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Crystal structure of the full-length ATPase GspE from the *Vibrio vulnificus* type II secretion system in complex with the cytoplasmic domain of GspL

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ABSTRACT

The type II secretion system (T2SS) is present in many Gram-negative bacteria and is responsible for secreting a large number of folded proteins, including major virulence factors, across the outer membrane. The T2SS consists of 11–15 different proteins most of which are present in multiple copies in the assembled secretion machinery. The ATPase GspE, essential for the functioning of the T2SS, contains three domains (N1E, N2E and CTE) of which the N1E domain is associated with the cytoplasmic domain of the inner membrane protein GspL.

Here we describe and analyze the structure of the GspE•cyto-GspL complex from *Vibrio vulnificus* in the presence of an ATP analog, AMPPNP. There are three such ~83 kDa complexes per asymmetric unit with essentially the same structure. The N2E and CTE domains of a single *V. vulnificus* GspE subunit adopt a mutual orientation that has not been seen before in any of the previous GspE structures, neither in structures of related ATPases from other secretion systems. This underlines the tremendous conformational flexibility of the T2SS secretion ATPase.

Cyto-GspL interacts not only with the N1E domain, but also with the CTE domain and is even in contact with AMPPNP. Moreover, the cyto-GspL domains engage in two types of mutual interactions, resulting in two essentially identical, but crystallographically independent, "cyto-GspL rods" that run throughout the crystal. Very similar rods are present in previous crystals of cyto-GspL and of the N1E•cyto-GspL complex. This arrangement, now seen four times in three entirely different crystal forms, involves contacts between highly conserved residues suggesting a role in the biogenesis or the secretion mechanism or both of the T2SS.

1. Introduction

Secretion of proteins into the extra-cellular milieu is important for many pathogenic and non-pathogenic bacteria, which have developed an impressive variety of often complex multi-protein assemblies to perform this task. One of these protein secretion machineries is the sophisticated type II secretion system (T2SS) that spans the inner and outer membranes from many Gram-negative bacteria (Korotkov et al., 2012). The T2SS is highly relevant for the pathogenicity of several major pathogens since it translocates major virulence factors in a folded form from the periplasm to the extracellular milieu. Examples of bacterial human pathogens where the T2SS plays an important role include:

(i) *Vibrio cholerae*, where the T2SS secretes the heterohexameric AB_5 cholera toxin (CT) and ~20 other proteins (Hirst et al., 1984; Sikora et al., 2011). *V. cholerae* is responsible for estimated 100,000–120,000 deaths per year, mainly in low-income countries and disaster areas (http://www.who.int/mediacentre/factsheets/fs107/en/).

(ii) Enterotoxigenic *Escherichia coli* (ETEC), where the T2SS translocates heat-labile enterotoxin (LT) (Hirst and Holmgren, 1987), a close structural and functional homolog of CT (Merritt and Hol, 1995). ETEC are an extremely important cause of diarrhea in the developing world (Qadri et al., 2005; Wenneras and Erling, 2004), and also are the most common cause of travelers' diarrhea (Steffen et al., 2005).

Abbreviations: AAS, archaella assembly system; BSA, buried surface area; Gsp, general secretion pathway; r.m.s.d, root-mean-square deviation; T2SS, type II secretion system; T4PS, type IV pilus system; AMPPNP, phosphoaminophosphonic acid-adenylate ester.

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(iii) Enterohemorrhagic *E. coli* (EHEC), which can cause severe foodborne disease, and even life-threatening renal failure in children and the elderly. The T2SS deletion mutant of EHEC shows defects in colonization. In addition, the zinc metalloprotease StcE and the metal binding protein YodA, which are crucial for EHEC adherence to host cells, are secreted by the T2SS encoded on plasmid pO157 (Goldwater and Bettelheim, 2012; Ho et al., 2008; Toshima et al., 2007).

(iv) Enteropathogenic *E. coli* (EPEC), is one of the most important pathogens affecting children worldwide with the infection resulting in persistent diarrhea (Ochoa and Contreras, 2011). The T2SS is required for EPEC virulence (Baldi et al., 2012).

(v) *Pseudomonas aeruginosa*, an opportunistic pathogen of major importance in cystic fibrosis patients, contains two distinct T2SS machineries (Ball et al., 2002; Jyot et al., 2011) and in certain strains even three (Cadoret et al., 2014).

(vi) The intracellular pathogen *Legionella pneumophila*, the causative agent of Legionnaire's disease, is dependent on the T2SS (DebRoy et al., 2006; Rossier et al., 2008).

The T2SS is made up from ~11–15 proteins, most of these present in multiple copies in the assembled secretion complex. As a result of numerous biochemical and structural studies, and from analogies to related systems, a generally accepted picture has emerged with the T2SS thought to consist of three subassemblies: the Inner Membrane Platform, the dynamic Pseudopilus, and the channel-forming Outer Membrane Complex (Cianciotto, 2013; Douzi et al., 2012; Filloux, 2004; Howard, 2013; Johnson et al., 2006; Korotkov et al., 2012; McLaughlin et al., 2012; Nivaskumar and Francetic, 2014). The Inner Membrane Platform (Py et al., 2001) is composed of the T2SS membrane proteins GspC, GspF, GspL, GspM and, in some species, GspN. The ATPase GspE resides in the cytoplasm interacting with the cytoplasmic domain of GspL (Abendroth et al., 2005; Sandkvist et al., 1995; Shiue et al., 2006) and with GspF (Arts et al., 2007; Py et al., 2001). The stoichiometry of the Inner Membrane Complex, the nexus of the T2SS since it interacts with all other subassemblies, is still a mystery. The Pseudopilus contains five different pseudopilins: GspK, GspI, GspJ, GspH and GspG. The tip is formed by a GspK•GspI•GspJ heterotrimer (Korotkov and Hol, 2008), most likely linked by one or a few GspH subunits (Douzi et al., 2011; Yanez et al., 2008a) to a helical filament made up of multiple copies of a calcium-requiring GspG (Campos et al., 2011; Kohler et al., 2004; Korotkov et al., 2009; Yanez et al., 2008a,b). (Note: the symbol "•" is used throughout to indicate non-covalent complexes). The Outer Membrane Complex is composed of a dodecamer of GspD subunits which form a gated channel of ~880 kDa (Chami et al., 2005; Reichow et al., 2010). Intriguingly, the T2SS is possibly only fully assembled transiently, perhaps triggered by the presence of exoproteins in the periplasm (Chen and Hu, 2013; Howard, 2013).

Over the decades, an increasing number of bacterial multiprotein machineries spanning the inner and outer membrane of Gram-negative bacteria have been uncovered. The system closest related to the T2SS is the type IV pilus system (T4PS) (Ayers et al., 2010). At least two types of T4PS exist, with the best studied the Type 4a Pilus system (T4aPS) that differs from the Type 4b Pilus system (T4bPS) in several ways, including a different major pilin subunit (Craig and Li, 2008) and a different protein and domain organization of the homolog of the T2SS inner membrane protein GspL (Supplementary Fig. S1B). T4PS variants perform a diversity of functions in a wide range of species (Craig and Li, 2008; Giltner et al., 2012; Pelicic, 2008). More distantly related systems are the bacterial transformation system and the archaellum assembly system (AAS) (Korotkov et al., 2011, 2012; Lassak et al., 2012). The critical functions of these systems in bacterial survival and pathogenicity increase the importance of our understanding of the T2SS.

Among the many protein components of the T2SS, the secretion ATPase GspE plays an essential role, and is likely responsible for providing energy for the protein translocation process (Camberg and Sandkvist, 2005; Camberg et al., 2007; Patrick et al., 2011; Sandkvist et al., 1995). GspE has several other, species-specific, names and is e.g. in *Vibrio* species called EpsE (Sandkvist et al., 1995). Here we will use the generic nomenclature, i.e. GspE. GspE is a protein of ~500 residues folding into three major domains, the N-terminal domains N1E and N2E, and the C-terminal domain CTE. The CTE can be subdivided into the subdomains C1E, CME and C2E (Supplementary Fig. S1A) (Lu et al., 2013; Robien et al., 2003), where the CME is the critical zinc-binding domain (Camberg and Sandkvist, 2005; Possot and Pugsley, 1997). In *Xanthomonas campestris*, an additional domain (N0E) occurs prior to N1E (Chen et al., 2005). However, this is an exceptional case in the T2SS GspE family. The amino acid sequences of *Vibrio vulnificus* and *V. cholerae* GspE share 48%, 94% and 90% identity for the N1E, N2E and CTE, respectively (a sequence alignment is provided in Supplementary Fig. S2).

Here we report the first crystal structure containing a full-length T2SS secretion ATPase, while previous structures of the T2SS GspE missed the N1E. The initial *V. cholerae* $^{\Delta N1E}$GspE structure contained an arrangement of molecules with 6_1 helical symmetry (Robien et al., 2003). Solution studies have provided evidence that GspE tends to form multimers, most likely hexamers (Camberg et al., 2007; Shiue et al., 2006). Recently, crystal structures of two different hexamers of *V. cholerae* $^{\Delta N1E}$GspE have been obtained by using an "assistant hexamer", Hcp1 (Lu et al., 2013), which served to induce multimer formation of the fused $^{\Delta N1E}$GspE chains. One of these *V. cholerae* $^{\Delta N1E}$GspE hexamers adopts an arrangement with quite regular, quasi C_6, symmetry, another hexamer is elongated exhibiting C_2 symmetry (Lu et al., 2013). These hexamers reveal considerable variability in the orientation of the N2E versus the CTE. In contrast, the association of a CTE and a N2E' (i.e. N2E from a neighboring subunit) is remarkably similar in both hexamers of $^{\Delta N1E}$GspE-Hcp1 fusion proteins as well as in the helical *V. cholerae* $^{\Delta N1E}$GspE structure. This CTE•N2E' "construction unit" has also been observed in ATPase hexamers from related systems such as in the retraction ATPase PilT from the *P. aeruginosa* and *Aquifex aeolicus* T4aPS (Misic et al., 2010; Satyshur et al., 2007), and in the ATPases from the AAS, *Archaeoglobus fulgidus* GspE2 and *Sulfolobus acidocaldarius* FlaI (Reindl et al., 2013; Yamagata and Tainer, 2007). These latter ATPases lack the CME, and contain either no N1E at all, or an N1E with a different fold from the T2SS N1Es (Supplementary Fig. S1A). Hence the T2SS, T4PS and AAS ATPases share two common core domains, the N2E and CTE. These domains display major variations in length and number of subdomains, and are often distantly related in sequence. While T4PS and AAS ATPases form hexamers readily, the T2SS ATPase has so far been captured only as a stable hexamer when fused to Hcp1 as assistant hexamer (Lu et al., 2013).

Another important T2SS protein is GspL, which in *Vibrio* species is also called EpsL (Sandkvist et al., 1995), and has additional, species-specific, names (Supplementary Fig. S3). We use here the generic name GspL. GspL is a bitopic inner membrane protein that plays a central role in T2SS function since it interacts with several other T2SS proteins, including (1) the inner membrane platform protein GspM (Sandkvist et al., 1999); (2) the major pseudopilin GspG (Gray et al., 2011); and (3) GspE (Abendroth et al., 2005; Sandkvist et al., 1995). The cytoplasmic domain of GspL (cyto-GspL) is responsible for the interactions with the first domain of GspE (Sandkvist et al., 2000) and consists of three subdomains with similarities to proteins belonging to the actin-like ATPase superfamily (Abendroth et al., 2004a).