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Research

Plipastatins Directly Inhibit Agrobacteria and Prevent Crown Gall Disease

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Abstract

Diseases severely impact plant growth and productivity. Here, we sought to identify new products for preventing agrobacteria from causing crown gall disease, which can affect many agriculturally important crop species. To this end, we characterized bacteria that fortuitously contaminated and antagonized a culture of plant-pathogenic bacterial species unrelated to agrobacteria. Analysis of genome sequences suggested that many of the isolated strains are members of the operational group *Bacillus amyloliquefaciens*. Of those tested, three strongly inhibited growth in a culture of agrobacteria. We focused on strain Z062 and showed that partially purified broth extracts inhibited growth in a culture of agrobacterial strains representative of taxonomic and virulence plasmid diversity. One of the inhibiting products was purified and identified as a mixture of plipastatin analogs, cyclic lipopeptides better known as antifungal products. Importantly, we demonstrated that broth extracts containing plipastatins or a mixture of plipastatins protected plants against crown gall disease. Last, the findings suggest that purified individual analogs of plipastatin vary in efficacy, as those with shorter fatty acid chains were generally more effective in a culture against agrobacteria. Plipastatins have potential as a preventive product to protect crop species against diverse genotypes of pathogenic agrobacteria.

Keywords: *Agrobacterium*, *Bacillus*, biocontrol, crown gall disease, natural products, plipastatins

Plant agricultural productivity is severely impacted by many plant diseases, with losses of major crops estimated to be 20 to 40% globally (Savary et al. 2019). Crown gall disease affects a wide range of plant species, including many economically important crops such as fruit and nut trees and ornamental plants (De Cleene and De Ley 1976). Crown gall is caused by members of the *Agrobacterium* genus and can result in up to 40% reductions in yield and vigor in some perennial crops (Tzfira and Citovsky 2008). The tumors associated with crown gall disease can result in impairment of root and vascular

tissue development, with compromises to nutrient and water translocation being most damaging and sometimes lethal to young plants.

Agrobacterium represents a diverse, polyphyletic group within the *Rhizobiaceae* family (Weisberg et al. 2020, 2023). The three major lineages are referred to as biovars (BVs) 1 to 3 and were once synonymous with *A. tumefaciens*, *A. rhizogenes*, and *A. vitis*, respectively, but have since been assigned revised Latin binomials (Keane et al. 1970). BV1 and BV3 represent multiple subgroups, and those of BV1 have been subclassified into so-called genomospecies, with some being recognized as species and assigned Latin binomials (Weisberg et al. 2020, 2023). In addition, pathogenic agrobacteria outside of the main biovars are continually being identified and named. From hereafter, we will use the vernacular term, agrobacteria, to embody each of its diverse lineages that can or are inferred to have the potential to gain the capacity to cause crown gall disease.

The pathogenicity of crown gall-causing agrobacteria is dependent upon oncogenic tumor-inducing (Ti) plasmids (Kerr 1969; Van Larebeke et al. 1975). Agrobacterial cells typically attach to plant surfaces and enter hosts through wounds. Once inside, plasmid-encoded virulence proteins process a fragment (transfer DNA; T-DNA) of the plasmid, pilot it into the plant cell, and incorporate the T-DNA sequence into the host genome (Nester 2014). Expression of genes introduced by the T-DNA causes misregulation of hormone biosynthesis and uncontrolled growth of affected plant cells, leading to the characteristic gall. Transformed host cells also produce opines, which are nutrients useable by the infecting bacteria. The Ti plasmids sequenced to date have been classified into evolutionarily distinct types that vary in gene composition and structure (Chou et al. 2022; Weisberg et al. 2020, 2022). Some types are broadly distributed across lineages of agrobacteria, whereas others are seemingly more restricted. For example, some types are present only within BV3 and are limited to causing disease on grapevine (Weisberg et al. 2022).

There is a limited number of products for preventing crown gall disease. Strains K84 and K1026 are successful biocontrol strains against some strains of agrobacteria (Brown et al. 2023). These two biocontrol strains are identical in chromosome sequence but differ in carrying pAgK84 or pAgK1026, respectively. These plasmids are similar in being non-oncogenic and necessary for the production of agrocin 84, a secondary metabolite that inhibits protein translation (New and Kerr 1972; Reader et al. 2005; Roberts et al. 1977; Slota and Farrand 1982). The pAgK1026 plasmid is derived from pAgK84 and modified to be incapable of conjugation (Jones et al. 1988). Though successful in prevention, neither strain is universally effective. Inhibition by agrocin 84 is influenced by the genetic identity of target strains, types of oncogenic plasmids they carry, and whether susceptibility genes are expressed (Kerr and Panagopoulos 1977; Kerr and Roberts 1976; Moore and Warren 1979). Notably, K84 and K1026 are ineffective against *A. vitis*, and crown gall disease remains a problem in nurseries and orchards (Burr and Otten 1999).

Some members of the *Bacillus* genus have uses in preventing plant disease. The *B. amyloliquefaciens* operational group is within the *Bacillus subtilis* species complex that circumscribes species adapted to plants (Borriss et al. 2011). One of these strains, FZB42 (aka *B. amyloliquefaciens* subsp. *plantarum*; *B. velezensis*), is used commercially as a biofertilizer and biocontrol agent (Chowdhury et al. 2015; Fan et al. 2018). These bacteria are intensively studied for their antifungal activities, which are often attributed to the production of cyclic lipopeptides, including surfactins, iturins, fengycins, and plipastatins (Sumi et al. 2015). Structurally, these cyclic lipopeptides consist of peptides varying in length, linked to a β-hydroxy fatty acid. For example, fengycins and plipastatins are similar in structure in being composed of a decapeptide (Honma et al. 2012). The first fengycin identified exhibited antifungal activities, whereas the first plipastatin identified inhibited phospholipase A2 (Nishikiori et al. 1986; Vanittanakom et al. 1986). Both products were identified from strains of *Bacillus*. Plipastatins have since been shown to also be active against filamentous fungi (Gong et al. 2015; Romero et al. 2007; Vanittanakom et al. 1986). Whether fengycins and plipastatins are equivalent is still under debate, though fengycin IX and plipastatin A1 have since been found to be identical in structure (Honma et al. 2012).

Findings have implied that *B. amyloliquefaciens* strains and cyclic lipopeptides have potential uses in controlling plant diseases caused by bacteria. For example, high concentrations of iturins were shown to cause morphological changes in plant-pathogenic bacteria of the *Xanthomonas* genus (Etchegaray et al. 2008). Likewise, fengycins from a strain of *B. amyloliquefaciens* can cause cell damage to the plant pathogen *Xanthomonas axonopodis* pv. *vesicatoria* (Medeot et al. 2020). Culture filtrate from *B. amyloliquefaciens* strain RC-2 inhibited growth in a culture of agrobacteria and several other tested bacterial and fungal pathogens of plants (Yoshida et al. 2001). However, the efficacy of control against bacterial pathogens was not evaluated on infected plants for any of these cases. Conversely, spores and culture supernatant from *B. amyloliquefaciens* strain 32A inhibited agrobacteria in a culture and significantly protected plants from crown gall disease (Abdallah et al. 2015). In a follow-up study, cyclic lipopeptides were identified from strain 32A and associated with biocontrol of agrobacteria and with protecting plants from crown gall disease (Abdallah et al. 2018). However, the class and identities of the cyclic lipopeptides that control against agrobacteria were not identified. Also, strain 32A was tested against only strain C58, so it is unknown how broadly the products control against the diversity of agrobacterial strains.

Here, we identified a new strain of *Bacillus* within the operational group *B. amyloliquefaciens* that can inhibit the growth of gram-negative and gram-positive plant-pathogenic bacteria. We further demonstrate that strain Z062 can inhibit the growth in cultures of diverse strains of agrobacteria. We purified plipastatins from Z062 and demonstrated that they are sufficient for controlling agrobacteria. Importantly, total extracts and a purified mixture of plipastatins are sufficient to prevent crown gall disease on different species of plants. Last, the findings show that Z062 produces several plipastatin analogs, and that those with shorter tail lengths have stronger inhibitory effects against agrobacteria grown in a culture. This discovery may lead to the development of new plipastatin-based products or reveal new applications for antifungal, cyclic lipopeptide-based products in controlling crown gall disease.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are described in Supplementary Table S1. Unless stated otherwise, strains of *Rhodococcus*, agrobacteria, and *Bacillus* were grown overnight on a solid LB, mannitol-glutamate-yeast, or LB medium, respectively, at 28°C or in liquid at 28°C with shaking at 200 rpm (Bertani 1951; Gordon et al. 2024).

Whole-genome sequencing and analyses

The Wizard Genomic DNA Purification Kit (Promega, Fitchburg, WI, U.S.A.) was used to extract genomic DNA from *Bacillus* strains. Directions for extracting DNA from gram-positive bacteria were followed. DNA samples were quantified

on a NanoDrop spectrophotometer and prepared as well as sequenced on an Illumina 3000 by the Center for Genome Research and Biocomputing (since renamed Center for Quantitative Life Sciences; Oregon State University, Corvallis, OR, U.S.A.) to generate 150mer paired-end sequencing reads. For long read sequencing on the Oxford Nanopore MinION, libraries were prepared using a Ligation Sequencing Kit (SQK-LSK110) and sequenced on Flongle flow cells (FLO-FLG001; Oxford Nanopore, Oxford, U.K.). Previously described methods were used to assemble short reads as well as hybrid assemble short and long reads (Weisberg et al. 2020).

A multilocus sequence analysis phylogeny was generated based on the genes *eutD*, *glpF*, *gmk*, *ilvD*, *purH*, *pycA*, and *tpiA* (Priest et al. 2004). Sequences from the strain *Bacillus subtilis* 168 were used as queries. AutoMLSA v.2.1.0 with the options "-prog blastn -relaxed -nocomplete -remote refseq_genomic -entrez_query 'Bacillus[ORGANISM]' -target 1000" and a local blast database were used to identify homologs and generate partitioned alignments (Davis et al. 2016). Sequences were retrieved in the summer of 2019. IQ-TREE v.1.6.12 with the options "-bb 1000 -alrt 1000 -nt 8" and the alignment and partition files were used to generate a maximum-likelihood phylogeny (Nguyen et al. 2015).

Previously described methods were used to calculate the average nucleotide identity (ANI; threshold $\geq$ 95%; Weisberg et al. 2020). The program get_assemblies v.0.8.3 was used to download publicly available genome sequences of *Bacillus* type strains from the NCBI database in August of 2022 (Davis 2022).

Disc diffusion assay

Cultures of target bacteria were washed in 10 mM $MgCl_2$ and resuspended in LB at an OD_{600} of 0.1, and 100 µl was either plated onto solid media or resuspended within solidifying media. Plates were dried for 20 min prior to use. Strains of *Bacillus* were washed in 10 mM $MgCl_2$ and resuspended in LB at an OD_{600} of 0.5. Sterile 5-mm paper discs with 5 µl of either 0.5 mM $MgCl_2$ or *Bacillus* strain were placed on the solid medium. For extracts, 5 µl of indicated concentrations were applied to sterilized discs. Methanol alone was used as the vehicle control. After drying, loaded discs were placed on the solid medium. Plates were incubated at 28°C overnight, and after approximately 24 h, they were examined and photographed. Some were stained with 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for better visualization (Gordon et al. 2024).

Plipastatin A1 (10 mg/ml) and surfactins (10 mg/ml), purified in this study, as well as commercially purchased tetracycline (0.1 mg/ml), were tested at a volume of 20 µl. Tetracycline was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

To determine the minimal inhibitory concentrations of purified plipastatin analogs, agrobacteria were grown on an agar plate (LB) overnight at 28°C and then added to a liquid LB medium (3 ml). After shaking at 200 rpm, 28°C, for 5 h, the culture was diluted with the LB medium to obtain an OD_{600} value of 0.1. The bacterial culture (100 µl) with the desired optical density value was added to the LB medium (50 ml) and then mixed gently. The bacterium-containing medium (98 µl) was then added to each well of 96-well plates containing DMSO solutions (2 µl) of the tested compounds at different concentrations to give final concentrations of 0.5, 0.25, 0.125, 0.062, 0.03, and 0.015 mM. DMSO was used as a vehicle control, and apramycin was used as a positive control. The 96-well plates were placed in a 28°C incubator for 17 to 24 h. An MTT solution (50 µl, 1 mg ml^{-1}) was then added to each well. After letting them stand for 30 min, the color change was observed. All experiments were performed in triplicate. The minimum inhibitory concentration values were determined as the lowest concentration required for bacterial growth inhibition.

Isolation and analyses of plipastatin

A 2× YT (15 g/liter tryptone, 5 g/liter NaCl, 10 g/liter yeast extract, 1.9 g/liter KH_2PO_4, 10 g/liter glucose, and 5 g/liter $MgSO_4$) seed medium (50 ml) was inoculated with a colony of Z062 and cultured for 18 to 20 h. The culture (1 ml) was dried by a lyophilizer (Labconco FreeZone), resuspended in MeOH (1 ml), and centrifuged at 10,000 rpm. The MeOH soluble portion was collected in a vial, and the solvent was evaporated by a rotary evaporator. The dried product was redissolved in MeOH to a final concentration of 20 mg/ml. The MeOH extract was used for preliminary antibacterial tests in a disc diffusion assay.

To isolate and identify active compounds, the seed culture (1 ml) was transferred to 500-ml Erlenmeyer flasks containing a modified 2× YT medium (100 ml) augmented with 1 ml of a trace element solution (0.42 g/liter $CaCl_2$, 2.29 g/liter $FeSO_4 \cdot 7H_2O$, 0.10 g/liter $MnCl_2 \cdot 4H_2O$, 0.17 g/liter $ZnCl_2$, 0.03 g/liter $CuAc_2$, 0.06 g/liter $CoCl_2 \cdot 6H_2O$, 0.06 g/liter $Na_2MoO_4 \cdot 2H_2O$) (1 ml) and grown for 2 days at 30°C with shaking (200 rpm). The culture broth was collected by centrifugation (3,500 rpm, 4°C, 30 min).

The first fractionation method was as follows: the culture broth was extracted with an equal volume of ethyl acetate (EtOAc) twice, and the aqueous layer was extracted with an equal volume of n-butanol (nBuOH). The EtOAc and nBuOH extracts were dried using a rotary evaporator, redissolved in methanol (MeOH) to a final concentration of 20 mg/ml, and tested against *Rhodococcus* strain D188 and agrobacterial strain C58 in a disc diffusion assay. Because the nBuOH extract showed stronger inhibitory activity than the EtOAc extract, it was then subjected to SiO_2 column chromatography, using a gradient solvent system of CH_2Cl_2–MeOH 20:1 to 7:3, followed by a 100% MeOH wash, and yielded 10 fractions. The fractions that were most active against agrobacterial strain C58 were combined and subjected to reverse-phase high-performance liquid chromatography (HPLC) on a Shimadzu system dual LC-20AD solvent delivery system with a Shimadzu SPD-M20A UV/vis photodiode array detector and with a YMC-Pak C18 column (250 × 10 mm ID) and eluted with a gradient solvent system of CH_3CN–H_2O (5:95) to CH_3CN (100%) over 60 min to yield plipastatin A1 and surfactins. The eluted compounds were monitored at 210 and 270 nm.

The second fractionation method was as follows: the culture broth (2 liters) was acidified by adding 2M HCl until the pH reached 2.0 (Roongsawang et al. 2002). The solution was stirred overnight at 4°C, and the formed precipitate was collected by centrifugation (3,500 rpm, 4°C, 30 min) and extracted with MeOH (5 × 5 ml). The MeOH extract was dried in vacuo. It was then either resuspended and used for testing against agrobacteria in a disc assay or in planta or further fractionated. For the disc diffusion assay, the extract was resuspended in MeOH, whereas for in planta assays, the extract was resuspended in phosphate buffer (see the next Materials and Methods section). For further fractionation, it was redissolved in MeOH (1 ml), passed through a C18 SPE column (Supelco), and eluted with MeOH–H_2O from 10:90 to 100:0. All 20 fractions were tested for antibacterial activity against agrobacterial strain C58, and the most active fraction was subjected to a reverse-phase HPLC on a Shimadzu dual LC-20AD solvent delivery system with a Shimadzu SPD-M20A UV/vis photodiode array detector and with a YMC-Pak C18 column (250 × 10 mm ID), eluted with a gradient solvent system of CH_3OH–CH_3COONH_4 10 mM pH 4.5 (82:18) to CH_3OH (100%) over 65 min. The eluted compounds were monitored

at 210 and 270 nm. This active fraction represents the mixture of plipastatins used for in vitro and in planta tests. One of the major products was isolated and structurally characterized by mass spectrometry (Agilent 6545 Quadrupole Time-of-Flight [Q-TOF]) and nuclear magnetic resonance (NMR; Bruker Avance III 700 MHz spectrometer equipped with a 5-mm ^{13}C cryogenic probe; Honma et al. 2012; Nishikiori et al. 1986).

For HPLC analysis of plipastatin analogs, the MeOH extract (from the second fractionation method described above) was loaded onto a HyperSep C18 column (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The column was washed with MeOH–aqueous CH_3COONH_4 solution (10 mM, pH 4.2) (1:1) and eluted with MeOH–aqueous CH_3COONH_4 solution (10 mM, pH 4.2) (9:1). The eluate was concentrated and injected into an HPLC system (Shimadzu dual LC-20AD solvent delivery system with a Shimadzu SPD-M20A UV/vis photodiode array detector) equipped with a YMC-Pack ODS-A column (250 × 10 mm, 5.0 μm). The system was run using a linear gradient, 0 min, 30% A/70% B; 40 min, 18% A/82% B, at a flow rate of 2.5 ml/min. Solvent A was an aqueous CH_3COONH_4 solution (10 mM, pH 4.2), and Solvent B was MeOH. The eluted compounds were monitored at 210 and 270 nm. The fractions were concentrated and applied to liquid chromatography mass spectrometry analysis using an Agilent 1260 HPLC and an Agilent 6545 Q-TOF. The separation used an InfinityLab Poroshell EC-C18 column (100 × 3.0 mm, 2.7 μm) at a flow rate of 0.4 ml/min and the following gradient method. Solvent A was an aqueous ammonium formate solution (10 mM), and Solvent B was acetonitrile. The column was pre-equilibrated with 10% A/90% B. Upon injection, the mobile phase composition was maintained for 2 min, after which the mobile phase was changed using a linear gradient to 90% A/10% B over a 20-min period. The Q-TOF mass spectrometer was run in Auto MS/MS mode using Agilent MassHunter software (version 10.1). The ion polarity was set for positive ions. The MS/MS was set for 5 Max Precursor per cycle with a mass range of 100 to 2,000 *m/z*. The collision energy was set for 20, 40, and 60 eV. The data were processed on an Agilent MassHunter workstation.

In planta assays

Tests for tumorigenesis were done on carrot root slices (Ryder et al. 1985). Fresh organic carrots purchased from a grocery store were washed with soap, rinsed with sterile distilled water, sprayed with 70% ethanol, air dried, then submerged in 20% bleach containing 0.1% sodium dodecyl sulfate for 20 min. Following this, the carrots were rinsed with sterile distilled water and sliced into segments 5 mm thick; each slice was placed on water agar (1.5%) with the basal side upward. The discs were treated with 25 μl of the MeOH extract (from the second fractionation method described above) at 100 or 200 mg/ml and resuspended in 50 mM potassium phosphate buffer (pH 7.2). One hour later, 25 μl ($OD_{600} = 0.1$) of agrobacterial strain C58, grown overnight at 28°C in the LB medium and resuspended in 50 mM potassium phosphate buffer (pH 7.2), was added to the extract-treated discs. Additional discs were treated with the bacteria first, then the two concentrations of extract. Controls were discs treated with agrobacteria alone and the two concentrations of extract alone. Carrot discs were incubated at 28°C for 7 days prior to visual inspection for tumor formation. All treatments consisted of five replicate discs. The experiment was repeated with similar results.

A sterile scalpel was used to decapitate the tip of newly rooted cuttings (5 to 8 cm tall) of chrysanthemum cultivars 'Copper Coin Bronze' and 'Jolly Cheryl Red'. The dried MeOH extract (from the second fractionation method described above) was resuspended at 20 mg/ml in 50 mM potassium phosphate buffer (pH 7.2) containing Tween 20 (0.1%) and sprayed, using an atomizer, onto the wounded portion of plants. After 1 h, an atomizer was used to apply a mixture of three plant-pathogenic agrobacterial strains (15-172, 15-173, and 15-174; $OD_{600} = 0.1$) to the wounded areas (Gordon et al. 2024; Weisberg et al. 2020). Controls included the following: wounded and treated with 20 mg/ml MeOH extract; wounded and treated with 20 mg/ml potassium phosphate buffer containing 0.1% Tween 20; wounded only; and wounded and inoculated with pathogenic agrobacterial strains. All treatment groups consisted of at least four plants each. Wounded plants did not show disease symptoms.

In a separate test, a sterile needle was used to wound stems immediately above the cotyledons of 4-week-old Rio Grande 76R tomato plants. A total of 5 μl of the purified plipastatin mixture resuspended at 25 or 50 mg/ml in MeOH was applied directly to wounds and dried for 1 h. A total of 5 μl of strain C58 ($OD_{600} = 0.1$) was then applied directly to wounded areas. Plants were grown in a walk-in growth chamber (25°C, 60% humidity, 12-/12-h light cycle). Plants were watered three times a week and treated with NPK 20-20-20 plant fertilizer weekly. Each treatment had at least five plants, and experiments were repeated two more times with similar results.

Disease symptoms were visually inspected at 2 weeks after inoculation. Efficacy of plipastatin control was determined by calculating an index where plipastatin control = 100% − [(% of disease plants in treatment × 100)/(% of diseased plants in control treatment)] (Penyalver and López 1999). The Shapiro-Wilk test was used to determine if a sample of data came from a normal distribution. The chi-square test was used to determine if there was variation across replicates. A Kruskal-Wallis test was used to analyze the variance between tested groups. Statistical analyses were done using R version 4.3.1 and relevant packages.

Results

Members of *Bacillus* inhibit gram-positive and gram-negative plant pathogens

We observed contaminating colonies on lawns of strain D188, a strain of gram-positive *Rhodococcus* that causes leafy gall disease to plants (Crespi et al. 1992). The contaminating colonies were white colored or translucent and surrounded by zones cleared of D188 colonies (Fig. 1A). We isolated 22 contaminating colonies for analysis ("Z" strains; Supplementary Table S1). Based on a multilocus phylogeny, the contaminating strains are diverse, with 13 clustering within the operational group *B. amyloliquefaciens* (Fig. 1B; Supplementary Table S1). Four strains are within unnamed groups sister to *B. altitudinis* (Shivaji et al. 2006). The remaining five strains formed distinct clades closer to the root of the tree and are outside of the *B. subtilis* species complex. We used the ANI (threshold ≥ 95%) and included genome sequences of type strains to operationally determine the species-level relationships of the Z strains (Supplementary Table S2). The Z strains are members of four different species-level groups. One of these groups is within the *B. subtilis* complex and belongs to the operational group *B. amyloliquefaciens*, and its members are closely related to reference strain *B. velezensis* FZB42 (Borriss et al. 2011). For these, the range of ANI values was consistent with the phylogeny in which five of the Z strains clustered in a different subclade than FZB42, whereas eight are in a subclade with FZB42. The strains sister to *B. altitudinis* are associated with members of *B. pumilus*, and the rest are related to members of *Bacillus cereus senso lato*, which includes human pathogens but also an atmospheric laboratory environment contaminant, *B. mobilis* (Liu et al. 2020).

To determine whether these strains were also effective against agrobacteria, we tested two strains from each of the subclades

within the operational group *B. amyloliquefaciens*. Strains Z025, Z030, and Z062 strongly inhibited growth of reference strain C58 (BV1, genomospecies G8; Fig. 2A). Strain Z010 behaved like a mock-inoculated disc and failed to show visible activity. We next used Z062 as a focal strain and tested its ability to inhibit a small panel of agrobacterial strains available in our collection and representative of the major taxonomic diversity of strains (Supplementary Table S1; Weisberg et al. 2020). We also tested against strains within genomospecies G1, selected because they differ in the presence or type of Ti plasmid they carry. A disc diffusion assay showed that strain Z062 inhibited the growth, to varying degrees, of all tested agrobacterial strains (Fig. 2B). The strain selected to represent BV2 showed a sharp and small clear area like that shown by strain C58, whereas the agrobacterial strain selected to represent BV3 showed a more diffuse but larger clear area. Among other members of BV1, strains representing genomospecies G4 and G7 showed distinct and large clear areas. Within genomospecies G1, all strains were cleared to some degree, suggesting that Ti plasmid status was not determinative of inhibition by Z062. We also demonstrated that the same three Z strains can inhibit at least one additional strain of *Rhodococcus*; we selected four to represent the different clades of plant-associated *Rhodococcus* (Supplementary Fig. S1; Supplementary Table S1; Savory et al. 2017). Like the case with agrobacteria, strain Z010 failed to show visible activity against any of the tested *Rhodococcus* strains, though a faint halo could be observed when tested against UNC23MFCrub1.1 of clade III.

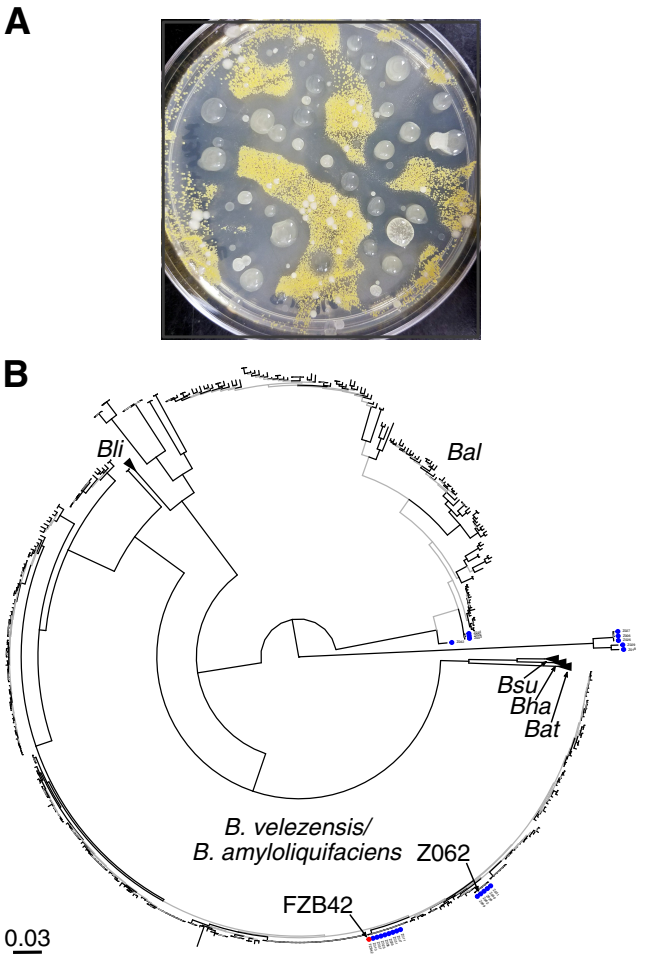

FIGURE 1
Strains associated with inhibiting *Rhodococcus* strain D188 are members of the *Bacillus* genus. **A,** Photograph of bacterial colonies (white colored to transparent) contaminating a lawn of *Rhodococcus* strain D188 (yellow-colored colonies). **B,** A multilocus sequence analysis maximum-likelihood tree shows the relationships of 22 isolated Z strains relative to approximately 900 strains of *Bacillus*. Circles at tips correspond to Z strains and strain *B. velezensis* FZB42. Major clades are labeled (abbreviations are *B. licheniformis* [*Bli*], *B. altitudinis* [*Bal*], *B. subtilis* [*Bsu*], *B. halotolerans* [*Bha*], and *B. atrophaeus* [*Bat*]), and those without Z strains were collapsed. Black lines indicate UFBoot and SH-aLRT bootstrap support of ≥95 and 80%, respectively. The tree is midpoint rooted. The scale bar indicates the average number of substitutions per site.

Plipastatins from strain Z062 inhibit agrobacteria

We next sought to identify the product or products from Z062 that inhibit agrobacteria. Culture broth was collected and subsequently extracted with ethyl acetate and n-butanol to give EtOAc and nBuOH extracts, respectively. Both extracts inhibited strain C58, with the nBuOH extract showing stronger inhibitory activity than the EtOAc extract (Supplementary Fig. S2A; 21-mm diameter versus 19-mm diameter). The nBuOH extract was then subjected to column chromatography and HPLC to yield two groups of lipopeptides, which, based on their NMR and mass spectral data, were identified as surfactins and plipastatins. The two purified products were then tested at the same concentration to determine which one of them exhibits anti-agrobacterial activity against strain C58. The result showed that the plipastatins have activity, whereas the surfactins did not show any activity against strain C58 (Supplementary Fig. S2A). Conversely, the surfactins, but not the plipastatins, showed activity against *Rhodococcus* (Supplementary Fig. S2B).

As the plipastatins were found to be the active metabolites against agrobacteria, we modified our extraction method by adapting the acid precipitation method for lipopeptides (extraction method 2; Roongsawang et al. 2002). The resulting methanol (MeOH) extract was subjected to C18 SPE column chromatography and HPLC to yield purified plipastatin A1 (peak 5, Fig. 3A), which is one of the major components of the plipastatin mixture, and related compounds. The proton NMR (^{1}H NMR) and high-resolution mass spectrometry of peak 5 fully matched reported values for plipastatin A1, a molecule with a 16-carbon long fatty acid tail and an alanine in the sixth position (Supplementary Figs. S3 and S4; Supplementary Table S3). High-resolution mass spectrometry analysis of the other peaks showed that they corresponded to plipastatin analogs having C14, C15, C16, and C17 fatty acids, as well as alanine or valine for each different chain length molecule (Fig. 3B; Supplementary Fig. S5; Supplementary Table S3). Therefore, strain Z062 produces a diversity of plipastatin analogs, and the MeOH extract contained a mixture of plipastatins.

A BLAST analysis of the Z062 genome sequence revealed homologs of the *ppsABCDE* operon, which encodes subunits of the plipastatin non-ribosomal peptide synthetase from *Bacillus subtilis* 3NA. The translated sequences of genes from Z062 have approximately 60% identity with those of the query sequences (Vahidinasab et al. 2020). Additionally, antiSMASH analysis was used to predict loci involved in the synthesis of antibiotics and secondary metabolites (Blin et al. 2023). This approach identified a region carrying the *ppsABCDE* homologs, showing 100% similarity to gene clusters encoding putative non-ribosomal peptide synthetases involved in fengycin biosynthesis.

We tested the sufficiency of the MeOH extract and the purified plipastatin mixture against diverse strains of agrobacteria (Fig. 4A and B). Both inhibited the growth of most strains of agrobacteria, with the plipastatin mixture often associated with very large

zones of inhibition (Fig. 4B). The one notable exception was with strain X1/95 (BV2), which was seemingly unaffected by either of the extracts despite showing susceptibility to strain Z062 (Figs. 2 and 4). Also notable was that strain LMG305 showed strong susceptibility to the plipastatin mixture but responded to the MeOH extract with a faint halo (Fig. 4).

Next, we evaluated the inhibitory effects of individually purified plipastatin analogs on agrobacterial strain C58. Because of the larger zone of inhibition observed in a disc diffusion assay, the shorter-chain length plipastatins, C14A, we suggested that it has greater activity in vitro compared with that of the longer-chain length plipastatins (Fig. 4C).

A total extract and plipastatin mixture from Z062 can prevent crown gall disease

The products extracted from Z062 prevent crown gall disease on multiple species of plants. We first used a carrot disc assay to evaluate the efficacy of the MeOH extract (Supplementary Fig. S6A). By 20 days postinoculation, discs treated with agrobacterial strains formed numerous galls, whereas those mock inoculated with buffer only showed no disease symptoms. Discs pretreated with 20 mg/ml (suspended in buffer), but not 10 mg/ml, of the MeOH extract were reliably protected from agrobacteria that were inoculated 1 h afterward. Discs failed to form galls after 20 days. Surprisingly, carrot discs also failed to show disease symptoms even when 20 mg/ml of the MeOH extract was applied 1 h after treating with agrobacterial strains.

We next evaluated the efficacy of the MeOH extract in protecting plants. We used chrysanthemum because it is a susceptible ornamental host plant species (Supplementary Fig. S6B; Smith and Townsend 1907). We wounded plants to mimic plant management practices used in nursery settings. Across replicates, all plants inoculated with pathogenic agrobacterial strains formed galls, whereas wounded plants mock inoculated with buffer only showed no disease symptoms. In contrast, no disease symptoms were observed on plants across replicates that were pretreated with 20 mg/ml of the MeOH extract (resuspended in buffer) 1 h before being inoculated with pathogenic agrobacterial strains.

In a final test, we evaluated the sufficiency of the plipastatin mixture in protecting tomato plants (Braun and Laskaris 1942). Wounded stems pretreated with 25 mg/ml of the plipastatin mixture (resuspended in MeOH) showed no galls (Fig. 5A). Conversely, wounded plants treated with only the MeOH vehicle showed no symptoms, whereas those first treated with the MeOH vehicle and subsequently inoculated with strain C58 formed galls (Fig. 5A). Therefore, these controls confirmed that prevention of crown gall disease was due to the plipastatin mixture and not the MeOH vehicle. Results were repeated with no variability across replicated experiments that used the same concentration of plipastatin (Supplementary Table S4). Nonetheless, we converted the data to an index of plipastatin control to further analyze results (Fig. 5B; Penyalver and López 1999). The Shapiro-Wilk test indicated that the data were not normally distributed ($W = 0.549$, $P < 0.001$). The chi-square test indicated that there was no replicate effect on disease ($X^2 = 1.737$, df = 2, $P = 0.420$) or treatment ($X^2 = 2.159$, df = 4, $P = 0.707$), confirming consistency across repeated trials. Therefore, we aggregated the data for the Kruskal-Wallis test, which, not surprisingly, indicated a significant difference ($P = 5.288 \times 10^{-15}$) in control between plants pretreated with the plipastatin mixture prior to inoculation with strain C58 versus plants pretreated with the MeOH vehicle prior to inoculation with strain C58 (Fig. 5B). Control by

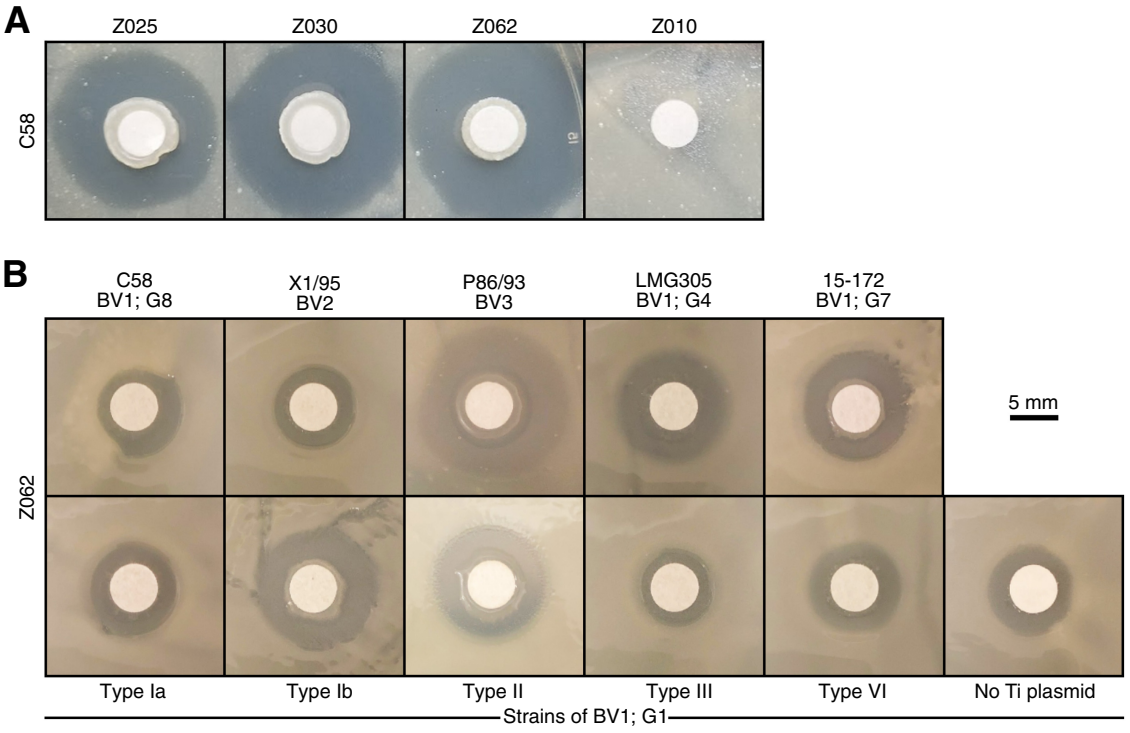

FIGURE 2
Three Z strains inhibit growth in cultures of agrobacteria. **A,** Three of the four Z strains inhibited the growth of agrobacterial strain C58 in a disc diffusion assay. C58 is a member of genomospecies 8 within biovar 1. **B,** Strain Z062 inhibits diverse strains of agrobacteria. In the top row, strain names of agrobacteria, as well as their designated biovar (BV) and genomospecies (G followed by a number), if applicable, are shown. In the bottom row, all strains are from biovar 1, genomospecies G1, and they vary in presence or type of tumor-inducing (Ti) plasmid. In both panels, photographs were taken after 24 h and show representative results. Filter discs are 5 mm in diameter.

plipastatin was significant regardless of whether 25 or 50 mg/ml was used (Supplementary Table S4). Results from statistical analysis supported the clearly observed and repeatable significant effect of the plipastatin mixture in protecting plants from strain C58.

The yields of individual and purified plipastatin analogs were too low for us to test their efficacy on plants. Regardless, the findings of multiple experiments involving three plant species and different pathogenic strains of BV1 demonstrated that applications of either the MeOH extract or plipastatin mixture from Z062 reproducibly protected plants when applied before pathogen infection.

Discussion

Crown gall is a significant problem in agriculture because there is zero tolerance for this disease, and its management requires a diversity of control methods. Here, the findings showed that against most tested strains of agrobacteria, a mixture of plipastatins is sufficient for preventing disease. Both the MeOH extract from the acid precipitated portion of the culture broth and the plipastatin mixture, when applied to plants or plant tissues prior to inoculation with agrobacteria, prevented the pathogen from causing crown gall disease. Importantly, there was absolute protection, as no disease symptoms were observed despite the high level of the pathogen inoculum used and regardless of host species, pathogenic strain tested, or inoculation method. The levels of the inoculum used in greenhouse-based studies likely greatly exceed bacterial loads present in agricultural settings. However, direct applications of Z062 to tomato plants showed no effect in preventing crown gall disease (data not shown).

These findings advance those of initial discoveries that a strain of *Bacillus* can be used to control agrobacteria (Abdallah et al. 2018). Prior work had implicated cyclic lipopeptides as the inhibitory compound. The work here revealed that plipastatins are one of the possible inhibitory products, whereas surfactins are ineffective at the concentrations tested (Supplementary Fig. S2). That plipastatins were effective was unexpected because they are less known for their antibacterial activities and are better known for antifungal activities (Gong et al. 2015; Romero et al. 2007;

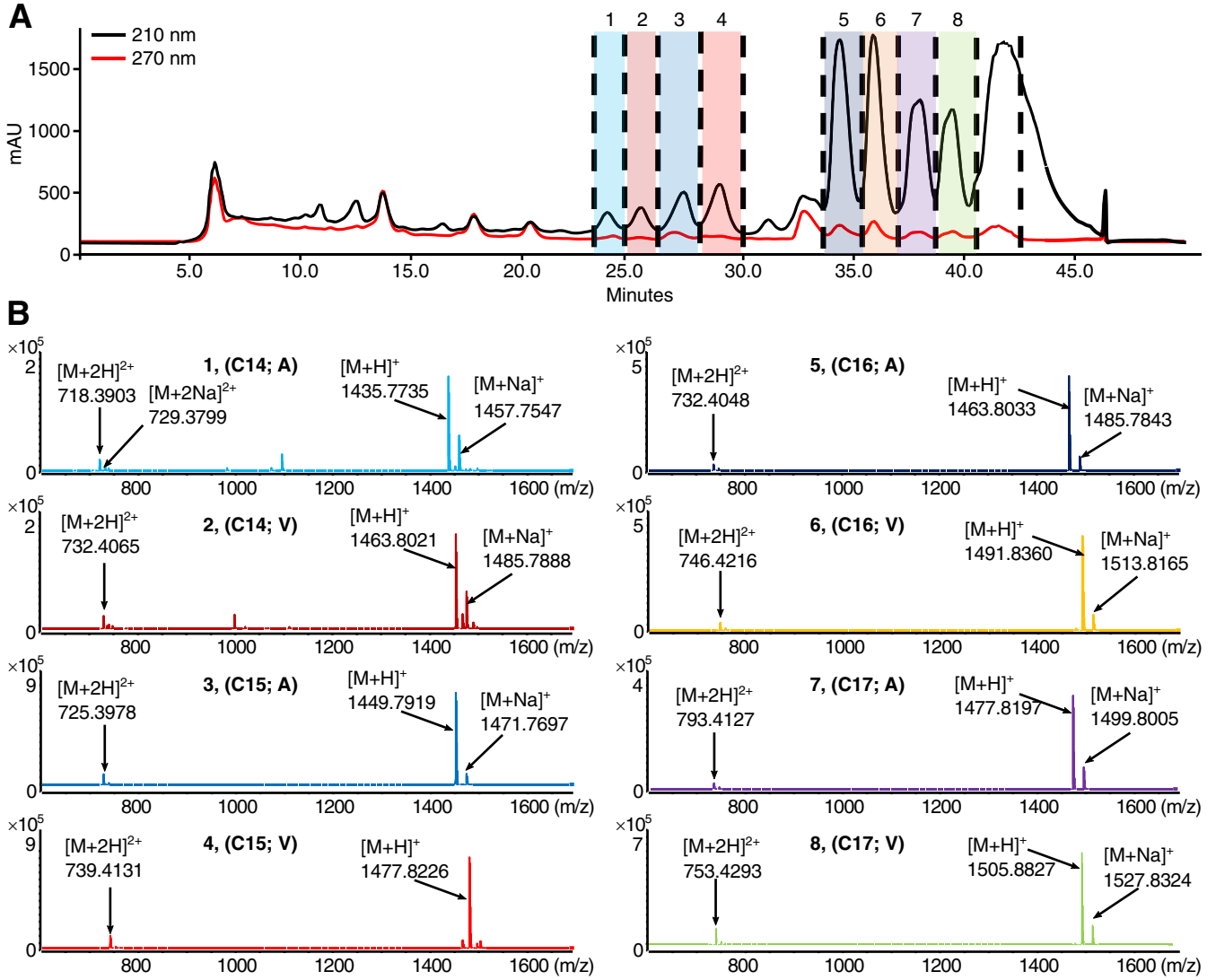

FIGURE 3

The active product of Z062 is a mixture of plipastatin molecules. **A,** High-performance liquid chromatography chromatogram of a mixture of plipastatin analogs purified from strain Z062. The dark (black) and light (red) traces correspond to 210 and 270 nm detection wavelengths, respectively. The eight highlighted peaks correspond to the mass spectrometry chromatograms shown in **B**. In panel B, C14 to C17 indicates the chain length of the plipastatin molecule, and alanine (A) or valine (V) indicates the identity of the sixth amino acid in the structure.

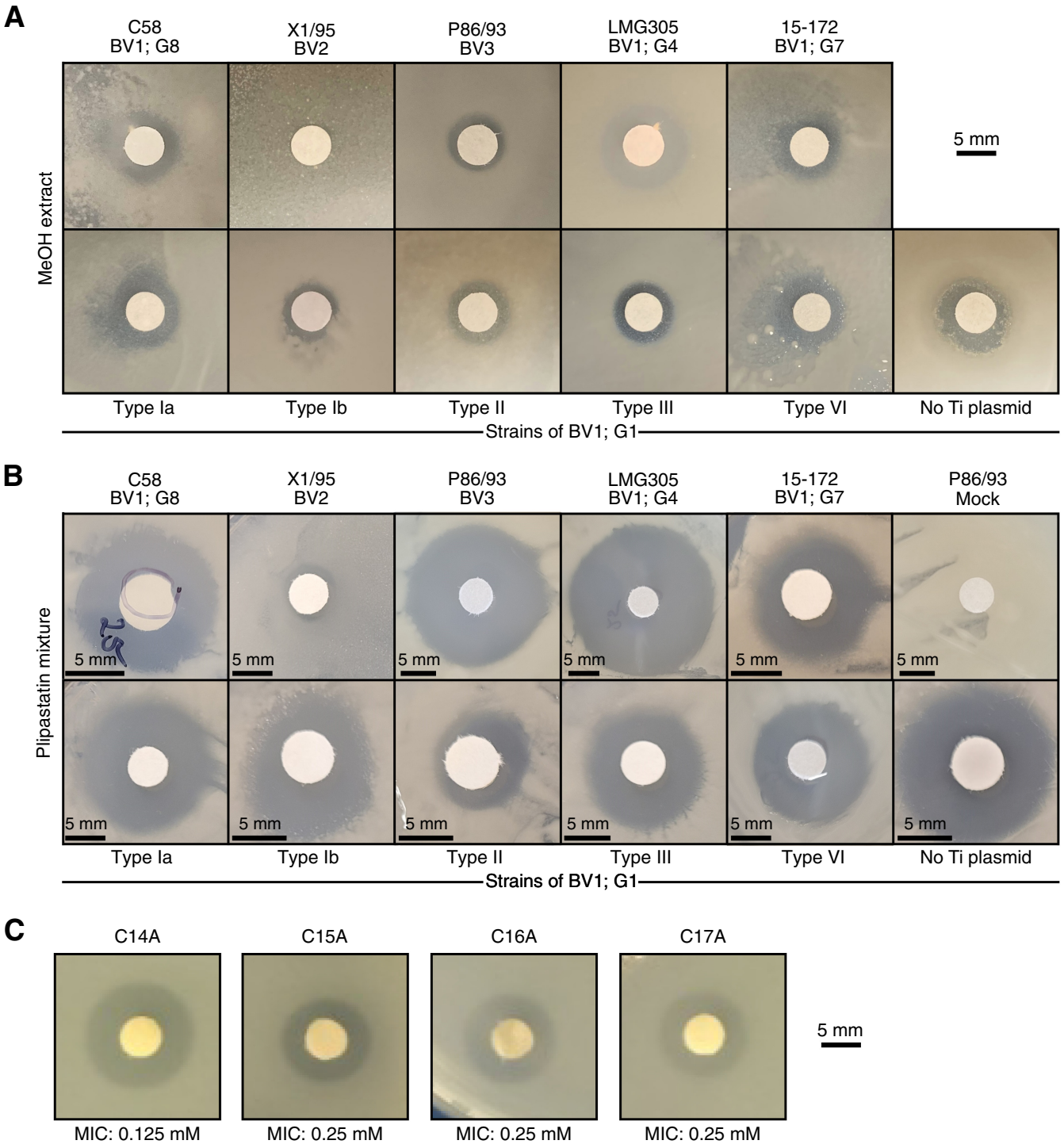

FIGURE 4

Isolated products from Z062 are variable in their inhibitory effect on agrobacterial strains. **A,** The MeOH extract from culture broth and **B,** plipastatin mixture from Z062 were tested against strains of agrobacteria. In the top rows for both panels, strain names of agrobacteria, as well as their designated biovar (BV) and genomospecies (G followed by a number), if applicable, are shown. In the bottom rows for both panels, all strains are from biovar 1, genomospecies G1, and they vary in presence or type of tumor-inducing (Ti) plasmid. The mock-loaded (MeOH) disc treatment for P86/93 is shown for comparative purposes. **C,** Purified plipastatin analogs, varying in chain length (C14 to C17), were tested at their minimum inhibitory concentration (MIC) against agrobacterial strain C58. Each isoform was applied at the same concentration of (10 mM, 20 µl). Photographs were taken after 24 h and show representative results. Filter discs are 5 mm in diameter.

Vanittanakom et al. 1986). Moreover, the efficacy of plipastatins in controlling bacterial diseases of plants has not been previously shown. Another important observation was that the plipastatin mixture has potential in controlling against diverse agrobacterial pathogens, regardless of the type of Ti plasmid they carry. This contrasts with the currently available biocontrol products, K84 and K1026, which are limited in the breadth of strains they control against (Kerr and Roberts 1976). Additional characterization of individual analogs within the plipastatin mixture suggested that they vary in their efficacy against culture-grown agrobacteria. The impact of chain lengths on the antimicrobial activity of cyclic lipopeptides has been previously reported. Surfactins with shorter lipid tails have lower antiviral effects than those with longer ones (Kracht et al. 1999). We observed the opposite, where plipastatins with shorter lipid tails exhibited stronger activities against culture-grown bacteria. Whether the side chain bears any effect on their ability to protect plants remains unknown. Methods need to be developed to produce or purify enough of each plipastatin analog from strain Z062 to test them individually on plants.

Another finding with potentially important implications was the inhibition of a BV3 (*A. vitis*) strain in vitro (Fig. 4). BV3 differs in three main aspects from the agrobacterial strains we tested on plants. First, BV3 members have a narrow host range and are limited to causing crown gall disease on grapevine (Burr and Otten 1999). Second, BV3 bacteria can cause systemic infection and reside within grapevines without causing disease symptoms (Lehoczky 1971). Third, and importantly, is that K84 or K1026 are not effective against BV3 bacteria (Burr and Otten 1999). Several potential biocontrol strains for BV3 have been identified, but we are not aware of any being commercially available (Filo et al. 2013). Plipastatin has potential use in managing crown gall disease on grapevine, though its efficacy on woody plants and effectiveness in preventing disease from systemic bacteria need to be tested (Johnson et al. 2016).

The mechanism by which plipastatins inhibit agrobacteria is unknown. Cyclic lipopeptides are structurally diverse and have been suggested to have multiple roles and remarkable ranges of functions that could influence the efficacy of biocontrol. One major proposed function is in disrupting cellular membranes to provide a competitive advantage against other microorganisms (Etchegaray et al. 2008). Based on modeling, it is predicted that the susceptibility of fungi may be due to phosphatidylcholine in membranes (Sur et al. 2018). In agrobacteria, phosphatidylcholine makes up almost 25% of the phospholipids (Geiger et al. 2013; Klüsener et al. 2009). However, a Δ*pmtA/pcs* mutant, previously shown to be compromised in phosphatidylcholine, was not affected in terms of sensitivity to a mixture of plipastatins (data not shown; Klüsener et al. 2009; Wessel et al. 2006). A second hypothesized function is that surfactins and plipastatins elicit immune responses in plants (Farace et al. 2015). A third is that lipopeptides can also function as biosurfactants that assist with colonization and biofilm formation (Raaijmakers et al. 2010). The findings here showed that the plipastatin mixture, as well as individual analogs, acts directly on agrobacteria in a culture, and it is therefore unlikely that their mode of action is to incite host immunity or give Z062 a competitive advantage over other bacteria for plant surfaces.

That Z strains were contaminants, likely from air samples, and can protect plants was not surprising. Several of the Z strains are related to strains previously identified from air samples (Liu et al. 2020; Shivaji et al. 2006). In addition, members of the *Bacillus* genus have shown significant ability to serve as biocontrol agents against a wide variety of pathogens (Shafi et al. 2017). Strains of *Bacillus* have repeatedly been demonstrated to be effective against *Fusarium graminearum*, a fungal pathogen that causes

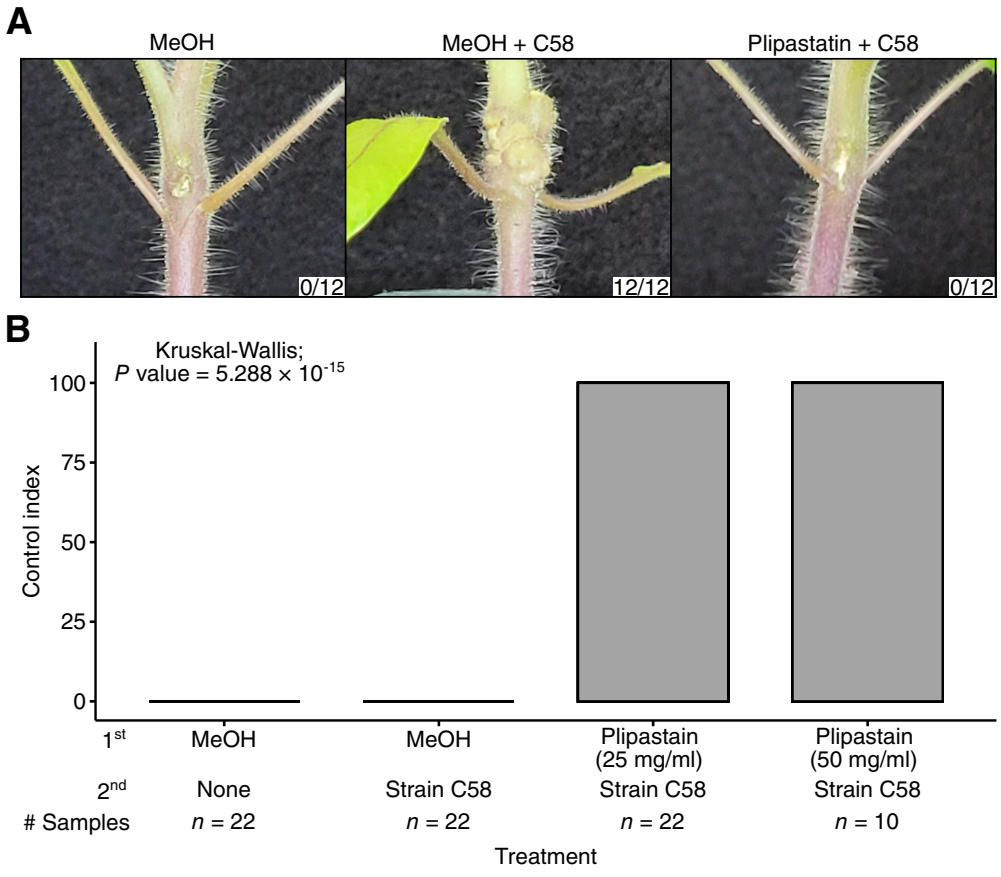

FIGURE 5
A plipastatin mixture from Z062 protects tomato plants against crown gall disease. **A,** Stems of tomato plants were wounded and first treated with the MeOH vehicle control or 25 mg/ml of a plipastatin mixture suspended in MeOH. One hour later, plants were untreated (left) or inoculated with strain C58 (middle and right). The ratio in the lower right of each photograph indicates the number of plants showing similar symptoms as those in the photographs relative to the number of plants treated. Plants were analyzed 2 weeks postinoculation. **B,** Data were converted to a control index. These are shown as a bar graph with data aggregated across three biological replicates that included pretreatments with 50 mg/ml of a mixture of plipastatin suspended in MeOH.

head blight in cereal crops (Ntushelo et al. 2019). Other strains of *Bacillus* have also been identified based on their potential antimicrobial activities against pathogenic fungi of plants and animals (Cai et al. 2017; Horng et al. 2019; Reiss and Jørgensen 2017; Romero et al. 2007). An extract derived from *B. amyloliquefaciens* strain PTA-4838 and enriched in cyclic lipopeptides has been developed into a biofungicide (Valent Bioscience, Libertyville, IL, U.S.A.). If they contain plipastatins, we suggest that commercially available cyclic lipopeptides derived from *Bacillus* have promise as a control product for bacterial pathogens such as agrobacteria.

Strain Z062 produces numerous and diverse compounds that collectively exhibit broad antibacterial activity. Notably, the fact that the bacterial strain Z062, but not a plipastatin mixture purified from it, inhibited *Rhodococcus* and agrobacterial strain X1/95 suggests that Z062 produces other compounds that are active and yet to be identified. Indeed, surfactins purified from Z062 were sufficient in inhibiting *Rhodococcus* in vitro (Supplementary Fig. S2). Additional compounds may also be present in the EtOAc or nBuOH extracts. Furthermore, though we did not isolate all the peaks, liquid chromatography tandem mass spectrometry data suggested that there are additional plipastatin analogs with unsaturated fatty acid tails.

The findings here show that plipastatins and members of the operational group *B. amyloliquefaciens* have strong promise in being developed for uses in controlling against diverse plant-pathogenic bacteria that afflict agriculture.

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