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Abstract:	The Gram-negative bacterial type VI Secretion System (T6SS) delivers toxins to kill or inhibit the growth of susceptible bacteria, while others target eukaryotic cells. Deletion of atsR, a negative regulator of virulence factors in B. cenocepacia K56-2, increases T6SS activity. Macrophages infected with a K56-2 ΔatsR mutant display dramatic alterations in their actin cytoskeleton architecture that rely on the T6SS, which is responsible for the inactivation of multiple Rho-family GTPases by an unknown mechanism. We employed a strategy to standardize the bacterial infection of macrophages and densitometrically quantify the T6SS-associated cellular phenotype, which allowed us to characterize the phenotype of systematic deletions of each gene within the T6SS cluster and ten vgrG encoding genes in K56-2 ΔatsR. None of the genes from the T6SS core cluster and the individual vgrGs were directly responsible for the cytoskeletal changes in infected cells. However, a mutant strain with all vgrG genes deleted was unable to cause macrophage alterations. Despite not being able to identify a specific effector protein responsible for the cytoskeletal defects in macrophages, our strategy resulted in the identification of the critical core components and accessory proteins of the T6SS assembly machinery and provides a screening method to detect T6SS effectors targeting the actin cytoskeleton in macrophages by random mutagenesis.

Quantification of Type VI secretion system activity in macrophages infected with *Burkholderia cenocepacia*

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Running title: Characterization of the *B. cenocepacia* T6SS

Key words: T6SS, core component, VgrG, effector protein, *Burkholderia cenocepacia*

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ABSTRACT

The Gram-negative bacterial type VI Secretion System (T6SS) delivers toxins to kill or inhibit the growth of susceptible bacteria, while others target eukaryotic cells. Deletion of *atsR*, a negative regulator of virulence factors in *B. cenocepacia* K56-2, increases T6SS activity. Macrophages infected with a K56-2 Δ*atsR* mutant display dramatic alterations in their actin cytoskeleton architecture that rely on the T6SS, which is responsible for the inactivation of multiple Rho-family GTPases by an unknown mechanism. We employed a strategy to standardize the bacterial infection of macrophages and densitometrically quantify the T6SS-associated cellular phenotype, which allowed us to characterize the phenotype of systematic deletions of each gene within the T6SS cluster and ten *vgrG* encoding genes in K56-2 Δ*atsR*. None of the genes from the T6SS core cluster and the individual *vgrG*s were directly responsible for the cytoskeletal changes in infected cells. However, a mutant strain with all *vgrG* genes deleted was unable to cause macrophage alterations. Despite not being able to identify a specific effector protein responsible for the cytoskeletal defects in macrophages, our strategy resulted in the identification of the critical core components and accessory proteins of the T6SS assembly machinery and provides a screening method to detect T6SS effectors targeting the actin cytoskeleton in macrophages by random mutagenesis.

INTRODUCTION

Burkholderia cenocepacia is an environmental Gram-negative opportunistic pathogen that causes persistent, often severe, lung infections in individuals with cystic fibrosis (CF) and other underlying diseases (Drevinek & Mahenthiralingam, 2010; Isles *et al.*, 1984; Mahenthiralingam *et al.*, 2008). Infections by this bacterium are difficult to treat due to the intrinsic and high-level multidrug resistance of *B. cenocepacia* to most clinically relevant antibiotics (Waters, 2012). Also, *B. cenocepacia* can be transmitted from patient to patient (Drevinek & Mahenthiralingam, 2010). *B. cenocepacia* is pathogenic in several plant and non-mammalian animal infection models (Khodai-Kalaki *et al.*, 2015; Thomson & Dennis, 2013; Uehlinger *et al.*, 2009; Vergunst *et al.*, 2010) and can survive intracellularly within epithelial cells (Burns *et al.*, 1996; Sajjan *et al.*, 2006), macrophages (Lamothe *et al.*, 2007; Martin & Mohr, 2000; Saini *et al.*, 1999) and amoebae (Lamothe *et al.*, 2004; Marolda *et al.*, 1999).

The Type VI secretion system (T6SS) is widely distributed among Gram-negative bacteria (Costa *et al.*, 2015; Zoued *et al.*, 2014). It forms an elongated protein complex, which is structurally related to the tail-tube and puncturing device of bacteriophages (Shneider *et al.*, 2013; Zoued *et al.*, 2014). The T6SS is an extremely dynamic contractile nanomachine (Basler *et al.*, 2012; Bonemann *et al.*, 2010; Clemens *et al.*, 2015; Kudryashev *et al.*, 2015) that attacks cells by initially penetrating them with a trimeric protein complex called the VgrG spike. The spike first assembles into a membrane-anchored complex formed of an inner tail tube made of Hcp proteins surrounded by an outer sheath VipA- and VipB-like proteins (Bonemann *et al.*, 2009). In turn, proteins

from the PAAR (proline-alanine-alanine-arginine) repeat superfamily bind to the VgrGs and are essential for T6SS-mediated secretion into other bacterial cells, forming a spike complex decorated with multiple effectors that are delivered simultaneously into target cells through a contraction-driven translocation event (Shneider *et al.*, 2013). The AAA+ ATPase ClpV disassembles the outer sheath complex, a process that requires ATP hydrolysis, and then the inner Hcp tube is detached and released into the medium (Bonemann *et al.*, 2009). The T6SS, now referred to as a bacterial poison dagger, is a versatile weapon, which requires intimate cell contact to deliver a wide range of toxins into bacterial competitors or eukaryotic cells. Most identified T6SS effector proteins act on bacterial cells and include peptidoglycan-degrading enzymes, membrane-degrading lipases, and nucleic acid targeting enzymes (Durand *et al.*, 2014; Russell *et al.*, 2014). In some cases, the same effector can function in bacterial antagonism and also alters cell-signaling pathways in eukaryotic cells (Jiang *et al.*, 2014). Also, "evolved" VgrGs have been described that contain various C-terminal extensions leading for instance to actin-crosslinking or actin-ADP-ribosylation in eukaryotic cells (Brooks *et al.*, 2013; Pukatzki *et al.*, 2007; Suarez *et al.*, 2010), and host cell fusion presumably to facilitate intercellular bacterial spreading (Schwarz *et al.*, 2014; Toesca *et al.*, 2014).

The T6SS of *B. cenocepacia* K56-2 was first identified in a signature-tagged mutagenesis study pointing out the importance of this secretion system for *B. cenocepacia* survival in a rat model of chronic respiratory infection (Aubert *et al.*, 2008; Hunt *et al.*, 2004). Study of *B. cenocepacia* T6SS *in vitro* was rendered possible by the discovery of AtsR (Adhesion and Type Six secretion system Regulator), a hybrid sensor kinase that

negatively regulates the expression of *B. cenocepacia* virulence factors including the T6SS (Aubert *et al.*, 2010; Aubert *et al.*, 2008; Aubert *et al.*, 2013; Khodai-Kalaki *et al.*, 2013). Deletion of *atsR* causes a significant increase in T6SS activity, as denoted by increased amounts of Hcp released into bacterial culture supernatant (Aubert *et al.*, 2008), induction of actin cytoskeletal rearrangements in infected macrophages (Aubert *et al.*, 2008; Flannagan *et al.*, 2012; Rosales-Reyes *et al.*, 2012), and delayed assembly of the NADPH oxidase complex at the membrane of the *B. cenocepacia*-containing vacuole (Keith *et al.*, 2009; Rosales-Reyes *et al.*, 2012). These cellular defects in infected macrophages are characteristic for *B. cenocepacia* and depend on T6SS-mediated defects in the activation of multiple Rho family GTPases by an unknown mechanism presumably via unknown T6SS effector molecules (Flannagan *et al.*, 2012; Rosales-Reyes *et al.*, 2012). Here we show that Hcp detection in bacterial culture supernatants and quantification of the morphological phenotype in infected macrophages allowed us to characterize the components of the T6S apparatus in *B. cenocepacia* required for T6SS function and to refine the boundaries of the T6SS cluster. The relevance of *B. cenocepacia* VgrG proteins for T6SS function and T6SS-related phenotype was also investigated. From our results, we propose that quantification of the morphological phenotype in macrophages is a sensitive and reproducible test that can serve as a screening tool to identify mutations denoting *B. cenocepacia* genes that are responsible for disturbing the actin cytoskeleton in infected macrophages.

METHODS

Bacterial strains, plasmids, and culture media. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria Broth (LB) (Difco) at 37ºC. *Escherichia coli* cultures were supplemented, as required, with the following antibiotics (final concentrations): 30 μg tetracycline ml^{-1}, 30 μg kanamycin ml^{-1}, and 50 μg trimethoprim ml^{-1}. *B. cenocepacia* cultures were supplemented, as required, with 100 μg trimethoprim ml^{-1} and 100 μg tetracycline ml^{-}.

General molecular techniques. DNA manipulations were performed as described previously (Sambrook *et al.*, 1990). T4 DNA ligase (Roche Diagnostics, Laval, Quebec, Canada) and Antarctic phosphatase (New England Biolabs, Pickering, Ontario, Canada) were used as recommended by the manufacturers. Transformation of *E. coli* DH5α and *E. coli* GT115 was done using the calcium chloride method (Cohen *et al.*, 1972). Mobilization of complementing plasmids and mutagenesis plasmids into *B. cenocepacia* was performed by triparental mating using *E. coli* DH5α carrying the helper plasmid pRK2013 (Craig *et al.*, 1989; Figurski & Helinski, 1979). DNA amplification by polymerase chain reaction (PCR) was performed using a PTC-221 DNA engine (MJ Research, Incline Village, Nevada) with Taq or HotStar HiFidelity DNA polymerases (Qiagen Inc., Mississauga, Ontario, Canada). DNA sequences of all primers used in this study are described in the Supplemental Table S2. DNA sequencing was performed at the DNA sequencing Facility of York University, Toronto, Canada. The KEGG database (Kanehisa & Goto, 2000) and the computer program BLAST (Altschul *et al.*, 1990) were used to analyze the sequenced genome of *B. cenocepacia* strains K56-2 and J2315.

Deletion mutagenesis of *B. cenocepacia* K56-2 and complementing plasmids. Oligonucleotide primers used for the construction of mutagenic and complementing plasmids are listed in Table S1, and the plasmids construction details are provided in Supplementary data. Unmarked and non-polar deletions were performed as described previously (Flannagan *et al.*, 2008; Hamad *et al.*, 2010). All deletion plasmids were introduced into *E. coli* GT115 by transformation and mobilized into *B. cenocepacia* by triparental mating. When gentamicin-sensitive strains were used, *E. coli* counter-selection was performed with 200 μg carbenicillin ml^{-1} and 10 μg polymyxin B ml^{-1} instead of 50 μg gentamicin ml^{-1}. Gene deletions were confirmed by PCR. Mutants were tested in a Bioscreen C automated microbiology growth curve analysis system at 37°C, with continuous shaking and OD_{600} measurements taken every hour as described previously (Aubert *et al.*, 2008).

Expression and purification of His-tagged Hcp and polyclonal antibody preparations. *hcp* was PCR amplified with primers 2143 and 2748 and cloned into plasmid pET30a using *Nde*I and *Hin*dIII restriction sites. This generated plasmid pDA44 encoding Hcp_{6xHis}, which was introduced into *E. coli* strain BL21 (DE3) by transformation. Overexpression of Hcp_{6xHis} was performed as follows. *E. coli* cells were grown to an OD_{600} of 0.6, induced with 0.05 mM isopropyl-β-d-1-thiogalactopyranoside (IPTG), and grown for another 2 h at 30°C. Cells were collected by centrifugation and resuspended in 50 mM sodium phosphate pH 7.4, 300 mM NaCl, and lysed using a French press. Debris were removed following centrifugation at 20 000 ×*g* for 20 min.

154 Hcp_{6xHis} was purified from filtered supernatant by FPLC (ÄKTA Basic instrument) using
155 a 5 ml HisTrap column (GE Healthcare). Elution was performed using a linear gradient
156 concentration of imidazole (10-400 mM). Fractions containing purified Hcp_{6xHis} were
157 pooled and dialyzed against 50 mM sodium phosphate, pH 7.4, 300 mM NaCl, and stored
158 at 4°C. The eluted Hcp_{6xHis} was judged >90% pure after this step. Polyclonal antibodies
159 recognizing Hcp were generated in New Zealand White rabbits by ProSci Inc. (Poway,
160 CA).
161
162 **Precipitation of culture supernatant proteins and immunoblot analysis.** Culture
163 supernatant proteins were precipitated as described previously (Aubert *et al.*, 2008) with
164 some modifications. Briefly, overnight cultures were diluted to an OD_{600} of 0.03 in pre-
165 warmed LB and grown until early exponential phase, at which time OD_{600} was also
166 recorded. Proteins from filter-sterilized culture supernatants were precipitated overnight
167 at 4 °C using 20% trichloroacetic acid (final concentration). Five µg of secreted proteins
168 were loaded on an 18% SDS-PAGE. The crude lysate sample (pellet fraction) was
169 prepared as follows: bacteria from 1 ml of exponential phase culture adjusted at an OD_{600}
170 of 0.5 were pelleted by centrifugation, resuspended into 30 µl of 1x protein loading dye
171 and boiled for 10 min. Samples were centrifuged for 3 min at 5 900 ×*g* and 3 µl of total
172 cell lysate were loaded on a 18% SDS-PAGE. After electrophoresis, gels were transferred
173 to nitrocellulose membranes for immunoblot analysis. After blocking (Roche), the
174 membranes were incubated with the following primary antibodies as required. 4RA2
175 monoclonal antibody (Neoclone), which cross-reacts with the *B. cenocepacia* and *B.*
176 *multivorans* RNA polymerase subunit alpha (cytosolic / cell lysis control) (dilution of

1:25 000), anti-Hcp polyclonal antiserum (ProSci-inc) (dilution of 1:1 000) and FLAG M2 monoclonal antibody (Sigma) (dilution of 1:50 000). Secondary antibodies Alexa Fluor 680 conjugated goat anti-mouse IgG (Molecular Probes) and IRDye800 conjugated goat anti-rabbit IgG (Rockland) were used at a dilution of 1:50 000. Detection was performed using the Odyssey Infrared Imager (LI-COR Biosciences).

Macrophage infections and quantification of the T6SS activity. Infections were performed as previously described (Aubert *et al.*, 2008) using the C57BL/6 murine bone marrow-derived macrophage cell line ANA-1 (Cox *et al.*, 1989). Bacteria were washed three times with DMEM 10% FBS and added to ANA-1 cells grown on glass coverslips at a MOI of 50:1. Plates were centrifuged for 2 min at 300 ×*g* to synchronize the infection and incubated at 37°C under 5% CO_2. Coverslips were analyzed by phase contrast microscopy at 4 h post-infection. T6SS activity was recorded as the ability of the bacteria to induce the formation of characteristic ectopic structures around the macrophages (Aubert *et al.*, 2008). An assay was developed to measure the extent of the formation of these structures around the macrophages. As "beads on a string-like" structures appear as dark objects on a clear background around macrophages in phase contrast microscopy the percentage of the area occupied by dark objects can be measured upon picture analysis using the Northern Eclipse software. For each infection, pictures with a 100x magnification were taken under the same conditions of light, gain and exposure. A threshold was applied to highlight the dark pixels on the images and the number of macrophages and Percent of object Area values for each image was recorded. The intensity of T6SS activity was calculated for each mutant by dividing the sum of the

Percent Area values measured over at least 21 fields of view by the total number of macrophages (over 300 macrophages). The ability to induce the formation of "beads on a string-like" structures around macrophages, which is representative of the T6SS activity in each mutant, was expressed in arbitrary units relative to Δ*atsR* set as 1. Experiments were repeated independently three times. Uninfected ANA-1 cells were used as a negative control to determine background levels. The negative control had 0.1±0.02 relative units. Therefore, experimental samples giving relative units equal or lower than 0.2 (corresponding to 5 standard deviation units from the mean of the negative control) were considered as indicative of cells lacking ectopic structures. One-way Anova (Prism 5.0a, GraphPad Software Inc.) was utilized to analyze the data from the quantification experiments. The Bonferroni Multiple Comparison test using a significance level of 0.01 was used to compare the relative units obtained from experimental samples and uninfected controls.

Gentamicin protection assay. Bacterial infection and bacterial intracellular survival were assayed as described previously (Schmerk & Valvano, 2013) with slight modifications. ANA-1 macrophages were seeded in 12-well plates at a density of $3x10^5$ cells per well and incubated overnight. Gentamicin sensitive strains were grown overnight in LB broth at 37°C with shaking. Bacteria were used to infect ANA-1 macrophages at a MOI of 50:1 as described above. One hour post-infection, macrophages were washed with PBS three times to remove extracellular bacteria. DMEM 10% FBS containing 100 µg gentamicin ml^{-1} was added to kill remaining extracellular bacteria. One hour later, macrophages were washed twice in PBS, and fresh medium containing 10

223 μg gentamicin ml^{-1} was added for the remainder of the experiment. To enumerate
224 intracellular bacteria, infected macrophages were lysed with 0.1% sodium deoxycholate
225 (w/v) at 4 h post-infection. Lysates were serially diluted in PBS and plated on LB agar.

RESULTS

Functional characterization of the T6SS components of *B. cenocepacia* K56-2

The genome of *B. cenocepacia* K56-2 contains only one T6SS locus on chromosome 1 (spanning 23.7 kilobase pairs) (Fig. 1a). The boundaries of the T6SS locus in *B. cenocepacia* K56-2 were initially set from BCAL0352 up to BCAL0333 because of its immediate location upstream of a tRNA sequence (as observed in pathogenicity islands) and based on the identification of three putative transcriptional units containing conserved T6SS components (Aubert *et al.*, 2010; Boyer *et al.*, 2009). Unlike many other bacteria (Boyer *et al.*, 2009), the putative T6SS cluster of *B. cenocepacia* K56-2 does not contain any *vgrG* (Fig. 1a). The predicted functions of the T6SS genes are listed in the Supplemental Table S2, and whenever possible the genes were named according to the proposed standard nomenclature for T6SS core components, *tss* (for T6SS gene) or *tag* (T6SS-associated gene) (Shalom *et al.*, 2007). Each of the genes in the T6SS cluster was systematically deleted in *B. cenocepacia* Δ*atsR* and the mutants investigated for T6SS related phenotypes. All mutants had similar growth rates compared to the wild type strain (data not shown). As previously demonstrated (Aubert *et al.*, 2008), infection of ANA-1 macrophages with Δ*atsR* induces the formation of "beads on a string-like" structures due to lamellipodia collapse and impairment of actin-tail retraction during macrophage migration (Flannagan *et al.*, 2012; Rosales-Reyes *et al.*, 2012) (Fig. 1b). This phenotype depends on a functional T6SS, as Δ*atsR*Δ*hcp* cannot disturb the cytoskeleton organization (Aubert *et al.*, 2008; Rosales-Reyes *et al.*, 2012). The nature of the secreted

effector eliciting changes in actin architecture is still unknown. In an attempt to determine whether a gene encoding an effector lied within the T6SS cluster, mutants were first evaluated for their ability to induce cytoskeletal rearrangements in infected macrophages and then for their ability to release Hcp into culture supernatants (denoting a functional T6SS). An assay was developed to quantify the T6SS activity by measuring the extent of the formation of "beads on a string-like" structures. Since these structures appear around macrophages as dark objects on a clear background in phase contrast microscopy (Fig. 1b) it is possible to measure the area they occupy per field of view using image analysis software. The "amount" of dark objects, which is representative of the intensity of T6SS activity, was calculated (see Methods) for each mutant tested and expressed relative to Δ*atsR* (Fig. 1c). Uninfected cells were used as negative control to determine the background level. Relative units below 0.2 (corresponding to 5 standard deviation units from the mean) were considered as indicative of cells lacking ectopic structures and consequently infected with T6SS-defective strains.

Deletion mutants lacking *tssM* (BCAL0351; *icmF*-like), *tssA* (BCAL0348), *tssH* (BCAL0347; *clpV*-like), *tssG* (BCAL0346), *tssF* (BCAL0345), *tssE* (BCAL0344), *tssD* (BCAL0343; *hcp*-like), *tssC* (BCAL0342; $bcsK_C$), *tssB* (BCAL0341; $bcsL_B$), *tssK* (BCAL0338) or *tssL* (BCAL0337) did not produce visible ectopic structures around macrophages. Therefore, the calculated relative units were not significantly different than those of uninfected cells, indicating that the deleted genes encode critical components for the T6SS activity (Fig. 1c). The mutant with a deletion of BCAL0340 was able to induce the formation of ectopic structures around macrophages at very low levels (0.27 ± 0.02

relative units), but the results did not show a significant difference compared to uninfected cells. In contrast, mutants carrying a deletion in BCAL0352, *tagF* (BCAL0350), *tagL* (BCAL0349), *tssJ* (BCAL0339), and BCAL0336-33 were able to induce the formation of ectopic structures around macrophages, resulting in significantly different relative units compared to the uninfected control ($p < 0.001$), suggesting that these genes encode proteins dispensable for T6SS activity under the conditions assayed here.

Mutants were also evaluated for their ability to export and release Hcp into culture supernatants. As previously demonstrated (Aubert *et al.*, 2010; Aubert *et al.*, 2008), Hcp is clearly detected in culture supernatants from Δ*atsR* denoting a functional T6S machinery (Fig. 1d). Most of the genes located within the T6SS cluster were required for Hcp export. Mutants carrying a deletion in *tssM*, *tssA*, *tssH*, *tssG*, *tssF*, *tssE*, *tssC*, *tssB*, BCAL0340, *tssK* or *tssL* were unable to export Hcp, while mutants with a deletion in BCAL0352, *tagL* or *tssJ* had reduced levels of Hcp exported into the culture supernatants as compared to Δ*atsR*. These results also agree with the observation that these three mutants reproducibly induced lower levels of ectopic structures in macrophages in comparison with Δ*atsR* (0.71, 0.72, and 0.55 relative units; Fig. 1c), suggesting that the encoded proteins probably have an effect on the overall efficiency of the T6SS. In contrast, similar levels of Hcp were detected in Δ*atsR*ΔBCAL0336-33 and Δ*atsR* culture supernatants. Besides Δ*atsR*Δ*hcp*, the intracellular levels of Hcp were similar for all mutants tested except for *ΔatsRΔtagF*, which repeatedly displayed lower levels of cytosolic Hcp but higher levels of exported Hcp compared to Δ*atsR* (Fig. 1d).

These results show that there is in most cases, a good correlation between Hcp export levels and T6SS activity as measured by the extent of the formation of "beads on a string-like" structures. Together, these assays identified *tssM*, *tssA*, *tssH*, *tssG*, *tssF*, *tssE*, *tssD*(*hcp*), *tssC*, *tssB*, BCAL0340, *tssK* and *tssL* as core components of the T6S machinery, which are critical for assembly and function, and BCAL0352, *tagL* and *tssJ* as accessory proteins likely involved in the stability of the T6SS complex or required for its proper functioning. None of the mutants tested were unable to elicit changes in macrophages morphology while retaining the ability to export Hcp, suggesting that the gene encoding the effector molecule responsible for cytoskeletal rearrangements is not located within the T6SS cluster.

Characterization of BCAL0345 paralogs

We investigated the presence of paralogs of the T6SS genes using the KEGG database for the *B. cenocepacia* strain J2315 (http://www.genome.jp/kegg-bin/show_organism?org=bcj), which is a clonal isolate with K56-2 (Mahenthiralingam *et al.*, 2000). For each gene of the T6SS, paralogs were investigated according to the threshold values given for the Smith-Waterman algorithm (Smith & Waterman, 1981), as automatically provided by the KEGG database. We found that the genes located in the T6SS locus were unique within *B. cenocepacia* except for *tssF* and *tssH*. Each of them has two paralogs BCAL1293/BCAS0668 (in chromosome 3) and BCAL1919/BCAL2730, respectively. BCAL1919 and BCAL2730 were not further investigated as they encode the well-characterized ClpB heat-shock protein and ATP-

binding subunit ClpA from the ATP-dependent Clp protease, respectively. Interestingly, BCAL1293 and BCAS0668 were located immediately next to a VgrG encoding gene (BCAL1294 and BCAS0667). The amino acid sequences of BCAL1293 and BCAS0668 are 42% and 40% identical (58% and 54% similar) to TssF, respectively (Fig. S1). BCAL1293 and BCAS0668 were individually deleted in Δ*atsR* and mutants were tested for T6SS activity in our macrophage infection model. In contrast to Δ*atsR*Δ*tssF*, Δ*atsR*ΔBCAL1293 and Δ*atsR*ΔBCAS0668 could elicit morphological changes in macrophages at similar levels to Δ*atsR* (Fig. 2). Introduction of plasmid pL0345 (encoding TssF) into Δ*atsR*ΔBCAL0345 restored T6SS activity, however introduction of plasmids pL1293 or pS0668 (expressing BCAL1293 or BCAS0668, respectively) did not (Fig. 2). These results suggest that although BCAL1293 or BCAS0668 are paralogs of the critical core component TssF, they are not required for T6SS activity under the conditions assayed here, as they cannot functionally replace TssF.

Identification and characterization of the VgrGs of *B. cenocepacia* K56-2

The N-terminus of a VgrG element contains two conserved domains, Phage_GPD and Phage_base_V (former DUF586), which are related to the bacteriophage T4 tail spike protein gp27 (Pukatzki *et al.*, 2007). Computer-assisted analysis using the sequence of the Phage_base_V motif from VgrG2 (VCA0018 from *V. cholerae*) as query identified ten VgrGs (BCAL1165, BCAL1294, BCAL1355, BCAL1359, BCAL1362, BCAL2279, BCAM0043, BCAM0148, BCAM2254, and BCAS0667) in the sequenced genome of *B. cenocepacia* K56-2 (Varga *et al.*, 2013)(GenBank accession number NZ_ALJA00000000.2) (Fig. 3). The *vgrGs* were scattered over the three chromosomes

and none of them was localized in or at the vicinity of the T6SS cluster. BCAM2279 has an insertion sequence (IS) inserted at the very end of the gene; however, the encoded VgrG might still be functional since only the last 17 amino acids are missing and replaced by 22 amino acids provided by the IS, which are followed by a stop codon. Psi-BLAST and PFAM analysis revealed that these VgrGs also contains the Phage_GPD domain and other motifs classically found in VgrG proteins such as T6SS_Vgr, Gp5_C repeats and DUF2345 (Fig. 3). None of them displayed C-terminal extensions with homologies to domains found in previously characterized "evolved" VgrGs (e.g. actin-crosslinking domain, ADP-ribosylation domain, peptidoglycan-binding domain). Instead, conserved domain predictions identified a C-terminal extension in the two largest VgrGs, BCAL1359 (1233 aa) and BCAS0667 (999 aa), which carry a M6 family metalloprotease domain with a characteristic HExxH motif (E-value: 7.18e-03) and a putative lipase_3 domain (GxSxG motif, E-value: 2.8e-02) found in triglyceride lipase, respectively.

Each *vgrG* gene was individually deleted in *B. cenocepacia* Δ*atsR* and growth curves analyses indicated that all mutants had similar growth rates (data not shown). Mutants were then tested for their ability to induce cytoskeleton rearrangements in infected macrophages and to export Hcp into culture supernatants (Fig. 4a-b). Single *vgrG* deletions did not affect either Hcp export or morphological changes in macrophages denoting T6SS activity, suggesting that none of the individual VgrGs are critical for T6SS function. Although we cannot rule out that the evolved VgrGs BCAL1359 and BCAS0667 are T6SS effectors, they can be excluded from an involvement in actin cytoskeleton alterations. A VgrG-less strain was created in *B. cenocepacia* Δ*atsR*.

Deletion of the 10 *vgrG* genes resulted in a non-functional T6SS unable to cause the actin cytoskeleton phenotype in infected macrophages or export Hcp (Fig. 4c), indicating that multiple VgrGs are nevertheless required for the changes in macrophage morphology that are characteristics of *B. cenocepacia* T6SS function.

DISCUSSION

This study aimed to characterize the *B. cenocepacia* T6SS using two assays indicative of T6SS function: Hcp secretion and quantification of the morphological defects in infected macrophages. Deletion of most genes in the *B. cenocepacia* T6SS cluster resulted in strains unable to export Hcp. These results were not surprising since with the exception of BCAL0340, orthologs of all the genes annotated as critical components in our study were also identified as genes encoding conserved core subunits essential for the T6 secretory functions in other bacteria (Durand *et al.*, 2012; English *et al.*, 2014; Zheng & Leung, 2007; Zheng *et al.*, 2011; Zoued *et al.*, 2013). However, one major difference concerned the *tssJ*-like BCAL0339. In enteroaggregative *E. coli*, *V. cholerae* and *Edwardsiella tarda*, *tssJ* encodes an outer membrane lipoprotein that interacts with TssM and is critical for Hcp export (Aschtgen *et al.*, 2008; Felisberto-Rodrigues *et al.*, 2011; Zheng & Leung, 2007; Zheng *et al.*, 2011). In *B. cenocepacia*, $TssJ_{Bc}$ is not essential, although it is required for optimum Hcp export and T6SS activity. While this remains to be tested, it is possible that an additional component encoded within the *B. cenocepacia* T6SS cluster exerts a function similar to that of $TssJ_{Bc}$. BCAL0340 (NOG73587) could be such a candidate. Like $TssJ_{Bc}$, BCAL0340 is predicted to be a lipoprotein, and contains tetratricopeptide repeats indicating that it is likely involved in protein-protein interaction. BCAL0340 is highly conserved among the T6SS clusters in *Burkholderia* species but infrequently found in T6SS clusters from other bacteria (Boyer *et al.*, 2009). It is also absent from the T6SS clusters from enteroaggregative *E. coli, V. cholerae* and *Edwardsiella tarda.* In contrast to $TssJ_{Bc}$, our results demonstrate that BCAL0340 is a critical component of the *B. cenocepacia* T6SS, since deletion of BCAL0340 abolished

392 Hcp export and also prevented the "beads on a string" phenotype in infected
393 macrophages.
394
395 Like BCAL0340, BCAL0352 is another gene not frequently found within T6SS clusters
396 (Boyer *et al.*, 2009), and it is also highly conserved among the T6SS clusters of
397 *Burkholderia* species. BCAL0352 (NOG43466) encodes a putative membrane anchored
398 M15C metallopeptidase possibly with L-alanyl-D-glutamate endopeptidase activity,
399 which suggests an involvement in peptidoglycan degradation that is reminiscent of the
400 Tse toxins involved in bacterial competition (Russell *et al.*, 2011). However, the effect of
401 BCAL0352 deletion on Hcp export, the presence of a N-terminal signal peptide and one
402 transmembrane domain in the encoded protein argue against a putative effector role.
403 Although previous data in our laboratory indicated that BCAL0352 and BCAL0351 are
404 co-transcribed (Aubert & Valvano, unpublished), it is also possible that deletion of
405 BCAL0352 affects additional promoter sequences required for optimum T6SS
406 expression.
407
408 TagF (BCAL0350) is conserved in 30% of the identified T6SS clusters (Boyer *et al.*,
409 2009), but it was dispensable for T6SS activity in *B. cenocepacia*. Interestingly, deletion
410 of *tagF* led to decreased endogenous Hcp pool and a corresponding increase in Hcp
411 release into culture supernatants. These observations suggest that TagF might have a role
412 in the turnover of the Hcp channel. TagL (BCAL0349) is conserved in 25% of the T6SS
413 clusters (Boyer *et al.*, 2009) and encodes a putative outer membrane protein with an
414 OmpA motif (E value 4.4e-25) and the PF0691 motif, which is characteristic of cell wall

binding protein components of T6SSs (Aschtgen *et al.*, 2010). TagL may have an accessory function, which can be important for the proper production, assembly, or activity of the T6S apparatus. Further, our results ruled out the small ORFs (BCAL0333-BCAL0336) as genes encoding either potential T6SS effectors involved in the eukaryotic phenotype or components influencing T6SS functioning. Therefore, we have reassigned the boundaries of the *B. cenocepacia* T6SS cluster to only include 16 genes (BCAL0352-*tssL*)(Fig. 1a).

Individual deletion of any of the 10 VgrGs identified in *B. cenocepacia* K56-2 did not alter Hcp export or the ability of the mutant strain to produce morphological changes in macrophages, suggesting these proteins have redundant function. Whether the two VgrGs with a C-terminal extension (BCAL1359 and BCAS0667) are effectors involved in other T6SS functions such as bacterial competition will require additional experimentation. VgrGs are also structural components of the T6S machinery and as part of the base-plate complex they are required for the initiation of the Hcp tube polymerization (Basler *et al.*, 2012). As expected, deletion of the 10 *vgrGs* in Δ*atsR* abolished Hcp export and T6SS activity. Although the effect of successive *vgrG* deletions was not investigated, these results confirm that at least several of the *B. cenocepacia* VgrGs are critical for the T6SS function. Most of the 10 VgrGs likely have redundant functions, suggesting their relevance in T6SS assembly presumably depends on their nature and relative expression levels. In summary, we have characterized core components of the *B. cenocepacia* T6SS. Our results indicate that none of these components are directly responsible for inducing actin cytoskeletal changes in macrophages. However, the quantitative approach we have

438 developed to investigate macrophage cell morphology can be adapted for screening
439 random mutants in the search for one or more T6SS effector proteins acting on Rho-type
440 GTPases, which is currently underway in our laboratory.
441
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447

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Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source and/or reference
B. cenocepacia		
K56-2	ET12 clone related to J2315, CF clinical Isolate	[b]BCRRC, (Mahenthiralingam *et al.*, 2000)
K56-2 Δ*atsR*	Deletion of *atsR* in K56-2	(Aubert *et al.*, 2010)
K56-2 Δ*atsR* Gm^S	Deletion of BCAL1674-76 in K56-2 Δ*atsR* - Gm^S	This study
K56-2 Δ*atsR* ΔBCAL1165	Deletion of BCAL1165 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL1293	Deletion of BCAL1293 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL1294	Deletion of BCAL1294 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL1355	Deletion of BCAL1355 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL1359	Deletion of BCAL1359 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL1362	Deletion of BCAL1362 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL2279	Deletion of BCAL2279in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAM0043	Deletion of BCAM0043 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAM0148	Deletion of BCAM0148 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAM2254	Deletion of BCAM2254 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAS0667	Deletion of BCAS0667 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAS0668	Deletion of BCAS0668 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL0352	Deletion of BCAL0352 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL0351	Deletion of BCAL0351 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL0350	Deletion of BCAL0350 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL0349	Deletion of BCAL0349 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL0348	Deletion of BCAL0348 *(bcsE)* in K56-2 Δ*atsR*	(Aubert *et al.*, 2010)
K56-2 Δ*atsR* ΔBCAL0347	Deletion of BCAL0347 *(bcsF)* in K56-2 Δ*atsR*	(Aubert *et al.*, 2010)
K56-2 Δ*atsR* ΔBCAL0346	Deletion of BCAL0346 *(bcsG)* in K56-2 Δ*atsR*	(Aubert *et al.*, 2010)
K56-2 Δ*atsR* ΔBCAL0345	Deletion of BCAL0345 *(bcsH)* in K56-2 Δ*atsR*	(Aubert *et al.*, 2010)
K56-2 Δ*atsR* ΔBCAL0344	Deletion of BCAL0344 *(bcsI*) in K56-2 Δ*atsR*	(Aubert *et al.*, 2010)
K56-2 Δ*atsR* Δ*hcp*	Deletion of BCAL0343 in K56-2 Δ*atsR*	(Aubert *et al.*, 2010)
K56-2 Δ*atsR* ΔBCAL0342	Deletion of BCAL0342 *(bcsK)* in K56-2 Δ*atsR*	(Aubert *et al.*, 2010)
K56-2 Δ*atsR* ΔBCAL0341	Deletion of BCAL0341 *(bcsL*) in K56-2 Δ*atsR*	(Aubert *et al.*, 2010)
K56-2 Δ*atsR* ΔBCAL0340	Deletion of BCAL0340 *(bcsM*) in K56-2 Δ*atsR*	(Aubert *et al.*, 2010)
K56-2 Δ*atsR* ΔBCAL0339	Deletion of BCAL0339 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL0338	Deletion of BCAL0338 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL0339	Deletion of BCAL0337 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* ΔBCAL0336-33	Deletion of BCAL0336-0333 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* Δ10*vgrGs*	Deletion of BCAL1165, 1294, 1355, 1359, 1362, 2279, BCAM0043, 0148, 2254 and BCAS0667 in K56-2 Δ*atsR* - VgrG-less strain	This study
E. coli		
BL21 (DE3)	F- *ompT hsdSB*(r_B^- m_B^-) *gal dcm*(λDE3)	Laboratory stock
DH5α	F- ϕ80*lacZ* M15 *endA1 recA1 supE44 hsdR*17(r_K^- m_K^+) *deoR thi-1 nupG supE44 gyrA96 relA1* Δ(*lacZYA-argF*)*U169*, λ–	Laboratory stock
GT115	F– *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80Δ*lacZ*ΔM15 Δ*lacX74 recA1 rpsL* (StrA) *endA1*Δ*dcm uidA*(ΔMluI)::*pir-116* Δ*sbcC-sbcD*	Invivogen
Plasmids		
pL0345	BCAL0345 cloned into pDA12	This study
pL1293	BCAL1293 cloned into pDA12	This study
pS0668	BCAS0668 cloned into pDA12	This study
pDA12	cloning vector, ori_{pBBR1}, Tet^R, mob^+, P_{dhfr}	(Aubert *et al.*, 2008)
pDA44	hcp_{6xHis} cloned into pET30a	This study

pDAI-SceI-SacB	ori_{pBBR1}, Tet^R, P_{dhfr}, mob^+, expressing I-SceI, SacB	(Hamad *et al.*, 2010)
pDelL0352	pGPI-SceI with fragments flanking BCAL0352	This study
pDelL0351	pGPI-SceI with fragments flanking BCAL0351	This study
pDelL0350	pGPI-SceI with fragments flanking BCAL0350	This study
pDelL0349	pGPI-SceI with fragments flanking BCAL0349	This study
pDelL0339	pGPI-SceI with fragments flanking BCAL0339	This study
pDelL0338	pGPI-SceI with fragments flanking BCAL0338	This study
pDelL0337	pGPI-SceI with fragments flanking BCAL0337	This study
pDelL0036-33	pGPI-SceI with fragments flanking BCAL0336-0333	This study
pDelL1165	pGPI-SceI with fragments flanking BCAL1165	This study
pDelL1293	pGPI-SceI with fragments flanking BCAL1293	This study
pDelL1294	pGPI-SceI with fragments flanking BCAL1294	This study
pDelL1355	pGPI-SceI with fragments flanking BCAL1355	This study
pDelL1359	pGPI-SceI with fragments flanking BCAL1359	This study
pDelL1362	pGPI-SceI with fragments flanking BCAL1362	This study
pDelL2279	pGPI-SceI with fragments flanking BCAL2279	This study
pDelM0043	pGPI-SceI with fragments flanking BCAM0043	This study
pDelM0148	pGPI-SceI with fragments flanking BCAM0148	This study
pDelM1857	pGPI-SceI with fragments flanking BCAM1857	This study
pDelM2254	pGPI-SceI with fragments flanking BCAM2254	This study
pDelS0667	pGPI-SceI with fragments flanking BCAS0667	This study
pDelS0668	pGPI-SceI with fragments flanking BCAS0668	This study
pET30a	Expression vector, Kan^R	Novagen
pGPI-SceI	$ori_{R6K,}$ Tp^R , mob^+, including an I-SceI restriction site	(Flannagan *et al.*, 2008)
pMH447	pGPI-SceI with fragments flanking BCAL1674-1676	(Hamad *et al.*, 2010)
pRK2013	$ori_{colE1,}$ RK2 derivative, Kan^R, mob^+, tra^+	(Figurski & Helinski, 1979)

[a] Gm^S, gentamicin sensitive, Kan^R, kanamycin resistance, Tet^R, tetracycline resistance, Tp^R, trimethoprim resistance.
[b] BCRRC, *B. cepacia* Research and Referral Repository for Canadian CF Clinics.

FIGURE LEGENDS

Fig.1. Investigation of the *B. cenocepacia* T6SS gene cluster. (a) Genetic map of the *B. cenocepacia* K56-2 T6SS gene cluster. The arrows represent the location and direction of transcription of each gene. The "BCAL" locus tags assigned by the Sanger Center are shown above and the standard *tss* or *tag* annotation of the genes (listed in Supplemental Table S2) is shown below. White, grey and black arrows represent the critical components, accessory proteins and dispensable elements for T6SS activity identified in this study, respectively. The genes encoding the well-characterized components IcmF (BCAL0351; $TssM_{Bc}$), ClpV (BCAL0347; $TssH_{Bc}$), and Hcp (BCAL0343; $TssD_{Bc}$) and location of the tRNA threonine (tRNA-Thr) sequence are shown. (b) Phase-contrast microscopy of infected ANA-1 macrophages and qualitative assessment of T6SS activity. The infections were performed at an MOI of 50:1 for 4 h. The presence of ectopic structures is indicative of expression and functionality of the T6SS. Characteristic 'beads' surrounding infected macrophages are shown by white arrows. Infections were repeated independently and reproducible results were obtained. The pictures shown are representatives. (c) Measurement of T6SS activity. The proportion of dark "beads on a string-like" structures around macrophages was measured using image analysis software. Results were expressed in arbitrary units relative to Δ*atsR* set as 1 (white bar). Values are mean ± standard deviation for at least 21 fields of view and are representative of three independent experiments. Uninfected cells were used as negative control to determine the background level. The dotted line indicates the 0.2 relative units threshold indicative of no "beads on a string-like" structures. ***, $p < 0.001$ compared to the relative units of

uninfected cells (see Methods for details on the statistical analysis). (d) Western blot analysis of total cell lysates (Pellet) and concentrated culture supernatants recovered from Δ*atsR* and T6SS mutants using anti-RNAP α subunit (cytosolic protein, cell lysis control) and anti-Hcp antibodies. The upper band seen in the pellet fractions with the anti-Hcp antibody corresponds to a cross-reacting unspecific protein.

Fig.2. Investigation of the *tssF* (BCAL0345) paralogs. Phase-contrast microscopy of infected ANA-1 macrophages and qualitative assessment of T6SS activity. The infections were performed at an MOI of 50:1 for 4 h with *B. cenocepacia* K56-2 Δ*atsR*, derivative mutants in *tssF* or paralogs (BCAL1293 and BCAS0668) and mutants carrying plasmids pL0345, pL1293 or pS0668, which express TssF, BCAL1293 and BCAS0668, respectively. Formation of ectopic structures indicates T6SS functionality. Infections were repeated independently and reproducible results were obtained. The pictures shown are representatives.

Fig. 3. Putative conserved domains detected within the VgrGs of *B. cenocepacia* K56-2. Conserved domains within VgrGs were detected using PFAM search (http://pfam.sanger.ac.uk). The VgrG2 (VCA0018) from *Vibrio cholerae* was used as query to identify the VgrGs from *B. cenocepacia* K56-2 and is shown here for comparison. Domains are as follows: Phage_GPD (green), Phage_base_V (red), T6SS_Vgr (blue), DUF2345 (yellow). The putative effector domains found at the C-terminus of BCAL1359 and BCAS0667 are represented with a grey (M6 family metalloprotease domain) and brown (lipase_3 domain) circle, respectively.

Fig. 4. Investigation of the *B. cenocepacia* VgrGs. (a) Measurement of T6SS activity. The infections were performed at an MOI of 50:1 for 4 h with *B. cenocepacia* K56-2 Δ*atsR* and derivative *vgrG* mutants. The proportion of dark "beads on a string-like" structures around macrophages was measured as described in Methods and in the Fig. 1 legend. The dotted line indicates the 0.2 relative units threshold indicative of no "beads on a string-like" structures. All the single *vgrG* deletion strains induced "beads on a string-like" structures at a similar level as Δ*atsR* (no statistically significant differences) and significantly different levels ($p < 0.001$) from uninfected cells. (b) and (c) Western blot analysis of total cell lysates (Pellet) and concentrated culture supernatants recovered from *B. cenocepacia* K56-2 Δ*atsR*, Δ*atsR*Δ*hcp*, *vgrG* mutants and from the *vgrG*-less strain K56-2 Δ*atsR*Δ10*vgrG*s using anti-RNAP α subunit (cytosolic protein, cell lysis control) and anti-Hcp antibodies.

Figure 1

Click here to download Figure: Aubert_T6SSactivity_m-Fig1-revised.eps

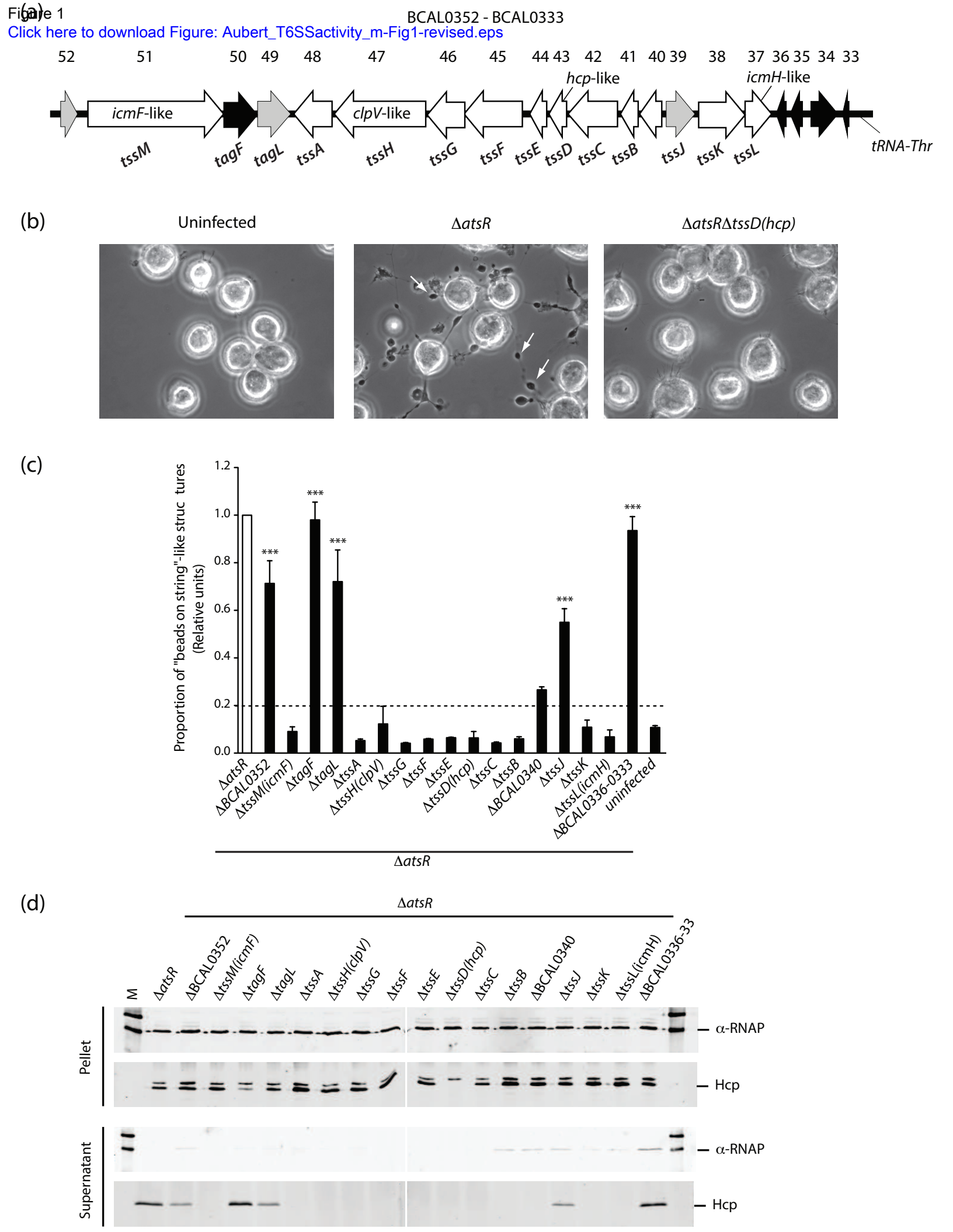

Figure 2

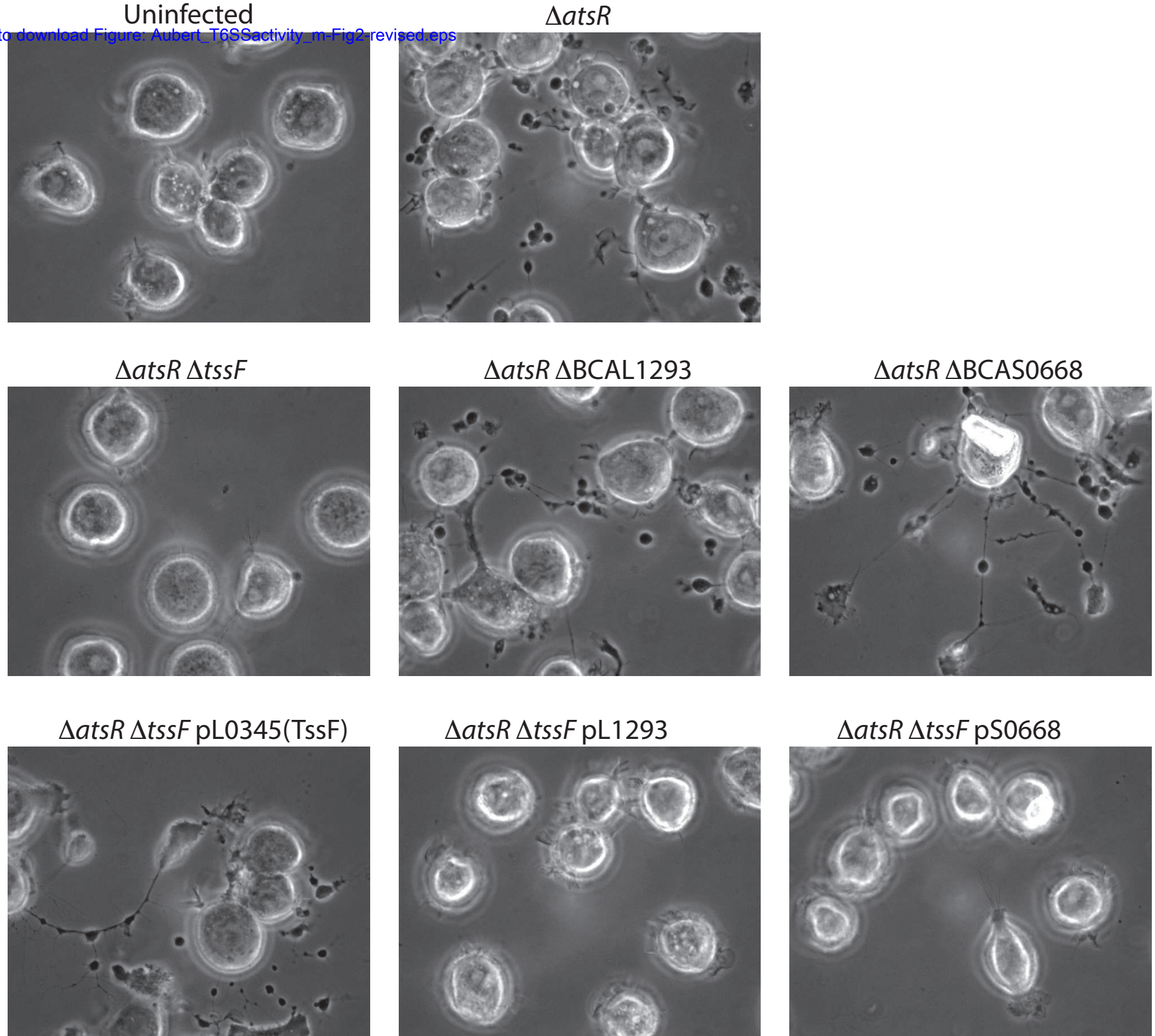

Figure 3
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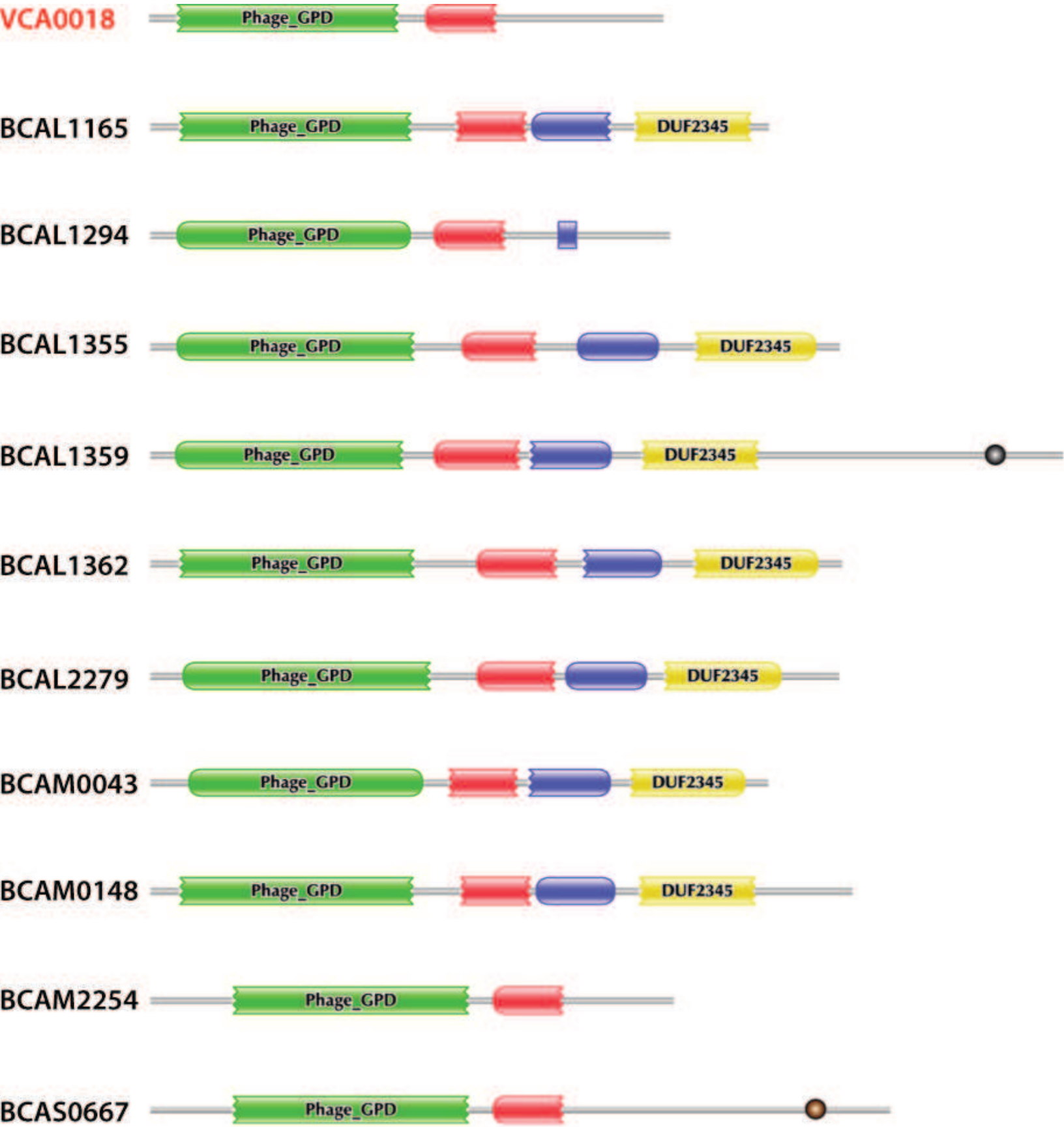

Figure 4

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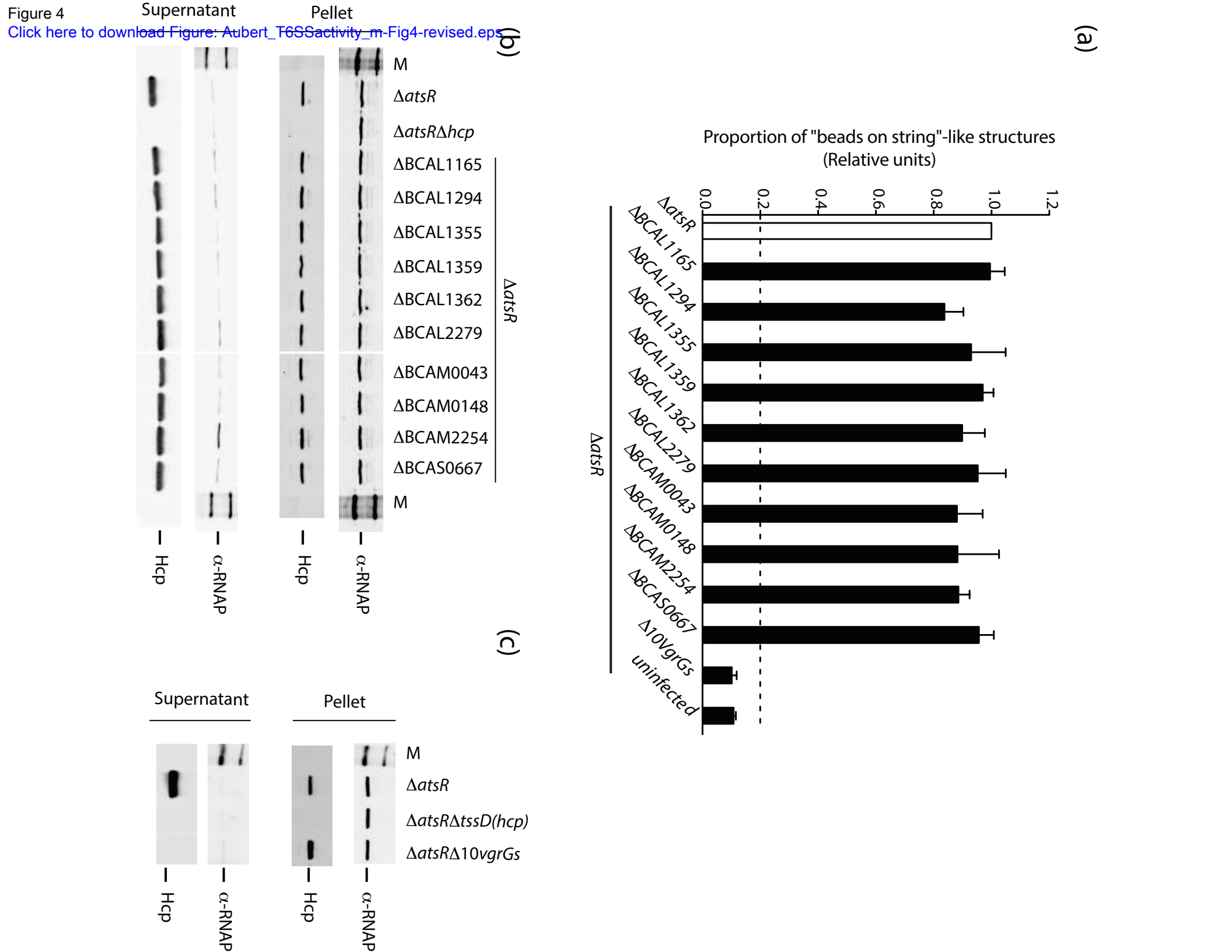

SUPPLEMENTAL DATA

Quantification of Type VI secretion system activity in macrophages infected with *Burkholderia cenocepacia*

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Table S1

Oligonucleotide primers

Primer No.	5'-3' Primer sequence	Restriction enzyme*
430	TGTGGCTGCACTTGAACG	N/A
2143	AGTCGAAGGCATATGTTACACATGCACTTGCAG	NdeI
2748	TCGAAAGCTTGACCGCGTAGGTCTTGTCGT	HindIII
2863	TTTACCCGGGTCTTGACAGGGACGATTTCC	SmaI
2867	TACGTCTAGAATGAGCTCAAGCGGCTGT	XbaI
3059	TTTTTCTAGAAGATACGCAACGGCGATAAC	XbaI
3065	TTTTCTCGAGATTGCTCTGGAGCTGGAAGA	XhoI
3066	TTTTCTCGAGATTGAGTTGCGAACCGGTAA	XhoI
3068	TTTTGAATTCGCAGTCCTTTGTCGATGACC	EcoRI
3077	TAGGTACCATTCACAAGAAGGATTCGA	KpnI
3080	TAGGTACCTTAGGCCACACGTTCAAG	KpnI
3177	TTAACTCGAGCGACGTCTTGATCGTCTTCA	XhoI
3201	GCTACGGTCTCGAGAAGCTG	XhoI
3202	AAAAGAATTCAGCCGAGAAACGCGAAAT	EcoRI
3203	TTTTTCTAGAATCTCACAGCGTCCTTTCGT	XbaI
3263	TTTACTCGAGTCGATGGTGAGAGCGTGAC	XhoI
3267	ATTACTCGAGATTCGGGGCGAAACTCAT	XhoI
3349	TTTATCTAGAAGGACGATGAAGGGTTTGGT	XbaI
3354	TAATCTCGAGATTGAATGCGCCAAAAAGAG	XhoI
3454	TTTTTCTAGAGGCGACGATGAACAGAATG	XbaI
3457	TTTTGAATTCGCCGGTTTAGTATCGGACAG	EcoRI
3458	TTTTCTCGAGCGTGTCGTCGTACTGGAACA	XhoI
3465	TTTTCTCGAGTACCAGTTGTACGGCCAGGT	XhoI
3759	TTTTTCTAGACGTCGCCTCTCTATTGCTCT	XbaI
3760	TTTTGCGGCCGCATGCGGGATCAAGACGTTAC	NotI
3761	AAAAGCGGCCGCGCGAATCAGGGGTTTCAGTA	NotI
3762	AAAAGAATTCTCAAATACCCGCTCCTCATC	EcoRI
3800	AAAAGAATTCGTTGGCATGTGACTGTCTGG	EcoRI
3801	AAAACTCGAGAGCTTAACCACGAAGCGAAA	XhoI
3947	TTTTCTCGAGTAGCGAATCATGGACGATCT	XhoI
3948	TTTTTCTAGATGTTGAACACTTCCGCAGTC	XbaI
4125	TAATGAATTCCCGTGTCGATGAAATTGATG	EcoRI
4126	TAATGCGGCCGCAAAACGGGGTTCGAATGAAT	NotI
4127	TTTAGCGGCCGCGCGTCGCATACGTTCATCG	NotI
4128	TTTTTCTAGAATCACGCTCTCCATCTGCTC	XbaI
4134	TTTTGAATTCCTGAGATCACACGCAAGGAA	EcoRI
4135	TTTTCTCGAGGACGTAGCCGTCGAAGTAGC	XhoI
4136	TTTTCTCGAGCTGGAAGCCAAGGAACTCTG	XhoI
4137	TTTTTCTAGAAGGTGAGCATCAGGACAACC	XbaI
4185	AAAACTCGAGGGTCAGTGTGCTCGACAAG	XhoI
4186	AAAATCTAGAATGCCAGCGCCTTCATATAG	XbaI
4305	TTTTGAATTCGCCGACTTCGAAAGAAAGTG	EcoRI
4306	TTTTGCGGCCGCTCAGCTGGTAGCAGGTCAAC	NotI
4308	TTTTGCGGCCGCAACCTGAGCCGAGTCTTCAA	NotI
4309	TTTTTCTAGAACCGCCCCAGTAATTCTTCT	XbaI
4893	TTTTGAATTCAATTGCCGAGGTTCACAATC	EcoRI
4894	TTTTCTCGAGGCTACGTTCAACTCGTCGTG	XhoI

4895	TTTTCTCGAGCAAGCAGTCCGACTTCACGTT	XhoI
4896	TAATTCTAGATACATCGCAAAACCACCATC	XbaI
4897	TTTTTTCATATGTGAATATTGCAGCGAATG	NdeI
4898	TTTTTCTAGACTATCTCCACTGACCTATAC	XbaI
4899	TTTTGAATTCCCTTTATTTTCGTCTCAGG	EcoRI
4900	TTTTCTCGAGGCGTTCGTCCAAGGATTG	XhoI
4901	TTTTCTCGAGCGAGTGCTCACCTGACATTGC	XhoI
4902	TTTATCTAGAATGCCGAGAAGTTTCGAGAG	XbaI
4903	TATATTCATATGGCGTGACCGTCTGAAACG	NdeI
4904	TTTTTCTAGACTCTTCCGTCACCAGTCA	XbaI
4905	AAAAAACATATGGCAGGAAATATTGAGGTTC	NdeI
4906	AAAATCTAGAACTACGCCAGGATCGATTC	XbaI
5015	TTATCTCGAGCAGTTTCCCGAAAAGGTCTG	XhoI
5016	TTATGAATTCGAGACGCACCAGAAATGCTT	EcoRI
5017	TTTTGAATTCTGGAACCACAACCAGATCAA	EcoRI
5018	TTTTCTCGAGATGCGGCAACTAGATCCAT	XhoI
5019	TTTTCTCGAGACATCAACTCGCGACATCTG	XhoI
5020	TTTTTCTAGAGTACGTGCCCTCGTAATGGT	XbaI
5021	GAACTCGAATTCAGGCGATG	EcoRI
5022	TTTTCTCGAGACCTGCCATTCGATGAGGAT	XhoI
5023	TTTTCTCGAGGATGGAAGGCAACGACATC	XhoI
5024	TTTTTCTAGAAGTTTCGGCACGAGTTCATC	XbaI
5025	TTTTCTCGAGGCGCGTTTCATCATCGAC	XhoI
5026	TTTTTCTAGAAGATCGCGCACATGAAGG	XbaI
5177	TTTTGAATTCGTCGGGCAACGTATCCAGT	EcoRI
5178	TTTTCTCGAGTATGCTGTCTTCGGTCTCCTG	XhoI
5179	TTTTCTCGAGGTCGGGCAACGTATCCAGT	XhoI
5180	TTTTGAATTCTATGCTGTCTTCGGTCTCCTG	EcoRI
5181	TTTTTCTAGAGATCCGCGTGCTGAACTC	XbaI
5182	TTTTCTCGAGGACGACGTCGACAAGCTC	XhoI
5183	TATAGAATTCGATCGACGACAAGACCGAGT	EcoRI
5184	TATACTCGAGAACCCGTCGTACAGCAAATC	XhoI
5185	TTTTTCTAGAACGTCAGCTGGTTCTTCCAC	XbaI
5186	TTTTCTCGAGGTACCTCGGGCTCAACACGTA	XhoI
5187	TTTTGAATTCATGGCACCTTCGACCTGAC	EcoRI
5188	TTTTCTCGAGGGCTTCGCATCCTCGTAGAC	XhoI
5189	TTTTCTCGAGCGTCAGCTACCTCGACCAACC	XhoI
5190	TTTTTCTAGAGTAGGTGTCGCGCAGCTTCT	XbaI
5191	TTTTGAATTCGTCAGCTACCTCGACCAACC	EcoRI
5192	TTTTCTCGAGGTAGGTGTCGCGCAGCTTCT	XhoI
5193	TTTTCTCGAGGGCAAGTTGATCCTCGACCAG	XhoI
5194	TTTTTCTAGAGGCTCGCTGTCGCATATT	XbaI
5333	TTTTGAATTCTCGTGCTCAACATCAGCTTC	EcoRI
5334	TTTTCTCGAGCCGTAACCCTTTTTCGGATT	XhoI
6066	TTTTGAATTCACCGTACTCGACCCTTCCTT	EcoRI
5887	CTGTCATGCCATCCGTAAGA	N/A
6207	TTTTTTCATATGGAAAGGAGCTATCCGTGCAG	NdeI
6208	TTTTTCTAGACCTACAGGCGCGACAGAT	XbaI

*****Restriction endonuclease sites incorporated in the oligonucleotide sequences are underlined.
N/A indicates absence of restriction site.

Table S2.

Annotation of T6SS cluster genes in *B. cenocepacia* K56-2

BCAL Number	Standard Annotation[a]	Predicted function[b]
0352		Putative bacteriophage-like L-alanyl-D-glutamate peptidase; highly conserved in *Burkholderia* T6SSs (Boyer *et al.*, 2009).
0351	tssM	IcmF-like, inner membrane protein with a cytoplasmic region that has ATPase activity. It makes a complex with TssL and TagL (Felisberto-Rodrigues *et al.*, 2011).
0350	tagF	SciT domain, DUF2094, unknown function.
0349	tagL	OmpA-like peptidoglycan binding domain lipoprotein; PF0691 (Aschtgen *et al.*, 2010)
0348	tssA	Cytoplasmic protein (Cascales & Cambillau, 2012).
0347	tssH	ClpV-ATPase for the sheath assembly/disassembly (Bonemann *et al.*, 2009).
0346	tssG	Recruited to the membrane by TssK. (English *et al.*, 2014; Zoued *et al.*, 2013).
0345	tssF	Recruited to the membrane by TssK
0344	tssE	Similarity with the phage gp25 baseplate protein
0343	tssD	hcp-like, hexameric inner tube (Zoued *et al.*, 2014)
0342	tssC	Phage-like sheath subunit (Aubert *et al.*, 2010)
0341	tssB	Phage-like sheath subunit (Aubert *et al.*, 2010)
0340		Outer membrane lipoprotein, NlpI lipoprotein domain. Highly conserved in *Burkholderia* T6SSs (Boyer *et al.*, 2009)
0339	tssJ	Outer membrane lipoprotein (Felisberto-Rodrigues *et al.*, 2011).
0338	tssK	ImpJ, VasE trimeric complex interacts with TssF and TssG (English *et al.*, 2014; Zoued *et al.*, 2013).
0337	tssL	IcmH-like, dot U, inner membrane (Durand *et al.*, 2012).
0336		DUF4262, conserved in many bacterial and viral proteins. Not part of the *B. cenocepacia* T6SS (this work)
0335		Conserved hypothetical protein. Not part of the *B. cenocepacia*

	T6SS (this work)
0334	Putative amino-acid transporter periplasmic solute protein. Not part of the *B. cenocepacia* T6SS (this work)
0333	Putative "winged helix" DNA binding protein. Not part of the *B. cenocepacia* T6SS (this work)

[a] Based on Shalom *et al.* (2007)
[b] Based on BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and HHpred (http://toolkit.tuebingen.mpg.de/hhpred) searches.

SUPPLEMENTARY METHODS

Details of the construction of mutagenic and complementing plasmids

Mutagenesis of *B. cenocepacia* K56-2. Unmarked and non-polar deletions were performed as described previously (Flannagan *et al.*, 2008). Amplicons were digested with the appropriate restriction enzymes and cloned into plasmid pGPI-SceI (see below for details). Mobilization of mutagenesis plasmids into *Burkholderia* strains was performed by triparental mating using *E. coli* DH5α carrying the helper plasmid pRK2013 (Craig *et al.*, 1989; Figurski & Helinski, 1979). Gene deletions were confirmed by PCR. Deletion of BCAL1674-76 was performed using plasmid pMH447 and resulted in K56-2 gentamicin sensitive strains.

Deletion of genes localized in the T6SS cluster. To delete BCAL0352, PCR amplifications of regions flanking BCAL0352 were performed using 3454-3465 and 3458-3457 primer pairs. The amplicons were digested with the restriction enzymes XbaI-XhoI and XhoI-EcoRI, respectively, and cloned into the mutagenic plasmid pGPI-SceI digested with XbaI and EcoRI giving rise to pDelL0352. Several deletion plasmids were created using a similar approach. To create pDelL0351, pDelL0350, pDelL0349, pDelL0339, pDelL0338, pDelL0337, and pDelL0336-33 (to delete BCAL0351, 0350, 0349, 0339, 0338, 0337 and BCAL0336-0333, respectively), PCR products were amplified using primers 3203-3177 and 3201-3202; 5190-5189 and 5188-5187; 5194-5193 and 5192-5191; 3948-3947 and 5178-5177; 5181-5182 and 5179-5180; 5185-5186 and 5184-5183; 5026-5025 and 5334-5333, respectively.

Deletion of BCAL0345 paralogs. To create pDelL1293 and pDelS0668 (to delete BCAL1293 and BCAS0668, respectively), PCR products were amplified using primers 4896-4895 and 4894-4893; 4902-4901 and 4900-4899, respectively.

Deletion of *vgrGs*. To create pDelL1165, pDelL1294, pDelL1355, pDelL1359, pDelL1362, pDelL2279, pDelM0043, pDelM0148, pDelM2254 and pDelS0667 (to delete BCAL1165, 1294, 1355, 1359, 1362, 2279, BCAM0043, 0148, 2254 and BCAS0667, respectively), PCR products were amplified using primers 3800-3801 and 4185-4186; 3059-3065 and 3066-3068; 3349-3354 and 5015-5016; 5017-5018 and 5019-5020; 5021-5022 and 5023-5024; 4305-4306 and 4308-4309; 4125-4126 and 4127-4128, 3759-3760 and 3761-3762; 4134-4135 and 4136-4137, 2863-3263 and 3267-2867, respectively.

Complementing plasmids. The complementing plasmids pL0345, pL1293, pM1857 and pS0668 were created as follows. BCAL0345, BCAL1293, BCAM1857 and BCAS0668 were PCR amplified using primer pairs 4905-4906, 4897-4898, 6207-6208 and 4903-4904, respectively. PCR products were digested with *Nde*I and *Xba*I and cloned into similarly digested pDA12.

SUPPLEMENTARY FIGURES

Fig. S1 Aubert *et al.*
Sequence alignment of BCAL0345 and paralogs (BCAL1293 and BCAS0668). Sequence alignment was generated using ClustalW (Larkin *et al.*, 2007). Identical (black) and similar (grey) residues were illustrated using BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html).

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