Mitigation of Pseudomonas syringae virulence by signal inactivation

Sieber, Simon; Mathew, Anugraha; Jenul, Christian; Kohler, Tobias; Bär, Max; Carrión, Víctor J; Cazorla, Francisco M; Stalder, Urs; Hsieh, Ya-Chu; Bigler, Laurent; Eberl, Leo; Gademann, Karl

Abstract: Pseudomonas syringae is an important plant pathogen of many valuable crops worldwide, with more than 60 identified pathovars. The phytotoxins produced by these organisms were related to the severity of the damage caused to the plant. An emerging strategy to treat bacterial infections relies on interference with their signaling systems. In this study, we investigated P. syringae pv. syringae, which produces the virulence factor mangotoxin that causes bacterial apical necrosis on mango leaves. A previously unknown signaling molecule named leudiazen was identified, determined to be unstable and volatile, and responsible for mangotoxin production. A strategy using potassium permanganate, compatible with organic farming, was developed to degrade leudiazen and thus to attenuate the pathogenicity of P. syringae pv. syringae.

DOI: https://doi.org/10.1126/sciadv.abg2293

The following work is licensed under a Creative Commons: Attribution-NonCommercial 4.0 International (CC BY-NC 4.0) License.

Originally published at:
Sieber, Simon; Mathew, Anugraha; Jenul, Christian; Kohler, Tobias; Bär, Max; Carrión, Víctor J; Cazorla, Francisco M; Stalder, Urs; Hsieh, Ya-Chu; Bigler, Laurent; Eberl, Leo; Gademann, Karl (2021). Mitigation of Pseudomonas syringae virulence by signal inactivation. Science Advances, 7(37):eabg2293. DOI: https://doi.org/10.1126/sciadv.abg2293
Mitigation of *Pseudomonas syringae* virulence by signal inactivation

Simon Sieber1, Anugraha Mathew2, Christian Jenu1, Tobias Kohler1, Max Bär1, Víctor J. Carrión3, Francisco M. Cazorla4, Urs Stalder1, Ya-Chu Hsieh1, Laurent Bigler1, Leo Eberl1*, Karl Gademann1*

*Pseudomonas syringae* is an important plant pathogen of many valuable crops worldwide, with more than 60 identified pathovars. The phytotoxins produced by these organisms were related to the severity of the damage caused to the plant. An emerging strategy to treat bacterial infections relies on interference with their signaling systems. In this study, we investigated *P. syringae* pv. *syringae*, which produces the virulence factor mangotoxin that causes bacterial apical necrosis on mango leaves. A previously unknown signaling molecule named leudiazen was identified, determined to be unstable and volatile, and responsible for mangotoxin production. A strategy using potassium permanganate, compatible with organic farming, was developed to degrade leudiazen and thus to attenuate the pathogenicity of *P. syringae* pv. *syringae*.

INTRODUCTION

*Pseudomonas* represent an excellent example of both benign and pathogenic plant-bacteria interactions (1). While some strains are beneficial for plants (2), others, such as members of the genus *Pseudomonas syringae* (*P. syringae*), are important pathogens, which infect almost all economically important crop species (3). Decades of studies have revealed the broad repertoire of virulence strategies used by *P. syringae*, which include large numbers of functionally redundant type III secretion system (T3SS) effectors and phytotoxins (4). A toxin that has a crucial ecological impact is mangotoxin (5), which is produced by *P. syringae* pv. *syringae* (Pss) and identified as the causal agent of bacterial apical necrosis (BAN) of mango trees (6). While the structure of the toxin remains unknown, the *mbo* gene cluster encoding mangotoxin biosynthesis genes was first described in Pss (7) and was later also identified in many other *P. syringae* pathovars (8, 9).

Investigations of the regulation of mangotoxin production have led to the identification of the *mgo* operon (*mgoBCAD*), which was shown to be required for the production of an unknown signaling molecule (Fig. 1A) (10, 11). The *mgo* gene cluster comprises four genes including a nonribosomal peptide synthetase (NRPS) complex (*MgoA*), and homolog clusters have been identified in other strains, such as *Pseudomonas entomophila* (puf cluster) (12), *Pseudomonas aurantiaca* PB-St2 (13), *Pseudomonas* sp. SH-CS2 (14, 15), several *Pseudomonas fluorescens* (16, 17), and various Burkholderia species (ham cluster) (18, 19). Recently, we found that the *ham* cluster directs the synthesis of the diazeniumdiolate validian (1), which was identified as a signal molecule that positively autoregulates its own expression (18). Furthermore, in 2018, Morgan et al. investigated the substrate specificity of the NRPS domain of *MgoA* and its homologs PvfC and HamD and concluded that the different strains produce at least two distinct signaling molecules (20). A comparison of the *mgo, puf*, and *ham* clusters (Fig. 1) reveals that the four genes required for the biosynthesis of the signals are highly conserved in *P. syringae*, *Pseudomonas entomophila*, and *Burkholderia cenocepacia*, respectively (tables S1 to S4). Here, we demonstrate that mangotoxin production is controlled by leudiazen (2, Fig. 1B), a new member of the diazeniumdiolate family of bacterial signal molecules. Furthermore, we speculated that the knowledge of the signaling molecule structure might pave the way to develop a strategy for plant protection.

RESULTS

Given the homology of the NRPS complexes *MgoA* and HamD (Fig. S1), we speculated that the *mgo* signal could be a related diazeniumdiolate compound. We used a transcriptional fusion of the promoter of the mangotoxin gene cluster (*mbo*) to the lacZ gene in a Pss Δ*mgoA* mutant background (Δ*mgoA*) to detect the signal-inducing mangotoxin production. The biosensor was activated by the wild-type extract but not by the Δ*mgoA* extract (Fig. S2). Valdiazen (1) only weakly induced the biosensor relative to the Pss extract, suggesting that the unknown signaling molecule may not be valdiazen (Fig. S2). Bioassay-guided fractionation under various conditions did not result in a pure product, as bioactivity was lost during the purification procedure, suggesting that the signal is either unstable or volatile.

In a complementary approach, we synthesized various derivatives of 1 and tested them for activation of the *mbo* promoter with the underlying anticipation that the indigenous signal shows the highest bioactivity. The choice of the derivatives was guided by a bioinformatic analysis of the predicted specificity of the MgoA NRPS (Fig. S1 and table S5) (20). On this basis, we selected the aliphatic amino acids glycine, alanine, leucine, and isoleucine as starting material. Leudiazen (2) and isoleudiazen (3) were synthesized using a four-step procedure, and glydiazen (4) and aladiazen (5) [also known as nitrosofungin (21)] were synthesized using an alternative route because of the instability of the intermediates (5, Fig. 2A). The five derivatives were tested, and leudiazen (2) was found to be the most potent inducer of the *mbo* promoter (Fig. 2B). No differences in the activities of the (R) and (S) enantiomer of leudiazen were observed (fig. S3). Using a targeted ultra-high-performance liquid chromatography (UHPLC)–MS analysis, we identified a new signaling molecule from Pss (Pss 18679) that was structurally similar to valdiazen (2) and when tested in the bioassay (Fig. 2C), it activated the *mbo* promoter similarly to valdiazen (2).

---

1Department of Chemistry, University of Zurich, 8057 Zurich, Switzerland. 2Department of Plant and Microbial Biology, University of Zurich, 8006 Zurich, Switzerland. 3Institute of Biology, Leiden University, 2333 BE Leiden, Netherlands. 4IHSM-UUM-CSIC, Department of Microbiology, University of Málaga, 29071 Málaga, Spain. 5Corresponding author. Email: karl.gademann@uzh.ch (K.G.); leberl@botinst.uzh.ch (L.E.). 6These authors contributed equally to this work.
chromatography–tandem mass spectrometry (UHPLC-MS/MS) approach (163 to 83 Da), we found 74 ± 7 nM leudiazen (2) in the Pss extract (fig. S4). No leudiazen (2) was detected in the extract of the ΔmgoA mutant (Fig. 2C).

The biosensor was activated in a dose-dependent manner and 10 μM leudiazen (S)-2, where the β-galactosidase activity was higher than the level observed for Pss::PmboA-lacZ (Fig. 3A and fig. S5). Leudiazen’s (2) ability to trigger mangotoxin production was evaluated...
using an Escherichia coli (E. coli) indicator strain. The antimicrobial activity, which indicates mangotoxin production, of the ΔmgoA mutant was restored to the level of the wild-type strain by the addition of 10 μM leudiazen ((S)-2) but not below this concentration (Fig. 3B and fig. S6). Likewise, 10 μM leudiazen ((S)-2) was needed to rescue the virulence of the mutant strain to the level of the wild-type in a tomato leaflet infection model (Fig. 3C).

Preliminary results suggested that leudiazen (2) could easily evaporate and be present in the gas phase (figs. S7 to S9). To confirm the volatility of 2, we performed a split plate assay, where one compartment contained the E. coli JM 105 (DSM 3949) reporter strain overlaid with the ΔmgoA mutant strain while the other compartment contained 20 μM leudiazen (2, Fig. 4A). After incubation for 24 hours, a halo was observed around the ΔmgoA mutant strain, suggesting mangotoxin production by the mbo gene cluster. In a control experiment, in which charcoal was added to a third compartment of the petri dish, growth of the E. coli JM 105 indicator strain was not inhibited, suggesting that leudiazen (2) present in the gas phase was adsorbed by the charcoal (fig. S10). Furthermore, the degradation of leudiazen (2) was studied by nuclear magnetic resonance spectroscopy, and the results indicated that the stability of 2 is solvent dependent (figs. S11 and S12).

We hypothesized that the mgo signaling system’s inhibition could be an effective way to control Pss pathogenicity. A chemical strategy was developed for leudiazen (2) degradation and potentially other diazeniumdilates. In agreement with previous work reporting that KMnO₄ efficiently decomposes the diazeniumdiolate functional group (22), we observed that leudiazen ((S)-2) was inactivated by treatment with 300 parts per million (ppm) of KMnO₄ within 10 min (Fig. 4B and fig. S13). Spraying of infected tomato leaves with an aqueous KMnO₄ solution (1000 ppm) suppressed necrotic symptoms (Fig. 4C). We recovered Pss cells from the treated and the untreated leaves (Fig. 4D), indicating that the chemical treatment abolished toxin production and did not inhibit bacterial growth.

**DISCUSSION**

Our study adds leudiazen (2) as only the second member of the diazeniumdiolate signal family. Homologs of the leudiazen gene cluster are widespread among Pseudomonas strains (tables S1 to S4), indicating that the signal may regulate the production of diverse secondary metabolites in these organisms. While valdiazen (1) controls the production of an antifungal agent in the opportunistic human pathogen H111 (18), we show that leudiazen (2) controls mangotoxin production in the plant pathogen Pss. The biosynthesis and chemical properties of diazeniumdiolate natural products have been recently investigated in several studies (18–20, 23–26). A remarkable aspect of leudiazen in contrast with other diazeniumdilates is its volatility and the ability to be transmitted through the air (Fig. 4A). Only very few volatile signals in bacteria have been described to date (27–29), and only two of those were determined to control the virulence in important plant pathogens. Volatile signals may be advantageous for long-distance communication, a feature that may be important for bacteria that live on plant surfaces.

Interference with bacterial signaling has emerged as a valuable strategy for developing novel anti-infective drugs that do not aim at killing the pathogen but attenuate their virulence (30). Our data...
demonstrate that leudiazen (2) can be inactivated by treatment with a dilute KMnO₄ solution without affecting the bacteria’s growth. A common strategy to interfere with bacterial communication is based on the enzymatic degradation of signaling molecules (31, 32). Here, we present the first example of a chemical approach to inactivate a bacterial signaling molecule to attenuate virulence. Notably, the concentration of the KMnO₄ solution used in our experiments is regulatorily approved for use in organic farming within the European Union (33).

**MATERIALS AND METHODS**

**Microbiological and analytical procedures**

Strains and plasmids used in this study are listed in table S6. Pss wild-type UMAF0158, Pss ΔmgoA mutant as well as strains carrying transcriptional lacZ fusions, Pss pMP/P₃mg0A::lacZ, and ΔmgoA pMP/P₃mg0A::lacZ strains were provided by V.J.C. and F.M.C. The construction of both Pss ΔmgoA mutant and the transcriptional fusions has been reported previously (7, 10). E. coli strains were routinely grown in LB medium (BD Difco, USA) at 37°C, while P. syringae strains were grown at 30°C. For the extraction of leudiazen (2), as well as for promoter activity measurements, P. syringae strains were grown in Pseudomonas minimal salts (PMS) medium [consists of 1 g of (NH₄)₂HPO₄ per liter, 0.2 g of KCl per liter, and 0.2 g of anhydrous MgSO₄ per liter] (34). The pH was adjusted to 7.0 before autoclaving, and 0.2% glucose was added as a carbon source. Yeast extract (0.005%) was routinely added to the PMS medium to boost growth. When required, media were supplemented with antibiotics at the following concentrations: kanamycin (100 μg/ml), gentamicin (20 μg/ml), tetracycline (20 μg/ml), and chloramphenicol (20 μg/ml).

**Assessment of promoter activity by β-galactosidase assay**

Promoter activity of the transcriptional lacZ fusions in liquid cultures was assessed by β-galactosidase assays as described before with minor modifications (35). Briefly, bacterial cells were grown overnight in PMS medium. Where indicated, synthetic valdizin (1) or leudiazen (2) or ((S)-2) or ((R)-2) and different valdizin derivatives dissolved in methanol were added to the cultures. Fifty to 200 μl of cells were harvested and resuspended in Z buffer (1 ml), and OD₆₀₀ (optical density at 600 nm) values were recorded. To permeabilize the cell membrane, chloroform (25 μl) and sodium dodecyl sulfate (SDS, 0.1%) were added to the residual bacterial suspension (1 ml), vortexed for 10 s, and incubated at 28°C for 10 min. The reaction was initiated by adding o-nitrophenyl-β-D-galactoside (200 μl) solution (4 mg/ml in Z buffer) to each sample, vortexed briefly, and incubated at room temperature (RT). The reaction was stopped by the addition of an aqueous Na₂CO₃ solution (500 μl, 1 M). The samples were centrifuged at 16,000 rpm for 10 min, and cell debris–free supernatant (1 ml) was used to measure the absorbance at 420 and 550 nm. The promoter activity (expressed as Miller units) was determined using the following formula:

\[ \text{Miller unit} = 1000 \times \frac{\text{OD}_{420} - 1.75 \times \text{OD}_{550}}{t \times v \times \text{OD}_{600}} \]

Where \( t \) = reaction time in minutes and \( v \) = volume of assayed sample in milliliters. Data were based on three independent biological replicates (\( n = 3 \)).

Sieber et al., Sci. Adv. 2021; 7 : eabg2293  8 September 2021  4 of 6
every β-galactosidase assay, a sample containing only the growth medium was processed as described above and used as a blank.

**Extraction procedure**

Bacterial strains were grown in PMS medium containing 0.005% yeast extract for 7 days with agitation (220 rpm) at 30°C. Bacterial cultures were centrifuged for 10 min at 8000 rpm in a Sorvall RC-5C plus centrifuge at 4°C. Culture supernatants were subsequently filtered with a Millipore Express Plus 0.22-μm system to remove the cell debris. The cell-free supernatant was acidified to pH 5 with an aqueous HCl solution (10 M) and extracted thrice with 0.5 volumes of dichloromethane. The organic phases were combined, dried with MgSO₄, filtered, and concentrated under reduced pressure to obtain the crude extract.

**Method for the detection and quantification of leudiazen (2)**

Leudiazen (2) was quantified by UHPLC–ESI (electrospray ionization)–MS/MS (Ultimate 3000 LC, Thermo Fisher Scientific; coupled to a TSQ Quantum Ultra, Thermo Fisher Scientific) using a Kinetex EVO C18 column (50 mm by 2.1 mm, 1.7 μm) at a flow rate of 0.4 ml/min. The solvent system was composed of A (H₂O with 0.1% HCO₃⁻) and B (MeCN with 0.1% HCO₃⁻). After isocratic elution at 5% B for 1 min, the gradient varied from 5% to 60% of B in 2.5 min, 60 to 95% of B in 1 min, and 95 to 100% of B in 0.05 min, and the column was finally washed with 100% B for 1.24 min. The detection was achieved in the selected reaction monitoring (SRM) mode using the specific fragmentation of the protonated molecules [M + H]⁺ at mass/charge ratio (m/z) 163 into the fragment ion at m/z 83 Da at a collision energy of 14 eV. The quantification was done using a calibration curve obtained from analytical standard solutions prepared in MeOH at the following concentrations: 1, 5, 10, 20, 50, and 100 μg/ml. A sample volume of 3 μl was injected. Cultures of Pss (2 liters) and the Δmgo mutant (2 liters) were prepared following the extraction procedure described above. The crude extract was resuspended in MeOH (1 ml). A concentration of 24 ± 3 μg/ml of leudiazen (2) was determined in the Pss extract using a calibration curve (fig. S4), which represents a leudiazen (2) concentration of 74 ± 7 nM in the supernatant of Pss. Leudiazen (2) was not detected in the Δmgo crude extract.

**Volatility experiment**

A glass vial (2 ml) containing leudiazen ((S)-2), ~5 mg] was placed into a vacuum chamber at 0.02 mbar, and another sample was placed under a gentle flow of nitrogen at 40°C. The samples were weighted each 2 hours for 8 hours (fig. S7). The headspace was performed by heating leudiazen [(S)-2], 0.9 mg] in a 10-ml vial for 10 min at 100°C (figs. S8 and S9). Then, 10 ml of the gas phase was transferred into another vial containing 1-ml acetonitrile with a gas-tight syringe. This vial was shaken for 30 s, and an aliquot of the acetonitrile layer (3 μl/min) was measured with ESI–high-resolution mass spectrometry (ESI-HRMS). ESI-HRMS: m/z 161.0930 (C₈H₁₂N₂O₂⁺ [M – H]⁺; calc. 161.0932, –1.2 ppm). A control experiment was performed by injecting a MeCN solution (fig. S9).

**Mangotoxin production assay**

Mangotoxin production was determined using an *E. coli* strain as an indicator using a previously described procedure (34) with minor modifications. Briefly, a double layer of the indicator microorganism, an *E. coli* K12 strain, was prepared, and after solidification, the Pss strains to be tested were stab-inoculated [Pss wild type, Δmgo mutant, and Δmgo mutant complemented with different amounts of leudiazen (2) or ((S)-2) or ((R)-2)] on to the agar seeded with *E. coli*. The plates were incubated at 30°C for 48 hours and observed for inhibition zones around the colony.

*E. coli* killing assay was also performed on split petri dishes to confirm the volatile-mediated effect of leudiazen. PMS medium mixed with *E. coli* [10⁻ colony-forming unit (CFU) final concentration] was included in one compartment of the petri dish, and the ΔmgoA mutant was stab-inoculated on to it while 10 μl of 20 μM leudiazen was spotted in the adjacent compartments. A control plate, containing charcoal in a third compartment to trap the volatile leudiazen, was also included in the assay (fig. S10).

**Virulence assay using tomato leaflets as model host**

To assess whether the putative signal molecules restored the virulence in the mutant strain, detached tomato leaflets (Hellfrucht Frühhstamm variety) were used as model hosts, and the virulence assay was performed as previously described (36) with some modifications. Exponentially growing cultures of Pss in PMS were adjusted to 10⁶ CFU ml⁻¹. In one set of virulence assay, 10-μl drops of the bacterial suspension were injected on three different points on one side of a leaflet, while three 10-μl drops of an aqueous MgSO₄ solution (10 mM) were injected on the other side of the same leaflet as a control. Alternatively, in another set of virulence assay, six 10-μl drops of the bacterial suspension were injected on six different points on the same leaflet, and detached leaflets inoculated with an aqueous MgSO₄ solution (10 mM) were included in all experiments as a control. Six tomato leaflets were used per strain, and the infected leaflets were maintained at 22°C and a 16:8-hour light:dark photoperiod for a period of 10 days. These experiments were repeated three times. The development and severity of necrosis on the leaflets were determined every 2 days for a period of 10 days. Bacterial strains were retrieved from the infected leaves, and colony counts were calculated on several days after inoculation.

**KMnO₄ treatment of tomato leaves infected with Pss**

To test whether KMnO₄ protects the tomato leaves from Pss infection, detached tomato leaves infected with Pss, as described above, were treated with an aqueous KMnO₄ solution. Leaflets (6) infected with Pss were dipped in an aqueous KMnO₄ solution (1000 ppm) with manual shaking at RT, allowing a total contact time of around 20 s. The solution was decanted, and the leaves were then dipped in fresh sterile distilled water to remove any traces of KMnO₄. The tomato leaflets (6) infected with Pss without KMnO₄ treatment were included in the assay. Both sets of leaflets were maintained for a period of 10 days, and development of necrotic symptoms was recorded. Bacterial strains were recovered from both sets of leaflets after 10 days, and presence of Pss was confirmed by polymerase chain reaction using Pss-specific primers.

**Degradation procedures**

Aqueous KMnO₄ solution (500 μl at a concentration of 20, 200, 600, or 1000 ppm) were added to 500 μl of an aqueous solution of leudiazen [(S)-2], 10 μg/ml] to obtain solutions having a concentration of KMnO₄ of 10, 100, 300, and 500 ppm. The samples were stirred at RT for 10 min, filtered, and 3 μl injected to the UHPLC-ultraviolet system (254 nm).
Mitigation of *Pseudomonas syringae* virulence by signal inactivation

Simon Sieber, Anugraha Mathew, Christian Jenul, Tobias Kohler, Max Bär, Victor J. Carrión, Francisco M. Cazorla, Urs Stalder, Ya-Chu Hsieh, Laurent Bigler, Leo Eberl, Karl Gademann

*Sci. Adv.*, 7 (37), eabg2293. • DOI: 10.1126/sciadv.abg2293

View the article online
https://www.science.org/doi/10.1126/sciadv.abg2293
Permissions
https://www.science.org/help/reprints-and-permissions