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Structural and Functional Analysis of the Type III Secretion System from *Pseudomonas fluorescens* Q8r1-96†§

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*Pseudomonas fluorescens* Q8r1-96 represents a group of rhizosphere strains responsible for the suppressiveness of agricultural soils to take-all disease of wheat. It produces the antibiotic 2,4-diacetylphloroglucinol and aggressively colonizes the roots of cereal crops. In this study, we analyzed the genome of Q8r1-96 and identified a type III protein secretion system (T3SS) gene cluster that has overall organization similar to that of the T3SS gene cluster of the plant pathogen *Pseudomonas syringae*. We also screened a collection of 30 closely related *P. fluorescens* strains and detected the T3SS genes in all but one of them. The Q8r1-96 genome contained *ropAA* and *ropM* type III effector genes, which are orthologs of the *P. syringae* effector genes *hopAA1*-1 and *hopM1*, as well as a novel type III effector gene designated *ropB*. These type III effector genes encoded proteins that were secreted in culture and injected into plant cells by both *P. syringae* and Q8r1-96 T3SSs. The Q8r1-96 T3SS was expressed in the rhizosphere, but mutants lacking a functional T3SS were not altered in their rhizosphere competence. The Q8r1-96 type III effectors RopAA, RopB, and RopM were capable of suppressing the hypersensitive response and production of reactive oxygen species, two plant immune responses.

Rhizosphere-inhabiting fluorescent *Pseudomonas* spp. have been studied extensively in recent years because they can provide biological control of soilborne pathogens of a wide range of agricultural crops and they have a key role in the suppressiveness of some soils to plant pathogens (66). Among the major phenotypic traits associated with biological control are the capacity to produce biologically active metabolites and the ability to colonize and persist in the plant rhizosphere. The latter trait is of critical importance because a successful biocontrol agent must establish and maintain a minimum threshold population density in order to provide effective protection of the host plant. Most rhizosphere isolates of *Pseudomonas* spp. readily colonize diverse plant species and establish populations large enough to suppress a variety of pathogens (66), but their populations decline over time to levels below the threshold needed for disease control. Others are characterized by extraordinarily competitive colonization of the roots of particular crops on which they maintain population sizes sufficient to provide extended disease suppression (66). Such superior root colonizers are exemplified by a group of strains of the *Pseudomonas fluorescens* complex that produce the polyketide antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) and belong to the rep-PCR D genotype (67). In order to gain insight into this superior root colonizer phenotype, we recently sequenced the genome of the D-genotype strain *P. fluorescens* Q8r1-96 and identified genes that encode, among traits potentially important in interactions with the host plant, a type III protein secretion apparatus.

Type III protein secretion systems (T3SSs) are found in many Gram-negative bacteria that live in close association with eukaryotes (18, 52). T3SSs act as molecular syringes that can inject bacterial proteins called type III effectors into eukaryotic cells (11, 14, 62, 71). Exactly how type III effectors are injected and what they target once inside host cells make up one of the most actively investigated areas of research in host-microbe interactions. The T3SSs of plant pathogens are encoded by the *hrp/hrc* gene cluster (2, 62). While the majority of plant targets for type III effectors remain unknown, it is becoming apparent that they are directed to components of the plant immune system (11, 25, 71).

Plant immunity can be broadly viewed as consisting of two main branches. One branch is triggered by conserved microbial compounds that are recognized outside the plant cell by extracellular receptors (12, 46). These molecules are found in both pathogenic and nonpathogenic microbes and are called pathogen (microbe)-associated molecular patterns (PAMPs/MAMPs) (7, 44). When a PAMP is recognized, it induces immune responses collectively referred to as PAMP-triggered immunity (PTI) (33). The other branch is induced by effector proteins from pathogens. These effectors are recognized by resistance (R) proteins, resulting in effector-triggered immunity (ETI). The majority of the research on plant immunity has been done on aerial plant parts. However, roots have recently been shown to induce PTI in response to specific PAMPs (45).
First investigated in the context of pathogenesis, T3SSs have recently been recognized as an essential feature of a wide range of mutualistic and commensal symbioses that arise from the colonization of eukaryotic hosts by Gram-negative bacteria (52). Pseudomonads represent an environmentally significant group of bacteria that often colonize higher organisms, and T3SSs are found in both pathogenic and saprophytic species of this genus. However, our understanding of the importance of the T3SS and its effectors for interactions of Pseudomonas spp. with eukaryotic hosts is largely limited to the opportunistic human and animal pathogen Pseudomonas aeruginosa (64) and the plant pathogen Pseudomonas syringae (3). The function of the T3SS in saprophytic members of this genus, and particularly in ubiquitous rhizosphere-dwelling Pseudomonas spp., remains poorly understood.

To date, the role of the T3SS in rhizosphere pseudomonads has been studied only in two isolates of the P. fluorescens complex. Rainey and Preston et al. (53) identified and characterized a 20-kb gene cluster resembling the hrp/hr loci of P. syringae in the sugar beet isolate P. fluorescens SBW25. While SBW25 has no known pathogenic activity, Preston et al. (53) showed that constitutive expression of its hrpL homolog enabled an AvrB-dependent hypersensitive response (HR) in Arabidopsis and a host-specific HR in Nicotiana clevelandii, suggesting that this T3SS is functional. Inactivation of T3SS genes compromised the ability of SBW25 to persist in the plant rhizosphere. A T3SS was also characterized by Rezzonico et al. (56) in P. fluorescens KD, which controls the oomycete Pythium ultimum and has no known phytopathogenic activity. Inactivation of this strain’s T3SS strongly reduced its activity against Pythium ultimum on cucumber but had no measurable effect on root colonization (56). No type III effectors were identified in P. fluorescens KD, and the exact function of type III effectors in SBW25 has remained elusive. The lack of information from other rhizosphere isolates has provided few clues about the diversity of T3SSs and type III effectors that are associated with the rhizosphere lifestyle.

Here, we bridge this gap in our knowledge by reporting the sequence and organization of the T3SS cluster from P. fluorescens Q8r1-96, which produces 2,4-DAPG and is representative of strains responsible for the suppressiveness of U.S. and European agricultural soils to take-all disease of wheat (66, 67). We describe novel type III effectors from P. fluorescens and present evidence that these effectors, like their P. syringae counterparts, are capable of suppressing PAMP- and effector-triggered plant immunity. Finally, we show that Q8r1-96-like T3SS genes are widely distributed and highly conserved among rhizosphere isolates of Pseudomonas spp. of diverse geographic and host plant origin.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** Strains and plasmids used in this study are listed in Table 1. Strains of 2,4-DAPG-producing Pseudomonas spp. screened for the presence of T3SS were described previously (40) and include CHA0, Pf-5, Q2-1, Q2-2, Q2-5, Q2-87, Q37-87, Q1T-5, Q1T-6, W2-6, W4-4, D2B1, JPM6, JPM7, FFL1R18, CV1-1, HT5-1, FTAD1R36, FL1R22, TMA12, MW1-1, MV4-2, MVP1-4, STAD384, FTAD1R34, FFL1R9, Q128-87, OC1-4, ATCC 49054, and F113. Pseudomonas spp. and Escherichia coli were grown at 28°C and 37°C, respectively, in Luria-Bertani (LB) (8), M9 (8), or one-third-strength King’s medium B (KMB) (34). For type III secretion assays, Pseudomonas spp. were grown in Hrp-inducing minimal medium at 22°C (30). Antibiotic supplementations were used at the following concentrations: chloramphenicol (Cm), 30 μg/ml; gentamicin (Gm), 10 or 12 μg/ml; rifampin (Rif), 100 μg/ml; spectinomycin (Sp), 50 μg/ml; tetracycline (Tc), 20 μg/ml; and kanamycin (Km), 25 μg/ml.

**Sequence analyses.** DNA sequence data were analyzed with Vector NTI Advance 9 (Invitrogen, Carlsbad, CA) and OMAIGA 2.0 (Accelrys, San Diego, CA). Database searches for similar protein sequences were performed using the NCBI’s BLAST network service (http://www.ncbi.nlm.nih.gov/BLAST). Database searches against collections of protein motifs and domains were carried out by using the MyHits (49) and CD-Search (39) web-based engines. Protein folds and subcellular localization were predicted by using Phyre (10) and PSORT II 2.0 (23), respectively. The Q8r1-96 genome assembly (I. T. Paulsen et al., unpublished data) was screened for potential Hrp boxes using hidden Markov models (HMMs) built from the compilation of P. syringae Hrp boxes using HMMER3 (http://hmmr.wustl.edu). Sequences of the Q8r1-96 opines gene cluster and effector genes were deposited in GenBank under accession numbers HM991502 through HM991505.

**Amplification of T3SS genes and phylogenetic inference.** To screen 2,4-DAPG-producing Pseudomonas spp. for the presence of T3SS, genomic DNA was extracted from bacteria by using a cell-labile ammonium bromide mini-prep procedure (8) and screened by PCR with HRCR0092–HRCR0086 and qtp–qillow primer sets (Table 1). Amplification was performed with a PCR-200 gradient thermal cycler (Bio-Rad, Hercules, CA) using GoTaq DNA polymerase (Promega, Inc., Madison, WI). The DNA fragments amplified by primers qtp and qillow were cleaned with QIAquick PCR purification spin columns (Qiagen, Valencia, CA) and sequenced with a BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The nucleotide sequences were aligned with Clustal X 2.0.11 (66), and Molecular Evolutionary Genetics Analysis (MEGA) software 4.0.2 (63) was used to infer neighbor-joining (NJ) trees. Reproducibility of clades within the inferred NJ trees was assessed by bootstrap resampling with 1,000 replicates.

**Construction of expression plasmids.** Standard techniques were used for plasmid DNA isolation, cloning, agarose gel electrophoresis, and electrotransformation (8). PCR amplifications were performed with KOD Hot Start DNA polymerase (Novagen, Madison, WI) according to the manufacturer’s recommendations. Oligonucleotides were designed with Oligo 6.65 software (Molecular Biology Insights, West Cascade, CO).

For construction of pLN615-based plasmids, target genes were amplified with the primers listed in Table 1 and cloned into a pENTR/D-TOPO Gateway entry vector (Invitrogen) by following the manufacturer’s recommendations. To clone genes srcB and ropB in tandem, the genes were amplified separately by PCR with primer sets srcB-up–srcB-low and ropB-up–ropB-low (Table 1), and the two partially overlapping fragments were gel purified and fused by overlap extension PCR (16) with primers srcB-up and ropB-low. All pENTR/D-TOPO-based recombinant plasmids were single-pass sequenced to confirm the integrity of the cloned genes, and LR Clonase II enzyme (Invitrogen) was used to transfer the cloned genes into pLN615 (Table 1). The pLN615-based plasmids were introduced into P. fluorescens strain 56S by electroporation.

**Construction of rcsp mutants and transposon tagging.** Mutations in rcls, rscV, rscC, ropL, and ropH were introduced as follows. For each gene, flanking the chosen cassette insertion site were amplified by PCR with corresponding m1-m2 and m3-m4 primer sets, and the Km cassette was amplified with primers Fkan and Rkan (Table 1). The partially overlapping fragments were gel purified with a QIAEX II gel extraction kit (Qiagen) and fused by overlap extension PCR (16) with KOD Hot Start DNA polymerase (Novagen) and primers m1 and m4. The resultant fragments were cloned into the Small site of the gene replacement vector pEX18Tc.

To create the ΔOPOR and rscR mutants, pCR-T3SG2 (Table 1) was treated with HindIII, BamHI, and Klenow DNA polymerase I fragment and religated, yielding pCR-T3SG2a. For the ΔOPOR mutation, the pCR-T3SG2Δa plasmid was treated with PsiI and T4 DNA polymerase and ligated with the 879-bp Gm cassette, giving pCR-T3SG2ΔaPm. To inactivate rscR, pCR-T3SG2Δa was treated with SacI and T4 DNA polymerase and then ligated with the Gm cassette, yielding pCR-T3SG2ΔaaSac. The interrupted inserts from pCR-T3SG2ΔaPm and pCR-T3SG2ΔaSac were subcloned into pEX18Tc. All pEX18Tc-based plasmids were mobilized in Q8r1-96 from E. coli S17-1 (λ-pir), and double crossover events were selected and verified by PCR as described earlier (42).

The ropH/lux–ΔABE transcription fusion was constructed by amplifying the ropH promoter with primers PrspJ1 and PrspJ4 (Table 1), digesting the resultant fragment with BamHI and PsiI, and cloning it into pUC18-miniTn7/Gm-lux (17). Q8r1-96 was tagged by electroporation of freshly prepared competent cells with 200 ng each of pUC18-miniTn7/Gm-lux-ropH and the helper plasmid pTNS2 (17). The tagged clones were isolated on LB agar amended with genta-
TABLE 1. Bacterial strains, plasmids, and oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid, or oligonucleotide primer</th>
<th>Description*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. fluorescens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q8r1-96</td>
<td>Wheat rhizosphere isolate from Quincy, WA; Rif r</td>
<td>54</td>
</tr>
<tr>
<td>Q8r1-96Gm</td>
<td>Q8r1-96 tagged with mini-Tn7-aph2; Rif r, Gm r</td>
<td>65</td>
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<tr>
<td>Q8r1-96Km</td>
<td>Q8r1-96 tagged with mini-Tn7-aph2; Rif r, Km r</td>
<td>65</td>
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<tr>
<td>Q8r1-96rsrPL</td>
<td>TSS mutant; rpsL::aph; Rif r, Km r</td>
<td>This study</td>
</tr>
<tr>
<td>Q8r1-96rsrCV</td>
<td>TSS mutant; rcfV::aph; Rif r, Km r</td>
<td>This study</td>
</tr>
<tr>
<td>Q8r1-96rsrCR</td>
<td>TSS mutant; rcsC::aph; Rif r, Gm r</td>
<td>This study</td>
</tr>
<tr>
<td>Q8r1-96ΔOPQR</td>
<td>TSS mutant; Δ(opQ-opR):::aph; Rif r, Gm r</td>
<td>This study</td>
</tr>
<tr>
<td>Q8r1-96rsrCC</td>
<td>TSS mutant; rcsC::aph; Rif r, Km r</td>
<td>This study</td>
</tr>
<tr>
<td>Q8r1-96ropB</td>
<td>TSS mutant; ropB::aph; Rif r, Km r</td>
<td>This study</td>
</tr>
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<td>Q8r1-96rsrPH</td>
<td>TSS mutant; rpsP::aph; Rif r, Km r</td>
<td>This study</td>
</tr>
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<td>Q8r1-96rsrpl</td>
<td>Q8r1-96 tagged with mini-Tn7-Gm::raf::lux; Rif r, Gm r</td>
<td>This study</td>
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<tr>
<td>55</td>
<td>Nal r</td>
<td>M. Sasser</td>
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<tr>
<td><strong>Pseudomonas syringae</strong></td>
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<td>DC3000</td>
<td>Wild type; spontaneous Rif r</td>
<td>19</td>
</tr>
<tr>
<td>DC3000 hrcC</td>
<td>hrcC mutant defective in TSS; Rif r, Cm r</td>
<td>70</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1(α-pir)</td>
<td>thi pro hsdM recA rpsL rpsL</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>DH5α</td>
<td>F' dm80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (r _- _m +) gal phoA supE44 λ - thi-1 gyrA96 relA</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>DB3.1</td>
<td>F' gyrA462 endA1 Δ(opQ-opR):::aph; mrr hsdS20 (r _- _m +) supE44 ara-14 galK2 lacY1 proA2 rpsL20 (Sm'; xyl-5 - leu-1)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pUC18-mini-Tn7-T-Gm-lux</td>
<td>Mini-Tn7-based transcriptional fusion vector with luxCDABE genes; Gm r, Amp r</td>
<td>17</td>
</tr>
<tr>
<td>pTNS2</td>
<td>Tn7 transposase donor; Amp r</td>
<td>17</td>
</tr>
<tr>
<td>pUC18-mini-Tn7-T-lux-rpsL</td>
<td>pUC18-mini-Tn7-T-Gm-lux derivative with P _psl-lux transcriptional fusion; Gm r, Amp r</td>
<td>This study</td>
</tr>
<tr>
<td>pHIR11</td>
<td>Cosmid pLAFR3 derivative carrying genomic DNA of P. syringae pv. syringae 61; Te r</td>
<td>29</td>
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<tr>
<td>pLN347</td>
<td>pML123 derivative containing nvrPSB-lux; Gm r</td>
<td>32</td>
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<tr>
<td>pLN1965</td>
<td>pHR11 derivative containing a deletion of srcA::hopA1 operon that is replaced with Sp r-resistant gene cassette; Te r, Sp r</td>
<td>25</td>
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<tr>
<td>pML2193</td>
<td>pML123-derived Gateway destination vector containing a CyaA tag for C-terminal fusions; Gm r, Gm'</td>
<td>M. Guo</td>
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<td>pCPP2318</td>
<td>Plasmid carrying blam lacking signal peptide sequence; Te r</td>
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<td>pLN4451</td>
<td>pML123 derivative containing hopU1::cya; Gm r</td>
<td>This study</td>
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<td>pLN4452</td>
<td>pML123 derivative containing hopA4::cya; Gm r</td>
<td>This study</td>
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<tr>
<td>pLN4453</td>
<td>pML123 derivative containing srcM and ropM::cya; Gm r</td>
<td>This study</td>
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<tr>
<td>pLN4454</td>
<td>pML123 derivative containing ropB::cya; Gm r</td>
<td>This study</td>
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<td>pLN4455</td>
<td>pML123 derivative containing srcB and ropB::cya; Gm r</td>
<td>This study</td>
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<td>pENTR/D-TOP0</td>
<td>Entry vector for Gateway system; ColE1 Kan r</td>
<td>Invitrogen</td>
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<tr>
<td>pLN615</td>
<td>pML123-derived destination vector for Gateway system for C-end HA tagging; Gm r, Cm r</td>
<td>25</td>
</tr>
<tr>
<td>pLN615-M</td>
<td>pLN615 containing srcM and ropM from Q8r1-96; Gm r</td>
<td>This study</td>
</tr>
<tr>
<td>pLN615-AA</td>
<td>pLN615 containing ropA4 from Q8r1-96; Gm r</td>
<td>This study</td>
</tr>
<tr>
<td>pLN615-B</td>
<td>pLN615 containing ropB from Q8r1-96; Gm r</td>
<td>This study</td>
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<tr>
<td>pLN615srcB</td>
<td>pLN615 containing a tandem of srcB and ropB genes from Q8r1-96; Gm r</td>
<td>This study</td>
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<tr>
<td>pUCGM</td>
<td>Source of Gm' cassette; ColE1 bla aacC1</td>
<td>26</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Source of Km' cassette; ColE1 bla aph</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>pEX18Tc</td>
<td>Gene replacement vector; Te r, srcF' susC r</td>
<td>26</td>
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<tr>
<td>pCR-Blunt</td>
<td>Cloning vector; Km' Zeo r ColE1 cedB r</td>
<td>Invitrogen</td>
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<td>5D8</td>
<td>pCPP47-based cosmide with TSS genes of Q8r1-96</td>
<td>This study</td>
</tr>
<tr>
<td>16E12</td>
<td>pCPP47-based cosmide with TSS genes of Q8r1-96</td>
<td>This study</td>
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<tr>
<td>pCR-T3SG2</td>
<td>pCR-Blunt containing the 6,866-bp fragment with rpsQ genes amplified by PCR with T3SG2 and 8up primers</td>
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<td>pCR-T3SG2Δ</td>
<td>pCR-T3SG2 containing the HindIII-BamHI deletion</td>
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<tr>
<td>pCR-T3SG2ΔPst</td>
<td>pCR-T3SG2Δ with rpsOP::pCQR replaced by the Gm r cassette</td>
<td>This study</td>
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<tr>
<td>pCR-T3SG2ΔSac</td>
<td>pCR-T3SG2Δ with rop interrupted by the Gm r cassette</td>
<td>This study</td>
</tr>
<tr>
<td>pEX18TcΔPst</td>
<td>pEX18Tc containing insert from pCR-T3SG2ΔPst</td>
<td>This study</td>
</tr>
<tr>
<td>pEX18TcΔSac</td>
<td>pEX18Tc containing insert from pCR-T3SG2ΔSac</td>
<td>This study</td>
</tr>
</tbody>
</table>

Oligonucleotide primers

PrspJ3 5'-CAT TGG ATC CTA GTG CTT TGG CTC TAT TTG TG-3'; upper primer for amplification of the rpsJ promoter region; T_m = 78.2°C | This study |
| PrspJ4 5'-CTT GCT GCA GGA TCA GGT CGG TA-3'; lower primer for amplification of the rpsJ promoter region; T_m = 73.5°C | This study |
| P_73R 5'-CAC AGC ATA ACT GGA CTG ATT TC-3'; primer for amplification of the region flanking attR7; T_m = 64.1°C | 17 |
| O8glmS 5'-AAC CTG GGG AAA TCT GTG AC-3'; primer for amplification of the region flanking attR7; T_m = 64.5°C | This study |
Phenotypic characterization of rsc/* mutants. Mutants were assayed for growth kinetics in King’s B and M9 media, swimming motility, and production of exoprotease, siderophore, and 2,4-DAPG as described earlier (41, 42). Long-term rhizosphere colonization assays were performed with wheat *Triticum aestivum.*
tivum L. cv. Penawawa and pea Pisum sativum L. cv. Columbia in raw soil as described by Landa et al. (35). Each mutant was tested in the colonization assay alone and in direct competition with the parental strain. For colonization assays, kanamycin- and gentamicin-resistant derivatives Q8r1-96Km and Q8r1-96Gm were used instead of the wild-type Q8r1-96 in order to distinguish strains in mixed inoculation treatments. Q8r1-96Km and Q8r1-96Gm were obtained by tagging the parental strain with disarmed transposons mini-Tn7/gfp2 and mini-Tn7/gfp2, which integrate into a specific neutral integenic attachment site and do not affect the competitiveness of Q8r1-96 in the plant rhizosphere (40, 41, 64).

Quincy virgin soil was inoculated with bacteria in a 1% suspension of methylcellulose to give 1 x 10⁶ CFU g⁻¹ of soil. Mixed inoculation treatments contained a 1:1 mixture (0.5 x 10⁶ CFU g⁻¹ of soil for each strain) of Q8r1-96Km or Q8r1-96Gm and mutant. Plants were inoculated in a growth chamber for four successive 2-week cycles at 15 ± 1°C (wheat) or 22 ± 1°C (pea) with a 12-h photoperiod. After each cycle, six randomly selected plants were harvested and root samples were prepared to determine the population size of the inoculated bacteria. Each treatment was replicated three times, and the entire experiment was conducted twice. Population densities of introduced bacteria were enumerated by using a PCR-based dilution endpoint assay (22). In mixed inoculation treatments, variants tagged with mini-Tn7s (42) and the rsc/rop mutant strains were distinguished by PCR with primers targeting Km⁺ and Gm⁺ genes. Bacterial growth was assayed after 72 h, with an optical density at 600 nm (OD₆₀₀) of at least 0.1 considered positive.

Take-all biocontrol assays were performed according to the method of Weller et al. (69). To prepare the oat kernel inoculum of Gaeumannomyces graminis var. tritici, 250 ml of oat grain and 300 ml of water were mixed in 1-liter flasks. The oat was autoclaved on two consecutive days, and each flask was inoculated with G. graminis var. tritici isolate ARS-A1 pregrown on 1/5 potato dextrose agar (PDA; BD, Sparks, MD) for 7 days. After 3 weeks of incubation at 23°C, the colonized oat grain was removed from the flasks, dried for 48 h under sterile airflow, and then stored at 4°C. Colonized oat kernels were fragmented in a blender and sieved into particles of 0.25 to 1.0 mm. For biocontrol assays, 0.7% (wt/vol) of the G. graminis var. tritici ARS-A1 oat kernel inoculum was added to sieved and pasteurized Quincy virgin soil. Plastic tubes (15 cm long, 2.5-cm diameter) (Stuewe and Sons Inc., Corvallis, OR) were plugged with cotton balls and covered with a 6-cm column of autoclaved vermiculite and 10 g of inoculated soil. Each tube was inoculated with 12 ml of water. After 7 days, the tubes were removed from the tubes and the roots were washed and inspected for take-all lesions. Plants then were scored for height and take-all symptom severity (0 to 8 scale, where “0” indicates no disease and “8” indicates a dead plant). The experiment was conducted twice.

Type III protein secretion assays. The plN615 derivatives with cloned rpa, srcR, ropAA, and srcR ropB genes were electroporated into DC3000 (pCPP2318), DC3000 hrcC (pCPP2318) or Q8r1-96 and used for type III protein secretion assays. The β-lactamases encoded by pCPP2318 and NPTII were used as lysis controls. Secretion assays were performed as previously described (31). Briefly, strains were grown on 6% h at 22°C in lyso-inducing conditions, after which the cultures were separated into cell and supernatant fractions by centrifugation. Secreted proteins in the supernatants were precipitated with trichloroacetic acid and washed with acetone. Proteins were separated by electrophoresis in a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes for immunoblotting. The following primary antibodies were used: anti-hemagglutinin (HA) (Roche Diagnostics Corp., Indianapolis, IN), anti-β-lactamase (Chemicon International, Temecula, CA), and anti-NPTII (Cortex Biochem, San Carlos, CA). Proteins on immunoblots were visualized using the CDP-Star chemiluminescence detection kit (Tropix, Bedford, MA) followed by autoradiography.

Adenylate cyclase injection assays. PENTR/D-TOPO plasmids ropA, srcR ropB, and ropAA ropB, and srcR ropB and ropAA were used in Gateway LR reactions with Gateway destination plasmid pLN2193, which fuses genes with cyaA. These resultant constructs were electroporated into P. fluorescens 55 (pLN1965). CyaA assays were performed as previously described (58). Briefly, bacteria were suspended in 5 mM morpholinocethanesulfonic acid (MES) (pH 5.6) amened with 100 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at an O₆₀₀ of 0.4 and infiltrated into leaves of Nicotiana tabacum cv. Xanthi. Disks of leaf tissue (0.7-cm diameter) were harvested 16 h after infiltration, and the concentration of cyclic AMP (cAMP) in each sample was measured using a direct CAMP enzyme immunoas-

say kit (Assay Designs, Ann Arbor, MI) by following the manufacturer’s instructions.

Plant hypersensitive response assays. Constructs containing ropAA, srcR ropB, or srcR ropM were electroporated into P. fluorescens 55(pHR11). Bacteria were suspended at 1 x 10⁶ CFU ml⁻¹ in 5 mM MES (pH 5.6) and used to prepare three 5-fold serial dilutions. The cell suspensions were infiltrated into Nicotiana tabacum cv. Xanthi leaves to determine the extent that they were able to suppress the HR caused by P. fluorescens 55(pHR11). Strains containing pLN615 (an empty vector) or pLN347, a construct encoding AvrPtoB, were used as negative and positive controls, respectively. The HR was recorded and photographed 48 h after infiltration.

Measurement of reactive oxygen species (ROS). P. fluorescens 55(pLN1965) was electroporated with constructs that encoded RopAα, RopB, or RopM, each fused with a hemagglutinin (HA) tag at their C termini. These strains were separately infiltrated into Nicotiana benthamiana at a cell density of 2 x 10⁶ CFU ml⁻¹. ROS measurements were done as described by Asai et al. (6). Briefly, disks of leaf tissue (0.4-cm diameter) were harvested from infiltrated leaf regions 15 h after infiltration and floated on water in wells of 96-well plates overnight. The water was replaced with a solution containing 0.5 mM chemiluminescence probe L-012 (Wako Pure Chemicals, Osaka, Japan). 10 mM morpholinepropanesulfonic acid (MOPS)/KOH (pH 7.4), and 1 mM tgl22. Luminescence was measured using a GloMax Multi luminometer (Promega, Madison, WI).

Statistical analyses. All treatments in the growth chambers were arranged in a randomized complete block design. Data were analyzed by using STATISTIX v.8.0 (Analytical Software, Tallahassee, FL). Population data were converted to log CFU g⁻¹ of root (fresh weight) or soil to satisfy the assumptions of analysis of variance (ANOVA). Differences in population densities among treatments were determined by standard ANOVA, and mean comparisons among treatments were performed by Fischer’s protected least significance difference test (P < 0.05) or, when appropriate, by the Kruskal-Wallis test (P < 0.05).

Nucleotide sequence accession numbers. Sequence data were deposited in GenBank under accession numbers HM852191 through HM852211 for rscRST and HM854217 for 16s rRNA genes from STAD384.

RESULTS

Characterization of the P. fluorescens Q8r1-96 T3SS. While analyzing T3SS genes of Q8r1-96 we followed the naming convention introduced in P. fluorescens SBW25 by Preston et al. (53). Accordingly, type III structural and regulatory genes are named rsp (rhizosphere-expressed secretion protein) or rsc (rsc conserved), whereas type III effectors and their type III chaperones are named rop (rhizosphere-expressed outer protein) and src (specific Rop chaperone), respectively.

In P. syringae, expression of T3SS-related genes is mediated by the alternative sigma factor HrpL, which is thought to activate transcription in an RpoN-dependent manner by binding to a consensus bipartite promoter element, the “hpR box” (30). The hpR box motif is highly conserved and has been used as a reliable way to pinpoint the location of T3SS regulon genes in genomes of P. syringae and other bacterial species (24). Q8r1-96 carries rpsL, a gene with similarity to hrpL, the product of which presumably forms the type III secretion regulatory circuit together with a putative RspL-specific response regulator, RspS, that is similar to the cognate response regulator HrPS of P. syringae pv. tomato DC3000 (Fig. 1; see Table S1 in the supplemental material). We manually examined regions upstream of Q8r1-96 rpsL, rspF, and rpa4 genes and identified additional putative rps box sequences (see Table S2 in the supplemental material). Since their structure closely resembled that of the consensus hrp box of P. syringae (KGGARCYN[S15-16]CCACNNA) (72), we built a hidden Markov model based on the aligned hrp boxes of strain DC3000 to screen the Q8r1-96 genome sequence.

The results of this screening yielded nine putative RspL-dependent promoters (see Table S2 in the supplemental ma-
type III effector gene cluster and related genes in *P. fluorescens* Q8r1-96 (top) and its comparison to the *hrp/hrp* cluster of *P. syringae* pv. tomato DC3000 (1) (middle) and the *rsc/rsp* cluster of *P. fluorescens* SBW25 (53) (bottom). Predicted open reading frames and their orientation are shown by large arrows. Thin solid arrows and small rectangles indicate predicted transcripts and *rsp* boxes, respectively. Homologous genes are connected with gray shading. Insertions of GmR and KmR cassettes in different mutants are indicated with inverted black and white triangles, respectively. Full gene names within *rsc/rsp* and *hrp/hrp* clusters were shortened and are indicated by a single letter preceded by “c” or “p” (corresponding to *rsc*/*hrp* or *rsp/hrp*, respectively). Some Q8r1-96 gene designations correspond to genome locus tags; e.g., the 5643 gene has locus tag PfQ8_5643.

![Genetic organization of the type III secretion gene cluster and related genes in *P. fluorescens* Q8r1-96](image)

**Fig. 1.** Genetic organization of the type III secretion gene cluster and related genes in *P. fluorescens* Q8r1-96 (top) and its comparison to the *hrp/hrp* cluster of *P. syringae* pv. tomato DC3000 (1) (middle) and the *rsc/rsp* cluster of *P. fluorescens* SBW25 (53) (bottom). Predicted open reading frames and their orientation are shown by large arrows. Thin solid arrows and small rectangles indicate predicted transcripts and *rsp* boxes, respectively. Homologous genes are connected with gray shading. Insertions of GmR and KmR cassettes in different mutants are indicated with inverted black and white triangles, respectively. Full gene names within *rsc/rsp* and *hrp/hrp* clusters were shortened and are indicated by a single letter preceded by “c” or “p” (corresponding to *rsc*/*hrp* or *rsp/hrp*, respectively). Some Q8r1-96 gene designations correspond to genome locus tags; e.g., the 5643 gene has locus tag PfQ8_5643.

### Type III effectors of *P. fluorescens* Q8r1-96

Three putative type III effectors, RopAA, RopB, and RopM, were identified in the genome of *P. fluorescens* Q8r1-96 using blast similarity searches (see Table S1 in the supplemental material) and the following lines of evidence. All three putative Q8r1-96 type III effectors have composition typical of substrate proteins recognized by the T3SS (i.e., an amphipathic N-terminal part rich in Ser and polar residues, an aliphatic amino acid in position 3 or 4, and only one acidic residue in the first 12 positions) (see Table S3 in the supplemental material). Two of these genes, *ropM* and *ropB*, are found in the immediate vicinity of the *rsp/rsp* cluster and are accompanied by genes (*srcM* and *srcB*, respectively) encoding proteins with properties typically exhibited by type III chaperones. Finally, the putative promoters of all three type III effector genes contain well-conserved *rsp* box motifs (see Table S2 in the supplemental material).

The Q8r1-96 type III effector protein sequences were used to query the nonredundant data set of GenBank CDS translations, and the retrieved sequences were subjected to phylogenetic analyses. Database searches revealed that two of these effectors are members of the *P. syringae* HopAA1 and HopM1 type III effector families (38). Based on the similarity, these Q8r1-96 effectors were named RopAA and RopM, respectively. The NJ phylogeny estimated using available HopAA1 sequences revealed three major clades corresponding to proteins of *P. syringae*/*Pseudomonas viridiflava*, *Pseudomonas mendocina*, and *Ralstonia* spp. (Fig. 2A). The analysis also revealed that Q8r1-96 RopAA is only distantly related to its well-characterized *P. syringae* counterparts (25.5% identity; 38.6% similarity to HopAA1-1 of *P. syringae* pv. tomato DC3000) and more closely resembles putative type III effectors from *Ralstonia* spp. (30.4% identity; 40.9% similarity to RS1PO_04287 of *Ralstonia solanacearum* IPO1609). The RopM type III effector of Q8r1-96 also did not group with sequences from *P. syringae*/*P. viridiflava* (23.9% identity; 35.3% similarity to HopM1 of *P. syringae* pv. tomato DC3000) but rather formed a well-supported distinct clade (Fig. 2B). Finally, the third putative type III effector encoded by *ropB* bears no resemblance to
known type III effectors. Iterative psi-blast searches revealed a very weak similarity to uncharacterized CRISPR-associated helicase Cas3 domain proteins from Fusobacterium spp. (data not shown).

**Q8r1-96 represents a ubiquitous group of P. fluorescens-like strains with a distinct T3SS.** We also investigated how the T3SS of Q8r1-96 is related to distinct variants of T3SSs previously described in plant-associated Pseudomonas spp. For this purpose, we estimated phylogenies of conserved HrcV, HrcR, HrcC, and HrcJ proteins, which each represent an individual operon of the T3SS gene cluster. The data set used for analyses (n = 13) included sequences from three P. fluorescens-like strains, eight strains of *P. syringae* and closely related species (i.e., *P. viridiflava* and *Pseudomonas cichorii*), and *P. mendocina*, a saprophytic soil- and rhizosphere-inhabiting organism closely related to *P. aeruginosa*. Neighbor-joining (NJ) phylogenies inferred from HrcV, HrcR, HrcC, and HrcJ protein sequences were highly congruent with each other and revealed that the *hrp/hrc* cluster of Q8r1-96 represents one of three distinct lineages (two other lineages are represented by entries from SBW25 and KD, respectively) of T3SSs found in *P. fluorescens*-like strains (see Fig. S1 in the supplemental material). Despite being diverse, individual Hrc sequences of Q8r1-96 always clustered together with those of SBW25, forming a well-supported clade. Comparison of Hrc-based phylogenies with that inferred from sequences of 16S rRNA genes revealed that strains Q8r1-96 and SBW25 are related but belong to two different groups of *P. fluorescens*-like species, thus agreeing with the results of Hrc-based analyses and suggesting that T3SSs of Q8r1-96 and SBW25 are likely evolved through vertical descent (Fig. S1). Our results also revealed that although *Pseudomonas* sp. strain KD is closely related to Q8r1-96, Hrc sequences of the former cluster consistently with their counterparts from *P. syringae*, *P. viridiflava*, and *P. mendocina* (Fig. S1). This finding strongly suggests the possibility of acquisition of the *hrp/hrc* gene cluster of *Pseudomonas* sp. KD via horizontal transfer and agrees with results previously reported by Rezzonico et al. (57).

The notion that the T3SSs of Q8r1-96 and SBW25 represent two distinct lineages is further supported by the fact that the *rsp/rsc* clusters of these strains are found in different chromosomal locations (see Fig. S2 in the supplemental material). In Q8r1-96, the cluster is flanked by genes *PflQ8_5613* and *PflQ8_5648*, which encode conserved hypothetical and penicillin amidase-like proteins, respectively. Examination of other sequenced *P. fluorescens* genomes revealed that in strain Pf-01 (60) this spot is occupied by an S-pyocin gene cluster, whereas in strains Pf-5 (50) and SBW25 (60) the homologues of *PflQ8_5613* and *PflQ8_5648* flank each other directly (Fig. S2A). Interestingly, the integration site of the *rsp/rsc* genes in SBW25 is also conserved in *P. fluorescens* and is occupied in strains Pf-5 and Pf-01 by a genomic island and a cluster of S-pyocin genes, respectively (Fig. S2B). In Q8r1-96, this site is flanked by genes *PflQ8_0753* and *yehF* (*PflQ8_5648*) and is occupied by a 30.3-kb genomic island (unrelated to the one from Pf-5) that has low G+C content (54.1%) and includes, among other genes, those encoding a putative group II intron and a bacteriophage-like site-specific integrase (*PflQ8_0767*). The average G+C content of the Q8r1-96 T3SS cluster (63.0%) and putative effector genes *ropB* (59.8%), *ropM* (62.3%), and *ropAA* (62.9%) is similar to that of the genome (61.0%), and no abrupt shifts in G+C content are observed between *rsp/rsc* genes and flanking sequences (Fig. S2A). This differs from SBW25, where the average G+C content of the part of the T3SS cluster spanning *rsp* to *rsc* is significantly lower (52.0%) than the average content of the genome (59.9%) (53).

Finally, we used PCR with primers targeting *rscRST* genes to screen a set of biocontrol strains that share with Q8r1-96 the capacity to produce the antibiotic 2,4-DAPG but have been isolated worldwide from different crops. Results of the screening revealed that of 21 strains tested, only 1, CHA0, lacks T3SS genes (data not shown). The amplified *rscRST* DNA fragments were sequenced and used in phylogenetic analyses along with homologous sequences from other plant-associated pseudomonads (n = 56). Phylograms inferred by NJ analysis confirmed the presence of the three distinct lineages in species of the *P. fluorescens* group. The analysis also revealed that *rscRST* sequences of most 2,4-DAPG-producing *Pseudomonas* spp. are tightly clustered with their homologues from Q8r1-96 (see Fig. S3 in the supplemental material), thus suggesting that the
Q8r1-96-like lineage of T3SS genes is widely distributed among rhizosphere Pseudomonas spp.

The rsc/rsp genes of Q8r1-96 are expressed in the rhizosphere. To confirm that the T3SS locus is functional in the plant rhizosphere, we tagged Q8r1-96 with a mini-Tn7 carrying the rspP promoter fused to the operon encoding luciferase of Photorhabdus luminescens and used the resultant Q8r1-96::rspP-lux strain to treat seeds of wheat T. aestivum L. cv. Penawawa. The treated seeds were sown in pots containing raw Quincy virgin soil, and plants were grown in a growth chamber as described in Materials and Methods. After 10 days, plants were gently removed from pots, wrapped in clear plastic film, and exposed to X-ray film. Results of the assay confirmed that the T3SS genes of Q8r1-96 are indeed expressed in the rhizosphere of wheat (Fig. 3). No signal was detected in plants inoculated with the control strain carrying mini-Tn7 with a promoterless luciferase operon (data not shown).

Effect of rsc/rsp mutations. To test the possible involvement of the T3SS in rhizosphere competence of Q8r1-96, we introduced mutations in rspH, rspL, rscV, rscR, ropB, and hrcC genes along with a deletion that spans rspOP-rscQR genes (Fig. 1). Homologs of each of these genes are required for a functional P. syringae T3SS (27, 28). The rsc/rsp mutant strains were tested in vitro for possible phenotypic changes in traits contributing to biocontrol by rhizosphere-dwelling Pseudomonas spp. All mutants were compared to the parental strain for the ability to grow in rich and minimal media, to produce exoprotease, siderophores, and the antibiotic 2,4-DAPG, and to spread on semisolid media. No phenotypic changes were associated with the introduced mutations, and the corresponding mutants did not differ significantly from Q8r1-96 in any of the aforementioned tests (data not shown). The mutant strains were also tested for the ability to persist in the plant rhizosphere by using competitive wheat root colonization assays in the greenhouse. Wheat and pea were sown into raw Quincy virgin soil inoculated with the mutant and parental strains introduced either individually or as a 1:1 mixture. The results indicated that inactivation of the rsc/rsp genes did not reduce the competitiveness of Q8r1-96, and populations of the mutants were similar to those of the parental strain (see Tables S4 and S5 in the supplemental material). We also tested whether the T3SS mutations impaired the ability of Q8r1-96 to suppress take-all disease of wheat. Wheat seeds were treated with Q8r1-96 or each of the five T3SS mutants and sown in pasteurized Quincy virgin soil inoculated with the take-all decline fungus. Disease severity ratings taken after 4 weeks of growth under controlled conditions indicated that plants colonized by the wild-type Q8r1-96 had significantly less (P = 0.05) disease (severity of ~4.0 on the 0-to-8 scale) than those from control treatments inoculated only with the take-all disease fungus G. graminis var. tritici (severity of 5.0). However, the inactivation of rsc/rsp genes did not result in a significant reduction in the biocontrol activity of Q8r1-96 (see Table S6 in the supplemental material).

Q8r1-96 type III effectors are secreted in culture from P. syringae and P. fluorescens. To determine whether the putative Q8r1-96 type III effectors RopAA, RopB, and RopM could be secreted in culture via a T3SS, we electroporated constructs carrying each of their genes fused at their 3′ termini to a hemagglutinin (HA) epitope into P. syringae pv. tomato DC3000 and P. fluorescens Q8r1-96. Importantly, the constructs encoding RopB-HA and RopM-HA also carried genes encoding their putative type III chaperones ScrB and ScrC, respectively. We included P. syringae secretion experiments because the protocols are well established whereas they are not for P. fluorescens. The strains were grown overnight in a rich liquid medium and then for 6 h in a minimal medium that induces the P. syringae type III secretion system (30). Cell-bound and supernatant fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses. RopAA-HA, RopB-HA, and RopM-HA were detected in supernatant fractions from wild-type DC3000 but not from a DC3000 hrcC mutant defective in type III secretion (Fig. 4A). These strains also expressed a mature form of β-lactamase, which was found only in the cell fractions, indicating that nonspecific cell lysis did not occur in significant amounts (Fig. 4A). RopAA-HA, RopB, and RopM were also found in the supernatant fractions in similar secretion experiments that used Q8r1-96 strains expressing these type III effectors (Fig. 4B). Taken together, the results indicate that these proteins are secreted in culture via the P. syringae and P. fluorescens T3SSs.

RopAA, RopB, and RopM are injected into plant cells by the P. syringae and P. fluorescens T3SSs. In the majority of cases, type III substrates that are secreted in culture by the T3SS of P. syringae are also injected into plant cells. To determine if Q8r1-96 type III effectors RopAA, RopB, and RopM are injected into leaf cells, we used an adenylyl cyclase (CyaA) injection assay (58). This assay can determine if a type III effector-CyaA fusion is injected into eukaryotic cells because the CyaA enzyme is dependent on calmodulin, a protein present in sufficient amounts only inside eukaryotic cells. When a CyaA fusion is injected into plant cells there is a substantial increase in cAMP, a product of the CyaA-catalyzed reaction. We made constructs that express Q8r1-96 type III effectors C terminally fused to CyaA and electroporated these constructs into P. fluorescens 55(pLN1965) and P. fluorescens Q8r1-96. P. fluorescens 55(pLN1965) encodes a functional P. syringae T3SS, and it has been used to inject P. syringae type III effectors into plant leaf cells (25). We included P. fluorescens 55(pLN1965) in these experiments because we did not know if the native T3SS of Q8r1-96 would be capable of injecting type III effectors into leaf cells. Bacterial strains expressing RopAA-CyaA, RopB-CyaA, or RopM-CyaA were infiltrated...
was not injected in large amounts based on the low levels of cAMP (Fig. 4C), demonstrating that RopB is dependent on SrcB for it to be type III secreted.

Tobacco leaf tissue infiltrated with Q8r1-96 strains expressing the different CyaA fusions contained low levels of cAMP (Fig. 4D). However, the strains that expressed RopAA-CyaA, RopB-CyaA (with SrcB), and RopM-CyaA (with SrcM) contained significantly higher levels of cAMP than samples infiltrated with the control strains, indicating that the Q8r1-96 T3SS can inject these type III effectors into leaf cells (Fig. 4D), albeit at a much lower level than the P. syringae T3SS. It is important to note that P. syringae normally resides in the apoplast between leaf mesophyll cells whereas P. fluorescens is a soil bacterium normally present in the rhizosphere near plant roots. Thus, the Q8r1-96 T3SS may not sense the proper plant signals in leaves to fully engage its contact-dependent injection system.

RopAA, RopB, and RopM can suppress PAMP-triggered and effector-triggered immune responses. To determine if the Q8r1-96 type III effectors RopAA-CyaA, RopB-CyaA, and RopM-CyaA were capable of suppressing the innate immune response, we tested if they were able to suppress an HR, an ETI response, and flg22-induced reactive oxygen species (ROS) production, a PTI response. We used an established assay using strain P. fluorescens 55(pH1101) to determine the extent that Q8r1-96 type III effectors could suppress the HopA1-dependent HR (25, 32). This strain can elicit an HR on tobacco and many other plants because it encodes a functional P. syringae T3SS and one type III effector, HopA1, which is recognized by tobacco and other plants inducing ETI (29). We introduced constructs that encoded these type III effectors (fused C terminally to an HA epitope) into P. fluorescens 55(pH1101) and confirmed that they were expressed with immunoblot analyses (data not shown). Bacterial strains expressing these Q8r1-96 type III effectors did not elicit the HopA1-dependent HR when they were infiltrated at 2 × 10⁷ cells/ml (Fig. 5A). However, they retained the ability to elicit the HopA1-dependent HR when the bacterial strains were infiltrated at 1 × 10⁸ cells/ml (data not shown). This would designate them as class II suppressors using the grouping system described by Guo et al. (25).

To determine if the Q8r1-96 type III effectors could suppress a PTI response, we infiltrated P. fluorescens 55(pLNI965) expressing RopAA-HA, RopB-HA, or RopM-HA into N. benthamiana leaf tissue and then 24 h later ROS levels were determined after flg22 treatment. ROS levels were determined using the chemiluminescence probe L-012, a luminol derivative that measures superoxide anion. We found that plant tissue infiltrated with bacterial strains expressing Q8r1-96 type III effectors produced smaller amounts of ROS after flg22 treatment than did a bacterial control strain carrying an empty vector (Fig. 5B). Thus, RopAA-HA, RopB-HA, or RopM-HA can suppress flg22-induced ROS production, suggesting that these type III effectors can suppress PTI.

**DISCUSSION**

The results of our study revealed that Q8r1-96 carries a full-fledged T3SS consisting of structural proteins, dedicated regulatory proteins RspL and RspS, a putative harpin-like translocator, RspZ, an accessory lytic murein transglycosylase,
FIG. 5. Q8r1-96 type III effectors can suppress PTI and ETI responses. (A) P. fluorescens 55(pHIR11) strains carrying constructs that encode Q8r1-96 type III effectors RopAA-HA, RopB-HA, or RopM-HA were infiltrated into N. benthamiana cv. Xanthi leaves at 1 x 10^7 cells/ml. Also infiltrated were P. fluorescens 55(pHIR11) strains with an empty vector (EV) or a construct that encoded the type III effector AvrPtoB, a P. syringae effector known to suppress the pHIR11-dependent HR. (B) ROS levels in N. benthamiana leaf tissue were determined 21 h after infiltration of P. fluorescens 55(pLN1965) strains carrying constructs that encode the Q8r1-96 type III effectors RopAA-HA, RopB-HA, or RopM-HA. The plant tissue was bathed in a flg22 solution while the ROS levels were being measured, and the relative light units (RLU) for the 10-min reading are shown. The standard errors are indicated with bars, and the results for the plant tissue infiltrated with the empty vector (EV) are significantly different from those for the strains expressing the Q8r1-96 type III effectors (P < 0.02).

RspH, and at least three type III effector proteins, RopM, RopAA, and RopB (see Table S1 in the supplemental material). The T3SS gene cluster appears to be stably fixed in the Q8r1-96 genome, a notion supported by the overall topological congruence among type III secretion and 16S rRNA genes and the lack of atypical G+C content, tRNAs, and site-specific integrase genes in the vicinity of the rsc/rsp genes (see Fig. S1 and S2 in the supplemental material). Our results also revealed that the T3SS of Q8r1-96 is different from its counterparts in the previously characterized biocontrol strains SBW25 (53) and KD (56) (Fig. S1) and that Q8r1-96-like T3SS genes are highly conserved among the majority of 2,4-DAPG-producing Pseudomonas spp. Taken together, these findings suggest that the rsc/rsp gene cluster of Q8r1-96 encodes a new distinct and conserved lineage of T3SSs, which is widely distributed among rhizosphere-dwelling Pseudomonas spp. that are associated with biological control of soilborne pathogens on a wide range of agricultural crops (66). The presence of stable divergent lineages of T3SSs in P. fluorescens-like strains is similar to the polymorphism of T3SSs observed in P. viridiflava, where the hrc/hrp genes are encoded by two distinct paralogous pathogenicity islands (PAIs), called T-PAI and S-PAI, that are integrated in different chromosome locations (4). The T-PAI- and S-PAI-carrying strains exhibit clear differences in virulence on Arabidopsis thaliana and tobacco, and it has been proposed that the stable maintenance of the two PAI types by selection may be beneficial for interaction of P. viridiflava with different hosts. Rhizosphere pseudomonads often strongly vary in their capacity to colonize roots of different plant species (66), and it is plausible that the presence of divergent lineages of T3SSs also reflects host specialization within P. fluorescens-like strains.

Over the past decade, considerable knowledge has been gained about the diversity and function of type III effectors in a wide range of animal- and plant-associated bacterial species (5). This makes comparative genome analyses useful and informative for providing clues about the identity of eukaryotic hosts targeted by a T3SS. The similarity of type III structural, regulatory, effector, and chaperone proteins of Q8r1-96 to their counterparts from P. syringae strongly suggests that the T3SS of Q8r1-96 is involved in interactions with the host plant. To test this hypothesis, we investigated whether the presence of functional rsp/rsc and effector genes is important for the ability of Q8r1-96 to multiply and persist in the plant rhizosphere and control the soilborne pathogen G. graminis var. tritici. Wheat and pea rhizosphere colonization assays performed in raw soil under greenhouse conditions revealed no difference between the population dynamics of several isogenic T3SS-deficient mutants and that of the parental strain (see Tables S4 and S5 in the supplemental material). Similarly, results of biocontrol assays revealed that the inactivation of T3SS genes did not impact the ability of Q8r1-96 to control take-all disease of wheat (see Table S6 in the supplemental material). The lack of a biocontrol phenotype associated with the T3SS mutants is surprising given the widespread occurrence and high level of conservation of the T3SS gene cluster in rhizosphere pseudomonads. We propose several explanations for these negative results. The type III effector-mediated suppression of PAMP recognition may be important for colonization of the plant rhizosphere only at early stages of the process, whereas our root colonization assays, which are tailored to estimate bacterial populations in the entire plant rhizosphere, may lack the sensitivity needed for detection of such transient changes in population levels. Root tip colonization assays may be more suitable for the detection of differences between the wild-type and mutant strains during initial steps in
rhizosphere colonization. We are currently establishing this assay to evaluate the Q8r1-96 T3SS mutants. It is also possible that type III effectors play a role in the interaction of Q8r1-96 with a plant host other than wheat and pea, which were evaluated in this study. The lack of an impact on the biocontrol capacity of Q8r1-96 may be due to the fact that production of the antibiotic 2,4-DAPG, which is the primary mechanism of inhibition of the take-all fungus in *P. fluorescens*, was unaffected in the *rep/spsc* mutants. These data partially agree with results reported by other groups studying the T3SSs of saprophytic plant-associated *Pseudomonas* spp. For example, the interruption of *hrcV* in *P. fluorescens* KD diminished biological control of the soilborne pathogen *Pythium ultimum* but did not affect the persistence of the bacterium in the rhizosphere of cucumber (56). Finally, the presence of a hemolysin-like gene preceded by a well-conserved *rap* box motif (see Tables S1 and S2 in the supplemental material) suggests another, yet unproven, role for the T3SS in Q8r1-96. Assuming that the aforementioned protein is indeed coexpressed with the T3SS and functions as a hemolysin, it is plausible that the resulting cytotoxic activity may enhance the ability of Q8r1-96 to resist bacteriophage predation in soil and this would not have been detected in our assays. Interestingly, a recent study by Spersandoio et al. (61) demonstrated the involvement of a *P. syringae*-like T3SS gene cluster in hemolysin secretion by an unusual clinical strain of *P. fluorescens*.

In spite of the fact that we were unable to identify a role for the Q8r1-96 T3SS in the rhizosphere colonization and biocontrol properties of Q8r1-96, the results of our reporter assays reveal that type III secretion genes are expressed in the rhizosphere of wheat—the natural habitat of this organism (Fig. 3). The results of our functional assays also prove that the T3SS of Q8r1-96 is fully functional and capable of secreting and translocating cognate type III effectors (Fig. 4). Most interestingly, the results of our functional assays also prove unequivocally that RopB, RopM, and RopAA operate in Q8r1-96 as type III effectors, are secreted via the T3SS, and, upon entry into the host plant cells, are capable of suppressing both PTI and ETI (Fig. 5).

These type III effectors are of particular interest since they may provide clues about the molecular signaling that takes place during root colonization by T3SS+ plant growth-promoting bacteria. RopM and RopAA belong to ancient type III effector families (38), and their *P. syringae* homologs (HopM1 and HopAA1-1) have been shown to suppress both ETI and PTI immune responses (9, 20, 25, 37, 48). While the enzymatic activities of HopM1 and HopAA1-1 are not known, HopM1 has been shown to induce the degradation of several host proteins via the plant 26S proteasome (47). One of these proteins targets is AtMIN7, a key component of the vesicle trafficking system in plants, which would likely be involved in the delivery of immunity-related products outside plant cells during the plant’s immune response. The similarity of RopM and RopAA to HopM1 and HopAA1-1, respectively, suggests that the Q8r1-96 type III effectors act inside plant cells and that they may possess activities similar to those of their *P. syringae* counterparts. Our results demonstrating that these proteins can also suppress plant immunity further support this possibility. Because the Q8r1-96 type III effector RopB is unlike any known type III effector and has no predicted homologs in the available sequenced bacterial genomes, less is known about its role in plants. However, since it has the ability to be secreted via the T3SS and it can suppress innate immune responses similarly to RopM and RopAA, it is likely to also act inside plant cells. Since RopB is not found in the type III effector inventory of plant pathogens, it is tempting to speculate that RopB may have targets that are root cell specific.

Since PAMPs of beneficial rhizobacteria act as common elicitors of induced systemic resistance (ISR) in plants (21), our data regarding the suppression of PTI and ETI immune responses by type III effectors are seemingly in conflict with the capacity of Q8r1-96 to induce the systemic resistance in *Arabidopsis thaliana* to *P. syringae* pv. tomato DC3000 (68). One plausible explanation for this contradiction is that the ISR effect of Q8r1-96 is mediated primarily by the polypeptide metabolite 2,4-DAPG. 2,4-DAPG produced by *Pseudomonas* spp. is primarily known as an antibiotic involved in the suppressiveness of some soils to plant pathogens (66). However, recent studies have also demonstrated that DAPG produced by *P. fluorescens* can induce resistance in plants against *P. syringae* (66), oomycetes (31), and plant-parasitic nematodes (59), presumably by interfering with auxin signaling (13).

To summarize, our findings suggest for the first time the T3SS-mediated suppression of innate immunity by a rhizosphere-inhabiting strain of *P. fluorescens*. We also demonstrated that genes within the T3SS cluster are conserved among a large and diverse group of rhizosphere pseudomonads. The results of this study add to our understanding of the complex effects exerted by saprophytic bacteria on their plant hosts and raise questions about the diversity in type III effector repertoires in rhizosphere T3SS+ bacteria and immune responses targeted by these effectors in root tissue.

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