A Type VI Secretion System of Pseudomonas aeruginosa Targets a Toxin to Bacteria

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SUMMARY

The functional spectrum of a secretion system is defined by its substrates. Here we analyzed the secretomes of Pseudomonas aeruginosa mutants altered in regulation of the Hcp Secretion Island-I-encoded type VI secretion system (H1-T6SS). We identified three substrates of this system, proteins Tse1–3 (type six exported 1–3), which are coregulated with the secretory apparatus and secreted under tight posttranslational control. The Tse2 protein was found to be the toxin component of a toxin-immunity system and to arrest the growth of prokaryotic and eukaryotic cells when expressed intracellularly. In contrast, secreted Tse2 had no effect on eukaryotic cells; however, it provided a major growth advantage for P. aeruginosa strains, relative to those lacking immunity, in a manner dependent on cell contact and the H1-T6SS. This demonstration that the T6SS targets a toxin to bacteria helps reconcile the structural and evolutionary relationship between the T6SS and the bacteriophage tail and spike.

INTRODUCTION

Secreted proteins allow bacteria to intimately interface with their surroundings and other bacteria. The importance and diversity of secreted proteins are reflected in the multitude of pathways bacteria have evolved to enable their export (Abdallah et al., 2007; Filloux, 2009). Large multicomponent secretion systems, including types III and IV secretion, have been the focus of a great deal of study because in many organisms they are specialized for effector export and they have the remarkable ability to directly translocate proteins from bacterial to host cell cytoplasm via a needle-like apparatus (Cambronne and Roy, 2006). The recently described type VI secretion system (T6SS) is another specialized system; however, its physiological role and general mechanism remain poorly understood (Bingle et al., 2008).

Studies of T6SSs indicate that a functional apparatus requires the products of approximately 15 conserved and closely linked genes and is strongly correlated to the export of a hexameric ring-shaped protein belonging to the hemolysin coregulated protein (Hcp) family (Filloux, 2009; Mougous et al., 2006). Hcp proteins are required for assembly of the secretion apparatus, and they interact with valine-glycine repeat (Vgr) family proteins, which are also exported by the T6SS. The function of the Hcp/Vgr complex remains unclear; however, it is believed that the proteins are extracellular structural components of the secretion apparatus. Recent X-ray crystallographic insights into Hcp and Vgr family proteins show that they are similar to bacteriophage tube and tailspike proteins, respectively (Leiman et al., 2009; Pell et al., 2009). These findings prompted speculation that the T6SS is evolutionarily, structurally, and mechanistically related to bacteriophage. According to this model, the T6SS assembles as an inverted phage tail on the surface of the bacterium, with the Hcp/Vgr complex forming the distal end of the cell-puncturing device. Another notable conserved T6S gene product is ClpV, a AAA+ family ATPase that has been postulated to provide the energy necessary to drive the secretory apparatus (Mougous et al., 2006). The roles of the remaining conserved T6S proteins remain largely unknown.

Nonconserved genes encoding predicted accessory elements are also linked to most T6SSs (Bingle et al., 2008). In the HSI-I-encoded T6SS of Pseudomonas aeruginosa (H1-T6SS) (Figure 1A), these genes encode elements of a posttranslational regulatory pathway that strictly modulates the activity of the secretion system through changes in the phosphorylation state of a forhead-associated domain protein, Fha1 (Mougous et al., 2007). Phosphorylation of Fha1 by a transmembrane serine-threonine Hanks-type kinase, PpkA, triggers Hcp1 secretion. PpPA, a PP2C-type phosphatase, antagonizes Fha1 phosphorylation.

The T6SS has been linked to a myriad of processes, including biofilm formation (Aschtgen et al., 2008; Enos-Berlage et al., 2005), conjugation (Das et al., 2002), quorum-sensing regulation (Webber et al., 2009), and both promoting and limiting virulence (Filloux, 2009). The P. aeruginosa H1-T6SS has been implicated in the fitness of the bacterium in a chronic infection; mutants in
How the apparently conserved T6SS architecture can participate in such a wide range of activities is not clear. At least one mechanism by which the secretion system can exert its effects on a host cell has been garnered from studies of *Vibrio cholerae*. A T6S-associated VgrG family protein of this organism contains a domain with actin-crosslinking activity that is translocated into host cell cytoplasm in a process requiring endocytosis and cell-cell contact (Ma et al., 2008; Pukatzki et al., 2007; Satchell, 2009). The subset of VgrG family proteins that contain nonstructural domains with conceivable roles in pathogenesis have been termed “evolved” VgrG proteins (Pukatzki et al., 2007). This configuration, wherein an effector domain is presumably translocated into host cell cytoplasm by virtue of its fusion to the T6S cell puncturing apparatus, is intriguing, but it is likely not general; a multitude of organisms containing T6SSs do not encode “evolved” VgrG proteins (Boyer et al., 2009; Pukatzki et al., 2009).

Key to understanding the function of the T6SS—as with any secretion system—is the identification and characterization of the protein substrates that it exports. EvpP from *Edwardsiella tarda* and RbsB from *Rhizobium leguminosarum* are proposed substrates of the system; however, inconsistent with anticipated properties of T6S substrates, RbsB contains an N-terminal Sec secretion signal, and EvpP stably associates with a component of the secretion apparatus (Bladergroen et al., 2003; Pukatzki et al., 2009; Zheng and Leung, 2007).

In this study, we identified three proteins, termed Tse1–3 (type VI secretion exported 1–3), that are substrates of the H1-T6SS of *P. aeruginosa*. We showed that one of these, Tse2, is the toxin component of a toxin-immunity system, and that it is able to arrest the growth of a variety of prokaryotic and eukaryotic organisms. Despite the promiscuity of toxin expressed intracellularly, we found that H1-T6SS-exported Tse2 was specifically targeted to bacteria. In growth competition experiments, immunity to Tse2 provided a marked growth advantage in a manner dependent on intimate cell-cell contact and a functional H1-T6SS. The ability of the secretion system to efficiently target Tse2 to a bacterium, and not to a eukaryotic cell, suggests that T6S may play a role in the delivery of toxin and effector molecules between bacteria.

**RESULTS**

**Design and Characterization of H1-T6SS**

**On- and Off-State Strains**

Under laboratory culturing conditions, activation of the H1–T6SS is strongly repressed at the posttranslational level by the phosphatase PppA (Figure 1A). We have shown that inactivation of *pppA* leads to Hcp1 export and that this could reflect triggering of the “on state” in the secretory apparatus (Hsu et al., 2009; Mougous et al., 2007). These observations led us to predict that additional components of the apparatus, and even substrates of the secretion system, are also exported in this state. To identify these proteins, we sought to compare the secretomes of Δ*pppA* and Δ*clpV1*. The latter lacks the H1-T6SS ATPase, ClpV1, and therefore remains in the “off state” (Figure 1A) (Mougous et al., 2006).

To probe whether the on-state and off-state mutations could modulate the activity of the H1-T6SS, we assayed their effect on Hcp1 secretion in *P. aeruginosa* PAO1 *hcp1–V* (where **Figure 1. Overview and Results of an MS-Based Screen to Identify H1-T6SS Substrates**

(A) Gene organization of *P. aeruginosa* HSI-I. Genes manipulated in this work are shown in color.

(B) Activity of the H1-T6SS can be modulated by deletions of *pppA* and *clpV1*. Western blot analysis of Hcp1–V in the cell-associated (Cell) and concentrated supernatant (Sup) protein fractions from *P. aeruginosa* strains of specified genetic backgrounds. The genetic background for the parental strain is indicated below the blot. An antibody directed against RNA polymerase α (α-RNAP) is used as a loading control in this and subsequent blots.

(C) Deletion of *pppA* causes increased p-Fha1–V levels. p-Fha1–V is observed by western blot as one or more species with retarded electrophoretic mobility.

(D) Spectral count ratio of C1 proteins detected in R1 and R2 of the comparative semiquantitative secretome analysis of Δ*pppA* and Δ*clpV1*. The position of Hcp1 in both replicates is indicated. Proteins within the dashed line have SC ratios of <2-fold and constitute 89% of C1 proteins.
We divided the proteins into three groups: category 1 (C1; H1-T6SS substrate, current study), category 2 (C2), and category 3 (C3; H1-T6SS substrate, current study). We measured the distribution of SC ratios (on state/position) within C1 proteins (Figure 1D). Since we did not anticipate that the H1-T6SS should exhibit a global effect on the secretome, we were encouraged by the approximate split (50% ± 2% in both replicates) between those proteins that were up- versus downregulated between the on and off states. Additionally, the change in average SCs between the states was low, and this value was similar in the replicates ([R1], 1.13 ± 1.04; [R2], 1.15 ± 0.90). Only 30 R1 and 33 R2 proteins yielded an SC ratio >2.

As expected, Hcp1 was overrepresented in the on-state samples. Indeed, Hcp1 was the most differentially secreted protein in both data sets (SC ratio: [R1], 13; [R2], 17) (Figure 1D). The presence of Hcp1 in the secretome of off-state cells suggests a certain extent of cellular protein contamination within the preparations. This contamination is also evidenced by the predicted or known functions of many of the detected proteins (Tables S2–S4). The high abundance of Hcp1 (119 SC average) relative to the average protein abundance (10.9 SC) is likely another factor contributing to its detection in the off-state samples.

Next we analyzed C2 proteins—those observed only in the on state. Similar numbers of these proteins were identified in R1 (19) and R2 (20), and five of these were found in both replicates (Table 1). The reproducibility of C2 versus C1 proteins is attributable to the difference in their average SCs; the average SC of C2 proteins was 2.6, versus 12 in C1. The C2 proteins identified in both R1 and R2 accounted for five of the six most abundant in C2–R1, and five of the ten most abundant in C2–R2. Each of these proteins lacked a secretion signal for known export pathways. The salinity of these proteins and the biochemical validation of their secretion is the subject of subsequent sections.

The number and abundance of C3 proteins in both R1 and R2 were slightly lower than the corresponding C2 values. Nonetheless, we did identify three C3 proteins in common between R1 and R2 (Table 1). The occurrence of these proteins in the off state

### Table 1. Select Parameters of Category 2 and 3 Proteins Detected in Both Replicates of MS Secretome Analyses

<table>
<thead>
<tr>
<th>Name</th>
<th>Locus Tag</th>
<th>R1</th>
<th>R2</th>
<th>ΔrES/WTC</th>
<th>Unique Peptides Detected</th>
<th>Descriptiona (COG Assignment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vgr4</td>
<td>PA2685</td>
<td>18</td>
<td>20</td>
<td>4.3 (0.002)</td>
<td>62</td>
<td>H1-T6SS apparatus component, current study (COG3501)</td>
</tr>
<tr>
<td>VgrG1</td>
<td>PA0091</td>
<td>6.9</td>
<td>3.9</td>
<td>30 (0.002)</td>
<td>18</td>
<td>H1-T6SS apparatus component, current study (COG3501)</td>
</tr>
<tr>
<td>Tse3</td>
<td>PA3484</td>
<td>2.6</td>
<td>1.7</td>
<td>7.9 (0.004)</td>
<td>7</td>
<td>H1-T6SS substrate, current study</td>
</tr>
<tr>
<td>Tse1</td>
<td>PA1844</td>
<td>2.0</td>
<td>1.7</td>
<td>13 (0.001)</td>
<td>6</td>
<td>H1-T6SS substrate, current study</td>
</tr>
<tr>
<td>Tse2</td>
<td>PA2702</td>
<td>2.0</td>
<td>1.8</td>
<td>9.0 (&lt;0.001)</td>
<td>6</td>
<td>H1-T6SS substrate, current study</td>
</tr>
<tr>
<td>PA3422</td>
<td>1.1</td>
<td>1.8</td>
<td>–</td>
<td>4</td>
<td>Hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>PA1888</td>
<td>1.5</td>
<td>1.7</td>
<td>–</td>
<td>6</td>
<td>Hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>PA3836</td>
<td>1.0</td>
<td>1.6</td>
<td>–</td>
<td>3</td>
<td>ABC-type transport system (COG2984)</td>
<td></td>
</tr>
</tbody>
</table>

a Protein names were extracted from the Integrated Microbial Genomes (IMG) database (http://img.jgi.doe.gov/). If no protein identifier was available, the gene nomenclature was extrapolated. Proteins with nomenclature put forth in this study (“Tse” and “Vgr”) are named accordingly.

b Ratio of gene expression measured by microarray analysis of P. aeruginosa PAK wild-type and ΔretS as determined by Goodman and colleagues (Goodman et al., 2004). The provided p value was determined by Goodman et al.

c Average of R1 and R2; numbers were based on Sequest search results (see the Supplemental Experimental Procedures).

d COG numbers and descriptions were extracted from the IMG database where available. A function is provided for proteins characterized in this study.

### Mass Spectrometric Analysis of On- and Off-State Secretomes

Next, we used MS and spectral counting to compare proteins present in the secretomes of the on- and off-state P. aeruginosa strains (Liu et al., 2004). Average spectral count (SC) values were used to identify whether each protein was differentially secreted between states. The results of our MS analyses are summarized in Table S1, available online. Importantly, the total number of spectral counts was comparable between the on- and off-states in both replicates. A total of 371 proteins that met our filtering criteria were identified between replicate experiments (Table S2). We divided the proteins into three groups: category 1 (C1; Tables S3 and S4), present in both the on- and off-states; category 2 (C2; Table S5), present only in the on state; and category 3 (C3; Table S6), present only in the off state. Overlap between the replicates was greatest among C1 proteins. A total of 314 C1 proteins were identified, of which 249 were shared between the replicates. A significant fraction of the C1 differences can be ascribed to the fact that 13% more proteins were identified in this category in replicate 1 (R1) than in replicate 2 (R2).

To assess the accuracy of the quantitative component of our data sets, we measured the distribution of SC ratios (on state/off state) within C1 proteins (Figure 1D). Since we did not anticipate that the H1-T6SS should exhibit a global effect on the secretome, we were encouraged by the approximate split (50% ± 2% in both replicates) between those proteins that were up- versus downregulated between the on and off states. Additionally, the change in average SCs between the states was low, and this value was similar in the replicates ([R1], 1.13 ± 1.04; [R2], 1.15 ± 0.90). Only 30 R1 and 33 R2 proteins yielded an SC ratio >2.

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Two VgrG Proteins Are Secreted by the H1-T6SS

Two VgrG family proteins, the products of open reading frames PA0091 and PA2685, were the most abundant C2 proteins in R1 and R2 (Table 1). Interestingly, earlier microarray work has shown that PA0091 and PA2685 are coordinately regulated with HSI-I by the RetS hybrid two-component sensor/response regulator protein; however, the participation of these proteins in the H1-T6SS was not investigated (Figure 2A) (Goodman et al., 2004; Laskowski and Kazmierczak, 2006; Zolfaghari et al., 2005). The PA0091 locus is located within HSI-I, while the PA2685 locus is found at an unrelated site that lacks other apparent T6S elements (Figures 1A and 2A). To remain consistent with previous nomenclature, these genes will henceforth be referred to as vgrG1 and vgrG4, respectively (Mougous et al., 2006).

To confirm the MS results, we compared the localization of VgrG1 and VgrG4 in wild-type bacteria to strains containing the on-state (ΔpppA) and off-state (ΔclpV1) mutations. Consistent with our MS findings, western blot analyses of cell and supernatant fractions in vgrG1–V and vgrG4–V backgrounds indicated that secretion of the proteins is strongly repressed by pppA and requires clpV1 (Figure 2B and 2C). These data show that the H1-T6SS exports at least two VgrG family proteins. For reasons not yet understood, VgrG4–V migrated as two major bands in the cellular fraction and a large number of high molecular weight bands in the supernatant.

Identification of Three H1-T6SS Substrates

The remaining C2 proteins identified in both R1 and R2 are proteins encoded by ORFs PA1844, PA2702, and PA3484. Interestingly, an earlier study identified the product of PA1844 as an immunogenic protein expressed by a P. aeruginosa clinical isolate (Wehmhoner et al., 2003). Bioinformatic analyses of the three proteins indicated that they do not share detectable sequence homology to each other or to proteins outside of P. aeruginosa. Each protein is encoded by an ORF that resides in a predicted two-gene operon with a second hypothetical ORF. Intriguingly, we noted that the three unlinked operons—like HSI-I (which includes vgrG1) and vgrG4—are negatively regulated by RetS (Figure 2A).

Based on our secretome analyses, we hypothesized that the proteins encoded by PA1844, PA2702, and PA3484, henceforth referred to as Tse1–3, respectively, are substrates of the H1-T6SS. To test this, we analyzed the localization of the proteins when ectopically expressed in a diagnostic panel of P. aeruginosa strains. The secretion profile of each protein was similar in these strains; relative to the wild-type, ΔpppA displayed dramatically increased levels of secretion, and secretion levels were at or below wild-type levels in ΔpppA strains containing additional deletions in either hcp1 or clpV1 (Figure 3A). Overexpression of the proteins was ruled out as a confounding factor, as the secretion profile of chromosomally encoded Tse1–V in related backgrounds was similar to that of the ectopically expressed protein (Figure 3B). Finally, we complemented Tse1–V secretion in ΔpppA ΔclpV1 tse1–V with a plasmid expressing clpV1.

To further distinguish the Tse proteins as H1-T6SS substrates rather than structural components, we determined their influence on core functions of the T6 secretion apparatus. Fundamental to each studied T6SS is the ability to secrete an Hcp-related protein. In a systematic analysis, Hcp secretion was shown to require all predicted core T6SS components, including VgrG family proteins (Pukatzki et al., 2007; Zheng and Leung, 2007). We generated a strain containing a deletion of all tse genes in the ΔpppA hcp1–V background and compared Hcp1 secretion in this strain to strains lacking both vgrG1 and vgrG4 or clpV1 in the same background. Western blot analysis revealed that Hcp1 secretion was abolished in both the ΔclpV1 and ΔvgrG1 ΔvgrG4 strains; however, it was unaffected by tse1–3 deletion (Figure 3C).

A multiprotein complex containing ClpV1 is essential for a functional T6S apparatus (Hsu et al., 2009). As a second indicator of H1-T6SS function, we used fluorescence microscopy...
to examine the formation of this complex in strains containing a chromosomal fusion of \textit{clpV1} to a sequence encoding the green fluorescent protein (\textit{clpV1–GFP}) (Mougous et al., 2006). In line with the Hcp1 secretion result, the punctate appearance of ClpV1–GFP localization, which is indicative of proper apparatus assembly, was not dependent on the \textit{tse} genes (Figure 3D). On the other hand, deletion of \textit{ppkA}, a gene required for assembly of the H1-T6S apparatus, disrupted ClpV1–GFP localization. Together, these findings provide evidence that the Tse proteins are substrates of the H1-T6SS.

**Tse Secretion Is Triggered by Derepression of the Gac/Rsm Pathway**

Earlier microarray experiments suggested that the \textit{tse} genes are tightly repressed by RetS, a component of the Gac/Rsm signaling pathway (Lapouge et al., 2008). In this pathway, the activity of RetS and two other sensor kinase enzymes, LadS and GacS, converge to reciprocally regulate an overlapping group of acute and chronic virulence pathways in \textit{P. aeruginosa} through the small RNA-binding protein RsmA (Brencic and Lory, 2009; Goodman et al., 2004; Ventre et al., 2006). To directly investigate the effect of the Gac/Rsm pathway on tse expression, we monitored the abundance of Tse proteins in the cell-associated and secreted fractions of strains containing the \textit{retS} deletion. Our data showed that activation of the Gac/Rsm pathway dramatically elevates cellular Tse levels and triggers their export via the H1-T6SS (Figure 3E). It is noteworthy that secretion of Tse proteins in \textit{ΔretS} is far in excess of that observed in \textit{ΔpppA} (Figure 3E, compare \textit{ΔpppA} and \textit{ΔretS}).

**Tsi2 Is an Essential Protein that Protects \textit{P. aeruginosa} from Tse2**

The lack of transposon insertions within the \textit{tse2/tsi2} locus in a published transposon insertion library of \textit{P. aeruginosa} PA01 suggested that these ORFs may be essential for viability of the organism (Jacobs et al., 2003). To test this possibility, we attempted to generate deletions of \textit{tse2} and \textit{tsi2}. While a \textit{Δtse2} strain was readily constructed, \textit{tsi2} was refractory to several methods of deletion. Based on genetic context and coregulation (Figure 2A), we predicted that Tse2 and Tsi2 could interact functionally and that the requirement for \textit{tsi2} could therefore depend on \textit{tse2}. Success in simultaneous deletion of both genes confirmed this hypothesis (Figure 4A).

Our findings implied that Tsi2 protects cells from Tse2. To probe this possibility further, we introduced \textit{tse2} to the \textit{Δtse2 Δtsi2} background. Induction of \textit{tse2} expression completely abrogated growth of \textit{Δtse2 Δtsi2}; however, it had only a mild effect on wild-type cells (Figure 4A). These data demonstrate that tse2 encodes a toxic protein capable of inhibiting the growth of \textit{P. aeruginosa}, and that tsi2 encodes a cognate immunity genetic backgrounds measured by fluorescence microscopy. TMA-DPH is a lipophilic dye used to visualize the position of cells.

(E) The production and secretion of Tse proteins is dramatically increased in \textit{ΔretS}. Western blot analysis of Tse levels from strains containing chromosomally encoded Tse-VSV-G epitope tag fusions prepared in the wild-type or \textit{ΔretS} backgrounds. Note that under conditions used to observe the high levels of Tse secretion in \textit{ΔretS}, secretion cannot be visualized in \textit{ΔpppA} as was demonstrated in (B).
Tse2 and Tsi2 Proteins Are a Toxin-Immunity Module

(A) Tse2 is toxic to P. aeruginosa in the absence of Tsi2. Growth of the indicated P. aeruginosa strains containing either the vector control (-) or vector containing tse2 (+) under noninducing (-IPTG) or inducing (+IPTG) conditions. (B) Tse2 and Tsi2 physically associate. Shown is western blot analysis of samples before (Pre) and after (Post) α-VSV-G immunoprecipitation from the indicated strain containing a plasmid expressing tsi2 (control) or tsi2-V. The glycogen synthase kinase (GSK) tag was used for detection of Tse2 (Garcia et al., 2006).

Figure 4. The Tse2 and Tsi2 Proteins Are a Toxin-Immunity Module

We named Tsi2 based on this property (type VI secretion immunity protein 2). Tsi2 could block the activity of Tse2 through a mechanism involving direct interaction of the proteins, or by an indirect mechanism wherein the proteins function antagonistically on a common pathway. To determine if Tse2 and Tsi2 physically interact, we conducted coimmunoprecipitation studies in P. aeruginosa. Tse2 was specifically identified in precipitate of Tsi2–V, indicative of a stable Tse2-Tsi2 complex (Figure 4B). These data provide additional support for a functional interaction between Tse2 and Tsi2, and they suggest that the mechanism of Tsi2 inhibition of Tse2 is likely to involve physical association of the proteins.

Intracellular Tse2 Is Toxic to a Broad Spectrum of Prokaryotic and Eukaryotic Cells

P. aeruginosa is widely dispersed in terrestrial and aquatic environments, and it is also an opportunistic pathogen with a diverse host range. As such, Tse2 exported from P. aeruginosa has the potential to interact with a range of organisms, including prokaryotes and eukaryotes. To investigate the organisms that Tse2 might target, we expressed tse2 in the cytoplasm of representative species from each domain. Two eukaryotic cells were chosen for our investigation, Saccharomyces cerevisiae and the HeLa human epithelial-derived cell line. Yeast were included primarily for diversity; however, these organisms also interact with P. aeruginosa in assorted environments and could therefore represent a target of the toxin (Wargo and Hogan, 2006). S. cerevisiae cells were transformed with a galactose-inducible expression plasmid for each tse gene or with an empty control plasmid (Mumberg et al., 1995). Relative to the other tse genes and the control, tse2 expression caused a dramatic decrease in observable colony-forming units following 48 hr of growth under inducing conditions (Figure 5A). To address the specificity of Tse2 effects on S. cerevisiae, we next tested whether Tsi2 could block Tse2-mediated toxicity. Coexpression of tsi2 with tse2 restored viability to levels similar to the control strain (Figure 5B). This result implies that the effects of Tse2 on S. cerevisiae are specific and that the toxin may act via a similar mechanism in bacteria and yeast. Our findings are consistent with an earlier screen for P. aeruginosa proteins toxic to yeast. Arnoldo et al. found Tse2 among nine P. aeruginosa proteins most toxic to S. cerevisiae within a library of 505 that included known virulence factors (Arnoldo et al., 2008).

The effects of Tse2 on a mammalian cell were probed using a reporter cotransfection assay in HeLa cells. Expression plasmids containing the tse genes were generated and mixed with a GFP reporter plasmid. Cotransfection of the reporter plasmid with tse1 and tse2 had no impact on GFP expression relative to the control; however, inclusion of the tse2 plasmid reduced GFP expression to background levels (Figures 5C and 5D). We also noted morphological differences between cells transfected with tse2 and control transfections, which was apparent in the fraction of rounded cells (Figure 5E). These were specific effects of Tse2, as the inclusion of a tsi2 expression plasmid restored GFP expression and lowered the fraction of rounded cells to that of the control. From these studies, we conclude that Tse2 has a deleterious effect on essential cellular processes in assorted eukaryotic cell types.

Next we asked whether Tse2 has activity in prokaryotes other than P. aeruginosa. We tested two organisms, Escherichia coli and Burkholderia thailandensis. Both organisms were transfected with plasmids engineered for inducible expression of either tse2 or, as a control, both tse2 and tsi2. In each case, tse2 expression strongly inhibited growth, and coexpression with tsi2 reversed this effect (Figures 5F and 5G). Taken together with the effects we observed in S. cerevisiae and HeLa cells, we conclude that Tse2 is a toxin that—when administered intracellularly—inhibits essential cellular processes in a broad spectrum of organisms.

P. aeruginosa Can Target Bacterial, but Not Eukaryotic, Cells with Tse2

Since tse2 expression experiments indicated that the toxin could act on eukaryotes (Figures 5A–5E), we asked whether P. aeruginosa could target these cells with the H1-T6SS. We measured cytotoxicity toward HeLa and J774 cells for a panel of
P. aeruginosa strains, including Tse2 hypersecreting (ΔretS) and nonsecreting (ΔretS ΔclpV1) backgrounds. Under all conditions analyzed, we were unable to observe Tse2-promoted cytotoxicity or a morphological impact on the cells as was observed in transfection experiments (Figure 6A and data not shown). Additionally, attempts to detect Tse2 or other Tse proteins in
mammalian cell cytoplasm yielded no evidence of translocation (data not shown). We also investigated Tse2-dependent effects on yeast cocultured with *P. aeruginosa*; again, no effect could be attributed to Tse2 (Figure S1). Based on our data, we concluded that *P. aeruginosa* is unlikely to utilize Tse2 as a toxin against eukaryotic cells. This is in line with results of earlier reports, which have shown that strains lacking *retS* are highly attenuated in acute virulence-related phenotypes, including macrophage and epithelial cell cytotoxicity (Goodman et al., 2004; Zolfaghar et al., 2005), and acute pneumonia and corneal infections in mice (Zolfaghar et al., 2006; Laskowski et al., 2004).

The influence of intracellular *tse2* expression on the growth of bacteria prompted us to next investigate whether its target could be another prokaryotic cell. To test this, we conducted a series of in vitro growth competition experiments with *P. aeruginosa* strains in the *ΔretS* background engineered with regard to their ability to produce, secrete, or resist Tse2. Competitions between these strains were conducted in liquid medium or following filtration onto a porous solid support. Neither production nor secretion of Tse2, nor immunity to the toxin, impacted the growth rates of competing strains in liquid medium (Figure 6B). On the contrary, a striking proliferative advantage dependent on *tse2*...
and tsi2 was observed when cells were grown on a solid support. In growth competition experiments between ΔretS and ΔretS Δtse2 Δtsi2, henceforth referred to as donor and recipient strains, respectively, donor cells were approximately 14-fold more abundant after 5 hr (Figure 6B). This was entirely Tse2 mediated, as a deletion of tse2 from the donor strain, or the addition of tsi2 to the recipient strain, abrogated the growth advantage. Inactivation of clpV1 within the donor strain confirmed that the Tse2-mediated growth advantage requires a functional H1-T6SS (Figure 6B). Importantly, the total proliferation of the donor remained constant in each experiment, indicating that Tse2 suppresses growth of the recipient strain.

In order to examine the extent to which Tse2 could facilitate a growth advantage, we conducted long-term competitions between strains with and without Tse2 immunity. The experiments were initiated with a donor-to-recipient cell ratio of approximately 10:1, raising the probability that each recipient cell will contact a donor cell. After 48 hr, the Tse2 donor strain displayed a remarkable 104-fold growth advantage relative to a recipient strain lacking immunity (Figure 6C). These data conclusively demonstrate that the P. aeruginosa H1-T6SS can target Tse2 to another bacterial cell. The differences observed between competitions conducted in liquid medium versus on a solid support suggest that intimate donor-recipient cell contact is required. We have not directly demonstrated that Tse2 is translocated into recipient cell cytoplasm; however, it is a likely explanation for our data, given that cell contact is required and Tsi2 is a cytoplasmic immunity protein that physically interacts with the toxin (Figure 4B).

**DISCUSSION**

The T6SS has been implicated in numerous, apparently disparate processes. With few exceptions, the mode of action of the secretion system in these processes is not known. Since the T6SS architecture appears highly conserved, we based our study on the supposition that the diverse activities of T6SSs, including T6SSs within a single organism, must be attributable to a diverse array of substrate proteins exported in a specific manner by each system. Our findings support this model; we identified three T6S substrates that lack orthologs outside of P. aeruginosa and that specifically require the H1-T6SS for their export (Figures 1 and 3).

Bacterial genomes encode a large and diverse array of toxin-immunity protein (TI) systems. These can be important for plasmid maintenance, stress response, programmed cell death, cell-fate commitment, and defense against other bacteria (Aoki et al., 2005; Gerdes et al., 2005). Tse2 differs from other TI toxins in that it is exported through a large, specialized secretion apparatus, while many TI system toxins are either not actively secreted, or they utilize the sec pathway (Riley and Wertz, 2002). This distinction implies that secretion through the T6S apparatus is required to target Tse2 to a relevant environment, cell, or subcellular compartment. Indeed, we have shown that targeting of Tse2 by the T6S apparatus is essential for its activity (Figure 6).

We found that Tse2 is active against assorted bacteria and eukaryotic cells when expressed intracellularly (Figures 4 and 5). Despite this, we found no evidence that P. aeruginosa can target Tse2 to a eukaryotic cell, including mammalian cells of epithelial and macrophage origin (Figure 6A and data not shown). Surprisingly, P. aeruginosa efficiently targeted the toxin to another bacterial cell (Figure 6). These findings, combined with the following recent observations, provide support for the hypothesis that the T6SS can serve as an interbacterial interaction pathway. First, the secretion system is present and conserved in many nonpathogenic, solitary bacteria (Bingle et al., 2008; Boyer et al., 2009). Second, there is experimental evidence supporting an evolutionary relationship between extracellular components of the secretion apparatus and the tail proteins of bacteriophages T4 and λ (Ballister et al., 2008; Leiman et al., 2009; Pell et al., 2009; Pukatzki et al., 2007). Finally, two recent reports have implicated the conserved T6S component, VgrG, in interbacterial interactions. A bioinformatic analysis of Salmonella genomes identified a group of “evolved” VgrG proteins bearing C-terminal effector domains highly related to bacteriophage T4-targeting S-type pyocins, and a VgrG protein from Proteus mirabilis was shown to participate in an intraspecies self/nonsense recognition pathway (Blondel et al., 2009; Gibbs et al., 2008).

It is also evident that in certain instances the T6SS has evolved to engage eukaryotic cells. In at least two reports, the T6S apparatus has been demonstrated to deliver a protein to a eukaryotic cell (Ma et al., 2009; Suarez et al., 2010). Moreover, the T6SSs of several pathogenic bacteria are major virulence factors (Bingle et al., 2008). Taken together with our findings, we posit that there are two broad groups of T6SSs, those that target bacteria and those that target eukaryotes. It is not possible at this time to rule out that a given T6SS may have dual specificity. However, our inability to detect the effects of Tse2 in an infection of a eukaryotic cell, and the fact that a Tse2 hypersecreting strain is attenuated in animal models of acute infection (Laskowski et al., 2004; Zolfaghar et al., 2006), suggests that the T6S apparatus can be highly discriminatory. In this regard, it is instructive to consider other secretion systems that have evolved from interbacterial interaction pathways. The type IVA and type IVB secretion systems are postulated to have evolved from a bacterial conjugation system (Burns, 2003; Christie et al., 2005; Lawley et al., 2003). These systems have become efficient at eukaryotic cell intoxication; however, measurements indicate that substrate translocation into bacteria occurs at a frequency of only ~1 × 10^-6/donor cell (Luo and Isberg, 2004). In contrast, Tse2 targeting to bacteria by the H1-T6SS appears many orders of magnitude more efficient, as the donor strain in our assays is able to effectively suppress the net growth of an equal amount of recipient cells. The host-adapted type IV secretion systems and the H1-T6SS represent two apparent extremes in the cellular targeting specificity of Gram-negative specialized secretion systems. Furthermore, they show that a high degree of discrimination can exist between pathways targeting eukaryotes and prokaryotes.

The physiologically relevant target bacteria of Tse2 and the H1-T6SS remains an open question. We have initiated studies to address the role of these factors in interspecies interactions; however, we have not yet identified an effect. This may be because diffusible antibacterial molecules released by P. aeruginosa dominate the outcome of growth competitions performed under the conditions used in Figure 6 (Hoffman et al., 2006; Kessler et al., 1993; Voggu et al., 2006). In future studies designed to allow free diffusion of these factors, and thereby more closely mimic a natural setting, their role may be mitigated.
All sequenced *P. aeruginosa* strains in the public databases encode clear orthologs of tse2 and tsi2. Additionally, we found the genes universally present within a library of 44 randomly selected CF patient clinical isolates (Figure S2). Despite these findings, it remains possible that Tse2-mediated inter-*P. aerugino-

osa* interactions could be relevant in a natural context. For instance, it may not be simply the presence or absence of the toxin or its immunity protein, but rather the extent and manner in which these traits are expressed that decides the outcome of an interaction. In prior investigations of clinical isolates, we noted a high degree of heterogeneity in H1-T6SS activation, as judged by Hcp1 secretion levels (Mougous et al., 2006, 2007). The wild-type strain used in the current study does not secrete Hcp1, and in this background the H1-T6SS does not provide a growth advantage against an immunity-deficient strain (data not shown). However, the H1-T6SS activation state of many clinical isolates resembles the ΔretS background, and therefore these strains are likely capable of using Tse2 in competition with other bacteria. In this context, it is intriguing that tse and HSI-I expression are subject to strict regulation by the Gac/Rsm pathway (Eng et al., 1994) and validated with Peptide/Protein Prophet (Keller et al., 2002). The relative abundance for identified proteins was calculated using spectral counting (Ji et al., 2004). See the Supplemental Experimental Procedures for additional MS procedures.

**Secretome Preparation**

Cells were grown to optical density 600 nm (OD600) 1.0 in Vogel-Bonner minimal medium containing 19 mM amino acids as defined in synthetic CF sputum medium (Palmer et al., 2007). The presence of amino acids was required for H1-T6SS activity (data not shown). Proteins were prepared as described previously (Wehmhoner et al., 2003).

**Mass Spectrometry**

Precipitated proteins were suspended in 100 μl of 6 M urea in 50 mM NH4HCO3, reduced and alkylated with dithiothreitol and iodoacetamide, respectively; and digested with trypsin (50:1 protein:trypsin ratio). The resultant peptides were desalted with Vydac C18 columns (The Nest Group) following the manufacturer’s protocol. Samples were dried to 5 μL, resuspended in 0.1% formic acid/5% acetonitrile, and analyzed on an LTQ-Orbitrap mass spectrometer (Thermo Fisher) in triplicate. Data were searched using Sequest (Eng et al., 1994) and validated with Peptide/Protein Prophet (Keller et al., 2002). The relative abundance for identified proteins was calculated using spectral counting (Ji et al., 2004). See the Supplemental Experimental Procedures for additional MS procedures.

**Preparation of Proteins and Western Blotting**

Cell-associated and supernatant samples were prepared as described previously (Hsu et al., 2009). Western blotting was performed as described previously (Mougous et al., 2006), with the exception that detection of the Tse proteins required primary antibody incubation in 5% BSA in Tris-buffered saline containing 0.05% v/v Tween 20 (TBST). The Gsk tag was detected using α-GSK (Cell Signaling Technologies).

**Immunoprecipitation**

Cells grown in appropriate additives were harvested at mid-log phase by centrifugation (6000 × g, 3 min) at 4°C and resuspended in 10 ml of buffer 1 (200 mM NaCl, 20 mM Tris [pH 7.5], 5% glycerol, 2 mM dithiothreitol, 0.1% triton) containing protease inhibitors (Sigma) and lysisyme (0.2 mg ml⁻¹). Cells were disrupted by sonication, and the resulting lysate was clarified by centrifugation (25,000 × g, 30 min) at 4°C. A sample of the supernatant material was removed (Pre), and the remainder was incubated with 100 μl of α-VSV-G agarose beads (Sigma) for 2 hr at 4°C. Beads were washed three times with 15 ml of buffer 1 and pelleted by centrifugation. Proteins were eluted with SDS-PAGE loading buffer.

**Fluorescence Microscopy**

Mid-log phase cultures were harvested by centrifugation (6000 × g, 3 min), washed with phosphate-buffered saline (PBS), and resuspended to OD600 5 with PBS containing 0.5 mM TMA-DPH (Molecular Probes). Microscopy was performed as described previously (Hsu et al., 2009). All images shown were manipulated identically.

**Yeast Toxicity Assays**

Saccharomyces cerevisiae BY4742 (MATa his3Δ1 leu2-3,112 lys2-801 ura3-50) was transformed with p426-GAL-L containing tse1, tse2, tse3, or the empty vector and grown o/n in SC – Ura + 2% glucose (Mumberg et al., 1995). Cultures were resuspended to OD600 1.0 with water and serially diluted 5-fold onto SC – Ura + 2% glucose agar or SC – Ura + 2% galactose + 2% raffinose agar. Plates were incubated at 30°C for 2 days before being photographed. The ts2 gene was cloned into p426-GAL-L and transformed into S. cerevisiae BY4742 harboring the p426-GAL-L plasmid. Cultures were grown o/n in SC – Ura – His + 2% glucose.

**Growth Competition Assays**

Overnight cultures were mixed at the appropriate donor-to-recipient ratio to a total density of approximately 1.0 × 10⁸ CFU/ml in 5 ml LB medium. In each experiment, either the donor or recipient strain contained lacZ inserted at the neutral phage attachment site (Vance et al., 2005). This gene had no effect on competition outcome. Cocultures were either filtered onto a 47 mm 0.2 μm nitrocellulose membrane (Nalgene) and placed onto LB agar or were

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**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**

The *P. aeruginosa* strains used in this study were derived from the sequenced strain PA01 (Stover et al., 2000). *P. aeruginosa* were grown on Luria-Bertani (LB) medium at 37°C supplemented with 30 μg ml⁻¹ gentamicin, 300 μg ml⁻¹ carbenicillin, and 25 μg ml⁻¹ iginas, 5% w/v sucrose, 0.5 mM IPTG, and 40 μg ml⁻¹ X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as required. Burkholderia thailandensis E264 and Escherichia coli BL21 were grown on LB medium containing 200 μg ml⁻¹ trimethoprim, 50 μg ml⁻¹ kanamycin, 0.2% w/v glucose, 0.2% w/v rhamnose, and 0.5 mM IPTG as required. E. coli SM10 used for conjugation with *P. aeruginosa* was grown in LB medium containing 15 μg ml⁻¹ gentamicin. Plasmids used for inducible expression include pPSV35, pPSV35CV, and pSW196 for *P. aeruginosa* (Baynham et al., 2006; Hsu et al., 2009; Rietsch et al., 2005), pET29b (Novagen) for E. coli, pSChraB2 (Cardona and Valvano, 2005) for B. thailandensis, and p426-GAL-L and p423-GAL-L for S. cerevisiae (Mumberg et al., 1995).

**Growth Competition Assays**

Overnight cultures were mixed at the appropriate donor-to-recipient ratio to a total density of approximately 1.0 × 10⁸ CFU/ml in 5 ml LB medium. In each experiment, either the donor or recipient strain contained lacZ inserted at the neutral phage attachment site (Vance et al., 2005). This gene had no effect on competition outcome. Cocultures were either filtered onto a 47 mm 0.2 μm nitrocellulose membrane (Nalgene) and placed onto LB agar or were...
inoculated 1:100 into 2 ml LB (containing 0.4% w/v L-arabinose, if required), and were incubated at 37°C with shaking. Filter-grown cells were resuspended in LB medium and plated on LB agar containing X-gal.

**Cell Culture and Infection Assays**

HeLa cells were cultured and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin or streptomycin as required. Incubations were performed at 37°C in the presence of 5% CO2. Infection assays were carried out using cells seeded in 96-well plates at a density of 2.0 × 10^5 cells/well. Following 0/n incubation, wells were washed in 1× Hank’s balanced salt solution and DMEM lacking thymidine pyruvate, and antibiotics were added. Bacterial inoculum was added to wells at a multiplicity of infection of 50 from cultures of OD600 1.0. Following incubation for 5 hr, the percent cytotoxicity was measured using the CytoTox-One assay (Promega).

**Transient Transfection, Cell-Rounding Assays, and Flow Cytometric Analysis**

HeLa cells were seeded in 24-well flat bottom plates at a density of 2.0 × 10^5 cells/well and incubated o/n in DMEM supplemented with 10% FBS. Reporter cotransfection experiments were performed using Lipofectamine according to the manufacturer’s protocol. Total amounts of transfected DNA were normalized using equal quantities of the GFP reporter plasmid (empty pEGFP-N1 [Clonetech]), one of the tss expression plasmids (pEGFP-N1-derived), and either a nonspecific plasmid or the tss2 expression plasmid where indicated. Cell rounding was quantified manually using phase-contrast images from three random fields acquired at 40× magnification. Prior to flow cytometry, HeLa cells were washed two times and resuspended in 1× PBS supplemented with 0.75% FBS. Analysis was performed on a BD FACSscan® cell analyzer, and mean GFP intensities were calculated using FlowJo 7.5 software (Tree Star, Inc.).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, two figures, and six tables and can be found with this article online at doi:10.1016/j.chom.2009.12.007.

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