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**Microbiology**

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| Corresponding Author: | Miguel A Valvano  
|                    | Queen's University Belfast  
|                    | Belfast, UNITED KINGDOM |
| First Author:      | Daniel F. Aubert |
| Order of Authors:  | Daniel F. Aubert  
|                    | Sherry Hu  
|                    | Miguel A Valvano |
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Quantification of Type VI secretion system activity in macrophages infected with

*Burkholderia cenocepacia*

Daniel F. Aubert¹, Sherry Hu¹ and Miguel A. Valvano¹,²

1 Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1, Canada;

2 Centre for Infection and Immunity, Queen's University Belfast, BT9 5GZ, Belfast, United Kingdom

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Correspondence: Miguel A. Valvano, m.valvano@qub.ac.uk
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.
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 37°C. *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⁻¹.

**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 OD₆₀₀ 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 *Hind*III restriction sites. This generated plasmid pDA44 encoding Hcp₆xHis, which was introduced into *E. coli* strain BL21 (DE3) by transformation. Overexpression of Hcp₆xHis was performed as follows. *E. coli* cells were grown to an OD₆₀₀ of 0.6, induced with 0.05 mM isopropyl-β-d-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.
Hcp$_{6x\text{His}}$ was purified from filtered supernatant by FPLC (ÄKTA Basic instrument) using a 5 ml HisTrap column (GE Healthcare). Elution was performed using a linear gradient concentration of imidazole (10-400 mM). Fractions containing purified Hcp$_{6x\text{His}}$ were pooled and dialyzed against 50 mM sodium phosphate, pH 7.4, 300 mM NaCl, and stored at 4°C. The eluted Hcp$_{6x\text{His}}$ was judged >90% pure after this step. Polyclonal antibodies recognizing Hcp were generated in New Zealand White rabbits by ProSci Inc. (Poway, CA).

Precipitation of culture supernatant proteins and immunoblot analysis. Culture supernatant proteins were precipitated as described previously (Aubert et al., 2008) with some modifications. Briefly, overnight cultures were diluted to an OD$_{600}$ of 0.03 in pre-warmed LB and grown until early exponential phase, at which time OD$_{600}$ was also recorded. Proteins from filter-sterilized culture supernatants were precipitated overnight at 4 °C using 20% trichloroacetic acid (final concentration). Five μg of secreted proteins were loaded on an 18% SDS-PAGE. The crude lysate sample (pellet fraction) was prepared as follows: bacteria from 1 ml of exponential phase culture adjusted at an OD$_{600}$ of 0.5 were pelleted by centrifugation, resuspended into 30 μl of 1x protein loading dye and boiled for 10 min. Samples were centrifuged for 3 min at 5 900 ×g and 3 μl of total cell lysate were loaded on a 18% SDS-PAGE. After electrophoresis, gels were transferred to nitrocellulose membranes for immunoblot analysis. After blocking (Roche), the membranes were incubated with the following primary antibodies as required. 4RA2 monoclonal antibody (Neoclone), which cross-reacts with the $B. \text{cen}ocepacia$ and $B. \text{multivorans}$ 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% CO₂. 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 ΔatsR 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
µg gentamicin ml\(^{-1}\) was added for the remainder of the experiment. To enumerate intracellular bacteria, infected macrophages were lysed with 0.1\% sodium deoxycholate (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* ΔatsR 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 ΔatsR induces the formation of “beads on a string-like” structures due to lamellipodia collapse and impairment of actin-tail retraction during macrophage migration (Flanagan *et al.*, 2012; Rosales-Reyes *et al.*, 2012) (Fig. 1b). This phenotype depends on a functional T6SS, as ΔatsRΔhcp 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 ΔatsR (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; bcsKc), tssB (BCAL0341; bcsLb), 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 ΔatsR 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 ΔatsR. These results also agree with the observation that these three mutants reproducibly induced lower levels of ectopic structures in macrophages in comparison with ΔatsR (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 ΔatsRΔBCAL0336-33 and ΔatsR culture supernatants. Besides ΔatsRΔhcp, 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 ΔatsR (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 \textit{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, \textit{tagL} and \textit{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 \textit{B. cenocepacia} strain J2315 (http://www.genome.jp/kegg-bin/show_organism?org=bcj), which is a clonal isolate with K56-2 (Mahenthiralingam \textit{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 \textit{B. cenocepacia} except for \textit{tssF} and \textit{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 ΔatsR and mutants were tested for T6SS activity in our macrophage infection model. In contrast to ΔatsRΔtssF, ΔatsRΔBCAL1293 and ΔatsRΔBCAS0668 could elicit morphological changes in macrophages at similar levels to ΔatsR (Fig. 2). Introduction of plasmid pL0345 (encoding TssF) into ΔatsRΔBCAL0345 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 ΔatsR 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 ΔatsR.
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.
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

BCAL0340 (NOG73587) could be such a candidate. Like TssJ

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

BCAL0340 is a critical component of the *B. cenocepacia* T6SS, since deletion of BCAL0340 abolished...
Hcp export and also prevented the "beads on a string" phenotype in infected macrophages.

Like BCAL0340, BCAL0352 is another gene not frequently found within T6SS clusters (Boyer et al., 2009), and it is also highly conserved among the T6SS clusters of *Burkholderia* species. BCAL0352 (NOG43466) encodes a putative membrane anchored M15C metallopeptidase possibly with L-alanyl-D-glutamate endopeptidase activity, which suggests an involvement in peptidoglycan degradation that is reminiscent of the Tse toxins involved in bacterial competition (Russell et al., 2011). However, the effect of BCAL0352 deletion on Hcp export, the presence of a N-terminal signal peptide and one transmembrane domain in the encoded protein argue against a putative effector role. Although previous data in our laboratory indicated that BCAL0352 and BCAL0351 are co-transcribed (Aubert & Valvano, unpublished), it is also possible that deletion of BCAL0352 affects additional promoter sequences required for optimum T6SS expression.

TagF (BCAL0350) is conserved in 30% of the identified T6SS clusters (Boyer et al., 2009), but it was dispensable for T6SS activity in *B. cenocepacia*. Interestingly, deletion of *tagF* led to decreased endogenous Hcp pool and a corresponding increase in Hcp release into culture supernatants. These observations suggest that TagF might have a role in the turnover of the Hcp channel. TagL (BCAL0349) is conserved in 25% of the T6SS clusters (Boyer et al., 2009) and encodes a putative outer membrane protein with an 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 ΔatsR 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
developed to investigate macrophage cell morphology can be adapted for screening random mutants in the search for one or more T6SS effector proteins acting on Rho-type GTPases, which is currently underway in our laboratory.

ACKNOWLEDGEMENTS

The authors thank W. Cladman for purification of the Hcp protein used for immunization and M.S. Saldías and C. Schmerk for critical review of the manuscript. This work was supported by grants from Cystic Fibrosis Canada and the U.K. Cystic Fibrosis Trust (to M.A.V).
REFERENCES


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pDAI-SceI-SacB

*ori*<sub>BBRI</sub>, *P<sub>abp</sub>, *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

pDelL0338

pGPI-SceI with fragments flanking BCAL0338

This study

pDelL0337

pGPI-SceI with fragments flanking BCAL0337

This study

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This study

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pDelS0668

pGPI-SceI with fragments flanking BCAS0668

This study

pET30a

Expression vector, Kan<sup>R</sup>

Novagen

pGPI-SceI

*ori*<sub>BK6</sub>, <sub>T</sub><sup>R</sup>, *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*<sub>BBR1</sub>, RK2 derivative, Kan<sup>R</sup>, *mob*, *tra*

(Figurski & Helinski, 1979)

---

<sup>a</sup> Gm<sup>R</sup>, gentamicin sensitive, Kan<sup>R</sup>, kanamycin resistance, Tet<sup>R</sup>, tetracycline resistance, Tp<sup>R</sup>, trimethoprim resistance.

<sup>b</sup> 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<sub>Bc</sub>), ClpV (BCAL0347; TssH<sub>Bc</sub>), and Hcp (BCAL0343; TssD<sub>Bc</sub>) 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 ΔatsR 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 ΔatsR, 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 ΔatsR 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 ΔatsR (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 ΔatsR, ΔatsRΔhcp, vgrG mutants and from the vgrG-less strain K56-2 ΔatsRΔ10vgrGs using anti-RNAP α subunit (cytosolic protein, cell lysis control) and anti-Hcp antibodies.
Figure 1

(a) Genetic map of BCAL0352 - BCAL0333

(b) Uninfected, ΔatsR, ΔatsRΔtssD(hcp)

(c) Proportion of "beads on string"-like structures (Relative units)

(d) Western blot analysis of Pellet and Supernatant samples under ΔatsR conditions
Figure 4

Proportion of "beads on string"-like structures (Relative units)

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Supernatant

Pellet

M
ΔatsR
ΔatsRΔhcp
ΔBCAL1165
ΔBCAL1294
ΔBCAL1355
ΔBCAL1359
ΔBCAL1362
ΔBCAL2279
ΔBCAM0043
ΔBCAM0148
ΔBCAM2254
ΔBCAS0667

Proportions:
0.0
0.2
0.4
0.6
0.8
1.0
1.2

0
2
4
6
8
10
12

ΔatsR
ΔatssD(hcp)
ΔatsRΔ10vgrGs
SUPPLEMENTAL DATA

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

Daniel F. Aubert¹, Sherry Hu¹ and Miguel A. Valvano¹,²

¹ Department of Microbiology and Immunology, University of Western Ontario, London,
Ontario N6A 5C1, Canada;
² Centre for Infection and Immunity, Queen's University Belfast, BT9 5GZ, Belfast,
United Kingdom
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<td>4306</td>
<td>TTTTGCGCGCCGCTCGAGCTGTGGACTCGAGGTCAAC</td>
<td>NotI</td>
</tr>
<tr>
<td>4308</td>
<td>TTTTGCAGCGAATCTGAGGGCAGGCTTCAA</td>
<td>NotI</td>
</tr>
<tr>
<td>4309</td>
<td>TTTTCTAGAATCCCGCCCGCTCTCTCATTACTC</td>
<td>XbaI</td>
</tr>
<tr>
<td>4893</td>
<td>TTTTGAATTCGACTGCTCGAATCTCGT</td>
<td>EcoRI</td>
</tr>
<tr>
<td>4894</td>
<td>TTTTCTCAGAGCTCGTCAACTCGTCTG</td>
<td>Xhol</td>
</tr>
</tbody>
</table>
**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**

<table>
<thead>
<tr>
<th>BCAL Number</th>
<th>Standard Annotation</th>
<th>Predicted function</th>
</tr>
</thead>
<tbody>
<tr>
<td>0352</td>
<td>352</td>
<td>Putative bacteriophage-like L-alanyl-D-glutamate peptidase; highly conserved in <em>Burkholderia</em> T6SSs (Boyer <em>et al.</em>, 2009).</td>
</tr>
<tr>
<td>0351</td>
<td>tssM</td>
<td>IcmF-like, inner membrane protein with a cytoplasmic region that has ATPase activity. It makes a complex with TssL and TagL (Felisberto-Rodrigues <em>et al.</em>, 2011).</td>
</tr>
<tr>
<td>0350</td>
<td>tagF</td>
<td>SciT domain, DUF2094, unknown function.</td>
</tr>
<tr>
<td>0349</td>
<td>tssA</td>
<td>OmpA-like peptidoglycan binding domain lipoprotein; PF0691 (Aschtgen <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>0348</td>
<td>tssC</td>
<td>Cytoplasmic protein (Cascales &amp; Cambillau, 2012).</td>
</tr>
<tr>
<td>0347</td>
<td>tssD</td>
<td>ClpV-ATPase for the sheath assembly/disassembly (Bonemann <em>et al.</em>, 2009).</td>
</tr>
<tr>
<td>0345</td>
<td>tssF</td>
<td>Recruited to the membrane by TssK</td>
</tr>
<tr>
<td>0344</td>
<td>tssG</td>
<td>Outer membrane lipoprotein, NlpI lipoprotein domain. Highly conserved in <em>Burkholderia</em> T6SSs (Boyer <em>et al.</em>, 2009).</td>
</tr>
<tr>
<td>0343</td>
<td>tssJ</td>
<td>Phage-like sheath subunit (Aubert <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>0342</td>
<td>tssK</td>
<td>Phage-like sheath subunit (Aubert <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>0341</td>
<td>tssL</td>
<td>Outer membrane lipoprotein (Felisberto-Rodrigues <em>et al.</em>, 2011).</td>
</tr>
<tr>
<td>0339</td>
<td>tssM</td>
<td>Outer membrane lipoprotein</td>
</tr>
<tr>
<td>0337</td>
<td>tssF</td>
<td>IcmH-like, dot U, inner membrane (Durand <em>et al.</em>, 2012).</td>
</tr>
<tr>
<td>0336</td>
<td>DUF4262, conserved in many bacterial and viral proteins. Not part of the <em>B. cenocepacia</em> T6SS (this work)</td>
<td></td>
</tr>
<tr>
<td>0335</td>
<td>Conserved hypothetical protein. Not part of the <em>B. cenocepacia</em> T6SS (this work)</td>
<td></td>
</tr>
</tbody>
</table>
0334 Putative amino-acid transporter periplasmic solute protein. Not part of the \textit{B. cenocepacia} T6SS (this work)

0333 Putative "winged helix" DNA binding protein. Not part of the \textit{B. cenocepacia} T6SS (this work)

\textsuperscript{a} Based on Shalom \textit{et al.} (2007)
\textsuperscript{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 \textit{B. cenocepacia} K56-2. Unmarked and non-polar deletions were performed as described previously (Flannagan \textit{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 \textit{Burkholderia} strains was performed by triparental mating using \textit{E. coli} DH5α carrying the helper plasmid pRK2013 (Craig \textit{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 and XhoI, 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, 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 3947-3946; 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 \textit{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 \textit{NdeI} and \textit{XbaI} and cloned into similarly digested pDA12.
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).
SUPPLEMENTARY REFERENCES


Additional Material for Reviewer

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