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Protein Flexibility and Coiled-Coil Propensity: New Insights Into Type III and Other Bacterial Secretion Systems

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1. Introduction

Secretion in unicellular species is the transport or translocation of molecules, for example proteins, from the interior of the cell to its exterior. In bacteria secretion is a very important mechanism, either modulating their interactions with their environment for adaptation and survival or establishing interactions with their eukaryotic hosts for pathogenesis or symbiosis. To overcome the physical barriers of membranes, Gram-negative bacteria use a variety of molecular machines which have been elaborated to secrete a wide range of proteins and other molecules; their functions include biogenesis of organelles (e.g. pili and flagella), virulence, efflux of toxins etc. As in some cases the secreted proteins are destined to enter host cells (effectors, toxins), some of the secretion systems include extracellular appendices to translocate proteins across the plasma membrane of the host.

With the rapid accumulation of bacterial genome sequences, our knowledge of the complexity of bacterial protein secretion systems has expanded and several secretion systems have been identified. Gene Ontology has been very useful for describing the components and functions of these systems, and for capturing the similarities among the diverse systems (Tseng et al., 2009). These analyses along with numerous biochemical studies have revealed the existence of at least six major mechanisms of protein secretion. These pathways are highly conserved throughout the Gram-negative bacterial species and are functionally independent with respect to outer membrane translocation; commonalities exist in the inner membrane transport steps of some systems, with most of them being terminal branches of the general secretion pathway (Sec). The pathways have been numbered Type I, II, III, IV, V and VI.

In Gram-negative bacteria, some secreted proteins are exported across the inner and outer membranes in a single step via the Type I, III, IV or VI pathways. Other proteins are first exported into the periplasmic space using the universal Sec or two-arginine (Tat) pathways

and then translocated across the outer membrane via the Type II, V or less commonly, the Type I or IV machinery. In Gram-positive bacteria, secreted proteins are commonly translocated across the single membrane by the Sec pathway, the two-arginine (Tat) pathway, or the recently identified type VII secretion system (Abdallah et al., 2007). In the following we will briefly survey the six Gram-negative bacterial secretion systems known to modulate interactions with host organisms:

Type I secretion system: This system (T1SS) forms a contiguous channel traversing the inner and outer membranes of Gram-negative bacteria. It is a simple system, which consists of only three major components: ATP-binding cassette transporters, Outer Membrane Factors, and Membrane Fusion Proteins (Holland et al., 2005). T1SS transports ions and various molecules including proteins of various sizes (20-900 kDa) and non-proteinaceous substrates like cyclic β -glucans and polysaccharides.

Type II secretion system: This system (T2SS) is encoded by at least 12 genes and supports the transport of a group of seemingly unrelated proteins across the outer membrane. In order for these proteins to enter the type II secretion pathway, they have to first translocate across the cytoplasmic membrane via the Sec-system and then fold into a translocation competent conformation in the periplasm. Proteins secreted by T2SS include proteases, cellulases, pectinases, phospholipases, lipases, and toxins which contribute to cell damage and disease. Although Sec-dependent translocation is universal (Cao & Saier, 2003), the T2SS is found only in Gram-negative proteobacteria phylum (Cianciotto, 2005; Filloux, 2004). A bacterial species may have more than one T2SS (Cianciotto, 2005; Filloux, 2004).

Type III secretion system: These systems (T3SS) are essential mediators of the interaction of many Gram-negative pathogenic proteobacteria (α , β , γ and δ subdivisions) with their human, animal, or plant hosts and are evolutionarily related to bacterial flagella. (Dale & Moran, 2006; Tampakaki et al., 2004; Troisfontaines & Cornelis, 2005). The machinery of the T3SS, termed the injectisome, appears to have a common evolutionary origin with the flagellum and translocates a diverse repertoire of effector proteins either to extracellular locations or directly into eukaryotic cells, in a Sec-independent manner (interkingdom protein transfer device). The T3SS effectors (T3EPs) modulate the function of crucial host regulatory molecules and trigger a range of highly dynamic cellular responses which determine pathogen-host recognition, pathogen/symbiont accommodation and elicitation or suppression of defense responses by the eukaryotic hosts. In some cases however, effector proteins are simply secreted out of the cell. T3SS have evolved into seven families (Troisfontaines & Cornelis, 2005). Some bacteria may harbor more than one T3SS, usually from different families. T3SS genes are encoded in pathogenicity islands and/or are located on plasmids, and are commonly subject to horizontal gene transfer.

Type IV secretion system: In comparison to other secretion systems, T4SS is unique in its ability to transport nucleic acids in addition to proteins into plant and animal cells, as well as into yeast and other bacteria. Usually T4SS comprises 12 proteins that can be identified as homologs of the VirB1-11 and VirD4 proteins of the *Agrobacterium tumefaciens* Ti plasmid transfer system (Christie & Vogel, 2000). T4SS spans both membranes of Gram-negative bacteria, using a specific transglycosylase, VirB1, to digest the intervening murein (Koraimann, 2003; Baron et al., 1997). While many organisms have homologous type IV secretion systems, not all systems contain the same sets of genes. The only common protein is VirB10 (TrbI) among all T4SS systems (Cao & Saier, 2003).

Type V secretion system: T5SS is the simplest protein secretion mechanism. Proteins are secreted via the autotransporter system (type Va or AT-1), the two-partner secretion pathway (type Vb), and the oligomeric autotransporters (type Vc or AT-2 system) (Yu et al., 2008; Desvaux et al., 2004). Proteins secreted via these pathways have similarities in their primary structures as well as striking similarities in their modes of biogenesis. There are three sub-classes of T5SS. The archetypal bacterial proteins secreted via the T5SS (T5aSS subclass) consist of a N-terminal passenger domain of 40-400 kD in size and a conserved C-terminal domain (Henderson et al., 2004). The proteins are synthesized with a N-terminal signal peptide that directs their export into the periplasm via the Sec machinery.

Type VI secretion system: In T6SS 13 genes are thought to constitute the minimal number needed to produce a functional apparatus (Boyer et al., 2009). The T6SS gene clusters (T6SS loci) often occur in multiple, non-orthologous copies per genome and have probably been acquired via horizontal gene transfer (Sarris & Skoulica, 2011; Sarris et al., 2011). Each T6SS probably assumes a different role in the interactions of the harbouring organism with others. Although the T6SS has been studied primarily in the context of pathogenic bacteria-host interactions, it has been suggested that it may also function to promote commensal or mutualistic relationships between bacteria and eukaryotes, as well as to mediate cooperative or competitive interactions between bacterial species. The T6SS machinery constitutes a phage-tail-spike-like injectisome that has the potential to introduce effector proteins directly into the cytoplasm of host cells, analogous to the T3SS and T4SS machineries.

Genetic, structural and biochemical studies of the above bacterial secretion systems along with massive *in silico* analyses of microbial genomes have been used to distinguish pathogens from their non-pathogenic relatives. These studies have established the presence of characteristic conserved features within individual types of secretion systems (e.g. Tampakaki et al., 2004), along with considerable sequence and structural diversities within each system at the level of specific components and effector proteins.

Despite the complexity of these systems however, the problem of identifying conserved features and properties within each secretion system type, or across several types of systems is of particular importance, going beyond a fundamental understanding of how bacterial secretion works. Even for well studied pathogens, not all virulence factors have been identified, making it possible that e.g. effector proteins that are associated with different diseases are still unknown. In less well characterized bacterial species there is certainly a wide spectrum of unknown effectors. This situation may be now changing through new approaches that use advanced machine learning algorithms to identify within individual types of secretion systems common themes for effectors and other system components that go beyond simple amino acid motifs (Arnold et al., 2009; Samudrala et al., 2009), or through the identification of important structural and physicochemical properties as universal signatures of virulence factors (Gazi et al., 2008; 2009).

This review will focus on the well-characterized T3SS proteins where the prevalence of coiled-coil domains along with pronounced structural flexibility/disorder have been proposed to be characteristic properties associated with a protein-protein interaction mode within T3SS and as essential requirements for secretion (Delahay and Frankel, 2002; Pallen et al., 1997; Gazi et al., 2008; 2009). Common themes with other secretion systems (T4SS, T6SS) will be also discussed.

2. Overview of the T3SS system: Architecture, conserved features and protein structures

Pathogenic bacterial strains are distinguished from non-pathogenic ones by the presence of specific set of genes that code for toxins, secretion systems, effectors that are meant to act extracellularly or effectors that should be delivered inside the host cell cytoplasm. These genes are usually tightly organized in operones that are located in chromosomal areas with a high distribution of mobile elements or can be found in virulence plasmids. Usually these chromosomal areas are called pathogenicity islands as they possess a different GC content from the rest of the genome, which implies recent acquisition through horizontal gene transfer events. One of the most profound cases was a set of approximately 20-25 genes which together encode one of the best characterized pathogenic mechanisms termed “type III secretion”. By this mechanism extracellularly located bacteria that are in a close contact with a eukaryotic cell deliver proteins into the host cell cytosol. While the T3S apparatus is conserved in pathogens across the plant/animal phylogenetic divide, the secreted proteins differ considerably. The genes coding for what are now recognized as structural T3SS components were first described as a contiguous cluster, designated “*hrp*” (hypersensitive response and pathogenicity) in plant pathogens. Important insights into fundamental questions of bacterial pathobiology came with the recognition, in subsequent years, of the T3SS as a complex multiprotein channel dedicated to translocate the effectors from the pathogen to the host. Although originally linked to pathogenesis, T3SS are also found in members of the phylum proteobacteria that are symbiotic, commensal or otherwise associated with insects, nematodes, fishes, plants, as well as in obligatory bacterial parasites of the phylum *Chlamydiae* (Dale and Moran, 2006; Marie et al., 2001).

T3SS is a multicomponent apparatus with the following characteristics: i) when fully developed it spans both bacterial membranes and the periplasmic space; ii) it possesses a large extracellular appendage (termed ‘pilus’ in plant pathogenic bacteria or ‘needle’ in animal pathogenic ones) that reaches the eukaryotic host cell contributing to bacterial adherence; iii) it forms the translocation pore in the host cell membrane to efficiently deliver proteins of bacterial origin inside the host cell; iv) a large number of T3SS cytosolic components form the export gate into the bacterial cytoplasm which sorts and prepares the substrates for secretion (Fig. 1).

The integral bacterial membrane part of the T3S apparatus consists of a series of rings. The protein that oligomerizes and forms the outer membrane and periplasmic rings (yellow parts in Fig. 1) belongs to the secretin family of proteins (which is also common to T2SS) and has a crucial role in T3S biogenesis (Diepold et al., 2010; Korotkov et al., 2011). Secretins consist of various domains with the C-terminal one integrated in the outer membrane. The N-terminal domains are less conserved among secretion systems and are responsible for the formation of the periplasmic rings. An N-terminal signal targets secretins to the periplasmic space through the Sec pathway. From there they are delivered to the outer membrane through a specific small lipidated protein, pilotin (Okon et al., 2008). Pilotins from various secretion systems possess different structures despite their common function, probably due to their interaction with the non-conserved C-terminal tail of various secretins. Thus, for example, the T3SS pilotin of *Shigella flexneri* possess an overall fold which differs from the fold of the T3SS pilotin of *Pseudomonas aeruginosa* or the T2SS pilotins of *Neisseria meningitidis* and *P. aeruginosa* (Izore et al., 2011).

The T3SS inner membrane (IM) rings are formed by the proteins SctD and SctJ [orange parts in Fig. 1; the unified nomenclature is followed here as proposed by Hueck (1998)]. SctD is a single-pass inner membrane protein that oligomerizes to form the most external inner membrane ring of the T3SS. Its N-terminal domain is facing the bacterial cytoplasm and its structure is homologous to forkhead-associated (FHA) domains (McDowell et al., 2011). The inner membrane part of the *Salmonella typhimurium* injectisome has been studied by EM (Marlovits et al., 2006; Marlovits et al., 2004). The inner membrane topology of six conserved components (HrcD^{SctD}, HrcR^{SctR}, HrcS^{SctS}, HrcT^{SctT}, HrcU^{SctU} and HrcV^{SctV}) of the T3SS from *Xanthomonas campestris* was recently studied (Berger et al., 2010) by translational fusions to a dual alkaline phosphatase- β -galactosidase reporter protein. Full IM rings have been modeled for PrgH^{SctD} and PrgK^{SctJ} [the species-specific name is followed by the standard T3SS nomenclature as proposed by Hueck (1998) in superscript] based on docking of atomic structures of individual domains to cryo electron microscopy maps (Schraidt & Marlovits, 2011). The central density observed in the inner membrane rings (socket region) of a T3SS needle complex cryo electron microscopy reconstruction map from *Salmonella enterica* sv. typhimurium (Fig. 1, red parts) is attributed to the SpaP^{SctR}, SpaQ^{SctT}, SpaR^{SctS}, SpaS^{SctU} and InvA^{SctV} proteins (Schraidt & Marlovits, 2011; Wagner et al., 2010).

In the socket region numerous cytosolic components are recruited to orchestrate the secretion of various T3SS substrates, like the ATPase SctN and its various subunits SctO, SctL. As biogenesis of the T3SS must take place before the secretion of the effectors, the first T3SS substrates to be secreted are the proteins that build the needle or pilus (SctF) and the inner rod (SctI), (green part in Fig. 1). The proteins that form the translocator pore in the eukaryotic membrane along with the proteins found in the needle tip are the next substrates to be secreted prior to effector proteins secretion.

An additional cytoplasmic ring is believed to be formed around the T3SS export gate as in the case of the flagellum (Thomas et al., 2006). Although never really observed by electron microscopy, recently Lara-Tejero and colleagues have reported the presence of a large platform in the T3SS of *S. enterica* sv. typhimurium that can sort substrates prior to secretion (Lara-Tejero et al., 2011). This platform consists of SpaO^{SctQ}, OrgA^{SctK} and OrgB^{SctL}.

Numerous crystal structure determinations of T3SS components have been reported: The structures of the C-terminal domain of HrcQ_B^{SctQ} (Fadouloglou et al., 2004; Fadouloglou et al., 2009), the C-terminal domain of FliN (Brown et al., 2005) and the central part of FliM (Park et al., 2006), all members of the SctQ/FliN,Y family and components of the cytoplasmic ring of the T3SS apparatus (C-ring) have been determined. Extended mutational and cross linking studies support a donut-shaped tetramer organization for the FliN protein which is localized at the bottom of the C-ring (Paul and Blair, 2006). A model where the FliN tetramers alterates with the C-terminal domain of FliM (FliM_C) seems to be in agreement with the major features observed in electron microscopic reconstructions. The side-wall of C-ring above the FliN₄FliM_C array is formed by the middle domain of FliM while the N-terminal domain interacts with the FliG which is localised in proximity with the inner membrane and is the connection unit between the C-ring and the inner membrane, MS-ring (Sarkar et al., 2010; Paul et al., 2011). FliG has no homolog in non-flagellar T3SS and the homolog SctQ proteins are interacting to the T3SS injectisome through the SctD proteins.

The structures of EscU^{SctU} and YscU^{SctU}, *EPEC* and *Yersinia* homologs of HrcU^{SctU} respectively (Zarivach et al., 2008; Lountos et al., 2009; Thomassin et al., 2011) provide

insights into the properties of conserved core components. The periplasmic domain of PrgH^{SctC} from *Salmonella* (Spreter et al., 2009) and the cytoplasmic domain of MxiD^{SctC} from *Shigella* (McDowell et al., 2011) have been recently determined. Structures of the periplasmic domains of the membrane components EscJ^{SctJ} from the enteropathogenic *Escherichia coli* (EPEC) are also available (Yip et al., 2005b; Spreter et al., 2009).

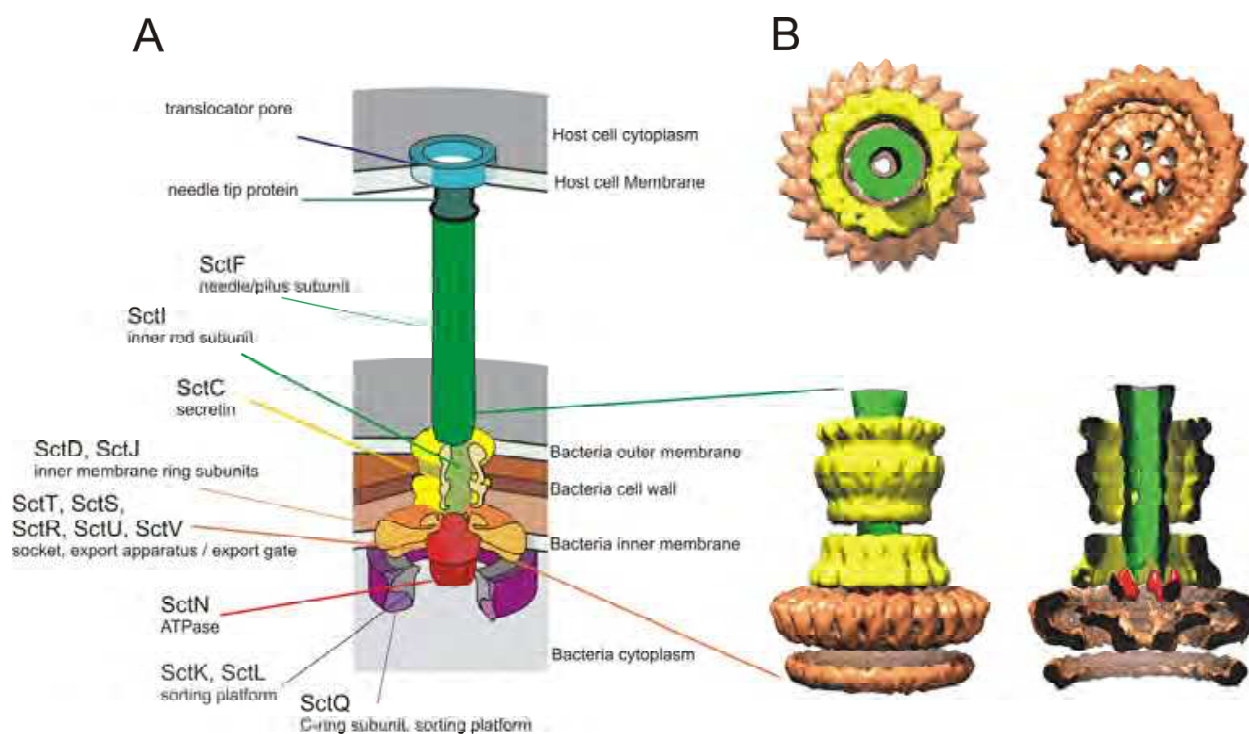


Fig. 1. (A) Overview of the T3S injectisome. (B) Different views of the *S. enterica* T3SS needle complex (Schraidt & Marlovits, 2011). Various parts of the needle complex are depicted in different colors using UCSF Chimera (Goddard et al., 2007). The colouring scheme used in (A) is followed. Top left: The T3SS needle complex viewed from top, Top right: View from the bottom, Lower left: side view, Lower right: a cross section of the side view. The inner membrane rings (orange) possess a 24-fold symmetry axis while the secretin rings (yellow) possess a 15-fold symmetry axis resulting in an overall 3-fold symmetry for the needle complex. The socket region (red parts and part of the orange area under the red parts) has a 6-fold symmetry (top right, icenter of the bottom view), which is also the symmetry of the T3SS ATPase that presumably docks in this area.

The T3SS utilizes an ATPase dedicated to drive secretion substrates through the central channel of the apparatus. Members of the SctN family (HrcN/FliI/YscN homologs) have a demonstrated ATP-hydrolysis activity, and exhibit extensive sequence and structural similarities with the F_0F_1 -ATPase α/β subunits. Biochemical and electron microscopy data suggest that as it is the case with F_0F_1 -ATPases, also the T3SS ATPases are hexamers anchored at the bacterial inner membrane. The crystal structure of FliI has been determined in the ADP-bound state and extensive structural similarities with to the α and β subunits of the F_0F_1 -ATP synthase have been found (Imada et al., 2007). The catalytic domain of EscN^{SctN} also shows structural similarity with F_0F_1 -ATPases (Zarivach et al., 2007). Recently the structure of FliJ, member of the SctO family (HrpO/FliJ/YscO homologs) has been reported as an α -helical coiled coil (Ibuki et al., 2011). Its structural

similarity to a subunit of the F_0F_1 -ATP synthase and its interactions with FliI will be presented in section 6.1.1. Proteins from the SctL family (HrpE/FliH/YscL homologs) interact both with the T3SS ATPase and with structural proteins from the YscQ/FliN family located at the cytoplasm/inner membrane i.e. YscQ^{SctQ}, EscQ^{SctQ} and FliN (Blaylock et al., 2006; McMurry et al., 2006; Biemans-Oldhinkel et al., 2011).

The outer supramolecular structure of the needle has been studied (Cordes et al., 2003), while structures of needle subunits from various bacteria have been recently determined (Wang et al., 2007; Deane et al., 2006; Zhang et al., 2006). At the tip of the T3SS needle resides an adaptor structure which mediates the interaction between the needle and the translocation pore at the eukaryotic membrane. The adaptor is formed through polymerization of a single protein. Information is available for the following needle tip proteins from three T3SS families: IpaD (*Shigella flexneri*), SipD (*Salmonella* spp.) and BipD (*Burkholderia pseudomallei*) from the *Inv-Mxi-Spa* T3SS family; LcrV (*Yersinia* spp.), PcrV (*Pseudomonas aeruginosa*, Sato et al., 2011; Sato & Frank, 2011) and AcrV (*Aeromonas salmonicida*) from the Ysc T3SS family; EspA (EPEC) from the *Ssa-Esc* family. The structures of IpaD, BipD, LcrV and part of the EspA structure have been elucidated (Espina et al., 2007; Johnson et al., 2007; Yip et al., 2005a; Derewenda et al., 2004), while a 3D-reconstruction of the MxiH filament is available (Deane et al., 2006).

Effectors are a large and structurally diverse group of virulence proteins which usually comprise a domain or a motif with a significant and proven role within the host cell during infection (for a review see Dean, 2011). Structures of several T3SS effectors from plant and animal pathogens are known (Desveaux et al., 2006; Stebbins, 2005). In addition, several structures for chaperones and chaperone-substrate complexes have been determined, including class I, class II and class III chaperones (Lilic et al., 2006; Buttner et al., 2008; Quinaud et al., 2007; Sun et al., 2008). Chaperones will be also presented in section 2.1.

2.1 The T3SS secretion signal

Type III effector proteins (T3EPs) possess non-cleavable secretion signals in the N-terminal protein regions, but no discernible amino acid or peptide similarities (Buttner and He, 2009) can be found. Three different types of potential secretion signals have been discussed: i) the N-terminus of the effector protein, ii) the ability of a chaperone to bind the effector before secretion, and iii) the 5'-end region of the mRNA; this hypothesis is very controversial (Gauthier et al., 2003; Anderson & Schneewind, 1997; Ramamurthi et al., 2002).

The prevailing view, supported by extensive bioinformatic analyses, is that the amino acid composition of the N-terminal region of the effectors serves as secretion signal (Lloyd et al., 2001; Buttner and He, 2009; Arnold et al., 2009; Samudrala et al., 2009). The required N-terminal peptide length for secretion is usually 10–15 residues, whereas the minimum length needed for translocation is 50–60 residues. Additional targeting information is contained within the first 200 residues which provide binding sites for secretion chaperones (Lilic et al., 2006). T3SS chaperones of mammal pathogens interact with their cognate effectors through a chaperone-binding domain (CBD) located within the first 100 amino acids of the effector, after the N-terminal export signal (Cornelis, 2006).

Analyses of effectors from pathogenic bacteria revealed that the 25 N-terminal residues are enriched in Ser and lack Leu (Buttner and He, 2009; Arnold et al., 2009; Samudrala et al.,

2009). The N-terminal regions of T3EPs are probably unfolded, which is an important prerequisite for their transport through the narrow inner T3SS channel of presumably only 2.8 nm in diameter as was previously shown for the T3SS of several animal pathogenic bacteria (Marlovits et al., 2004, 2006; Galan and Wolf-Watz, 2006; Gazi et al., 2009).

For some effectors however, the N-terminal secretion signal is not sufficient for maximal secretion (Buttner & He, 2009) and specific chaperone proteins are needed; these are usually located adjacent to the cognate effector genes, suggesting strong selection for their coexistence in the genome. T3S chaperones are proposed to play a role in targeting secretory cargo to the injectisome, either by providing targeting information (Birtalan et al., 2002), or facilitating the exposure of the N-terminal export signal (Cornelis et al., 2006). Some chaperones are involved in the translocation of many substrate proteins, e.g. the global HpaB chaperone from *Xanthomonas campestris* pv. *vesicatoria* or Spa15 of *S. flexneri* (Hachani et al., 2008; Parsot et al., 2003; 2005; Buttner et al., 2004; 2006). Class I chaperones (the chaperones of effectors) are soluble small, usually homodimeric proteins that bind effector proteins. Although diverse in their sequences, they belong to the structural class of α/β proteins with a two-layer-sandwich architecture. For the chaperone-effector interaction a β -strand of the effector is added to extend the β -sheet layer of the chaperone (Lilic et al., 2006). Class I chaperones have been further subclassified depending on whether they associate with one (class Ia) or several (class Ib) effectors (Page & Parsot, 2002). Class II chaperones are T3SS chaperones of the translocators (Neyt & Cornelis, 1999). Experimental determinations of their structures (Buttner et al., 2008; Lunelli et al., 2009; Job et al., 2010; Priyadarshi & Tang, 2011) have confirmed earlier sequence analyses (Pallen et al., 2003) predicting an all- α -helical domain structure, with the bulk of the protein consisting of three tandem tetratricopeptide repeats (TPRs) which are involved in protein-protein interactions. Their substrate is recognised and bound into a concave site of the chaperone. Class III chaperones prevent the premature polymerization of needle components in the bacterial cytoplasm. They are predicted to adopt extended α -helical structures; this was confirmed by the crystal structure of the Cesa which binds the EspA filament protein (Yip et al., 2005a).

Many functions have been attributed to T3SS chaperones, but the exact role(s) of the entire family of chaperones remain to be determined. However, it has been proposed that one of the main roles of the T3SS chaperones is the stabilization of at least some effector proteins inside bacterial cell, as well as their maintainance in a secretion-competent state, i.e. a partially folded or unfolded conformation.

3. The coiled-coil motif in proteins and α -helical bundles

The coiled-coil motif in protein structures consists of amphipathic α -helices that twist around each other to form a supercoiled bundle (Burkhard et al., 2001). It represents one of the efficient geometric solutions to packing helices in a stable way. The motif was one of the earliest protein structures discovered, first described for the hair protein alpha keratin (Crick, 1952). Coiled-coils are associated with all types of protein structure (globular, fibrous, membrane) and frequently provide a structural scaffold linked with molecular recognition interactions and oligomerization. Coiled-coil interactions play a major role in the formation of protein complexes in transcription, cell divisions, host-pathogen interactions etc. (Rackham et al., 2010). Coiled-coil helices may run parallel or antiparallel, and may form homo- or heterocomplexes (Grigoryan and Keating, 2008). The structures range from simple

dimers through pentamers to more complex assemblies of many helices or bundles of bundles. Sequences of regular, left-hand twisted coiled-coils are characterized by a seven-residue periodicity (heptad repeat). If the heptad positions are labeled *a-g*, then positions *a* and *d* are hydrophobic and form the core of the bundle (Fig. 2). Positions *b, c, e, f* and *g* are more solvent-exposed and their amino acid preferences reflect constraints which are specific to each type of helical bundle (Paliakasis & Kokkinidis, 1992; Lupas et al., 1991). The hydrophobic residues *a* and *d* from one helix form 'knobs' that pack into 'holes' formed by residues *g, a, d, e* on neighbouring helices. Coiled-coil helices are distinguished from other amphipathic helices by the periodicity of hydrophobic residues (3.5 vs. 3.65 residues per turn), the length (long vs. short) and the packing interactions. Some proteins are induced to form coiled-coils upon association with a binding partner (Lupas, 1996).

Coiled-coil predictions at the genome level have explored the 'coilomes' of individual organisms (Barbara et al., 2007; Newman et al., 2000, Rose et al., 2004). In addition, genomewide analyses of coiled-coils evolution have been performed (Rackham et al., 2010) and shown that coiled-coils do not change their oligomeric state over evolution, and do not evolve from rearrangements of α -helices in protein structures. An analysis of proteomes (Liu and Rost, 2001) showed that twice as many coiled-coils are found in eukaryotes (10%) as in prokaryotes and archaea (4%–5%). The size of coiled-coil proteins ranges from short domains of 6-7 heptad repeats, e.g. Leucine zippers, serving as homo-/heterodimerization motifs in transcription factors (Jakoby et al., 2002; Vinson et al., 2002), to long domains of several hundreds amino acids found in functionally distinct proteins, often involved in attaching protein complexes to larger cellular structures (e.g. the Golgi, centrosomes, centromeres, or the nuclear envelope).

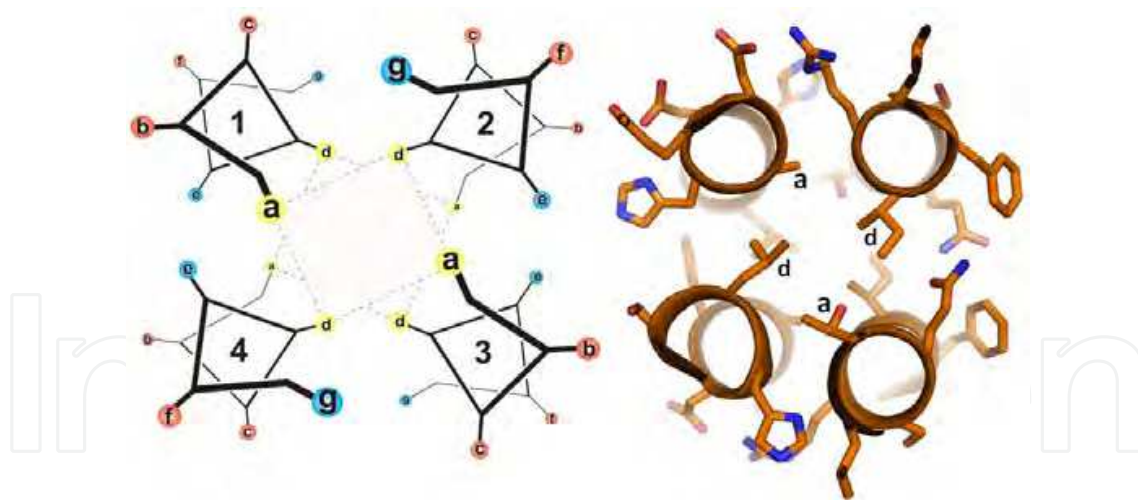


Fig. 2. Left: Antiparallel coiled-coil structure (4- α -helical bundle) and assignment of heptad positions *a-g*. Hydrophobic and hydrophilic positions are coloured yellow and red respectively. Right: Positions *a, d* and their packing in the core of the 4- α -helical ColE1 Rop (Banner et al., 1987). Positions *a, d* form slices perpendicularly to the bundle axis.

4. Flexibility and disorder in proteins

Until recently the classical structure–function paradigm which states that protein function is dependent on a defined, if flexible, three-dimensional polypeptide structure was widely

accepted in protein science (Anfinsen, 1973). However, even in the early days of structural biology, with only approx. 20 protein crystal structures determined, some protein segments were known which yield weak or non-detectable electron density and yet they may be essential for function (Bloomer et al., 1978; Bode et al., 1978). A common reason (apart from crystal defects) for missing electron density is that the unobserved region fails to scatter X-rays coherently due to variation in position from one atom to the next, i.e. the unobserved atoms are disordered. In addition, during the last decade, many proteins have been described that fail to adopt a stable tertiary structure under physiological conditions and yet display biological activity (Dunker et al., 2008a; Uversky & Dunker, 2010). This state of the proteins, defined as intrinsic disorder, has been found to be rather widespread; disordered regions lacking stable secondary and tertiary structure are often a prerequisite for biological activity, suggesting that structure-function relationships can be frequently only understood in a dynamic context in which function arises from conformational freedom. Fully or partly nonstructured proteins are described as intrinsically disordered (IDPs) or intrinsically unstructured proteins. The term natively unfolded proteins indicates that protein function is associated with a dynamic ensemble of different conformations (Gazi et al., 2008).

Structural plasticity and flexibility is believed to represent a key functional feature of IDPs (Dunker et al., 2008a, 2008b; Dunker & Uversky, 2008; Xie et al., 2007; Cortese et al., 2008), enabling them to interact with numerous binding partners, e.g. proteins, membranes, nucleic acids and small molecules (Durand et al., 2008; Uversky et al., 2009). Because of their functional importance, intrinsically disordered domains are very common in proteomes and play crucial roles in signaling, recognition, regulation and self-assembly (Namba, 2001). The extreme flexibility of IDPs has been suggested to represent a strategy for optimizing the search and interaction with their targets (Sugase et al., 2007). Intrinsically disordered proteins are substantially depleted in W,C,F,Y,V,L,N (order-promoting) and enriched in A,R,G,Q,S,P,E,K (disorder-promoting residues) (Dunker et al., 2002; Uversky, 2010). These biases in the amino acid compositions of IDPs (which result in low overall hydrophobicity and low net charge) are used in various methods for the prediction of the ID propensities (Prilusky et al., 2005). Such analyses suggest that approx. 45% of proteins within a eukaryotic proteome contain a disordered region (Pentony & Jones, 2010). As a result of their frequent node positions in interactoms, many disordered proteins are tightly regulated at the levels of their synthesis, degradation and posttranslational modifications (Gspaner, 2008). It is noteworthy that extreme structural plasticity and ensembles of different conformations has been occasionally observed for coiled-coils and α -helical bundles (Glykos et al., 1999, 2004); as is the case with other proteins, the plasticity of coiled coils may have functional implications, e.g. in the establishment of macromolecular assemblies based on coiled-coil interactions (Gazi et al., 2008).

5. Tools for the analysis of coiled-coils and intrinsic disorder

5.1 *In silico* prediction and analysis of coiled-coil domains

Prediction of coiled coils from sequence: The 'COILS' webserver assesses the probability that a residue in a sequence is part of a coiled-coil structure by comparison of its flanking sequences with sequences of known coiled-coil proteins (Lupas et al., 1991) (http://www.ch.embnet.org/software/COILS_form.html). In the 'Paircoil2' algorithm

(McDonnell et al., 2006), pairwise residue probabilities are used to detect coiled-coil motifs in protein sequences (<http://groups.csail.mit.edu/cb/paircoil2/paircoil2.html>). 'Matcher' (<http://cis.poly.edu/~jps/>) determines whether a given sequence contains heptads and assigns heptad positions to residues (Fischetti et al., 1993). To predict the oligomerization states of coiled coils 'Multicoil2' (Trigg et al., 2011) uses pairwise correlations and Hidden Markov Models (HMMs). For distinguishing dimers, trimers and non-coiled-coil oligomerization states the algorithm integrates sequence features through a multinomial logistic regression and devises an optimized scoring function that incorporates pairwise correlations localized in the sequence. A database comprising 2015 sequences with reliable structural annotation from experimental data is used (<http://multicoil2.csail.mit.edu>). 'SCORER' (Armstrong et al., 2011) also provides predictions of coiled-coil oligomerization (<http://coiledcoils.chm.bris.ac.uk/Scorer>).

Assignment of the coiled coil packing: COILCHECK (Alva et al., 2008) can be used for analysis and validation of coiled-coil structures through calculation of the strength of interhelical interactions in coiled coils; it can be used to rationalize the behaviour of single residue mutations and to design mutations (<http://caps.ncbs.res.in/coilcheck/>). SOCKET (Walshaw & Woolfson, 2001) can be used to identify coiled coils through an analysis of the knobs-into-holes side chain packing (<http://coiledcoils.chm.bris.ac.uk/socket/>).

Databases: For genomewide predictions the 'SpiriCoil' algorithm (Rackham et al., 2010) is employed which uses hundreds of HMMs representing coiled-coil-containing domain families. Their results are available through the SpiriCoil Database (<http://supfam.org/SUPERFAMILY/spiricoil>). It includes results from all completely sequenced genomes. The CC+ database is a detailed, searchable repository accessible via the SOCKET program (Testa et al., 2009) (<http://coiledcoils.chm.bris.ac.uk/ccplus/>).

Several of the above tools have been used in sections 6 and 7 of this chapter. In addition, protein sequences were retrieved from the NCBI/GenBank and specialized databases e.g. PPI: *P.syringae* Genome Resources (www.pseudomonas-syringae.org) and the Kyoto Encyclopedia for Genes and Genomes (KEGG) (Kanehisa & Goto, 2000). Secondary structure predictions were performed with 'PSIPRED' (Jones, 1999). Protein structures were retrieved from the Protein Data Bank (PDB).

5.2 *In silico* analysis of T3SS effectors and secretion signals

A selection of bioinformatics tools is available for T3SS effector and secretion signal prediction: 'Effective' is an on-line tool for sequence-based prediction of secreted proteins available from the TUM Genome Oriented Bioinformatics, University of Vienna (Arnold et al., 2009; Jehl et al., 2011), which can be used for the effector prediction in bacterial protein-sequences (<http://www.effectors.org/>). 'Effective' provides pre-calculated predictions on bacterial effectors in all publicly available pathogenic and symbiotic genomes or using sequence data provided by the user. T3SS secretion signal predictions from amino acid sequences, is available from 'moblab' (http://gecco.org.chemie.uni-frankfurt.de/T3SS_prediction/T3SS_prediction.html). The basic concepts of this tool are described by Lower & Schneider (2009). The 'SIEVE' Server (<http://www.sysbep.org/sieve/>) for the prediction of type III secreted effectors was originally described by Samudrala et al., (2009) and recently reviewed by McDermott et al., (2011). Potential T3SS effectors are scored using a computational model developed via Machine-Learning Methodologies.

5.3 Experimental and *in silico* analysis of disordered domains

Disordered regions may be detected in protein structures determined by X-ray crystallography through missing electron density. Heteronuclear multidimensional NMR is a powerful tool for the characterization of protein disorder and provides direct measurement of the mobility of unstructured regions (Eliezer, 2007). Loss of secondary structure may be detected (among other methods) by far-UV CD (Kelly & Price, 1997) and Fourier transform infra-red spectroscopy (FTIR) (Uversky et al., 2000). Hydrodynamic parameters obtained from techniques such as gel filtration, SAXS (Gazi et al., 2008), dynamic and static light scattering provide information on whether a protein is unfolded since the unfolding results in an increase in protein hydrodynamic volume. The degree of globularity, which reflects the presence of a well-packed hydrophobic core may be estimated by a special analysis of small angle X-ray scattering (SAXS) data in form of a Kratky plot. Kratky plots are obtained by plotting $I(s)xs^2$ against s (scattering intensity: I ; momentum transfer: $s=4\pi\sin(\theta)/\lambda$; 2θ : scattering angle; wavelength of X-rays: λ). They are used to judge the folding of the protein, as the shape of the curve is sensitive to the conformational state of the scattering molecules (Gazi et al., 2008).

Several algorithms have been developed to predict protein disorder on the basis of specific biochemical properties and biased amino acid compositions. These tools include PONDR (Romero et al., 2001; Peng et al., 2005), DisEMBL (Linding et al., 2003), IUPred (Dosztanyi et al., 2005), FoldUnfold (Galzitskaya et al., 2006) and PrDOS (Ishida & Kinoshita, 2007).

The main tool used in sections 6 and 7 for the *in silico* prediction of protein disorder from sequences is FoldIndex© (Prilusky et al., 2005). The propensity of N-termini of proteins for disorder was analyzed on the basis of their biased content of order-/disorder- promoting residues (Dunker et al., 2002).

6. The occurrence of coiled-coils and intrinsic disorder in T3SS proteins

Analyses of T3SS protein sequences (Table 1) reveal an unusually frequent occurrence of predicted heptad repeats, which is indicative of a high propensity for coiled-coil formation (Delahay and Frankel, 2002; Pallen et al., 1997; Gazi et al., 2009; Knodler et al., 2011). Structural studies have confirmed the unusual prevalence of coiled-coils among T3SS proteins (Gazi et al., 2009; Ibuki et al., 2009; Lorenzini et al., 2010). In addition, coiled-coil interactions occur frequently in crystal structures of T3SS protein complexes, e.g. in the macromolecular assembly TyeA-YopN that regulates type III secretion in *Yersinia pestis* (Schubot et al., 2005) or in the complex of the filament protein EspA from the enteropathogenic *E. coli* T3SS with its chaperone CesaA (Yip et al., 2005a). In a recent report, the interactions of the *Salmonella typhimurium* needle protein PrgI, an α -helical hairpin, with the tip protein SipD which comprises a long, central coiled coil (Rathinavelan et al., 2011) were studied using NMR paramagnetic relaxation enhancement. A specific region on the SipD coiled-coil was identified as the binding site for the α -helix of PrgI. Crystallographic studies of the PrgI-SipD complex have revealed coiled-coil interactions via the formation of an intermolecular 4- α -helical bundle structure (Lunelli et al., 2011). These studies also showed the importance of the structural flexibility of SipD (introduced by a π -bulge structure) in complex formation. Coiled-coil interactions of HrpO and FliJ with their cognate protein targets have been also reported (Gazi et al., 2008).

Predicted coiled-coil domains have been shown by mutagenesis to enhance membrane association of *Salmonella* T3SS effectors (Knodler et al., 2011). T3SS proteins and coiled-coil domains are frequently predicted to be structurally disordered (Table 1, 2). For many T3SS effectors disorder in their N-terminal region, as well as an increased overall flexibility have been also noted (Table 1, Gazi et al., 2009). In the following, these aspects of T3SS proteins will be elaborated with specific examples from various protein families. Structures of T3SS proteins with increased coiled-coil content are shown in Fig. 3

Protein	% heptad repeats	% order-promoting aa among the 50 N-terminal residues	% disorder-promoting aa among the 50 N-terminal residues	% overall disorder
EFFECTORS				
AvrPto1	27 (~70)	32	52	58
HopE1	23	24	44	50
HopH1	24	26	49	61
HopY1	26	30	62	47
PILUS				
HrpA1	43	40	46	26
OTHER SECRETED/PUTATIVE SECRETED				
HrpJ	41	32	54	30
HrpF	60	28	48	57
HrpB	23	26	58	18
CYTOPLASMIC				
HrpO	79	22	58	85
HrpE	51	30	54	17
HrcN	21	36	54	21
HrcQ _B	53	14	54	46

Table 1. Heptad repeats prediction and disorder analysis for selected proteins from the T3SS of *P. syringae* pv. tomato DC3000. Only proteins with coiled-coil content above 20% are given. For the AvrPto1 protein the crystallographically determined coiled-coil content is given in parentheses. The overall disorder was calculated using FOLDINDEX. N-terminal protein disorder calculations used Dunker's et al. (2002) definition of order-/ disorder-promoting residues. HrcQ_B does not include the disordered N-terminal domain.

6.1 Cytoplasmic proteins

Several cytoplasmic T3SS proteins exhibit a significant coiled coil propensity and intrinsic disorder (Table 1, Fig. 3, 4). Evidence from some cytoplasmic proteins (see section 6.1.1) suggests that these properties might be essential elements in the establishment of key protein-protein interaction networks required for T3SS function (Gazi et al., 2008).

6.1.1 The SctO family (HrpO/FliJ/YscO homologs)

The most extensive heptad repeat pattern occurs in the HrpO/FliJ/YscO family of T3SS proteins (Gazi et al., 2008). Despite the absence of significant homologies, the family members share specific characteristics, e.g. increased propensity for coiled coil formation and intrinsic disorder (Gazi et al., 2008). The extreme flexible nature of HrpO^{SctO} from

Pseudomonas syringae pv. phaseolicola (Gazi et al., 2008), a property shared with FliJ from *S. typhimurium*, has prevented its crystallization and determination of its 3D-structure by X-ray crystallography. A variant form of the FliJ protein from *S. enterica* sv. typhimurium was crystallized however (Ibuki et al., 2009), and its structure was found to be remarkably similar (Fig. 5) to that of the two-stranded α -helical coiled-coil part of the γ subunit of F₀F₁-ATP synthase (Ibuki et al., 2011). A similar coiled coil structure (Fig. 5) consisting of two long α -helices was also reported for the crystal structure of the CT670^{SctO} protein (a YscO homolog) from *Chlamydia trachomatis* (Lorenzini et al., 2010).

Protein	% total disorder	Protein	% total disorder	Protein	% total disorder
NEEDLE		CHAPERONES		TIP	
MxiH	52	SycD	12	LcrV	45
PrgI	21	PscE	0	IpaD	41
BsaL	30	PscG	7	BipD	26
		YscE	27	EspA	6
		YscG	39		
		CesA	66		

Table 2. Disorder analysis for T3SS proteins of known 3D-structures with coiled-coil content exceeding 30%. The overall protein disorder was calculated from sequence data using the FoldIndex program with a window of 21 residues.

Small angle X-ray scattering (SAXS) and circular dichroism (CD) characterization of HrpO^{SctO} from *P. syringae* pv. phaseolicola revealed a high α -helical content with coiled-coil characteristics and molten globule-like properties (Gazi et al., 2008). HrpO^{SctO} like its flagellar counterpart FliJ is essential for export, but its function remains obscure. HrpO^{SctO} interacts, probably via intermolecular coiled-coil formation, with HrpE, a highly α -helical T3SS protein which belongs to the HrpE/FliH/YscL family. FliH, the flagellar counterpart of HrpE is a regulator of the FliI ATPase (Lane et al., 2006). Evidence from HrpO^{SctO} and its analogs in various flagellar or non-flagellar T3S systems suggests that the extreme flexibility (Fig. 4) and propensity for coiled-coil interactions observed in members of the HrpO/FliJ/YscO family might be important factors for increased interactivity and the establishment of functional protein-protein interaction networks in T3SS. This is consistent with the observation that several members of the HrpO/FliJ/YscO family were found to interact with other cytosolic T3SS components or self-associate via coiled-coil interactions: The flagellar FliJ protein, a key player in a chaperone escort mechanism that recruits unloaded chaperones for the minor filament-class subunits of the filament cap and hook-filament junction substructures (Evans et al., 2006) binds to the same chaperone site as the cognate export substrate of the chaperone, albeit with a much lower affinity. Similarly, YscO^{SctO} from *Yersinia enterocolitica* and InvI^{SctO} from *Salmonella typhimurium* do not bind to export substrates but recognize a subset of export chaperones that are specialized to deliver the T3SS translocators to the export apparatus (Evans & Hughes, 2009). In all of these cases the interaction partners of the HrpO/FliJ/YscO family members exhibit a very high α -helical/coiled-coil content (Fig. 5). FliJ was also found to interact with structural cytoplasmic components of the T3SS like the FliM^{SctQ} protein, even in the absence of FliH, suggesting a docking mechanism for export substrates, chaperones and the ATPase to the T3SS machinery (Gonzalez-Pedrajo et al., 2006). The CT670^{SctO} protein exists in monomeric and

dimeric forms, with the monomeric form dominating at low protein concentrations. For self-association and dimer formation the involvement of coiled-coil interactions is predicted (Lorenzini et al., 2010). CT670^{SctO} interacts with CT671^{SctP}, a T3SS protein, with a predicted coiled-coil domain in its C-terminal region. CT671^{SctP} is a homolog of the YscP protein which has been characterized as a molecular ruler and as a switch for T3SS substrate specificity in *Yersinia* species (Agrain et al., 2005). The two coiled-coil containing proteins CT670^{SctO} and CT671^{SctP} have been suggested to form a chaperone-effector-like pair with CT670^{SctO} acting as chaperone (Lorenzini et al., 2010).

The HrpO/FliJ/YscO family members are encoded by genes located always downstream of the gene coding for T3SS ATPases (the SctN family of T3SS proteins which includes HrcN/FliI/YscN homologs); this implies a close connection between these proteins and the ATPase. In flagellar T3SS the FliI protein is an ATPase that has extensive structural similarity to the α - and β - subunits of the F_oF₁-ATP synthase (Imada et al., 2007), while also the structure of FliJ from *S. enterica* sv. typhimurium (Fig. 4, 5) is remarkably similar to that of the two-stranded α -helical coiled-coil part of the γ -subunit of F_oF₁-ATP synthase (Ibuki et al., 2001). FliJ promotes the formation of FliI hexamer rings by binding to the center of the ring in a similar way to the γ -subunit penetrating into the central channel of the $\alpha_3\beta_3$ ring in F_oF₁-ATPase. Moreover, the HrpE/FliH/YscL family of proteins (interaction partners of the HrpO/FliJ/YscO family) are distant homologs to both β - and δ - subunits of the F_oF₁-ATP synthase (Pallen et al., 2006). In flagellar systems the docking of the ATPase to the T3S machinery is mediated by the FliJ/FliH pair (Minamino et al., 2009). These results strongly suggest that T3SS and F- and V-type ATPases share a similar mechanism and an evolutionary relationship. It is thereby striking that extensive coiled-coil domains (e.g. FliJ, FliH) have been conserved between the two systems.

Overall, the above remarkable findings support our earlier suggestions (Gazi et al., 2008, 2009) that T3SS proteins, and in particular members of the SctO family, with long disordered/flexible coiled coil structures occupy node positions in the T3SS interactome, being capable of interacting with different partners and possess various roles in the secretion mechanism. These roles are to a large extent poorly understood and remain to be elucidated experimentally.

6.1.2 The SctL family (HrpE/FliH/YscL homologs)

In terms of predicted heptad repeats content (Gazi et al., 2008) the HrpE/FliH/YscL family of proteins comes second after the HrpO/FliJ/YscO family. These proteins are distant homologs of the second-stalk components of the F_o-F₁ ATPases (Pallen et al., 2006). FliH is a regulator of the FliI ATPase (Evans et al., 2006) and was found to interact with the 18 N-terminal residues of FliI that are predicted to form an amphipathic α -helix upon interaction with FliH (Lane et al., 2006).

The HrpE/FliH/YscL family members possess glycine-rich repeats of the form AxxxG(xxxG)_mxxxA with m representing a non constant value between FliH proteins from different bacteria and x standing for any residue. The amino acid sequence distribution of each of the three x positions was found to differ significantly from the overall amino acid composition of the HrpE/FliH/YscL proteins. The high frequency of Glu, Gln, Lys and Ala residues in the repeat positions suggests the presence of α -helical

structure for this motif (Trost et al., 2009). When the Protein Data Bank was searched for GxxxG repeats similar in length to those found in FliH, no helices containing more than three contiguous glycine repeat segments were found implying that long GxxxG repeats are presumably quite rare in nature.

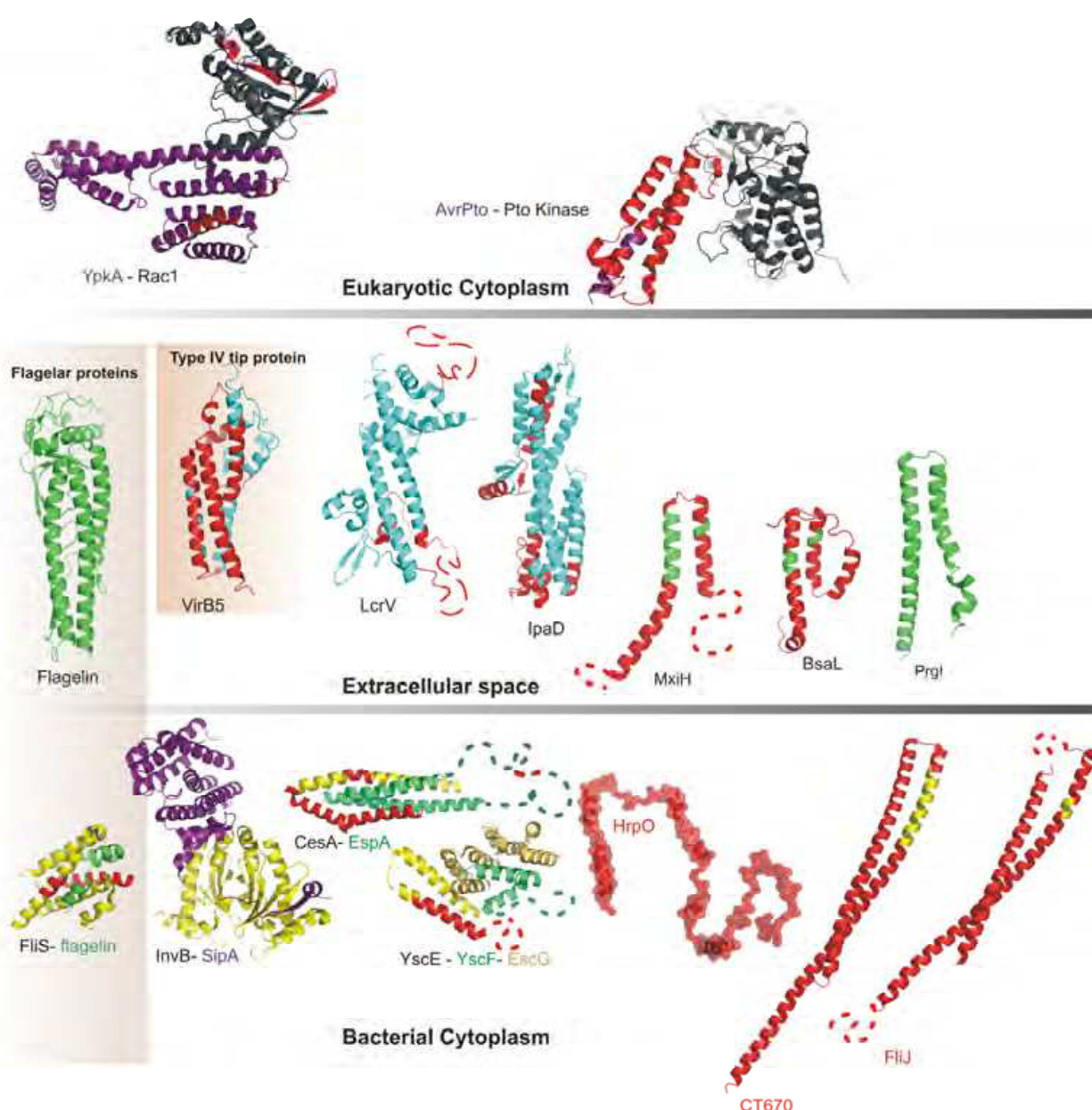


Fig. 3. 3D-structures of T3SS proteins with significant coiled-coil content and their locations. The structure of the intrinsically disordered HrpO is based on SAXS data (Gazi et al., 2008).

6.2 T3SS needle and pilus proteins

The major extracellular T3SS component is the needle with a length of 60 nm and an external diameter of 7 nm for animal pathogens; a much longer structure (up to 2 μ m) named the Hrp pilus is the needle counterpart in phytopathogenic bacteria (Barrett et al., 2008; Cordes et al., 2003; He and Jin, 2003; Alfano & Collmer, 1997; Roine, 1997). The needle

appears to play a major role in host sensing and signal transmission from the distal to the basal end of T3SS (Deane et al., 2006). The needle structures are formed through the helical assembly of multiple copies of a small α -helical protein. Along the needle axis runs a narrow (2.5 nm) conduit which is used for the passage of needle components, tip proteins, translocators and effectors, whereby a partially unfolded form of the substrate is required. Structures are available (Wang et al., 2007; Deane et al., 2006; Zhang et al., 2006) for three needle components from animal pathogens: MxiH (*S. flexneri*), BsaL (*B. pseudomallei*) and PrgI (*S. typhimurium*). These structures are highly α -helical, with a central coiled-coil which is essential for needle assembly. Outside this coiled coil, all three proteins have highly mobile N-termini and C-termini, although these regions may retain some degree of helical structure in solution (Blocker et al., 2008). The sequences of the coiled-coil parts of the needle proteins show strong similarities, suggesting that they all share a common fold and pattern of interactions (Wang et al., 2007; Zhang et al., 2006). Analysis of structures and sequences of needle components from various pathogens suggests that the majority has a propensity for structural disorder (Table 2). An analysis of needle/pilus components predicts a mean overall disorder of approx. 30%. The predicted disorder has been confirmed by CD and thermal unfolding studies of MxiH, BsaL and PrgI which reveal that under conditions resembling the physiological ones, all three C-terminally truncated proteins adopt a molten globule-like state; at temperatures above 37° C their tertiary structure collapses while the secondary structure is largely retained (Barrett et al., 2008); this behaviour is strongly reminiscent to the one observed for the cytoplasmic HrpO^{SctO} protein (Gazi et al., 2008). A partially unfolded state of the needle components could be functionally important e.g. for transversing the needle channel and for the extracellular assembly. Signal transmission for host cell sensing is suspected to utilize the flexibility of needle subunits (Deane et al., 2006).

The major subunits of the Hrp pilus (HrpA) are generally predicted to be almost entirely α -helical, with the exception of the *Pseudomonas syringae* species, for which the 50 N-terminal amino acids are predicted to contain β -strands (He and Jin, 2003; Koebnik, 2001). The major subunits of the Hrp-dependent pili, like the needle structural proteins, are all small proteins (of 6 to 11 kDa), but their sequences are surprisingly hypervariable, even within *P. syringae* pathovars. This hypervariability may reflect the evolutionary adaptations to evade plant defense systems. The predicted secondary structures of the major pilus subunits however, are remarkably similar, almost entirely α -helical. Insights into the structure of Hrp pilus components have been obtained from the recent investigation of the HrpA protein from the *P. syringae* pv. phaseolicola T3SS pilus (Kotzabasaki & Kokkinidis, unpublished). The C-terminal part of the 11 kDa protein is responsible for the assembly of multiple HrpA copies in the pilus (Roine, 1997). No chaperons for HrpA have been identified. The secondary structure of HrpA is predicted to be highly α -helical, with a propensity for coiled-coil formation in its functionally important C-terminal region. Surprisingly, experimental characterization of the HrpA protein using CD, Raman, FTIR and SAXS provides strong evidence that HrpA does not adopt a helical structure, but rather a highly disordered state with β -strand features. High resolution transmission electron microscopy (TEM) of purified HrpA samples reveals a pronounced propensity for polymerization and formation of two types of fibrils with nano-to micro scale features, one of which has comparable geometrical parameters with the Hrp pilus.

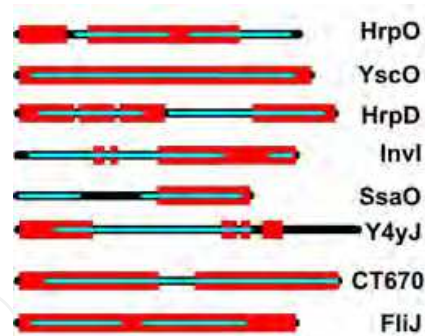


Fig. 4. Predicted coiled-coil regions and structural disorder for the HrpO/FliJ/YscO family. Disordered segments (predicted by FoldIndex) are colored red, coiled-coil domains cyan. All sequences were predicted to be almost entirely α -helical, which was confirmed for HrpO (Gazi et al., 2008), FliJ (Ibuki et al., 2011) and CT670 (Lorenzini et al., 2010). Sequences are drawn to scale, and vary from 166 (HrpD) to 125 residues (SsaO).

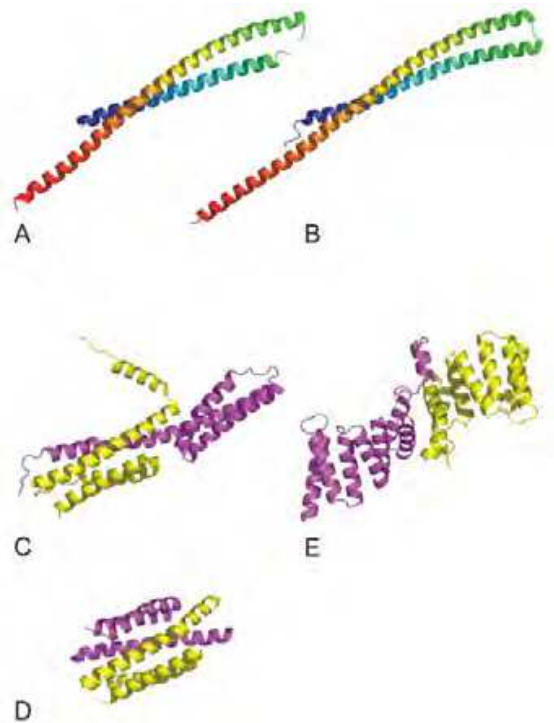


Fig. 5. T3SS proteins involved in coiled-coil interactions: (A) FliJ (Ibuki et al., 2011) from *S. typhimurium*, (B) CT670^{SctO} (Lorenzini et al., 2010) from *Ch. trachomatis* colored from N- (blue) to C-terminus (red). The dimeric FliT of *S. enterica* sv. *typhimurium* (C) and *Bordetella bronchiseptica* (D), each monomer differently coloured. In (C) the C-terminal helix adopts a different conformation in each monomer (Imada et al., 2010). (E) The *Y. enterocolitica* SycD (Buttner et al., 2008) which is recognized by YscO^{SctO} (Evans & Hughes, 2009).

6.3 T3SS effector flexibility and coiled-coil propensity

Approximately a third of the T3SS effector structures known forms regular coiled-coils with knob-into-holes packing, while several others exhibit short heptad repeat patterns in their sequences which give rise to coiled-coil interactions and short, distorted α -helical

bundles: A YpkA subdomain folds in two 3-helical bundles (Prehna et al., 2006). Coiled-coils are also observed in the N-terminal domains of YopH and SptP (Khandelwal et al., 2002; Stebbins and Galan, 2000) and in MxiC and YopN-TyeA (Deane et al., 2008; Schubot et al., 2005). The *S. flexneri* effector IpaH is a E3 ubiquitin ligase with a C-terminal which consists entirely of α -helical bundles and carries the catalytic activity for ubiquitin transfer (Singer et al., 2008). Helical bundle structures are also adopted by AvrPto (Wulf et al., 2004) and AvrPtoB (Dong et al., 2009), although both interact with their target protein kinase Pto via β -strand addition (Xing et al., 2007). AvrPto (PDB ids: 1R5E, 2QKW) displays considerable structural plasticity which is consistent with its predicted increased flexibility (Table 1). In a recent analysis, 49% of the *S. typhimurium* effectors have been predicted to possess at least one coiled-coil domain which enhances membrane association in mammalian cells (Knodler et al., 2011).

Apart from the frequent occurrence of coiled-coils, a further common feature among T3SS effectors are disorder effects established both as localized disorder of their extreme N-terminal peptide where the secretion signal resides, or frequently as an overall structural disorder. Usually, the 15-20 N-terminal residues of effectors are highly disordered. Truncation of N- and C-terminal residues was necessary for the NMR study of the AvrPto effector (Wulf et al., 2004), while in the crystal structure of the AvrPto-Pto kinase complex 28 N-terminal residues are missing (Xing et al., 2007). In the case of ExsE the N-terminal fifteen residues had to be omitted for crystallization and structure determination (Vogelaar et al., 2010). Although, the full length AvrB and AvrPphF ORF2 were crystallized, electron density was not observed for the 27 N-terminal residues due to disorder (Lee et al., 2004; Singer et al., 2004). The N-terminal region of the SipA effector in the complex with the chaperone InvB is highly disordered (Lilic et al., 2006). In the CBD of effectors bound to class IA chaperones, there is a prevalent localized disorder for the part of the effector that crosses the interface of the dimeric chaperone; thus, this part of the effector cannot be modelled, as can be seen not only for SipA but also for ExsE and YopN (Vogelaar et al., 2010). Moreover, a large majority (~75%) of *P. syringae* pv. tomato effectors show a significant propensity for structural disorder in the region of their 50 N-terminal residues based on the ratio of order- vs. disorder-promoting residues (selected effectors are shown in Table 1) which has an average value of 0.45 (M. Kokkinidis, unpublished). Other secreted proteins have an average value of 0.50. The ratio for cytoplasmic T3SS proteins is 0.55; the average ratio in proteomes is 0.58 based on the amino acid frequencies of order- and disorder-promoting residues (Brooks et al., 2002); significantly lower values, as in the case of the N-termini of T3SS effectors, indicate a propensity for disorder. The only structured N-terminal region of a T3SS effector is that of YopH. The 129 residue N-terminal domain has two functions: the first 70 residues contain the CBD domain for chaperone SycH, while the full 129 N-terminal domain binds to phosphotyrosine-containing proteins and adopts an overall globular fold (Khandelwal et al., 2002). The N-terminal region of VirA is partially disordered (Davis et al., 2008). Apart from the N-terminal disorder, a propensity for overall disorder is predicted for T3SS effectors, which may reflect an increased structural flexibility. An average value of 35% of disordered residues is predicted by FOLDINDEX for the T3SS effectors of *P. syringae* pv. tomato DC3000, which is to be contrasted with an average value of 28% for cytoplasmic T3SS proteins, if the extensively disordered members of the HrpO/FliJ/YscO family (some of which could be classified as IDPs) are excluded. For other secreted/putatively secreted T3SS proteins the values are 30-37%.

7. Comparison with other secretory systems: T2SS, T4SS and T6SS

To compare with T3SS, earlier analyses for T2SS and T4SS were updated and an analysis of T6SS protein sequences was performed. Coiled-coil predictions and disorder analysis were carried out for *Helicobacter pylori* (T4SS), *Legionella pneumophila* (T2SS and T4SS), the Type-4-pili (T4P) of *L. pneumophila* and *Pseudomonas aeruginosa* and *P. aeruginosa* strain PA14 (T6SS). For 2878 proteins encoded in the *L. pneumophila* genome (GenBank accession number NC_006369), the predicted coiled-coil content is 4%.

Using the Virulence Factor Database (Yang et al., 2008) for the classification of *L. pneumophila* proteins, a coiled-coil content of 14% is predicted for T2SS (11 proteins), 19% for T4SS (50 proteins), and 13% for T4P (3 proteins). For 1573 proteins of *H. pylori* (NC_000915) a coiled-coil content of 3% is predicted, with 26% for T4SS (24 proteins). For 5571 proteins of *P. aeruginosa* (NC_002516) the predicted content is 4%, 8% for T2SS (11 proteins) and 10% for T4P (32 proteins). A high coiled-coil content is predicted for the T4SS of *H. pylori* and the T4SS effectors of *L. pneumophila*. The latter (Table 3) exhibit a particularly high propensity for structural disorder (on the average 46% disordered regions) and coiled-coil content (30%), thus strongly resembling T3SS effectors. The analysis of T6SS protein sequences retrieved from the KEGG database is in Table 4. Hcp and Vgr proteins are proposed effectors (Mougous, et al., 2006), although they may also act as T6SS machine components (Zheng & Leung, 2007). Non-secreted components include ClpV an AAA+ Clp-like ATPase (Cascales, 2008) and various other core components. The analysis suggests a low coiled-coil content for most secreted proteins (on the average 6%) and a higher one (12% on the average) for core proteins. Interestingly, the mean overall disorder of secreted T6SS components is very high (41%) and comparable to T3SS effectors or to T4SS effectors of *L. pneumophila*. Core components of T6SS display a significantly lower degree disorder (28%).

T4SS effectors	% heptad repeats	% overall disorder	T4SS effectors	% heptad repeats	% overall disorder
Ceg9	0	56	SidJ	5	35
VipA	29	36	LaiA/SdeA	28	42
AnkX/AnkN/LegA8	15	35	YlfA/LegC7	60	40
LidA	47	68	DrrA/SidM	42	47
Ceg19	33	30	LepB	49	50
LegC3	62	60	SidC	36	62
LegC2	67	61	SidF	28	56
RalF	0	43	LepA	7	16
SetA	26	42	LubX/LegU2	10	46
AnkB/Cag27/LegAU13	20	51	VipD	42	35

Table 3. Predictions (using MATCHER) of the heptad repeats content and disorder analysis (using FOLDINDEX) for effectors from the T4SS of *L. pneumophila* strain Philadelphia 1.

Locus No (Protein)	% heptad repeats	% overall disorder	Locus No (Protein)	% heptad repeats	% overall disorder
SECRETED COMPONENTS			CORE COMPONENTS		
PA14_01030 (Hcp)	14	37	PA14_00875 (PpkA)	7	25
PA14_01110 (VgrG)	6	42	PA14_00890 (PppA)	0	14
PA14_01160 (VgrG)	6	59	PA14_00910 (ImpL)	18	22
PA14_33960 (VgrG)	0	34	PA14_00925 (ImpK)	10	29
PA14_34030 (Hcp)	0	20	PA14_00940 (ImpJ)	0	20
PA14_03220 (VgrG)	4	38	PA14_00960 (VasD)	0	35
PA14_03240 (Hcp)	0	52	PA14_00970 <i>unknown</i>	45	29
PA14_18985 (VgrG)	0	45	PA14_00980 (HFA)	0	30
PA14_21450 (VgrG)	0	41	PA14_00990 (ImpA)	12	20
PA14_29390 (VgrG)	8	45	PA14_01060 (ImpF)	14	31
PA14_44900 (VgrG)	5	42	PA14_01070 (ImpG)	5	28
PA14_67230 (VgrG)	0	46	PA14_01100 (ClpV)	18	21
PA14_69550 (VgrG)	0	40	PA14_34050 (ImpC)	8	19
PA14_29190 (tse2)	44	27	PA14_34070 (ImpB)	22	49

Table 4. T6SS proteins (T6S system HSI-I, HSI-III) of the *P. aeruginosa* strain PA14.

8. Conclusions

In conclusion, structural studies and *in silico* analyses of bacterial genomes have confirmed the occurrence of coiled-coil domains and protein flexibility in the T3SS and provide a more consolidated insight into the occurrence of such features in other secretory systems, e.g. T4SS and T6SS. In the case of T3SS the occurrence of coiled-coils is considerably higher than the average predicted occurrence in prokaryotic proteomes (Schubot et al., 2005). Coiled-coils occur in all types of T3SS proteins, including in proteins from the T3SSs of plant pathogens, for which in earlier studies no coiled-coils could be predicted (Delahay and Frankel, 2002). Apart from coiled-coils, a further widespread feature in T3SS proteins is a considerable structural flexibility which may range from localized to extensive disorder effects. At the level of experimental observations, disorder manifests itself as missing stretches of electron density in crystallographically determined structures (e.g. in the case of the N-termini of effectors), or occasionally as establishment of a molten-globule-like state at conditions resembling the physiological ones. Examples for the latter include the IDPs HrpO (Gazi et al., 2008) and HrpA from *P. syringae* pv. phaseolicola or the needle subunits MxiH, BsaL and PrgI (Barrett et al., 2008). The flexibility of T3SS proteins is frequently associated with a plasticity of coiled-coil domains; this becomes evident in the case of multiple structural studies of the same protein, e.g. AvrPto (PDB ids: 2QKW, 1R5E) or in differences between subunits of oligomeric proteins, e.g. in the FliT dimer (PDB id: 3A7M).

The combination of coiled-coiled interactions and structural plasticity are frequently essential prerequisites for the establishment of interaction networks within T3SS, as exemplified by the interactions of proteins of the HrpO/FliJ/YscO family with members of the HrpE/FliH/YscL family (Gazi et al., 2008), the SipD/PrgI (Lunelli et al., 2011; Rathinavelan et al., 2011) or the CT670/CT671 interaction (Lorenzini et al., 2010). In addition, the assembly of the T3SS supramolecular structures frequently requires a combination of coiled-coils and conformational flexibility: T3SS needle assembly occurs through the stepwise polymerization of a major subunit (e.g. MxiH, BsaL and PrgI) via a flexible or partially disordered C-terminal helix which exhibits a propensity for coiled-coil interactions. For the IDP HrpA polymerization into pilus-like fibrils has been observed, although no experimental evidence for the involvement of coiled-coil interactions could be obtained, despite the high α -helical content predicted by sequence analysis.

The propensity for disorder is frequently reflected the amino acid composition of T3SS protein sequences. The vast majority N-terminal sequences of T3SS effectors and other secreted proteins exhibits specific biases (Table 1) in their composition with respect to order- and disorder-promoting residues (Dunker et al., 2002; Uversky, 2010), from which a disorder propensity can be predicted, usually in agreement with experimental observations. Interestingly, these disorder-associated biases (as reflected in the ratio of order- vs. disorder- promoting residues), result in sequence preferences for the N-termini which are similar to those determined for T3SS effectors from various bacterial species (Greenberg and Vinatzer, 2003). The structural disorder of the N-termini may thus play a role as a secretion signal, a suggestion made earlier by Akeda & Galan (2005) and confirmed by subsequent analyses (Gazi et al., 2009). However, as N-terminal structural disorder does not ensure specificity of substrate recognition (e.g. the cytoplasmic HrcQ_B protein is predicted to possess a highly flexible N-terminus), it may be assumed that N-terminal flexibility could be one of multiple secretion signals (Marlovits et al., 2006), with other signals, e.g. chaperones, ensuring specificity. Analysis of effectors and other secreted/non-secreted T3SS components strongly suggests that the overall disorder of T3SS proteins is a further parameter strongly correlated with secretion (Table 1, 2). Flexible or disordered T3SS domains could facilitate rapid unfolding which is necessary for secretion. Both N-terminal and overall flexibility might be thus considered in prediction algorithms for the identification of universal T3SS effectors signatures; this would complement recent efforts based on machine learning approaches (Arnold et al., 2009; Samudrala et al., 2009). Interestingly, sequence stretches with coiled-coils propensities are suitable tertiary motifs to provide the necessary flexibility which is proposed to be associated with secretion. In fact, coiled-coil proteins are frequently viewed as a specific set of intrinsically disordered proteins (Gaspari & Nyitray, 2011) and occasionally they have been observed to display molten globule characteristics (Glykos & Kokkinidis, 2004). A further advantage of coiled-coils might be associated with specific features of their disordered state: As shown in the case of the HrpO protein (Gazi et al., 2008), proteins exhibiting coiled-coil propensity are capable of adopting highly non-globular conformations, while maintaining a considerable α -helical content. The geometrical dimensions of such non-globular helical conformations permit passage through the narrow needle/pilus channel if the appropriate secretion signal is present. It is intuitive to assume that after passing this conduit, such preformed and folding-competent helices encompassing a few turns may form a nucleation site which promotes

fast assembly of a globular coiled-coil domain. Flexible coiled-coil domains are thus particularly suitable as secretion substrates as they can easily unfold into secretion-competent α -helices, which in turn may refold in the host cell into a native structure following a relatively fast pathway, and thus avoid degradation of the unfolded polypeptide by host defences. In addition, coiled-coils of effectors may also be a particularly suitable structural motif for interactions in the host cell, as many key processes in the eukaryotic cell involve coiled-coil domains, a fact already noted by Pallen (1997), and confirmed by recent experiments (Knodler et al., 2011). It might be thus hypothesized that the selective evolutionary pressure for optimization of bacterial effectors favours coiled-coil domains and increased flexibility, and this in turn creates a basis for the overall prevalence of coiled-coil domains in T3SS, as this helps establish interaction networks within the T3SS, which may be exploited by even partially unfolded effectors or other secretion substrates.

The predicted high occurrence of coiled-coil domains and structural disorder in T4SS effectors of *L. pneumophila* (Table 3) indicate that the concepts outlined above for T3SS effectors might also have some validity in other Gram-negative secretory mechanisms. In addition, the analysis of T6SS secreted components (Table 4) strongly supports the concept of structural flexibility of proteins being an important prerequisite for bacterial secretion. We still have a long way to go to decipher the full complexity of bacterial secretion, even for extensively studied systems such as T3SS. However, the elegant genetic, biochemical, genetic and computational studies which were reviewed in this contribution may open ways to resolve this issue.

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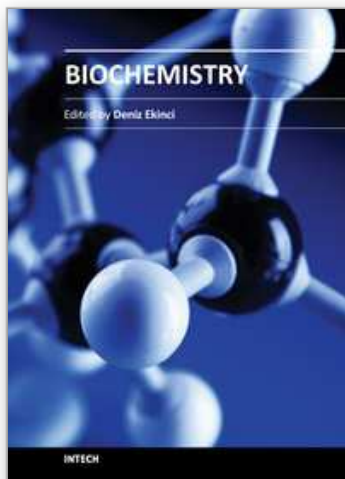
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Over the recent years, biochemistry has become responsible for explaining living processes such that many scientists in the life sciences from agronomy to medicine are engaged in biochemical research. This book contains an overview focusing on the research area of proteins, enzymes, cellular mechanisms and chemical compounds used in relevant approaches. The book deals with basic issues and some of the recent developments in biochemistry. Particular emphasis is devoted to both theoretical and experimental aspect of modern biochemistry. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in biochemistry, molecular biology and associated areas. The book is written by international scientists with expertise in protein biochemistry, enzymology, molecular biology and genetics many of which are active in biochemical and biomedical research. We hope that the book will enhance the knowledge of scientists in the complexities of some biochemical approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of biochemistry.

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