Polar localization of the ATPase ClpV-5 occurs independent of type VI secretion system apparatus proteins in *Burkholderia thailandensis*

Jan Lennings¹, Christian Mayer², Munira Makhlouf¹, Heike Brötz-Oesterhelt² and Sandra Schwarz¹*

**Abstract**

**Objective:** ClpV, the ATPase of the type VI secretion system (T6SS) recycles cytoplasmic T6SS proteins following effector translocation. Fluorescent protein fusions to ClpV showed that it localizes to discrete and dynamic foci. ClpV-1-sfGFP of the bacterial cell targeting T6SS-1 of *Burkholderia thailandensis* exhibits a virtually random localization, whereas ClpV-5-sfGFP of the T6SS-5 targeting host cells is located at one or both poles. The mechanisms underlying the differential localization pattern are not known. Previous analysis of T6SSs, which target bacterial cells revealed that ClpV foci formation is dependent on components of the T6SS. Here, we investigated if the T6SS-5 apparatus confers polar localization of ClpV-5.

**Results:** ClpV-5-sfGFP foci formation and localization was examined in a *B. thailandensis* mutant harboring a deletion of the entire T6SS-5 gene cluster. We found that ClpV-5-sfGFP localization to discrete foci was not abolished in the absence of the T6SS-5 apparatus. Furthermore, the number of ClpV-5-sfGFP foci displaying a polar localization was not significantly different from that of ClpV-5-sfGFP expressed in the wild type genetic background. These findings suggest the presence of a T6SS-independent localization mechanism for ClpV-5 of the T6SS-5 targeting host cells.

**Keywords:** *Burkholderia thailandensis*, Host cell, Type VI secretion system, ClpV ATPase

**Introduction**

The type VI secretion system (T6SS) is a widespread nanomachine bearing considerable structural and mechanistic similarity to contractile phage tails [1–3]. T6SSs are employed as offensive or defensive tools to kill prokaryotic or eukaryotic target cells or to acquire metal ions to overcome stress conditions, respectively [4–6]. The T6S apparatus is comprised of a cytoplasmic tubule—made of an inner tube enclosed in a contractile sheath—and an envelope spanning membrane complex that are connected by a base plate [7]. Contraction of the sheath acts as a molecular spring that pushes the inner tube tipped with a spike protein into the extracellular milieu or into the target cell, thereby translocating effector proteins [8].

The contracted sheath remains in the cytoplasm and is recycled by the ring-forming AAA⁺ ATPase ClpV enabling a new assembly-contraction-disassembly cycle [9–11]. ClpV of *Vibrio cholerae* interacts directly with the N-terminus of the sheath protein TssC that is exposed in its contracted state [11–14]. Fluorescent protein fusions to ClpV of T6SSs targeting bacterial cells showed that the protein localizes to dynamic foci, which is dependent on components of the T6SS [9, 15, 16]. Localization dynamics of ClpV foci are associated with lysis or blebbing of the neighboring target cell indicating a prior translocation event [9, 17].

*Burkholderia thailandensis* is a soil saprophyte able to switch to an intracellular life style upon contact with phagocytic and non-phagocytic cells [18, 19]. The bacteria encode multiple T6SSs belonging to the canonical T6SSi subtype [20], [21]. The T6SS-5, which is expressed during infection of host cells, induces the formation of multinucleated giant cells (MNGCs) for...
intercellular spread of the bacteria and the T6SS-1 is involved in interbacterial interactions [18, 22, 23]. This work extends a previous study showing that the ATPase of the T6SS-1 (ClpV-1) and T6SS-5 (ClpV-5) localizes to distinctly different sites inside B. thailandensis [16]. In contrast to ClpV-5-GFP foci, which were predominantly found at one or both cell poles, ClpV-1-GFP foci localized in a nearly random manner along the cell length, similar to ClpV of other bacterial cell targeting T6SS. In addition, ClpV-1-GFP foci are more dynamic than ClpV-5-sfGFP foci [16]. The mechanism underlying the differential localization pattern is not known. Here, we imaged a ClpV-5-sfGFP fusion protein in B. thailandensis harboring a deletion of the entire T6SS-5 gene cluster to investigate if T6SS-5 apparatus proteins direct ClpV-5 to the pole. We found that the polar localization of ClpV-5 is not dependent on the interaction with other T6SS-5 proteins.

Main text

Methods

Bacterial strains and growth conditions

Escherichia coli DH5α and SM10λpir and B. thailandensis E264 were routinely grown in Luria-Bertani (LB) medium at 37 °C supplemented with 200 μg/ml trimethoprim and 0.02% rhamnose when necessary.

Construction of plasmids and B. thailandensis mutants

Burkholderia thailandensis ΔT6SS-5 carrying an unmarked deletion of the T6SS-5 gene cluster (BTH_II0855-BTH_II0873) and B. thailandensis expressing a chromosomal fusion of mCherry to clpV-5 at the native site (clpV-5-mCherry) were generated using the suicide vector pRCL115 as described previously [23, 24]. The mini-Tn7 transposon delivery plasmid pUC18T-mini-Tn7T-Tp was used for expression of clpV-5-sfGFP from a neutral chromosomal site in ΔT6SS-5. The gene clpV-5 (BTH_II0864) fused to sfGFP was cloned into pUC18T-mini-Tn7T-Tp::P<sub>512</sub> to express clpV-5-sfGFP under control of the constitutive ribosomal promoter P<sub>512</sub> (BaseClear) [23, 25]. Transformation of B. thailandensis ΔT6SS-5 with this and the transposase helper plasmid pTNS3 yielded the mutant ΔT6SS-5 attTn7::P<sub>512</sub>-clpV-5-sfGFP, which we termed T6SS-5<sup>−</sup> clpV-5-sfGFP. B. thailandensis expressing a chromosomal fusion of clpV-5-sfGFP (T6SS-5<sup>−</sup> clpV-5-sfGFP) was generated in previous work [16]. The genes virA (BTH_II0871) and virG (BTH_II0872) encoding the two component system VirAG were cloned into the expression vector pSCRhab2 carrying a rhamnose inducible promoter (p::virAG) [26].

Infection of RAW 264.7 macrophages and fluorescence microscopy

The RAW 264.7 murine macrophage cell line (ATCC) and the Hela epithelial cell line (ATCC) were maintained in high glucose DMEM supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum (Gibco) at 37 °C and 5% CO<sub>2</sub>. On the day before the experiment 1 × 10<sup>5</sup> macrophages and 5 × 10<sup>4</sup> Hela cells were seeded on glass cover slips in 24 well plates. The cells were infected with B. thailandensis wild type and mutants harvested from exponential phase cultures at multiplicity of infection (MOI) 2 (macrophages) and 50 (Hela cells) and incubated for 1 h. The medium was replaced with fresh DMEM containing 100 μg/ml imipenem followed by 13 h incubation. At this time point the macrophages were stained with Giemsa (Sigma) and viewed with an Olympus BX51 microscope and a 20 × (for quantification of MNGC formation) and 40 × (for images shown in Fig. 1b) objective. MNGC formation was quantified using the formula: (number of nuclei within MNGCs/total number of nuclei) × 100. For epifluorescence microscopy, macrophages were infected with B. thailandensis at MOI 50 for 6 h and fixed with 4% formaldehyde. F-actin and DNA was stained with Texas Red-X Phalloidin or Alexa Fluor 488 Phalloidin and 4',6-diamidino-2-phenylindole (DAPI), respectively. Images of cells were acquired with a Nikon Eclipse Ti-E equipped with a CCD Hamamatsu Orca Flash 4.0 camera and a CFI Plan-Apo DM 100×/1.45 Oil Ph3 objective.

Western blot

Burkholderia thailandensis strains were grown in LB broth supplemented with 0.02% rhamnose and 200 μg/ml trimethoprim where necessary. A 1 ml aliquot of bacterial cultures grown to an OD<sub>600nm</sub> of 1.0 was centrifuged and the cell pellet was resuspended in H<sub>2</sub>O and Laemml sample buffer. Following SDS PAGE the samples were transferred to nitrocellulose membranes, which were blocked with 5% skim milk and probed with the primary antibodies mouse monoclonal anti-GFP (Thermo Fisher; MA5-15256) or mouse monoclonal anti-RNA polymerase beta (Thermo Fisher; MA1-25425). HRP-conjugated rabbit anti-mouse (Thermo Fisher; 31457) was used as secondary antibody. Blots were developed using Clarity Western ECL substrate (Biorad).

Statistical analysis

The t-test or Welch’s t-test was performed to test the difference between two means as indicated in the figure legends. A P value of ≤0.05 was considered statistically significant.
Results and discussion
To examine whether ClpV-5 localizes to the pole by interacting with protein(s) of the T6SS-5 apparatus, we constructed an unmarked deletion mutant of the entire T6SS-5 gene cluster (BTH_II0855–BTH_II0873) in *B. thailandensis*. This mutant lacks all components of the T6SS-5 secretion apparatus. ClpV-5 localization analysis in the absence of T6SS-5 components was performed by integrating a *clpV-5-sfgfp* fusion gene into the chromosome of the ΔT6SS-5 mutant under control of a constitutive promoter using the mini-Tn7 system (T6SS-5*− clpV-5-sfgfp*). Deletion of the T6SS-5 gene cluster was confirmed by PCR and the inability of the T6SS-5*− clpV-5-sfgfp* mutant to induce MNGC formation of RAW264.7 macrophages (Fig. 1a–c). As control, bacteria expressing a chromosomal *clpV-5-sfgfp* fusion in the wild type genetic background were used (T6SS-5*++ clpV-5-sfgfp*). The capability of this mutant to mediate MNGC formation of RAW264.7 macrophages infected with the indicated *B. thailandensis* strains and stained as described in B. The ability of the T6SS-5*++ clpV-5-sfgfp* mutant to induce MNGC formation shows that ClpV-5 is functional when fused to sfGFP. The data shown are mean values ±SD based on two experiments performed in triplicate and three randomly selected microscopic fields per sample (N > 7578 nuclei). ns, not significant/P = 0.498 (t-test); ****P < 0.0001 (Welch’s t-test).

**Detection of ClpV-5-sfGFP fusion proteins in whole cell lysates of the indicated *B. thailandensis* strains by Western blot analysis using α-GFP antibody (MW: GFP, 27 kDa; ClpV-5, 101 kDa) and as loading control α-RpoB antibody (MW: RpoB, 153 kDa).** The bacteria were grown to an OD*600nm* of 1.0 in LB broth. The genes BTH*II0871* and BTH*II0872* encoding the two component system VirAG were overexpressed (p::virAG) in *B. thailandensis* T6SS-5*++ clpV-5-sfgfp* to induce production of the fusion protein outside the host cell environment.

To this end, cell lysates of bacteria grown in LB broth were used. Transcription of *clpV-5-sfgfp* in T6SS-5*++ clpV-5-sfgfp* was achieved by overexpression of the two component regulatory genes *virAG* (p::virAG) previously shown to stimulate the T6SS-5 under conditions that lack the native host cell derived activation signal [16, 27, 28]. Furthermore, we investigated the localization of ClpV-5 fused to the monomeric fluorescent protein mCherry during infection of host cells. ClpV-5-mCherry localizes to single discrete foci at the pole of *B. thailandensis* thus verifying the localization pattern observed for ClpV-5-sfGFP (Additional file 1: Figure S1).

**Given that the native environment for T6SS-5 expression and activity is the intracellular milieu of the host cell, we studied the localization of ClpV-5 within *B. thailandensis* during infection of RAW264.7 macrophages.** The cells were infected with *B. thailandensis* T6SS-5*− clpV-5-sfgfp* and as control with T6SS-5*++ clpV-5-sfgfp*. In the T6SS-5*++* genetic background giving rise to a complete and functional T6SS-5, the ClpV-5-sfGFP fusion protein displayed the formation of discrete foci and diffuse cytoplasmic localization.
as reported previously (Fig. 2a) [16]. The majority of ClpV-5-sfGFP foci (81%) exhibited a polar localization (Fig. 2a, c). Interestingly, in the absence of all T6SS-5 components ClpV-5-sfGFP still assembled into foci located at the pole (Fig. 2b). Furthermore, the number of polar ClpV-5-sfGFP foci did not significantly differ between T6SS-5+ and T6SS-5− bacteria (Fig. 2c). The non-native chromosomal position of clpV-5-sfgfp in the T6SS-5− mutant did not abolish polar localization of the fusion protein. The data demonstrate that T6SS-5 apparatus protein(s) are not required for polar localization of ClpV-5. Instead, a non-T6SS protein may serve for example as a polar anchor for ClpV-5. The finding that T6SS-5 components are dispensable for ClpV-5 localization is consistent with reports showing that ClpV specifically interacts with the T6 apparatus following a secretion event, i.e. with the contracted sheath [9]. Moreover, ClpV1 of the bacterial cell targeting T6SS of E. coli does not directly interact with any of the T6SS apparatus components except for TssC [12]. On the other hand, the deletion of components of bacterial cell targeting T6SSs in V. cholerae, Pseudomonas aeruginosa and Serratia marcescens abrogated ClpV foci formation and resulted in a diffuse cytoplasmic localization [9, 15, 29–31]. Likewise, the ATPase ClpB of the anti-host but non-canonical T6SS in Francisella novicida requires the T6SS protein PdpB for localization [32]. Altogether, the results suggest that complex and distinct localization mechanisms underlie the positioning of the ATPase of bacterial and host cell targeting T6SS.

**Limitations**

Fluorescent protein fusion, a widely used method for protein localization studies, was used to determine the subcellular localization of ClpV-5. A limitation of this technique is that the fluorescent protein might alter localization and activity of ClpV-5. The clpV-5-sfgfp fusion was expressed from the native chromosomal clpV-5 locus activated by the native host cell signal and we confirmed that ClpV-5 was functional when fused to sfGFP. Moreover, a previous study utilizing ClpV specific antibodies showed that –like ClpV-5-sfGFP– the native untagged ClpV protein assembles into discrete foci in V. cholerae [29].

**Additional file**

Additional file 1: Figure S1. A ClpV-5-mCherry fusion protein localizes to the bacterial cell pole during infection of host cells. Phase contrast and fluorescence microscopy images of Hela cells infected with B. thailandensis expressing a chromosomal clpV-5-mCherry fusion at MOI 50 for 6 h. Host cell actin and DNA was stained with Texas Red-X Phalloidin and DAPI, respectively. Scale bars, 1 μm for main figure and insets, respectively.

**Abbreviations**

att Tn7: attachment site of Tn7; HRP: horseradish peroxidase; MNGC: multinucleated giant cell; MW: molecular weight; RpoB: RNA polymerase subunit beta; sfGFP: superfolder GFP.
Authors' contributions
SS conceived the study, JL, CM and MM performed experiments, JL, CM, MM, HBO and SS and analyzed the data and JL and SS wrote the manuscript. All authors read and approved the final manuscript.

Author details
1 Interfaculty Institute of Microbiology and Infection Medicine, Department of Medical Microbiology and Hygiene, University of Tübingen, Tübingen, Germany. 2 Interfaculty Institute of Microbiology and Infection Medicine, Department of Microbial Bioactive Compounds, University of Tübingen, Tübingen, Germany.

Acknowledgements
We thank Maria Haug for excellent technical assistance and Peter Sass for helpful discussions and technical support.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

Funding
JL and this work were funded by the German Research Foundation (SFB766/Project B16). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 27 November 2018 Accepted: 21 February 2019
Published online: 28 February 2019

References
1. Ge P, Scholl D, Leiman PG, Yu X, Miller JF, Zhou ZH. Atomic structures of a bacteriophage contractile nanotube in its pre- and post-contractile states. Nat Struct Mol Biol. 2015;22(9):377–82.
2. Leiman PG, Shneider MM. Contractile tail machines of bacteriophages. Adv Exp Med Biol. 2012;726:93–114.
3. Veerler D, Cambillau C. A common evolutionary origin for tailed-bacteriophage functional modules and bacterial machineries. MMBR. 2011;75(3):423–33.
4. Galligue M, Bouteiller M, Memria A. The type VI secretion system: a dynamic system for bacterial communication? Front Microbiol. 2017;8:1454.
5. Hachani A, Wood TE, Filloux A. Type VI secretion and anti-host effectors. Curr Opin Microbiol. 2016;29:81–93.
6. Russell AB, Peterson SB, Mougouw JD. Type VI secretion system effectors: poisons with a purpose. Nat Rev Microbiol. 2014;12(2):137–48.
7. Zoued A, Brunet YR, Durand E, Aschtgen MS, Loggger L, Douzi B, et al. Architecture and assembly of the type VI secretion system. Biochem Biophys Acta. 2014;1843(8):1664–73 PubMed PMID: 24681160.
8. Brackmann M, Nazarov S, Wang J, Basler M. Using force to punch holes: mechanics of contractile nanomachines. Trends Cell Biol. 2017;27(7):623–32 PubMed PMID: 28602424.
9. Basler M, Pilhofer M, Henderson GP, Jensen GJ, Mekalanos JJ. Type VI secretion requires a dynamic contractile phage tail-like structure. Nature. 2012;483(7388):182–6.
10. Bonemann G, Petrissik A, Diemard M, Zentgraf H, Mogg A. Remodelling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion. EMBO J. 2009;28(4):315–25.
11. Pietrosiuk A, Lenherr ED, Falt S, Bonemann G, Kopp J, Zentgraf H, et al. Molecular basis for the unique role of the AAA+-chaperone ClpV in type VI protein secretion. J Biol Chem. 2011;286(34):30010–21.
12. Douzi B, Brunet YR, Spinelli S, Leni V, Legrand P, Blangy S, et al. Structure and specificity of the type VI secretion system ClpV-TssC interaction in enteropathogenic Escherichia coli. Sci Rep. 2016;6(46):34405.
13. Kube S, Kapitein N, Zimniak T, Herzog F, Mogg A, Wendler P. Structure of the VipA/B type VI secretion complex suggests a contraction-state-specific recycling mechanism. Cell Rep. 2014;8(1):20–30.
14. Kudryashov M, Wang RY, Brackmann M, Scherer S, Maier T, Baker D, et al. Structure of the type VI secretion system contractile sheath. Cell. 2015;160(5):952–62.
15. Gerc AJ, Diepold A, Trunk K, Porter M, Rickman C, Armitage JP, et al. Visualization of the serrata type VI secretion system reveals unprovoked attacks and dynamic assembly. Cell Rep. 2015;12(2):2131–42.
16. Schwarz S, Singh R, Robertson JD, Leffoux M, Skerrrett SJ, Goodlett DR, et al. VgrG-S is a Burkholderia type VI secretion system-exported protein required for multinucleated giant cell formation and virulence. Infect Immun. 2014;82(4):1445–52.
17. Basler M, Ho BT, Mekalanos JJ. Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. Cell. 2013;152(4):884–94.
18. French CT, Toesca U, Wu TH, Teslata T, Beatty SM, Wong W, et al. Dissection of the Burkholderia intracellular life cycle using a photothermal nanoblade. Proc Natl Acad Sci USA. 2011;108(29):12095–100.
19. Whiteley L, Meffert T, Haug M, Wendemanna C, Hofv V, Bitschar K, et al. Intracellular survival, and multinucleated-giant-cell-forming activity of Burkholderia pseudomallei in human primary phagocytic and nonphagocytic cells. Infect Immun. 2017;85(10).
20. Russell AB, Wexler AG, Harding BN, Whitney JC, Bohn AJ, Goo YA, et al. A type VI secretion-related pathway in Bordetella mediates interbacterial antagonism. Cell Host Microbe. 2014;16(2):227–36.
21. Lemmens J, West TE, Schwarz S. The Burkholderia type VI secretion system: composition, regulation and role in virulence. Front Microbiol. 2018;9:3339.
22. Leffoux M, De Leon JA, Kuvada NJ, Russell AB, Pinto-Santini DJ, Hood RD, et al. Quantitative single-cell characterization of bacterial interactions reveals type VI secretion is a double-edged sword. Proc Natl Acad Sci USA. 2012;109(48):19824–9.
23. Whiteley L, Meffert T, Haug M, Wendemanna C, Hofv V, Bitschar K, et al. Entry, intracellular survival, and multinucleated-giant-cell-forming activity of Burkholderia pseudomallei in human primary phagocytic and nonphagocytic cells. Infect Immun. 2017;85(10).
24. Chandler JR, Duerskop BA, Hinz A, West TE, Herman JP, Churchill ME, et al. Mutational analysis of Burkholderia thailandensis quorum sensing and self-aggregation. J Bacteriol. 2009;191(19):5901–9.
25. Choi KH, DeShazer D, Schweizer HP. mini-Ttn insertion in bacteria with multiple glmS-linked attTn sites: example Burkholderia mallei ATCC 23344. Nat Protoc. 2006;1(1):162–9.
26. Cardona ST, Valvano MA. An expression vector containing a rhamnose-inducible promoter provides tightly regulated gene expression in Burkholderia cenocepacia. Mol Genet Genomics. 2005;274(5):219–28.
27. Schell MA, Ulrich RL, Riber JW, Bruegmann EE, Hines HB, Chen D, et al. Type VI secretion is a major virulence determinant in Burkholderia mallei. Mol Microbiol. 2007;64(6):1466–85.
28. Toesca U, French CT, Miller JF. The Type VI secretion system spike protein VgrG5 mediates membrane fusion during intercellular spread by pseudomallei group Burkholderia species. Infect Immun. 2014;82(4):1436–44.
29. Kaptein N, Bonemann G, Pietrosiuk A, Seyffert F, Hauser J, Locker JK, et al. ClpV recycles VipA/VipB tubules and prevents non-productive tubule formation to ensure efficient type VI protein secretion. Mol Microbiol. 2013;87(5):1013–28.
30. Mougouw JD, Cuff ME, Rauner S, Shen A, Zhou M, Gifford CA, et al. A virulence locus of Pseudomonas aeruginosa encodes a protein secretion apparatus. Science. 2006;312(5779):1526–30.
31. Stevens MB, Stevens JM, Jeng RL, Taylor LA, Wood MW, Havel P, et al. Identification of a bacterial factor required for actin-based motility of Burkholderia pseudomallei. Mol Microbiol. 2005;56(1):40–53.
32. Brodmann M, Dreier RF, Broz P, Basler M. Francisella requires dynamic type VI secretion system and ClpP to deliver effectors for phagosomal escape. Nat Commun. 2017;8:15853.