

# **Lokalisationsmechanismen der ATPase des Virulenz-assoziierten Typ VI Sekretionssystems in *Burkholderia thailandensis***

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## Abkürzungsverzeichnis

A22	S-(3,4-Dichlorbenzyl)isothioharnstoff
AAA+	ATPases associated with various cellular activities
Ca.	circa
CDC	Centers for Disease Control and Prevention
DCS	d-Cycloserin
EAEC	Entero-aggregativer <i>Escherichia coli</i>
GFP	grün fluoreszierendes Protein
Hcp	Haemolysin co-regulated protein
MNGC	multi nucleated giant cell
MOI	multiplicity of infection
N/D	nicht detektiert
PFA	Paraformaldehyd
PG	Peptidoglykan
PGS	Peptidoglykansynthese
T3SS	Typ III Sekretionssystem
T5SS	Typ V Sekretionssystem
T6SS	Typ VI Sekretionssystem
T6SS-1 <sup>-</sup>	komplettes T6SS-1-Gencluster deletiert
T6SS-5 <sup>+</sup>	komplettes T6SS-5-Gencluster vorhanden
T6SS-5 <sup>-</sup>	komplettes T6SS-5-Gencluster deletiert
Tag	Type six associated gene
Tss	Type six secretion gene
VgrG	Valine glycine repeat G protein
WT	Wildtyp
z. B.	zum Beispiel

## Zusammenfassung

Das Typ VI Sekretionssystem (T6SS) besteht aus einem in der Membran verankerten kontraktilen zytoplasmatischen Tubus, der eine Nadel in Nachbarzellen treibt um toxische Effektoren zu sekretieren. Der kontrahierte Tubus wird von der AAA+ ATPase ClpV entfaltet und recycelt. Immer mehr Studien zeigen, dass die Lokalisation des T6SS in der Bakterienzelle von dem Typ der Zielzelle bedingt wird. T6SS, die gegen eukaryotische Zellen wirken, zeigen eine polare Lokalisation, wohingegen T6SS, die Bakterien angreifen, eine zufällige Lokalisation aufweisen. Wie die T6SS-Proteine den Assemblierungsort erreichen, ist jedoch unbekannt. Ziel dieser Arbeit war es, mithilfe des Modellorganismus *Burkholderia thailandensis* erste Informationen über die grundlegenden Lokalisationsmechanismen der T6SS-Komponenten zu erlangen. *B. thailandensis* besitzt mehrere T6SS. Unter anderem das T6SS-1, welches Effektoren in andere Bakterien transloziert und das T6SS-5, ein essentieller Virulenzfaktor der Wirtszellen manipuliert. Vorausgegangene Arbeiten haben gezeigt, dass die ATPasen dieser beiden Sekretionssysteme, ClpV-1 und ClpV-5, diskrete Fokusse bilden, die eine zufällige bzw. polare Lokalisation aufweisen. ClpV-1-GFP-Fokusse weisen eine hohe Dynamik auf, die vermutlich den Abbau von kontrahierten Tubuli anzeigt. Im Gegensatz dazu sind ClpV-5-sfGFP-Fokusse statisch am Pol lokalisiert, was auf eine niedrigere Aktivität schließen lässt. Zunächst wurde die Rolle des T6SS-Apparates bei der Lokalisation von ClpV bestimmt. Dazu wurden *B. thailandensis* Mutanten mit vollständigen T6SS-1 und T6SS-5 Gencluster-Deletionen hergestellt. Es konnte gezeigt werden, dass die diskrete, singuläre Fokusbildung von ClpV-1-GFP in der  $\Delta$ T6SS-1-Mutante signifikant reduziert war, was bedeutet, dass ClpV-1 ohne T6SS-1-Proteine nicht lokalisieren kann. Im Gegensatz dazu lokalisiert ClpV-5-sfGFP auch in Abwesenheit der T6SS-5-Proteine am Pol. Das könnte bedeuten, dass es mit einem anderen Faktor interagiert, um zum Pol zu gelangen. Die Ergebnisse zeigen, dass die T6SS ATPasen unterschiedliche Informationen über die subzelluläre Lokalisation enthalten können. Weiterhin konnte die polare Lokalisation von ClpV-5 in der  $\Delta$ T6SS-5 Mutante sowohl während der Infektion von Wirtszellen als auch außerhalb in Kulturmedium beobachtet werden. Ein intrazelluläres Signal der Wirtszelle ist daher nicht in Lokalisation von ClpV-5 involviert. Andere zytoplasmatische T6SS-5-Proteine wie TssK-5 und TssC-5 zeigten eine diffuse Verteilung in der  $\Delta$ T6SS-5-Mutante. Die Beeinträchtigung von zellulären Faktoren, die in die räumliche Organisation von Bakterienzellen involviert sind, wie beispielsweise das Zytoskelettprotein MreB, hatte keinen Einfluss auf die Fokusbildung von ClpV-5 und ClpV-1. Da das T6SS ein kontaktabhängiges System ist und das Signal zur Kontraktion von T6SS-5 und T6SS-1 unbekannt ist, wurde der Einfluss von Oberflächenkontakt auf die Verteilung von ClpV-5 und ClpV-1 untersucht. Dafür wurden die Bakterien für unterschiedliche Zeitintervalle auf Agarosepads aufgetropft, um Oberflächenkontakt zu generieren. Als Kontrolle wurden die Bakterien in Flüssigkultur mit Paraformaldehyd fixiert. Die Ergebnisse zeigen, dass Oberflächenkontakt nicht essentiell für die polare Lokalisation von ClpV-5 ist, diese aber induziert. Nach längerer Inkubation änderte sich die diffuse Verteilung von TssK-5-sfGFP zu diskreter Fokusbildung. Die Fokusse waren zufällig lokalisiert und scheinen unspezifisch zu sein. In *B. thailandensis*-Bakterien, die eine chromosomale *clpV-1-gfp* Fusion exprimierten und länger auf Agarosepads aufgetropft waren, konnte ein ClpV-1-GFP-Signal beobachtet werden. Die gleiche Mutante ohne Oberflächenkontakt zeigte kein GFP-Signal. Hieraus lässt sich schließen, dass Oberflächenkontakt ein Signal zur Expression von *clpV-1* und vermutlich des gesamten T6SS-1-Genclusters darstellt. Zusammenfassend sind in dieser Arbeit neue Erkenntnisse zur Lokalisation von ClpV ATPasen von T6SS, die gegen Bakterien und Wirtszellen gerichtet sind, gewonnen worden.

## Summary

The type VI secretion system (T6SS) is comprised of a membrane-anchored cytoplasmic tubular sheath which contracts to expel an inner needle thereby puncturing neighboring target cells to translocate toxic effector proteins. The contracted sheath proteins are recycled by the AAA<sup>+</sup> ATPase ClpV. There is accumulating evidence that the localization of the T6SS inside the bacterial cells is associated with the type of target cell: T6SS specialized in targeting eukaryotic cells localize to the pole of the bacteria whereas T6SS that deliver effector proteins to bacteria display a random distribution. It is unknown, how T6SS proteins reach the site destined for T6SS apparatus assembly. The aim of this work was to gain insight into the mechanisms underlying the localization of T6SS components using *Burkholderia thailandensis* as a model organism. Of the multiple T6SS *B. thailandensis* encodes, the T6SS-1 translocates effector proteins into competing bacteria and the T6SS-5 is a major virulence factor that targets host cells. Previous work showed that the ATPases of these secretion systems, ClpV-1 and ClpV-5, exhibit a random and polar distribution, respectively, thus recapitulating the localization patterns observed in other bacteria. Moreover, assembled ClpV-1-GFP foci rapidly changed their localization presumably reflecting disassembly of the contracted tubular sheath. In contrast, ClpV-5-GFP foci were more static and confined to the pole suggesting that the T6SS-5 is less active. First, the role of the T6SS apparatus in ClpV localization was analyzed in this work. To this end, *B. thailandensis* mutants harboring deletions of the entire T6SS-1 and T6SS-5 gene cluster, respectively, were generated. The data show that single discrete ClpV-1-GFP foci formation is significantly decreased in the  $\Delta$ T6SS-1 mutant indicating that ClpV-1 requires the T6SS-1 apparatus for localization. Interestingly, however, ClpV-5-GFP still localizes to the pole in the absence of all T6SS-5 components suggesting that the ATPase interacts with a non T6SS factor to reach the pole. The results show that T6SS ATPases can harbor distinct information for subcellular localization. Polar distribution of ClpV-5-GFP in the  $\Delta$ T6SS-5 mutant was observed during infection of host cells but also in *B. thailandensis* grown in culture medium demonstrating that the host cell environment – containing the signal that induces T6SS-5 gene expression – is not required for ClpV-5 localization. Unlike ClpV-5, the cytoplasmic T6SS-5 proteins TssC-5 and TssK-5 displayed a diffuse localization in the  $\Delta$ T6SS-5 mutant. Furthermore, the disruption of cellular factors known to be involved in the spatial organization of the bacterial cell, such as the cytoskeleton protein MreB, did not abrogate focal assembly of both ClpV-1 and ClpV-5. Given that the T6SS is a contact dependent injection device and the signal triggering T6SS-1 and T6SS-5 activity is not known, the influence of surface contact on ClpV-1 and ClpV-5 distribution was investigated. For this, *B. thailandensis* was spotted for different periods of time onto agarose pads for surface contact. As control, the bacteria were fixed during growth in suspension culture with Paraformaldehyde. The results show that surface contact is not essential for ClpV-5-GFP foci formation at the pole but stimulates it. Elongated incubation times on agarose pads induced a marked change in the localization of TssK-5-GFP from diffuse distribution to formation of discrete foci, which rarely localized to the pole and appear to be T6SS-5 unrelated. A ClpV-1-GFP signal was detected in *B. thailandensis* expressing a chromosomal *clpV-1-gfp* fusion and spotted onto agarose pads. This signal was not detected in bacteria isolated from suspension cultures, therefore surface contact appears to be a signal critical for T6SS-1 gene cluster expression. In summary, this work provided novel insights into the localization of ClpV ATPases from a prokaryotic and eukaryotic cell targeting T6SS.

## Liste der Publikationen

### Akzeptierte Publikationen

1. „The Burkholderia Type VI Secretion System 5: Composition, Regulation and Role in Virulence”

*Jan Lennings, T. Eoin West and Sandra Schwarz*

PMID: 30687298

10. Januar 2019, *Frontiers in Microbiology*, Volume 9, Article 3339

DOI: 10.3389/fmicb.2018.03339

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

*Jan Lennings, Christian Mayer, Munira Makhlouf, Heike Brötz-Oesterhelt and Sandra Schwarz*

PMID: 30819219

28. Februar 2019, *BMC Research Notes*, Volume 12, Article 109

DOI: 10.1186/s13104-019-4141-3

3. „Environmental and cellular factors affecting the localization of T6SS proteins in *Burkholderia thailandensis*“

*Jan Lennings, Munira Makhlouf, Przemysław Olejnik, Christian Mayer, Heike Brötz-Oesterhelt and Sandra Schwarz*

PMID: 31378704

September 2019, *Int J Med Microbiol.* 309(6):151335.

DOI: 10.1016/j.ijmm.2019.151335

## **Eigenanteil an den Publikationen**

### **Publikation 1: „The Burkholderia Type VI Secretion System 5: Composition, Regulation and Role in Virulence”**

Ich war direkt an der Literaturrecherche, Konzeptualisierung und dem Schreibprozess des Manuskriptes sowie der graphischen Darstellung der Daten beteiligt.

### **Publikation 2: „Polar localization of the ATPase ClpV-5 occurs independent of type VI secretion system apparatus proteins in *Burkholderia thailandensis*”**

Fast alle Plasmide und Bakterienstämme wurden von mir entworfen und hergestellt. Die Durchführung der Experimente und Auswertung und Analyse der Daten erfolgte fast ausschließlich durch mich. Ich war am Schreibprozess des Manuskriptes und der graphischen Darstellung der Daten direkt beteiligt.

### **Publikation 3: „Environmental and cellular factors affecting the localization of T6SS proteins in *Burkholderia thailandensis*“**

Fast alle Plasmide und Bakterienstämme wurden von mir entworfen und hergestellt. Sämtliche Experimente wurden von mir durchgeführt und ggf. etabliert. Die Analyse und Auswertung der Daten erfolgte größtenteils durch mich. Ich war an der Konzeptualisierung, dem Schreibprozess des Manuskriptes sowie der graphischen Darstellung der Daten beteiligt.



# 1. Einleitung

## 1.1 *Burkholderia pseudomallei*

*Burkholderia pseudomallei* ist ein motiles, stäbchenförmiges, Gram negatives Bakterium, welches zu den Betaproteobakterien gezählt wird. Der Lebensraum erstreckt sich über die tropischen und subtropischen Klimate, speziell Südostasien, aber auch Indien, China, Afrika, Australien, Nord- sowie Südamerika (White, 2003, Cheng et al., 2007, Currie et al., 2008). Das saprophytische Bakterium tritt in einer Vielzahl ökologischer Nischen auf, z.B. in feuchten Böden und stehenden Gewässern (Brett and Woods, 2000). Das Bakterium kann unter limitierten Sauerstoffkonzentrationen, erhöhten Salzkonzentrationen und erhöhtem oxidativem Stress überleben (Korbsrisate et al., 2005, Pumirat et al., 2009, Pumirat et al., 2010, Hamad et al., 2011, Jitprasutwit et al., 2014). Die Bakterien sind in der Lage, in eukaryotischen Wirtszellen zu überleben und sich zu teilen. Das Spektrum erstreckt sich von Einzellern, wie beispielsweise Vertretern der Gattung *Acanthamoeba*, bis zu Makrophagen und Epithelzellen vielzelliger Organismen (Pruksachartvuthi et al., 1990, Harley et al., 1994, Egan and Gordon, 1996, Jones et al., 1996, Harley et al., 1998, Kespichayawattana et al., 2000). Eine Infektion mit *B. pseudomallei* kann die Krankheit Melioidose auslösen. Aufgrund des pathogenen Potenzials, der effektiven Anpassung an verschiedene Lebensräume und der erheblichen Resistenzen gegenüber antibiotischen Substanzen wird *B. pseudomallei* in die Biosicherheitsstufe 3 eingeteilt.

## 1.2 Melioidose

Die Inhalation von Aerosolen, die Aufnahme kontaminierter Nahrung und perkutanes Eindringen durch Wunden stellen die Hauptinfektionswege von *B. pseudomallei* dar (Barnes and Ketheesan, 2005, Cheng and Currie, 2005, Limmathurotsakul and Peacock, 2011). Durch die Aufnahme der Bakterien erkranken weltweit jährlich Schätzungen zufolge 165.000 Menschen. Weitere Schätzungen gehen von 89.000 Todesfällen pro Jahr aus (Limmathurotsakul et al., 2016a). Melioidose kann eine Mortalitätsrate von bis zu 40% aufweisen. Entwickelt sich eine Sepsis, kann sie auf 90% steigen (Wiersinga et al., 2012, Limmathurotsakul et al., 2016b). Betroffen sind besonders Menschen, die bereits an immunsupprimierenden Vorerkrankungen wie Diabetes mellitus, Alkoholismus, und chronischen Lungen- und

Urogenitalerkrankungen leiden (Suputtamongkol et al., 1999, Simpson et al., 2003, Currie et al., 2010, Lecube et al., 2011, Casqueiro et al., 2012).

Das klinische Bild der Krankheit variiert stark: akute, chronische und latente Infektionen wurden beschrieben (Ngauy et al., 2005, Wiersinga et al., 2006). Letztere können noch nach Jahren zu einem akuten Zustand führen (Ngauy et al., 2005). Ausgehend vom befallenen Gewebe sind verschiedene Symptome zu beobachten. Eine akute Pneumonie verbunden mit Bakteriämie ist bei Erwachsenen die häufigste Form (Wiersinga et al., 2018). Über den Blutstrom können die Bakterien nahezu jedes Gewebe, auch das Knochenmark und das Gehirn, erreichen und eine systemische Infektion auslösen (White, 2003, Bommakanti et al., 2010). Zudem sind die Bakterien in der Lage, wandernde Zellen wie Makrophagen und dendritische Zellen zu infizieren und sich so unter anderem über das Lymphsystem auszubreiten (Pandey et al., 2010, Sulaiman et al., 2013, Williams et al., 2014). Die Diversität der Symptome erschwert die korrekte Diagnose der Krankheit enorm (Wiersinga et al., 2006). Die chronische pulmonale Melioidose ähnelt in Symptomen und Verlauf stark der durch *Mycobacterium tuberculosis* ausgelösten Lungentuberkulose. Eine Falschdiagnose ist daher möglich und oft tödlich, da die gängige Tuberkulosetherapie gegen *B. pseudomallei* wirkungslos ist (Yee et al., 1988, Vishnu Prasad et al., 2012, Kunnathuparambil et al., 2013). Genaue Zahlen hinsichtlich Prävalenz und Inzidenz sind aufgrund der genannten Problematik schwer zu erheben. Bei korrekter Diagnose bleibt eine Therapie schwierig, da *B. pseudomallei* aufgrund von amrAB-oprA Effluxpumpen (Bianco et al., 1997) gegenüber vielen Antibiotikaklassen Resistenzen aufweist. Dazu zählen Penicilline, Cephalosporine, Aminoglycoside und Makrolide (Moore et al., 1999, Piliouras et al., 2002). Die Medikation laut CDC besteht aus einem zehn- bis 14-tägigem Intervall von intravenös verabreichtem Ceftazidim alle 6 bis 8 Stunden oder Meropenem alle 8 Stunden, gefolgt von 3 bis 6 monatiger oraler Einnahme von Trimethoprim-Sulfamethoxazol alle 12 Stunden oder Amoxicillin/Clavulansäure alle 8 Stunden (CDC 2012). Bei korrekter Einhaltung dieser Intervalle besteht ein Risiko von 20%, einen Rückfall zu erleiden (Chaowagul et al., 1993, Currie et al., 2000, White, 2003, Cheng and Currie, 2005). Eine wirksame Impfung existiert nicht (Titball et al., 2017).

Der schwere Verlauf der Krankheit, die Toleranz und Anpassungsfähigkeit des Erregers an Umweltfaktoren, seine Resistenzen gegenüber Antibiotika und die

Schwierigkeit der Erhebung genauer Zahlen verdeutlicht die Dringlichkeit alternativer Behandlungsmethoden. Hierfür ist ein umfassendes Verständnis der Virulenz von *B. pseudomallei* von zentraler Bedeutung.

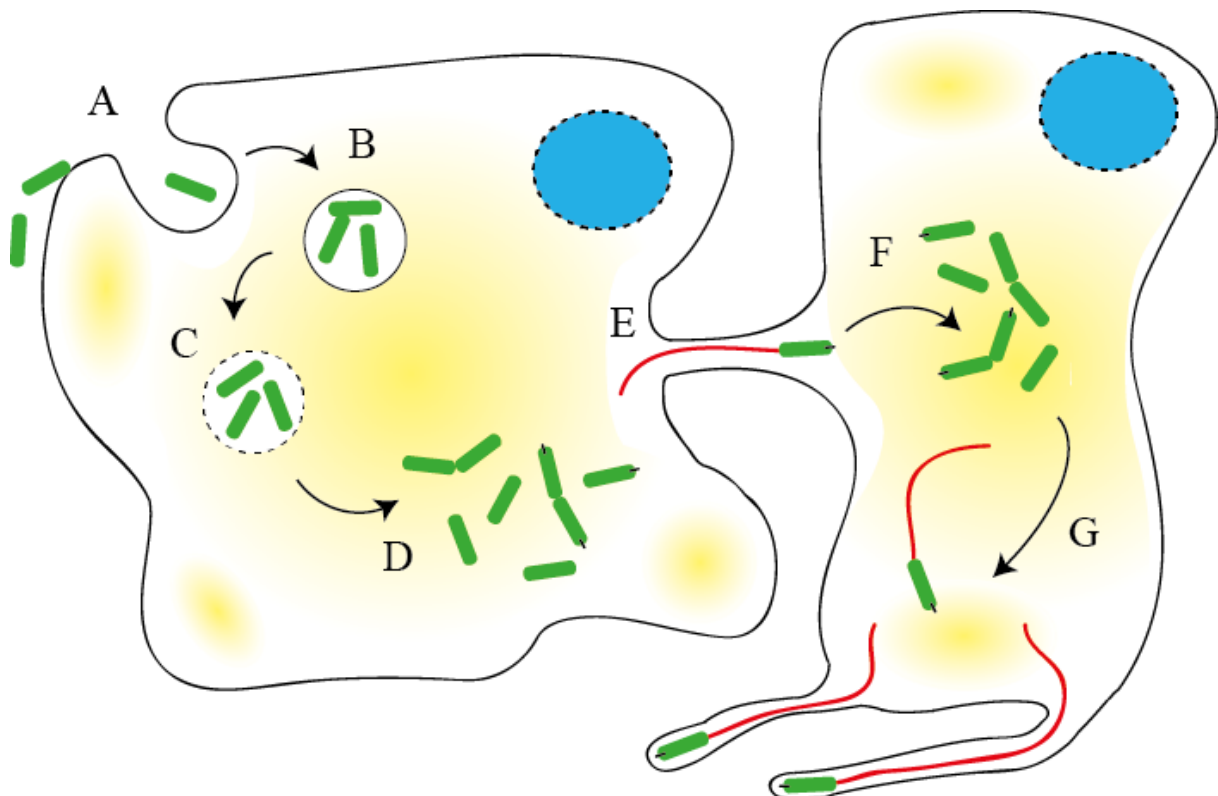
### **1.3 Burkholderia thailandensis**

*Burkholderia thailandensis* ist nah mit *B. pseudomallei* verwandt und belegt die gleichen ökologischen Nischen (Brett et al., 1998). *B. thailandensis* weist eine hohe Sequenzähnlichkeit zu *B. pseudomallei* auf und ist ein etablierter Modellorganismus zur Untersuchung der Virulenz von *B. pseudomallei* (Haraga et al., 2008, Schwarz et al., 2014, Garcia and Cotter, 2016). Ungefähr 70% der Virulenzfaktoren von *B. pseudomallei* sind in *B. thailandensis* präsent (Yu et al., 2006). Die Virulenz von *B. thailandensis* ist verglichen mit *B. pseudomallei* im syrischen Hamster um den Faktor  $10^5$  und in BALB/c Mäusen um  $10^7$  geringer (Brett et al., 1997, Brett et al., 1998, Smith et al., 1997). In der Literatur ist ein einziger Fall einer reinen *B. thailandensis*-Infektion beim Menschen bekannt (Chang et al., 2017). Co-Infektionen mit anderen Bakterien wie z. B. *Stenotrophomonas maltophilia* oder *Serratia marcescens* sind häufiger beschrieben (Glass et al., 2006). In Deutschland wird *B. thailandensis* in die Biosicherheitsstufe 2 eingeteilt. Alle in dieser Arbeit aufgeführten Experimente wurden mit *B. thailandensis* durchgeführt.

### **1.4 Der intrazelluläre Lebenszyklus von B. pseudomallei und B. thailandensis**

*B. pseudomallei* und *B. thailandensis* sind fakultativ intrazelluläre Bakterien. Sie können über diverse Virulenzfaktoren wie beispielsweise Typ III Sekretionssysteme (T3SS), Typ IV Pili und Typ V Sekretionssystem-Adhesine (T5SS) aktiv in nicht-phagozytische Wirtszellen eindringen (Sun and Gan, 2010, Stevens et al., 2002, David et al., 2015, Balder et al., 2010). Ebenso können sie passiv von Makrophagen oder polymorph-nukleären Leukozyten phagozytiert werden (Abb. 1) (Pruksachartvuthi et al., 1990, Harley et al., 1994, Egan and Gordon, 1996, Jones et al., 1996, Harley et al., 1998, Kespichayawattana et al., 2000). Beim Eintritt werden die Bakterien von einer Vakuole aus Wirtszellmembran umschlossen. Die Vakuole wird durch ein T3SS aufgelöst, bevor die Bakterien vom lysosomalen System verdaut werden können (Cullinane et al., 2008, Gong et al., 2011). Liegen die Bakterien frei

im Zytoplasma vor, beginnen sie mit der Zellteilung. Zudem erfolgt die Expression und Aktivierung verschiedener Virulenzfaktoren, die der intrazellulären Motilität dienen. Dazu zählen das Fla2-Flagellensystem und der trimere Autotransporter BimA zur Polymerisation von monomerem Aktin der Wirtszelle (Kespichayawattana et al., 2000, Stevens et al., 2005, French et al., 2011). Durch die unipolare Polymerisation des Aktins können lange Schweife gebildet werden, durch welche die Bakterien sich fortbewegen und Protrusionen bilden (Kespichayawattana et al., 2000, Stevens et al., 2005). Die interzelluläre Verbreitung der Bakterien erfolgt durch die Fusionierung der Plasmamembranen von infizierten mit benachbarten uninfizierten Wirtszellen. Dadurch entstehen mehrkernige Riesenzellen, sogenannte multi-nucleated giant cells (MNGC) (Suparak et al., 2005). Die Bildung von MNGCs ist bei *in vitro* Versuchen und im Lungengewebe von Melioidosepatienten nachgewiesen worden (Kespichayawattana et al., 2000, Wong et al., 1995). Die Bakterien können sich dadurch lokal verbreiten und weiteren Zugang zu Nährstoffen erhalten. Es wird vermutet, dass die Bildung von MNGCs zusätzlich Schutz vor extrazellulärer Immunabwehr bietet (Kespichayawattana et al., 2000). Als zentraler Virulenzfaktor für die Zell-Zell Fusionen ist das Typ VI Sekretionssystem 5 (T6SS-5) identifiziert worden (Shalom et al., 2007, Brett et al., 2011, French et al., 2011, Schwarz et al., 2014).

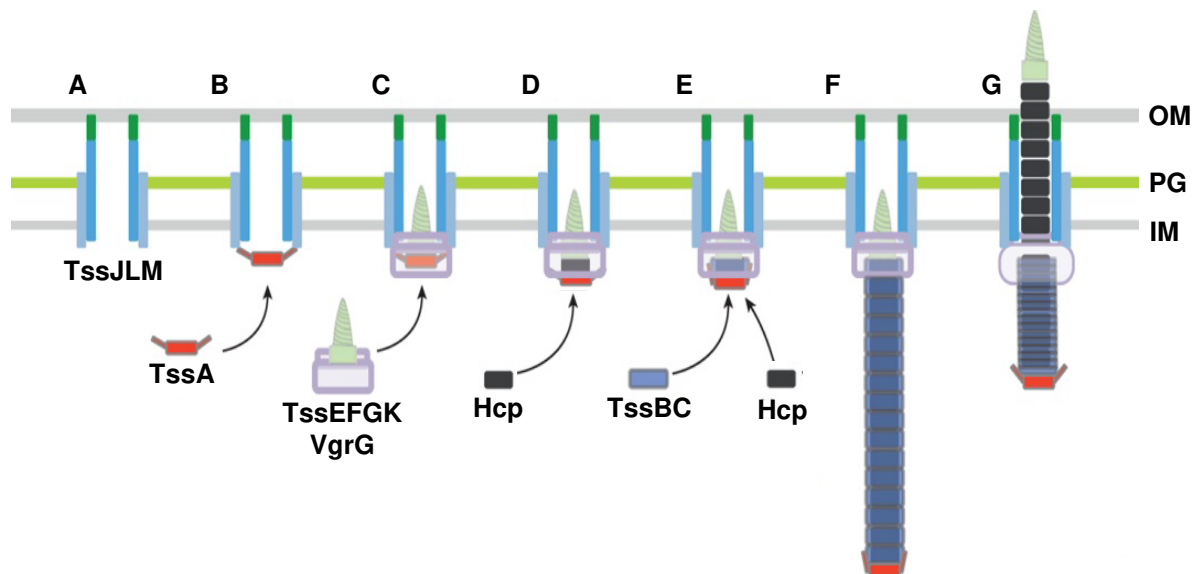


**Abbildung 1: Der intrazelluläre Lebenszyklus von *B. thailandensis* und *B. pseudomallei***

Die Abbildung zeigt den intrazellulären Lebenszyklus von *Burkholderia*. Die Bakterien (grün) adhäreren an die Wirtszelle (**A**) und dringen aktiv oder passiv in die Wirtszelle ein (**B**). Über das T3SS-3 wird die Vakuole aufgelöst (**C**). Die Bakterien liegen frei im Zytoplasma vor und beginnen mit der Zellteilung und der Expression des T6SS-5 (schwarz) sowie anderer Virulenzfaktoren (**D**). Durch BimA werden Aktinschweife (rot) gebildet. Das T6SS-5 induziert Zell-Zell Fusionen, die eine interzelluläre Verbreitung der Bakterien ermöglichen (**E**). In den mehrkernigen Riesenzellen vermehren sich die Bakterien im Zytoplasma (**F**) und bilden weiter Aktinschweife (**G**). Zellkerne sind blau dargestellt.

## **1.5 Das Typ VI Sekretionssystem**

Das T6SS wurde 2006 in *Vibrio cholerae* und *Pseudomonas aeruginosa* erstmalig beschrieben (Pukatzki et al., 2006, Mougous et al., 2006). Es ist in 25% der sequenzierten Gram negativen Bakterienspezies nachgewiesen worden (Bingle et al., 2008). Das T6SS ist ein kontraktiler Injektionsapparat, mit dem Effektoren in prokaryotische oder eukaryotische Zielzellen transloziert werden können, um diese zu töten (Abb. 2) (Pukatzki et al., 2007, Suarez et al., 2010, Brooks et al., 2013). Auf chromosomaler Ebene ist es in Genclustern angeordnet, welche für Tss- (type six secretion gene) und Tag- (type six associated gene) Proteine codieren. Die Tss-Proteine sind die zur Sekretion essentiellen dreizehn Kernkomponenten des T6SS. Einige haben andere Namen erhalten, beispielsweise VipA (TssB), VipB (TssC), Hcp (TssD), ClpV (TssH) und VgrG (TssI). Subkomplexe des T6SS sind der Membrankomplex (TssJLM), der die innere und äußere Membran umspannt (Aschtgen et al., 2010, Felisberto-Rodrigues et al., 2011, Coulthurst, 2013, Ho et al., 2014, Zoued et al., 2014, Durand et al., 2015). Des Weiteren die Basisplatte (TssEFGK) und der zytoplasmatische Tubus, der aus einer starren Nadel (Hcp) besteht, die von einem kontraktilen TssBC-Tubus umschlossen ist (Durand et al., 2015), (Park et al., 2018, Cascales and Cambillau, 2012, Kudryashev et al., 2015, Wang et al., 2017). Der Tubus ist über die Basisplatte mit dem Membrankomplex verankert. Das TssK der Basisplatte bindet direkt an die zytoplasmatischen Domänen von TssLM, von ihr ausgehend wird dann ein VgrG-Trimer eingesetzt und der Hcp- sowie der TssBC-Tubus werden ausgehend von der Basis in das Zytoplasma assembliert (Nguyen et al., 2018, Cherrak et al., 2018, Nazarov et al., 2018). Das Protein TssA verbindet den TssBC- und den Hcp-Tubus als eine Art Kappenstruktur am distalen Ende (Zoued et al., 2017, Dix et al., 2018).



**Abbildung 2: Modell der Assemblierung und Sekretion des T6SS**

Die Abbildung zeigt ein Modell der Assemblierung und des Sekretionsmechanismus des T6SS. **A)** Der TssJLM-Membrankomplex durchspannt das Periplasma und ist in beiden Membranen verankert. **B)** Das Kappenprotein TssA bindet an TssL. **C)** Die Basisplatte aus TssEFGK und VgrG bindet an TssLM. Anschließend wird der Hcp-Tubus einzeln (**D**) oder simultan mit TssBC (**E**) synthetisiert, bis er die finale Länge erreicht hat (**F**). **G)** Die schnelle Kontraktion des VipAB-Tubus ( $\leq 2$  ms) schießt die starre Hcp-Nadel, die mit Effektorproteinen beladen ist, aus der Zelle. Verändert mit Erlaubnis nach: (Zoued et al., 2016)

Viele der T6SS-Proteine weisen strukturelle Homologien zu Phagenproteinen auf. Die Kristallstruktur von VgrG ist bei einer Aminosäuresequenzähnlichkeit von lediglich 13% nahezu deckungsgleich mit gp27/gp5 des T4 Phagen, (Pukatzki et al., 2007). Das Nadel-bildende Protein Hcp ist verwandt mit den Proteinen gpV aus dem Phagen  $\lambda$  und gp19 des T4-Phagen (Mougous et al., 2006, Pell et al., 2009). Weitere Ähnlichkeiten bestehen zwischen TssE und gp25 (Hood et al., 2010, Lossi et al., 2011) sowie zwischen TssBC und gp18 (Lossi et al., 2013, Kudryashev et al., 2015). Die deutlichen Übereinstimmungen der räumlichen Strukturen ließen auf einen ähnlichen Mechanismus von T6SS und Bakteriophagen schließen. In der Tat ist mittels Timelapse-Fluoreszenzmikroskopie gezeigt worden, dass der T6SS-Tubus im Zytoplasma der Bakterien kontrahiert, analog zu kontraktile Phagen (Basler et al., 2012, Basler and Mekalanos, 2012).

Die Effektoren können an die sekretierten Proteine Hcp und/oder VgrG gebunden sein oder sie sind bereits Teil des Proteins. Eine Vielzahl an Effektoren wurde bereits beschrieben, beispielsweise die Amidase Tse1 aus *P. aeruginosa* oder die Phospholipasen Tle1 und Tle2, die gegen Bakterien wirken (Hood et al., 2010,

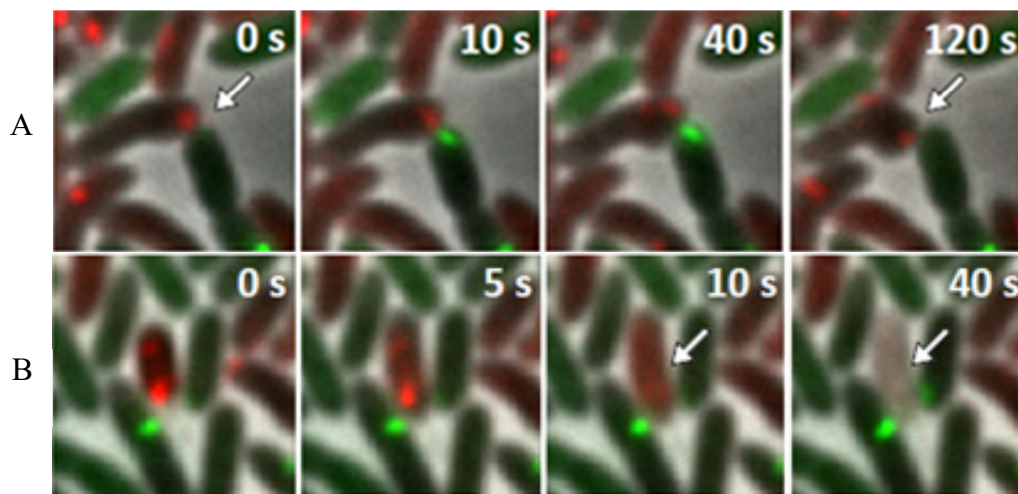
Russell et al., 2013). Damit die Bakterien sich nicht durch eigene Effektoren vernichten, verfügen sie über Immunitätsproteine (Russell et al., 2011, Whitney et al., 2013, Srikanthasani et al., 2013, Altindis et al., 2015). VgrG-1 aus *V. cholerae* zum Beispiel wirkt gegen eukaryotische Zellen. Es besitzt eine C-terminale Domäne, die homolog zu RtxA ist, einem G-Aktin quervernetzenden Toxin aus *V. cholerae* (Pukatzki et al., 2007). Des Weiteren ist gezeigt worden, dass VgrG-5 aus *B. thailandensis* und *B. pseudomallei* für die Bildung von MNGCs essentiell ist. Der Mechanismus ist jedoch unbekannt (Schwarz et al., 2014, Toesca et al., 2014, Whiteley et al., 2017b).

### **1.6 Dynamik der ATPase ClpV**

Nach vollständiger Assemblierung des T6SS führt ein bisher unbekanntes Signal zu einer Kontraktion des TssBC-Tubus, von der hochenergetischen ausgedehnten Form zur niederenergetischen kontrahierten Form (Kudryashev et al., 2015, Renault et al., 2018, Wang et al., 2017). Dadurch wird die Länge des Tubus deutlich reduziert (Basler et al., 2012). Die starre Hcp-Nadel wird durch die Verkürzung des TssBC-Tubus aus der Zelle herausgeschleudert. Der kontrahierte TssBC-Tubus verbleibt im Zytoplasma und wird für eine erneute Kontraktion abgebaut und neu assembliert. Für den Abbau ist die zytoplasmatische, hexamere ATPase ClpV essentiell (Bonemann et al., 2009). ClpV gehört zur Proteinfamilie der AAA+ Hsp100/Clp Proteasen (ATPases associated with various cellular activities) (Iyer et al., 2004). Kennzeichen der Familie sind ringförmige AAA-Domänen mit denen ATP hydrolysiert und chemische in mechanische Energie umgewandelt werden kann (Wang, 2004). Diese Energie wird dann genutzt, um andere Proteine zu entfalten oder abzubauen. Durch die Kontraktion von TssBC und die damit einhergehende Konformationsänderung wird der N-Terminus von TssC exponiert. An diesen bindet der N-Terminus von ClpV (Bonemann et al., 2009, Pietrosiuk et al., 2011). Eine hohe Substratspezifität verhindert, dass ClpV an nicht kontrahierte TssBC-Dimere oder TssC-Monomere bindet (Pietrosiuk et al., 2011).

Die Fusion von ClpV mit Fluoreszenzproteinen zeigt, dass das Protein singuläre Fokuse in den Bakterien ausbildet (Mougous et al., 2007). Die ClpV-Fokuse kolokalisieren mit anderen T6SS-Proteinen, wie z. B. TssB (Basler and Mekalanos,

2012). In *P. aeruginosa* konnte gezeigt werden, dass die Lokalisation der ClpV-Fokuse mit der Lyse von benachbarten *V. cholerae* assoziiert ist (Abb. 3) (Basler et al., 2013). Dies zeigt, dass zwischen der Lokalisation von ClpV und der Aktivität des T6SS ein direkter Zusammenhang besteht. Daher ziehen viele Studien ClpV als verlässlichen und funktionalen Reporter für die Lokalisation des gesamten T6SS heran (Mougous et al., 2007, Bonemann et al., 2009, Basler and Mekalanos, 2012, Schwarz et al., 2014). Die Studien zeigten unterschiedliche Dynamiken der Lokalisation von ClpV: in *V. cholerae* kolokalisieren ClpV und TssC unmittelbar nach der Kontraktion. In *P. aeruginosa* konnte gezeigt werden, dass ClpV exakt an der Position lokalisierte, wo zuvor T6SS-Aktivität der Nachbarzelle auftrat (Basler et al., 2013). Eine neuere Studie konnte unbewegliche ClpV-GFP-Fokuse mit wechselnder Intensität im H1-T6SS von *P. aeruginosa* nachweisen (Corbitt et al., 2018). Aufgrund dieser Beobachtung und dem Fehlen einer TssC-Dynamik postulierten die Autoren einen neuen Sekretionsmechanismus.



**Abbildung 3: Die Lokalisation von ClpV markiert T6SS-Aktivität**

Gezeigt sind fluoreszenzmikroskopische Aufnahmen von *V. cholerae* *clpV-mCherry2* (rot) und *P. aeruginosa* *clpV1-gfp* (grün) in Mischkultur. **A)** Ein roter Fokus in *V. cholerae* zeigt T6SS-Aktivität gegen eine benachbarte *P. aeruginosa*-Bakterienzelle. *P. aeruginosa* bildet an der gleichen Stelle ein T6SS aus, sichtbar durch den ClpV1-GFP-Fokus. Kurz darauf werden morphologische Veränderungen an *V. cholerae* sichtbar. **B)** Nach erfolgter Sekretion des T6SS1 von *P. aeruginosa* wird die *V. cholerae*-Bakterienzelle innerhalb von 40 Sekunden lysiert. Verändert mit Erlaubnis nach: (Basler et al., 2013)



### **1.7 T6SS in *B. pseudomallei* und *B. thailandensis***

In *B. pseudomallei* sind sechs verschiedene T6SS-Gencluster identifiziert worden (T6SS-1-6), fünf davon finden sich homolog auch in *B. thailandensis*. Drei davon sind ansatzweise charakterisiert.

Durch Experimente von *B. thailandensis* und *P. putida* in gemischtem Biofilm konnte gezeigt werden, dass das T6SS-1 einen selektiven Vorteil vermittelt. *B. thailandensis* konnte *P. putida* innerhalb von vier Tagen überwachsen. Der Kontrollstamm, *B. thailandensis*  $\Delta tssK-1$  war nicht in der Lage, in der Mischkultur zu überleben (Schwarz et al., 2010). Durch Versuche mit C57BL/6-Mäusen konnte gezeigt werden, dass das T6SS-5 essentiell für das Überleben der Bakterien im Lungengewebe ist. *B. thailandensis* mit intaktem T6SS-5 tötete alle Versuchstiere innerhalb von drei Tagen. Eine Infektion durch *B. thailandensis*  $\Delta tssK-5$  überlebten 100% der Versuchstiere, bei durchgehender Symptombefreiheit. Die Anzahl an Bakterien stieg in der Lunge infizierter Mäuse innerhalb von zwei Tagen um den Faktor 100. Bei einer Infektion mit der  $\Delta tssK-5$  Mutante sank die Bakterienzahl um den Faktor 100 in der gleichen Zeit (Schwarz et al., 2010). Entsprechende Ergebnisse wurden mit einer T6SS-5 Mutante in *B. pseudomallei* erzielt (Burtnick et al., 2011, Toesca et al., 2014). Somit konnte klar gezeigt werden, dass das T6SS-5 von *B. pseudomallei* und *B. thailandensis* ein essentieller Virulenzfaktor ist, der für eine erfolgreiche Infektion und ein Vermehren der Bakterien im Wirt unerlässlich ist. Weiterhin konnte gezeigt werden, dass Glutathion im Zytoplasma der Wirtszelle das Signal zur Expression des T6SS-5 darstellt (Wong et al., 2015). Das Signal zur Aktivierung des T6SS-5 in der Wirtszelle ist jedoch unbekannt.

Das Fusionsprotein ClpV-1-GFP zeigte in mikroskopischen Aufnahmen distinkte, singuläre Fokuse in *B. thailandensis*. Es zeigte sich, dass das T6SS randomisiert entlang der gesamten Bakterienzelle lokalisiert sein kann. Ebenso konnte eine hohe Dynamik der ClpV-1 Fokuse beobachtet werden: die Fokuse bewegten sich in Intervallen von unter einer Minute in der Zelle (Schwarz et al., 2014). Damit verhält sich ClpV-1 des gegen Bakterien gerichteten T6SS-1 aus *B. thailandensis* wie ClpV des gegen Bakterien gerichteten T6SS aus *V. cholerae* (Basler et al., 2012, Basler et al., 2013, Basler and Mekalanos, 2012). Mikroskopische Studien des chromosomal exprimierten Fusionsproteins ClpV-5-sfGFP zeigten, dass das T6SS-5 nur exprimiert und aktiv ist, wenn die Bakterien im Zytoplasma eukaryotischer Wirtszellen vorliegen.

ClpV-5-sfGFP formt singuläre und distinkte Fokusse, wie auch für ClpV-1-sfGFP beschrieben. Die Lokalisation der Fokusse ist zu 80% polar (Schwarz et al., 2014). Die beobachtete Dynamik von ClpV-5-Fokussen unterscheidet sich grundlegend von ClpV-1-Fokussen: Die Fokusse bleiben über Intervalle, die länger als drei Minuten sind, stationär am Pol. Das T6SS aus *Francisella* ist das einzige weitere bekannte, polar lokalisierte, gegen eukaryotische Wirtszellen gerichtete T6SS. Der Mechanismus dieser polaren Lokalisation ist vollkommen unbekannt.

### **1.8 Polare Lokalisation von Virulenzfaktoren**

Alle bisher beschriebenen, „antibakteriellen“ T6SS besitzen eine randomisierte Lokalisation. Es ist naheliegend, dass eine biologische Funktion mit diesem Muster verbunden ist. Im Naturzustand ist ein Bakterium von Artgenossen und Konkurrenten umgeben. Während die Artgenossen durch die Immunproteine vor den T6SS-Effektoren geschützt sind, werden die konkurrierenden Bakterien getötet. Die Chancen, ein konkurrierendes Bakterium per T6SS auszuschalten, steigen vermutlich mit zunehmender Dynamik des Systems. Durch die randomisierte Lokalisation ist zudem die Orientierung des Bakteriums zu den Konkurrenten unerheblich.

Das T6SS-5 aus *Burkholderia* lokalisiert ausschließlich am Pol. Andere Virulenzfaktoren, die gegen eukaryotische Wirtszellen wirken, zeigen ebenfalls eine polare Lokalisation. Beispielsweise der trimere Autotransporter BimA aus *Burkholderia* oder die periplasmatische Apyrase PhoN2 aus *Shigella flexneri* (Scribano et al., 2014). Beide Faktoren ermöglichen den Bakterien intrazelluläre Motilität durch unipolare Polymerisation des Wirtszellaktins. Andere polar lokalisierte Sekretionssysteme sind die Typ IV Pili von *P. aeruginosa*, deren ATPase PilT über Interaktion mit MreB am Pol lokalisiert (Cowles and Gitai, 2010). Ein weiteres Beispiel ist das Esx-1 Typ VII Sekretionssystem aus *Mycobacterium tuberculosis* und *M. marinum* (Carlsson et al., 2009). Dieses System ist für das Auflösen des Phagosoms und damit auch für das Überleben der Bakterien in Wirtszellen essentiell (Gao et al., 2004, Conrad et al., 2017). Das T6SS aus *Francisella novicida* ist zur Auflösung des Phagosoms und für das intrazelluläre Wachstum von *Francisella* von zentraler Bedeutung (Clemens et al., 2015). Das T6SS-5 aus *Burkholderia* und das

T6SS aus *Francisella* sind die einzigen beschriebenen T6SS, die polar lokalisiert sind und gegen Wirtszellen gerichtet sind.

Es ist anzunehmen, dass mit der polaren Lokalisation von gegen Wirtszellen gerichteten Virulenzfaktoren und Sekretionssystemen auch eine biologische Funktion verbunden ist.

## 2. Wissenschaftliche Zielsetzung

Bakterielle Faktoren und Sekretionssysteme, die gegen andere Bakterien wirken, lokalisieren oft randomisiert. Gegen eukaryotische Zellen gerichtete Sekretionssysteme weisen in der Regel eine polare Lokalisation auf. Durch fluoreszierende Fusionen der jeweiligen ATPasen konnte gezeigt werden, dass das auch auf die Typ VI Sekretionssysteme (T6SS) des fakultativ intrazellulären Bakteriums *B. thailandensis* zutrifft. Das antibakterielle T6SS-1 ist essentiell für das Abtöten von Konkurrenz Bakterien. Es zeigt eine randomisierte Lokalisation. Das für das Überleben in Wirtszellen essentielle T6SS-5 hingegen ist am bakteriellen Pol lokalisiert. Es ist davon auszugehen, dass die Unterschiede der Lokalisationsmuster der T6SS von entscheidender Bedeutung für die jeweilige biologische Funktion sind. Die genauen Mechanismen, die dieser Lokalisation zugrunde liegen, sind bislang jedoch unbekannt.

In dieser Arbeit sollten erste Erkenntnisse zu den Lokalisationsmechanismen von T6SS Proteinen gewonnen werden.

Folgende Fragen sollten beantwortet werden:

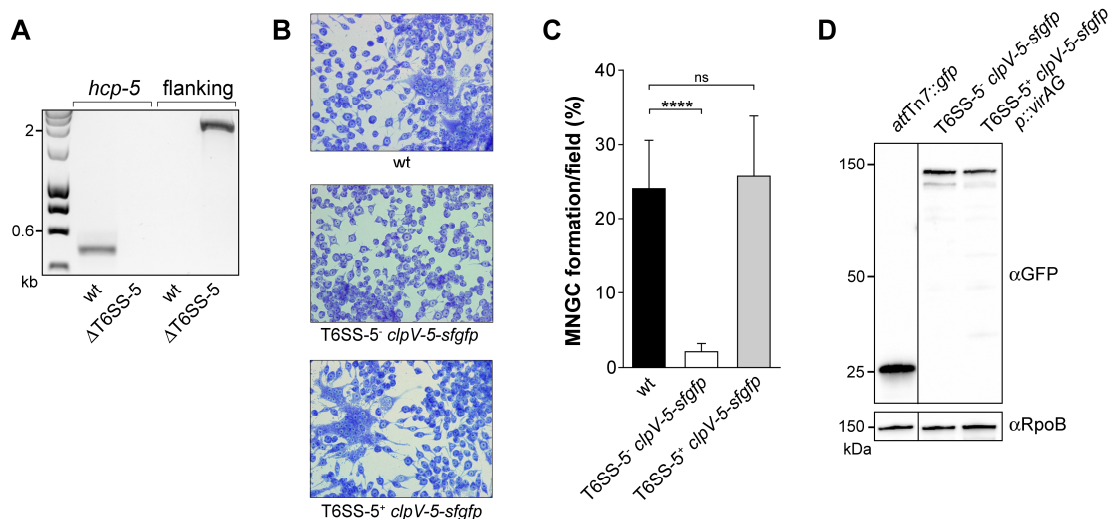
1. Ist die Lokalisierung von ClpV-1 und ClpV-5 abhängig von der Interaktion mit Komponenten des Sekretionsapparats?
2. Ist die Infektion der Wirtszelle, die das Signal für die Expression der T6SS-5 Gene produziert, für die Lokalisierung von ClpV-5 erforderlich?
3. Welche Rolle spielen zelluläre Faktoren, die an der räumlichen Organisation von Bakterien beteiligt sind (z.B. das Zytoskelettprotein MreB oder die Peptidoglykansynthese) in der Lokalisierung von ClpV-1 und ClpV-5?
4. Welchen Einfluss hat Oberflächenkontakt auf die Lokalisierung von Proteinen des T6SS, das auf Kontakt von Donor- und Zielzelle angewiesen ist?

### 3. Ergebnisse und Diskussion

#### **3.1 Publikation 2: ClpV-5-sfGFP weist eine vom T6SS-5 unabhängige polare Lokalisation auf**

Der Mechanismus der polaren Lokalisation des T6SS-5 ist vollständig unbekannt. Die Unterschiede zwischen ClpV-1 und ClpV-5 hinsichtlich der Lokalisation deuten auf unterschiedliche Lokalisationsmechanismen des gesamten Systems hin. Bisherige Studien zeigten klare Abhängigkeiten der Lokalisation von ClpV-Fokussen von anderen Proteinen des Sekretionsapparates. In einer *Serratia marcescens*  $\Delta tssE$  Mutante konnten keine ClpV-mCherry-Fokusbildung mehr beobachtet werden (Gerc et al., 2015). Durch Immunofluoreszenz konnte gezeigt werden, dass in *V. cholerae* V52 ohne intakten TssBC-Tubus ClpV diffus in der gesamten Zelle verteilt ist (Kapitein et al., 2013). Für das gegen Wirtszellen gerichtete T6SS aus *Francisella tularensis* konnte gezeigt werden, dass Lokalisation und Dynamik der AAA+ ATPase ClpB von dem Protein PdpB (TsiX) abhängig sind (Brodmann et al., 2017). Für die ATPase ClpV1 des H1-T6SS aus *P. aeruginosa* konnte gezeigt werden, dass die Bindung von ClpV1 an HsiC (TssC) nach erfolgter Kontraktion des Systems durch HsiE (TagJ) vermittelt wird (Forster et al., 2014). Über die ClpV-Proteine aus *Burkholderia* ist in dieser Hinsicht keinerlei Vorwissen existent.

In dieser Arbeit sollte zunächst untersucht werden, ob die Lokalisation von ClpV-5 von anderen Proteinen der jeweiligen Sekretionsapparate abhängig ist. Dafür wurde eine *B. thailandensis*-Mutante hergestellt, in welcher das gesamte T6SS-5-Gencluster durch unmarkierte Deletion entfernt wurde, inklusive ClpV-5. ClpV-5 wurde konstitutiv von einem neutralen Lokus auf dem Chromosom in der Mutante exprimiert (T6SS-5<sup>-</sup> *clpV-5-sfgfp*). Die T6SS-5<sup>-</sup> Mutante war wie erwartet nicht in der Lage, Wirtszellfusionen auszulösen (Abb. 4 B & C). Als Positivkontrolle wurde *B. thailandensis* *clpV-5-sfgfp* (T6SS-5<sup>+</sup> *clpV-5-sfgfp*) verwendet. Diese Mutante diente als Kontrolle für die weiteren Experimente und war in der Lage, in Zellkultur Makrophagen mit der gleichen Effektivität wie der Wildtyp (WT) zu fusionieren. Dies zeigt, dass die Fusion von ClpV-5 und sfGFP weder die ATPase noch die T6SS-5-Aktivität beeinträchtigt.

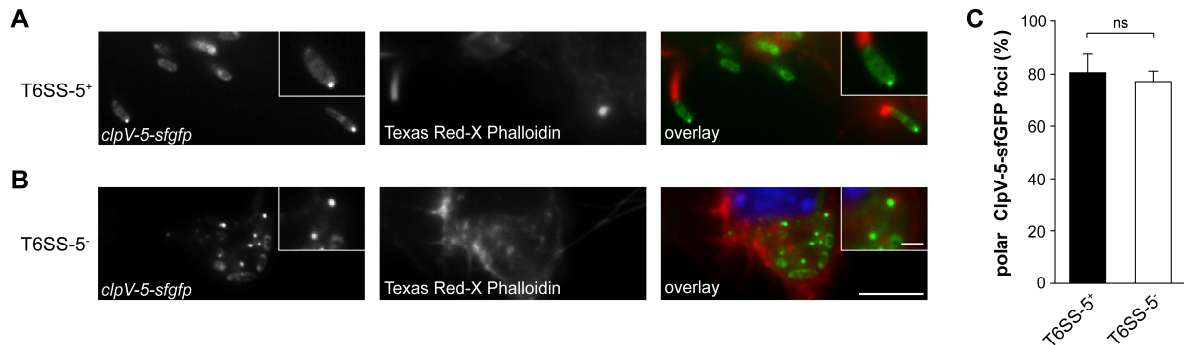


**Abbildung 4: Verifizierung der *B. thailandensis* T6SS-5<sup>-</sup> *clpV-5-sfgfp* und T6SS-5<sup>+</sup> *clpV-5-sfgfp* Mutanten**

**A)** Nachweis der Deletion des T6SS-5-Genclusters in *B. thailandensis* mittels PCR. Verwendet wurden genomische DNA der angegebenen Stämme als Template und Primerpaare die spezifisch *hcp-5* (489 bp langes Produkt für den Wildtyp, kein Produkt für die T6SS-5<sup>-</sup>Mutante) und flankierende Gene des T6SS-5 (28201 bp langes Produkt für den Wildtyp, 1885 bp langes Produkt für die T6SS-5<sup>-</sup>Mutante) amplifizieren. **B)** Repräsentative Aufnahmen von RAW264.7 Makrophagen, die mit den angegebenen Stämmen bei MOI 2 für 13 Stunden infiziert wurden. **C)** Quantifizierung der MNGC-Bildung von RAW264.7 Makrophagen, die mit den angegebenen Stämmen infiziert wurden. Dargestellt sind Mittelwerte mit Standardabweichung aus zwei Experimenten, die in Triplikaten durchgeführt wurden ( $N > 7578$  Zellkerne). ns, nicht signifikant,  $p = 0,498$  (T-Test); \*\*\*\*,  $p < 0,0001$  (Welch's T-Test). **D)** Detektion von ClpV-5-sfGFP-Fusionsproteinen in Ganzzell-Lysaten der angegebenen *B. thailandensis*-Stämme durch Western Blot unter Verwendung eines  $\alpha$ -GFP Antikörpers (MW, sfGFP: 27 kDa, ClpV-5: 101 kDa) und eines  $\alpha$ -RpoB Antikörpers als Ladekontrolle (MW, RpoB: 153 kDa). Durch *p::virAG* wird das Zwei-Komponenten-Signalsystem, welches die Expression des T6SS-5 aktiviert, überexprimiert, sodass das Fusionsprotein außerhalb von Wirtszellen produziert wird. (Lennings et al., 2019b)

Es wurden RAW 264.7 Makrophagen mit *B. thailandensis* T6SS-5<sup>-</sup> *clpV-5-sfgfp* und T6SS-5<sup>+</sup> *clpV-5-sfgfp* infiziert. Die Untersuchung durch Fluoreszenzmikroskopie ergab, dass sowohl in der T6SS-5<sup>+</sup> wie auch der T6SS-5<sup>-</sup>Mutante ca. 80% der beobachteten ClpV-5-sfGFP-Fokusse am Pol lokalisiert waren (Abb. 5 C). Damit ist gezeigt, dass die Lokalisation von ClpV-5-sfGFP unabhängig von der Präsenz anderer T6SS-5 Proteine bzw. des ganzen Systems erfolgt. Eine unabhängig vom System lokalisierende ATPase ist bisher noch für kein T6SS beschrieben worden. ClpV-5 könnte demnach mit einem anderen Faktor interagieren, um am Pol zu lokalisieren. Es ist bekannt, dass Glutathion im Zytoplasma der Wirtszelle das Signal zur Expression der T6SS-5-Gene darstellt (Wong et al., 2015). Darüber hinaus

konnte gezeigt werden, dass Wirtszellfaktoren in den Vorgang der MNGC-Bildung involviert sind (Whiteley et al., 2017a). Daher scheint es möglich, dass Wirtszellfaktoren auch einen Einfluss auf die Lokalisation von T6SS-5 und ClpV-5 haben.



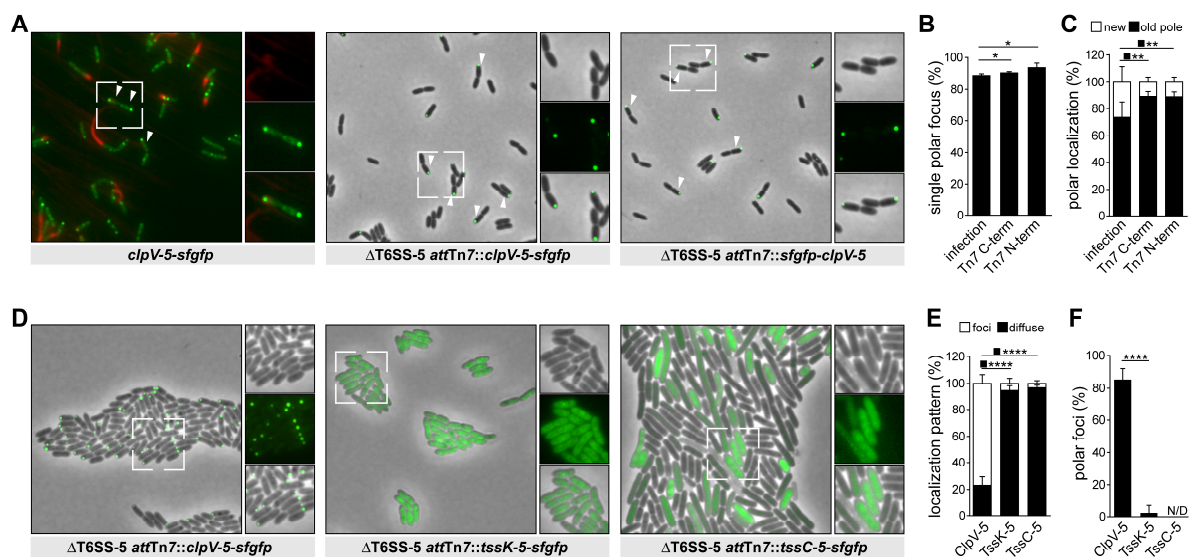
**Abbildung 5: Die polare Lokalisation von ClpV-5-sfGFP wird durch das Fehlen aller T6SS-5-Komponenten nicht beeinträchtigt**

**A, B)** RAW264.7 Makrophagen wurden mit *B. thailandensis* **A** mit chromosomaler *clpV-5-sfgfp*-Fusion im Wildtyp-Hintergrund (T6SS-5<sup>+</sup> *clpV-5-sfgfp*) und **B** mit vollständiger T6SS-5-Genclusterdeletion und *clpV-5-sfgfp*-Expression von einem neutralen chromosomalem Locus aus (T6SS-5<sup>-</sup> *clpV-5-sfgfp*) bei MOI 50 für 6 Stunden infiziert. Das Wirtszellaktin und die DNA wurden mit Texas Red-Phalloidin und DAPI gefärbt. **c)** Quantifizierung von polarer, singularer ClpV-5-sfGFP-Fokusbildung in den angegebenen *B. thailandensis*-Stämmen während der Infektion von RAW264.7 Makrophagen. Gezeigt sind Mittelwerte mit Standardabweichung aus zwei Experimenten, die in Duplikaten durchgeführt wurden (N ≥ 223 Fokusse). ns, nicht signifikant,  $p = 0,156$  (T-Test). (Lennings et al., 2019b)

### **3.2 Publikation 3: Die Lokalisation von ClpV-5-sfGFP erfolgt unabhängig von Wirtszellfaktoren**

Um den Einfluss von Wirtszellfaktoren auf die Lokalisation von ClpV-5 zu untersuchen, wurde *B. thailandensis* T6SS-5<sup>-</sup> *clpV-5-sfgfp* lebend auf Agarosepads getropft und mikroskopiert. Als Kontrollen wurden *B. thailandensis* T6SS-5<sup>-</sup> *tssK-5-sfgfp* und *tssC-5-sfgfp* verwendet. TssK-5 und TssC-5 wurden jeweils vom gleichen neutralen Locus exprimiert wie ClpV-5. Es zeigte sich, dass ClpV-5-sfGFP außerhalb von Wirtszellen in singularen Fokussen vorliegt, ebenso wie während der Infektion von Makrophagen (Abb. 6 A) (Lennings et al., 2019a). Die Fokusse waren in 90% der Fälle unipolar lokalisiert, ähnlich zur Lokalisation während der Infektion von Makrophagen (Abb. 6 B). Das Ergebnis zeigt, dass ClpV-5-sfGFP unabhängig von möglichen Wirtszellfaktoren lokalisiert. Die Expression des Basisplattenproteins TssK-5-sfGFP in *B. thailandensis* T6SS-5<sup>-</sup> führte zu 100% Signal-positiven Bakterien. Es konnte jedoch keine spezifische Lokalisation von TssK-5-sfGFP beobachtet

werden. In allen Bakterien war das Protein gleichmäßig und diffus verteilt (Abb. 6 D & E). Die Expression von *tssC-5-sfgfp* in *B. thailandensis* T6SS-5<sup>-</sup> zeigte ebenfalls eine unspezifische und diffuse Verteilung in manchen Bakterien. Im Vergleich zu TssK-5-sfGFP zeigten viele Bakterien jedoch kein Signal (Abb. 6 D & E). Im Gegensatz zu ClpV-5-sfGFP sind weder TssK-5-sfGFP noch VipB-5-sfGFP in der Lage, ohne andere T6SS-5 Komponenten zum Pol zu gelangen. Die polare Lokalisation ohne T6SS-5-Komponenten ist demnach eine Eigenschaft von ClpV-5-sfGFP, die nicht auf



**Abbildung 6: Die Fokusbildung und polare Lokalisation von ClpV-5-sfGFP ist unabhängig von Wirtszellfaktoren**

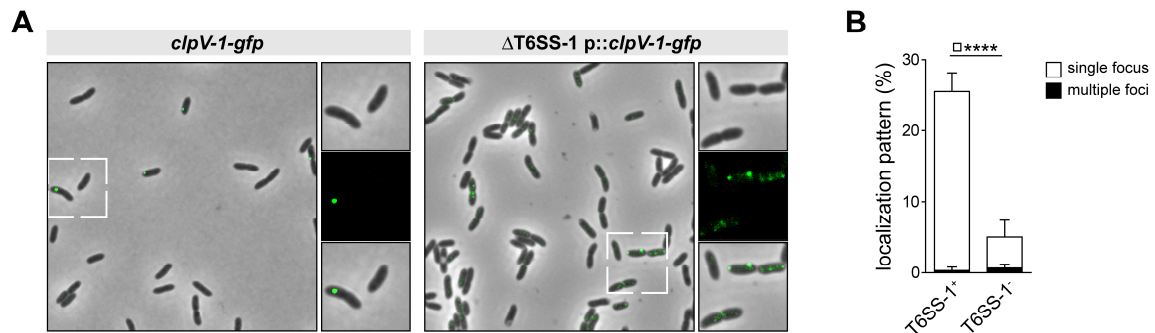
**A)** Repräsentative fluoreszenzmikroskopische Aufnahmen von *B. thailandensis* mit chromosomaler Expression von *clpV-5-sfgfp* während der Infektion von Hela-Zellen (links) und einer *B. thailandensis* T6SS-5<sup>-</sup> Mutante, in der *clpV-5-sfgfp* (mitte) und *sfgfp-clpV-5* (rechts) exprimiert wurde. Die T6SS-5<sup>-</sup> Mutanten wurden in LB-Medium angezogen und auf Agarosepads aufgetropft. **B)** Quantifizierung von Bakterien mit singulärem polarem ClpV-5-sfGFP- Fokus. *B. thailandensis clpV-5-sfgfp* während der Infektion (infection), T6SS-5<sup>-</sup> attTn7::*clpV-5-sfgfp* (Tn7 C-term) und ΔT6SS-5 attTn7::*sfgfp-clpV-5* (Tn7 N-term) außerhalb von Wirtszellen (infection: N = 480, Tn7 C-term: N = 573, Tn7 N-term: N = 532 GFP-Signal positive Bakterien). \*,  $p = 0.025 - 0.035$  (Mann-Whitney-Test). **C)** Quantifizierung des Lokalisationsmusters einzelner polarer ClpV-5-sfGFP-Fokusse in *B. thailandensis* (infection: N = 361, Tn7 C-term: N = 560, Tn7 N-term: N = 556 Bakterien mit singulärem polarem Fokus). \*\*,  $p = 0.002 - 0.003$  (Mann-Whitney-Test) **D)** Repräsentative fluoreszenzmikroskopische und Phasenkontrast-Aufnahmen der angegebenen *B. thailandensis* Stämme. Die Stämme wurden in LB-Medium angezogen und auf Agarosepads getropft. **E)** Quantifizierung der Lokalisationsmuster der angegebenen T6SS-5-Proteine, fusioniert mit GFP und exprimiert in *B. thailandensis* T6SS-5<sup>-</sup> (ClpV-5: N = 1482, TssK-5: N = 1497, TssC-5: N = 919 GFP-Signal positive Zellen). \*\*\*\*,  $p < 0.0001$  (Mann-Whitney-Test) **F)** Quantifizierung polarer Fokusbildung der angegebenen T6SS-5-Proteine, fusioniert mit GFP und exprimiert in *B. thailandensis* T6SS-5<sup>-</sup> (ClpV-5: N = 591, TssK-5: N = 77, TssC-5: 45 fokuspoitive Zellen). \*\*\*\*,  $p < 0.0001$  (Mann-Whitney-Test); N/D, nicht detektiert. Gezeigt sind Mittelwerte mit Standardabweichung aus zwei unabhängigen Experimenten die in Duplikaten durchgeführt wurden. (Lennings et al. 2019a)



andere zytoplasmatische T6SS-5-Komponenten zutrifft. Viele Proteine der AAA+/Hsp-Familie interagieren mit unterschiedlichen Adapterproteinen. Dadurch werden spezifische Interaktionen mit den Zielproteinen ermöglicht (Iyer et al., 2004, Erzberger and Berger, 2006). Die Vielseitigkeit dieser Adapterproteine kann auch innerhalb der T6SS-Familie beobachtet werden. So wird die Bindung von ClpV1 an HsiC (TssC) in *P. aeruginosa* durch HsiE (TagJ) vermittelt (Forster et al., 2014). Dem einzigen anderen polar lokalisierten und gegen Wirtszellen gerichteten T6SS, von *Francisella tularensis*, fehlt das *clpV*-Homolog (Filloux et al., 2008). Die Entfaltung von TssBC wird von dem AAA+/Hsp-Protein ClpB vollzogen. Es konnte gezeigt werden, dass die Toleranz von *F. tularensis* und *F. holarctica* gegenüber Hitze und pH-Stress durch ClpB vermittelt wird (Meibom et al., 2008, Alam et al., 2018). ClpB interagiert dem zufolge mit weiteren Ziel- und Adapterproteinen und besitzt Funktionen, die über die Entfaltung von TssBC hinausgehen. Zudem ist auch gezeigt worden, dass ClpB-mCherry2 in Abwesenheit von PdpB (TssM) keine Fokuse mehr ausbildet (Brodmann et al., 2017). ClpB zeigt also wie die ATPasen von gegen Bakterien gerichteten T6SS eine von T6SS-Proteinen abhängige Lokalisation. Möglicherweise interagiert ClpV-5 aus *Burkholderia* mit einem bisher unbekanntem, polar lokalisierten Adapterprotein, welches kein Bestandteil des T6SS-5 Genclusters ist. Eine andere Möglichkeit wie ClpV-5 den Pol erreichen könnte, wäre die Interaktion mit einem polaren Organisatorprotein. Da ClpV-5-sfGFP unipolar lokalisiert, müsste der Organisator ebenfalls unipolar vorliegen, ähnlich wie beispielsweise PopZ aus *C. crescentus* (Bowman et al., 2010). Es konnte gezeigt werden, dass PopZ eine wichtige Rolle bei der Regulation der AAA+ ATPase ClpXP einnimmt (Joshi et al., 2018). Um mögliche Interaktionspartner von ClpV-5 zu identifizieren, könnte ClpV-5-sfGFP mittels eines „GFP pulldown assays“ aufgereinigt werden. Potentielle Interaktionspartner könnten aufgrund verschiedener Bindungen ebenfalls aufgereinigt werden. Eine Analyse der Elutionsprofile von ClpV-5-sfGFP und sfGFP als Kontrolle mittels Massenspektrometrie könnte die Interaktionspartner identifizieren. Sollten die Interaktionen zu schwach sein, um eindeutige Ergebnisse zu erzielen, könnten sie mit „cross linking“ stabilisiert werden.

Zum Vergleich wurde eine *B. thailandensis* T6SS-1-Mutante nach dem gleichen Prinzip wie die T6SS-5-Mutante hergestellt. Von einem Rhamnose-induzierbarem Promotor wurde *clpV-1-sfgfp* exprimiert. Als Kontrolle diente *B. thailandensis clpV-1-sfgfp*. Wie in vorigen Experimenten zeigte ClpV-1-sfGFP im Kontrollstamm singuläre

Fokuse. Ca. 25% der signalpositiven Bakterien wiesen einen randomisiert lokalisierten Fokus auf. Im T6SS-1<sup>-</sup> Hintergrund hingegen wiesen nur knapp 5% der signalpositiven Bakterien einen diskreten Fokus auf (Abb. 7 A & B). Dieses Ergebnis zeigt, dass ClpV-1-sfGFP wie bereits beschriebene ATPasen von „antibakteriellen“ T6SS in Abhängigkeit anderer Proteine des Sekretionsapparates lokalisiert (Kapitein et al., 2013, Forster et al., 2014, Gerc et al., 2015).



**Abbildung 7: Die distinkte Fokusbildung von ClpV-1-GFP wird durch Abwesenheit des T6SS-1 signifikant reduziert**

**A)** Repräsentative fluoreszenzmikroskopische und Phasenkontrastaufnahmen von *B. thailandensis*-Mutanten, eine exprimiert eine chromosomale *clpV-1-gfp*-Fusion (*clpV-1-gfp*), bei der anderen wurde der T6SS-1-Gencluster deletiert. Die T6SS-1<sup>-</sup> Mutante exprimiert *clpV-1-gfp* von einem Plasmid ( $\Delta$ T6SS-1 p::*clpV-1-gfp*). **B)** Quantifizierung des Lokalisationsmusters von ClpV-1-GFP Fokussen in GFP-Signal-positiven *B. thailandensis clpV-1-gfp* und  $\Delta$ T6SS-1 p::*clpV-1-gfp* (T6SS-1<sup>-</sup>) Zellen (T6SS-1<sup>+</sup>: N = 1501, T6SS-1<sup>-</sup>: N = 1235 GFP-Signal positive Zellen). \*\*\*\*,  $p < 0.0001$  (T-Test). Gezeigt sind Mittelwerte mit Standardabweichung aus zwei unabhängigen Experimenten die in Duplikaten durchgeführt wurden. (Lennings et al. 2019a)

### **3.3 Publikation 3: Der Einfluss zellulärer Funktionen auf die Lokalisation einzelner T6SS Komponenten**

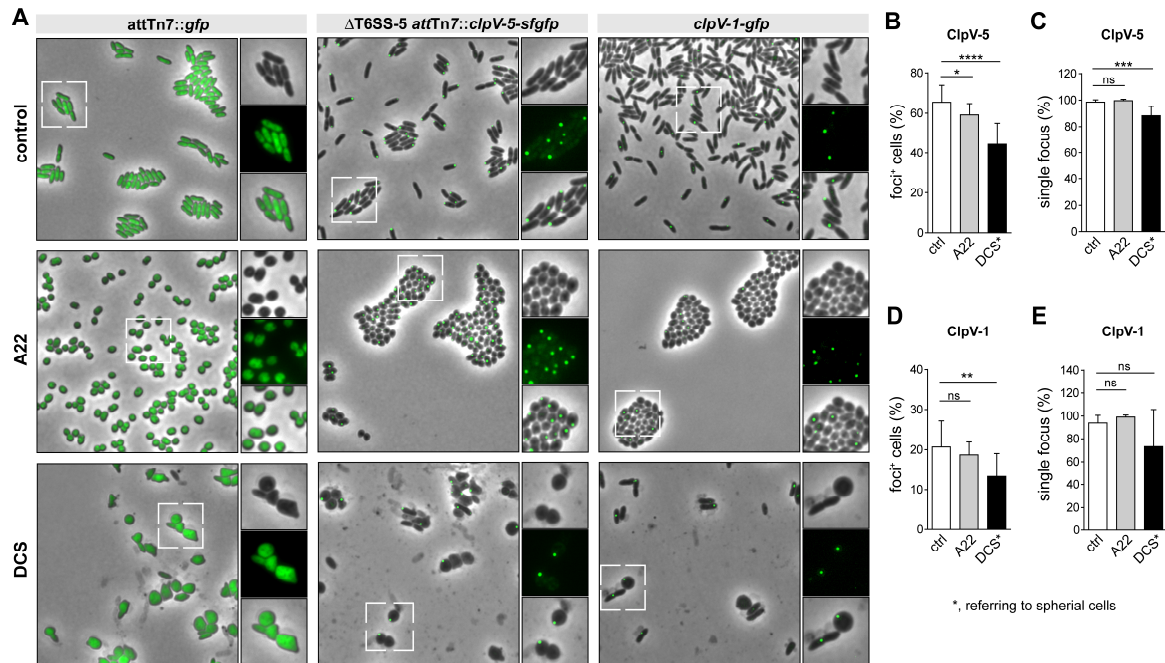
Der Membrankomplex des T6SS ist in der inneren und äußeren Membran verankert (Zoued et al., 2014). Es müssen also Berührungspunkte zwischen Membrankomplex und der Peptidoglykanschicht existieren. Die Peptidoglykansynthese und das mit ihr verbundene bakterielle Zytoskelett zählen zu grundlegenden Funktionen der bakteriellen Zelle. Mehrere Studien konnten zeigen, dass die polare Lokalisation von anderen Sekretionssystemen an grundlegende zelluläre Prozesse gebunden ist. Beispielsweise ist die bipolare Lokalisation des T4BSS aus *Legionella* an die Zellteilung gebunden (Jeong et al., 2017). Der trimere Autotransporter IcsA aus *Shigella* wird über MreB, einem bakteriellen Aktin-Homolog, am Pol lokalisiert

(Krokowski et al., 2019). Die Lokalisation der T6SS und deren ATPasen in *Burkholderia* ist noch nicht auf die Abhängigkeit vom bakteriellen Zytoskelett oder der Peptidoglykansynthese untersucht worden.

Um den Einfluss dieser grundlegenden zellulären Funktionen auf die Lokalisation von ClpV-1- und ClpV-5-sfGFP zu untersuchen, wurden *B. thailandensis clpV-1-sfgfp* und T6SS-5<sup>-</sup> *clpV-5-sfgfp* mit pharmakologischen Inhibitoren behandelt. Als Kontrolle diente *B. thailandensis* mit konstitutiv exprimiertem *sfgfp*. Die Polymerisation von MreB wurde durch Zugabe des pharmakologischen Inhibitors A22 gehemmt. Im Zuge des Experiments konnte gezeigt werden, dass *B. thailandensis* auf A22 sensibel reagiert (Abb. 8 A). Bei einer Konzentration von 15 µg/ml ging die Stäbchenform verloren und die Zellen nahmen eine rundliche, eiförmige Morphologie an. ClpV-5-sfGFP lokalisierte nach der A22-Behandlung weiterhin zu 99% in singulären Fokussen, gleichbleibend zur Kontrolle ohne A22-Zugabe (Abb. 8 C). Durch die veränderte Morphologie der Bakterien ist die Polregion nicht mehr eindeutig bestimmbar. Aus diesem Grund konnte nicht zwischen polarer und apolarer Lokalisation unterschieden werden. Eine Quantifizierung der ClpV-5-sfGFP-Fokusse ergab, dass ohne Zugabe von A22 65% der Bakterien Fokusse ausbildeten. Nach Zugabe von A22 sank die Anzahl fokuspositiver Bakterien auf 59% (Abb. 8. B). Der gleiche Vorgang wurde für *B. thailandensis clpV-1-gfp* durchgeführt. Auch im Fall von ClpV-1-GFP war die Fokusbildung nicht beeinträchtigt. In An- und Abwesenheit von A22 wiesen 20% der Bakterien Fokusse auf. Wie bei ClpV-5-sfGFP hatte die A22-Zugabe keinen Effekt auf die Fokusanzahl pro Bakterium. 95% der Bakterien wiesen einzelne Fokusse auf (Abb. 8 A, D & E). Diese Ergebnisse zeigen, dass die Lokalisation der ATPase ClpV-5 und die des gesamten T6SS-1 nicht von einem intakten MreB-Zytoskelett abhängig ist. Das würde bedeuten, dass der mögliche Interaktionspartner von ClpV-5 ebenfalls unabhängig von MreB lokalisiert.

Um die Peptidoglykansynthese (PGS) von *B. thailandensis* zu beeinträchtigen, wurde der pharmakologische Inhibitor d-Cycloserin (DCS) verwendet. DCS inhibiert die zytoplasmatischen Proteine D-Alanyl-D-Alanin-Synthetase und die Alanin-Racemase (Neuhaus and Lynch, 1964). Dadurch ist die Synthese der PG-Vorstufen drastisch eingeschränkt. Durch den beständigen Zyklus von Synthese und Hydrolyse des PG wird das PG-Gerüst dünner und instabiler. Zur Prüfung der Sensibilität wurde *B. thailandensis sfgfp* mit DCS behandelt und anschließend mikroskopiert. Bei einer

Konzentration von 50 µg/ml konnten stäbchenförmige Bakterien beobachtet werden, die keine sichtbaren morphologischen Änderungen zum Wildtyp aufwiesen. Zusätzlich konnten vollständig sphärische Bakterien sowie diverse Übergangsstadien beobachtet werden (Abb. 8 A).



**Abbildung 8: Störungen des MreB-Zytoskeletts, der Peptidoglykansynthese und stäbchenförmiger Morphologie können die distinkte, singuläre Fokusbildung von ClpV-5 nicht unterbinden**

**A)** Repräsentative fluoreszenzmikroskopische und Phasenkontrastaufnahmen der angegebenen *B. thailandensis*-Stämme, die nicht (control), oder mit dem MreB-Polymerisationsinhibitor A22 oder dem Peptidoglykansynthese-Inhibitor D-Cycloserin (DCS) behandelt wurden. **B)** Quantifizierung von ClpV-5-sfGFP fokuspositiven *B. thailandensis* nach Zugabe von A22 oder DCS (control: N = 922, A22: N = 1156, DCS: N = 462 Zellen). \*\*\*\*,  $p < 0.0001$  (Mann-Whitney-Test), \*,  $p = 0.042$  (T-Test). **C)** Quantifizierung von *B. thailandensis* mit singulären ClpV-5-sfGFP-Fokussen nach Zugabe von A22 oder DCS (control: N = 606, A22: N = 686, DCS: N= 202 fokuspositive Zellen). \*\*\*,  $p = 0.0003$  (Mann-Whitney-Test); ns, nicht signifikant (Mann-Whitney-Test). **D)** Quantifizierung von *B. thailandensis* mit ClpV-1-GFP Fokusbildung nach Zugabe von A22 oder DCS (control: N = 2748, A22: N = 2786, DCS: N = 360 Zellen). \*\*,  $p = 0.0086$  (Mann-Whitney-Test); ns, nicht signifikant (Mann-Whitney-Test). **E)** Quantifizierung von *B. thailandensis* mit singulärer ClpV-1-GFP-Fokusbildung (control: N = 638, A22: N = 526, DCS: N = 46 fokuspositive Zellen). ns, nicht signifikant (Mann-Whitney-Test). Gezeigt sind Mittelwerte mit Standardabweichung aus zwei unabhängigen Experimenten die in Duplikaten durchgeführt wurden. (Lennings et al. 2019a)

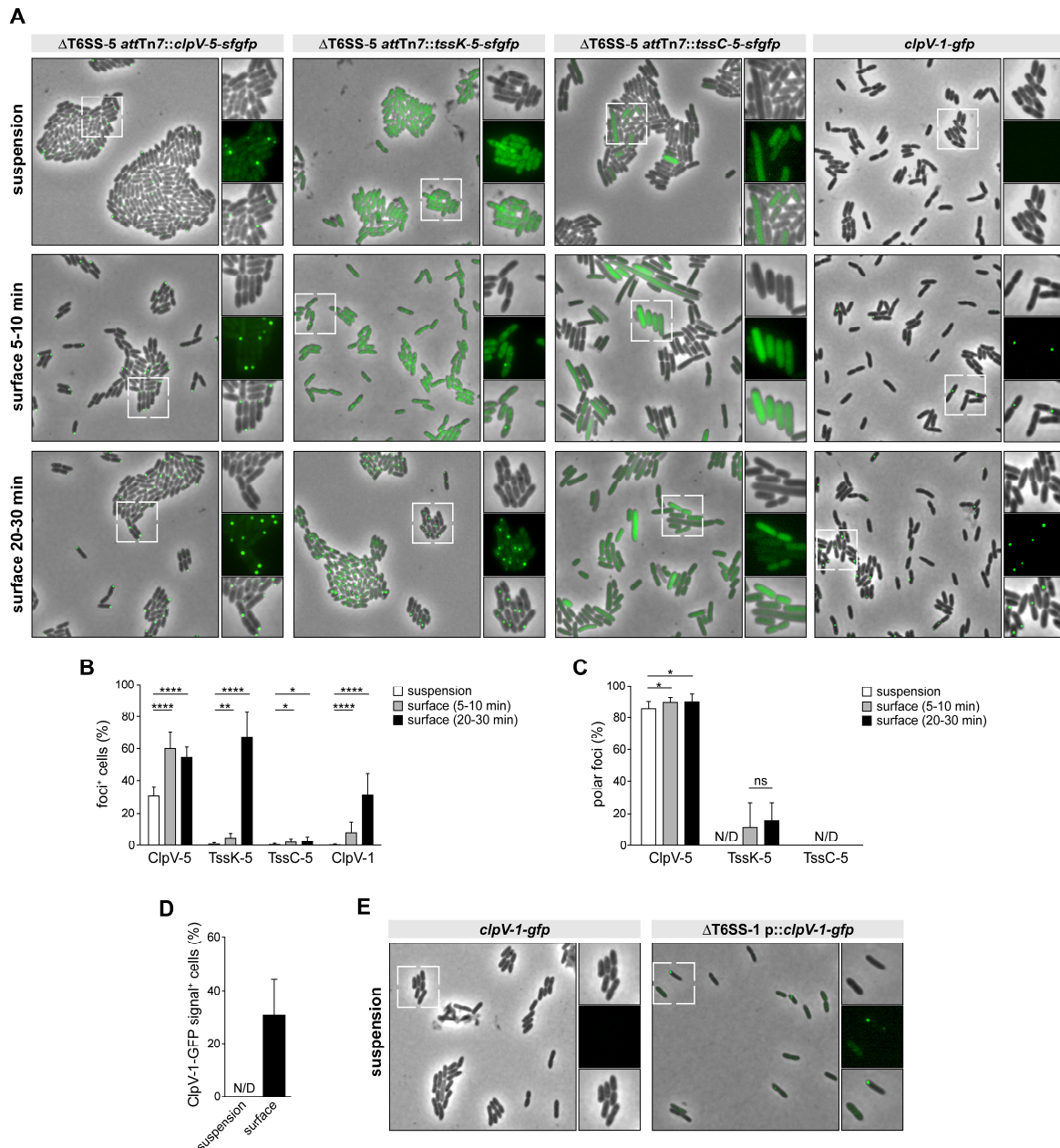
ClpV-5-sfGFP formte weiterhin distinkte Fokusse. Die Anzahl fokuspositiver Bakterien sank von 65% auf 45% nach Zugabe von DCS. Die Anzahl Bakterien mit einzelner Fokus sank von 99% bei unbehandelten auf 88% bei behandelten Bakterien (Abb. 8 B & C). Über 10% der fokuspositiven Bakterien wiesen damit

multiple Fokusse auf. Durch DCS wird zum einen die Fokusbildung von ClpV-5-sfGFP reduziert. Zum anderen wird auch die Integrität der Fokusse beeinträchtigt. Das könnte bedeuten, dass sowohl das ClpV-5-sfGFP direkt beeinträchtigt wird, wie auch sein möglicher polarer Interaktionspartner. Für *B. thailandensis clpV-1-gfp* wurden die gleichen Experimente mit gleicher DCS-Konzentration durchgeführt. Nach DCS-Zugabe konnte weiterhin die Bildung von distinkten Fokussen beobachtet werden. Die Anzahl fokuspositiver Bakterien sank signifikant von 20 auf 14%. Die Anzahl von Bakterien mit individuellem Fokus veränderte sich nicht signifikant zur Kontrolle, wies aber eine sehr große Schwankung auf. Verglichen mit ClpV-5-sfGFP ist der Effekt der Inhibition der PG-Synthese auf die Fokusbildung von ClpV-1-GFP weniger deutlich. Möglicherweise ist die randomisierte Lokalisation des T6SS-1 weniger anfällig als die spezifische polare Lokalisation des T6SS-5 bzw. der ATPase ClpV-5. Für das T6SS aus *E. coli* konnte gezeigt werden, dass die lytische Transglykosylase MltE und ihre Funktion entscheidend sind, um den Membrankomplex assemblieren zu können (Santin and Cascales, 2017). Ohne lokalen Abbau von Peptidoglykan kann der Membrankomplex nicht in das bestehende Gerüst eingebettet werden. Es ist wahrscheinlich, dass ein ähnlicher Vorgang auch in *B. thailandensis* stattfindet, um den Membrankomplex des T6SS-5 am alten Pol in die Zellwand integrieren zu können. Dieser Vorgang könnte auch einen Einfluss auf die Lokalisation von ClpV-5 haben. Es ist denkbar, dass eine Störung der Synthese auch lokale Prozesse wie den exakt regulierten Abbau des PGs beeinträchtigt.

### **3.4 Publikation 3: Die Lokalisation von T6SS Komponenten wird durch Oberflächenkontakt beeinflusst**

Schon mehrfach ist gezeigt worden, dass die Detektion von Oberflächen großen Einfluss auf den Stoffwechsel und die Lebensweise der Bakterien hat. Biofilme werden nach der Detektion und Anheftung an Oberflächen gebildet (Costerton, 1995, Costerton et al., 1999). Vertreter der Gattung *Caulobacter* zeigen eine asymmetrische Zellteilung. Sessile, an der Oberfläche haftende, und motile frei schwimmende Tochterzellen, welche sich unterschiedlich verhalten (Bodenmiller et al., 2004, Levi and Jenal, 2006). Für *P. aeruginosa* konnte gezeigt werden, dass durch Oberflächenkontakt eine Vielzahl von Virulenzfaktoren aktiviert wird (Siryaporn

et al., 2014). In *F. novicida* konnte beobachtet werden, dass IglA-GFP (TssB) während der Infektion von Makrophagen innerhalb von 15 Minuten erste Fokusse ausbildet. Wurden die Bakterien aus Flüssigkultur auf Agarosepads aufgetropft, traten die IglA-GFP-Fokusse erst nach einiger Zeit des Kontaktes mit dem Agarosepad auf (Clemens et al., 2018). Um mögliche Einflüsse von Oberflächenkontakt auf die Lokalisation von ClpV-5-sfGFP zu untersuchen, wurde *B. thailandensis* T6SS-5<sup>-</sup> *clpV-5-sfgfp* auf Agarosepads aufgetropft und nach Intervallen von fünf bis zehn und 20 bis 30 Minuten mikroskopiert. Als Negativkontrolle wurden die Bakterien vor dem Auftropfen in Flüssigkultur mit Paraformaldehyd fixiert (planktonisches Wachstum). Zum Vergleich wurde das Experiment für *B. thailandensis* T6SS-5<sup>-</sup> *tssK-5-sfgfp* und *tssC-5-sfgfp* sowie für *B. thailandensis* *clpV-1-sfgfp* durchgeführt. Die fixierten *B. thailandensis* T6SS-5<sup>-</sup> *clpV-5-sfgfp* zeigten zu 35% singuläre ClpV-5-sfGFP-Fokusse. In 85% der Fälle war dieser Fokus am Pol lokalisiert. In nicht fixierten Bakterien stieg die Anzahl fokuspositiver Bakterien nach fünf bis zehn Minuten Oberflächenkontakt auf 60%. Die Anzahl polarer Fokusse stieg auf 90%. Diese Werte blieben auch nach 20 bis 30 Minuten Oberflächenkontakt stabil (Abb 9 A, B & C). Das Ergebnis zeigt, dass die Fokusbildung und die Lokalisation von ClpV-5-sfGFP unabhängig von Oberflächenkontakt erfolgen. Dennoch verdoppelt sich die Anzahl fokuspositiver Bakterien nach längerem Oberflächenkontakt. Diese Ergebnisse unterscheiden sich deutlich von den Beobachtungen aus *F. novicida* (Clemens et al., 2018). Der Oberflächenkontakt hat vermutlich keinen Einfluss auf die Expression der T6SS-5-Komponenten. Zum einen wurde Glutathion als Expressionssignal identifiziert, zum anderen wurde *clpV-5-sfgfp* in Abwesenheit des T6SS-5 konstitutiv exprimiert, unabhängig von der Zeit des Oberflächenkontakts. Es ist jedoch unklar, ob das T6SS-5 direkt nach der Expression assembliert wird, oder ob dazu zusätzlich ein weiteres Signal nötig ist. Ebenso ist das Signal zur Kontraktion des T6SS-5 unbekannt. Möglicherweise wird während der Infektion die Wirtszellmembran bei entsprechendem Gegendruck als Oberfläche von den Bakterien wahrgenommen. Das könnte ebenso auf die Komponenten des Endomembransystems zutreffen. *B. thailandensis* könnte darauf mit erhöhter T6SS-5-Aktivität bzw. mit T6SS-5-Assemblierung reagieren. Es ist ebenso denkbar, dass der polare Marker, mit dessen Hilfe ClpV-5 möglicherweise am Pol lokalisiert, durch den Oberflächenkontakt beeinflusst wird. Eine erhöhte Abundanz am bakteriellen Pol



**Abbildung 9: Oberflächenkontakt aktiviert die Expression von ClpV-1-GFP, ist jedoch für die polare Fokusbildung von ClpV-5-sfGFP nicht essentiell**

**A)** Repräsentative fluoreszenzmikroskopische und Phasenkontrastaufnahmen der angegebenen *B. thailandensis*-Stämme, die mit PFA in Flüssigkultur fixiert wurden (suspension), oder für die angegebenen Zeiträume auf dem Agarosepad inkubiert wurden. **B)** Quantifizierung von Bakterien mit fluoreszenten Fokussen der angegebenen T6SS-5-GFP-Fusionsproteine während des planktonischen Wachstums und verschiedenen Intervallen des Oberflächenkontakts (ClpV-5: N = 778/871/1068, TssK-5: N = 901/1085/1191, TssC-5: N = 674/636/567, ClpV-1: N = 676/1145/1005 Zellen). \*\*\*\*,  $p < 0.0001$  (ClpV-5: T-Test; TssK-5/ClpV-1: Mann-Whitney-Test), \*\*,  $p = 0.0018$  (Mann-Whitney-Test), \*,  $p = 0.03$  (Mann-Whitney-Test) **C)** Quantifizierung polarer Fokusse der angegebenen T6SS-5-GFP-Fusionsproteine mit und ohne Oberflächenkontakt (ClpV-5: N = 233/502/578, TssK-5: N = 7/55/768, TssC-5: N = 2/13/19 fokuspositive Zellen). \*,  $p > 0.0153$  (Mann-Whitney-Test), ns, nicht signifikant (Mann-Whitney-Test); N/D, nicht detektiert **D)** Quantifizierung von Bakterien mit chromosomaler Expression von *clpV-1-gfp* und detektiertem Fluoreszenzsignal während des planktonischen Wachstums und 20 bis 30 Minuten Oberflächenkontakt (planktonic: N = 676, surface: N = 320 Zellen). N/D, nicht detektiert **E)** Repräsentative fluoreszenzmikroskopische und Phasenkontrastaufnahmen der angegebenen *B. thailandensis*-Stämme, die mit 4% PFA während des planktonischen Wachstums fixiert wurden. (Lennings et al. 2019a)

könnte zu erhöhter Rekrutierung von ClpV-5 und so zu mehr fokuspositiven Bakterien führen. Während der mikroskopischen Untersuchung von *B. thailandensis clpV-1-gfp* war zunächst kein GFP-Signal detektierbar. Erst nach fünf bis zehn Minuten Oberflächenkontakt mit dem Agarosepad zeigten 7% der Bakterien ClpV-1-GFP-Fokusse. Nach 20 bis 30 Minuten konnten in 35% der Bakterien Fokusse beobachtet werden. Das Lokalisationsmuster war randomisiert, entsprechend voriger Studien (Abb. 9 A, B & C). Die verzögerte Bildung von ClpV-1-GFP-Fokussen entspricht den Beobachtungen aus *F. novicida* (Clemens et al., 2018). Bisher ist bekannt, dass das antibakterielle T6SS aus *V. cholerae* permanent aktiv ist (Pukatzki et al., 2006, Basler and Mekalanos, 2012). Das antibakterielle T6SS aus *P. aeruginosa* hingegen reagiert spezifisch auf vorausgegangene T6SS-Aktivität der Nachbarzelle (Basler and Mekalanos, 2012, Basler et al., 2013). Für das T6SS-1 aus *Burkholderia* ist bekannt, dass es nur aktiv ist, wenn die Bakterien außerhalb von Wirtszellen lokalisiert sind (Schwarz et al., 2014). Es ist denkbar, dass das T6SS-1 von *B. thailandensis* allgemein auf den Kontakt mit anderen Bakterien reagiert, oder dass Oberflächenkontakt zum gleichen Effekt führt. Die schnelle Aktivierung des T6SS-1 nach Oberflächenkontakt könnte bereits sessile Konkurrenten abtöten, bevor *B. thailandensis* mit dem Ausbilden eines Biofilms beginnt.

Fixierte *B. thailandensis* T6SS-5 *tssK-5-sfgfp* zeigten zu 100% ein starkes GFP-Signal, jedoch keine spezifische Lokalisation von TssK-5-sfGFP. Nach fünf bis zehn Minuten konnten in 4% der signalpositiven Bakterien singuläre Fokusse beobachtet werden. 10% dieser Fokusse waren am Pol lokalisiert, was einer zufälligen Verteilung entsprechen könnte. Nach 20 bis 30 Minuten Oberflächenkontakt wiesen über 70% der signalpositiven Bakterien einen Fokus auf. Die Lokalisation der Fokusse war dabei unverändert zu 10% polar (Abb 9 A, B & C). Das bedeutet, dass auch TssK-5-sfGFP Fokusse ausbilden kann, diese jedoch nicht wie ClpV-5 am Pol lokalisieren. Dieses Ergebnis deutet darauf hin, dass die Lokalisation von TssK-5-sfGFP in Abwesenheit des T6SS-5 von anderen Faktoren beeinflusst wird als die von ClpV-5. Im Vergleich zu ClpV-1 fällt auf, dass die Fokusse von TssK-5-sfGFP zeitgleich mit den ClpV-1-sfGFP-Fokussen ansteigen. Zudem ist das zufällige Lokalisationsmuster deckungsgleich. Das könnte darauf hindeuten, dass TssK-5-sfGFP an den Membrankomplex des T6SS-1 binden kann. In neueren Studien konnte gezeigt werden, dass TssK als Teil der Basisplatte diese mit dem zytoplasmatischen Teil des Membrankomplexes verbindet (Durand et al., 2015,



Nazarov et al., 2018, Cherrak et al., 2018). Die konstitutive Expression von TssK-5-sfGFP ohne den passenden T6SS-Membrankomplex könnte die Substratspezifität von TssK reduzieren, sodass es an den Membrankomplex des T6SS-1 bindet und mit TssK-1 konkurriert. Da in natürlicher Umgebung die Systeme nicht zeitgleich aktiv sind, ist dieser Fall durchaus denkbar. Zur Überprüfung müsste eine  $\Delta tssK-1$  Mutante mit *tssK-5* komplementiert werden. Im Anschluss müsste dann die Funktionalität des T6SS-1 getestet werden, beispielsweise in gemischtem Biofilm mit *P. putida* (Schwarz et al., 2014). Es konnte gezeigt werden, dass Bakteriengemeinschaften translozierte T6SS-Komponenten untereinander austauschen können (Vettiger and Basler, 2016). Ob die Komponenten unterschiedlicher T6SS innerhalb des gleichen Organismus austauschbar sind, ist hingegen unbekannt. Die Expression von *tssC-sfgfp* in *B. thailandensis* T6SS-5<sup>-</sup> zeigte im Vergleich zu TssK-5-sfGFP eine geringe Zahl signalpositiver Bakterien. Die Anzahl fokuspositiver Bakterien lag bei unter 1%. Es konnten keine polar lokalisierten Fokuse beobachtet werden. Nach fünf bis zehn Minuten stieg die Anzahl Bakterien mit Fokus auf 3%, davon war keiner am Pol lokalisiert. Diese Werte änderten sich nach 20 bis 30 Minuten Oberflächenkontakt nicht weiter (Abb 9 A, B & C). Im Gegensatz zu ClpV-5 und -1 sowie TssK-5 ist VipB-5 nicht in der Lage, in Abwesenheit des T6SS-5 Fokuse auszubilden. Grundsätzlich scheint die konstitutive Expression von VipB problematisch zu sein. Es ist keine spezifische Lokalisation sichtbar, darüber hinaus treten elongierte Zellen auf. Es konnte bereits gezeigt werden, dass Deletionen von ClpV und anderen T6SS-Proteinen zur Akkumulation von VipB und einem Verlust der Tubus-Bildung in *V. cholerae* führen (Kapitein et al., 2013). Möglicherweise entstehen filamentöse Multimere, die zelluläre Funktionen beeinträchtigen.

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## **Anhang**

### **Anhang 1: Manuskript „The Burkholderia Type VI Secretion System 5: Composition, Regulation and Role in Virulence”**



# The *Burkholderia* Type VI Secretion System 5: Composition, Regulation and Role in Virulence

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The soil saprophyte and Tier I select agent *Burkholderia pseudomallei* can cause rapidly fatal infections in humans and animals. The capability of switching to an intracellular life cycle during infection appears to be a decisive trait of *B. pseudomallei* for causing disease. *B. pseudomallei* harbors multiple type VI secretion systems (T6SSs) orthologs of which are present in the surrogate organism *Burkholderia thailandensis*. Upon host cell entry and vacuolar escape into the cytoplasm, *B. pseudomallei* and *B. thailandensis* manipulate host cells by utilizing the T6SS-5 (also termed T6SS1) to form multinucleated giant cells for intercellular spread. Disruption of the T6SS-5 in *B. thailandensis* causes a drastic attenuation of virulence in wildtype but not in mice lacking the central innate immune adapter protein MyD88. This result suggests that the T6SS-5 is deployed by the bacteria to overcome innate immune responses. However, important questions in this field remain unsolved including the mechanism underlying T6SS-5 activity and its physiological role during infection. In this review, we summarize the current knowledge on the components and regulation of the T6SS-5 as well as its role in virulence in mammalian hosts.

**Keywords:** *B. pseudomallei*, type VI secretion system, intracellular life cycle, multinucleated giant cell formation, virulence

## INTRODUCTION

*Burkholderia pseudomallei* is a soil dwelling Gram-negative bacterium that causes the potentially fatal disease melioidosis in humans and animals. Infection with *B. pseudomallei* may affect virtually any organ and may encompass a wide array of non-specific clinical manifestations ranging from acute pneumonia and sepsis to localized abscess formation, making diagnosis difficult (Currie, 2015; Wiersinga et al., 2018). The mortality rate can reach 40% despite appropriate antibiotic therapy. Southeast Asia and Northern Australia are hyperendemic regions (Cheng and Currie, 2005). However, reports of environmental *B. pseudomallei* isolates or melioidosis cases from Central and South America, Africa and South Asia indicate that the bacteria are found in the tropics worldwide (Mukhopadhyay et al., 2018; Rolim et al., 2018; Steinmetz et al., 2018; Torres et al., 2018). Furthermore, a recent comprehensive modeling study suggests vast underreporting of melioidosis cases and highlights the need to assess the true global burden and epidemiology of the disease (Limmathurotsakul et al., 2016).

*Burkholderia thailandensis* is used as a surrogate model for the Tier I Select Agent *B. pseudomallei*. Reports of human infections with *B. thailandensis* are exceedingly rare and the LD<sub>50</sub> of *B. thailandensis* in mammalian animal models is at least 100 fold higher than that of *B. pseudomallei* (Smith et al., 1997; Brett et al., 1998; Lertpatanasuwan et al., 1999; Glass et al., 2006; West et al., 2008; Chang et al., 2017; Gee et al., 2018). Yet, at higher inocula via the respiratory tract, *B. thailandensis* causes rapidly fatal infections in mice and the manifestations such as neutrophil influx to the lungs, pulmonary inflammatory cytokine response, multifocal pneumonia and extra-pulmonary dissemination are similar to *B. pseudomallei* infections (West et al., 2008, 2012; Wiersinga et al., 2008a). Furthermore, both bacteria are facultative intracellular parasites and important regulatory systems and virulence factors of *B. pseudomallei* such as quorum sensing, type III and type VI secretion systems are conserved in *B. thailandensis* (Haraga et al., 2008; Majerczyk et al., 2014; Toesca et al., 2014). *B. pseudomallei* encodes six type VI secretion systems (T6SSs) and orthologs of five of them are present in *B. thailandensis* (Schell et al., 2007; Shalom et al., 2007). The analysis, so far, of three of the *Burkholderia* T6SSs revealed a high functional diversity: while the T6SS-1 and T6SS-4 are involved in interbacterial competition and metal ion acquisition, respectively, the T6SS-5 plays a central role in the intracellular life cycle of the bacteria (Schwarz et al., 2010; French et al., 2011; Russell et al., 2012; Si et al., 2017).

## THE INTRACELLULAR LIFE CYCLE OF *BURKHOLDERIA PSEUDOMALLEI* AND *BURKHOLDERIA THAILANDENSIS*

Since *B. pseudomallei* is only sporadically transmitted between humans and *B. thailandensis* infections of humans are extremely rare, the capacity of the bacteria for survival and virulence in mammals likely has its origin in the exposure of the bacteria to soil dwelling predators such as protozoa (Abbink et al., 2001; Ralph et al., 2004; Fang et al., 2016). *B. pseudomallei* is able to survive phagocytosis by protozoa, which has been suggested as pre-adaptation to avoid killing by mammalian phagocytes (Gao et al., 1997; Inglis et al., 2000; Strassmann and Shu, 2017). Indeed, *B. pseudomallei* and *B. thailandensis* are able to survive inside a range of mammalian phagocytic and non-phagocytic host cells (Jones et al., 1996; Sim et al., 2009; Bast et al., 2011; Lu et al., 2012; Whiteley et al., 2017).

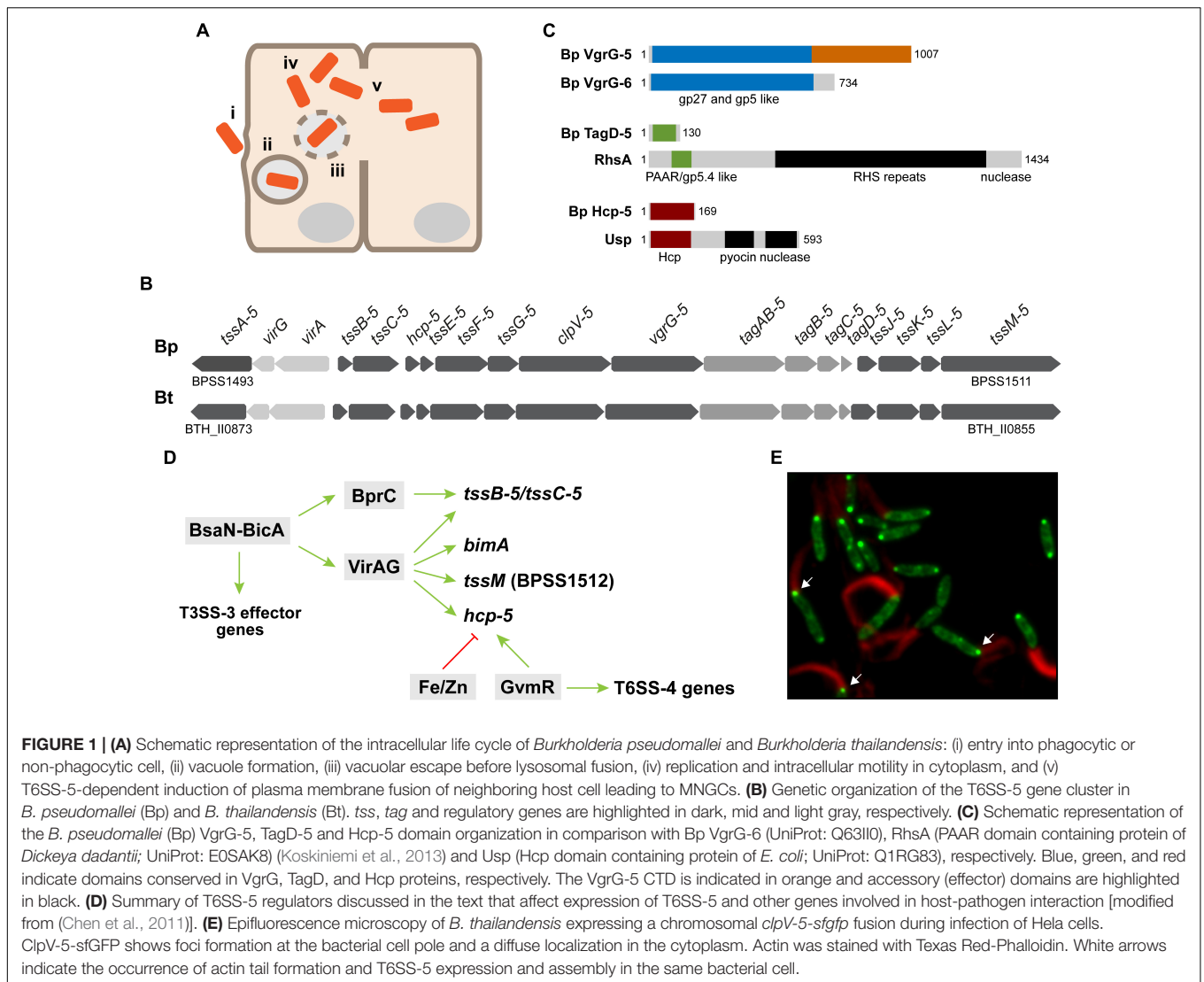
A detailed discussion on the intracellular life cycle is beyond the scope of this review and we refer the reader to several comprehensive overviews on this topic (Allwood et al., 2011; Stone et al., 2014; Willcocks et al., 2016). In brief, upon passive or active entry into the host cell the bacteria are located in a membrane-bound vacuole (Jones et al., 1996; **Figure 1A**). Before lysosomal fusion *B. pseudomallei* and *B. thailandensis* escape the endocytic vacuole, a process that is significantly impaired in T3SS-3 mutants (Stevens et al., 2002; Vander Broek and Stevens, 2017). Once in the cytosol of the host cell the bacteria replicate and employ BimA to facilitate actin tail formation or the flagella *fla2* system for intracellular motility

(Stevens et al., 2005; French et al., 2011; Sitthidet et al., 2011). Intercellular spread of *B. pseudomallei* and *B. thailandensis* can occur directly without exposure of the bacteria to the extracellular milieu by the formation of multinucleated giant cells (MNGCs). MNGCs are the result of plasma membrane fusion and subsequent cytoplasmic mixing of the infected and neighboring host cell (Kespichayawattana et al., 2004; Boddey et al., 2007; French et al., 2011). These cell-cell fusions have been detected in lung tissue samples of melioidosis patients and mice infected with a low dose of *B. pseudomallei* (Wong et al., 1995; Conejero et al., 2011). Essential to MNGC formation is the *Burkholderia* T6SS-5 (also named cluster 1 T6SS) whose mechanism of action is still unknown (Pilatz et al., 2006; Burtnick et al., 2011; French et al., 2011; Suparak et al., 2011; Schwarz et al., 2014). Furthermore, findings on the T6SS-5 of *Burkholderia mallei*, which is closely related to *B. pseudomallei*, are discussed in the review (Schell et al., 2007; Losada et al., 2010).

## COMPONENTS OF THE T6SS-5 APPARATUS

The T6SS is a complex contractile injection system (CIS) exhibiting close structural and functional resemblance with other CIS such as myophage tails and R type pyocins (Veesler and Cambillau, 2011; Leiman and Shneider, 2012; Ge et al., 2015). The T6S apparatus is composed of 14 core components termed Tss (type VI secretion system) and PAAR, and variably present Tag (type VI secretion system associated) proteins serving regulatory, structural or effector functions (Shalom et al., 2007; Hsu et al., 2009; Aschtgen et al., 2010; Shneider et al., 2013; Cianfanelli et al., 2016a). Tss proteins assemble into three T6SS subcomplexes: a tubular system located in the cytoplasm consisting of the contractile sheath proteins TssB and TssC surrounding an inner tube formed by the Hcp (TssD) protein sharpened at one end by the TssI (VgrG) and PAAR proteins, an envelope spanning membrane complex (TssM, TssL, and TssJ) and a base plate (TssE, TssF, TssG, and TssK) anchoring tube and sheath to the membrane complex (Cascales and Cambillau, 2012; Zoued et al., 2014; Nguyen et al., 2018). TssA was shown to initiate and coordinate sheath and tube polymerization during T6SS biogenesis (Zoued et al., 2016, 2017; Dix et al., 2018). The force-generating contraction of TssB and TssC acts as a molecular spring that pushes the inner Hcp tube tipped with the VgrG and PAAR spike proteins across the cell envelope into the target cell (Basler et al., 2012; Basler, 2015). Following translocation, the contracted sheath proteins are recycled by the ATPase ClpV (TssH) (Bonemann et al., 2009; Pietrosiuk et al., 2011; Kube et al., 2014; Kudryashev et al., 2015).

The vast majority of characterized T6SSs are employed by bacteria to inject toxic effector proteins into other prokaryotes (Russell et al., 2014a; Cianfanelli et al., 2016b; Hood et al., 2017; Sana et al., 2017). In addition, T6SSs specialized for effector protein delivery into eukaryotic cells including fungi and for the acquisition of metal ions have been described (Wang et al., 2015; Si et al., 2017; Trunk et al., 2018). This functional diversity



**FIGURE 1 | (A)** Schematic representation of the intracellular life cycle of *Burkholderia pseudomallei* and *Burkholderia thailandensis*: (i) entry into phagocytic or non-phagocytic cell, (ii) vacuole formation, (iii) vacuolar escape before lysosomal fusion, (iv) replication and intracellular motility in cytoplasm, and (v) T6SS-5-dependent induction of plasma membrane fusion of neighboring host cell leading to MNGCs. **(B)** Genetic organization of the T6SS-5 gene cluster in *B. pseudomallei* (Bp) and *B. thailandensis* (Bt). *tss*, *tag* and regulatory genes are highlighted in dark, mid and light gray, respectively. **(C)** Schematic representation of the *B. pseudomallei* (Bp) VgrG-5, TagD-5 and Hcp-5 domain organization in comparison with Bp VgrG-6 (UniProt: Q631I0), RhsA (PAAR domain containing protein of *Dickeya dadantii*; UniProt: E0SAK8) (Koskiniemi et al., 2013) and Usp (Hcp domain containing protein of *E. coli*; UniProt: Q1RG83), respectively. Blue, green, and red indicate domains conserved in VgrG, TagD, and Hcp proteins, respectively. The VgrG-5 CTD is indicated in orange and accessory (effector) domains are highlighted in black. **(D)** Summary of T6SS-5 regulators discussed in the text that affect expression of T6SS-5 and other genes involved in host-pathogen interaction [modified from (Chen et al., 2011)]. **(E)** Epifluorescence microscopy of *B. thailandensis* expressing a chromosomal *clpV-5-sfgfp* fusion during infection of HeLa cells. ClpV-5-sfGFP shows foci formation at the bacterial cell pole and a diffuse localization in the cytoplasm. Actin was stained with Texas Red-Phalloidin. White arrows indicate the occurrence of actin tail formation and T6SS-5 expression and assembly in the same bacterial cell.

extends to the mechanisms facilitating effector loading onto the T6SS. For instance, VgrG and PAAR (TagD) proteins can contain domains with effector function or act as carriers by binding to effector proteins while the Hcp tube can serve to translocate small (<25 kDa) effector proteins (Pukatzki et al., 2007; Silverman et al., 2013; Durand et al., 2014; Unterweger et al., 2015; Bondage et al., 2016; Ma et al., 2017; Quentin et al., 2018).

In addition to the canonical T6SS<sup>i</sup> subtype described above, which is predominantly found in Proteobacteria, other pathways (T6SS<sup>ii-iv</sup>) have been identified that differ in composition and taxonomic distribution (Boyer et al., 2009; Broms et al., 2010; Russell et al., 2014b; Eshraghi et al., 2016; Bock et al., 2017). The *Burkholderia* T6SSs belong to the T6SS<sup>i</sup> pathway. Two different nomenclatures exist for naming the T6SS gene clusters and components: cluster 1–6 T6SS (Schell et al., 2007) and T6SS-1–T6SS-6 (Shalom et al., 2007). We adopted the nomenclature T6SS-1–T6SS-6 by Shalom et al., which is also universally used to name individual T6SS proteins. The T6SS-5 [cluster 1 T6SS

according to the nomenclature by Schell et al. (2007)] consists of the 13 Tss core components that are encoded by the same gene cluster (Table 1 and Figure 1B). Furthermore, four *tag* genes, *tagA/B-5*, *tagB-5*, *tagC-5* and *tagD-5*, are present in the T6SS-5 gene cluster whose role in T6SS-5 function is currently unknown. Primary sequence analysis indicates that TagA/B-5 and TagB-5 belong to the family of pentapeptide repeat proteins (PRP) (Shalom et al., 2007). Examples of characterized PRPs are the cytoplasmic quinolone resistance protein Qnr in *E. coli* and PipB2, a kinesin-recruiting T3SS effector protein in *S. enterica* sv. Typhimurium (Tran et al., 2005; Henry et al., 2006). TagA/B-5 is essential for MNGC formation, full virulence in mice and Hcp-5 secretion indicating a critical role in T6SS-5 activity but not as an effector protein (Hopf et al., 2014). TagD-5 is a PAAR-like protein comprising 130 amino acids that appears to lack effector domains and TagC-5 is a hypothetical protein of unknown function (DUF3540) (Figure 1C). Lastly, two regulatory genes are located within the T6SS-5 cluster encoding the two component regulator VirAG, which is required for

**TABLE 1** | Components of the T6SS-5 gene cluster in *B. pseudomallei* and *B. thailandensis*.

Gene ID Bp <sup>a</sup>	Gene ID Bt <sup>b</sup>	Tss/Tag nomenclature	Alternative name	T6SS <sup>i</sup> description/subcomplex
BPSS1493	BTH_I10873	<i>tssA-5</i>		Sheath/tube assembly coordination <sup>c</sup>
BPSS1494	BTH_I10872	<i>virG</i>		Two component regulator VirAG; response regulator
BPSS1495	BTH_I10871	<i>virA</i>		Two component regulator VirAG; sensor kinase
BPSS1496	BTH_I10870	<i>tssB-5</i>		Contractile sheath
BPSS1497	BTH_I10869	<i>tssC-5</i>		Contractile sheath
BPSS1498	BTH_I10868	<i>tssD-5</i>	<i>hcp-5</i>	Tail tube/needle
BPSS1499	BTH_I10867	<i>tssE-5</i>		Base plate
BPSS1500	BTH_I10866	<i>tssF-5</i>		Base plate
BPSS1501	BTH_I10865	<i>tssG-5</i>		Base plate
BPSS1502	BTH_I10864	<i>tssH-5</i>	<i>clpV-5</i>	Sheath recycling AAA <sup>+</sup> ATPase
BPSS1503	BTH_I10863	<i>tssI-5</i>	<i>vgrG-5</i>	Spike protein
BPSS1504	BTH_I10862	<i>tagA/B-5</i>		Pentapeptide repeat protein
BPSS1505	BTH_I10861	<i>tagB-5</i>		Pentapeptide repeat protein
BPSS1506	BTH_I10860	<i>tagC-5</i>		Hypothetical
BPSS1507	BTH_I10859	<i>tagD-5</i>		PAAR like protein/spike tip
BPSS1508	BTH_I10858	<i>tssJ-5</i>		Membrane complex
BPSS1509	BTH_I10857	<i>tssK-5</i>		Base plate
BPSS1510	BTH_I10856	<i>tssL-5</i>		Membrane complex
BPSS1511	BTH_I10855	<i>tssM-5</i>		Membrane complex

<sup>a</sup>*B. pseudomallei* isolate K96243; <sup>b</sup>*B. thailandensis* isolate E264; <sup>c</sup>Dix et al. (2018).

transcriptional activation of T6SS-5 genes during infection (Chen et al., 2011).

## REGULATION OF T6SS-5 GENE EXPRESSION

The first evidence of the induction of T6SS-5 gene expression by a host cell derived signal has been provided by an *in vivo* expression technology (IVET) study (Shalom et al., 2007). The subsequent finding that the capability of vacuolar escape into the cytoplasm is a prerequisite for the activation of T6SS-5 genes suggested a cytoplasmic localization of the signal (Wong et al., 2015). Indeed, glutathione (GSH) and other low molecular weight (LMW) thiols such as cysteine have been identified to induce T6SS-5 gene expression (Wong et al., 2015). GSH is an antioxidant present at millimolar concentrations in the host cell cytoplasm. It contains one thiol group that acts as a reducing agent. Exposure of *B. pseudomallei* outside of host cells to reduced but not oxidized glutathione stimulated *hcp-5* expression by approximately 1000 fold (Wong et al., 2015). However, it is important to note that so far LMW thiols have been shown to induce transcription of T6SS-5 genes but not secretory activity of the T6SS-5. At present, the signal(s) necessary to elicit T6SS-5 contraction and secretion are not known.

Low molecular weight thiols are sensed by the sensor histidine kinase VirA of the two component system VirAG, which forms a dimer that is reduced by thiols to the active monomeric form (Wong et al., 2015). During infection of host cells VirAG positively regulates expression of *bimA* and T6SS-5 genes (Chen et al., 2011). *In trans* overexpression of *virAG* in *B. pseudomallei* and *B. thailandensis* activates the T6SS-5 and leads to Hcp-5

secretion in culture media (Schell et al., 2007; Burtnick et al., 2011; Schwarz et al., 2014; Toesca et al., 2014). Furthermore, the transcription of T6SS-5 and T3SS-3 genes is co-regulated by BsaN encoded in the T3SS-3 gene cluster (Figure 1D). BsaN activates T3SS-3 effector and translocon genes, *virAG* and the regulatory gene *bprC*. BprC in turn induces expression of *tssB-5* and *tssC-5* (Chen et al., 2011, 2014). Expression of T6SS-5 genes was shown to be co-regulated with that of T6SS-4 and secondary metabolite genes as well as a gene located next to the T6SS-5 gene cluster encoding a deubiquitinase that is secreted by the type II secretion system (Shanks et al., 2009; Burtnick et al., 2014; Duong et al., 2018). Lastly, transcription of T6SS-5 genes is inhibited in the presence of iron and zinc (Burtnick and Brett, 2013).

## PROTEINS SECRETED BY THE T6SS-5

Taking advantage of the fact that *virAG* overexpression induces secretion of T6SS-5 in bacteria grown in culture medium, comparative mass spectrometric analysis of culture supernatants was performed to identify T6SS-5 effector proteins in *B. thailandensis*. Two proteins have been identified that were absent or of significantly lower abundance in the supernatant of a  $\Delta tssK-5$  mutant relative to the wildtype: Hcp-5, the inner tube forming protein of T6SSs and VgrG-5, the needle spike protein (Schwarz et al., 2014). Hcp-5 does not appear to carry effector domains (Figure 1C). VgrG-5 contains an N-terminal and middle domain related to gp5 and gp27 bacteriophage spike forming proteins that are conserved in all T6SS<sup>i</sup> VgrG proteins (Leiman et al., 2009). However, VgrG-5 possesses an additional domain, located at the C-terminus (VgrG-5 CTD),

that is unique to the *Burkholderia* genus (Figure 1C; Burtneck et al., 2011; Schwarz et al., 2014; Toesca et al., 2014). Deletion of the VgrG-5 CTD abrogated cell-cell fusions and virulence in mice but did not affect secretion of Hcp-5 (Burtneck et al., 2011; Schwarz et al., 2014; Toesca et al., 2014). This result suggests that VgrG-5 is a specialized VgrG protein and that its CTD has essential effector function (Pukatzki et al., 2007; Durand et al., 2014; Schwarz et al., 2014). At present, VgrG-5 is the only T6SS-5 secreted protein identified with putative effector activity. Many other VgrG proteins containing additional domains at the C-terminus that display enzymatic activity, such as cross-linking of monomeric actin, have been described (Pukatzki et al., 2007; Ma et al., 2009; Suarez et al., 2010; Brooks et al., 2013). However, the VgrG-5 CTD lacks significant sequence similarity to proteins of known function and further studies will be required to determine whether the protein exhibits membrane fusion activity. Furthermore, it cannot be excluded that the protein acts as a carrier for as yet unidentified T6SS-5 effectors.

## ROLE OF THE T6SS-5 IN THE INTRACELLULAR LIFE CYCLE AND IN VIRULENCE *IN VIVO*

Several studies established a principal role of the *B. pseudomallei* and *B. thailandensis* T6SS-5 in inducing MNGC formation (Pilatz et al., 2006; Burtneck et al., 2011; French et al., 2011; Schwarz et al., 2014; Toesca et al., 2014). The function of host cell fusion in the pathogenesis of melioidosis, however, has yet to be determined. *In vitro*, *B. pseudomallei* and *B. thailandensis* are capable of stimulating MNGC formation in a range of primary and immortalized cells (Kespichayawattana et al., 2004; Welkos et al., 2015; Whiteley et al., 2017). Obvious potential benefits of this host cell manipulation are access to nutrients provided by uninfected host cells, and localized spread and replication without exposure to extracellular immune defense mechanisms.

Like T6SS-5 mutants, T3SS-3 mutants of *B. pseudomallei* display a host cell fusion defect (Suparak et al., 2005; Muangsombut et al., 2008; Gong et al., 2011). Since T3SS-3 mutants are impaired in vacuolar escape into the cytoplasm – a requirement for the induction of T6SS-5 gene expression – the role of T3SS-3 in MNGC formation could be indirect. To clarify the function of the T3SS-3 in MNGC formation, French et al. utilized a photothermal nanoblade to place a *B. thailandensis* T3SS-3 mutant from the extracellular milieu directly into the cytoplasm of the host cell thereby bypassing endocytic vesicle enclosure and escape (French et al., 2011). The finding that the mutant was able to induce host cell fusion following nanoblade delivery conclusively demonstrated that the T3SS-3 is not involved in this process.

In addition to T6SS-5 genes and *bimA* being co-regulated by VirAG, it has been shown that the deletion of structural components of the T6SS-5 reduced actin tail formation in *B. pseudomallei* and *B. mallei* (Burtneck et al., 2010; Chen et al., 2011). The underlying basis of this effect is currently

unclear. Interestingly, however, the ability of the bacteria to move in the host cell cytoplasm is a prerequisite for the stimulation of cell-cell fusion. Disruption of intracellular motility of *B. pseudomallei* and *B. thailandensis* almost entirely abolished MNGC formation (French et al., 2011). This observation suggests a site-specific induction of T6SS-5 secretion inside the host cell that leads to cell-cell fusion. Alternatively, intracellular motility was proposed to be required to bring the plasma membrane of neighboring host cells into close proximity before they are punctured by the T6SS-5 to create a hemifusion zone leading to cell-cell fusion (Toesca et al., 2014). In support of these notions, fluorescence microscopy of *B. thailandensis* expressing *clpV-5-sfgfp* during infection showed actin tail formation and T6SS-5 expression in the same bacterial cell (Figure 1E; Schwarz et al., 2014).

The deletion of essential T6SS-5 genes drastically decreased virulence of *B. pseudomallei* and *B. thailandensis* in mammalian models of acute infection (Pilatz et al., 2006; Schwarz et al., 2010, 2014; Burtneck et al., 2011; Hopf et al., 2014). Intranasal inoculation of mice with *B. pseudomallei* wildtype and *tssK-5* and *tagA/B-5* mutants showed a significant attenuation of virulence upon T6SS-5 disruption (Pilatz et al., 2006; Hopf et al., 2014). CFU measurements of lung, liver, and spleen revealed that T6SS-5 mutants were able to disseminate to distant sites although the bacterial load in the organs was significantly lower compared with wildtype challenged mice. Likewise, the LD<sub>50</sub> of a *B. pseudomallei* *hcp-5* mutant in hamsters after intraperitoneal challenge was 1000 fold higher than that of the wildtype (Burtneck et al., 2011). Furthermore, after high dose pulmonary infection with *B. thailandensis* wildtype all mice succumbed whereas a *tssK-5* mutant failed to cause lethal infections and to proliferate in the lung, liver and spleen (Schwarz et al., 2010). However, the *tssK-5* mutant caused rapidly fatal infections in mice lacking the innate immune adapter molecule MyD88, which contributes to neutrophil recruitment and activation in mice infected with *B. pseudomallei* (Wiersinga et al., 2008b). The finding that the *B. thailandensis* *tssK-5* mutant is highly virulent in MyD88<sup>-/-</sup> mice indicates that the T6SS-5 is required to overcome MyD88-dependent immune responses to establish an infection (Schwarz et al., 2010). *In vitro*, T6SS-5 mutants are able to multiply in the host cell cytoplasm (Shalom et al., 2007). Thus, the mere ability to replicate in the intracellular compartment appears to be a necessary but not sufficient trait of *B. pseudomallei* to cause disease. Lastly, virulence of T6SS-5 mutants of *B. pseudomallei*, *B. thailandensis* and *B. mallei* was attenuated in a cockroach model of infection (Fisher et al., 2012).

## CONCLUSION AND FUTURE PERSPECTIVE

Many fundamental questions remain unanswered since the discovery of the vital role of the T6SS-5 in *B. pseudomallei*-host cell interaction over 10 years ago. Critically, deciphering the mode of action of the T6SS-5 poses a challenge for the field as it still remains elusive although an essential candidate effector



has been identified. In particular, important unsolved questions are: What is the exact subcellular localization of translocated VgrG-5 and does it function as membrane fusion protein? Is the VgrG-5 CTD sufficient for mediating cell-cell fusion or are other (T6SS-5) proteins involved in the process? Does the T6SS-5 employ host cellular factors to exert its function? In addition, investigating the molecular and cellular details of the MyD88-dependent immune response that facilitates control of T6SS-5 mutant bacteria will improve our understanding of T6SS-5 function. To answer these questions *B. thailandensis* will be an ideal model organism as work with this bacterium is less laborious and less restricted with respect to for example high throughput and *in vivo* imaging techniques compared with *B. pseudomallei*. Advancing knowledge on the molecular basis of the T6SS-5 – a key virulence determinant of *B. pseudomallei* – will benefit the development of strategies to disable the capacity of the pathogen to survive and proliferate in humans.

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## AUTHOR CONTRIBUTIONS

JL and TW wrote the manuscript. SS conceived and wrote the manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Anhang 2: Manuskript „Polar localization of the ATPase ClpV-5 occurs independent of type VI secretion system apparatus proteins in *Burkholderia thailandensis*”**

RESEARCH NOTE

Open Access



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

Jan Lennings<sup>1</sup>, Christian Mayer<sup>2</sup>, Munira Makhoulouf<sup>1</sup>, Heike Brötz-Oesterhelt<sup>2</sup> and Sandra Schwarz<sup>1\*</sup> 

## 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<sup>+</sup> 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 T6SS<sup>i</sup> subtype [20], [21]. The T6SS-5, which is expressed during infection of host cells, induces the formation of multinucleated giant cells (MNGCs) for

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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* DH5 $\alpha$  and SM10 $\lambda$ pir and *B. thailandensis* E264 were routinely grown in Luria–Bertani (LB) medium at 37 °C supplemented with 200  $\mu$ g/ml trimethoprim and 0.02% rhamnose when necessary.

#### Construction of plasmids and *B. thailandensis* mutants

*Burkholderia thailandensis*  $\Delta$ T6SS-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 pJRC115 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  $\Delta$ T6SS-5. The gene *clpV-5* (BTH\_II0864) fused to *sfgfp* was cloned into pUC18T-mini-Tn7T-Tp::P<sub>S12</sub> to express *clpV-5-sfgfp* under control of the constitutive ribosomal promoter P<sub>S12</sub> (BaseClear) [23, 25]. Transformation of *B. thailandensis*  $\Delta$ T6SS-5 with this and the transposase helper plasmid pTNS3 yielded the mutant  $\Delta$ T6SS-5 *att*Tn7::P<sub>S12</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

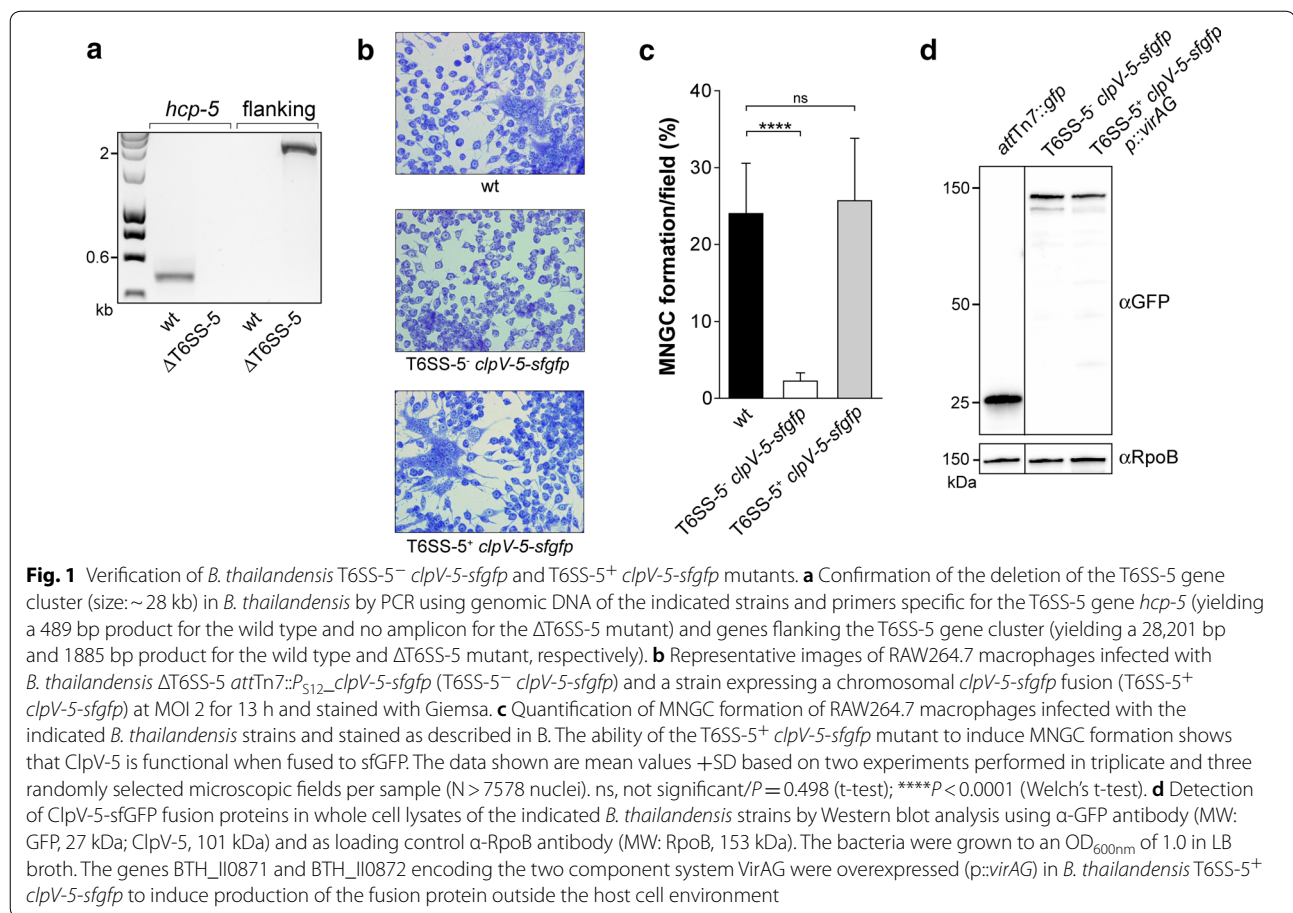
The RAW 264.7 murine macrophage cell line (ATCC) and the HeLa epithelial cell line (ATCC) were maintained in high glucose DMEM supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum (Gibco) at 37 °C and 5% CO<sub>2</sub>. On the day before the experiment 1  $\times$  10<sup>5</sup> macrophages and 5  $\times$  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  $\mu$ g/ml imipenem followed by 13 h incubation. At this time point the macrophages were stained with Giemsa (Sigma) and viewed with a Olympus BX51 microscope and a 20  $\times$  (for quantification of MNGC formation) and 40  $\times$  (for images shown in Fig. 1b) objective. MNGC formation was quantified using the formula: (number of nuclei within MNGCs/total number of nuclei)  $\times$  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 $\times$ /1.45 Oil Ph3 objective.

#### Western blot

*Burkholderia thailandensis* strains were grown in LB broth supplemented with 0.02% rhamnose and 200  $\mu$ g/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 Laemmli 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  $\leq$  0.05 was considered statistically significant.



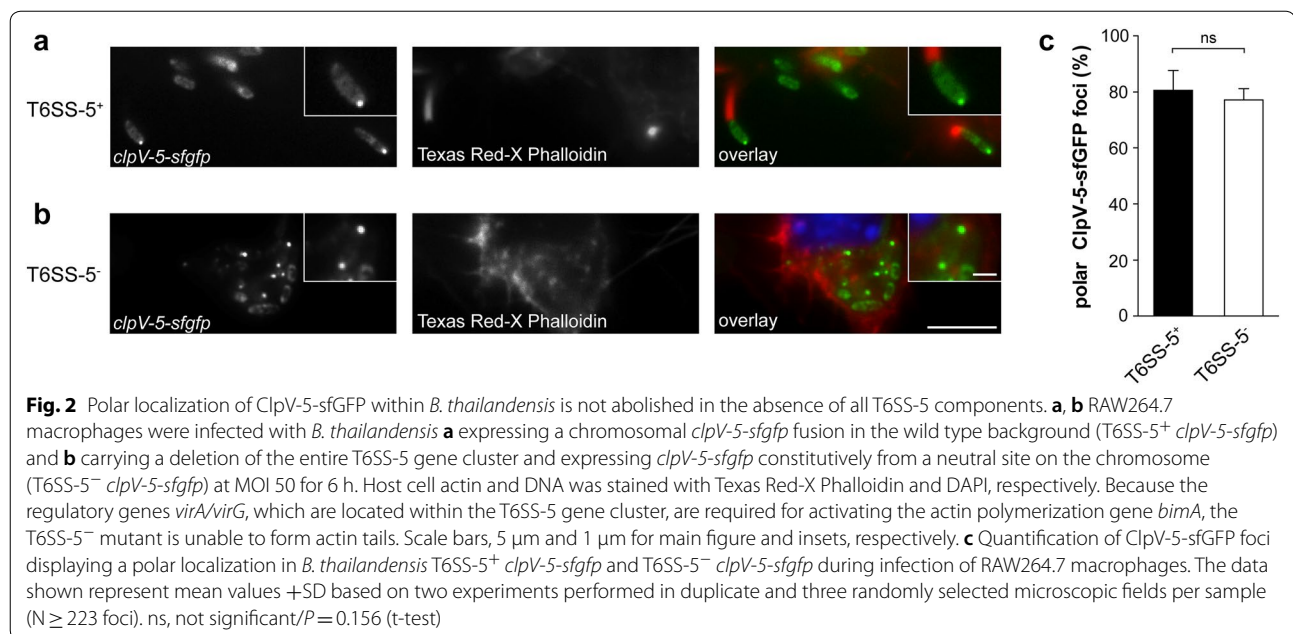
## Results and discussion

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  $\Delta$ T6SS-5 mutant under control of a constitutive promoter using the mini-Tn7 system (T6SS-5<sup>-</sup> *clpV-5-sfgfp*). Deletion of the T6SS-5 gene cluster was confirmed by PCR and the inability of the T6SS-5<sup>-</sup> *clpV-5-sfgfp* mutant to induce MNGC formation of RAW 264.7 macrophages (Fig. 1a–c). As control, bacteria expressing a chromosomal *clpV-5-sfgfp* fusion in the wild type genetic background were used (T6SS-5<sup>+</sup> *clpV-5-sfgfp*). The capability of this mutant to mediate MNGC formation at levels similar to the wild type demonstrates that ClpV-5 is functional when fused to sfGFP (Fig. 1b, c). Production of the ClpV-5-sfGFP fusion protein by *B. thailandensis* T6SS-5<sup>-</sup> *clpV-5-sfgfp* and T6SS-5<sup>+</sup> *clpV-5-sfgfp* was confirmed by Western blot analysis (Fig. 1d).

To this end, cell lysates of bacteria grown in LB broth were used. Transcription of *clpV-5-sfgfp* in T6SS-5<sup>+</sup> *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<sup>-</sup> *clpV-5-sfgfp* and as control with T6SS-5<sup>+</sup> *clpV-5-sfgfp*. In the T6SS-5<sup>+</sup> 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<sup>+</sup> and T6SS-5<sup>-</sup> bacteria (Fig. 2c). The non-native chromosomal position of *clpV-5-sfgfp* in the T6SS-5<sup>-</sup> 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-sfGFP. 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 13 h. Host cell actin was stained with Alexa Fluor 488 Phalloidin. Scale bar, 2 μm.

### Abbreviations

*attTn7*: 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 analyzed the data and JL and SS wrote the manuscript. All authors read and approved the final manuscript.

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**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.

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**Anhang 3: Manuskript „Environmental and cellular factors affecting the localization of T6SS proteins in *Burkholderia thailandensis*“**



## Environmental and cellular factors affecting the localization of T6SS proteins in *Burkholderia thailandensis*

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*Burkholderia thailandensis*  
Type VI secretion  
AAA + ATPase ClpV  
Polar localization

### ABSTRACT

The type VI secretion system (T6SS) injects effector proteins into neighboring bacteria and host cells. Effector translocation is driven by contraction of a tubular sheath in the cytoplasm that expels an inner needle across the cell envelope. The AAA + ATPase ClpV disassembles and recycles the contracted sheath. While ClpV-1-GFP of the *Burkholderia* T6SS-1, which targets prokaryotic cells, assembles into randomly localized foci, ClpV-5-GFP of the virulence-associated T6SS-5 displays a polar distribution. The mechanisms underlying the localization of T6SSs to a particular site in the bacterial cell are currently unknown. We recently showed that ClpV-5-GFP retains its polar localization in the absence of all T6SS-5 components during infection of host cells. Herein, we set out to identify factors involved in the distribution of ClpV-5 and ClpV-1 in *Burkholderia thailandensis*. We show that focal assembly and polar localization of ClpV-5-GFP is not dependent on the intracellular host cell environment, known to contain the signal to induce T6SS-5 gene expression. In contrast to ClpV-5-GFP, localization of ClpV-1-GFP was dependent on the cognate T6SS. Foci formation of both ClpV5-GFP and ClpV-1-GFP was decreased by D cycloserine-mediated inhibition of peptidoglycan synthesis while treatment of *B. thailandensis* with A22 blocking the cytoskeletal protein MreB did not affect assembly of ClpV-5 and ClpV-1 into single discrete foci. Furthermore, we found that surface contact promotes but is not essential for localization of ClpV-5-GFP to the pole whereas expression of *clpV-1-gfp* appears to be induced by surface contact. In summary, the study provides novel insights into the localization of ClpV ATPases of T6SSs targeting prokaryotic and eukaryotic cells.

### 1. Introduction

The type VI secretion system (T6SS) is a bacteriophage related microinjection device, which evolved to contract in the cytoplasm of the bacterial cell to propel a needle loaded with effector proteins into neighboring target cells (Brunet et al., 2014; Kube et al., 2014; Brackmann et al., 2017; Nguyen et al., 2018; Taylor et al., 2018). T6SSs are widely distributed among  $\gamma$ -Proteobacteria but are also found in other phyla such as Bacteroidetes (Bingle et al., 2008; Russell et al., 2014a). The majority of bacteria deploy the T6SS to deliver toxic effectors to other bacteria, while few T6SSs were shown to kill or manipulate host cells and fungi (Russell et al., 2014b; Hachani et al., 2016; Coulthurst, 2019). The T6SS apparatus consists of a cytoplasmic tail anchored via a baseplate to a membrane complex spanning the cell envelope. The baseplate is formed by the proteins TssE, F, G, K and VgrG and the components TssJ, L and M assemble into the membrane complex (Brunet et al., 2015; Durand et al., 2015; Rapisarda et al.,

2019). The tail is composed of the proteins TssB and TssC assembling into an extended tubular sheath, which surrounds an inner needle formed by the protein Hcp and tipped with the spike protein VgrG (Kudryashev et al., 2015; Wang et al., 2017). The sheath rapidly contracts to expel the needle across the cell envelope (Basler et al., 2012). The contracted TssBC sheath remains in the cytoplasm of the bacterial cell and is disassembled and recycled by ClpV, a ring-forming unfoldase belonging to the Hsp100/Clp subfamily of AAA + ATPases (Bonemann et al., 2009; Pietrosiuk et al., 2011; Kapitein et al., 2013). Unique N-terminal domains of AAA + proteins mediate substrate specificity via direct or indirect interactions. The N-terminal domain of ClpV of *Vibrio cholerae* and enteroaggregative *Escherichia coli* was shown to directly interact with the N-terminus of TssC, which is exposed specifically in the contracted conformation of the sheath (Pietrosiuk et al., 2011; Douzi et al., 2016). In *Pseudomonas aeruginosa* the H1-T6SS component TagJ of appears to function as an adapter protein to promote indirect binding of ClpV with the sheath protein TssB indicating that the

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interaction partner and mechanism of T6SS ATPases can differ (Lossi et al., 2012; Forster et al., 2014). Time lapse microscopy of fluorescent protein fusions to ClpV in *V. cholerae* showed that it assembles into a discrete dynamic focus at varying locations inside the bacterial cell that co-localizes with the contracted but not extended sheath (Basler et al., 2012). However, in *P. aeruginosa* ClpV cycles between focal assembly and disassembly at the same location (Basler et al., 2012; Corbitt et al., 2018). Together, the data suggest the existence of different mechanisms of T6SS assembly, disassembly or translocation.

*Burkholderia thailandensis* is closely related to the human pathogen and BSL-3 agent *Burkholderia pseudomallei*. Both bacteria are motile soil saprophytes capable of infecting host cells and multiplying in the cytoplasm (Jones et al., 1996; Harley et al., 1998; Horton et al., 2012; Whiteley et al., 2017). Six T6SSs are encoded by the genome of *B. thailandensis* and *B. pseudomallei*, five of which are homologous between the species. Of the latter, two T6SSs were shown to play a critical role in interbacterial and bacteria-host interaction: The T6SS-1 confers a growth advantage for *B. thailandensis* in competition with other bacteria and persistence of *B. thailandensis* and *B. pseudomallei* in the lung of mice requires the T6SS-5, which could be explained by the fact that *in vitro* a T6SS-5 deletion mutant is unable to induce the formation of multinucleated giant cells (MNGC) for intercellular spread (Pilatz et al., 2006; Schwarz et al., 2010; Burtneck et al., 2011; French et al., 2011; Russell et al., 2012; Toesca et al., 2014). While the signal stimulating expression of the T6SS-5 gene cluster in the host cell cytoplasm has been identified, the mechanism of the T6SS-5 remains unknown (Wong et al., 2015). In previous work, a functional chromosomal *clpV-5-sfgfp* fusion was generated in *B. thailandensis* to analyze T6SS-5 localization and activity during infection of host cells. ClpV-5-sfGFP assembled into discrete foci with a predominantly polar localization (Schwarz et al., 2014). Likewise, the *Francisella* T6SS, which targets eukaryotic cells, localizes to the pole (Clemens et al., 2015; Brodmann et al., 2017). In contrast, ClpV-1-GFP assembled into foci at random locations in *B. thailandensis* (Schwarz et al., 2014). This localization pattern is consistent with that of other T6SSs that translocate effectors into prokaryotic cells (LeRoux et al., 2012; Russell et al., 2014a; Brackmann et al., 2017; Saak et al., 2017). Furthermore, ClpV-1 and ClpV-5 foci displayed a dynamic localization in *B. thailandensis* (Schwarz et al., 2014). However, ClpV-5 foci were considerably less dynamic than ClpV-1 foci suggesting that T6SS-5 firing events occur at a relatively low rate. At present, focal assembly and dynamic localization of ClpV proteins were found to depend on individual components of the T6SS apparatus (Mougous et al., 2006; Basler et al., 2012; Kapitein et al., 2013; Gerc et al., 2015; Brodmann et al., 2017). However, we have recently shown that the deletion of all T6SS-5 genes in *B. thailandensis* did not disrupt foci formation and polar localization of ClpV-5-sfGFP during host cell infection (Lennings et al., 2019). This observation raises the question of how localization of the protein is achieved. In general, the mechanism underlying the localization of T6SS to a particular site in the bacterial cell is not known. In the present study, we examined the effect of external and cellular factors on the distribution of ClpV-5 and ClpV-1. We report that the intracellular host cell environment is dispensable for polar positioning of ClpV-5 and found that disruption of peptidoglycan synthesis reduced discrete foci formation of both ClpV-5 and ClpV-1. Furthermore, we show that surface contact induces but is not essential for focal assembly of ClpV-5 at the pole whereas surface contact appears to stimulate expression of *clpV-1*.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*B. thailandensis* E264 and *E. coli* DH5 $\alpha$  and SM10 $\lambda$ .pir were cultured in LB-Miller broth shaking at 200 rpm or on LB agar plates supplemented with 200  $\mu$ g/ml trimethoprim and 0.02% rhamnose when necessary.

### 2.2. Plasmid and mutant construction

An unmarked deletion of the entire T6SS-1 gene cluster (BTH\_I2954 – BTH\_I2968) in *B. thailandensis* was generated using the suicide vector pJRC115 as described previously (Chandler et al., 2009; Schwarz et al., 2010). A *B. thailandensis*  $\Delta$ T6SS-5 mutant harboring an unmarked deletion of the entire T6SS-5 gene cluster (BTH\_II0855 – BTH\_II0873) was constructed previously (Lennings et al., 2019). The transposon vector pUC18T-mini-Tn7T-Tp was used to integrate *sfGFP-clpV-5*, *clpV-5-sfgfp*, *tssK-5-sfgfp* and *tssC-5-sfgfp* fusion constructs under control of the constitutive  $P_{S12}$  promoter into a neutral site on the chromosome of *B. thailandensis*  $\Delta$ T6SS-5 (Choi et al., 2006; Schwarz et al., 2010). A *clpV-1-sfgfp* fusion construct was generated using gene synthesis and cloned into the expression vector pSCRhaB2 under control of a rhamnose inducible promoter (BaseClear) (Cardona and Valvano, 2005). Plasmid inserts were verified by Sanger sequencing (GATC). *B. thailandensis* mutants expressing a fusion of *gfp* and *sfgfp* to *clpV-1* and *clpV-5*, respectively, from the native chromosomal site have been described previously (Schwarz et al., 2014).

### 2.3. Infection of HeLa cells

HeLa cells were propagated 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>. HeLa cells were seeded on glass cover slips in a 24 well plate at a density of  $5 \times 10^4$  cells per well and infected with *B. thailandensis* *clpV-5-sfgfp* at MOI 50 the next day. After 1 h incubation the medium was replaced with DMEM supplemented with imipenem (100  $\mu$ g/ml) and infection was allowed to proceed for 13 h. The cells were fixed with 4% formaldehyde, permeabilized with 0.5% Triton-X 100 and stained with Texas Red-X Phalloidin.

### 2.4. Treatment of *B. thailandensis* with A22 and DCS

Overnight cultures of *B. thailandensis* were diluted to an OD<sub>600nm</sub> of 0.05 and grown to an OD<sub>600nm</sub> of approximately 0.4. The inhibitors A22 (S-(3,4-dichlorobenzyl)isothiourea hydrochloride) (Sigma) and DCS (D-cycloserine; 4-amino-3-isoxazolidinone) (Sigma) were added at 15 and 50  $\mu$ g/ml, respectively. After 1 h incubation the bacteria were washed and immobilized on agarose pads for 15 min before image acquisition.

### 2.5. Fluorescence microscopy

Unless stated otherwise live bacteria grown to an OD<sub>600nm</sub> of approximately 0.4 were used for fluorescence microscopy. For some experiments the bacteria were fixed during growth in LB medium at an OD<sub>600nm</sub> of approximately 0.4 or after 20 min incubation in PBS at 23 °C with 4% paraformaldehyde (PFA). Imaging of *B. thailandensis* was performed using Gene Frames (Thermo) filled with 1% agarose re-suspended in PBS and sealed with a cover slip. After spotting the bacteria onto the agarose pads they were incubated for 5–10 min or 20–30 min before acquisition of randomly selected microscopic fields. For this, a Nikon Eclipse Ti-E microscope equipped with a CFI Plan-Apo DM 100x/1.45 Oil Ph3 objective or a Leica DMRE microscope equipped with a HCX PL Apo 100x/1.35 Oil Ph3 objective was used.

### 2.6. Western blot

Bacteria were grown to an OD<sub>600nm</sub> of 1.0 and the pellet of a 1 ml aliquot was resuspended in Laemmli sample buffer. The samples were separated by 10% SDS PAGE, transferred to nitrocellulose membranes and probed with antibodies against GFP (Thermo Fisher) or RNA polymerase beta (Thermo Fisher). Proteins were detected using HRP-conjugated rabbit anti-mouse antibody (Thermo Fisher) and Clarity Western ECL substrate (Biorad).

2.7. Statistical analysis

The Mann-Whitney test or *t*-test was performed to determine the difference between two means as indicated in the figure legends. A *P* value of < 0.05 was considered statistically significant. Shown are mean values + SD.

3. Results

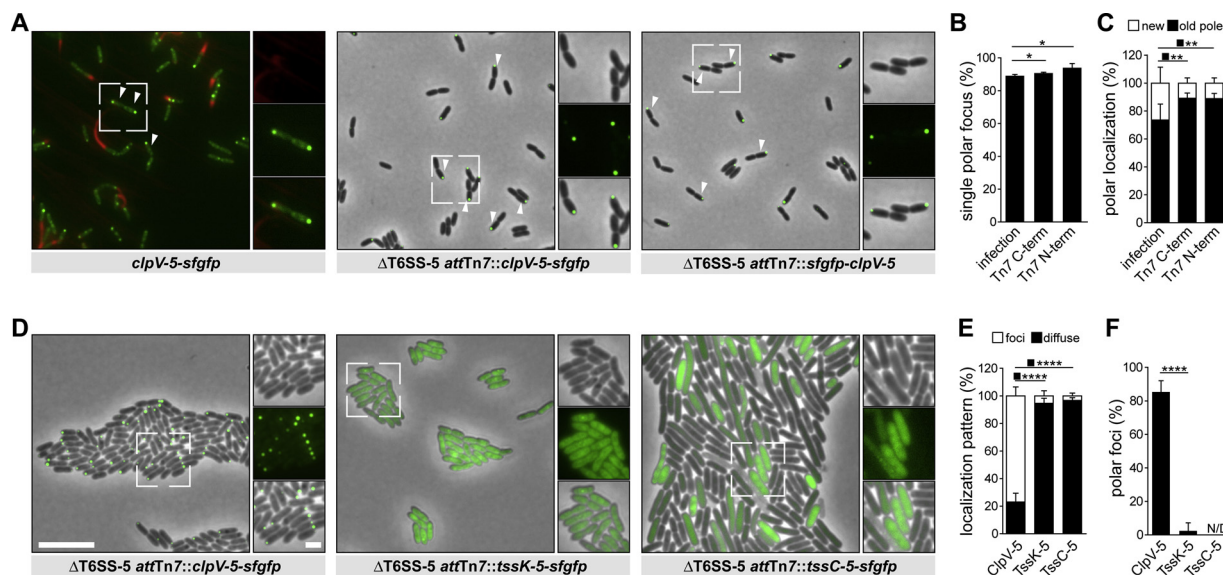
3.1. Focal assembly and polar localization of ClpV-5-sfGFP is not dependent on the intracellular host cell environment

A ClpV-5-sfGFP fusion expressed from the native chromosomal location of *clpV-5* localizes to discrete and predominantly static foci at the pole of *B. thailandensis* during infection of host cells (Schwarz et al., 2014). The fusion protein is functional as indicated by similar MNGC formation efficiencies of *B. thailandensis* wildtype and *clpV-5-sfgfp* (Lennings et al., 2019). Recently, we showed that polar foci formation of ClpV-5-sfGFP does not require any of the T6SS-5 components in *B. thailandensis* located in macrophages (Lennings et al., 2019). To gain insight into ClpV-5 localization we first investigated if the intracellular host cell environment – which is required for induction of T6SS-5 gene expression – is necessary for focal assembly and polar positioning of the protein. As control, Hela cells were infected with *B. thailandensis clpV-5-sfgfp*. As described previously, ClpV-5-sfGFP formed discrete unipolar foci in the majority of bacteria (89 ± 1%) located in the host cell cytoplasm (Fig. 1 A and B). Bacterial cells that remain associated after a recent division allowed us to distinguish between the old and new pole. We found that the ClpV-5-sfGFP fusion protein preferably localizes to the old pole after division (74 ± 11%) (Fig. 1 C and Fig. S2). Having specified the localization pattern of ClpV-5-sfGFP in the presence of a

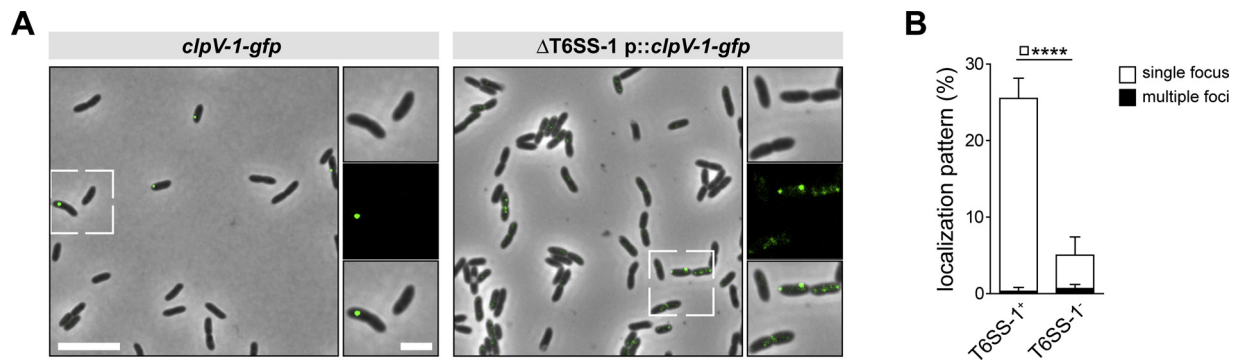
complete T6SS-5 apparatus during infection, we next asked whether it is dependent on host cell derived factors or signals in the absence of the T6SS-5. To this end, *clpV-5-sfgfp* and *sfgfp-clpV-5* were expressed constitutively from a neutral site on the chromosome in a mutant lacking the T6SS-5 gene cluster ( $\Delta$ T6SS-5 attTn7::*clpV-5-sfgfp* and  $\Delta$ T6SS-5 attTn7::*sfgfp-clpV-5). The bacteria were grown in LB medium and spotted onto agarose pads for imaging. 91 ± 0.8% and 94 ± 3% of the  $\Delta$ T6SS-5 attTn7::*clpV-5-sfgfp* and  $\Delta$ T6SS-5 attTn7::*sfgfp-clpV-5 mutants, respectively, displayed single unipolar localization of the fusion proteins (Fig. 1 A and B). In addition, ClpV-5-sfGFP and sfGFP-ClpV-5 still predominantly localized to the old pole (Fig. 1 C). The results show that a host cell derived factor is dispensable for polar localization of the ATPase. Next, we analyzed the dependence of other cytoplasmic T6SS-5 components on the T6SS-5 apparatus for localization. For this, the baseplate protein TssK-5 and the sheath protein TssC-5, a protein displaying a diffuse localization in *V. cholerae* when expressed in the absence of TssB-5, were fused to sfGFP in the  $\Delta$ T6SS-5 background ( $\Delta$ T6SS-5 attTn7::*tssK-5-sfgfp* and  $\Delta$ T6SS-5 attTn7::*tssC-5-sfgfp* (Kapitein et al., 2013). Expression of the fusion proteins was verified by Western blot (Fig. S1). Unlike the  $\Delta$ T6SS-5 attTn7::*tssK-5-sfgfp* mutant a GFP signal was not detected in all  $\Delta$ T6SS-5 attTn7::*tssC-5-sfgfp* mutant, which displayed an altered cell morphology. In contrast to ClpV-5, TssK-5-sfGFP and TssC-5-sfGFP fusion proteins exhibited an almost exclusively diffuse localization suggesting that TssK-5 and TssC-5 interact with other T6SS-5 components for positioning (95 ± 4% and 97 ± 2%, respectively) (Fig. 1 D and E). The few TssK-5-sfGFP foci that formed did not display a specific polar localization (Fig. 1 F).**

3.2. ClpV-1-GFP foci formation is dependent on the T6SS-1 apparatus

Previous studies using fluorescent protein fusions to ClpV in other



**Fig. 1. The host cell environment is not required for focal assembly of ClpV-5-sfGFP and localization to the pole.** A. Left: Representative fluorescence microscopy images of *B. thailandensis* expressing a chromosomal *clpV-5-sfgfp* fusion during infection of Hela cells (left) and a *B. thailandensis*  $\Delta$ T6SS-5 mutant expressing *sfgfp-clpV-5* (middle) and *clpV-5-sfgfp* (right) and grown in LB broth and spotted onto agarose pads. Host cell actin was stained with Texas Red-X phalloidin. White arrows indicate cells used for quantification of ClpV-5-sfGFP localization to the old or new pole. B. Quantification of bacteria displaying a single polar focus of ClpV-5-sfGFP fusion proteins. *B. thailandensis clpV-5-sfgfp* during infection (infection),  $\Delta$ T6SS-5 attTn7::*clpV-5-sfgfp* (Tn7 C-term) and  $\Delta$ T6SS-5 attTn7::*sfgfp-clpV-5* (Tn7 N-term) outside the host cell environment (infection: N = 480, Tn7 C-term: N = 573, Tn7 N-term: N = 532 GFP signal<sup>+</sup> bacteria). \*, *p* = 0.025 - 0.035 (Mann-Whitney test). C. Quantification of the polar localization pattern of single ClpV-5-GFP fusion protein foci in *B. thailandensis* (infection: N = 361, Tn7 C-term: N = 560, Tn7 N-term: N = 556 bacteria with a single polar focus). \*\*, *p* = 0.002 - 0.003 (Mann-Whitney test) D. Representative phase contrast and fluorescence microscopy images of the indicated *B. thailandensis* mutants grown in LB broth and spotted onto agarose pads. E. Quantification of the localization pattern of the indicated T6SS-5 proteins fused to sfGFP in *B. thailandensis* (ClpV-5: N = 1482, TssK-5: N = 1497, TssC-5: N = 919 GFP signal<sup>+</sup> cells). \*\*\*\*, *p* < 0.0001 (Mann-Whitney test) F. Quantification of polar foci localization of the indicated T6SS-5 proteins fused to sfGFP in *B. thailandensis* (ClpV-5: N = 591, TssK-5: N = 77, TssC-5: 45 foci<sup>+</sup> cells). \*\*\*\*, *p* < 0.0001 (Mann-Whitney test); N/D, not detected. Shown are mean values + SD of two independent experiments performed in duplicate. Scale bar, 10  $\mu$ m (overview) and 2  $\mu$ m (insets) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).



**Fig. 2.** Assembly of ClpV-1-GFP into single discrete foci is significantly decreased in the absence of the T6SS-1 secretion apparatus. **A.** Representative phase contrast and fluorescence microscopy images of a *B. thailandensis* mutant expressing a chromosomal *clpV-1-gfp* fusion and a mutant harboring a deletion of the T6SS-1 gene cluster and expressing *clpV-1-gfp* from a plasmid ( $\Delta$ T6SS-1 p::*clpV-1-gfp*). **B.** Quantification of the localization pattern of discrete ClpV-1-GFP foci in GFP signal positive cells of *B. thailandensis clpV-1-gfp* and  $\Delta$ T6SS-1 p::*clpV-1-gfp* (T6SS-1<sup>-</sup>) (T6SS-1<sup>+</sup>: N = 1501, T6SS-1<sup>-</sup>: N = 1235 signal<sup>+</sup> cells). \*\*\*\*,  $p < 0.0001$  (*t*-test). Shown are mean values + SD of two independent experiments performed in duplicate. Scale bar, 10  $\mu$ m (overview) and 2  $\mu$ m (insets).

bacteria demonstrated a diffuse localization pattern of the ATPase in the absence of other T6SS proteins. To determine if ClpV-1 of the *Burkholderia* T6SS-1 targeting bacterial cells requires the T6SS-1 for focal assembly, *clpV-1-gfp* was expressed from a plasmid in bacteria carrying a deletion of the entire T6SS-1 gene cluster ( $\Delta$ T6SS-1 p::*clpV-1-gfp*). *B. thailandensis* expressing a chromosomal *clpV-1-gfp* fusion in the wild type genetic background was used as control (*clpV-1-gfp*). Under the conditions used,  $26 \pm 3\%$  of *B. thailandensis clpV-1-gfp* showed discrete foci formation (Fig. 2 A and B). In the absence of T6SS-1 components, however, ClpV-1-GFP localized to discrete foci in only  $5 \pm 2\%$  of the bacteria and the majority of cells displayed a diffuse or punctate GFP signal (Fig. 2 A and B). This result suggests that ClpV-1 requires one or more T6SS-1 proteins for discrete focal assembly and presumably proper localization consistent with reports on ClpV proteins of T6SSs in other bacteria.

### 3.3. Surface contact stimulates but is not essential for ClpV-5-sfGFP focal assembly at the pole while expression of *clpV-1-gfp* appears to depend on surface contact

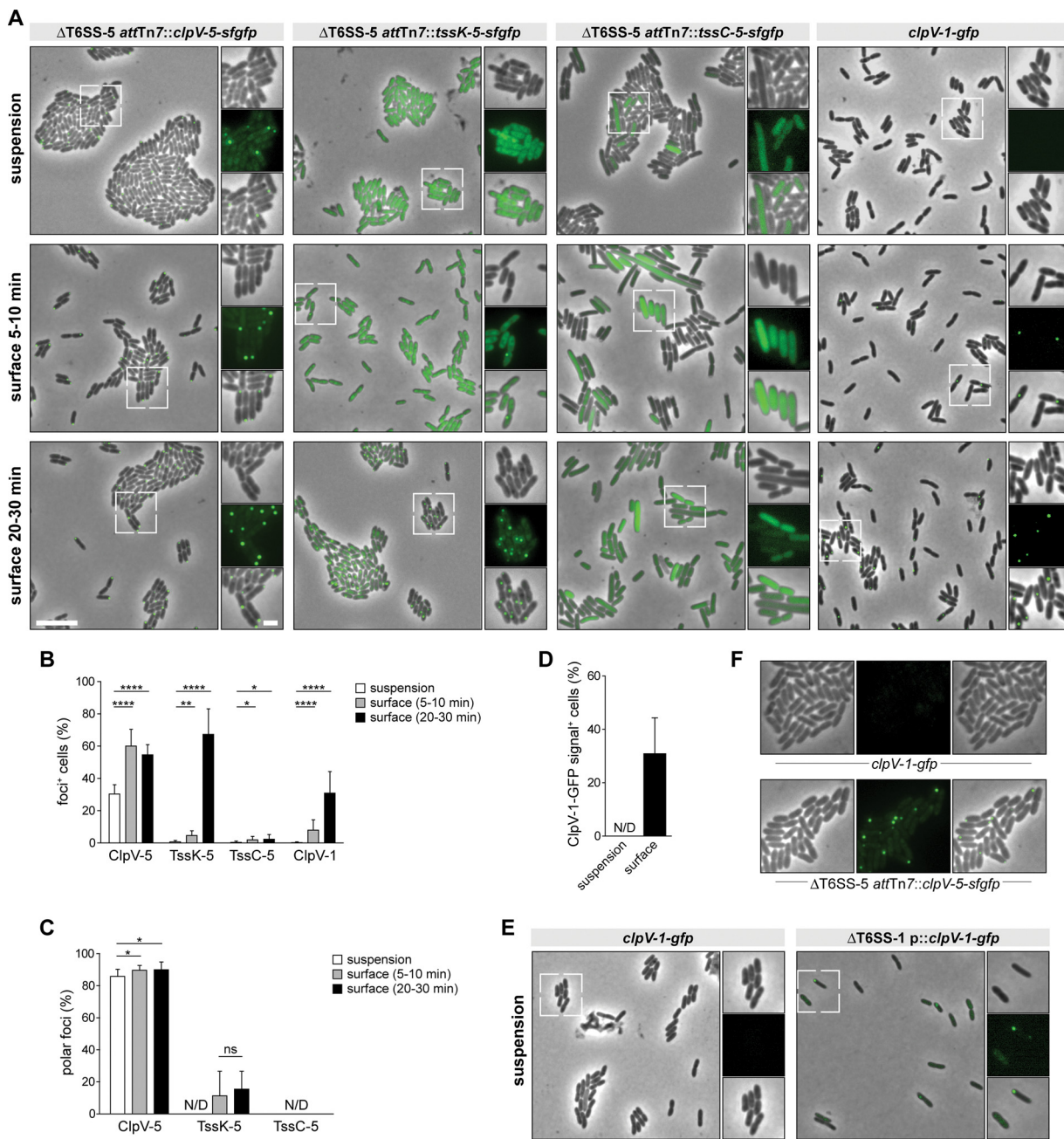
Given that the mechanism of ClpV-5-sfGFP localization does not involve the T6SS-5 apparatus and the intracellular host cell environment, we performed the following experiments in a  $\Delta$ T6SS-5 mutant cultured in LB medium ( $\Delta$ T6SS-5 attTn7::*clpV-5-sfGFP*). ClpV-1-GFP, however, was analyzed in the wild type genetic background (*clpV-1-gfp*) because its localization is dependent on the T6SS-1. While performing the experiments described in Fig. 1D, we noticed that longer incubation time of the bacteria on the agarose pad was accompanied by a change in the localization pattern of TssK-5-sfGFP. Thus, the influence of surface contact on the localization of ClpV-5 and ClpV-1 was investigated. To study the distribution of the proteins in the absence of surface contact *B. thailandensis* was fixed with 4% PFA during growth in LB broth. Fixed cells were spotted onto agarose pads for imaging. ClpV-5-sfGFP showed foci formation in  $30 \pm 6\%$  of bacteria grown in suspension indicating that surface contact is not essential for ClpV-5 localization (Fig. 3A and B). By contrast, TssK-5-sfGFP and TssC-5-sfGFP virtually never displayed focal assembly in suspension bacteria (Fig. 3A and B). Next, the effect of surface contact on protein localization was examined by incubating live bacteria from liquid cultures on agarose pads before image acquisition. Bacteria incubated for 5–10 min showed a two fold increase in foci formation of ClpV-5-sfGFP compared with fixed suspension bacteria (Fig. 1B). The result indicates that the localization of ClpV-5 to foci is stimulated by surface contact. Increasing incubation time on the agarose pad to 20–30 min did not enhance foci formation of the ClpV-5-sfGFP fusion protein (Fig. 3A and B). Similarly, incubating *B. thailandensis* on agarose pads for 5–10 min induced discrete TssK-5-sfGFP foci formation by sevenfold relative to suspension cells. However,

unlike ClpV-5-sfGFP extended surface contact further promoted TssK-5 foci formation 15-fold. Furthermore, the ClpV-5 fusion protein displayed a polar localization in the majority of suspension bacteria ( $86 \pm 5\%$ ) showing that surface contact is not required for the ClpV-5 localization pattern observed during host cell infection and in the presence of a complete T6SS-5 apparatus (Fig. 3A and C). In contrast, TssK-5-sfGFP foci appeared to localize randomly and only  $16 \pm 11\%$  of bacteria following 20–30 min incubation on the agarose surface had TssK-5-sfGFP positioned at the pole.

In contrast to ClpV-5 virtually no ClpV-1-GFP foci were detected in suspension *B. thailandensis* cells expressing *clpV-1-gfp* from the native chromosomal locus of *clpV-1* (Fig. 3A and B). Furthermore, weak *clpV-1-gfp* expression was detected in suspension bacteria by Western blot (Fig. S1). However, ClpV-1-GFP foci were observed in bacteria after incubation on the agarose pad (Fig. 3A and B). The number of ClpV-1-GFP foci positive cells increased with elongated incubation on the agarose pad to  $31 \pm 13\%$  (Fig. 3A and B). We did not detect a fluorescent signal in *B. thailandensis clpV-1-gfp* grown in suspension (Fig. 3A and D). This finding suggests that expression of *clpV-1* and presumably other T6SS-1 genes is stimulated by surface contact. Increased ClpV-1-GFP foci assembly in bacteria located on agarose pads could be attributed to increasing expression levels of the fusion construct. To test if ClpV-1-GFP requires surface contact for focal assembly, we expressed *clpV-1-gfp* ectopically in a mutant lacking the T6SS-1 gene cluster, induced expression during growth in liquid medium and added PFA to the culture. Discrete formation of ClpV-1-GFP foci was observed in suspension bacteria suggesting that it does not require surface contact (Fig. 3E). In this study agarose was resuspended in PBS to prepare the pads on which the bacteria were incubated at room temperature before image acquisition. To exclude a potential effect of PBS and room temperature on ClpV-5-sfGFP and ClpV-1-GFP we incubated the bacteria in PBS solution at 23 °C for 20 min followed by PFA fixation of the suspension bacteria. The results show that exposure to PBS and lower temperature did not affect ClpV-5-sfGFP localization and *clpV-1-gfp* expression (Fig. 3F).

### 3.4. Inhibition of peptidoglycan synthesis but not MreB polymerization decreases formation of single and discrete ClpV-5-sfGFP and ClpV-1-GFP foci

ClpV-5 localizes to the pole in *B. thailandensis* grown outside the host cell. To gain further insight into the mechanisms by which ClpV-5 and ClpV-1 localize, we investigated the role of core cellular factors known to be involved in the spatial organization of bacterial cells such as the MreB cytoskeleton. The genome of *B. thailandensis* E264 contains two genes annotated as *mreB* both encoding proteins with significant similarity to MreB proteins (BTH\_10146 and BTH\_III1780). To study

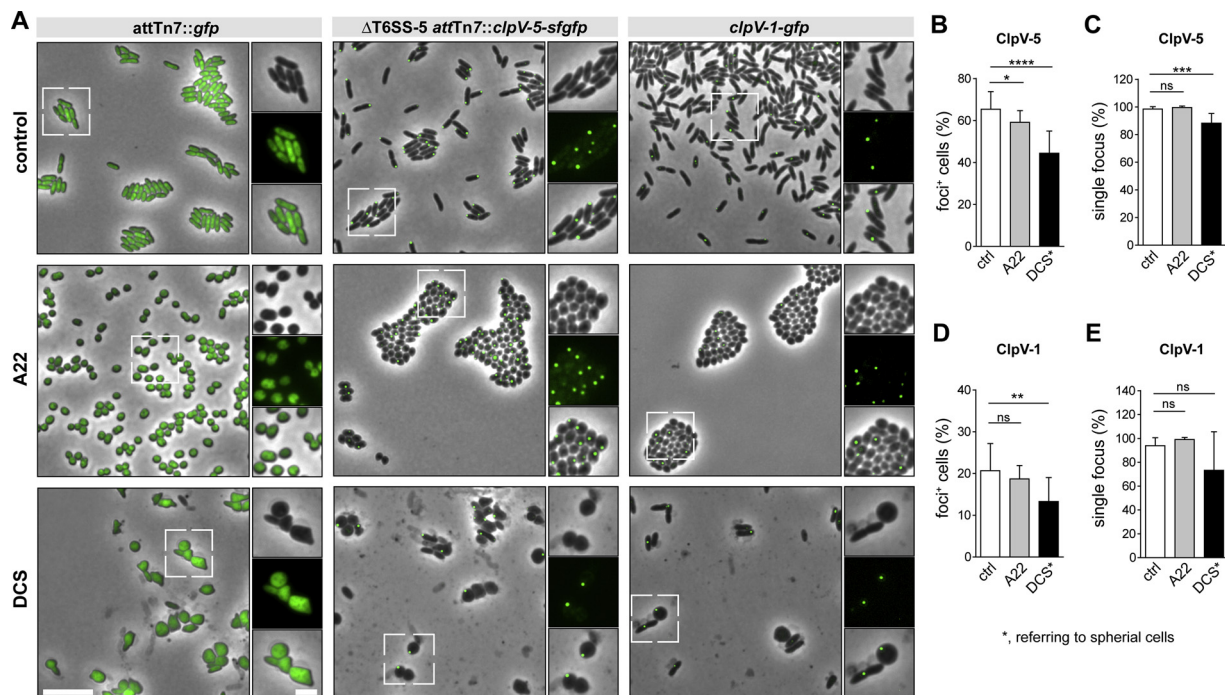


**Fig. 3.** Surface contact is required for expression of *clpV-1-gfp* but is not essential for ClpV-5-sfGFP formation and polar localization. **A.** Representative phase contrast and fluorescence microscopy images of the indicated *B. thailandensis* mutants fixed with PFA during growth in liquid medium or incubated for different periods of time on agarose pads before image acquisition. **B.** Quantification of bacteria displaying fluorescent foci of the indicated T6SS proteins fused to GFP during growth in suspension and after surface contact (ClpV-5: N = 778/871/1068, TssK-5: N = 901/1085/1191, TssC-5: N = 674/636/567, ClpV-1: N = 676/1145/1005 cells). \*\*\*\*,  $p < 0.0001$  (ClpV-5: t test; TssK-5/ClpV-1: Mann-Whitney test), \*\*,  $p = 0.0018$  (Mann-Whitney test), \*,  $p = > 0.03$  (Mann-Whitney test) **C.** Quantification of polar foci of the indicated T6SS proteins fused to GFP in foci<sup>+</sup> bacteria without surface contact and following surface contact (ClpV-5: N = 233/502/578, TssK-5: N = 7/55/768, TssC-5: N = 2/13/19 foci<sup>+</sup> cells). \*,  $p > 0.0153$  (Mann-Whitney test), ns, not significant (Mann-Whitney test); N/D, not detected **D.** Quantification of bacteria expressing *clpV-1-gfp* from the chromosome showing a fluorescent ClpV-1-GFP signal during growth in liquid media (suspension) and after 20–30 min of surface contact (suspension: N = 676, surface: N = 320 cells). N/D, not detected **E.** Representative phase contrast and fluorescence images of the indicated *B. thailandensis* mutants fixed with 4% PFA during in growth in liquid medium. Induced expression of *clpV-1-gfp* during growth in suspension indicates that focal assembly of ClpV-1 occurs independent of surface contact. Data represent mean values + SD of two independent experiments performed in duplicate. **F.** Representative phase contrast and fluorescence microscopy images of the indicated *B. thailandensis* mutants incubated for 20 min in PBS at 23 °C and fixed in solution with PFA. Scale bar, 10  $\mu$ m (overview) and 2  $\mu$ m (insets).

whether an intact MreB cytoskeleton is required for the localization of the ATPases, *B. thailandensis*  $\Delta T6SS-5$  attTn7::clpV-5-sfgfp and *clpV-1-gfp* were treated with the MreB polymerization inhibitor A22. This caused pronounced cell rounding indicating that *B. thailandensis* is sensitive against A22. ClpV-5-sfGFP assembled into discrete foci in 59 ± 6% of

A22 treated bacteria and in 65 ± 8% of untreated cells (Fig. 4 A and B). Of untreated bacteria positive for ClpV-5-sfGFP foci 99 ± 2% displayed a single focus (Fig. 4 A and C). The fusion protein still formed a single and discrete focus in A22 treated cells at numbers very similar to that of untreated bacteria (Fig. 4 A and C). No delocalization of ClpV-5-





**Fig. 4.** The effect of disrupting the MreB cytoskeleton and peptidoglycan synthesis on the formation of single discrete ClpV-5 and ClpV-1 foci. **A.** Representative phase contrast and fluorescence images of the indicated *B. thailandensis* mutants treated with the MreB polymerization inhibitor A22 and the peptidoglycan synthesis inhibitor D cycloserine (DCS). **B.** Quantification of bacteria displaying ClpV-5-sfGFP foci formation following A22 and DCS treatment (control: N = 922, A22: N = 1156 cells, DCS: N = 462 cells). \*\*\*\*,  $p < 0.0001$  (Mann-Whitney test), \*,  $p = 0.042$  (*t*-test) **C.** Quantification of single ClpV-5-sfGFP foci in foci positive bacteria treated with A22 and DCS (control: N = 606, A22: N = 686, DCS: N = 202 foci<sup>+</sup> cells). \*\*\*,  $p = 0.0003$  (Mann-Whitney test); ns, not significant (Mann-Whitney test) **D.** Quantification of bacteria harboring ClpV-1-GFP foci after treatment with A22 and DCS (control: N = 2748, A22: N = 2786, DCS: N = 360 cells). \*\*,  $p = 0.0086$  (Mann-Whitney test); ns, not significant (Mann-Whitney test) **E.** Quantification of single ClpV-1-GFP foci in foci positive bacteria after treatment with A22 and DCS (control: N = 638, A22: N = 526, DCS: N = 46 foci<sup>+</sup> cells). ns, not significant (Mann-Whitney test). Shown are mean values + SD of two independent experiments performed in duplicate. Scale bar, 10  $\mu$ m (overview) and 2  $\mu$ m (insets).

sfGFP such as diffuse or multiple foci localization was observed. These findings suggest that an intact MreB cytoskeleton of the bacteria is dispensable for localization of ClpV-5-sfGFP to discrete foci. Furthermore, no significant reduction of ClpV-1-GFP foci formation was observed following A22 treatment (Fig. 4 A and D). The number of bacteria having a single ClpV-1-GFP focus was not significantly different between untreated and A22 treated bacteria ( $94 \pm 7\%$  and  $99 \pm 2\%$ , respectively) (Fig. 4 A and E). Thus, similar to ClpV-5-sfGFP, localization of ClpV-1-GFP to single discrete foci was not affected by MreB perturbation (Fig. 4 A and E).

ClpV-5-sfGFP predominantly localizes to the old pole, which presumably is a site of no or little peptidoglycan synthesis in *B. thailandensis*. Thus, the possibility that the spatially heterogeneous synthesis of peptidoglycan acts as localization cue for ClpV-5 was investigated. To inhibit peptidoglycan synthesis *B. thailandensis* was incubated with D cycloserine (DCS), a structural analog of D-alanine, which inhibits two cytoplasmic enzymes of the peptidoglycan synthesis pathway. The formation of spherical cells following DCS treatment demonstrated the effectiveness of the inhibitor in *B. thailandensis* (Fig. 4 A). Spherical but not rod shaped bacteria were included in the analysis to ensure that only cells with blocked cell wall synthesis were considered. DCS treatment caused a significant decrease in bacteria displaying ClpV-5-sfGFP foci compared with untreated cells (Fig. 4 A and B). Furthermore, the localization of ClpV-5-sfGFP to discrete single foci was 10% lower in DCS treated bacteria relative to the control although  $88 \pm 7\%$  of the cells still contained foci (Fig. 4 A and C). The presence of discrete ClpV-5-sfGFP foci in spherical cells suggests that the strong negative curvature at the pole of rod shaped bacteria is not required to maintain focal assembly of the ATPase. Similar to ClpV-5, DCS treatment reduced the number of ClpV-1-GFP foci positive cells but did not abolish focal assembly of the fusion protein (Fig. 4 A and D).

#### 4. Discussion

During infection of host cells ClpV-5-sfGFP retains its polar position in *B. thailandensis* in the absence of all T6SS-5 apparatus components (Lennings et al., 2019). This finding suggests that ClpV-5 recognizes a spatial cue or interacts with a polar protein unrelated to T6SS-5 for polar localization. Here we show that a functional ClpV-5-sfGFP fusion protein expressed in the wild type background predominantly localizes to the old pole of *B. thailandensis* located inside host cells. Focal assembly and the unipolar localization pattern of ClpV-5-sfGFP were not abrogated in bacteria grown in LB medium indicating that the intracellular host cell environment is dispensable for proper localization of ClpV-5. The result is consistent with a report showing that expression and focal assembly of the TssB/C orthologs of the eukaryotic cell targeting *Francisella* T6SS can be induced outside the host cell by 5% KCl or placement of the bacteria between cover slips (Clemens et al., 2015).

In agreement with previous localization studies on ClpV in other bacteria, we found that focal assembly of ClpV-1-GFP is dependent on T6SS-1 protein(s). This finding implies that T6SS independent localization of ClpV proteins, as observed for ClpV-5, is not a general feature of *B. thailandensis* T6SSs. Similar to ClpV-5, the polar AAA + ATPase PilT of *P. aeruginosa* assumed to depolymerize type IV pili localizes independent of type IV pilus and associated proteins (Chiang et al., 2005; McCallum et al., 2017). However, a localization of T6SS-associated ATPases independent of secretion apparatus components has not been described to date. This includes ClpB recycling the *Francisella* T6SS sheath, which assembles into single foci in wildtype bacteria but delocalizes in the absence of the TssM ortholog PdpB (Brodmann et al., 2017). In addition to the contracted T6SS sheath, ClpB appears to interact with other substrates for disaggregation as it also contributes to heat stress tolerance and maintains the level of several proteins under

stress conditions (Meibom et al., 2008). The molecular basis for these interactions is as yet unknown. The ability of ClpV-5 to localize without interacting with other T6SS-5 proteins might be ascribed to regions specific for the protein such as its C-terminus, which contains a repeat region that appears to be unique among T6SS ATPases. In fact, the C-terminus of the Cdc48 AAA + ATPase in yeast can interact with different co-factors (Bodnar and Rapoport, 2017). In addition to ClpV-5 other T6SS-5 protein(s) are presumably able to localize to the pole in a manner independent of other T6SS-5 components to initiate assembly of the secretion apparatus. Several studies indicate that the biogenesis of the T6SS begins with the assembly of the membrane complex TssLMJ (Brunet et al., 2015; Durand et al., 2015; English et al., 2014). Consequently, one or more proteins of this complex might contain the information to localize in the absence of all other T6SS components. Interestingly, TssL and TssM display homology to the type IVB secretion system (T4BSS) proteins DotU and IcmF, respectively, both of which mediate polar recruitment of the T4BSS in *Legionella pneumophila* (Ghosal et al., 2019). However, with the exception of the present study, localization analyses of individual T6SS proteins in a mutant lacking all other secretion system components have not been performed.

The influence of the absence of surface contact on assembly and localization of T6SSs has so far not been investigated. The finding that 30% of *B. thailandensis* bacteria grown in suspension contained discrete ClpV-5-sfGFP foci of which 86% exhibited unipolar localization indicates that surface contact is not essential for correct ClpV-5 localization. Thus, following escape from the vacuole into the host cell cytoplasm during infection *B. thailandensis* may not require surface contact with the plasma membrane of the host cell for example for proper T6SS-5 localization. Whether contact with a surface stimulates T6SS-5 secretion remains to be determined. Furthermore, the absence of a ClpV-1-GFP signal in *B. thailandensis* isolated from suspension cultures suggests that surface contact triggers expression of *clpV-1* and potentially other T6SS-1 genes.

Incubation of *B. thailandensis* with DCS led to marked changes in the cell morphology including cell rounding and our data might indicate that PGN synthesis and/or its spatial organization or composition promotes ClpV-5-sfGFP localization to a discrete subcellular site. MreB forms filaments rotating around the periphery of the cell and is implicated in establishing polarity amongst others (Shih et al., 2005; Carballido-Lopez, 2006). Different results have been obtained with respect to the effect of A22 treatment in protein localization: Foci formation of the type II secretion system protein EspC in *Vibrio cholerae* for example was not disrupted whereas Dot proteins of the T4BSS and the autotransporter protein IcsA mislocalized (Lybarger et al., 2009; Jeong et al., 2017; Krokowski et al., 2019). Incubation of *B. thailandensis* with A22 caused pronounced cell rounding but did not abrogate discrete formation of single ClpV-5-sfGFP foci suggesting that disruption of MreB polymerization does not affect proper localization of ClpV-5. Altogether, this study provided novel insights into the role of environmental and cellular factors in the localization of ATPases of T6SSs targeting prokaryotic and eukaryotic cells.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.ijmm.2019.151335>.

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