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Hcp2, a Secreted Protein of the Phytopathogen *Pseudomonas syringae* pv. Tomato DC3000, Is Required for Fitness for Competition against Bacteria and Yeasts

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When analyzing the secretome of the plant pathogen *Pseudomonas syringae* pv. tomato DC3000, we identified hemolysin-coregulated protein (Hcp) as one of the secreted proteins. Hcp is assumed to be an extracellular component of the type VI secretion system (T6SS). Two copies of *hcp* genes are present in the *P. syringae* pv. tomato DC3000 genome, *hcp1* (PSPTO_2539) and *hcp2* (PSPTO_5435). We studied the expression patterns of the *hcp* genes and tested the fitness of *hcp* knockout mutants in host plant colonization and in intermicrobial competition. We found that the *hcp2* gene is expressed most actively at the stationary growth phase and that the Hcp2 protein is secreted via the T6SS and appears in the culture medium as covalently linked dimers. Expression of *hcp2* is not induced *in planta* and does not contribute to virulence in or colonization of tomato or *Arabidopsis* plants. Instead, *hcp2* is required for survival in competition with enterobacteria and yeasts, and its function is associated with the suppression of the growth of these competitors. This is the first report on bacterial T6SS-associated genes functioning in competition with yeast. Our results suggest that the T6SS of *P. syringae* may play an important role in bacterial fitness, allowing this plant pathogen to survive under conditions where it has to compete with other microorganisms for resources.

Gram-negative bacteria possess a number of secretion systems to transport proteins through the inner and outer membranes to the surrounding milieu to carry out a wide range of functions, such as biogenesis of organelles (i.e., flagella), nutrient acquisition, pathogenesis, and efflux of toxins or drugs (36, 51). The type VI secretion system (T6SS) was only recently discovered and found to be conserved in many Gram-negative bacterial genera (6, 31, 38). The T6SS secretory machinery includes an ATPase (ClpB/ClpV), a serine/threonine protein kinase (PpkA), a phosphatase (PppA), a regulatory forkhead-associated domain protein (Fha), tubulus-forming structural proteins VipA and VipB, and IcmF/IcmH (DotU)-like proteins that are homologous to the T4SS membrane components (3, 4, 8, 44). The proteins secreted by this route are still largely uncharacterized. The T6SS has been demonstrated to have a role in the ecological success or virulence of a bacterial pathogen during its interaction with animal host cells (21, 38, 46). It has also been shown to contribute to virulence or fitness in plant hosts (27, 54). The T6SS is also important for the survival of attacks by predatory amoeba (29, 38). In some cases, T6SS was found to function as the secretion route for toxins that inhibit the growth of other bacteria (16, 26, 32, 42). The common T6SS-secreted proteins encoded by all T6SS-harboring bacteria are Hcp (hemolysin coregulated) and VgrG (valine-glycine rich) protein variants. Hcp and VgrG proteins are assumed to be extracellular components of the secretion machinery (37). The C-terminal part of some VgrG proteins has been associated with virulence-related functions such as actin cross-linking and ADP-ribosylation in eukaryotic host cells (37, 50). The N-terminal part of VgrG proteins shows structural similarity to phage tail tip proteins gp27 and gp5, and Hcp resembles the phage tail tube proteins, which suggests that the T6SS might have evolved from bacteriophages (20, 34, 37, 52). The finding of phage tail homology led to the hypothesis that the T6SS may also function like the T4 bacteriophage tail and puncture the target cell membrane to deliver effector proteins (37). Hcp proteins have been observed to form hexameric donut-like structures and VgrG proteins were found to form trimers (31, 37). The Hcp hexamers could theoretically form larger tubular structures which could serve as a conduit for secretion, and tube-like Hcp multimers have been observed in *in vitro* studies (20). However, no T6SS-associated stable cell surface structure resembling the pili of T3SS or T4SS (12, 41) or a phage tail has been observed thus far. Instead, an intracellular tubular structure consisting of VipA/B proteins and functioning like a contractile phage tail was recently found (3).

Pseudomonas syringae is a plant-pathogenic bacterium found in a wide variety of agricultural and nonagricultural environments such as clouds, rivers, and snowpack (30). More than 50 pathogenic varieties have been described that can infect numerous plant species, causing diseases such as leaf spots, blight, and speck and cankers and galls in shoots. Although it is typically regarded as a leaf- and shoot-infecting pathogen, it can also infect the roots of plants (2). Because of the economic importance of the pathogen in causing disease in so many plant species, it has emerged as the model bacterial pathogen for studying plant pathogenicity. Stud-

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ies of *P. syringae* have revealed that the T3SS and its effector proteins are the major virulence determinants of this phytopathogen, allowing the bacteria to suppress plant defenses and multiply inside the host tissues (13). However, the plant signal for induction of the virulence factors is still unknown. To further understand how *Pseudomonas* reacts to the conditions inside the host plants and therefore define factors important for colonization and virulence, a proteomic approach was conducted using an *hrp*-inducing medium supplemented with tomato cell extracts (15; this study). In this modified minimal medium, Hcp2 was identified as one of the most abundant secreted proteins and its secretion was shown to be dependent on T6SS. The three *P. syringae* strains whose full genomic sequences are available were all found to carry putative T6SS gene clusters, and the two chromosomal T6SS gene clusters in *P. syringae* pv. tomato DC3000 were named HSI-I and HSI-II, after Hcp secretion island (44). Here we show that only one of the two Hcp proteins is secreted in *P. syringae* pv. tomato DC3000. By using deletion mutants, we also found that neither of the *hcp* genes is involved in virulence in host plants. Instead, a novel activity was revealed in this phytopathogenic bacterium suggesting that Hcp2 plays a role in intermicrobial competition.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *P. syringae* were grown in King's medium B (KB) (19) with appropriate antibiotics at 22 to 28°C or in Hrp-inducing minimal medium (HIM) (17) at 18°C unless otherwise specified. *Escherichia coli* strains and the other enterobacterial species were cultured in Luria-Bertani (LB) medium. The concentrations of the antibiotics used for selection were as follows: kanamycin, 50 μg/ml; rifampin, 100 μg/ml; tetracycline, 25 μg/ml.

Analysis of secreted proteins under Hrp-inducing conditions. The *P. syringae* pv. tomato DC3000 wild-type and *hrcC* mutant strains were first cultured in KB starting at an optical density at 600 nm (OD_{600}) of 0.1 to 0.3 (3.5 h at 28°C) and then in HIM starting at an OD_{600} of 0.3. After 19 h of incubation at 18°C, the cultures were centrifuged at 8,000 × *g* for 15 min at 4°C. The culture supernatants were filtered through a 0.45-μm filter to abolish all of the bacterial cells, and phenylmethylsulfonyl fluoride was added to a 0.5 mM concentration. Proteins from the cell-free supernatants of 100-ml liquid cultures were concentrated by using Amicon ultrafiltration devices and extracted with phenol as previously described (33). Protein samples were focused overnight on isoelectric focusing strips in a pH range 3 to 10 and then run on 12% polyacrylamide gels, which were subsequently silver stained. Samples of induced proteins were picked for matrix-assisted laser desorption ionization–time of flight (TOF) mass spectrometry (MS) and quadrupole TOF MS. Protein identification and data analysis were performed in a manner similar to that previously described (28).

Purification of Hcp1 and Hcp2 proteins for antiserum preparation. The genes that encode Hcp1 and Hcp2 were cloned by PCR from *P. syringae* pv. tomato DC3000 genomic DNA. The primers used for amplification of the coding region of *hcp1* as an NdeI-HindIII fragment were 5′-GGCCCATATGCCTACACCCGCATTTC-3′ (forward) and 5′-GTCAAGCTTCAGTCCAGACCTAAGTCG-3′ (reverse), and *hcp2* was amplified as an NdeI-BamHI fragment with primers 5′-CAGGCATATGGCTACGCCAGCGTA-3′ (forward) and 5′-CGAGGATCCAGACTTAGCCAGCGAC-3′ (reverse). PCR products were ligated into the expression vector pJC40 (10) and transformed first into *E. coli* DH5α and then to *E. coli* BL21(DE3). Hcp overproduction in *E. coli* BL21(DE3) was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After probe sonication of the cells nine times with 5-s pulses and 3-s breaks, proteins were precipitated and dissolved in 6 M guanidine hydrochloride solution. Hcp proteins with an N-terminal His_{10} tag were purified by Ni-nitrilotriacetic acid affinity chromatography under denaturing conditions according to the Qia-Expressionist protocol (Qiagen, Valencia, CA). Rabbit antisera against the Hcp1 and Hcp2 proteins were prepared by LabAs Ltd. in Tartu, Estonia.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
Escherichia coli DH5α	F^- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Invitrogen
Escherichia coli BL21(DE3)	Lambda DE3 lysogen	Invitrogen
Escherichia coli S17-1	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn*7*	49
Pectobacterium carotovorum subsp. *carotovorum* SCC1	Field isolate from Finland; old name, *Erwinia carotovora* subsp. *carotovora*	43
Pectobacterium wasabiae SCC3193	Field isolate from Finland; old name, *Erwinia carotovora* SCC3193	35
Pseudomonas syringae pv. tomato DC3000 strains		
Wild type	Field isolate (rifampin sensitive)	D. Cupples
Rif^r mutant	Rifampin-resistant derivative of field isolate	D. Cupples
Δ*hcp1* mutant	In-frame deletion of *hcp1* (Pto_2539); Rif^r	This study
Δ*hcp2* mutant	In-frame deletion of *hcp2* (Pto_5435); Rif^r	This study
Δ*hcp1* Δ*hcp2* mutant	In-frame deletion of *hcp1* and *hcp2;* Rif^r	This study
Δ*icmF1* mutant	In-frame deletion of *icmF1* (Pto_2554); Rif^r	This study
Δ*icmF2* mutant	In-frame deletion of *icmF2* (Pto_5418); Rif^r	This study
Δ*icmF1* Δ*icmF2* mutant	In-frame deletion of *icmF1* and *icmF2*; Rif^r	This study
Plasmids		
pK18mobsac	RP4*mob*; Km^r	45
pPP	Promoter probe plasmid with beetle luciferase gene; Tet^r	14
phcp2-LUC	*hcp2* promoter in pPP	This study

Construction of *hcp1*, *hcp2*, *icmF1*, and *icmF2* single and double deletion mutants of *P. syringae* pv. tomato DC3000. To make in-frame deletions of *hcp1* and *hcp2* in *P. syringae* pv. tomato DC3000, pΔhcp1 and pΔhcp2 were constructed as previously described (53), with some modifications. First, a 1.6-kb fragment containing the upstream region of *hcp1* was amplified by PCR using primers Pto_2539mt-1 and Pto_2539mt-2 (Table 2). A 2-kb DNA fragment containing the downstream region of *hcp1* was amplified using primers Pto_2539mt-3 and Pto_2539mt-4. Then, a crossover PCR was performed to amplify a 3.6-kb fragment containing both the upstream and downstream fragments of *hcp1* using the PCR products from the first two steps and primers Pto_2539mt-1 and Pto_2539mt-4. This fragment was digested with EcoRI and HindIII and cloned into the EcoRI/HindIII sites of pK18mobsac (45) to generate pΔhcp1, which was then transformed into *E. coli* S17-1 λ *pir* (49). The kanamycin-resistant *P. syringae* pv. tomato DC3000 strain with pΔhcp1 inserted into the upstream or downstream region by homologous recombina-

TABLE 2 Primers used in PCRs to create plasmid constructs for generation of in-frame chromosomal deletions in *P. syringae* pv. tomato DC3000

Primer	Sequence (5′→3′)	Feature of underlined sequence
Pto_2539mt-1	CCGAATTCGCGGAAAAACGCCTTGGAAAGG	EcoRI site
Pto_2539mt-2	GCGAGATTATTTCGGACTGCATTGGTTTCTC CTGGAAGGTTA	Region complementary to primer Pto_2539mt-3
Pto_2539mt-3	ATGCAGTCCGAAATAATCTCGC	
Pto_2539mt-4	CCAAGCTTTATCCA TTGCGCGACCTCT	HindIII site
Pto_5435mt-1	CCGGATCCTTCCAGCTCGACCAAGAGATC	BamHI site
Pto_5435mt-2	TGATTGGCCGGCGCGAACATGGAGGTGCTCCTTGCTGGAT	Region complementary to primer Pto_5435mt-3
Pto_5435mt-3	ATGTTCGCGCCGGCCAATCA	
Pto_5435mt-4	CCTCTAGATCAGATCGCACACGCACCGT	XbaI site
Pto_2554mt-1	CCGGATCCATGAATTCGCACAAGGTTATCTG	BamHI site
Pto_2554mt-2	CTAAAGATCCCGTGACATCAGACTTTTCTCTTGTCAGGATCGAG	Region complementary to Pto_2554mt-3
Pto_2554mt-3	TGATGTCACGGGATCTTTAGAACCG	
Pto_2554mt-4	CCTCTAGATCAACCCGCGGTACTGGTCG	XbaI site
Pto_5418mt-1	CCTCTAGAACACCCATGAACCGCTCGCC	XbaI site
Pto_5418mt-2	CAAGGGCTGGCTACCAGCATTTACGGCTGTGACTGCGGCTGGAC	Region complementary to Pto_5418mt-3
Pto_5418mt-3	ATGCTGGTAGCCAGCCCTTGGCGC	
Pto_5418mt-4	CCAAGCTTGCTGCGCCGACATCTGCTGA	HindIII site

tion was obtained via conjugation and subsequently plated onto KB plates containing 20% sucrose to counterselect the integration. The Δ*hcp1* mutant was first screened by PCR with primers Pto_2539mt-1 and Pto_2539mt-4 and further confirmed by Southern blot analysis (data not shown). Similarly, pΔhcp2 was constructed by using the corresponding primers Pto_5435mt-1, Pto_5435mt-2, Pto_5435mt-3, and Pto_5435mt-4. pΔhcp2 was transferred from *E. coli* S17-1 λ *pir* into the *P. syringae* pv. tomato DC3000 wild-type and Δ*hcp1* mutant strains via conjugation, and the deletion of *hcp2* was screened for by PCR with primers Pto_5435mt-1 and Pto_5435mt-4, followed by confirmation by Southern blot analysis. This produced the Δ*hcp2* and Δ*hcp1* Δ*hcp2* mutants.

In the *P. syringae* pv. tomato DC3000 genome, PSPTO_2554 and PSPTO_5418 were identified as two genes that encode proteins homologous to the T6SS membrane protein IcmF (44), which is required for the secretion of proteins that belong to the Hcp family (24, 58). The genes were named *icmF1* and *icmF2*, respectively. In-frame deletions of *icmF1* and *icmF2* in *P. syringae* pv. tomato DC3000 were obtained by using an approach similar to that used to construct the Δ*hcp1* and Δ*hcp2* mutants. The primers and restriction sites used to generate pΔicmF1 and pΔicmF2 for deletion of the chromosomal *icmF1* and *icmF2* genes are listed in Table 2. After the Δ*icmF1* mutant was acquired, it was used to construct the Δ*icmF1* Δ*icmF2* double mutant. All of the mutations were confirmed by PCR and Southern blot analysis.

Luciferase fusion construct. A region 558 bp upstream from the first ATG codon of the *hcp2* gene was amplified as a KpnI-BamHI fragment by using primers 5′-GTGGTACCTGACTATCTAAGCGAATG-3′ (forward) and 5′-GGCGGATCCATGGAGGTGCTCCTTG-3′ (reverse) and cloned into the promoter probe plasmid pPP (14) 5′ of the luciferase gene *LUCGR* coding region. The resulting construct was named phcp2-LUC.

***In vivo* luciferase activity assay.** To study luciferase expression *in vivo* from the phcp2-LUC fusion construct carried on a plasmid, susceptible tomato (cv. Agriset) leaves were infiltrated with a *P. syringae* pv. tomato/phcp2-LUC cell suspension at an OD_{600} of 1.0 (10^9 CFU/ml) in 10 mM potassium phosphate buffer (PPB), pH 7.2. For sample preparation, the leaves with infiltrated areas were detached and surface sterilized with 15% H_2O_2 in phosphate-buffered saline (PBS) for 10 min and then rinsed with sterile PBS. Two leaf discs 10 mm in diameter were removed from the infiltrated leaf areas with a cork borer and homogenized in 1.5 ml ice-cold 10 mM PPB, pH 7.2, with an Ultra-Turrax T25 grinder (IKA, Stauffen, Germany). Luciferase activity expressed by the *P. syringae LUC* transformant strains was determined by a method similar to one previously described (15). Briefly, a 100-μl bacterial sample, in this case, the undiluted leaf disc homogenate, was quickly mixed with 100 μl of cell lysis mix (Promega, Madison, WI) and 100 μl of luciferin substrate solution (luciferin from Sigma-Aldrich, St. Louis, MO) in a luminometer cuvette. Photon counts were recorded immediately after the insertion of the cuvette into a Biocounter M1500 luminometer (Lumac, St. Paul, MN). To determine the number of viable bacteria, the homogenized leaf samples were diluted 10-fold in a series in 100 mM PPB, pH 7.2, with 0.1% soybean peptone and plated on KB agar supplemented with rifampin at 75 μg/ml. Colonies were counted after 3 days of incubation at 28°C.

Virulence assay on tomato plants. Tomato (*Solanum lycopersicum*) cultivar Moneymaker was grown in a growth chamber set at 24°C/22°C (day/night) with a 16-h photoperiod and 70% relative humidity. Four-week-old tomato plants were dip inoculated with different *P. syringae* pv. tomato DC3000 strains at a level of 2×10^7 CFU/ml in a 10 mM $MgCl_2$ solution containing 0.02% Silwet-77. Determination of bacterial populations in tomato leaves was done as described previously (22).

***Arabidopsis* colonization assay.** *Arabidopsis thaliana* ecotype Colombia plants were spray inoculated separately with the *P. syringae* pv. *tomato* DC3000 parent strain and the Δ*hcp1* Δ*hcp2* double mutant at an OD_{600} of 0.1 (10^8 cells/ml) in 10 mM $MgCl_2$; six plants per strain were sprayed until runoff. After 7 days, the leaf rosettes were cut and weighed and each rosette was homogenized in 3 ml of ice-cold PPB with soybean peptone without surface sterilization. Dilutions for plating were made in the same buffer. Samples were spread on KB agar with rifampin, and colonies were counted after 3 days of incubation at 22°C. To test the competitive fitness of the *hcp* double mutant with the parent strain, eight *Arabidopsis* plants were spray inoculated with a 1:1 mixture of the *P. syringae* pv. *tomato* DC3000 rifampin-sensitive strain and the Δ*hcp1* Δ*hcp2* double mutant strain, both transformed with the pPP plasmid carrying tetracycline resistance genes, at an OD_{600} of 0.1 (total cell density, 10^8/ml) in 10 mM $MgCl_2$. After 7 days, inoculated plant rosettes were homogenized without surface sterilization. Bacteria from the plant samples were plated on KB agar with tetracycline (12.5 μg/ml) and with or without rifampin. Colonies were counted after 4 days of incubation at 22°C.

Bacterial competition assay. The *P. syringae* pv. tomato DC3000 rifampin-resistant parent strain and its mutant derivatives were precultured on KB agar. Enterobacterial strains were precultured on LB agar and then suspended in KB broth to an OD of 0.1, and 100 μl of the suspension was spread onto each KB agar plate to obtain an even cell layer. Droplets of a similarly prepared *P. syringae* cell suspension were added on top with the aid of paper discs to prevent the droplets from spreading too wide. After overnight incubation at 25°C, both the enterobacterial growth inhibition and the fluorescence under UV light of *P. syringae* were detected visually. To obtain quantitative results, the double-inoculated area of the nutrient agar was cut out with a sterilized 10-mm cork borer and the bacteria were resuspended in 5 ml KB broth, diluted, and spread onto fresh KB agar

plates for CFU counting. The enterobacteria were cultured at 37°C overnight, and *P. syringae* strains were cultured with rifampin (75 μg/ml) at 25°C for 3 to 4 days.

Yeast competition assay. The *P. syringae* pv. tomato DC3000 rifampin-resistant parent strain and its mutant derivatives were precultured on KB agar, and the *Cryptococcus carnescens* yeast was cultured in liquid PYG medium (per liter, 15 g proteose peptone, 2.5 g yeast extract, 18 g glucose, 280 mg $NaHPO_4$, 272 mg KH_2PO_4, 240 mg NaCl, 8 mg $MgSO_4$, and 12 mg $CaCl_2$). *P. syringae* pv. tomato cells were suspended in 10 mM $MgCl_2$ and diluted to an OD of 0.01, corresponding to 10^7 cells/ml. Ten microliters of a *P. syringae* pv. tomato cell suspension and 10 μl of yeast cell culture were applied together to PYG agar in 24-well cell culture plates. After 7 days of incubation at 23°C, all of the cells in a well were suspended in 5 ml of salt solution (per liter, 280 mg $NaHPO_4$, 272 mg KH_2PO_4, 240 mg NaCl, 8 mg $MgSO_4$, 12 mg $CaCl_2$) and the yeast cells were counted with a Bürker cell counting chamber. For each sample, six 0.04-mm squares with a 0.1-mm depth were counted.

RESULTS

Hcp2 is expressed and secreted into the culture medium. To achieve our goal of identifying bacterial factors that contribute to plant colonization and virulence, we specifically aimed to find plant-inducible proteins secreted by *P. syringae* pv. tomato DC3000 in HIM—a minimal medium that mimics the conditions in the plant apoplast—and in the same medium supplemented with tomato cell exudates. Among the proteins secreted into the minimal medium, with and without tomato exudates, several known T3SS-dependent proteins were identified by MS, i.e., the pilin HrpA; accessory proteins HrpZ, HrpK, HopAK1, HopP1, and HrpW; effector proteins AvrPto and HopAM1-1; the flagellin FliC; and the flagellar hook protein FliD. The only protein whose production was clearly upregulated by the tomato cell exudates was the T3SS pilin HrpA. In addition to these previously characterized T3SS-secreted proteins, the T6SS-related hemolysin-coregulated protein Hcp2 was identified in two silver-stained spots on the gel (Fig. 1 and Table 3). Other T6SS-associated proteins were not found in the culture medium. A comparison of the two-dimensional gel spots and MS identification of the proteins from the *P. syringae* pv. tomato DC3000 wild-type and *hrcC* mutant strains showed that Hcp2 secretion was not dependent on T3SS and not enhanced or repressed by tomato exudates. Because of the discovery of a T6SS-related protein being expressed in HIM, we extended our focus to characterize Hcp and to determine if it also plays a role in virulence or plant colonization.

***P. syringae* pv. tomato DC3000 Hcp2 is expressed as covalently linked dimers.** Because of the detection of several presumably intracellular proteins in the culture supernatant, which suggests the occurrence of cell lysis during sample preparation, we needed to confirm whether Hcp2 is actively secreted into the extracellular medium. The *P. syringae* pv. tomato DC3000 genome harbors two putative *hcp* genes (1, 44), but only Hcp2 was detected in the culture medium. To find out if only one or both of the *hcp* genes were actively expressed, antisera against the Hcp1 and Hcp2 proteins were produced. The proteins were overproduced in *E. coli*, purified, and used to immunize rabbits. Both the Hcp1 and Hcp2 antisera recognized a 19-kDa protein from the culture supernatants of *P. syringae* pv. tomato DC3000, and each antiserum prepared against one of the two proteins also recognized the other affinity-purified protein by cross-reaction (data not shown). The two Hcp proteins of *P. syringae* pv. tomato DC3000 show 53% amino acid sequence identity, so it is not surprising that the antiserum prepared against one Hcp cross-reacted with the other. Besides that, the sizes of the two proteins differ by only one amino acid and they cannot be distinguished by standard gel electrophoresis. Hence, single deletion (Δ*hcp1* or Δ*hcp2*) and double deletion (Δ*hcp1* Δ*hcp2*) chromosomal mutants were created to study the expression of Hcp1 and Hcp2 separately. Protein immunoblot assays revealed that the Δ*hcp2* mutant did not produce any protein recognizable by either of the two antisera and thus resembled the Δ*hcp1* Δ*hcp2* double mutant (Fig. 2, left panels). Since the amount of recognized protein produced and secreted by the Δ*hcp1* mutant seemed equal to that of the parent strain, we concluded that the Hcp1 protein was not produced by *P. syringae* pv. tomato DC3000 under these experimental conditions whereas Hcp2 was abundantly produced and secreted.

Further analysis of the Hcp2 protein was carried out to determine whether it is monomeric or oligomeric. In a Tris-glycine-SDS-polyacrylamide gel system with β-mercaptoethanol (β-ME) in the sample buffer, Hcp2 always appeared as a 19-kDa monomer (Fig. 2, left panel). When the reducing agent was omitted from the sample buffer, the antiserum no longer detected a 19-kDa band but instead recognized a 38-kDa band, corresponding to a Hcp2 dimer, from the culture supernatant (Fig. 2, right panel). The dimer was not detectable in the cell fractions, suggesting that the reducing conditions in the cytoplasm do not allow Hcp2 dimerization. In nondenaturing gels using perfluorooctanoic acid instead of SDS, Hcp2 also appeared as a dimer and no higher-order oligomers were detectable (data not shown).

Secretion of Hcp2 is dependent on the T6SS membrane component IcmF2. To confirm that the observed release of Hcp2 into the culture medium is dependent on T6SS, we created new mutants of *P. syringae* pv. tomato DC3000 in which the genes homologous to the T6SS membrane protein component IcmF are deleted. We found that *P. syringae* pv. tomato Δ*icmF2* mutant was unable to secrete Hcp2 protein into the culture medium (KB) (Fig. 3). In contrast, the Δ*icmF1* mutant was still fully capable of secreting Hcp2. These results suggest that the secretion of Hcp2 is dependent on the function of the T6SS built up of the components encoded by HSI-II.

The Hcp2 expression level is dependent on the bacterial growth phase. *P. syringae* pv. tomato DC3000 secretome analysis showed that Hcp2 was expressed in minimal medium. We sought to confirm this by examining the expression of the *hcp2* gene and also to determine whether this gene is constitutively expressed or regulated by the culture conditions or bacterial growth phase. We constructed a plasmid with the *hcp2* promoter linked to a luciferase reporter gene, called phcp2-LUC, and transformed it into *P. syringae* pv. tomato DC3000. The luciferase activity under the control of the *hcp2* promoter was determined in both HIM and a rich medium, KB. Luminescence counts and OD_{600} were measured at 24, 48, and 72 h. The relative expression level in KB was lower than that in the minimal medium containing fructose or mannitol, but it increased with culture age and finally reached a higher level when the cultures reached the late stationary phase (Fig. 4A). *hcp2* promoter activity in minimal medium supplemented with glucose was expressed at the same levels as with mannitol (data not shown). The *hcp2* promoter was also active in *P. syringae* pv. tomato DC3000 growing on solid KB, and the luminescence per unit of OD_{600} was slightly higher in plate cultures than in liquid cultures (Fig. 4B). In contrast to *hcp2*, reporter gene

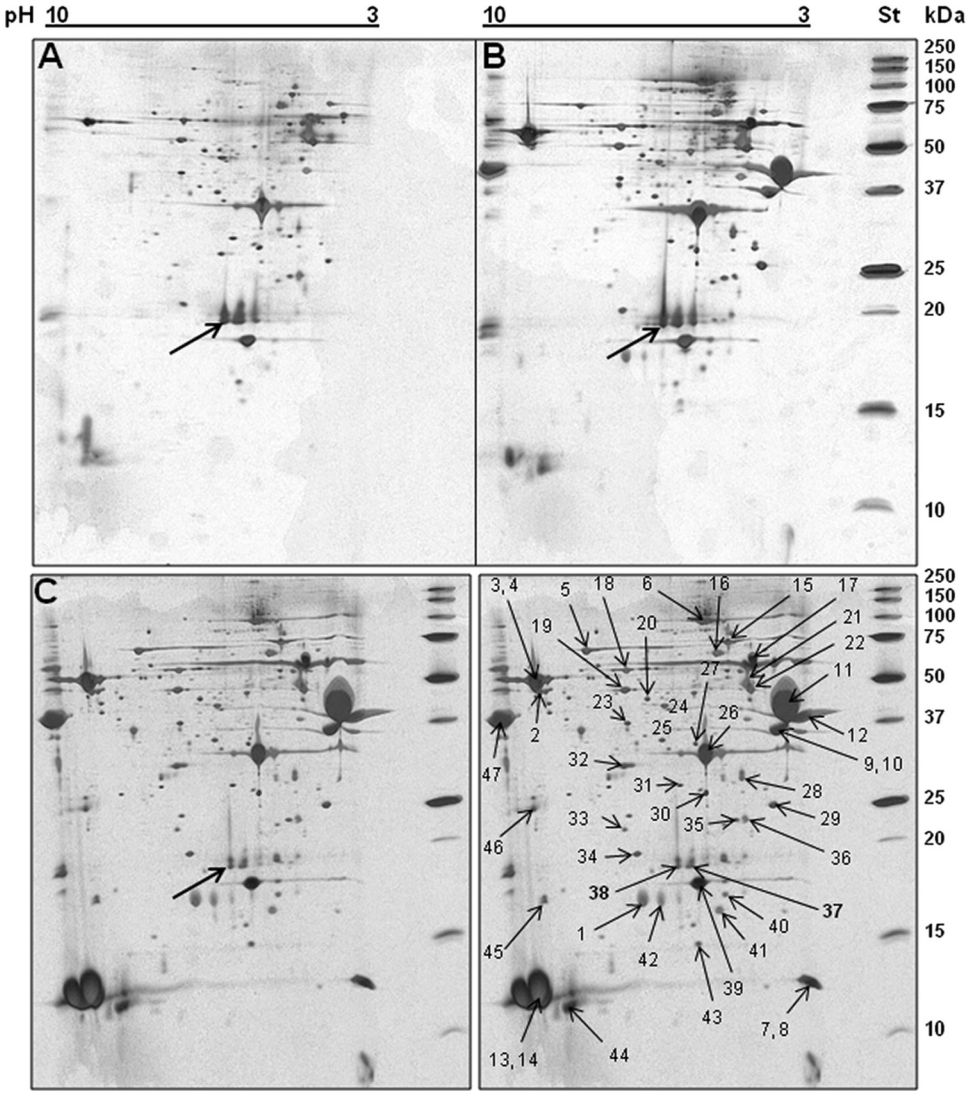

FIG 1 Proteins detected in the culture supernatant of *P. syringae* pv. tomato DC3000 grown in HIM. (A) The *P. syringae* pv. tomato DC3000 *hrcC* mutant is unable to secrete the T3SS-dependent proteins. (B) The *P. syringae* pv. tomato DC3000 parent strain cultured in HIM. (C) The *P. syringae* pv. tomato DC3000 parent strain cultured in HIM supplemented with tomato cell culture exudates. At the right edge of the gels, in a separate lane, are the standard proteins. In the right half of panel C, the spots analyzed by MS are indicated by arrows and the numbering corresponds to that in Table 2. The single arrows in the other panels point to the spots at which Hcp2 was identified.

constructs with the *hcp1* promoter failed to detect any activity *in vitro* (data not shown).

Because putative RhlR binding sites were identified in both the *hcp1* and *hcp2* promoter sequences (see Fig. S1 and Table S1 in the supplemental material), suggesting that *hcp* gene expression could be regulated by quorum-sensing signals, we studied more closely whether *hcp2* expression is growth phase dependent. The activity of the *hcp2* promoter, observed as a function of luciferase activity per unit of cell density at several time points in liquid culture in KB, was at the lowest level during the early logarithmic phase and increased during the late logarithmic phase (Fig. 4C). Surprisingly, *hcp2* promoter activity did not increase at a constant rate and oscillated between expression and repression during population growth. From the lowest level detected during the early logarithmic phase, the activity increased >3-fold by the mid-logarithmic phase and then declined before increasing again in the late logarithmic phase. Because cultures started at different cell densities had the same relative expression level at the same time points (data not shown), a higher cell density *per se* does not seem to enhance *hcp2* promoter activity. Also, culture supernatant taken from a dense *P. syringae* pv. tomato DC3000 culture and mixed into fresh culture medium before starting a new culture did not enhance *hcp2* expression, suggesting that the *hcp2* expression level is not regulated by a diffusible quorum-sensing signal. However, dilution of a culture with fresh medium always caused a drop in the relative expression level.

Altogether, the results indicate that *hcp2* expression was highest after 24 h of growth, when the bacterial population entered the stationary phase, and higher in minimal medium or on a solid surface than in rich medium or in a liquid culture with shaking. This may indicate that *hcp2* expression is linked to the depletion of nutrients.

TABLE 3 Proteins identified by MS in supernatant of *P. syringae* pv. tomato DC3000 culture in HIM

Sample(s)	ID code	Protein	Characterization	% Sequence coverage[b]	Observed mass (kDa)	Calculated pI	Predicted localization
1	PSPTO_4001	AvrPto	T3SS-secreted effector	63	18.309	6.02	Secreted
2, 3, 4	PSPTO_1951	FliD	Flagellar hook protein	48, 26	49.290	8.99	Membrane
5	PSPTO_4101	HopAK1	T3SS helper, putative pectate lyase	37	59.048	6.85	Secreted
6	PSPTO_1405	HrpK1	T3SS putative translocase	11	81.044	5.27	Secreted
7, 8	PSPTO_1589	AAO55109	Putative lipoprotein	30	33.749	7.66	Membrane[a]
9, 10	PSPTO_2678	HopP1	T3SS helper, putative lytic transglycosylase	39, 25	32.349	4.33	Secreted
11, 12	PSPTO_1382	HrpZ1	T3SS-secreted harpin	28, 22	36.463	4.01	Secreted
13, 14	PSPTO_1381	HrpA1	T3SS pilin	70	11.580	8.96	Secreted
15	PSPTO_2841	Transposase	ISPsy14 transposase	19	58.143	9.46	Intracellular
16	Not identified						
17	PSPTO_4504	DnaJ	Chaperone (Hsp40)	15	41.009	6.34	Cytoplasm[a]
18	PSPTO_2678	HopP1	T3SS helper	37	32.349	4.33	Secreted
19	PSPTO_1534	RpsB	Ribosomal protein S2	22	27.441	8.56	Intracellular
20	Not identified						
21	PSPTOA0032	Lsc-3	Levansucrase	29	47.970	4.74	Secreted
21	PSPTO_1373	HrpW1	T3SS-secreted harpin	29	42.885	4.63	Secreted
22, 23	Not identified						
24	PSPTO_3615	Recombinase	Site-specific recombinase	14	60.488	7.29	Intracellular
25	Not identified						
26	PSPTO_1949	FliC	Flagellin	52	29.158	5.25	Secreted
27-31	Not identified						
32	PSPTO_1022	HopAM1-1	T3SS-secreted effector	61	31.340	6.26	Secreted
33	PSPTO_2713	CheR	Putative chemotaxis methyltransferase	18	33.582	6.26	Intracellular
34	PSPTO_5098	Hypothetical	Putative acyltransferase	21	36.400	9.38	Membrane[a]
35	PSPTO_0945	TerE	Tellurium resistance protein	59	20.267	4.84	Membrane[a]
36	PSPTO_0944	TerD	Tellurium resistance protein	38	20.495	4.69	Cytoplasm[a]
37	PSPTO_4001	Hcp2	Secreted protein Hcp	38	19.132	5.53	Secreted
	PSPTO_5435	AvrPto	T3SS-secreted effector	34	18.375	5.92	Secreted
38	PSPTO_4001	Hcp2	Secreted protein Hcp	38	19.132	5.53	Secreted
	PSPTO_5435	AvrPto	T3SS-secreted effector	34	18.375	5.92	Secreted
39	PSPTO_4001	AvrPto	T3SS-secreted effector	45	18.375	5.92	Secreted
40	PSPTO_2713	CheR	Putative methyltransferase (fragment?)	21	33.382	6.46	Intracellular
41	PSPTO_1950	FlaG	Flagellar protein	33	14244	5.19	Secreted
42	PSPTO_4001	AvrPto	T3SS-secreted effector	63	18375	5.92	Secreted
43	Not identified						
44	PSPTO_1925	FlgM	Negative regulator of flagellin synthesis	63	10.979	8.04	Cytoplasm[a]
45, 46	Not identified						
47	PSPTO_1382	HrpZ1	T3SS-secreted harpin	19	36.463	4.01	Secreted

[a] Prediction by PSORT algorithm.
[b] Two values indicate sequence coverage for the second spot from which the same protein was identified, when different from the first spot sample.

***hcp2* expression is not upregulated *in planta* and does not significantly contribute to colonization of or virulence in the plant host.** The discovery that Hcp2 was expressed in a minimal medium that mimics the plant apoplast raised the possibility that *hcp2* expression is upregulated in the plant. To analyze the expression of the *hcp2* gene in *P. syringae* pv. tomato DC3000 during host plant infection, we injected the phcp2-LUC strain into the leaf tissue of susceptible tomato plants (cv. Agriset). Leaf disc samples were taken from the infiltrated areas, luciferase activity was measured, and bacteria were plated on KB agar to determine viable cell numbers. We found that the activity of the *hcp2* promoter in bacteria inoculated into tomato leaves was lower than the expression level in culture medium and declined over time (Fig. 5). Thus, differently from the T3SS components, *hcp2* expression was not promptly induced in the plant apoplast. Because of the localized necrosis of leaf tissue caused by the high bacterial inoculum concentration, it was not possible to obtain samples at later time points to see if *hcp2* expression would increase after the initial decline.

To determine whether *hcp1* or *hcp2* of *P. syringae* pv. tomato DC3000 plays a role in plant colonization or virulence, bacterial populations and disease development in the tomato cultivar Mon-

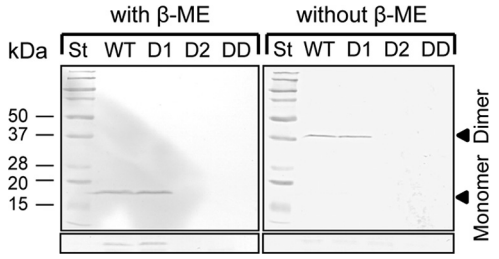

FIG 2 The Hcp2 protein is secreted by *P. syringae* pv. tomato DC3000. Protein samples were prepared from supernatants and cell pellets of fresh overnight cultures in KB. β-ME was added to the samples on the left and omitted from the samples on the right. The proteins were detected by using anti-Hcp1 antiserum and an anti-rabbit monoclonal antibody conjugated with alkaline phosphatase, which degrades the Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIP) substrate, producing the color violet. The lower panels show immunodetection of Hcp from the cell fractions of the same samples with and without β-ME. Only the monomeric form could be detected in the cell fractions treated with β-ME. St, standard proteins; WT, *P. syringae* pv. tomato DC3000 parent strain; D1, Δ*hcp1* deletion mutant; D2, Δ*hcp2* deletion mutant; DD, Δ*hcp1* Δ*hcp2* double deletion mutant.

eymaker were monitored over 7 days after dip inoculation with the *P. syringae* pv. tomato DC3000 wild-type or Δ*hcp1*, Δ*hcp2*, or Δ*hcp1* Δ*hcp2* mutant strain at 2×10^7 CFU/ml. Deletion of *hcp1* or *hcp2* did not significantly affect bacterial growth in tomato plants, indicating that these two genes play no or minor roles in *P. syringae* pv. tomato DC3000 virulence (see Fig. S2 in the supplemental material). The experiment was repeated three times with similar results, and data analysis was performed using Duncan's multiple-range test.

A similar result was obtained with another host of *P. syringae* pv. tomato DC3000, *A. thaliana*, when the population sizes of the parent strain and the Δ*hcp1* Δ*hcp2* mutant were determined 7 days after spray inoculation of *A. thaliana* plants with pure and mixed cultures. After separate spray inoculations with pure cultures, the average population size of the *P. syringae* pv. tomato DC3000 parent strain in *Arabidopsis* leaves was 1.0×10^8 (standard deviation [SD], 7.19×10^7) CFU/g of fresh weight and that of the Δ*hcp1*

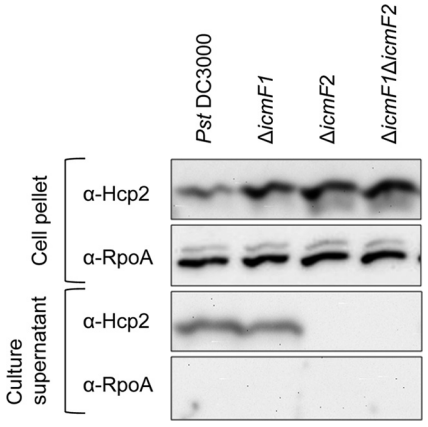

FIG 3 Secretion of Hcp2 is dependent on IcmF2. The Hcp2 protein was immunologically detected in the cell fraction of wild-type *P. syringae* pv. tomato DC3000 and all three of the Δ*icmF* mutants grown in KB overnight. In the culture medium, Hcp2 was detected only in the wild-type strain and the Δ*icmF1* mutant, suggesting that the secretion of Hcp2 requires IcmF2 function. RpoA is a nonsecreted reference protein.

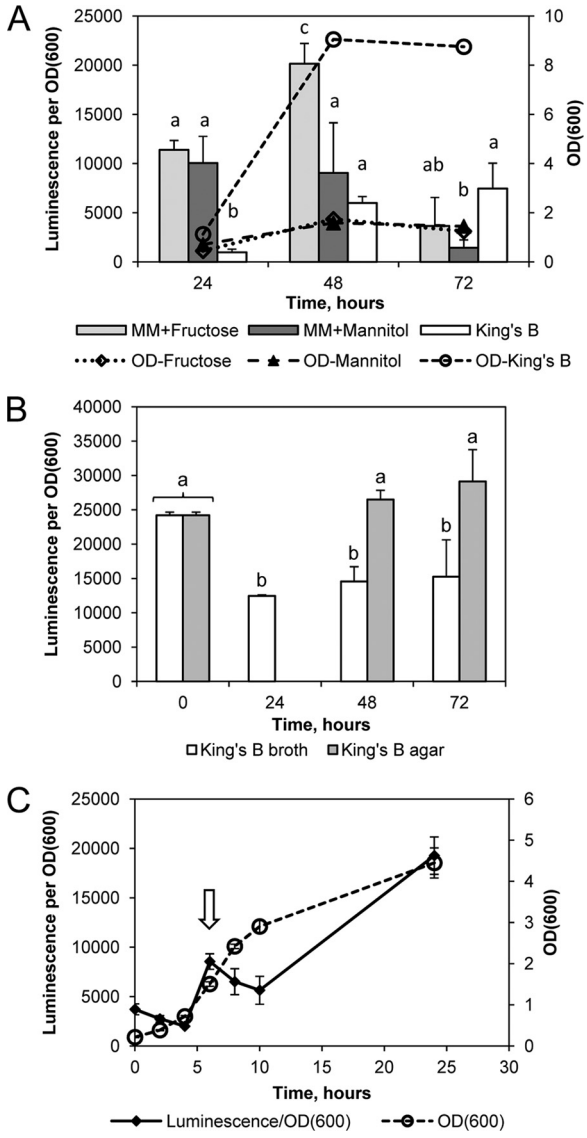

FIG 4 The level of *hcp2* expression shows variation depending on the culture conditions and growth phase. (A) After 24 h, luciferase expression under the control of the *hcp2* promoter was significantly higher in the minimal medium (MM) than in KB ($P < 0.0001$, Student's *t* test). After 48 h, luminescence counts per unit of OD_{600} were significantly higher in MM plus fructose than in the two other media ($P < 0.0001$ in comparison with KB, $P < 0.01$ in comparison with MM plus mannose), and after 72 h, both growth and luminescence were declining in the minimal medium while they were still maintained in KB. Three parallel cultures were started in each medium, KB and MM with 10 mM fructose or 10 mM mannitol at an OD_{600} of 0.3, and incubated with shaking for 3 days at 22°C. Error bars indicate SDs, and statistically significant differences are indicated by different letters. (B) The activity of the *hcp2* promoter was higher on solid medium than in liquid medium. The *P. syringae* pv. tomato/phcp2-LUC strain and the promoterless control *P. syringae* pv. tomato/pPP strain were precultured on KB agar and inoculated at an OD_{600} of 0.1 into fresh KB liquid medium or spread as a lawn on KB agar. The relative luminescence of the control strain stayed at a constant low level below 100. In agar cultures, luciferase activity could not be reliably determined before 48 h because the cell numbers were too low. Two separate cultures of each kind were sampled, and error bars indicate SDs. Results that are different at a confidence level of $P < 0.05$ (Student's *t* test) are marked with different letters. (C) The activity of the *hcp2* promoter changed with the growth phase. Five parallel liquid cultures of the *P. syringae* pv. tomato/phcp2-LUC strain were started at an OD_{600} of 0.2 in KB and grown overnight at 22°C. $n = 5$; error bars indicate SDs. The arrow points to the peak of *hcp2* promoter activity detected in the mid-logarithmic growth phase.

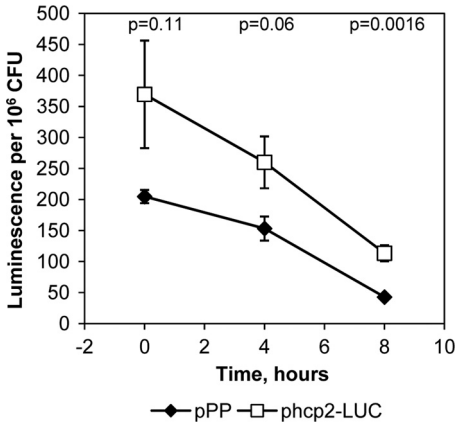

FIG 5 *hcp2* promoter activity is very low in bacteria inoculated into tomato leaves. pPP, luciferase gene with no promoter; phcp2-LUC, promoter fusion construct. Luminescence counts per 10^6 viable cells, determined as CFU, are shown. $n = 4$; error bars indicate SDs, and P values indicate the statistical significance of the difference between the *hcp2* promoter and the promoterless construct (Student's t test).

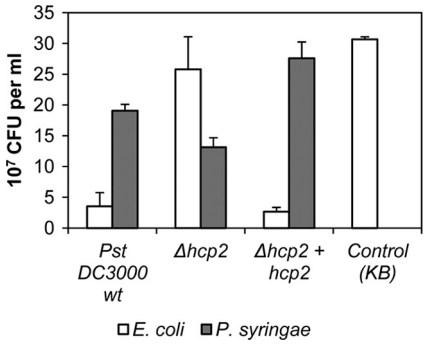

FIG 6 The ability of *P. syringae* pv. tomato DC3000 to inhibit *E. coli* growth in a mixed culture is dependent on *hcp2*. Reintroduction of the *hcp2* gene into the Δ*hcp2* mutant resulted in functional complementation of the mutation. The *P. syringae* strains and *E. coli* BL21 were cocultured on KB agar plates for 16 h at 25°C before samples were taken. The control sample is *E. coli* cultured alone. For detection of CFU, the cocultures were suspended in liquid medium and replated. *E. coli* colonies were counted after 20 h of incubation at 37°C, and *P. syringae* colonies were counted after 4 days of incubation at 21°C with rifampin. The results shown are averages of samples from three separate cocultures, and error bars indicate SDs. The negative correlation between *P. syringae* pv. tomato growth and that of *E. coli* in a mixed culture is significant at a level of $P = 0.01$ (Pearson two-tailed correlation test). *wt*, wild type.

Δ*hcp2* mutant was 7.25×10^7 (SD, 5.13×10^7) CFU/g of fresh weight (see Fig. S2 in the supplemental material). A comparison of means by Student's t test indicated that the difference in the mean population sizes was not statistically significant. Variations in fitness can be revealed by the more sensitive competitive index (CI) assay that uses mixed inoculations (25). The CI of the Δ*hcp1* Δ*hcp2* double mutant was 0.893 (SD, 0.482), which is not significantly different from 1 according to a one-sample t test ($P = 0.27$ with 95% confidence). Thus, the Δ*hcp1* Δ*hcp2* mutant did not significantly differ from the wild type in plant colonization efficiency under these conditions. These data therefore suggest that *hcp1* and *hcp2* of *P. syringae* pv. tomato DC3000 might have a function other than to promote host plant colonization.

Hcp2 of *P. syringae* is required for survival in competition with enterobacteria. Since the T6SS is commonly present in Gram-negative bacteria and was recently discovered to have a role in suppressing the growth of competitive bacteria (16, 32, 47), we hypothesized that T6SS in phytopathogenic bacteria may be used to enhance competitive fitness instead of virulence or pathogenicity. When *P. syringae* pv. tomato DC3000 was cocultured with a range of different bacteria on solid medium, we found that it was able to inhibit the growth of several species of enterobacteria, including *E. coli*, *Proteus vulgaris*, and the plant pathogens *Pectobacterium carotovorum* subsp. *carotovorum* (SCC1) and *Pectobacterium wasabiae* (SCC3193). Deletion of *hcp2* abolished the ability of *P. syringae* pv. tomato to inhibit enterobacterial growth and also decreased its growth in the mixed culture (Fig. 6; see Fig. S3 in the supplemental material). Complementation of the Δ*hcp2* mutation fully restored the ability to inhibit enterobacterial growth and allowed the complemented strain to grow well in the mixed culture and be more competitive. The inverse correlation between cell counts of *E. coli* and *P. syringae* pv. tomato DC3000 strains carrying the *hcp2* gene is significant ($P < 0.01$) according to a Pearson two-tailed correlation test and a paired-sample test. In pure culture without competition, the Δ*hcp2* mutant grew as well as the *P. syringae* pv. tomato DC3000 parent strain (data not shown), indicating that the mutation does not noticeably impair any basic cellular functions of *P. syringae* pv. tomato DC3000. Suppression of enterobacterial growth could be visually detected as a zone with a thinner translucent cell layer at the *P. syringae* pv. tomato application site, and since a viable *P. syringae* pv. tomato population emits strong greenish fluorescence under UV light due to siderophore production, it was easy to confirm which bacterial species was dominating in a mixed culture (Fig. 7A). Later, after 2 days in coculture, *E. coli* was able to grow despite the inhibitory activity of *P. syringae* pv. tomato. This delayed growth of *E. coli* could not displace *P. syringae* pv. tomato at the site of inoculation, but the two bacterial species reached approximately equal cell densities. While the parent and Δ*hcp1* mutant strains survived the competition, the Δ*hcp2* mutant population declined. The complemented Δ*hcp2* strain was able to recover the ability to compete with *E. coli* (Fig. 7B). In contrast to the Gram-negative species, the Δ*hcp2* mutant was not impaired in the ability to compete with the Gram-positive *Arthrobacter* and *Bacillus* species on solid KB (data not shown).

When the *P. syringae* pv. tomato DC3000 parent and the Δ*hcp2* mutant strains were cocultured with *E. coli* in liquid KB with shaking at 200 rpm, no differences in the viable cell numbers of the two strains could be detected (data not shown). Also, cell-free culture filtrates from a *P. syringae* pv. tomato DC3000 overnight liquid culture in KB did not inhibit *E. coli* growth when added to an *E. coli* cell layer on solid medium. To test whether the *hcp2*-associated function requires direct contact between the cells of the two competing species, a cell-impermeable filter (pore size, 0.2 μm) was placed between the two bacterial cell layers on solid KB. Both the *P. syringae* pv. tomato DC3000 parent strain and the *hcp2* mutant grew equally well on the filter laid on top of the *E. coli* cell layer. When wild-type *P. syringae* pv. tomato DC3000 or the *hcp2* mutant was first spread on the agar, *E. coli* grew equally well on the filter regardless of which *P. syringae* pv. tomato strain was growing beneath it. Without the filter, if *P. syringae* pv. tomato was spread first on KB agar and *E. coli* added on top, the Δ*hcp2* mutant was cleared away within the *E. coli* application area whereas parent strain DC3000 was not cleared away (see Fig. S4 in the supplemen-

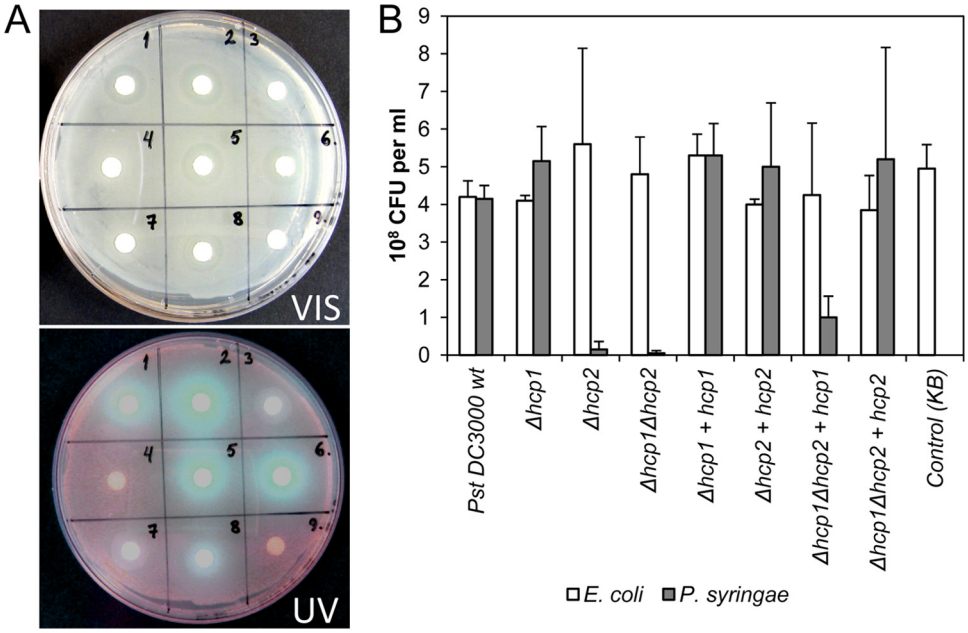

FIG 7 Hcp2 is required for the survival of *P. syringae* pv. tomato DC3000 in a coculture with *E. coli*, whereas Hcp1 is not required. *P. syringae* pv. tomato strains were added as droplets on top of an *E. coli* cell layer in the following order: 1, DC3000 parent strain; 2, Δ*hcp1* mutant; 3, Δ*hcp2* mutant; 4, Δ*hcp1* Δ*hcp2* double mutant; 5, Δ*hcp1* + *hcp1* complemented mutant; 6, Δ*hcp2* + *hcp2* complemented mutant; 7, Δ*hcp1* Δ*hcp2* + *hcp1* complemented double mutant; 8, Δ*hcp1* Δ*hcp2* + *hcp2* complemented double mutant; 9, control (KB only). The bacteria were cocultured on KB agar plates at 25°C for 48 h before photographs were taken, and samples were subsequently prepared for the determination of viable counts. (A) In daylight, the inhibition of *E. coli* BL21 growth by *P. syringae* can be detected as a thinner cell layer zone (VIS). Under UV light, *P. syringae* cells are fluorescent whereas *E. coli* cells are not. (B) For CFU detection, cocultures were suspended in liquid medium and replated. *E. coli* colonies were counted after 20 h of incubation at 37°C, and *P. syringae* colonies were counted after 3 days of incubation at 25°C with rifampin. The results shown are averages of samples from two separate cocultures, and error bars indicate SDs. *wt*, wild type.

tal material). Thus, the difference in competitive fitness between the parent strain and the Δ*hcp2* mutant was observed only when *P. syringae* pv. tomato cells were in contact with *E. coli* on solid medium.

***P. syringae hcp* genes are required for competition against eukaryotic microbes.** In other studies, active T6SSs have been found to influence the growth of eukaryotic organisms such as amoebae (38). We therefore tested the *P. syringae* pv. tomato DC3000 parent strain and the Δ*hcp1* Δ*hcp2* double deletion mutant in cocultures with a range of eukaryotic microorganisms. We found that deletion of the *hcp* genes from *P. syringae* pv. tomato DC3000 resulted in increased susceptibility to grazing by the amoeba *Acanthamoeba polyphaga* (Fig. 8A). In contrast, no difference between wild-type *P. syringae* pv. tomato DC3000 and the *hcp* double mutant was seen when they were tested for nematode grazing (data not shown). Of the yeast species tested, *Saccharomyces cerevisiae* (baker's yeast) was unable to compete against either the wild-type or the Δ*hcp1* Δ*hcp2* double mutant *P. syringae* pv. tomato strain in a mixed culture. However, competition against *Cryptococcus carnescens* and *Rhodotorula glutinis* revealed a clear difference between the two *P. syringae* pv. tomato strains. Competitive fitness assays against the *Cryptococcus* yeast were done with both a rich medium (PYG agar) and a minimal medium (HIM). On PYG agar, the *P. syringae* pv. tomato strains grew quickly and then finally lost their viability during the 7-day incubation while the yeast still slowly multiplied in the cultures. In contrast, on HIM agar, the *P. syringae* pv. tomato strains remained viable for several weeks, which allowed us to determine the effect of competition with the yeast on the population size of *P. syringae* pv. tomato in a mixed culture. The Δ*hcp1* Δ*hcp2* double mutant strain displayed reduced fitness in a mixed culture with the yeast (Fig. 8B). We found that wild-type *P. syringae* pv. tomato DC3000 was able to inhibit yeast growth, whereas the Δ*hcp1* Δ*hcp2* mutant strain was dramatically impaired in this activity (Fig. 8C). Thus, the *hcp* genes seem to be required for yeast growth suppression.

To further characterize the impact of the *P. syringae* pv. tomato DC3000 T6SS on eukaryotes, we focused on the yeast competition phenotype. *P. syringae* pv. tomato DC3000 strains with individual deletions of *hcp1* and *hcp2* were tested for fitness for competition against the *Cryptococcus* yeast. Deletion of *hcp2* resulted in a significant ($P < 0.0001$) decrease in *P. syringae* pv. tomato fitness, which was observed as increased yeast growth. Surprisingly, deletion of *hcp1* also resulted in a small but statistically significant ($P = 0.0008$) increase in yeast growth. The reduced inhibitory activity of the mutants was restored to the wild-type level by genetic complementation of the *hcp* gene mutations (Fig. 8D). When both of the *hcp* genes were present, as in the parent strain and in the Δ*hcp1* + *hcp1* and Δ*hcp2* + *hcp2* complementation strains, the yeast growth was effectively suppressed. According to a Pearson two-tailed correlation test, the correlation between yeast growth inhibition and the presence of *hcp* genes is significant at $P < 0.01$ (see Fig. S5 in the supplemental material).

DISCUSSION

We used a proteomic approach to analyze the secretome of *P. syringae* pv. tomato DC3000 for the identification of potential

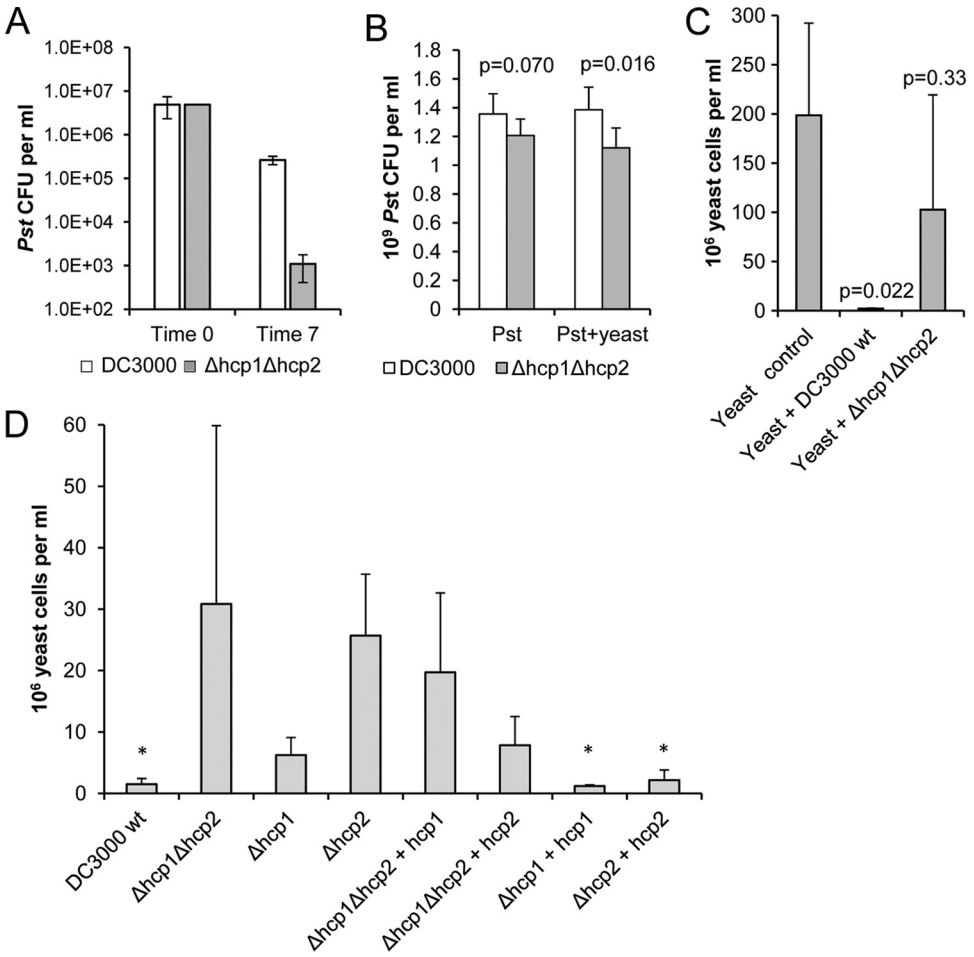

FIG 8 Survival of *P. syringae* pv. tomato DC3000 (*Pst*) in mixed cultures with amoebae and yeast cells is dependent on Hcp function. (A) The *hcp* genes are required for full survival of predation by amoebae. Bacterial populations of the *P. syringae* pv. tomato DC3000 wild-type and *hcp* double mutant strains were enumerated at time point 0 and after 7 days of coincubation with 3×10^5 *A. polyphaga* cells on PYG agar. The values shown are means of three replicates, and error bars indicate the standard errors of the means. At time point 7, the difference between the wild-type (wt) and mutant *P. syringae* pv. tomato strains is significant at a level of $P = 0.0053$ (5 df) by analysis of variance. (B) The *P. syringae* pv. tomato Δ*hcp1* Δ*hcp2* double mutant has reduced fitness in a mixed culture with yeast cells. *P. syringae* pv. tomato strains were inoculated onto HIM agar with the *Cryptococcus* yeast (10^5 *P. syringae* pv. tomato cells and 7.4×10^4 yeast cells per spot), and after 7 days of incubation, the cells were collected and plated onto KB agar. $n = 6$; error bars indicate SDs. *P* values (Student's *t* test) indicate the significance of differences between the *P. syringae* pv. tomato parent strain and the *hcp* mutant. (C) *hcp* genes are required for yeast growth inhibition. Yeast cells were counted after 7 days of culture on PYG agar. Each culture was started with 7.4×10^4 yeast cells, and in the mixed cultures, 10^5 *P. syringae* pv. tomato cells were added. $n = 3$; error bars show SDs. *P* values (*t* test) indicate the significance of differences between the mixed culture and the control yeast culture. (D) Hcp2 is a major factor in yeast growth inhibition. Yeast cells were counted after 7 days of incubation on PYG agar together with one of the *P. syringae* pv. tomato strains. Each of the mixed cultures was started with 10^5 *P. syringae* pv. tomato cells and 5.8×10^4 yeast cells. $n = 3$; error bars show SDs. The asterisks indicate cultures in which yeast growth was inhibited by *P. syringae* pv. tomato. According to one-way analysis of variance, the differences between the *P. syringae* pv. tomato strains are significant at a level of $P = 0.04$ ($F = 23.9$). *wt*, wild type.

novel plant colonization and virulence factors. In addition to known T3SS effectors, Hcp2, probably a component of the T6SS, was found abundantly in *hrp*-inducing medium. Unlike T3SS effectors whose expression is tightly controlled under the regulation of an unknown plant signal(s) and the alternate sigma factor *hrpL* (55), the expression of *hcp2* could be detected in *P. syringae* pv. tomato DC3000 grown in both rich medium and minimal medium. Immunoblotting results suggest that, unlike Hcp2, the Hcp1 protein is not produced in any of the culture media used in this study or it is produced at a very low level that is under the detection limit. We also performed Northern blotting to confirm expression at the transcript level, and while *hcp2* mRNA was clearly visible, *hcp1* mRNA could not be detected, suggesting that no *hcp1* transcript was present in *P. syringae* pv. tomato DC3000 cells (data not shown). Thus, *hcp1* may be a silent gene or it is expressed under as-yet-uncharacterized conditions. Hence, *P. syringae* pv. tomato DC3000 differs from the more complex pattern discovered in *Pectobacterium atrosepticum*, which was previously found to secrete four different Hcp proteins into the culture medium (27). *In vitro*, *P. syringae* pv. tomato DC3000 *hcp2* expression was enhanced by culture aging, nutrient depletion, and growth on a solid surface, and thus, Hcp2 function could be associated with bacterial growth in a nutrient-poor environment. Induction of *hcp2* by quorum sensing could not be verified in liquid culture, possibly because the cell densities were not high enough. When the bacteria were inoculated into host plants, *hcp2* expres-

sion in *P. syringae* pv. tomato was not upregulated, which also suggests that T6SS is regulated differently from T3SS and thus may be expressed at a different stage of the *P. syringae* pv. tomato life cycle. Interestingly, the *hcp2* promoter contains an 18-nucleotide-long perfect inverted repeat (see Fig. S1 in the supplemental material). Such a long palindromic structure implies a role as a target sequence for a dimeric regulator protein. Only one "arm" of this repeat sequence is found in the *hcp1* promoter, and apart from this motif, the promoter regions of the *hcp1* and *hcp2* genes show no sequence homology.

When the virulence and fitness of the *hcp* deletion mutants of *P. syringae* pv. tomato DC3000 were tested on host plants, none of the mutants were found to be significantly affected in colonization. This result is in agreement with the previous finding of Records and Gross (40) that the *P. syringae* pv. syringae B728a *clpV* (T6SS) mutant multiplied *in planta* and produced disease symptoms similar to those caused by the wild-type strain. In contrast to *P. syringae* pv. tomato DC3000, which multiplies mainly inside the host plant leaf tissues (5), *P. syringae* pv. syringae B728a resides primarily as an epiphyte on bean plants. The *P. syringae* pv. syringae B728a genome was shown to encode a functional T6SS and secrete Hcp under regulation by the sensor kinases RetS, LadS, and GacS (40). Records (39) proposed that the lack of an obvious phenotype *in planta* may indicate that the T6SS functions in other aspects of *P. syringae* pv. syringae B728a fitness, possibly in host specificity or intermicrobial interactions. This could include competition with other bacteria for space and nutrients, as well as acting as an antipredation mechanism. In *Vibrio cholerae* V52, a constitutively active T6SS has been shown to confer virulence toward phagocytic eukaryote cells, including the social amoeba *Dictyostelium discoideum* and murine macrophages (29, 38). We found evidence to suggest that the Hcp protein(s) of *P. syringae* pv. tomato DC3000 also plays a role in defense against predation by amoebae. However, the growth phase-dependent expression pattern of *P. syringae* pv. tomato *hcp2* suggests a regulation different from that previously observed for T6SS genes in *V. cholerae*, *Pseudomonas aeruginosa*, and *P. atrosepticum* (18, 21, 23, 27). In *P. syringae* pv. tomato DC3000, *hcp2* expression reached the highest level in the stationary growth phase, suggesting that the function could be important in the late stages of plant infection. In a heavily infected plant, secondary infections by yeasts and molds occur in the damaged plant tissues. Supporting this hypothesis, we found that the T6SS-associated *hcp* genes of *P. syringae* are required for competition against another type of eukaryotic organism, i.e., yeasts. Various species of yeast are common in soil and on plant surfaces, and some of them are parasites (56) or symbionts (9) while others live in a more intimate relationship with plants as endophytes (7). Among others, *Cryptococcus* and *Rhodotorula* species grow well on plant surfaces, including tomato plants, the host of *P. syringae* pv. tomato DC3000 (11). *P. syringae* pv. tomato, as well as other plant-associated bacteria, may have to compete with yeasts for sugar and other nutrients available in plants. Our results, associating *hcp2* function with survival in a nutrient-limited environment and yeast growth suppression, suggest that T6SS might be the decisive component to win the battle for nutrients against yeasts. Sessitsch et al. (48) detected a high abundance of T6SS-encoding genes in the metagenome of a rice endophytic bacterial community and estimated that every endophytic bacterium would harbor T6SS (*hcp*) genes. At present, the molecular mechanism of yeast growth suppression by the T6SS of *P. syringae* pv. tomato is still unclear. T6SS could secrete toxins that kill the yeast or somehow deplete the environment of a growth factor essential for yeast cells.

When we tested the competitive fitness of *P. syringae* pv. tomato in cocultures with different bacteria, we found that *P. syringae* pv. tomato was able to suppress the growth of enterobacteria in a mixed culture and that the survival of *P. syringae* pv. tomato in competition with enterobacteria was completely dependent on a functional *hcp2* gene. Of the bacterial species with which *P. syringae* pv. tomato was found to be able to compete, *E. coli* and *P. vulgaris* are common in surface waters and soil and *P. carotovorum* is well known to cause soft rot in numerous plants, including tomato plants. Thus, it is likely that *P. syringae* pv. tomato encounters these bacteria in nature at different stages of its life cycle. *In vitro*, this competitive activity against enterobacteria was detected only on solid medium and not in a liquid culture with shaking, which is similar to the result previously obtained with *P. aeruginosa* in a coculture with *E. coli* (16). Altogether, our findings on interbacterial competition suggest that the observed suppression of *E. coli* growth in a mixed culture with *P. syringae* pv. tomato DC3000 probably is not caused by a diffusible compound secreted into the culture medium by *P. syringae* pv. tomato but rather is a result of a more intimate interaction between the living cells of the two species. Also, the fact that no T6SS-dependent proteins other than Hcp2 were found in the secretome analysis gives support to the idea that these proteins, e.g., VgrG, could be directly translocated into the cells of the competing microorganism. Russell et al. (42) presented evidence of direct cell contact-dependent delivery of T6SS-secreted bacteriolytic effector proteins from *P. aeruginosa* to other bacteria. However, despite the similarity of *P. syringae* pv. tomato DC3000 and *P. aeruginosa* T6SS gene clusters (44), BLAST searches of the genomes of *P. syringae* strains revealed no sequences clearly homologous to the T6SS-secreted *P. aeruginosa* effectors Tse2 and Tse3, showing toxin and muramidase activities, respectively (16, 42). The third identified T6SS-secreted effector, Tse1, an amidase-cleaving peptidoglycan (42), shows only partial homology to the NlpC/P60 family bacterial cell wall hydrolase also found in *P. syringae*. Thus, although the secretion machinery seems to be highly conserved, the effectors *P. syringae* secretes to survive interbacterial competition are probably different from *P. aeruginosa* effectors. *Burkholderia thailandensis* T6SS-1 (47) and *Serratia marcescens* T6SS (32) were also found to have an essential role in fitness for competition against several other Gram-negative species in a mixed culture, suggesting that this function mediated by T6SS is both important and common.

On the basis of our results, it is clear that none of the *hcp* deletion mutants of *P. syringae* pv. tomato DC3000 has any discernible loss of fitness or pathogenicity in plants. Instead, the Hcp function is required for full fitness for competition against other Gram-negative bacteria, including *Pectobacterium* species, the opportunistic, soft-rot-causing plant pathogens, and against yeasts and amoebae, under biofilm-like conditions on a solid surface. Only *hcp2* is required for interbacterial competition, but in competition with yeast, *hcp1* also seems to play a minor role. As the two T6SSs of *P. syringae* pv. tomato DC3000 seem to be differently regulated, they might be specialized in interactions with different organisms. However, we cannot rule out the possibility that HSI-I is a silent operon, dominated by HSI-II. Functional divergence of different T6SS clusters in a bacterial species was discovered in *Burkholderia* (47), with one cluster specialized in interaction with

the eukaryotic host and another cluster required for interbacterial competition. Moreover, in *V. cholerae* V52, which carries only one T6SS cluster, different T6SS-secreted VgrG proteins have different impacts on antibacterial and antiamoeba functions (57). In the context of interaction with plants and plant-associated microbes, we can postulate that the T6SS of *P. syringae* pv. tomato DC3000 functions to protect the bacterium from a range of competitors and predators. Further identification of T6SS effectors and mechanisms underlying this competitive fitness of *P. syringae* pv. tomato DC3000 should help us understand why *P. syringae* pv. tomato DC3000 possesses two divergent T6SS clusters. It will be of particular interest to determine whether the T6SS enhances bacterial survival in soil or leaf litter or prevents competitor ingress at sites of infection.

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