Multiple pathways allow protein secretion across the bacterial outer membrane
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Secretion of proteins across the bacterial outer membrane takes place via a variety of mechanisms from simple one-component systems to complex multicomponent pathways. Secretion pathways can be organized into evolutionarily and functionally related groups, which highlight their relationship with organelle biogenesis. Recent work is beginning to reveal the structure and function of various secretion components and the molecular mechanisms of secretion.

Introduction
Protein secretion is required for numerous aspects of the bacterial life cycle, including organelle biogenesis, nutrient acquisition, and virulence-factor expression. Gram-negative bacteria face a special challenge in this regard, as secreted proteins must cross the periplasm and the outer membrane (OM) in addition to the cytoplasmic or inner membrane (IM). Secretion is defined in this review as any process in which a protein crosses the OM barrier. Thus, in addition to export of proteins to the extracellular medium, secretion includes export of proteins that remain anchored to the cell surface or are assembled into cell-surface organelles.

Secretion across the OM represents a different problem from export of unfolded proteins across the IM. Proteins acquire structure in the periplasm, including formation of disulfide bonds, and may completely fold before crossing the OM. In addition, ATP or other sources of energy are not known to be present at the OM. Export systems must therefore be self-energized or have mechanisms for harnessing the energy available at the IM. Gram-negative bacteria have evolved a number of pathways for protein secretion that can be organized into six overarching groups. Four of these pathways represent terminal branches of the general secretory pathway (GSP), exporting proteins with cleavable amino-terminal signal sequences that require the Sec system for translocation across the IM (see [1] for a recent Sec review). The other pathways are Sec-independent and capable of exporting substrates directly from the cytoplasm outside the bacteria. At least three pathways share secretion mechanisms with, and probably evolved from, organelle biogenesis systems, and one pathway is dedicated to the assembly of surface structures. This review will present a brief overview of each secretion pathway, focusing on secretion mechanisms and will highlight important advances over the past year. Readers are referred to recent reviews for in-depth discussions of each pathway [2–8].

Autotransporters and proteins requiring single accessory factors
The autotransporter secretion pathway is a terminal branch of the GSP that exports proteins with diverse functionalities, including proteases, toxins, adhesins and invasins [2]. The prototypical member of the autotransporter family is the Neisseria gonorrhoeae IgA1 protease [9]. This pathway has also been termed type IV secretion. However, the type IV nomenclature has also been claimed for the much more complex DNA- and protein-exporting system described below. To avoid confusion, this review will use the more descriptive autotransporter designation.

Autotransporters do not require accessory factors for transit from the periplasm to the bacterial surface (Figure 1). A typical autotransporter contains three domains: an amino-terminal signal sequence for secretion across the IM by the Sec system, an internal passenger or functional domain, and a carboxy-terminal β-domain [2]. The β-domain inserts into the OM to form what is predicted to be a β-barrel pore structure, similar to the bacterial porins, through which the passenger domain passes to the cell surface. A linker region connecting the passenger and β-domains is also essential for export and may guide the passenger region through the β-domain channel [10]. Secretion across the OM does not appear to require input of external energy. Once secreted, the passenger domain is either retained on the bacterial surface or released into the environment by proteolysis. The nature of the passenger domain does not appear to be important, as foreign proteins can be substituted for this domain and successfully secreted [11,12].

Work on secretion of foreign proteins indicates that formation of disulfide bonds by the passenger domain hinders export to the cell surface, pointing to a requirement for unfolded or partially folded substrates [11,12]. However, this is contradicted by recent research showing export of a functional, disulfide-bonded antibody fragment fused to the IgA β-domain [13*]. Much attention has been focused on the structure of the β-domain. Computer modeling and gel-mobility studies support the formation
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of a β-barrel structure [2,10]. The β-domain of the Bordetella pertussis autotransporter BrkA was recently shown to form pores in experimental lipid bilayers [14]. It is unknown whether a single β-domain forms the channel or if oligomerization is required.

A separate pathway has been identified that requires a single accessory factor for secretion across the OM. Examples include export of B. pertussis filamentous hemagglutinin and Serratia marcescens hemolysin by FhaC and ShlB, respectively [15,16]. The accessory protein is thought to form a β-barrel pore in the OM for secretion of the substrate to the cell surface (Figure 1). Foreign proteins can be secreted by this pathway, but acquisition of disulfide bonds appears to block export to the cell surface [17]. Recent work demonstrated that both FhaC and ShlB form channels in experimental lipid bilayers [15,16]. The channels may be gated and evidence suggests that ShlB acts as a monomer. This single accessory pathway appears to be functionally similar to the autotransporters, except that separate proteins encode the passenger and β-domains. However, there is no sequence homology between the two systems and the accessory factors appear instead to be related to a group of OM proteins that share homology with a protein-translocating porin found in the outer envelope of chloroplasts [18]. Thus, the autotransporter and single accessory pathways probably evolved independently and may, in fact, employ completely different mechanisms.

Chaperone/usher pathway

The chaperone/usher pathway is a branch of the GSP dedicated to the assembly and secretion of a broad range of adhesive virulence structures on the Gram-negative bacterial surface [3]. Secretion across the OM by this pathway requires only two components: a periplasmic chaperone and an OM protein termed an usher (Figure 2). The prototypical organelles assembled by this pathway are P and type I pili, expressed by uropathogenic Escherichia coli [19,20]. These pili are composite structures consisting of a thin, flexible tip fibrillum connected to a rigid, helical rod (Figure 2) [21]. Following export across the IM via the Sec pathway, pilus subunits interact with the periplasmic chaperone via a conserved carboxy-terminal motif present on each of the pilus subunits. The chaperone facilitates release of pilus subunits into the periplasm, guides their proper folding, and caps subunit-interactive surfaces to
prevents premature interactions in the periplasm [22].

Next, periplasmic chaperone–subunit complexes are targeted to the OM usher [23,24].

Interaction with the usher triggers chaperone dissociation from the subunit by an unknown mechanism, exposing subunit-assembly surfaces and allowing incorporation of the subunit into the pilus fiber. The usher provides a translocation channel through the OM for secretion of the pilus. The P pilus usher, PapC, has been imaged by electron microscopy [3] and revealed as ring-shaped oligomeric structures containing central pores 2–3 nm in diameter. Pore formation was confirmed by reconstitution of purified usher protein into liposomes [3]. Interestingly, OM components of the unrelated type II and type III secretion systems also form ring-shaped oligomeric complexes with central pores (see below). A 2–3 nm usher channel would be sufficient to allow passage of folded subunits assembled into a linear fiber, such as the pilus tip fibrillum, but not the helical pilus rod. Therefore, the pilus rod would be constrained to a linear fiber of folded subunits while traversing through the usher. It would only be able to coil into its final helical conformation once it reached the cell surface (Figure 2). The chaperone/usher pathway does not appear to require input of external energy for secretion of pili across the OM [25] and coiling of the rod outside the cell may facilitate the outward translocation of pili.

Recently, crystal structures of FimC–FimH and PapD–PapK (corresponding to chaperone-subunit complexes from type I and P pili, respectively) have been solved (Figure 2) [26**,27**]. The chaperone (green) consists of two Ig folds. The subunit (blue) consists of a single Ig fold that lacks the usual seventh β strand, resulting in exposure of its hydrophobic core. The G1 β strand of PapD binds to the conserved carboxy-terminal motif of PapK, donating its hydrophobic residues to complete the structure of the subunit in a mechanism termed donor strand complementation. Chaperone–subunit complexes are targeted to the OM usher for assembly into pili and secretion across the OM. Subunit–subunit interactions are thought to take place by interaction of conserved amino-terminal (black box) and carboxy-terminal (white box) motifs. The amino-terminal motif of one subunit may complete the structure of the preceding subunit in a mechanism termed donor-strand exchange to build the pilus fiber. The usher channel is only able to allow passage of a linear fiber of folded subunits, forcing the pilus rod to adopt its final helical conformation at the cell surface. The location of Pap subunits in the pilus is indicated.

In the chaperone–subunit complexes, the chaperone donates its G1 β-strand and a portion of its F1-G1 loop to complete the Ig fold of the subunit by occupying the groove (Figure 2). This donor-strand complementation interaction stabilizes the subunit by shielding its hydrophobic core. Indeed, alternating hydrophobic residues of the G1 strand become an integral part of the hydrophobic core of the subunit. Part of the groove is formed by the conserved carboxy-terminal subunit motif, which has also been
implicated in subunit–subunit interactions [28]. Thus, the donor-strand complementation interaction caps an interactive surface and prevents premature pilus formation in the periplasm. Indeed, the folding, stabilization, and capping of the subunit may be part of a single dynamic process that ensures subunit interactive surfaces are not accessible until pilus biogenesis at the usher.

The crystal structures suggest a model for pilus assembly. Subunits also have an amino-terminal extension with a conserved alternating hydrophobic motif that participates in subunit–subunit interactions [28]. This motif is similar to the alternating hydrophobic motif present in the chaperone G1 β-strand. The amino-terminal extension projects away from the subunit, where it would be free to interact with another subunit [27••]. During pilus biogenesis, the amino-terminal extension of one subunit may displace the chaperone G1 β-strand from its neighboring subunit in a mechanism termed donor-strand exchange. The mature pilus would thus consist of an array of Ig domains, each of which contributes a strand to the fold of the preceding subunit to produce a highly stable organelle.

**Type I secretion**

Type I or ATP-binding cassette (ABC) protein exporters are employed by a wide range of Gram-negative bacteria for secretion of toxins, proteases and lipases [4]. Secretion of α-hemolysin by *E. coli* represents the prototypical type I exporter [29]. Related ABC systems are conserved from prokaryotes to eukaryotes and export a variety of toxic compounds and antibiotics. The type I pathway is Sec independent and secretes proteins directly from the cytoplasm across the OM without a periplasmic intermediate. Substrates of this pathway lack a cleavable amino-terminal signal sequence, instead, they possess a carboxy-terminal ~60 amino acid secretion signal [4]. The type I export apparatus consists of three proteins: an IM ABC exporter, an IM-anchored protein that spans the periplasm, termed a membrane fusion protein (MFP), and an OM protein (OMP) (Figure 3).

TolC, the OMP for hemolysin export, assembles as a trimeric complex in the OM and is predicted to consist of a porin-like β-barrel membrane domain with a carboxy-terminal hydrophilic region that extends into the periplasm [30]. However, recent sequence analysis indicates that TolC and other OMPs are unrelated to porins [31•]. The OMP presumably functions as the OM secretion channel. Indeed, pore-forming activity by TolC oligomers has been demonstrated in experimental lipid bilayers [32]. The periplasmic MFP component also assembles as a trimer and interacts with both the OMP and ABC exporter [29,33]. MFPs typically contain a hydrophobic amino terminus believed to span the IM or are anchored in the IM by lipid modification of the amino terminus [31•]. The bulk of the MFP is thought to extend across the periplasm to contact the OMP and/or the OM itself. The MFP is thought to facilitate substrate secretion without a periplasmic intermediate by forming a closed bridge or channel across the periplasm or by fusing the inner and outer membranes, allowing direct contact of the ABC exporter and OMP channel.

Experimental evidence has led to two related models for secretion by the type I pathway. Work on *E. coli* hemolysin secretion [29] indicates that the ABC exporter and MFP associate before substrate binding. Substrate binding to this complex then triggers contact of the MFP with the OMP. This bridging is transient, collapsing after export of the substrate. ATP hydrolysis by the ABC exporter drives substrate release outside the cell and is not required for substrate binding or assembly of the complex.

Work on *S. Marcescens* hemoprotein and *Erwinia chrysanthemi* metalloprotease secretion points towards a slightly different order of events [33]. In this model, the ABC exporter and MFP do not associate before substrate
binding. Instead, an ordered set of associations takes place in which the substrate first binds to the ABC exporter, which then triggers binding of the MFP. This complex in turn interacts with the OMP, allowing secretion of the substrate. Future work is needed to resolve these two models or to determine if they reflect a variation among individual type I mechanisms.

**Type II secretion**

The type II secretory pathway represents a third terminal branch of the GSP, also referred to as the main terminal branch. This pathway is responsible for secretion of extracellular enzymes and toxins by a wide variety of Gram-negative bacteria [6,34,35]. Pullulanase (pul) secretion by *Klebsiella oxytoca* represents the prototypical type II pathway [35]. The type II secretory pathway is closely related to biogenesis of type 4 pili — long, polarly localized pili important for the virulence of many bacterial pathogens [5]. It should be clarified that type II does not refer to the GSP in general, but is one of several terminal branches as described in this review.

Secretion across the OM by the type II branch is much more complex than the autotransporter and chaperone/usher branches, requiring between 12 and 16 accessory proteins, collectively referred to as the secretin (Figure 4) [35]. A unified type II nomenclature has been established, using the designation Gsp (perhaps reinforcing confusion of type II secretion with the GSP) and giving each homologous secreton component a letter based on the *pull* system. The type II secreton includes two OM components: GspD and GspS [34,35]. GspD is an integral OM protein and GspS is a small lipoprotein required in at least some type II systems for proper targeting and insertion of GspD in the OM [36]. GspD secretins form highly stable ring-shaped complexes of 12–14 subunits with central channels ranging from 5–10 nm in diameter, large enough to accommodate folded substrates [38–41]. Secretins share structural similarity with the ring-shaped usher proteins, but there is no sequence homology between these two families. Whether they utilize similar secretion mechanisms remains to be determined.

The PulD secretin was recently imaged by cryo-electron microscopy as a complex with the PulS lipoprotein [36]. Three-dimensional reconstruction revealed a 7.5 nm central channel formed by two stacked rings, with a set of radial spokes, presumably formed by PulS, emanating from one ring. The PulDS complex, as well as isolated secretins from *Pseudomonas* and *fI* phage, form channels in experimental lipid bilayers and there is strong evidence for channel gating [36,42,43]. The amino-terminal half of secretins may serve as the channel gate, whereas the carboxyl terminus appears to direct oligomerization and contain the channel-forming activity [43,44].
Surprisingly, most secreton components are associated with the IM (Figure 4) [34]. At least four IM proteins, GspG, H, I, and J, exhibit limited homology to the type 4 pilus structural subunit, pilin. These ‘pseudopilins’ undergo processing by the GspO IM protein, a prepilin peptidase interchangeable with the type 4 pilus prepilin peptidase (Figure 4) [45]. The pseudopilins have been proposed to assemble into a pilus-like structure that spans the periplasm, although evidence for such a structure has not been found [46,47].

Secreton components GspC, F, K, L, M and N are also IM proteins, all with extensive periplasmic domains. GspE is a cytoplasmic protein that localizes to the IM via interaction with GspL [48,49]. GspE contains a conserved ATP-binding motif and has autokinase activity [48,50]. GspE may regulate secretion or energize the secretion process and/or assembly of the secreton. Accumulated data points towards extensive protein–protein interactions among the IM components [47,49,51]. This leads to a model in which GspE uses ATP to effect conformational changes in the IM proteins that are transmitted to the periplasmic domains and then to the OM. GspC, which fractionates with both the IM and OM, may be responsible for energy transduction to GspD in the OM in a manner similar to that proposed for the role of TonB in siderophore-mediated iron import [52,53].

**Type III secretion**

Type III secretion pathways capable of translocating anti-host factors into the cytosol of target eukaryotic cells have been identified in a number of animal and plant pathogens [7]. Secretion of *Yersinia* outer proteins (Yops) by *Yersinia* spp. represents the prototypical type III export pathway. Translocation of Yops into the cytosol of target cells disrupts signaling pathways, allowing the bacteria to evade host defense mechanisms [54]. Type III secretion is Sec independent, may take place without a periplasmic intermediate, and requires ~20 secretion components that assemble into a large structure that spans both bacterial membranes and possibly the host cell membrane as well.
Type III secretion shares one homologous component with the type II pathway: secretion across the OM requires a member of the secretin family of channel-forming proteins. The *Yersinia* and *Salmonella typhimurium* secretins YscC and InvG, respectively, have been imaged as ring-shaped complexes with large central pores [39,40]. Proper targeting of InvG to the OM requires the InvH lipoprotein, analogous to the role of GspS in the type II pathway.

The majority of type III components are thought to localize to the IM and are closely related to components of the flagellar basal body (Figure 5) [7]. The flagellar basal body spans the inner and outer membranes and provides an anchor for the flagellar filament [55]. The cytoplasmic face of the basal body is thought to contain the machinery that drives secretion and assembly of flagella via ATP hydrolysis. This machinery exports flagellin monomers through a central channel within the basal body and filament for assembly at the distal end of the growing flagellum. Interestingly, the flagellar apparatus was recently shown to function as a protein secretion system [56].

The *S. typhimurium* and *Shigella flexneri* type III secretion structures have recently been visualized by electron microscopy [57,58‡]. The *S. typhimurium* structure is strikingly similar to the flagellar basal body, with a hollow projection extending out from the bacterial surface, termed the needle, in place of a flagellar filament. In addition, the *S. flexneri* structure revealed a large bulb region that presumably extends into the bacterial cytoplasm. This bulb may represent the intact IM machinery that drives assembly and secretion in both the type III and flagellar systems. Both plant and animal type III systems assemble long pili [59,60], possibly through extension of the needle structure. By analogy with flagella, this type III pilus may serve as a secretion tube allowing protein transport within its length.

Type III secretion is highly regulated and two amino-terminal secretion signals have been identified for export of Yop proteins (Figure 5). The first signal appears to reside in the mRNA and may target the RNA–ribosome complex to the type III machinery for coupled translation and secretion, as recently demonstrated for YopQ [61‡]. The second Yop secretion signal serves as the binding site for cytoplasmic chaperones termed Syc proteins and may specifically target Yops to the type III machinery for translocation into host cells [62].

Of great interest is the mechanism of translocation across the host cell membrane. Most models postulate pore
formation in the target cell by type-III-secreted proteins. The *Yersinia* YopB and YopD proteins are good candidates for such an activity [63••,64]. YopB and YopD are substrates of the secretion apparatus, yet are required for translocation of Yop effector proteins into host cells. *Yersinia* and *S. flexneri* expressing type III systems create pores in red blood cell membranes, causing hemolysis [58••,65]. Close contact is required for lysis, which is dependent on YopB in *Yersinia* and the YopB and YopD homologs, IpaB and IpaC, in *S. flexneri*. YopB and YopD are both required for pore formation in macrophage membranes, and both proteins were inserted by the type III system into liposomes [63••,64]. Fusion of these liposomes with experimental lipid bilayers resulted in channel formation, which was dependent on YopB and modified by YopD [63••]. Thus, YopB and YopD may be inserted by the type III secretion apparatus into the plasma membrane of target cells to form a pore for translocation of effector Yops into the host cytosol (Figure 5). However, a recent report questions the role of YopB in translocation [66] and this model remains to be proven.

**Type IV secretion**

Type IV secretion is a recently identified protein secretion pathway homologous to the IncP and IncN bacterial conjugation systems and the VirB system of *Agrobacterium tumefaciens* that facilitates translocation of oncogenic T-DNA into plant cells [8]. Type IV homologs have been identified in *Legionella pneumophila* and *Helicobacter pylori*. A type IV system is also required for pertussis toxin (PT) secretion by *B. pertussis* [67-70]. PT export requires nine gene products of the *ptl* locus, all with homologs in the *virB* locus of *A. tumefaciens*, which contains 11 genes. DNA export by the VirB and conjugal systems is postulated to take place in a single step from the cytoplasm to the outside of the cell. However, evidence strongly suggests PT export occurs in two steps, with the toxin subunits first traversing the IM via the Sec system [8]. Thus, protein export by the type IV pathway may represent a fourth branch of the GSP.

Most information about the type IV secretory apparatus comes from studying the VirB system (Figure 6). A number of VirB proteins are membrane-associated, interact with each other and themselves, and are present in multiple copies. Proteins VirB7-10 fractionate with both the inner and outer membranes and are proposed to contain large periplasmic domains, suggesting the formation of a complex that spans the periplasm [71,72]. VirB7 is an OM-associated lipoprotein that forms a disulfide-bonded heterodimeric complex with VirB9 [73]. VirB7 and B9 are important for the stability of other VirB proteins, which leads to a model in which the B7–B9 complex at the OM nucleates and stabilizes assembly of the secretion apparatus [73]. There is no secretin homolog in the type IV system and the protein or proteins that form the OM secretion channel have yet to be defined. VirB4 and B11 localize to the IM and possess ATPase and/or autokinase activity [74,75].

Mutations in the consensus nucleotide-binding domain of VirB4 exert a dominant-negative effect on secretion, whereas VirB11 mutants are recessive [76]. However, both proteins are thought to act as multimers and to interact with transport components. A subset of VirB proteins consisting of VirB3,4 and B7–10 facilitates uptake of conjugal plasmid DNA [77] and may represent a core type IV transport complex. Interestingly, VirB4 oligomerization, but not nucleotide-binding activity, is required for function of this minimal complex [76]. Thus, protein–protein interactions may drive assembly of a minimal transport complex, and nucleotide-binding activity may be required to configure this complex for secretion.

Reminiscent of the type III pathway, the VirB system assembles pili. VirB2 constitutes the major pilus subunit [78]. VirB2 and the IncP conjugative pilus subunit TbhC are post-translationally processed to a cyclic form via intramolecular head-to-tail peptide bonds [79•]. This novel configuration may confer stability to the pilus, analogous to the donor-strand exchange mechanism described above for chaperone/usher pili. It is tempting to speculate that the VirB pilus mediates cell–cell contact and provides a channel for direct translocation of the T-DNA complex into the host. Whether type IV protein exporters, such as the *ptl* secretion system, assemble pili or mediate direct transfer of proteins into host cells, akin to injection by the type III pathway, remains to be determined.

**Conclusions**

A vast amount of information has been gathered about protein secretion in Gram-negative bacteria, spurred on by the realization of the importance of these pathways in bacterial pathogenesis. A wide range of secretion strategies has been uncovered, from simple one-component systems to complex multicomponent assemblies. Relationships with organelle biogenesis mechanisms have informed our understanding of a number of these pathways. However, much is still unknown about the structure of the various secretion machineries, their molecular mechanisms of action, and how the machineries themselves are assembled. Future work in this direction will answer important cell biological questions and will also present attractive targets for the development of antimicrobial therapeutics.

**Update**

The link between type II secretion and type 4 pilus assembly was demonstrated in a recent publication by Sauvonnet *et al.* [80]. They showed that expression of the pullulanase type II secretion system of *K. oxytoca* in *E. coli* resulted in assembly of the PulG ‘pseudopilin’ into pilus-like bundles on the bacterial surface. In addition, this system could assemble the *E. coli* type 4 pilin protein PpdD into pili.

Recent work by Odenbreit *et al.* [81] on the type IV secretion system of *H. pylori* demonstrated that this system is able to translocate the CagA protein into the cytosol of gastric epithelial cells where it modulates host cell proteins.
References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest


This paper describes the purification of the secretin PulC as a stable complex with the PulS lipoprotein and analysis of this complex by cryoelectron microscopy. A three-dimensional reconstruction of the complex is presented, together with evidence that the complex forms channels in lipid bilayers.


This paper presents lipid-bilayer analysis of the secretin pIV and a pIV mutant with altered permeability characteristics. The paper provides strong evidence that secretins contain large gated channels.


