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A DODECAMERIC RING-LIKE STRUCTURE OF THE N0 DOMAIN OF THE TYPE II SECRETIN FROM ENTEROTOXIGENIC *ESCHERICHIA COLI*

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Abstract

In many bacteria, secretins from the type II secretion system (T2SS) function as outer membrane gated channels that enable passage of folded proteins from the periplasm into the extracellular milieu. Cryo-electron microscopy of the T2SS secretin GspD revealed previously the dodecameric cylindrical architecture of secretins, and crystal structures of periplasmic secretin domains showed a modular domain organization. However, no high-resolution experimental data has as yet been provided about how the entire T2SS secretin or its domains are organized in a cylindrical fashion. Here we present a crystal structure of the N0 domain of the T2SS secretin GspD from enterotoxigenic *Escherichia coli* containing a helix with 12 subunits per turn. The helix has an outer diameter of ~125 Å and a pitch of only 24 Å which suggests a model of a cylindrical dodecameric N0 ring whose dimensions correspond with the cryo-electron microscopy map of *Vibrio cholerae* GspD. The N0 domain is known to interact with the HR domain of the inner membrane T2SS protein GspC. When the new N0 ring model is combined with the known N0•HR crystal structure, a dodecameric double-ring of twelve N0-HR heterodimers is obtained. In contrast, the previously observed compact N0-N1 GspD module is not compatible with the N0 ring. Interestingly, a N0-N1 T3SS homolog is compatible with forming a N0-N1 dodecameric ring, due to a different N0- vs-N1 orientation. This suggests that the dodecameric N0 ring is an important feature of T2SS secretins with periplasmic domains undergoing considerable motions during exoprotein translocation.

Keywords

type II secretion; secretin; N0 domain; EpsD; PulD; XcpQ; type IV pilus

1. Introduction

Secretins are large, cylindrical, multimeric, outer membrane proteins which serve as protein transport conduits in several sophisticated protein secretion and/or fimbriae-generating

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5. Accession numbers

The atomic coordinates and structure factors (code 4JTM) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

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molecular machines, including the type II secretion system (T2SS), the type IV pili system (T4PS), the type III secretion system (T3SS) and filamentous phage assembly systems (Korotkov et al., 2011a). In addition, some proteins from the type IV secretion system (T4SS) contain domains that have the same fold as the N-terminal domain of the T2SS secretin GspD, which suggests a relationship in some aspects of the architecture of the T4SS with the T2SS (Nakano et al., 2010; Souza et al., 2011). In the T2SS, the secretin interacts with the inner membrane protein GspC (Korotkov et al., 2006; Korotkov et al., 2011b; Login et al., 2010; Lybarger et al., 2009) and also with exoproteins (Bouley et al., 2001; Francetic and Pugsley, 2005; Reichow et al., 2011). Here we focus on the T2SS, which is a multi-protein machinery that in many species transports a wide variety of folded proteins across the outer membrane of Gram-negative bacteria (Douzi et al., 2012; Korotkov et al., 2012; McLaughlin et al., 2012). The T2SS studied here is from enterotoxigenic *Escherichia coli* (ETEC), a major pathogen responsible for the death of hundreds of thousands victims, mainly young children in low-income countries (Fleckenstein et al., 2010). ETEC's T2SS secretes many folded proteins across the outer membrane, including the large 89 kDa AB₅ heat-labile enterotoxin (LT), a major virulence factor (Tauschek et al., 2002). LT is closely related to cholera toxin, which is secreted by the T2SS from *Vibrio cholerae* (Hirst et al., 1984; Sandkvist et al., 1997).

Secretins from different systems have a modular architecture with a variable number of N-terminal domains, followed by a conserved secretin domain (Korotkov et al., 2011a). The T2SS secretins, with the generic name GspD, start with an N-terminal N0 domain, followed by three repeat domains N1, N2 and N3 that have the same so-called KH-fold, different from the fold of the N0 domain (Fig. 1) (Korotkov et al., 2009). The remainder of the secretin contains the part embedded in the outer membrane that includes the secretin domain, which presumably consists mainly of α -strands (Pugsley, 1993). Previous studies have shown the outline of T2SS secretin multimers (Chami et al., 2005; Nouwen et al., 1999). Subsequently, a 19 Å resolution cryo-electron microscopy reconstruction of *V. cholerae* GspD revealed a dodecameric channel with a periplasmic vestibule and an extracellular chamber separated by a gate (Reichow et al., 2010). Also several crystal structures of periplasmic GspD domains have been reported. These include the structures of the N0-N1-N2 domains of ETEC GspD in complex with a nanobody (Korotkov et al., 2009), the N0-N1 domains of ETEC GspD in complex with the HR-domain of GspC (Korotkov et al., 2011b), and the N0-N1-N2 domains of *Pseudomonas aeruginosa* GspD^{XcpQ} (Van der Meeren et al., 2013). These structures all share a similar compact N0-N1 module. A combination of the crystal structures of individual secretin domains with the cryo-electron microscopy reconstruction resulted in dodecameric models with C₁₂ symmetry for the organization of the periplasmic domains of GspD (Korotkov et al., 2011b; Reichow et al., 2010). In contrast, a hexameric assembly of dimers has been suggested based on the basis of cysteine scanning mutagenesis and disulfide mapping analysis of *Dickeya dadantii* (previously *Erwinia chrysanthemi*) GspD^{OutD} (Wang et al., 2012), and also on the basis of dimers occurring in the crystal structure of the N0-N1-N2 domains of *P. aeruginosa* GspD^{XcpQ} (Van der Meeren et al., 2013). So far, however, high-resolution experimental information on the multimeric structure of a secretin is still missing.

The periplasmic domains of the outer membrane channel GspD are responsible for contacts with the periplasmic HR domain of the inner membrane protein GspC in several species. This is the only known contact between inner and outer membrane T2SS proteins, which is of critical importance for the functioning of the T2SS and subject of intensive investigations (Douzi et al., 2011; Gu et al., 2012; Korotkov et al., 2006; Korotkov et al., 2011b; Login et al., 2010; Shevchik et al., 1997; Wang et al., 2012). Here we report a crystal structure of the N0 domain of secretin from the best studied of the two T2SSs of enterotoxigenic *Escherichia coli* (ETEC), which is also called T2SS (Strozen et al., 2012), but for

simplicity is here referred to as T2SS. This structure reveals an intriguing dodecameric helical arrangement of N0 domains. Based on this helical organization, a model of a N0 ring with C_{12} point group symmetry is obtained whose dimensions agree with the cryo-electron microscopy reconstruction (Reichow et al., 2010). The dodecameric N0-GspD ring is analyzed in the light of numerous recent reports regarding the structure and function of the N0 domain and the interactions of the N0 domain of GspD with the HR-domain of the inner membrane T2SS protein GspC (Douzi et al., 2011; Gu et al., 2012; Korotkov et al., 2009; Korotkov et al., 2011b; Login et al., 2010; Van der Meeren et al., 2013; Wang et al., 2012). Our analysis suggests that the N0 domain of the T2SS secretin has an intrinsic tendency to form a cylindrical dodecamer, and that, consequently, T2SS and related secretins are multimers with cylindrical 12-fold symmetry. It is likely that the N0 and other periplasmic domains of T2SS secretin undergo considerable motion when the secretion apparatus performs its function of exoprotein translocation.

2. Materials and methods

2.1. Protein expression and purification

The gene fragments corresponding to the N0 and N1-N2 domains of ETEC GspD (residues 3–80 and 99–237) were cloned into a modified pRSF-Duet1 vector (EMD Millipore) under control of two T7 promoters. The N0 domain gene was extended by an N-terminal His₆-tag and a tobacco etch virus (TEV) protease cleavage site. The proteins were expressed in *E. coli* BL21(DE3) cells (EMD Millipore) at 30° C for 4 h after induction with 0.5 mM IPTG. The N0 and N1-N2 domains were co-purified via a Ni-NTA column (Qiagen) followed by His₆-tag removal with TEV protease and a second Ni-NTA purification step. The N0 and N1-N2 domains were separated by size-exclusion chromatography using Superdex 75 column (GE Healthcare).

2.2. Crystallization, data collection and structure determination

The best crystals of the N0 domain of GspD were obtained at 4°C by vapor diffusion method using 0.1 M Tris-HCl pH 8.5, 30% PEG2000mme as precipitant. Crystals were cryoprotected using 10% glycerol and flash-cooled for data collection. Diffraction data were collected at beamline BL12-2 of the Stanford Synchrotron Radiation Laboratory (SSRL). Data were integrated and scaled using XDS and XSCALE (Kabsch, 2010). The structure was solved by molecular replacement using Phaser (McCoy et al., 2007) and the N0 domain structure from the GspD•Nb7 complex (PDB 3EZJ) as search model (Korotkov et al., 2009). The structure was rebuilt and solvent molecules added using ARP/wARP and Coot (Emsley et al., 2010; Langer et al., 2008), further refined with REFMAC5 using TLS groups defined by the TLSMD server (Murshudov et al., 2011; Painter and Merritt, 2006), and validated using Coot and the Molprobtity server (Chen et al., 2010).

2.3. Construction a dodecameric ring of N0 subunits

We used the dodecameric ETEC N0 helix in the crystals to construct a dodecameric N0 ring model by translating subunits in the 6_1 superhelix by a 0, 2, 4, 6 etc Å translation per subunit parallel to the helix axis. This method is similar to modeling of flattened ring of the inner membrane protein EscJ from the inner membrane platform of the enteropathogenic *E. coli* T3SS (Yip et al., 2005).

2.4. Figure preparation

Figures were prepared using PyMOL (Schrodinger, 2010) and Chimera (Pettersen et al., 2004). Sequence alignments were prepared using ClustalW (Larkin et al., 2007) and rendered using ESPript (Gouet et al., 2003). The sequence conservation in GspD homologs was analyzed using the ConSurf server (Ashkenazy et al., 2010).

3. Results

3.1. Structure determination

Previously determined structures of the N-terminal domains of GspD secretin from ETEC relied on the use of nanobodies as crystallization chaperones or on the interacting partner protein GspC (Korotkov et al., 2009; Korotkov et al., 2011b). While crystals of ETEC N0-N1-N2 domains without an additional protein were obtained (Korotkov et al., 2009), their poor quality did not allow a structure determination. We hypothesized that a disordered flexible linker between the N0 and N1 domain prevented the formation of highly ordered crystal contacts since in all the structures of ETEC periplasmic GspD domains (PDB codes 3EZJ, 3OSS), the N0 and N1 domains form a compact module with the N0 domain connected to the N1 domain by a flexible disordered linker (Korotkov et al., 2009; Korotkov et al., 2011b). To study the mutual interaction and orientation, or orientations, of ETEC N0-GspD and N1-N2-GspD with respect to each other, we designed an expression construct for co-expression of the N0 and N1-N2 domains. We co-purified the N0 and N1-N2 domains by immobilized metal affinity chromatography using an N-terminal His₆-tag on the N0 domain. However, the two fragments separated in a subsequent size-exclusion chromatography step, with the N0 and the N1-N2 domains appearing each as monomers. Encouragingly, the isolated N0 domain readily produced crystals diffracting to 1.43 Å, much better than previously determined structures containing this GspD domain. The crystals exhibit space group P6₁ with two monomers per asymmetric unit. The new N0 structure was solved by molecular replacement using PHASER (McCoy et al., 2007) and the N0 domain from the N0-N1-N2-GspD structure in complex with a nanobody (PDB 3EZJ) (Korotkov et al., 2009) as probe. Subsequent refinement gave a model with all residues in excellent density and good statistics (Table 1).

3.2. The dodecameric N0-GspD helix

The structures of the two monomers per asymmetric unit are similar and superimpose with an r.m.s.d. of 1.2 Å for 81 C atoms. The two monomers in the asymmetric unit are related by a rotation of 30.0 degrees about a non-crystallographic screw-axis coinciding with the crystallographic 6₁ screw axis and a shift of 2.04 Å along that axis. As a consequence, the crystallographic 6₁ screw axis generates, from these two domains in the asymmetric unit, a superhelix in the crystal with twelve N0 domains per turn, ~125 Å in diameter, and a pitch of only 24 Å (Fig. 2).

3.3. The N0•N0 contacts

Interactions between the two crystallographically independent A1 and B1 N0 monomers in the asymmetric unit, and between the B1 monomer and the A2 monomer in the adjacent asymmetric unit, are very similar (Fig. 3). Therefore, we will describe here only the A1•B1 interface. This N0•N0 interface buries 1100 Å² solvent-accessible surface area according to the PISA server (Krissinel and Henrick, 2007). Subunit A1 contributes Surface Area I, which comprises mainly residues from helix 2 plus a few residues prior and after this helix. Subunit B1 contributes Surface Area II, which consists of residues from two different regions: strand 2 and the 2-3 loop, and residues from strand 5 and the loop between 4 and 5 (Fig. 3, 4). Major contributing residues, i.e. with more than 30 Å² buried solvent-accessible surface in both A1•B1 and B1•A2 interfaces (with *** indicating residues with more than 50 Å² solvent-accessible surface buried) are, for Surface Area I: Ser40, Met44***, Leu55, Asn58***, Ala62*** and Gln63; and for Surface Area II: Glu19, Thr28***, Ile30***, Pro33, Glu71 and Asn72*** (Figs. 3C and D; Fig. 4). Strand 2 from subunit A1 and strand 3 from subunit B1 are approaching each other but are not forming main chain to main chain hydrogen bonds (Fig. 3C,D). Overall the interface is mainly hydrophobic with Met44 and many aliphatic and methionine side chains, including those

from Ile41, Met44, Leu55, Leu59 and Ala62, of Interface I, and Ala23, Ile29, Ile30, Met31 and Pro33 of Interface II, involved in central interface contacts.

3.4. Degree of conservation of residues in the N0•N0 helix interface

The residues engaged in the N0•N0 interface of the helical N0 dodecamer observed in our crystals are highly conserved among nine homologous species (Fig. 4). For the most important contact residues in Surface Area I Met44*** is always large and hydrophobic, except in *P. aeruginosa* where it is a Lys; Asn58*** is a Ser or an Asn; and Ala62*** is an Ala, Val or Thr. For the major contact residues in Surface Area II Glu 19*** is a Glu, Asp or Asn; Thr28*** is completely conserved; Ile30*** is either an Ile or a Val; and Asn72*** is always an Asn, except in *P. aeruginosa* where it is an Asp. Clearly the interface residues are highly conserved, and only slightly less so if *P. aeruginosa* is included. When we expanded the sequence analysis to include 74 homologs of the T2SS secretin, we obtained similar results showing that the aliphatic interface residues are more conserved than the surface residues outside of the interface observed in the super-helix (Supplementary Figs. S1 and S2). Interestingly, some of these aliphatic residues correspond to aromatic residues in a broader set of secretin homologs (Supplementary Fig. S2). In positions 44 and 55 aromatic residues could be easily accommodated in the context of interfaces that we observed in our crystal structure. In the case of Gln63, a preference for Tyr at this position may be explained by proximity of Pro33 (almost universally conserved) from the next N0 domain, i.e. stacking interaction between Tyr63 and Pro33 might actually strengthen the interface. Therefore, the sequence variations in the T2SS secretins are all compatible with the observed domain-domain interfaces in the N0 superhelix.

3.5. Structural comparisons

Structures of the N0 domain of the T2SS, from various species, have been reported only with N0 linked to N1 or more periplasmic GspD domains: ETEC N0-N1-N2-GspD•Nb7 (Korotkov et al., 2009); ETEC HR-GspC•N0-N1-N2-GspD•Nb3 (Korotkov et al., 2011b), ETEC HR-GspC•N0-N1-GspD•Nb3 (Korotkov et al., 2011b), ETEC HR-GspC•N0-N1-GspD (Korotkov et al., 2011b), and *P. aeruginosa* N0-N1-N2 of GspD^{XcpQ} (Korotkov et al., 2009; Korotkov et al., 2011b; Van der Meeren et al., 2013). The structure of the current ETEC N0 monomer is the first structure of N0 not linked to other domains. The ETEC N0 domains from previous ETEC structures superimpose onto the new ETEC N0 domain with an r.m.s.d. of 0.8–0.9 Å. The N0-N1 modules of ETEC N0-N1 GspD (Korotkov et al., 2009) and *P. aeruginosa* N0-N1 GspD^{XcpQ} (Van der Meeren et al., 2013) are very similar and superimpose with an r.m.s.d. of 2.4 Å for 124 residues, while the superposition of the N0 domains yields in r.m.s.d. of 1.2 Å and the superposition of the N1 domain an r.m.s.d. of 1.6 Å, a result of a difference of ~17 degrees in orientation of the N1 versus N0 domains in these two cases (Supplementary Fig. S3). In addition, a structure of the N0-N1 domains from the enteropathogenic *E. coli* T3SS secretin EscC has been reported (Spreter et al., 2009). Also, when our manuscript was submitted, a structure of the N0-N1 domains the *Salmonella enterica* serovar Typhimurium T3SS secretin InvG has been reported, as well as a new ring model based on the current electron microscopy reconstruction of the T3SS injectisome (Bergeron et al., 2013; Schraidt and Marlovits, 2011). The current ETEC N0 domain can be superimposed onto the N0 domain of EscC with an r.m.s.d. of 1.7 Å for 81 residues and 11 % sequence identity. However, the orientation of the N1 domain of EscC with respect to the N0 domain differs substantially from the N0-*vs*-N1 orientation in the ETEC T2SS N0-N1 module (Korotkov et al., 2009; Spreter et al., 2009). Similarly, for the novel InvG structure, the ETEC N0 domain can be superimposed onto the N0 domain of InvG with an r.m.s.d. of 2.1 Å for 75 residues and 14% sequence identity. The relative orientations of N0-*vs*-N1 domains are similar, although not identical, in the EscC and InvG structures (Bergeron et al., 2013; Spreter et al., 2009) and, therefore the N0-*vs*-N1

orientation in InvG is also significantly different from the N0-*vs*-N1 orientation in GspD. In summary, the N0-N1 module is highly similar in all known T2SS N0-N1 secretin structures despite a low degree of sequence identity. However, according to the currently available structures, the orientation between N0 and N1 differs considerably between T2SS and T3SS secretins.

3.6. Model of a N0 dodecameric ring

Members of the T2SS secretin family have been reported to form dodecameric structures according to electron microscopy studies (Chami et al., 2005; Reichow et al., 2010). Therefore we used the dodecameric ETEC N0 helix in the crystals to construct a dodecameric N0 ring model as described in Methods. The organization of N0 domains in this ETEC N0-GspD ring is different from the N0 ring in the N0-N1-GspD dodecamer model obtained previously on the basis of the structure of ETEC N0-N1-GspD (Korotkov et al., 2009). Whereas the N0-rings in these models have similar dimensions, in the new N0-ring the axes of the two helices per N0 domain are oriented in a more radial direction (Fig. 5).

The N0-N0-domain interface in the dodecameric N0-ring is essentially unchanged with respect to the interfaces in the N0-helix observed in the crystal structure. The resultant dodecameric N0-ring has an outer diameter of ~125 Å, an inner diameter of ~67 Å and a height of ~18 Å (Fig. 5A). The ETEC N0-GspD ring agrees approximately with the dimensions of the cryo-electron microscopy reconstruction of *V. cholerae* GspD (Reichow et al., 2010) although the internal diameter of the N0-ring is slightly less, by ~8 Å, than suggested by the cryo-electron microscopy study (Fig. 5B). In the new dodecameric N0-ring, subunit A1 contributes for Interface I mainly residues from helix 2 and Subunit A2 from Interface II residues from strand 2 and the 2-3 loop, and residues from strand 5 and the loop between 4 and 5. The α -helices are making a considerable angle with the twelve-fold axis of the ring. In contrast, in the SymmDock-generated N0-N1 dodecameric ring (Korotkov et al., 2009; Korotkov et al., 2011b; Schneidman-Duhovny et al., 2005), the contacts between N0 domains are mediated by loop residues leading to tail-to-head orientation of the domains and the α -helices aligned almost perpendicular to the C_{12} symmetry axes. Interestingly, in both models, strand 1 of the N0 domain is fully accessible and available for interactions with strand 1 of the HR domain as observed in the HR-GspC•N0-GspD complex (Korotkov et al., 2011b).

4. Discussion

It is striking that we obtained a dodecameric helix of N0 domains, which is almost a ring with C_{12} point group symmetry, while the symmetry of the T2SS secretins is believed to be a dodecamer with C_{12} symmetry (Chami et al., 2005; Korotkov et al., 2011a; Reichow et al., 2010). Moreover, the interface residues of the N0 dodecameric helix are highly conserved among the T2SS secretins (Fig. 4). Therefore it is worthwhile to investigate several characteristics of the newly obtained N0 ring, in particular regarding its interactions with partner proteins and GspD domains.

The N0 domain of T2SS secretins is known to interact with the HR domain of the inner membrane platform protein GspC in the T2SS from ETEC, *V. vulnificus* and *D. dadantii* (Gu et al., 2012; Korotkov et al., 2006; Korotkov et al., 2011b; Wang et al., 2012). SPR studies suggest that the N3 domain of GspD^{XcpQ} is the major periplasmic GspD domain that interacts with the HR domain from GspC^{XcpP} and not N0 (Douzi et al., 2011). However, this could be the result of weak interactions between individual N0 and HR domains in the *P. aeruginosa* T2SS, or due to species differences (note that the C-terminal GspC domain in *P. aeruginosa* is a coiled-coil domain and not a PDZ domain as e.g. in all other species shown

in Fig. 4). Crystal structures of several ETEC N0-N1 GspD•HR-GspC complexes have recently been reported (Korotkov et al., 2011b) and these are the first crystallographically elucidated complexes between outer membrane and inner membrane T2SS proteins. The importance of the interface observed in these crystal structures was confirmed by substitutions of contact residues, which blocked protein secretion *in vivo* (Korotkov et al., 2011b). The N0•HR interface seen in the crystal structure partially agrees with the contacts between these domains deduced from NMR studies in *D. dadantii* homologs, with residues on strand 1 and helix 2 of the N0 domain interacting with strand 1 of the HR domain, although a model involving strand 3 of the N0 domain has been proposed (Gu et al., 2012). Therefore, it is of great interest to investigate where the HR-GspC domains will be located with respect to the new N0 ring when HR-GspC domains are added twelve times to the N0 ring in the same orientation and position as observed in the N0-GspD•HR-GspC complex (Korotkov et al., 2011b).

The result of adding twelve HR domains to the N0 ring in this manner is a “double dodecamer” (Fig. 5C). In this 24-meric assembly, there are neither clashes between the twelve HR domains nor clashes between the HR domains and N0 domains. The outer diameter of the N0•HR double-ring dodecamer is 125 Å, i.e. the same as of the N0 ring by itself (Fig. 5A). Hence, when the double N0•HR dodecamer is placed into its likely position in the EM reconstruction (Fig. 5B), the opening of the periplasmic vestibule of full-length dodecameric GspD would be left unaltered. This means that exoproteins as large as the 89 kDa cholera toxin, or even larger, could position themselves into this vestibule as an early step in the translocation process (Reichow et al., 2010; Reichow et al., 2011).

The N0-GspD domain not only interacts with HR-GspC but also with the N1-GspD domain, forming the compact N0-N1-GspD module mentioned above, with a buried surface area of 1180 Å² and a flexible linker of ~18 residues between the N0 and N1 domains (Supplementary Fig. S3) (Korotkov et al., 2009; Korotkov et al., 2011b; Van der Meeren et al., 2013). This leads to the question if this N0-N1-GspD module is compatible with the N0 ring derived from our helical N0 structure. Intriguingly, this appears not to be the case. When twelve N0 domains of an N0-N1 module are superimposed onto the twelve N0 domains of the ETEC N0 ring, the N1 domains clash with the adjacent N0 domain (Supplementary Fig. S4). This result has potential implications for the dynamics of the periplasmic domains of GspD as will be discussed below.

Secretin channels occur in multiple important bacterial protein secretion machineries (Korotkov et al., 2011a). Hence, it is of interest that the homologous N0-N1 module from the T3SS secretins EscC and InvG (Bergeron et al., 2013; Spreter et al., 2009) have N0-vs-N1 orientation, which is 143 and 149 degrees, respectively, different from that in the T2SS N0-N1 module (Korotkov et al., 2009; Korotkov et al., 2011b; Van der Meeren et al., 2013). When twelve ETEC GspD N1 domains are added in the “T3SS N0- vs-N1 orientation” to the ETEC GspD dodecameric N0 ring derived from our current structure, then there are far fewer clashes between the hypothetical positions of the N1 domains and the N0 GspD ring than in the previous case when twelve N1 GspD domains were added to the N0 GspD ring in the “T2SS N0- vs-N1 orientation” (Fig. 5D). It is well possible that small adjustments in the N0-N1 linker could relieve the short contacts in the model. This suggests that the new N0 T2SS ring is compatible with a N1 T2SS ring with an N1- vs-N0 orientation approximately observed as in the T3SS secretins EscC and InvG. It should be noted, however, that the N0 domain-domain contacts are different in our current dodecameric GspD N0 ring model with extensive contacts between the domains (Fig. 2) and the InvG ring model with C15 symmetry where N0-N0 contacts are limited to only a few residues (Bergeron et al., 2013).

It is obvious that the N0 ring with cyclic C_{12} point group symmetry derived from our current crystal structure is distinctly different from proposals that dimers of N0-N1-N2-GspD exist. One type of dimer of these periplasmic domains has been suggested based on cysteine scanning studies on *D. dadantii* GspD^{OutD} (Wang et al., 2012), and another type of dimer on the basis of the presence a *P. aeruginosa* N0-N1-N2-GspD^{XpcQ} dimer in a crystal structure (Van der Meeren et al., 2013). In the latter, but not the former, case it was suggested that the entire GspD dodecamer would have C_6 symmetry, i.e. the secretin would consist of six full length GspD dimers. The dimers of periplasmic N0-N1-N2-GspD domains proposed in these two studies are entirely different, however. This can be the result of species differences, of differences in the methodologies used, or due to the fact that studies on components from such a large system as the T2SS are prone to form dimers when “carved” out from the full complex. After all, dimers have been frequently occurring in crystallographic studies on T2SS domains (Abendroth et al., 2009a; Abendroth et al., 2004a; Abendroth et al., 2004b; Abendroth et al., 2005; Abendroth et al., 2009b) and it has been suggested that such dimers might be the result of high local protein concentrations during crystal formation (Korotkov et al., 2012).

There is little doubt that the T2SS secretins have to undergo dramatic structural changes during the secretion process (Douzi et al., 2012; Korotkov et al., 2012; McLaughlin et al., 2012; Reichow et al., 2011; Shevchik et al., 1997). This has been demonstrated once again by the periplasmic gate observed in the cryo-electron microscopy reconstruction of *V. cholerae* GspD^{EpsD} (Reichow et al., 2010). Folded exoproteins cannot move from the periplasm through the GspD channel to the extracellular milieu without this gate opening, and, actually, also extracellular gate of GspD needs to change considerably during exoprotein secretion. There are therefore likely a number of quite different conformations of GspD. When limiting the discussion to periplasmic GspD domains, some states represent conformations without exoprotein or HR domain bound; other states may have one to twelve HR domains bound to N0 domains; others with an exoprotein bound to one or more N0 domains only; and yet other states may have an exoprotein bound near the interface of one or more N0•HR complexes (Douzi et al., 2011; Lindeberg et al., 1996; Shevchik et al., 1997). It has recently been suggested that exoprotein recognition is a key factor in completing the assembly of the T2SS (Chen and Hu, 2013). After initial binding to one or more N0-GspD domains, HR-GspC domains, or near N0•HR interfaces, an exoprotein might transiently occupy different positions inside the periplasmic vestibule of GspD. While the exoprotein is pushed through the secretin channel by a growing pseudopilus piston, it interacts successively with different periplasmic GspD domains, (Campos et al., 2013; Korotkov et al., 2012; McLaughlin et al., 2012; Shevchik et al., 1997). Exoproteins are also vastly different in ternary and quaternary structure ternary and quaternary structure (Korotkov et al., 2012; Sikora, 2013). Hence, the periplasmic domains of GspD might transiently adopt multiple, quite different, exoprotein-dependent, conformations, with the linkers between the N0, N1, N2 and N3 domains playing a critical role in allowing exoprotein-specific conformational flexibility in the initial stages of the secretion process.

Hence, it might be that the N0 dodecameric ring (Fig 5A), the N0-N1 dodecameric ring (Korotkov et al., 2009), and N0-N1-N2-GspD dimers (Van der Meeren et al., 2013; Wang et al., 2012), may all play a role in the mechanism of T2SS secretins. However, the dodecameric ring-like nature of the N0 helix in the current study, combined with electron microscopy studies (Chami et al., 2005; Reichow et al., 2010) provides evidence that C_{12} point group symmetry is a major subunit arrangement in T2SS secretins, both in the periplasm and for full-length subunits. Given the similarities in sequence and domain architecture, most likely also the secretins in the T4PS, T3SS and the filamentous phage assembly machinery have the same cylindrical twelve-fold symmetry (Korotkov et al.,

2011a). Obviously, further studies will be required to unravel the high-resolution architecture of full-length secretins in their various conformational states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

T2SS	Type II secretion system
T3SS	Type III secretion system
T4PS	type IV pili system

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Fig. 1. Domain structure and interactions of GspD secretin with GspC

(A) Schematic representation of the position of the periplasmic GspD domains and the interactions between GspD and GspC in the periplasm. The GspD secretin is represented by the cryo-EM reconstruction map of *V. cholerae* GspD (Reichow et al., 2010).

(B) Domain structure of GspD and GspC, with interactions between the N0 domain of GspD and the HR domain of GspC indicated by a blue arrow.

Fig. 2. The N0 domain of GspD secretin forms a superhelix in the crystal

(A) Cartoon representation of one full turn of the superhelix that contains 12 subunits. The outer diameter is ~125 Å with a pitch of 24 Å. The two crystallographically independent copies of the N0 domain are shown in two different shades of blue.

(B) The packing of the N0 helices viewed parallel to the 6₁ crystallographic screw axis, with the same coloring scheme as in A. The *a* and *b* unit cell axes are indicated.

Fig. 3. Structure of the N0-GspD domain and intersubunit interactions

(A) Structure of the N0 domain from ETEC GspD. Secondary structure elements are labeled.

(B) A close up view of four N0 subunits in the crystal. The rotation angle between 2 neighboring subunits and intersubunit interfaces shown in *C* and *D* are indicated.

(C) Close-up stereo view of interface I. Conserved interface residues are shown in stick representation.

(D) Close-up stereo view of interface II. Conserved interface residues are shown in stick representation.

Fig. 4. Sequence alignment of T2SS secretins

Sequence alignment of N0-GspD with N0-N0 interface residues and HR contact residues highlighted with blue and green arrows, respectively. From top to bottom: enterotoxigenic *E. coli* (ETEC), *Vibrio cholerae* (54% sequence identity to GspD ETEC for full-length protein), *Vibrio parahaemolyticus* (55%), *Vibrio vulnificus* (55%), *Aeromonas hydrophila* (48%), enterohemorrhagic *E. coli* (EHEC) (44%), *Klebsiella oxytoca* (44%), *D. dadantii* (43%) and *P. aeruginosa* (33%). While residues Glu19 and Asn26 engage both in N0•N0 and N0•HR interactions, there are no clashes in the double-dodecamer N0•HR ring (Fig. 5). Supplementary Fig. S2 shows a more extensive alignment of 74 unique T2SS secretins.

Fig. 5. The N0-GspD secretin ring and interactions with partner proteins

(A) Model of the ETEC N0-GspD ring. Left: cartoon representation. Right: surface representation. Note the radial directions of the N0 helices and β -strands. Subunits are colored as in Figs. 2 and 3.

(B) The ETEC N0 domain ring model placed into the cryo-EM map (EMDB 1763) of homologous *V. cholerae* secretin GspD^{EpsD} (Reichow et al., 2010).

(C) The double-dodecamer N0•HR ring obtained by adding to the ETEC N0-GspD ring twelve ETEC HR-domains (green) according to the structure of the ETEC N0-GspD•HR-GspC complex (Korotkov et al., 2011b).

(D) Model of the triple-dodecamer ETEC N1-N0•HR ring obtained by adding to the double-dodecamer N0•HR ring as shown in C above, the N1 domains of ETEC N1-GspD (purple) with the N1-vs-N0 conformation from the T3SS secretin EscC (Spreter et al., 2009).

Modeling of the N1 domains of ETEC N1-GspD based on the N1-vs-N0 conformation from the T3SS secretin InvG (Bergeron et al., 2013) leads to a similar model. See text for further details.

TABLE 1

Data collection and refinement statistics.

Data collection	
Space group	$P6_1$
Cell dimensions	
a, b, c (Å)	113.46, 113.46, 24.45
α, β, γ (°)	90, 90, 120
Resolution (Å)	49.2-1.43 (1.51-1.43) ¹
R_{sym}	0.046 (0.61)
$I / \sigma I$	19.1 (2.58)
Completeness (%)	92.7 (65.3)
Redundancy	9.2 (6.4)
Refinement	
Resolution (Å)	49.2-1.43
No. reflections	29882
R_{work} & R_{free}	0.1852 & 0.2192
No. atoms	
Protein	1263
Water	208
B -factors (Å ²)	
Protein	20.9
Water	31.9
Wilson B	25.3
R.m.s. deviations	
Bond lengths (Å)	0.0152
Bond angles (°)	1.694
Ramachandran distribution (%) ²	
Favored	99.4
Outliers	0

¹Values in parentheses are for the highest-resolution shell.²Calculated using Molprobit (Chen et al., 2010).