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AAA+ proteases and their role in distinct stages along the *Vibrio cholerae* lifecycle

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ABSTRACT

The facultative human pathogen *Vibrio cholerae* has to adapt to different environmental conditions along its lifecycle by means of transcriptional, translational and post-translational regulation. This study provides a first comprehensive analysis regarding the contribution of the cytoplasmic AAA+ proteases Lon, ClpP and HslV to distinct features of *V. cholerae* behaviour, including biofilm formation, motility, cholera toxin expression and colonization fitness in the mouse model. While absence of HslV did not yield to any altered phenotype compared to wildtype, absence of Lon or ClpP resulted in significantly reduced colonization in vivo. In addition, a Δ*lon* deletion mutant showed altered biofilm formation and increased motility, which could be correlated with higher expression of *V. cholerae* flagella gene class IV. Concordantly, we could show by immunoblot analysis, that Lon is the main protease responsible for proteolytic control of FliA, which is required for class IV flagella gene transcription, but also downregulates virulence gene expression. FliA becomes highly sensitive to proteolytic degradation in absence of its anti-sigma factor FlgM, a scenario reported to occur during mucosal penetration due to FlgM secretion through the broken flagellum. Our results confirm that the high stability of FliA in the absence of Lon results in less cholera toxin and toxin corgulated pilus production under virulence gene inducing conditions and in the presence of a damaged flagellum. Thus, the data presented herein provide a molecular explanation on how *V. cholerae* can achieve full expression of virulence genes during early stages of colonization, despite FliA getting liberated from the anti-sigma factor FlgM.

1. Introduction

The facultative human pathogen *Vibrio cholerae* causes the severe life-threatening secretory diarrheal disease cholera, characterized through a massive loss of water causing rapid dehydration (Koch, 1884). Along its lifecycle, *V. cholerae* is capable of transiting between two different habitats, the aquatic environment, which acts as a natural reservoir between the epidemic outbreaks, and the gastrointestinal tract of the human host (Nelson et al., 2009; Sack et al., 2004).

A key factor for survival and persistence of *V. cholerae* in the nutrient poor aquatic environment is the ability to form biofilms on chitinous surfaces (Huq et al., 2008; Tamplin et al., 1990). Formation of these bacterial communities is a stepwise process comprising surface adhesion, formation of a monolayer, maturation to a three-dimensional biofilm and finally detachment and release into the planktonic stage (Heithoff and Mahan, 2004; Silva and Benitez, 2016). Not surprisingly, *V. cholerae* induces a distinct set of genes along the different stages of the biofilm (Moorthy and Watnick, 2005; Schoolnik et al., 2001; Seper et al., 2014). Noteworthy, chitin and nucleic acids are not only the attachment surface or matrix component, respectively, but can also serve as nutrient sources via a set of degradative enzymes and uptake systems, which facilitate the survival fitness of *V. cholerae* in such nutrient poor conditions (Gumpenberger et al., 2016; Meibom et al., 2004; Pruzzo et al., 2008; Seper et al., 2011). *V. cholerae* aggregates detached from biofilms might comprise the agent for initial infection via oral ingestion. The cells associated to biofilm clumps might be better protected against acidic conditions, temperature change and high osmolarity, therefore increasing transmission efficiency of the disease (Huq et al., 2008; Tamayo et al., 2010; Zhu and Mekalanos, 2003).

Upon entry in the human host, *V. cholerae* substantially changes its expression profile and induces a set of virulence genes controlled by a complex regulatory network. These changes include the decrease of the second messenger cyclic di-GMP (c-di-GMP), the

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activation of ToxR- and TcpP-regulons, which induce transcription of ToxT, the essential transcriptional activator of many virulence genes including the cholera toxin (CTX) and the toxin coregulated pilus (TCP) (Childers and Klose, 2007; Nelson et al., 2009). In addition, *V. cholerae* uses flagella-dependent motility and mucinases to penetrate through the mucus gel in order to efficiently attach to the epithelial cells in the small intestine (Butler and Camilli, 2005; Freter and Jones, 1976; Freter and O'Brien, 1981). The impact of motility is highlighted by reports demonstrating that non-motile *V. cholerae* mutants colonize 10–25 times less efficiently than wild-type (WT) strains (Lee et al., 2001; Liu et al., 2008; Morris et al., 2008). Interestingly studies proved, that the *V. cholerae* flagella breaks during mucin penetration, which allows secretion of the anti-sigma factor FlgM through its flagellar apparatus (Correa et al., 2004; Liu et al., 2008). This decrease of intracellular FlgM releases the alternative sigma factor FliA, which subsequently will activate FliA-dependent genes (Correa et al., 2004; Liu et al., 2008). Noteworthy, work by Syed et al. demonstrated that FliA can be a potent inhibitor of virulence gene expression. To avoid repression of virulence genes FliA must be effectively inactivated during early stages of colonization to allow full virulence gene expression (Syed et al., 2009). At the late stage of infection *V. cholerae* alters its gene expression again in order to detach from the epithelium by activating a RpoS-dependent mucosal escape response and to prepare for its transition into the aquatic environment by activating c-di-GMP synthesis and nutrient accumulation (Gumpenberger et al., 2016; Moisi et al., 2013; Nielsen et al., 2006; Schild et al., 2007).

In the past, gene expression analyses revealed several interesting and physiological relevant mechanisms for adaptation and fitness of *V. cholerae* along its lifecycle using a diverse set of reporter-based technologies, microarrays or RNAseq (Bina et al., 2003; Camilli and Mekalanos, 1995; Hsiao et al., 2006; Lee et al., 1999; Mandlik et al., 2011; Moorthy and Watnick, 2005; Nielsen et al., 2006; Papenfort et al., 2015; Schild et al., 2007; Seper et al., 2014). Besides such global changes in the transcriptome, *V. cholerae* also has to perform rapid adaptations via posttranslational regulation, e.g. proteolysis. For example, the membrane bound transcriptional virulence gene regulators ToxR and TcpP are subjects to regulated intramembrane proteolysis by RseP (YaeL) (Almagro-Moreno et al., 2015a,b; Matson and DiRita, 2005).

Furthermore, *V. cholerae* harbors, as many other bacteria, members of the AAA+ (ATPase associated with a variety of cellular activities) protein family representing proteases active in the cytosol (Neuwald et al., 1999). These include homologs of ClpA/P, ClpX/P, HslU/V (also known as ClpYQ), Lon and FtsH (also known as HflB), which have been best studied in *Escherichia coli* and generally target misfolded, truncated or mutated proteins and affect the global protein turnover in the cell (Gottesman, 2003; Schmidt et al., 2009). Notably, there is also growing evidence that these proteases have conserved roles in specific, controlled proteolysis in response to environmental stimuli and thereby may contribute to bacterial pathogenesis (Ingmer and Brondsted, 2009).

ClpA/P, ClpX/P and HslU/V proteases consist of functional units, with ClpP and HslV being the proteolytic domain, respectively. ClpP, which assembles with two heptameric rings involving the active site, can either associate with the ATPase ClpA or ClpX (Gottesman et al., 1993; Kessel et al., 1995; Maurizi et al., 1998; Wang et al., 1997; Wojtkowiak et al., 1993). Similarly, the protease HslU/V consists of an ATPase domain (HslU) and a proteolytic subunit (HslV) (Kessel et al., 1996; Missiakas et al., 1996; Rohrwild et al., 1996). While HslU/V has not been correlated with virulence of bacterial pathogens so far, Clp-dependent proteolysis plays several important roles in pathogenesis, especially in Gram-positive bacteria via controlling virulence factor production (Ingmer and Brondsted, 2009). For example, in *Listeria monocytogenes* ClpP degrades an inhibitor of the virulence factor listeriolysin O, which causes food poisoning. In addition a deletion in *clpP* leads to a decreased ability to multiply in macrophages (Gaillot et al., 2000). In the human pathogen *Staphylococcus aureus,* which causes a variety of serious infections, a deletion in either *clpP* or *clpX* leads to a reduced transcription of hemolysin α-toxin (Frees et al., 2003; Lowy, 1998). In case of *Streptococcus pneumoniae*, causing pneumonia, bacteremia and meningitis a *clpP* mutant is not only deficient in colonization of the nasopharynx and survival in the lungs of mice, but also exhibits shorter survival than the WT in presence of murine macrophages (Kwon et al., 2004).

Contrary to the above mentioned AAA+ proteases, FtsH and Lon harbor the ATPase and proteolytic activity on a single polypeptide chain (Langklotz et al., 2012; Licht and Lee, 2008; Park et al., 2006). Furthermore, FtsH is unique as it possesses an inner membrane anchor and has been shown to be essential for viability in *E. coli* (Langklotz et al., 2012; Ogura et al., 1999). In *S. aureus*, *ftsH* mutants exhibit reduced colonization fitness in a murine model and FtsH degrades MgtC in *Salmonella*, which is required for intramacrophage survival (Alix and Blanc-Potard, 2007; Lithgow et al., 2004).

Finally, Lon plays a major role in the protein quality control of the cell by degradation of misfolded or unstable proteins and has also been shown to be important for virulence in several pathogens, including Gram-positive as well as Gram-negative bacteria (Phillips et al., 1984) (Boddicker and Jones, 2004; Cohn et al., 2007; Gottesman, 1996; Takaya et al., 2003). For example, Lon affects the type III secretion system (T3SS), which is used to translocate virulence proteins into host cells. In *Salmonella enterica* serovar Typhimurium and *Pseudomonas syringae,* Lon acts as negative regulator of the T3SS (Takaya et al., 2005). In contrast, absence of Lon in *Yersinia pestis* results in repression of T3SS genes, since Lon rapidly degrades YmoA, being a transcriptional repressor of T3SS (Jackson et al., 2004). In *Pseudomonas aeruginosa,* Lon controls production of homoserine lactones, which mediate quorum sensing, and thereby interferes with virulence gene expression (Takaya et al., 2008).

Evidence for the importance of the AAA+ proteases along the *V. cholerae* lifecycle is scarce. This study investigates the role of the AAA+ proteases of a *V. cholerae* El Tor isolate in distinct stages of its lifecycle including biofilm formation, motility and colonization fitness. Our results demonstrate that Lon and ClpP contribute to colonization fitness in vivo. In addition, biofilm formation, motility and CTX expression are significantly altered in a *lon*-mutant. In a more detailed analysis the alternative sigma-factor FliA was revealed to be a target of Lon mediated proteolysis. Finally, the high stability of FliA in the absence of Lon was correlated with altered expression levels of class IV flagellar genes and CTX, providing an explanation of the increased motility and colonization defect of a *lon*-mutant.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S1; oligonucleotides are listed in Table S2. *V. cholerae* SP27459, a spontaneous streptomycin (Sm)-resistant mutant of the clinical isolate O1 El Tor Inaba P27459 was used as WT strain (Pearson et al., 1993). *E. coli* strains DH5α*λpir* and SM10*λpir* were used for genetic manipulations (Hanahan, 1983; Kolter et al., 1978; Miller and Mekalanos, 1988). If not noted otherwise, strains were cultured in Luria Bertani (LB) broth (1% tryptone; 1% NaCl; 0.5% yeast extract), on LB broth agar plates with aeration at 37 °C, or for biofilm formation under static conditions at room temperature (RT). If required, antibiotics or other supplements were used in the following final concentrations: streptomycin (Sm), 100 μg/ml; ampicillin (Ap), 100 μg/ml