Secretion Systems of *Acinetobacter baumannii*

Subjects: Microbiology

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Infections led by *Acinetobacter baumannii* strains are of great concern in healthcare environments due to the strong ability of the bacteria to spread through different apparatuses and develop drug resistance. Secretion systems have recently been demonstrated to be involved in the pathogenic process, and five types of secretion systems out of the known six from Gram-negative bacteria have been found in *A. baumannii*. They can promote the fitness and pathogenesis of the bacteria by releasing a variety of effectors. Additionally, antibiotic resistance is found to be related to some types of secretion systems.

Acinetobacter baumannii | secretion systems | pathogenicity

1. Introduction

Acinetobacter baumannii is a strictly aerobic, non-fermenting, Gram-negative coccobacillus with pili and capsule, but no flagella. It is ubiquitous in nature, and used to be considered to be of negligible significance due to its low virulence [1]. However, the rapidly increasing nosocomial infections and high mortality caused by *A. baumannii*, as well as its strong drug resistance, have raised people's attention [2]. Taken together with *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Enterobacter*, *Acinetobacter baumannii* has been enrolled as a member of ESKAPE by the Infectious Diseases Society of America (IDSA) in order to emphasize the importance of these pathogens in causing hospital infections and resisting the effects of a variety of antimicrobial drugs [3][4].

The high frequency of *A. baumannii* nosocomial infections is closely related to its strong environmental persistence. *A. baumannii* can survive in nutrient-limited and desiccation environments, and is capable of resisting disinfections [5]. Moreover, it is able to survive for long periods of time on both biotic and abiotic surfaces [6]. Based on these advantages, *A. baumannii* can be easily transmitted patient to patient by air, water, and contact with medical personnel's hands and equipment, thus colonizing multiple sites and finally leading to a variety of infections, such as pneumonia, septicemia, urinary tract infections, meningitis, and skin and wound infections [7][8][9].

Antibiotic resistance is another key factor that contributes to *A. baumannii* infections and outbreaks. The increasing rate of infections caused by drug-resistant *A. baumannii* is a significant issue in hospitals all over the world [10]. The continued overuse and misuse of antibiotics enable *A. baumannii* to develop different types of resistance

mechanisms, e.g., the acquisition of multiple antibiotic resistance genes to produce degradative enzymes, a decrease in bacterial membrane permeability, the alteration of antibiotic targets, the overexpression of efflux pumps, a change in metabolic status, and the formation of biofilms [11]. Therefore, this bacterium can escape the killing of antibiotics and conquer the stress conditions, further leading to infections. *A. baumannii* has an extraordinary genetic plasticity that results in a high capacity to acquire antimicrobial resistance traits [2], thus producing many multidrug-resistant (MDR), extensively drug-resistant (XDR), and even pan-drug-resistant (PDR) strains, representing a significant challenge for therapy in clinics.

Infections are also dependent on virulence factors. Various genes have been revealed to be involved in the pathogenic procedures of iron acquisition, nutrient uptake, adhesion, biofilm formation, invasion, hemolytic activity, and cytolytic activity [12][13]. Among them, protein secretion systems have received much attention. They can transport the virulence factors produced by bacteria into extracellular environments, meaning that the latter will manipulate the host's defenses and facilitate pathogen infection [14][15]. Until recently, six secretion systems from Gram-negative bacteria have been revealed and studied; namely, type I secretion system (T1SS) to type VI secretion system (T6SS). Some of these have been characterized and reported to have specific roles in the pathophysiology of *A. baumannii*, whereas the gene and protein structures of some secretion systems in *A. baumannii* are still not clear and are being explored. Moreover, the association between secretion systems and drug resistance has been discovered in some bacteria, e.g., the T3SS in *Pseudomonas aeruginosa* correlates with a fluoroquinolone resistance phenotype, and the T4SS in many Gram-negative pathogens mediates antibiotic resistance via conjugation [16][17][18]. Meanwhile, the contribution of secretion systems to antibiotic resistance in *A. baumannii* is poorly understood.

2. Type I Secretion System (T1SS)

The T1SS is a highly conserved secretion system in pathogenic Gram-negative bacteria. However, it is less reported in *A. baumannii*. In 2017, the T1SS was first identified in the pathogenic *Acinetobacter nosocomialis* strain M2 upon bioinformatic analysis by Harding et al. [19]. Until now, only two reports have described the structure and function of the T1SS in *Acinetobacter* [19][20].

Gene and Structure

The locus that is homologous to the prototype T1SS of *Escherichia coli* containing the *tolC*, *hlyB*, and *hlyD* genes is found in the M2 chromosome, as well as in *A. baumannii*. In contrast to *E. coli*, these genes are found in three gene clusters, and are most likely in an operon, given that the open reading frame (ORF) for *hlyB* overlaps with both *tolC* and *hlyD* [19] (**Figure 1**a).

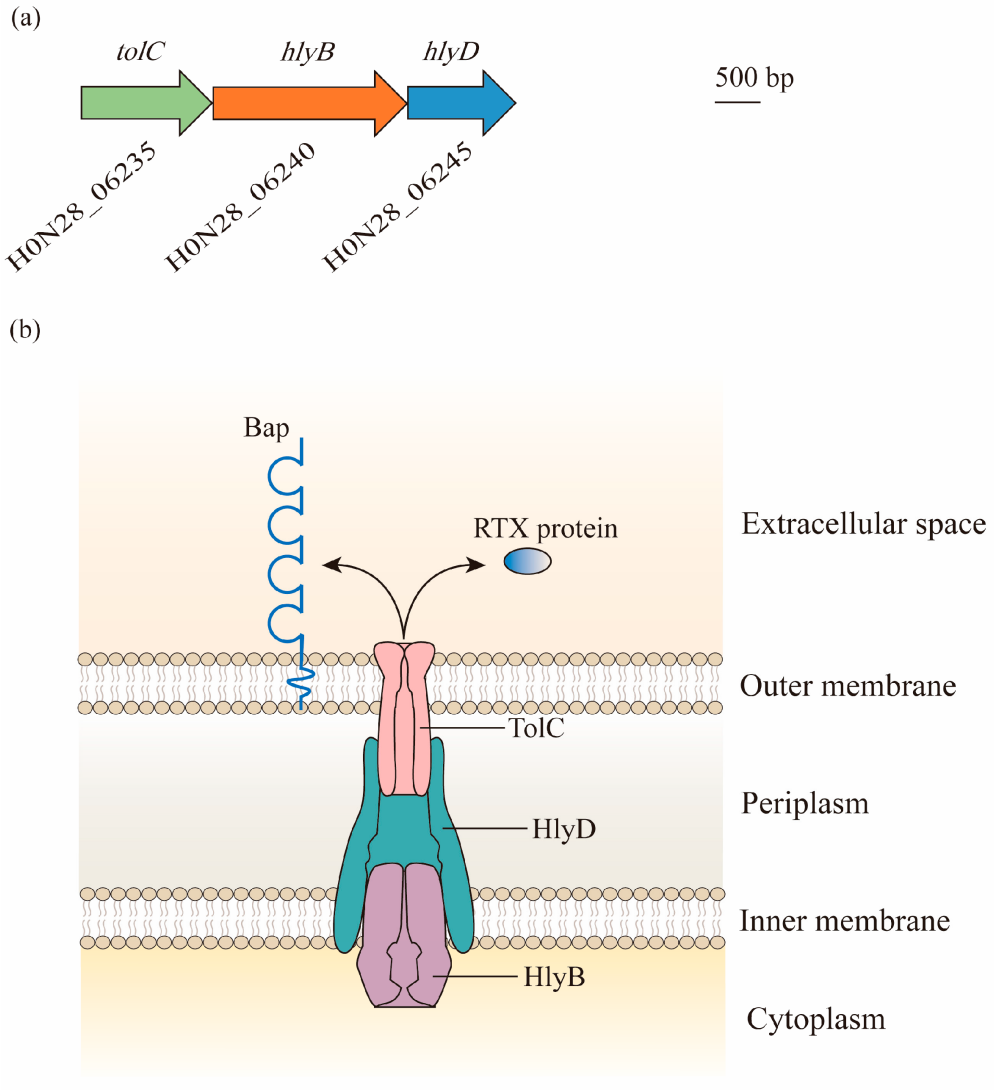


Figure 1. Composition and structure of the type I secretion system (T1SS) in *A. baumannii*: (**a**) Bioinformatic analysis has led to the identification of the T1SS in genomes of *A. baumannii*. Gene locus tags are cited from ATCC 17978. Genes predicted to encode proteins required for the biogenesis of the T1SS are found in three gene clusters, with *hlyB* overlapping with *tolC* and *hlyD*. (**b**) The three components of the T1SS act together to facilitate the secretion of effectors. TolC is a trimeric outer membrane protein with the α-helical barrel forming a tunnel through the periplasm, and it interacts with HlyD. HlyD has a large periplasmic domain linked by a single transmembrane helix, which anchors in the inner membrane. The energy required for the export of specific T1SS substrates is provided by HlyB, which is an ATP-binding protein. Two putative T1SS effectors, namely, Repeats-in-Toxin (RTX)-serralysin-like toxin and biofilm-associated protein (Bap), are involved in the formation and stability of biofilm.

This *tolC-hlyB-hlyD* gene cluster produces three proteins with high molecular weights of 130 kDa, 250 kDa, and 70 kDa. They form a secretion system with the elements of TolC, which is localized in the outer membrane, HlyB, which is anchored in the inner membrane as an ATP-binding cassette transporter, and HlyD as a periplasmic adaptor [19] (**Figure 1**b).

3. Type II Secretion System (T2SS)

The T2SS is a multiprotein secretion system that is widely distributed in Gram-negative bacteria, including enterotoxigenic *Escherichia coli*, *Legionella pneumophila*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* [21][22][23][24][25][26][27]. It was first reported in *A. baumannii* in 2014 and was subsequently shown to be active in ATCC 17978 by Johnson et al. [28][29]. Further, the T2SS is found in the majority of *A. baumannii* genomes.

Gene and Structure

In *Acinetobacter* spp., the T2SS is encoded by 12 essential genes, namely, *gspC-M* and *pilD*, and forms an apparatus spanning both the inner membrane and outer membrane [30][31] (**Figure 2**). In contrast to other Gram-negative pathogens, the core *gsp* genes are not organized in one or two operons, but are grouped into five distinct gene clusters scattered throughout the *Acinetobacter* genome [32] (**Figure 2**a).

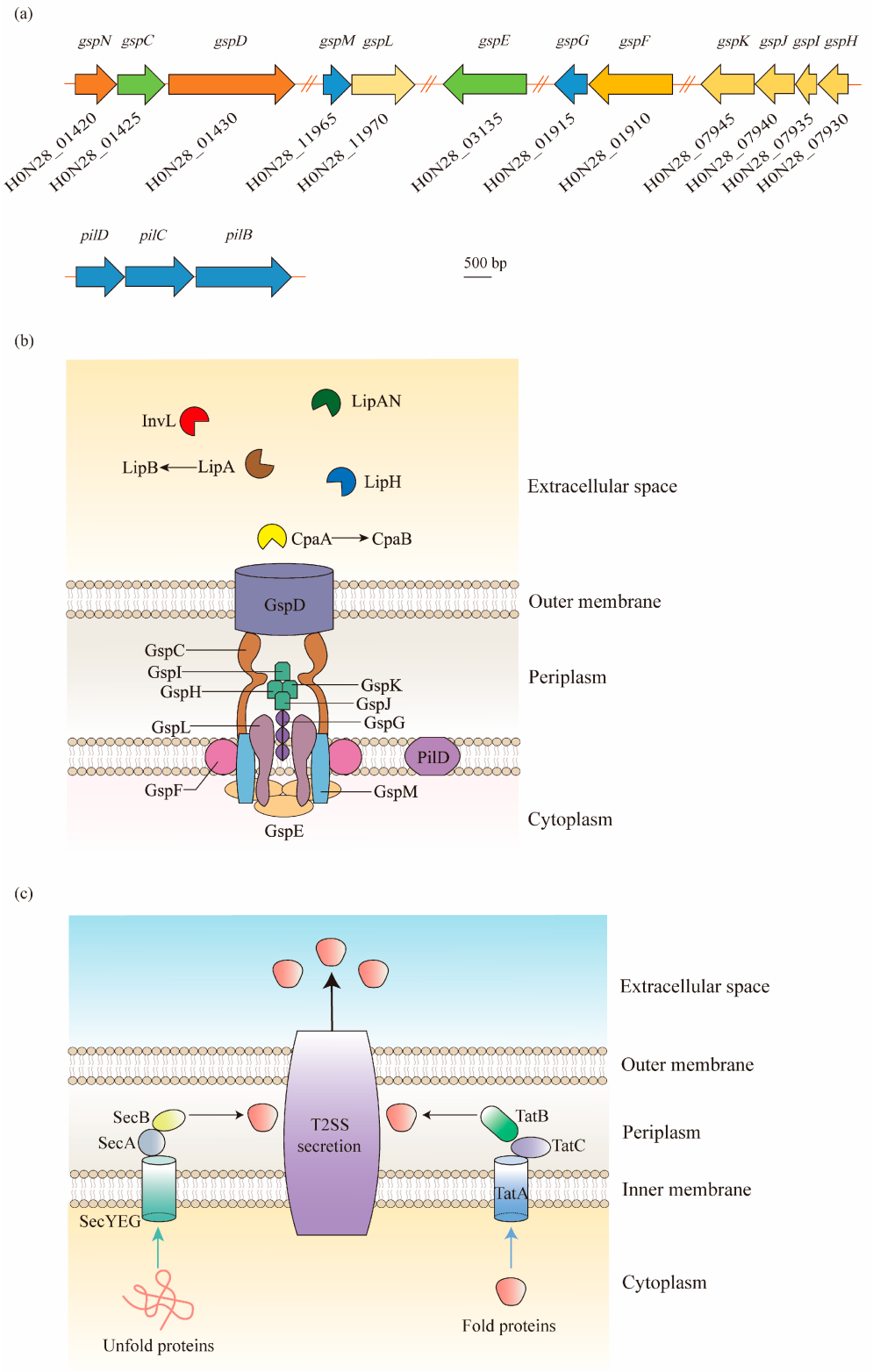


Figure 2. Type II secretion system (T2SS) structure of *A. baumannii* and its protein secretion mechanism: (**a**) As shown in the ATCC 17978 genome, the *gsp* genes required for the T2SS are located in five distant loci, and a single prepilin/pre-pseudopilin peptidase homolog is located in the *pilBCD* cluster. (**b**) The T2SS is composed of an outer membrane (OM) complex (GspD), a periplasmic pseudopilus (GspG, GspH, GspI, GspJ, and GspK), and an inner membrane (IM) platform (GspC, GspF, GspL, and GspM), which relates to the cytoplasmic ATPase GspE. In *A. baumannii*, the T2SS shares a processing protein, PilD, with type IV pili. The T2SS secretes a large number of

effectors required for virulence, including the metallopeptidase CpaA (chaperone CpaB), the lipoyl synthases LipA (chaperone LipB), LipH, and LipAN, and a novel lipoprotein, InvL. (**c**) The T2SS-dependent proteins are first exported across the IM to the periplasm via the Sec or Tat pathways in *A. baumannii*. The Sec pathway primarily translocates unfolded proteins, relying on a hydrophobic signal sequence at the N-terminus. On the contrary, the Tat pathway, consisting of TatA, TatB, and TatC, primarily secretes folded proteins. Afterwards, the signal sequence is cleaved, followed by the folding of proteins. Finally, the folded proteins are expelled extracellularly through the OM channel.

In general, the T2SS consists of four parts: (1) an outer membrane (OM) complex; (2) a periplasmic pseudopilus; (3) an inner membrane (IM) complex called the assembly platform (AP); and (4) a cytoplasmic ATPase [33]. The OM complex is composed of GspD, which forms a secretin channel across the outer membrane to transport substrates from the periplasm to the extracellular milieu [34]. The IM platform is composed of GspC, GspF, GspL, and GspM, in which GspC is joined to the periplasmic domains of GspD, thereby connecting the IM platform with the OM complex. In between the OM and IM complexes, the periplasmic pseudopilus, a structure homologous to the type IV pilus, is attached to the IM platform with the composition of major pseudopilin GspG and minor pseudopilins GspH, GspI, GspJ, and GspK. Before the assembly of these subunits, PilD is involved in the cleavage and methylation procedure. Additionally, the cytoplasmic ATPase is formed by a hexamer protein, GspE, to provide ATP to the T2SS for the secretion of effector proteins [33][35] (**Figure 2**b).

4. Type IV Secretion System (T4SS)

T4SSs are multiprotein nanomachines, widespread in Gram-negative and Gram-positive bacteria, that deliver macromolecules, e.g., DNA and protein, to bacterial recipients or eukaryotic target cells [36]. They are generally divided into three groups; namely, type F and P (IVA), IVB, and GI systems [37][38]. However, T4SSs are less reported in *A. baumannii*. The information can be summarized from five studies, as discussed below. By using the high-density pyrosequencing method, the elements homologous to the Legionella/Coxiella T4S apparatus were first discovered in *A. baumannii* ATCC17978 [39]. Later, in a pathogenic isolate, ACICU, the plasmid pACICU2 was found harboring a complete *tra* locus, which encoded the conjugative apparatus and an F-type T4SS (based on the F-plasmid of *Escherichia coli*) [40]. However, the structure and function of the *A. baumannii* T4SS were not illustrated in these two studies. Furthermore, the plasmid replicase (*rep*) gene *rep*Aci6 from pACICU2 was found widely distributed in *A. baumannii* clinical strains, which carried the T4SS protein TraC coding gene [41][42]. Thus, *rep*Aci6 served as a candidate for screening the F-type T4SS, and the plasmid carried the genes required for the biogenesis of the T4SS, such as *traC*, *traD*, and *traU*, which were identified in clinical carbapenem-resistant *A. baumannii* (CRAB) isolates [43].

Gene and Structure

The F-type T4SS in *A. baumannii* contains a series of *tra* operon genes, including *traA*, *traB*, *traC*, *traD*, *traE*, *traF*, *traG*, *traH*, *traI*, *traK*, *traL*, *traM*, *traN*, *traU*, *traV*, and *traW*, as well as another two genes, *trbC* and *finO*. Through the alignment of seven F-like *A. baumannii* plasmids, it was observed that the core genes involved in pilus

biosynthesis (*traA*, *traB*, *traC*, *traF*, *traH*, *traK*, *traU*, *traV*, *traW*, and *trbC*), nicking (*traI*), the initiation of transfer (*traM* and *traD*), mating aggregate stabilization (*traN* and *traG*), and regulation (*finO*) were highly conserved [43] (**Figure 3**a).

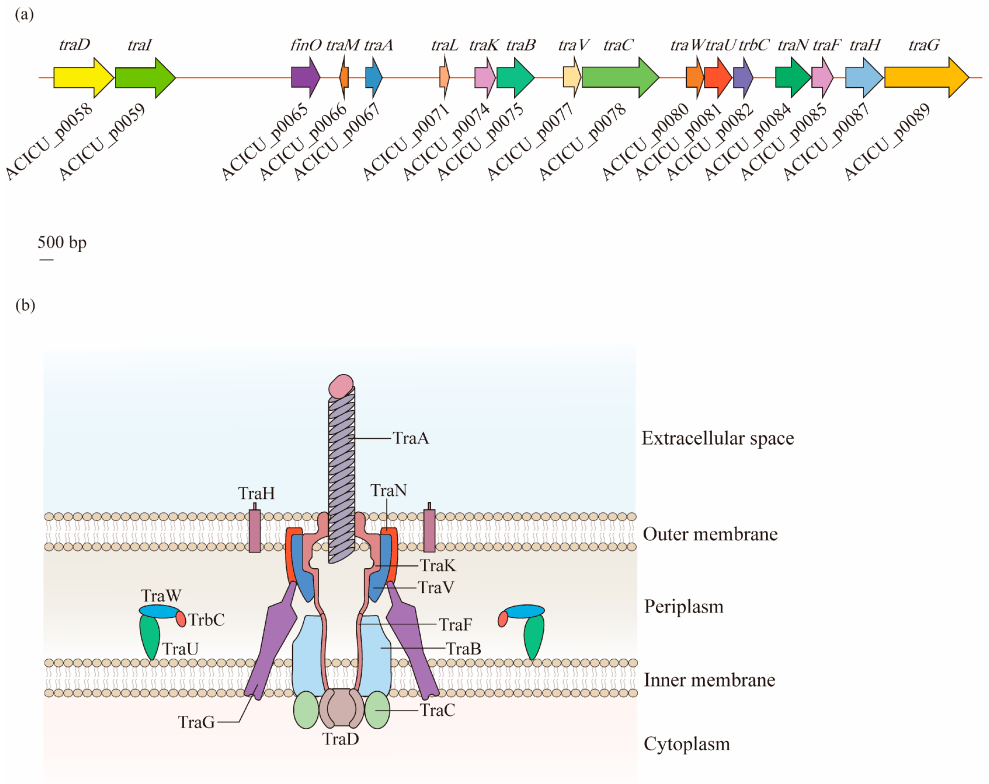


Figure 3. Structural organization of the type IV secretion system (T4SS) in *A. baumannii*: (**a**) Discovered in the *A. baumannii* ACICU plasmid pACICU2, the F-type T4SS contains a series of *tra* operon genes, and two other genes, *trbC* and *finO*. (**b**) The T4SS is a highly sophisticated nanomachine spanning the entire bacterial cell envelope in *A. baumannii*. The F-like T4SS apparatus is composed of a pilus assembly component (TraA), a core complex (TraK, TraV, TraN, and TraH) embedded in the outer membrane (OM), an inner membrane (IM) platform (TraF, TraB, TraG, TraU, TraW, and TrbC), and components of the cytoplasm (TraC and TraD).

According to the analysis of Liu et al. [43], the T4SS of *A. baumannii* is a symmetrical barrel-shaped structure that is divided into the following units: (1) the pilus assembly component localized in the extracellular space across the OM (TraA); (2) the core complex embedded in the OM (TraK, TraV, TraN, and TraH); (3) the constituents of an IM platform (TraF, TraB, TraG, TraU, TraW, and TrbC); and (4) the components of the cytoplasm (TraC and TraD). This structure is similar to that of the typical VirB/D4 T4SS, which exists on the *Agrobacterium tumefaciens* Ti plasmid, and has gene consistency with *tra* operons as *traB*/*virB10*, *traC*/*virB4*, and *traD*/*virD4* [36][44] (**Figure 3**b).

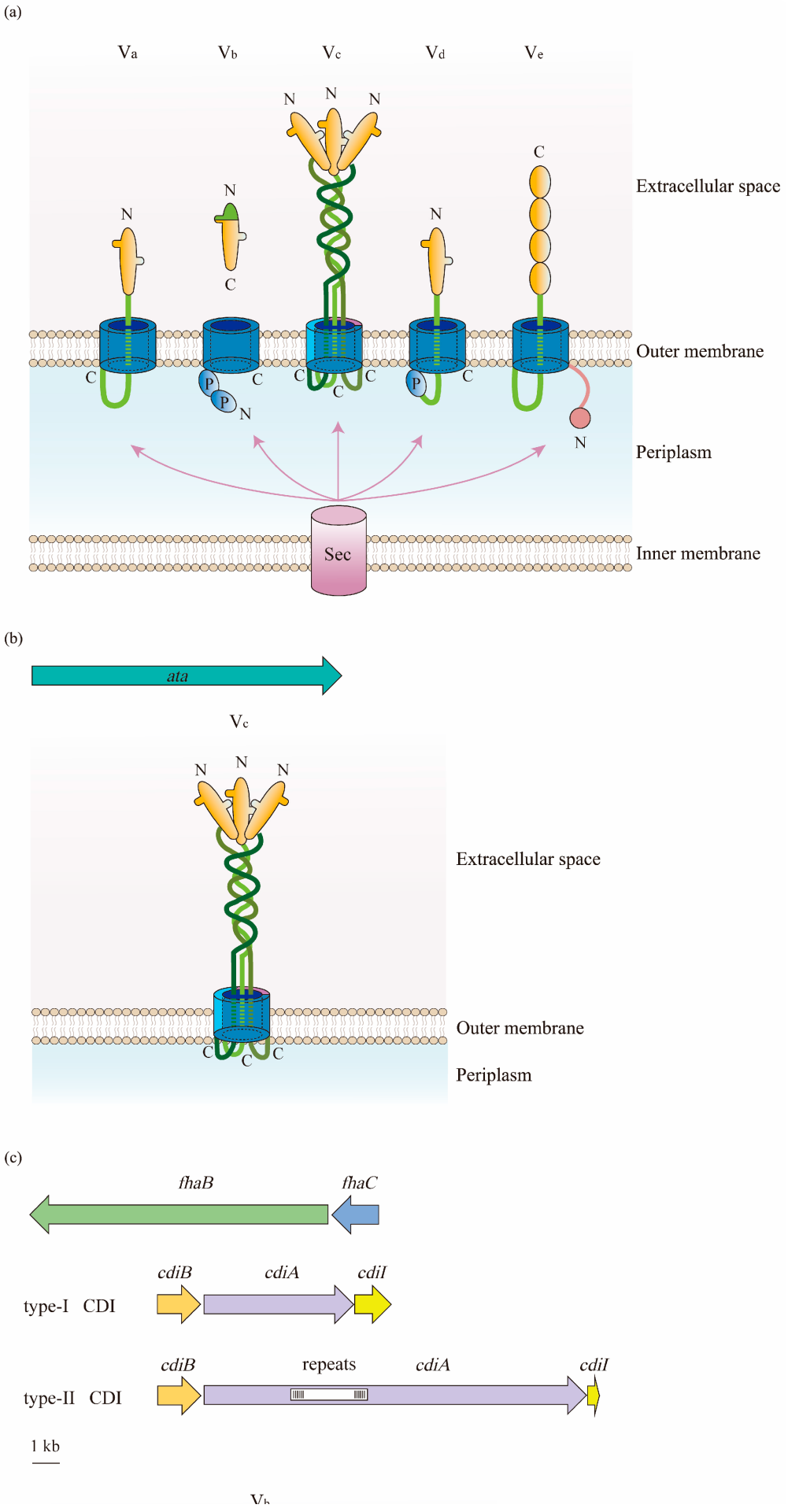
5. Type V Secretion System (T5SS)

The T5SS, also known as the autotransporter, is a series of simple protein export pathways that are distributed in a large range of Gram-negative bacteria [45]. They are classified into monomeric autotransporters (MA), trimeric

autotransporters (TA), and two-partner secretion systems (TPSS), with the composition of a single polypeptide for MA and TA, and separate polypeptide chains for TPSS [46][47]. Depending on the different structural features and domain organization, the T5SS is divided into five known subclasses, so-called types Va to Ve, and possibly another recently identified type, Vf [47]. However, only two types, Vb and Vc, have been identified in *A. baumannii* [27].

Gene and Structure

In contrast to other types of secretion systems that span the entire cell envelope with a syringe-shape structure, the T5SS only spans the OM. The T5SS consists of three major regions; namely, a signal sequence at the N-terminus, an extracellular secreted passenger, and a β-barrel domain (transporter) at the C-terminal that anchors the protein to the bacterial OM [47][48] (**Figure 4**a). Being produced in the cytoplasm, the protein is recognized at the N-terminal signal peptide, which targets the Sex complex to mediate the inner-membrane translocation of the protein to the periplasm [27]. Thereafter, the C-terminal transporter domain inserts into the OM and secretes the protein to the external environment through its OM pore. Finally, the passenger domain located between the signal peptide and the β-barrel domain displays the specific effector function extracellularly after proteolytic cleavage [33].


(a)
Va
Vb
Vc
Vd
Ve
Extracellular space
Outer membrane
Periplasm
Inner membrane
Sec
(b)
ata
Vc
Extracellular space
Outer membrane
Periplasm
(c)
fhaB
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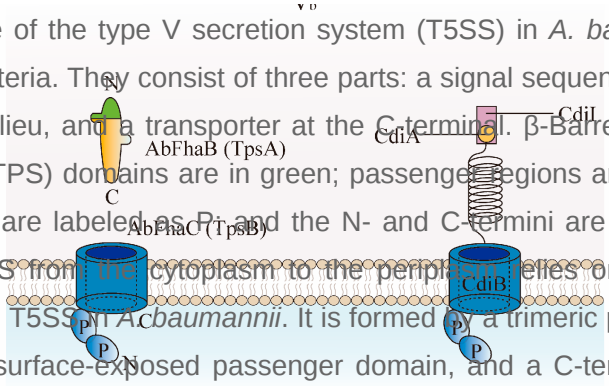

Figure 4. Structure of the type V secretion system (T5SS) in *A. baumannii*: (**a**) There are five types of T5SS in Gram-negative bacteria. They consist of three parts: a signal sequence at the N-terminus, a secreted passenger in the extracellular milieu, and a transporter at the C-terminal. β-Barrels are displayed in blue; linkers and the two-partner secretion (TPS) domains are in green; passenger regions are in orange; polypeptide transport-associated (POTRA) domains are labeled as P, and the N- and C-termini are indicated. The translocation of substrates for subclasses of T5SS from the cytoplasm to the periplasm relies on the Sec pathway. (**b**) Type Vc is the most frequently identified T5SS in *A. baumannii*. It is formed by a trimeric protein, Ata, which contains a signal peptide at the N-terminus, a surface-exposed passenger domain, and a C-terminal domain. (**c**) Two forms of type Vb are found in *A. baumannii*. The one belonging to the TPSS is constructed of AbFhaB and AbFhaC, which represent TpsA and TpsB in other Gram-negative bacteria, respectively. AbFhaB (TpsA) is the passenger domain that is secreted out of cells through the outer membrane (OM) by AbFhaC (TpsB), which is the translocator domain located in the OM. Another one is the contact-dependent inhibition (CDI) system composed of CdiA and CdiB. Similar to TpsA, the toxin CdiA is released from the periplasm to the cell surface by the OM transporter CdiB.

Type Vc is the most popular T5SS in the *A. baumannii* chromosome that belongs to the TA family. Therefore, the protein of type Vc in this bacterium is designated as the *Acinetobacter* trimeric autotransporter (Ata) [49]. Encoded by the *ata* gene, the autotransporter Ata contains a long signal peptide followed by an N-terminus, a surface-exposed passenger domain, and a C-terminal domain encoding four β-strands [49] (**Figure 4**b).

In contrast to classical autotransporters, type Vb belongs to TPSS, where the passenger and translocator (β-barrel) domains locate in two distinct polypeptide chains that are formed by TpsA and TpsB [46]. TpsA and TpsB are encoded in one operon, and the former connects at the polypeptide transport-associated (POTRA) domain of the latter for secretion through the OM to either be surface-displayed or transported extracellularly [50]. In this way, when releasing the passenger out of the cells after being transported by the β-barrel domain, there is no need for release by proteolytic cleavage [47]. In the *A. baumannii* strain AbH12O-A2, AbFhaB and AbFhaC were found to represent TpsA and TpsB, respectively, due to the highly conserved structure of these proteins [51] (**Figure 4**c).

Another type of Vb recently observed in *A. baumannii* is the CDI system composed of CdiA and CdiB. CdiA is a large multi-domain protein that forms a filament folded as a β-helix, similarly to TpsA, and has a C-terminal toxin domain. The CdiA protein in the periplasm is released to the cell surface by the OM transporter CdiB, and its β-helix presents the toxin domain to the neighboring bacteria, finally inhibiting their growth [52]. A cytoplasmic immunity protein, CdiI, is also expressed by the CDI operon to protect bacteria from fratricide and auto-inhibition by CdiA toxins [53][54][55] (**Figure 4**c).

6. Type VI Secretion System (T6SS)

The T6SS is a multiprotein transmembrane nanomachine discovered in numerous Gram-negative bacteria, including *Vibrio cholerae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Francisella tularensis*, and *Yersinia pseudotuberculosis* [56]. It is syringe-shaped and is commonly used by bacteria to inject toxic effectors into competitors or host cells [57]. Several parts of this secretion system are structurally and

functionally homologous to the T4 bacteriophage tail, suggesting a common evolutionary origin of this apparatus [58]. In recent years, an increasing number of studies have reported various aspects of the T6SS from *A. baumannii*, including its composition, structure, regulation, and function, confirming it as an important virulence factor.

Gene and Structure

The T6SS in *A. baumannii* is found in a cluster located in the genome that contains 18 genes, arranged as *asaA-tssBC-hcp*(*tssD*)*-tssEFG-asaB-tssM-tagFN-asaC-tssHAKL-asaDE*, while genes of *vgrG*, also known as *tssI*, which are scattered in various numbers throughout the genome [59][60]. In these genes, 12 encode the core T6SS proteins (Tss, Hcp, and VgrG), two encode the TagF and TagN that are associated with the T6SS in other bacteria, and five encode the Asa proteins that only appear in *Acinetobacter* spp. [59] (**Figure 5**a). Based on the Tss core proteins, the T6SS is composed of three main parts: a membrane complex, a cytoplasmic baseplate, and a contractile tail tube/sheath complex (**Figure 5**b).

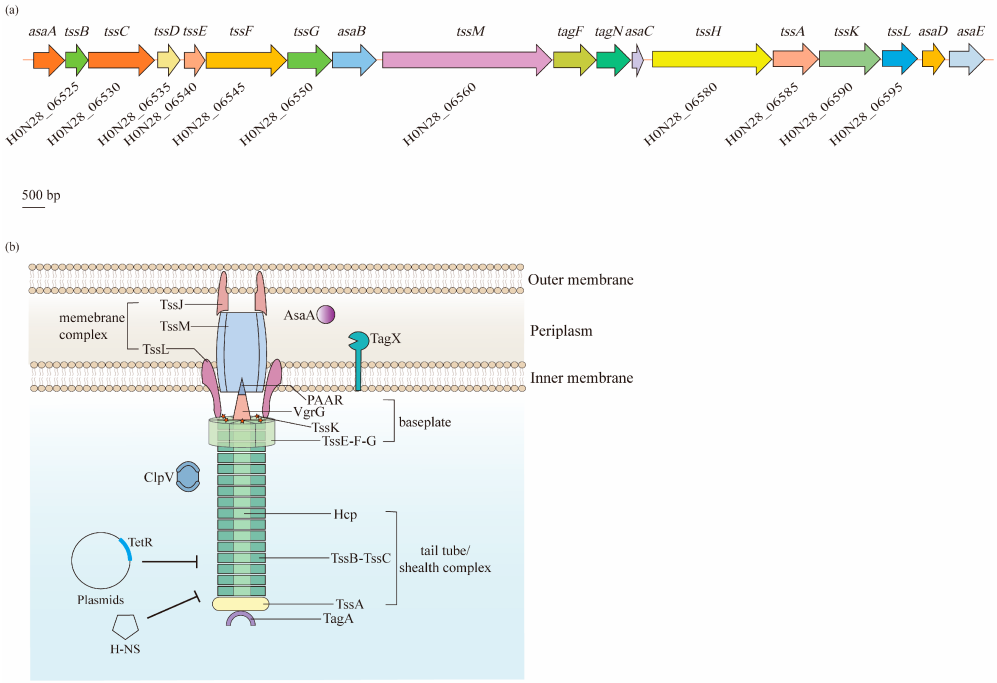

Figure 5. Biogenesis and regulation of the type VI secretion system (T6SS) in *A. baumannii*. The T6SS is a class of macromolecular secretion machines, which translocate proteins into a variety of recipient cells: (**a**) A single gene cluster carries 18 putative genes that are predicted to encode components of the T6SS. Among them, 12 core genes (*tss*) are coded on the chromosome of *A. baumannii* ATCC 17978. (**b**) The T6SS is composed of three main parts: a membrane complex (TssJ, TssL, and TssM), a cytoplasmic baseplate (TssK, TssF, TssG, TssE, VgrG, and PAAR), and a contractile tail tube/sheath complex (Hcp, TssB, TssC, and TssA). The expression of the T6SS is negatively regulated by the TetR-like proteins encoded on the large, conjugative plasmid pAB3 and proteins within the H-NS family.

Normally, in a wide range of bacteria, the membrane complex consists of the TssJ, TssL, and TssM proteins that span the cell envelope, with the complex anchored in the IM and the tip embedded in the OM, but not crossing it [61]. Notably, TssJ, an OM lipoprotein interacting with TssM, is absent in *A. baumannii* [60]. TssM and TssL have strong homology with the T4bSS proteins IcmF and IcmH (or DotU), respectively [62][63]. TssM is a core component of the T6SS that anchors to the IM through three transmembrane segments [63]. Similarly, the cytoplasmic protein TssL is also bound to the IM, but through a single transmembrane helix. Two residues of TssL in *A. baumannii*, Asp98 and Glu99, are strongly conserved among T6SS-encoding Gram-negative bacteria, and remarkably impact the dynamics, expression, and functionality of this protein [64]. TssM and TssL are involved in the recruitment and secretion of Hcp, and are important for the activity of the T6SS [65].

The baseplate complex is a central piece of the T6SS machinery that consists of six $(TssK)_6$-$(TssF)_2$-$(TssG)_1$-$(TssE)_1$ wedges around a central $(VgrG)_3$-PAAR spike. It connects the tail to the membrane complex and initiates the polymerization of the tail tube/sheath complex [66]. TssG is the core component of a baseplate wedge, where its C-terminal domain acts as an adaptor to interact with both TssF and TssK. VgrG, which binds to the PAAR-repeat protein at its distal extremity, is essential for the assembly of the Hcp tube, thus significantly contributing to the structure of the T6SS in various bacteria, including *A. baumannii* [67][68][69].

The tail tube/sheath complex is a contractile structure formed by the Hcp tube, TssBC sheath, and TssA cap. Although VgrG locates in the center of the baseplate complex, it is identified as an extension of the Hcp tube, as the central density of the latter is uniform from the first ring docked on top of the $(VgrG)_3$-PAAR spike [68]. Normally, the inner Hcp tube assembles onto the base of VgrG and extends into the cytoplasm. Simultaneously, the TssBC helical sheath polymerizes around the Hcp tube in an extended, high-energy “primed” conformation [70]. Additionally, its proximal ring has been suggested to interact with the TssK-TssF-TssG complex [67]. After contraction, the sheath is disassembled by the AAA^+ ATPase ClpV for a new assembly cycle of an extended sheath [71]. Lastly, TssA is involved in the assembly of Hcp-TssBC, and caps the distal end of this structure [70][72].

In addition to the core components, additional auxiliaries are required for the *A. baumannii* T6SS to ensure the correct assembly and full activity. For example, TagF and TagN were identified to negatively regulate the activity of the T6SS, where the absence of these two proteins increased the secretion of Hcp [73]. Moreover, AsaA was demonstrated to localize in the periplasmic space and affect the assembly or stability of the T6SS by interacting with TssM [74]. Additionally, a novel peptidoglycan hydrolase, TagX, was proposed to be required for the transit of the T6SS machinery across the peptidoglycan layer, thus finally allowing the assembly of the T6SS [73].

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