chez les agents pathogènes humains est un enjeu majeur et allant croissant. De nombreuses bactéries deviennent résistantes aux antibiotiques les plus couramment prescrits, aboutissant à des infections de plus en plus prolongées et des frais médicaux toujours plus conséquents [78]. Un facteur potentiel et majeur contribuant à l'antibiorésistance chez les organismes pathogènes humains est le traitement à grande échelle, typiquement par vaporisation, d'antibiotiques sur les arbres fruitiers tels que les pommiers et les poiriers [1, 37]. Plus l'arbre et le sol sont pulvérisés par ces produits, plus le consortium bactérien du sol développera une résistance et fournira, par conséquent, le matériel génétique responsable de la résistance aux pathogènes humains [78]. À titre d'exemple, plusieurs études ont montré que le miel produit par les abeilles est souvent contaminé par les antibiotiques vaporisés sur les pommiers et les poiriers utilisés en routine pour combattre la maladie du feu bactérien causée par la bactérie *E. amylovora* [39]. Ainsi, l'utilisation d'antibiotiques en agriculture ne peut plus être considérée dans un contexte de développement durable, étant responsable de pressions de sélection conduisant inévitablement à la résistance de certaines bactéries. [31, 32]. À moyen et long terme, l'utilisation croissante et intempestive d'antibiotiques pourrait avoir des conséquences redoutables pour la santé humaine, animale et environnementale [8].

À ce jour, aucune méthode efficace et durable de lutte contre la brûlure bactérienne n'a été mise au point [79]. Les microbicides à large spectre les plus utilisés dans le domaine agricole sont ceux à base de cuivre. Parmi ces produits, on retrouve l'hydroxyde de cuivre et le sulfate de cuivre. Toutefois, ces formulations ne peuvent pas être utilisées sur toutes les plantes en raison d'une phytotoxicité attribuée aux ions de cuivre [41, 80]. Pour pallier à cette phytotoxicité chez les pommiers et poiriers, les producteurs agricoles utilisent parfois l'oxytétracycline, souvent la streptomycine et maintenant de plus en plus la kasugamycine. Ces produits sont utilisés à grande échelle et en concentration élevée pour combattre le feu bactérien sur les pommiers et les poiriers en culture conventionnelle. Cependant, ces pratiques sont de plus en plus limitées en raison de préoccupations relatives à la phytotoxicité cuivre-dépendante et le développement croissant de l'antibiorésistance dans les populations d'*E. amylovora* [79, 81]. De plus, la Commission Nationale des Normes Biologiques (NOSB) des États-Unis a banni depuis 2016 l'emploi de la streptomycine dans les vergers des pommes et des poires en culture biologique [38].

Besoins pour une solution biologique, stable, efficace et spécifique contre *Erwinia amylovora.*

La majorité des produits à base de microorganismes homologués à ce jour ont été développés pour contrôler plusieurs infections touchant les plantes, peu importe si le pathogène est une bactérie ou un champignon. Ils peuvent donc manquer d'efficacité spécifique. Dès lors, il peut très intéressant d'instaurer un programme de recherche et développement spécifique contre *E. amylovora*.

De plus, la majorité des produits à base de microorganismes homologués dépendent de leur viabilité et multiplication une fois épandues. Il faut que les microorganismes puissent se reproduire sur les plantes pour inhiber les pathogènes car leurs modes d'actions sont par compétition et exclusion des niches écologiques [62, 82]. Mais cela dépend du succès de la croissance et de la propagation de ces microorganismes sur les plantes.

Il existe un besoin réel et urgent de développer des solutions plus acceptables du point de vue écologique et éthique et qui soient en mesure de contrôler efficacement *E. amylovora*, afin de lutter contre l'antibiorésistance. Un outil alternatif considéré aussi performant que les antibiotiques pour le contrôle des maladies des plantes est la lutte biologique (biocontrôle), qui consiste en l'utilisation des microorganismes dits "bénéfiques'' pour traiter les maladies des plantes [83-88].

Il est aussi fondamental de développer un criblage spécifique contre *E. amylovora* en isolant des souches de fleurs des pommiers et poiriers afin qu'elles survivent et colonisent bien les fleurs une fois vaporisées. Finalement, les souches intéressantes et actives contre *E. amylovora* seront criblées une deuxième fois afin de sélectionner les souches dont l'activité est extraite à partir du surnageant, dans l'optique de développer un produit contenant à la fois des souches qui colonisent les fleurs et des métabolites extracellulaires qui inhibent *E. amylovora.*

Ainsi, le but de ce projet était de développer une solution à large spectre basée sur des souches bactériennes actives et l'emploi de certains métabolites bactériens ayant moins d'effets phytotoxiques que les produits cuivrés, tout en étant aussi performant dans la lutte contre la maladie du feu bactérien.

3. HYPOTHÈSE ET OBJECTIFS

3.1. Hypothèses

On peut trouver des bactéries dans l'environnement présentant une activité inhibitrice envers *E. amylovora*. Les fleurs des pommiers et poiriers recueillies à partir de plusieurs cultivars et à plusieurs reprises durant la période de floraison sont une bonne source d'échantillons pour isoler ces bactéries compétitives qui peuvent bien coloniser les fleurs. Finalement, il est possible de développer un produit efficace et stable qui peut inhiber la bactérie *E. amylovora* via 1) les métabolites des bactéries actives isolées une fois appliqués sur les pommiers et poiriers et 2) qui peut, par ailleurs, coloniser les fleurs via les souches vivantes afin de prévenir la croissance d' *E. amylovora*.

3.2. Objectif général

L'objectif général de cette thèse était d'isoler des microorganismes qui possèdent une activité antagoniste et antibactérienne spécifique contre le phytopathogène E. *amylovora*. Idéalement, cette activité doit être présente dans le milieu extracellulaire sécrétée.

3.3. Objectifs spécifiques

Les objectifs spécifiques sont :

1. Isoler des microorganismes des fleurs des pommiers et des poiriers
2. Cribler ces isolats pour une activité antagoniste et antibactérienne contre le phytopathogène *E. amylovora*.
3. Sélectionner les isolats dont l'activité contre le pathogène-cible est la plus forte, étant due aux métabolites extracellulaires.
4. Charactériser le mode d'action de chacun des isolats sélectionnés pour leur activité contre *E. amylovora*.
5. Mettre au point d'une formulation bactérien à base de souches vivantes et de métabolites anti-*Erwinia* extraits à partir du surnageant.
6. Mettre en évidence l'efficacité antimicrobienne de la formulation sur les fleurs et les feuilles des pommiers et poiriers infectés par *E. amylovora.*
7. Séquencer le génome des isolats les plus prometteurs contre *E. amylovora*
8. Identifier les gènes codant pour les métabolites actifs contre *E. amylovora*.

4. ARTICLES

4.1. Présentation de l'article «Development of a novel biological control agent targeting the plant phytopathogen *Erwinia amylovora* ».

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Soumission : 12 décembre 2019 ; **Acceptation :**18 mars 2020 ; **Publication :** 8 octobre 2020

Journal : Heliyon. www.cell.com/Heliyon. Volume 6, page e05222 (10 pages); https://doi.org/10.1016/j.heliyon.2020.e05222

Résumé

Les antibiotiques sont largement utilisés pour lutter contre les agents pathogènes animaux, végétaux et humains. Ils sont pulvérisés dans les vergers de pommiers et de poiriers pour lutter contre la bactérie *Erwinia amylovora* , l'agent responsable du feu bactérien. Ce phytopathogène développe une résistance aux antibiotiques et les alternatives sont soit moins efficaces, soit phytotoxiques, soit plus lourdes à gérer. L'objectif de notre étude était de développer un agent de contrôle biologique efficace colonisant la plante hôte et concurrençant *Erwinia amylovora* . Il ne doit pas être phytotoxique, doit avoir un large spectre d'activité et être peu susceptible d'induire une résistance chez le pathogène. À cette fin, plusieurs isolats bactériens provenant de divers échantillons environnementaux ont été criblés afin d'identifier des candidats appropriés qui sont antagonistes à *Erwinia amylovora* . Nous avons prélevé des bactéries sur les fleurs, les feuilles et le sol de vergers de pommiers et de poiriers, de la période de floraison au printemps jusqu'à l'été. Les bactéries les plus efficaces, dont des isolats de *Pseudomonas poae*, *Paenibacillus polymyxa*, *Bacillus amyloliquefaciens* et *Pantoea agglomerans*, ont été testées *in vitro* et *in vivo* et formulées en produits stables contenant à la fois les souches

vivantes et leurs métabolites. Les arbres traités avec le produit à base de *Pantoea agglomerans* NY60 avaient moins de brûlure bactérienne que le témoin non traité et n'étaient pas significativement différents des arbres témoins traités à la streptomycine. Avec *Pantoea agglomerans* NY60, le feu bactérien ne s'est jamais étendu au-delà de la veine centrale de la feuille inoculée. Aucun dommage chimique n'a été observé avec ce traitement. Le score médian de gravité de la maladie, 10 jours après l'inoculation, était jusqu'à 70 % moins élevé sur les arbres traités avec *Pantoea agglomerans* NY60 que sur les arbres non traités.

4.1.1. 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 *Pantoea agglomerans*, were tested *in vitro* and *in vivo* and formulated into stable products containing both the live strains and their metabolites. Trees treated with the product based on *P. agglomerans* NY60 had less fire blight than the untreated control and were not significantly different from streptomycin-treated control trees. With *P. agglomerans* NY60, fire blight never extended beyond the central vein of the inoculated leaf. No chemical injury was observed with the treatment. 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 ones.

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 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.

4.1.2. 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" [78]. 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 [1, 37]. 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 [78].

The bacterial plant pathogen responsible for fire blight is *Erwinia amylovora* [2, 3, 89] a rod-shaped Gram-negative bacterium capable of infecting different hosts in the *Rosacea* family, including all species of the *Maloideae* subfamily, containing apple and pear trees [12, 90]. Primary infections caused by this pathogen affect initially the flowers, which constitute the main entry point of the bacterium into the host tree. However, the presence of *E. amylovora* does not necessarily cause an infection of apple and pear unless the bacterial population exceeds 10,000 bacteria per flower, and infection conditions are favorable [12], including: 1) wetting of flowers by rain or dew and 2) a higher temperature which favors bacterial multiplication. In general, the temperature must exceed 18°C during the flowering period for the infection by *E. amylovora* to lead to fire blight [16, 91]. In addition, the wetting of the flowers allows the formation of an

aqueous film which transports the bacteria from the stigma where they multiply to the nectariferous glands. These nectaries are the entry point of infection in the tree. Effective disease control therefore focuses on preventing the growth of the pathogen on flower surfaces before infection occurs [31].

Antibiotics sprayed on apple and pear orchards were banned in European countries to reduce bacterial resistance development and eliminate the traces of antibiotics contaminating foods. For instance, honey produced by bees was often found to be contaminated with the antibiotic sprayed to fight fire blight disease on apple and pear trees [39]. Moreover, the National Organic Standards Board (NOSB) in the United States has banned the use of streptomycin in organic apples and pears orchards in 2014 [38]. Further, the use of antibiotics in agriculture is not sustainable because it inevitably leads to resistance [31, 32]. In the long term, this use could potentially lead to health problems for everyone if the consumption of antibiotics keeps increasing in humans, animals and plants [92].

While the most widely used formulations to control bacterial phytopathogens in crop protection are based on copper, e.g. copper hydroxide and copper sulfate, these products cannot be used on all plants because of phytotoxicity issues [41, 80] and copper accumulation in soils is detrimental. Thus, there are major restrictions on the use of copper in Europe.

This phytotoxicity can be partially alleviated with "fixed" copper, designed to leave a poorly soluble residue on the surface of the leaves [40]. When the leaves are wet, copper ions slowly escape from these deposits to attack the bacteria. The greater the seasonal application of copper, the higher the risk of phytotoxicity on flowers and the possibility of unacceptable russeting of the fruits of certain apple cultivars, such as McIntosh and Empire, as well as a decrease in fruit yields [41]. Since copper efficiency and phytotoxicity are closely related, it is difficult to maximize the former while limiting the latter. For this reason, antibiotics are considered by growers as the standard bacterial control method on some crops [41]. Thus, antibiotics such as streptomycin and oxytetracycline are widely used on apple and pear trees to fight bacterial diseases [92, 93].

Annual losses due to fire blight can be significant in many countries [92]. In the United States, losses are estimated at over $ 100 million per year [9]. In Michigan, during the year 2000, a fire blight epidemic resulted in economic losses of approximately $ 42 million due to the elimination of 350,000 to 400,000 apple trees [10]. In 1998, apple and pear producers in

Washington and northern Oregon suffered a loss of an estimated $ 68 million due to fire blight [31].

Antagonism between bacteria can be based not only on antibiosis, but also on competition for niche and nutrients as well as on parasitism. A few biological products have shown commercial antagonistic potential against fire blight, but they all suffer from limitations. For instance, the aureobasidium yeast marketed as Blossom protect™ is incompatible with many fungicides and can sometimes result in unacceptable russetting of fruits. The timing of application must also be very precise to insure efficacy. Many bacterial products are now commercially available based on *Bacillus* spp. (*B. amyloliquefaciens, B. subtilis*) as well as *Pseudomonas fluorescens* and *Pantoea agglomerans* but these registered products in Canada are of limited efficacy [94].

Thus, the objective of this research was to develop a more ecologically acceptable and ethically responsible product as compared to copper 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*.

4.1.3. Materials and Methods

Media

Two nonselective media (Tryptic Soy Agar (TSA), and Plate Count Agar (PCA)) and three selective media (Benedict (for isolation of *Streptomyces* spp.) [95], BCSA (for isolation of *Burkholderia* spp.) [96] and Gould (for isolation of *Pseudomonas* spp.) [97] supplemented with 50 mg mL^{-1} cycloheximide (to limit the growth of fungi) were used to isolate bacteria from environmental samples.

Isolation of microorganisms from plants

Ten seeds and three segments (0.5 cm^2) 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 2). To isolate bacteria, the plates were incubated in the dark for 2-5 days at room temperature (~21 °C).

Isolation of microorganisms 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 2). 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.

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.

Strain used to evaluate antimicrobial activity

The pathogenic strains used as indicators for antimicrobial activity were *E. amylovora* S435, 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).

First screening step: antagonistic activity assays

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

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* S435 growing on TSA plates. These lawns were first

made by incubating 50 µL of *E. amylovora* S435 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.

Table 2: **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) Québec, Canada, September 2014	4
Agricultural field soil	[B]: Apple and pear orchards, Oka, Québec, Canada, September, 2014	3
Agricultural field soil	[C]: Sherrington, Québec, Canada, September, 2014	4
Agricultural field soil	[D]: Wimauma, Florida, USA, April, 2013	1
Agricultural field soil	[E]: Sherrington, Québec, 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), Québec, Canada, September, 2014	4

Tomato leaves and fruits	[J]: Sherrington, Québec, Canada, November, 2011	4
Leaves, stems, and fruits of various plants	[K]: Orchard, Laval, Québec, 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), Québec, Canada, May-September, 2015	30
Apple leaves, stems, and soil	[M]: Apple trees, Laval, Québec, Canada, June, 2015	5
Rome apple leaves and stems	[N]: Geneva, New York, USA, July, 2015	5
Pear flowers, leaves, and stems	[O]: Laval, Québec, Canada, May, 2015	5
Total		**79**

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 *E. amylovora* S435 were incubated overnight in 3 mL TSB at 30°C and 150 rpm and then resuspended in sterile water (OD_{620} = 0.2). The suspensions were spread onto TSA plates and left to air-dry. Wells were bored into the agar with a sterile glass tube (d = 10 mm) and filled with 200 µL 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.

***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} = 0.02) 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^{-6} 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.

Identification of bacterial isolates

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_2O and maintained at -20 °C [98-100].

16S rRNA gene sequence analysis

To identify the isolates of interest, PCR amplification of the gene encoding the 16S rRNA was performed (Supplemental Table 2). 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 Feldan 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 [101].

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 [102] to predict an accurate consensus lineage of the active isolates.

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 2). 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* fragments were analyzed by agarose gel electrophoresis (Supplemental Tables 1 and 2).

To refine the identification of the *Paenibacillus* spp. isolates, the *rpoB* gene was amplified (Supplemental Table 1) [103, 104]. 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 Feldan 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 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 *Paenibacillus* sp. isolates. The *rpoB* fragments from the *Bacillus* sp. isolates were cloned into a pGEM-T-Easy Vector™ (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.

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.

Formulation of spore-forming bacteria

To formulate two products based on strains of *P. polymyxa* 273 and *B. amyloliquefaciens* subsp. *plantarum* FL50S, Schaeffer's sporulation medium (SSM) was used [105]. 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 as described above in the antimicrobial activity assays section.

Formulation of vegetative bacteria

For the formulation of products based on vegetative bacteria, *P. poae* FL10F, *P. fluorescens* IRDA4F, *P. agglomerans* NY60 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 [106]. 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% w/v to preserve the bacterial products [107]. 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.

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% w/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.

Potted tree validation

A trial was conducted at the IRDA research station in Saint-Bruno-de-Montarville, Québec 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* S435 in potassium phosphate buffer (pH = 6.5) in King B agar which favors the growth of this phytopathogen [108, 109]. The bacterial density was adjusted to ~1 x 10^9 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. 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; 3) Sterile culture medium; 4) *P. polymyxa* 273; 5) *P. poae* FL10F; 6) *B. amyloliquefaciens* FL50S; 7) *P. fluorescens* IRDA 4F; 8) *P. agglomerans* NY130 and 9) *P. agglomerans* NY60.

Inoculated controls were sprayed either 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* FL9F, *P. poae* FL10F, *P. agglomerans* NY60, *P. agglomerans* NY130, and *P. fluroescens* 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 [110]. 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 [111]. Treatment and observation date were used as fixed effects. Individual trees were used as a random effect.

Statistical data

The standard error of the mean (SEM) was used for inhibition zones and colony size measurement. SEM quantifies the precision of the mean. It is a measure of how far your sample mean is likely to be from the true population mean. It is expressed in the same units as the data. Severity scores for the *in planta* study were analyzed as a cumulative link mixed model (clmm) using the "ordinal" package.

4.1.4. Results

Characteristics of the most efficient bacterial strains isolated from the screening
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 2).

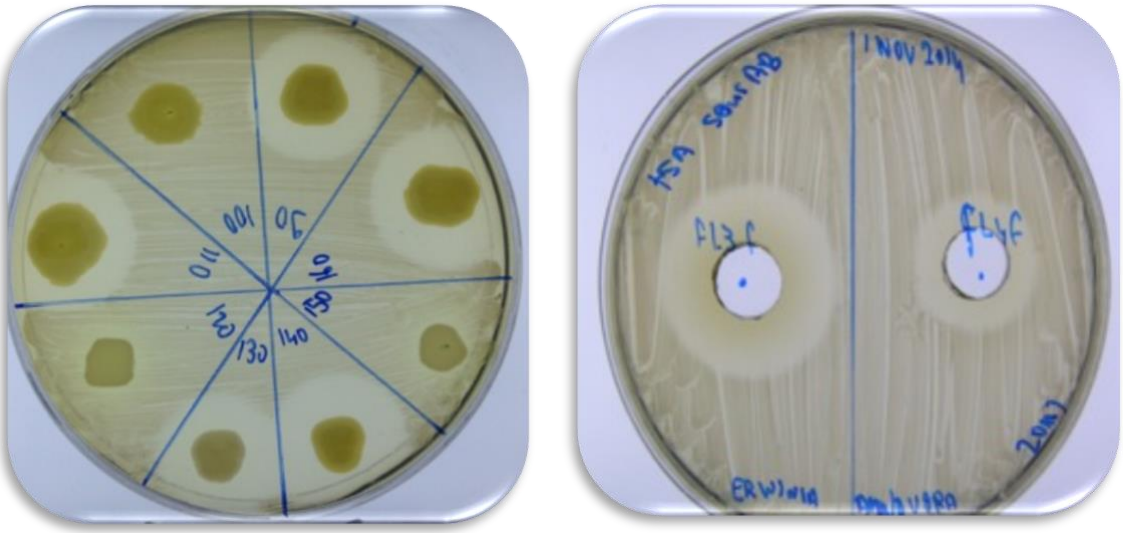
Isolation of microorganisms from environmental samples

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 (Fig. 7A). 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 (Fig. 7B and Table 3). 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 4). 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 5) produced CFS with no visible inhibitory activity against *E. amylovora* S435 (Table 3, bottom).



7A **7B**

Figure 7: 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.

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 inhibitory zones, Table 3) 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 3).

Table 3: **Activities of cell-free culture supernatants against *Erwinia amylovora* S435**

Bacterial isolates / 16S rRNA gene sequence identification = 99% similarity	**Source**	**Medium of isolation**	**Diameter of growth inhibition zone** (including well diameter)*, mm	**2-fold diluted CFS**	**10-fold diluted CFS**
P. polymyxa 273	[E]	Benedict	26.66 ± 0.34	21.00 ± 0.36	17.58 ± 0.50
P. polymyxa 344	[J]	TSA	23.67 ± 0.21	17.08 ± 0.27	no inhibition
B. amyloliquefaciens subsp. *plantarum* FL50S	[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. FD308	[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 FL3F	[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. agglomerans NY50	[N]	TSA	No activity	No activity	No activity
P. agglomerans NY60	[N]	TSA	No activity	No activity	No activity
P. agglomerans NY130	[N]	TSA	No activity	No activity	No activity
P. agglomerans IRDA 36	[L]	TSA	No activity	No activity	No activity
P. agglomerans 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 4: **Efficacies of the extracellular metabolites of *P. polymexa* 273, *B. amyloliquefaciens* FL50S 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	*Paenibacillus polymyxa* 273	25.00 ± 0.25	25.58 ± 0.37
2	*Bacillus amyloliquefaciens* subsp. *plantarum* FL50S	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 5: **Direct antagonistic activity of *Pantoea* isolates against *E. amylovora* S435**

N	Bacterial isolates	*Pantoea* colony diameter (mm) / 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

Identification of active isolates

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 3), 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.

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 6).

Table 6: Summary of further *Pseudomonas* and *Pantoea* strains identification for isolates FL10F, FL9F, FL3F, IRDA 4F, NY60 and NY130.

Isolates	**FL10F CFSAN034337**	**FL9F CFSAN055119**	**FL3F CFSAN034336**	**IRDA 4F CFSAN055120**
Isolates identification (% similarity)				
Resphera Insight	*Pseudomonas poae* (99%)	*Pseudomonas poae* (99%)	*Pseudomonas poae* (98%)	Fluorescent *Pseudomonas* spp. (99%)
Summary of further *Pantoea* strains identification for isolates NY60 and NY130				
Isolates	**NY60 CFSAN047153**	**NY130 CFSAN047154**		
Isolates identification (% similarity)				
Resphera Insight	*Pantoea agglomerans* (99%)	*Pantoea agglomerans* (99%)		

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.

***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* strains. 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.

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*.

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. polymyxa* 273 and *B. amyloliquefaciens* FL50S by ~1 log (Table 7). The mean results of three replicates are presented in Table 7. Furthermore, the inhibitory activity of the various CFS and the direct cell-to-cell antagonism of bacteria in the formulations remained active after > 9 mo storage at room temperature as shown in Tables 8 and 9. Heating during the formulation of spore-forming bacteria-based products had no effect on the activity of CFS (Table 8).

Survival of formulated bacteria sprayed on apple leaves (McIntosh) was then assessed. Table 10 describes the mean of the three replicates which were performed for each treatment, showing that formulated products remained viable on apples leaves for 7 d.

Table 7: **Stability of the biological control product formulations**

		Concentration of bacteria (CFU/ml)		
N	**biological control product**	**Time:Zero**	**3 months**	**9 months**
1	*Paenibacillus polymyxa* 273	$1.10\pm0.02 \times 10^7$	$1.01\pm0.01 \times 10^7$	$2.00\pm0.11 \times 10^6$
2	*Bacillus amyloliquefaciens* subsp. *Plantarum* FL50S	$2.10\pm0.10 \times 10^7$	$2.00\pm0.10 \times 10^7$	$1.03\pm0.06 \times 10^7$
3	*Pseudomonas poae* FL10F	$1.06\pm0.08 \times 10^9$	$1.96\pm0.14 \times 10^8$	$1.96\pm0.12 \times 10^7$
4	*Pantoea agglomerans* NY60	$2.63\pm0.06 \times 10^9$	$1.93\pm0.06 \times 10^9$	$1.00\pm0.10 \times 10^7$
5	*Pantoea agglomerans* NY130	$2.56\pm0.03 \times 10^9$	$1.96\pm0.03 \times 10^9$	$0.96\pm0.13 \times 10^7$
6	*Pseudomonas fluorescens* IRDA4F	$2.00\pm0.10 \times 10^9$	$1.96\pm0.03 \times 10^9$	$1.96\pm0.12 \times 10^7$

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

Table 8: **Antimicrobial activity of CFS of the biological control product formulations against *E. amylovora* S435**

		Diameter of growth inhibition zone (including well diameter) *, mm		
N	**biological control product**	**Time Zero**	**3 months**	**9 months**
1	*Paenibacillus polymyxa* 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	23.50 ± 0.34
3	*Pseudomonas poae* FL10F	35.08 ± 0.27	33.00 ± 0.50	29.16 ± 0.30
4	*Pseudomonas fluorescens* IRDA 4F	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 9: **Antagonistic activity of the biological control product formulations**

N	**biological control product**	**Time Zero***	**3 months***	**9 months***
1	*Paenibacillus polymyxa* 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* IRDA 4F	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* NY60	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

Table 10: Survival of the biological control product formulations on leaves

N	Microbial-based products	Time Zero *CFU per leaf	After 7 days *CFU per leaf
1	*Paenibacillus polymyxa* 273	1.25±0.006 x10^3	1.53±0.033 x10^3
2	*Bacillus amyloliquefaciens* subsp. *Plantarum* FL50S	1.90±0.057 x10^4	1.76±0.003 x10^4
3	*Pseudomonas poae* FL10F	1.20±0.088 x10^5	1.04±0.000 x10^5
4	*Pseudomonas fluorescens* IRDA 4F	1.35±0.005 x10^5	1.35±0.005 x10^5
5	*Pantoea agglomerans* NY60	2.03±0.066 x10^4	2.20±0.000 x10^4
6	*Pantoea agglomerans* NY130	1.95±0.009 x10^4	2.00±0.000 x10^4
7	Control Leaves (disinfected with ethanol)	0.00±0.000	0.00±0.000

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

Potted tree validation

Formulations based on *P. polymyxa* 273 and *B. amyloliquefaciens* 50S were prepared as described in "Formulation of spore-forming bacteria". Those based on *P. poae* FL9F, *P. poae* FL10F, *P. fluorescens* IRDA 4F, *P. agglomerans* NY60, and *P. agglomerans* NY130 were formulated as described in "Formulation of vegetative bacteria". They were diluted to 20% (v/v) before application. Trees treated with *P. agglomerans* strains NY60 *and* NY130 had much less fire blight than the control after 10 days, with Disease Scores (DS) not significantly different from those treated with streptomycin. Fire blight never extended beyond the central vein of the inoculated leaf in trees receiving *P. agglomerans* NY60. The fire blight median severity score, 10 days after inoculation, was reported to be 71.4% less severe on trees treated with *P. agglomerans* NY60 as compared to untreated ones, calculated based on the median score for water=3.5; whereas for *P. agglomerans* NY60=1. No evidence of phytotoxicity appeared in either treatment (Table 11).

Table 11: **Effects of various treatments on potted McIntosh apple trees experimentally inoculated with *E. amylovora* S435**

N	Treatment	Median score	Score range	Tukey**
1	Streptomycin (100 ppm)	0	[0, 2]	a
2	Water	3.5	[2, 4]	c
3	Sterile culture medium	3	[2, 4]	c
4	*P. polymyxa* 273	3	[1, 4]	bc
5	*P. poae* FL10F	2	[1, 3]	bc
6	*B. amyloliquefaciens* FL50S	3.75	[3, 4]	c
7	*P. fluorescens* IRDA 4F	3	[2, 4]	c
8	*P. agglomerans* NY130	1	[0, 3]	ab
9	*P. agglomerans* NY60	1	[0, 1]	ab

** Values followed by the same letter are not significantly different ($P \leq 0.05$) according to lsmeans [112] with an adjustment for Tukey's HSD to control for family-wise error.

4.1.5. Discussion

From 2011-2015, bacteria suppressing the fire blight pathogen *E. amylovora* were isolated from 79 environmental samples. On lawns of *E. amylovora* S435 cultures, 205 bacterial isolates created inhibition zones of various diameters and were, therefore, considered antagonistic against this phytopathogen. Culture supernatants from only 32 of these bacteria clearly inhibited the growth of S435 cultures on agar plates. These were retained for further experimentation as well as five isolates of *P. agglomerans* displaying strong cell-cell antagonistic activity against the phytopathogen, but not through extracellular metabolites. These most active strains were identified by 16S rRNA, *gyrA*, *gyrB* and *rpoB* and were found to be belonging to *Bacillus*, *Paenibacillus*, *Pseudomonas* and *Pantoea* genus. Interestingly there are many publications reporting microorganisms belonging to the above genera for their ability to control *E. amylovora*. An experiment on a strain of *P. agglomerans* which produces, by antibiosis, a complex zone of growth inhibition of *E. amylovora* was performed. This activity was attributed to two antibiotics produced, called pantocin A and B [113]. It was also reported that polymyxin P is the main active ingredient in the suppression of *Erwinia* species by *Paenibacillus polymyxa* M-1

[64]. It was also found that culture filtrates of a strain of *Pseudomonas aeruginosa* strongly inhibited the growth of *E. amylovora* [73]. A strain of *Bacillus amyloliquefaciens* subsp. *plantarum* with a high activity against *E. carotovora* which infects vegetables in post-harvest was isolated [66]. Another study on a *Pantoea vagans* strain indicated two main factors contributing to the biological activity against *E. amylovora*: 1) competition for limiting substrates and 2) production of antibacterial metabolites. They also demonstrated that the genes encoding that antibacterial pantocin A were found on a 28 kb chromosomal genomic island [69].

Among the 32 strains with active CFS, *P. polymyxa* 273, *B. amyloliquefaciens* subsp. *plantarum* FL50S, 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 Bacillus amyloliquefaciens* subsp. *plantarum* FL50S 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 not likely to be developed.

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 [68, 69, 108, 114].

Six strains were chosen based on their abilities to control *E. amylovora in vitro* and i*n 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 (for the sporulating species) or 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 trees inoculation with *E. amylovora*, formulations based on *P. poae* FL10F, *P. agglomerans* NY60 or NY130 were effective

had significantly less fire blight than the control. Importantly, trees treated with formulations based on *P. agglomerans* NY60 or NY130, isolated from an apple orchard, were similar to from those treated with streptomycin, the standard treatment for fire blight indicating that the *P. agglomerans* NY60 or NY130 formulations have a probable curative effect in controlling *E. amylovora* [115].

Our bacteria did not have to be exposed to phytopathogenic bacteria such as *Erwinia amylovora* in order to produce Cell-Free Supernatant (CFS). These active CFS are produced when our bacteria were cultivated in TSB even in the absence of a phytopathogen. The activity of our CFS is attributed to the presence of secondary metabolites and not hydrolytic enzymes. Furthermore, we have recently identified, by mass spectrometry, the inhibitory secondary metabolites present in active fractions of some CFS of our bacteria; these results will be published soon in a follow-up article.

A single formulation of consortia based on the combination of the six formulations could be developed and this is an avenue that could be considered in a follow-up experiment. However, we 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 and will be published in a follow-up article.

Although our active strains are natural isolates, more work is required on the safety of the biocontrol formulations as the whole genome of the most active strains were sequenced.

4.1.6. Conclusions

A major screening was conducted to select bacteria with strong 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.

Acknowledgments

The authors would like to thank Valentin Joubert of IRDA, Saint-Bruno-de-Montarville, Québec, Canada, for his collaboration on this project.

Funding sources

This work was funded by a Consortium de recherche et innovation en bioprocédés industriels du Québec (CRIBIQ) grant and a NSERC-Collaborative Research and Development (CRD) grant

Conflicts of interest

Authors have no conflict of interest to declare.

4.1.7. References

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4.2. Présentation de l'article « Characterization of the biocontrol activity of three bacterial isolates against the plant phytopathogen *Erwinia amylovora* »

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Soumission : 12 mars 2021 ; **Acceptation :** 13 mai 2021 ; **Publication :** 17 mai 2021

Journal : MicrobiologyOpen Volume 10, page e1202 (12 pages); DOI: 10.1002/mbo3.1202

Résumé

Les antibiotiques sont pulvérisés dans les vergers de pommiers et de poiriers pour lutter, entre autres, contre la bactérie *Erwinia amylovora* , l'agent responsable du feu bactérien. Comme pour de nombreux autres agents pathogènes, on observe l'émergence de souches d'*E. amylovora* résistantes aux antibiotiques. Par conséquent, les producteurs recherchent des solutions alternatives pour combattre le feu bactérien. Dans un effort pour trouver des alternatives aux antibiotiques contre ce pathogène, nous avons précédemment isolé trois souches bactériennes avec une activité antagoniste et extracellulaire contre *E. amylovora*, à la fois *in vitro* et *in planta*, correspondant à trois genres bactériens différents : Ici, nous avons identifié le mode d'action inhibiteur de chacun des trois isolats contre *E. amylovora*. L'isolat *Bacillus amyloliquefaciens* subsp. *plantarum* (*B. velezensis*) FL50S produit plusieurs métabolites secondaires dont des surfactines, des iturines et des fengycines. Plus précisément, nous avons identifié l'oxydifficidine comme étant la plus active contre *E. amylovora* S435. *Pseudomonas poae* FL10F produit un composé extracellulaire actif contre *E. amylovora* S435 qui peut être attribué au principe d'induction de la ligne blanche (WLIP), un lipopeptide cyclique appartenant à la sous-famille des viscosines (massétolide E, F, L ou viscosine). *Pantoea agglomerans* NY60 a un effet antagoniste direct de cellule à cellule contre *E. amylovora* S435. En criblant des mutants de cette souche

générés par insertion aléatoire de transposons avec une activité antagoniste réduite contre la souche S435, nous avons identifié plusieurs transposants défectueux. Un mutant d'un gène codant pour un transporteur de la Superfamille des Facilitateurs Majeurs (MFS), correspondant à une protéine transmembranaire supposée être impliquée dans la localisation extracytoplasmique de l'acide griséolutéique, un intermédiaire dans la biosynthèse de l'antibiotique phénazine à large spectre, l'acide D-alanylgriséolutéique, présentait un intérêt particulier.

4.2.1. 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. In an effort 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 biosynthesis of the broad-spectrum phenazine antibiotic D-alanylgriseoluteic acid.

4.2.2. Introduction

The bacterial plant pathogen responsible for fire blight is *Erwinia amylovora* [116, 117]. It is a rod-shaped Gram-negative bacterium capable of infecting different host plants in the *Rosaceae* family, including apple and pear trees [94]. Annual losses due to fire blight can be significant in many countries [92]. In the United States alone, apple and pear producers' losses were estimated in millions of dollars during the year 2000 in Michigan [118, 119] and during 1998 in Washington and northern Oregon due to fire blight [120]. Growers have been spraying antibiotics on apple and pear orchards, but are looking for alternatives due to resistance development [8, 31, 32]. 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 [67]. Giddens *et al.* (2002) characterized a novel phenazine antibiotic gene cluster in *P. agglomerans* Eh1087 effective in the suppression of the fire blight pathogen [121]. 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 [108]. Niu *et al.* (2013) showed that polymyxin P is the main active ingredient in the suppression of *Erwinia* species by *Paenibacillus polymyxa* M-1 [64]. Lee *et al.* (1987) found that culture filtrates of a strain of *Pseudomonas aeruginosa* strongly inhibited the growth of *E. amylovora* [73]. Zhao *et al.* (2013) isolated a strain of *Bacillus amyloliquefaciens* subsp. *plantarum* with a high activity against *E. carotovora* which infects vegetables in post-harvest [66].

Several species belonging to the genera *Pseudomonas*, *Pantoea*, *Bacillus* and *Paenibacillus* are used to control several plant infectious diseases [11, 58]. They have been tested against phytopathogenic bacteria and have shown antagonistic activity against most phytopathogens [59-62]. Several effective inhibitory metabolites produced by the above active bacteria have been described, especially in *Bacillus* and *Paenibacillus* [122]. Polyketides are one important class of microbial secondary metabolites with a wide spectrum of antibacterial and antifungal activities in an agricultural context [42, 123]. 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 [124]. Several species of fluorescent *Pseudomonas* are recognized for their ability to produce NRP-type secondary metabolites with antibacterial and antifungal characteristics [125]. 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 [126, 127].

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 [128]. Here we characterized the inhibitory mode-of-action of each of these strains against *E. amylovora*.

4.2.3. Materials and Methods

Bacterial strains and culture conditions

The bacterial strains used in this study are as follows: *B. amyloliquefaciens* subsp. *plantarum* FL50S was isolated from an 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 [128]. 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.

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 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). Taxonomy of each isolate was assigned using Kraken [129].

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 [130, 131].

The resulting assembled sequences were polished with Illumina reads using a combination of BWA version 0.7.17-r1188 [49], SAMtools version 1.9 [49] and Pilon version 1.22 [132]. The whole genome for the three strains were sequenced and assembled as outlined above. Moreover, the sequences of strain *P. agglomerans* NY60 and *B. velezensis* FL50S have been deposited in GenBank under the successive accession numbers: CP034469 (ncbi.nlm.nih.gov/nuccore/CP034469) and LYNC00000000 (ncbi.nlm.nih.gov/nuccore/LYNC00000000).

Secondary metabolite gene cluster prediction and analysis

Genome mining of biosynthetic gene clusters including non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), hybrid PKS/NRPS, siderophores, and bacteriocins were predicted with antibiotics & Secondary Metabolite Analysis SHell (antiSMASH) [133] web server version 5 (http://antismash.secondarymetabolites.org/).

Production of secondary metabolites

The production of metabolites by bacterial isolates was carried out in 2 L conical flasks with 500 ml of respective medium. *P. agglomerans* NY60 was cultivated in Difco Nutrient Broth (Becton, Dickinson and Co., Franklin Lanes, NJ, USA). King's B medium was used as a production medium for *P. poae* FL10F isolate [109] while Landy medium was used for cultivation of *B. amyloliquefaciens* subsp. *plantarum* FL50S [134, 135]. 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 48h at 200-250 rpm.

To obtain cell-free supernatants, cultures of all three strains were centrifuged at 17,700 × *g* for 1 hour. The pellets were discarded and the supernatants were filtered using 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 one week, or at -20°C for 6 months, while keeping their activity.

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 (Milli-Q system; Millipore, Bedford, MA). 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^{-1}. 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 disc diffusion assays.

Activity of cell-free supernatants and fractions against *Erwinia amylovora* S435

In order to estimate the activity of bacterial metabolites and fractions against *E. amylovora* S435, an agar disc diffusion assay was performed. Blank paper discs (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 disc on a lawn of freshly inoculated *E. amylovora* S435 TSB agar plate and measuring the growth inhibition area (mm^2) after 48 hrs of cultivation at room temperature (~21 °C). This assay was performed in triplicate.

LC-ESI-MS/MS analyses

The cell-free supernatants and active fractions were further analyzed by high-performance liquid chromatography (HPLC; Waters 2795, Mississauga, ON, Canada) equipped with a 250 x 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.

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 [136]. Transposants were selected on TSA plates containing spectinomycin (15 µg/ml). The plates were incubated at room temperature (~21°C) for 2 d. Thereafter, a lawn of lab-derived streptomycin-resistant *E. amylovora* S435 were spread onto TSA 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 determination of transposon insertion site.

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' TACGGTAGCAGAGACTTGGTCTCTAGCATAGAGTGCGTAGCTCTGCT 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 (AGATCGGAAGAGCACACGTCTGAACTCCAGTCACACACTGACGACATGGTTCTACA) and primer 2.

Thereafter, sequencing was performed on an Illumina MiSeq using the MiSeq Reagent Kit v2 Kit (500-cycles). In order to find the transposon insertion sites, 288,889 reads were mapped to *P. agglomerans* NY60 genome (CP034469.1).

4.2.4. Results

Whole-genome sequencing

We have previously reported the identification of three bacterial isolates presenting an effective inhibitory activity against the phytopathogen *E. amylovora* [128]. 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. Its details are oulined in: (https://www.ncbi.nlm.nih.gov/nuccore/NZ_LYNC00000000.1). 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. Its details are oulined in: (https://www.ncbi.nlm.nih.gov/nuccore/CP034469.1) [128].

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 12 and 13 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 biosynthesis of surfactin, iturin and fengycin, and polyketide synthases (PKSs) encoding for difficidin, macrolactin and bacillaene (Table 12). 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 biosynthesis of cyclic lipopeptides (CLP) were identified (Table 13).

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 [128],

the antiSMASH anlysis did not reveal much secondary metabolite genes with anti-bacterial properties.

Table 12: Identified biosynthetic gene cluster regions in the genome of *B. velezensis* FL50S

Region on genome	Biosynthetic gene cluster type	Most similar known cluster	Similarity	MIBiG BGC-ID
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

NRPS: Nonribosomal peptides synthetases
transAT-PKS: Polyketide synthase
MIBiG: The Minimum Information about a Biosynthetic Gene cluster https://mibig.secondarymetabolites.org/
*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 [137].

Table 13: Identified biosynthetic gene cluster regions in the genome of *P. poae* FL10F.

Region on genome	Biosynthetic gene cluster type	Most similar known cluster	Similarity	MIBiG BGC-ID
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

NRPS: Nonribosomal peptides synthetases
MIBiG: The Minimum Information about a Biosynthetic Gene cluster https://mibig.secondarymetabolites.org/
*Similarity shows the % of sequence similarity of the region on the genome to the entries in the MIBiG database using the ClusterBlast algorithm

Identification of metabolites and their inhibitory activity

The antiSMASH analysis suggests that *B. velezensis* FL50S has the biosynthetic machinary 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 analysed 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. CLP were identified by comparing the detected peaks with those from literature and the actual mass of CLP [138, 139]. 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 (**Fig. 8**). Regarding polyketides, only the oxidized form of difficidin, oxydifficidin, could be identified (**Fig. 9**). 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 [123]; in positive mode, we detected oxydifficidin in its dephosphorylated (m/z 463.4) and dimer (m/z 1121.3) species (**Fig. 9**) [140].

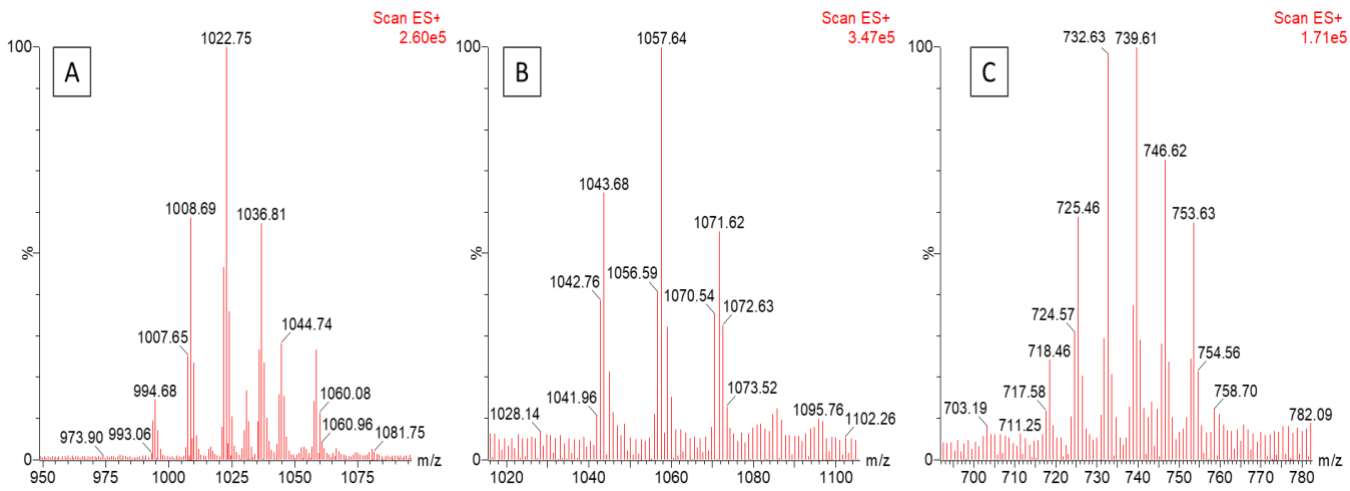

Figure 8 : MS spectra of three families of cyclic lipopeptides produced by *B. velezensis* FL50S
A) surfactins B) iturins and C) fengycins detected in positive ionization mode. In case of fengycins, peaks correspond to the doubly charged ions.

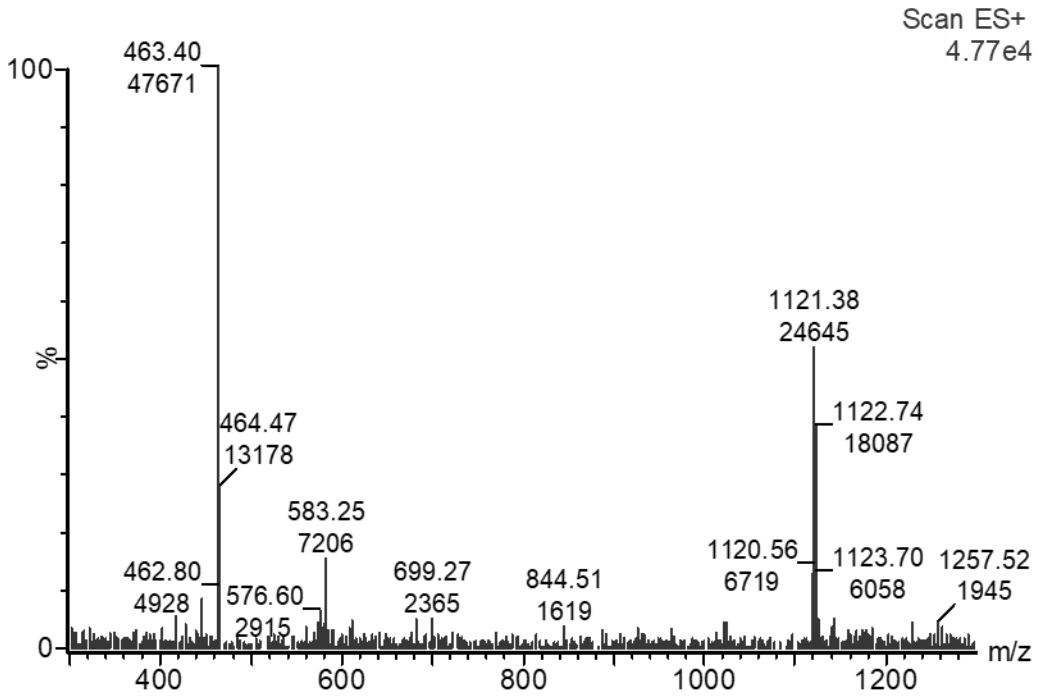

Figure 9 : MS spectra of produced oxydifficidin in supernatant of *B. velezensis* FL50S culture detected in positive ionization mode.

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 (Supplemental Table S1), with fraction #21 causing the largest inihibiton 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 (Supplemental Table S2) with fraction #20 causing the largest inihibiton zone. Analysing the MS spectra of the fractions of *P. poae* FL10F 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 (Fig. 10). 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 putative product of two unlinked NRPS gene clusters involved in biosynthesis of cyclic lipopeptides, suggest that the 1127.1 peak corresponds to the white-line-inducing principle (WLIP) [141], a cyclic lipodepsipeptide belonging to the viscosin subfamily [142] (Fig. 11).

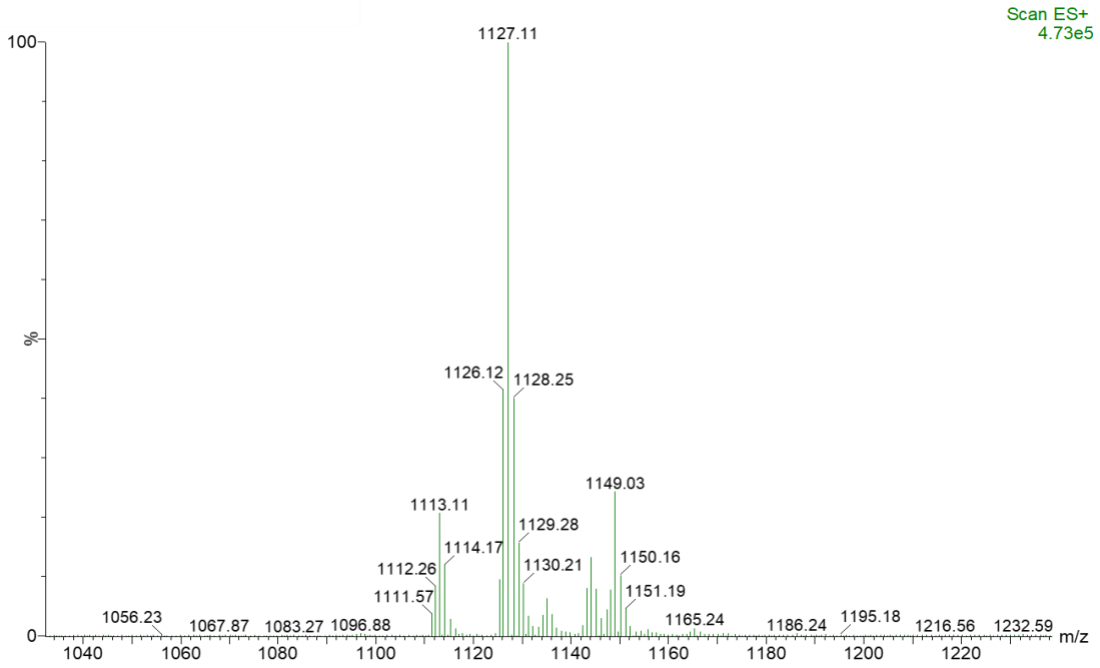

Figure 10 : MS spectra of fraction of *P. poae* FL10F culture extract active against *E. amylovora* S435

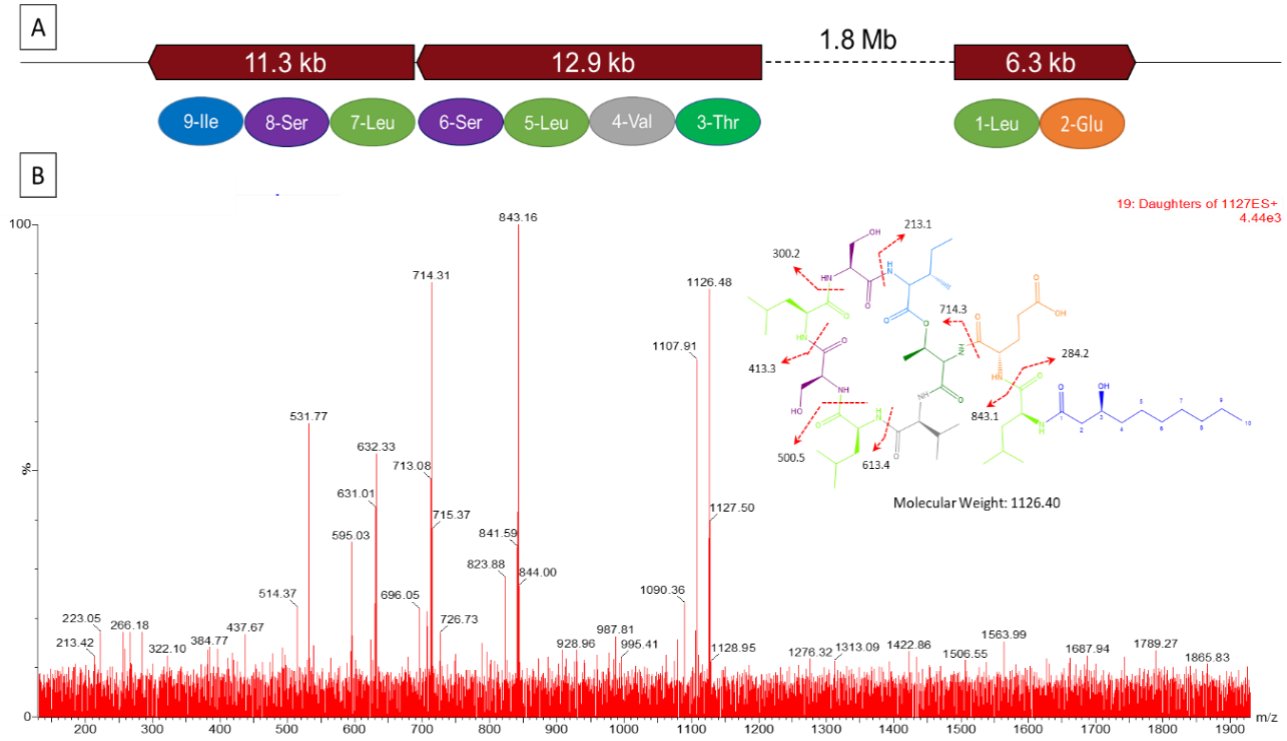

Figure 11: Biosynthetic gene clusters of *Pseudomonas poae* FL10F corresponding to the viscosin and bananamide 1 by antiSMASH A) Organization of CLP biosynthetic gene clusters of *Pseudomonas poae* FL10F **corresponding to the viscosin and bananamide 1 gene clusters (BGC0001312 and BGC0001346 respectivly)** as identified by antiSMASH. B) MS/MS fragmentation spectrum of 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.

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 lawn of *E. amylovora* S435 on TSA agar plates [128]. 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 [128].

In order 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 (Supplemental Table S3). Using transposon insertion sequencing (Tn-seq), the transposon insertion sites of these mutants were mapped, leading to the identification of few genes that apparently altered the production of antagonistic functions (Table 14).

Table 14: Identified transposon insertion sites in the genome of P. agglomerans NY60.

Genome	Transposon insertion site	Locus tag	Putative *P. agglomerans* ortholog[a]
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

Interestingly, one of the transposants has an insertion in a gene predicted to code for a MFS transporter located on the 211 kbp plasmid (CP034471) carried by strain NY60. BLASTing the

sequence indicated that it matches the sequence for EhpJ gene in *Pantoea agglomerans* (*Erwinia herbicola*) Eh1087 which is a transmembrane protein predicted to be involved in the extra cytoplasmic localization of griseoluteic acid, an intermediate in biosynthesis of the potent broad-spectrum phenazine antibiotic D-alanylgriseoluteic acid (AGA) [121].

4.2.5. 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. Accordlingly, 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 [143]; however, to the best of our knowledge, this study is the first to show the specific activity of oxydifficidin on *E. amylovora*.

Our results demonstrates 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 lipononadepsipeptides featuring a Glu2 amino acid with both antifungal as well as antibacterial activity (against gram-positive bacteria) [144, 145]. 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 [139]. Pseudomonads produce molecules that scavenge nutrients, sense population density and enhance or inhibit growth of competing microorganisms [142]. 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 [128]. 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 a random mutagenenesis 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).

Phenazine-producing species like *P. agglomerans* Eh1087, in which the *phz* genes are in a plasmid. Its main phenazine product, D-alanylgriseoluteic acid (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 [146, 147]. 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* [148]. Our data are also consistant with the literature and the emerging view of the antimicrobial activity of AGA against the phytopathogen *E. amylovora* [149].

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

Acknowledgments

The authors would like to thank Marie-Christine Groleau for her help on this project.

Funding sources

This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC)-Collaborative Research and Development (CRD) grant (CRDPJ 488896-15).

Conflicts of interest

Authors have no conflict of interest to declare.

4.2.6. References

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5. DISCUSSION GÉNÉRALE

L'objectif principal de ce projet de recherche était de développer une solution biologique pour protéger les pommiers et des poiriers contre la bactérie phytopathogène *E. amylovora* dans le cadre de la lutte biologique.

Pour ce faire, des tests d'inhibition d'*E. amylovora* (souche S435) ont été effectués avec différentes bactéries isolées à partir de 79 échantillons environnementaux. Dans l'ensemble, 205 isolats ont été avancés comme candidats antagonistes de ce phytopathogène, au vu de leurs zones d'inhibition de diamètres variés. Seulement 32 de ces bactéries ont clairement et sans ambage inhibé la croissance des cultures de S435 sur milieu de culture gélosé via leurs métabolites extracellulaires. Ces dernières ont été retenues pour la suite des travaux, ainsi que cinq isolats de *P. agglomerans* présentant une forte activité antagoniste cellule-cellule contre le phytopathogène, cet antagonisme n'étant pas sous l'influence de métabolites extracellulaires. Les souches les plus actives ont été identifiées par séquençage des gènes *gyrA*, *gyrB* et *rpoB* et celui codant pour l'ARNr 16S, et se sont révélées appartenir aux genres *Bacillus, Paenibacillus, Pseudomonas* et *Pantoea*, déjà tous connus pour inclure dans leur famille des souches au pouvoir inhibiteur d'*E. amylovora*.

Inhibition par des molécules

L'effet inhibiteur de la croissance d'*E. amylovora* par *P. agglomerans* a été relié à deux antibiotiques produits par cette dernière, à savoir la pantocine A et B [140]. Il a également été signalé que la polymyxine P est la molécule active liée à la suppression des espèces d'*Erwinia* par *Paenibacillus polymyxa* M-1 [62]. Une autre étude sur une souche de *Pantoea vagans* a mis en exergue les deux facteurs principaux contribuant à l'activité biologique contre *E. amylovora,* à savoir : 1) la compétition pour les substrats limitants et 2) la production de métabolites antibactériens. Les gènes codant pour la pantocine A sont localisés sur un îlot génomique chromosomique de 28 kb [66].

Inhibition par des souches

Les filtrats de culture d'une souche de *P. aeruginosa* ont démontré une forte inhibition de la croissance d'*E. amylovora* [70]. D'autres études ont mis en évidence que des souches

de *Bacillus amyloliquefaciens* subsp*. plantarum* pouvaient avoir un effet antagoniste contre *E. carotovora,* connu pour infecter les légumes après leur récolte [63].

Parmi les 32 souches identifiées ayant un surnageant actif sans cellules, *P. polymyxa* 273, *B. amyloliquefaciens* subsp*. plantarum* FL50S, ainsi quatre souches de *P. poae* (FL10F, FL3F, FL4F, FL9F) ont toutes conservé leur activité d'inhibition de l'*E. amylovora,* même lorsque diluées 10 fois, ce qui indique que les métabolites anti-*Erwinia* sont produits à des concentrations élevées ou sont très efficaces même à des concentrations faibles de l'ordre de 1/10ème. Il est à noter que le surnageant sans cellules de *P. poae* FL10F, *P. polymyxa* 273, et *B. amyloliquefaciens* subsp. *plantarum* FL50S a démontré une très forte activité antimicrobienne contre trois souches différentes d' *E. amylovora*, dont deux sont résistantes à la streptomycine. Cette activité est attribuable à des métabolites et il est donc peu probable qu'une résistance se développe.

Bien que de nombreuses souches aient produit des métabolites inhibiteurs extracellulaires, ce sont les isolats de *Pantoea* qui ont été montré l'activité antagoniste la plus notable contre *E. amylovora* S435. Par ailleurs, seules les souches de *Pantoea* NY60 et NY130, lorsqu'elles étaient co-cultivées avec *E. amylovora* S453, S153 et S1605*,* étaient capables d'inhiber de manière totale la croissance des agents phytopathogènes cibles, probablement par interaction directe. Une telle activité inhibitrice efficace de *P. agglomerans* contre *E. amylovora* n'avait jamais été signalée auparavant [65, 66, 136, 141].

Dans le but de développer des formulations, six isolats ont été choisies sur la base de leur capacité à contrôler *E. amylovora in vitro* et *in vivo*. Ces dernières appartenaient à différents genres bactériens, ce choix ayant été fait dans l'optique d'augmenter la diversité des souches formulées et d'accroître leur efficacité sur les pommiers par la mise en jeu de mécanismes d'action multiples. Chacune de ces formulations était composée de cultures entières stabilisées par chauffage (pour les espèces sporulantes) ou par ajout de NaCl. Les métabolites actifs non identifiés présents dans le milieu extracellulaire ont maintenu leur activité et une proportion significative des souches bactériennes est restée vivante même après un stockage de 9 mois à température ambiante, ce qui laisse présager que ces souches peuvent probablement être formulées et stockées à température ambiante et sur une durée de temps significative.

Un essai, *in planta*, a été réalisé pour évaluer l'efficacité des formulations bactériennes contre le feu bactérien. Bien que les formulations aient été appliquées deux heures après l'inoculation des arbres avec *E. amylovora*, les formulations à base de *P. poae* FL10F, *P.*

agglomerans NY60 ou NY130 se sont révélées efficaces et ont beaucoup moins souffert du feu bactérien comparativement aux témoins. Il est important de noter que les arbres traités avec des formulations à base de *P. agglomerans* NY60 ou NY130*,* isolées d'un verger de pommiers, étaient similaires à ceux traités avec de la streptomycine, le traitement standard pour le feu bactérien, ce qui indique que les formulations de *P. agglomerans* NY60 ou NY130 ont un effet curatif probable dans la lutte contre *E. amylovora* [142].

Nos bactéries n'ont pas eu besoin d'être en contact direct avec des bactéries phytopathogènes comme *Erwinia amylovora* pour produire du surnageant sans cellules (SSC) actif. Ces SSC actifs sont produits lorsque ces bactéries sont cultivées dans du TSB, même en l'absence d'un phytopathogène. L'activité de notre SSC est attribuée à la présence de métabolites secondaires.

Nous avons alors décidé d'identifier le principe actif inhibiteur de chacun des trois isolats contre *E. amylovora*. La souche *Bacillus amyloliquefaciens* subsp. *plantarum* (maintenant *B. velezensis*) FL50S produit plusieurs métabolites secondaires, notamment des surfactines, des iturines et des fengycines. Plus précisément, nous avons identifié l'oxydifficidine comme la plus active contre *E. amylovora S435.*

La souche *Pseudomonas poae* FL10F produit un composé extracellulaire actif contre *E. amylovora S435* qui peut être attribué au principe (WLIP), un lipopeptide cyclique appartenant à la sous-famille des viscosines (massétolide E, F, L ou viscosine).

La souche *Pantoea agglomerans* NY60 a un effet antagoniste direct de cellule à cellule contre *E. amylovora S435*. En criblant des mutants de cette souche générés par insertion de transposon aléatoire avec une activité antagoniste diminuée contre la souche S435, nous avons identifié plusieurs transposants défectueux. D'un intérêt particulier était un mutant dans un gène codant pour un transporteur de la superfamille des facilitateurs majeurs (MFS), et correspondant à une protéine transmembranaire supposée être impliquée dans la localisation extracytoplasmique de l'acide griséolutéique, un intermédiaire dans la biosynthèse de l'antibiotique phénazine à large spectre d'acide D-alanylgriséolutéique.

L'activité de biocontrôle de trois bactéries inhibitrices efficaces contre le phytopathogène *E. amylovora* a été caractérisée. Pour la souche *B. velezensis* FL50S, grâce aux analyses HPLC-ESI MS sur les fractions obtenues par fractionnement guidé par l'activité du métabolite extracellulaire brut, nous avons découvert que le polykétide oxydifficidine est le principal métabolite actif contre *E. amylovora S435*. De même, les gènes de biosynthétiques correspondants ont été identifiés sur le génome de FL50S. L'oxydifficidine était jusqu'ici connue pour son efficacité contre d'autres phytopathogènes des plantes tels que *Ralstonia solanacearum*, un agent causal du flétrissement bactérien de la tomate [150]; cependant, à notre

connaissance, cette étude est la première à montrer l'activité spécifique de l'oxydifficidine contre *E. amylovora.*

Nos résultats démontrent que *P. poae* FL10F produit un composé extracellulaire actif contre *E. amylovora S435* dont l'activité peut être attribuée au principe inducteur de la ligne blanche (WLIP), un lipodepsipeptide appartenant à la sous-famille des viscosines, qui comprend les massétolides E, F, L et la viscosine. Le WLIP est un membre du groupe des viscosines, des lipononadepsipeptides cycliques présentant un acide aminé Glu2 et ayant une activité antifongique et antibactérienne (à savoir, contre les bactéries à Gram-positif) [151, 152]. En plus de relier les phénotypes connus de production de lignes blanches et d'activité hémolytique d'un producteur de WLIP à la biosynthèse de WLIP, des propriétés supplémentaires d'importance écologique conférées par la production de WLIP ont été publiées, à savoir l'antagonisme contre *Xanthomonas* ainsi que l'implication dans la motilité *swarming* et la formation de biofilms [153]. Les Pseudomonades produisent des molécules qui attirent les nutriments tout en inhibant la croissance des microorganismes concurrents [154]. Ils produisent plusieurs lipopeptides tensioactifs présentant des propriétés antimicrobiennes, mais qui facilitent également la motilité de la surface et influencent la formation de biofilms.

Contrairement aux deux autres bactéries, les surnageants de culture de *P. agglomerans* NY60 n'inhibent pas la croissance de *E. amylovora* [155]. Par conséquent, nous n'avons pas pu utiliser une approche basée sur le fractionnement des fluides de culture guidé par l'activité pour identifier le mode d'action de cet isolat. Au lieu de cela, j'ai effectué une mutagenèse aléatoire couplée à un criblage pour identifier les mutants présentant des activités inhibitrices défectueuses. Parmi les cinq transposants ayant une activité réduite contre *E. amylovora* que nous avons identifiés, le mutant que nous avons relevé comme le plus intéressant est doté d'un transposon inséré au milieu d'un opéron codant pour la biosynthèse de l'AGA, un antibiotique phénazine à large spectre.

P. agglomerans Eh1087 produit une phénazine, AGA, agissant comme un antibiotique et employé en compétition avec des espèces bactériennes étroitement apparentées dans sa niche écologique. Il est intéressant de noter que l'AGA lui-même est toxique pour *P. agglomerans* Eh1087, et que la voie *phz* code une protéine spéciale de liaison à la phénazine, EhpR, qui empêche l'auto-empoisonnement du producteur [156, 157]. Mechan et ses collaborateurs [158] ont également indiqué que le groupe de gènes de synthèse d'AGA est à la base de l'activité antimicrobienne de l'isolat de *P. agglomerans* contre *E. amylovora*.

Selon la littérature, l'oxydifficidine, un polykétide produit par la souche *B. amyloliquefaciens* subsp. *plantarum* FL50S, joue un rôle dans l'inhibition de la biosynthèse des protéines. La surfactine, un lipopeptide produit aussi par la souche FL50S, joue un rôle dans la perméabilisation et la perturbation des membranes.

La viscosine, un lipodepsipeptide cyclique (CLP) produit par la souche *P. poae* FL10F, joue un rôle dans l'interaction avec la membrane cellulaire de l'agent phytopathogène.

La phénazine, un antibiotique produit par la souche *P. agglomerans* NY60, agit comme un agent réducteur une fois diffusé à travers ou inséré dans la membrane, entraînant le découplage de la phosphorylation oxydative et la génération de radicaux superoxydes intracellulaires toxiques et de peroxyde d'hydrogène qui sont nocifs pour l'agent phytopathogène. À notre connaissance, cette étude est la première qui caractérise le mode d'action probable des métabolites secondaires de trois espèces bactériennes différentes appartenant à trois genres bactériens différents efficaces contre *E. amylovora*.

6. CONCLUSION GÉNÉRALE

Bien que nos souches actives soient des isolats naturels, des travaux supplémentaires sont nécessaires pour s'assurer de la sécurité et de l'innocuité des formulations de biocontrôle. Néanmoins, le génome entier des souches les plus actives a été séquencé. De plus, ces produits biologiques possèdent un spectre d'activité plus large et une multiplicité de modes d'action contre *E. amylovora* qui a l'avantage de réduire la probabilité de développement d'une résistance. En utilisant une approche de criblage adaptée et spécifique pour isoler les bactéries efficaces contre *E. amylovora*, nous avons développé un inoculum de lutte biologique contenant à la fois des souches bactériennes actives ainsi que leurs métabolites respectifs, moins phytotoxiques que le cuivre mais tout aussi efficaces pour contrôler la maladie induite par *E. amylovora*.

Les résultats obtenus serviront de base aux expériences futures, notamment aux éventuels mélanges synergiques. Il s'agit toutefois d'un processus complexe, car les bactéries de bio-contrôle peuvent également être antagonistes entre elles.

Une formulation unique de consortia basée sur la combinaison des six formulations pourrait être développée, une piste envisageable dans une expérience de suivi. Cependant, nous avons développé et comparé l'activité de formulations distinctes pour deux raisons principales; premièrement, nous voulions rester le plus proches possibles d'une situation réelle, la meilleure option était donc d'utiliser des formulations bactériennes plutôt que des bactéries vivantes. Egalement, et en particulier, nous envisagions d'identifier quelle formulation/souche de bio-contrôle était la plus efficace.

De plus, nous avons récemment identifié, par spectrométrie de masse, les métabolites secondaires inhibiteurs présents dans les fractions actives de certains SSC de ces bactéries; toutefois, les détails sur les mécanismes antimicrobiens nécessiteront d'autres expérimentations. Des recherches ultérieures seront nécessaires à une meilleure compréhension du mode d'action de nos souches contre *E. amylovora*, ainsi que les facteurs contribuant à l'expression et l'optimisation de ces fonctions inhibitrices.

L'ensemble de l'approche utilisée et des résultats a abouti à une plateforme de développement de solutions biologiques spécifiques afin de contrôler des agents phytopathogènes spécifiques *E. amylovora.* Nous avons pu soumettre un brevet présenté dans l'annexe 1 qui détaille la plateforme utilisée pour développer des solutions biologiques spécifiques contre des agents phytopathogènes particuliers.

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ANNEXE 1: BIOLOGICAL CONTROL AGENTS AGAINST FIRE BLIGHT

Patent: US-2020178540-A1

Inventors: Dagher Fadi and Déziel Éric

Assignee: INRS

Date: Priority 2018/12/07

The present invention relates to biological control agents having anti-microbial activity against the fire blight phytopathogen *Erwinia amylovora*.

BACKGROUND

Fire blight is a contagious disease affecting a number of agriculturally important crops such as apples, pears, and some other members of the *Rosaceae* family, and annual losses due to fire blight are significant in many countries. The bacterial plant pathogen responsible for fire blight is *Erwinia amylovora* , a rod-shaped Gram-negative bacterium capable of infecting different hosts in the *Rosaceae* family, including all species of the *Maloideae* subfamily.

While the most widely used formulations to control bacterial phytopathogens in crop protection are copper-based (e.g., copper hydroxide and copper sulphate), these formulations are not suitable for all plants because of phytotoxic effects, such as unacceptable rusting of the fruits of certain apple cultivars, and decreased fruit yields. Thus, the large-scale application of antibiotics (e.g., streptomycin and oxytetracycline) in agricultural fields have been widely used by growers to fight bacterial diseases, which has contributed to the rise of antibiotic resistant bacteria. In fact, antibiotics sprayed on apple and pear orchards were banned in some European countries to not only reduce the development of resistant bacteria, but also to eliminate the traces of antibiotics contaminating foods.

Presently, there are few effective biological products available to growers to fight diseases such as fire blight. Furthermore, some of the few biological products available were extensively tested as substitutes for antibiotics for more than 7 years in different regions of the United States, and they have generally been shown to have low efficacy and variability in results from one geography to another and from one year to the next (Granatstein, 2011). Thus, there is a need for effective and ecologically responsible products for controlling bacterial phytopathogens that cause fire blight and other plant diseases.

SUMMARY

Described herein are biopesticides having activity against *Erwinia* species, particularly *Erwinia amylovora* , the phytopathogen responsible for fire blight.

About 5,000 individual bacterial isolates from a total of 79 different North American environmental samples from a variety of geographic locations were collected, cultured and screened for potential direct antagonistic activity against *Erwinia amylovora* , based on their ability to produce inhibition zones on a lawn of *E. amylovora* S435. Of the thousands of isolates screened, 205 candidate isolates were identified as exhibiting some degree of inhibition and cell-free supernatants from these 205 isolates were subsequently screened for potential activity of their extracellular metabolites. These screening efforts revealed a plurality of isolates whose undiluted cell-free supernatants exhibited anti-*Erwinia amylovora* activity, with cell-free supernatants from several isolates exhibiting activity even after 10-fold dilution. In fact, cell-free supernatants from several isolates even showed activity against streptomycin-resistant *E. amylovora*. Interestingly, the cell-free supernatants from several *Pantoea agglomerans* strains identified after initial screenings did not exhibit any detectable anti-*Erwinia amylovora* activity, but viable cells of these isolates exhibited potent anti-*Erwinia amylovora* activity, which in some cases were comparable to streptomycin treatment following *in planta* experiments on apple trees with minimal or no phytotoxicity observed. The identity of each of the active isolates was identified through DNA sequencing and other high-resolution taxonomic assignment methods/tools. Formulations of biopesticides comprising the active ingredients from the bacterial species/stains described herein were prepared having increased stability and/or shelf-life. Finally, the efficacy of selected biopesticides described herein were validated in field trials performed on McIntosh and Rome apple trees inoculated with *E. amylovora*, and compared to streptomycin treatment.

In some aspects, described herein is a biopesticide comprising intact or vegetative cells, endospores, spores, and/or metabolites from one or more bacterial species as active ingredients, wherein the vegetative cells, endospores, spores, and/or metabolites exhibit antimicrobial activity against phytopathogenic *Erwinia* species or *Erwinia amylovora* . In some embodiments, the one or more bacterial species may comprise spore-forming bacterial species, and/or the metabolites may comprise secondary and/or extracellular metabolites. In some embodiments, the one or more bacterial species may comprise or consist of: (a) a *Bacillus* species, a *Paenibacillus* species, a *Pantoea* species, a *Pseudomonas* species, or any combination

thereof; (b) *Bacillus velezensis* (formerly known as *Bacillus amyloliquefaciens*), *Bacillus velezensis* subsp. *plantarum*, *Bacillus subtilis*, *Paenibacillus polymyxa*, *Pantoea agglomerans*, *Pseudomonas fluorescens*, *Pseudomonas poae*, or any combination thereof; (c) *Bacillus velezensis* 304, *Bacillus velezensis* 417, *Bacillus velezensis* 431, *Bacillus velezensis* subsp. *plantarum* FL50S, *Bacillus* sp. 331, *Bacillus* sp. 418, *Bacillus* sp. FD308, *Bacillus* sp. FD402, *Bacillus* sp. FD604, *Bacillus* sp. IRDA27, *Bacillus* sp. IRDA618, *Bacillus* sp. IRDA619, *Bacillus* sp. IRDA627, *Bacillus* sp. IRDA63, *Bacillus* sp. IRDA672, *Bacillus* sp. IRDA675, *Bacillus* sp. IRDA683, *Bacillus* sp. IRDA684, *Bacillus* sp. IRDA685, *Bacillus* sp. IRDA687, *Bacillus subtilis* 421, *Paenibacillus polymyxa* 273, *Paenibacillus polymyxa* 344, *Pantoea agglomerans* IRDA36, *Pantoea agglomerans* IRDA59, *Pantoea agglomerans* NY130, *Pantoea agglomerans* NY50, *Pantoea agglomerans* NY60, *Pseudomonas fluorescens* IRDA4, *Pseudomonas poae* FL10F, *Pseudomonas poae* FL3F, *Pseudomonas poae* FL4F, *Pseudomonas poae* FL9F, *Pseudomonas* sp. 41, *Pseudomonas* sp. 42, *Pseudomonas* sp. 43, *Pseudomonas* sp. NY1238, or any combination thereof; (d) *Pseudomonas poae*, *Bacillus velezensis* subsp. *plantarum*, *Paenibacillus polymyxa*, or any combination thereof; (e) *Pseudomonas poae* FL10F, *Pseudomonas poae* FL4F, *Pseudomonas poae* FL3F, *Bacillus velezensis* subsp. *plantarum* FL50S, *Paenibacillus polymyxa* 273, *Pseudomonas poae* FL9F, or any combination thereof; or (f) *Pantoea agglomerans* NY60 or *Pantoea agglomerans* NY130. In some embodiments, the one or more bacterial species comprise or consist of: (g) *Paenibacillus polymyxa, Bacillus velezensis subsp. plantarum, Pseudomonas poae, Pantoea agglomerans, Pantoea agglomerans, Pseudomonas fluorescens, Pseudomonas poae*, *Paenibacillus polymyxa*, *Bacillus velezensis* subsp. *plantarum*, or any combination thereof; and/or (h) *Paenibacillus polymyxa* 273*, Bacillus velezensis subsp. plantarum* FL50S*, Pseudomonas poae* FL10F*, Pantoea agglomerans* NY60*, Pantoea agglomerans* NY130*, Pseudomonas fluorescens* IRDA4*,* or any combination thereof.

In some embodiments, the biopesticide may comprise or consist of: (a) vegetative bacteria; (b) killed vegetative bacteria; (c) heat-killed vegetative bacteria; (d) sporulated bacteria; (e) bacterial spores or endospores; (f) bacterial extracellular metabolites; or (g) any combination thereof, from the one or more bacterial species as defined herein.

In some aspects, described herein is the use of the biopesticide as defined herein as an anti-microbial agent against a plant and/or human pathogenic microorganism, a phytopathogenic *Erwinia* species, *Erwinia amylovora* , or for the prevention and/or treatment of fire blight on a growing plant (e.g., a fruit plant, nut, cereal, vegetable, or flower).

In some aspects, described herein is a method for controlling the growth of a pathogenic microorganism on a target plant or tissue, the method comprising contacting the target plant or tissue with the biopesticide as defined herein.

In some aspects, described herein is a kit for preparing an aqueous solution for use in controlling a pathogenic microorganism (e.g. a phytopathogenic *Erwinia* species or *Erwinia amylovora*) on a plant tissue of a growing plant, the kit comprising: (a) the biopesticide as defined herein; and (b) a suitable container.

In some aspects, described herein is a method for producing the biopesticide as defined herein, the method comprising: for a biopesticide comprising spore-forming bacteria without vegetative cells as active ingredients, culturing vegetative cells from the one or more bacterial species in a sporulation medium for inducing sporulation; inactivating, heat-inactivating, or removing vegetative bacteria from the culture; or for a biopesticide comprising vegetative cells as active ingredients, culturing vegetative cells from the one or more bacterial species in a growth-promoting medium; and formulating the culture to improve viability.

In some aspects, described herein is a biopesticide comprising a vegetative *Pantoea agglomerans* strain as an active ingredient, wherein the vegetative *Pantoea agglomerans* strain: (a) is from the same subspecies as closely related *Pantoea agglomerans* strains NY60 (CFSAN047153) and NY130 (CFSAN047154); (b) has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% overall sequence identity at the genomic DNA level to CFSAN047153 and/or corresponding to the chromosomal sequence as set forth in **SEQ ID NO: 14** and plasmids thereof (**SEQ ID NOs: 15-18**); and/or to CFSAN047154 and/or corresponding to the chromosomal sequence as set forth in **SEQ ID NO: 19** and plasmids thereof (**SEQ ID NOs: 20-23**); (c) comprises genes encoding a bacteriocin, an aryl polyene, an acyl-homoserine lactone (hserlactone), a turnerbactin, a carotenoid, desferrioxamine B, a phenazine (e.g., pyocyanine and/or D-alanylgriseoluteic acid (AGA)), and a microcin; and/or (d) completely kills *E. amylovora* strains S435, streptomycin-resistant S153, and/or streptomycin-resistant S1605, when co-cultivated together *in vitro*.

In some aspects, described herein is a biopesticide comprising a live *Pseudomonas poae* strain and/or cell-free supernatant therefrom, as active ingredient, wherein the cell-free supernatant exhibits anti-*Erwinia amylovora* activity upon 10-fold dilution. In some embodiments, the *Pseudomonas poae* strain: (a) is from the same subspecies as *Pseudomonas poae* strain FL10F (CFSAN034337); (b) has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%,

98%, 99%, 99.5%, or 100% overall sequence identity at the genomic DNA level to CFSAN034337 and/or corresponding to the contig sequences as set forth in any one of **SEQ ID NOs: 24-103** or any combination thereof; and/or (c) comprises genes encoding a bacteriocin, an aryl polyene, a safracin, a pyoverdine, a mangotoxin, a poaeamide, a pyochelin, or any combination thereof.

In some aspects, described herein is a biopesticide comprising a live *Bacillus velezensis* strain and/or cell-free supernatant therefrom, as active ingredient, wherein the cell-free supernatant exhibits anti-*Erwinia amylovora* activity upon 10-fold dilution. In some embodiments, the *Bacillus velezensis* strain: (a) is from the same subspecies as *Bacillus velezensis* strain FL50SF (CFSAN034340); (b) has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% overall sequence identity at the genomic DNA level to CFSAN034340 and/or corresponding to contig sequences as set forth in any one of **SEQ ID NOs: 104-173** or any combination thereof; and/or (c) comprises genes encoding difficidin, oxydifficidin, a bacteriocin, an aryl polyene, a macrolactin, a bacillaene, an iturin, a bacilysin, a surfactin, a bacillibactin, a fengycin, a plipastatin, a teichuronic acid, a locillomycin, a citrulline, or any combination thereof.

In some aspects, described herein is a method for manufacturing a biopesticide against fire blight, the method comprising: cultivating a bacterial strain which is the *Pantoea agglomerans* strain as defined herein, the *Pseudomonas poae* strain as defined herein, or the *Bacillus velezensis* strain as defined herein, under growth conditions; isolating active ingredients from the culture, the active ingredients comprising or consisting of intact or vegetative cells, endospores, spores, and/or metabolites from the bacterial species; and formulating the active ingredients for increased shelf-life, as compared to corresponding unformulated active ingredients.

General definitions

The use of the word "**a**" or "**an**" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one" but it is also consistent with the meaning of "one or more", "at least one", and "one or more than one".

As used herein, the term "**metabolites**" refers to any compound, substance, or by-product obtainable by the culture or fermentation of a microorganism as described herein. In some embodiments, the metabolites of the present description may be produced by culturing microorganisms and harvesting extracellular metabolites produced therefrom (e.g., released

into the culture supernatants). In other embodiments, the metabolites of the present description may be produced using recombinant DNA technology (e.g., recombinant proteins). In some embodiments, the metabolite may be a proteinaceous substance (i.e., a substance comprising a linear polymer chain of at least 3 amino acids bonded together by peptide bonds), bacteriocins, lantibiotics, lipopeptides and/or polyketides. In some embodiments, the metabolites may be extracellular bacterial and/or extracellular fungal secondary metabolites. As used herein, "secondary metabolites" refers to compounds that are not directly involved in normal growth, development, or reproduction. Unlike primary metabolites, the absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of survivability, fecundity or aesthetics.

As used herein, the expression "**antimicrobial**" refers to the ability of the metabolites of the present description to prevent, inhibit, and/or destroy the growth of pathogenic microbes such as pathogenic bacteria and/or pathogenic fungi. In some embodiments, the expression "antimicrobial" encompasses agents or compounds exhibiting antagonistic activity against pathogenic microbes. In some embodiments, the antimicrobial activity may be *in vitro* antimicrobial activity or *in vivo* antimicrobial activity.

In some embodiments, the present description relates to metabolites having antimicrobial activity against phytopathogenic *Erwinia* species, particularly *Erwinia amylovora* . As used herein, term "**pathogen**" or "**pathogenic**" refers to an organism capable of producing a disease in a plant or animal. The term "**phytopathogen**" as used herein refers to a pathogenic organism that infects a plant.

In some embodiments, the present description relates to metabolites which may be extracellular bacterial metabolites. As used herein, the term "**extracellular**" refers to the compounds that are secreted or released (either actively or passively) into the extracellular medium upon culture of viable cells, but may also include compounds that contact the extracellular medium, but which remain associated with the cell membrane.

In some embodiments, the present description relates to metabolites from a *Bacillus* species, a *Paenibacillus* species, a *Pantoea* species, a *Pseudomonas* species, or any combination thereof. As used herein, the expression "**from a [genus] species**" or "**obtainable from a [genus] species**", refers to a compound that may be obtained (i.e., that is obtainable) from the culture or fermentation of a species belonging to the recited genus, but does not necessarily mean that the metabolite must be obtained from that particular species or from the culture of a

microorganism *per se*. For example, compounds produced recombinantly or synthetically, but which have a structure substantially corresponding to the metabolite from the recited species, are also encompassed in the aforementioned expressions. In contrast, as used herein, the expression "**produced from**" is intended to refer to a compound which is obtained from the culture or fermentation of a microorganism of the present description.

In some embodiments, the compositions described herein may be used as an anti-microbial (e.g., bactericidal and/or fungicidal) agent against a plant and/or human pathogenic microorganism, or for the manufacture of an anti-microbial agent for same. In some embodiments, the pathogenic microorganism may be a phytopathogenic *Erwinia* species, particularly *Erwinia amylovora* . In some embodiments, antimicrobial compositions, bacterial species/strains described herein may be used for biological control. As used herein, the expression "**biological control**" refers to the control of a pathogen or any other undesirable organism by the use of at least a second organism other than man. In some embodiments, the compositions defined herein may be a biopesticide or biological pesticide. As used herein, the expressions "**biopesticide**" and "**biological pesticide**" refer to non-naturally occurring commercial products that may include naturally occurring metabolites/microorganisms which are formulated to have anti-microbial activity when applied to plants. Such formulations increase the stability and/or concentrations of the metabolites/microorganisms to levels that are not found in nature, which enable them to be useful as plant pesticides.

SEQUENCE LISTING

This application contains a Sequence Listing in computer readable form created December 5, 2019 having a size of about 25,800 kb. The computer readable form is incorporated herein by reference.

SEQ ID NO:	**Description**
1	rpoB-f primer
2	rpoB-r primer
3	rpoB1698f primer
4	rpoB2041r primer
5	gyrA-f primer
6	gyrA-r primer
7	UP-1 primer

8	UP-2r primer
9	UP-1S primer
10	UP-2Sr primer
11	T7 primer
12	pA-27f-YM primer
13	pH primer
14	*P. agglomerans* NY60 chromosome
15-18	*P. agglomerans* NY60 plasmids 1-4
19	*P. agglomerans* NY130 chromosome
20-23	*P. agglomerans* NY130 plasmids 1-4
24-103	*P. poae* FL10F contigs 1-80
104-173	*B. velezensis* FL50S contigs 1-70
174	Tn-seq primer 1
175	Tn-seq primer 2
176	Tn-seq primer 3

BRIEF DESCRIPTION OF THE DRAWINGS

In the appended drawings:

Fig. 1A shows the antagonistic activity of several active bacterial isolates against *E. amylovora* S435. Bacterial isolates such as those indicated with arrows were selected because they formed clear haloes (inhibition zones indicated by dotted circles) around their colonies on an *E. amylovora* S435 lawn. **Fig. 1B** shows antimicrobial activity of two bacterial isolate cell-free supernatants (CFS) against a lawn of *E. amylovora* S435, wherein inhibition zones are indicated by dotted circles. Left: isolate *P. poae* FL3F; Right: isolate *P. poae* FL4F.

Figs. 2A-2G show representative images of apples inoculated with *E. amylovora* and treated with various antimicrobial control agents or antibiotics. Apples were cut 10 d after treatment. **Fig 2A**: One milliliter sterile water (no inoculation). **Fig 2B**: One milliliter *E. amylovora* S1605 at OD_{620} = 0.2. **Fig. 2C**: One milliliter *E. amylovora* S1605 at OD_{620} = 0.2, followed by 1 mL of Tryptic Soy Broth (TSB) medium 30 min later. **Fig 2D**: One milliliter *P. agglomerans* NY60 (OD_{620} = 0.2) only. **Fig 2E**: One milliliter *E. amylovora* S1605 at OD_{620} = 0.2, followed by 1 ml NY60 culture (OD_{620} = 0.2) 30 min later. **Fig 2F**: One milliliter sterile water containing 100 ppm streptomycin. **Fig 2G**: One milliliter *E. amylovora* S1605 at OD_{620} = 0.2, followed by 1 mL sterile

water containing 100 ppm streptomycin 30 min later.

Figs. 3A-3G show apple leaves cut with scissors inoculated with *E. amylovora* S1605 at OD_{620} = 0.2 and treated with various antimicrobially active isolates. Observations were made 10 d after inoculation. **Fig. 3A**: One milliliter sterile water (no inoculation). **Fig. 3B**: Apple leaves cut with sterile scissors inoculated with 1 mL *E. amylovora* S1605 (OD_{620} = 0.2). **Fig. 3C**: Apple leaves cut with sterile scissors inoculated with 1 mL *E. amylovora* S1605 (OD_{620} = 0.2) followed by 1 mL TSB 30 min later. **Fig. 3D**: Apples leaves treated with 1 mL strain *P. agglomerans* NY60 (OD_{620} = 0.2). **Fig. 3E**: Apple leaves cut with sterile scissors inoculated with 1 ml *E. amylovora* S1605 (OD_{620} = 0.2), then treated with 1 mL strain *P. agglomerans* NY60 (OD_{620} = 0.2) 30 min later. **Fig. 3F**: Apple leaves injected with 1 mL sterile water containing 100 ppm streptomycin. **Fig. 3G**: Apple leaves cut with sterile scissors inoculated with 1 mL *E. amylovora* S1605 (OD_{620} = 0.2) then treated with 1 mL sterile water containing 100 ppm streptomycin 30 min later.

Figs. 4A-4C show MS spectra of three families of cyclic lipopeptides produced by *B. velezensis* FL50S. **Fig. 4A:** surfactins detected in positive ionization mode. **Fig. 4B:** iturins detected in positive ionization mode. **Fig. 4C:** fengycins detected in positive ionization mode. In the case of fengycins, peaks correspond to the doubly charged ions.

Fig. 5 shows MS spectra of produced oxydifficidin in supernatant of *Bacillus velezensis* FL50S culture detected in positive ionization mode.

Fig. 6 shows MS spectra of fraction of *P. poae* FL10F active against *E. amylovora* S435.

Fig. 7A shows organization of cyclic lipopeptide (CLP) biosynthetic gene clusters of *Pseudomonas poae* FL10F as identified by antiSMASH. **Fig. 7B** shows MS/MS fragmentation spectrum of 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.

Fig. 8 shows schematic representation of the three loci with genes encoding proteins involved in T6SS found in strain *P. agglomerans* NY60. The red, green, and blue arrows represent the core, accessory, and other genes, respectively.

DETAILED DESCRIPTION

In some aspects, described herein is a biopesticide comprising active ingredients from one or more bacterial species and/or strains that exhibit antimicrobial activity against phytopathogenic *Erwinia* species, particularly against *Erwinia amylovora* , the bacterial plant pathogen responsible for fire blight.

In some embodiments, the one or more bacterial species and/or strains described

herein may comprise or consists of: (a) bacteria from the genus *Bacillus*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, or any combination thereof; (b) bacteria from the species *Bacillus velezensis* (i.e. *Bacillus amyloliquefaciens*), *Bacillus velezensis* subsp. *plantarum*, *Bacillus subtilis*, *Paenibacillus polymyxa*, *Pantoea agglomerans*, *Pseudomonas fluorescens*, *Pseudomonas poae*, or any combination thereof; or (c) the bacterial strains *Bacillus velezensis* 304, *Bacillus velezensis* 417, *Bacillus velezensis* 431, *Bacillus velezensis* subsp. *plantarum* FL50S, *Bacillus* sp. 331, *Bacillus* sp. 418, *Bacillus* sp. FD308, *Bacillus* sp. FD402, *Bacillus* sp. FD604, *Bacillus* sp. IRDA27, *Bacillus* sp. IRDA618, *Bacillus* sp. IRDA619, *Bacillus* sp. IRDA627, *Bacillus* sp. IRDA63, *Bacillus* sp. IRDA672, *Bacillus* sp. IRDA675, *Bacillus* sp. IRDA683, *Bacillus* sp. IRDA684, *Bacillus* sp. IRDA685, *Bacillus* sp. IRDA687, *Bacillus subtilis* 421, *Paenibacillus polymyxa* 273 (also referred to as *Paenibacillus peoriae* 273), *Paenibacillus polymyxa* 344, *Pantoea agglomerans* IRDA36, *Pantoea agglomerans* IRDA59, *Pantoea agglomerans* NY130, *Pantoea agglomerans* NY50, *Pantoea agglomerans* NY60, *Pseudomonas fluorescens* IRDA4, *Pseudomonas poae* FL10F, *Pseudomonas poae* FL3F, *Pseudomonas poae* FL4F, *Pseudomonas poae* FL9F, *Pseudomonas* sp. 41, *Pseudomonas* sp. 42, *Pseudomonas* sp. 43, *Pseudomonas* sp. NY1238, or any combination thereof. Bacteria from the above-mentioned genera/species/strains were found to produce inhibition zones on a lawn on *E. amylovora* after screenings for antimicrobial activity of cell-free supernatants (see **Tables 3 and 4**) and/or direct antagonistic activity (see **Table 5**), as described in **Example 2**.

In some embodiments, the one or more bacterial species and/or strains described herein may comprise or consists of: (d) bacteria from the species *Pseudomonas poae*, *Bacillus velezensis* subsp. *plantarum*, *Paenibacillus polymyxa*, or any combination thereof; or (e) bacteria from the strains *Pseudomonas poae* FL10F, *Pseudomonas poae* FL4F, *Pseudomonas poae* FL3F, *Bacillus velezensis* subsp. *plantarum* FL50S, *Paenibacillus polymyxa* 273, *Pseudomonas poae* FL9F, or any combination thereof. Cell-free supernatants from the above-mentioned species/strains were found to produce inhibition zones on a lawn on *E. amylovora* even following a 10-fold dilution, as shown in **Table 3**.

In some embodiments, the one or more bacterial species and/or strains described herein may comprise or consists of: (f) bacteria from the strains *Pantoea agglomerans* NY60 or *Pantoea agglomerans* NY130. Strikingly, these strains were the only ones that exhibited the ability of completely killing *E. amylovora* strains S435, streptomycin-resistant S153, and streptomycin-resistant S1605 when co-cultivated together *in vitro*, as described in **Examples 1.8 and 5**. Such effective inhibitory activity of *P. agglomerans* against *E. amylovora* is not believed to

have been previously reported (Pusey et al., 2011; Smits et al., 2011; Stockwell et al., 2010; Kim et al., 2012), and is particularly unexpected because, for example, of the inactivity of their extracellular metabolites against *E. amylovora* (see **Table 3**).

In some embodiments, the one or more bacterial species and/or strains described herein may comprise or consists of: (g) bacteria from *Paenibacillus polymyxa, Bacillus velezensis subsp. plantarum, Pseudomonas poae, Pantoea agglomerans, Pantoea agglomerans, Pseudomonas fluorescens, Pseudomonas poae*, *Paenibacillus polymyxa*, *Bacillus velezensis* subsp. *plantarum*, or any combination thereof; and/or (h) bacteria from the strains *Paenibacillus polymyxa* 273*, Bacillus velezensis subsp. plantarum* FL50S*, Pseudomonas poae* FL10F*, Pantoea agglomerans* NY60*, Pantoea agglomerans* NY130*, Pseudomonas fluorescens* IRDA4*,* or any combination thereof. These species/strains were shown to be amendable to formulation for improved shelf-life, as described in **Example 6**. Furthermore, the cell-free supernatants from *Pseudomonas poae* FL10F*, Paenibacillus polymyxa* 273, *Bacillus velezensis subsp. plantarum* FL50S*, Pseudomonas*, demonstrated the strongest antimicrobial activity against three different *E. amylovora* strains, including two streptomycin-resistant strains.

In some embodiments, one or more bacterial species and/or strains described herein may comprise or consists of the following strains: ***Paenibacillus polymyxa* 273**, which is the strain corresponding to the Center for Food Safety and Applied Nutrition (CFSAN) strain CFSAN034343, BioSample: SAMN04990134, GenBank assembly accession: GCA_001707685.1, Genbank assembly name: ASM170768v1, GenBank sequence accession no: NZ_LYND01000206; ***Pseudomonas poae* FL10F**, which is the strain corresponding to CFSAN strain CFSAN034337 and/or corresponding to the contig sequences as set forth in any one of **SEQ ID NOs: 24-103** or any combination thereof; ***Pseudomonas poae* FL9F**, which is the strain corresponding to CFSAN strain CFSAN055119; ***Pseudomonas poae* FL3F**, which is the strain corresponding to CFSAN strain CFSAN034336; **Fluorescent *Pseudomonas* spp. IRDA4**, which is the strain corresponding to CFSAN strain CFSAN055120**; *Bacillus velezensis* FL50S** which is the strain corresponding to CFSAN strain CFSAN034340 and/or corresponding to contig sequences as set forth in any one of **SEQ ID NOs: 104-173** or any combination thereof; ***Pantoea agglomerans* NY60**, which is the strain corresponding to CFSAN strain CFSAN047153, BioSample: SAMN09909699 and/or corresponding to the chromosomal sequence as set forth in **SEQ ID NO: 14** and plasmids thereof (**SEQ ID NOs: 15-18**); or ***Pantoea agglomerans* NY130**, which is the strain corresponding to CFSAN strain CFSAN047154, BioSample: SAMN09909698 and/or corresponding to the

chromosomal sequence as set forth in **SEQ ID NO: 19** and plasmids thereof (**SEQ ID NOs: 20-23**). In some embodiments, the one or more bacterial species and/or strains described herein may comprise or consists of a strain (e.g. a subspecies) having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% overall sequence identity at the genomic DNA level to one of the strains identified herein by a public CFSAN strain number and/or GenBank accession number.

In some embodiments, a *Pseudomonas poae* strain described herein may comprise native genomic and/or recombinantly-introduced genes encoding one or more of the metabolites a bacteriocin, an aryl polyene, a safracin, a pyoverdine, a mangotoxin, a poaeamide, a pyochelin, and a tolaasin; preferably a bacteriocin, an aryl polyene, a safracin, a pyoverdine, a mangotoxin, a poaeamide, a pyochelin, or any combination thereof.

In some embodiments, a Fluorescent *Pseudomonas* spp. strain described herein may comprise native genomic and/or recombinantly-introduced genes encoding one or more of the metabolites a bacteriocin, an aryl polyene, a safracin, a pyoverdine, a mangotoxin, a poaeamide, a pyochelin, a syringafactin, or any combination thereof.

In some embodiments, a *Pantoea agglomerans* strain described herein may comprise native genomic and/or recombinantly-introduced genes encoding one or more of the metabolites a bacteriocin, an aryl polyenes, an acyl homoserine lactone, a turnerbactin, a carotenoid, desferrioxamine B, a phenazine (e.g., pyocyanine and/or D-alanylgriseoluteic acid (AGA), a microcin, or any combination thereof.

In some embodiments, a *Bacillus velezensis* strain herein may comprise native genomic and/or recombinantly-introduced genes encoding one or more proteins involved in the synthesis, upregulation, or extra-cellular export of difficidin, oxydifficidin, a bacteriocin, an aryl polyene, a macrolactin, a bacillaene, an iturin, a bacilysin, a surfactin, a bacillibactin, a fengycin, a plipastatin, a teichuronic acid, a locillomycin, a citrulline, or any combination thereof.

As used in the context of biopesticides described herein, the expression "**active ingredient**" refers mainly to bacteria-derived agents that are present in sufficient concentrations and formulated to possess the desired antimicrobial activity (e.g., against *Erwinia* species, particularly against *Erwinia amylovora*). In some embodiments, the active agents may include intact cells (e.g., live vegetative cells, sporulated bacteria), killed vegetative bacteria (e.g., heat-killed vegetative bacteria), bacterial endospores/spores (e.g., heat-treated endospores/spores), metabolites (e.g., primary, secondary, intracellular, extracellular/secreted

metabolites), or any combination thereof, from one or more bacterial species or strains described herein that exhibit antimicrobial activity against *Erwinia amylovora* . In some embodiments, the active ingredients are obtainable from or produced by a bacterial species/strain described herein.

In some embodiments, the biopesticide described herein may comprise active agents from one or more spore-forming bacterial species described herein (e.g., *P. polymyxa*, *B. velezensis* subsp. *plantarum*). For example, the biopesticide may be prepared as described in **Example 1.11** by culturing the spore-forming bacteria in a sporulation medium (e.g., Schaeffer's sporulation medium) under conditions (e.g., temperature, time, and agitation conditions) to achieve the desired level of sporulation. The cultures may then be heated at a temperature and for a sufficient period of time to inactivate or kill substantially all vegetative bacteria in the culture, thereby producing a product devoid of vegetative bacteria that comprises spores and/or metabolites (e.g., extracellular metabolites) as active ingredients. In some embodiments, biopesticides lacking live, vegetative bacteria may present advantages in terms of product storage, shelf-life, as well as more favorable paths for regulatory approval.

In some embodiments, the biopesticides described herein may comprise live vegetative bacteria from one or more bacterial species/strains described herein as an active ingredient. Such biopesticide formulations may be advantageous particularly for bacterial species/strains that exhibit their antimicrobial activity via direct antagonism and/or competition (e.g., as shown in **Table 5** for various *Pantoea agglomerans* strains such as NY50, NY60, NY130, IRDA36, and IRDA59; and in **Example 5**), and/or bacteria that produce/secrete antimicrobial extracellular metabolites. Such vegetative bacteria-containing biopesticide formulations may also be advantageous to achieve, for example, longer-lasting products that can colonize, compete with *E. amylovora*, and/or provide extended protection to target plants susceptible to fire blight or other diseases. Indeed, candidate bacteria in the present study 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.

In some embodiments, the biopesticides described herein may comprise live vegetative bacteria from one or more bacterial species/strain described herein as an active ingredient, wherein the live vegetative bacteria have a functional type VI secretion system. Without being bound by theory, such systems may be mainly responsible for interbacterial antagonism (e.g.,

for *P. agglomerans*).

In some embodiments, the biopesticides described herein may comprise live vegetative bacteria from only one bacterial species/strain described herein (or in total) as an active ingredient. Such biopesticide formulations may be advantageous particularly for bacterial species/strains that show antagonism not only with *E. amylovora*, but also with other antimicrobial (e.g., anti-*Erwinia*) bacterial species/strains. Furthermore, a biopesticide comprising only a single bacterial species/strain as an active ingredient may be easier and/or less costly to produce and formulate that biopesticides comprising combinations of different bacterial species/strains.

In some embodiments, preparation of such formulation may comprise a step of culturing the one or more bacterial species under conditions to promote or increase production of secondary metabolites and/or extracellular metabolites (e.g., as compared to under standard growth conditions). For example, the one or more bacterial species are cultured in the presence of glycerol (e.g., about 0.5% w/w) to promote production of extracellular metabolites. Accordingly, the biopesticides described herein may comprise glycerol.

In some embodiments, the cultures comprising live vegetative bacteria and their metabolites may be treated (e.g., heated) to kill remaining vegetative bacteria, leaving the metabolites as active ingredients. As mentioned above, biopesticides lacking live, vegetative bacteria may present advantages in terms of product storage, shelf-life, as well as more favorable paths for regulatory approval. In some embodiments, the intact or vegetative cells may be removed (e.g., via centrifugation and/or filtration) to obtain a cell-free product.

In some embodiments, the biopesticides described herein may comprise one or more agriculturally-suitable additives or preservatives for increased stability/shelf-life. For example, one or more agriculturally-suitable salts may be added as a preservative (e.g., NaCl at about 4% w/w).

In some embodiments, the biopesticides described herein may comprise more than one type of active ingredients (e.g., vegetative bacteria, sporulated bacteria, endospores/spores, and/or extracellular metabolites) and/or active ingredients originating from at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least ten different bacterial species/strains described herein. In some embodiments, the combination use of different types of active ingredients and/or from different bacterial species may reduce the likelihood that the pathogenic microorganism (e.g., *E. amylovora*) develops resistance.

In some embodiments, the biopesticides described herein may lack (not comprise) vegetative bacteria as an active ingredient or is a cell-free composition; and may comprise bacterial spores or endospores, and/or bacterial extracellular metabolites from one or more bacterial species as defined herein, as active ingredients. Alternatively, in some embodiments, the biopesticides described herein may comprise vegetative bacteria (and extracellular metabolites) from the one or more bacterial species as defined herein, as active ingredients. In some embodiments, biopesticides described herein comprise vegetative *Pantoea agglomerans*, *Pantoea agglomerans* NY50, *Pantoea agglomerans* NY60, *Pantoea agglomerans* NY130, *Pantoea agglomerans* IRDA36, *Pantoea agglomerans* IRDA59, or any combination thereof, as active ingredients.

In some embodiments, the biopesticides described herein may comprise active ingredients (e.g., cells, spores, metabolites) from a microorganism that is naturally-occurring and/or that has not been genetically modified using recombinant DNA technology, and thus qualifies as a natural biopesticide and/or natural bioproduct. In alternative embodiments, the biopesticides described herein may comprise active ingredients (e.g., cells, spores, metabolites) from a microorganism that is or has been genetically modified using recombinant DNA technology, for example to improve efficacy and/or stability. In some embodiments, the active ingredients (e.g., cells, spores, metabolites) may be from a mutant microorganism derived from a bacterial species/strain described herein, wherein the mutant retains anti- *Erwinia amylovora* activity.

In some embodiments, the present description relates to genetically modified or mutant antimicrobial bacterial or fungal strains as defined herein. As used herein, the term "**mutant**" making reference to a microorganism refers to a modification of the parental strain in which the desired biological activity (e.g., ability to produce antimicrobial metabolites as defined herein) is similar to or higher than that expressed by the parental strain.

In some embodiments, the biopesticides described herein have the ability to completely kill *Erwinia amylovora* S453, *Erwinia amylovora* S153, *Erwinia amylovora* S1605, or any combination thereof, when co-cultured together *in vitro* (e.g., as described in **Example 5**).

In some embodiments, the biopesticides described herein may exhibit antimicrobial activity against a further plant and/or human pathogenic microorganism, or a pathogenic virus, bacteria, fungus, yeast, mold, or any combination thereof. In some embodiments, biopesticides described herein may have broad spectrum antimicrobial activity. In some embodiments, the

biopesticides described herein may exhibit higher antimicrobial activity against *Erwinia* species (e.g., *Erwinia amylovora*), as compared to other phytopathogenic species.

In some embodiments, biopesticides described herein may comprise at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least ten different bacterial metabolites (e.g., secondary metabolites and/or extracellular metabolites). In some embodiments, the metabolites may comprise lipopeptides and/or siderophores from the one or more bacterial species as defined herein. In some embodiments, the metabolites may comprise metabolites selected from non-ribosomal lipopeptides (NRPs), bananamide, poaeamide, poacin, viscosin, massetolide, orfamide, arthrofactin, surfactin, fengycin, plipastatin, iturin, bacilysin, bacillibactin, bacillomycin, locillomycin, paenilarvin, pelgipeptin, polymyxin, paenibacterin, fusaricidin, bacitracin, tridecaptin, bacteriocin, arylpolyene, acyl homoserine lactone (hserlactone), turnerbactin, carotenoid, desferrioxamine B, pyocyanine, microcin, pantocin, safracin, pyoverdine, mangotoxin, pyochelin, tolaasin, syringafactin, or any combination thereof.

In some embodiments, biopesticides described herein may comprise one or more polyketides. In some embodiments, polyketides could comprise difficidin and/or oxydifficidin. In some embodiments, said polyketides can be oxidized and/or dephosphorylated. In some embodiments, said polyketides may be from one or more bacterial species described herein. In some embodiments, said polyketides may be from *B. velezensis* FL50S.

In some embodiments, biopesticides described herein may comprise isolated, purified, or enriched oxydifficidin (e.g., as the major or principal against against phytopathogenic *Erwinia* species (e.g., *Erwinia amylovora*). In some embodiments, the levels of oxydifficidin in the biopesticides described herein may be present at levels or concentrations that do not occur in nature. In some embodiments, biopesticides described herein may comprise a fraction of cell-free supernatant comprising concentrated oxydifficidin, and optionally lower levels of difficidin. In some embodiments, a cell-free supernatant (or fraction thereof) comprising difficidin may be subjected to an oxidation reaction to convert the difficidin to oxydifficidin, thereby producing a composition comprising increased oxydifficidin. In some embodiments, the present description relating to oxydifficidin may comprise chemical variants of oxydifficidin (e.g., dephosphorylated oxydifficidin).

In some embodiments, biopesticides described herein may comprise one or more lipopeptides. In some embodiments, said lipopeptides could be cyclic lipopeptides. In some

embodiments, biopesticides described herein may comprise one or more lipopeptides from the viscosin subfamily, such as white-line-inducing principle (WLIP), massetolide E, massetolide F, massetolide L, viscosin, or any combination thereof. In some embodiments, said cyclic lipopeptides comprise massetolide and/or viscosin. In some embodiments, said massetolide and/or viscosin is white line-inducing principle (WLIP). In some embodiments, said cyclic lipopeptides may be from one or more bacterial species described herein. In some embodiments, said cyclic lipopeptides may be from *P. poae* FL10F.

In some embodiments, biopesticides described herein may comprise one or more metabolites, antibiotics, or proteins involved in the synthesis and/or release of antibiotics. In some embodiments, the antibiotic is a phenazine. In some embodiments, the phenazine is D-alanylgriseoluteic acid (AGA). In some embodiments, said protein involved in the synthesis and/or release of antibiotics is the bacterial protein EhpJ. In some embodiments, said metabolites, antibiotics, or proteins may be from one or more bacterial species described herein. In some embodiments, said metabolites, antibiotics, or proteins may be from *P. agglomerans* NY60 or NY130.

In some embodiments, biopesticides described herein may comprise an agriculturally acceptable excipient, additive, and/or preservative (e.g., non-toxic carriers, surfactants, preservatives, nutrients, UV protectants, stickers, spreaders, and chelating agents). As used herein, the phrase "**agriculturally acceptable excipient**" refers to an essentially inert substance that can be used as a diluent and/or carrier for an active agent (e.g., antimicrobial metabolites of the present description) in a composition or biopesticide for treatment of plants.

In some embodiments, biopesticides described herein may be formulated as a liquid, concentrate, powder, tablet, gel, pellets, granules, or any combination thereof.

In some embodiments, biopesticides described herein, once applied to a target plant, may have no detectable phytotoxic effect on said target plant, or on the fruits, nuts, or leaves thereof.

In some embodiments, the biopesticides described herein comprise effective amounts of each active ingredient. An "**effective amount**", as used herein, is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations. In terms of treatment, inhibition or protection, an effective amount is that amount sufficient to ameliorate, stabilize, reverse, slow or delay progression of the target infection or disease states.

In some embodiments, biopesticides described herein may comprise one or more active ingredients at concentrations of at least about 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm, 600 ppm, 700 ppm, 800 ppm, 900 ppm, 1000 ppm, 1500 ppm, 2000 ppm , 2500 ppm, 3000 ppm, 3500 ppm, 4000 ppm, 4500 ppm, 5000 ppm, 5500 ppm, 6000 ppm, 6500 ppm, 7000 ppm, 8000 ppm, 8500 ppm, 9000 ppm, 9500 ppm, or 10 000 ppm. In some embodiments, biopesticides described herein may comprise one or active ingredients at concentrations of between about 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm, 600 ppm, 700 ppm, 800 ppm, 900 ppm, 1000 ppm, 1500 ppm, 2000 ppm, 2500 ppm, 3000 ppm, 3500 ppm, 4000 ppm, 4500 ppm, 5000 ppm, to about 10 000 ppm. The term "**about**" is used herein to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value. In general, the terminology "about" is meant to designate a possible variation of up to 10%. Therefore, a variation of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% of a value is included in the term "about".

In some embodiments, biopesticides described herein are for use as an anti-microbial agent against a plant and/or human pathogenic microorganism, a phytopathogenic *Erwinia* species, *Erwinia amylovora* , or for the prevention and/or treatment of fire blight on a growing plant. In some embodiments, the present description relates to the use of the biopesticide as defined herein as an anti-microbial agent against a plant and/or human pathogenic microorganism, a phytopathogenic *Erwinia* species, *Erwinia amylovora* , or for the prevention and/or treatment of fire blight on a growing plant. In some embodiments, the growing plant is a fruit plant, nut, cereal, vegetable, or flower. In some embodiments, the fruit is pome fruit trees and related plants, Pear (*Pyrus* species), quince (*Cydonia*), apple, crabapple (*Malus* species), firethorns (*Pyracantha* species), hawthorn (*Crataegus* species), *Spiraea*, *Cotoneaster*, toyon (*Photinia* species), juneberry, serviceberry (*Amelanchier* species), loquat (*Eriobotria*), mountain ash (*Sorbus* species), blossom (*Prunus*) peach, apricot, cherry, banana, blackberry, blueberry, cantaloupe, cherry, cranberry, currant, grapes, greengage, gooseberry, honeydew, lemon, mandarin, melon, orange, peach, pears, pineapple, plum, raspberry, strawberry, tomatoes, watermelon, grapefruit, pepper, olive, or lime; the nut is: almond, beech nut, Brazil nut, butternut, cashew, chestnut, chinquapin, filbert, hickory nut, macadamia nut, pecan, walnut, or pistachio; the cereal is: amaranth, breadnut, barley, buckwheat, canola, corn, fonio, kamut, millet, oats, quinoa, cattail, chia, flax, kañiwa, pitseed goosefoot, wattleseed, rice, rye, sorghum, spelt, teff, triticale, wheat, or colza; the vegetable is: artichoke, bean, beetroot, broad bean,

broccoli, cabbage, carrot, cauliflower, celery, chicory, chives, cress, cucumber, kale, dill, eggplant, kohlrabi, lettuce, onion, pepper, parsnip, parsley, pea, potato, pumpkin, radish, shallot, soybean, spinach, turnip, or peanut; or the growing plant is an apple tree, pear tree, an apricot plant, stone fruit tree, a plant from the rose family (*Rosaceae* family, or the *Maloideae* subfamily).

In some aspects, described herein is a method for controlling the growth of a pathogenic microorganism on a target plant or tissue, the method comprising contacting said target plant or tissue with the biopesticide as defined herein in sufficient/effective amounts to achieve the desired antimicrobial effect. In some embodiments, the pathogenic microorganism is a phytopathogenic *Erwinia* species or *Erwinia amylovora* . In some embodiments, the contacting method comprises spraying, irrigating, painting, daubing, and/or fogging, onto and/or into the target plant or tissue, the target plant or tissue's hydroponic substrate, and/or the target plant or tissue's agricultural earth (e.g., a growing plant as defined herein).

In some aspects, described herein is a kit for preparing an aqueous solution for use in controlling a pathogenic microorganism (e.g., a phytopathogenic *Erwinia* species or *Erwinia amylovora*) on a plant tissue of a growing plant, the kit comprising a biopesticide as defined herein; and a suitable container (e.g., a pouch, a tablet, or bucket).

In some aspects, described herein is a method for producing the biopesticide as defined herein. For a biopesticide comprising spore-forming bacteria without vegetative cells as active ingredients, the method may comprise culturing vegetative cells from the one or more bacterial species/strain described herein in a sporulation medium for inducing sporulation; and inactivating, heat-inactivating, or removing vegetative bacteria from the culture. For a biopesticide comprising vegetative cells as active ingredients,
The method may comprise culturing vegetative cells from the one or more bacterial species/strain described herein in a growth-promoting medium; and formulating the culture to improve viability. In some embodiments, the aforementioned methods may further comprise culturing the one or more bacterial species/strains described herein under conditions to promote production of secondary metabolites and/or extracellular metabolites (e.g., in the presence of glycerol, such as about 0.5% w/w glycerol). In some embodiments, the method may further comprise adding a preservative, additive or stabilizer to improve the shelf-life of the active ingredients. In some embodiments, the method may comprise combining one or more active ingredients from at least two, at least three, at least four, at least five, at least six, at least

seven, at least eight, at least nine, or at least ten different bacterial species/strains described herein.

EXAMPLES

Example 1: Materials and Methods

Media

Two nonselective media (Tryptic Soy Agar (TSA), and Plate Count Agar (PCA)) and three selective media (Benedict, for isolation of *Streptomyces* spp., Porter et al., 1960; BCSA, for isolation of *Burkholderia* spp., Henry et al., 1997); and Gould, for isolation of *Pseudomonas* spp., Fromin et al., 2001) supplemented with 50 mg/mL cycloheximide (to limit the growth of fungi) were used to isolate bacteria from environmental samples.

Isolation of microorganisms from plants

Ten seeds and three segments (0.5 cm^2) randomly excised from each leaf, stem, root, and fruit were vortexed in 5 mL sterile 0.85% (w/w) 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. To isolate bacteria, the plates were incubated in the dark for 2-5 days at room temperature (~21°C). Microorganisms were isolated from plant samples in triplicate.

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. 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. This assay was performed in triplicate.

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% w/w glycerol.

Strains used to evaluate antimicrobial activity

The pathogen strains used as indicators for antimicrobial activity were *E. amylovora* S435 (IRDA, Quebec, Canada), streptomycin-resistant *E. amylovora* S153 (Botany and Plant Pathology, Oregon State University, USA), streptomycin-resistant *E. amylovora* S1605 (MAPAQ, Quebec, Canada).

First screening step: antagonistic activity assays

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

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* S435 growing on TSA plates. These lawns were first made by incubating 50 µL of *E. amylovora* S435 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; see **Fig. 1A**) on the *E. amylovora* S435 lawns were selected for the second screening step.

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 *E. amylovora* S435 were incubated overnight in 3 mL TSB at 30°C and 150 rpm and then resuspended in sterile water (OD_{620} = 0.2). The suspensions were spread onto TSA plates and left to air-dry. Wells were bored into the agar with a sterile glass tube (d = 10 mm) and filled with 200 µL 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.

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} = 0.02) 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^{-6} 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.

Identification of bacterial isolates

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 ddH2O and maintained at -20 °C.

16S rRNA gene sequence analysis

PCR amplification of the gene encoding the 16S rRNA was performed to identify the isolates of interest according to the **Table 1A** below.

Table 1A: Primers used to determine 16S rRNA and specific genes of active bacterial isolates

Target	Primers	Sequence 5'→3'	Approx. product size (bp)
rpoB gene	rpoB-f	AGGTCAACTAGTTCAGTATGGAC (**SEQ ID NO: 1**)	579
	rpoB-r	AAGAACCGTAACCGGCAACTT (**SEQ ID NO: 2**)	
rpoB gene	rpoB1698f	AACATCGGTTTGATCAAC (**SEQ ID NO: 3**)	
	rpoB2041r	CGTTGCATGTTGGTACCCAT (**SEQ ID NO: 4**)	
gyrA gene	gyrA-f	CAGTCAGGAAATGCGTACGTCCTT (**SEQ ID NO: 5**)	1025
	gyrA-r	CAAGGTAATGCTCCAGGCATTGCT (**SEQ ID NO: 6**)	
gyrB gene (amplificatio	UP-1	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA	1200

n)		(**SEQ ID NO: 7**)	
	UP-2r	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNG TCAT (**SEQ ID NO: 8**)	
gyrB gene (sequencing)	UP-1S	GAAGTCATCATGACCGTTCTGCA (**SEQ ID NO: 9**)	1200
	UP-2Sr	AGCAGGGTACGGATGTGCGAGCC (**SEQ ID NO: 10**)	
	T7	TTGTAATACGACTCACTATAGGG (**SEQ ID NO: 11**)	
16 sRNA	pA-27f-YM	AGAGTTTGATYMTGGCTCAG (**SEQ ID NO: 12**)	1500
	pH	AAGGAGGTGATCCARCCGCA (**SEQ ID NO: 13**)	

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 Feldan 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) and visualized under UV illumination.

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 to predict an accurate consensus lineage of the active isolates.

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 (**Table 1A**). 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 fragments were analyzed by agarose gel electrophoresis (**Tables 1A and 1B**).

Table 1B: Temperature program for amplification of DNA fragments of specific genes by PCR

Step	Protocol			
	rpoB	***gyrA***	***gyrB***	
1	5 min @ 95 ˚C	5 min @ 95 ˚C	5 min @ 95 ˚C	initial denaturation
2	1 min @ 95 ˚C	30 s @ 95 ˚C	1 min @ 95 ˚C	denaturation
3	1 min @ 51 ˚C	45 s @ 51 ˚C	1 min @ 60 ˚C	annealing
4	1 min @ 72 ˚C	1 min @ 72 ˚C	2 min @ 72 ˚C	elongation
5	Repeat steps 2-4	Repeat steps 2-4	Repeat steps 2-4	29×
6	10 min @ 72 ˚C	10 min @ 72 ˚C	10 min @ 72 ˚C	final elongation
7	∞ 4 ˚C	∞ 4 ˚C	∞ 4 ˚C	stop PCR reaction and refrigerate DNA products
8	end	end	end	

To refine the identification of the *Paenibacillus* spp. isolates, the *rpoB* gene was amplified (**Table 1B**). 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 Feldan 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 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 *Paenibacillus* sp. isolates. The *rpoB* fragments from the *Bacillus* sp. isolates were cloned into a pGEM-T-Easy Vector™ (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%, it was considered 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.

In planta testing

Treatment of apple leaves and fruits

For leaf inoculation, *E. amylovora* S435 was grown for 24 h in 3 mL TSB with shaking at 200 rpm. The culture was diluted with fresh sterile TSB to OD_{620} = 0.2 which was equivalent to 2 x 10^8 CFU/mL. Silwet L-77 surfactant at a final concentration of 0.025% (v/v) was added to the cell suspension to facilitate penetration of the pathogenic bacteria. McIntosh leaves were collected from apple orchards (IRDA) and stored at 4°C for 1-5 days prior to treatment. Sterile scissors were soaked in 1 mL *E. amylovora* S435 suspension and used to cut three McIntosh apple leaves replicates per strain in order to inoculate them with *E. amylovora* S435 (one cut of 2-3 cm per leaf). The test strains were grown in 10 mL TSB medium for 2 d at 30°C and 200 rpm. About 30 min after leaf inoculation, 1 mL per leaf of test strains culture were sprayed with sterile sprayers. They were then stored in Petri dishes unto sterile wet Whatman filter papers and incubated in a growth chamber (25°C, 40% relative humidity (RH), 16-h light / 8-h dark photoperiod) for 10 d to determine the efficacy of the isolates against fire blight infection by comparing treated vs. untreated infected leaves which were sprayed with sterile TSB only.

For fruit inoculation, 1 mL *E. amylovora* S435 culture, diluted with fresh sterile TSB to OD_{620} = 0.2, was injected into McIntosh apples (IRDA orchard) with three fruit replicates per strain using a sterile syringe (one injection 3-5 cm deep per apple). About 30 min after the *Erwinia* inoculation, the test isolates were prepared as described above. One milliliter of each isolate was injected into the same inoculation site on the fruit. The fruits were then incubated in a growth chamber as described above to determine the efficacy of the isolates by comparing treated vs. untreated infected fruit injected with sterile TSB only. The leaves and fruits were evaluated for disease severity by visually rating the percentage of leaf tissue and fruit with lesions 2-10 d after inoculation.

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.

Formulation of spore-forming bacteria

To formulate two products based on strains of *P. polymyxa* 273 and *B. velezensis* (formerly known as *Bacillus amyloliquefaciens*) subsp. *plantarum* FL50S, Schaeffer's sporulation medium (SSM) was used. 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 and found to not significantly affect antimicrobial activity.

Formulation of vegetative bacteria

For the formulation of products based on vegetative bacteria, *P. poae* FL10F, *P. fluorescens* IRDA4, *P. agglomerans* NY60 and NY130 were cultivated in Difco Nutrient Broth (Becton, Dickinson and Co., Franklin Lanes, NJ, USA) supplemented with 0.5% w/w glycerol to promote the production of secondary metabolites. 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% w/w to preserve the bacterial products. 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.

Stability and survival of stored isolates *in vivo*

Apple leaves (McIntosh) were disinfected 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 months were diluted at a rate of one part product in twenty parts sterile water. One milliliter of this solution 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% w/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.

Management of fire blight on shoots in apple trees

A trial was conducted at the IRDA research station in Saint-Bruno-de-Montarville,

Québec to evaluate the efficacies of the best isolates previously screened. To simulate fire blight, *Malus* x *domestica* 'McIntosh' cultivar trees were inoculated then treated 2 h later with 20% (w/w) isolate dilutions. Inoculum was obtained by suspending a 5-d culture of the virulent strain *E. amylovora* S435 in potassium phosphate buffer (pH = 6.5) in King B agar which favors the growth of this phytopathogen. The bacterial density was adjusted to ~1 x 10^9 CFU/mL. Actively growing potted trees (cv. McIntosh grafted onto M26) were inoculated by transversely bisecting the two youngest leaves with scissors dipped in the inoculum. 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.

Inoculated controls were sprayed either with water or 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* FL9F, *P. poae* FL10F, *P. agglomerans* NY60, *P. agglomerans* NY130, and *P. fluroescens* IRDA4. It consisted of Nutrient Broth NB supplemented with 0.5% w/w glycerol and a 4% w/w NaCl. 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. 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. Treatment and observation date were used as fixed effects. Individual trees were used as a random effect.

Field trial to control fireblight on Rome cultivars in Geneva NY (USA)

A field trial was conducted to determine the performance of the formulated product based on the vegetative cells of *Pseudomonas poae* FL10F and its extracellular metabolites produced in the supernatants and was compared to antibiotic (FireWall 17 WP Streptomycin), copper fungicide (Cueva, Certis, USA) and the biological product Blossom Protect based on two strains of *Aureobasidium pullulans*. The strain (*P. poae* FL10F) was selected because it demonstrated the strongest *in vitro* antagonistic and antimicrobial activity against *E. amylovora* S435, S153 and S1605. Both antibiotic and copper that were used in this trial were EPA registered products. *Pseudomonas poae* FL10F was cultivated for 2 days at 30°C and 200 rpm and reached an OD_{600nm}= 2.50, which was empirically determined to represent 2.50×10^9 CFU/mL

by plating serial dilutions of the suspension and counting colonies. A 4L of product based on *Pseudomonas poae* FL10F and its metabolites was produced as described above and was sprayed more than 2 months later on apple Rome trees. Three samples of this formulated product were retained in sterile 50 mL polypropylene centrifuge tubes at room temperature for long-term activity and bacterial count studies.

The 4L product was used during in a field trial conducted in Phelps, (Geneva) NY to evaluate the product against fire blight. Apple Rome were used for this study that had 5 to 12 blossom clusters per tree. These were 1-year-old trees from Waffler Nursery, Wolcott, NY.

The experimental treatments were applied using a CO_2 Backpack sprayer that was calibrated to deliver 100 gallons per acre (Trees were sprayed to first drip with a "dilute" solution of products.) The sprayer was calibrated at 42 PSI and used 2 Hollow Cone (TXVK18) Nozzles.

An evaluation of treatments was conducted using the method where scissors were dipped into a solution of a streptomycin sensitive *Erwinia amylovora* strain S01, and the two smallest leaves on each terminal or shoot were cut in half. This severe test is used to simulate infection that can occur following hailstorms. The scissors were dipped into the *Erwinia amylovora* S01 solution prior to each cut. The apical terminal shoot and the terminal leaves on any side shoots were cut. For the size of these trees, the number of terminals that were cut varied from 9 to 18. To evaluate the treatments for this method, each treatment was applied as before to all leaves and shoots of seven individual trees. The inoculations occurred near dusk, and 1.15 inches of rain occurred during the overnight period. All treatments were applied 30 minutes before the infection and then re-applied 24 hours after the initial inoculation. The second application of treatments was applied to the same trees. The product based on Fluorescent *Pseudomonas poae* FL10F and its metabolites was first diluted at a concentration of 20% (w/w) with water then it was mixed properly and sprayed.

At 3 weeks following the scissor inoculation, each terminal shoot was rated for the length in inches with typical symptoms of fire blight (rated as SEVERITY). Then the percentage of all shoots that had any symptoms of fire blight was calculated as INCIDENCE).

Example 2: Isolation of bacterial isolates and *in vitro* screening for anti-*E. amylovora* activity

The leaves, flowers, stems, and fruits of apples, pears, tomatoes, and strawberries, and agricultural field soil samples, were collected from various locations from 2011-2015, as described in **Table 2**.

Table 2: 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) Québec, Canada, September 2014	4
Agricultural field soil	[B]: Apple and pear orchards, Oka, Québec, Canada, September, 2014	3
Agricultural field soil	[C]: Sherrington, Québec, Canada, September, 2014	4
Agricultural field soil	[D]: Wimauma, Florida, USA, April, 2013	1
Agricultural field soil	[E]: Sherrington, Québec, 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), Québec, Canada, September, 2014	4
Tomato leaves and fruits	[J]: Sherrington, Québec, Canada, November, 2011	4
Leaves, stems, and fruits of various plants	[K]: Orchard, Laval, Québec, Canada, September, 2014	4
Flowers, leaves, stems, and soil of Empire, Marshall McIntosh, Cortland,	[L]: Apple and pear orchards, Mont-Saint- Bruno (IRDA), Québec, Canada, May-September, 2015	30

Mcintosh, Paula Red, Honeycrisp, Royal Court and Lobo apple cultivars		
Apple leaves, stems, and soil	[M]: Apple trees, Laval, Québec, Canada, June, 2015	5
Rome apple leaves and stems	[N]: Geneva, New York, USA, July, 2015	5
Pear flowers, leaves, and stems	[O]: Laval, Québec, Canada, May, 2015	5
Total		**79**

A total of 79 environmental samples were analyzed. 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 (**Fig. 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 (**Fig. 1B** and **Table 3**). The CFS of *P. polymyxa* 273, *P. poae* FL10F, and *B. velezensis* subsp. *plantarum* FL50S, displayed the strongest activity against *E. amylovora* S435. They also inhibited the growth of streptomycin-resistant *E. amylovora* S153 and *E. amylovora* S1605 (**Tables 3 and 4**). Cell-free supernatants from these three isolates formed inhibition halos 25.0-35.0 mm in diameter on *E. amylovora* S435 cultures and were subjected to further analyses in **Table 4**.

Table 3. Activities of CFS against *Erwinia amylovora* S435

Bacterial isolates / 16S rRNA gene sequence identification = 99% similarilty	**Source**	**Medium of isolation**	**Diameter of growth inhibition zone** (including well diameter)*, mm	**2-fold diluted CFS**	**10-fold diluted CFS**
Paenibacillus polymyxa 273	[E]	Benedict	26.66 ± 0.34	21.00 ± 0.36	17.58 ± 0.50
Paenibacillus polymyxa 344	[J]	TSA	23.67 ± 0.21	17.08 ± 0.27	no inhibition
Bacillus velezensis subsp. *plantarum* FL50S	[F]	PCA	28.58 ± 0.33	27.16 ± 0.21	19.08 ± 0.27

Bacillus velezensis 304	[E]	Benedict	27.25 ± 0.30	20.16 ± 0.30	No inhibition
Bacillus sp. FD308	[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
Bacillus velezensis 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
Bacillus subtilis 421	[E]	TSA	25.16 ± 0.21	22.66 ± 0.42	No inhibition
Bacillus velezensis 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 FL3F	[G]	PCA	28.10 ± 0.27	25.16 ± 0.30	19.50 ± 0.18
Pseudomonas poae FL4F	[G]	PCA	29.92 ± 0.20	23.75 ± 0.31	21.50 ± 0.34
Pseudomonas poae FL9F	[G]	PCA	26.00 ± 0.26	23.50 ± 0.34	16.50 ± 0.22
Pseudomonas poae FL10F	[G]	PCA	35.08 ± 0.27	30.41 ± 0.37	25.10 ± 0.27
Pseudomonas fluorescens IRDA4	[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
Pantoea agglomerans NY50	[N]	TSA	No activity	No activity	No activity
Pantoea agglomerans NY60	[N]	TSA	No activity	No activity	No activity

Pantoea agglomerans NY130	[N]	TSA	No activity	No activity	No activity
Pantoea agglomerans IRDA36	[L]	TSA	No activity	No activity	No activity
Pantoea agglomerans IRDA59	[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 4. Efficacies of the extracellular metabolites of *P. polymyxa* 273, *B. velezensis* FL50S and *Pseudomonas poae* FL10F against *E. amylovora* S153 and S1605

Bacterial isolates	**Diameter of growth inhibition zone of *E. amylovora* strains (including well diameter)*, mm**	
	S153	**S1605**
Paenibacillus polymyxa 273	25.00 ± 0.25	25.58 ± 0.37
Bacillus velezensis subsp. *plantarum* FL50S	27.30 ± 0.42	27.83 ± 0.40
Pseudomonas poae FL10F	29.58 ± 0.52	33.08 ± 0.27

± Standard error of the mean (SEM) of three replicates, * Well diameter = 10 mm.

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 inhibitory zones, **Table 3**) were diluted by 2-fold and 10-fold, and the *E. amylovora* S435 inhibition zone assays were repeated. CFS from six of the isolates retained some inhibitory activity even at a 10-fold dilution: *P. polymyxa* 273; *B. velezensis* subsp. *plantarum* FL50S; *P. poae* FL3F; *P. poae* FL4F; *P. poae* FL9F; and *P. poae* FL10F (see **Table 3**).

Surprisingly, a subgroup of five *P. agglomerans* isolates (NY50, NY60, NY130, IRDA36, IRDA59) selected because of their strong inhibitory activity in direct antagonism assays (see e.g., **Table 5**), even in comparison to a plurality of other isolates from the same species eliminated following the first screenings, produced CFS with no visible inhibitory activity against *E. amylovora* S435 (see **Table 3**).

Table 5. Direct antagonistic activity of *Pantoea* isolates against *E. amylovora* S435

Bacterial isolates	***Pantoea* colony diameter (mm) / inhibition zone on *E. amylovora* S435 lawn (including *Pantoea* diameter in mm)**
P. agglomerans NY50	11.33±0.33 / 28.33±0.33
P. agglomerans NY60	10.00±0.57 / 30.66±0.33
P. agglomerans NY130	10.66±0.66 / 31.33±0.66
P. agglomerans IRDA36	10.66±0.66 / 25.33±0.66
P. agglomerans IRDA59	10.33±0.66 / 26.33±0.88

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

Example 3: *In planta* testing of apples and leaves inoculated with *E. amylovora*

Two *in planta* experiments were then performed: (1) infections of apples by *E. amylovora* and subsequent injection of an active strain, and (2) infection of apple leaves by *E. amylovora* via scissor-cutting and subsequent spraying of active strains, to assess their biocontrol potential. The 37 active strains were tested in these *in planta* experiments.

Strikingly, some *P. agglomerans* strains (e.g., NY60) showed comparable efficacy to the streptomycin antibiotics, controlling around 80% disease severity in apples and around 40% in leaves as shown in **Figs. 2 and 3**. No phytotoxicity was observed with the *P. agglomerans* NY60 treatment. Disease symptoms on the leaves were observed and recorded independently by two separate technicians. Disease severity and incidence assessments were made based on the results compiled from three separate experiments.

Example 4: Identification of active isolates

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 3**), 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 other 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.

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 *Pseudomans* 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 (**Tables 6 and 7**).

Table 6: Summary of further *Pseudomonas* strains identification for isolates FL10F, FL9F, FL3F and IRDA4

Isolates	**FL10F CFSAN034337**	**FL9F CFSAN055119**	**FL3F CFSAN 034336**	**IRDA4 CFSAN055120**
Isolates identification (% similarity)				
Resphera Insight	*Pseudomonas poae 99%*	*Pseudomonas poae 99%*	*Pseudomonas poae 98%*	Fluorescent *Pseudomonas spp. 99%*

Table 7: Summary of further *Pantoea* strains identification for isolates NY60 and NY130

Isolates	**NY60 CFSAN047153**	**NY130 CFSAN047154**
Isolates identification (% similarity)		
Resphera Insight	*Pantoea agglomerans 99%*	*Pantoea agglomerans 99%*

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 velezensis*; 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.

Whole-genome sequencing

Genomic DNA was isolated from an overnight culture of each strain using a QiagenDNeasy™ blood and tissue kit (Qiagen Inc., Valencia, CA). Genome sequencing was performed using Illumina MiSeq™ sequencing system (Illumina, San Diego, CA), achieving > 50X average genome coverage. *De novo* assembly was created 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). Taxonomy of each isolate was assigned using Kraken.

The two-way ANI calculator (http://enve-omics.ce.gatech.edu/ani/index) was used to estimate the average nucleotide identity between genomic datasets of the best anti-*Erwinia* bacterial isolates and already known type strains of *Pantoea, Pseudomonas, Paenibacill*us and *Bacillus* species from NCBI database (http://www.ncbi.nlm.nih.gov/nuccore). If the ANI value between two genomes is above 95%, it means that two isolates belong to the same species.

Complete Genome Sequencing

Genome mining of biosynthetic gene clusters including non-ribosomal peptide synthetases (NRPSs) and other secondary metabolites were predicted with antibiotics & Secondary Metabolite Analysis SHell (antiSMASH) web server (http://antismash.secondarymetabolites.org/).

For the strain 273, the genome coverage was as high as 88% (N50 = 364,236 bp) and the resulting number of contigs was calculated to be 206. Only 7.37% of the reads were unclassified which reflect the accuracy of the species identification. The confidence score and species check percentage was as high as 90.91% indicating that the strain 273 is indeed a *Paenibacillus polymyxa* species.

Concerning the strain FL50S, the genome coverage was as high as 78% (N50 = 132,866 bp) and the resulting number of contigs was calculated to be 105. Only 5.20% of the reads were unclassified which reflect the accuracy of the species identification. The confidence score and species check percentage was as high as 93.26% indicating that the strain FL50S is indeed a *Bacillus velezensis* species with a percentage of 11.63 for the subsp. *Plantarum*. The gene clusters for non-ribosomal peptide synthetases (NRPSs) and other secondary metabolites for isolates 50S, 273, FL3F, FL9F, FL10F, IRDA4, NY60 and NY130, are illustrated in **Tables 7A-7C**. Strikingly, although the NY60 and NY130 strains were isolated from independent environmental samples, their genomic sequences – particularly with respect to non-ribosomal peptide

synthetases and other secondary metabolites – were extremely similar suggesting that such genomic sequences may be used to identify these strains, as well as other *P. agglomerans* strains of having potent anti-*Erwinia* activity.

Table 7A: Summary of the complete genome sequencing and major gene clusters for non-ribosomal peptide synthetases (NRPSs) and secondary metabolites for isolates 50S and 273

Isolate	**50S** **CFSAN034340** LYNC00000000: accession numbers NCBI assigned after annotation Annotated as '*Bacillus velezensis*'	**273** **CFSAN034343** LYND00000000: accession numbers NCBI assigned after annotation
Genome sequencing and species check		
Genome coverage	78%	88%
N50 (bp)	132,866	364,236
Number of contigs	105	206
Unclassified reads	5.20%	7.37%
Confidence score and species check	93.26% for *Bacillus velezensis*	90.91% for *Paenibacillus polymyxa*
Subsp.	11.63% for *Plantarum*	-
Biosynthetic gene clusters % similarity for non-ribosomal peptide synthetases (NRPSs) and other secondary metabolites		
Macrolactin	100%	-
Bacillaene	85%	28%
Difficidin	53%	-
Iturin	22%	-
Bacilysin	100%	-
Surfactin	47%	-
Bacteriocin	-	-
Bacillibactin	92%	-
Kalimantacin/batumin	-	-
Fengycin	80%	-
Nosperin	-	46%
Bacillomycin	-	-
Fusaricidin	-	100%
Paenibacterin	-	60%

Polymyxin	-	100%
Bacitracin	-	22%
Tridecaptin	-	80%
Plipastatin	23%	-
Teichuronic acid	100%	-
Subtilin	-	-
Locillomycin	28%	-
Myxovirescin	-	8%
Citrulline	27%	-
Bacitracin	-	-
Paenibacillin	-	-

Table 7B: Summary of the complete genome sequencing and major gene clusters for non-ribosomal peptide synthetases (NRPSs) and secondary metabolites for isolates FL10F, FL9F, FL3F and IRDA4

Isolates	**FL10F CFSAN034337**	**FL9F CFSAN055119**	**FL3F CFSAN034336**	**IRDA4 CFSAN055120**
Isolates identification (% similarity)				
-16S ribosomal RNA -Resphera Insight	*Pseudomonas poae* *99%*	*Pseudomonas poae* *99%*	*Pseudomonas poae* *98%*	Fluorescent *Pseudomonas spp.* *99%*
Complete genome sequencing - Biosynthetic gene clusters % similarity for non-ribosomal peptide synthetases (NRPSs) and other secondary metabolites				
Bacteriocin	+	+	+	+
Arylpolyene	45%	15%	45%	20%
Safracin	90%	70%	90%	90%
Pyoverdine	11%	3%	11%	10%
Mangotoxin	57%	-	57%	57%
Poaeamide	100%	-	100%	100%
Pyochelin	100%	-	80%	80%
Tolaasin	-	50%	-	-
Syringafactin	-	-	-	83%

+: gene cluster is present on the genome, but the percentage similarity is not determined.

Table 7C: Summary of the complete genome sequencing and major gene clusters for non-ribosomal peptide synthetases (NRPSs) and secondary metabolites for isolates NY60 and NY130.

Isolates	**NY60 CFSAN047153**	**NY130 CFSAN047154**
Isolates identification (% similarity)		
-16S ribosomal RNA	*Pantoea agglomerans*	*Pantoea agglomerans*
-Resphera Insight	*99%*	*99%*
Complete genome sequencing - Biosynthetic gene clusters similarity for non-ribosomal peptide synthetases (NRPSs) and other secondary metabolites		
Bacteriocin	+	+
Arylpolyene	73%	73%
Acyl homoserine lactone (Hserlactone)	+	+
Turnerbactin	30%	30%
Carotenoid	100%	100%
Desferrioxamine B	40%	60%
Pyocyanine	28%	28%
Microcin	+	+

+: gene cluster is present on the genome, but the percentage similarity is not determined.

Example 5: *In vitro* competition in co-cultures

The *in vitro* competition was assessed by co-culturing active strains (i.e., those characterized in **Tables 7A-7C**) with *E. amylovora* S435, S153, and S1605 strains. Not all active strains completely killed all colonies of *E. amylovora* strains. Interestingly, only *Pantoea agglomerans* NY60 and NY130 completely killed *E. amylovora* strains S435, S153, and S1605 when they were co-cultivated together.

Example 6: Formulations of products: stability and activity

The objective of this work 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 potent 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. Without being bound by theory, when the product is sprayed on the trees, the active metabolites are intended to control *E. amylovora*, while the live bacteria in the formulation are intended to grow, colonize, and outcompete any remaining or forthcoming *E. amylovora*.

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. polymyxa* 273 and *B. velezensis* FL50S by ~1 log, (see **Table 8**, which presents the means of three replicates).

Table 8. Stability of the biological control product formulations

Biological control product	**Concentration of bacteria(CFU/mL)**		
	Time: Zero	**3 months**	**9 months**
Paenibacillus polymyxa 273	1.10±0.02 x 10^7	1.01±0.01 x 10^7	2.00±0.11 x 10^6
Bacillus velezensis subsp. *Plantarum* FL50S	2.10±0.10 x 10^7	2.00±0.10 x 10^7	1.03±0.06 x 10^7
Pseudomonas poae FL10F	1.06±0.08 x 10^9	1.96±0.14 x 10^8	1.96±0.12 x 10^7
Pantoea agglomerans NY60	2.63±0.06 x 10^9	1.93±0.06 x 10^9	1.00±0.10 x 10^7
Pantoea agglomerans NY130	2.56±0.03 x 10^9	1.96±0.03 x 10^9	0.96±0.13 x 10^7
Pseudomonas fluorescens IRDA4	2.00±0.10 x 10^9	1.96±0.03 x 10^9	1.96±0.12 x 10^7

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

Furthermore, the inhibitory activity of the various CFS (**Table 9**) and the direct cell-to-cell antagonism (**Table 10**) of bacteria in the formulations remained active after >9 months storage at room temperature. Heating during the formulation of spore-forming bacteria-based products had no effect on the activity of CFS (**Table 9**).

Table 9. Antimicrobial activity of CFS of the biological control product formulations

Biological control product	**Diameter of growth inhibition zone (including well diameter) *, mm**

	Time Zero	3 months	9 months
Paenibacillus polymyxa 273	26.66 ± 0.34	23.33 ± 0.35	21.67 ± 0.21
Bacillus velezensis subsp. *Plantarum* FL50S	28.58 ± 0.33	26.67 ± 0.16	23.50 ± 0.34
Pseudomonas poae FL10F	35.08 ± 0.27	33.00 ± 0.50	29.16 ± 0.30
Pseudomonas fluorescens IRDA4	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 10. Antagonistic activity of the biological control product formulations

Biological control product	Time Zero*	3 months*	9 months*
Paenibacillus polymyxa 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
Bacillus velezensis 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
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
Pseudomonas fluorescens IRDA4	11.00±0.57 / 29.66±0.33	10.00±0.00 / 28.30±0.66	9.00±0.00 / 26.00±0.57
Pantoea agglomerans NY60	10.00±0.57 / 30.66±0.33	10.66±0.33 / 29.50±0.28	10.00±0.57 / 29.30±0.16
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

Survival of formulated bacteria sprayed on apple leaves (McIntosh) was then assessed. **Table 11** describes the mean of the three replicates which were performed for each treatment, showing that formulated products remained viable on apples leaves for 7 d.

Table 11. Survival of the biological control product formulations on leaves

Microbial-based products	Time Zero *CFU per leaf	After 7 days *CFU per leaf
Paenibacillus polymyxa 273	1.25±0.006 x10^3	1.53±0.033 x10^3
Bacillus velezensis subsp. *Plantarum* FL50S	1.90±0.057 x10^4	1.76±0.003 x10^4
Pseudomonas poae FL10F	1.20±0.088 x10^5	1.04±0.000 x10^5
Pseudomonas fluorescens IRDA4	1.35±0.005 x10^5	1.35±0.005 x10^5
Pantoea agglomerans NY60	2.03±0.066 x10^4	2.20±0.000 x10^4
Pantoea agglomerans NY130	1.95±0.009 x10^4	2.00±0.000 x10^4
Leaves (disinfected with ethanol)	0.00±0.000	0.00±0.000

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

Example 7: Field trial for the management of fire blight on *Malus* x *domestica* 'McIntosh' apple cultivar tree shoots

A trial was conducted at the IRDA research station in Saint-Bruno-de-Montarville (Québec) to evaluate the effectiveness of selected active strains against shoot blight. One or two shoots were inoculated with *E. amylovora* per tree. Two hours after inoculation, various active strain preparations were applied to the shoots until runoff using a low-pressure atomizer. Five trees were used per treatment, and the experimental design was completely randomized. Formulations based on *P. polymyxa* 273 and *B. velezensis* 50S were prepared as described herein in **Example 1.11** ("Formulation of spore-forming bacteria". Those based on *P. poae* FL9F, *P. poae* FL10F, *P. fluorescens* IRDA4, *P. agglomerans* NY60, and *P. agglomerans* NY130 were formulated as described in "Formulation of vegetative bacteria". They were diluted to 20% (v/v) before application. Trees treated with *P. agglomerans* strains NY60 and NY130 had much less fire blight than the control after 10 days, with Disease Scores (DS) not significantly different from those treated with streptomycin. Fire blight never extended beyond the central vein of the inoculated leaf in trees receiving *P. agglomerans* NY60. No evidence of phytotoxicity appeared in either treatment (**Table 12**).

Table 12. Effects of various treatments on McIntosh apple trees experimentally inoculated with *E. amylovora* S435

Treatment	Median score	Score range	Tukey**
Streptomycin (100 ppm)	0	[0, 2]	a
Water	3.5	[2, 4]	c
Sterile culture medium	3	[2, 4]	c
P. polymyxa 273	3	[1, 4]	bc
P. poae FL10F	2	[1, 3]	bc
B. velezensis FL50S	3.75	[3, 4]	c
P. fluorescens IRDA4	3	[2, 4]	c
P. agglomerans NY130	1	[0, 3]	ab
P. agglomerans NY60	1	[0, 1]	ab

** Values followed by the same letter are not significantly different ($P \leq 0.05$) according to lsmeans (Lenth, 2016) with an adjustment for Tukey's HSD to control for family-wise error.

Example 8: Apple trees trial to control fire blight on Rome cultivars in Geneva NY (USA)

A field trial was conducted to determine the performance of the formulated product based on the living cells of *Pseudomonas poae* FL10F (see **Example 1.14**). This strain was selected to conduct the trial because of its both: 1) strong cell-free supernatant and 2) antagonistic activity against *E. amylovora* strains.

The objective of this trial was to compare the activity of *P. poae* FL10F with antibiotic (Streptomycin), copper fungicide (Cueva, Certis, USA) and the biological product Blossom Protect based on two strains of *Aureobasidium pullulans* (Porter et al., 1960). Apple trees were infected with 1 x 10^7 CFU/mL solution of *Erwinia amylovora* . The concentration of various treatments applied are represented in **Table 13**. The medium used in Treatment 7 is the same one used to cultivate the *P. poae* FL10F composed of Nutrient Broth NB (Difco) supplemented with 0.5% (w/w) glycerol and a concentration of 4% NaCl (w/w). Neither the medium nor the treatments used were phytotoxic to apple leaves and no phytotoxicity was noted on the apple trees in this study.

Table 13. Performance of the *P. poae* FL10F product compared to antibiotic and copper products

				Fire blight Severity Average length of shoot with symptoms of Fire blight (INCHES)		Fire blight Incidence Average percentage of shoots with symptoms of Fire blight following inoculation %		Phytotoxicity Percentage of leaves with atypical symptoms %	
Trt No.	Treatment Name	Rate	Rate Unit		1		2		3
1	Untreated			6.12	a	82.78	a	0.00	a
2	Strepmycin	8	oz wt/a	3.89	bc	52.39	bc	0.00	a
3	Strepmycin	2.4	oz wt/a	5.53	ab	81.73	a	0.00	a
4	Medium	20	% v/v	6.13	a	82.80	a	0.00	a
5	Cueva	64	fl oz/a	3.90	bc	71.14	ab	0.00	a
6	*P. poae* FL10F	20	% v/v	2.49	c	47.70	c	0.00	a
7	Blossom Protect	20	oz wt/a	3.25	c	52.95	bc	0.00	a
LSD (P=.05)					1.403		12.909		0.000
Standard Deviation					1.312		12.075		0.000

Means followed by same letter do not significantly differ (P=.05, Student-Newman-Keuls)

The above severe test was conducted in the field to determine if the product based on *Pseudomonas poae* FL10F and its metabolites can control trauma blight which refers to fire blight infection caused when major weather events (i.e., late frost accompanied by hail or high winds) causing injury to the plant tissue. Each treatment was performed on 7 different trees and each tree was 7 feet tall. More than 82% of all cuts with scissors had infection indicating that the infection of the trees was successful. The above results (**Table 13**) demonstrated that the *P. poae* FL10F product was even more efficacious than antibiotics and copper products.

Example 9: Identification of active metabolites in *B. velezensis* FL50S, *P. poae* FL10F, and *P. agglomerans* NY60

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this example are as follows: *B. velezensis* strain FL50S was isolated from an agricultural field soil in Wimauma, Florida, USA, *P. poae* FL10F was isolated from strawberry leaves in Dover, Florida, USA, *P. agglomerans* NY60 was isolated from Rome apple leaves in Geneva, New York, USA, and *E. amylovora* S435 was supplied by IRDA, Quebec, Canada). Unless otherwise specified, the bacteria were routinely grown from frozen glycerol stock by culturing in 3 ml tryptic soy broth (TSB) (BD, New Jersey, USA) 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, the 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 almost 8×10^6 CFU/ml.

Production of secondary metabolites produced by *Bacillus* and *Pseudomonas* isolates

The production of metabolites by bacterial isolates was carried out in 2L conical flasks with 500 ml of respective medium. *P. agglomerans* NY60 was cultivated in Difco Nutrient Broth (Becton, Dickinson and Co., Franklin Lanes, NJ, USA) and was incubated for 2 d at 30°C with agitation at 200 rpm. King's B medium was used as a production medium for *P. poae* FL10F isolate, while Landy medium was used for cultivation of *B. velezensis* FL50S. Cultures were shaken at 30°C for 48h at 250 rpm. Landy medium contains: glucose 20 g/L, L-glutamic acid 5.0 g/L, yeast extract 1.0 g/L, K_2HPO_4 1.0 g/L, $MgSO_4$ ($7H_2O$) 0.5 g/L, KCl 0.5 g/L, $CuSO_4$ 1.6 mg/L, $Fe_2(SO_4)_3$ 1.2 mg/L, $MnSO_4$ 0.4 mg/L.

To obtain cell-free supernatants, cultures of both strains were centrifuged at 25 000 rpm for 1 hour. The pellets were discarded and the supernatants were filtered using 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 one week, or at -20°C for 6 months, while keeping their activity.

Secondary metabolite gene cluster prediction and analysis

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

Whole-genome sequencing

Genomic DNA was isolated from an overnight culture of each strain using a QiagenDNeasy™ blood and tissue kit (Qiagen Inc., Valencia, CA). Genome sequencing was performed using Illumina MiSeq™ sequencing system (Illumina, San Diego, CA), achieving > 50X average genome coverage. *De novo* assembly was created 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). Taxonomy of each isolate was assigned using Kraken.

Bioassay-guided fractionation and isolation of active metabolites

1 L of each bacterial culture was combined, and biomass was removed by centrifugation at 17 700 × *g* for 20 min. 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 (Milli-Q system; Millipore, Bedford, MA). The concentrated solution was then applied onto a 12 gram Biotage® *SNAP Ultra C18* column to be fractionate using reverse-phase flash chromatography on a Biotage (Stockholm, Sweden) Isorela One instrument. This chromatography was performed using a linear gradient acetonitrile from 5% to 100% over 50 min at 15 ml min^{-1}. To identify column fractions containing active compound, each fraction was evaporated to dryness by rotary evaporation and was redissolved in 2 ml of milliQ™ water. The bioactivity of these fractions was further determined by performing agar disc diffusion assay.

Activity of cell-free supernatants and fractions against *Erwinia amylovora* S435

In order to estimate the activity of bacterial metabolites and fractions against *E. amylovora* S435, agar disc diffusion assay was performed. Paper blank discs (d=6 mm) were saturated by cell-free supernatants or fractions (20 µl), air dried overnight in the biological cabinet at room temperature (22°C) for 30 min. The antimicrobial activity was then tested by placing saturated disc on a lawn of *E. amylovora* S435 TSB agar plate and measuring the inhibition area (mm^2) of pathogen. This assay was performed in triplicate.

LC-ESI-MS/MS analysis

The cell-free supernatants and active fractions were further analyzed by high-performance liquid chromatography (HPLC; Waters 2795, Mississauga, ON, Canada) equipped with a 250 x 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 window ranging from m/z 130-1930. Collision-induced dissociation (CID) MS/MS experiments were performed using argon gas at various collision energies.

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.

The resulting assembled sequences were polished with Illumina reads using a combination of BWA version 0.7.17-r1188, SAMtools version 1.9 and Pilon version 1.22. SAMtools version 1.9 and Pilon version 1.22. The sequences of strain *P. agglomerans* NY60 have been deposited in GenBank under the accession number CP034469.

The sequences of strain *P. agglomerans* NY60 were annotated using the DFAST web server and the eggnog-mapper web server. The genes involved in a type VI secretion system (T6SS) were subsequently investigated with Artemis version 17.0.0 [159][158][159][158][159][158][157][157][158][157][157][157][157][161][161][161][161][160][159][152][159][159][158][157][157][157][156][155][154][153][152][151][150][149][148][148][148][148][147][147][146][146][145][144][144][143][142][142][142][141][141][175][175][175][175][175][175][175][174][174][173][172][172][172][171][171]and the BLAST suite following the nomenclature of a study describing this system in *Pantoea ananatis*. Finally, a graphical representation of the T6SS loci was made with EasyFig version 2.2.2.

Random transposon mutagenesis and colony selection

Transposon insertions in *P. agglomerans* NY60 chromosome were generated by mating *P. agglomerans* NY60 with DAP-dependent *E. coli* strain χ7213 carrying pUT/mini-Tn5 Sm/Sp on Lysogeny broth (LB) agar supplemented with DAP at 37°C. Mutagenized cells were plated on TSA plates containing spectinomycin (15 µg/ml). The plates were incubated at room temperature (~21°C) for 2 d. Thereafter, a lawn of streptomycin-resistant *E. amylovora* S435 were spread onto TSA plates. After incubation for another 2 days at 21°C Bacterial isolates which did not form clear haloes (inhibition zones) on the *E. amylovora* S435 lawns were selected for determination of transposon insertion site.

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

Genomic DNA of 8 clones was extracted from cultures using phenol-chloroform and pooled together. DNA was sequenced at the Génome Québec Innovation Centre (McGill University, Montreal, QC). DNA concentrations were determined using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies). DNA samples were generated using the NEBNext® 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 SPRIselect™ 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' **(SEQ ID NO: 174)** [transposon-specific sequence is in Bold])), and the Illumina adapter with primer 2 (5' TACGGTAGCAGAGACTTGGTCTCTAGCATAGAGTGCGTAGCTCTGCT 3') **(SEQ ID NO: 175)** 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 (AGATCGGAAGAGCACACGTCTGAACTCCAGTCACACACTGACGACATGGTTCTACA) **(SEQ ID NO: 176)** and primer 2. Thereafter, sequencing was performed on an Illumina MiSeq using the MiSeq Reagent Kit v2 Kit (500-cycles). In order to find the transposon insertion sites, reads were mapped to *P. agglomerans* NY60 genome (CP034469.1).

Results

Genome mining studies

Genome mining of biosynthetic gene clusters (BGC) including NRPSs and other secondary metabolites were predicted using antiSMASH 5.0. The summary of the identified secondary metabolites for strains *B. velezensis* FL50S and *P. poae* FL10F are shown in **Tables 14 and 15**.

For the *B. velezensis* FL50S, through genome mining, a total of 14 putative BGCs were found of which, 8 were identified, including non-ribosomal peptide synthetases (NRPSs) atrributed to biosynthesis of surfactin, iturin and fengycin, and polyketide synthases (PKSs) encoding for difficidin, macrolactin and bacillaene (**Table 14**).

Interestingly, crude extracellular metabolite studies showed that the polyketide oxydifficidin is the major active metabolite produced by *B. velezensis* FL50S against *E. amylovora* S435, with this extracellular metabolite fraction producing inhibition halos on a lawn of *E. amylovora* S435 that were comparable in diameter to that of the live strain itself or crude extracellular metabolites therefrom.

Table 14. Identified biosynthetic gene cluster regions in the genome of *B. velezensis* FL50S

Biosynthetic gene cluster type	Most similar known cluster	Similarity	MIBiG BGC-ID
NRPS	Surfactin	82%	BGC0000433
NRPS, transAT-PKS	Fengycin	80%	BGC0001095
NRPS, transAT-PKS	Iturin	88%	BGC0001098
transAT-PKS	Difficidin	100%	BGC0000176
transAT-PKS	Macrolactin	100%	BGC0000181
transAT-PKS, NRPS	Bacillaene	85%	BGC0001089
NRPS	Bacillibactin	100%	BGC0000309
other	Bacilysin	100%	BGC0001184

For the *pseudomonas sp.* FL10F, a total of 5 NRPSs gene clusters were identified. Among them, two unlinked NRPS gene clusters with 100% similarity to gene clusters coding for NRPS responsible for biosynthesis of cyclic lipopeptide (CLP) Poaeamide were identified. Crude extracellular metabolite studies showed that the cyclic lipopeptide white line-inducing principle (WLIP), a cyclic lipopeptide from Viscosin subfamily (Massetolide E, F, L or Viscosin), is the major active metabolite produced by *P. poae* FL10F against *E. amylovora* S435, with this extracellular metabolite fraction producing inhibition halos on a lawn of *E. amylovora* S435 that were comparable in diameter to that of the live strain itself or crude extracellular metabolites therefrom.

Table 15. Identified biosynthetic gene cluster regions in the genome of *P. poae* FL10F

Biosynthetic gene cluster type	Most similar known cluster	Similarity	MIBiG BGC-ID
NRPS-like	Mangotoxin	71%	BGC0000387
NRPS	Poaeamide	100%	BGC0001208
NRPS	Pyochelin	100%	BGC0000412
NRPS	Poaeamide	100%	BGC0001208
NRPS	Safracin	100%	BGC0000421

Mass spectrometry identification of metabolites

The antiSMASH results suggest that *B. velezensis* FL50S has the biosynthetic machinery for the production of three classes of cyclic lipopetides as well as three polyketides. To verify the antiSMASH results and see if these gene clusters are functional, cell-free supernatant of

B. velezensis FL50S was analysed by HPLC-ESI MS. In accordance with antiSMASH results, MS spectra of *B. velezensis* FL50S indicated that it produces three families of cyclic lipopeptides, surfactins, iturins and fengycins (**Fig. 4**). Regarding polyketides, only the oxidized form of difficidin, oxydifficidin, could be detected (**Fig. 5**). Performing the HPLC-ESI MS analyses on fractions obtained from activity guided fractionation of the crude extracellular metabolite showed that the polyketide, oxydifficidin, is the major active metabolite against *E. amylovora* S435. While it is 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, in positive mode, oxydifficidin could be detected in its dephosphorylated (m/z 463.4) and dimmer (m/z 1121.3) states.

Analysis of the MS spectra of the active fraction of *P. poae* FL10F 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 a 14 unit mass difference with the major peak (**Fig. 6**). 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 the putative product of two unlinked gene clusters coding for the cyclic lipopeptide poaeamide, suggest that 1127.1 peak could be attributed to white-line-inducing principle (WLIP), a cyclic lipopeptides from Viscosin subfamily (Massetolide E, F, L or Viscosin) (**Fig. 7**).

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 showed no activity against *E. amylovora* S435, it was still able to produce an inhibition zone on lawn of *E. amylovora* S435 on TSA agar plates. This suggest that the metabolites can only be produced on solid agar medium. In order to identify the active metabolites, random transposon mutants of *P. agglomerans* NY60 were generated using mini-Tn5 Sp/Sm transposon and screened on TSA plates against streptomycin resistant *E. amylovora* S435. Transposon-generated mutants of *P. agglomerans* NY60 were screened and 8 mutants that has lost their antagonistic activity were obtained. Using transposon insertion sequencing (Tn-seq), the insertion sites in these were mapped and lead to the identification of few genes that significantly altered the production of antagonistic metabolites (**Table 16**).

Table 16: Identified transposon insertion sites in the genome of *P. agglomerans* NY60

Insertion site	Locus tag/ Gene product description
CP034469	-
474908-474967	TonB-dependent siderophore receptor CDS
569998-570191	*dndD* CDS
1416125-1416319	glycosyltransferase CDS
1526586-1526815	*flgL* CDS
4028399-4028630	16S rRNA
plasmid CP034471	-
97482-97585	MFS transporter CDS

Genomes of *P. agglomerans* NY60 and NY130, *P. poae* FL10F, and *B. velezensis* FL50S were determined by whole genome sequencing (**Table 17**).

Table 17: Whole genome sequencing of *P. agglomerans* NY60 and NY130, *P. poae* FL10F, and *B. velezensis* FL50S

Strain	Genome	SEQ ID NO:
	Chromosome	**14**
	Plasmids	**15-18**
	Chromosome	**19**
	Plasmids	**20-23**
***P. poae* FL10F**	Contig sequences	**24-103**
***B. velezensis* FL50S**	Contig sequences	**104-173**

Conclusions

***B. velezensis* FL50S**

In accordance with antiSMASH results, MS spectra of *B. velezensis* FL50S indicated that it produces three families of cyclic lipopeptides, surfactins, iturins and fengycins (**Fig. 4**). Regarding polyketides, only the oxidized form of difficidin, oxydifficidin, could be detected (**Fig. 5**). Performing the HPLC-ESI MS analyses on fractions obtained from activity guided fractionation of the crude extracellular metabolite showed that the polyketide, oxydifficidin, is the major active metabolite against *E. amylovora* S435. While it is 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, in positive mode, oxydifficidin could be detected in its dephosphorylated (m/z 463.4) and dimmer (m/z 1121.3) species.

***P. poae* FL10F**

Our results demonstrate that *P. poae* FL10F produce an active extracellular compound against *E. amylovora* S435 that can be attributed to a cyclic lipopeptide, white line-inducing principle (WLIP), a CLP from the Viscosin subfamily (Massetolide E, F, L or Viscosin).

It is interesting to note that by indexing the *Pseudomonas* specialized metabolome, in the literature, the molecular-networking-based discovery of four molecules and their evolutionary relationships were reported: a poaeamide analogue and a molecular subfamily of cyclic lipopeptides, bananamides 1, 2 and 3. Analysis of their biosynthetic gene cluster showed that it constitutes a distinct evolutionary branch of the *Pseudomonas* cyclic lipopeptides.

***P. agglomerans* NY60**

The strain *P. agglomerans* NY60 had a direct cell-to-cell antagonistic effect against *E. amylovora* S435. The whole genome was sequenced by SMRT PacBio and three loci with genes known to be involved in T6SS were found. Two of the three loci include all the core genes needed to form a functional system, in addition to a few accessory genes. The third locus only harbor two core genes and is likely the remnant of an ancient T6SS locus. It is interesting to note that the three loci have important structural similarities with the T6SS-1, T6SS-2 and T6SS-3 loci found in the bacterium *P. ananatis*. However, the T6SS-2 locus is known to be plasmid-borne in *P. ananatis* while it is located on the chromosome in strain *P. agglomerans* NY60.

By screening for transposon mutants of *P. agglomerans* NY60 with decreased antagonist activity against *E. amylovora* S435, it was interestingly discovered, one of the transposons is inserted in MFS transporter gene. BLASTing the sequence indicated that it matches the sequence for EhpJ gene in *Pantoea agglomerans* (*Erwinia herbicola*) Eh1087 which is a transmembrane protein predicted to be involved in the extra cytoplasmic localization of griseoluteic acid, an intermediate in biosynthesis of broad-spectrum phenazine antibiotic D - alanylgriseoluteic acid (AGA).

Although the present invention has been described herein by way of specific embodiments thereof, it can be modified without departing from the spirit and nature of the subject invention as defined in the appended claims. The scope of the claims should not be

limited by the preferred embodiments set forth in the examples but should be given the broadest interpretation consistent with the description as a whole.

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All references mentioned in the present document are hereby incorporated herein by reference.

CLAIMS:

1. A biopesticide comprising intact or vegetative cells, endospores, spores, and/or metabolites from one or more bacterial species as active ingredients, wherein said intact or vegetative cells, endospores, spores, and/or metabolites exhibit antimicrobial activity against phytopathogenic *Erwinia* species or *Erwinia amylovora* .

2. The biopesticide of claim 1, wherein the one or more bacterial species comprise spore-forming bacterial species.

3. The biopesticide of claim 1 or 2, wherein the metabolites comprise secondary and/or extracellular metabolites.

4. The biopesticide of any one of claims 1 to 3, wherein the one or more bacterial species comprise or consists of:

(a) a *Bacillus* species, a *Paenibacillus* species, a *Pantoea* species, a *Pseudomonas* species, or any combination thereof;

(b) *Bacillus velezensis*, *Bacillus velezensis* subsp. *plantarum*, *Bacillus subtilis*, *Paenibacillus polymyxa*, *Pantoea agglomerans*, *Pseudomonas fluorescens*, *Pseudomonas poae*, or any combination thereof;

(c) *Bacillus velezensis* 304, *Bacillus velezensis* 417, *Bacillus velezensis* 431, *Bacillus velezensis* subsp. *plantarum* FL50S as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 104-173 or any combination thereof, *Bacillus* sp. 331, *Bacillus* sp. 418, *Bacillus* sp. FD308, *Bacillus* sp. FD402, *Bacillus* sp. FD604, *Bacillus* sp. IRDA27, *Bacillus* sp. IRDA618, *Bacillus* sp. IRDA619, *Bacillus* sp. IRDA627, *Bacillus* sp. IRDA63, *Bacillus* sp. IRDA672, *Bacillus* sp. IRDA675, *Bacillus* sp. IRDA683, *Bacillus* sp. IRDA684, *Bacillus* sp. IRDA685, *Bacillus* sp. IRDA687, *Bacillus subtilis* 421, *Paenibacillus polymyxa* 273, *Paenibacillus polymyxa* 344, *Pantoea agglomerans* IRDA36, *Pantoea agglomerans* IRDA59, *Pantoea agglomerans* NY130 as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%,

98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 19 and 20-23 or any combination thereof, *Pantoea agglomerans* NY50, *Pantoea agglomerans* NY60 as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 14 and 15-18 or any combination thereof, *Pseudomonas fluorescens* IRDA4, *Pseudomonas poae* FL10F as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 24-103 or any combination thereof, *Pseudomonas poae* FL3F, *Pseudomonas poae* FL4F, *Pseudomonas poae* FL9F, *Pseudomonas* sp. 41, *Pseudomonas* sp. 42, *Pseudomonas* sp. 43, *Pseudomonas* sp. NY1238, or any combination thereof;

(d) *Pseudomonas poae*, *Bacillus velezensis* subsp. *plantarum*, *Paenibacillus polymyxa*, or any combination thereof;

(e) *Pseudomonas poae* FL10F as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 24-103 or any combination thereof, *Pseudomonas poae* FL4F, *Pseudomonas poae* FL3F, *Bacillus velezensis* subsp. *plantarum* FL50S as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 104-173 or any combination thereof, *Paenibacillus polymyxa* 273, *Pseudomonas poae* FL9F, or any combination thereof; or

(f) *Pantoea agglomerans* NY60 as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 14 and 15-18 or any combination thereof, or *Pantoea agglomerans* NY130 as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%,

99.9% or 100% identity to any one of SEQ ID NOs: 19 and 20-23 or any combination thereof.

5. The biopesticide of any one of claims 1 to 4, wherein the one or more bacterial species comprise or consist of:

(g) *Paenibacillus polymyxa, Bacillus velezensis subsp. plantarum, Pseudomonas poae, Pantoea agglomerans, Pantoea agglomerans, Pseudomonas fluorescens, Pseudomonas poae*, *Paenibacillus polymyxa*, *Bacillus velezensis* subsp. *plantarum*, or any combination thereof; and/or

(h) *Paenibacillus polymyxa* 273*, Bacillus velezensis subsp. plantarum* FL50S as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 104-173 or any combination thereof, *Pseudomonas poae* FL10F as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 24-103 or any combination thereof, *Pantoea agglomerans* NY60 as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 14 and 15-18 or any combination thereof, *Pantoea agglomerans* NY130 as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 19 and 20-23 or any combination thereof*, Pseudomonas fluorescens* IRDA4*,* or any combination thereof.

6. The biopesticide of any one of claims 1 to 5 comprising or consisting of:

(a) vegetative bacteria;

(b) killed vegetative bacteria;

(c) heat-killed vegetative bacteria;

(d) sporulated bacteria;

(e) bacterial spores or endospores;

(f) bacterial extracellular metabolites; or

(g) any combination thereof,

from the one or more bacterial species as defined in claim 4 or 5.

7. The biopesticide of any one of claims 1 to 6 comprising active ingredients from at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least ten different bacterial species selected from the one or more bacterial species as defined in claim 4 or 5.

8. The biopesticide of any one of claims 1 to 7, which:

(a) lacks vegetative bacteria as an active ingredient, or is a cell-free composition; and

(b) comprises bacterial spores or endospores, and/or bacterial extracellular metabolites from one or more bacterial species as defined in claim 4 or 5, as active ingredients.

9. The biopesticide of any one of claims 1 to 7, which comprises vegetative bacteria from the one or more bacterial species as defined in any one of claims 1 to 6, as active ingredients.

10. The biopesticide of claim 9, which comprises only one type of vegetative bacteria species/strain.

11. The biopesticide of claim 9 or 10, which completely kills *E. amylovora* strains S435, streptomycin-resistant S153, and/or streptomycin-resistant S1605, when co-cultivated together *in vitro*.

12. The biopesticide of any one of claims 9 to 11, wherein the vegetative bacteria comprise *Pantoea agglomerans*, *Pantoea agglomerans* NY50, *Pantoea agglomerans* NY60 as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 14 and 15-18 or any combination thereof, *Pantoea agglomerans* NY130 as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID

NOs: 19 and 20-23 or any combination thereof, *Pantoea agglomerans* IRDA36, *Pantoea agglomerans* IRDA59, or any combination thereof.

13. The biopesticide of any one of claims 1 to 12, exhibiting antimicrobial activity against antibiotic-resistant *Erwinia amylovora* , *Erwinia amylovora* S453, *Erwinia amylovora* S153, *Erwinia amylovora* S1605, or any combination thereof.

14. The biopesticide of any one of claims 1 to 13 exhibiting antimicrobial activity against a further plant and/or human pathogenic microorganism, or a pathogenic virus, bacteria, fungus, yeast, mold, or any combination thereof.

15. The biopesticide of any one of claim 1 to 14 exhibiting higher antimicrobial activity against *Erwinia* species, as compared to other phytopathogenic species.

16. The biopesticide of any one of claims 1 to 15, wherein said metabolites comprise lipopeptides and/or siderophores from the one or more bacterial species as defined in claim 4 or 5.

17. The biopesticide of claim 16, wherein said lipopeptides are non-ribosomal lipopeptides (NRPs), and/or said lipopeptides and/or siderophores comprise: bananamide, poaeamide, poacin, viscosin, massetolide (e.g., massetolide E, F, or L), orfamide, arthrofactin, surfactin, fengycin, plipastatin, iturin, bacilysin, bacillibactin, bacillomycin, locillomycin, paenilarvin, pelgipeptin, polymyxin, paenibacterin, fusaricidin, bacitracin, tridecaptin, bacteriocin, arylpolyene, acyl homoserine lactone (hserlactone), turnerbactin, carotenoid, desferrioxamine b, pyocyanine, microcin, pantocin, safracin, pyoverdine, mangotoxin, pyochelin, tolaasin, syringafactin, or any combination thereof.

18. The biopesticide of any one of claims 1 to 17, wherein said metabolites comprise polyketides from the one or more bacterial species as defined in claim 4 or 5.

19. The biopesticide of claim 18, wherein said polyketides comprise difficidin and/or oxydifficidin.

20. The biopesticide of claim 19, wherein said polyketides comprising difficidin and/or oxydifficidin are from *B. velezensis* FL50S.

21. The biopesticide of any of claims 1 to 20, wherein said metabolites comprise cyclic lipopeptides from the viscosin subfamily, such as white-line-inducing principle (WLIP), massetolide E, massetolide F, massetolide L, viscosin, or any combination thereof.

22. The biopesticide of claim 21, wherein said metabolites comprise white line-inducing principle (WLIP).

23. The biopesticide of claim 21 or 22, wherein said metabolites comprise cyclic lipopeptides from the viscosin subfamily (e.g., such as white-line-inducing principle (WLIP), massetolide E, massetolide F, massetolide L, viscosin, or any combination thereof) produced from *P. poae* FL10F.

24. The biopesticide of any one of claims 1 to 23, wherein said metabolites comprise an antibiotic.

25. The biopesticide of claim 24, wherein said antibiotic is phenazine.

26. The biopesticide of claim 25, wherein said phenazine is D-alanylgriseoluteic acid (AGA).

27. The biopesticide of any one of claims 24 to 26, wherein said antibiotic or phenazine is from *Pantoea agglomerans* NY60 or *Pantoea agglomerans* NY130.

28. The biopesticide of claim 27, wherein said *Pantoea agglomerans* NY60 or *Pantoea agglomerans* NY130 produce a transmembrane protein EhpJ for the synthesis and/or release of phenazine.

29. The biopesticide of any one of claims 1 to 28, further comprising an agriculturally acceptable excipient, additive, and/or preservative.

30. The biopesticide of any one of claims 1 to 29, further comprising one or more of: non-toxic carriers, surfactants, preservatives, nutrients, UV protectants, stickers, spreaders, chelating agents, or any combination thereof.

31. The biopesticide of any one of claims 1 to 30, formulated as a liquid, concentrate, powder, tablet, gel, pellets, granules, or any combination thereof.

32. The biopesticide of any one of claims 1 to 31, once applied to a target plant, has no detectable phytotoxic effect on said target plant, or on the fruits, nuts, or leaves thereof.

33. The biopesticide of any one of claims 1 to 33, which comprises:

(a) at least 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm, 600 ppm, 700 ppm, 800 ppm, 900 ppm, 1000 ppm, 1500 ppm, 2000 ppm , 2500 ppm, 3000 ppm, 3500 ppm, 4000 ppm, 4500 ppm, 5000 ppm, 5500 ppm, 6000 ppm, 6500 ppm, 7000 ppm, 8000 ppm, 8500 ppm, 9000 ppm, or 9500 ppm of one or more active ingredients; or

(b) between about 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm, 600 ppm, 700 ppm, 800 ppm, 900 ppm, 1000 ppm, 1500 ppm, 2000 ppm, 2500 ppm, 3000 ppm, 3500 ppm, 4000 ppm, 4500 ppm, 5000 ppm, to about 10 000 ppm of one or more active ingredients.

34. The biopesticide of any one of claims 1 to 33, for use as an anti-microbial agent against a plant and/or human pathogenic microorganism, a phytopathogenic *Erwinia* species, *Erwinia amylovora* , or for the prevention and/or treatment of fire blight on a growing plant.

35. Use of the biopesticide as defined in any one of claims 1 to 34, as an anti-microbial agent against a plant and/or human pathogenic microorganism, a phytopathogenic *Erwinia* species, *Erwinia amylovora* , or for the prevention and/or treatment of fire blight on a growing plant.

36. The biopesticide of claim 34 or the use of claim 35, wherein said growing plant is a fruit plant, nut, cereal, vegetable, or flower.

37. The biopesticide of claim 34 or the use of claim 35 or 36, wherein:

(a) said fruit is pome fruit trees and related plants, Pear (*Pyrus* species), quince (*Cydonia*), apple, crabapple (*Malus* species), firethorns (*Pyracantha* species), hawthorn (*Crataegus* species), *Spiraea*, *Cotoneaster*, toyon (*Photinia* species), juneberry, serviceberry (*Amelanchier* species), loquat (*Eriobotria*), mountain ash (*Sorbus* species), blossoms (*Prunus*), peach, apricot, cherry, banana, blackberry, blueberry, cantaloupe, cherry, cranberry, currant, grapes, greengage, gooseberry, honeydew, lemon, mandarin, melon, orange, peach, pears, pineapple, plum, raspberry, strawberry, tomatoes, watermelon, grapefruit, pepper, olive, or lime;

(b) said nut is: almond, beech nut, Brazil nut, butternut, cashew, chestnut, chinquapin, filbert, hickory nut, macadamia nut, pecan, walnut, or pistachio;

(c) said cereal is: amaranth, breadnut, barley, buckwheat, canola, corn, fonio, kamut, millet, oats, quinoa, cattail, chia, flax, kañiwa, pitseed goosefoot, wattleseed, rice, rye, sorghum, spelt, teff, triticale, wheat, or colza;

(d) said vegetable is: artichoke, bean, beetroot, broad bean, broccoli, cabbage, carrot, cauliflower, celery, chicory, chives, cress, cucumber, kale, dill, eggplant, kohlrabi, lettuce, onion, pepper, parsnip, parsley, pea, potato, pumpkin, radish, shallot, soybean, spinach, turnip, or peanut; or

(e) the growing plant is an apple tree, stone fruit tree, pear tree, an apricot plant, or a plant from the rose family.

38. A method for controlling the growth of a pathogenic microorganism on a target plant or tissue, the method comprising contacting said target plant or tissue with the biopesticide as defined in any one of claims 1 to 33.

39. The method of claim 38, wherein the pathogenic microorganism is a phytopathogenic *Erwinia* species or *Erwinia amylovora* .

40. The method of claim 38 or 39, wherein said contacting comprising spraying, irrigating, painting, daubing, and/or fogging, onto and/or into the target plant or tissue, the target plant or tissue's hydroponic substrate, and/or the target plant or tissue's agricultural earth.

41. The method of any one of claims 38 to 40, wherein the target plant or tissue is from a growing plant as defined in claim 36 or 37.

42. A kit for preparing an aqueous solution for use in controlling a pathogenic microorganism on a plant tissue of a growing plant, said kit comprising:

(a) the biopesticide as defined in any one of claims 1 to 33; and

(b) a suitable container.

43. The kit of claim 42, wherein said container is a pouch, a tablet, or a bucket.

44. The kit of claim 42 or 43, wherein the pathogenic microorganism is a phytopathogenic *Erwinia* species or *Erwinia amylovora* .

45. A method for producing the biopesticide as defined in any one of claims 1 to 33, said method comprising:

for a biopesticide comprising spore-forming bacteria without vegetative cells as active ingredients,

culturing vegetative cells from the one or more bacterial species in a sporulation medium for inducing sporulation; inactivating, heat-inactivating, or removing vegetative bacteria from the culture; or

for a biopesticide comprising vegetative cells as active ingredients,

culturing vegetative cells from the one or more bacterial species in a growth-promoting medium; and formulating the culture to improve viability.

46. The method of claim 45, wherein the culturing step further comprises culturing the one or more bacterial species under conditions to promote production of secondary metabolites and/or extracellular metabolites.

47. The method of claim 46, wherein the one or more bacterial species are cultured in the presence of glycerol.

48. The method of any one of claims 46 or 47, further comprising adding a preservative to improve the shelf-life of the active ingredients.

49. A biopesticide comprising a vegetative *Pantoea agglomerans* strain as an active ingredient, wherein said vegetative *Pantoea agglomerans* strain:

(a) is from the same subspecies as closely related *Pantoea agglomerans* strains NY60 (CFSAN047153) as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 14 and 15-18 or any combination thereof, and NY130 (CFSAN047154) as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 19 and 20-23 or any combination thereof;

(b) has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% overall sequence identity at the genomic DNA level to CFSAN047153 and/or CFSAN047154;

(c) comprises genes encoding EhpJ, a phenazine (e.g., pyocyanine), D-alanylgriseoluteic acid (AGA), a bacteriocin, an aryl polyene, an acyl homoserine lactone (hserlactone), a turnerbactin, a carotenoid, desferrioxamine B, a pyocyanine, a microcin, or any combination thereof; and/or

(d) completely kills *E. amylovora strains* S435, streptomycin-resistant S153, and/or streptomycin-resistant S1605, when co-cultivated together *in vitro*.

50. A biopesticide comprising a live *Pseudomonas poae* strain and/or cell-free supernatant therefrom, as active ingredient, wherein the cell-free supernatant exhibits anti-*Erwinia amylovora* activity upon 10-fold dilution.

51. The biopesticide of claim 50, wherein said *Pseudomonas poae* strain:

(a) is from the same subspecies as *Pseudomonas poae* strain FL10F (CFSAN034337) as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 24-103 or any combination thereof;

(b) has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% overall sequence identity at the genomic DNA level to CFSAN034337; and/or

(c) comprises genes encoding a massetolide (e.g., massetolide E, massetolide F, and/or massetolide L), viscosin, white line-inducing principle (WLIP), a bacteriocin, an aryl polyene, a safracin, a pyoverdine, a mangotoxin, a poaeamide, a pyochelin, or any combination thereof.

52. A biopesticide comprising a live *Bacillus velezensis* strain and/or cell-free supernatant therefrom, as active ingredient, wherein the cell-free supernatant exhibits anti-*Erwinia amylovora* activity upon 10-fold dilution.

53. The biopesticide of claim 52, wherein said *Bacillus velezensis* strain:

(a) is from the same subspecies as *Bacillus velezensis* strain FL50S (CSFAN034340) as defined by a microorganism comprising a polynucleotide sequence at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 104-173 or any combination thereof;

(b) has at least 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% identity to any one of SEQ ID NOs: 104-173 or any combination thereof, and/or

(c) comprises genes encoding difficidin, oxydifficidin, a bacteriocin, an aryl polyene, a macrolactin, a bacillaene, an iturin, a bacilysin, a surfactin, a bacillibactin, a fengycin, a plipastatin, a teichuronic acid, a locillomycin, a citrulline, or any combination thereof.

54. The biopesticide of any one of claims 49 to 53, which is the biopesticide as defined in any one of claims 1 to 33.

55. The biopesticide of any one of claims 49 to 54, for the use as defined in any one of claims 35 to 37.

56. A method for manufacturing a biopesticide against fire blight, the method comprising

cultivating a bacterial strain which is the *Pantoea agglomerans* strain as defined in claim 49or 54, the *Pseudomonas poae* strain as defined in any one of claims 50 to 55, or the *Bacillus velezensis* strain as defined in any one of claims 52 to 55 under growth conditions;

isolating active ingredients from said culture, the active ingredients comprising or consisting of intact or vegetative cells, endospores, spores, and/or metabolites from said bacterial species; and

formulating said active ingredients for increased shelf-life, as compared to corresponding unformulated active ingredients.

57. The method of claim 56, further comprising additional steps as defined in any one of claims 45 to 48.

58. A biopesticide formulated for use against a phytopathogenic *Erwinia* species (e.g., *Erwinia amylovora*) or for treating fire blight in plants, the biopesticide comprising a mixture of bacterial metabolites comprising: oxydifficidin; a cyclic lipopeptide from the viscosin subfamily (e.g., white-line-inducing principle (WLIP), massetolide E, massetolide F, massetolide L, viscosin, or any combination thereof); a phenazine antibiotic (e.g., D-alanylgriseoluteic acid (AGA)); a surfactin; an iturin; a fengycin; difficidin; a macrolactin; a bacillaene; or any combination thereof.

59. A biopesticide formulated for use against a phytopathogenic *Erwinia* species (e.g., *Erwinia amylovora*) or for treating fire blight in plants, the biopesticide comprising oxydifficidin; a cyclic lipopeptide from the viscosin subfamily (e.g., white-line-inducing principle (WLIP), massetolide E, massetolide F, massetolide L, viscosin, or any combination thereof); a phenazine antibiotic (e.g., D-alanylgriseoluteic acid (AGA)); or any combination thereof.

60. A biopesticide formulated for use against a phytopathogenic *Erwinia* species (e.g., *Erwinia amylovora*) or for treating fire blight in plants, the biopesticide comprising isolated, purified, or enriched oxydifficidin.

61. The biopesticide of any one of claims 58 to 60, which comprises a combination of metabolites that do not occur naturally, or wherein the combination of metabolites are from at least two different bacterial species, or wherein the concentrations of one or more of the metabolites in the biopesticide are concentrations which do not occur in nature.

62. The biopesticide of any one of claims 58 to 61 formulated as a dry particulate composition or as a concentrate (e.g., for dissolution or dilution at the time of use).

63. The biopesticide of any one of claims 58 to 62, which lacks viable bacteria or which is a cell-free biopesticide.

64. Use of the biopesticide as defined in any one of claims 58 to 63 for treating phytopathogenic *Erwinia* species (e.g., *Erwinia amylovora*) or fire blight in plants.

65. A method for treating a phytopathogenic *Erwinia* species (e.g., *Erwinia amylovora*) or fire blight in plants, the method comprising contacting the plant with the biopesticide as defined in any one of claims 58 to 63.

Abstract

Described herein are biopesticides having activity against *Erwinia* species, particularly against the fire blight phytopathogen *Erwinia amylovora* . The biopesticides comprise intact or vegetative cells, endospores, spores, and/or metabolites from one or more bacterial species or strains as active ingredients, wherein the vegetative cells, endospores, spores, and/or metabolites exhibit antimicrobial activity against phytopathogenic *Erwinia* species or *Erwinia amylovora* .