Discovery and Characterization of Low-Molecular Weight Inhibitors of *Erwinia tracheiphila*

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ABSTRACT

Plant pathogenic bacteria in the genus *Erwinia* cause economically important diseases, including bacterial wilt of cucurbits caused by *Erwinia tracheiphila*. Conventional bactericides are insufficient to control this disease. Using high-throughput screening, 464 small molecules (SMs) with either cidal or static activity at 100 μ M against a cucumber strain of *E. tracheiphila* were identified. Among them, 20 SMs (SM1 to SM20), composed of nine distinct chemical moiety structures, were cidal to multiple *E. tracheiphila* strains at 100 μ M. These lead SMs had low toxicity to human cells and honey bees at 100 μ M. No phytotoxicity was observed on melon plants at 100 μ M, except when SM12 was either mixed with Silwet L-77 and foliar sprayed or when delivered through the roots. Lead SMs did not inhibit the growth of beneficial *Pseudomonas* and *Enterobacter* species but inhibited the growth of *Bacillus* species. Nineteen SMs

were cidal to *Xanthomonas cucurbitae* and showed >50% growth inhibition against *Pseudomonas syringae* pv. *lachrymans*. In addition, 19 SMs were cidal or static against *Erwinia amylovora* in vitro. Five SMs demonstrated potential to suppress *E. tracheiphila* when foliar sprayed on melon plants at 2 $\times$ the minimum bactericidal concentration. Thirteen SMs reduced *Et* load in melon plants when delivered via roots. Temperature and light did not affect the activity of SMs. In vitro cidal activity was observed after 3 to 10 h of exposure to these five SMs. Here, we report 19 SMs that provide chemical scaffolds for future development of bactericides against plant pathogenic bacterial species.

Keywords: bacteriology, disease control and pest management, discovery, *Erwinia tracheiphila*, small molecule

Bacterial wilt is considered one of the most destructive diseases of cucurbits grown in the midwestern and northeastern United States, and losses can reach 80% (Rojas et al. 2015). The causal agent, *Erwinia tracheiphila*, survives in the alimentary canal of both spotted (*Diabrotica undecimpunctata howardi* Barber) and striped (*Acalymma vittatum* [F]) cucumber beetles (Brust 1997). Cucumber beetles diapause in the soil during the winter and emerge to feed on cucurbits in the spring (Shapiro 2012), coinciding with the transplanting period of cucurbit crops. The pathogen is transmitted via insect feces or contaminated mouthparts. Beetle feeding wounds are ports of entry for *E. tracheiphila* (Yao et al. 1996). Systemic infection of cucurbits occurs after the bacteria enter the xylem, multiply, and interrupt the water flow in the vascular tissue, leading to wilting symptoms (Koike et al. 2007; Sasu et al. 2010).

There are no systemic compounds available to aid in preventive or curative management programs for bacterial wilt of cucurbits. Bacterial plant pathogen control through conventional antibiotics is also difficult because of the limited number available in agriculture and the high risk of resistance development to these materials within bacterial pathogen populations (Thanner et al. 2016). Therefore,

novel compounds with potency to inhibit bacterial growth are needed for management of bacterial diseases of various crops.

Small molecules (SMs) are low-molecular weight (<500 Da) compounds. Some SMs have shown inhibitory activity against a number of bacterial pathogens. SM compounds were shown to inhibit the activity of the type III secretion system (T3SS) in *Pseudomonas aeruginosa*, affecting the secretion or translocation of effectors (Aiello et al. 2010), as well as inhibit quorum sensing in *P. aeruginosa* and *Escherichia coli*, which consequently inhibits biofilm formation (Geske et al. 2005; Helmy et al. 2018). Two synthetic SMs with activity against the type IV secretion system (T4SS) were identified for *Helicobacter pylori* and *E. coli*; T4SS-active molecules inhibit DNA transfer via conjugation mediated by the T4SS (Shaffer et al. 2016). Only a few studies have addressed SM effects on bacterial plant pathogens. Twelve novel molecules with activity against *Clavibacter michiganensis* subsp. *michiganensis* were identified from a yeast-active library of 4,182 SMs (Xu et al. 2015). In another study, a group of sulfanilamide molecules decreased *Pseudomonas syringae* populations in infected *Arabidopsis* plants (Schreiber et al. 2008). SMs in the group of salicylidene acylhydrazides were identified as inhibitors of the T3SS in *Erwinia amylovora*; compounds in this group also inhibited synthesis of the pathogenicity factor amylovoran. One compound in this group reduced disease development in pistils of crab apple (Yang et al. 2014). SMs with activity against the *Agrobacterium tumefaciens* T4SS were also identified; these molecules impaired transfer DNA (T-DNA) delivery via the T4SS (Shaffer et al. 2016). In another study, Worthington et al. (2012) selected 2-aminoimidazole, an SM that enhanced copper-mediated control of copper-resistant *Xanthomonas euvesicatoria* on pepper. The minimum inhibitory concentration (MIC) of copper was reduced by nearly half when mixed with 2-aminoimidazole at 100 μ M. Reduction in *X. euvesicatoria* colony forming units (CFU) was greater when this SM was applied in combination with copper hydroxide than for copper hydroxide applications alone. Decreased biofilm formation was also observed with the use of

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2-aminoimidazole. This SM also enhanced the control efficacy of copper hydroxide, potassium phosphite, kasugamycin, and the resistance-inducing giant knotweed extract Regalia under field conditions (Worthington et al. 2012).

The objectives of this study were to identify potential growth inhibitors of *E. tracheiphila* among a population of candidate small inhibitory molecules; characterize their activity against beneficial bacteria, other cucurbit bacterial pathogens, pollinators, and human model cell lines; and determine their safety to plants and efficacy in controlling bacterial wilt of cucurbits in planta through different delivery methods.

MATERIALS AND METHODS

Chemical library and bacterial strains. A library containing 4,182 bioactive SMs (Deblais et al. 2018; Kumar et al. 2016; Wallace et al. 2011; Xu et al. 2015) obtained from Chembridge (San Diego, CA) was used. The molecular weight of SMs in this library varied between 177.3 and 645.3 Da, and clogP varied between 0.63 and 14.69; the clogP value is an estimation of the log *P* (the log of the 1-octanol/water partition coefficient), which measures the lipophilicity of a compound (Ghose et al. 1998). Ninety-three percent of the library followed Lipinski's rule of five (Lipinski et al. 1997), and 30% of the library is present in the National Institutes of Health (<https://ncats.nih.gov/smr>). The library was supplied in powder form in 96-well plates, and compounds were dissolved in 100% dimethyl sulfoxide (DMSO) to a 10 mM final concentration. The library was stored at -80°C . Bacterial strains used in this study are listed in Supplementary Table S1.

Primary screen. Primary screening was performed using *E. tracheiphila* wild-type strain TedCu10, originally isolated from cucumber (*Cucumis sativus*) in Iowa (Rojas et al. 2013). Fresh bacterial culture was inoculated into 5 ml of full-strength nutrient broth yeast extract (NBY) and incubated at 28°C with shaking at 200 rpm. After 12 h, cells were harvested by centrifugation at $3,000 \times g$ for 7 min at room temperature. Supernatant was discarded, and bacterial cells were resuspended in half-strength NBY (50% NBY:50% distilled water; dH_2O) to a final concentration of approximately 10^7 CFU/ml (optical density at 600 nm; $\text{OD}_{600} = 0.05$). A 99- μl aliquot of the adjusted suspension was added to each well of a 96-well plate followed by addition of 1 μl of SM using a slotted pin tool (V and P Scientific, San Diego, CA) for a final concentration of 100 μM . Controls were six replicates per plate of a bacterial suspension with 1 μl of 100% DMSO or 1 μl of kanamycin (30 $\mu\text{g}/\mu\text{l}$) and four replicates of 100 μl of cell-free half-strength NBY medium. Plates were incubated at 28°C with shaking at 160 rpm in the dark for 24 h. Initial and final optical densities (OD_{600}) were measured using a Sunrise Tecan kinetic microplate reader (Tecan Group Ltd., San Jose, CA). Percentage bacterial growth inhibition was calculated for each SM using the following formula: inhibition = $((\mu_{\text{C}+} - X)/(\mu_{\text{C}+} - \mu_{\text{C}-})) \times 100$, where $\mu_{\text{C}+}$ and $\mu_{\text{C}-}$ are the average final OD_{600} values of positive (100% DMSO) and negative (kanamycin) controls, respectively, and *X* is the final OD_{600} of culture treated with each molecule. After incubation, the culture in each well was spotted onto fresh full-strength non-amended NBY agar medium, and growth recovery was monitored for 7 days. The SMs were grouped as “no effect” if no growth inhibition was detected, “cidal” if calculated growth inhibition was $\geq 99\%$ and no colonies grew on nonamended NBY agar, or “static” if calculated growth inhibition was $\geq 99\%$ but bacteria were recovered after plating on nonamended NBY agar. The activity of selected SMs against *E. tracheiphila* was confirmed in a separate experiment as described above. Fifty-seven SMs were selected to test multiple *E. tracheiphila* strains isolated from different *Cucumis* and *Cucurbita* hosts (Supplementary Table S1).

SM efficacy spectrum against *E. tracheiphila* strains. Twenty *E. tracheiphila* strains (Supplementary Table S1) isolated from plants in the genera *Cucumis* (10 strains) and *Cucurbita* (10

strains) were used to assess the antimicrobial efficacy of the selected 57 SMs with cidal effect against TedCu10 at 100 μM . These strains were collected from Ohio (SM838-12, SM924-13, and SM782-12), Indiana (TPINCu, HFMusk, and Musk1IN), Kentucky (KYMusk and BHKY), New York (LiMusk2, NYAcSq1, GZ4, LiSumSq1, NYZuch1, and NYZuch2), Oklahoma (MBrut1), Iowa (TedCu10), Maryland (MDCuke), Michigan (MISpSq), and Pennsylvania (PPHow1 and PPHow2). Each strain was grown individually, and suspensions were prepared as described above. Three to four *E. tracheiphila* strains were pooled by the genus from which they were isolated and mixed in equal volumes at a final OD_{600} of 0.05. Ninety-nine microliters of the pooled cultures were transferred to each well of a 96-well plate. To each well, 1 μl of selected SMs were added for a final concentration of 100 μM . Controls were used as described above. Growth inhibition was assessed as described above. Twenty SMs were selected for downstream analysis.

Chemical structural similarity analysis of 20 selected SMs. A free chemical structure-clustering tool offered from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) was used to assess the two-dimensional structure similarity of the 20 selected SMs inhibitory to all *E. tracheiphila* strains tested. A Tanimoto distance of 0.3 and a complete hierarchical clustering method were used to identify SMs with similar chemical structure (Bartlett's test; $P < 0.01$; JMP Pro 14) (Yu et al. 2017). The PubChem chemical identifications used for structure analysis are listed in Supplementary Table S2.

SM dose-response assays. The dose-response effect of 20 lead SMs against TedCu10 (*Cucumis* derived) and BHKY (*Cucurbita* derived) was determined. Individual bacterial suspensions were prepared as described above. Compounds were twofold diluted from 100 μM to 50, 25, and 12.5 μM final concentrations. For SM13, an additional concentration of 6.25 μM was tested. One μl of diluted SM was added to each well. The same controls as mentioned above were used. Plates were incubated in a Sunrise Tecan microplate reader for kinetic growth measurements every 30 min for 24 h at 28°C . Inhibition was assessed as described above. The MIC and minimum bactericidal concentration (MBC) were determined based on percentage growth inhibition and survival postincubation on nonamended NBY agar plates. The experiment was conducted twice.

Spectrum of SM activity against beneficial bacteria and other cucurbit bacterial pathogens. SM activity against 12 beneficial bacteria, 5 other bacterial pathogens of cucurbits, and *E. amylovora* (Supplementary Table S1) was assessed as described in the primary screening using the 20 lead SMs at a final concentration of 100 μM . The same controls as mentioned above were used. Plates were incubated in a Sunrise Tecan microplate reader, and growth inhibition was assessed as described above.

Toxicity of SMs to melon plants. To determine if selected 20 SMs were phytotoxic to cucurbits, 1 μl of 10 mM compound was diluted into 99 μl of water to a final concentration of 100 μM . Three droplets of 10 μl each were randomly placed on the first true leaf of 13-day-old melon cultivar Athena F1 plants. Control seedlings were treated with water, 1% DMSO, 1.2% thymol, 2% 2,4-dichlorophenoxyacetic acid (2,4-D), or 0.75% glyphosate. Plants were incubated in the dark at room temperature. Toxicity, abnormal growth, and plant death were monitored daily for 5 consecutive days. Each treatment was replicated three times, and the experiment was conducted twice.

SM toxicity to honey bees. The protocol for testing for toxicity to honey bees was adapted from Organisation for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals (OECD 1998a) and performed as follows. Three-day-old honey bees were allocated to cages with 20 bees per cage. A 50% sucrose solution (in water) was provided as needed during the experiment; feeders were refilled every 24 h. Each cage was a Styrofoam ice cream cup covered with cheesecloth. Honey bees

were anesthetized with carbon dioxide for 15 to 30 s prior to application of treatments. The 20 lead compounds were tested at 100 μ M (1 μ l of 10 mM stock in 99 μ l of acetone). Controls were 1% DMSO in acetone, acetone alone, and the insecticide thiamethoxam at 0.35 μ g of active ingredient per bee. Treatments were applied to bees topically. One microliter of each SM or control was applied to the dorsal side of the thorax of each bee. After treatment, bees were reallocated to cages. Cages were incubated in the dark. Bee mortality was monitored daily for 7 days. When necessary, Abbott's correction for natural mortality was used. The experiment was conducted twice.

Cytotoxicity of selected SMs to human cells. Caco-2 (human colonic carcinoma; ATCC HTB-37) cells were cultured in minimal essential medium (MEM; Gibco, Gaithersburg, MD) supplemented with 1% nonessential amino acid glycine, L-alanine, L-asparagine, L-L-aspartic acid, L-glutamic acid, L-proline, and L-serine (Invitrogen Life Technologies, Carlsbad, CA); 1 mM sodium pyruvate (Gibco, Gaithersburg, MD); and 20% fetal bovine serum (FBS; Gibco, Gaithersburg, MD) (Helmy et al. 2018). Cells were incubated at 37°C in 5% CO₂. To each well of a 96-well plate, 100 μ l of Caco-2 cell suspension was transferred to a final concentration of approximately 1.4×10^5 cells per well. Plates were incubated at 37°C in a humidified 5% CO₂ incubator. Forty-eight hours after incubation, when a complete monolayer formed, cells were carefully washed twice with media containing no antibiotic or FBS. Wells were refilled with 150 μ l of MEM without FBS or antibiotic. SMs were added to each well for a final concentration of 100 μ M. After 24 h of incubation at 37°C in a humidified 5% CO₂ incubator, 50 μ l of the supernatant was collected and added to a new 96-well plate, where the lactate dehydrogenase (LDH) release was measured and the cytotoxicity level was determined using the Pierce LDH Cytotoxicity Assay Kit (ThermoFisher Scientific, Waltham, MA). For this experiment, phosphate buffer saline (PBS), 1% DMSO, 1 $\times$ LDH lysis buffer, and 10 $\times$ LDH lysis buffer were used as controls. Each compound and controls were replicated three times, and the experiment was conducted twice.

Effectiveness of foliar-applied SMs as protectants against *E. tracheiphila* in plants. Melon Athena F1 plants were grown in 50-cell trays containing Baccto professional growers' potting mix (Baccto, Houston, TX). Plants were maintained in a greenhouse at 28°C under a 14-h photoperiod. Ten-day-old melon plants were sprayed with 1.4 ml per plant (equivalent to approximately 655 liters per hectare) of each SM (SM1 to S20) at 2 $\times$ MBC using an iGo 2-oz. Fingertip Sprayer (The Bottle Crew, Farmington Hills, MI). The solution containing each SM was prepared in water at a final concentration of 0.2% DMSO. Controls for this experiment were 0.2% DMSO, kanamycin (30 μ g/ μ l), and noninoculated plants. Compounds or controls were sprayed on the cotyledons only, and the nozzle was held approximately 5 cm from cotyledons during application. Plants remained in the dark for 12 h before inoculation. Cotyledons were inoculated with bioluminescent *E. tracheiphila* TedCu10-BL#9 (Vrisman et al. 2016) as follows: one 10- μ l droplet of approximately 10^7 CFU/ml was placed on each cotyledon, after which the cotyledon was wounded with a sterile razor blade through each droplet. Plants were incubated in the greenhouse at 28°C with a 14-h photoperiod. The number of wilted leaves and the total number of leaves per plant were recorded daily for 14 days postinoculation (DPI), with one final rating at 21 DPI; these data were used to calculate percentage wilt per plant. Plants were photographed for detection of bioluminescent bacteria using the In Vivo Imaging System (IVIS) Lumina Series III (PerkinElmer, Shelton, CT) with a 570 filter at 7 DPI.

The protective effect of the five selected SMs was also tested in a growth chamber with 28°C controlled temperature and a 14-h photoperiod. Ten-day-old melon Athena F1 plants were sprayed with SMs or controls, inoculated, and rated, and data were analyzed as described above. Plants remained for 7 days after inoculation in the growth chamber and then, were transferred to a greenhouse. In the

repeat of the growth chamber experiment, the surfactant Silwet L-77 was added to each spray bottle at 0.01% vol/vol. Plants were sprayed, inoculated, and rated, and data were analyzed as described above.

The experiments were established using a randomized complete block design with five replications treated as random effect. Each treatment within a replication was composed of individual plants. The experiments were performed twice. Data for percentage wilting per plant were arcsine transformed $\left[\left(\arcsine \left(\sqrt{\text{percentage wilting} / 100} \right) \right) \right]$ prior to analysis. Areas under the wilting progress curve (AUWPCs) were calculated based on the following formula: $\sum [(x_i + x_{i+1}) / 2] (t_{i+1} - t_i)$, where x_i is the percentage of wilted leaves per plant at each evaluation time and $(t_{i+1} - t_i)$ is the number of days between evaluations. Data for each experiment were analyzed individually using PROC GLIMMIX in SAS (version 9.4; SAS Institute, Cary, NC), and transformed means were compared with the DMSO control using Dunnett's multiple comparison test.

Effectiveness of SMs to control *E. tracheiphila* when delivered to plants via the roots. A bioassay was developed to assess the efficacy of SMs when delivered via the roots. Melon Athena F1 plants were grown as described above. Thirteen-day-old plants were removed from the potting mix, and roots were washed with deionized water and trimmed to approximately 1 cm³ to increase SM absorption into the vascular system. Plants were then transferred to 5.0-ml Five-O screw cap macrotubes (MTC Bio, Metuchen, NJ) containing 1.4 ml of sterile water in which roots were immersed. Twenty-four hours later, the remaining water was replaced with 1.4 ml of each of the SMs (SM1 to SM20) at 2 $\times$ MBC prepared as described above. Controls for this experiment were 0.2% DMSO, kanamycin (30 μ g/ μ l), and noninoculated plants. After 12 h, plants were inoculated with bioluminescent *E. tracheiphila* TedCu10-BL#9 as follows: a 100- μ l aliquot of inoculum adjusted to approximately 10^6 CFU/ml was pipetted in five approximately equal volumes on the surface of the first true leaf of each melon plant ($n = 5$). Leaves were slightly perforated through each drop of inoculum with a pipette tip. Plants were incubated at room temperature with an approximately 12-h photoperiod and watered with deionized water as needed. Plants were rated at 5, 7, 10, and 14 DPI, and data were analyzed as described above. Plants were photographed for detection of bioluminescent bacteria using IVIS with a 570 filter at 7 DPI. The experiment was conducted twice.

Contact time assessment for SM cidal activity. To determine the contact time required for cidal activity against *E. tracheiphila* cells, a suspension of bioluminescent *E. tracheiphila* TedCu10-BL#9 (Vrisman et al. 2016) was prepared as described in the primary screening. A 99- μ l aliquot of the suspension was added to individual wells of a 96-well black with clear flat bottom microtiter plate (Corning, Corning, NY). One microliter of SM2, SM15, SM16, SM17, or SM20 was added to a final concentration of 2 $\times$ MBC in three replicate wells. Controls were bacteria plus 1% DMSO, bacteria plus kanamycin (30 μ g/ μ l), and half-strength NBY alone. The plate was photographed using an IVIS Lumina Series III (PerkinElmer, Shelton, CT) with open filter immediately before and after adding molecules, 30 min later, and then, hourly for 10 h. The plate was wrapped with aluminum foil and incubated on a shaker at 100 rpm and 28°C. Starting 3 h after incubation and continuing hourly until the end of the experiment, an aliquot of the culture in each well was spotted onto fresh full-strength nonamended NBY agar medium to monitor cidal activity. The experiment was performed once.

Effects of temperature and light on the activity of the lead SMs. Three microliters of SM2, SM15, SM16, SM17, or SM20 (2 $\times$ MBC) was added to wells of 0.2-ml low-profile eight-tube PCR strips (Bio-Rad, Hercules, CA). Controls were 3 μ l of 100% DMSO and 3 μ l of kanamycin (30 μ g/ μ l). Individual strips containing SMs and controls were incubated for 12 h at 28, 35, 40, or 45°C in a thermocycler (Bio-Rad, Hercules, CA). In another experiment, SMs and controls in individual strips were exposed to light in a growth chamber for 1, 2, 4, 8, or 12 h at 28°C. After completion of each period of light exposure, strips were transferred

to a box inside the growth chamber at 28°C until completion of the 12-h exposure treatment. A suspension of *E. tracheiphila* TedCu10 was prepared as described in the primary screening. A 99- μ l aliquot of this suspension was added to individual wells of a 96-well plate. A separate plate was used for each experiment. One microliter of each SM exposed to the different conditions was added to duplicate wells. SMs maintained at -80°C were used as nonexposed controls. Plates were incubated in a Sunrise Tecan microplate reader for kinetic growth measured every 30 min for 24 h at 28°C. At the end of each experiment, an aliquot of the culture in each well was spotted onto fresh full-strength nonamended NBY agar medium to monitor activity as described for the primary screening. The experiment was performed once for each condition.

Potential of *E. tracheiphila* to acquire resistance to selected SMs. Resistance assays using sequential passages at sublethal doses and a single step at lethal doses were conducted as previously described (Deblais et al. 2018; Kumar et al. 2016; Xu et al. 2015). The five SMs (SM2, SM15, SM16, SM17, and SM20) were tested in these experiments using sublethal (0.75 $\times$ MBC) and lethal (5 $\times$ MBC) concentrations.

Evaluation of resistance to selected SMs using sequential passages at sublethal doses. A suspension of *E. tracheiphila* TedCu10 was incubated in 100% NBY broth for 12 h. Cells were centrifuged at 3,000 $\times$ g for 7 min, resuspended in fresh 50% NBY, and adjusted to an OD₆₀₀ of 0.01. In a 96-well plate, 99 μ l of the suspension was mixed with 1 μ l of SM to a final concentration demonstrating approximately 70% growth inhibition (Deblais et al. 2018; Kathayat et al. 2018; Kumar et al. 2016; Xu et al. 2015). Controls were bacterial suspension plus 1% DMSO or kanamycin (0.005 μ g/ml) and medium alone. SMs and controls were replicated three times. The plate was incubated in a Sunrise Tecan microplate reader in the dark for 14 h at 28°C, after which cells were harvested as described above. Supernatant was discarded, and bacteria were resuspended in 100 μ l of fresh half-strength NBY medium at the appropriate concentration for each SM. Following 15 passages, the remaining bacteria in each replicated well were assessed for resistance to each of the SMs by challenging them in half-strength NBY amended with the 0.5 $\times$, 1 $\times$, 2 $\times$, or 5 $\times$ MBC of SM. Controls were DMSO alone or kanamycin (30 μ g/ml). The experiment was conducted twice.

Evaluation of resistance to selected SMs using a single step lethal dose. A suspension of *E. tracheiphila* TedCu10 was incubated in full-strength NBY broth for 12 h. Cells were centrifuged at 3,000 $\times$ g for 7 min and resuspended in fresh half-strength NBY broth to approximately 10⁹ CFU/ml. In each well of a 24-well plate, 1 ml of molten half-strength NBY agar was mixed with individual SMs at a final concentration of 5 $\times$ MBC. The medium was allowed to solidify overnight in the dark. A 50- μ l aliquot of the 12-h bacterial suspension was spread in each well, and plates were allowed to dry under a hood. Plates were incubated in the dark at 28°C for 15 days. Bacteria spread on half-strength NBY agar containing 30 μ g/ μ l of kanamycin or half-strength NBY agar alone were used as controls. Compounds and controls were replicated twice. Colonies that appeared were challenged in duplicate in half-strength NBY amended with 0.5 $\times$, 1 $\times$, 2 $\times$, or 5 $\times$ MBC of SM as described above. The experiment was conducted twice.

Three-dimensional image of *E. tracheiphila*-treated cells. *E. tracheiphila* TedCu10 cells were grown overnight in full-strength NBY to exponential phase. Cells were harvested as described above, resuspended in half-strength NBY, and adjusted to an OD₆₀₀ of 0.8. A 600- μ l aliquot of bacterial suspension was mixed with each of the five lead SMs separately to a final concentration of 5 $\times$ MBC in 1.5-ml Eppendorf tubes. The control was the bacterial suspension mixed with DMSO at a final concentration of 1%. Tubes were wrapped in aluminum foil and incubated at 28°C with 200 rpm shaking for 5 h. After incubation, one volume of each specimen was mixed with one volume of fixative (3% glutaraldehyde and 1% paraformaldehyde in 0.1 M potassium phosphate, pH 7.2) and

incubated at 4°C overnight. The fixed bacterial cells were centrifuged at 1,200 $\times$ g for 5 min, washed twice with PBS, and suspended in 1% osmium tetroxide for 1 h at room temperature in the dark. Samples were serially dehydrated in ethanol and platinum sputter coated. Samples were visualized using a Hitachi S-3500N scanning electron microscope (SEM; Hitachi, Chiyoda, Tokyo, Japan) at the Molecular and Cellular Imaging Center at The Ohio State University (Wooster, OH).

RESULTS

Twenty SMs completely inhibited *E. tracheiphila* growth at 100 μ M. From the initial high-throughput screening of 4,182 SMs against *E. tracheiphila* TedCu10, 231 SMs exhibited cidal activity at 100 μ M in limited nutrient conditions. Another 233 SMs were identified with static activity against this strain. In silico comparison of screening assays performed using this same library of SMs against other plant, animal, and foodborne pathogens (Deblais et al. 2018; Helmy et al. 2018; Kathayat et al. 2018; Kumar et al. 2016; Xu et al. 2015) identified 16 SMs that had cidal activity specific to *E. tracheiphila* at 100 μ M. Eighty-three SMs showed cidal activity against *E. tracheiphila* and at least one other phytopathogen tested against this library (unpublished data). Further confirmation using these 83 SMs at a final concentration of 100 μ M identified only 57 SMs that were cidal, and the remainder showed static activity against *E. tracheiphila* TedCu10.

The 57 confirmed SMs were screened against multiple strains of *E. tracheiphila* isolated from plants in the *Cucumis* and *Cucurbita* genera at a concentration of 100 μ M in limited nutrient conditions, which yielded the 20 lead SMs (Supplementary Table S2). The remaining 37 SMs inhibited the growth of all of the strains in liquid medium, but at least one of the strains was recovered after plating on nonamended medium, and thus, these were classified as static and were not used for downstream analysis.

Structure analysis of the selected 20 SMs. Based on the two-dimensional structure similarity with a Tanimoto distance of 0.3, the 20 SMs were segregated into five clusters (A to E) or four singletons (SM2, SM3, SM13, and SM16) with characteristic chemical structures (Fig. 1). Overall, all of the SMs possessed amine-based functional groups. The majority ($n = 12$) were in the piperidine group: cluster A (SM14, SM15, and SM18) characterized by a benzyl-butyl-piperidinecarboxamide moiety; cluster B (SM4, SM5, SM9, SM10, and SM11) characterized by a 1,3,5-trimethyl-1H-pyrazol-4-ylamine moiety, cluster C (SM1, SM6, SM7, and SM20) characterized by a biphenyl-piperidinecarboxamide moiety; cluster D (SM8 and SM17) characterized by phenyl-isoxazolyl-piperidinol moiety; and cluster E (SM12 and SM19) characterized by benzyl-methylpiperidinol-benzyl moiety. Detailed chemical structures of the 20 SMs are displayed in Figure 2.

Based on the dose-response study, 4 of the 20 lead SMs were cidal to both *E. tracheiphila* strains TedCu10 and BHKY at a concentration of 100 μ M, 9 were cidal at a concentration of 50 μ M, 6 were cidal at a concentration of 25 μ M, and 1 was cidal at a concentration of 6.25 μ M (Table 1).

Antimicrobial specificity of the 20 SMs against other plant pathogenic and beneficial bacteria at 100 μ M. No cidal activity was observed with the 20 SMs tested at 100 μ M in limited nutrient conditions against beneficial bacteria in the genera *Pseudomonas* and *Enterobacter*, except SM13 with *Pseudomonas protegens* (Fig. 3). All of the lead SMs were either cidal or static to bacteria in the genus *Bacillus* at a concentration of 100 μ M (Fig. 3). Interestingly, all of the SMs were cidal to one strain of *Bacillus subtilis* (BS1) but static to the other (GB03) at 100 μ M in limited nutrient conditions. A static effect was also observed against *Bacillus amyloliquefaciens* with all lead SMs. Only two SMs (SM15 and SM16) were cidal to *Mitsuaria* sp. at 100 μ M. Eight SMs (SM2, SM4, SM7, SM9, SM13, SM14, SM15, and SM17) were cidal to

Lysobacter enzymogenes at 100 μ M. Five SMs (SM1, SM3, SM5, SM9, and SM13) were cidal to *Streptomyces* sp. at 100 μ M (Fig. 3).

Compound SM13 was cidal to the cucurbit bacterial pathogens *Acidovorax citrulli*, *Agrobacterium rhizogenes*, *P. syringae* pv. *lachrymans*, *Serratia marcescens*, and *Xanthomonas cucurbitae* when tested at a final concentration of 100 μ M in vitro (Fig. 4). All SMs were cidal to *X. cucurbitae* at 100 μ M, except SM3, which was static to the test strain of *X. cucurbitae* at this concentration (Fig. 4). SM2 was cidal to *X. cucurbitae* and static at 100 μ M to all of the other cucurbit bacterial pathogens (Fig. 4). Nineteen molecules inhibited >50% of the growth of *P. syringae* pv. *lachrymans*. None of the SMs tested at 100 μ M showed cidal activity to both *P. syringae* pv. *lachrymans* and *Serratia marcescens*, except SM13 (Fig. 4). Seven SMs (SM4, SM7, SM9, SM13, SM14, SM15, and SM17) were cidal to *Agrobacterium rhizogenes* (Fig. 4). Both SM13 and SM17 were cidal to *Acidovorax citrulli* (Fig. 4). Eleven of the SMs were cidal to *E. amylovora*, the causal agent of fire blight of apple and pear; eight were static, and one inhibited 26% of the growth (Fig. 4).

Minimal toxicity to melon tissues, honey bees, and human colon cells at 100 μ M observed for selected 20 SMs. No phytotoxicity was observed on melon Athena F1 plants when SMs were applied to leaves at 100 μ M in water (Fig. 5). However, control plants treated with 1.2% thymol exhibited localized necrotic lesions, whereas control plants treated with 0.75% glyphosate showed systemic necrosis. Control plants treated with 2% 2,4-D had abnormal growth and stunting. Systemic necrosis was observed in melon plants sprayed with SM12 plus the surfactant Silwet L-77 or when roots were exposed to a solution containing SM12.

Low toxicity to honey bees (<12% mortality) was observed with SMs tested via topical application at a concentration of 100 μ M. SM2, SM5, SM8, SM12, and SM13 caused mortality >10%; however, the average mortality rate was <12% with these SMs (Table 1). Thiamethoxam at 0.35 μ g of active ingredient per bee caused 100% mortality in all of the tests. Cytotoxicity to human Caco-2 cells was <30% for all 20 lead SMs when tested at 100 μ M; the average cytotoxicity of 15 of the SMs was <20% (Table 1).

Five SMs reduced wilt caused by *E. tracheiphila* in melon plants. No wilting was observed at 21 DPI in plants treated with SM16 in the first experiment. Percentage wilting of plants treated with SM2, SM15, or SM16 was significantly lower than that of the DMSO control ($P < 0.05$) at 7 DPI (Supplementary Fig. S1). Percentage wilting at 10 and 14 DPI (Supplementary Fig. S1) and AUWPC (Supplementary Fig. S2) of plants treated with SM2, SM15, SM16, SM17, or SM20 were significantly lower than those of the DMSO control ($P < 0.05$). The percentage wilting of plants treated with SM16 or SM17 was significantly lower than that of the DMSO control ($P < 0.05$) at 21 DPI. Only one of five plants treated with SM2, SM15, SM17, or SM20 wilted. Wilting was first observed at 6 DPI in the control plants treated with DMSO and the only plant treated with SM20 that wilted. The symptomatic plant treated with SM17 showed initial wilting symptoms at 7 DPI. Among plants treated with SM2 or SM15, the one symptomatic plant in each treatment showed initial wilting symptoms at 9 and 10 DPI, respectively. SM10 was no longer available for purchase, and thus, it was not tested in planta using either foliar-applied application or root delivery. The same level of suppression was not observed in the second experiment conducted in greenhouse or in the first experiment conducted in a growth chamber (data not shown). No symptoms of bacterial wilt were observed in the repeat of the growth chamber experiment in which plants were sprayed with SMs plus Silwet. Therefore, a different delivery method was investigated.

The SMs SM1, SM2, SM3, SM5, SM7, SM8, SM11, SM12, SM13, SM17, SM18, SM19, and SM20 demonstrated potential disease preventive activity when delivered via the roots of melon plants in a single application in the first experiment. Percentage wilting (Supplementary Fig. S3) of plants treated with these SMs was significantly lower than that of DMSO-treated control plants

(80% wilt; $P < 0.05$) at 7 DPI. Additionally, the percentage wilting of plants treated with SM5 and SM8 was significantly lower than that of DMSO-treated control plants ($P < 0.05$) at 10 DPI. No differences in percentage wilting were observed between plants treated with any of the SMs delivered to the roots and DMSO-treated control plants at 14 DPI. AUWPC values (Supplementary Fig. S4) for plants treated with SM8 and SM20 were significantly lower than those of DMSO-treated control plants ($P < 0.05$). AUWPC of plants treated with SM5 was not significantly different from that of DMSO-treated control plants ($P = 0.0592$). No bioluminescence signal was observed in the leaf petiole or main stem of any of the plants treated via the roots with SM8 or in four of five plants treated with SM12 or kanamycin (Supplementary Fig. S5). Percentage wilting of the control (DMSO) plants in the second experiment was low (20% wilt).

Short exposure of *E. tracheiphila* in vitro to the five most potent SMs was sufficient for cidal activity. The five lead SMs required different lengths of exposure for complete cidal activity in vitro. Reductions in bioluminescence signal were observed immediately after cells were exposed to $2\times$ MBC of each SM (Fig. 6). SM2 was cidal to bioluminescent *E. tracheiphila* TedCu10-BL#9 in approximately 5 h across all replicates. Cidal activity was observed after 5 to 6 h of exposure to SM15, 5 to 8 h of exposure to SM16, 3 to 6 h of exposure to SM17, and 5 to 10 h of exposure to SM20.

SM2, SM7, SM15, SM16, and SM20 shared a maximum of 70% structural similarity based on the analysis of their two-dimensional fingerprints using the Tanimoto coefficient (Table 2). SM2 was classified as a pyrimidine, and SM15, SM16, SM17, and SM20 were classified as piperidines. However, in SM15 and SM16, a pyrazole group was present, and in SM17, an isoxazole group was present (Fig. 2).

The five selected SMs affected *E. tracheiphila* cell envelope integrity. Differences in *E. tracheiphila* TedCu10 cell

Fig. 1. Two-dimensional structural similarity analysis of 20 small molecules inhibiting *Erwinia tracheiphila* growth at 100 μ M. The constellation plot was generated using JMP Pro 14 (complete hierarchical clustering method) after extracting the Tanimoto distance score from the free online PubChem clustering tool (<https://pubchem.ncbi.nlm.nih.gov/>). Assignment of a Tanimoto distance of 0.3 resulted in eight different clusters (colored nodes except black). **A to E,** The five most representative moiety chemical structures were generated using Hit2Lead (<https://www.hit2lead.com/search.asp?db=SC>). Small molecules are listed by their small molecule number (Fig. 2 and Supplementary Table S2). R, radical fluctuating between small molecules of a same cluster.

morphology were observed after cells were exposed to each of the five lead SMs at 5× MBC for 5 h compared with the 1% DMSO control (Fig. 7) when visualized using SEM. Cidal activity of these five lead SMs was not affected after exposure to different temperatures (Supplementary Fig. S6) or light regimes (Supplementary Fig. S7).

No resistance in *E. tracheiphila* was detected to the five SMs. Identical MBCs were observed between *E. tracheiphila* TedCu10 before and after exposure for 15 passages to sublethal doses of SMs in half-strength NBY broth. Additionally, *E. tracheiphila* TedCu10 growth was detected in wells containing solid half-strength NBY amended with 5× MBC of SM15 or SM20

Fig. 2. Chemical structures of the top 20 lead small molecules (SMs) inhibitory to *Erwinia tracheiphila* identified in this study.

TABLE 1. Summary of minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of 20 small molecules (SMs) to approximately 10⁷ CFU/ml of *Cucumis*-derived (TedCu10) and *Cucurbita*-derived (BHKY) *Erwinia tracheiphila* strains, and percent mortality (± standard deviation [SD]) of honey bees 7 days after topical application of 1 µl of SMs at 100 µM and percent mammalian cytotoxicity (± SD) using Caco-2 cells exposed to SMs at 100 µM for 24 h^a

SM	<i>Cucumis</i> strain		<i>Cucurbita</i> strain		Toxicity (%)	
	MBC (µM)	MIC (µM)	MBC (µM)	MIC (µM)	Honey bees ± SD	Caco-2 ± SD
SM14	25	25	25	25	5.0 ± 7.1	10.9 ± 6.2
SM15	50	50	50	50	2.5 ± 3.5	14.9 ± 2.7
SM18	50	25	50	25	7.8 ± 3.1	18.6 ± 1.3
SM4	50	50	50	50	9.5 ± 13.5	19.3 ± 2.3
SM5	100	50	100	50	10.4 ± 7.6	16.9 ± 4.1
SM9	25	25	25	25	0.0 ± 0.0	17.6 ± 1.6
SM10	50	50	100	50	8.3 ± 11.8	17.7 ± 4.5
SM11	50	25	50	25	2.8 ± 3.9	17.2 ± 6.0
SM1	50	50	50	25	7.3 ± 3.9	18.6 ± 1.5
SM6	50	25	25	25	5.6 ± 7.9	19.6 ± 1.8
SM7	25	25	25	25	9.8 ± 0.3	14.9 ± 8.7
SM20	25	25	25	25	5.3 ± 7.4	22.9 ± 9.3
SM8	100	50	100	50	10.7 ± 0.6	13.7 ± 4.9
SM17	25	12.5	25	12.5	9.9 ± 0.5	15.6 ± 6.6
SM12	100	100	100	100	10.8 ± 1.8	21.6 ± 13.9
SM19	50	50	50	50	7.5 ± 2.8	27.2 ± 7.9
SM2	50	50	50	50	11.9 ± 16.8	23.3 ± 14.3
SM3	25	12.5	50	12.5	2.6 ± 3.7	-0.7 ± 1.2
SM13	6.25	6.25	6.25	6.25	10.5 ± 14.9	12.6 ± 3.5
SM16	25	25	25	25	7.6 ± 5.0	20.4 ± 4.4

^a SMs are organized according to the clusters shown in Figure 1.

but not with 5× MBC of SM2, SM16, or SM17 after incubation for 5 days. However, identical MBCs were observed between *E. tracheiphila* TedCu10 and cultures originating from bacterial cells growing in wells containing half-strength NBY agar with 5× MBC of SM15 or SM20. No *E. tracheiphila* colonies were observed in wells containing solid half-strength medium amended with 5× MBC of SM2, SM16, or SM17 after incubation for 15 days.

DISCUSSION

Currently, there are no effective means of directly suppressing *E. tracheiphila* in cucurbits, and losses because of this pathogen can reach 80% (Rojas et al. 2015). Therefore, the identification of novel antimicrobials effective against *E. tracheiphila* is needed. This study is the first to screen and characterize novel SMs against *E. tracheiphila*. Nineteen drug-like molecules with potential to provide chemical scaffolds for the development of future bactericides against bacterial wilt and other bacterial diseases of cucurbits were identified. The initial screening in this study showed a hit rate of approximately 5.5% for cidal SMs and 5.5% for static SMs when *E. tracheiphila* was challenged with 100 µM SM in limited nutrient conditions. Eighty-three of the 231 SMs were cidal to multiple phytopathogens, and they were selected for downstream characterization. Selecting SMs with activity on multiple phytopathogens is attractive because it allows for development of novel bactericides for use in multiple crops. Fifty-seven of the 83 SMs were cidal to *E. tracheiphila* TedCu10 at 100 µM after confirming the results of our primary screening, and the remaining 26 SMs were static to this strain. The static activity observed could be explained by the fact that the MBC for these 26 SMs is very close to 100 µM. Of the 57 SMs with confirmed cidal activity against *E. tracheiphila* TedCu10, 20 exhibited broad spectrum activity against multiple *E. tracheiphila* strains. The remaining 37 SMs were static against multiple *E. tracheiphila* strains. This could also be explained by an MBC very close to 100 µM, or it could be because of genetic differences among the strains. These strains were collected from different states

and from different hosts within the Cucurbitaceae family (Rojas et al. 2013), and they are grouped in three distinct genetic clusters (Shapiro et al. 2018). Difference in sensitivity of multiple strains to SMs was

Fig. 4. Heat map of percentage growth inhibition of five species of cucurbit bacterial pathogens and *Erwinia amylovora* observed 24 h after incubation on half-strength nutrient broth yeast extract broth with each small molecule at a final concentration of 100 µM. Molecules causing >99.9% growth inhibition were classified as either static when the test strain was recovered on non-amended solid medium or cidal when the strain was not recovered. Color represents growth inhibition according to scale. Descriptions of strains used are as follows: *A.c.*, *Acidovorax citrulli* strain XU22-15; *A.r.*, *Agrobacterium rhizogenes* strain K599; *E.a.*, *Erwinia amylovora* strain MLI90-15; *P.s.l.*, *Pseudomonas syringae* pv. *lachrymans* strain SM317-11; *S.m.*, *Serratia marcescens* strain SM1794; *X.c.*, *Xanthomonas cucurbitae* strain SM622-11.

Fig. 3. Heat map of percentage growth inhibition of 12 strains of beneficial bacteria observed 24 h after incubation on half-strength nutrient broth yeast extract (NBY) broth with each small molecule (SM) at a final concentration of 100 µM. Molecules causing >99.9% growth inhibition were classified as either static when the test strain was recovered on nonamended NBY agar medium or cidal when the strain was not recovered. Color represents growth inhibition according to the scale. Descriptions of strains used are as follows: *B.a.1*, *Bacillus amyloliquefaciens* strain BA1; *B.s.1*, *Bacillus subtilis* strain BS1; *B.s.2*, *B. subtilis* strain GB03; *E.s.*, *Enterobacter* sp. strain SM1796; *L.e.*, *Lysobacter enzymogenes* strain C3; *M.s.*, *Mitsuaria* sp. strain H244L5A; *P.c.1*, *Pseudomonas chlororaphis* strain 14B11; *P.c.2*, *P. chlororaphis* strain 48B8; *P.c.3*, *P. chlororaphis* strain 48G9; *P.f.*, *Pseudomonas fluorescens* strain SM98-08; *P.p.*, *Pseudomonas protegens* strain 12H11; *S.s.2*, *Streptomyces* sp. strain S2.

also considered to be likely as it has been shown with *Clavibacter michiganensis* subsp. *michiganensis* (Xu et al. 2015).

None of the SMs in this study were cidal to any of the beneficial *Pseudomonas* strains tested, except SM13 to *P. protegens*. Similar results were identified for SMs tested against *Pseudomonas fluorescens* by Xu et al. (2015). The 20 lead SMs identified in this study do not seem to affect the viability of bacterial species commonly used or with potential use in biocontrol, hence these SMs could be considered safe for use in combination with biocontrol agents. Roberts (2015) identified zones of inhibition in *E. tracheiphila* in vitro studies with a commercial strain of *P. fluorescens*. In in vivo studies, this commercial strain showed promising inhibitory activity during establishment and maintenance of *E. tracheiphila* populations in the gut of striped cucumber beetles. Our 20 lead SMs were not cidal to *P. fluorescens*. However, SM1, SM2, SM9, SM13, SM14, and SM20 were static to *P. fluorescens* after incubation at a concentration of 100 μ M. SMs could also be considered in mixtures with potential biocontrol

agents to increase efficacy in suppressing *E. tracheiphila* populations in cucurbit plants and vectors.

The lead SMs were also effective against other cucurbit bacterial pathogens at 100 μ M. All of the SMs identified in this study were cidal to *X. cucurbitae*, except SM3, which was static. In addition, 19 SMs caused >50% growth inhibition of *P. syringae* pv. *lachrymans*; 5 of these 19 molecules were static, and 1 was cidal to this bacterium. This result indicates that products developed from these SMs could aid in the management of other important bacterial diseases of cucurbits that occur through the cropping season. However, in planta studies are needed to confirm if these SMs can suppress other bacterial pathogens in cucurbit crops.

All of the lead SMs, except SM12, were either static (8 molecules) or cidal (11 molecules) to *E. amylovora*, indicating that these SMs are promising to be used across different cropping systems and enhancing the potential utility of the SMs identified in this study. Resistance to currently used antibiotics is common in *E. amylovora* populations (McGhee and Sundin 2011). The primary site of infection for *E. amylovora* is the flower, which coincides with pollination, making it more difficult to control insect vectors without harming pollinators. The SMs identified in this study could be studied as a novel approach to manage fire blight through flower application.

Lead SMs were considered nontoxic at 100 μ M when compared with the insecticide thiamethoxam. A bee mortality <10% is required for product registration (EPA 2012). Based on our assessment, these molecules do not represent a high risk for pollinators, and future commercial products originating from these molecules could be considered safe for use throughout the entire season to manage bacterial wilt of cucurbits. Colonization by *E. tracheiphila* also occurs through flowers (Sasu et al. 2010), and flowers of healthy plants are more attractive to the vectors of this pathogen than flowers of wilting plants (Shapiro et al. 2012). Therefore, protecting flowers from bacterial infection is a potential strategy that needs to be tested for these molecules. In addition to the acute contact toxicity presented here, acute oral toxicity assays should also be performed on these SMs (OECD 1998b). The effects of these SMs on cucumber beetles

Fig. 5. Phytotoxicity of selected small molecules (SMs) at 100 μ M in water on 13-day-old melon cultivar Athena F1 plants 96 h after application of three 10- μ l droplets on the first true leaf. Control seedlings were treated with water, 1% dimethyl sulfoxide (DMSO), 1.2% thymol, 2% 2,4-Dichlorophenoxyacetic acid (2,4-D), or 0.75% glyphosate.

TABLE 2. Tanimoto coefficient for two-dimensional similarities between the top five lead small molecules (SMs) identified in this study

	SM2	SM15	SM16	SM17	SM20
SM2	1	0.646	0.498	0.426	0.458
SM15		1	0.677	0.502	0.574
SM16			1	0.63	0.703
SM17				1	0.62
SM20					1

Fig. 6. Bioluminescence imaging of *Erwinia tracheiphila* strain TedCu10-BL#9 immediately before (–1 m), immediately after (+1 m), 30 min after (+30 m), and every hour after exposure to 2 $\times$ minimum bactericidal concentration of small molecules (SMs) SM2, SM15, SM16, SM17, and SM20. A 99- μ l aliquot of a 10⁷-CFU/ml *E. tracheiphila* TedCu10-BL#9 suspension in half-strength nutrient broth yeast extract (NBY) was added to each individual well before challenge with SMs. Controls were dimethyl sulfoxide (DMSO) at a 1% final concentration and kanamycin at 30 μ g/ μ l (Kan³⁰). The plate was photographed using IVIS Lumina System III, and all images were corrected to the same light scale. The rainbow scale represents light intensity in photons per second (on the right).

remain unknown. It is also unknown if ingestion of cucurbit plants treated with these SMs could affect *E. tracheiphila* survival in its vectors and thus, contribute to bacterial wilt management.

Although a piperidine group was present in most SMs, we identified one compound classified as an imidazole (SM13). This SM showed promising results in our *in vitro* studies, and its MBC was the lowest identified in this study. This SM showed cidal activity to all of the other cucurbit bacterial pathogens and *E. amylovora* when tested at 100 μ M. Worthington et al. (2012) identified a molecule in the 2-aminoimidazole group with efficacy in reducing *in vitro* *X. euvesicatoria* multiplication and bacterial recovery from pepper leaves when used in combination with copper-containing products only. This molecule was thus considered a promising additive and synergistic to suppress resistance to existing products. This molecule has shown capacity to inhibit biofilms of a diverse number of bacterial species, including *P. aeruginosa* (Rogers and Melander 2008; Rogers et al. 2010). Copper-containing products are used in cucurbits to control some of the bacterial species tested in this study. Therefore, the additive or synergistic effect of this SM could be tested in combination with currently available products.

Five potential candidates for managing bacterial wilt on melon plants via preventive application were identified in initial *in planta* efficacy screening. These SMs significantly reduced percentage wilting and AUWPC in melon seedlings inoculated with *E. tracheiphila*. In our plant experiments, we used a 10- μ l droplet of inoculum that contained approximately 10^5 *E. tracheiphila* cells. This concentration was chosen because it was the highest number of bacteria detected in frass and in whole beetles (Shapiro et al. 2014). Nonetheless, this concentration may be higher than the concentration of bacterial cells that enter wounds. The inoculation efficiency

for this pathogen is unknown and could have possibly contributed to the variability observed.

The temperatures and light regimes tested did not affect the antimicrobial activity of these SMs; light activity was tested inside a growth chamber to achieve controlled temperature. The effects of the rising temperature inside the greenhouse as light exposure increased as well as the effects of ultraviolet light on the stability of these compounds were not tested but should be considered as a potential inactivation mechanism. Other parameters not investigated in this study are the retention, penetration, and mobility of these SMs on the sprayed tissue.

Delivery methods that bring bacterial cells in close contact with these molecules in plants need further investigation because morphological changes in bacterial cells were visualized after *in vitro* treatment with SMs for a short period of 5 h, strengthening the inhibitory potential of these molecules against *E. tracheiphila*. A pilot experiment with melon plant roots immersed in SM suspensions revealed that SM12 caused necrotic lesions in the stem in areas that were not in contact with the SM suspension, a potential indication that SM12 translocated in the plant. Bio-luminescent *E. tracheiphila* was restricted to the inoculated leaf in all of the plants treated with SM8 and four of five plants treated with SM12 and kanamycin via roots. Delivering these SMs through the soil for root absorption and mobility through the xylem could represent a potential approach for combating this xylem-inhabiting bacterium. Additional experiments to test the frequency and concentration with which these SMs should be supplied to the roots, simulating drip irrigation delivery, need to be conducted to optimize control strategies. Interactions of these molecules with the soil after delivery through drip irrigation remain unknown.

Fig. 7. Scanning electron microscopy of *Erwinia tracheiphila* TedCu10 after treatment with small molecules (SMs) SM2, SM15, SM16, SM17, or SM20 at 5 $\times$ minimum bactericidal concentration for 5 h in half-strength nutrient broth yeast extract medium. The phenotype displayed is typical of at least 70% of the cells observed. Specimens were prepared and visualized using a Hitachi S-3500N scanning electron microscope (Hitachi, Chiyoda, Tokyo, Japan) at the Molecular and Cellular Imaging Center at The Ohio State University (Wooster, OH). White arrows indicate vesicles on the surface of the cell envelope. Scale bar: 1 μ m. DMSO, dimethyl sulfoxide.

In conclusion, 19 molecules with potential to be developed into bactericides that could be used for controlling multiple bacterial diseases in cucurbits were identified after multiple *in vitro* tests. Furthermore, selected SMs showed promising results in plants. The Tanimoto coefficients for the five lead SMs indicated that they are not similar in structure. The Tanimoto two-dimensional similarity coefficient remains the best indicator of chemical fingerprint-based similarities (Willett 2006). The two-dimensional structure fingerprint showed that these compounds are <70% similar, except SM16 and SM20, which were classified in the same group with 70.3% similarity. Therefore, based on *in silico* analysis, testing different combinations of SMs might increase the potential to control *E. tracheiphila* in plants. Additional *in planta* studies are necessary to characterize the effect of these SMs to treat *E. tracheiphila* in cucurbit plants, improve delivery methods for these SMs, and assess the impact on marketable yield and fruit quality. *In vitro* data combined with SEM images suggest that these compounds are promising against bacterial wilt of cucurbits with the development of an optimal delivery method that brings them into the xylem, where bacteria are present. In addition, the information provided on these molecules offers an opportunity for a more in-depth biochemical analysis, modification and/or generation of derivatives and analogs, studies of SM combinations, and combination of SMs with other antimicrobial products currently available or with potential bio-control agents for management of pathosystems evaluated in this study.

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LITERATURE CITED

- Aiello, D., Williams, J. D., Majgier-Baranowska, H., Patel, I., Peet, N. P., Huang, J., Lory, S., Bowlin, T. L., and Moir, D. T. 2010. Discovery and characterization of inhibitors of *Pseudomonas aeruginosa* type III secretion. *Antimicrob. Agents Chemother.* 54:1988-1999.
- Brust, G. E. 1997. Interaction of *Erwinia tracheiphila* and muskmelon plants. *Environ. Entomol.* 26:849-854.
- Deblais, L., Helmy, Y. A., Kathayat, D., Huang, H. C., Miller, S. A., and Rajashekara, G. 2018. Novel imidazole and methoxybenzylamine growth inhibitors affecting *Salmonella* cell envelope integrity and its persistence in chickens. *Sci. Rep.* 8:13381.
- EPA. 2012. White paper in support of the proposed risk assessment process for bees. White Paper Docket ID: EPA-HQ-OPP-2012-0543. EPA, Washington, D.C., United States.
- Geske, G. D., Wezeman, R. J., Siegel, A. P., and Blackwell, H. E. 2005. Small molecule inhibitors of bacterial quorum sensing and biofilm formation. *J. Am. Chem. Soc.* 127:12762-12763.
- Ghose, A. K., Viswanadhan, V. N., and Wendoloski, J. J. 1998. Prediction of hydrophobic (lipophilic) properties of small organic molecules using fragmental methods: An analysis of alogp and CLOGP methods. *J. Phys. Chem. A* 102:3762-3772.
- Helmy, Y. A., Deblais, L., Kassem, I. I., Kathayat, D., and Rajashekara, G. 2018. Novel small molecule modulators of quorum sensing in avian pathogenic *Escherichia coli* (APEC). *Virulence* 9:1640-1657.
- Kathayat, D., Helmy, Y. A., Deblais, L., and Rajashekara, G. 2018. Novel small molecules affecting cell membrane as potential therapeutics for avian pathogenic *Escherichia coli*. *Sci. Rep.* 8:15329.
- Koike, S. T., Gladders, P., and Paulus, A. O. 2007. Diseases of vegetable crops: Cucurbitaceae. Pages 222-223 in: *Vegetable Diseases: A Color Handbook*. Academic Press, Boston, MA.
- Kumar, A., Drozd, M., Pina-Mimbela, R., Xu, X., Helmy, Y. A., Antwi, J., Fuchs, J. R., Nislow, C., Templeton, J., Blackall, P. J., and Rajashekara, G. 2016. Novel anti-*Campylobacter* compounds identified using high throughput screening of a pre-selected enriched small molecules library. *Front. Microbiol.* 7:405.
- Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J. 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* 23:3-25.
- McGhee, G. C., and Sundin, G. W. 2011. Evaluation of kasugamycin for fire blight management, effect on nontarget bacteria, and assessment of kasugamycin resistance potential in *Erwinia amylovora*. *Phytopathology* 101:192-204.
- OECD. 1998a. Guideline for testing of chemicals. Honeybees. Acute oral toxicity test. OECD Report 213. OECD, Paris, France.
- OECD. 1998b. Guideline for testing of chemicals. Honeybees. Acute contact toxicity test. OECD Report 214. OECD, Paris, France.
- Roberts, D. C. 2015. *Erwinia tracheiphila* pathosystem: Vector-pathogen interactions. Master's thesis. The Pennsylvania State University, University Park, PA.
- Rogers, S. A., Huigens, R. W., Cavanagh, J., and Melander, C. 2010. Synergistic effects between conventional antibiotics and 2-aminoimidazole-derived antibiofilm agents. *Antimicrob. Agents Chemother.* 54:2112-2118.
- Rogers, S. A., and Melander, C. 2008. Construction and screening of a 2-aminoimidazole library identifies a small molecule capable of inhibiting and dispersing bacterial biofilms across order, class, and phylum. *Angew. Chem.* 120:5307-5309.
- Rojas, E. S., Batzer, J. C., Beattie, G. A., Fleischer, S. J., Shapiro, L. R., Williams, M. A., Bessin, R., Bruton, B. D., Boucher, T. J., Jesse, L. C. H., and Gleason, M. L. 2015. Bacterial wilt of cucurbits: Resurrecting a classic pathosystem. *Plant Dis.* 99:564-574.
- Rojas, E. S., Dixon, P. M., Batzer, J. C., and Gleason, M. L. 2013. Genetic and virulence variability among *Erwinia tracheiphila* strains recovered from different cucurbit hosts. *Phytopathology* 103:900-905.
- Sasu, M. A., Seidl-Adams, I., Wall, K., Winsor, J. A., and Stephenson, A. G. 2010. Floral transmission of *Erwinia tracheiphila* by cucumber beetles in a wild *Cucurbita pepo*. *Environ. Entomol.* 39:140-148.
- Schreiber, K., Kukurshumova, W., Peek, J., and Desveaux, D. 2008. A high-throughput chemical screen for resistance to *Pseudomonas syringae* in *Arabidopsis*. *Plant J.* 54:522-531.
- Shaffer, C. L., Good, J. A. D., Kumar, S., Krishnan, K. S., Gaddy, J. A., Loh, J. T., Chappell, J., Almqvist, F., Cover, T. L., and Hadjifrangiskou, M. 2016. Peptidomimetic small molecules disrupt type IV secretion system activity in diverse bacterial pathogens. *MBio* 7:e00221-16.
- Shapiro, L. 2012. A to ZYMV guide to *Erwinia tracheiphila* infection: An ecological and molecular study. Doctoral dissertation. The Pennsylvania State University, University Park, PA.
- Shapiro, L., De Moraes, C. M., Stephenson, A. G., and Mescher, M. C. 2012. Pathogen effects on vegetative and floral odours mediate vector attraction and host exposure in a complex pathosystem. *Ecol. Lett.* 15:1430-1438.
- Shapiro, L. R., Paulson, J. N., Arnold, B. J., Scully, E. D., Zhaxybayeva, O., Pierce, N., Rocha, J., Klepac-Ceraj, V., Holton, K., and Kolter, R. 2018. An introduced crop plant is driving diversification of the virulent bacterial pathogen *Erwinia tracheiphila*. *bioRxiv*. doi:10.1101/345009.
- Shapiro, L. R., Seidl-Adams, I., de Moraes, C. M., Stephenson, A. G., and Mescher, M. C. 2014. Dynamics of short- and long-term association between a bacterial plant pathogen and its arthropod vector. *Sci. Rep.* 4:4155.
- Thanner, S., Drissner, D., and Walsh, F. 2016. Antimicrobial resistance in agriculture. *MBio* 7:e02227-e15.
- Vrismar, C. M., Deblais, L., Rajashekara, G., and Miller, S. A. 2016. Differential colonization dynamics of cucurbit hosts by *Erwinia tracheiphila*. *Phytopathology* 106:684-692.
- Wallace, I. M., Urbanus, M. L., Luciani, G. M., Burns, A. R., Han, M. K. L., Wang, H., Arora, K., Heisler, L. E., Proctor, M., St. Onge, R. P., Roemer, T., Roy, P. J., Cummins, C. L., Bader, G. D., Nislow, C., and Giaever, G. 2011. Compound prioritization methods increase rates of chemical probe discovery in model organisms. *Chem. Biol.* 18:1273-1283.
- Willett, P. 2006. Similarity-based virtual screening using 2D fingerprints. *Drug Discov. Today* 11:1046-1053.
- Worthington, R. J., Rogers, S. A., Huigens, R. W., III, Melander, C., and Ritchie, D. F. 2012. Foliar-applied small molecule that suppresses biofilm formation and enhances control of copper-resistant *Xanthomonas euvesicatoria* on pepper. *Plant Dis.* 96:1638-1644.
- Xu, X., Kumar, A., Deblais, L., Pina-Mimbela, R., Nislow, C., Fuchs, J. R., Miller, S. M., and Rajashekara, G. 2015. Discovery of novel small molecule modulators of *Clavibacter michiganensis* subsp. *michiganensis*. *Front. Microbiol.* 6:1127.
- Yang, F., Korban, S. S., Pusey, P. L., Eloffson, M., Sundin, G. W., and Zhao, Y. 2014. Small-molecule inhibitors suppress the expression of both type III secretion and amylovoran biosynthesis genes in *Erwinia amylovora*. *Mol. Plant Pathol.* 15:44-57.
- Yao, C., Zehnder, G., Bauske, E., and Kloepper, J. 1996. Relationship between cucumber beetle (Coleoptera: Chrysomelidae) density and incidence of bacterial wilt of cucurbits. *J. Econ. Entomol.* 89:510-514.
- Yu, J., Lan, L., Lewin, S. J., Rogers, S. A., Roy, A., Wu, X., Gao, P., Karanickolas, J., Aube, J., Sun, B., and Xu, L. 2017. Identification of novel small molecule Beclin 1 mimetics activating autophagy. *Oncotarget* 8: 51355-51369.