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

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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 γ-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; Burtnick 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* DH5α and SM10λpir were cultured in LB-Miller broth shaking at 200 rpm or on LB agar plates supplemented with 200 μg/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* ΔT6SS-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-Tn*7*T-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* ΔT6SS-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_2. Hela cells were seeded on glass cover slips in a 24 well plate at a density of 5×10^4 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 μg/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_{600nm} of 0.05 and grown to an OD_{600nm} 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 μg/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_{600nm} of approximately 0.4 were used for fluorescence microscopy. For some experiments the bacteria were fixed during growth in LB medium at an OD_{600nm} 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 resuspended 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_{600nm} 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 (ΔT6SS-5 attTn*7*::*clpV-5-sfgfp* and ΔT6SS-5 attTn*7*::*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 ΔT6SS-5 attTn*7*::*clpV-5-sfgfp* and ΔT6SS-5 attTn*7*::*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 ΔT6SS-5 background (ΔT6SS-5 attTn*7*::*tssK-5-sfgfp* and ΔT6SS-5 attTn*7*::*tssC-5-sfgfp* (Kapitein et al., 2013). Expression of the fusion proteins was verified by Western blot (Fig. S1). Unlike the ΔT6SS-5 attTn*7*::*tssK-5-sfgfp* mutant a GFP signal was not detected in all ΔT6SS-5 attTn*7*::*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

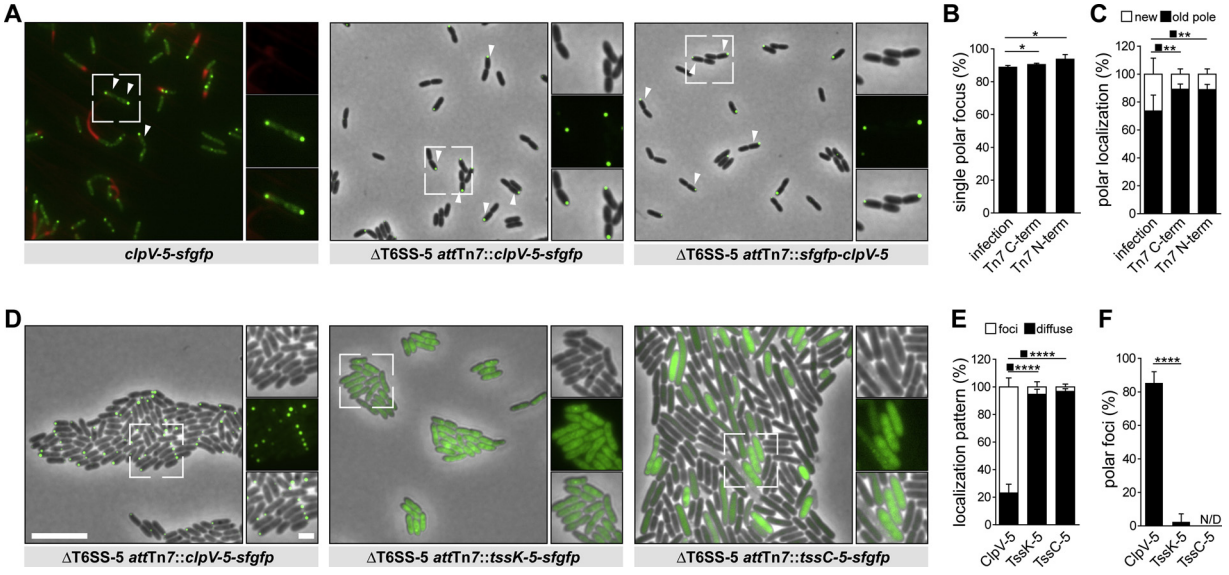

Fig. 1. The host cell environment is not required for focal assembly of ClpV-5-sfGFP and localization to the pole. A. Left: Representative fluorescence microscopy images of *B. thailandensis* expressing a chromosomal *clpV-5-sfgfp* fusion during infection of Hela cells (left) and a *B. thailandensis* ΔT6SS-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 and polar focus of ClpV-5-sfGFP fusion proteins. *B. thailandensis clpV-5-sfgfp* during infection (infection), ΔT6SS-5 attTn*7*::*clpV-5-sfgfp* (Tn7 C-term) and ΔT6SS-5 attTn*7*::*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$^+$ 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$^+$ 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$^+$ 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 μm (overview) and 2 μm (insets) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

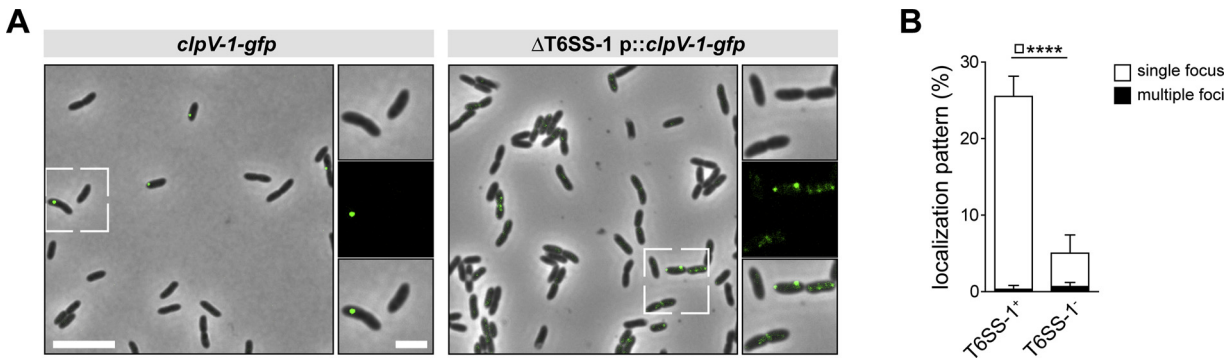

Fig. 2. Assembly of ClpV-1-GFP into single discrete foci is significantly decreased in the absence of the T6SS-1 secretion apparatus. A. Representative phase contrast and fluorescence microscopy images of a *B. thailandensis* mutant expressing a chromosomal *clpV-1-gfp* fusion and a mutant harboring a deletion of the T6SS-1 gene cluster and expressing *clpV-1-gfp* from a plasmid (ΔT6SS-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 ΔT6SS-1 p::*clpV-1-gfp* (T6SS-1$^-$) (T6SS-1$^+$: N = 1501, T6SS-1$^-$: N = 1235 signal$^+$ cells). ****, $p < 0.0001$ (*t*-test). Shown are mean values +SD of two independent experiments performed in duplicate. Scale bar, 10 μm (overview) and 2 μm (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 (ΔT6SS-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 ± 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 ± 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 ΔT6SS-5 mutant cultured in LB medium (ΔT6SS-5 attTn*7*::*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 ± 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. 1 B). 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 ± 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. 3 A and C). In contrast, TssK-5-sfGFP foci appeared to localize randomly and only 16 ± 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. 3 A 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. 3 A and B). The number of ClpV-1-GFP foci positive cells increased with elongated incubation on the agarose pad to 31 ± 13% (Fig. 3 A and B). We did not detect a fluorescent signal in *B. thailandensis clpV-1-gfp* grown in suspension (Fig. 3 A 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. 3 E). 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. 3 F).

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_ I0146 and BTH_II1780). To study

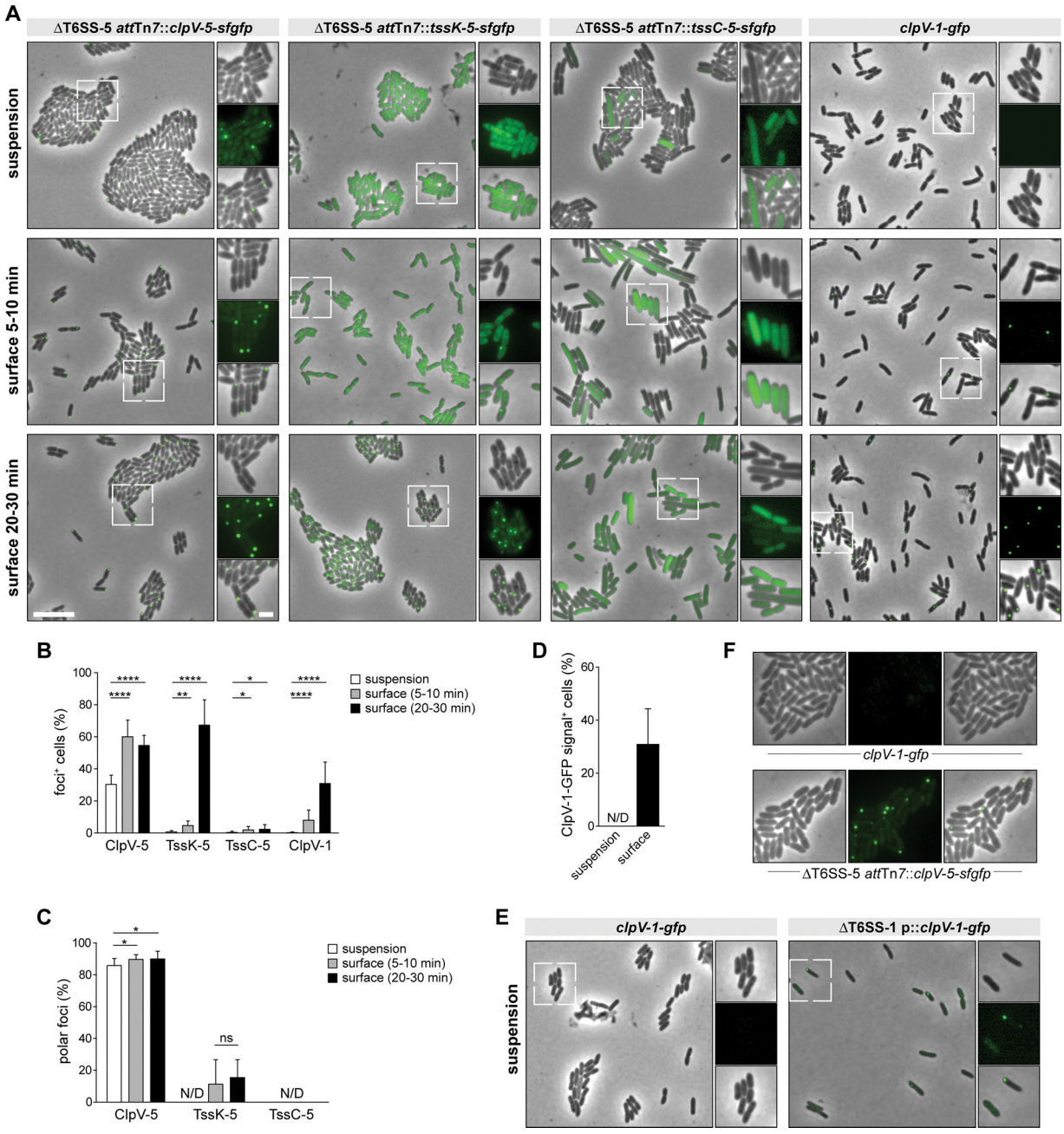

Fig. 3. Surface contact is required for expression of *clpV-1-gfp* but is not essential for ClpV-5-sfGFP formation and polar localization. A. Representative phase contrast and fluorescence microscopy images of the indicated *B. thailandensis* mutants fixed with PFA during growth in liquid medium or incubated 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 + 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$^+$ 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 µm (overview) and 2 µm (insets).

whether an intact MreB cytoskeleton is required for the localization of the ATPases, *B. thailandensis* ΔT6SS-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-

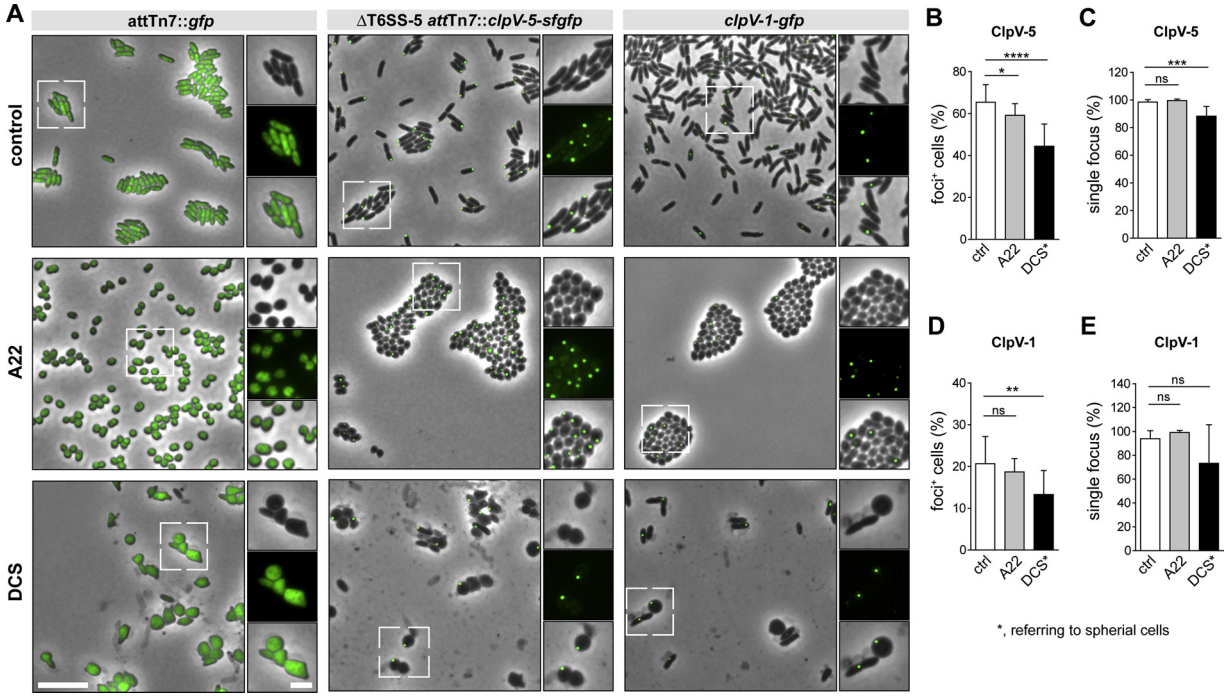

Fig. 4. The effect of disrupting the MreB cytoskeleton and peptidoglycan synthesis on the formation of single discrete ClpV-5 and ClpV-1 foci. A. Representative phase contrast and fluorescence images of the indicated *B. thailandensis* mutants treated with the MreB polymerization inhibitor A22 and the 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$^+$ 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$^+$ cells). ns, not significant (Mann-Whitney test). Shown are mean values + SD of two independent experiments performed in duplicate. Scale bar, 10 μm (overview) and 2 μm (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 ± 7% and 99 ± 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 ± 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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