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Proteomic Identification of Novel Secreted Antibacterial Toxins of the *Serratia marcescens* Type VI Secretion System*[S]

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It has recently become apparent that the Type VI secretion system (T6SS) is a complex macromolecular machine used by many bacterial species to inject effector proteins into eukaryotic or bacterial cells, with significant implications for virulence and interbacterial competition. "Antibacterial" T6SSs, such as the one elaborated by the opportunistic human pathogen, *Serratia marcescens*, confer on the secreting bacterium the ability to rapidly and efficiently kill rival bacteria. Identification of secreted substrates of the T6SS is critical to understanding its role and ability to kill other cells, but only a limited number of effectors have been reported so far. Here we report the successful use of label-free quantitative mass spectrometry to identify at least eleven substrates of the *S. marcescens* T6SS, including four novel effector proteins which are distinct from other T6SS-secreted proteins reported to date. These new effectors were confirmed as antibacterial toxins and self-protecting immunity proteins able to neutralize their cognate toxins were identified. The global secretomic study also unexpectedly revealed that protein phosphorylation-based post-translational regulation of the *S. marcescens* T6SS differs from that of the paradigm, H1-T6SS of *Pseudomonas aeruginosa*. Combined phosphoproteomic and genetic analyses demonstrated that conserved PpkA-dependent threonine phosphorylation of the T6SS structural component Fha is required for T6SS activation in *S. marcescens* and that the phosphatase PppA can reverse this modification. However, the signal and mechanism of PpkA activation is distinct from that observed previously and does not appear to require cell–cell contact. Hence this study has not only demonstrated that new and species-specific portfolios of antibacterial effectors are secreted by the T6SS, but also shown for the first time that PpkA-dependent post-translational regulation of the T6SS is tailored to fit the needs of different bacterial species. ***Molecular & Cellular Proteomics 12: 10.1074/mcp.M113.030502, 2735–2749, 2013.***

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Gram-negative bacteria have evolved several specialized protein secretion systems to secrete a wide variety of substrate proteins into the extracellular milieu or to inject them into other, often eukaryotic, cells (1). Secreted proteins and their associated secretion systems are very important in bacterial virulence and interactions with other organisms (2). One of the most recent discoveries in this field is the Type VI secretion system (T6SS),[1] which occurs widely across bacterial species (3, 4) and can target proteins to both bacterial and eukaryotic cells (5). The significance of the T6SS is becoming increasingly apparent. It has been implicated in virulence, commensalism, and symbiosis with eukaryotes (5, 6). Additionally, in many bacteria, the T6SS is now implicated in antibacterial activity. T6SS-mediated antibacterial killing appears to be important for competition between bacterial species, for example within the resident microflora of a eukaryotic host (5, 7).

Secretion by the T6SS relies on 13 conserved core components which are predicted to form a large machinery associated with the cell envelope, including membrane-bound and bacteriophage tail-like subassemblies (8, 9). The membrane bound subassembly consists of inner membrane proteins (TssLM) and an outer membrane lipoprotein (TssJ) and is anchored to the cell wall. The phage tail-like assembly consists of several proteins that show structural homology with T4 phage tail proteins or are organized in similar structures (10). Hcp (TssD) proteins form hexameric rings and are thought to stack into tube-like structures (11, 12). This Hcp tube is believed to be capped by a trimer of VgrG (TssI) proteins, which share structural homology with the needle of the T4 phage tail (10, 13). In addition, VipA (TssB) and VipB (TssC) form a large tubular structure highly reminiscent of the T4 phage tail sheath (14, 15). Such similarities have led to the idea that the T6SS resembles an inverted contractile bacteriophage infection machinery and injects substrates via an Hcp/VgrG needle into other cells. Recent models propose that the VipA/B sheath surrounds the Hcp/VgrG needle and contraction of the VipA/B tube pushes the Hcp/VgrG needle

[1] The abbreviations used are: T6SS, Type VI secretion system; FDR, false discovery rate; LFQ, label-free quantitation; ORF, open reading frame; TCEP, tris(2-carboxyethyl)phosphine.

out of the cell (16–18). It has been postulated that this mechanism can be triggered by close contact with other neighboring cells (19–21).

Assembly, localization, and remodelling of VipA/B tubules *in vivo* depend on the AAA+ ATPase ClpV (TssH), another essential core component of the T6SS (14, 16, 17). ClpV also interacts with the accessory component Fha (TagH) (22, 23), which is found in a subset of T6SSs (4). The Fha protein has an N-terminal domain with a forkhead associated motif, which is predicted to bind phospho-threonine peptides (24). In *Pseudomonas aeruginosa*, Fha1 is phosphorylated by the Thr/Ser kinase PpkA (TagE) and dephosphorylated by the phosphatase PppA (TagG), and the phosphorylation state of Fha1 regulates the activity of the T6SS (22, 23). Phosphorylation of Fha in *P. aeruginosa* is also controlled by additional components, which act upstream of PpkA and form a regulatory cascade for T6SS activation (22, 25). Although homologs of PpkA and PppA have been identified in the T6SS gene clusters of certain other bacteria (3), the regulation of the T6SS by post-translational protein phosphorylation has not yet been experimentally investigated outside of *Pseudomonas*.

To understand how the T6SS affects eukaryotic and bacterial cells, it is critical to identify substrate proteins secreted by the T6SS. The VgrG and Hcp proteins were the first identified T6SS substrates and appear to be generally secreted to the external milieu by all T6SSs (26). However, as mentioned above, Hcp and VgrG are core components of the T6SS machinery and therefore represent extracellular components of the secretion apparatus rather than genuine secreted effector proteins. Nonetheless, a limited number of VgrG homologs with extra functional effector domains at the C terminus have been identified or predicted, which account for some of the T6SS dependent effects seen against bacteria and eukaryotes. For example, the C-terminal domain of VgrG-1 from *Vibrio cholerae* shows actin crosslinking activity in eukaryotic cells (13, 27) and the C-terminal domain of *V. cholerae* VgrG-3 has bacterial cell wall hydrolase activity (28, 29).

Recently, following much effort in the field, a small number of proteins secreted by the T6SS, but not structural components, have been experimentally identified. These proteins are regarded as true secreted substrates of the T6SS, with effector functions in target cells (29–35). For example, antibacterial T6SS-secreted effector proteins with peptidoglycan amidase (cell wall hydrolysis) function, the Type VI amidase effector (Tae) proteins, have been identified in *Burkholderia thailandensis* (32), *P. aeruginosa* (31), and *Serratia marcescens* (30). These Tae proteins play a role in T6SS-mediated antibacterial killing activity and genes encoding four families of Tae protein have been widely identified in other bacteria with T6SSs (32). T6SS-secreted effector proteins which are not peptidoglycan hydrolases have also been reported, including Tse2 secreted by *P. aeruginosa*, which acts in the bacterial cytoplasm (31), and the VasX and TseL proteins secreted by the *V. cholerae* T6SS, which are suggested to target membrane lipids (29, 34, 35). In the case of antibacterial T6SSs, the secreting bacterial cells are protected from their own T6SS effector proteins by specific immunity proteins (29–32, 35). However, given the large number of T6SSs in different bacterial species and their apparent ability to secrete multiple substrates, experimentally identified T6-secreted effector proteins still remain surprisingly scarce.

Here we report the identification of multiple T6SS-secreted effector proteins in *S. marcescens*. *S. marcescens* is an opportunistic pathogen, for example causing ocular infections, nosocomial septicemia and pneumonia (36). Previously, we have identified a T6SS in *S. marcescens* Db10, which targets and efficiently kills other bacterial cells and plays a role in antibacterial competition (37). We have recently demonstrated that this T6SS secretes two antibacterial effectors, the Tae4 homologs Ssp1 and Ssp2, with cognate immunity proteins Rap1a and Rap2a (30).

In this work, we report the analysis of the T6SS-dependent secretome of *S. marcescens* by label-free quantitation (LFQ) mass spectrometry and describe the identification and characterization of four novel T6SS-secreted effector proteins. These were confirmed as antibacterial toxins and specific immunity proteins were identified. Additionally, this global secretomic analysis, in combination with genetic and phosphoproteomic analyses, demonstrated that a post-translational phosphorylation system influences the ability of the *S. marcescens* T6SS to secrete effector proteins. Although this system uses homologs of the *P. aeruginosa* PpkA, PppA and Fha components, the circumstances and impact of Fha phosphorylation were shown to vary between organisms.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—Strains and plasmids used in this study are given in supplemental Table S1 and all *S. marcescens* strains are derivatives of *S. marcescens* Db10 (38). Allelic exchange to construct in-frame gene deletions or chromosomal hemagglutinin-tag (HA-tag) fusions at the C terminus of Fha in *S. marcescens* Db10 was performed using the pKNG101 suicide vector as described previously (37, 39). To conserve the polycistronic organization of the *fha* locus in the HA-tagged fusion strains, the HA-tag was followed by the last ten codons of native *fha* in the wild-type genetic background (MJF16; *fha*-HA) and the kinase mutant (SJC48; *fha*-HA, Δ*ppkA*). However, the HA-tag fusion in the phosphatase mutant (SJC49; *fha*-HA, Δ*pppA*) was followed by the last six codons of *pppA* to accommodate deletion of *pppA*, which is directly adjacent to the *fha* gene. This construct resulted in a slightly smaller fusion protein in the *pppA* deletion background compared with the wild type and *ppkA* deletion backgrounds and slightly altered mobility in SDS-PAGE (supplemental Fig. S7*A*). A streptomycin-resistant version of strain JAD01, JAD06, was generated by generalized transduction as described (30). Expression of *S. marcescens* genes in *E. coli* MG1655 from derivatives of plasmid pBAD18-Kn was repressed or induced with D-glucose or L-arabinose, respectively, at concentrations as specified. Cultures of *S. marcescens* were grown at 30 °C in LB (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl, 1.5 g l^{-1} agar for solid media; 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl for liquid media) or minimal media (40 mM K_2HPO_4, 15 mM KH_2PO_4,

0.1% (w/v) $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 0.2% (w/v) glucose). *Escherichia coli* cultures were grown at 37 °C in LB or M9 minimal media (M9) (40). Media were supplemented with appropriate antibiotics as follows: ampicillin (Ap) 100 μg ml^{-1}, kanamycin (Kn) 100 μg ml^{-1}, streptomycin (Sm) 100 μg ml^{-1}. M9 media were supplemented with 0.5% (w/v) glycerol and expression from pBAD18-Kn constructs was repressed with 0.5% (w/v) D-glucose during cloning and culture maintenance.

Antibacterial Competition and Coculture Assays—Antibacterial competition assays were described previously (37). In brief, the *S. marcescens* attacker strain and the *P. fluorescens* target strain (both at OD_{600} 0.5) were mixed at an attacker to target ratio of 5:1, cocultured on solid LB medium for 4 h at 30 °C and the surviving target cells were subsequently enumerated by serial dilution and viable counts on streptomycin-supplemented media. For competition between *S. marcescens* attacker strains and the *S. marcescens* Δ*sip4* target strain, an initial ratio of 1:1 and incubation time of 7.5 h was used.

Immunodetection of Secreted Proteins—SDS-PAGE and immunoblotting were carried out according to the methods of Laemmli (41) and Towbin *et al.* (42), respectively. Polyclonal antisera against Hcp1 and Ssp2 were used for immunoblotting as described previously (30, 37). Samples were prepared from 25 ml liquid cultures grown for 8 h at 30 °C. Whole cell samples were prepared from 100 μl culture and cell pellets were suspended in 100 μl 2× Laemmli buffer and 100 μl LB media. Supernatant samples for Hcp1 secretion were prepared from 100 μl cell culture supernatant and mixed with 2× Laemmli buffer. For Ssp2 secretion, precipitated supernatant samples were prepared from 20 ml cell culture supernatant and precipitated using chloroform/methanol precipitation as described previously (43), and the protein precipitate was dissolved in 200 μl 2× Laemmli buffer.

Proteomic Secretome Sample Preparation and Mass Spectrometry—Four biological replicates of *S. marcescens* Db10 wild-type strain, SJC3 (Δ*clpV*), and SJC19 (Δ*pppA*) were grown in 50 ml minimal media at 30 °C with vigorous agitation for 8 h. Cells were separated from the culture supernatant by four cycles of centrifugation at 5000 × *g* for 30 min at 4 °C. Supernatant proteins in 40 ml of culture supernatant were precipitated overnight on ice with 6% (w/v) trichloroacetic acid (TCA, Sigma), recovered by centrifugation (5000 × *g*, 4 °C, 30 min), washed five times in 1 ml ice-cold 80% (v/v) acetone, and air dried. Precipitated proteins were redissolved in 8 M urea, 100 mM Tris-HCl pH 8.0, 1 mM TCEP. Cysteines were alkylated by addition of 20 mM chloroacetamide and incubation for 20 min at 25 °C and the reaction quenched by addition of 20 mM DTT. Samples were diluted to 2 M urea with 100 mM Tris-HCl pH 8.0 and trypsin (sequencing grade, Promega, Madison, WI) was added at a 1:50 ratio. Proteins were digested for 4 h at 37 °C under constant shaking. 0.5 μg of digest was injected in an interleaved manner onto a 2 cm × 100 μm trap column and separated on a 15 cm × 75 μm Pepmap C_{18} reversed-phase column (Thermo Fisher Scientific) on a Dionex 3000 Ultimate RSLC. Peptides were eluted by a linear 2 h gradient of 95% A/5% B to 35% B (A: H_2O, 0.1% Formic acid (FA); B: 80% acetonitrile (ACN), 0.08% FA) at 300 nl/min into a LTQ Orbitrap Velos (Thermo Fisher Scientific). Data was acquired using a data-dependent "top 20" method, dynamically choosing the most abundant precursor ions from the survey scan (350–1650 Th, 60,000 resolution, target value 10^6). Precursors above the threshold of 500 counts were isolated within a 2 Th window and fragmented by CID in the LTQ Velos using normalized collision energy of 35 and an activation time of 10 ms. Dynamic exclusion was defined by a list size of 500 features and exclusion duration of 60 s. Lock mass was used and set to 445.120025 for ions of polydimethylcyclosiloxane (PCM).

Sample Preparation and Mass Spectrometry of the S. marcescens Phosphoproteome—Four biological replicates of *S. marcescens* Db10 wild-type strain, SJC19 (Δ*pppA*), and SJC25 (Δ*ppkA*) were grown with vigorous agitation in 100 ml LB at 30 °C for 5 h. Cells were harvested by centrifugation at 5000 × *g* for 15 min at 4 °C, washed with 10 ml 50 mM Tris-HCl, pH 8.0 and suspended in 0.5 ml 8 M urea, 50 mM Tris-HCl pH 8.0, 1 mM TCEP with phosphatase inhibitors (1.15 mM Na_2MoO_4, 1 mM Na_3VO_4, 4 mM Sodium tartrate, 5 mM glycerol 3-phosphate), and protease inhibitors (complete, Mini, EDTA-free Protease Inhibitor Mixture; Roche). Cells were disrupted by 5 cycles of sonication with 5 s pulse and 30 s pause on ice-ethanol slush and the cell lysate was cleared from cell debris by centrifugation, 16000 × *g* for 30 min at 4 °C. One milligram of cell lysates were reduced, alkylated, and digested as described above. After sample clean-up using solid phase extraction cartridges (HPE, Waters), phosphopeptides were enriched using TiO_2 chromatography (44–46). Phosphopeptides were analyzed on an Orbitrap Velos Pro, using above LC and mass spectrometry settings.

Affinity Isolation ("pull-down") of Fha-HA Fusions and Mass Spectrometry—Three biological replicates of *S. marcescens* Db10 wild-type strain, MJF16 (*fha*-HA), SJC48 (*fha*-HA, Δ*ppkA*), and SJC49 (*fha*-HA, Δ*pppA*) were grown with vigorous agitation in 100 ml LB at 30 °C for 5 h. Cells were harvested by centrifugation at 5000 × *g* for 15 min at 4 °C, washed with 10 ml phosphate buffered saline (PBS) pH 7.4, and suspended in 1 ml PBS pH 7.4, 1% (v/v) Triton X-100 with phosphatase inhibitors (1.15 mM Na_2MoO_4, 1 mM Na_3VO_4, 4 mM Sodium tartrate, 5 mM glycerol 3-phosphate) and protease inhibitors (complete, Mini, EDTA-free Protease Inhibitor Mixture; Roche). Cells were disrupted by 5 cycles of sonication with 5 s pulse and 30 s pause on ice-ethanol slush, and the cell lysate was cleared from cell debris by centrifugation at 4 °C, 16000 × *g* for 30 min. Cleared cell lysates were incubated with 30 μl anti-HA agarose conjugate (Sigma) for 1 h and the anti-HA agarose conjugate was washed 5 times with 1 ml PBS pH 7.4, 0.2% (v/v) Triton X-100. Proteins were eluted from the anti-HA conjugate in 50 μl 50 mM Tris-HCl pH 8.0, 1% (w/v) RapiGest (Waters Analytical), 1 mM TCEP. Cysteines were alkylated by addition of 5 mM iodoacetamide and incubation for 20 min at 37 °C and the reaction quenched by addition of 5 mM DTT. Samples were diluted to 0.1% (w/v) RapiGest with 50 mM Tris-HCl pH 8.0 and digested with 250 ng trypsin (sequencing grade, Promega) for 16 h at 30 °C under constant shaking. Rapigest was cleaved by acidification with 1% TFA at 37 °C for 1 h. Samples were desalted using StageTips (ThermoFisher Scientific) and lyophilized by vacuum centrifugation.

Mass Spectrometry Data Analysis and Label-free Quantitation—Label-free quantitation was performed using MaxQuant v1.2.2.5 (47). Mass spectrometric runs of four biological replicates of *S. marcescens* Db10 (WT), SJC19 (Δ*pppA*) and SJC3 (Δ*clpV*) (secretome experiments) or Db10 (WT), SJC19 (Δ*pppA*) and SJC25 (Δ*ppkA*) (phosphoproteome experiments) or Db10 (WT), MJF16 (*fha-HA*, SMA2267-HA), SJC48 (*fha-HA,* Δ*ppkA,* SMA2267-HA), SJC49 (*fha-HA,* Δ*pppA,* SMA2267-HA), were searched against a combined database of *S. marcescens* containing 4720 sequences (generated using the complete *S. marcescens* Db11 genome sequence and preliminary gene prediction available from the Sanger Institute repository (http://www.sanger.ac.uk/resources/downloads/bacteria/serratia-marcescens.html)) and a list of common contaminants in proteomics experiments (247 entries). The following settings were used: enzyme trypsin, allowing for one missed cleavage, fixed modifications were carbamidomethyl (C), variable modifications were set to Acetyl (Protein N-term) and Oxidation (M). In case of the phosphoproteome experiments, Phospho (STY) was added as variable modification. MS/MS tolerance was set to 0.5 Da, precursor tolerance was set to 6 ppm. Peptide and Protein FDR was set to 0.01, minimal peptide length was 6, and one unique peptide was required. Requantify and retention time alignment (2 min) were enabled. For the phosphoproteome experiment, peptides were also searched using Mascot

v2.2. For this, peaks were detected using Mascot Distiller 2.4.3.1 (using standard Orbitrap low resolution MS2 settings) and searched against the same database using the same settings except for precursor mass tolerance of 10 ppm. Any "bold red," unique phosphopeptide with a MOWSE score >25 was considered significant.

For the secretome experiment, MaxQuant provided LFQ data for 1020 proteins, 841 of which had ≥ 2 unique peptides. Significant protein hits required a minimum of two unique peptides, a fold-change of at least four between Db10 and Δ*clpV*, and measurable quantitative data from at least two of the four biological replicates. A student's *t* test was performed on the LFQ intensities and only proteins with $p < 0.05$ were considered significant. For three proteins (SMA2274, SMA4628 and SMA4673), no peptide intensities could be obtained in the Δ*clpV* mutant, but reproducible LFQ intensities were obtained in the secretomes of the other strains. To obtain ratios, the Δ*clpV* LFQ intensities for these proteins were set arbitrarily to 100000.

Analysis of Fha Phosphopeptide Occupancy—Peptide ion intensities for all peptides of Fha (SMA2267) were extracted from the evidence file of the MaxQuant analysis of three biological replicates of the immunoprecipitated HA-tagged Fha from Db10, MJF16, SJC48, SJC49. Peptide and phosphopeptide ion intensities were normalized for differences in protein abundance.

RESULTS

Identification of T6SS-Secreted Proteins—To identify the secreted substrates of the *S. marcescens* T6SS in a non-biased manner, we adopted a secretomic approach which has been used successfully in the past (48). The secretome was analyzed from cultures grown in liquid minimal media to provide sufficient and reproducible material. Because the T6SS is a contact dependent secretion system and likely requires cell–cell contact for efficient secretion of effectors (19, 20), we anticipated only low levels of T6SS substrates in the medium under these conditions. Therefore, the total secretome was analyzed using a highly sensitive mass spectrometry approach coupled with label-free quantitative (LFQ) analysis. Because a T6SS$^-$ mutant strain would not secrete any T6SS substrates, comparison of the secretome of a T6SS$^-$ mutant strain with that of the wild-type strain would permit identification of those proteins that are secreted by the T6SS of *S. marcescens*. For this secretomic analysis, we utilized a T6SS$^-$ mutant strain bearing an in-frame deletion of the core component *clpV*, previously shown to be essential for T6 secretion in *S. marcescens* (37). In addition, we also included a *S. marcescens* mutant strain with a deletion of the predicted phosphatase *pppA* (SMA2268). It was reported previously that a deletion of *pppA* in *P. aeruginosa* caused a T6SS hypersecreting phenotype, which was used in a mass spectrometry-based approach to identify successfully *P. aeruginosa* T6SS substrates (31). Therefore, we anticipated a different secretion profile in a *pppA* mutant strain compared with the wild-type, perhaps facilitating identification of candidate substrates.

The proteins present in the culture supernatant of four biological replicates of each strain were analyzed by mass spectrometry and the average LFQ data was compared between each strain. To identify T6SS substrates, those protein hits were considered candidates that had at least two peptides, were reproducibly detected in at least two out of the four biological replicates and whose average LFQ intensities were decreased more than fourfold (p value < 0.05) in the T6SS$^-$ mutant compared with the wild-type strain. Samples from each replicate showed good technical and biological reproducibility (average coefficient of variation 19.9–22.8%) (supplemental Fig. S1*A*, Fig. S2) and the majority of proteins found in the secretome did not show any significant changes between strains (supplemental Fig. S1*C*). Comparison of the wild-type with the T6SS$^-$ mutant strain and analysis of the LFQ data identified 841 proteins with at least two unique peptides (supplemental Table S2), and of these proteins, 14 proteins matched the aforementioned criteria defining candidate T6-secreted proteins (Fig. 1*B*, Table I). In contrast, comparison of the secretion profile between the wild-type and the phosphatase mutant did not show any significant differences (Table I, supplemental Fig. S1*B*). Similarly, the number and identities of differentially secreted proteins in the wild-type strain and phosphatase mutant, when each was compared with the T6SS$^-$ mutant, was the same (Table I). Hence, unexpectedly, deletion of *pppA* in *S. marcescens* did not yield a hypersecreting phenotype.

The list of T6SS substrate candidates contained all three Hcp homologs (SMA2263, SMA3455, SMA3456) and both VgrG homologs (SMA2244, SMA2276) encoded within the genome of *S. marcescens* (Fig. 1*B*, Table I). As described above, the Hcp and VgrG proteins are extracellular components of the secretion apparatus but not true secreted effectors. However, because they depend on a functional T6SS for their secretion to the external milieu, their identification in the T6SS secretome acted as proof-of-principle, validating our experimental approach. Importantly, Ssp1 (SMA2261) and Ssp2 (SMA2264) were also identified as T6SS substrates. In a parallel study, we recently demonstrated these to be T6SS-secreted antibacterial effectors using genetic and biochemical approaches (30). The successful identification of Ssp1 and Ssp2 further validates the secretomic data.

The list of T6SS substrate candidates also included two other proteins encoded within the T6SS cluster: the ClpV homolog SMA2274 and the Rhs-family protein SMA2278. Use of Δ*clpV* as the T6SS$^-$ mutant meant that traces of this intracellular protein resulting from low level lysis could only be observed in the supernatant of the wild type and Δ*pppA* strains, hence it passed the criteria as a "substrate". SMA2278 seemed an unlikely candidate for a T6-secreted effector because its molecular mass of 165 kDa is far in excess of other effectors identified to date (32). However only N-terminal peptides of SMA2278 were identified, perhaps suggesting secretion of a cleaved isoform. Penicillin-binding protein 2 (PBP2; SMA0444) was also identified as a potential candidate, however the reason for its identification is unclear, because PBP2 homologs have a known role in the periplasm and are generally localized in the inner membrane via a transmembrane helix (49, 50).

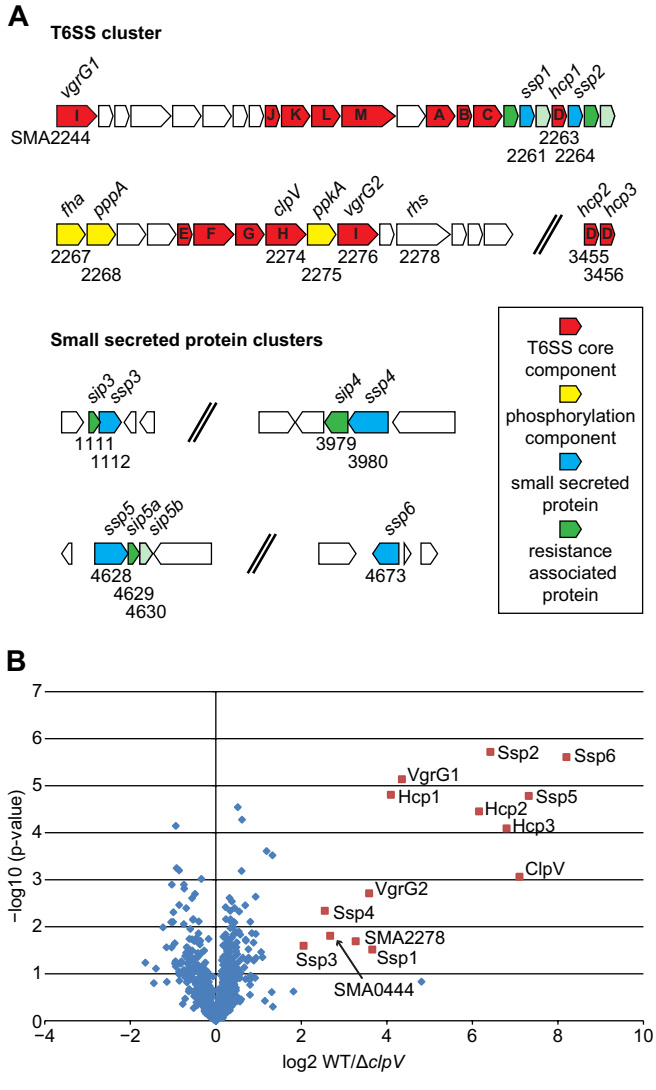

FIG. 1. **T6SS-dependent secretome of *S. marcescens* Db10.** *A*, Schematic representation of the T6SS gene cluster of *S. marcescens* Db10 and the genetic context of the identified T6-secreted proteins. Arrows representing ORFs encoding T6SS core components are shown in red, post-translational phosphorylation system components in yellow, T6-secreted proteins in blue, and associated immunity proteins in green (pale green indicates a gene encoding a "silent" immunity protein with homology to the adjacent immunity protein). The direction of transcription for each ORF is identified by the orientation of the solid arrow, with *S. marcescens* gene identifiers below. Letters corresponding to the systematic Tss nomenclature (60) are shown inside the arrows. *B*, Volcano plot summarizing the secretomic comparison between wild type and T6SS$^-$ mutant (Δ*clpV*) strains of *S. marcescens* Db10. The log2 of the ratios of peptide intensities between the wild-type strain and the T6SS$^-$ mutant (Δ*clpV*) are plotted against the *p* values of label-free quantitation data intensities. Red squares correspond to proteins with significant changes between the wild-type strain and the T6SS$^-$ mutant (WT/Δ*clpV* $>$ 4; $p < 0.05$) whereas blue diamonds correspond to proteins without significant changes.

Of greatest interest, four proteins (SMA1112, SMA3980, SMA4628, and SMA4673) encoded outside the main T6SS gene cluster were identified as candidates for new T6-secreted effector proteins. No known functional domains at the amino acid sequence level could be predicted for any of these proteins and all were relatively small, in common with the limited number of other T6SS substrates identified to date. These proteins seemed strong candidates for T6SS substrates of *S. marcescens* and thus SMA1112, SMA3980, SMA4628 and SMA4673 were named according to the *S. marcescens*-specific *s*mall *s*ecreted *p*rotein (Ssp) nomenclature as Ssp3, Ssp4, Ssp5, and Ssp6, respectively.

The Identified T6SS-secreted Proteins are Antibacterial Toxins and Have Associated Immunity Proteins—The newly identified Ssp proteins were found to be encoded in four different loci throughout the *S. marcescens* Db10 genome. Except for *ssp6*, the *ssp* genes appeared to be organized in polycistronic operons together with one or two neighboring ORFs encoding small proteins (10–20 kDa) with no known function (Fig. 1*A*). This organization was reminiscent of previously described T6SS effector and immunity protein pairs (30–32). For example, Ssp1 and Ssp2 are encoded next to cognate Rap immunity proteins within the T6SS cluster of *S. marcescens* (Fig. 1*A*). We hypothesized that Ssp3, Ssp4, Ssp5, and Ssp6 represented new cytotoxic effector proteins and that the neighboring ORFs encoded cognate immunity proteins.

To test this hypothesis, the newly identified *ssp* genes were cloned into an L-arabinose inducible vector, either alone or in different combinations with the candidate immunity genes. These constructs were used to heterologously express the candidate proteins in the cytoplasm of *E. coli* cells. First, the *ssp* genes were expressed on their own in order to test if the Ssp proteins have a toxic effector function. Induction of expression of the *ssp* genes did indeed lead to severe impairment of the growth of *E. coli* (Fig. 2). The toxic effect appeared to be strongest for *ssp*5 and *ssp*6, where *E. coli* cells expressing the respective protein exhibited negligible growth, even when high cell densities were spotted on the plates. Next, it was tested whether the toxic effect of the Ssp proteins on *E. coli* could be alleviated by the adjacently encoded candidate immunity proteins. Growth of *E. coli* was fully restored when the ORFs *SMA1111*, *SMA3979*, and *SMA4629* were coexpressed with *ssp3*, *ssp4* and *ssp5*, respectively (Fig. 2). At least in the case of Ssp4+SMA3979 and Ssp5+SMA4629, this immunity seemed to be highly specific, because coexpression of *SMA3978* and *SMA4630* with *ssp4* and *ssp5*, respectively, did not abolish the growth defect associated with the Ssp protein. However, despite the fact that SMA4630 showed no immunity effect against Ssp5, amino acid sequence alignment and *in silico* secondary structure predictions of SMA4629 and SMA4630 showed similarity between the two proteins (supplemental Fig. S3). This indicates that SMA4629 and SMA4630 are paralogs of each other and

TABLE I
Candidates for T6SS-dependent secretion in S. marcescens identified by label-free mass spectrometry

Identifier	Name[a]	Description	Peptides	WT/Δ*clpV* Ratio	WT/Δ*clpV* *p* value	Δ*pppA*/Δ*clpV* Ratio	Δ*pppA*/Δ*clpV* *p* value	WT/Δ*pppA* Ratio	WT/Δ*pppA* *p* value	Sequence coverage	Mol. weight
SMA3456	Hcp3 (TssD3)	Hcp homolog	8	111.4	8.1E-05	123.4	5.9E-04	0.9	4.7E-01	48%	17443
SMA2264[c]	Ssp2 (Tae4.2^{SM})	T6-secreted toxin, predicted amidase	11	85.7	1.9E-06	99.9	3.3E-04	0.9	3.6E-01	64%	17867
SMA3455	Hcp2 (TssD2)	Hcp homolog	9	71.4	3.5E-05	97.8	1.0E-04	0.7	7.8E-02	63%	17170
SMA4673	Ssp6	Function unknown	2[b]	29.4	2.5E-06	26.4	1.8E-07	1.1	1.7E-01	10%	24463
SMA2244[c]	VgrG1 (TssI1)	VgrG homolog	18	20.4	7.3E-06	50.0	8.5E-05	0.4	2.0E-04	33%	87546
SMA2263[c]	Hcp1 (TssD1)	Hcp homolog	18	17.1	1.6E-05	13.9	1.0E-04	1.2	1.5E-01	94%	16803
SMA4628	Ssp5	Function unknown	2[b]	16.0	1.7E-05	14.3	1.6E-03	1.1	5.7E-01	6%	29398
SMA2274[c]	ClpV (TssH)	T6SS ATPase	3[b]	13.7	8.6E-04	13.7	8.9E-05	1.0	9.9E-01	5%	96881
SMA2261[c]	Ssp1 (Tae4.1^{SM})	T6-secreted toxin, predicted amidase	9	12.6	3.1E-02	17.8	1.4E-02	0.7	3.4E-01	53%	18179
SMA2276[c]	VgrG2 (TssI2)	VgrG homolog	16	12.0	1.9E-03	21.9	5.9E-03	0.5	1.2E-01	39%	71336
SMA2278[c]		Rhs-family protein	3	9.7	2.0E-02	13.1	1.0E-02	0.7	2.0E-01	2%	165501
SMA0444		Predicted penicillin-binding protein 2	2	6.4	1.6E-02	3.3	7.1E-02	1.9	1.6E-01	3%	70772
SMA3980	Ssp4	Function unknown	6	5.9	4.6E-03	13.3	1.3E-01	0.4	3.3E-01	19%	35500
SMA1112	Ssp3	Function unknown	2	4.1	2.5E-02	3.1	1.3E-01	1.3	3.3E-01	10%	20574

[a] The systematic Tss (Type six secretion) (60) and Tae (Type VI amidase effector) (32) nomenclature is given in parentheses. The Ssp (small secreted protein) nomenclature for secreted effector proteins is specific for *S. marcescens*.
[b] No peptides detected in the T6SS$^-$ mutant strain (Δ*clpV*), ratio determined by setting Δ*clpV* LFQ to 100000.
[c] Encoded within the main T6SS gene cluster of *S. marcescens* Db10 (*SMA2244–2281*).

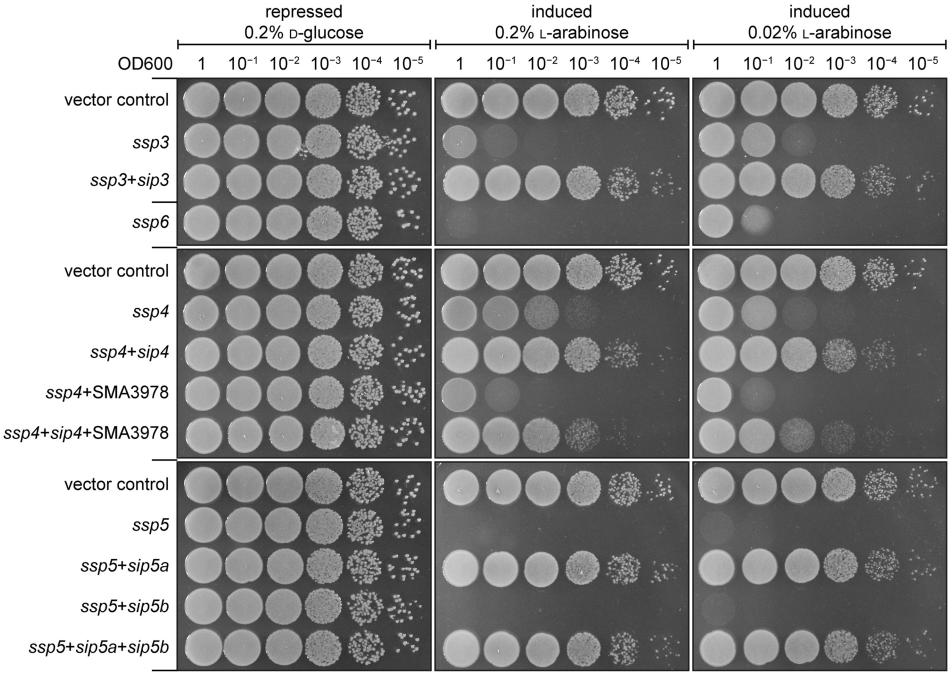

FIG. 2. **Heterologous expression of newly identified T6-secreted proteins is toxic in *E. coli* and alleviated by coexpression of associated immunity proteins.** Genes encoding *S. marcescens* T6-secreted proteins with or without associated candidate immunity proteins were heterologously expressed in *E. coli* MG1655 from an inducible plasmid. Serial dilutions of *E. coli* strains were spotted on M9 minimal media plates and gene expression was repressed or induced with D-glucose or L-arabinose, respectively. The expressed genes are indicated on the left and the density of the inoculum is given along the top of the images.

SMA4630 represents a "silent" immunity protein of SMA4629. In contrast, SMA3978 had no sequence or secondary structure similarities with SMA3979. Hence, *SMA1111*, *SMA3979*, and *SMA4629* encode specific immunity proteins and these proteins were named Sip3, Sip4, and Sip5a (Ssp-associated *i*mmunity *p*rotein), respectively. SMA4630 was renamed Sip5b to reflect the sequence and secondary structure similarity between Sip5a (SMA4629) and SMA4630. This nomenclature was chosen to distinguish the newly identified immunity proteins from the previously identified Rap (*r*esistance *a*ssociated *p*rotein) proteins encoded within the T6SS gene cluster of *S. marcescens* (30).

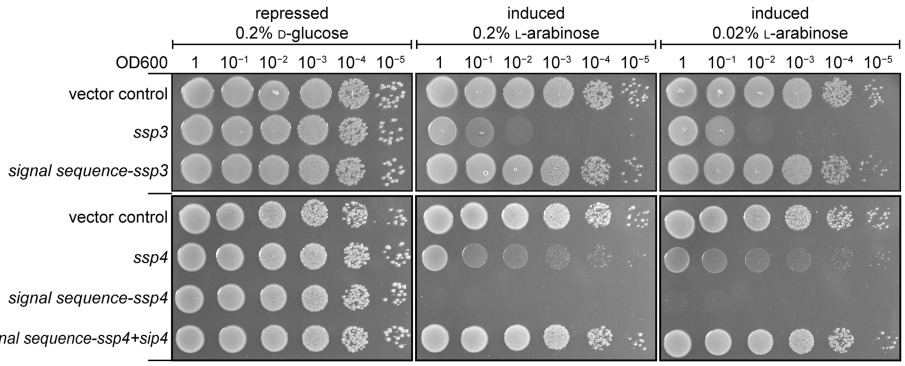

FIG. 3. **Directed export of T6SS-secreted proteins Ssp3 and Ssp4 to the periplasm of *E. coli* alters effector toxicity.** Genes encoding *S. marcescens* Ssp3 and Ssp4, with or without the OmpA Sec signal sequence, were heterologously expressed in *E. coli* MG1655 from an inducible plasmid. Serial dilutions of *E. coli* strains were spotted on M9 minimal media plates and gene expression was repressed or induced with D-Glucose or L-arabinose, respectively. The expressed genes are indicated on the left and the density of the inoculum is given along the top of the images.

Because Ssp3-Ssp6 appeared to be novel effector toxins, their site of action in the target cell was of great interest. The majority of T6SS antibacterial effectors described to date appear to act in the periplasm of the target cell (30, 32). Evidence for this site of action includes toxicity only being observed when an effector is targeted to the periplasm, for example by fusion to a Sec signal sequence, and the immunity protein being localized in the periplasm. Sip3, Sip4, and Sip5a immunity proteins lack any detectable N-terminal Sec signal sequences (SignalP 4.1 (51)). This, together with Ssp3-Ssp6 all showing toxicity when expressed in the cytoplasm of *E. coli* (Fig. 2), initially suggested that all the newly identified Ssp proteins exert their toxic effect in the cytoplasm. However, we noticed that Ssp4 did not show complete toxicity in the cytoplasm and that the amino acid sequence of Sip4, the immunity protein of Ssp4, was predicted to have four transmembrane domains (MEMSSAT (52); OCTOPUS (53)), indicating that Sip4 might reside in the inner membrane between the cytoplasm and the periplasm. Therefore, N-terminal fusions of the Ssp proteins to a Sec signal sequence, which directs export from the cytoplasm to the periplasm, were constructed and expressed heterologously in *E. coli*. The fusion of Ssp3 to a Sec signal sequence alleviated toxicity (Fig. 3), indicating that export of Ssp3 from the cytoplasm into the periplasm removed the toxin from its target and confirming Ssp3 as a cytoplasmic-acting effector protein. In contrast, expression of the signal sequence-Ssp4 fusion protein had a stronger toxic effect than Ssp4 without a signal sequence fusion (Fig. 3), suggesting that the toxic activity of Ssp4 is directed against a target that is most accessible from the periplasm. The toxicity of the signal sequence-Ssp4 fusion was fully relieved by coexpression of Sip4, whose predicted transmembrane domains of Sip4 could indicate an inner membrane localization with access to the periplasm.

Expression of signal sequence fusions to *ssp5* and *ssp6* inhibited growth of *E. coli* cells, in a manner comparable with constructs lacking the signal sequence (supplemental Fig. S4*A*). This result cannot confirm whether toxic activity of Ssp5 and Ssp6 is restricted to either the cytoplasm or periplasm; either the proteins can cause harm from both compartments or they cannot be efficiently targeted to the periplasm via the Sec export pathway before causing harm in the cytoplasm. The toxic effect of the signal sequence fusion to *ssp5* was alleviated by coexpression with *sip5a* (supplemental Fig. S4*A*). Because the immunity protein Sip5a appears to be localized in the cytoplasm, because of a lack of any signal sequences or transmembrane domains, it is most likely that Ssp5 was inactivated by Sip5a in the cytoplasm before export to the periplasm. Given the localization of Sip5a and the high potency of Ssp5 in the cytoplasm, this effector almost certainly acts on a cytoplasmic target.

T6SS-dependent Antibacterial Activity of Ssp4—To initially investigate the role of these new effectors in T6SS-mediated antibacterial activity of *S. marcescens*, we constructed four chromosomal mutants, each lacking one of the Ssp3–6 toxins, and determined the ability of each mutant to kill the susceptible target strain *Pseudomonas fluorescens* (37). Each mutant was still able to kill *P. fluorescens* similarly to the wild-type (supplemental Fig. S4*B*). This result was not unexpected, however, because each of these single mutants still possessed five potent antibacterial toxins and was also observed previously for mutants lacking Ssp1 and Ssp2 (30). Therefore, to provide additional support for the conclusion that the proteomic study had successfully identified secreted antibacterial effectors of the *S. marcescens* T6SS, we chose Ssp4 for further analysis. In-frame deletion mutants Δ*ssp4* and Δ*sip4*,Δ*ssp4* of *S. marcescens* were constructed (unsurprisingly, a single immunity mutant could not be isolated, presumably because of Ssp4-mediated self-killing). An antibacterial competition assay was used to determine whether the Δ*sip4*,Δ*ssp4* immunity mutant was susceptible to T6SS-mediated killing by the wild type (Fig. 4). Coculture of the Δ*sip4*,Δ*ssp4* immunity mutant (target) with wild-type *S. marcescens* (attacker) resulted in >100x reduction in viable target

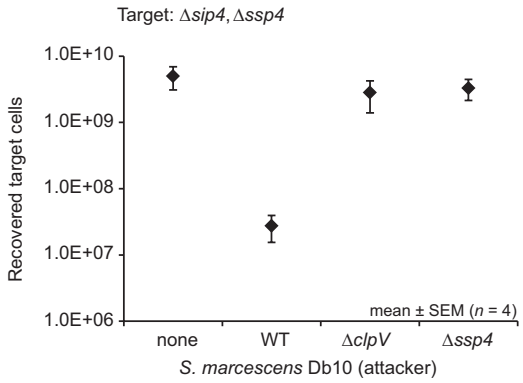

FIG. 4. **T6SS-mediated antibacterial activity against a sensitive Δ*sip4* mutant depends on Ssp4.** Recovery of Δ*sip4* mutant JAD06 (Δ*sip4*, Δ*ssp4*) as the target strain following coculture with different attacking strains: wild-type *S. marcescens* Db10 (WT), T6SS$^-$ mutant SJC3 (Δ*clpV*) and effector mutant MJF8 (Δ*ssp4*). As a negative control, the target was incubated with sterile media alone (none). Error bars show standard error of the mean (S.E.M.) with $n = 4$.

cells recovered, compared with when the immunity mutant was cultured with media alone. This loss of target cells was dependent on the T6SS of the attacker, being eliminated when the immunity mutant was cocultured with a T6SS-inactive mutant. Critically, killing of the sensitive Δ*sip4*,Δ*ssp4* immunity mutant was also eliminated when the attacker lacked the effector Ssp4 (Δ*ssp4* mutant), as shown in Fig. 4. Hence Ssp4 is indeed an antibacterial effector delivered by the T6SS and capable of efficiently killing or inhibiting susceptible target bacterial cells. Given that Ssp1 and Ssp2 have also been confirmed to act as antibacterial effectors when delivered by the T6SS in *S. marcescens* (30), it is apparent that the proteomic study did indeed successfully identify such effectors.

Post-translational Phosphorylation Components Regulate the T6SS in S. marcescens—Beyond T6SS substrate identification, the second, unexpected, finding of the global secretome analysis was that there were no significant differences between the secretomes of the wild type and the *pppA* mutant of *S. marcescens*. In addition to PppA, a homolog of the corresponding Thr/Ser kinase PpkA (SMA2275) is also encoded within the T6SS gene cluster of *S. marcescens*. However, the secretomic outcome raised the question of whether the *S. marcescens* post-translational phosphorylation components are functional and whether they do form a post-translational regulatory system which influences the activity and effector secretion of this T6SS.

To answer this question, in-frame deletions of *pppA* and *ppkA* were constructed in *S. marcescens* and T6SS secretion activity was determined. First, secretion of Hcp1 and the effector protein Ssp2 was monitored by immunoblotting of culture supernatant fractions from *pppA* and *ppkA* mutants, compared with the wild type and inactive T6SS$^-$ strains (carrying deletions of the essential *clpV* or *tssE* genes (37)). Deletion of the phosphatase gene *pppA* did not give a significant increase in Hcp1 or Ssp2 secretion (Fig. 5*A*), which is in agreement with the result of the mass spectrometry analysis above. However, complementation of the *pppA* deletion *in trans* reduced secretion of Hcp1 and Ssp2 indicating that the phosphatase PppA is able to repress T6SS activity when overexpressed from a plasmid vector (Fig. 5*B*). In contrast, deletion of the kinase gene *ppkA* or generation of a Δ*pppA*,Δ*ppkA* double mutant abolished Hcp1 and Ssp2 secretion (Fig. 5*A*), demonstrating that PpkA is essential for T6 secretion in *S. marcescens*. This result was further confirmed with the secretion defect of the *ppkA* deletion mutant and the Δ*pppA*,Δ*ppkA* double mutant being restored to the wild-type phenotype by complementation with *ppkA in trans* (Fig. 5*B*).

Next, T6SS-mediated antibacterial killing activity of the *pppA* and *ppkA* mutants was tested by coculture assays between *S. marcescens* "attacker" and *P. fluorescens* "target" strains. Coculture of the *S. marcescens* wild-type strain with *P. fluorescens* results in a massive decrease in the number of recovered target cells, whereas coculture with a T6SS$^-$ mutant does not affect the amount of recovered target cells, showing the potent antibacterial activity of the *S. marcescens* T6SS documented previously (37). In this assay, deletion of the phosphatase gene *pppA* resulted in modestly reduced killing activity (shown by a 50x increase in the number of recovered target cells compared with a wild-type attacker) (Fig. 5*C*). Consistent with the secretion assays, deletion of *ppkA* abolished antibacterial killing activity (Fig. 5*C*). Complementation of the *ppkA* mutant *in trans* restored killing activity, although not quite to wild-type levels, and attempted complementation of the *pppA* mutant similarly resulted in killing activity moderately less efficient than the wild-type strain (Fig. 5*D*). Hence PpkA is essential for T6 secretion in *S. marcescens* and PppA does have the ability to impact on T6SS activity. The *in trans* complementation data further indicate that the expression levels of *ppkA* and *pppA* are critical and affect the regulation of the T6SS, because T6SS-dependent effector secretion was reduced by overexpression of *pppA* and antibacterial killing activity was reduced by overexpression of *ppkA*. The former also explains why complementation of the *pppA* mutant by overexpression of *pppA* did not restore killing activity to wild-type levels.

Finally, we tested whether the kinase activity of PpkA was required for its function in regulating T6SS activity. The highly conserved residue D165 in the kinase domain of PpkA represents the predicted catalytic aspartate residue in the active site loop (supplemental Fig. S5). Although the Hcp secretion defect of the *ppkA* mutant could be fully complemented by expression of wild-type PpkA *in trans*, expression of the point mutant D165N, lacking this catalytic residue, completely failed to restore Hcp secretion (Fig. 5*E*). This indicates that the kinase activity of PpkA is essential for T6SS activity in *S. marcescens*.

PpkA and PppA Control Phosphorylation of Fha in S. marcescens—Having shown genetically that the kinase PpkA and, to some extent, the phosphatase PppA can influence the

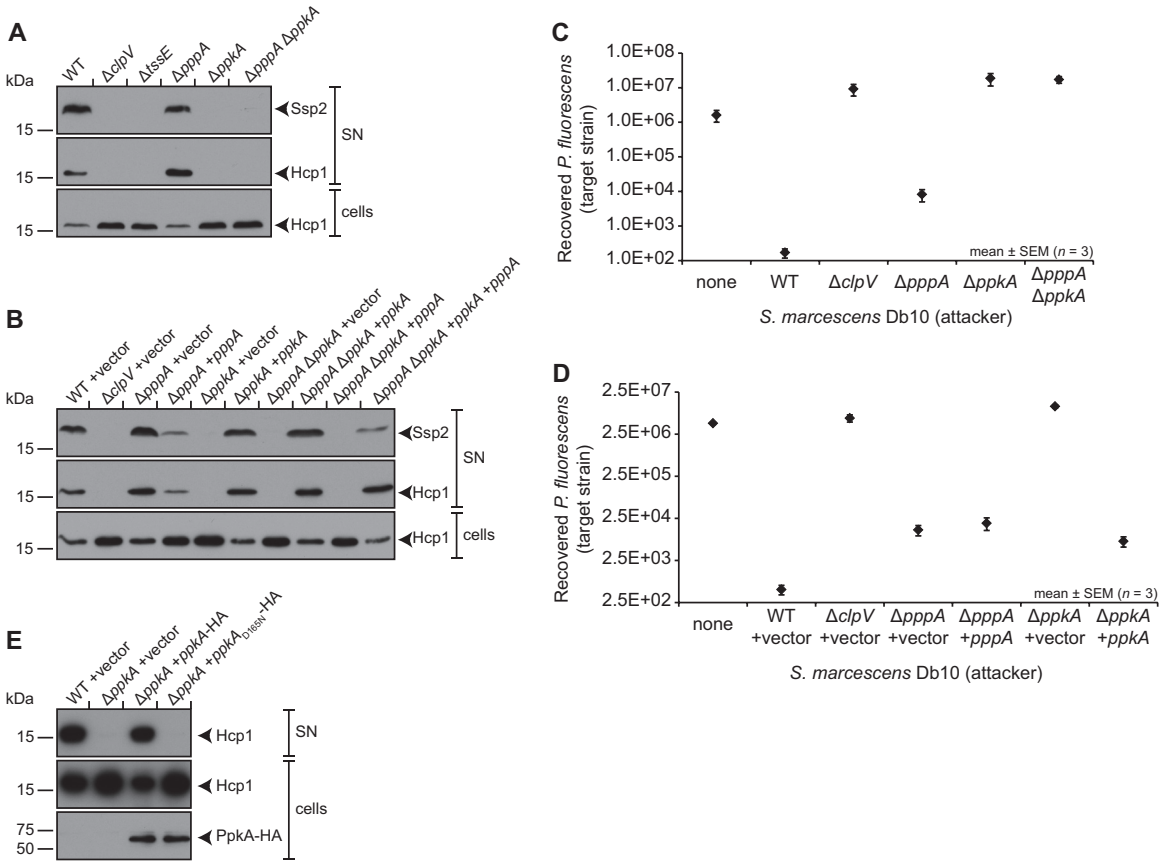

FIG. 5. **Components of the post-translational phosphorylation system are functional and essential for T6SS activity in *S. marcescens*.** *A*, *B*, T6SS-mediated secretion by *S. marcescens* strain Db10 (WT), T6SS$^-$ mutant strains SJC3 (Δ*clpV*) and SJC11 (Δ*tssE*), phosphatase mutant strain SJC19 (Δ*pppA*), kinase mutant strain SJC25 (Δ*ppkA*), and double mutant strain MJF4 (Δ*pppA*, Δ*ppkA*) was monitored by immunoblotting cell and supernatant (SN) samples using anti-Hcp1 (*middle* and *bottom* panel) and anti-Ssp2 (*top* panel) antisera. *C*, *D* Antibacterial killing activity of *S. marcescens* strains Db10 (WT), SJC3 (Δ*clpV*), SJC19 (Δ*pppA*), SJC25 (Δ*ppkA*), and MJF4 (Δ*pppA*, Δ*ppkA*) against *P. fluorescens*. Error bars show standard error of the mean (S.E.M.) with $n = 3$. In parts (*B*, *D*) mutant strains were complemented with plasmids expressing the cognate gene *in trans*: pSC812 (*ppkA*), pSC815 (*pppA*), or pSC830 (*pppA*, *ppkA*), and pSUPROM was the empty vector control. *E*, T6SS-dependent Hcp secretion (*top* and *middle* panel) or levels of PpkA protein (*bottom* panel) in the strain SJC25 (Δ*ppkA*) carrying plasmids expressing wild-type PpkA (pSC814) or PpkA D165N (pSC863) fused with a C-terminal HA tag, or the empty vector control (pSUPROM). Cellular and supernatant samples were subjected to immunoblotting using anti-Hcp1 (*top* and *middle* panel) or anti-HA (*bottom* panel) antisera.

activity of the *S. marcescens* T6SS, we wanted to identify any proteins subject to PpkA/PppA phosphorylation/dephosphorylation. As an initial experiment, we attempted to identify PpkA-dependent phosphorylation targets using a non-biased mass spectrometry based approach, namely by comparing the entire *in vivo* phosphoproteome of the *S. marcescens* wild-type strain with that of the phosphatase and kinase mutant strains. We applied a thorough setup and searched the data both with MaxQuant/Andromeda as well as Mascot. Using conservative settings, we required peptides to be positively identified in both Andromeda (PEP$<$0.05, 22 peptides) and Mascot (MOWSE score $>$25, 40 peptides). Overall this experiment detected 15 phosphopeptides belonging to 15 different proteins in *S. marcescens* (supplemental Tables S3, S4). To qualify as a substrate candidate for PpkA, peptides needed to show increased intensity in the Δ*pppA* phosphatase mutant and loss of the signal in the Δ*ppkA* kinase mutant. Only one peptide followed this pattern: the peptide AEMpTMILDEANNPFK of the *S. marcescens* Fha protein (SMA2267) containing the phosphorylation site Thr_{438} (Fig. 6*A*). Previously, it has been shown that the Fha1 homolog of *P. aeruginosa* is essential for Hcp secretion (54) and is phosphorylated and dephosphorylated at residue Thr_{362} by PpkA and PppA, respectively (23). Deletion of the Fha homolog SMA2267 in *S. marcescens* also abolished Hcp secretion, demonstrating an essential role for Fha in this T6SS (Fig. 6*B*). A global sequence alignment based on *S. marcescens* Fha showed high sequence conservation with the phosphorylation and phospho-peptide binding sites of Fha homologs from other Gram-negative bacteria (supplemental Fig. S6). The Thr_{438} residue of *S. marcescens* Fha aligned directly with conserved threonine residues of other bacterial Fha ho-

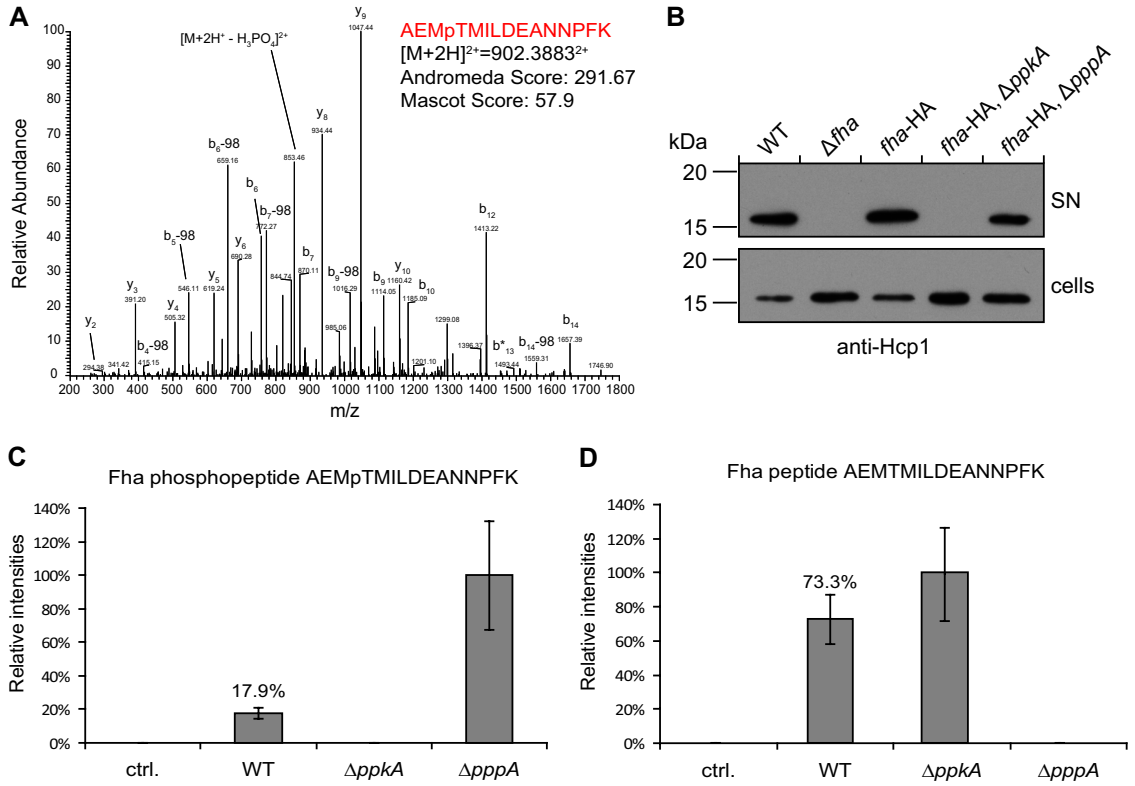

FIG. 6. **Phosphorylation of the essential T6SS component Fha in *S. marcescens*.** *A*, MS/MS spectrum of the phosphorylated peptide AEMpTMILDEANNPFK of Fha. *B*, T6SS functionality of strains carrying an in-frame deletion of *fha* or a chromosomal *fha-HA* fusion. T6SS activity was monitored by immunoblotting of cell and culture supernatant (SN) samples from *S. marcescens* strains Db10 (WT), KK2 (Δ*fha*), MJF16 (*fha*-HA), SJC48 (*fha*-HA, Δ*ppkA*) and SJC49 (*fha*-HA, Δ*pppA*) using anti-Hcp1 antiserum. *C*, *D*, Relative intensities of the phosphorylated (*C*) and unmodified (*D*) Fha peptide containing Thr_{348} in immunoprecipitation samples from *S. marcescens* strains Db10 (ctrl.), MJF16 (WT), SJC48 (Δ*ppkA*) and SJC49 (Δ*pppA*). Peptide intensities are normalized to the intensities of total Fha peptides and expressed relative to the *fha*-HA, Δ*pppA* phosphatase mutant (*C*) or the *fha*-HA, Δ*ppkA* kinase mutant (*D*). Error bars show standard error of the mean (S.E.M.) with $n = 3$.

mologs, including with the Thr_{362} residue representing the phosphorylation site of *P. aeruginosa* Fha1 (Supplemental Fig. S6*B*). Hence this residue, Thr_{438} in *S. marcescens* Fha, represents a highly conserved phosphorylation site. No other PppA/PpkA-dependent phosphopeptides were detected in our global analysis, suggesting that Thr_{438} of Fha might be the sole target of this phosphorylation system. The Thr_{438} phosphorylated peptide was also detected in the wild-type strain, although not in every replicate. Because *S. marcescens* was grown in liquid culture, this result appeared to contrast with the observation in *P. aeruginosa* that Fha1 is essentially only phosphorylated when grown on solid medium, supporting the idea of cell–cell contact dependent activation of the T6SS (54). Therefore, our *in vivo* proteomic data suggested that phosphorylation of Fha in *S. marcescens* might be at least partially independent of cell–cell contact.

To analyze the phosphorylation of Fha in more detail under planktonic growth conditions in liquid cultures, C-terminal hemagglutinin tag (HA-tag) fusions of Fha were introduced in the native *fha* locus on the chromosome of the wild-type strain, the phosphatase mutant and the kinase mutant. The Fha-HA fusion proteins were fully functional for Hcp secretion in the wild-type and *pppA* mutant backgrounds, confirming that the C-terminal HA-tag was not interfering with the function of Fha (Fig. 6*B*). The presence of the Fha-HA fusion proteins in immunoprecipitated samples was confirmed by SDS-PAGE and label-free mass spectrometry (Fig. 6, supplemental Fig. S7). Further mass spectrometry analysis identified the Thr_{438} phosphorylated Fha peptide, and comparison of the unmodified and phosphorylated Fha peptide was used to determine the levels of Fha phosphorylation between the different strains (Fig. 6*C, D,* supplemental Fig. S7). No phosphorylated peptides were found in the kinase mutant whereas the unmodified species of this peptide was not found in the phosphatase mutant (Fig. 6*C*, *D*). This result confirmed that the kinase PpkA is responsible for Fha phosphorylation and, because essentially 100% of Fha is phosphorylated in the PppA mutant, also confirms that PppA is the sole phosphatase for this phosphorylation site. Wild-type samples also contained phosphorylated Fha but the amount of phosphorylated peptide species was, at about 20%, less than the phosphatase mutant (Fig. 6*C*). This result confirms that the

PppA phosphatase homolog of *S. marcescens* is capable of dephosphorylating Fha and shows that Fha is phosphorylated and active, at least at a moderate level, under planktonic growth conditions.

DISCUSSION

Knowledge of secreted substrates is critical for understanding the biological role of the T6SS and for dissecting T6SS-dependent interactions of bacterial cells with eukaryotes and within bacterial communities. Our analysis of the T6SS secretome of *S. marcescens* by label-free quantitative mass spectrometry has provided a unique, global snapshot of T6SS substrates in *S. marcescens*. It not only confirmed secretion of known extracellular T6SS components but also identified six effector proteins. These included four completely novel T6SS protein substrates, Ssp3–6, in addition to the previously characterized Ssp1 and Ssp2 peptidoglycan amidase effectors. Heterologous expression of Ssp3, Ssp4, Ssp5, or Ssp6 in a bacterial host demonstrated that these novel T6SS substrates are cytotoxic antibacterial effectors. Moreover T6SS-dependent antibacterial activity of Ssp4 (and, previously, Ssp1 and 2) in the context of *S. marcescens* cell–cell interactions has been demonstrated. These findings significantly expand the repertoire of known T6SS effectors and further reinforce the growing idea that T6SSs can secrete multiple, diverse effector proteins to provide highly efficient killing and to protect against target strains acquiring resistance to a single toxin. Indeed, similar to what we observed for Ssp1 and 2 (30), single effector mutants lacking Ssp3, Ssp4, Ssp5 or Ssp6 showed no significant loss of antibacterial killing activity against *P. fluorescens*. However this was expected, because these single effector mutants are still able to secrete at least five other potent antibacterial toxins, and confirms the "belt and braces" nature of T6SS-dependent bacterial killing by *S. marcescens*.

T6-secreted effectors have only been experimentally identified in a small number of bacterial species, particularly compared with the large number of organisms possessing T6SSs. These include *P. aeruginosa* (effectors Tse1–3, PldA/Tle5^{PA}), *V. cholerae* (VasX and TseL/Tle2^{VC}) and *B. thailandensis* (Tae2^{BT}, Tle1^{BT}, Tle3^{BT}) (29, 31, 32, 34, 35). Additionally, elegant bioinformatic studies have recently identified large families of candidate peptidoglycan amidase and phospholipase T6-secreted effectors (32, 35). However, analysis of the primary amino acid sequence of Ssp3, Ssp4, Ssp5, and Ssp6 did not show significant sequence similarity to any of these previously identified T6SS effectors. In addition, no functional domains could be readily predicted from their amino acid sequences (data not shown). Thus, the toxicity mechanism of these effectors is currently unclear. Examination of the predicted cellular localization of the immunity proteins and the toxicity of the effectors in two different cellular compartments shows that Ssp3 acts on a cytoplasmic target whereas Ssp4 acts on a periplasmic target. These experiments further strongly indicate that Ssp5 acts on a cytoplasmic target. The site of action of Ssp6 is less obvious, although it clearly shows potent toxicity when expressed in the cytoplasm. The precise cellular targets of these new toxins will be the subject of future research.

We have also identified novel immunity proteins, which neutralize effector activity, encoded next to the coding sequences of their specific effector proteins. This organization has also been found for other effector-immunity pairs and is probably because of horizontal gene transfer as one genetic unit between different bacterial species (29, 30, 32, 35). In addition, "silent" immunity proteins next to active immunity proteins, for example Sip5b next to Sip5a identified in this study and Rap2b next to Rap2a associated with Ssp2, have been found in several effector protein loci (30, 32). Such silent immunity proteins may have evolved as protection against similar effector proteins from closely related species. It is likely that the set of six effector-immunity units identified in *S. marcescens* Db10 during this study might provide a specific toolbox allowing this organism to distinguish itself from, and kill, other closely related strains. A graphical summary of the full set of T6SS effector proteins of *S. marcescens* with their cognate immunity proteins and their proposed subcellular activities is shown in Fig. 7. We propose that the immunity protein Sip4 might be the first integral membrane immunity protein identified to date and this might indicate the cellular compartment targeted by Ssp4.

The mass spectrometry analysis of the secretome has also provided the first experimental evidence in *S. marcescens* for the T6SS-dependent secretion of both VgrG homologs (VgrG1, VgrG2) and the two Hcp homologs encoded outside the main T6SS gene cluster (Hcp2, Hcp3). Secretion of Hcp1 by the T6SS was demonstrated previously (37). The results here indicate that all VgrG and Hcp homologs play an active part in the T6SS of *S. marcescens* and this raises the question of whether the different homologs can complement each other or have specific, distinct functions. For example, the VgrG1a and VgrG1c homologs of *P. aeruginosa* can complement each other but have different multimer stabilities (55). Another question is whether the VgrG trimer at the tip of the Hcp/VgrG needle complex consists of a homotrimer or a heterotrimer. In addition, do specific VgrG homologs only interact with specific Hcp homologs? It might be possible that particular effector proteins require specific combinations of Hcp and VgrG homologs in the needle complex for secretion. The T6SS of *V. cholerae* secretes three different VgrG homologs and recent experiments have shown that the *V. cholerae* VgrG-3 directly interacts with the TseL effector protein and is required for secretion of this effector (29). Therefore, having a set of different VgrG and Hcp homologs in *S. marcescens* might be required to secrete a range of different T6SS effector proteins.

The list of T6SS candidates also included a homolog of an Rhs-family protein (SMA2278); genes encoding such proteins

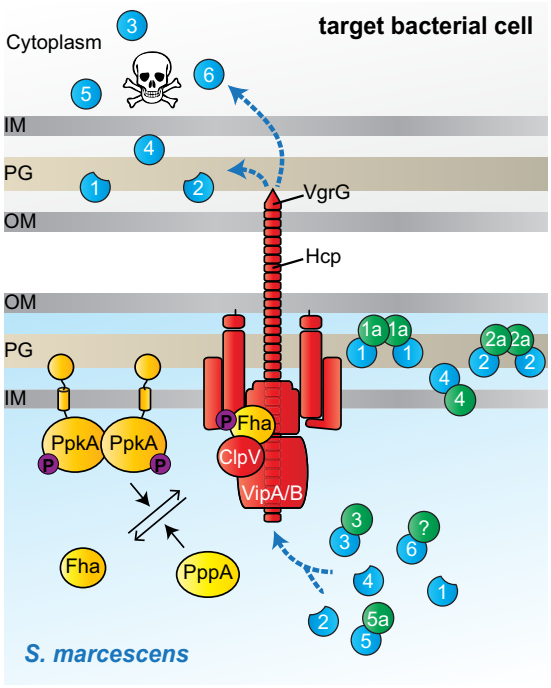

FIG. 7. **Model: *S. marcescens* utilizes its T6SS, activated by PpkA, to secrete a range of toxic effector proteins into neighboring bacterial cells and is protected by cognate immunity proteins.** *S. marcescens* injects toxic effectors into neighboring cells via the Hcp/VgrG needle complex of the T6SS. Cognate immunity proteins provide protection against those effectors (whether produced within the secreting cell or injected by neighboring sibling cells). PpkA is activated in response to a ubiquitous cue and phosphorylates Fha. Fha phosphorylation results in activation of the T6SS by inducing assembly of the VipAB sheath and Hcp/VgrG needle onto a baseplate-like complex and thus allowing recruitment of ClpV to the machinery. PppA-mediated dephosphorylation of Fha may keep this activation under control or reset the system, allowing disassembly and reassembly at new cellular sites. Effector proteins are shown as blue circles with numbers corresponding to Ssp1–6 and immunity proteins as green circles with numbers corresponding to Rap1a/2a or Sip3–5a; a question mark indicates the unidentified immunity protein of Ssp6. Core components of the T6SS complex are shown in red and components of the post-translational phosphorylation system are shown in yellow; IM, inner membrane; PG, peptidoglycan cell wall; OM, outer membrane.

are frequently located with T6SS-associated genes. However, our data provide the first experimental evidence of T6SS dependent secretion of an Rhs-family protein. Interestingly, a subset of Rhs-family proteins with C-terminal toxin domains and cognate immunity proteins has been linked with bacterial contact-dependent growth inhibition (56). More importantly, a recent study in the Gram-negative bacterium *Dickeya dadantii* showed that contact-dependent growth inhibition caused by an Rhs homolog was lost in the absence of genetically linked *vgrG* genes (57). Thus Rhs proteins with effector domains might constitute a new family of T6SS effectors. It should be noted that there is no readily identifiable effector function predicted for the C-terminal domain of the SMA2278 Rhs-family protein of *S. marcescens*. Therefore, whether the Rhs-family SMA2278 is a T6SS effector protein remains to be experimentally determined. It is also unclear if the whole SMA2278 protein is secreted by the T6SS, given its large size compared with other effectors, or if it is processed and only part of it secreted. Perhaps in support of processing, only peptides from the N-terminal region of SMA2278 were detected in the secretome.

Overall, our approach by LFQ mass spectrometry proved to be very sensitive and specific for the identification of T6SS substrates. Fourteen T6SS substrate candidates were identified out of over 800 robustly detected proteins by using strict criteria within a quantitative analysis. Only two previous studies have reported successful mass spectrometry based identification of T6SS substrates, using a spectral counting approach to analyze the T6SS-dependent secretomes of *B. thailandensis* and *P. aeruginosa* (31, 32). Among the many proteins found in the culture supernatant of *S. marcescens,* our approach not only detected T6SS substrates with high abundance, for example Hcp1, but also proteins with much lower LFQ intensities (supplemental Fig. S1*B*). It was expected that effector proteins were likely to be present at very low abundance, reflecting highly inefficient secretion into the media rather than a target cell. Our selection criteria proved to be very specific, because out of 14 candidates, there is good evidence that at least 11 are indeed secreted by the T6SS, whereas only the Rhs protein and penicillin-binding protein remain ambiguous. Therefore, this experimental set-up could also be adopted to identify substrates of T6SSs in other Gram-negative organisms or indeed substrates of other bacterial secretion systems.

The outcome of the secretome analysis also prompted investigation of whether effector secretion by the *S. marcescens* T6SS depends on a PpkA/PppA-dependent post-translational regulatory system. The main components of this phosphorylation system have been well characterized in *P. aeruginosa* (22, 23, 25, 54). Here we have shown for the first time that Fha, PpkA, and PppA homologs are also involved in T6 secretion by another organism, *S. marcescens*. Similar to the situation in *P. aeruginosa*, the kinase PpkA is essential for the activation of the *S. marcescens* T6SS and is able to phosphorylate the conserved threonine residue Thr_{438} of Fha, whereas PppA removes this modification. However, although deletion of *pppA* did lead to total phosphorylation of Fha, this did not cause an increase in T6 secretion in liquid media. Additionally, a lower, but readily detectable, level of Fha phosphorylation was observed in the wild-type in liquid, concomitant with detectable levels of effector secretion. This is in contrast with *P. aeruginosa*, where Fha is not significantly phosphorylated nor effectors secreted when wild-type cells are grown in liquid media, but phosphorylation is observed if cells are grown on solid media or PppA is inactivated, leading to effector secretion (54). Recently, it has been proposed that the phosphorylation system of *P. aeruginosa* is activated by the T6SS activity of neighboring cells to trigger reciprocal

T6SS secretion (21). Therefore, phosphorylation of Fha in *P. aeruginosa* would only occur on solid media when cells are in close proximity. In addition, the proteins TagQ, TagR, TagS, and TagT also regulate phosphorylation of Fha, upstream of PpkA, suggesting that these components are involved in sensing cell–cell contact and T6SS activity from neighboring cells (25). However, these upstream components are absent in *S. marcescens* and many other Gram-negative bacteria harboring T6SSs. Therefore, cell–cell contact sensing might be different or deficient in *S. marcescens*; certainly, T6SS regulation by PpkA/PppA modulation of Fha phosphorylation appears to operate slightly differently.

The lack of difference between the wild type and phosphatase mutant secretome might suggest two possible scenarios in *S. marcescens*. First, in liquid media the contribution of the phosphorylation system is very minor and other inputs are able to activate assembly of the T6SS such that the phosphorylation state of Fha does not significantly affect the observed secretion activity. Indeed it has been proposed previously that PpkA might have a structural role in the T6SS, independent of its phosphorylation activity (22). However the observation that not only the complete PpkA deletion, but also the kinase-inactivating D165N mutant, cannot support any T6SS activity suggests that this is not the case and that the kinase activity of PpkA is essential to activate the *S. marcescens* T6SS. The second possible scenario, which we favor, is that the level of Fha phosphorylation observed in the wild-type in liquid is sufficient to activate the T6SS and that a signal other than cell–cell contact is able to induce this activation via PpkA. In support of this idea, overexpression of PppA, which would reduce Fha phosphorylation, did decrease secretion of Hcp and the effector Ssp2. Additionally, the putative signal sensing domain of PpkA is not conserved between *P. aeruginosa* and *S. marcescens*. The periplasmic C-terminal domain of *P. aeruginosa* PpkA contains a large von Willebrand Factor A domain and has been proposed to be involved in binding of the periplasmic protein TagR, which activates PpkA-dependent phosphorylation of Fha in concert with TagQST (22, 25). In contrast, the periplasmic domain of *S. marcescens* PpkA is shorter than the *P. aeruginosa* homolog and does not have a von Willebrand Factor A domain (supplemental Fig. S5*A*). However, protein structure prediction using Phyre2 (58) identified homology with a pre-albumin fold in the C-terminal domain of *S. marcescens* PpkA (supplemental Fig. S5*B*). Hence, the differences in post-translational phosphorylation-dependent activation of the T6SS between *S. marcescens* and *P. aeruginosa* are likely explained by the difference in the PpkA C-terminal domains, which strongly suggests that different signals are sensed.

It is noteworthy that the only significant phenotype observed for the *pppA* mutant in *S. marcescens* was a modest loss of antibacterial killing activity. This suggests that PppA and the phosphorylation system do play a modulatory role under conditions of cell–cell contact on a solid surface; perhaps under these conditions total Fha phosphorylation in a *pppA* mutant is deleterious, interfering with activation of the T6SS by other cues or leading to harmful overactivation. Another, attractive possibility is that the reduced antibacterial activity of the *pppA* mutant can be explained by a reduction in T6SS dynamics, namely a reduced frequency of T6SS disassembly and subsequent reassembly at different cellular sites, as shown recently in *P. aeruginosa* (21). Unfortunately, Fha phosphorylation under sessile growth conditions could not be investigated by mass spectrometry because cultures grown on solid media did not yield sufficient material for quantification.

Our data suggest that the phosphorylation system of *S. marcescens* has a "fine-tuning" role, especially in a competition scenario, but that T6SS shows at least some constitutive activity in a non-contact scenario. Constitutive activation of the T6SS, not dependent on incoming T6SS attacks from other cells, has also been shown in *Vibrio cholerae* (21, 59), which has an Fha homolog but lacks homologs of PppA and PpkA. Constitutive activation probably confers a fitness advantage for an environmental organism, such as the insect pathogenic isolate *S. marcescens* Db10, which needs to compete persistently with other microorganisms within its ecological niche. In contrast, for a pathogen such as *P. aeruginosa* it might be advantageous to avoid activation of an immune response within its host organism by regulating its T6SS secretion activity via post-translational phosphorylation at an early stage of infection. It will be interesting to see whether clinical isolates of *S. marcescens* also show constitutive activation of the T6SS.

In conclusion, we have successfully adopted a sensitive LFQ mass spectrometry-based approach to considerably expand the set of known secreted effectors of the T6SS system and have found novel proteins not previously identified in studies of T6SSs in other organisms. Significantly, the functions of new effectors identified in this study appear to be distinct from previously characterized T6SS effector proteins. Determination of the functions and targets of the effector proteins described herein will be the focus of future work. Identification and characterization of these and other effectors is crucial to understand the function of T6SSs in general and also to elucidate the contribution of T6SSs in inter-bacterial competition at polymicrobial infection sites and during direct interaction with eukaryotic cells. Perhaps more importantly, characterizing the cellular targets of T6SS effectors, like the ones identified in this study, which cause toxin-mediated death or stasis of bacterial cells might also reveal new targets for novel antimicrobials.

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