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# Derivatives of Plant Phenolic Compound Affect the Type III Secretion System of *Pseudomonas aeruginosa* via a GacS-GacA Two-Component Signal Transduction System

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**Antibiotic therapy is the most commonly used strategy to control pathogenic infections; however, it has contributed to the generation of antibiotic-resistant bacteria. To circumvent this emerging problem, we are searching for compounds that target bacterial virulence factors rather than their viability. *Pseudomonas aeruginosa*, an opportunistic human pathogen, possesses a type III secretion system (T3SS) as one of the major virulence factors by which it secretes and translocates T3 effector proteins into human host cells. The fact that this human pathogen also is able to infect several plant species led us to screen a library of phenolic compounds involved in plant defense signaling and their derivatives for novel T3 inhibitors. Promoter activity screening of *exoS*, which encodes a T3-secreted toxin, identified two T3 inhibitors and two T3 inducers of *P. aeruginosa* PAO1. These compounds alter *exoS* transcription by affecting the expression levels of the regulatory small RNAs RsmY and RsmZ. These two small RNAs are known to control the activity of carbon storage regulator RsmA, which is responsible for the regulation of the key T3SS regulator ExsA. As RsmY and RsmZ are the only targets directly regulated by GacA, our results suggest that these phenolic compounds affect the expression of *exoS* through the GacSA-RsmYZ-RsmA-ExsA regulatory pathway.**

The type III secretion system (T3SS) is a highly specialized protein secretion apparatus that facilitates the translocation of effector proteins from the bacterial cytoplasm directly into host cells. The structure, as well as the function of this system, is conserved among several Gram-negative bacteria, including human pathogens and phytopathogens (20). *Pseudomonas aeruginosa*, a bacterial pathogen which causes opportunistic infections in humans, possesses a single set of genes encoding the structural components and effectors for the T3SS (16). Along with biofilm formation and quorum sensing, the T3SS is one of the major virulence factors of *P. aeruginosa* (45). Through the T3SS, this human pathogen secretes and injects four known effectors into host cells: ExoS, ExoT, ExoU, and ExoY. ExoS and ExoT are closely related bifunctional proteins with N-terminal GTPase activating protein (GAP) activity and C-terminal ADP ribosylase (ADPRT) activity (14, 43). The GAP domains of both ExoS and ExoT are responsible for the disruption of the actin cytoskeleton, the inhibition of bacterial internalization into epithelial cells and macrophages, the induction of host cell rounding, and the prevention of wound healing (15). Along with these functions, ExoT inhibits cell division and can induce apoptosis in epithelial cells (39, 40). ExoU has phospholipase A2 activity and causes rapid cell death (42). ExoY is an adenylate cyclase and may disrupt the actin cytoskeleton (10). By secreting these effector proteins via the T3SS, *P. aeruginosa* efficiently inhibits wound repair and the host innate immune response to facilitate its colonization and its ability to cause injury.

The T3SS of *P. aeruginosa* consists of 43 genes that encode the T3 machinery, regulatory functions, T3 effectors, and effector-specific chaperones (16). All T3SS genes, including the genes encoding T3 effectors, such as *exoS*, are under the direct transcriptional control of ExsA, an AraC-type transcriptional activator. The function of ExsA as a transcriptional activator is thought to be regulated by a coupling model in which three interacting proteins (ExsC, ExsD, and ExsE) play roles in controlling ExsA activity (see Fig. 6). ExsD, the antiactivator, inhibits the transcription of the T3SS by directly binding to ExsA (28). ExsC functions as an anti-antiactivator by binding to and sequestering ExsD, thereby inhibiting the negative effect of ExsD on ExsA (13). Finally, ExsE antagonizes the regulatory effect of ExsC by physical binding (37). This protein interaction regulatory system is controlled by T3 secretory activity, which is greatly induced upon host cell contact or calcium depletion (24, 28). Besides ExsA activation by the induction of the T3 secretory activity, *exsA* expression is regulated primarily by three pathways: CyaB-cAMP/Vfr, GacSA-RsmYZ-RsmA, and PsrA-RpoS. When cells encounter low calcium conditions, an adenylate cyclase (CyaB) is activated (50) and produces cyclic AMP (cAMP). Together with the cAMP-regulatory protein Vfr, a high level of cAMP controls *exsA* expression along with quorum sensing (12, 34). The *exsA* expression of *P. aeruginosa* also is positively regulated by a carbon storage regulator, RsmA. GacS, a tripartite sensor histidine kinase, senses environmental stimuli and activates its cognate response regulator GacA by phosphorylation,

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which in turn induces the expression of regulatory small RNAs RsmY and RsmZ (5, 46). RsmY and RsmZ transcripts then bind to and sequester RsmA, which eventually reduces the expression of *exsA* (31). In addition to these regulatory cascades, PsrA also has been reported to regulate T3SS. PsrA, a long-chain fatty acid sensory regulator, directly binds to the promoter region of the *exsCEBA* operon and positively regulates the expression of these genes (41). Along with this, PsrA also binds to the promoter region of *rpoS* and positively regulates its transcription, which in turn represses *exsA* expression and other T3SS genes (18).

Controlling diseases in humans, animals, and plants is important to sustain our society, and the most commonly used strategy is traditional antibiotic therapy. However, most antibiotics kill bacteria by inhibiting cellular processes essential for survival, which leads to a strong selective pressure to develop resistance against antibiotics. The long-term use of large quantities of antibiotics has contributed to the acquisition of antibiotic resistance in both agriculturally and medically important bacteria, including *P. aeruginosa* (2). Therefore, society urgently needs alternative approaches to develop effective therapies against pathogens without affecting their growth in order to avoid creating antibiotic-resistant bacteria (23). The T3SS is an attractive target for the development of therapeutic agents, because it is the major virulence factor of many animal and plant pathogenic bacteria and is not necessary for their viability (20). Until now, studies have been focused on the identification of small molecules that block the secretion of T3SS of human pathogens (1, 19, 22, 33, 44). MBX 1641 is a potent blocker of the *P. aeruginosa* T3SS (1). Although the mode of action of this compound remains unknown, the authors demonstrated that MBX 1641 was able to block the T3-mediated secretion of ExoS, and this blockage of T3SS resulted in the reduction of the pathogenicity of *P. aeruginosa* and other Gram-negative bacteria (1). Recently, we identified a plant phenolic compound, *p*-coumaric acid (PCA), which inhibits the T3SS of the phytopathogen *Dickeya dadantii* (27). Given that the human opportunistic pathogen *P. aeruginosa* also can infect plant species (48), we hypothesized that some of the plant phenolic compounds and/or their analogues also affect *P. aeruginosa* virulence factors, such as the T3SS. In this study, we describe novel derivatives of plant phenolic compounds that alter the promoter activity of *exoS*, which encodes a T3SS effector of *P. aeruginosa* strain PAO1. Our results indicate that these compounds affect T3SS gene expression primarily through the GacSA-RsmYZ-RsmA-ExsA pathway.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PAO1 and *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 6.8) at 37°C. LB broth supplemented with 200 mM NaCl and 10 mM nitrilotriacetic acid (NTA) was used for the induction of *P. aeruginosa* PAO1 T3SS (38). When required, antibiotics were added as follows: 100 $\mu$g/ml of ampicillin (Ap) and 200 $\mu$g/ml of carbenicillin (Car).

**Recombinant DNA techniques.** The preparation of genomic or plasmid DNA, PCR, restriction digestion, ligation, DNA electrophoresis, and electroporation were performed as described by Ausubel and associates (3).

**Green fluorescent protein (GFP) transcriptional reporter screening.** To screen compounds that induce or inhibit the expression of *P. aeruginosa* PAO1 T3SS, a 510-bp fragment containing the promoter region of *exoS* was PCR amplified using the primer set PexoS-F (5′-cagGGATCCttgttcgagttgatggtgga) and PexoS-R (5′-ccgGAATTCgatg gttgccttctcctgatg). Capitalized letters in the forward and reverse primer sequences indicate the recognition sites for BamHI and EcoRI, respectively. The amplified fragment and pPROBE-AT, a broad-host-range vector carrying promoterless *gfp* (29), were digested with EcoRI and BamHI, gel purified, and ligated to create pAT-exoS. This plasmid then was transferred to *P. aeruginosa* PAO1 by electroporation (8).

**TABLE 1** Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s)[a] | Source or reference |
|---|---|---|
| Strains | | |
| *P. aeruginosa* PAO1 | Wild-type *P. aeruginosa* | |
| *E. coli* DH10B | F$^-$ *endA1 recA1 galE15 galK16 nupG rpsL* Δ*lacX74* Φ*80lacZ*ΔM15 *araD139* Δ(*ara,leu*)*7697 mcrA* Δ(*mrr-hsdRMS-mcrBC*) λ$^-$ | Invitrogen |
| Plasmids | | |
| pPROBE-AT | Broad-host-range vector containing promoterless *gfp*, Ap$^r$ | 29 |
| pAT-exoS | pPROBE-AT carrying a 510-bp fragment containing *exoS* promoter, Ap$^r$ | This work |

[a] Ap$^r$, resistant to ampicillin.

For T3 induction and screening, an overnight culture of PAO1 carrying pAT-exoS was diluted 1:200 into fresh LB broth supplemented with 200 mM NaCl and grown for 2 h at 37°C. The cultures then were diluted into an equal volume with fresh medium supplemented with 10 mM NTA (38). At this time, compounds were added to the culture at a final concentration of 250 $\mu$M. Bacteria then were incubated with aeration for another 6 h at 37°C and subsequently chilled on ice for at least 10 min (38). The bacterial cultures were diluted with PBS and assayed for fluorescence using fluorescence-activated cell sorter (FACS) flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ). The *exoS* expression levels in the presence of compounds were standardized using the dimethylsulfoxide (DMSO) treatment group as the reference. The z score was calculated according to the formula $z = (x - \mu)/\sigma$, where $x$ is a raw score to be standardized, $\mu$ is the mean of the population, and $\sigma$ is the standard deviation of the population. Two independent experiments were performed, and four replicate samples were used in each experiment.

**Sources of the screened compounds.** Compounds TS001 to TS035, TS108 to TS113, and TS134 to TS136 were purchased from commercial sources (Aldrich, Alfa Aesar, or TCI). The remaining compounds were synthesized via the routes described in the supplemental material and other reported procedures (9, 26, 32, 36, 54). All compounds were dissolved in DMSO.

**Measurement of bacterial growth.** *P. aeruginosa* PAO1 carrying pAT-exoS was cultured in LB supplemented with 200 mM NaCl, and the T3SS was induced by the addition of NTA as mentioned above in the presence or absence of compounds. At the time points indicated, the optical density at 600 nm ($OD_{600}$) of the culture was measured using a Synergy HT multimode microplate reader (BioTek, Winooski, VT). Two independent experiments were performed, and three replicate samples were used in each experiment.

**Western blotting.** For Western blotting experiments, *P. aeruginosa* PAO1 cells grown overnight in LB broth were subcultured 1:1,000 in fresh LB supplemented with 200 mM NaCl and 10 mM NTA and grown for 4 h at 37°C with aeration in the presence or absence of either inhibitors or inducers at a final concentration of 250 $\mu$M. The culture supernatant was collected by centrifugation, and proteins were concentrated by ultrafiltration. Proteins were separated by SDS-PAGE, blotted to a polyvinylidene fluoride (PVDF) membrane using a Trans-blot SD semidry transfer cell (Bio-Rad Laboratories, Hercules, CA), and subjected to immunodetec-

**TABLE 2** Screening of inhibitors and inducers of *P. aeruginosa* T3SS

| Compound no. | *exoS* expression (MFI) | z score[b] | Compound no. | *exoS* expression (MFI) | z score[b] |
|---|---|---|---|---|---|
| DMSO[a] | 116.27 ± 6.81 | | TS034 | 93.68 ± 8.27 | 0.55 |
| TS001 | 95.02 ± 1.87 | 0.51 | TS035 | 96.30 ± 1.18 | 0.47 |
| TS002 | 102.78 ± 9.94 | 0.28 | TS100 | 115.06 ± 14.42 | 0.08 |
| TS003 | 107.73 ± 1.28 | 0.13 | TS101 | 78.03 ± 1.50 | 1.01* |
| TS004 | 112.32 ± 9.96 | 0.00 | TS102 | 104.35 ± 5.11 | 0.23 |
| TS005 | 107.77 ± 6.97 | 0.13 | TS103 | 246.35 ± 20.79 | 4.16** |
| TS006 | 92.89 ± 4.77 | 0.57 | TS104 | 102.49 ± 3.68 | 0.29 |
| TS007 | 93.54 ± 7.91 | 0.55 | TS105 | 112.04 ± 2.79 | 0.00 |
| TS008 | 106.43 ± 3.65 | 0.17 | TS106 | 97.85 ± 2.30 | 0.42 |
| TS009 | 98.85 ± 4.26 | 0.39 | TS107 | 100.90 ± 2.42 | 0.33 |
| TS010 | 104.61 ± 12.16 | 0.22 | TS110 | 106.23 ± 0.67 | 0.18 |
| TS011 | 90.24 ± 9.57 | 0.65 | TS111 | 97.61 ± 5.62 | 0.43 |
| TS012 | 98.19 ± 6.09 | 0.41 | TS112 | 102.20 ± 5.98 | 0.30 |
| TS013 | 107.72 ± 10.83 | 0.13 | TS113 | 104.26 ± 9.41 | 0.24 |
| TS014 | 109.59 ± 14.70 | 0.08 | TS114 | 98.41 ± 3.24 | 0.41 |
| TS015 | 102.41 ± 11.54 | 0.29 | TS115 | 107.97 ± 7.04 | 0.13 |
| TS016 | 120.09 ± 16.79 | 0.23 | TS117 | 90.84 ± 3.67 | 0.63 |
| TS017 | 99.76 ± 7.08 | 0.37 | TS120 | 105.16 ± 8.52 | 0.21 |
| TS018 | 105.31 ± 8.90 | 0.20 | TS121 | 107.08 ± 3.92 | 0.15 |
| TS019 | 126.51 ± 6.63 | 0.42 | TS122 | 100.87 ± 10.45 | 0.34 |
| TS020 | 99.15 ± 7.65 | 0.39 | TS123 | 109.04 ± 8.37 | 0.09 |
| TS021 | 96.07 ± 7.20 | 0.48 | TS124 | 105.06 ± 2.10 | 0.21 |
| TS022 | 114.22 ± 16.66 | 0.06 | TS125 | 159.61 ± 10.65 | 1.40 |
| TS023 | 99.28 ± 9.59 | 0.38 | TS126 | 105.77 ± 8.29 | 0.19 |
| TS024 | 105.98 ± 9.66 | 0.18 | TS127 | 83.66 ± 10.42 | 0.84 |
| TS025 | 121.31 ± 6.06 | 0.27 | TS129 | 136.48 ± 13.99 | 0.72 |
| TS026 | 124.18 ± 4.43 | 0.35 | TS130 | 162.74 ± 4.58 | 1.49 |
| TS027 | 71.48 ± 8.14 | 1.20* | TS131 | 123.90 ± 7.51 | 0.35 |
| TS028 | 110.43 ± 14.76 | 0.05 | TS132 | 182.50 ± 15.46 | 2.08 |
| TS029 | 120.76 ± 2.02 | 0.25 | TS133 | 134.51 ± 16.00 | 0.66 |
| TS030 | 92.74 ± 2.97 | 0.58 | TS134 | 293.96 ± 39.43 | 5.37** |
| TS031 | 87.00 ± 5.39 | 0.75 | TS135 | 120.40 ± 8.62 | 0.23 |
| TS033 | 104.96 ± 8.59 | 0.21 | TS136 | 106.71 ± 8.86 | 0.16 |

[a] The *exoS* expression level in the presence of DMSO is the average of all subsets of experiments.
[b] An asterisk represents a z score of ≥1.00 for T3 inhibitors, and two asterisks represents a z score of ≥4.00 for T3 inducers.

tion. The blotted membrane then was washed three times with phosphate-buffered saline (PBS) and incubated in blocking solution (5% [wt/vol] skim milk in PBS) overnight. After washing three times with the washing buffer (PBS containing 0.3% [vol/vol] Triton X-100), the membrane was incubated in washing buffer supplemented with an anti-ExoS polyclonal antibody (Accurate Chemical & Scientific Corp., Westbury, NY) for 1.5 h and then in the washing buffer supplemented with an anti-chicken IgG (H+L) conjugated with alkaline phosphatase (AP) (SouthernBiotech, Birmingham, AL) for another 1.5 h. After each incubation, the membrane was washed three times with washing buffer. The membrane then was incubated in AP reaction buffer (100 mM Tris base, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$) for 5 min, and ExoS proteins were detected by the chromogenic detection method. The results were analyzed using ImageJ (http://imagej.nih.gov/ij/index.html) to obtain the relative intensity of the secreted ExoS protein level.

**Quantitative reverse transcription-PCR (qRT-PCR).** *P. aeruginosa* PAO1 was cultured and treated by the same procedure as mentioned for the GFP transcriptional reporter screen. Total bacterial RNA was isolated using an RNeasy minikit (Qiagen, Valencia, CA), and cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA) with random hexamer as a primer by following the manufacturer's instructions. Real-time PCR then was performed using RealMasterMix SYBR ROX (5 prime; Gaithersburg, MD) and a DNA Engine OPTICON2 (MJ Research, Waltham, MA). The comparative threshold cycle ($C_T$) method was used for the analysis of transcriptional levels for the tested genes as described previously (4). To calculate the relative expression level of target genes, the expression level of 16S rRNA was used as an internal control. All PCR experiments were performed in three replicates to obtain standard deviations, and the Tukey-Kramer method was used for the statistical analysis.

**Quantification of biofilm formation.** Biofilm formation was quantified according to a method previously described (35), with minor modifications. Briefly, overnight *P. aeruginosa* PAO1 cells were subcultured 1:100 into fresh LB broth in microtiter wells (nontreated, polystyrene 96-well plate) supplemented with compounds at a final concentration of 250 $\mu$M or with DMSO as a control. After 12 h of incubation at 37°C without agitation, one-fourth of the culture volume of the crystal violet solution (1%, wt/vol) was added to each sample well, and the mixture was incubated at room temperature for 15 min. Wells were washed twice with distilled water and were filled with 200 $\mu$l of 95% ethanol to solubilize crystal violet in the solvent. The eluent (125 $\mu$l) was transferred to a new microtiter well, and the absorbance was determined with a Synergy HT multimode microplate reader (BioTek, Winooski, VT) at 600 nm. Two independent experiments were performed, and three replicate samples were used in each experiment.

**Statistical analysis.** Means and standard deviations were calculated using Excel (Microsoft, Redmond, WA), and the statistical analysis was performed using R version 2.8.1 (http://www.r-project.org/).

## RESULTS AND DISCUSSION

**Screening of inhibitors and inducers of *P. aeruginosa* T3SS.** Our inventory of compounds consists of salicylic acid, its precursors,

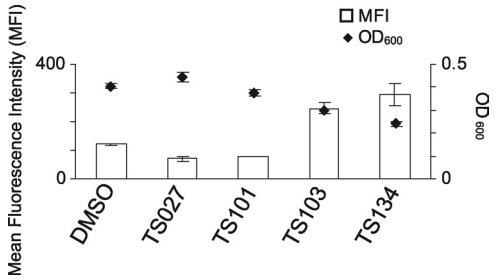


**FIG 1** Effect of compounds on *exoS* expression and bacterial growth. The promoter activity of *exoS* and growth of *P. aeruginosa* PAO1 were measured by flow cytometry and spectrophotometry, respectively, at 12 h after inoculation. Bacterial cells were grown in LB broth supplemented with 200 mM NaCl, 10 mM NTA, and 250 $\mu$M compounds (or the same volume of DMSO). Bars represent *exoS* expression as MFI, and dots represent cell density as the $OD_{600}$.

and their analogues (27) (see the supplemental material). These compounds were screened for inhibitors and inducers of *P. aeruginosa* PAO1 T3SS by monitoring the promoter activity of *exoS*, which encodes a T3SS effector. Sixty-five out of 72 compounds did not form a precipitate when added to the culture medium (data not shown). These compounds then were screened for the alteration of *exoS* promoter activity using pAT-exoS and FACS. *P. aeruginosa* PAO1 carrying pAT-exoS was grown in T3SS inducing conditions (200 mM NaCl and 10 mM NTA) in the presence of compounds at a final concentration of 250 $\mu$M, and the mean fluorescence intensity (MFI) was measured. TS027 and TS101 were identified as potential T3 inhibitors, with an inhibition of MFI by at least 1 standard deviation below the sample population average (z score, $\geq$1.00) (Table 2). In addition to inhibitors, TS103 and TS134 were identified as potential T3 inducers by at least 4 standard deviations above the sample population average (z score, $\geq$4.00) (Table 2).

We measured the bacterial growth 12 h postinoculation in the presence or absence of compounds. T3 inhibitors TS027 and TS101 had almost no effect on the growth of *P. aeruginosa* PAO1 at a concentration of 250 $\mu$M, whereas T3 inducers TS103 and TS134 showed some growth inhibition at the same concentration (Fig. 1). These four compounds were subjected to further investigation.

**Dose-dependent effects of compounds on *exoS* promoter activity and bacterial growth.** The *exoS* expression and bacterial growth of *P. aeruginosa* PAO1 were examined in medium supplemented with different concentrations of compounds. TS027 began to show an inhibitory effect on *exoS* expression at 20 $\mu$M, whereas TS101 did not ($P < 0.01$) (Fig. 2A). At 50 $\mu$M, both TS027 and TS101 inhibited *exoS* expression at a similar level, and this inhibitory effect increased as the compound concentration increased (Fig. 2A). These T3 inhibitors showed little alteration of bacterial growth when added to the medium at final concentrations of 250 $\mu$M or less ($P < 0.01$) (Fig. 2C). TS027 had almost no effect on the bacterial growth even at 500 $\mu$M, whereas TS101 showed a significant difference in growth at the same concentration ($P < 0.01$) (Fig. 2C). On the other hand, significant differences in *exoS* expression were observed at all concentrations (20 to 250 $\mu$M) when two T3 inducers, TS103 and TS134, were added to the culture ($P < 0.01$) (Fig. 2B), whereas these compounds had relatively strong growth inhibition compared to that of other T3 inhibitors (significant differences at 250 and 500 $\mu$M for TS103 and 100, 250, and 500 $\mu$M for TS134; $P < 0.01$) (Fig. 2D). Since our goal is to identify and characterize potential T3 inhibitors of *P. aeruginosa* PAO1, compounds were added to the culture at a final concentration of 250 $\mu$M for subsequent experiments. Although potential T3 inducers showed the growth inhibition of *P. aeruginosa* PAO1 cells, they were used in the following experiments to examine the regulatory mechanism by which T3 inhibitors altered the promoter activity of *exoS*.

**Validation of T3 inhibitors and inducers of ExoS secretion.** To evaluate the effect of T3 inhibitors/inducers on the secretion of the

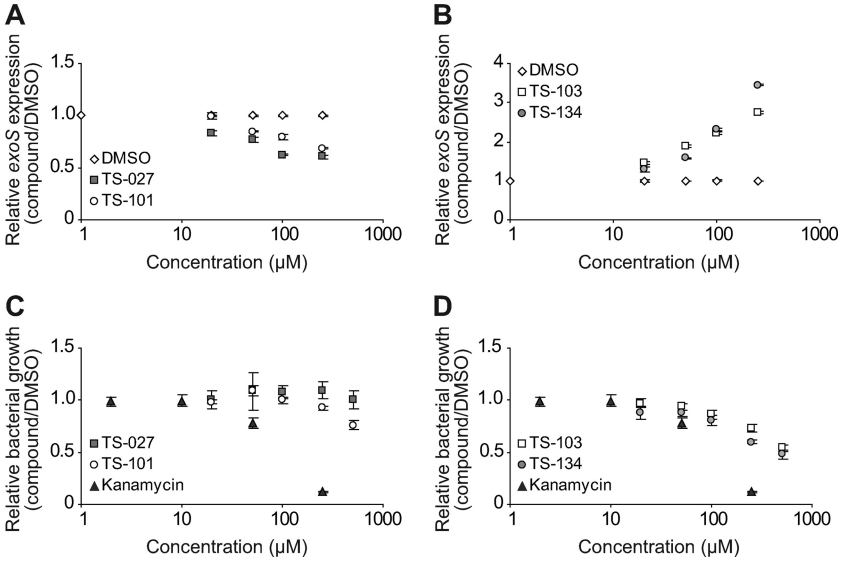


**FIG 2** Evaluation of the dose-dependent effect of compounds on *exoS* expression (A and B) and bacterial growth (C and D). *P. aeruginosa* PAO1 cells carrying pAT-exoS were precultured in LB broth supplemented with 200 mM NaCl and transferred to fresh LB broth with 200 mM NaCl, 10 mM NTA, and various concentrations of compounds or DMSO for 6 h. (A and C) Potent T3 inhibitors TS027 and TS101. (B and D) Potent T3 inducers TS103 and TS134. *y* axes represent the relative *exoS* expression level (A and B) or the relative bacterial growth (C and D). Kanamycin was used as a bactericidal control (C and D).

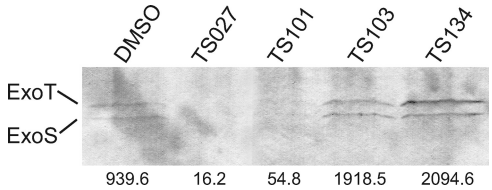


**FIG 3** Alteration of the secretion level of T3 effector ExoS by T3 inhibitors/inducers. *P. aeruginosa* PAO1 cells were grown in LB broth supplemented with 200 mM NaCl, 10 mM NTA, and 250 $\mu$M either TS027 (lane 2), TS101 (lane 3), TS103 (lane 4), or TS134 (lane 5). The same volume of DMSO was added to the culture as a negative control (lane 1). The Western blot was performed using an anti-ExoS polyclonal antibody. Numbers below the Western blot indicate the relative intensity of secreted ExoS provided by ImageJ.

T3 effector ExoS, Western blotting using an anti-ExoS polyclonal antibody was performed. In agreement with the *exoS* promoter activity results (Fig. 1), the level of secreted ExoS was reduced in the presence of T3 inhibitors TS027 and TS101 at a final concentration of 250 $\mu$M, whereas the same concentration of T3 inducers TS103 and TS134 increased the level of ExoS secreted from the cells (Fig. 3). Similarly to an earlier report, along with ExoS, another T3 effector ExoT was detected in the supernatant (Fig. 3) (11). This could be because the polyclonal anti-ExoS antibody used in this study cross-reacts with ExoT as described previously (11).

**TS027 and TS103 affect T3SS through the Rsm-ExsA pathway.** The T3SS of *P. aeruginosa* is regulated by a key T3 regulator, ExsA, and several regulatory cascades control *exsA* expression and ExsA activity (51). To determine which pathways were affected by the compounds and how this affected the expression level of *exoS*, the mRNA levels of seven genes responsible for the T3SS regulation in *P. aeruginosa* (*rsmA*, *rsmY*, *rsmZ*, *psrA*, *rpoS*, *cyaB*, and *vfr*), and also the T3 master regulator *exsA*, were examined (see Fig. 6). Since there are some structural similarities between TS027/TS101 and TS103/TS134 (see the supplemental material), TS027 and TS103 were used for qRT-PCR as representatives of T3 inhibitors and inducers, respectively. In the presence of the T3 inhibitor TS027, the *exsA* transcripts were reduced to the T3SS noninduced level (Fig. 4). On the other hand, the *exsA* mRNA level increased almost 3-fold in the presence of the potential T3 inducer TS103 compared to the level of the DMSO control (Fig. 4). These results

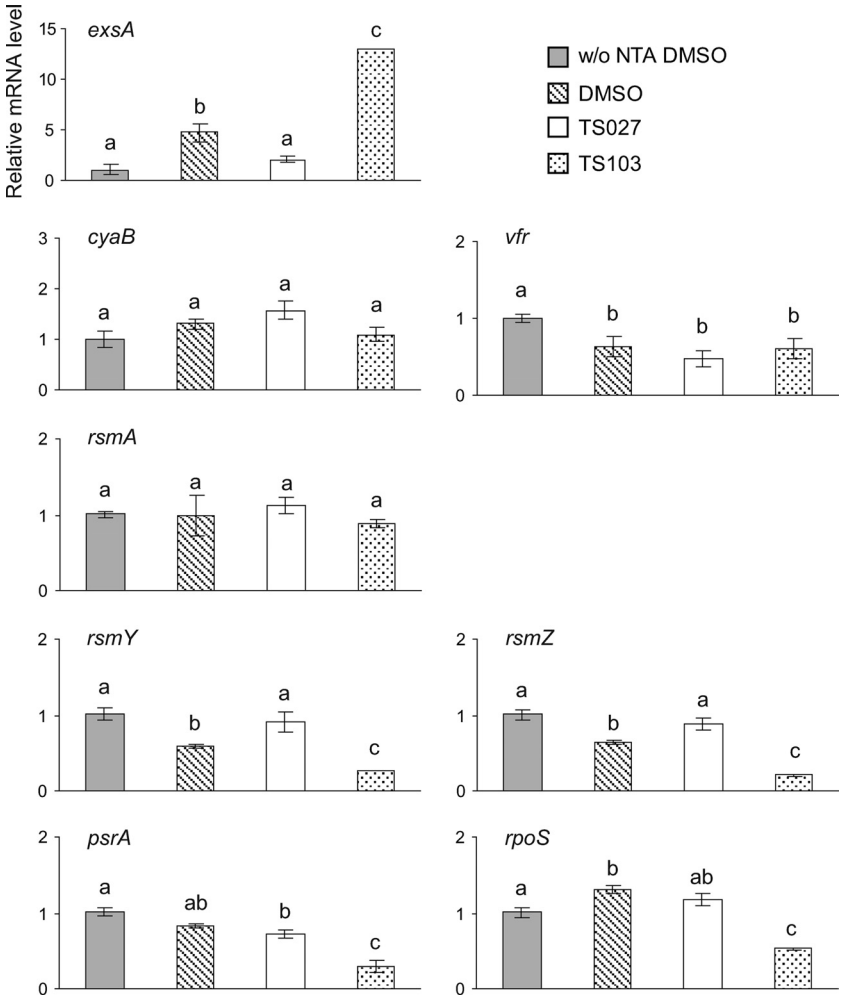


**FIG 4** mRNA levels of known T3 regulatory factors in the presence of compounds. *P. aeruginosa* PAO1 cells were grown in T3 noninduction medium with DMSO (gray), T3 induction medium with DMSO (hatched line), T3 induction medium with TS027 (open), or T3 induction medium with TS103 (dotted). Compounds were applied to the medium at a final concentration of 250 $\mu$M. *y* axes represent the relative expression level of each gene (qRT-PCR). Bars labeled with different letters represent significantly different means as revealed by the Tukey-Kramer method at $P < 0.01$.

indicate that the effect of the compounds on the *exoS* promoter activity is due to the alteration of the *exsA* mRNA level. In *P. aeruginosa*, RsmA positively regulates the expression of *exsA*. Additionally, regulatory small RNAs RsmY and RsmZ bind to the RsmA protein and neutralize its effect on *exsA* expression. Significant differences were observed in mRNA levels of *rsmY* and *rsmZ* in the presence of the compounds (Fig. 4). The reduction of *rsmY* and *rsmZ* transcripts was observed in the presence of TS103, whereas TS027 increased the mRNA levels of both genes (Fig. 4). Given that the regulatory small RNAs RsmY and RsmZ regulate the activity of RsmA protein (7, 46), our results indicate that TS027 and TS103 affect *exsA* expression through the RsmYZ-RsmA regulatory pathway. Since RsmY and RsmZ regulate RsmA activity at the protein level, it is not surprising to observe similar mRNA levels of *rsmA* in the presence or absence of compounds (Fig. 4). Additionally, as Brencic and colleagues found that the phosphorylated GacA binds to the promoter regions of *rsmY* and *rsmZ*, which are direct targets of the GacS-GacA two-component signal transduction system (6), our results suggest that TS027 and TS103 affect the activation of GacA through GacS and/or other two-component sensor proteins that cross-talk to GacS-GacA, such as RetS or LadS (5, 7) (see Fig. 6).

**TS103 affects the expression of *psrA* and *rpoS*.** The mRNA levels of *psrA* and *rpoS* were reduced in the presence of the T3 inducer TS103 (Fig. 4). As a major part of the promoter activity of *rpoS* relies on a TetR family regulator (PsrA) in *P. aeruginosa* (25), the decrease in the *rpoS* transcripts caused by TS103 may be due to the reduction in *psrA* mRNA. Meanwhile, no significant difference in *psrA* or *rpoS* mRNA levels was observed in the presence of the T3 inhibitor TS027 (Fig. 4). While GacA positively controls *psrA* expression in *Pseudomonas chlororaphis* (17, 47), our result suggests that the *psrA* expression of *P. aeruginosa* PAO1 is independent of GacS-GacA, since there was no significant influence on the expression of *psrA* or *rpoS* when the T3 inhibitor TS027 was applied to the culture (Fig. 4). Alternatively, it still is possible that GacA controls *psrA* expression in *P. aeruginosa* PAO1; however, the effect of TS027 on GacA might not be strong enough to cause a significant change in *psrA* and *rpoS* mRNA levels. In addition to the positive regulation of the *exsCEBA* operon by PsrA via direct binding to its promoter region, PsrA also has a negative effect on *exsA* expression through RpoS (41). These two regulatory pathways may partially offset their effects on *exsA* expression. Besides the PsrA-RpoS pathway, the expression of *exsA* is controlled by a membrane-bound adenylate cyclase (CyaB) and a cAMP-dependent transcriptional regulator (Vfr) (12, 50). A decrease in *vfr* mRNA was observed in *P. aeruginosa* PAO1 when cells were grown in T3 inducing conditions compared to that in noninducing conditions by an unknown mechanism (Fig. 4). Similar mRNA levels of *cyaB* and *vfr* were observed in the presence or absence of compounds, suggesting that TS027 and TS103 had no significant effect on the *cyaB* and *vfr* mRNA level compared to that of the DMSO treatment (Fig. 4).

**TS103 and TS134 reduce biofilm formation.** In *P. aeruginosa*, the expression of two reciprocally controlled phenotypes, T3SS and biofilm formation, are known to be predominately coordinated by a single regulatory cascade, GacSA-RsmYZ-RsmA (6, 7). Since several compounds were shown to inhibit or induce the expression of T3SS, the effect of those compounds on the biofilm formation of *P. aeruginosa* PAO1 also was investigated. The biofilm formation of *P. aeruginosa* PAO1 was significantly diminished in the presence of potential T3 inducers TS103 and TS134 (Fig. 5), which is consistent with the fact that the compound TS103 induced T3SS expression via the GacSA-RsmYZ-RsmA regulatory cascade (Fig. 1 and 4). Along with RsmA, RpoS also is known to control biofilm formation by regulating the transcription of *pslA*, whose product functions as a bacterial sugar transferase (21). Therefore, the reduction in the *rpoS* transcript can partially account for the reduction of the biofilm formation in the presence of T3 inducers (Fig. 4). Additionally, reduction in biofilm formation in the presence of T3 inducers may be due partially to their inhibitory effect on bacterial growth (Fig. 1 and 5). Interestingly, the presence of T3 inhibitors TS027 and TS101 did not, however, result in significant differences in biofilm formation compared to that with DMSO (Fig. 5). This could be due to the effect of those inhibitors on the *rsmY* and *rsmZ* transcripts, which were relatively low compared to that when the cultures were supplemented with inducers (Fig. 5). As a result, these inhibitors did not reach the threshold concentration that is required to alter the biofilm formation.

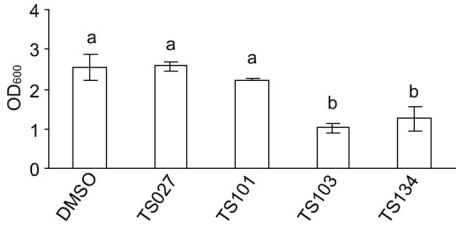


**FIG 5** Effect of compounds on the biofilm formation of *P. aeruginosa* PAO1. Cells were inoculated to LB supplemented with compounds (250 $\mu$M) or DMSO as a control and incubated for 12 h. Bars labeled with different letters represent significantly different means as revealed by the Tukey-Kramer method at $P < 0.01$.

To date, a couple of T3 inhibitors of human pathogens have been identified by screening-based approaches (1, 22). Instead of applying a random library containing a vast number of chemicals for the screening of T3 inhibitors, in this study we used a library of small molecules derived from plant phenolic compounds based on evidence that *P. aeruginosa* is able to infect several plant species, such as *Arabidopsis* and lettuce (48). Using the subset of small molecules and their derivatives, we have already found several candidates which either inhibit or induce the T3SS of phytopathogens (27, 52) (unpublished data). Interestingly, TS027 (*trans*-4-aminocinnamic acid), a potential T3 inhibitor of *P. aeruginosa* found in this study, has been reported as a T3 inducer of the soft-rot phytopathogen *D. dadantii* (27). Unlike that of *P. aeruginosa*, the RsmA protein of *D. dadantii* negatively regulates the T3SS expression by promoting the degradation of *hrpL* mRNA, which encodes a master regulator of the T3SS (49). RsmB, a regulatory small RNA molecule, binds to RsmA and neutralizes the degradative effect of RsmA on *hrpL* mRNA (30). The expression of *rsmB* is upregulated by the GacS-GacA two-component signal transduction system (53). Since the chemical structure of TS027 is very similar to that of *trans*-cinnamic acid (TCA), except for the presence of an amine group on the phenol ring (27), and because TCA induces the expression of T3 genes, probably through the GacS-GacA two-component signal transduction system (52), it is possible that TS027 upregulates the T3SS of *D. dadantii* by increasing the RsmB RNA level through the GacS-GacA system. Overall, this antithetical effect of TS027 in different bacterial spe-

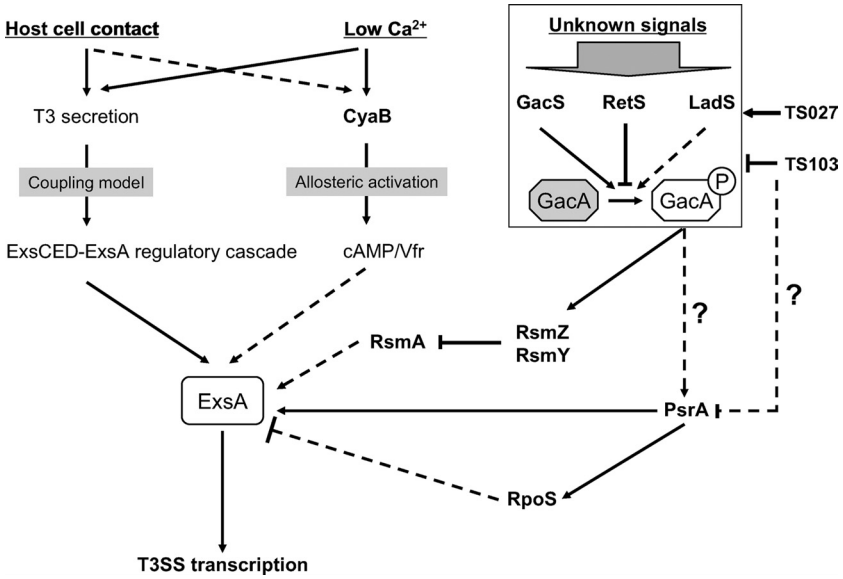


**FIG 6** Schematic of the known *P. aeruginosa* T3 regulation. Solid lines indicate direct connections (protein-protein interaction, direct binding to the promoter region, or compound synthesis), and dashed lines indicate indirect connections or hypothetical regulatory links. Host cell contact and $Ca^{2+}$ depletion induce T3SS in two independent cascades, the activation of ExsE secretion via a T3SS to relieve ExsA from the antiactivator ExsD and the activation of CyaB, an adenylate cyclase, to increase cAMP levels in cells, which in turn activates the cAMP binding regulator Vfr. Besides these cascades, *P. aeruginosa* has other pathways to perceive unknown environmental signals. Upon contact with environmental stimuli, GacS phosphorylates GacA, which in turn activates the expression of small regulatory RNAs RsmY and RsmZ. The expression of *exsA* is induced by RsmA, a carbon storage regulator, which can be sequestered by those small regulatory RNAs. The GacA-PsrA regulatory pathway has been demonstrated in *P. chlororaphis* and likely exists in *P. aeruginosa* as well. A transcriptional regulator, PsrA, directly binds to the promoter region of the *exsCEBA* operon to activate *exsA* expression. Simultaneously, PsrA negatively regulates *exsA* expression through RpoS. In this study, we found that TS027 and TS103 alter the promoter activity of *exoS* (ExsA regulon) through GacSA-RsmYZ-RsmA-ExsA. TS103 may induce *exsA* expression through PsrA-RpoS independently of GacSA.

cies may be attributed to the fact that in both *D. dadantii* and *P. aeruginosa* T3SS expression is influenced by the regulatory system that consists of a carbon storage regulator, RsmA, and a small regulatory RNA that acts as the antagonist of RsmA, and these have opposing consequences on T3SS expression (31, 53).

In summary, we discovered small phenolic molecules that affect the T3SS of *P. aeruginosa* via the GacSA-RsmYZ-RsmA-ExsA regulatory pathway (Fig. 6). In addition, TS103 may negatively affect the expression of *psrA* independently of GacSA-RsmYZ-RsmA, which in turn induces the expression of *exsA* through RpoS (Fig. 6), although this pathway is thought to have only a minor effect on the T3SS in *P. aeruginosa* (41). Based on the results obtained in this study, new compounds will be synthesized according to the structure-activity relationships (SARs) to create potent T3 inhibitors.

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## REFERENCES

1. **Aiello D, et al.** 2010. Discovery and characterization of inhibitors of *Pseudomonas aeruginosa* type III secretion. Antimicrob. Agents Chemother. **54**:1988–1999.
2. **Ash C.** 1996. Antibiotic resistance: the new apocalypse? Trends Microbiol. **4**:371–372.
3. **Ausubel FM, et al.** 1987. Current protocols in molecular biology. John Wiley, New York, NY.
4. **Babujee L, Venkatesh B, Yamazaki A, Tsuyumu S.** 2007. Proteomic analysis of the carbonate insoluble outer membrane fraction of the soft-rot pathogen *Dickeya dadantii* (syn. *Erwinia chrysanthemi*) strain 3937. J. Proteome Res. **6**:62–69.
5. **Bordi C, et al.** 2010. Regulatory RNAs and the HptB/RetS signalling pathways fine-tune *Pseudomonas aeruginosa* pathogenesis. Mol. Microbiol. **76**:1427–1443.
6. **Brencic A, et al.** 2009. The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. Mol. Microbiol. **73**:434–445.
7. **Brencic A, Lory S.** 2009. Determination of the regulon and identification of novel mRNA targets of Pseudomonas aeruginosa RsmA. Mol. Microbiol. **72**:612–632.
8. **Choi KH, Kumar A, Schweizer HP.** 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J. Microbiol. Methods **64**:391–397.
9. **Corey EJ, Chaykovsky M.** 1965. Dimethyloxosulfonium methylide [(CH3)2SOCH2] and dimethylsulfonium methylide [(CH3)2SCH2]. Formation and application to organic synthesis. J. Am. Chem. Soc. **87**: 1353–1364.
10. **Cowell BA, Evans DJ, Fleiszig SM.** 2005. Actin cytoskeleton disruption by ExoY and its effects on *Pseudomonas aeruginosa* invasion. FEMS Microbiol. Lett. **250**:71–76.
11. **Cowell BA, Twining SS, Hobden JA, Kwong MSF, Fleiszig SMJ.** 2003. Mutation of *lasA* and *lasB* reduces *Pseudomonas aeruginosa* invasion of epithelial cells. Microbiology **149**:2291–2299.
12. **Dasgupta N, Ashare A, Hunninghake GW, Yahr TL.** 2006. Transcriptional induction of the *Pseudomonas aeruginosa* type III secretion system by low $Ca^{2+}$ and host cell contact proceeds through two distinct signaling pathways. Infect. Immun. **74**:3334–3341.
13. **Dasgupta N, Lykken GL, Wolfgang MC, Yahr TL.** 2004. A novel anti-

anti-activator mechanism regulates expression of the *Pseudomonas aeruginosa* type III secretion system. Mol. Microbiol. **53**:297–308.
14. **Deng Q, Barbieri JT.** 2008. Molecular mechanisms of the cytotoxicity of ADP-ribosylating toxins. Annu. Rev. Microbiol. **62**:271–288.
15. **Engel JN.** 2003. Molecular pathogenesis of acute *Pseudomonas aeruginosa* infections, p. 201–203. *In*Hauser A and Rolle JK (ed), Severe infection caused by *Pseudomonas aeruginosa*. Plenum Press, New York, NY.
16. **Frank DW.** 1997. The exoenzyme S regulon of *Pseudomonas aeruginosa.* Mol. Microbiol. **26**:621–629.
17. **Girard G, van Rij ET, Lugtenberg BJJ, Bloemberg GV.** 2006. Regulatory roles of *psrA* and *rpoS* in phynazine-1-carboxamide synthesis by *Pseudomonas chlororaphis* PCL1391. Microbiology **152**:43–58.
18. **Hogardt M, Roeder M, Schreff AM, Eberlt L, Heesemann J.** 2004. Expression of *Pseudomonas aeruginosa exoS* is controlled by quorum sensing and RpoS. Microbiology **150**:843–851.
19. **Hudson DL, et al.** 2007. Inhibition of type III secretion in *Salmonella enterica* serovar Typhimurium by small-molecule inhibitors. Antimicrob. Agents Chemother. **51**:2631–2635.
20. **Hueck CJ.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. **62**:379–433.
21. **Irie Y, et al.** 2010. *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. Mol. Microbiol. **78**:158–172.
22. **Kauppi AM, Nordfelth R, Uvell H, Wolf-Watz H, Elofsson M.** 2003. Targeting bacterial virulence: inhibitors of type III secretion in *Yersinia.* Chem. Biol. **10**:241–249.
23. **Keyser P, Elofsson M, Rosell S, Wolf-Watz H.** 2008. Virulence blockers as alternatives to antibiotics: type III secretion inhibitors against Gram-negative bacteria. J. Intern. Med. **264**:17–29.
24. **Kim J, et al.** 2005. Factors triggering type III secretion in *Pseudomonas aeruginosa.* Microbiology **151**:3575–3587.
25. **Kojic M, Venturi V.** 2001. Regulation of *rpoS* gene expression in *Pseudomonas*: involvement of a TetR family regulator. J. Bacteriol. **183**: 3712–3720.
26. **Krawczyk H, Albrecht Ł.** 2005. Knoevenagel reaction of diethylphosphonoacetic acid: a facile route to diethyl (*E*)-2-arylvinylphosphonates. Synthesis **17**:2887–2896.
27. **Li Y, et al.** 2009. The plant phenolic compound *p*-coumaric acid represses gene expression in the *Dickeya dadantii* type III secretion system. Appl. Environ. Microbiol. **75**:1223–1228.
28. **McCaw ML, Lykken GL, Singh PK, Yahr TL.** 2002. ExsD is a negative regulator of the *Pseudomonas aeruginosa* type III secretion regulon. Mol. Microbiol. **46**:1123–1133.
29. **Miller WG, Leveau JHJ, Lindow SE.** 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. Mol. Plant Microbe Interact. **13**: 1243–1250.
30. **Mukherjee A, Cui Y, Liu Y, Chatterjee AK.** 1997. Molecular characterization and expression of the *Erwinia carotovora* $hrpN_{Ecc}$ gene, which encodes an elicitor of the hypersensitive reaction. Mol. Plant Microbe Interact. **10**:462–471.
31. **Mulcahy H, O'Callaghan J, O'Grady EP, Adams C, O'Gara F.** 2006. The posttranscriptional regulator RsmA plays a role in the interaction between *Pseudomonas aeruginosa* and human airway epithelial cells by positively regulating the type III secretion system. Infect. Immun. **74**:3012–3015.
32. **Niimi T, et al.** 2001. Design and synthesis of non-peptidic inhibitors for the Syk C-terminal SH2 domain based on structure-based in-silico screening. J. Med. Chem. **44**:4737–4740.
33. **Nordfelth R, Kauppi AM, Norberg HA, Wolf-Watz H, Elofsson M.** 2005. Small-molecule inhibitors specifically targeting type III secretion. Infect. Immun. **73**:3104–3114.
34. **Nouwens AS, et al.** 2003. Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. Microbiology **149**:1311–1322.
35. **O'Toole GA, Kolter R.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. **28**:449–461.
36. **Patel BA, et al.** 1977. Palladium-catalyzed vinylic substitution reaction with carboxylic acid derivatives. J. Org. Chem. **42**:3903–3907.
37. **Rietsch A, Vallet-Gely I, Dove SL, Mekalanos JJ.** 2005. ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa.* Proc. Natl. Acad. Sci. U. S. A. **102**:8006–8011.
38. **Rietsch A, Mekalanos JJ.** 2006. Metabolic regulation of type III secretion gene expression in *Pseudomonas aeruginosa.* Mol. Microbiol. **59**:807–820.
39. **Shafikhani SH, Morales C, Engel J.** 2008. The *Pseudomonas aeruginosa* type III secreted toxin ExoT is necessary and sufficient to induce apoptosis in epithelial cells. Cell Microbiol. **10**:994–1007.
40. **Shafikhani SH, Engel J.** 2006. *Pseudomonas aeruginosa* type III-secreted toxin ExoT inhibits host-cell division by targeting cytokinesis at multiple steps. Proc. Natl. Acad. Sci. U. S. A. **103**:15605–15610.
41. **Shen DK, Filopon D, Kuhn L, Polack B, Toussaint B.** 2006. PsrA is a positive transcriptional regulator of the type III secretion system in *Pseudomonas aeruginosa.* Infect. Immun. **74**:1121–1129.
42. **Stirling FR, Cuzick A, Kelly SM, Oxley D, Evans TJ.** 2006. Eukaryotic localization, activation and ubiquitinylation of a bacterial type III secreted toxin. Cell Microbiol. **8**:1294–1309.
43. **Sun J, Maresso AW, Kim JJ, Barbieri JT.** 2004. How bacterial ADP-ribosylating toxins recognize substrates. Nat. Struct. Mol. Biol. **11**: 868–876.
44. **Veenendaal AKJ, Sundin C, Blocker AJ.** 2009. Small-molecule type III secretion system inhibitors block assembly of the *Shigella* type III secretion. J. Bacteriol. **191**:563–570.
45. **Veesenmeyer JL, Hauser AR, Risboa T, Rello J.** 2009. *Pseudomonas aeruginosa* virulence and therapy: evolving translational strategies. Crit. Care Med. **37**:1777–1786.
46. **Ventre I, et al.** 2006. Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. Proc. Natl. Acad. Sci. U. S. A. **103**:171–176.
47. **Venturi V.** 2003. Control of rpoS transcription in *Escherichia coli* and *Pseudomonas*: why so different? Mol. Microbiol. **49**:1–9.
48. **Walker TS, et al.** 2004. *Pseudomonas aeruginosa*-plant root interactions. Pathogenicity, biofilm formation, and root exudation. Plant Physiol. **134**: 320–331.
49. **Wei ZM, Beer SV.** 1995. *hrpL* activates *Erwinia amylovora hrp* gene transcription and is a member of the ECF subfamily of $\sigma$ factors. J. Bacteriol. **177**:6201–6210.
50. **Wolfgang MC, Lee VT, Gilmore ME, Lory S.** 2003. Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. Dev. Cell **4**:253–263.
51. **Yahr TL, Wolfgang MC.** 2006. Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. Mol. Microbiol. **62**: 631–640.
52. **Yang S, et al.** 2008. Type III secretion system genes of *Dickeya dadantii* 3937 are induced by plant phenolic acids. PLoS One **8**:e2973.
53. **Yang S, et al.** 2008. Dynamic regulation of GacA in type III secretion, pectinase gene expression, pellicle formation, and pathogenicity of *Dickeya dadantii* (*Erwinia chrysanthemi* 3937). Mol. Plant Microbe Interact. **21**:133–142.
54. **Zhang X, et al.** 2002. A new procedure for preparation of carboxylic acid hydrazides. J. Org. Chem. **67**:9471–9474.