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JOURNAL REVIEW

Recombinant protein secretion via the type III secretion system

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Abstract−The type III secretion system (T3SS) is a mechanism by which bacteria export proteins from the cytoplasm, through the membranes, to the extracellular environment. T3SS is made up of more than 20 different proteins, about half of which maintain conserved sequences. This review summarizes the features of this novel apparatus and discusses the potential of utilizing T3SS to export recombinant proteins into the external environment, and the impact this system will have on protein production technology.

Key words: Type III Secretion System (T3SS), Secretion, Recombinant Protein

INTRODUCTION

The type III secretion system (T3SS) is found in around 25 gram-negative bacterial species. The most studied species containing T3SS are *Shigella*, *Salmonella*, *Escherichia coli*, *Burkholderia*, *Yersinia*, *Chlamydia*, *Pseudomonas*, *Erwinia*, *Ralstonia*, *Rhizobium* and *Xanthomonas*. The structure and function of the supramolecular apparatus have been clarified through many previous studies [1-9]. This review presents the most typical features of T3SS and discusses the capability of this system to export recombinant proteins to the extracellular environment.

STRUCTURE OF TYPE III SECRETION SYSTEM

The T3SS is a macromolecular complex spanning the inner bacterial membrane, peptidoglycan layer, periplasmic space, outer bacterial membrane, extracellular space, and host cellular membrane (Fig. 1). In other words, T3SS may be described as a base spanning both membranes with a needle [1].

1. Basal Body

The basal body is assembled first. Next, secretion of the major needle subunit through the basal body results in assemblage of the extracellular needle, which is tightly controlled by regulatory mech-

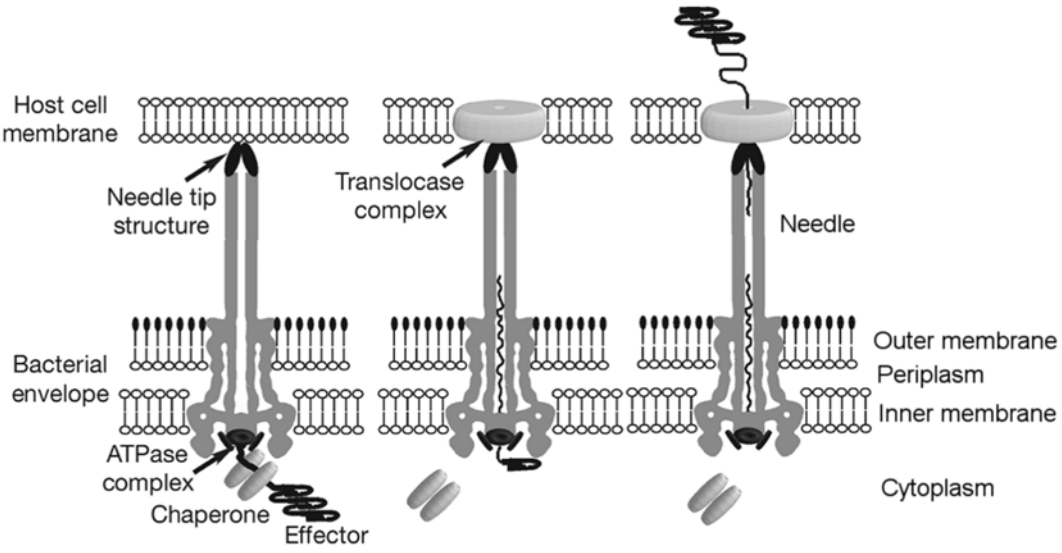

Fig. 1. Model for substrate recognition and delivery of proteins by type III secretion machines. The effector-chaperone complex is recognized by the secretion machinery, including a type-III-secretion-associated ATPase. The ATPase 'strips' the chaperone from the complex, which remains within the bacterial cell, and mediates the unfolding and 'threading' of the effector protein through the central channel of the needle complex. A 'translocator complex' made up of proteins also secreted by the T3SS is assembled on the host cell membrane and mediates the passage of the effector proteins through the target cell membrane. The translocated effectors refold within the host cell to carry out their function [5].

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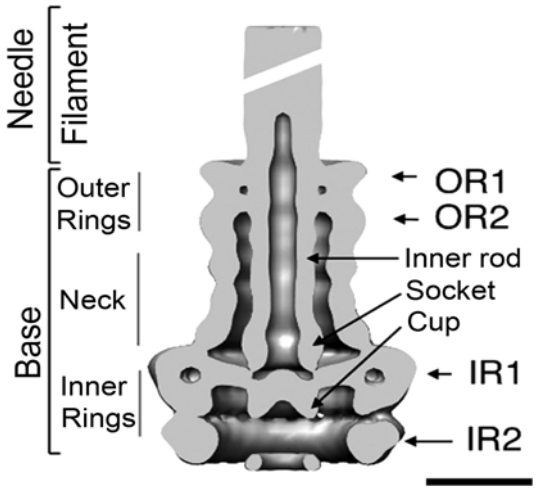

Fig. 2. Cut-away view and description of individual substructures of the needle complex from *Salmonella typhimurium* (OR outer ring; IR inner ring). Bar=10 nm [72].

Table 1. T3SS apparatus proteins of *S. typhimurium* (adapted from references [9,29])

Structure	T3SS protein	Ref.
Needle	PrgI	[61]
Inner rod	PrgJ	[15-16]
Needle length determinant	InvJ	[29]
Outer membrane secretin	InvG	[12]
Inner membrane rings	PrgK	[2]
	PrgH	[2]
ATPase	InvC	[29]

anisms which determine its length. After the needle assembly is completed, other proteins are secreted through the needle and assembled at the tip (Fig. 2) [1].

The basal body of the T3SS consists of three proteins (Table 1). In *Salmonella typhimurium*, the base proteins are composed of equimolar amounts: InvG makes up the outer rings, while PrgH and PrgK form the rest of the structure [2].

2. Needle Complex (NC)

Utilizing cryo-EM and a combination of particle sorting and symmetry particle averaging, it has been shown that the needle complex (NC) consists of a cylindrical basal body and a hollow, extended needle-like structure, which is incorporated into the base during assembly [2,9,10]. In addition, the NC extends from the outer-membrane portion of the apparatus which runs a ~25 Å channel, forming the secretion conduit [1,11]. Thus, the NC is composed of a multiring base anchoring the structure to the bacterial envelope with a needle-like projection protruding several nanometers from the bacterial surface [5]. The base is traversed by the inner rod, a cylindrical substructure connecting the needle to the basal side of the base substructure. The entire needle complex is traversed by a narrow channel (~25 Å in diameter), likely to serve as a conduit for proteins travelling through the secretion pathway [5].

Assembly of the needle complex is a sequential process. First, the base substructure is assembled. Second, the inner rod and needle are assembled. In some T3SSs, the assembly of the outer rings requires an accessory outer membrane protein that serves as a chaperone to facilitate the proper folding and multimeric arrangement of the outer ring subunit [12,13]. Assembly of the base substructure presumably causes the recruitment of a subset of accessory inner membrane proteins to facilitate the passage of secreted proteins through the inner membrane. Another compartment, the 'C ring,' may be assembled in the bacterial cytoplasm immediately beneath the needle complex [14]. When completely assembled, the base substructure begins to function as a 'type III protein secretion machine', especially serving in the secretion of proteins which are components of, or necessary for, the assembly of the inner rod and needle substructures [5]. In *S. typhimurium* these proteins are PrgJ (the putative inner rod protein), PrgI (the needle protein), and InvJ (a regulatory protein required for efficient assembly of the needle complex) (Table 1). On completion of the needle complex assembly, the secretion machine changes specificity and becomes competent for secreting effector proteins [15,16].

Substrate switching and reprogramming of the export machinery is observed during flagellar assembly [17]. In this stage, once the hook substructure reaches a certain length, the export machinery switches substrate specificity in order to export proteins necessary for the assembly of the flagellar filament. However, the mechanisms by which these secretion machines change substrate specificity are poorly understood, although some models explaining the mechanism have been proposed [5].

The final length of the needle varies among different bacteria species and strains, but needle length is a regulated process. In *Y. enterocolitica* E40, the needle length is 58 nm±10 nm. In *S. typhimurium* flagellum, the length of the hook is controlled at approximately 55 nm [3]. In the two systems, the loss of one protein - FliK (in the flagellar system of *S. typhimurium*) and YscP (in the *Y. enterocolitica* injectisome) - results in uncontrolled growth of the hook and needle, respectively [16,18-20]. There are some mechanisms proposed to explain the control of the length of the needle and hook, such as the cup model and the ruler model [3].

The needle culminates in a tip complex containing multiple copies of an adaptor protein required in several species for regulation of secretion activity and insertion of the translocation pore [21-25]. This adaptor may function to mediate the interaction between the needle and the translocon (translocator or translocation channel) and may be involved in sensing the host cell [1].

3. Chaperone

The assembly and operation of the T3SS requires the assistance of small bacterial cytosolic proteins called chaperones. Chaperones are small, acidic, mostly alpha-helical proteins that facilitate the efficient secretion and translocation of specific effector proteins [7]. There are several mechanisms by which chaperones may function. Chaperones may regulate the cytoplasmic concentration of effector proteins by affecting transcription or protein stability. On the other hand, chaperones may function as molecular shuttles to deliver specific effector proteins to the type III machine for secretion [7]. Moreover, chaperones may serve as antifolding factors, secretion signals, or stability factors (Table 2).

Chaperones are grouped into three classes, according to their functions. The first (class I) assists the effector proteins, the second (class II) functions as pore forming proteins, and the third (class III) serves

Table 2. T3SS related chaperones of *Salmonella* (adapted from reference [4])

Chaperone	Size (aa)	p*I*	Substrate	Function	Ref.
FlgN	140	5.3	FlgK, FlgL, FlgM	Substrate stabilization	[62-63]
FliS	135	4.7	FliC	Anti-polymerization	[64]
FliT	122	4.9	FliD	Substrate stabilization	[65]
InvB	135	4.4	SicP/SspA		[66]
SicA	165	4.6	SipB/SspB, SipC/SspC, InvF	Substrate stabilization, regulation	[67-68]
SicP	116	3.9	SptP	Substrate stabilization, anti-folding	[69]
SigE	113	3.9	SidgD/SopB		[70]
SseA	108	10.2	SseB, SseD	Substrate stabilization	[71]

as subunits of substructures that polymerize outside of the bacterial cytosol. These chaperone proteins are never exported from the bacterial cell and are often recycled [3].

Together, these chaperones bind a 50-100 amino acid domain of secreted protein, which is located immediately downstream from the N-terminal secretion signal [26,27]. Although a given chaperone is normally dedicated to a single secreted protein, some chaperones can bind multiple targeted proteins. One of the functions of these chaperones is to guide the secreted proteins in rapid unfolding before secretion [28]. (Fig. 1). Another proposed function of the chaperones is to prevent the premature and undesirable interactions of the cognate secreted protein with other components of the T3SS within the bacterial cell [27]. It is obvious that these chaperones play key roles in targeting the secreted protein to the type III secretion apparatus. The chaperones are removed from the effector proteins in the bacterial cytosol before the delivery of the effectors to the secretion apparatus [5,29,30].

What are the substrates for T3SS? Obviously, T3SS must select protein substrates that can travel this pathway. Once recognized by the machine, substrates are unfolded before secretion. Most type III secreted proteins possess a secretion signal located within the first 20-30 amino acids [31,32]. However, T3SS signals are not cleaved at secretion unlike Sec-dependent signal sequences.

TRANSCRIPTIONAL REGULATION OF T3SS

The T3SS can be activated by temperature, pH, osmolarity, oxygen levels, or by lowering the concentrations of calcium ions in the growth medium. These regulators either regulate secretion directly or through a genetic mechanism [6]. Transcription of type III secretion genes is controlled by a multicomponent regulatory system, which senses environmental cues that induce T3SS, including temperature, pH, osmolarity, and oxygen levels.

The transcriptional control systems of T3SS are different among various pathogens. However, there are some common systems including two-component response regulators, AraC-like transcriptional activators, or systems involving histone-like proteins that regulate gene expression in response to temperature and osmolarity by controlling DNA superhelicity.

In the case of transcriptional regulation of T3SS genes in *S. typhimurium*, some genes in the *Salmonella* Pathogenicity Island 1 (SPI-1) including *invF*, *sspC*, *sspA*, *hilA*, *prgH*, *prgK*, and *orgA* are regulated by environmental factors such as temperature, pH, osmolarity, and oxygen levels. They are expressed at high levels only under low-oxygen, high-osmolarity, and slightly alkaline (pH 8) conditions. If any of the conditions change, then the secretion gene expression is repressed 10 to 200-fold [33]. Unlike T3SS genes in other enteropathogens, SPI-1 gene transcription in *S. typhimurium* is not regulated by temperature [6].

Several factors involved in transcriptional regulation of SPI-1 genes have been studied. Among them, HilA [34] and InvF [35] are encoded within the pathogenicity island, whereas SirA [36] and PhoP [37,38] are encoded in different locations in the chromosome. HilA is required for the expression of all genes in SPI-1 [33,34].

SirA is a protein required for transcriptional activation of type III secretion genes [36]. PhoP negatively regulates the expression of *hilA*, and shuts off expression of the SPI-1 encoded secretion system [33,37].

T3SS AND OTHER SECRETION SYSTEMS

Several secretion systems are capable of exporting proteins through both the inner and outer membrane. The *Sec* and *Tat* pathways deliver proteins to the bacterial periplasm. The *Sec* pathway threads polypeptides in an unfolded state across the inner membrane in an ATP-dependent manner [39,40]. The *Tat* pathway uses the proton motive force to drive the transport of folded proteins into the periplasm [41-43]. Both *Sec* and *Tat* export pathways require an N-terminal signal peptide for secretion [44,45]. These types of signal peptides typically end with a signal peptidase I cleavage site, allowing the cleavage of the tag on translocation to the periplasm [46]. Table 3 shows similarities and differences of T3SS among some other protein secretion systems.

1. Type I *sec*-Independent Pathway

Unlike the type II and IV secretion pathways, type I and type III secretion systems are independent of the *sec* system, and therefore amino-terminal processing of the secreted proteins is not involved. Three secretory proteins are necessary for type I secretion systems: including an inner membrane transport ATPase providing energy for protein secretion; an outer membrane protein exported via the *sec* pathway; and a membrane fusion protein anchored in the inner membrane and spanning the periplasmic space. Genes encoding the secretion apparatus are usually clustered with secreted proteins. The proteins that are secreted do not cause proteolytic cleavage, and the secretion signal is located within the carboxy-terminal (60 amino acids) of the secreted protein [6].

2. Type III *sec*-Independent Pathway

Like the type I secretion pathway, type III secretion is indepen-

Table 3. Comparison of T3SS with other protein secretion systems [6]

Features	Type I (T1SS)	Type II (T2SS)	Type III (T3SS)	Type IV (T4SS)
Sec pathway dependence	Independent	Dependent	Independent	Dependent
Secretion signal location	Carboxy terminal	Amino terminal	Amino terminal	Amino terminal
Secretion signal cleavage	Not cleaved	Cleaved	Not cleaved	Cleaved
Energy-providing protein for secretion	ATPase	Not mentioned	ATPase	Not mentioned
Application	Secrete enzymes active in the extracellular space	Secrete extracellular degradative enzymes	Translocate of pathogenic proteins, translocate recombinant proteins directly into culture medium	Not mentioned
Genes encoding secretion apparatus	Clustered	Clustered	Clustered	Not mentioned

dent of the *sec* system. The apparatus is composed of approximately 20 proteins, most of which are located in the inner membrane. Type III secretion requires a cytoplasmic, membrane-associated ATPase. Similar to type I and type II secretion, the genes encoding the type III secretion apparatus are clustered. As in type I secretion, the proteins secreted via the type III pathway are not subjected to amino-terminal processing during secretion [6].

Most T3SS proteins posses a secretion signal that is usually located within the first 20-30 amino acids [31]. This region supports and directs the secretion of effector proteins. However, the signal sequences of proteins secreted via the T3SS do not share any recognizable structural similarities in functioning as a common secretion signal. Unlike *sec* dependent signal sequences, T3SS signal sequences are not cleaved on secretion. It has been proposed that the secretion signal resides in the 5′ region of mRNA, encoding secreted proteins [47].

In contrast to type I secretion, in which the secreted enzymes are active in the extracellular supernatant, type III secretion systems are dedicated to the translocation of pathogenic proteins into the cytosol of eukaryotic cells. The secreted proteins require small cytoplasmic proteins with chaperone functions.T3SS differs from other secretion pathways in gram-negative bacteria in three ways. First, the secretion signals do not share any structural similarities in the secreted proteins. Second, there is no cleavage of signal sequence in secreted polypeptides. Finally, there are no periplasmic secretion intermediates [48], partly because T3SS consists of a basal body spanning both bacterial membranes which allows secretion of substrates without a periplasmic intermediate [1]. One of the most noticeable advantages of T3SS is that proteins can be exported into growth culture directly from cytoplasm. However, the main disadvantage of the system is that no signal sequences have been identified so far.

3. Type II and Type IV *sec*-Dependent Secretion Pathways

In both systems the transport occurs via the *sec* system. Both Type II and Type IV pathways are involved in protein translocation through the outer membrane, but they differ in the way in which proteins are transported across the outer membrane. In T2SS, the transport across the outer membrane needs an additional set of inner and outer membrane proteins. For instance, in the case of pullulanase secretion by *Klebsiella oxytoca*, 14 additional secretion factors, encoded by a continuous gene cluster, are necessary and sufficient for secretion. At least seven of these proteins are located in the cytoplasmic membrane, whereas the others such as PulS and PulD are outer membrane proteins [5]. The signal sequence in T2SS is 30 amino acids, mainly hydrophobic, with an amino-terminal. This signal sequence aids protein export and is cleaved by a periplasmic signal peptidase when the exported protein reaches the periplasm. T2SS is dedicated to the secretion of extracellular degradative enzymes by gram-negative bacteria [5].

Type IV secretion pathway consists of a group of autotransporters [49]. The information required for the transport across the outer membrane is located completely within the secreted protein. The autotransporters are specialized to form pores in the outer membrane through which they pass, and autoproteolytic cleavage releases the proteins into the supernatant. The secretion signal in Type IV secretion pathway is a short (30 aa), mainly hydrophobic, amino-terminal.

Type V secretion pathway is also one of the protein secretion pathways in gram-negative bacteria. Since this pathway has been the least well-studied pathway so far, it is not well-presented in this work.

RECOMBINANT PROTEIN SECRETION BY T3SS

So far, the most used and efficient fusion strategy to export protein through T3SS is making an operon containing the secreted protein fused to the N-terminal of secretion tag (effector protein) and its cognate chaperone. Then the fused gene is cloned into expression vector to express and export the target protein. Next is choosing the optimal culture condition such as temperature, pH, aeration, etc. to export the target protein with highest efficiency.

Fig. 3 shows strategy for recombinant protein secretion by T3SS and Table 4 presents some typical chaperones and their specific secretion tags in *Salmonella typhimurium.*

The secretion of silk monomers is a good example of utilizing the T3SS for the production of recombinant proteins, involving the construction of a system containing all of the necessary genetic parts

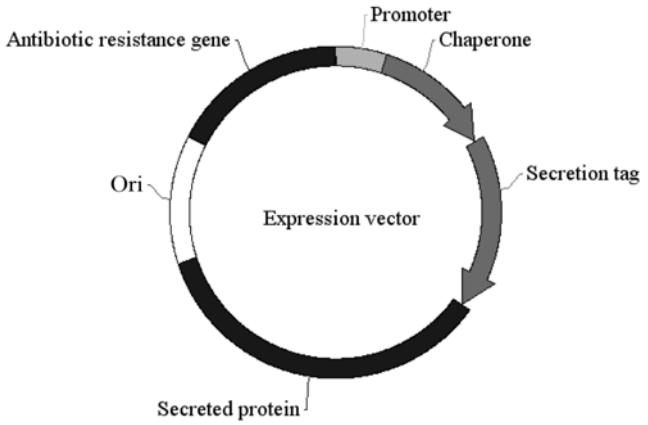

Fig. 3. Strategy for recombinant protein secretion by T3SS. Construction of fused gene including promoter, chaperone, secretion tag and secreted protein. The fused gene was coloned into expression plasmid to express and export the target protein.

Table 4. Some typical chaperones and their specific secretion tags in *Salmonella typhimurium* [51]

Chaperone	Secretion tag
sicA	sipC
sigE	sopB
sicP	sptP
invB	sipA

to secrete silk monomers. Secretion rates up to 1.8 mg/(L·h) were reported with up to 14% of expressed protein secreted [51].

The *Salmonella* T3SS functions as a molecular syringe to inject effector proteins into mammalian host cells to facilitate invasion and pathogenesis [52]. The *E. coli Salmonella* flagellum and *Yersinia enterocolitica* ysc T3SSs are able to secrete recombinant proteins [53-55]. In addition, these systems inject foreign proteins and peptides into mammalian cells as a mechanism to confer immunity [55-57].

The *Salmonella* T3SS has some features that make it a useful tool for the secretion of recombinant proteins. The apparatus is highly expressed under standard laboratory conditions (Luria-Bertani Broth at 37 ºC), with 10-100 needles per cell [58]. Under these conditions, effector proteins are secreted into the media in significant quantities without the need to co-culture with mammalian cells or expensive media components [59].

Fusion of the 5′ untranslated region of the flagellin protein FliC was sufficient for the export of several fusion proteins (up to 424 amino acids in length) into the extracellular culture medium via a modified flagellar (type III) machinery [60]. Using this system, 1-15 mg/L of extracellular protein was achieved.

In 2006, Konjufca [57] developed recombinant attenuated *Salmonella* vaccines (RASV) against avian coccidiosis by delivering *Eimeria* species antigens to the lymphoid tissues of chickens via the T3SS. In this research, Konjufca used two effector proteins, SptP and SopE, so that RASV strains were able to translocate heterologous antigens into the cytoplasm of host cells in order to induce antigen-specific cytotoxic T-lymphocyte responses. Development of this protective vaccine against the disease is a challenge but an important approach to reduce potentially negative economic impact. RASV strains are considered as an attractive approach since they are safe, easy to control, economical, and do not contain antibiotic resistance genes.

These achievements raise the possibility of exporting highly specific heterologous proteins into a growth medium at levels useful for commercial purposes using the T3SS.

CONCLUSIONS

Protein secretion in bacteria has been conducted in numerous academic and industrial laboratories for several decades. The choice of bacterial strain, medium formulation, promoter and expression system are critical for determining protein secretion yields. Recent advances in this area show great promise of secreting recombinant proteins using new systems. Exporting proteins directly to the extracellular environment utilizing T3SS is still new and challenging. Efforts to engineer T3SS for protein export at high levels into growth medium have so far met with little success. Furthermore, these systems cannot deal with folding matter, such as the disulfide-bond formation found in many recombinant molecules. Obtaining authentic bioactive polypeptides remains a significant challenge.

The discovery of the T3SS in bacterial pathogens has been considered as one of the most significant discoveries in recent years. The analysis of T3SS supports the understanding of the molecular secretion mechanisms as well as evolution of pathogenic bacteria. Pathogenic bacteria use these systems for delivering effector proteins directly into the cytosol of eukaryotic host cells. Structurally, T3SS consists of a basal body that spans both bacterial membranes [1], allowing secretion of substrates without any periplasmic intermediates. This makes T3SS a useful tool for direct secretion of recombinant proteins into growth media.

However, more biochemical methods are necessary to identify protein-protein interactions, as well as binding sites used for assembly of the apparatus. More efforts to engineer T3SS for protein export at high levels into growth medium should be made. Understanding the actual mechanism of protein secretion, and which steps in the secretion process could be controlled, would support studies in secretion regulation, as well as applicable uses of T3SS in producing valuable recombinant proteins.

We can now visualize the NC structure and ATPase at a resolution of approximately 1.6-1.7 nm. However, the detailed structure of the C ring should be analyzed. Some constituents of the T3SS such as the needle and the MS ring should be modeled at a better resolution based on available structures.

What would be the next challenge in our ongoing efforts to understand the T3SS systems? Structurally, the precise locations of many proteins in the system remain unavailable. For the time being, elucidation of the complete structure of the T3SS at an atomic level would be important. Deciphering the hierarchy of protein export during the assembly of the system and the assembly of the pores still remains challenging. These goals are very ambitious, and undoubtedly, would take several more years of concerted efforts to achieve. The importance and unique structure of the T3SS would attract more scientists to further discovery, as well as exploiting applicable uses of the apparatus in the near future.

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