Genomic and Functional Analysis of the Type VI Secretion System in Acinetobacter

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Abstract

The genus Acinetobacter is comprised of a diverse group of species, several of which have raised interest due to potential applications in bioremediation and agricultural purposes. In this work, we show that many species within the genus Acinetobacter possess the genetic requirements to assemble a functional type VI secretion system (T6SS). This secretion system is widespread among Gram negative bacteria, and can be used for toxicity against other bacteria and eukaryotic cells. The most studied species within this genus is A. baumannii, an emerging nosocomial pathogen that has become a significant threat to healthcare systems worldwide. The ability of A. baumannii to develop multidrug resistance has severely reduced treatment options, and strains resistant to most clinically useful antibiotics are frequently being isolated. Despite the widespread dissemination of A. baumannii, little is known about the virulence factors this bacterium utilizes to cause infection. We determined that the T6SS is conserved and syntenic among A. baumannii strains, although expression and secretion of the hallmark protein Hcp varies between strains, and is dependent on TssM, a known structural protein required for T6SS function. Unlike other bacteria, A. baumannii ATCC 17978 does not appear to use its T6SS to kill Escherichia coli or other Acinetobacter species. Deletion of tssM does not affect virulence in several infection models, including mice, and did not alter biofilm formation. These results suggest that the T6SS fulfills an important but as-yet-unidentified role in the various lifestyles of the Acinetobacter spp.

Introduction

The diversity of the Gram-negative Acinetobacter spp. is exemplified by the wide range of environments from which these bacteria can be isolated from. These environments include soils, [1], activated sludge [2], food [3], and colonized human carriers [4]. The traits of several species of this genus have been recognized as potentially having important implications to the field of biotechnology, including roles for degradation of hydrocarbons [5] and plant growth-promoting traits [6]. A. baumannii is recognized as one of the most clinically important species of Acinetobacter [7]; thus, much attention has been directed towards the ability of some members of this genus to cause severe infections. As a primarily nosocomial pathogen, A. baumannii causes a wide-range of infections in immunocompromised people, most often pneumonia and bloodstream infections [8], and, in contrast with most other Acinetobacter spp., it is rarely isolated outside of the hospital environment [7]. The treatment of A. baumannii infections has become increasingly difficult due to the widespread dissemination of multi- and pan-drug resistant strains [9]. Antibiotic resistance and epidemiology have been the focus of much of the scientific work on A. baumannii, but little is known about the strategies this bacterium uses for pathogenesis. Potential virulence mechanisms employed by A. baumannii are, however, beginning to be uncovered [10,11,12]. Well characterized iron-and zinc-acquisition systems are involved in A. baumannii persistence within the host [13,14,15], and capsule has been shown to be essential for resistance to serum killing and for survival in a rat model of infection [16]. A. baumannii phospholipases have also been implicated in interactions with epithelial cells and serum resistance [17,18]. Furthermore, the propensity of A. baumannii to resist desiccation and form biofilms may contribute to endemic disease within a healthcare setting [19,20,21,22]. An outer membrane protein A (OmpA) has been proposed to mediate interactions with epithelial cells and induce dendritic cell death [23,24,25]. It was recently shown that a conserved protein glycosylation system in A. baumannii is critical for full virulence in several infection models, as well as for biofilm formation [26].
Results

The T6SS is Operational in Several Species within the Acinetobacter Genus

Bioinformatic analysis of the genomes from several sequenced species of *Acinetobacter* revealed the presence of genes resembling a typical T6SS gene cluster (Figure 1) [36,37]. These putative T6SS loci contain homologs of 12 core T6SS genes (Figure 1 and Table 1); herein, T6SS genes are referred to by their generic names or by the proposed *ts* nomenclature of Shalom et al [47]. The gene clusters encode the hallmarks *hcp*, *clpV*, and *tssM* as well as accessory components and genes with unknown function. Varying numbers of genes located outside the clusters encode putative VgrG proteins, which are often secreted via the T6SS [49]. Many of the identified VgrG sequences are greater than 750 amino acids in length, indicating they may contain evolved effector domains in their C-termini; however, apart from N-terminal homology to bacteriophage components gp4 and gp5 that is typical of VgrG proteins [33], we were unable to identify conserved protein domains that could be indicative of possible functions.

All sequenced *A. baumannii* strains appear to have the core T6SS genes in a syntenic organization. *A. calcoaceticus* RUH2202, *A. oleivorans* DR1, *A. baylyi* ADP1, *A. johnsonii* SH046, *A. radiodurans* SH164, and *A. lwoffii* WJJ10621 were all found to possess the same 12 core genes present in *A. baumannii*; however, as shown in Figure 1, the organization differed slightly in some strains, with an opposite orientation of the final four genes in the cluster observed for *A. baylyi*, *A. johnsonii*, *A. radiodurans*, and *A. lwoffii*. The conserved T6SS proteins encoded by these clusters generally show high sequence identity (70% or greater) with *A. baumannii*, although the sequences of the VgrG proteins are slightly more divergent (60% or greater sequence identity) (Table S1). Each cluster, however, lacks an obvious homolog of *tsf*, an outer membrane anchored lipoprotein [50]. Interestingly, as shown in Table S1, several *Acinetobacter* species are not predicted to encode a functional T6SS (*A. fittii*, *A. nasocomialis*, *A.baumuyllicus*, and *A. junii*) due to the absence of several conserved proteins, yet still encode *vgrG* genes and in some cases *tssM* or *tsl* homologs.

We next wanted to determine whether the T6SSs encoded in these loci were active under laboratory conditions. The presence of Hcp in culture supernatants is used as a reliable indicator of an active T6SS [49]; therefore, we developed a polyclonal antibody raised against a purified, recombinant Hcp protein from *A. baumannii* ATCC 17978. Hcp expression and secretion was analyzed in several strains of *A. baumannii* and non-*baumannii* species. The *A. baumannii* strains studied included four well-characterized and sequenced strains (17978, 19606, SDF and AYE; Table S2) and three uncharacterized clinical isolates of *A. baumannii* (strains 1375, 1224, and 1225; Table S2). Although protein levels varied, Hcp was detected in the whole cell samples of all strains (Figure 2A). Interestingly, supernatants showed a greater variation; Hcp secretion was more pronounced in strains SDF, 19606, and 1224, compared to 17978. Strains AYE and 1375 did not show detectable levels of secreted Hcp under the conditions tested. Strain 1225 showed minimal Hcp secretion; however, all supernatant samples prepared from this strain had detectable levels of the cytoplasmic control protein RNA polymerase zeta-subunit, indicating lysis may account for the small amount of Hcp protein detected. In agreement with available genome sequence data, our results indicate that the T6SS, and Hcp expression, is conserved among *A. baumannii* strains; however, the secretion of Hcp protein varied among isolates. Furthermore, our results agree with previous reports showing widespread T6SS distribution and activity in several species from the genus *Acinetobacter*, and, in particular, *A. baumannii*. We report that under standard laboratory conditions, *A. baumannii* ATCC 17978 encodes a constitutively active T6SS that secretes the conserved component Hcp via a T6SS-dependent mechanism.
Table 1. Identification of conserved T6SS components in selected Acinetobacter spp.

| ts designation | Gene name/ COG id | A. baumannii ATCC 17978 | A. baumannii ATCC 19066 | A. baumannii SDF | A. baumannii AYE | A. calcoaceticus RUH2202 | A. radioreistant ABWJ10621 | A. lwoffii SHO146 | A. johnsonii DR1 | A. eoleivorans VCA0107 | V. cholerae V52 | P. aeruginosa PAO1 (HsI-I) | B. pseudomallei K96243 (T6SS-1) |
|----------------|------------------|--------------------------|-------------------------|-----------------|-----------------|--------------------------|--------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| tssA           | vpaA/3516        | A1S_1293, A1S_1294       | A1S_1301                | A1S_1287, A1S_1288 | A1S_1305       | A1S_1308                  | A1S_1309                  | A1S_1286, A1S_1289 | A1S_1308 | A1S_1301       | A1S_1302       | A1S_1303       | A1S_1304       | A1S_1305       |
| tssB           | hmp0021          | SH046                    | SH164                   | SH046           | SH046          | SH046                    | SH046                   | SH046           | SH046          | SH046          | SH046          | SH046          | SH046          |
| tssC           | hmp0021          | A1S_129, A1S_1299        | A1S_1301                | A1S_1287, A1S_1288 | A1S_1305       | A1S_1308                  | A1S_1309                  | A1S_1286, A1S_1289 | A1S_1308 | A1S_1301       | A1S_1302       | A1S_1303       | A1S_1304       | A1S_1305       |
| tssD           | hcp/3157         | A1S_129, A1S_1299        | A1S_1301                | A1S_1287, A1S_1288 | A1S_1305       | A1S_1308                  | A1S_1309                  | A1S_1286, A1S_1289 | A1S_1308 | A1S_1301       | A1S_1302       | A1S_1303       | A1S_1304       | A1S_1305       |
| tssE           | hmp0021          | A1S_129, A1S_1299        | A1S_1301                | A1S_1287, A1S_1288 | A1S_1305       | A1S_1308                  | A1S_1309                  | A1S_1286, A1S_1289 | A1S_1308 | A1S_1301       | A1S_1302       | A1S_1303       | A1S_1304       | A1S_1305       |
| tssF           | hmp0021          | A1S_129, A1S_1299        | A1S_1301                | A1S_1287, A1S_1288 | A1S_1305       | A1S_1308                  | A1S_1309                  | A1S_1286, A1S_1289 | A1S_1308 | A1S_1301       | A1S_1302       | A1S_1303       | A1S_1304       | A1S_1305       |
| tssG           | hmp0021          | A1S_129, A1S_1299        | A1S_1301                | A1S_1287, A1S_1288 | A1S_1305       | A1S_1308                  | A1S_1309                  | A1S_1286, A1S_1289 | A1S_1308 | A1S_1301       | A1S_1302       | A1S_1303       | A1S_1304       | A1S_1305       |
| tssH           | hmp0021          | A1S_129, A1S_1299        | A1S_1301                | A1S_1287, A1S_1288 | A1S_1305       | A1S_1308                  | A1S_1309                  | A1S_1286, A1S_1289 | A1S_1308 | A1S_1301       | A1S_1302       | A1S_1303       | A1S_1304       | A1S_1305       |
| tssI           | hmp0021          | A1S_129, A1S_1299        | A1S_1301                | A1S_1287, A1S_1288 | A1S_1305       | A1S_1308                  | A1S_1309                  | A1S_1286, A1S_1289 | A1S_1308 | A1S_1301       | A1S_1302       | A1S_1303       | A1S_1304       | A1S_1305       |

Locus tag identifiers are shown for the conserved ts components of several T6SS-containing Acinetobacters, as well as their homologs in V. cholerae, P. aeruginosa, and B. pseudomallei.

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with a recent report in which Hcp was found in culture supernatants from strain 19606 [59].

The non-
baumannii strains investigated were clinical isolates of A. calcoaceticus (strains A and B), A. pittii (strains A and B), and A. junii. The sequenced strain of the non-pathogenic soil isolate A. baylyi ADP1 [60,61] was also included (Table S2). Both A. calcoaceticus strains and A. baylyi ADP1 showed robust Hcp expression and secretion (Figure 2B), correlating with the presence of predicted T6SS genes in their respective genomes. The A. pittii and A. junii strains, which are not predicted to encode T6SSs (Table S1) and do not contain a Hcp homolog, did not react against the anti-Hcp antibody. Thus, while the T6SS is not universally conserved among Acinetobacter species, all tested strains with a predicted T6SS express and/or secrete Hcp.

To help visualize the differences in Hcp secretion we developed an ELISA assay to detect Hcp in supernatants. The T6SS-positive strains identified in Figure 2 were cultured in 96-well plates and supernatants were collected. These supernatants were incubated in 96-well ELISA plate overnight and the secreted Hcp was detected using an anti-Hcp antibody by an indirect ELISA approach (as described in Materials and Methods). The results of a typical assay are shown in Figure S1. Due to differences in growth of different strains observed in this assay, it is not possible to directly compare secretion rates. However, this assay clearly separates the strains into “high secretors” and “low secretors” (Figure 2C and Figure S2A). The high secretor strains generally reach a lower final optical density (Figure S2B) and therefore the high levels of Hcp in supernatants can be attributed to higher rates of Hcp secretion and not a larger number of cells. The results from this ELISA are in agreement with the data obtained via Western blots.

A. baumannii ATCC 17978 Secretes Hcp in a T6SS-dependent Manner

While Hcp was detected in the supernatants of several species of Acinetobacter, we wanted to determine whether this was a process dependent on other genes within the cluster. Due to the importance of A. baumannii as a nosocomial pathogen, and because our lab has previously employed 17978 in molecular studies of pathogenesis [26], we chose to use this strain as our model organism. We generated a hcp mutant (17978 Δhcp) by allelic exchange with a gentamicin resistance cassette and probed whole cells and culture supernatants with the anti-Hcp antibody. Hcp was detected by Western blot in the whole cell extract and cell-free supernatant of the wild type strain (Figure 3A), but the band

Figure 1. Genetic organization of T6SS loci. Selected genomes of sequenced Acinetobacter strains were probed for the presence of T6SS colored and identified below the figure. Gene accession numbers are provided in Table 1.
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corresponding to Hcp was absent from pellet and supernatant fractions from the 17978 Δhcp strain. Constitutive expression of Hcp from a plasmid restored Hcp expression and secretion in the mutant strain. As before, cytoplasmic RNA polymerase was used as a lysis and loading control, and was only seen in whole cell fractions, indicating that the presence of Hcp in culture supernatants was not due to cell lysis, and instead is actively exported by the bacterium.

To determine if Hcp secretion by 17978 is dependent on a functional T6SS, we generated an unmarked tssM deletion strain (17978 ΔtssM). TssM, a structural component of the T6SS, has been shown to be required for T6SS activity, and is therefore required for Hcp secretion [31,32]. In agreement with these previous results, whole cell samples from the 17978 ΔtssM strain contained Hcp, but its secretion was completely abrogated (Figure 3B). Expression of TssM from a plasmid complemented secretion of Hcp to the supernatant, indicating the lack of Hcp secretion was due to mutation of tssM. Taken together, these results suggest that the T6SS of 17978 is functional, and that its ability to secrete the conserved component Hcp is dependent upon at least one other gene in the cluster.

The tssM Mutant of A. baumannii ATCC 17978 is not Attenuated for Virulence Against Amoebae, Waxworms, or Mice

Dictyostelium discoideum amoebae have been widely used as a host model to study bacterial virulence factors [62], and was used as a
model system for assessing T6SS-mediated virulence in *V. cholerae* [31]. An active T6SS of *B. cenocepacia* has also been found to be important for mediating resistance to *D. discoideum* and for macrophage actin rearrangements [63]. When mixed and plated on agar containing ethanol, *A. baumannii* ATCC 17978 has been shown to kill *D. discoideum* and prevent plaque formation, the indicator of amoeboid feeding on the bacteria [30], and has been used to identify *A. baumannii* virulence factors [26]. When we co-plated *D. discoideum* with 17978 or 17978 ΔtssM on SM/5 agar, no plaques were observed in the bacterial lawns, indicating the T6SS mutant retained a virulent phenotype towards the amoebae (data not shown).

*Galleria mellonella* waxworms have also been used as non-mammalian eukaryotic models for assessing virulence defects of *A. baumannii* [14,26,64]. Injection of *A. baumannii* bacteria into the insect results in a dose-dependent killing, with the inoculum required for efficient killing varying between *A. baumannii* strains and species. We injected *G. mellonella* wax moth larvae with

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**Figure 3.** *A. baumannii* ATCC 17978 requires the conserved TssM protein for T6SS activity. A) Whole cell and supernatant samples prepared from cultures of wild type 17978, the T6SS mutant 17978 Δhcp, and its complemented (pHcp) or vector control (pWH1266) derivatives, were separated by SDS-PAGE and probed by Western blot with anti-Hcp (upper panel) or an anti-RNA polymerase (RNAP; lower panel) antibodies. B) Western blot of whole cell and supernatant samples prepared from cultures of wild type 17978, the T6SS mutant 17978 ΔtssM, and its complemented (pTssM) or vector control (pWH1266) derivatives probed for Hcp (upper panel) and RNAP (lower panel).

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approximately $10^6$ and $10^7$ CFUs of wild type 17978 and 17978
\(\Delta_{tsm}\) (Figure 4A). As previously reported, we observed a dose-
dependent killing of \textit{G. mellonella} by \textit{A. baumannii}, however the \(tsm\)
mutant retained virulence levels comparable to wild type bacteria.

We next assessed whether the T6SS of 17978 played a role in a
murine pneumonia model. This model has proven useful to
discriminate between wild type and attenuated \textit{A. baumannii}
strains [15,17,65]. Mice were intranasally infected with wild type 17978
or \(\Delta_{tsm}\) bacteria. After 36 hours, the bacterial burden in the lungs
and liver of infected animals was quantified, which revealed no
significant difference in colonization between the two strains
(Figure 4B). Taken together, these results suggest that the T6SS of
\textit{A. baumannii} ATCC 17978 does not play a role in virulence against
eukaryotic systems.

\textit{A. baumannii} ATCC 17978 Appears not to Kill Other
Bacteria via the T6SS, nor Employs this System for Biofilm
Formation

Recently, the T6SS of several bacterial pathogens has been
shown to mediate killing of other bacteria [39,40,41,45]. To
determine if \textit{A. baumannii} also exhibits T6SS-mediated antibacterial
activity, we initially used a rifampicin resistant derivative of \textit{E. coli}
strain MG1655, a strain susceptible to killing by \textit{V. cholerae} [39], as
a target in bacterial killing assays. Co-incubation of wild type
17978 or 17978 \(\Delta_{tsm}\) with \textit{E. coli} MG1655 showed no differences
in killing of \textit{E. coli}, while a drastic reduction in viable \textit{E. coli}
was seen when confronted with \textit{V. cholerae} (Figure 5). Of note, 17978
seemed to slightly reduce \textit{E. coli} growth in a T6SS-independent
fashion as compared to the avirulent \textit{V. cholerae} strain.

The conditions employed in this assay were optimized for \textit{V.
cholerae}. It is possible that \textit{A. baumannii} is prey-specific, or that

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{The T6SS is not required for virulence towards \textit{G. mellonella} or in a mouse model of pneumonia. A) Groups of 10 larvae were injected with approximately $10^6$ or $10^7$ CFU of wild type 17978 or the \(tsm\) mutant, incubated at 37°C, and monitored for survival. No significant difference (p>0.05) in log-rank test. B) Bacterial burden of lung and liver tissue from mice infected intranasally with either wild type 17978 or \(\Delta_{tsm}\) 36h post infection. No significant difference (p>0.05; two-tailed, unpaired Student’s \(t\) test) in bacterial burden of the two strains was observed in either tissue. doi:10.1371/journal.pone.0055142.g004}
\end{figure}
Comparison post-test).

![Figure 5. The T6SS of 17978 is not used for killing of E. coli MG1655. Survival of E. coli was determined by plate counts after exposure to wild type 17978, 17978 with vector control (17978/pWH1266), the 17978 ΔtsSM T6SS mutant, and its complemented (pTssM) and vector control (pWH1266) derivatives. Wild type V. cholerae (V52), and the isogenic tsSM mutant derivative (V52 ΔtsSM), were used as positive and negative controls for bacterial killing, respectively. The data presented correspond to three independent experiments and are plotted as means ± SD. Comparison of the 17978 strains shows no significant differences in killing (n.s.; p>0.05; Tukey's multiple comparison post-test).](https://plosone.org/doi/10.1371/journal.pone.0055142.g005)

We therefore tested different incubation times (4 hours or 20 hours), agar concentrations (0.5 and 1.5%), and other bacteria for bacterial killing. The alternative prey tested were another A. baumannii strain (A. baumannii ATCC 19606), and two non-baumannii Acinetobacter species, one containing a T6SS (A. baigyi ADP1) and the other lacking a T6SS in its genome (A. nosocomialis 1221). There were no significant differences in the survival of any of these preys in all the conditions tested (data not shown). These results suggest that A. baumannii ATCC 17978 may be highly specific for its target, or that it may require particular conditions to kill other bacteria. Alternatively, A. baumannii may use the T6SS for a different function. It has been shown that mutation of the T6SS of enteraggregative E. coli (EAEC) results in diminished biofilm formation [58], and the ability of A. baumannii to form biofilms may contribute to its pathogenicity and long term survival in hospital environments. Using a continuous flow-cell system, we determined that the biofilms formed by 17978 ΔtsSM were indistinguishable from wild type 17978 (Figure S3), suggesting the T6SS does not play a role in biofilm formation. Similar to our results, B. thailandensis does not require its T6SS-1 for biofilm formation [45].

**Discussion**

Inspection of the genome of several sequenced species of Acinetobacter revealed 12 genes conserved in all T6SSs, including the previously identified “hallmarks” of T6SSs [37]. Notably, the T6SS cluster of all Acinetobacter species lacked obvious homologs to tssJ, an outer membrane lipoprotein shown to be essential for Hcp secretion by the EAEC T6SS. However, this lipoprotein is also absent from the Rhizobium leguminosarum T6SS, which has demonstrated T6SS activity [66], and suggests that the T6SS can still function in the absence of tssJ. The organization of the T6SS was identical among all A. baumannii genomes analyzed, with nearly 99% nucleotide sequence identity between strains (data not shown), suggesting that this secretion system is conserved. Our analysis also uncovered the genetic components of T6SSs in several other species of Acinetobacter, including A. calcoaceticus, A. deicononas, A. baylyi, A. johnsonii, A. radioreistant, and A. baigyi. Additionally, genomic analysis of sequenced A. putii, A. junii, A. nosocomialis, and A. haemolyticus strains indicate that they lack homologs to conserved T6SS components, including Hcp (Table S1). Interestingly, all genome sequences we analyzed for this study showed the presence of VrgG-like proteins, even those strains not predicted to encode a T6SS. Moreover, A. putii and A. nosocomialis seem to have homologs of the T6SS component TssL, and A. haemolyticus possesses a homolog of TssM. This may indicate that functionality of the T6SS, as evidenced by a lack of core components, may have been lost in these strains, while the VrgG’s, which are located outside the T6SS cluster in Acinetobacter species, and TssL or TssM have been retained for an as yet unknown reason.

Through immunoblotting and mutational analysis we showed that Hcp is secreted by 17978 and that the TssM protein is necessary for Hcp secretion. Previous work [31,32] has established that TssM is an essential structural component of the secretory apparatus. Similarly, our results showed that TssM is also essential for T6SS activity in A. baumannii. While the remaining genes of the cluster remain to be functionally characterized, our results demonstrate that 17978 encodes a bona-fide T6SS.

We analyzed Hcp expression and secretion in several A. baumannii strains, both sequenced (17978, SDF, AYE, 19606) and unsequenced (1375, 1224, 1225), as well as other species within the genus Acinetobacter. We developed an ELISA-based method to detect Hcp in the culture supernatants. With this method, together with Western blot analysis, we observed wide variation in the actual secretion of Hcp to culture supernatants, with some isolates showing robust Hcp secretion (SDF, 19606, 1224, A. calcoaceticus A/B), and others with little (17978) or no (AYE, 1225) secretion. The ELISA method described in this work could be employed in the future for screening of T6SS inhibitors or to identify mutations affecting T6SS functionality. Clinical strains of P. aeruginosa have also been shown to display differences in their secretory profiles of Hcp [32,67]. In these cases, expression of the T6SS-activating or T6SS-repressing PpkA or PppA regulatory proteins could induce secretion in non-secreting isolates or repress secretion in Hcp secreting isolates, respectively, indicating that some clinical isolates may undergo mutations in their regulatory components [67]. Of note, we were unable to identify homologs of the P. aeruginosa ppkA/pppA post-translational regulatory system in A. baumannii, indicating that a different regulatory mechanism is likely involved. Indeed, other regulatory mechanisms have been described in other bacteria [68]. Although the elements which regulate T6SS in A. baumannii are not known, in a recent study the transcriptional profile of a LPS-deficient A. baumannii ATCC 19606 strain was analyzed [59]. It was shown that this strain upregulated expression of genes involved in cell-envelope and membrane biogenesis. Interestingly, the authors found that several genes encoding the T6SS locus analyzed in this study were down-regulated, which correlated with a loss of Hcp in culture supernatants. This suggests that the T6SS may be turned off under stress conditions. It is tempting to speculate that the strains that do not secrete Hcp constitutively may sense environmental signals and activate their T6SS.
Several T6SSs have been shown to facilitate killing of competing bacterial species [39,40,45,69]. In the case of P. aeruginosa, this is mediated by T6SS-directed intoxication of other bacteria with protein effectors as part of a toxin-antitoxin system [40,41]. We determined that 17978 is unable to utilize its T6SS for antibacterial activity against E. coli MG1655, a strain previously shown to be susceptible to the V. cholerae T6SS [39]. 17978 ΔtssM showed no difference in ability to affect E. coli survival compared to wild type. However, compared to the negative control V. cholerae ΔtssM, E. coli survival was decreased more than 100-fold when co-incubated with the A. baumannii strains, suggesting inhibition of E. coli growth through an unknown, T6SS-independent mechanism. This may be the result of an unidentified inhibitory factor produced by A. baumannii, or alternatively, a consequence of competition for nutrients. We tested other conditions and additional bacterial prey, obtaining the same results. It is possible that the A. baumannii ATCC 17978 T6SS is prey-selective or requires specific growth conditions that we were unable to determine. Alternatively, A. baumannii ATCC 17978 may not use its T6SS against other bacteria.

In an attempt to determine the biological function of the T6SS in A. baumannii, we tested 17978, and its isogenic tssM mutant derivative, in non-mammalian infection models. D. discoideum are unicellular amoebae which feed on bacteria through phagocytic mechanisms analogous to macrophages [70], and have become a widely used host model for studying bacterial pathogenesis [62]. Recently, A. baumannii was shown to be virulent towards amoebae, but required the presence of ethanol-stimulated virulence genes to kill D. discoideum [30]. A. baumannii ATCC 17978 and 17978 ΔtssM were equally virulent towards D. discoideum, indicating that the T6SS of this strain does not play a role in ethanol-stimulated virulence. A previous study identified several genes up-regulated by the presence of ethanol in A. baumannii; however, none of the genes presumed to be involved in the T6SS, including vsgS, were significantly affected [18]. We also tested the wild type and tssM mutant in the G. mellonella insect infection model, which has previously been used to assess the pathogenesis of Acinetobacter [14,26,64], and provides an alternative to the challenges associated with mammalian models. In this assay, the killing of G. mellonella larvae is dose-dependent [64]. We observed no statistically different survival of the insects by the two strains at either inoculum. Our results suggest that the T6SS of 17978 does not contribute to pathogenicity in these two non-mammalian models of infection. We then utilized an established mouse model of pneumococcal pneumonia to assess any potential role of the T6SS in mammalian infection. The bacterial burden in the lungs and liver was similar between wild type-infected and mutant-infected mice, indicating similar infectivity between the two bacterial strains in this model.

It should be noted that the T6SS is not exclusively harboured by pathogenic Acinetobacter species. A. calcoaceticus and A. baylyi, which we have experimentally demonstrated to have active T6SSs, are rarely implicated in serious human disease [7], and the specific strain of A. baylyi used in this study, ADP1, was derived from a soil isolate [60,61]. Two of the most clinically relevant species of Acinetobacter, A. pittii and A. nosocomialis (formerly Acinetobacter genomosp. 3 and Acinetobacter genomosp. 13TU, respectively [71]), appear not to have functional T6SSs (Table S1 and Fig. 2B). Also, as shown, the T6SS does not play a role in biofilm formation for A. baumannii ATCC 17978. Taken together, our results suggest that presence of a T6SS does not correlate with virulence in the genus Acinetobacter, at least in the models analyzed. The finding that several non-pathogenic, environmental Acinetobacter species possess T6SSs may indicate another function. Most of the proposed roles for T6SS systems of other bacteria do not seem to be applicable to A. baumannii. Considering the plasticity of the A. baumannii genome [72,73], it is unlikely that the system has been functionally conserved in so many strains for no reason, and therefore we believe it likely provides some advantage to the bacterium. Future work in our laboratory will attempt to define the role T6SS plays in the Acinetobacter genus.

Materials and Methods

Bacterial Strains and Growth Conditions

The A. baumannii reference strains used in this study were obtained from American Type Culture Collection. All strains and plasmids used are listed in Table S2. Strains were grown in Luria-Bertani (LB) medium at 37°C with shaking. Where necessary, antibiotics were added to the medium at the following concentrations: gentamicin (50 μg ml⁻¹), kanamycin (50 μg ml⁻¹), ampicillin (100 μg ml⁻¹), and tetracycline (50 μg ml⁻¹).

Purification of A. baumannii Hcp for Antibody Development

Purification of the histidine tagged Hcp was performed essentially as described elsewhere [74]. Briefly, the A. baumannii hcp gene (A1S_1296) was cloned into pEXT20 with a 10 histidine tag using HcpFwd and HcpRev10His, creating pEXT20-Hcp10His, and electroporated into E. coli DH5α. 1L of fresh LB was inoculated with 20 mL of an overnight culture of E. coli containing this vector, and grown for 4 h with 1 h induction by addition of 1 mM IPTG. Cells were harvested and resuspended in binding buffer (10 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0) and lysed using a French pressure cell, followed by centrifugation. Supernatants were collected, and pellets were resuspended in binding buffer for a second round of lysis followed by centrifugation. Inclusion bodies were solubilized as previously described [75] by resuspending the pellets obtained above in binding buffer containing 6M urea. Supernatants and solubilized inclusion bodies were mixed and loaded onto a HisTrap HP column (Amersham Pharma Biosciences) equilibrated with 10 column volumes of binding buffer with a flow rate of 1 mL min⁻¹ for Ni²⁺-affinity chromatography. The column was washed with 25 column volumes of washing buffer (20 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, 6M urea pH 8.0). Bound protein was eluted using elution buffer (250 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, 6M urea pH 8.0). Protein purity was determined by Coomassie stain following SDS-PAGE, and mass spectrometry analysis was performed to confirm protein ID. Sample was then transferred to PBS buffer by buffer exchange using a PD-10 column (GE Healthcare). Protein concentration was determined by the Bradford assay (Bio-Rad), and purified protein was sent to SACRI antibody services (University of Calgary, Alberta, Canada) for development of rabbit-derived polyclonal antibodies.

Preparation of Cell-free Supernatants for SDS-PAGE

The OD₆₀₀ of overnight Acinetobacter cultures were determined and fresh LB was inoculated with OD-normalized volumes of bacterial culture. Antibiotics were not added to diluted cultures in order to avoid potential cell lysis. After approximately 4 hours, bacteria were harvested by centrifugation (10 min at 5,000 × g) and supernatants collected and filtered through 0.22 μm syringe filters (Millipore Corporation, Billerica, MA) to obtain cell-free supernatants. Supernatant proteins were precipitated by the addition of 1:4 volumes trichloroacetic acid and incubation at 4°C for 20 min. Protein pellets were obtained by centrifugation at 14,000 × g for 5 min. The samples were then washed twice with...
ice-cold acetone, centrifuged to pellet, and supernatant removed. The pellets were dried in a heat block at 95°C and resuspended in loading buffer. OD_{600} normalized volumes of whole cells or supernatants were loaded onto 15% SDS-PAGE gels for separation, transferred to a nitrocellulose membrane, and probed by Western immunoblot with polyclonal rabbit anti-Hcp (1:1500) and mouse monoclonal anti-RNA polymerase (1:2500, RNAP z-subunit; Neoclon). Membranes were then probed with IRDye conjugated anti-mouse and anti-rabbit antibodies and visualized on an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

ELISA Assay for Hcp Secretion

250 μl of LB in a 96-well plate was inoculated in triplicate with individual colonies of the Acinetobacter strains used in this study. The plates were incubated in a humidified container (to prevent evaporation) at 37°C in a shaking incubator at 200 rpm for ~9 h to allow sufficient growth of all strains. Following incubation, the optical density at 600 nm was determined for each well by a plate reader, and then plates were centrifuged at 4 k rpm for 10 min. Fifty μl of supernatants were transferred to high-binding ELISA 96-well plates containing 50 μl of binding buffer (100 mM sodium bicarbonate/carbonate) and incubated at 4°C overnight. The plates were washed with PBS, blocked with a solution of 5% skim milk in PBS for 1.5 h, and then probed with a solution of 2.5% skim milk in PBST containing 1:7500 dilution of the anti-Hcp antibody for 1 h. The plates were washed with PBST and probed with a 1:5000 dilution of horse radish peroxidase conjugated goat anti-rabbit antibody (Bio-Rad) in 2.5% skim milk-PBST solution for 1 h. The plates were again washed with PBST, and then 100 μl of TMB substrate (Cell Signaling Technology, Danvers, MA) was added to each well. The plates were allowed to develop for ~5 mins before absorbance at 650 nm was measured by a plate reader. Alternatively, STOP (Cell Signaling Technology, Danvers, MA) solution could be added to end the colorimetric reaction, and absorbance at 450 nm measured.

Construction of Mutants and Complemented Strains

Primers are listed in Table S3, with restriction sites underlined where relevant. Approximately 1000 bp of DNA flanking either side of the 17978 hcp gene (A1S_1296) was amplified and individually cloned into pEXT20 using the primers 5’-hcpFwd and 5’-hcpRev for the upstream region, and 3’-hcpwdF and 3’-hcpRev for the downstream region. These segments were subcloned into a single plasmid to generate pWEB02. A gentamicin resistance cassette (aacC1) was excised from pSPG1 by SmaI digest, and subsequently ligated with pWEB02 to generate pWEB03. An EcoR1/XhoI double digest removed the entire fragment from pEXT20, and this was ligated to a similarly cut pFLP2 plasmid, which encodes a sacB counter selection gene and does not replicate in A. baumannii. The resultant pWEB04 plasmid was electroporated into 17978 cells followed by selection for those cells that had integrated the plasmid by plating on gentamicin. Gentamicin resistant colonies were used to inoculate 5 ml of binding buffer (100 mM sodium bicarbonate/carbonate) and incubated at 4°C overnight. The plates were washed with PBS, blocked with a solution of 5% skim milk in PBS for 1.5 h, and then probed with a solution of 2.5% skim milk in PBST containing 1:7500 dilution of the anti-Hcp antibody for 1 h. The plates were washed with PBST and probed with a 1:5000 dilution of horse radish peroxidase conjugated goat anti-rabbit antibody (Bio-Rad) in 2.5% skim milk-PBST solution for 1 h. The plates were again washed with PBST, and then 100 μl of TMB substrate (Cell Signaling Technology, Danvers, MA) was added to each well. The plates were allowed to develop for ~5 mins before absorbance at 650 nm was measured by a plate reader. Alternatively, STOP (Cell Signaling Technology, Danvers, MA) solution could be added to end the colorimetric reaction, and absorbance at 450 nm measured.

Similarly digested pWH1266 shuttle plasmid, creating pHcp. pHcp was electroporated into 17978 Δhcp for complementation analysis.

For the unmarked mutation of tssM, primer pairs tssMUpFwd, tssMUpRev, and tssMDwFwd, tssMDwRev were used to amplify approximately 500 bp of DNA upstream and downstream of tssM, respectively. The two PCR products were then mixed in equimolar amounts and nested overlap-extension PCR was performed using primers tssMFwdNest and tssMRenRev. The product was cloned into pABK, a derivative of pFLP2 with a kanamycin cassette inserted into its NheI sites, generating pWEB05. The vector was then transformed into wild type 17978 and plated on kanamycin to select for integration. Following the procedure described above, cells were then plated on sucrose containing plates to select for double recombinants. Colonies which were sucrose resistant but kanamycin sensitive were selected for PCR screening and sequencing to confirm generation of 17978 ΔtssM. The growth curve of 17978 ΔtssM was identical to parental 17978. For complementation, primers tssMFwd and tssMRen10His were used to amplify the full-length tssM gene and cloned into pEXT20. The product was then amplified out of pWEB07 plasmid using tssMFwd1 and tssMRen10His primers and cloned into the PstI site of pWH1266, generating pTssM.

D. discoideum Plaque Assay and G. mellonella Killing Assay

The D. discoideum plaque assay was performed essentially as described previously [31]. Mid-logarithmic growth phase amoebae were mixed with overnight cultures of bacteria to a final concentration of 1 × 10^9 cells ml^-1. 0.2 ml of the suspension was then plated on SM/5 agar containing 1% ethanol [30]. Plates were incubated at room temperature and monitored for D. discoideum plaques for up to 7 days. For G. mellonella killing assays, the experiments were performed as previously described [64]. Briefly, PBS-washed bacterial cells were normalized by OD_{600} and 5-μl aliquots were injected into G. mellonella larvae (Dr. Andrew Keddie, University of Alberta). For each group, 10 G. mellonella were used, and colony counts on LB agar were used to determine the CFUs injected. Larvae were incubated at 37°C after injection and survival was plotted using the Kaplan-Meier method and analyzed using the log-rank test [64]. Experiments comparing wild type and mutant were discarded if the difference in CFU counts were >0.5Log [64]. PBS injected G. mellonella were used as a negative control and showed 100% survival for the duration of the experiment. Figure 4A shows representative results from two separate experiments.

Animal Infections

For assessing pathogenesis in vivo, we utilized a murine model of A. baumannii pneumonia previously developed in our laboratory with a few modifications [17]. Briefly, 7-week-old female C57BL/6 mice were anesthetized followed by intranasal inoculation with 5–5 × 10^6 CFU A. baumannii in 40 μl PBS. At 36 hours post-infection mice were euthanized, and CFU were enumerated in lungs and livers following tissue homogenization and plating serial dilutions on LB agar plates. All of the infection experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee. Mice were obtained from Jackson Laboratories.

Bacterial Killing Assay

Killing assays were performed as described previously [39]. Bacterial strains were grown overnight on LB agar plates with the...
appropriate antibiotics. V. cholerae strains V32 and V52 ΔptoM were used as positive and negative controls for bacterial killing, respectively. The E. coli K-12 strain MG1655 (rifampicin resistant, derivative) was used as prey in initial assays. Intra-species competition assays were performed with A. baumannii ATCC 19606 and A. baylyi ADP1 transformed with pBAV1K-T5-gfp, a plasmid conferring kanamycin resistance and allowing for selection against A. baumannii ATCC 17978, as well as a clinical isolate A. nosocomialis 1221 that was naturally gentamicin resistant. Cells were harvested, resuspended in LB and mixed at a 1:10 ratio (predator:prey). Bacterial mixtures were spotted onto LB agar for four hours at 37°C, unless otherwise noted. Cells were harvested and seven serial dilutions were performed. Each serial dilution was plated in 10 μL spots on LB with appropriate antibiotic to select for surviving prey. Plates were incubated overnight at 37°C and the surviving prey were enumerated the following day. Statistical analysis was performed by one-way ANOVA and Tukey’s multiple comparisons post-test.

Supporting Information

Figure S1 Visual results of a typical Hcp secretion ELISA assay with different strains of Acinetobacter. An example of the distinction between strains that are “high” or “low” Hcp secretors are indicated by arrows (See Figure S2 for quantification of secretion).

Figure S2 Quantification of ELISA results. A) Absorbance at 650 nm following ELISA assay for Hcp secretion. The grey broken line indicates an arbitrary cut-off between “high” and “low” secreting strains. B) Optical density at 600 nm of strains shown in part A prior to isolation of supernatants and ELISA assay. The grey broken line indicates an arbitrary cut-off between “fast” and “slow” growing strains. A. pestis A, a strain which does not encode hcp, was used as a control.

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