JOURNAL OF BACTERIOLOGY, Nov. 2005, p. 7805–7814 Vol. 187, No. 22
0021-9193/05/$08.00+0 doi:10.1128/JB.187.22.7805–7814.2005

Novel Virulence Gene of *Pseudomonas syringae* pv. tomato Strain DC3000

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Received 3 June 2005/Accepted 28 August 2005

Previously, we conducted a mutant screen of *Pseudomonas syringae* pv. tomato strain DC3000 to identify genes that contribute to virulence on *Arabidopsis thaliana* plants. Here we describe the characterization of one mutant strain, DB4H2, which contains a single Tn*5* insertion in PSPTO3576, an open reading frame that is predicted to encode a protein belonging to the TetR family of transcriptional regulators. We demonstrate that PSPTO3576 is necessary for virulence in DC3000 and designate the encoded protein TvrR (*T*etR-like *v*i*r*ulence *r*egulator). TvrR, like many other TetR-like transcriptional regulators, negatively regulates its own expression. Despite the presence of a putative HrpL binding site in the *tvrR* promoter region, *tvrR* is not regulated by HrpL, an alternative sigma factor that regulates the expression of many known DC3000 virulence genes. *tvrR* mutant strains grow comparably to wild-type DC3000 in culture and possess an intact type III secretion system. However, *tvrR* mutants do not cause disease symptoms on inoculated *A. thaliana* and tomato plants, and their growth within plant tissue is significantly impaired. We demonstrate that *tvrR* mutant strains are able to synthesize coronatine (COR), a phytotoxin required for virulence of DC3000 on *A. thaliana*. Given that *tvrR* mutant strains are not defective for type III secretion or COR production, *tvrR* appears to be a novel virulence factor required for a previously unexplored process that is necessary for pathogenesis.

The infection of host plants by *Pseudomonas syringae* is a complex process involving epiphytic colonization of plant surfaces, entry into host tissue, bacterial colonization of the intercellular spaces between plant cells (the apoplast), water and nutrient acquisition, and evasion or suppression of general antimicrobial defense responses (1). Numerous studies have identified *P. syringae* virulence factors necessary for the successful infection of plant hosts. These include the *hrp* (*HR* and *p*athogenicity) and *hrc* (*HR* and *c*onserved) genes, which encode the structural components of the type III secretion system (TTSS) required for the translocation of bacterial effector proteins into the host cell; effector proteins secreted by the TTSS that presumably function within plant cells to promote disease; and phytotoxins such as coronatine (COR) (1, 31, 41). COR has been shown to contribute to the growth and disease lesion development of some *P. syringae* strains on several host plants (6). These virulence factors are expressed during infection, and many have been shown to be regulated by HrpL, an alternative RNA polymerase sigma factor (17, 54, 56). GacA has also been implicated as a regulator of *P. syringae* virulence factors (11). It is likely that additional, unidentified factors also contribute to the regulation of virulence in *P. syringae*.

Microorganisms, including *P. syringae*, possess the ability to sense their surrounding environment and regulate gene expression accordingly. The tetracycline repressor (TetR) family of bacterial regulatory proteins is one group of proteins that allow bacteria to sense and respond quickly to their environment. TetR-like regulators bind DNA via a highly conserved amino-terminal helix-turn-helix (HTH) motif (23). For example, TetR and AcrR sense the presence of antibiotics in the environment and respond by regulating the expression of antibiotic-secreting efflux pumps, thereby protecting bacteria that harbor these genes (7, 34). BetI, another TetR-like regulator, induces the synthesis of betaine in response to changes in environmental osmolarity (32). Recently, the TetR-like AefR (*A*HL and *e*piphytic *f*itness *r*egulator) protein was demonstrated to regulate the synthesis of *N*-acyl homoserine lactone (AHL) and contribute to the epiphytic fitness of *P. syringae* pv. syringae strain B728a on soybean leaf surfaces (43). Given that TetR-like regulators are known to modulate the expression of genes in response to environmental signals, TetR-like regulators may also play a role in pathogen adaptation to the plant host environment.

P. syringae pv. tomato strain DC3000 is both an important agricultural pathogen and a valuable model organism for studying plant-pathogen interactions (44). DC3000 causes disease on tomato and *Brassica* species, including turnip, mustard, collard, and cauliflower plants (13, 55; S. Elizabeth and C. Bender, unpublished data). DC3000 is also of interest to researchers investigating molecular mechanisms governing plant-pathogen interactions because it causes disease on *Arabidopsis thaliana*, a genetically tractable host plant (44). Furthermore, the DC3000 genome has been sequenced (9), which facilitates comparative analyses between genomes (26) as well as detailed investigations of individual genes.

In this study, we report the identification and characterization of a newly discovered gene required for virulence of *P. syringae* pv. tomato strain DC3000 on *A. thaliana* and tomato plants. This gene encodes a member of the TetR family of

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TABLE 1. Bacterial strains and vectors used for this study

Strain or plasmid	Relevant characteristics	Source
Strains		
P. syringae pv. tomato		
DC3000	Derivative of NCPPB1106; Rif^r	13
DB4H2	*tvrR*::Tn*5 uidA* Km^r	This study
tvrR::Ω strain	*tvrR*::Ω (Sm^r Sp^r)	This study
DC3000(pTvrR)	DC3000 + pTvrR complementing plasmid	This study
tvrR::Ω(pTvrR) strain	*tvrR*::Ω + pTvrR complementing plasmid	This study
PRE51	DC3000 with integrated p*tvrR-uidA*	This study
PRE97	*tvrR*::Ω (Sm^r Sp^r) with integrated p*tvrR-uidA*	This study
JB200	Integrated *avrPto* promoter-*uidA* reporter	5
hrpL::Ω strain	*hrpL*::Ω (Sm^r Sp^r) (VJ202)	56
hrpL::Ω *tvrR*::KO strain	*hrpL* and *tvrR* double deletion strain	This study
PRE53	*hrpL*::Ω with integrated p*tvrR-uidA*	This study
PRE90	DC3000 with p*tvrR*::Ω integrated into genome	This study
PRE105	*hrpL*::Ω with integrated p*tvrR*::KO	This study
E. coli		
DH5α	*recA lacZΔM15*	Invitrogen
MM294A(pRK600)	Cm^r Km^s triparental mating helper strain	16
Plasmids		
pCR BluntII-TOPO	Cloning vector; Km^r	Invitrogen
pJP5603	Suicide vector; Km^r	39
pME6031	Stable broad-host-range vector; Tc^r	21
pHP45Ω	Omega cassette source; Sm^r Sp^r	42
pIPET	*uidA* source for cloning; Km^r	5
pTvrR	*tvrR* complementing clone in pME6031; Tc^r	This study
p*tvrR-uidA*	*tvrR* promoter-*uidA* construct in pJP5603; Km^r	This study
p*tvrR*::Ω	*tvrR*::Ω in pJP5603; Km^r Sm^r Sp^r	This study
p*tvrR*::KO	*tvrR*::KO in pJP5603; Km^r	This study

transcriptional regulators which has been designated TvrR (for *T*etR-like *vir*ulence *r*egulator). We investigate regulation of TvrR and its role in the virulence of DC3000 on *A. thaliana*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used for this study are described in Table 1. The *P. syringae* pv. tomato DC3000 mutant strain DB4H2 was identified during a previously described screen for mutants with reduced virulence (6). All *P. syringae* strains were grown at 30°C in King's B (KB) medium (27), minimal medium (13 mM potassium phosphate, pH 7, 17 mM sodium chloride, 1.7 mM sodium citrate, 30.3 mM ammonium sulfate, 2.8 mM magnesium sulfate, and 10 mM sucrose), or *hrp*-derepressing medium (HDM; 50 mM potassium phosphate, pH 5.7, 1.7 mM sodium chloride, 7.6 mM ammonium sulfate, and 1.7 mM magnesium chloride) (24) supplemented with 10 mM fructose, unless otherwise specified. *Escherichia coli* strains were maintained on Luria-Bertani (LB) (47) medium at 37°C. The following antibiotics were added to growth media as appropriate (in μg/ml): rifampin, 100; kanamycin, 10; spectinomycin, 10; and tetracycline, 10. *E. coli* DH5α (Invitrogen) containing λ*pir* (36) was used to propagate all constructs made for this study. Plasmids were introduced into *P. syringae* recipient strains via triparental matings (45) using the helper strain MM294A(pRK600) (16).

Plant material and inoculation procedures. *A. thaliana* ecotype Columbia (Col-0) plants were used for this study. Plants were grown from seed in growth chambers maintained at 22°C with 75% relative humidity and 8 h of light daily. Dip-inoculation and growth curve experiments were conducted as described previously, using 4-week-old *A. thaliana* plants (6). *Lycopersicon esculentum* (tomato) cv. Rio Grande 76S plants were also used for dip-inoculation experiments.

For hypersensitive response (HR) assays, *Nicotiana tabacum* cv. Xanthi N/N plants were used. As described by Whalen et al. (52), tobacco plants were pipette infiltrated with 1×10^5 to 1×10^8 CFU/ml in 10 mM $MgCl_2$, and the ability to cause macroscopic tissue collapse was assayed at 18 h postinoculation. Macroscopic tissue collapse indicative of an HR was clearly visible on leaves inoculated with 1×10^7 and 1×10^8 CFU/ml of either strain DC3000 or DB4H2. No symptoms were readily detectable at 18 h postinoculation when lower inoculum concentrations of either strain were used.

β-Glucuronidase detection in vitro and in planta. Detection of β-glucuronidase (GUS) activity of DC3000 strains grown in culture medium was conducted as follows. Bacterial strains were grown to mid-log phase (optical density at 600 nm, 0.5 to 0.7) in 10 ml of KB medium, harvested by centrifugation, and resuspended in 2 ml of HDM. One milliliter of each bacterial culture was added to 10 ml fresh KB medium or 10 ml fresh HDM plus 10 mM fructose and shaken at 200 rpm for 90 min at 30°C. Approximately 5×10^9 CFU from each culture were collected by centrifugation and resuspended in 1 ml 10 mM $MgCl_2$ (final volume) to be used for β-glucuronidase activity determination. β-Glucuronidase activity was determined using 1.25 mM *p*-nitrophenyl β-D-glucuronide (PNPG) as a substrate, as described by Wilson et al. (53). GUS activity was calculated as μmol of 4-nitrophenol (measured at A_{405}) produced per min per 1×10^9 CFU of bacteria.

To detect β-glucuronidase activity in infected plant tissue, bacterial strains were grown in KB medium to mid-log phase (optical density at 600 nm, 0.5 to 0.7). Bacterial strains were washed once with 10 mM $MgCl_2$, resuspended to a final concentration of 1×10^8 CFU/ml in 10 mM $MgCl_2$, and used to vacuum infiltrate *A. thaliana* plants as previously described (52). Twelve and 22 h after inoculation, plant tissue (four disks per sample) was excised from leaves using a 0.5-cm cork borer and then ground in 10 mM $MgCl_2$. The bacterial population size was determined by plating serial dilutions of the sample onto KB plates. β-Glucuronidase activity was determined by using 1 mM 4-methylumbelliferyl-D-glucuronide (MUG) as a substrate, with 0.4 M Na_2CO_3 used to terminate the reaction, as described by Salmeron and Staskawicz (46). GUS activity was calculated as μmol of 4-methylumbelliferone produced per min per 1×10^9 CFU of bacteria, using 365 nm and 455 nm as the excitation and emission wavelengths, respectively.

Biochemical detection of coronatine. DC3000 strains were grown in Hoitink-Sinden medium plus sucrose (38) in four replicate 10-ml cultures at 18°C for 7 days. Organic acids were extracted from the culture supernatants, and high-performance liquid chromatography (HPLC) fractionation and detection of COR were performed as described previously (6). COR production was normalized to ng COR per 1×10^8 CFU. In multiple experiments, we did not observe

any difference in the HPLC retention times of purified COR and COR secreted by the wild type or the *tvrR*::Ω mutant.

Plasmid constructs. To generate pTvrR, the plasmid containing the wild-type DC3000 *tvrR* gene, primer 1.1 (5′ GCGCGGATCCGCGCTGTTCACTCCATC GTCG 3′) and primer 2 (5′ GAAAACTGCAGCCAATGCATTGGAAACGC TGTTGATTGCTCGC 3′) were used to amplify a 0.8-kb fragment containing *tvrR* and 224 nucleotides of upstream sequence, using genomic DC3000 DNA as template. The 0.8-kb PCR fragment was cloned into pCR-BluntII-TOPO (Invitrogen). The resulting plasmid was digested with KpnI and NsiI, the 0.8-kb *tvrR*-containing sequence was gel purified, and the fragment was cloned into pME6031 (21) digested with KpnI and PstI to yield pTvrR. DNA sequencing was used to confirm that the *tvrR*-containing insert within pTvrR did not contain any mutations.

To generate the *tvrR* deletion strain (*tvrR*::Ω), a 2-kb DNA fragment upstream of *tvrR* was amplified from genomic DC3000 DNA using primers 4H2 KO7 (5′ CAGGAGCTAGCGCCCAGCACTCTGTCG 3′; NheI site is underlined) and 4H2 KO3 (5′ GCTTCGGATCCGACCTTCATTTATATCCTCAATCCC 3′; BamHI site is underlined). A 1.7-kb DNA fragment downstream of *tvrR* was amplified using primers 4H2 KO6 (5′ CAGGAGGTACCGTGTCAACTGCTC TACAGC 3′; KpnI site is underlined) and 4H2 KO2 (5′ TGCTGGGATCCGA AAAACGCTGATCCGACGCGCCGC 3′; BamHI site is underlined). The upstream fragment was digested with NheI and BamHI, and the downstream fragment was digested with BamHI and KpnI. Both fragments were cloned into pJP5603 (39), which was digested with KpnI and XbaI to form p*tvrR*::KO. The omega fragment from pHP45Ω (42), which contains the spectinomycin/streptomycin resistance gene flanked by transcription and translation termination signals at both ends, was cloned into the BamHI site of p*tvrR*::KO to construct p*tvrR*::Ω. p*tvrR*::Ω was integrated into the DC3000 genome by triparental mating (45) using the *E. coli* helper strain MM294A(pRK600) to yield strain PRE90. PRE90 was subcultured seven times in KB medium containing spectinomycin. One resulting Sp^r Km^s colony was selected for PCR analysis, which verified that the *tvrR* gene was replaced with the Ω cassette. This strain was designated the *tvrR*::Ω deletion strain.

The *hrpL*::Ω *tvrR*::KO strain was constructed as follows. p*tvrR*::KO was integrated into the *hrpL*::Ω strain (56) by triparental mating to yield strain PRE105. PRE105 was passaged seven times in KB medium containing spectinomycin. One resulting Sp^r Km^s colony was selected for PCR analysis, which verified that the *tvrR* gene had been deleted. This strain was designated the *hrpL*::Ω *tvrR*::KO strain.

The *tvrR* promoter-*uidA* (p*tvrR-uidA*) reporter construct was assembled as follows. Primers 4H2 promof (5′ GTTTGGTACCCACCGTAGCCGATG CGTT 3′; KpnI site is underlined) and 4H2 promor (5′ GGAACCATGGCCA TTTATATCCTCAATCCCTTAGTG 3′; NcoI site is underlined) were used to amplify 1 kb of DC3000 genomic DNA upstream of the *tvrR* ATG start codon, which was then digested with KpnI and NcoI. The *uidA* gene was obtained by digesting pIPET (5) with NcoI and XbaI and gel purifying the *uidA*-containing 1.8-kb DNA fragment. The suicide vector pJP5603 was digested with KpnI and XbaI, and the DNAs were ligated to form the reporter construct p*tvrR-uidA*. The resulting plasmid was introduced into the appropriate DC3000 strains via triparental mating. Single-copy integration of p*tvrR-uidA* by homologous recombination adjacent to the *tvrR* gene was confirmed by PCR analysis.

DNA manipulation and sequencing. Routine DNA manipulations were performed as described by Sambrook et al. (47). DNA sequencing reactions were performed using ABI Prism Big Dye Terminator premix (version 2.1), as recommended by the supplier (Advanced Biosystems Incorporated, Foster City, CA). Automated sequencing was performed at the Protein and Nucleic Acid Chemistry Laboratory's DNA sequencing facility (Washington University, St. Louis, MO).

Bioinformatic analysis. BLASTP searches were performed using the NCBI and Swiss EMBnet servers (2). Searches conducted with the NCBI server used versions 2.2.5, 2.2.8, and 2.2.10 (04/05) to search the nonredundant and bacteria-only databases. Searches conducted with the EMBnet server used versions 2.2.5 and 2.2.8 to search the SWISS-PROT/TrEMBL and nonredundant databases. Theoretical mass was determined with NiceProt View (ExPASy). The TetR-like helix-turn-helix DNA binding domain signature was identified according to PROSITE PS01081 (ExPASy) (49).

NCBI conserved domain (CD) searches were performed using NCBI server versions 2.2.3 and 2.2.5 to search the oasis_sap. v1.57 and cdd. v1.60 databases (02/03), respectively (35). Pfam domain searches were performed using the Wellcome Trust Sanger Institute server, version 7.1, and the SWISS-PROT database (3).

Annotation of the DC3000 genome was provided by The Institute for Genomic Research (TIGR) (9). Other potential open reading frame (ORF) identifications and restriction enzyme analyses of the DC3000 genome were conducted using Lasergene software (version 3.08) by DNASTAR, Inc. (Madison, WI).

Nucleotide sequence accession numbers. The TvrR (PSPTO3676) sequence accession number is Q87Z55. The accession numbers for DC3000 ORFs are PSPTO3575 (Q87Z56), PSPTO3577 (Q87Z54), and PSPTO3578 (Q87Z53) (9). Accession numbers for hypothetical TetR-like proteins referred to in this study are as follows: *P. syringae* pv. syringae B728a Psyr_3347, Q4ZR43 (Department of Energy [DOE] Joint Genome Institute, Walnut Creek, CA); *Vibrio cholera* El Tor biotype strain N16961 VC1408, Q9K552 (22); *Xanthomonas campestris* pv. campestris ATCC 33913 XCC2684, Q8P7C7 (14); *P. syringae* pv. syringae B728a AefR, Q7WU46 (43); and DC3000 PSPTO3549, Q87Z81 (9). Accession numbers of known TetR-like transcriptional regulators referred to in this study are as follows: *E. coli* tetracycline repressor protein class C [TetR(C)], P03039 (7); *E. coli* AcrR, P34000 (33); *E. coli* UidR, Q59431 (K. J. Wilson and R. A. Jefferson, direct submission); and *E. coli* BetI, P17446 (32). The accession numbers for other proteins mentioned in the text are as follows: *Pseudomonas putida* strain G7 NahY, Q9Z429 (19); and *P. syringae* pv. syringae B728a Psyr03004306, ZP_00127519 (DOE Joint Genome Institute).

RESULTS

Characterization of a DC3000 mutant with decreased virulence. We previously identified several Tn*5* insertion mutants of *P. syringae* pv. tomato strain DC3000 in a screen to identify genes required for virulence on *A. thaliana* (6, 28). One mutant, designated DB4H2, resulted in significantly reduced disease symptoms when dip inoculated onto *A. thaliana* plants and was chosen for further study. Disease symptoms of *A. thaliana* plants inoculated with the DC3000 parent strain consisted of small, water-soaked lesions surrounded by yellow regions of chlorosis at 4 days postinoculation (Fig. 1A). In contrast, *A. thaliana* plants inoculated with the DB4H2 mutant did not develop water-soaked lesions or chlorosis at 4 days postinoculation (Fig. 1B) but did occasionally develop flecking and pitting symptoms (data not shown) similar to those previously observed on plants inoculated with coronatine-deficient (COR^-) mutant strains (6). We also observed that tomato plants inoculated with DB4H2 exhibited reduced disease symptoms (data not shown).

We examined whether the reduced disease symptoms observed on plants inoculated with DB4H2 correlated with a reduced ability to grow within plant tissue by measuring the internal population of bacteria growing within inoculated *A. thaliana* leaves. As shown in Fig. 1C, the internal population of wild-type DC3000 bacteria increased by 4 to 5 orders of magnitude over the course of 4 days. In contrast, DB4H2 growth in plant tissue was reduced by 1 to 3 orders of magnitude at 2 days postinoculation and by 2 to 3 orders of magnitude 4 days after inoculation compared to that of the DC3000 strain. The reduced disease phenotype of plants inoculated with DB4H2 correlated with its decreased ability to grow within infected plant tissue.

The dip-inoculation method used in our studies requires bacteria to enter plants through natural openings in the plant surface in order to establish infection sites within the apoplast. Because the decreased virulence of strain DB4H2 might be due to an impaired ability to enter leaf tissue through natural entry routes, we infected *A. thaliana* plants via vacuum infiltration, whereby bacteria are forcibly introduced into plant leaves. This inoculation method circumvents the need for bacteria to enter plant tissue on their own. The population of wild-type strain DC3000 increased approximately 4 orders of magnitude over the course of the 4-day experiment (Fig. 1D).

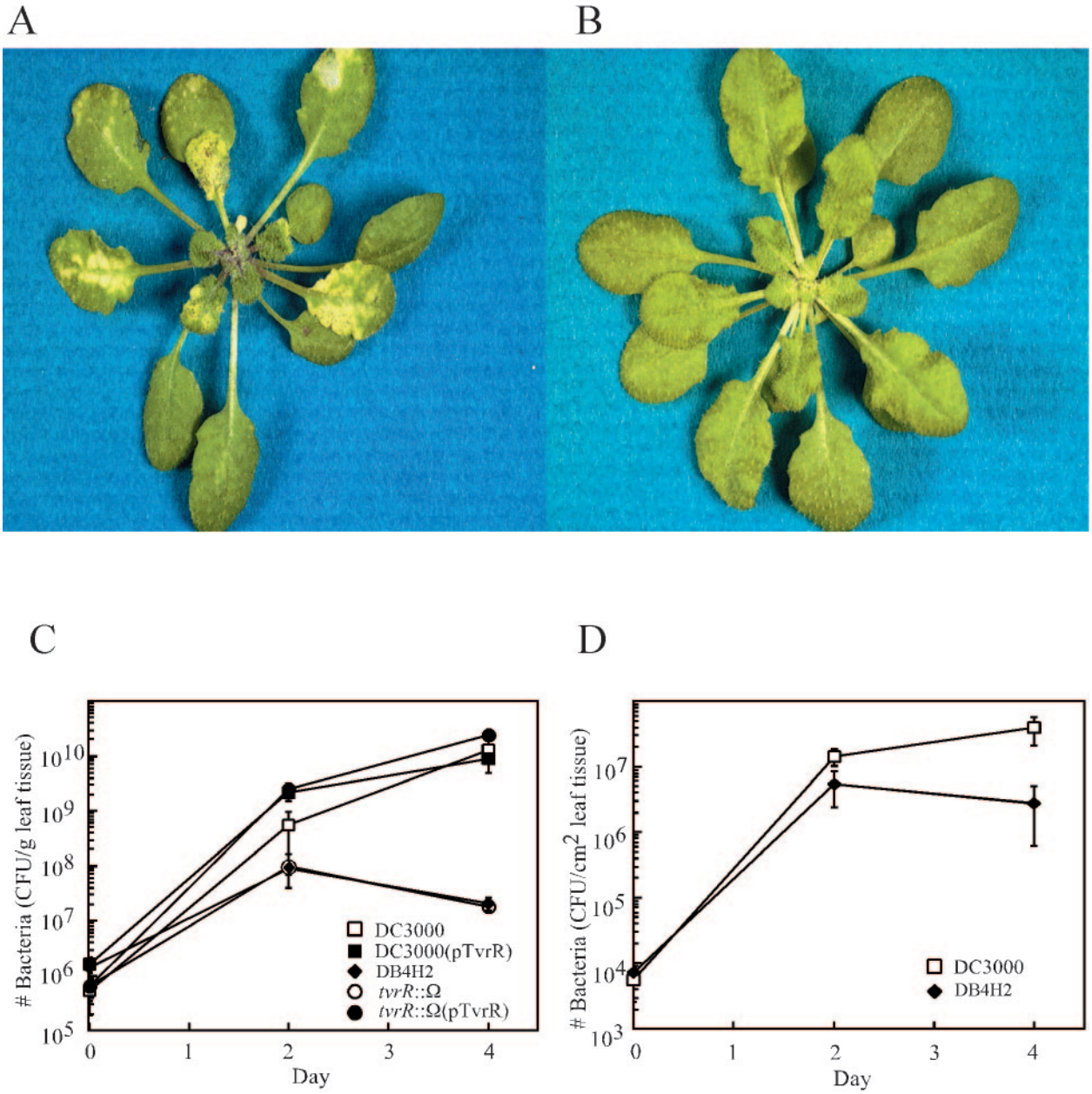


FIG. 1. Disease symptoms resulting from dip inoculation of 4-week-old Col-0 *A. thaliana* plants at 4 days postinoculation with DC3000 (A) or DB4H2 (B). (C) Growth of indicated dip-inoculated strains within 4-week-old Col-0 *A. thaliana* plants. Data points represent mean numbers of CFU per gram of leaf tissue ± standard deviations from three separate plant samples. Similar results were obtained for two additional experiments. Open squares, DC3000; closed squares, DC3000(pTvrR); diamonds, DB4H2; open circles, *tvrR*::Ω strain; closed circles, *tvrR*::Ω(pTvrR) strain. (D) Growth of indicated vacuum-infiltrated strains within 4-week-old Col-0 *A. thaliana* plants. Data points represent mean numbers of CFU per cm^2 of leaf tissue ± standard deviations from three separate plant samples. Similar results were obtained for two additional experiments. Open squares, DC3000; diamonds, DB4H2.

In contrast, growth of the DB4H2 strain was reproducibly lower (~1 order of magnitude) than that of the DC3000 parent strain at 2 days postinfiltration and 1 to 2 orders of magnitude lower at 4 days postinfiltration. Although the DB4H2 mutant strain grew slightly better when inoculated by vacuum infiltration into plant hosts than when dip inoculated, its growth was still impaired. Therefore, the impaired growth of mutant strain DB4H2 within dip-inoculated plant tissue is not solely due to a defect in entering plant tissue through natural entry routes (see Discussion).

The growth defect of DB4H2 within plant tissue led us to test whether DB4H2 is deficient in producing or utilizing some required nutrient by assessing its ability to grow in various culture media. The growth of strain DB4H2 in culture was comparable to that of strain DC3000 in both nutrient-rich KB medium and a variety of nutrient-poor minimal media, includ-

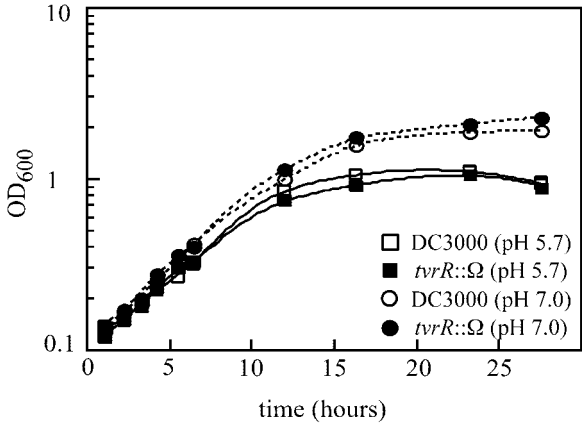


FIG. 2. Growth of DC3000 and the *tvrR*::Ω strain in HDM supplemented with 10 mM sucrose at various pHs. Open squares, DC3000 at pH 5.7; closed squares, the *tvrR*::Ω strain at pH 5.7; open circles, DC3000 at pH 7.0; closed circles, the *tvrR*::Ω strain at pH 7.0. Similar results were observed for two additional experiments. OD_{600}, optical density at 600 nm.

ing HDM plus 10 mM sucrose at pH 7.0 and 5.7 (Fig. 2 and data not shown). Because DB4H2 showed no growth defect when grown in culture media, we concluded that DB4H2 is not an auxotrophic mutant, can grow as well as DC3000 at pH 5.7 (the pH of the plant apoplast), and has the ability to catabolize sucrose, a carbon source presumed to be readily available within the plant apoplast (8). These data demonstrate that strain DB4H2 does not have a general growth defect. Rather, DB4H2 appears to be specifically impaired during plant pathogenesis.

Characterization of genomic sequences surrounding the transposon insertion site in DB4H2. Southern hybridization analysis confirmed that DB4H2 contains a single Tn*5 uidA* Km^r insertion (data not shown) (6). The DNA sequences flanking the transposon insertion site were determined by sequencing outward from the transposon, using primers complementary to the *uidA* and aminoglycoside 3′-phosphotransferase (Km^r) genes (6). The gene disrupted in the DB4H2 mutant strain is predicted to encode a putative 207-amino-acid protein that has been designated PSPTO3576 by the DC3000 genome sequencing project conducted by TIGR (Fig. 3A) (9). PSPTO3576 has a theoretical molecular mass of approximately 23 kDa, as determined by NiceProt View (18).

BLASTP sequence comparisons using the NCBI and EMBnet servers indicated that the PSPTO3576 protein is similar to the TetR family of transcriptional regulators (Fig. 3B) (2). PSPTO3576 is most similar to a putative TetR-like transcriptional regulator of *P. syringae* pv. syringae strain B728a designated Psyr_3347 (86% identity). PSPTO3576 also shares significant similarity with putative TetR-like proteins of the *V. cholera* El Tor biotype (58% similarity) and *X. campestris* pv. campestris ATCC 33913 (55% similarity). These putative proteins are most similar in the region containing the TetR-like HTH DNA binding motif located at the amino terminus (amino acids 24 through 54 [PROSITE PS01081] [Fig. 3B]) (49), although substantial similarity also extends through the carboxy termini of these proteins. A BLASTP search using PSPTO3576 amino acid residues 59 through 207 identified the same putative proteins among the closest matches (data not shown).

PSPTO3576 also shares significant similarity (52%) with the AefR protein of *P. syringae* pv. syringae strain B728a (Fig. 3B) (43). AefR positively regulates AHL production and contributes to epiphytic survival on plant surfaces (43). However, it is unlikely that PSPTO3576 is the DC3000 homolog of AefR, as another predicted DC3000 protein, PSPTO3549, shares 90% identity with AefR and is most likely the DC3000 AefR homolog (Fig. 3B).

BLASTP analysis revealed that PSPTO3576 is also similar to several known TetR-like transcriptional regulators. Of the many tetracycline repressor proteins, TvrR is most similar (40%) to TetR(C) (Fig. 3C) (7). PSPTO3576 is 41% similar to the multidrug efflux pump repressor AcrR (34), 44% similar to the β-glucuronidase repressor UidR (4), and 42% similar to the betaine synthesis regulator BetI (32). These proteins are most similar throughout their HTH DNA binding motifs (Fig. 3C) but diverge substantially at their carboxy termini (data not shown). Because PSPTO3576 is similar to known and putative TetR-like transcriptional regulators and is necessary for DC3000 virulence (see below), we designated this protein *T*etR-like *vir*ulence *r*egulator (TvrR).

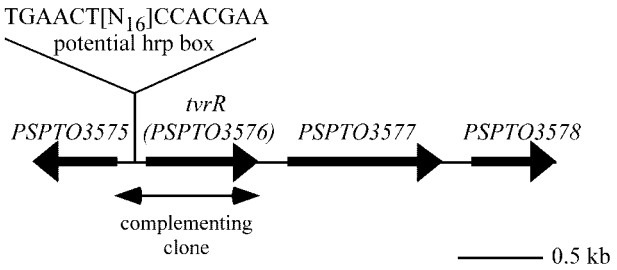
We also conducted several protein domain searches to investigate the possible function of TvrR. The NCBI CD search engine indicated that TvrR contains the TetR bacterial regulatory domain (E value, 6e−8) (35). Additionally, Pfam domain searches confirmed that TvrR contains the TetR domain Pfam-A 00440 (E value, 1.4e−16) and Pfam-B 48323 (E value, 1.5e−11), which is always found associated with Pfam-A 00440 (3). These protein domain analysis programs further support the hypothesis that TvrR is a member of the TetR family of transcriptional regulators.

In DC3000, the transcription of many virulence genes is activated by the HrpL alternative sigma factor (25, 48, 54). HrpL is believed to interact with promoter "hrp box" sequences located within the upstream regulatory regions of virulence genes to activate their transcription. Our analysis of the *tvrR* promoter region revealed a potential hrp box sequence (TGGAACT[N_{16}]CCACGAA) 94 nucleotides upstream of the predicted translation start site (Fig. 3A) (56). The presence of this potential regulatory element suggested that *tvrR* expression may be regulated by HrpL, as are many other DC3000 virulence genes.

Recently, two groups (17, 56) used computer algorithms to search the DC3000 genome for promoters containing potential hrp box consensus sequences. The genome search algorithm used by Zwiesler-Vollick et al. detected the potential hrp box sequence upstream of *tvrR* (designated HCO51 in their study). However, using microarray analysis, they failed to detect significantly higher levels of *tvrR* expression in cells grown in HDM than in cells grown in LB, and they did not further investigate this gene (56). The computer algorithm utilized by Fouts et al. used a more stringently defined hrp consensus sequence and did not report identification of the potential hrp box upstream of the *tvrR* gene (17).

We also examined the DC3000 genome in the vicinity of *tvrR* for possible clues to the function of TvrR. PSPTO3575, the divergently transcribed ORF upstream of *tvrR*, is predicted to encode a 173-amino-acid protein which is most similar to a

A



B

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TvrR          ---MKVRTEARREAIIDAAASVFLEMGYERASMNEVTKRMGGSKATIYSYFPSKEELFIA 57
Psyringae     ---MKVRTEARREAIIDAAASVFLEMGYERASMNEVTKRMGGSKATIYSYFPSKEELFIA 57
Vcholerae     ---MRVKSEEKRQAILEIAKDSFTKQGFEQTSMSHIAKMLGGSKATLYNYFSSKEEIFTA 57
Xcampestris   ---MRVRTEEKRDTIVQAASEVFLELGFEGASMSQIAARVGGSKRTLYGYFGSKEELFVA 57
AefR          MTEPMRLTDQKRESIVQAAIAEFGDRGFEITSMDRIAARAKVSKRTVYNHFPSKEELFAE 60
PSPTO3549     ----MRLTDQKRESIVQAAIAEFGDRGFEITSMDRIAARAEVSKRTVYNHFPSKEELFAE 56
                    :: :*::*:: *   * . *:* :**..::     ** *:*.:* ****:*

TvrR          VVNRLATAHLADAVSELTTYNGADTNAELRTLFTRFGERMLMVLINDDNALAVYRMVVAE 117
Psyringae     VVNRLATAHLADAVSELAIQD--DKPIDLRALFTRFGERMLMVLINDDKALAVYRMVVAE 115
Vcholerae     VMESSATEQIALSFLSLDHNR------ELKPALLEFGYNFLNSVLTQD-AMSIYRMAIHE 110
Xcampestris   VAKDMSDRYFDPLLDALSRSSG-----PIADALQRFGEDVLTFLCAPP-NITSWQTIIGV 111
AefR          MLQRLWSCAPPQAEVVFRPQIG------LREQLRELLWGKMRNLTNSH-FIDLARVVVGA 113
PSPTO3549     MLQRLWNCAPPQSEVVYRPLVS------LREQLLELLWGKMRNLTDSS-FLDLARVVVGA 108
              : :                        :   : .:    :  :      :   :  :

TvrR          SGHSAIGMMFYDSGPRECLQTVTALMTAAMQRGQLRETDPHIAALQLTSLLTAETDIRLY 177
Psyringae     SGHSDIGMMFYESGPSQCLQTVTSLMTLAMRKGQLRETDPHIASLQLTSLLTAETDIRLY 175
Vcholerae     ADRSAIGRHFYENGPKRGWARLSRYITCQIECGSLKECDPWIAAMHFKALLSAQYFEPFS 170
Xcampestris   SGRSDVGALFFSAGQEEGLKRFAEYLQTQVDCGQLRCDDTMLAAQQFAALVEAETLMPCL 171
AefR          TIHSPERAQVWMARINEREETFSAWIRAAQKDGRLKPVDPAFAATQMHALLKSFAFWPQV 173
PSPTO3549     TIHSPERAQVWLARINEREETFSAWIRAAQKDGRLKPVDPGFAATQMHALLKSFAFWPQV 169
              : :*     .:     .    .:  :      * *:  *. :*: :: :*: :

TvrR          QQTPARLSVEQISAMVERAVDTFMLGMEKR---- 207
Psyringae     QQAPMPLSIEEIRAMVERAVDTFMLGMEKR---- 205
Vcholerae     LGAIEKPTSSELKATTQRAVEAFLCLYAAD---- 200
Xcampestris   FGALKNPSPEYLRDATRRAVELFLVGYACKPAAA 205
AefR          TFSAALLTPEEQHTVVESTLDMFLGWYEITR--- 204
PSPTO3549     TFNAALLTPQEQSNVVESALNMFLGWYEIPG--- 200
                    : .     .. ::: *:
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C

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TvrR      ----MKVRTEARREAIIDAAASVFLEMGYERASMNEVTKRMGGSKATIYSYFPSKEELFIAV----- 58
TetR(C)   -------MNKLQREAVIRTALELLNDVGMEGLTTRRLAERLGVQQPALYWHFKNKRALLDALAEAML 60
AcrR      MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAAGVTRGAIYWHFKDKSDLFSEI----- 62
UidR      MMDNMQTEAQPTRTRILNAAREIFSENGFHSASMKAICKSCAISPGTLYHHFISKEALIQAIIL--- 64
BetI      --MPKLGMQSIRRRQLIDATLEAINEVGMHDATIAQIARRAGVSTGIISHYFRDKNGLLEAT----- 60
                    .  *  :: .    : : *    :   : . .     :  :* .*  ::
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FIG. 3. (A) Schematic diagram of the *tvrR* region of the DC3000 genome, including adjacent ORFs (bold arrows). *tvrR* (PSPTO3576 [accession no. Q87Z55]) is predicted to encode a 207-amino-acid protein with significant similarity to TetR-like regulatory proteins. The sequence of a putative hrp box located 94 nucleotides upstream of the predicted *tvrR* translation start site is indicated. The portion of the genome used to construct the complementing clone (pTvrR) is indicated by the double-headed arrow. PSPTO3575 (Q87Z56) is predicted to encode a 173-amino-acid protein of unknown function. PSPTO3577 (Q87Z54) is predicted to encode a 542-amino-acid protein with similarity to methyl-accepting chemotaxis proteins. PSPTO3578 (Q87Z53) is predicted to encode a 173-amino-acid protein of unknown function. (B) ClustalW (50) alignment of TvrR with similar putative proteins. The horizontal line indicates the PROSITE PS01081 TetR-type helix-turn-helix domain signature. TetR-like transcriptional regulators are indicated as follows: Psyringae, *P. syringae* pv. syringae strain B728a (Psyr_3347); Vcholera, *Vibrio cholerae* El Tor biotype strain N16961 (Q9K552); Xcampestris, *Xanthomonas campestris* pv. campestris ATCC 33913 (Q8P7C7); AefR, *P. syringae* pv. syringae strain B728a (Q7WU46); and PSPTO3549, the presumed DC3000 *aefR* homolog (Q87Z81). (C) ClustalW (50) alignment of TvrR with several known TetR-like transcriptional regulators, i.e., TetR(C) from *E. coli* (P03039), AcrR from *E. coli* (P34000), UidR from *E. coli* (Q59431), and BetI from *E. coli* (P17446). The horizontal line indicates the PROSITE PS01081 TetR-type helix-turn-helix domain signature.

hypothetical *P. syringae* pv. syringae strain B728a protein of unknown function (Psyr_3346) (Fig. 3A). ORF PSPTO3577, which is located 181 bp downstream of *tvrR*, is predicted to encode a 542-amino-acid protein with significant similarity (59%) to the NahY chemotaxis transducer protein of *P. putida* G7 (19). Both NahY and PSPTO3577 are nearly identical throughout the highly conserved bacterial chemotaxis signaling domain and share a significant degree of conservation throughout the naphthalene-sensing periplasmic domain (data not shown). PSPTO3578, which is adjacent to PSPTO3577 (Fig. 3A), is predicted to encode a 173-amino-acid protein of unknown function. TIGR annotation of this region indicates that

tvrR, PSPTO3577, and PSPTO3578 may form a single transcriptional unit spanning 3 kb (9).

***tvrR* is required for DC3000 virulence.** We verified that *tvrR* is required for DC3000 virulence by first generating a deletion mutant (*tvrR*::Ω) in which the *tvrR* gene was replaced with the streptomycin and spectinomycin resistance-conferring omega fragment (see Materials and Methods) (42). We then assessed the ability of the *tvrR*::Ω strain to grow and cause disease symptoms within *A. thaliana* plants. Plants dip inoculated with the deletion mutant developed no signs of chlorosis or necrosis by 4 days postinoculation, similar to what was observed for plants inoculated with the original DB4H2 mutant strain (data not shown). As shown in Fig. 1C, the growth of *tvrR*::Ω within plant tissue was significantly reduced compared to that of the DC3000 parent strain 4 days after inoculation. The decrease in growth of the *tvrR*::Ω strain was highly reproducible and nearly identical to that of the mutant strain DB4H2 in three separate experiments.

To demonstrate that the decrease in virulence seen for *tvrR* mutant strains was due to the loss of TvrR activity, we introduced the wild-type *tvrR* gene in *trans* into the *tvrR*::Ω deletion strain. The *tvrR* ORF, including 224 nucleotides of upstream sequence (Fig. 3A), was cloned into the stable broad-host-range vector pME6031 (21) and transformed into strains DC3000 and *tvrR*::Ω to create DC3000(pTvrR) and the *tvrR*::Ω(pTvrR) strain, respectively (Table 1; see Materials and Methods). The pTvrR construct fully restored wild-type disease symptom development to the *tvrR*::Ω deletion strain (data not shown) and restored growth within plant tissue to wild-type levels 4 days after inoculation (Fig. 1C). We consistently observed that both the DC3000 and *tvrR*::Ω strains carrying pTvrR grew to slightly higher levels than the DC3000 parent strain at 2 days postinoculation. As determined by Heeb et al. (21), pME6031 is present at approximately 14 copies per bacterial cell. Multiple copies of the *tvrR* ORF apparently increase virulence early in infection (Fig. 1C). Although these data do not allow us to conclude whether *tvrR* is part of an operon that includes PSPTO3577 and PSPTO3578, they do indicate that the *tvrR* gene is sufficient to complement the *tvrR*::Ω mutant strain when provided in *trans*. We therefore conclude that *tvrR* is required for DC3000 virulence.

Regulation of *tvrR* expression. Because *tvrR* is required for virulence, we investigated how *tvrR* expression is regulated to gain insight into its role during DC3000 infection. To accomplish this, we fused the *tvrR* promoter region to the promoterless *uidA* (GUS) reporter gene (p*tvrR-uidA*) and monitored the β-glucuronidase activity of DC3000 strains possessing a single copy of this reporter gene construct integrated into the genome adjacent to the *tvrR* locus (see Materials and Methods).

We began analyzing the regulation of *tvrR* expression by monitoring p*tvrR-uidA* expression levels in DC3000 grown in nutrient-rich KB medium and nutrient-poor HDM. Prior studies have demonstrated that many virulence genes are upregulated in HDM, so we hypothesized that *tvrR* would also be upregulated when bacteria are grown in HDM (5, 24, 46). As summarized in Table 2, *tvrR* was expressed when bacteria were grown in KB medium (27.5 units/10^9 CFU). *tvrR* expression in HDM, which is thought to mimic the nutritional conditions present in the plant apoplast, was only slightly elevated (42.8 units/10^9 CFU) compared to expression in KB medium (Table 2). As a control, we also measured the expression of an HrpL-dependent *avrPto* promoter-*uidA* fusion construct, which was previously shown to be upregulated in HDM. Consistent with previous reports, the *avrPto-uidA* fusion construct was upregulated in HDM compared to its expression in KB medium. From these data, we conclude that *tvrR* is constitutively expressed in cells grown in KB medium and is not significantly further induced when cells are transferred to HDM. We also examined *tvrR* expression in several other minimal media and consistently found that *tvrR* expression was similar to that observed in KB medium (data not shown). Consistent with *tvrR* being required for the growth of *P. syringae* within plants, we also observed that *tvrR* is expressed within infected plant tissue (data not shown).

TABLE 2. Expression of *tvrR* promoter-*uidA* fusion in culture

Genetic background	Promoter fusion	Presence of TvrR protein	Growth medium	GUS activity[a]
DC3000	*tvrR-uidA*	+	KB	27.5 ± 2.0
DC3000	*tvrR-uidA*	+	HDM	42.8 ± 4.0
Δ*hrpL*	*tvrR-uidA*	+	KB	28.7 ± 5.7
Δ*hrpL*	*tvrR-uidA*	+	HDM	35.9 ± 3.2
Δ*tvrR*	*tvrR-uidA*	−	KB	155.5 ± 17.7
Δ*tvrR*	*tvrR-uidA*	−	HDM	205.2 ± 18.3
Δ*hrpL* Δ*tvrR*	*tvrR-uidA*	−	KB	153.5 ± 26.0
Δ*hrpL* Δ*tvrR*	*tvrR-uidA*	−	HDM	198.5 ± 26.8
DC3000	*avrPto-uidA*[b]	+	KB	34.4 ± 5.9
DC3000	*avrPto-uidA*	+	HDM	104.0 ± 22.8

[a] β-Glucuronidase activity of *P. syringae* strains containing *tvrR* promoter-*uidA* gene fusion (mean ± standard error) grown in KB medium or HDM. GUS activity was calculated as μmol 4-nitrophenol produced per minute per 1×10^9 CFU of bacteria. Values shown were derived by averaging data from three to six independent experiments.

[b] The strain contains an *avrPto* promoter-*uidA* gene fusion and was used as a positive control for this experiment (5).

Many TetR-like transcriptional regulators, including TetR, AcrR, and UidR, negatively regulate their own expression (4, 7, 34). Therefore, it was conceivable that TvrR negatively regulates its own expression. To test this hypothesis, we introduced the p*tvrR-uidA* fusion into the *tvrR*::Ω strain and assessed its expression. *tvrR* expression in the *tvrR*::Ω deletion strain in KB medium was approximately fivefold higher than expression in wild-type DC3000 (Table 2). *tvrR* expression in HDM was also approximately fivefold higher in the deletion mutant strain than in the DC3000 parent strain. Therefore, TvrR, like other TetR-like transcriptional regulators, negatively regulates its own expression.

The promoter region of *tvrR* contains a potential hrp box sequence (Fig. 3A), which led us to hypothesize that *tvrR* expression is also regulated by HrpL. To test this hypothesis, we introduced the p*tvrR-uidA* fusion construct into the *hrpL*::Ω deletion strain (56) and monitored GUS expression in KB medium and HDM. *tvrR* expression in the *hrpL*::Ω mutant grown in KB medium was nearly identical to expression in the DC3000 strain grown in the same medium (Table 2). Similar results were seen when strains were grown in HDM. These data suggest that *tvrR* expression is not dependent on HrpL. However, because TvrR is present in both DC3000 and *hrpL*::Ω strains, it was conceivable that TvrR negative autoregulation masked any contribution HrpL made to *tvrR* regula-

tion. Alternatively, HrpL may not regulate *tvrR* expression, despite the potential hrp box sequence in its promoter region.

To discern whether TvrR negative autoregulation was masking any contribution by HrpL to the regulation of *tvrR* expression, we constructed an *hrpL*::Ω *tvrR*::KO double mutant (see Materials and Methods), transformed it with the p*tvrR-uidA* fusion construct, and assessed its expression in KB medium and HDM. Expression levels in the double mutant strain were essentially identical to expression levels in the *tvrR* deletion strain, regardless of the growth medium (Table 2). From these data, we conclude that HrpL does not regulate *tvrR* expression.

Effect of TvrR on two required aspects of virulence. Ultimately, we wish to understand why TvrR is necessary for virulence in DC3000. Our data indicated that TvrR negatively regulates its own expression, and we hypothesize that it also regulates the expression of one or more genes involved in DC3000 virulence. Two important aspects of DC3000 virulence are type III secretion and production of the phytotoxin COR, and we examined the effect of TvrR on both.

The TTSS is responsible for delivering effector proteins, including virulence factors and defense elicitors, into plant cells. TTSS mutants are nonpathogenic to host plants and unable to elicit the macroscopic tissue collapse that is characteristic of an HR on nonhost plants (1). Some mutants, such as the *dsbA* mutant (28), possess a partially impaired TTSS, which affects their virulence and causes a delayed HR. We investigated whether the decreased virulence of strain DB4H2 was caused by an impaired TTSS by testing the strain's ability to elicit plant defense responses in tobacco plants, which are not susceptible to DC3000 infection. With respect to both timing and inoculation concentration, DB4H2 elicited an HR indistinguishable from that elicited by DC3000 (see Materials and Methods). These data indicated that DB4H2 is not impaired for type III secretion.

Since COR is a necessary DC3000 virulence factor, we hypothesized that if TvrR were to regulate the expression of COR biosynthesis genes, then an inability to synthesize or secrete COR might account, at least in part, for the decreased virulence phenotype of the *tvrR* mutant strains. To test this, we measured COR production by DC3000, the *tvrR*::Ω strain, and the *tvrR*::Ω(pTvrR) strain grown in culture by using HPLC (6). DC3000 bacteria grown in Hoitink-Sinden medium plus sucrose for 7 days secreted approximately 80 ng COR per 10^8 CFU into the culture supernatant, while the *tvrR* deletion strain secreted approximately 50% more COR during the same period (~120 ng COR per 10^8 CFU). The *tvrR*::Ω(pTvrR) complemented strain secreted less COR (~30 ng COR per 10^8 CFU) than DC3000 into the culture medium. Similar results were obtained when we measured COR secreted by these strains grown in HDM (data not shown).

Given the observation that COR production was slightly higher in the *tvrR*::Ω strain and lower in the *tvrR*::Ω(pTvrR) complemented strain than in DC3000, we conclude that the decreased virulence of the *tvrR*::Ω deletion strain is not due to an inability to synthesize and secrete COR. We also conclude that TvrR is not a positive regulator of COR synthesis in vitro. In fact, the data suggest that TvrR may negatively regulate some aspect of COR synthesis or secretion or that TvrR may regulate another pathway that influences the production of COR.

DISCUSSION

In this study, we have demonstrated that *tvrR* is required for DC3000 virulence on *A. thaliana* plants. Plant hosts inoculated with *tvrR* mutant strains do not develop the characteristic disease symptoms of water-soaked lesions and chlorosis (compare Fig. 1A and B) which are the physical hallmarks of DC3000 infection. TvrR was also shown to be required for bacterial growth to high levels within inoculated plants (Fig. 1C and D). Complementation of *tvrR* mutant strains restored wild-type growth (Fig. 1C) and disease symptom development (data not shown). We also demonstrated that *tvrR* is constitutively expressed in HDM and KB medium (Table 2). Consistent with *tvrR* being an important virulence factor, we also saw expression of *tvrR* within inoculated plants (data not shown).

The expression of *tvrR* is not dependent upon HrpL, the alternative sigma factor that regulates the expression of many known DC3000 virulence genes (Table 2). This is intriguing because the upstream regulatory sequence of *tvrR* contains a putative hrp box consensus sequence (Fig. 3A). Zwiesler-Vollick et al. identified this potential hrp box sequence upstream of *tvrR* but failed to detect significantly higher levels of expression in HDM than in LB medium (56). Consistent with their findings, we did not see significant upregulation of *tvrR* expression in strains grown in HDM versus those grown in KB medium. A study by Fouts et al. (17), who utilized a more stringently defined hrp consensus sequence, did not report the identification of the potential hrp box sequence upstream of *tvrR.* Our study demonstrates that the presence of a putative bipartite hrp box consensus sequence within the regulatory region of a gene may not accurately predict whether that gene is regulated by HrpL.

BLASTP and protein domain search programs indicated that TvrR is a member of the TetR family of transcriptional regulators (Fig. 3B and C). As with many other proteins of the TetR family, we demonstrated that TvrR negatively regulates its own expression (Table 2). Although some TetR-like proteins regulate the expression of adjacent genes, which frequently encode efflux pumps (e.g., TetA and AcrAB) (7, 34), *tvrR* is not adjacent to genes encoding an efflux pump. Improper regulation of the TetA efflux pump in the form of either constitutive expression (37) or overexpression (15) is detrimental to *E. coli* due to loss of cell membrane potential (15). TvrR does not appear to fit this paradigm, as we observed that the growth of *tvrR* mutant strains in culture was indistinguishable from that of DC3000 in all media tested (Fig. 2 and data not shown). In contrast, the growth of *tvrR* mutant strains was significantly impaired within plant tissue (Fig. 1C and D). These data suggest that the virulence defect of *tvrR* mutant strains is not due to a general growth defect or aberrant regulation of an efflux pump. The virulence defect of the *tvrR* mutant strains becomes apparent only when the strains attempt to colonize plant host tissue.

Many DC3000 mutants with decreased virulence reported to date are known to disrupt the TTSS, an effector protein, or COR production (1, 6, 31, 41). The data presented in this study indicate that the *tvrR* mutant strains are not deficient in any of these. Our HR experiments indicate that type III secretion is not impaired and that the mutant strains are presumably able to deliver effector proteins as efficiently as the wild-type

DC3000 strain. It is unlikely that TvrR is an effector protein because (i) previous screens for effector proteins have not identified TvrR as a known or suspected bacterial effector (10, 20, 40); (ii) TvrR contains two negatively charged amino acids within its first 12 residues, while most secreted effector proteins lack negatively charged amino acids at their amino terminus (40); and (iii) we have demonstrated that TvrR functions in the bacterial cell, as it negatively regulates its own expression within DC3000 (Table 2). Thus, while we cannot formally conclude that TvrR is not an effector protein, we believe it unlikely that TvrR is secreted through the TTSS. We also show that the decreased virulence of *tvrR* mutant strains is clearly not due to a defect in COR synthesis or secretion. Therefore, we hypothesize that *tvrR* regulates a previously unexplored mechanism of pathogenesis.

At this point, the role of TvrR in DC3000 virulence is not clear. Because *tvrR* regulates its own expression, the function of TvrR might be to sense changes within the bacterial environment and respond appropriately via transcriptional regulation of as yet unknown genes that influence pathogenesis. Precedence supporting a potential role for TetR-like proteins in responding to environmental cues in *Pseudomonas* species exists. For example, the *psrA* gene (*Pseudomonas s*igma *r*egulator) encodes a TetR-like transcriptional regulator that negatively regulates itself and positively activates the expression of *rpoS*, encoding an alternative sigma factor, in *P. putida* and *Pseudomonas aeruginosa* (30). Upon entry into stationary phase, PsrA upregulates RpoS expression, which helps the bacteria to adapt to nutrient-limiting conditions and other environmental stresses (30, 51). Furthermore, it was demonstrated that PsrA is a DNA binding protein that binds to its own promoter DNA and to the *rpoS* promoter (29). Recently, Chin-A-Woeng et al. demonstrated that the *psrA* gene of *Pseudomonas chlororaphis* regulates the production of an antifungal metabolite in response to environmental signals to aid in establishing a niche within the rhizosphere (12).

Plant surfaces and the apoplast are two specific environments to which DC3000 must adapt. Strain DB4H2 grew better (but not to wild-type levels) when vacuum infiltrated into host plants than when dip inoculated (Fig. 1C and D). These data suggest that *tvrR* may play some role in the epiphytic colonization of plant surfaces and/or bacterial entry into plant leaves. Precedence for this hypothesis exists in *P. syringae* pv. syringae strain B728a, for which *aefR* was shown to contribute to epiphytic survival on plant leaf surfaces (43). However, *tvrR* is unlikely to function as the DC3000 homolog of *aefR*, because another predicted DC3000 protein, PSPTO3549, is nearly identical to AefR. Nonetheless, epiphytic colonization of leaf surfaces is an important step in the pathogenesis of DC3000, and TvrR may play a role in regulating this process. However, TvrR must be required for more than epiphytic colonization and/or bacterial entry, since *tvrR* deletion strains still do not grow to wild-type levels within vacuum-infiltrated plants.

Our data suggest that *tvrR* regulates a previously unexplored process required for DC3000 pathogenesis. We hypothesize that the decreased virulence of the *tvrR* mutant strains might be caused by the overexpression of genes detrimental to virulence, a reduced expression of genes required for virulence, or a combination thereof. For example, TvrR could downregulate genes necessary for survival within the soil. If these genes were overexpressed by *tvrR* mutant strains, then improper resource allocation caused by inappropriate gene expression could interfere with the expression of factors required for efficient colonization of plant tissue. It is also conceivable that TvrR could upregulate genes necessary for phytopathogen virulence. In this scenario, the reduced level of one or more virulence factors could result in unsuccessful pathogenesis of the plant host by *tvrR* mutant strains. Studies are under way to identify genes regulated by *tvrR* and to elucidate the role of these genes in pathogenesis.

ACKNOWLEDGMENTS

We thank Justin Richner for assistance in generating the *hrpL*::Ω *tvrR*::KO strain. We thank Agnes Demianski, Neva Laurie-Berry, Eve Mellgren, and Justin Richner for critical discussions and helpful comments on the manuscript.

This work was supported by National Science Foundation grant IBN 0130693, awarded to B. N. Kunkel and C. L. Bender.

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