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Insights into the Bacterial Type III Secretion System

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ABSTRACT

Bacterial type III secretions system (T3SS) is a membrane embedded needle like macromolecular complex structure present in Gram negative bacteria and mostly found in *Yersinia*, *Salmonella*, *Shigella*, Entero Pathogenic *E. coli* (EPEC), Entero Haemorrhagic *E. coli* (EHEC) and *Pseudomonas aeruginosa*. The infection dynamic involves several sub cellular components for easy delivery of the effector molecules from bacteria to the host cells to survive the host immune mechanism. Mainly three categories of proteins are involved- structural, translocator and effector proteins. T3SSs can be classified into seven phylogenetic families, based on the genetic analysis of their components. Multiple T3SSs are found in the same bacteria with the purpose of causing infection in multiple steps.Their contribution to virulence mechanism is mainly through modification of the host cytoskeleton system or interfering with the signaling Pathways in the host cellular events related to the defensive mechanism. The infection requires multi-step regulatory strategies which include spatiotemporal regulation of a different set of effector proteins encoded genes. Despite their contribution in virulence mechanism, they can be utilized by re-engineering them to deliver either various therapeutic protein agents or could be used as an alternative novel approach for antigen delivery into the host. Apart from its use as the delivery platform they can be targeted using broad spectrum inhibitors against diverse sets of T3SS mediated diseases. Therefore, this review summarizes the basic structure, its regulatory mechanism in different bacteria and the future perspective.

KEYWORDS: Antigen delivery platform, effector molecules, gene regulation, type III secretion system

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1. INTRODUCTION

Type III secretions system (T3SS) is a complex macromolecular mechanism present in different pathogenic as well as commensal Gram negative bacteria. They are present over the surface of these bacteria as appendages which are fineneedle like structures hence called as"injectisome". They are mostly found in *Yersinia*, *Salmonella*, *Shigella*, Entero Pathogenic *E. coli* (EPEC), Entero Haemorrhagic E. coli (EHEC) and *Pseudomonas aeruginosa*. Recently they have been also found in *Bordetella*, *Bulkholderia* and *Chlamydia*. Involvement of several categories of proteins occurred to carry out their respective functions such as- structural proteins are involved to form the apparatus, once the apparatus is formed then further translocators and effector molecules are secreted (Mota et al., 2005; Kendall and Melissa, 2017; Kudryashev et al., 2016). This T3SS is highly conserved in such gram negative Bacteria (Feng et al., 2019). The translocators form the pore complex and effectors molecules contribute tothe virulence mechanism (Bulmer et al., 2012; Du et al., 2016). Once the secretion is activated few new injectosomes are built up next to the existing complex (Kudryashev et al., 2015). Protein domains present in the basal body contribute to the flexibility of the length of the needle. Strong contraction of the basal body is noticed in case of *Chlamydia* upon contact with host cell. This strong contraction is a result of structural stabilization In the cytoplasmic part of the injectisome (Nans et al., 2015). Pilus encoding protein (HrpA) present in plant pathogens is an adaptation to the plant cells which are thick to penetrate. It shares several traits with the needle forming proteins present in the animal pathogens. The structure of the outer membrane decides the length of the needle length,for example: the needle length of *Chlamydia* is half the length of *Salmonella* (Nans et al., 2015).

T3SSs can be classified into seven phylogenetic families, based on the genetic analysis of their components. The first family is represented by the secretion apparatus of *Yersinia* spp. (pathogenic species) and *Pseudomonas aeruginosa* (Horna et al., 2021), the second family is represented by *Shigella flexneri* and *Salmonella typhimurium* Pathogenicity Island 1 (SPI-1), whereas the third family includes the T3SSs from EPEC, EHEC and the *S. typhimurium* Pathogenicity Island 2 (SPI-2). T3SS was first observed in *Yersinia* spp. by Rosqvist and his colleagues. Since then, several studies have led to a comprehensive understanding of the assembly and function of the T3SS injectisomes, including the role of the effector proteins.

The number of effector proteins delivered through this complex helps the bacteria in their invasion into the host cell and escape the host immune system. Blockage of these effector proteins just before contact is regulated by many other regulatory proteins such as gatekeeper proteins which sometimes even exist as two separate polypeptide chains that can determine substrate hierarchy (Pallen et al., 2005, Lai et al., 2013). The architecture of this complex is conserved in both structural as well as a functional level (Puhar and Sansonetti, 2014; Katsowich et al., 2017). Recently few studies demonstrated that the efector molecules interact with the outside of the needle complex to gain access to the host cell cytoplasm. Although secretion of auto transporter proteisns is mediated mainly *via* type five secretion system but few autotransporters require type III secretion complex for internalization in the host cell, eg.: EspC protein (serine protease autotransporters) present in *Enterobacteriaceae* family (Tejeda et al., 2017). The non flagellar type III secretion complexes are expressed on the bacterial surface hence they are mostly targeted by the immune system present in the host. Therefore these targets are utilized either as targets for the vaccines or discovering new antibacterial drugs (Abby et al., 2012; Hussain et al., 2021; Moir et al., 2021). Phylogenetic analysis came up with evidence that bacterial Type III secretion system was originated to favour the interaction with early unicellular eukaryotes. Reportedly the effector proteins which are plant associated can be secreted via animal associated type III secretion complex.

2. STRUCTURE

The structure is comprised of a cytoplasmic bulb, a basal body spanning both the inner and outer membrane of the bacteria and one long extracellular Needle (Deane et al., 2008). Outer rings of 20 nm in diameter comprise the base structure and 18 nm in thickness that helps in anchoring the structure to the outer membrane as well as the peptidoglycan layer. The inner rings of 40 nm in diameter usually anchor the base to the cytoplasmic membrane (Galan, 1999). The needle complex is about 3.5 MDa in size and a total of 30 different proteins are involved inits assembly. In the case of plants, the bacteria interacting with the plant cells have pilus which are few micrometres in length and translocate a protein called hairpin to modify the plant cell wall for its easy Penetration (Puhar and Sansonetti, 2014). In the case of bacteria infecting animals cells, tip proteins are present at the needle tip to block the secretion until it comes in contact with the host cell. However, a common secretion mechanism exists for the secretion of the flagellar component as well as proteins implicated in the secretion of virulence factors (Galan and Collmer, 1999).

3. FEATURES OF T3SS

Three important features of T3SS are a) absence of cleavable signal peptide on the secreted proteins, b)need of chaperons,c) requirement of contact by the host cell for fully functioning of the needle complex. Needle length is

maintained by the ruler proteins.The first supramolecular structure was isolated from *Salmonella typhimurium* and visualized under an electron microscope revealed that the hollow needle is almost 120 nm in length, 8 nm width and with two domains, composed of atleast 3 proteins InvG (a member of the secretin family) and two lipoproteins, PrgH and PrgK (Galan and Collmer, 1999) which bear limited or no sequence similarity with flagellar basal body. Conserved proteins such as InvA and InvC are required for the full assembly of the needle complex. InvA has sequence similarity to the flagellar FlhA protein and InvC is an ATPase with amino acid sequence similarity to the flagellar FliI protein and other members of the F0F1-related family of ATPases. Mutation in either result in defective needle complex formation without affecting the assembly of the base (Galan et al., 1992; Eichelberg et al., 1994). All the identified base structures have sec dependent signal sequence. PrgI acts as the main subunit for needle structure formation in *Salmonella* and while many proteins exhibit sequence similarity with PrgI are also found in *Yersinia* spp., S*higella*, *E. coli* and *P. aeruginosa*. The presence of InvJ proteins with several glutamines at its carboxyl terminus determines the length of the needle by controlling the switch from one effector molecule to another (Kubori et al., 2000). InvJ exhibits little similarity to any type III secretion-associated protein from other bacteria except for Spa32 protein of *Yersinia*, however, the similarity of InvJ with Spa32 is consistent with the similarity of several *S. typhimurium* and *Shigella* spp. secreted proteins (Kaniga et al., 1995; Kaniga et al., 1995a).

In some bacteria, apart from needle complex, some other extracellular supramolecular structures are found that are secreted via T3SS required for further pathogenesis. For example, EPEC and *S. typhimurium* have few filamentous appendages on their surface; *Pseudomonas syringae* produces a thinner structure (6 to 8 nm in diameter) known as pilus to penetrate the plant cell wall. The chaperons involved are of relatively small size (15 to 18 kDa) with predominantly alpha helix secondary structure and low isoelectric point (Ginocchio et al., 1994; Roine et al., 1997). Translocon pores are heterooligomers made of two bacterial proteins induced after coming in contact with the host cell.The *Shigella* protein IpaC, pore protein that interact with the host cell intermediate filament for docking or attachment of the needle onto the pore on the membrane (Russo et al., 2019) which in turn leads to docking dependent type III secretion. Discrete mutations in these translocation proteins do not usually hamper the pore formation efficiency, rather the docking or translocation efficiency of those effector molecules are restricted (Adams et al., 2015). Even few T3SSs carrying bacteria can induce hemolysis in red blood cells, where the hemolytic activity is due to the assembly of the T3SS translocon in a host cell membrane (Gentschev et al., 2002).

Multiple T3SSs are found in the same bacteria with the purpose of causing infection in multiple steps. For example- Salmonella Pathogenicity Islands-1(SPI-1) and Salmonella Pathogenicity Islands-1 (SPI-2) code for two different T3SSs, inv/spa and spi/ssa, respectively in *Salmonella enteritica* depending on common post translational isomerise (Hensel et al., 1995); *Yersinia spp.* contains plasmid encoded Ysc-Yop system as well as a chromosomally encoded T3SS (Ysa, SPI-2 like) with the presence of overlapping function (Young and Young, 2002); *Burkholderia pseudomallei* and related species can harbor up to three T3SSs: two systems, TTS1 and TTS2, are homologous to T3SSs of plant pathogens and a third system (TTS3 or bsa) is similar to SPI-1 of *S. enteric*a (Stevens et al., 2002). While Chlamydiales are the only non-Proteobacteria to harbor a T3SS but in the case of Chlamydiaceae instead of having two separate T3SSs, the translocators (CopB and CopD) and their specific chaperone are encoded twice by the genome, where CopB1 and CopD1 are required for translocation of effectors mediating internalization of the pathogen while CopB2 and CopD2 are built into the inclusion membrane, to allow transport of effectors to maintain intracellular Growth (Ouellette et al., 2005).

4. ASSEMBLY OF ITS STRUCTURE

The basal body assembly requires different proteins that involve sec dependent pathway and following assembly of the basal body the inner rod and the needle assemble which is sec independent. The C ring is composed of few cytoplasmic proteins which are associated with either AAA+ATPase or F1-ATPase *i.e* required for dissociation of chaperone–effector complexes and subsequent unfolding of the effector proteins. Apart from that, it supplies energy to transport the effector molecules via the needle however the exact mechanism is yet to be known (Akeda and Galan, 2005; Galan and Wolf-Watz, 2006; Pozidiz et al., 2003). The components of the C ring and ATPase are first assembled into the Sorting Platform(SP). This platform recruits further the components for needle complex, translocon and effector proteins in a sequence dependent manner. Such as Spa33, MxiK and MxiN C ring components form complexes with the ATPase Spa47 in *S. flexneri* and these protein complexes are said to be conserved according to the spp. (Johnson and Blocker, 2008; Jouihri et al., 2003). SP proteins required for needle formation have auto proteolytic activity in their cytoplasmic domain and this auto cleavage occurs immediately after translation but the cleavage does not trigger for needle component secretion or switching the secreted components. Needle length is regulated by Spa32-like proteins (Journet et al., 2003; Wagner et al., 2010). Few

strains of *S. flexneri*, *S. typhimurium* (Kubori et al., 2000) and *Yersinia* (Tamano et al., 2002) lacks these proteins so they are seen to have long T3SS needles. These proteins act as molecular rulersby inserting the N terminal in the base of the needle and C terminal in the tip and after attending an extended conformation further polymerization of the needle subunits is stopped. Once the Spa-40 like proteins are attached to the sorting platform the ATPase rod is loaded along with the needle components. After completion of the needle assembly, Spa-32 like protein is activated and interact with Spa-40 like proteins to stop the secretion of needle components (Barison et al., 2013). Different translocators like IpaD, LcrV and SipD are positioned at the distal end of the needle respectively in *S.* flexneri, *Yersinia* spp. and *S. typhimurium* SPI-1 (Mueller et al., 2005; Lara-Tejero and Galan, 2009; Olive et al., 2007). However, Cleavage of Spa40-like protein is necessary for efficient transfer of tip proteins in the case of *Yersinia* spp. and *S.* flexneri. In some bacteria, this tip complex is displayed on the tip of the needle before contact with the host cell and in some after contact. In some studies, it has been reported that interaction of the cytoplasmic domain of phosphorylated SP component Spa33 and forkhead-associated (FHA) phosphothreonine-binding domain of proximal ring is there for effective delivery of effectorand translocator proteins (Moriya et al., 2006). The switch from translocators to early effectors is induced by the secretion and subsequent depletion of MxiC from the cytoplasm as cytoplasmic MxiC inhibits the secretion of the effectors.Different homologous proteins are also found like InvE, SepL and YopN–TyeA complex respectively in *Salmonella typhimurium*, EPEC and *Yersinia* spp. (Muschiol et al., 2006; WolfK et al., 2006; Zhang et al., 2007).

5. REGULATION OF ITS SECRETION

Regulation of secretion of different molecules takes place at transcriptionalas well as post translational level; negative control of regulation also can be seen in some bacteria like in *Yersinia* spp. Moreover, different environmental factors can induce its secretion like change in the oxygen level, osmolarity, pH, bivalent cations, availability of nutrients and growth phase, the addition of bile salts and congo red can trigger secretion. The bile salts can either activate or inactivate T3SS in *S. flexneri* and *S. typhimurium* SPI-1 (Ye et al., 2018) respectively. In some enteric bacteria like *Shigella*, *Salmonella* and *Yersinia* contact with the host cells induces the secretion of molecules (Russo et al., 2019; Adams et al., 2015; Bulmer et al., 2012). In *Y. enterocolitica* Ca^{2+} deprived media results in the secretion of proteins (Sory et al., 1994) while in *Shigella* addition of small amphipathic dye results in the secretion of proteins, in case of *Salmonella* aeration can induce its secretion *in vitro* (Bahrani et al., 1997; Platenkamp and Mellies, 2018). The key regulation mechanism for LEE (Locus of Enterocyte Effacement) present in enteropathogenic *E. coli* Is at the transcriptional level but recently post transcriptional as well as post translational level of regulation also has been found. Different environmental conditions like temperature in the host cells,envelope stress, quorum sensing autoinducers send signals to LEE encoded regulators (Ler) to activate promoters of LEE operon for positive regulation of its expression, on the other hand,these promoters are repressed by histone-like nucleoid proteins (Platenkamp and Mellies, 2018; Turner et al., 2019; Umanski et al., 2002). Cpx envelope stress response decreases expression of LEE4 and LEE5 that ultimately decreases secretion of translocators and Effectors (Vogt et al., 2010). The role of small RNAs have been found to be post transcriptional regulators as they target mRNA both in EHEC and EPEC (Bhatt et al., 2016). The LEE5 operon encodes for attaching and effacing lesion causing proteins involve mainly three genes: tir, cesT and eae, encoding Tir, CesT and intimin, respectively (Mellies et al., 1999; Sanchez-SanMartin et al., 2001). CesT (homodimer chaperons) binds to two regions in Tir (Translocated intimin receptor) at the N- and C terminus and the delivery of these effectors into the host liberates CesT resulting in rapidly increased levels of free CesT in the cytoplasm. They in turn interact with an alternative binding partner, the carbon storage regulator A (CsrA). Since CsrA binds to the mRNA of numerous genes and regulates the stability and/or translation of these mRNAs, therefore, the elevated levels of free CesT, upon effectors injection, competitively inhibit CsrA-mRNA interaction result in massive remodeling of gene expression (Little et al., 20018; Lai et al., 2013; Katsowich et al., 2017; Goddard et al., 2019; Ye et al., 2018). ExsA, a transcriptional activator under AraC/XylS family, activates transcription of its own as well as that of a set of genes encoding T3SS apparatus proteins, chaperones and effectors in response to host cell contact or in vitro inducing cues (e.g., calcium limitation); further ExsA activity is controlled by the anti-activator ExsD, which binds and inhibits ExsA when T3SS inducing cues are absent in case of *Pseudomonas aeruginosa* (Diaz et al., 2011; Wolfgang et al., 2003; Brutinel et al., 2010). *Salmonella* has two types of T3SS, encoded in pathogenicity islands at centisome 63(SPI-1) and 31(SPI-2) but SPI-2 requires systemic infection to be expressed as it cannot be expressed under standard laboratory growth condition. The regulation of many T3SS genes often requires the input of multiple signals for maximal expression and it has been seen that SPI-1 T3SS is completely down-regulated in the absence of a cytoskeleton by an unidentified regulatory factor where in contrast, the SPI-2 T3SS remains functional (Bulmer et al., 2012). Roine et al. (1997) reported that

many transcriptional activators are present to regulate the expression of SPI-1 genes such as HilC, HilD, HilA and InvF where HilCas well as HilD activate expression of SPI-1 genes by binding upstream of the master regulatory gene hilA to induce its expression (Ellermeier et al., 2005). This in turn activates genes encoding the type 3 secretory apparatus by binding the promoters of SPI-1 operon, several secreted effectors and the transcriptional regulator InvF. InvF can activate the expression of effector genes present both inside as well as outside of SPI-1 such as sopB and sopE. T3SS in the case of marine bioluminescent bacteria *Vibrio harveyi* is regulated by quorumsensing (QS) in a density dependent manner through repression of ExcA. LuxR (transcriptional regulator of QS) functions indirectly to control T3SS gene expression by binding to a promoter upstream of the exsBA operon, repressing the expression of both exsB and exsA when there is high cell density (Waters et al., 2010). Although expression of *Yersinia* mainly induced by the shift of temperature from 25°C to 37°C due to the presence of activator (LcrF/VirF) which is an AraC-like transcriptional activatoralong with it negative feedback is also observed due to the presence of activity of an anti-activator protein (LcrQ/YscM) (Cornelis et al., 1998). The secretion in *Yersinia* is also stimulated by Ca^{+2} depletion but it has a negative impact on overall bacterial growth.

6. DIFFERENT EFFECTS IN THE HOST

The main effect of these secreted proteins isthe modification of the host cytoskeleton system through modification of Rho GTPase activity or directly acting on the actin filaments via involvement of guanine nucleotide exchange factors (GEFs) and GTPase-activated proteins (GAPs) for activation and down regulation respectively (Ridley, 2006).

6.1. Adherence and invasion of the eukaryotic cells

Mainly EHEC and EPEC are involved inproducing attaching and effacing lesions on the intestinal epithelium where EPEC tir proteins are phosphorylated for recruitment of Arp2/3 complex to drive actin polymerization in the other hand EHEC depends on TccP/EspFU effector proteins, for Arp2/3 recruitment. Down regulation of filopodia is an important factor for effective pedestal formation (Campellone and Leong, 2005; Garmendia et al., 2004). Two chromosomal loci within the LEE, eaeA and eaeB (eae for *E. coli* attaching and effacing), have been characterized where eaeA encodes for outer membrane protein called intimin shares sequence homology with invasion protein present in *Yersinia* spp. (Jerse et al., 1990). It has been seen that the eaeB deletion mutant is capable of producing intimin but cannot produce the AE lesion in the host (Donnenberg et al., 1993). Another sep genes are essential for secretion of virulence factors causing AE lesions and are highly conserved among AE lesion causing bacteria including *E. coli* 0157:H7 (Jarvis et al., 1995). *Salmonella* pathogenicity-I encoded proteins (SipA, SipC) along with activatingsecond messengers (SopB/SigD, a phosphatidylinositol phosphatase and mimic the function of their cognate GEFs (SopE and SopE2) cause membrane ruffling on the intestine (Jarvis et al., 1995; Schlumberger and Hardt, 2006). *Shigella* gains entry through the M cells present in the submucosa of the intestine and different effectors have a role in spreading infection such as IpaC, IpaB1, IpaB2 with Rho GTPase activity, IpgI inducing inositol influx, VirA causes destabilization of microtubules (Zhou and Galan, 2001; Alto et al., 2006; Marcus et al., 2001; Ohya et al., 2005). However, Biosurfactant Like Molecules (BLM) are seen to be secreted in the extra cellular medium by *Shigella flenxneri* which is T3SS dependent and is correlated with quorum sensing (Pearson et al., 1997). These BLMs play an important role in the adhesion of the cell-cell interface that allows to reduce the cell tension thereby helping the translocon and tip component to be close to the host cell membrane. Even the swarming ability of *Shigella* can promote biosurfactant production. This phenomenon often associated with biofilm production and antibiotic resistance are observed in few bacteria like *Proteus mirabilis*, *Salmonella enterica* serovar Typhimurium and *Serratia* (Belas and Suvanasuthi, 2005; Williamson et al., 2008; Butler et al., 2010).

6.2. Direct cytotoxicity

Mostly this hallmark is observed in infection caused by *Pseudomonas* in lungs where only ExoU protein is involved and independent of other effector molecules and known to have a potent cytosolic phospholipase A2 (cPLA2)-like activity (Horna et al., 2021). ExoS also acts as a major cytotoxin present in *Pseudomonas* that helps in dissemination following the invasionand also acts on Ras like proteins. Apart from these two toxins another ExoT toxin mainly targets different kinases that are involved in phagocytosis in the host and ExoY acts as adenyl cyclase that ultimately leads to rounding of host cells (Tang et al., 2016; Feng et al., 2019). One interesting fact has been found that about T3SS secreted effector proteins that in lungs, mutations of CFTR (CF trans-membrane conductance regulator genes) cause depletion of airway surface liquid and mucus dehydration that ultimately provides a niche favourable for chronic infections by opportunistic pathogens i.e. infection switch from *S. aureus* to *P. aeruginosa*. This usually occurs due to sufficient levels of ExoU proteins i.e. sPLA2-IIA to kill *S. aureus* with no or only minor effects on *P. aeruginosa* (Pernet et al., 2014). *Vibrio parahaemolyticus* causes inflammatory and systemic spread and according to the recent demonstration, VP1680 is found to be a central

effector protein that causes apoptosis which ultimately leads to cytotoxicity. It has been demonstrated that the T3SS of non O1 and non-O139 of V cholera is homologous to T3SS2 of *V. parahaemolyticus* that encodes proteins to cause cytotoxicity (Dziejman et al., 2005; Ono et al., 2006).

6.3. Disruption of epithelial tight junction

There are many proteins that are present in the transmembrane such as occluding, claudin and junctional adhesion molecules along with some adaptor proteins like zona occludens that are present in the cytoplasm linking to the actin cytoskeleton. Disruption of tight junction is mainly seen during *Citrobacter rodentium* infection due to EspF protein however EHEC also can induce disruption due to the presence of another protein U-EspF but not as quickly as EPEC. For EPEC Map, EspG and EspG2 are involved effector molecules for crossing the intestinal barrier (Dean and Kenny, 2004; Ma et al., 2006; Tomson et al., 2005; Viswanathan et al., 2004). *Salmonella enterica* serovar Typhimurium secreted effector molecules are involved in fluid accumulation in the intestinal epithelium due to destruction of the TJs, that are SPI1 enoded SipA and SopA, -B, -D, and –E. Another effector molecule YopE secreted from *Yersinia pseudotuberculosis* binds to β1-integrins and disturbs ZO-1 and occludin localization that further promotes paracellular translocation (Tafazoli et al., 2000).

7. CONCLUSION

T3SS study gives us important information about the evolutionary aspect about the virulent strains of bacteria.Broad-spectrum inhibitors that target conserved T3SS components and secretion mechanisms are therefore desired as therapeutics for T3SS-mediated diseases. T3SS also provides new insight into potential candidates that will target a broad spectrum of pathogens.T3SS can be considered as a target for antimicrobial drug design especially targeting the activity of T3SS ATPase but due to its high degree of conservative nature may lead to toxicity problem.

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