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Identification of Benzyloxy Carbonimidoyl Dicyanide Derivatives as Novel Type III Secretion System Inhibitors *via* High-Throughput Screening

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The type III secretion system (T3SS) in many Gram-negative bacterial pathogens is regarded as the most critical virulence determinant and an attractive target for novel anti-virulence drugs. In this study, we constructed a T3SS secretion reporter containing the β-lactamase gene fused with a signal peptide sequence of the T3SS effector gene, and established a high-throughput screening system for T3SS inhibitors in the plant pathogenic bacterium *Acidovorax citrulli*. From a library of 12,000 chemical compounds, we identified a series of benzyloxy carbonimidoyl dicyanide (BCD) derivatives that effectively blocked T3SS-dependent β-lactamase secretion. Substitution of halogens or nitro groups at the para-position on the benzene ring contributed to an increased inhibitory activity. One representative compound, BCD03 (3,4-dichloro-benzyloxy carbonimidoyl dicyanide), dramatically reduced pathogenicity of *A. citrulli* on melon seedlings, and attenuated hypersensitive responses in the non-host *Nicotiana tabacum* caused by pathogenic bacteria *A. citrulli*, *Xanthomonas oryzae* pv. *oryzae* and *Pseudomonas syringae* pv. *tomato* at sub-MIC concentrations. Western blotting assay further confirmed that BCD03 inhibited effector secretion from the above bacteria *via* T3SS in the liquid medium. Taken together, our data suggest that BCD derivatives act as novel inhibitors of T3SS in multiple plant bacterial pathogens.

Keywords: *Acidovorax*, *Xanthomonas*, *Pseudomonas*, type III secretion system, hypersensitive response, virulence inhibitor, benzyloxy carbonimidoyl dicyanide

INTRODUCTION

Antibiotic resistance is becoming a major concern in agricultural industries. Traditional antibiotics, which target growth and survival, induce high selective pressure on pathogenic bacteria. One promising way to reduce bacterial resistance is to target virulence factors, rather than growth (Rasko and Sperandio, 2010). The type III secretion system (T3SS) is highly conserved among Gram-negative bacteria and is directly related to pathogenicity during host cell invasion. The needle-like

T3SS penetrates the cell membrane where it injects T3SS effectors (T3Es), suppressing host immunity and promoting invasion and dissemination (Cornelis, 2006). T3SS is not necessary for bacterial survival, making it an attractive target for novel antibacterial drugs (Büttner, 2012). To date, a number of chemical compounds acting as T3SS inhibitors have been identified in a wide range of important plant and animal bacterial pathogens, such as *Erwinia*, *Pseudomonas, Xanthomonas, Escherichia*, *Yersinia* and *Salmonella* species (Felise et al., 2008; Wang et al., 2011; Jessen et al., 2013; Yang et al., 2014; Anantharajah et al., 2016; Fan et al., 2017).

Most of the known T3SS inhibitors were screened out by using transcriptional reporters that fused T3SS-related genes to the genes encoding reporter proteins like luciferase, phospholipase, and β-lactamase. (Kauppi et al., 2003; Li et al., 2005; Felise et al., 2008; Pan et al., 2009; Duncan et al., 2014). A few screenings were performed by using translational or translocational reporters (Kim et al., 2009; Pan et al., 2009; Swietnicki et al., 2011). The known inhibitors, which include alicylates, benzoates, thiazoles, phenolic compounds and specific antibodies, affect T3SS functions by directly targeting T3SS injectisome (Lynch et al., 2010; Jessen et al., 2013; Bowlin et al., 2014), regulating T3SS gene expression (Yang et al., 2014; Marsden et al., 2015; Fan et al., 2017), or through other indirect interactions (Wang et al., 2011; Bowlin et al., 2014; Faure et al., 2014).

In many plant bacterial pathogens, T3SS and T3Es are essential virulence factors during infection. T3SSs in plant bacteria fall into two groups, hypersensitive reaction and pathogenicity (Hrp) groups I and II, based on their nucleotides, operon structures, and regulatory systems (Alfano and Collmer, 1996; Cornelis, 2006; Nilles and Condry, 2017). Representatives of group I include *Pseudomonas syringae* and *Erwinia amylovora*, while *Ralstonia solanacearum* and *Xanthomonas oryzae* belong in group II (Tampakaki et al., 2010; Diepold and Armitage, 2015). Although these two groups share similar *hrc* (HR and conserved) genes, some of their *hrp* genes appear completely different. One significant difference is that group I T3SSs are activated by HrpL, an ECF family sigma factor activated by HrpS to promote gene expression (Lidell and Hutcheson, 1994; Alfano and Collmer, 1996), while group II T3SSs are regulated by AraC family transcriptional regulators, HrpB in *R. solanacearum* and HrpX in *X. oryzae* and *Acidovorax citrulli*. *HrpB* and *hrpX* are activated by the OmpR-type transcriptional regulator HrpG, a response regulator protein of a two-component signal transduction system, which induces expression of T3SS (Wengelnik and Bonas, 1996; Wengelnik et al., 1996; Tampakaki et al., 2010). However, expression of T3SS is not constitutive. Transcription of T3SS genes in both Hrp groups is repressed in full nutrient medium, but induced *in planta* or in medium mimicking the intracellular space of plants (Bonas, 1994; Nilles and Condry, 2017). Induction of T3SS expression tends to involve low-nutrient culture media; for example, XVM2 and XOM2 medium for *X. oryzae* (pH 6.5 and 5 mM Mg^{2+}), 1/4 M63 medium for *R. solanacearum* (1 mM $Mg^{2+}$and 3.9 mM Fe^{2+}) and MG medium for *P. syringae* (pH 7.0 and 2 mM Mg^{2+}) (Keane et al., 1970; Boucher et al., 1985; Tsuge et al., 2002). Meanwhile, with bacteria such as *A. citrulli*, T3SS expression is induced in modified lysogenic broth (LB) medium (pH5.8, 10 mM Mg^{2+}) (Zhang et al., 2018).

A. citrulli is the causal agent of bacterial fruit blotch in cucurbit crops such as watermelons (*Citrullus lanatus*) and melons (*Cucumis melo*) (Schaad et al., 2008). T3SS is required for pathogenicity and hypersensitive response (HR) induction in host and non-host plants, respectively (Bahar and Burdman, 2010; Johnson et al., 2011). In this study, we established a high-throughput screening system for T3SS inhibitors in *A. citrulli* strain MH21 and successfully screened a large number of chemical compounds under the optimized T3SS-inducing conditions. Chemicals showing inhibitory activity were further tested using different plant bacterial pathogens to explore their effective target spectrum.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in **Table 1**. *Escherichia coli* was grown in LB medium at 37°C, *A. citrulli* MH21 and its derivatives were grown in LB medium at 28°C, *X. oryzae* pv. *oryzae* PXO99^{A} and its T3SS mutant were grown in M210 medium (0.8% casein enzymatic hydrolysate, 0.5% sucrose, 0.4% yeast extract, 17.2 mM K_2HPO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$) at 28°C. XOM2 medium (0.18% D-(+) xylose, 670 μM L-methionine, 10 mM sodium L-(+) glutamate, 14.7 mM KH_2PO_4, 40 μM $MnSO_4$, 240 μM Fe(III)-EDTA and 5 mM $MgCl_2$, pH adjusted to 6.5 with KOH) was used for *hrp*-inducing (Tsuge et al., 2002). *P. syringae* pv. *tomato* DC3000 and its T3SS mutant were grown in King's B medium at 28°C. MG medium (1% Mannitol, 0.2% L-glutamic acid, 3.6 mM KH_2PO_4, 3.4 mM NaCl and 1.6mM $MgSO_4{\cdot}7H_2O$, pH adjusted to 7.0 with NaOH) was used for *hrp*-inducing (Keane et al., 1970). The following antibiotics were used when required: 50 μg/mL ampicillin (Ap), 50 μg/mL streptomycin (Sm), 25 μg/mL spectinomycin (Sp), 50 μg/mL kanamycin (Kan), 10 μg/mL gentamycin (Gm), and 50 μg/mL rifampicin (Rif).

Sources of Screened Compounds

Chemical compounds used for T3SS inhibitor screening were provided by Shenyang Sinochem Agrochemicals R&D Co. Ltd (Shenyang, China) and were dissolved in dimethyl sulfoxide (DMSO).

Construction of the T3SS Secretion Reporter Strain

To construct the T3E secretion reporter pZAC-3502sig-*penAC*, the *penAC* (GenBank accession MK576102) fragment without its 57-bp 5' Type II secretion system (T2SS) signal peptide sequence was PCR amplified from MH21 using primers *penAC*-F and *penAC*-R (**Table S1**). A 180-bp T3SS-dependent signal peptide sequence from the predicted effector *Aave_3502*, a homologue of *P. syringae* effector HopAO1 (Bretz et al., 2003; Underwood et al., 2007), was PCR amplified using primers Aave-3502sig-F and Aave-3502sig-R (**Table S1**) then in-frame fused to the 5'end of the *penAC* fragment digested

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strain		
Escherichia coli		
DH5α	F⁻ *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1Δ (argFlacZYA) I169Φ80lacZ ΔM15*	Invitrogen
Acidovorax citrulli		
MH21	Wild-type, Ap^R	Ren et al. (2014)
MH21Δ*hrcC*	*hrcC* in MH21 mutated; Ap^R, Rif^R	Lab collection
MH21Δ*penAC*	*penAC* in MH21 mutated; Rif^R	This study
MH21Δ*penAC*Δ*hrcC*	*hrcC* and *penAC* double mutant; Rif^R	This study
X. oryzae pv. *oryzae*		
$PXO99^A$	Wild-type strain, Philippine race 6	Hopkins et al. (1992)
$PXO99^A$Δ*hrcU*	*hrcU* in $PXO99^A$ mutated	Liu et al. (2010)
P. syringae pv. tomato		
DC3000	Wild type; Rif^R	Buell et al. (2003)
DC3000Δ*hrcQ-U*	*hrcQ-U* in DC3000 mutated; Rif^R	Badel et al. (2006)
Plasmids		
pBBR1MCS-2	pBBR1MCS-2 containing pBluescript II KS-*lacZ*α; Km^R	Kovach et al.(1994)
pRK600	Helper plasmid; ColE1 replicon TraRK⁺ Mob⁺	Finan et al. (1986)
pZLQ-Flag	Expression vector; Km^R	Luo and Farrand (1999)
pZAC	Expression vector; Km^R	This study
pZAC-3502sig-*penAC*	Expression vector pZAC carrying *penAC* fused with N-signal peptide sequence of a T3SS effector; Km^R	This study
pZAC-3502-FLAG	Expression vector pZAC carrying the full-length *hopAO1* gene with a C-FLAG tag; Rif^R, Km^R	This study
pHZY-*avrXa27*	Expression vector pHM1 carrying *avrXa27* with a C-FLAG tag; Ap^R, Sp^R	Ji et al. (2014)
pCPP5372-*avrPto*-HA	Expression vector pCPP5372 carrying *avrPto* with a C-HA tag; Gm^R	Wei et al. (2015)

[a] *Ap^R, Cm^R, Km^R, Rif^R, Sp^R and Gm^R indicate resistance to ampicillin, chloramphenicol, kanamycin, rifampin, spectinomycin and gentamycin, respectively.*

with *Eco*RI. The modified *β*-lactamase sequence was cloned into pZLQ (Luo and Farrand, 1999) under control of the P_{trc} promoter. The reporter plasmid was introduced from *E. coli* into *A. citrulli* MH21 *via* mating with an *E. coli* strain carrying the helper plasmid (pRK600) (Finan et al., 1986). The pZAC-3502-FLAG plasmid containing the FLAG-tagged *hopAO1* gene under the P_{trc} promoter was generated and introduced into *A. citrulli* MH21 for Western blotting analysis. The *X. oryzae* pv. *oryzae* carrying plasmid pHZY-*avrXa27* (Ji et al., 2014) and *P. syringae* pv. *tomato* DC3000 carrying plasmid pCPP5372-*avrPto*-HA (Wei et al., 2015) were also used for Western blotting analysis.

High-Throughput Screening of T3SS Inhibitors

A. citrulli MH21 reporter strain containing the pZAC-3502sig-*penAC* plasmid was grown overnight in LB medium at 28°C then the bacterial cells were harvested by centrifugation. After washing twice with sterilized distilled water (sdH_2O), the bacterial cells were resuspended in sdH_2O to OD_{600} = 1.0 and inoculated into LB medium (pH 5.8) supplemented with 10 mM $MgCl_2$ (Zhang et al., 2018) in a 96-well microplate at a ratio of 4%. Test compounds were added at a final concentration of 25 μg/mL. An identical concentration of DMSO was used as a control. Nitrocefin (TOKU-E, WA, USA) was added at a concentration of 250 μg/mL when the bacteria grew to OD_{600} = 0.4–0.6 (20 h) followed by incubation for 15 min. Color measurements of the medium (OD_{500}) were then taken using a Microplate Reader (SpectraMax i3x; Molecular Devices, Sunnyvale, CA). The screening experiment was performed with three technical replicates.

Minimum Effective Concentration (MEC) Assay and Half Maximal Inhibitory Concentration (IC_{50})

Bacterial strain MH21 harboring the pZAC-3502sig-*penAC* plasmid was prepared as above in a 96-well microplate. The MEC assay was carried out by diluting benzyloxy carbonimidoyl dicyanide (BCD) derivatives at a final concentration of 70, 60, 50, 40, 30, 20, 10, 5, 2.5, or 1.25 μg/mL, respectively. Nitrocefin was then added and the OD_{500} was determined as described above. The molarity of chemical compounds was converted based on the MECs and molecular weights (MWs). IC_{50} of these compounds was calculated based on the OD_{500} values and molarity (Sebaugh, 2011). Experiments were performed twice with three biological replicates.

Minimum Inhibitory Concentration (MIC) Assay

A MIC assay was carried out using strains MH21, $PXO99^A$ and DC3000 and their T3SS mutants. Bacteria were cultured in their own favorable medium until OD_{600} reached 1.0 then inoculated in 96-well microplates with the same medium at a ratio of 1:1,000, supplemented with BCD derivatives at a final concentration of 200, 100, 50, 25, 12.5, 6.25 or 3.125 μg/mL, respectively. The microplates were placed at 28 °C and OD_{600} was measured using a microplate reader after 48 h incubation. The experiments were performed twice with three biological replicates.

Pathogenicity Inhibition Assay

Germinated seeds of melon (*Cucumis melo* INV192) were dipped in MH21 bacterial suspension at 3×10^8 CFU, supplemented with 25 or 50 μg/mL BCD03 for 20 min. Δ*hrcC* mutant strain was used as a control. Cotyledons of the seedlings were collected 12 days after sowing and disease severity was assessed on a scale of 1 to 9: 1, no symptoms; 3, small, necrotic lesions on cotyledons, <20% necrotic cotyledon; 5, necrotic lesions with chlorosis on cotyledons, 20–50% necrotic cotyledon; 7, large spreading lesions, >50% of cotyledon necrotic; 9, >90% necrosis of the cotyledon or a dead plant (Hopkins and Thompson, 2002). The experiments were repeated twice with three biological replicates. Each replicate contained 5 seedlings.

Hypersensitive Response Assays

Nicotiana tabacum seedlings were grown under greenhouse conditions for 4–5 weeks. Bacterial cells were then harvested from precultured medium and suspended in 10 mM $MgCl_2$. Leaves of *N. tabacum* were subsequently inoculated with bacterial strains at 3×10^8 cfu/mL (MH21, PXO99^A and their T3SS mutants) or 3×10^6 cfu/mL (DC3000 and its T3SS mutant) by infiltration with a blunt syringe and assayed 18 h post inoculation (Alfano et al., 1996). BCD03 was added at a final concentration of 25 μg/mL 2 h before infiltration. BCD03 alone was used as a control. The experiment was performed with three biological replicates.

Effector Secretion and Immunodetection

Strains MH21, PXO99^A and DC3000 harboring reporter plasmids pZAC-3502-FLAG, pHZY-*avrXa27* and pCPP5372-*avrPto*-HA, respectively, were cultured in their own *hrp*-inducing medium supplemented with 25 μg/mL T3SS inhibitor BCD03 (or DMSO as a control) at 28°C for 8 h. Bacterial cultures were diluted to OD_{600} = 0.5 and centrifuged at 4000 × g for 10 min then the supernatant was filter sterilized, mixed with cold 100% trichloroacetic acid to a final concentration of 15% and incubated overnight at 4°C. Samples were then centrifuged again at 6000 × g for 30 min at 4°C, the supernatant was discarded, and the protein pellet was washed twice with 2 mL cold acetone. The bacterial pellet was resuspended in 50 μl phosphate buffered saline (PBS) and boiled for 10 min then HopAO1 and AvrXA27 were detected by Western blotting using a primary anti-FLAG mouse monoclonal antibody (Transgen Biotech, Beijing, China), diluted 1:2,000, and a goat anti-mouse IgG (H+L) secondary antibody conjugated to HRP (Invitrogen, Beijing, China), diluted 1:5,000. AvrPto was detected by Western blotting using a primary anti-HA mouse monoclonal antibody conjugated to HRP (MBL, Beijing, China) and diluted 1:5,000. Antibodies used in this study were all diluted in 40 ml PBS buffer supplemented with 0.5% tween 20 and 1% skimmed milk. Immunodetected HopAO1-FLAG, AvrXA27-FLAG, and AvrPto-HA were developed using Immobilon ECL (Beyotime, Shanghai, China) and membranes were photographed using the ChemiDoc Imaging System (BIO-RAD, Beijing, China).

Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics 17.0 (SPSS, Chicago, IL, USA) or GraphPad 7.0 (GraphPad Software, La Jolla, CA, USA) as specified.

RESULTS

Development of a T3SS Inhibitor Screening System in *Acidovorax citrulli*

We constructed a T3E secretion reporter plasmid containing a T3E signal peptide sequence fused with the β-lactamase gene in *A. citrulli* MH21 to screen for T3SS inhibitors. The β-lactamase gene was used as a reporter because it hydrolyzes nitrocefin and gives a clear color change readout from yellow to red, fulfilling the purpose of high through-put screening. However, wild-type *A. citrulli* MH21 is naturally resistant to ampicillin and hydrolyzes nitrocefin, indicating the presence of a gene with β-lactamase activity on its chromosome (**Figure S1A**). Genome sequence analysis subsequently revealed a potential β-lactamase gene named *penAC* in MH21, which had 39.8% identity and 83.0% coverage with the β-lactamase gene *penP* in *Bacillus subtilis* (GenBank accession BG11016). Further phylogenetic analysis (**Figure S2**) and conserved structure analysis (**Table S2**) revealed that PenAC belongs to β-lactamase class A (Ambler, 1980; Matagne et al., 1999; Eiamphungporn et al., 2018). In addition, mutation of *penAC* in *A. citrulli* MH21 led to loss of ampicillin resistance (**Figures S1A, B**), suggesting that PenAC is indeed a β-lactamase. We therefore constructed pZAC-3502sig-penAC, a reporter plasmid containing the Ptrc promoter to drive a recombined β-lactamase gene *penAC*. The original N-terminal T2SS signal peptide of *penAC* was replaced by the T3SS-signal peptide of effector gene *Aave_3502*, a homologue of the *P. syringae* effector HopAO1. (**Figure 1A**). The mutant MH21Δ*penAC* was then used as a host for the reporter plasmid pZAC-3502sig-*penAC* to remove the background β-lactamase activity. A double gene mutant, MH21Δ*penAC*Δ*hrcC*, was also constructed as a T3SS-negative control (**Figure S1C**).

When cultured in T3SS-inducing LB medium (pH 5.8, 10 mM $MgCl_2$), MH21Δ*penAC* containing the reporter plasmid pZAC-3502sig-*penAC* secreted β-lactamase in a T3SS-dependent manner. Extracellular β-lactamase activity was therefore used to indicate the effect of each inhibitor. Because over-cultured bacteria release undesired β-lactamase *via* cell lysis, we identified the most suitable bacterial state for reporter detection by comparing extracellular β-lactamase activity between the wild-type MH21 and its T3SS mutant throughout growth. As a result, no growth differences were observed between MH21 mutants Δ*penAC* and Δ*penAC*Δ*hrcC* carrying pZAC-3502sig-*penAC* when cultured in T3SS-inducing medium for 28 h. However, extracellular β-lactamase activity was significantly higher with MH21Δ*penAC* than the T3SS mutant MH21Δ*penAC*Δ*hrcC* at 20 h (OD_{600} = 0.4– 0.6) post inoculation (**Figures 1B**, **C**), indicating secretion of β-lactamase *via* T3SS. These culture conditions were therefore used to screen the T3SS inhibitors.

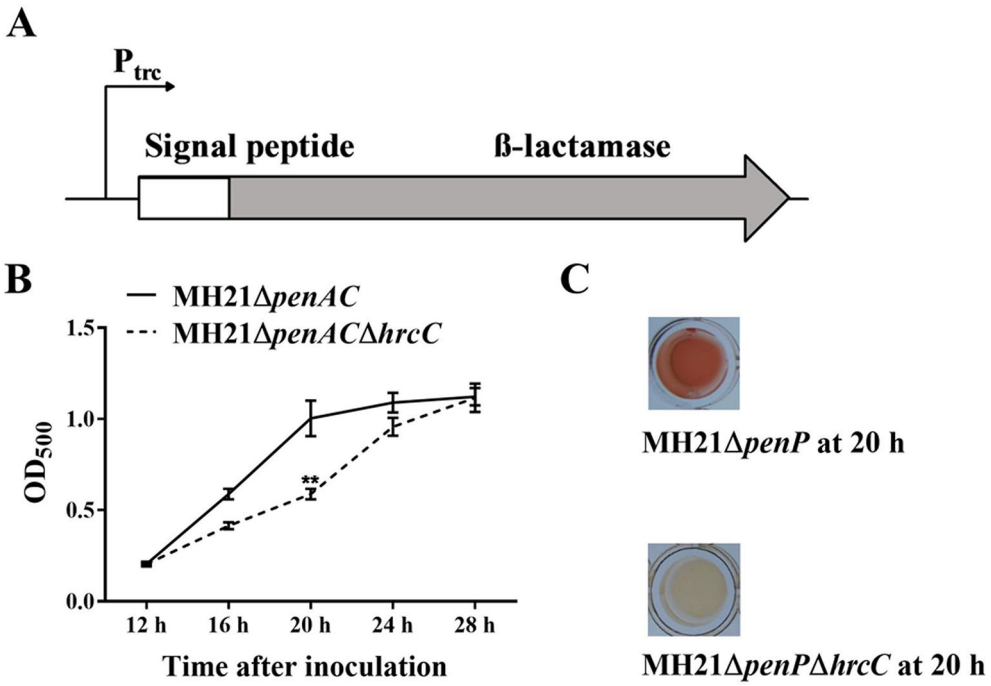

FIGURE 1 | Reporter structure and suitable growth state of reporter strain *Acidovorax citrulli* MH21Δ*penAC* (pZAC-3502sig-*penAC*). **(A)** Reporter structure on plasmid pZAC-3502sig-*penAC*. The P_{trc} promoter driven *β*-lactamase gene was fused with a signal peptide sequence of the T3SS effector gene (*Aave_3502*, a *hopAO1* homologous gene). **(B)** Extracellular *β*-lactamase activity of MH21Δ*penAC* and its T3SS mutant MH21Δ*penAC*Δ*hrcC* cultured in *hrp*-inducing medium. *β*-lactamase activity was measured spectrophotometrically by determining the OD_{500}. Experiments were performed twice with three biological replicates. Statistically significant differences (Student's t-test) were observed at 20 h post inoculation. P-value < 0.05 (**). **(C)** The reaction mixture containing the reporter plasmid in the T3SS mutant MH21Δ*penAC*Δ*hrcC* remained yellow, but changed into red in mixture containing the T3SS wild-type strain MH21Δ*penAC*.

Benzyloxy Carbonimidoyl Dicyanide as a Novel Inhibitor of Bacterial T3SS

More than 12,000 compounds at a final concentration of 25 μg/mL were screened in 96-well microplates using the above method (**Figure 2A**). A series of BCD derivatives prevented the color change from yellow to red in the reaction mixture, indicating potential T3SS inhibitory activity. These compounds shared the same BCD backbone as a number of substituents, including halogen, hydro, nitro, and methoxycarbonimidoyl dicyanide (MD), on its benzene ring (**Figure 2B**). The MICs of the BCD derivatives was always higher than 50 μg/mL, and with BCD03, BCD10, and BCD12 was as high as 100 μg/mL (**Table S3**). These findings indicate specific inhibition of T3SS by these compounds. The BCD derivatives were further tested with the wild- type MH21, which produced T2SS-dependent *β*-lactamase PenAC using the system described above. Accordingly, no color variation was observed, indicating that the compounds did not directly inhibit *β*-lactamase activity (data not shown).

Structure-Function Relationship of the BCD Derivatives

The MECs of effective inhibitors of T3SS from *A. citrulli* MH21 were subsequently measured at concentrations below their MICs. BCD01, BCD02, BCD03, BCD04, and BCD05 had relatively low MEC (**Table 2**). Moreover, using BCD03 as an example, the color of the reaction changed from red to yellow with increasing addition, indicating a dosage effect of the inhibition on the function of T3SS (**Figure 2C**).

Benzene substituted by halogens as in BCD01, BCD02 (containing an additional cyan group), BCD03 and BCD04, had relatively low IC_{50} values that mean the high T3SS-inhibitory activities. BCD13 and BCD14 also possessed a halogen substitution, but not directly on the benzene, resulting in a decreased inhibitory activity. Of the three nitro-substituted BCDs (BCD05, BCD06, and BCD07), BCD05, which had two nitro substitutes at both a para- and ortho-position, had the highest inhibitory activity, suggesting that two substitutions are better than one. The increased inhibitory activity of BCD06 compared to BCD07 further suggests a para-position substitution is also beneficial under single substitutional situation. A similar position-activity relationship was observed with BCD10-12, whereby ortho-, meta- and para-position substitutions resulted in IC_{50} values of 153.6, 149.7, and 117.7 μM, respectively. However, cyan Substitutions at para- and meta-positions did not result in any significant differences between BCD08 and BCD09. Overall, with the single substitution, para-position of the compounds resulted in the highest T3SS inhibitory activity, and the replacement of halogens or nitro substitutional groups resulted in an increase of T3SS inhibitory effect.

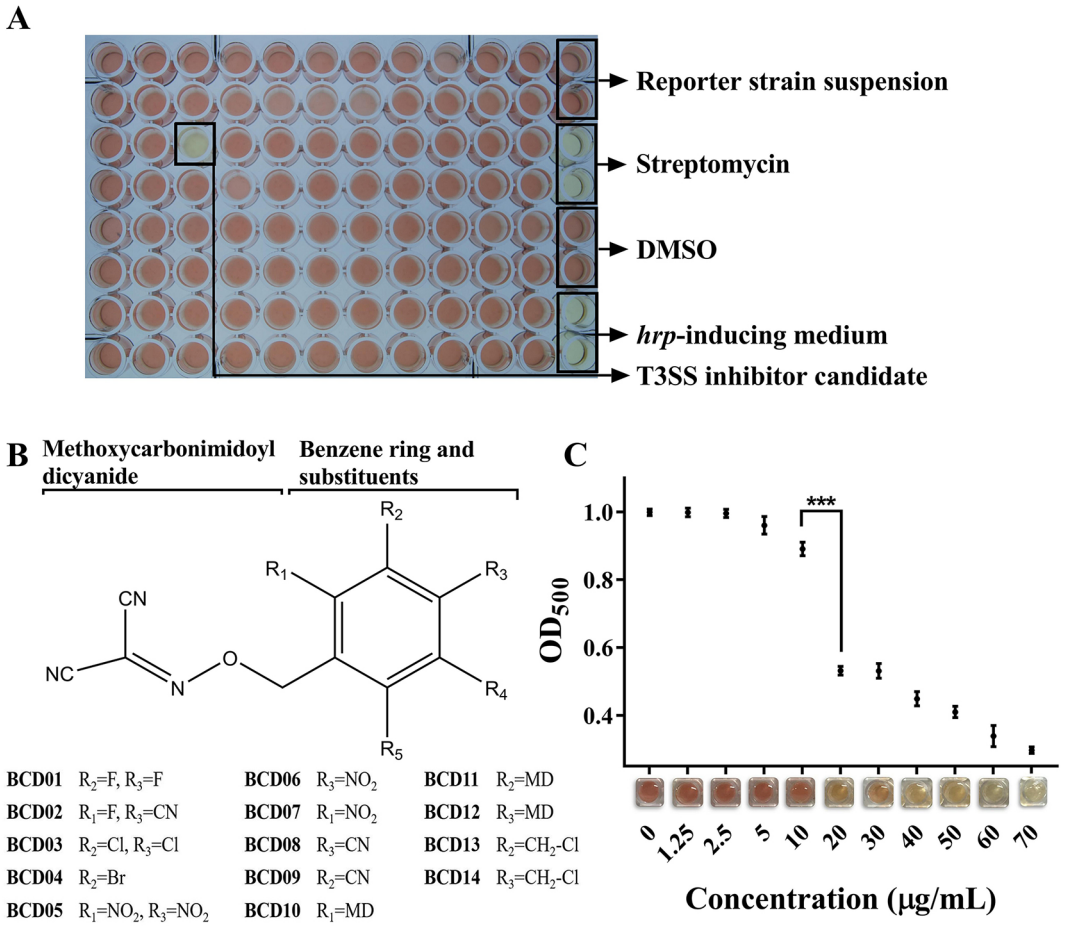

FIGURE 2 | Benzyloxy carbonimidoyl dicyanide derivatives (BCDs)-induced inhibition of T3SS function in *Acidovorax citrulli* MH21. **(A)** High-throughput screening in a 96-well microplate. Each well contained the reporter strain MH21Δ*penAC* harboring pZAC-3502sig-*penAC* in 200 μL *hrp*-inducing medium (LB with 10 mM $MgCl_2$, pH 5.8) supplemented with candidate chemicals and nitrocefin. Control groups were arranged in the rightmost row, including reaction mixture without candidate chemicals, reaction mixture with the antibiotic streptomycin, reaction mixture with solvent DMSO, and *hrp*-inducing medium only. **(B)** Chemical structures of BCD derivatives that had an inhibitory effect on T3E reporter secretion. The benzene ring substituted by one BCD is stabilized as a core. R1-R5 substitutions on the benzene ring were evaluated. **(C)** Inhibitory effect of different concentrations of BCD03 on T3SS function. The readout of OD_{500} decreased as the concentration of BCD03 increased, as shown by the color variation from red to yellow. Experiments were performed twice with three biological replicates. Statistically significant differences (Student's t-test) were observed between 10 and 20 μg/mL. P-value < 0.01 (***).

BCD03 Suppresses the HR in *Nicotiana tabacum* and the Bacterial Pathogenicity in Melon Seedlings

The HR-inducing capacities of bacteria belonging to Hrp groups I (*P. syringae* pv. tomato DC3000) and II (*X. oryzae* pv*., oryzae* PXO99^A, and *A. citrulli* MH21), and their T3SS mutants were examined in *N. tabacum* in the presence of BCD03. MICs of BCD03 in strains DC3000, PXO99^A, and MH21 were 200, 50 and 100 μg/mL, respectively (**Figure S3**). BCD03 was therefore used at the sub-MIC concentration of 25 μg/mL for all the above bacteria. HR symptoms were observed on leaves of *N. tabacum* 18 h after inoculation with wild-type bacteria (MH21, PXO99^A, and DC3000), but were dramatically suppressed when BCD03 was added (**Figure 3A**). No cell death symptoms developed on leaves treated with the T3SS mutants or BCD03 alone (**Figure 3A**). These results suggest that BCD03 inhibits T3SS-based HR induction in bacteria of at least three genera belonging to two Hrp groups.

To test the inhibitory activity of BCD03 on bacterial pathogenicity, the wild type *A. citrulli* MH21 with or without BCD03 at its sub-MICs was inoculated on the germinated melon seeds before sowing. Disease investigation at seedling stage showed a sever disease caused by the wild type MH21, but not its T3SS mutant Δ*hrcC*, indicating the critical role of T3SS in bacterial infection (**Figure 3B**). A dramatic decrease of disease severity was observed on seedlings treated with the wild type MH21 and BCD03 at concentrations 25 μg/mL and 50 μg/mL (**Figure 3B**). As a result, BCD03 reduced the T3SS-dependent pathogenicity of *A. citrulli* MH21 on its host plant at sub-MICs.

TABLE 2 | Derivatives of benzyloxy carbonimidoyl dicyanide (BCD) with their minimum effective concentrations (MEC), molecular weights (MW) and half maximal inhibitory concentrations (IC_{50}).

	R^1	R^2	R^3	MEC (μg/mL)	MW	IC_{50} (μM)
BCD01	–	F	F	20.0	221.2	57.5 ± 3.8
BCD02	F	–	CN	20.0	228.2	79.8 ± 6.9
BCD03	–	Cl	Cl	20.0	254.1	67.3 ± 2.5
BCD04	–	Br	–	20.0	264.1	52.1 ± 5.3
BCD05	NO_2	–	NO_2	20.0	275.2	47.6 ± 0.7
BCD06	–	–	NO_2	30.0	230.2	90.0 ± 4.5
BCD07	NO2	–	–	40.0	230.2	134.0 ± 8.8
BCD08	–	–	CN	30.0	210.2	100.9 ± 8.1
BCD09	–	CN	–	30.0	210.2	121.1 ± 5.4
BCD10	MD	–	–	50.0	292.3	153.6 ± 7.6
BCD11	–	MD	–	50.0	292.3	149.7 ± 9.8
BCD12	–	–	MD	40.0	292.3	117.7 ± 9.4
BCD13	–	CH_2–Cl	–	50.0	233.7	191.2 ± 7.6
BCD14	–	–	CH_2–Cl	40.0	233.7	163.3 ± 8.2

R^1–R^3: ortho-, meta- and para-position of the substitutional group on the benzene ring. F, Cl, Br: halogen substitutional group. CN: cyan group. NO_2: nitro group. "–": no substitutional group. Experiments were performed twice with three biological replicates.

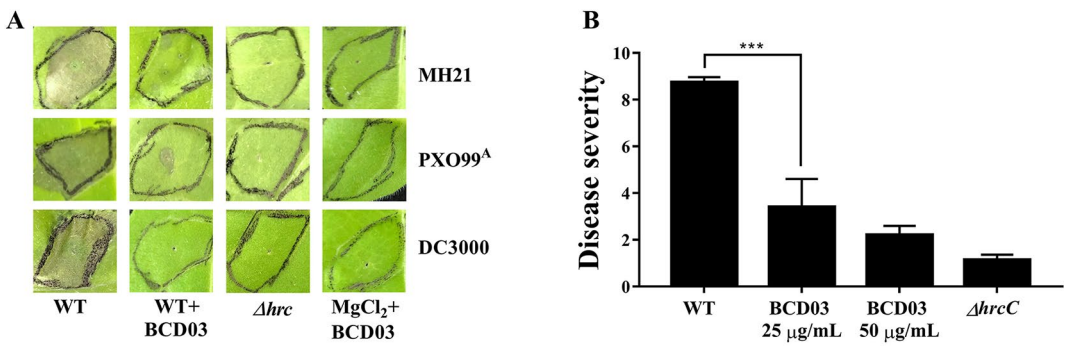

FIGURE 3 | (A) BCD03 suppression of hypersensitive responses in *Nicotiana tabacum* triggered by pathogenic bacteria. *A. citrulli* MH21, *X. oryzae* pv. *oryzae* PXO99^A, *P. syringae* pv. tomato DC3000 and their T3SS mutants, with or without BCD03 (25 μg/mL added 2 h before infiltration), were infiltrated into the leaves (3×10^8 cfu/mL for MH21 and PXO99^A, 3×10^6 cfu/ml for DC3000) then the response was photographed at 18 h post inoculation. The control group was treated with 10 mM $MgCl_2$ mix with 25 μg/mL BCD03. Photos from one of three biological replicate experiments are shown. **(B)** Effect of BCD03 on infection of *A. citrulli* MH21 on melon seedlings. Germinated melon seeds were dipped in bacterial suspension at 3×10^8 CFU, supplemented with 0, 25 or 50 μg/mL BCD03, respectively. Disease severity was investigated 12 days after sowing. Disease ratings are defined using a scale-based symptom appearance as described in Methods section. The experiments were repeated twice with three biological replicates. Each replicate contained 5 seedlings. Statistically significant differences (Two-way ANOVA) were observed between wild type treated groups and other groups. P-value < 0.01 (***).

BCD03 Inhibits T3SS Effector Secretion

When cultured in *hrp*-inducing media, plant pathogenic bacteria deliver T3Es to extracellular spaces *via* T3SS. We therefore examined the inhibitory effect of BCD03 on T3SS-dependent effector secretion in three different bacteria, *A. citrulli* MH21, *X. oryzae* PXO99^A, and *P. syringae* DC3000. All three wild-type bacteria and their T3SS mutants produced T3Es inside the cell, but only those of the wild-type bacteria were detectable in intracellular medium by Western blotting analysis, indicating the requirement of T3SS for effector secretion (**Figure 4**). BCD03 treatment suppressed secretion of effector HopAO1 and most of effector AvrXa27 in the Hrp group II bacterial strains MH21 and PXO99^A, respectively (**Figures 4A**, **B**). Similarly, secretion of effector AvrPto was suppressed in Hrp group I strain DC3000 (**Figure 4C**). It is remarkable that intracellular production of T3Es in all three bacteria was not reduced, which suggests that only secretion but not expression of T3Es was inhibited by BCD03. Overall, the Western blotting results suggest that BCD03 has a broad-spectrum inhibitory effect on plant pathogen bacterial T3SSs.

Discussion

In this study, we developed a high-throughput system based on a T3E secretion reporter to screen bacterial T3SS inhibitors, which is distinguished from most previous studies using transcriptional reporters of T3SS structure genes, regulatory genes or T3E genes (Kauppi et al., 2003; Gauthier et al., 2005; Li et al., 2005; Nordfelth et al., 2005; Pan et al., 2007; Felise et al., 2008; Pan et al., 2009; Aiello et al., 2010; Enquist et al., 2012; Fan et al., 2017). Inhibitors obtained from previous screening commonly affect transcription of T3SS structural gene clusters and T3E genes (Gauthier et al.,

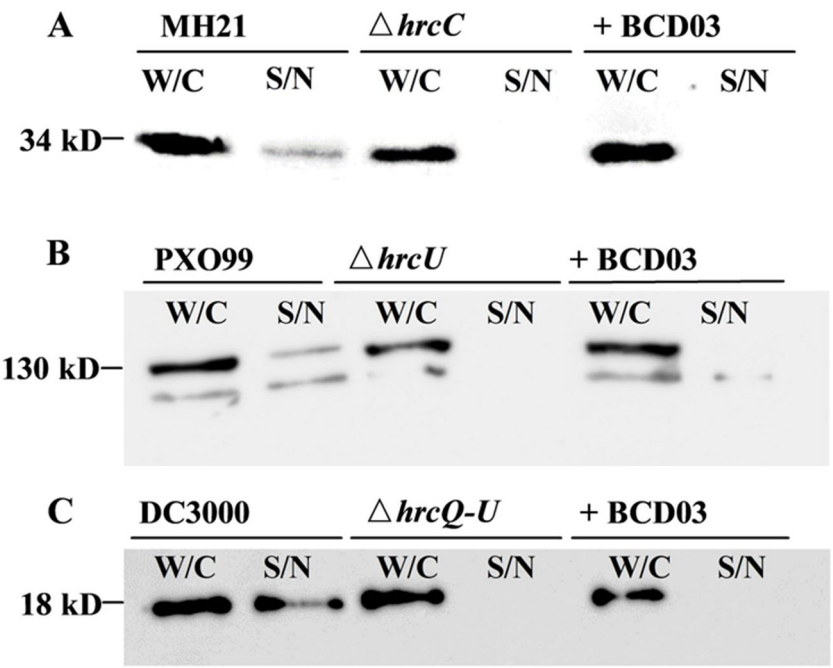

FIGURE 4 | BCD03 inhibition of effector secretion *via* bacterial T3SS. **(A)** *A. citrulli* MH21 bearing the *hopAO1*-FLAG construct. **(B)** *X. oryzae* pv. *oryzae* PXO99^{A} bearing the *avrXa27*-FLAG construct. **(C)** *P. syringae* pv. *tomato* DC3000 bearing the *avrPto*-HA construct. All bacteria were grown for 8 h in their own *hrp*-inducing media with or without BCD03 at a finial concentration of 25 μg/mL. Whole cell extracts (W/C) and supernatants (S/N) were separated by centrifugation, and their secreted proteins were detected with anti-FLAG or anti-HA antibodies.

2005; Lee et al., 2007; Arnoldo et al., 2008; Kim et al., 2009; Garrity-Ryan et al., 2010; Grier et al., 2010; Swietnicki et al., 2011; Yamazaki et al., 2012; Kim et al., 2014; Williams et al., 2015; Marsden et al., 2016), while potential inhibitors targeting post-transcriptional regulation or effector secretion were missed. In this study, we constructed a T3E secretion reporter for inhibitor screening since effector secretion is the end result of T3SS expression, which is delicately regulated by a complex network, including transcriptional and post-transcriptional regulation of T3SS components and regulatory genes, post-translational modification of T3SS structural proteins and assembly of the needle or pilus structure, as well as interactions between T3Es and T3SS structural proteins (Keyser et al., 2008; Harmon et al., 2010; Kimura et al., 2011; Warrener et al., 2014). A similar screening system was developed in *Yersinia* using β-lactamase fused with the T3E YopE, revealing a group of compounds that specifically inhibited translocation of effectors into the host cells without altering the synthesis and secretion of T3Es (Harmon et al., 2010). Revealing T3SS inhibitors with this kind of action model is difficult by using transcriptional reporters, highlighting the importance of secretion reporters in large-scale screening.

Because our screening system detected T3SS-induced extracellular activity of β-lactamase, cell growth had to be tightly controlled to avoid β-lactamase leakage during bacterial cell lysis. With *A. citrulli* MH21, a narrow window of time was revealed when the bacteria were grown in suitable medium for 20 h (OD_{600} = 0.4–0.6), during which extracellular β-lactamase activity was significantly higher in the wild-type strain than its T3SS mutant. This difference was less obvious with further incubation, suggesting that β-lactamase release occurs following cell lysis in the T3SS mutant (**Figure 1B**). β-lactamase was used as the reporter because of its high sensitivity, ease of operation and clear color change readout from yellow to red; however, special attention is required when using it in T3SS inhibitor screening. β-lactamase is not applicable when screening dark-colored compounds, as it can disturb the color readout of the reaction mixture. In addition, the positive compounds obtained using β-lactamase reporter need to be re-tested to avoid false positive results caused by potential lactamase-inhibitory activity.

Structure–function analysis of the BCD derivatives revealed that the single substitution at para-position on the benzene ring had the greatest effect on T3SS inhibition. It is likely that para-position substitution resulted in a stronger binding ability with the targets, or was similar to its competitors. However, although halogen substitutions of the BCD derivatives resulted in an increased T3SS inhibitory effect, it remains unknown whether other substitutions such as hydroxy, alkyl, and sulfo, also play a role in T3SS inhibition, suggesting the possible existence of other highly-efficient BCD derivatives.

The BCD compound BCD03 inhibited T3SS of plant pathogenic bacteria in distinct Hrp groups, suggesting that it targets a common component shared by T3SSs in different bacteria. Some broad-spectrum T3SS inhibitors have already been reported, such as salicylidene, acylhydrazides, and thiazolidinones, both of which affect T3SS in multiple bacterial species (Aiello et al., 2010; Duncan et al., 2012).

Salicylidene acylhydrazides were first found using *Yersinia* as the screening model, and later proved effective against *Chlamydia, Pseudomonas, Escherichia, Salmonella* and *Shigella* bacteria (Kauppi et al., 2003; Gauthier et al., 2005; Muschiol et al., 2006; Hudson et al., 2007; Veenendaal et al., 2009). Similarly, thiazolidinone inhibits the pathogenicity of *Yersinia* by inhibiting the formation of T3SS injectisome, preventing effector secretion of *Francisella* by targeting the type IV secretion system (Felise et al., 2008). Moreover, thiazolidinone was the first T3SS inhibitor reported to inhibit the plant pathogen *P. syringae* (Felise et al., 2008). Accordingly, the inhibitory effect of BCD derivatives on animal pathogenic bacteria is an attractive subject of future study.

The discovery of T3SS inhibitors has become a significant topic in recent years, and a number of inhibitors with different action models have subsequently been examined. Salicylidene acylhydrazides derivatives have diverse inhibitory mechanisms. For example, INP0007 blocks the translocation of Yop T3Es and protects HeLa cells from *Yersinia* infection (Nordfelth et al., 2005), while INP1750 binds to YscN, the ATPase of *Yersinia*, resulting in the suppression of T3Es secretion (Swietnicki et al., 2011). Meanwhile, other inhibitors specifically target T3Es. For example, exosin, which was identified in *P. aeruginosa*, directly blocks the ADP-ribosyltransferase activity of T3E ExoS, while pseudolipasin A suppresses the phospholipase A_2 activity of T3E ExoU (Lee et al., 2007; Arnoldo et al., 2008). Other compounds have also been screened as T3SS inhibitors, such as caminoside A, aurodox, and o-coumaric acid; however, their molecular targets remain unknown (Linington et al., 2002; Kimura et al., 2011; Fan et al., 2017). The BCD derivatives identified in this study had a significant inhibitory effect on T3Es secretion in several plant pathogenic bacteria and showed the ability to suppress the pathogenicity of *A. citrulli* on its natural host. To express sufficient T3Es for Western blotting analysis, the T3E gene *hopAO1* in *A. citrulli* and *avrXa27* gene in *X. oryzae* were placed under control of constitutive expression promoters, making it hard to distinguish between transcriptional differences by comparing Western Blotting bands. Meanwhile, expression of the T3Es gene *avrPto* under its own T3SS-inducing promoter had no obvious influence by BCD03 in terms of transcription of the reporter gene. It enhances our speculation that the BCD derivatives directly blocks T3E secretion through the T3SS complex. Our future study is to identify the potential targets of the BCD compounds in bacteria, which may help us explain their T3SS inhibitory mechanism and determine their applicability.

DATA AVAILABILITY

All datasets for this study are included in the manuscript and the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

LC, Y-NM, J-LL and L-QZ designed the research; J-LL and L-QZ supervised the study; LC and Y-NM performed all the experiments and analyzed data. Y-NM and L-QZ wrote the paper. All authors revised the manuscript and approved the final version for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01059/full#supplementary-material

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