Bacterial Systems for Assembly, Secretion and Targeted Translocation of Proteins and Protein/DNA Complexes

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Pathogenic bacteria of animals and plants have developed an astonishing set of tools that allow them to survive in their hosts. These effectors of infection or virulence factors are often delivered into eukaryotic host cells where they interfere with host cell signalling, thereby causing a variety of cellular changes which result in the disease symptoms in affected host organisms. The effectors can be large protein complexes and even DNA, such as in the case of the Ti plasmid of *Agrobacterium tumefaciens*. Effectors are delivered by dedicated bacterial secretion systems, some of which are evolutionarily related to ancient bacterial structures such as pili or flagella, or systems for assembly/secretion of filamentous phage.

For successful secretion of proteins from pathogenic bacteria and their and delivery to the host's cells, two main problems had to be solved by bacterial cells:

1. Crossing the barriers

Several barriers must be crossed in order for effector proteins to exit the bacterial cytoplasm and be delivered to the cytoplasm of a host cell. All bacteria have an inner or cytoplasmic membrane and Gram negative bacteria have a second, outer membrane, which must be traversed. For delivery into host cells, the host cell membrane(s) presents the final barrier.

2. Selection and targeting of proteins

Proteins destined for export must be distinguished from all other bacterial proteins and their fates precisely determined in order to fulfil their role in bacterial attack on the host.

Bacteria have developed not one, but several systems to solve these two requirements. These systems, which range from very low to very high complexity, have been the focus of intense research during last dozen years. Sequencing of a large number of bacterial genomes has revealed that their secretion systems are ubiquitous and that several systems can exist in the same organism. Many of the systems are coded by a compact set of genes located on the chromosome in so-called pathogenicity islands or on dedicated plasmids. This organisation enables rapid spread of secretion systems between organisms by horizontal gene transfer.

Protein secretion from Gram positive bacteria

Gram positive bacteria use the sec pathway, the ubiquitous bacterial system for secretion of proteins across the cytoplasmic membrane. The N-terminally-located hydrophobic sequence that targets proteins to the sec system is called a signal or leader sequence and is similar to that found in eukaryotic proteins as a signal for targeting proteins into the lumen of the endoplasmic reticulum.

After passage through the single membrane of Gram positive bacteria, proteins can either diffuse across or attach to the cell wall, the outer layer of the cell envelope. Members of the latter group carry a special hexapeptide tag which directs their covalent linkage to cell wall proteoglycans. Untethered effector proteins are released into the extracellular milieu and reach the host cell by diffusion. The efficiency of delivery is enhanced when the bacterium attaches to the host eukaryotic cell (1). Some soluble effector proteins interact with a receptor on the surface of the eukaryotic cell and become internalised. In other cases, a two-component system is secreted, in which one component, upon contact with the host membrane, forms a pore in its membrane which allows entry of the second component, the effector, into the host cell (2).

Secretion systems of Gram negative bacteria

Because of their outer membrane, Gram negative bacteria have a more complex task to deliver proteins to eukaryotic cells. That may well be the explanation for the variety of secretion systems used by this group – types I to IV as well as the auto-transporter and two-partner secretion systems. The type IV system can also deliver DNA into host cells. In addition, there are dedicated systems for assembly of several types of surface appendages (pili and flagella) and systems for assembly/secretion of filamentous bacteriophage.

The secretion systems can be as simple as a single protein (auto-transporter), or very complex, involving twenty or more accessory proteins and elaborate surface structures (type III, IV). Some of the systems rely on the sec pathway to translocate proteins across the inner membrane and others provide components to mediate secretion through the outer membrane, while others translocate proteins through both membranes simultaneously, bypassing the sec pathway altogether **(Fig. 1).**

Type I system

The Type I system traverses both bacterial membranes and directly exports proteins from the cytoplasm, bypassing the *sec* pathway **(Fig. 1G)**. It consists of three proteins: an inner membrane protein from the family of ABC (ATP-binding cassette) transporters; a periplasmic (inner-membrane-anchored) protein, and an outer membrane tunnel-channel (3). In the best studied example, the *Escherichia coli* system for secretion of the toxin haemolysin (HlyA), specificity of the system is provided by the ABC transporter, which interacts with the substrate, while the outer membrane channel, TolC, is used by several different pairs of inner membrane/periplasmic components. The structure of TolC has been determined recently. Like other outer membrane proteins, TolC forms a trans-membrane β barrel. Uniquely, its periplasmic portion forms an α barrel with a tapered end, which effectively closes this large channel in the absence of substrate. TolC associates with the complex of inner membrane/periplasmic components only after they have bound the substrate. Because the substrate binds the cytosolic face of the complex, it has been proposed that upon binding, the ABC transporter conveys a conformational change to the periplasmic component that results in the

recruitment of TolC (4). Other ABC transporters that work with the TolC are drug efflux pumps that can eliminate substances such as organic solvents or detergents from the bacterial cytoplasm.

Type II system

The most significant toxin secreted by this system is cholera toxin of *Vibrio cholerae*, the major virulence factor of cholera **(Fig. 1B)**. In plant pathogens such as *Klebsiella* and *Erwiniae*, type II systems secrete cellulases and other enzymes that degrade the plant cell wall (5). The type II system is designed to export the effector proteins or substrates from the periplasm through the outer membrane. The effector proteins are transferred to the periplasm via the sec pathway. A single type II system can transport a variety of seemingly unrelated, fully folded and, in some cases, multimeric proteins. Nonetheless, these systems show close to absolute preference for their proper cargo relative to other periplasmic proteins. This might be a reason for the complexity of type II systems. Depending on the species, they consist of 12 to 15 proteins that span the cell envelope – a set of approximately 11 inner membrane proteins (one of which is an ATPase), an outer membrane lipoprotein, and an outer membrane channel protein termed a secretin. Secretins are a large protein family, members of which also serve as the outer membrane component of type III secretion systems and filamentous phage assembly.

An interesting feature of type II systems is a short pilus-like structure that spans the periplasm, but normally does not extend outside the outer membrane. It is too narrow to transport proteins and too wide to fit into the secretin pore, and is anchored in the inner membrane by interaction with the inner membrane ATPase component of the system. It has therefore been proposed that this pilus-like structure serves as a piston to push secreted proteins through the secretion pore (6).

Efforts to explain the selectivity of type II systems have been frustrating. No common sequence motif is found in all substrates, but protein fusion experiments with a few substrates (exotoxin A, Cel5 cellulase and pectate lyase) suggest that two non-contiguous but correctly positioned regions are required for secretion. In contrast, in the case of the B subunit of cholera toxin, the protein in its entirety is required for secretion. These observations suggest that tertiary structural motifs are recognised by type II systems. Most of the substrates for which structures have been determined are rich in β sheet content, and that may be the feature recognised by the type II system (6).

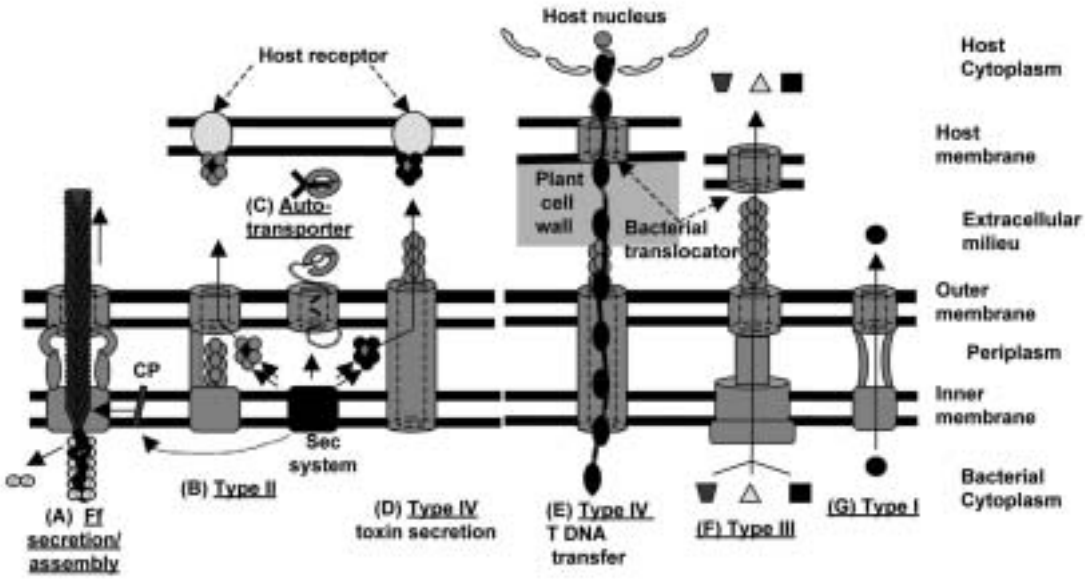

Fig. 1. Secretion systems of Gram negative bacteria.
A, During the filamentous phage (Ff) secretion/assembly, the Sec system is required for targeting the coat protein (CP) to the inner membrane. The phage-encoded secretion/assembly machinery at the inner membrane further translocates the coat protein to the ssDNA liberated from a cytosolic ssDNA binding protein. In the type II, protein-secreting type IV and auto-transporter systems, the Sec system of the inner membrane is required for translocation of proteins from cytoplasm to the periplasm.
B. The type II system has a short periplasmic pilus that is proposed to serve as a piston to push the substrates from the periplasm through the outer membrane channel into the extracellular milieu.
C. The C-terminal domain of auto-transporter forms an outer membrane channel that mediates secretion of the N-terminal domain. Once secreted, the N-terminal domain releases itself from the bacterium by auto-proteolysis and then digests the host antibodies (represented as horizontal Y).
D. The type IV systems secrete toxins from the periplasm to the extracellular milieu. In some systems, the type IV pilus can directly contact the host cell membrane. The type IV DNA transfer system and the type III and type I secretion systems directly translocate the substrates or effectors from the bacterial cytoplasm to their final destination, bypassing the periplasmic space.
E. The type IV DNA/protein complex transfer system translocates the donor DNA-protein complex to the nucleus of the host plant cell.
F. The type III system provides the machinery for complete journey from the cytoplasm of the bacterium to the cytoplasm of the host. The components of the type III system which traverse the bacterial envelope form a compact supramolecular structure called the needle complex.
G. The type I system for protein secretion belongs to the family of the ABC transporters. The inner membrane component is specific for the substrate, while the outer membrane channel is less specific and can pair with the inner membrane components of the ABC drug efflux systems.

Type III system

A battery of toxins of human enteropathogens, such as *Yersiniae*, *Salmonellae* and enteropathogenic *Escherichia coli*, use this system to carry out the invasion of host intestinal epithelial cells **(Fig. 1F)**. A Type III system is also used by *Pseudomonas syringae* and other plant pathogens to deliver a set of proteins, called avirulence proteins, which induce apoptosis in the host cells (7). The type III system is the most complex bacterial protein secretion system. Its main characteristic is its ability to translocate the substrates, independently of the sec system, directly from bacterial cytoplasm into the cytoplasm of the host eukaryotic cell. This system therefore spans three membranes: inner and outer of the bacterium and the cell membrane of the host. The Type III system is structurally complex, forming a remarkable structure that resembles a syringe and is named the needle complex (8). The needle complex is related (by appearance and sequence homology) to the basal body of an ancient bacterial structure, the flagellum – 9 of more than 20 proteins of the type III systems are conserved among different subtypes and flagella. A conserved membrane-associated cytosolic protein with a consensus ATP-binding motif, and related to the catalytic subunits of bacterial F_0F_1-ATPases is a part of the complex and might provide the energy for export.

The needle complex has morphological features very similar to those of the flagellar basal body and hook-filament. It is a large supramolecular structure, 77 nm in length, with a 30 nm base and 8.5 nm wide tip. Two sets of ring-like structures match the location of the inner and outer membrane. Above the outer membrane rings, a 45 nm long hollow needle protrudes into the surroundings. Type III systems carry out two types of secretion: that of the components for the assembly of the needle complex itself and that of the proteins to be exported from the bacterium. The latter group consists of translocators, which form the pore in the eukaryotic cell membrane, and effector proteins, which are translocated into the host, presumably through the pore (9). Like type II systems, type III systems secrete a number of diverse substrates, and the question is how the system distinguishes proper substrates from resident cytosolic proteins. There are a few possible mechanisms for selection of substrates: the 5´ untranslated region of mRNA, two regions within the N-terminal 15 and 100 amino acids, or specific cytosolic chaperonins that enable recognition. The most likely scenario is that the mechanism of selection depends on the stage at which the protein is required and the fate of the protein (10).

Type IV system

This system encompasses at least two related systems **(Fig. 1D,E)**. One of these translocates DNA-protein complexes into the cytoplasm of a wide variety of recipient cells, which can be other bacteria, fungi or plants (11; **Fig. 1E)**. Another system translocates proteins directly into the cytoplasm of eukaryotic cells. A few simpler variants, however, secrete effector proteins into the extracellular milieu **(Fig. 1D)**. These systems have conserved set of 10 different core components that form a trans-envelope complex.

The T-DNA transfer system of *A. tumefaciens* is related to conjugation systems in other Gram negative bacteria. However, instead of mediating DNA transfer to another bacterial cell, it delivers Ti plasmid DNA to plant cells. This system is extremely promiscuous, targeting many plant species and even yeast.

Proteins of the trans-envelope complex, pilus and all accessory proteins required for transfer and targeting of T-DNA into the host nucleus are coded by the Ti plasmid. To initiate transfer, a Ti plasmid-encoded protein cuts one strand of T-DNA and covalently associates with it. This is followed by unwinding and transfer of the one strand of the DNA and the single-strand DNA-binding protein VirE2 into the host cytoplasm. A nuclear localisation signal in VirE2 directs the DNA to the nucleus, where it inserts into the chromosome. There, the expression of a battery of T-DNA genes induces neoplastic growth of the affected tissue. The assembly of the export complex and pilus as well as the export of T-DNA has been a subject of intense research efforts.

In animal pathogens, the other major subgroup of type IV secretion systems serves to transfer a large group of effector proteins across the bacterial envelope and host cell membrane, such as CagA of *Helicobacter pylori*, a human pathogen that causes gastritis. Interestingly, this subtype IV system uses the sec pathway to transport the proteins across the inner membrane, and a complex with a short pilus-like structure for delivery of the effector proteins into the host cell (12). The simplest type IV subsystem is that for secretion of the pertusis toxin of *Bordatella pertusis* (agent of whooping cough), which is secreted into the extracellular milieu and binds directly to receptors on the surface of the cell (13).

Other secretion systems

A very simple system suffices to export some effector proteins of pathogenic bacteria **(Fig. 1C)**. Most such effectors are proteases that cut and inactivate the host proteins that participate in an immune response against invading bacteria, such as immunoglobulin IgA (14). These bacterial effector proteins are transported by either the auto-transporter or the two-partner secretion system, both of which also use the *sec* pathway. The auto-transporter system, the simplest, is an escape artist: the effector and the outer membrane channel are the same protein. It is believed that the channel portion of the protein (C-terminal) first inserts into the outer membrane, then the substrate (N-terminal) domain passes through the channel portion, and an autocatalytic proteolytic cleavage releases the N-terminal domain into the medium. A similar strategy is employed by the two-partner secretion system, except that the substrate and the outer membrane channel are coded by two separate but adjacent genes in an operon, rather than by a single gene (15).

Filamentous phage assembly

Filamentous phage are long filaments, composed of a single-stranded DNA genome wrapped in a tube formed by thousands of subunits of the major coat protein, pVIII. The ends of the tubes are asymmetric, made of two different pairs of minor coat proteins. One pair (pVII and pIX) acts as the initiator and the other (pIII and pVI) as terminator of assembly/secretion (16,17). Filamentous phage do not lyse the bacterial cell but are secreted through a phage-encoded secretion system that consists of two inner membrane proteins and one outer membrane protein of the secretin family **(Fig. 1A)**. The major coat protein spans the inner membrane prior to assembly and, with the help of the inner membrane components of the

assembly machinery, it associates with the DNA as the phage filament is secreted. The phage secretin (pIV) has served as a prototype for secretin structure and function. It is a large gated channel that is closed when substrate is absent (18,19). Future research will focus on direct observation of the substrate-assembly complex interactions and detailed architecture of the assembly machinery.

Several years ago it was discovered that the cholera toxin genes are encoded by a filamentous phage of *Vibrio cholerae*, ctxΦ, which mediates horizontal transfer of itself and the cholera toxin genes. Curiously, the genome of this phage does not encode a pIV secretin, but rather borrows the secretin of the *V. cholearae* type II system, which secretes phage-encoded cholera toxin (20).

Future directions

Although there is a lot of information about the components of various secretion systems, many questions remain. Secretion mechanisms have been deduced mostly from indirect evidence and need confirmation. The basis for secretion of diverse substrates in a defined temporal order also remains to be elucidated. A deeper understanding of the mechanism of secretion and of the interaction of secretion systems with their substrates will be invaluable in designing specific inhibitors, which in turn will help to combat bacterial diseases.

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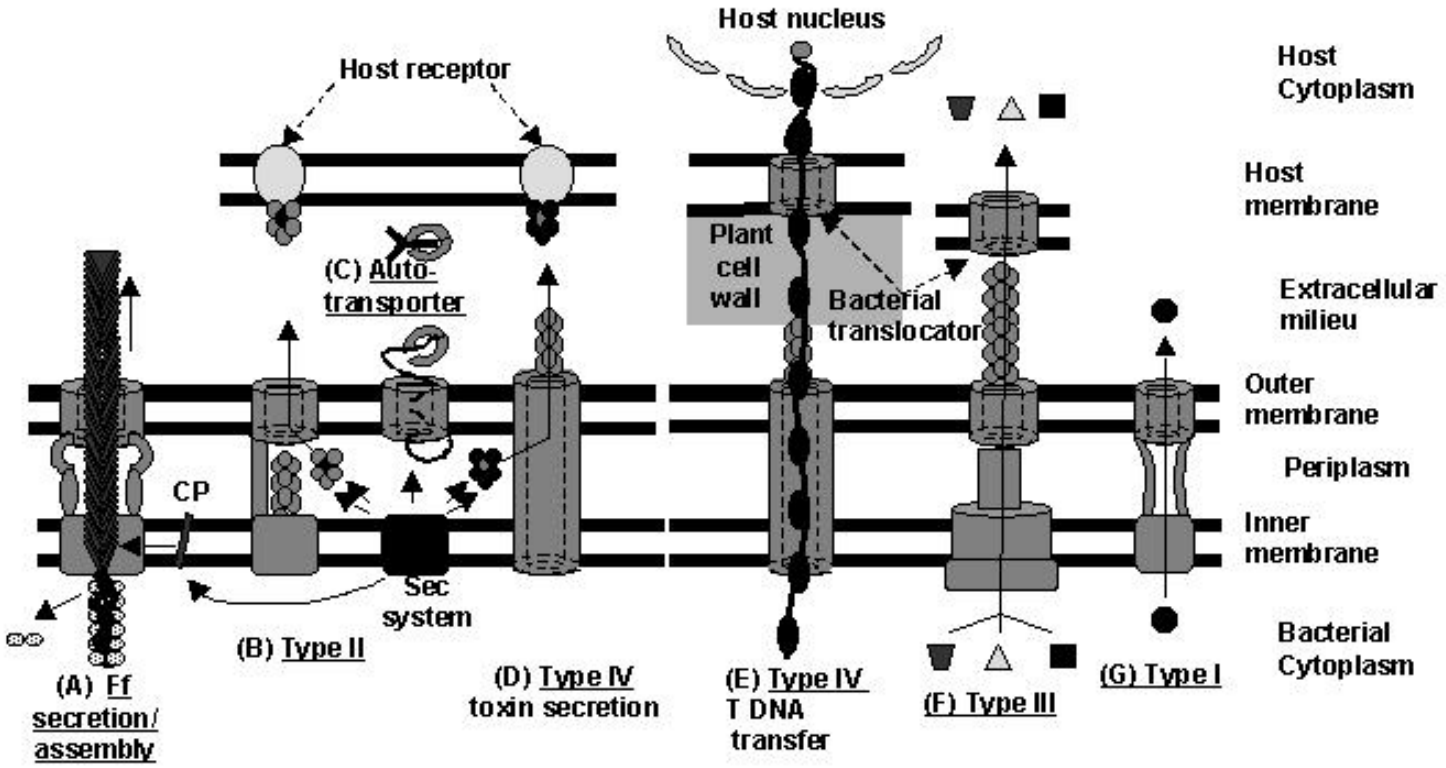
Host nucleus
Host receptor
Host Cytoplasm
Host membrane
Plant cell wall
(C) Auto-transporter
Bacterial translocator
Extracellular milieu
Outer membrane
Periplasm
CP
Inner membrane
Sec system
Bacterial Cytoplasm
(A) Ff secretion/ assembly
(B) Type II
(D) Type IV toxin secretion
(E) Type IV T DNA transfer
(F) Type III
(G) Type I

Fig. 1