Review

Secretion of virulence determinants by the general secretory pathway in Gram-negative pathogens: an evolving story

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ABSTRACT – Secretion of proteins by the general secretory pathway (GSP) is a two-step process requiring the Sec translocase in the inner membrane and a separate substrate-specific secretion apparatus for translocation across the outer membrane. Gram-negative bacteria with pathogenic potential use the GSP to deliver virulence factors into the extracellular environment for interaction with the host. Well-studied examples of virulence determinants using the GSP for secretion include extracellular toxins, pili, curli, autotransporters, and crystalline S-layers. This article reviews our current understanding of the GSP and discusses examples of terminal branches of the GSP which are utilized by factors implicated in bacterial virulence. © 2000 Éditions scientifiques et médicales Elsevier SAS

Gram-negative bacteria / general secretory pathway / Sec translocase / pullulanase / toxins / pili / curli / autotransporters / S-layers

1. Introduction

Gram-negative bacteria have evolved several secretion pathways for protein export to the extracellular environment [1]. (i) The type I or ABC transporter pathway, exemplified by the secretion of Escherichia coli hemolysin A, exports substrates directly across the inner (IM) and outer membranes (OM) and does not include periplasmic intermediates. (ii) The type II pathway, exemplified by the secretion of pullulanase of Klebsiella oxytoca [2, 3], is a two-step process that involves the Sec translocase and cleavable amino-terminal signal peptides for translocation across the IM, and a separate apparatus, termed the secretion, for translocation across the periplasm and the OM. (iii) The type III or contact-dependent pathway, exemplified by the secretion of Yop proteins in Yersinia spp., is used exclusively by pathogenic bacteria to deliver virulence factors into host cells. Surprisingly, these protein secretion pathways have been conserved among Gram-negative pathogens of wide host range, including animal and plant pathogens [4]. A schematic overview of the main secretion systems is shown in figure 1.

The type II pathway is also known as the main terminal branch of the general secretory pathway (GSP). More proteins are secreted by the GSP than by any other secretion pathway. GSP systems have been identified in almost all pathogenic and nonpathogenic Gram-negative bacteria, including E. coli K-12 [3] which has not been known to secrete extracellular proteins. Although the type III pathway appears to be used exclusively for secretion of virulence factors (perhaps with the possible exception of some flagellar assembly proteins), it has become apparent that many virulence determinants, including extracellular toxins, pilus and nonpilus adhesins, invasins, and proteases, are secreted by the GSP. The importance of the GSP in bacterial pathogenesis is underscored by the fact that some components of type I and type III secretion machineries, such as the OM secretins and ushers discussed later, are exported by the GSP route. This article summarizes recent advances in understanding variations in the GSP used to export virulence factors.
2. Main features of the general secretory pathway

2.1. Secretion across the IM

Periplasmic, OM, and many extracellular proteins of Gram-negative bacteria are synthesized in the cytoplasm as precursor proteins having an amino-terminal signal peptide and are exported into the periplasm with the help of the Sec secretion system (Sec translocase). The Sec translocase of *E. coli* (Table I; reviewed in [5, 6]) consists of a cytoplasmic secretion specific-chaperone (SecB), a protein translocation ATPase (SecA), and an integral membrane protein complex formed by at least six different protein subunits (SecY, SecE, SecD, SecF, SecG, and YajC). *In vivo* studies show that only SecY and SecE are essential, whereas the other subunits are required only at low temperatures [7].

The SecB chaperone functions to maintain precursor proteins in a secretion-competent state and to target them to SecA. SecA then targets the precursor protein-SecB complex to the Sec complex in the membrane for export across the IM. Precursor protein translocation is initiated by the binding of ATP to SecA which promotes insertion of the signal peptide and part of the mature protein sequence together with a SecA domain into the translocation channel comprised of SecYEG [8]. SecA translocates ~2.5 kDa polypeptide cargo across the IM. The translocated polypeptide is then cleaved by signal peptidase (SP) or DsbA to leave a mature extracellular protein.
of the exported protein upon ATP binding [5]. ATP hydrolysis releases SecA from the partially translocated protein and the Sec complex in the IM. SecA then binds another region of the precursor protein, promoting translocation of another ~2.5-kDa sequence. Translocation intermediates are driven forward by the proton-motive force (PMF), which also promotes the release of SecA from the Sec complex. ATP and the PMF serve as energy sources at different stages of the translocation process, with ATP being required first. However, ATP is essential for Sec-mediated secretion across the IM, whereas the PMF only enhances translocation rates [6]. After several cycles, the precursor protein is translocated across the IM but remains bound to the IM by its amino-terminal signal peptide. This translocation mechanism has many similarities to the mechanism by which protein translocation across the endoplasmic reticulum membrane occurs in eukaryotes.

A typical signal sequence contains a stretch of approximately 18 to 30 amino acids divided into 3 general domains: an N domain containing positively charged amino acids that associate the presecretory protein with the IM and correctly orients the protein for translocation; an H domain containing a core of hydrophobic amino acids that inserts the signal sequence into the IM; and a C domain containing a cleavage site recognized by the signal peptidase.

Recently, a number of secreted proteins have been identified that contain an atypical N-terminal signal sequence. These signal sequences are longer (>45 amino acids) than a typical signal sequence due to an extended region of amino acids (called an N-terminal extension) located between the first methionine and the N domain [9, 10]. The function of the N-terminal extension remains obscure, but one possibility is that this region functions as a cytoplasmic chaperone, preventing premature folding and degradation.

After translocation across the IM, signal peptides are cleaved by the appropriate membrane-bound signal peptidase. There are three types of signal peptidases (table I) and each type has a different substrate specificity [2]. Signal peptidase I (LepB) of E. coli is an integral membrane protein having two transmembrane helical segments and a large periplasmic C-terminal domain [11]. The proteolytic activity appears to be localized in the periplasmic C-terminal domain. Recent data from site-directed mutagenesis and sequence alignment studies suggest that signal peptidase I proteins may constitute a new family of serine proteases that do not require a histidine residue and are mechanistically related to the β-lactamases [12]. Signal peptidase II is an integral IM protein that cleaves signal peptides only from precursor lipoproteins. Signal peptidase II is modeled with four membrane-spanning α-helical segments and with terminal ends that are localized in the cytoplasm [13]. It has been suggested that signal peptidase II may belong to a novel family of aspartic proteases that are active around neutral pH [13]. Notable features of E. coli.
coli signal peptidase II include its thermostability and its noncompetitive inhibition by the antibiotic globomycin. The prepilin peptidase for type 4 pilis is a polytopic IM protein having two enzymatic activities: endoproteolytic activity, which is needed for the removal of the signal peptides of type 4 pilin subunits, and N-methylation activity for posttranscriptional modification of type 4 pilins [14]. Another unique feature of these types of signal peptidases is that they cleave signal sequences on the cytoplasmic side of the IM.

After proteolytic processing by the appropriate signal peptidase, the mature polypeptide is usually released into the periplasm, whereas its signal peptide is further degraded by protease IV [15].

2.2. Protein folding in the periplasm

Protein stability in the periplasm is an extremely important issue in the secretion of proteins via the GSP. Secreted proteins exposed temporarily to the periplasmic environment may need to remain partially unfolded before they are transported across the OM. A number of periplasmic enzymes have been identified that catalyze folding and turnover of cell envelope proteins.

Formation of disulfide bonds can be vital for the stability of proteins in the periplasm. In E. coli, formation of disulfide bonds in the periplasm is catalyzed by the Dsb system [16]. DsbA is a soluble, monomeric periplasmic protein that belongs to the thioredoxin superfamily. Although DsbA is the predominant dithiol oxidant in the periplasm, DsbC is the main periplasmic disulfide isomerase. DsbA is not an essential protein; however, dsbA mutations in E. coli have been shown to display reduced or blocked secretion of certain proteins by the GSP [17, 18].

Periplasmic proteins containing proline residues may require isomerization of peptidyl-proline bonds to fold correctly. There are at least four periplasmic proteins catalyzing peptidyl-prolyl isomerization (PPIases) in E. coli: SurA, PpiA, PpiD, and Ppal [19, 20]. The enzymatic activity is the same for all periplasmic E. coli PPIases; however, gene regulation and substrate specificity are probably different. It is also possible that some periplasmic PPIases, such as SurA, may have more than one function. The cell can tolerate absence of one PPIase protein, but double ppiD surA mutations are lethal [20].

Beyond containing folding catalysts like disulfide-bond isomerases and peptidyl-prolyl isomerases, the periplasm contains factors that can prevent secreted proteins from folding into their final conformation or can assist proteins in folding correctly. This process is mediated by molecular chaperones and is essential for the secretion of extracellular proteins which must be maintained in a properly folded state in the periplasm before transport across the OM. Chaperones recognize hydrophobic domains that are surface exposed in the unfolded/misfolded protein and bind noncovalently to those regions of the substrate, preventing aggregation or proteolysis. In other cases, chaperones can also target secreted proteins from the IM to their secretion apparatus in the OM, and function as ‘shuttles’ that facilitate protein transport across the periplasm. Known periplasmic chaperones include the P pilus-specific chaperone PapD [21, 22], porin-specific chaperone Skp [23], and LolA, a chaperone that is required for proper localization of OM lipoproteins [24].

Finally, the periplasm also contains several proteases that play important roles in the physiology of the bacterial cell. One major function of the periplasmic proteases is to activate turnover of damaged or misfolded cell envelope proteins. DegP is considered to be among the primary proteases responsible for turnover of misfolded proteins in the periplasm [25]. DegP proteins play an essential role in the virulence of some facultative intracellular pathogens [25]. In addition to being a protease, it was recently shown that DegP can function as a general molecular chaperone [26]. The chaperone function of DegP is mainly displayed at low temperatures, while the protease function is displayed at high temperatures.

2.3. Secretion across the OM

The final step of the general secretion pathway is translocation across the OM. Whereas all proteins secreted by the GSP traverse the IM via the Sec translocation complex (with the possible exception of type 4 pilis; see reference [27]), the terminal step can take several different routes, referred to as terminal branches. In most cases the terminal branches involve periplasmic intermediates that are partially or fully folded. Therefore, translocation of these intermediates across the OM represents a different problem than translocation of unfolded proteins across the IM. Furthermore, there is no known source of energy (ATP) available at the OM.

2.3.1. The main terminal branch of the GSP

2.3.1.1. The pullulanase example

The main terminal branch of the GSP, also referred to as type II secretion, exports the majority of Gram-negative bacterial exoenzymes and toxins. Type II secretion is exemplified by pullulanase export in K. oxytoca (reviewed in [2, 3]). Pullulanase (PulA) of K. oxytoca is an oligomeric extracellular lipoprotein that belongs to the α-amylase family. PulA is secreted across the Gram-negative cell envelope and then remains temporarily anchored on the cell surface by its fatty acylated amino terminus before being released into the extracellular medium. Secretion of PulA across the cell envelope involves the products of approximately 25 genes [3]. The second, terminal, step of PulA secretion is known to involve the products of at least 14 specific genes located in the pul gene cluster as well as the DsbA protein. This ‘pullulanase secretion’ consists of the following proteins.

(i) Cytoplasmic protein PulE. PulE contains an ATP-binding site and a tetracysteine motif that resembles the zinc-binding motif found in adenylate kinases [28]. PulE interacts with PulL, a monotypic IM protein that spans the membrane by its C-terminal end. PulE is a potential energy provider for PulA secretion and/or assembly of the pullulanase secretion apparatus.

(ii) Integral IM proteins PulC, PulM, PulN, PulK, and PulF. All, with the exception of PulF, are predicted to be anchored in the IM by a single N-terminal transmembrane-spanning segment followed by a large C-terminal periplasmic domain [29]. These proteins might be involved in the formation of the basal body of the secretion apparatus that
is anchored on the periplasmic side of the IM. Moreover, PulC, which has also been shown to interact with the OM and may form a bridge between the two membranes, is a potential energy transducer for protein translocation across the OM in a manner similar to that proposed for TonB [30].

(iii) Four type 4 pilin-like proteins (pseudopilins) designated PulG, PulH, PulI, and PulJ. All four proteins include prepilin peptidase cleavage and methylation sites and are most likely localized in the periplasm. It has been suggested that they may assemble into a pilus-like structure across the periplasm (a ‘secretion tube’), facilitating PulA transport to the OM [31]. In addition to the pilin-like proteins, the pullulanase secretion system includes a type 4 prepilin-like signal peptidase designated PulO which is essential for PulA secretion [3].

(iv) OM proteins PulD and PulS. PulD is a large integral OM protein that belongs to the secretin family. Secretins form highly stable multimers of 10-14 subunits that are presumed to provide an aqueous translocation channel through the OM [32]. Several members of the secretin family have been imaged by electron microscopy (figure 2) [33–36]. Each secretin was visualized as a ring-shaped complex possessing a large apparent central pore ranging from 5–10 nm in diameter, large enough to allow secretion of folded proteins. The majority of secretins require an OM lipoprotein (PulS for pullulanase secretion) for proper targeting and insertion into the OM [32]. PulS interacts with a C-terminal domain of the PulD secretin. The N-terminal domain of PulD appears to be responsible for substrate recognition and possibly binding to PulC. The N-terminal domain may serve as a channel gate, controlling the secretion of PulA and preventing the non-specific release of other periplasmic proteins. Evidence for formation of gated aqueous channels was recently shown for the filamentous phage secretin, pIV [37].

2.3.1.2. Virulence determinants secreted via the main terminal branch

Although most information on the type II secretion pathway has been derived from studies with pullulanase, there is an increasing number of secretion systems found in a variety of Gram-negative bacteria whose structural components display strong sequence homologies to the components of the pullulanase secreton [3]. These are substrate-specific secretion machineries that presumably share a similar architecture and secretion mechanism with the type II system discussed above. Proteins secreted by these systems are mainly virulence determinants, such as exotoxins, pili, and S-layer components.

Cholera toxin: Many bacterial pathogens produce toxins that cause damage to the eukaryotic target cells by a variety of mechanisms. The majority of bacterial toxins need to be actively secreted in order to acquire their complete toxic activity. Cholera toxin (CT) is the major virulence factor of the enteric pathogen Vibrio cholerae,
eliciting the severe diarrheal symptoms associated with cholera. CT is an oligomeric protein consisting of a single A subunit (27.2 kDa) and five identical B subunits (11.6 kDa each), adopting an A-BS architecture [38].

Secretion of CT to the extracellular space occurs by a type II secretory pathway. First, the individual subunits cross the IM, presumably mediated by the Sec translocase of V. cholerae. During this process, the amino-terminal signal peptides are removed and the mature subunits are released in the periplasm where they fold and assemble to form the cholera holotoxin. The second step is translocation of the holotoxin through the periplasm and then across the OM. This step requires the products of the eps gene cluster and the vcpD gene [39]. The eps gene cluster contains 12 genes, designated eps-C-N. These genes are similar to the pullulanase secreton of K. oxytoca. At least six of these genes encode proteins essential for CT secretion [39]. Similar to the pullulanase secretion system, four Eps components are pseudopilins. The vcpD gene encodes a type 4 prepilin peptidase which is required for CT secretion [40]. A DsbA homolog is also essential for CT secretion [41]. The putative export signal for CT secretion across the OM is thought to be located on the B subunit [42]. In addition to CT, the Eps machinery can facilitate extracellular secretion of at least two other V. cholerae proteins, as well as cloned heat-labile enterotoxin of enterotoxinogenic E. coli [42, 43].

Type 4 pili: Type 4 pili are long, polarly localized pili important for the virulence of many bacterial pathogens [44]. These organelles promote adhesion to target tissues and produce a type of movement termed twitching motility. The 6-nm diameter pilus fiber consists primarily of repeating subunits of pilin protein. The crystal structure of pilin monomer from Neisseria gonorrhoeae was solved, revealing a highly asymmetric protein with a globular head and a long α-helical tail [45]. Pilins possess a short, positive N-terminal signal sequence followed by the long, hydrophobic α-helical tail domain. After translocation across the IM, pilins remain anchored in the IM via their N-terminus. The pilin signal sequence remains on the cytoplasmic side of the IM and is cleaved by a specialized prepilin peptidase that also N-methylates the resulting N-terminal amino acid [14]. The bulk of the pilin is exposed to the periplasm. Pilins contain two conserved cysteines near their C-terminus that form a disulfide bond. The DsbA periplasmic disulde isomerase has been shown to be required for stability of enteropathogenic E. coli pilin, and in V. cholerae, a DsbA homolog is required for proper pilus function [46]. Assembly of pilins into the pilus fiber may occur through interactions of the hydrophobic α-helical domains in the IM bilayer rather than through association of soluble periplasmic intermediates [47]. Assembly and secretion of the pilus fiber across the OM requires at least 14 components, many with homology to components of the type II secretory pathway [48]. As with type II secretion, type 4 pilus biogenesis requires multiple pilin-like proteins that are processed by prepilin peptidase. Translocation of pilus to the cell surface requires a secretin, PilQ in N. gonorrhoeae and Pseudomonas aeruginosa, which presumably functions as an OM secretion channel. In P. aeruginosa, biogenesis and function of type 4 pili require three putative nucleotide-binding proteins, PilB, PilT and PilU. Mutation of PilB results in loss of surface pili, whereas mutation of PilT or PilU results in hyperpiliated bacteria, but these pili are unable to produce twitching motility [49].

S-layers: Surface-layers (S-layers) are two-dimensional paracrystalline arrays found on the outermost surface of the cell envelope of bacteria and archaea (reviewed in reference [50]). Most S-layers are composed of a single protein or glycoprotein subunit with a molecular mass ranging between 46 000 and 170 000. S-layers cover the entire cell surface and appear to have roles in cell shape determination, cell adhesion, and cell protection from damaging environmental agents. Most Gram-negative bacteria secrete S-layer subunits via the type II pathway [50].

The genus Aeromonas contains several species that are pathogenic for humans and animals. Aeromonas S-layers appear to have a number of roles in pathogenesis, including resistance to serum killing, protection from proteases and phagocytic cells, and binding to extracellular matrix proteins and immunoglobulins [51]. The S-layer of Aeromonas is a tetragonally arranged surface protein array consisting of a single protein subunit, having a lattice constant of 12–12.5 nm. It is anchored on the cell surface via Ca²⁺ mediated interactions with O-polysaccharide side chains of LPS [52]. The structural subunit has been designated VapA in A. salmonicida (a 53-kDa protein) and ApsE in A. hydrophila (a 46-kDa protein). Both the VapA and AhsA structural proteins contain cleavable amino-terminal signal peptides. So far, only one component of each secretory apparatus has been identified: SpsD of A. hydrophila [53], a homolog of the OM secretin PulD, and ApsE of A. salmonicida [54], a cytoplasmic ATP-binding protein and a homolog of PulE. Inactivation of either the spsD or apsE gene results in accumulation of S-layer subunits in the periplasm, without affecting the secretion of other extracellular or OM proteins. Most S-layer subunits, including VapA and AhsA, lack cysteine residues and the ability to form disulfide bridges and multimers in the periplasm.

2.3.2. Alternate terminal branches of the GSP

2.3.2.1. The chaperone/usher pathway

In contrast to type 4 pili, another major class of pili are assembled by the alternate terminal branch referred to as the chaperone/usher pathway. These pili are typified by P and type 1 pili of uropathogenic E. coli. P and type 1 pili allow binding to the kidney and bladder, respectively, and are major virulence determinants for colonization of the urinary tract [55]. These pili are composite structures consisting of a thin, flexible tip fibrillum connected to a rigid, helical rod [56].

Work on P pili has elucidated many molecular details of the chaperone/usher assembly pathway. Pili subunits emerging from the Sec translocation complex into the periplasm must interact with the PapD periplasmic chaperone and require the DsbA disulde isomerase for proper disulfide bond formation [57]. PapD facilitates release of pilus subunits into the periplasm and guides their proper folding [58]. PapD is also required to prevent premature interaction of subunits in the periplasm. In the absence of the chaperone, subunits form aggregates that are degraded
by the DegP periplasmic protease. Off pathway interactions of pilus subunits activate the Cpx and the oSO (RpoE) signal transduction pathways, both of which activate degP transcription as well as a number of other periplasmic chaperones and assembly factors [59]. Targeting of pilus subunits to periplasmic chaperones such as PapD requires specific motifs in the N-terminal and C-terminal regions of the subunits [60, 61].

The crystal structure of PapD and more recently the crystal structures of the PapD-PapK chaperone-subunit complex and the FimC-FimH chaperone-adenhesin complex of type 1 pili have all been solved [21, 62, 63]. The chaperone consists of two Ig-like domains oriented in an L-shape. The PapK subunit and the subunit domain of FimH also have Ig folds; however, they lack the seventh β-strand present in canonical Ig folds. The absence of this strand produces a deep groove along the surface of the pilin domain, exposing its hydrophobic core—hence the instability of pilins when expressed without the chaperone. In the chaperone-subunit complex, the chaperone donates one of its β-strands to complete the Ig fold of the subunit in a mechanism called donor strand complementation [62, 63]. This interaction stabilizes the subunit by shielding its hydrophobic core. In addition, the chaperone simultaneously caps one of the subunit’s interactive surfaces, preventing premature pilus formation in the periplasm. Subunits assembled by the chaperone/usher pathway have an N-terminal extension that does not contribute to the Ig fold of the subunit but rather projects away from the rest of the pilin domain where it would be free to interact with another subunit [63]. During pilus biogenesis, the N-terminal extension of one subunit may displace the donated chaperone 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 the organelle.

Periplasmic PapD-subunit complexes target to the PapC usher in the OM. Interaction of a chaperone-subunit complex with the usher triggers dissociation of the chaperone from the subunit, exposing subunit assembly surfaces and allowing incorporation of the subunit into the pilus fiber. In the absence of the usher, chaperone-subunit complexes accumulate in the periplasm, but no pili are assembled. Both PapC and the type 1 pilus usher called FimC have been imaged by electron microscopy ([64]; Saulino et al., unpublished). As with the secretins, the ushers assemble into ring-shaped oligomeric complexes with central pores (figure 2). The usher complexes are smaller than the secretin complexes, having only 2 to 3-nm diameter channels. A 2-nm diameter channel would be sufficient to allow passage of a linear fiber of folded pilus subunits. Pilus assembly is proposed to occur at the periplasmic face of the usher, concomitant with secretion through the usher pore [22]. Therefore, a nascently assembled pilus rod would be constrained to a linear fiber while traversing through the usher channel. Once reaching the cell surface, the rod could coil into its final 6.8-nm diameter helical conformation. This coiling of the rod on the cell surface may facilitate the outward translocation of pili. The chaperone/usher pathway does not appear to require input of external energy for assembly and secretion of pili across the OM [57]. Energy for secretion by this pathway is likely derived solely from the protein-protein interactions involved in assembling the pilus organelle. The usher family shares no sequence homology with the secretin family. However, ushers and secretins share obvious structural similarities and likely share functional similarities as well.

2.3.2.2. The autotransporter pathway

Bacterial proteins that are targeted to the microbial surface or released into the environment often depend on periplasmic proteins and almost always require OM proteins to promote their secretion. The autotransporter family of Gram-negative bacterial proteins is a unique subset of secreted proteins that do not rely on other proteins for transit from the periplasm to the bacterial surface [10]. These proteins possess a C-terminal domain that mediates targeting to and translocation across the OM. The prototype member of the autotransporter family is the N. gonorrhoeae IgA1 protease [65]. Other members include the Haemophilus influenzae Hap adhesin [66], the E. coli Tsh hemagglutinin [67], and the Shigella flexneri IcsA protein (also called VirG) [68], to name a few.

A typical autotransporter is synthesized as a precursor protein that can be divided into three domains: an N-terminal signal sequence, an internal passenger domain, and a C-terminal β-domain [10]. Once translated, the protein is exported across the IM, presumably using the N-terminal signal sequence and the Sec translocase. After cleavage of the signal sequence, the C-terminus of the protein inserts into the OM. Secondary structure analysis performed on the C-terminus of a number of autotransporter proteins predicts formation of a β-barrel. This β-barrel is believed to function as a porin, with a central hydrophilic channel [69]. It is unknown whether a single β-domain forms the transporter or if multiple domains are required. Whether the passenger domain is extruded across the OM, presumably through the β-barrel channel, in an unfolded or folded conformation needs further investigation [70, 71]. As with the chaperone/usher pathway, the autotransporter pathway appears to require no outside energy source.

Once surface-localized, the passenger domain is either retained on the bacterial surface or released into the environment by a proteolytic event. Cleavage of the passenger domain from the β-domain occurs via autoproteolysis or is mediated by another OM protease, depending on the particular autotransporter. For many autotransporters, the passenger domain contains a serine protease motif. In the case of the N. gonorrhoeae IgA1 protease and the H. influenzae Hap adhesin, the serine protease motif is responsible for autoproteolysis and appears to gain catalytic activity once exposed on the surface of the organism [65, 66]. IcsA is an example of an autotransporter that depends on another OM protease for cleavage [68] and is cleaved by SopA (also called IcsP), which displays a high level of homology to the OmpT and OmpP outer membrane serine proteases of E. coli. Interestingly, the Hap passenger domain mediates adherence to epithelial cells and formation of microcolonies when it is surface-localized and still connected to HapN (i.e., before

Microbes and Infection
2000, 1061-1072
autoproteolysis occurs) \[72\]. Whereas autoproteolysis interferes with Hap-mediated adhesive activity, proteolysis of IcsA by SopA is essential for proper intracellular movement of S. flexneri \[68, 73\]. Interestingly, if proteolysis of IcsA is blocked, the protein is no longer distributed in a unipolar fashion and host cell actin is not polymerized. Autotransporters represent an interesting family of proteins in which secretion and processing of the proteins determines and perhaps regulates the biological activity of the functional domains.

### 2.3.2.3. The ShlA-like secretion system pathway

The haemolysin of Serratia marcescens (ShlA) is a 165-kDa pore-forming toxin that is secreted into the culture medium \[74\]. The precursor ShlA is secreted across the IM by the Sec translocase. Its amino-terminal signal peptide is then cleaved off, releasing mature ShlA into the periplasm.

Translocation across the OM and activation of the toxic activity of ShlA requires a 62-kDa OM protein, designated ShlB \[75\]. Similar to ShlA, ShlB contains a cleavable N-terminal signal peptide. It has been proposed that ShlB folds as a \(\beta\)-barrel in the membrane, forming a gated channel large enough to accomodate the export of unfolded ShlA. The ShlA secretion exemplifies a novel terminal branch of GSP, since it only requires a Sec translocase in the IM and a channel-forming protein in the OM. This simple secretion system shows similarity to the secretion model described for the autotransporters \[10\]. However, there is no sequence homology between ShlB and the \(\beta\)-domains of autotransporters. Instead, ShlB appears to be related to the ushers and to a protein-translocating porin found in chloroplast membranes \[76\].

Additional virulence proteins have been identified which use a secretion mechanism similar to the one described for the ShlA haemolysin, including the HMW1 and HMW2 nonpilus adhesins of H. influenzae, the HpmA haemolysin of Proteus mirabilis, and the Bordetella pertussis filamentous haemagglutinin (FHA) \[77, 78\]. There is at least one reported case of complementation between substrates and OM proteins derived from different ShlA-like secretion systems \[78\]. As with the autotransporters, folding of the FHA protein in the periplasm blocks export across the OM \[79\]. The C-terminal domain of the FHA protein appears to function as an intramolecular chaperone that prevents premature folding of the secreted protein \[80\].

### 2.3.2.4. The extracellular nucleation-precipitation pathway

Several \(E. coli\) and \(Salmonella\) strains express thin, irregular, flexible, and highly aggregative cell surface fibres known as curli (also called thin aggregative fimbriae) \[81-84\]. These surface organelles are distinct from other types of pili in terms of both their morphology and mechanism of assembly \[85\]. Curli bind several matrix and plasma proteins, including fibronectin, plasminogen, tissue plasminogen activator, laminin, and surfaces such as agar and plastic. In addition, they have been shown to interact with the light chain of MHC class I molecules \[81\]. Cells expressing curli on their surface tend to autoaggregate, indicating that curli may mediate adhesion to other bacterial cells, a property that may be important for curli-mediated biofilm formation \[86\]. Nevertheless, the exact role of curli in bacterial pathogenesis has not been determined.

In \(E. coli\), two operons, csgDEFG and csgBA, are required for biogenesis of curli. csgA encodes the major structural component of curli, csgB encodes a minor component (nucleator) of curli, csgG encodes a lipoprotein located in the OM, and csgD encodes a transcription regulator belonging to the LuxR/UhpA family. The precise roles of the proteins expressed by csgE and csgF remain to be determined. Inactivation of csgE does not significantly affect formation of curli fibres, while mutation of csgF eliminates curli, without disrupting CsgA secretion into the extracellular environment. Both CsgE (14-kDa precursor) and CsgF (15-kDa precursor) are potentially secreted proteins, and mature CsgF shows sequence homology to a nitorgenase iron protein. The \(Salmonella\) curli operons are very similar to their counterparts in \(E. coli\) with respect to structure and regulation \[83, 84\].

The two components of curli, CsgA and CsgB, have significant sequence homology and structural similarity. Both proteins contain sec-like amino-terminal signal peptides, and their mature domains have 4-5 repeats of a consensus motif with a \(\beta\)-strand-turn-\(\beta\)-strand-turn structure \[87\]. Only the mature forms of CsgA and CsgB (13-kDa polypeptides) can be detected in the curli fibres. As with CsgA and CsgB, the CsgG lipoprotein contains a signal peptide that is removed during secretion. Loefer et al. have shown that CsgG is located on the periplasmic side of the OM, and mutants lacking CsgG accumulate CsgA and CsgB in the periplasmic space \[88\]. Consequently, CsgG is needed for export of the two components of curli across the OM. However, it remains to be determined if CsgG functions as the OM channel or as a chaperone that prevents misfolding and proteolysis of the curli subunits in the periplasm. CsgE and CsgF are also candidate chaperones of the curli secretion system. In the absence of CsgB, CsgA does not polymerize on the cell surface, but is released into the culture medium. It has been proposed that CsgB functions as either a nucleator required for the polymerization of CsgA or as a platform for the assembly of curli fibres \[89\]. Unlike P and type 4 pili that undergo assembly from the base, the formation of curli fimbriae seems to occur from both the tops and the cell-associated bases of the fibers. Curli production appears to involve a separate terminal branch of the GSP referred to as the extracellular nucleation-precipitation pathway.

### 2.3.2.5. The adapted conjugation system pathway

Pertussis toxin (PT) is an essential virulence factor of \(B. pertussis\), the etiologic agent of pertussis (whooping cough). Like CT, PT belongs to the A-B5 toxin family but contains five different subunits (S1, S2, S3, S4, and S5, found in a 1:1:2:1:2 ratio) \[38\]. S1 represents the A-subunit, which is the enzymatically active portion of the holotoxin and displays ADP-ribosylase activity. S1 is surrounded by a B-oligomeric ring-shaped structure, involved in target cell binding and delivery of the toxin into the eukaryotic cell.

To be effective, PT must be secreted from the \(B. pertussis\) cell. As is the case for CT, PT secretion is thought to occur in two steps. The first step involves export of the
individual S1-S5 subunits across the IM with the help of the Sec translocase. The second step involves export across the OM by products of the ptl locus. The ptl locus is located downstream of the PT structural genes and comprises nine genes [90]. Surprisingly, the ptl genes are homologous to the genes of the virB cluster of Agrobacterium tumefaciens, which encode the components of a secretion apparatus that facilitates transport of oncogenic single-stranded T-DNA to plant cells, and to the genes of the pKM101 pilus cluster that encodes the components of a conjugal transfer system for self-transmissible bacterial plasmids [91]. Presumably, PT secretion represents a unique terminal branch of the GSP that evolved as a hybrid pathway, combining the sec-dependent export of proteins across the IM and the VirB-like export of DNA across the OM.

There exists at least one other protein secretion system, found in Gram-negative pathogen Helicobacter pylori, that is homologous to the PT secretion system [92]. The secretion system exemplified by PT is referred to as the adapted conjugation or type IV secretion system.

3. Concluding remarks and future research

In Gram-negative bacteria, secretion of extracellular proteins involves passage through the inner and outer membranes and the intervening periplasm. Several distinct mechanisms of protein secretion, along with numerous variations, have been developed in bacteria during evolution. Here we have provided a brief overview of the GSP, the major secretion pathway in Gram-negative bacteria. GSP systems display the following three common elements: (i) protein secretion always occurs in two steps; the first step requires the Sec translocase and the second step requires a substrate-specific apparatus; (ii) secretion substrates are synthesized as precursors with N-terminal extensions, termed signal peptides, which are removed during the secretion; and (iii) secretion substrates remain temporarily in the periplasm before translocation across the OM. There is more variation in transport across the OM than transport across the IM. At least six different general terminal branches of GSP have been described, as exemplified by the secretion mechanisms of pullulanase, P pili, IgA1 protease, ShiA hemolysin, curli, and PT.

GSP systems have been identified in almost every Gram-negative species, including major human pathogens such as H. influenzae, V. cholerae, uropathogenic E. coli, and H. pylori. There is evidence indicating that secretion by the GSP plays an important role in bacterial pathogenesis: (i) many virulence factors are secreted to the extracellular environment by the GSP; (ii) many virulence determinants that are localized in the outer membrane of Gram-negative pathogens, such as the plasminogen activator Pla of Yersinia pestis and the Opc adhesin/invasin of Neisseria meningitidis [93, 94], are exported via the first step of the GSP; (iii) secretion by the GSP is required for assembly of the type I and type III secretion machineries [1] which are known to secrete a number of essential virulence factors; (iv) mutants of certain pathogens missing secretion components of the GSP such as DegP (HtrA), DsbA, or prepilin peptidase for type 4 pili are attenuated [25, 95–98], implying that the GSP is directly or indirectly involved in bacterial virulence, and (v) components and secretion substrates of the GSP, such as the H. influenzae DegP/HtrA protease [99] and the FimH adhesin of type 1 pili of uropathogenic E. coli [100], can function as protective vaccines.

Despite the tremendous amount of information that has been generated over the last fifteen years concerning secretion by the GSP, there are many questions that still need to be addressed. What is the architecture of the type II secretion? Do the Sec translocase and the secretion interact during any stage of the export process? What is the actual mechanism of substrate translocation across the cell envelope? How is the substrate recognized, and which components of the secretory pathway are involved? What is the energy requirement for the second step of secretion? What is the importance of extended signal sequences in some precursor substrate proteins? How are the autotransporter amino-terminal domains maintained in the unfolded state during transit through the periplasm and across the OM? What is the role of pseudopilins in protein secretion, and why have these proteins been conserved in several cell envelope-associated macromolecular complexes? How did bacterial secretion pathways evolve, and why are most bacterial secretion machines structurally related to bacterial surface appendages [1]? Answers to these questions will enhance our understanding of a biological process of major importance for molecular microbiology and will also provide us with more tools to prevent and treat bacterial diseases in humans, animals, and plants.

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References


Secretion of virulence factors by the general secretory pathway

Review


