REVIEW

Protein secretion and the pathogenesis of bacterial infections

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Instruments of bacterial warfare

The skin, the oral cavity, and the gastrointestinal tract of humans are colonized with bacteria. Microbial entry into the blood or blood-circulated tissues is hindered by anatomical barriers. The barriers consist of epithelia as well as membranes that are fortified by layers of collagen and other connective tissues. Following the breakdown of a barrier, the dwindling of an immune system, or an attack by particularly virulent bacteria, microbes gain entry into deeper tissues and multiply within newly conquered space. Human disease is the result of such bacterial multiplication. Microbial entry into circulated tissue is accompanied by an immune response. Immune cells recognize bacterial products (lipids, carbohydrates, peptidoglycan, or protein decorations) and respond by attracting macrophages, polymorph-nuclear leukocytes, or other immune cells in an effort to kill the invading pathogen (Medzhitov and Janeway 1999; Aderem and Ulevitch 2000). Many bacterial pathogens have evolved to enter and multiply within blood-circulated tissues (Finlay and Falkow 1997). The underlying pathogenic strategies are remarkably diverse and often result in unique disease symptoms. Nevertheless, all mechanisms of bacterial manipulation of the host organisms can be viewed in three principal categories: microbial adhesion, secretion of toxins into the extracellular milieu, and injection of virulence factors into host cells. There are three rules of thumb that bacterial pathogens must consider if they want to mount a successful infection.

If you want to invade a host—stick to it

Vibrio cholerae adhere to human intestinal tissues and secrete a toxin that, once engulfed by epithelial cells, causes a fulminant diarrhea. Mutants lacking the *Vibrio* surface adhesin (TCP pili) cannot stick to the intestines and fail to cause disease (Herrington et al. 1988). Microbial adhesion to host tissues can be mediated by individual proteins or by sophisticated organelles such as pili. Pili form fibrous structures that emanate from the bacterial surface and display an adhesive property at the tip. Pili are often distributed over the bacterial surface (pertrichious pili, e.g., type I pili) or they are located at a single site (polar pili, e.g., type IV pili). Some pili are retractable (type IV pili) and provide for adhesion and bacterial movement. Other pili are involved in conjugation, that is, the exchange of genetic information between cells, and provide for adhesion, retraction, and the transport of DNA. Flagella, another surface organelle of bacteria, are more flexible and longer than pili. Flagella do not display adhesive properties but function as a rotating propeller that allow bacteria to swim during infection.

If you don't want to get killed—bring your weapons and fight

Invasin is an adhesive outer membrane protein of *Yersinia pseudotuberculosis*, a microbe that colonizes lymphoid tissues after invading the intestines of humans (Isberg and Falkow 1985). Expression of invasin in *Escherichia coli* K-12 is sufficient for a nonpathogenic microbe to invade epithelial cells (Isberg et al. 1987). Nevertheless, invasin alone can not confer the ability to cause disease. What does it take to generate a potent pathogen such as *Y. pseudotuberculosis*? Protein secretion machines are the instruments of microbial warfare! Many bacteria secrete toxins into the extracellular milieu during infection. For example, *Staphylococcus aureus* secretes several exotoxins that damage host cell membranes (Dinges et al. 2000). Although these compounds diffuse within extracellular fluids, their concentration is highest in the immediate vicinity of staphylococci, generating "mine fields" that keep immune cells at bay. Other toxins act at a distance from the invading pathogen much like a bullet or an "intelligent" missile. Tetanus toxin circulates in blood and adheres to neuronal receptors (Schiavo et al. 2000). Following endocytosis, the engulfed toxin exerts its pathogenic property inside the target cell and cleaves proteins to prevent the fusion of synaptic vesicles with the plasma membrane. The infected host dies from respiratory paralysis while the underlying infection itself, typically contamination of a minor wound with *Clostridium tetani*, is located elsewhere and plays only an indirect role in the outcome of the disease. Bacteria have evolved a bounty of mechanisms for toxin secretion: the Sec pathway, autotransporter, type I–IV secretion, and toxin release systems

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(e.g., tetanus toxin). The type III and type IV secretion systems of Gram-negative pathogens can be viewed as bacterial arms for close combat with immune cells. A protein conduit is formed between the pathogen and the host cell that provides for the rapid and massive deposition of proteins (type III and type IV) or for the transport of DNA (type IV).

If you want to invade another host—get out if you can

The killing of a human host allows *Clostridium tetani* to multiply as these anaerobic microbes can only grow in tissues that are not circulated with oxygenated blood. But the killing doesn't provide microbes with a mechanism for transmission to a new host. Several enteric pathogens, for example, *Salmonella*, *Shigella*, or *V. cholerae*, have elegantly solved the problem by spreading via fecal–oral contamination. During *V. cholera* infections enough cholera toxin is secreted to cause a fulminant diarrhea with large amounts of infectious microbes while carefully avoiding the rapid killing of the host. Again, it is protein secretion that matters as too much of a toxin is not always a good thing and can limit the success of a pathogen. This article describes the principles of protein secretion by pathogenic microbes.

Gram-positive and Gram-negative microbes

Bacteria can be classified with the staining procedure of Christian Gram. Gram-positive bacteria retain the dye crystal violet, which is removed with ethanol from the envelope of Gram-negative bacteria (Popescu and Doyle 1996). The different staining properties are caused by differences in envelope structure. Gram-positive microbes elaborate a single plasma membrane (also called inner membrane [IM]; Fig. 1A) followed by a thick cell wall layer (CW) that functions as a surface organelle for the display of carbohydrates and proteins (Fig. 1; Ghuysen and Hackenbeck 1994). A double membrane surrounds Gram-negative bacteria, enclosing the periplasmic space and peptidoglycan layer between two lipid bilayers (Fig. 1; Inouye 1979). The outer membrane (OM) of Gram-negative bacteria functions as a surface organelle for the display of proteins and carbohydrates (Inouye 1979). Assembly of some bacterial envelope structures, for example, outer membrane or cell wall, is essential for the growth and the replication of microbes (Ghuysen and Hackenbeck 1994). In contrast, the secretion of proteins into the extracellular milieu appears to be essential for bacterial multiplication in infected host tissues, but it is not required for microbial growth under laboratory conditions (Pugsley 1993a).

Secretion across the plasma membrane

Protein secretion into the extracellular milieu by Gram-positive pathogens requires transport of polypeptides across the plasma membrane and the cell wall envelope (Fig. 1A; Smith et al. 1978, 1980, 1981; Schneewind et al. 1992). This process has been studied very little. Genome sequencing of a variety of Gram-positives showed that many of the secretion genes, which were initially identified in *E. coli* (Schatz and Beckwith 1990), are also present in these organisms. Transport of proteins across the plasma membrane of Gram-negative organisms leads to secretion into the periplasm but not to secretion into the extracellular milieu envelope (Fig. 1B,C; Pugsley 1993a). The thin peptidoglycan layer of Gram-negative organisms is not thought to function as a permeability barrier

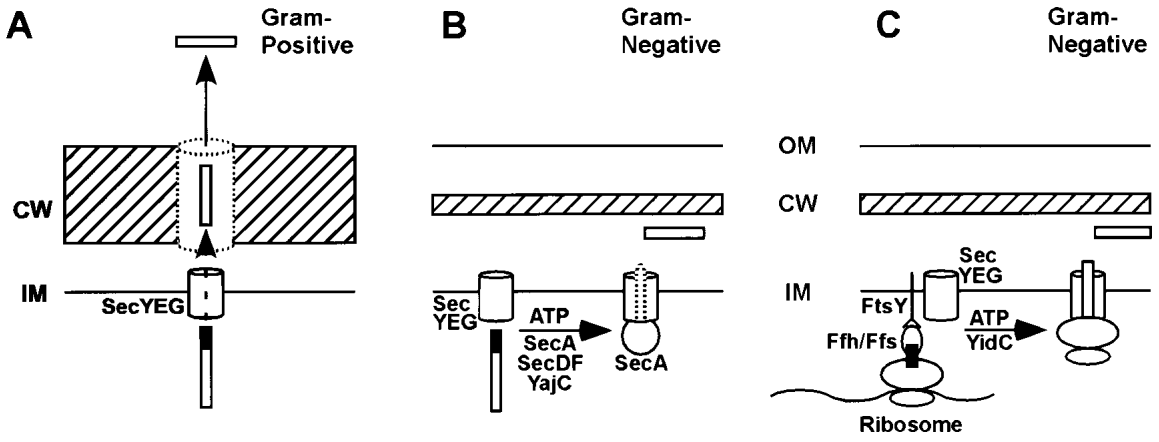

Figure 1. The Sec pathway transports proteins across the bacteria plasma membranes. (*A*) The SecA and SRP pathway of Gram-positive bacteria translocates proteins across the plasma membrane. The cell wall envelope (CW) of some organisms is thought to be impermeable for polypeptides, however factors for protein transport across the cell wall have hitherto not been identified. Proteins are translocated across the plasma membrane of Gram-negative bacteria in a post-translational (*B*) or cotranslational manner (*C*). (IM) Inner membrane. (*B*) Signal peptide (black box) bearing precursors are recognized by SecA and SecYEG. Upon substrate binding and ATP hydrolysis, SecA pushes the precursor through the SecYEG translocon in a manner that also requires SecDF and YajC. After the signal peptide is removed by signal peptidase, the translocated protein is released into the periplasm. (*C*) Signal peptides of nascent polypeptides are recognized by the signal recognition particle, SRP, which is composed of Ffh and Ffs. This interaction stalls ribosomal translation, which is restored once the SRP complex interacts with the SRP receptor, FtsY, and the ribosomes is docked on the translocon. The membrane protein YidC is also thought to be involved in the cotranslational secretion of membrane proteins. (OM) Outer membrane. Drawings adapted from Duong et al. (1997).

for folded proteins. Gram-negative organisms have evolved dedicated secretion systems that transport polypeptides beyond the outer membrane.

The Sec pathway has evolved to translocate polypeptides across the plasma membrane. Proteins destined for transport by this pathway are synthesized as signal peptide bearing precursors (Lingappa and Blobel 1980; Silhavy et al. 1983; Gennity et al. 1990). Signal peptides are generally positioned at the N terminus and consist of one or more positively charged amino acids followed by a stretch of 10–20 hydrophobic amino acids (Silhavy et al. 1983; Gennity et al. 1990). Once the precursors have been translocated across the membrane, the signal peptides are removed by signal peptidase (Chang et al. 1982; Dalbey and Wickner 1985). Some polypeptides insert into the plasma membrane using a noncleavable signal peptide, also referred to as a signal/anchor sequence (type I membrane protein; Davis and Model 1985; Davis et al. 1985; Singer 1990). Other polypeptides insert into the plasma membrane by using both a cleavable signal peptide and a second hydrophobic sequence that is located downstream (stop transfer or membrane anchor sequence–type II membrane protein; Lingappa et al. 1978). The Sec pathway was first characterized by searching for *E. coli* suppressor mutants that restore secretion of an outer membrane protein with a defective signal (Emr et al. 1981). Another search used LacZ fusions to the C terminus of secreted proteins, resulting in hybrids that jam the Sec pathway as LacZ folds rapidly into a nonsecretable conformation (Silhavy et al. 1976, 1977). Temperature sensitive mutations in *sec* genes allowed the internalization of a larger fraction of LacZ hybrids, thereby conferring a Lac-up phenotype (Oliver and Beckwith 1981). These genetic approaches have been complemented by biochemical studies measuring the Sec-mediated translocation of precursor proteins into membrane vesicles (Muller and Blobel 1984; Brundage et al. 1990). The membrane-embedded translocation machinery is composed of SecYEG and YajC, which together with the membrane proteins SecDF and cytoplasmic ATPase SecA, are sufficient to promote the translocation of precursor proteins into the lumen of membrane vesicles in vitro (Fig. 1B; Duong and Wickner 1997).

The translocation pore of the *E. coli* Sec pathway is composed of three membrane proteins, SecYEG, which can accept substrates in two ways (Duong et al. 1997; Pohlschroder et al. 1997). Signal peptide-bearing precursor proteins are maintained in a secretion-competent state by binding to chaperones, for example, SecB (Randall 1992). *Bacillus subtilis* and other Gram-positive bacteria make do without SecB, and other chaperones presumably function as substitute (Kunst et al. 1997). Translocation substrate is transferred to SecA, an ATPase that undergoes conformational rearrangements upon interacting with the secretion machinery, a process that is thought to push precursor proteins through the translocation pore (Economou and Wickner 1994). Once polypeptides have been translocated, signal peptidase removes the signal peptide from the precursor and mature protein is released into the periplasmic space (Dalbey and Wickner 1985). A second mechanism of protein secretion involves the cotranslational translocation of membrane proteins (Ulbrandt et al. 1997). The signal recognition particle (SRP), Ffh and 4.5 S RNA (*ffs*) in *E. coli*, binds to signal peptide-bearing nascent polypeptides, an interaction that is thought to stall ribosomal translation (Poritz et al. 1990). Once the SRP complex is bound to its receptor (FtsY in *E. coli*) and the ribosome has docked on the translocation pore, translation resumes and presumably provides the force to translocate polypeptides across the membrane (Miller et al. 1994). Three membrane proteins, SecD, SecF, and YajC, associate with the SecYEG pore and appear to regulate SecA-dependent translocation activity (Fig. 1C; Duong and Wickner 1997). The membrane protein YidC appears to be required for the insertion of polytopic membrane proteins into the plasma membrane (Samuelson et al. 2000); an association of YidC with the SecYEG translocase has not yet been revealed.

The genome of the Gram-positive pathogen *S. aureus* contains *secAYEG* and *yajC* similar to *E. coli* and *B. subtilis* (Blattner et al. 1997; Kunst et al. 1997). A *secB* gene could not be found, however, a second set of secretion genes, *secA-2* and *secY-2*, was identified. As observed for *B. subtilis*, the *S. aureus* genome encodes for a *secDF* fusion gene but not for single *secD* and *secF* genes (Bolhuis et al. 1998). Two signal peptidase genes (*spsA* and *spsB*) are present in the *S. aureus* chromosome (Cregg et al. 1996), whereas *ffh* and *ftsY* are present in single copy only. It is not clear whether the presence of two sets of secretion genes results in the assembly of two secretion pathways in *S. aureus*. The cell wall envelope of some Gram-positive bacteria acts as a diffusion barrier for the movement of proteins. Few studies have addressed whether additional factors are required to facilitate secretion of proteins across the bacterial cell wall. *B. subtilis* PrsA is a peptidyl–prolyl isomerase and tethered to the bacterial plasma membrane via thioether diacylglyceride modification (Jacobs et al. 1993; Kontinen and Sarvas 1993). PrsA is thought to act on translocated proteins by catalyzing their folding and release into the extracellular medium (Vitikainen et al. 2001).

Bacterial surface organelles

Surface proteins of Gram-negative organisms are inserted into the outer membrane. The outer membrane is an asymmetric bilayer with an inner phospholipid leaflet and an outer leaflet that is largely composed of lipopolysaccharide (LPS; Raetz 1987). Five or six acyl side chains of LPS create the hydrophobic environment and are attached to the phosphorylated saccharide KDO (Imoto et al. 1983; Qureshi et al. 1983; Takayama et al. 1983). The phosphoryl groups of neighboring LPS molecules are complexed with magnesium ions, an ionic interaction that is important for the integrity of the membrane envelope. Attached to KDO and protruding on the bacterial surface are long polysaccharide chains, whose composition is highly variable among bacterial species (Rietschel 1984) and even subject to regulated variation within organisms (Ernst et al. 1999). Proteins destined for the

outer membrane are translocated across the inner membrane via the Sec pathway. A large number of folding factors appear to act on outer membrane proteins (Missiakas and Raina 1997; Danese and Silhavy 1998), which assume β-barrel structures that expose the alternating hydrophobic residues of β-sheets at the interface with membrane lipids (Cowan et al. 1992). The outer membrane is tethered to the peptidoglycan layer via murein (Braun) lipoprotein, a short helical polypeptide that is covalently linked to the peptidoglycan at the C-terminal end (Braun and Hantke 1974). The N terminus of lipoprotein is inserted into the inner leaflet of the outer membrane with its diacylglyceride decoration (Hantke and Braun 1973). Electron microscopic studies suggest the existence of adhesion zones between inner and outer membranes (Bayer 1979; Lopez and Webster 1985). The physiological relevance of these adhesions has been under debate for a long time. It is not yet clear whether the adhesions truly represent interacting lipid bilayers and whether adhesions play a role in the assembly of the outer membrane and its proteins. Pulse-labeling experiments revealed the appearance and disappearance of soluble intermediates in pathways that leads to the insertion of outer membrane proteins (Stader and Silhavy 1988; Brissette and Russel 1990). These observations certainly do not support a model whereby outer membrane proteins are transported via lipid adhesions.

Several outer membrane proteins have been characterized as receptors for host proteins or tissues (Fig. 2A; Isberg and Falkow 1985; Isberg et al. 1987). One well-studied example is *Yersinia pseudotuberculosis* invasin. The N-terminal domain of invasin is inserted into the outer membrane (Leong et al. 1991), whereas the C-terminal domain protrudes 180 Å on the bacterial surface using a string of IgG-like domains as a folding module (Hamburger et al. 1999). Positioned at the C-terminal end of invasin is the adhesive part of the molecule with a folded structure resembling that of C-type lectins (Kelly et al. 1999; Luo et al. 2000). Invasin binds β1 integrin surface receptors of host cells (Isberg and Leong 1990) and this mechanism is thought to be instrumental during the intestinal uptake of *Y. pseudotuberculosis* and *Y. enterocolitica*, leading to bacterial infection of lymphoid tissues within the intestines (Pepe and Miller 1993). Intimins are another group of receptors displayed on the surface of *E. coli* as well as several other Gram-negative pathogens and provide a function that allows bacteria to inject virulence factors via the type III pathway (see below) into host cells (Kenny and Finlay 1997). Intimin binds to Tir, a secreted bacterial protein that inserts into host cell membranes where it is phosphorylated (Kenny et al. 1997). The mechanism of target cell selection for Tir insertion has not been established. The structures of intimin and the intimin/Tir complex have been solved and consist of three immunoglobulin folds connecting a C-type lectin-like receptor binding domain to the outer membrane (Kelly et al. 1999; Luo et al. 2000).

Surface proteins of many different Gram-positive bacteria are tethered to the cell wall envelope by a mechanism requiring a C-terminal sorting signal with an LPXTG motif (Fig. 2B; Schneewind et al. 1992, 1993). After initiation of surface proteins into the Sec pathway, the sorting signal is recognized and cleaved by sortase, a membrane-associated transpeptidase (Mazmanian et al. 1999; Ton-That et al. 1999). The carboxyl group of threonine within the LPXTG motif is amide linked to peptidoglycan cross-bridges, thereby tethering the C-terminal end of surface proteins to the cell wall envelope (Schneewind et al. 1995; Ton-That et al. 1997; Navarre et al. 1998). A second targeting mechanism for surface proteins involves the binding of polypeptides to designated envelope structures, cell wall teichoic acids, or lipoteichoic acids (Sanchez-Puelles et al. 1990; Baba and Schneewind 1996, 1998; Jonquieres et al. 1999; Varea et al. 2000). Teichoic acids are often composed of polyribitolphosphate or polyglycerophosphate backbone structures (Fischer 1997). Species-specific esterified decorations of these molecules appear to be required for the anchoring of some surface proteins to the cell wall envelope (Holtje and Tomasz 1975). Bacterial crystalline layers are surface organelles that are assembled from secreted polypeptides (Sleytr et al. 1993). After secretion, the polypeptides bind to carbohydrate receptors on the bacterial surface and aggregate into regularly shaped arrays, which form the new surface layer (Ries et al. 1997; Egelseer et al. 1998). For example, surface layer proteins of *B. anthracis* adhere to pyruvylated cell wall polysac-

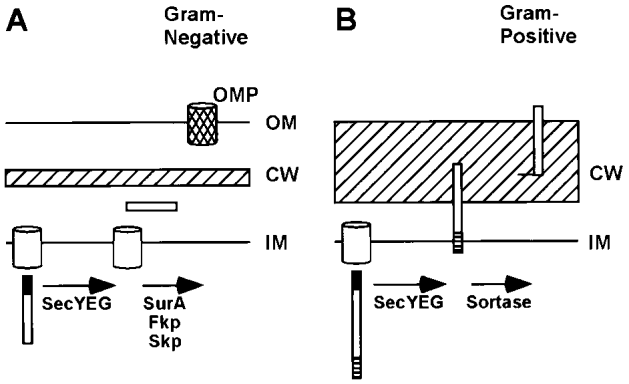

Figure 2. Display of proteins on the bacterial surface. (*A*) Gram-negative bacteria anchor surface proteins in the outer membrane (OM). After translocation and signal peptide cleavage of precursor proteins, outer membrane proteins (OMPs) are folded in a manner that requires the peptidyl–prolyl isomerases SurA and Fkp. Skp and the presumed cofactor lipopolysaccharide are thought to promote membrane insertion of OMPs. (CW) Cell wall; (IM) inner membrane. (*B*) Gram-positive bacteria anchor surface proteins to the cell wall envelope. Signal peptide bearing surface proteins are initiated into the Sec pathway. The C-terminal sorting signal (hatched box), which is composed of an LPXTG motif, a hydrophobic domain, and charged tail, retains translocated polypeptides within the secretory pathway. Sortase, a membrane anchored transpeptidase, cleaves the sorting signal between the threonine and the glycine of the LPXTG motif and links the C-terminal carboxyl group to the amino group of peptidoglycan cross-bridges within the cell wall envelope. Drawings adapted from Duong et al. (1997) and Navarre and Schneewind (1999).

charide (Mesnage et al. 2000). The Gram-negative organism *Aeromonas hydrophila* employs a type II secretion pathway (see below) to assemble an array of paracrystalline protein on the bacterial surface (Thomas and Trust 1995a,b).

Secretion pathways of Gram-negative bacteria

Research over the past two decades has identified a large number of secreted proteins that play important roles during the pathogenesis of infections caused by Gram-negative bacteria. The pathways whereby these proteins are transported have been separated into several classes: (1) type I pili (Soto and Hultgren 1999; Sauer et al. 2000); (2) auto-transporters; (3) type I secretion (Koronakis and Hughes 1996; Binet et al. 1997); (4) type II secretion (general secretory pathway [GSP]; Russel 1998) and type IV pili (Nunn 1999); (5) type III secretion (Hueck 1998; Galan and Collmer 1999) and flagella (Macnab 1992); and (6) type IV secretion (Christie and Vogel 2000) and DNA conjugation (Pansegrau and Lanka 1996). The classification is based on the molecular nature of the transport machineries and their catalyzed reactions. Many excellent reviews that provide a more detailed and in-depth treatise of the various subjects have been published recently for each of the six classes of secretion pathways (Macnab 1992; Koronakis and Hughes 1996; Pansegrau and Lanka 1996; Binet et al. 1997; Hueck 1998; Russel 1998; Galan and Collmer 1999; Soto and Hultgren 1999; Sauer et al. 2000; Christie 2001; Plano et al. 2001; Sandkvist 2001). This review will emphasize recent findings about secretion machines and the various mechanisms whereby proteins can be transported.

Assembly of type I pili

Type I pili (Duguid et al. 1955; Brinton 1959) allow *E. coli* and other Gram-negative bacteria to attach to host cells during the initial stages of infection (for detailed review, see Soto and Hultgren 1999; Sauer et al. 2000). Pili are essential for bacterial colonization of the urinary tract as the invading microbes are confronted with an additional defense barrier, the flow of urine. Employing adhesion and retraction properties of pili, bacteria advance against the flow of urine and colonize the lower (cystitis) or upper (pyelonephritis) urinary tract of humans. *E. coli* strains associated with pyelonephritis display Pap pili, structures that are composed of three parts: (1) the tip adhesin; (2) the fibrillum; and (3) the pilus rod (Lindberg et al. 1987; Kuehn et al. 1992). The base of the pilus rod is embedded in the outer membrane of Gram-negative bacteria. Pap (type I) pili are assembled from six pilins, all of which are encoded by a single large operon (Soto and Hultgren 1999). To identify the position of pilins within the pilus, wild-type *E. coli* and mutants lacking single pilin genes were analyzed by electron microscopy and immuno-gold labeling (Norgren et al. 1984; Baga et al. 1987; Lindberg et al. 1987; Kuehn et al. 1992; Jacob-Dubuisson et al. 1993). PapG pilin (G in Fig. 3) functions as an adhesive molecule and is located at the pilus tip (Fig. 3; Lindberg et al. 1987; Lund et al. 1987). The N-terminal domain of PapG binds digalactoside, a glycolipid that is located on the surface of host cells (Kallenius et al. 1980; Leffler and Svanborg-Eden 1981; Bock et al. 1985). The C-terminal domain of PapG is incorporated into the fibrillum (Hultgren et al. 1989) via the adapter protein PapF (Lindberg et al. 1987; Kuehn et al. 1992; Jacob-Dubuisson et al. 1993). The fibrillum, which consists mostly of PapE subunits, is attached to the pilus rod via a second adapter protein, PapK (Jacob-Dubuisson et al. 1993). PapA is the major pilin subunit and assembles to form the pilus rod (Normark et al. 1983; Norgren et al. 1984). PapH terminates the polymerization of PapA and is presumably positioned at the pilus base within the plane of the outer membrane bilayer (Baga et al. 1987). PapJ is required to prevent the release of growing pili into the extracellular milieu (Tennent et al. 1990). Deletion of *papJ*, encoding a periplasmic pilin-like subunit, results in the release of pili into the culture medium, a phenotype that is similar to that of *papH* mutants (Tennent et al. 1990). Plasmid-encoded *papJ* complements the phenotype of *papJ* but not that of *papH* mutants, suggesting that PapJ and PapH fulfill distinct functions during pilus assembly (Tennent et al. 1990). PapJ encompasses a Walker box domain (ATP-binding site), however, its significance is not clear as the periplasm is not believed to contain nucleotides.

Assembly of Pap pili occurs in two stages (Fig. 3). Pilins are first translocated by the Sec pathway into the periplasmic space (Baga et al. 1984, 1987; Norgren et al. 1984; Lindberg et al. 1986; Tennent et al. 1990). Each pilin is engaged by the PapD chaperone (D), thereby preventing premature aggregation of subunits within the periplasm (Kuehn et al. 1991, 1993). During the second

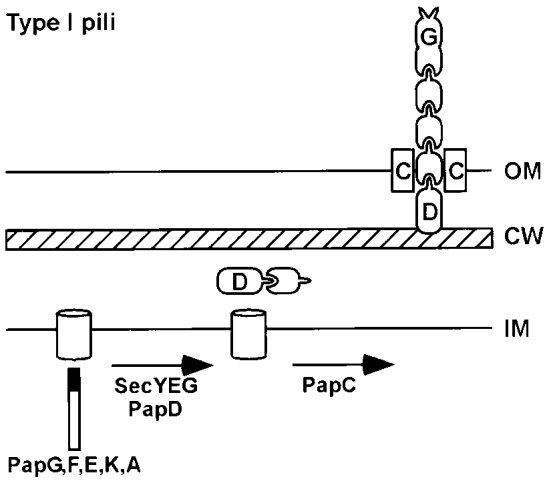

Figure 3. Assembly pathway of type I pili. Six different pilins assemble into a structure that is composed of a tip protein PapG (G), a fibrillum consisting of PapF, PapE, and PapK, as well as the pilus rod consisting of PapA. After Sec-mediated translocation across the plasma membrane, pilin associate with the periplasmic chaperone PapD (D). Pilin–PapD complexes dissociated at the assembly site, that is, the outer membrane usher PapC (C), in a series of donor strand exchange reactions. Drawing adapted from Sauer et al. (2000).

stage of pilus assembly, pilin-chaperone complexes associate with PapC (C), the usher protein in the outer membrane (Dodson et al. 1993). PapC forms a pore structure with an inner diameter of 2–3 nm that is large enough to accommodate folded pilin subunits (Thanassi et al. 1998). Pilin-chaperone complexes are thought to occupy the pore structure and newly formed pili emanate on the bacterial surface, assembled by successive addition of pilins at the outer membrane base (Saulino et al. 2000).

How is an ordered assembly of pilins achieved? A model of kinetic partitioning has emerged that assumes that assembly sites (PapC) display altered affinity for subunits when engaged with different pilins. PapG, the fibrillar tip component, complexed with the PapD chaperone displays the highest affinity for the empty PapC usher (Dodson et al. 1993). The chaperone of PapC:PapG–PapD complexes could be dissociated by incoming PapF–PapD complexes. The chaperone of PapC:PapG/PapF–PapD could in turn be dissociated by PapE–PapD and so forth, until PapA–PapD dissociates PapC:PapG/F/E_n/K/A_n–PapD to form the PapG/F/E/K/A_{n+1}–PapD product.

Structural analysis of pilins and pilin-chaperone complexes has provided a more detailed understanding of pilus assembly. PapD is composed of two globular domains, each with an IgG-like fold (Holmgren and Branden 1989). Pilin subunits assume a similar IgG-like fold, however the seventh β-sheet of the IgG-barrel is absent, resulting in the exposure of a hydrophobic groove on the molecular surface (Choudhury et al. 1999; Sauer et al. 1999). Exposure of this hydrophobic surface likely causes pilin aggregation (Lindberg et al. 1989; Kuehn et al. 1991). PapD stabilizes bound pilins by providing the missing β-sheet in a mechanism that has been referred to as "donor strand complementation" or "domain swapping". The N terminus of pilin subunits consist of alternating hydrophobic amino acids much like the complementing strand of the PapD chaperone and has been implicated in subunit interactions. The PapD chaperone of PapG–PapD, complexed with the outer membrane usher PapC, could be dissociated by the N terminus of PapF, thereby completing the IgG-like fold (Eisenberg 1999; Sauer et al. 1999). Pilus assembly can thus be viewed as a series of domain swapping reactions in which the seventh β-sheet of the pilin β-barrel is provided by either the periplasmic PapD or the N-terminal extension of incoming pilins. The specificity of pilin selection into the growing pilus presumably is determined by the interaction of the hydrophobic groove of the pilin subunit complexed with the usher and the structure of the N terminus in subsequent pilin subunits.

How is pilus assembly terminated? PapA is the most abundant pilin both in the bacterial periplasm and in the assembled pilus. PapH is present only in small amounts (Normark et al. 1983; Baga et al. 1987). Experimental increase in the cellular concentration of PapH shortens the length of pili dramatically. In contrast, a reduced concentration of PapH results in elongated pili and in loss of pili into the extracellular milieu (Baga et al. 1987). It is not yet known whether PapH, the terminator of assembly, assumes a complete IgG-like fold or an altered hydrophobic surface that prevents other pilin subunits from binding (Barnhart et al. 2000). As assembled subunits exit the periplasmic space, pilins are sequestered from the periplasm and cannot be removed from the structure by additional domain swapping reactions. What is the energy source that drives pilus assembly? It appears that assembly or disassembly may be fueled solely by gradients of concentration. During assembly, the concentration of pilin-chaperone complexes is high and that of outer membrane assembly sites low, favoring the dissociation of PapD and pilins. During disassembly, which has not yet been studied extensively, the concentration of "empty" chaperone may be high, promoting the removal of pilins from the pilus base. Alternatively, PapJ may discharge pili out of the usher into the extracellular medium when concentrations of other pilins are low.

The inner diameter of the PapC usher pore (2–3 nm) permits assembly and extrusion of folded pilins. In contrast, the 7-nm pilus rod is too large to exit the usher (Thanassi et al. 1998). Assembled rods can be unraveled into linear fibers with a diameter of 2 nm (Abraham et al. 1983; Bullitt and Makowski 1995; Thanassi et al. 1998). Presumably, polymerized PapA subunits undergo a rearrangement, forming a wider assembly that may stabilize the pilus rod. Knowledge of the atomic structure of PapA has been used to build a molecular model for a 7-nm-wide rod (Choudhury et al. 1999; Sauer et al. 1999). The pilus is generated by helical right-handed rotation, with 3.3 PapA subunits per turn, and a central cavity of 1.5–2.5 nm, dimensions that have been observed in native pili (Gong and Makowski 1992; Bullitt and Makowski 1995).

Autotransporter

Pathogenic *Neisseria*, *N. gonorrhea*, and *N. meninigitis*, infect humans and survive in for prolonged periods of time their tissues. Within their host *Neisseria* must escape the immune response, in particular secretory IgA antibodies in urinary and oral mucosa fluids that facilitate complement-mediated killing and opsonization of microbes. *Neisseria* secrete IgA protease, an enzyme that cleaves antibodies on mucosal surfaces (Halter et al. 1984). IgA protease is synthesized as a pre-proenzyme (1528 residues; Pohlner et al. 1987). An N-terminal signal peptide initiates the precursor into the Sec pathway (Fig. 4). After cleavage of the signal peptide by signal peptidase, the proenzyme resides in the bacterial periplasm and is presumed to be only partially folded. The C-terminal β-domain of IgA protease (residues 1254–1528) assumes a β-barrel structure that inserts into the outer membrane and functions as an autotransporter for the N-terminal domain. Once the N-terminal protease domain is exposed on the bacterial surface, it cleaves the proenzyme at the junction between the N-terminal and the C-terminal domain (residues P1117–A1118). The cleaved N-terminal domain of the proenzyme is released from the bacterial surface and acts as a diffusible viru-

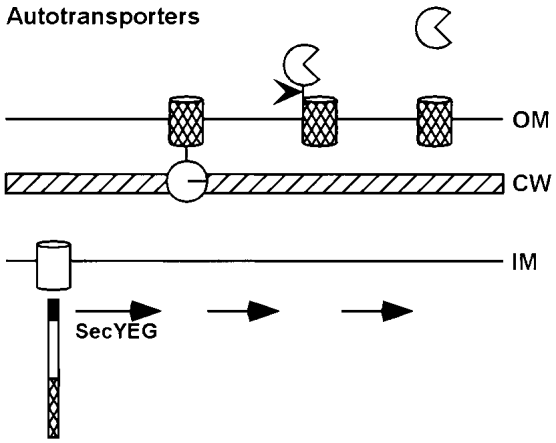

Figure 4. Autotransporters. *Neisseria* IgA protease is synthesized as a pre-proenzyme. After Sec-mediated translocation across the cytoplasmic membrane, the C-terminal domain of the proenzyme inserts into outer membrane and translocates the N-terminal domain through its lumen. The N-terminal domain acts as a protease that cleaves its peptide tether with the C-terminal domain (arrowhead) and is thereafter released into the extracellular medium. Drawing adapted from Pohlner et al. (1987).

lence factor, which matures into the 106-kD IgA protease and the small stable α-protein. Fusion of signal peptide bearing polypeptides to the N terminus of the β-domain results in autotransport of the hybrid polypeptide across the outer membrane (Klauser et al. 1990). Expression of IgA protease or β-domain fusions in *E. coli* also result in autotransport, suggesting that this pathway requires no specific machinery factors other than the Sec pathway (Klauser et al. 1992). Autotransporters with a C-terminal β-domain similar to that of *Neisseria* IgA protease have been found in several other Gram-negative organisms.

Type I secretion

Bacteria secrete pore-forming toxins and degradative enzymes as a mechanism of countering host defenses. Several of these proteins are transported across the bacterial envelope using ATP-binding cassette (ABC) containing transporters (type I secretion). *E. coli* HlyA is a well-studied example for type I secretion and is discussed here (Fig. 5). Pathogenic *E. coli* secrete hemolysin (HlyA) into the extracellular milieu (for detailed review, see Koronakis and Hughes 1996; Binet et al. 1997). HlyA is a lipid-modified polypeptide with a domain that is composed of 11–17 nine-amino-acid repeats (LxGGxGND; Felmlee and Welch 1988). The repeat domains bind calcium and are thought to interact with host cells, triggering HlyA insertion into the plasma membrane and leakage of the cytoplasmic contents of target cells (Ludwig et al. 1988; Boehm et al. 1990a,b; Ostolaza et al. 1995). Similar repeat domains have been identified in secreted proteins of other Gram-negative bacteria and these polypeptides are collectively referred to as the family of repeat toxins (RTX; Coote 1992).

After synthesis in the bacterial cytoplasm, HlyA is modified by N-acylation of two lysine residues with myristate or palmitate (Stanley et al. 1994, 1998) in a reaction that requires the product of *hlyC*, the acyl carrier protein (ACP), and ATP (Issartel et al. 1991). HlyC functions as an acyl-transferase to decorate the ε-amino groups of lysine within HlyA using thioester-linked fatty acids (ACP) as substrate (Hardie et al. 1991; Issartel et al. 1991). Knockout mutations of *hlyC* and *acp* abolish lipid modification and the hemolytic property of HlyA but do not affect secretion of the unmodified polypeptide across the double membrane envelope of *E. coli* (Ludwig et al. 1987). HlyA polypeptide is not cleaved during the secretion process (Felmlee et al. 1985a,b). Fusion of HlyA to the C terminus of cytoplasmic reporter proteins resulted in secretion of the hybid proteins into the extracellular milieu (Fig. 5; Gray et al. 1986; Ludwig et al. 1987; Mackman et al. 1987). Deletion of HlyA sequences from the hybrid polypeptides identified a 20–60 residue C-terminal peptide signal that is necessary and sufficient for secretion of reporter proteins (Gray et al. 1989; Koronakis et al. 1989; Hess et al. 1990). Further mutational analysis of the C-terminal secretion signal revealed several amino acids involved in the signal recognition (Kenny et al. 1992, 1994).

Knockout mutations of *hlyB*, *hlyD*, and *tolC* abolish secretion and cause HlyA to reside in the bacterial cytoplasm (Wagner et al. 1983; Koronakis et al. 1988; Wandersman and Delepelaire 1990). *hlyB* encodes an inner membrane protein with an ATP-binding cassette (ABC transporter; Delepelaire and Wandersman 1991; Wang et al. 1991; Gentschev and Goebel 1992). TolC is a trimeric

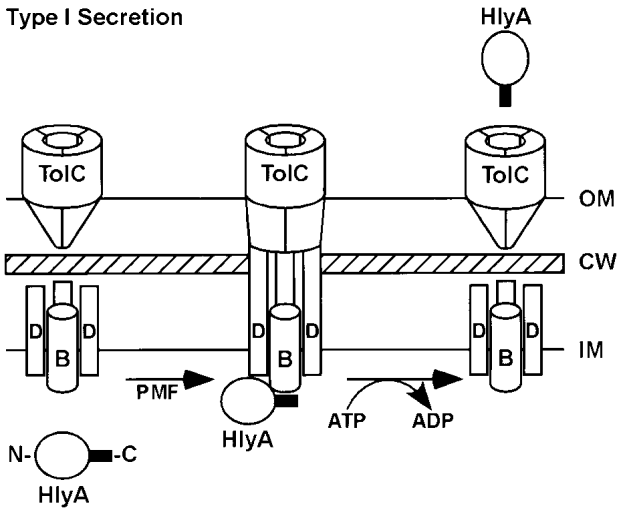

Figure 5. Type I secretion of repeat toxins. *E. coli* HlyA interacts with its cognate ABC transporter (HlyB, ATP-binding cassette) and the trimeric membrane fusion protein HlyD in a proton motive force (PMF) dependent manner. HlyA is translocated simultaneously across the inner and outer membrane in a reaction that requires ATP hydrolysis in the bacterial cytoplasm and interaction of trimeric HlyD with the trimeric outer membrane transporter TolC. Drawing adapted from Stanley et al. (1998) and Zgurskaya and Nikaido (2000).

outer membrane protein that forms a central, water-filled cavity. The crystal structure of TolC revealed two domains, an N-terminal domain with four β-sheets typical of outer membrane proteins and a C-terminal domain with four 10-nm α-helices that extend into the periplasm (Koronakis et al. 2000). Assembled trimeric TolC forms a 12-stranded β-barrel in the outer membrane with an inner diameter of 3.5 nm (Koronakis et al. 2000). Unlike outer membrane porins, the interior of the β-barrel is occluded as the C-terminal α-helices of TolC taper to closure and prevent leakage of periplasmic contents across the outer membrane (Koronakis et al. 2000). TolC appears to be involved in several *E. coli* transport processes that are similar to the type I secretion mechanism, including the efflux of antibiotics, heavy metal ions, detergents, and solvent (multi-drug efflux). HlyD spans the inner and the outer membrane of *E. coli* and binds directly to both TolC and HlyB (Schulein et al. 1992; Letoffe et al. 1996; Thanabalu et al. 1998).

During secretion, HlyA forms a complex with HlyB and HlyD (Fig. 5). Assembly of trimeric HlyD with HlyB occurs in the presence and absence of secretion substrate (Thanabalu et al. 1998). HlyD trimerization is a requirement for its interaction with TolC (Thanabalu et al. 1998). In the initial phases of transport, HlyA polypeptide is bound to the ABC transporter and the membrane-fusion complex leading to a transient complex with the outer membrane TolC trimer. Several different transport steps can be envisaged: (1) formation of a complex between HlyA and HlyB/HlyD; (2) binding of ATP by $HlyA/HlyB_n/HlyD_3$, presumably the first committed step of transport; (3) Conformational change of HlyD resulting in the formation of a $HlyA/HlyB_n/HlyD_3/TolC_3$ complex; (4) HlyB-mediated movement of HlyA across the plasma membrane and ATP hydrolysis; (5) movement of HlyA across the outer membrane. Catalysis of steps 1–4 could occur by alterations in the folding state of HlyB and could be triggered by the binding, hydrolysis, and release of ATP (Koronakis et al. 1993). Further, the proton motive force across the plasma membrane of *E. coli* plays a critical role in secretion as reagents that disrupt the proton gradient also abolish the initial stages of HlyA transport (Koronakis et al. 1991).

Similar ABC transporters, membrane fusion proteins, and outer membrane pore proteins have been identified in the secretion pathways for RTX toxins and other proteins of many Gram-negative bacteria. It seems that Gram-negative pathogens use this system to transport virulence factors across the bacterial envelope (Fleischmann et al. 1995; Stover et al. 2000). Gram-positive organisms also employ ABC transporters (Kunst et al. 1997). As Gram-positive bacteria lack the outer membrane permeability barrier, the HlyD and TolC components of the Gram-negative apparatus are not required for secretion in Gram-positive organisms. In addition to secretion of proteins in bacteria, ABC transporter are also found in eukaryotes from yeast to humans and are responsible for the efflux and influx of a large number of ions and small molecules (Holland and Blight 1999; Zgurskaya and Nikaido 2000).

Type II secretion, type IV pili, and filamentous phage

Type II secretion

Secretion of type II substrates occurs in two stages (Fig. 6). First, the Sec machinery translocates signal peptide bearing type II substrates across the plasma membrane. Folded secretion substrates are then transported by the type II machinery across the outer membrane. Some well-known bacterial toxins are secreted in this manner. *V. cholerae* secrete cholera toxin, which is composed of CtxA and CtxB subunits (Lonnroth and Holmgren 1973). Mature CtxB assembles into a pentameric ring structure within the bacterial periplasm (Hirst and Holmgren 1987) and associates with the C-terminal domain of mature CtxA to form cholera toxin, $CtxA:CtxB_5$ (Cuatrecasas et al. 1973; Lonnroth and Holmgren 1973; Finkelstein et al. 1974; Heyningen 1974). CtxA forms an intramolecular disulfide bond and is proteolytically processed (Cuatrecasas et al. 1973; Lonnroth and Holmgren 1973; Heyningen 1974). Secreted $CtxB_5$ binds to G_{m1} ganglioside , a sphingoglycolipid, on the surface of human intestinal cells (Cuatrecasas 1973; Holmgren et al. 1973; King and Van Heyningen 1973), causing toxin uptake and reduction of CtxA by cytosolic thioredoxin. The reduced CtxA fragment is released from the membrane-bound receptor complex (Sattler and Wiegandt 1975; To-

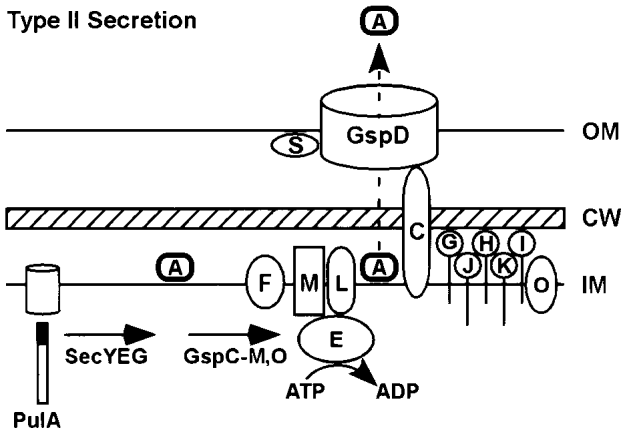

Figure 6. Type II secretion across the outer membrane. *Klebsiella oxytoca* PulA precursor protein is transported by the Sec pathway across the plasma membrane. After substrate recognition by the type II machinery (GSP, general secretory pathway), the fully folded and enzymatically active PulA (A) is translocated across the outer membrane. The outer membrane secretin, GspD, acts as a translocator for PulA and requires binding of the GspS chaperone for insertion into the outer membrane. The type II secretion machinery is assembled from one cytoplasmic component (GspE, an ATPase), five inner membrane proteins (GspC, GspF, GspM, GspL, and GspO), and five pseudopilins (GspG, GspH, GspI, GspJ, and GspK). Pseudopilins harbor a pilin signal peptide (see Fig. 7) and are cleaved by prepilin peptidase (GspO) during Sec-mediated translocation across the plasma membrane. The type II secretion machinery appears to be multifunctional, allowing secretion of proteins across the outer membrane and assembly or retraction of type IV pili. Drawing adapted from Russel (1998) and Sandkvist (2001).

masi et al. 1979) to activate host cell adenylate cyclase (Cassel and Selinger 1977; Cassel and Pfeuffer 1978; Gill and Meren 1978), resulting in massive diarrhea (Greenough et al. 1970; Schafer et al. 1970; Sharp and Hynie 1971; Field et al. 1972). *E. coli* enterotoxin (LT), *Shigella dysenteriae* shiga toxin, and *E. coli* shiga-like toxins are composed of a similar structural assembly (AB toxins), albeit that the mode of action for shiga toxins is to prevent ribosomal translation of host cells (Schmitt et al. 1999). Type II secretion substrates of other Gram-negative organisms include proteases, alkaline phosphatase, pectate lyase, and elastase, as well as the lipoprotein pullulanase (for further details, see Russel 1998).

Klebsiella oxytoca secretes pullulanase, a lipoprotein that forms micelles once it has been secreted into the extracellular milieu (Fig. 6; Wallenfels et al. 1966; Wohner and Wober 1978). Pullulan is a large complex carbohydrate that can not diffuse through bacterial outer membrane pores, whereas the product of pullulanase cleavage, maltotriose, can diffuse (Bender and Wallenfels 1966). When *Klebsiella* are plated on pullulan as the sole carbon source, the type II secretion of pullulanase is required for bacterial growth (Michaelis et al. 1985; d'Enfert et al. 1987). Cloning of a cluster of 16 *Klebsiella* genes (*pulA–O* and *pulS*) into *E. coli* can promote the growth of recombinant strains that use pullulan as the sole carbon source (d'Enfert et al. 1987). These and other results suggest that expression of *pulC–G*, *pulI–M*, *pulO*, and *pulS* is sufficient to promote the type II secretion of pullulanase (PulA) in heterologous organisms (Possot et al. 2000). Once PulA has been translocated by the Sec machinery, its precursor is di-acyl glyceride modified at a cysteine residue and cleaved by type II signal peptidase (Pugsley et al. 1986). An aspartyl residue at position 2 of mature PulA is thought to retain lipid-modified PulA in the outer leaflet of the inner membrane (Poquet et al. 1993b). This mechanism is a prerequisite for type II secretion, as PulA mutants bearing a serine substitution at position 2 are transported by LolAB (Yamaguchi et al. 1988; Matsuyama et al. 1995, 1997) to the inner leaflet of the outer membrane and are not substrate for the secretion machinery (Seydel et al. 1999). Sec translocation and type II secretion of pullulanase can be uncoupled. Expression of PulA without the type II secretion machine results in the accumulation of N-terminally processed PulA in the periplasm (Pugsley et al. 1991). Subsequent expression of the type II secretion machine restores the secretion of PulA into the extracellular milieu (Pugsley et al. 1991; Poquet et al. 1993a).

Type II secretion machines have been referred to as secretons or general secretory pathways and their components have been assigned a unified Gsp nomenclature (Pugsley 1993a). One component, GspD or secretin, is inserted into the outer membrane and oligomerizes into a dodecameric ring structure with an inner diameter of 7.6 nm (Nouwen et al. 1999). Type II machinery-catalyzed transport is thought to move polypeptides through the lumen of GspD. The secretin is not only conserved among type II machines but is also a component of type III machines, the assembly pathway of filamentous phage, and the polymerization of type IV pili (Russel 1994). *K. oxytoca* GspD requires binding to the outer membrane chaperone PulS (GspS) for proper folding and activity in vivo (Hardie et al. 1996a). Not all GspD homologs require a GspS chaperone for folding (Pugsley et al. 1997). The GspS requirement correlates with the presence of a GspS-binding domain in *Klebsiella* GspD (Hardie et al. 1996b). *Erwinia carotovora* and *E. chrysanthemi* each secrete cellulases via the type II pathway. Expression of cellulase (PelB) in the heterologous organism does not lead to type II secretion (He et al. 1991a; Py et al. 1991). However, replacement of *E. carotovora* GspD with *E. chrysanthemi* GspD allowed secretion of *E. chrysanthemi* PelB by *E. crysanthemi*, suggesting that GspD may be involved in substrate recognition during type II secretion (Lindeberg et al. 1996). Experiments exchanging GspD from *K. oxytoca* and *E. caratovora* corroborated this notion (Possot et al. 2000). Coprecipitation experiments suggest an interaction between PelB and the N-terminal domains of GspD (Shevchik et al. 1997); however, expression of hybrids of PulD and Erwinia OutD in *Klebsiella* failed to restore secretion of PulA, suggesting that a different region of GspD is responsible for substrate recognition (Guilvout et al. 1999).

Twelve type II machinery proteins (GspBCFGHIJKLMNO) are positioned in the inner membrane (for review, see Russel 1998). One machinery component (GspE) resides in the cytoplasm (Possot et al. 1992; Pugsley and Dupuy 1992; Nunn and Lory 1993; Pugsley 1993b; Reeves et al. 1994; Sandkvist et al. 1995; Thomas et al. 1997). How can cytoplasmic or plasma membrane proteins catalyze secretion when transport of type II substrates across the plasma membrane is accomplished by the Sec pathway? A definitive answer is not yet in sight.

Type IV pili

Several studies have suggested specific functions for several Gsps during the assembly of type IV pili. Type IV pili are expressed by *Pseudomonas aeruginosa*, *Neisseria gonorrhea*, and other Gram-negative pathogens (Henrichsen 1975; Fussenegger et al. 1997; Tonjum and Koomey 1997). The pili are thought to retract in a coordinated fashion (Bradley 1980), a phenomenon that has been associated with bacterial gliding motility (Wu and Kaiser 1995). Presumably, pilus adhesion to an immobilized surface and pilus retraction can pull *Myxobacteria* or *Neisseria* in one direction. *P. aeruginosa* type IV pili are involved in adhesion to respiratory epithelia (Alm and Mattick 1997; Hahn 1997) and are composed of the major pilin subunit PilA (Fig. 7; Pasloske et al. 1985). PilA is synthesized as a precursor with a signal anchor sequence that also contains a unique N-terminal prepilin signal sequence (Strom et al. 1991). After translocation of PilA by the Sec machinery, the prepilin signal sequence is cleaved and the liberated amino-group is N-methylated. Both reactions are catalyzed by PilD (GspO) and require S-adenosyl-methionine as a cofactor (Nunn and Lory 1991; Dupuy et al. 1992; Strom et al. 1993). Mature, translocated type IV pilin is thought to polymerize at the

plasma membrane (Parge et al. 1995) and the assembled pilus may be extruded through the central cavity of the outer membrane secretin XcpQ (GspD; Parge et al. 1995).

Filamentous phage

The assembly pathway of type IV pili shares several features with the extrusion of filamentous bacteriophages, for example, coliphage f1 (Russel 1991). After replication of single stranded phage DNA in the bacterial cytoplasm, the DNA packaging and export signal (Dotto and Zinder 1983) is recognized by the plasma membrane ATPase pI (Russel and Model 1989), a morphogenic phage protein that is not incorporated into infectious particles (Fig. 8). pI, together with its host cofactor Fib (thioredoxin; Russel and Model 1985), displaces the cytoplasmic DNA binding protein (pV; Gray et al. 1982) and loads coat proteins (pIII, VI, VII, VIII, IX) from the plasma membrane onto bacteriophage DNA (Feng et al. 1999). pVII and pIX are loaded at the tip of the phage, presumably close to the DNA packaging signal, followed by the addition of many subunits of the major coat protein (pVIII; Lopez and Webster 1983). The end of the phage is completed by the addition of pIII and pVI, which mediate stability and infectivity of the phage (Lopez and Webster 1982). The resulting phage particle assumes a cylindrical (filamentous) structure and is extruded across the outer membrane (Russel 1994). Outer membrane translocation is catalyzed by pIV protein, the founding member of the family of outer membrane secretins (GspD; Brissette and Russel 1990). pIV forms a dodecameric donut-shaped ring that has been visualized by electron microscopy (Linderoth et al. 1997). When inserted into artificial membranes, pIV functions as a membrane channel with a pore size of 6 nm, the approximate size of the emanating bacteriophage (6–7 nm; Marciano et al. 1999).

V. cholerae ctxA and *ctxB* are encoded by a filamentous phage that can insert into the bacterial chromosome (Waldor and Mekalanos 1996). In contrast to the 10 proteins encoded by the filamenous coliphage f1, the *ctx* phage encodes only five genes. Most notably the gene encoding the morphogenic protein pIV is missing, whereas a pI homolog is provided by the *ctx* phage genome (Waldor and Mekalanos 1996). *V. cholerae* secretes CtxA and CtxB via a type II pathway that is encoded by *eps* genes (Sandkvist et al. 1997). EpsD is an outer membrane secretin (GspD) and is required for type II secretion of cholera toxin and the assembly of *ctx* bacteriophage (Davis et al. 2000). Another example of shared components in secretion and assembly pathways is the prepilin peptidase PilD (GspO). *P. aeruginosa* type II secretion and type IV pilus assembly use the same prepilin peptidase (PilD/XcpA–GspO; Bally et al. 1992; Nunn and Lory 1993).

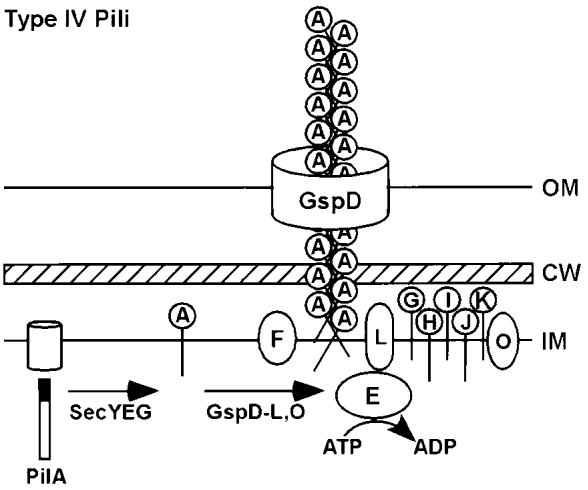

Figure 7. Assembly of type IV pili. *Pseudomonas aeruginosa* PilA assembles into type IV pili and is synthesized as a precursor with a prepilin signal peptide. During Sec-mediated translocation, the prepilin signal peptide is cleaved and the liberated amino-group is N-methylated by PilD (GspO), a reaction that requires S-adenosyl methionine as cofactor. Pilin polymerization requires components of the *Pseudomonas* type II secretion machinery. The growing of pilus is thought to emanate through the outer membrane secretin (PilQ–GspD) onto the bacterial surface. Pilus polymerization requires cytoplasmic PilB (GspE), plasma membrane PilN, PilC, PilD (GspL, GspF, GspO), and pseudopilins PilE, PilX, PilV, PilW. FimU, and FimT (GspG, GspH, GspI, GspJ, and GspK). Drawing adapted from Nunn (1999).

Components of the general secretory pathway and substrate recognition

Five Gsp proteins (GspGIHJK—pseudopilins) are exported with a prepilin signal sequence and are processed by GspO (Bally et al. 1992; Nunn and Lory 1992, 1993; Pugsley and Dupuy 1992). *K. oxytoca* and *E. chrysanthemi* GspG, GspI, GspJ, and GspO are each essential for type II secretion (Lindeberg et al. 1996; Possot et al. 2000). Cross-linking studies in *Pseudomonas* revealed interactions between PilA and pseudopilins (Lu et al. 1997). Furthermore, knockout mutations of *pilA* reduce the transport of several *Pseudomonas* type II substrates, suggesting that the interaction between PilA and pseudopilins may facilitate the secretion process (Lu et al. 1997). Overexpression of one pseudopilin, GspG, results in the assembly of a pilus-like structure on the surface of *Klebsiella* (Sauvonnet et al. 2000). Nevertheless, the precise role of pseudopilins and pseudopili in type II secretion has thus far remained elusive. GspE, a cytoplasmic ATPase, interacts with the inner membrane protein GspL (Sandkvist et al. 1995, 2000; Housby et al. 1998; Py et al. 1999). The localization of GspE to the plasma membrane appears to be essential for type II secretion and may provide the energy for translocation or assembly of pilins and pseudopilins. GspL interacts with a second plasma membrane protein, GspM (Sandkvist et al. 1999, 2000). GspC, another protein predicted to reside in the plasma membrane, can be coimmunoprecipitated with GspD (the outer membrane secretin; Possot et al. 1999). Further, GspC requires expression GspD for stability in vivo (Bleves et al. 1999). Does the type II machinery assume a supra-molecular structure that spans

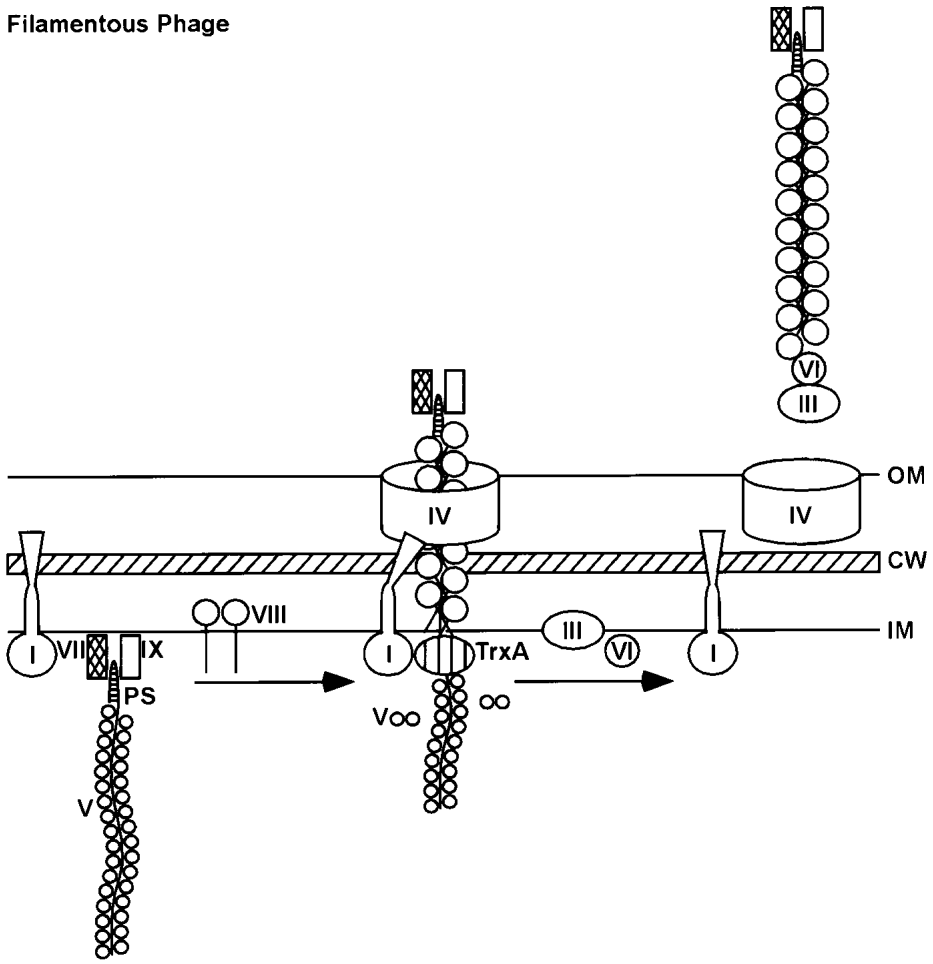

Figure 8. Assembly of filamentous phage. The packaging signal of single-stranded DNA phages interacts with phage proteins pVII, pIX, and the morphogenetic protein pI. The pI ATPase, in conjunction with the host factor thioredoxin (TrxA), displaces cytoplamic pV while loading the coat protein (pVIII) from the membrane on the DNA. An interaction between pI and pIV leads to the opening of pIV (the outer membrane secretin) and to the extrusion of assembled phage. pIII and pVI are incorporated at the very tip of the filamentous structure leading to the release of phage into the extracellular milieu. Drawing adapted from Russel (1991).

the distance between the plasma membrane and the outer membrane? Evidence for this hypothesis is still incomplete as such a machinery complex has not yet been purified or visualized by electron microscopy.

What is the signal(s) that allows the type II machinery to recognize its substrates? Studies on many different type II substrates have identified a large variety of domains and amino acids that are required for secretion (Hamood et al. 1989; Kornacker and Pugsley 1990; He et al. 1991b; Wong and Buckley 1991; Py et al. 1993; Palomaki and Saarilahti 1995; Sauvonnet et al. 1995; Lu and Lory 1996). For example, two spatially separated domains of pullulanase (PulA) seem required for substrate recognition (Sauvonnet and Pugsley 1996). In contrast, *P. aeruginosa* exoprotein A appears to display two surface domains each of which is sufficient to promote secretion of fused reporter proteins (Hamood et al. 1989; Lu and Lory 1996). The difficulty in identifying a universal secretion signal in type II substrates has led to the proposal that traits of folded tertiary structure are recognized by the machinery. Recent work in *Erwinia* has taken advantage of the fact that highly homologous exoproteins can be secreted in a species-dependent manner. Although pectate lyase (PelC) of *E. chrysanthemi* is 71% identical in sequence to Pel1 of *E. carotovora*, secretion of each pectate lyase requires the corresponding secretion machinery (He et al. 1991a; Py et al. 1991). Swapping regions of PelC and Pel1 identified multiple regions of the protein that are required for secretion (Lindeberg et al. 1998). The regions were located on the crystal structure of PelC, which assumes an extended helix consisting of β-sheets with α-helical extensions at the N and C terminus (Lindeberg et al. 1998). The C-terminal portion of the β-helical core and the loop extending from the core were proposed to act as primary, surface-exposed recognition sites (Lindeberg et al. 1998). An internal part of the β-helical core is thought to provide a scaffold for the proper display of the secretion signal.

Type III machines

Many laboratories sought to identify genes that are required for the pathogenesis of *Yersinia* and *E. coli* infections or for the invasion of *Salmonella* and *Shigella* into tissue culture cells (Hueck 1998; Galan and Collmer 1999). These studies isolated genetic loci that encode type III secretion machines and their transport substrates. Type III secretion systems are activated by bacterial contact with host cells. The binding of bacterial and host cell surface receptors appears to be one mechanism that activates type III machines (Aldon et al. 2000). *Yersinia* and *Salmonella* species sense the ion concentration in the cytoplasm of host cells as a mechanism to trigger type III secretion (Straley et al. 1993; Wösten et al. 2000). Whatever the mechanism of activation, type III machines have evolved to inject proteins into host cells. Such a pathway can be exploited for several different purposes. *Salmonella* species employ a type III machinery for bacterial entry into epithelial cells and inject factors that first activate and then stabilize the polymerization of actin filaments in the host cytoplasm (Galan and Zhou 2000). A second type III pathway is used for *Salmonella* survival within macrophages and manipulates the cell's ability to promote vesicle fusion (Uchiya et al. 1999). *Yersinia* type III machines prevent the phagocytic killing of microbes that have adopted an extracellular life style (Cornelis 2000). The type III pathway of *enteropathogenic E. coli* damages epithelial cells and provides a microbial growth advantage in the intestines (Donnenberg et al. 1997). Much of this work progressed rapidly as several type III genes display striking homology to basal body genes, specifying a machinery that is dedicated to the assembly of flagella.

Flagellar assembly

Basal bodies with attached flagella can be dislodged from bacteria by detergent and alkali extraction of the membrane envelope (DePamphilis and Adler 1971b; Dimmitt and Simon 1971). Cesium chloride density centrifugation is used to obtain a pure sample, which can be viewed in an electron microscope (DePamphilis and Adler 1971a). Basal bodies display rotational symmetry and several ring structures appear embedded within the plasma membrane (MS ring), the peptidoglycan layer (P ring), and the outer membrane (L ring; Fig. 9; DePamphilis and Adler 1971a). A central shaft, composed of a proximal and a distal rod, is positioned within the center of rings, and continued as a bent "hook," a joint to connect the basal body with the flagellar filament (DePamphilis and Adler 1971a). Rotation of the flagellar filament is fueled by the proton motive force as well as ATP hydrolysis and propels bacteria in liquid media (Manson et al. 1977). Basal body assembly has been studied with electron microscopy and purification of structural components purified from various mutant backgrounds (Komeda et al. 1978; Suzuki et al. 1978; Aizawa et al. 1985). The MS ring is the first visible structure in the plasma membrane and is required for the assembly of rods, P rings, and L rings (Jones et al. 1987; Ohnishi et al. 1987; Kubori et al. 1992). The hook is assembled in a second phase (Kagawa et al. 1976; Kutsukake et al. 1979) and, after completion, triggers polymerization of the filament (Homma and Iino 1985; Homma et al. 1985). Two underlying principles of flagellar assembly have emerged. (1) The structure is built by adding its bricks at the tip, that is, in a manner opposite to that of pili and phage, which add building blocks at the base. (2) The

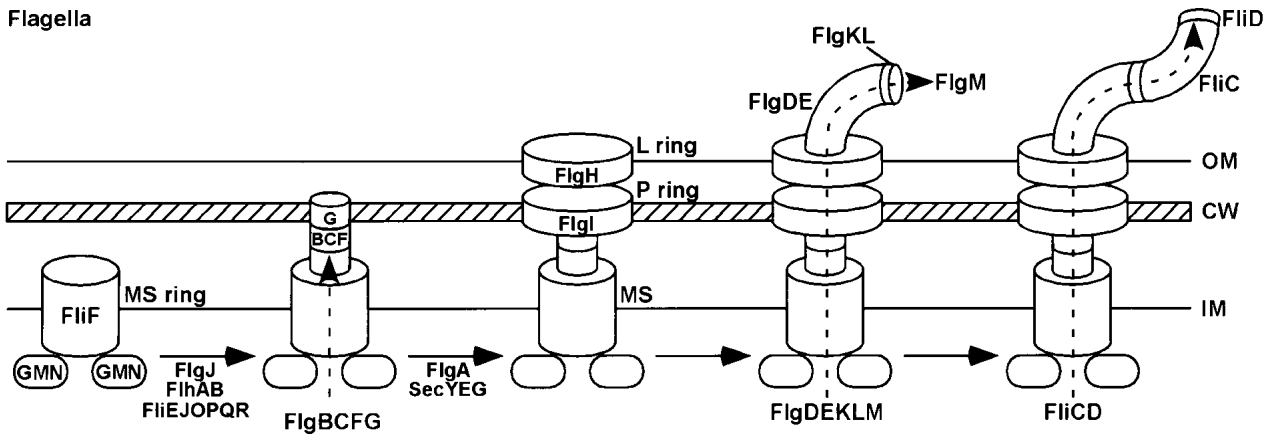

Figure 9. Flagellar assembly. FliF is inserted into the plasma membrane via the Sec pathway to generate MS ring structures. The addition of FlgJ, FlhAB, and FliEGJMNOPQR results in the assembly of a functional secretion apparatus, a structure that supports the transport of FlgBCFG into the periplasm, thereby forming the central rod. FlgI and FlgH are transported across the plasma membrane via the Sec pathway and assemble to generate the P and L rings, respectively. Hook proteins, FlgDE, are presumed to travel through the basal body complex and assemble into the hook structure. Completion of the hook allows export of FlgM, an anti-σ factor that binds FliA, and allows transcription of genes encoding filament proteins (*flgKL* and *fliCD*). FlgKL act as a capping protein to seal the hook. FliD forms the flagellar cap and functions as an assembly site for flagellin (FliC), the major component of the growing filament. Drawing adapted from Macnab (1992).

completion of rings, rods, or hook serve as checkpoints to promote expression and transport of new building blocks for subsequent parts of the structure (Macnab 1992).

Flagellar building blocks are transported by the basal body complex, a machinery assembled from twelve gene products (*fliFGHIJMNOQR* and *flhAB*). Knockout mutations in 11 of these genes arrest assembly of the basal body at the MS-ring stage (Suzuki et al. 1978; Jones and Macnab 1990; Kubori et al. 1992). Knockout mutations in the twelfth gene, *fliF*, altogether prevent MS ring assembly (Kubori et al. 1992). This and other evidence suggests that the MS ring, which is composed of FliF alone, functions as an entry site for basal body proteins (Homma et al. 1987; Ueno et al. 1992). Unlike type III secretion systems, flagellar transport seems solely dedicated to structural assembly. Basal body rods, the hook, and the filament are believed to form a channel for the transport of substrates to the tip of the structure (Wagenknecht et al. 1982; Mimori et al. 1995; Morgan et al. 1995). Capping proteins, used for either the hook or the flagellum, are positioned at the growing tip and may occlude the channel and catalyze filament polymerization (Homma and Iino 1985; Ikeda et al. 1987; Ohnishi et al. 1994). Eleven flagellin subunits complete two right-handed helical upward turns of the filament and are sealed by a pentamer of capping protein, with five protrusions for contacting the flagellins in the filament (Yonekura et al. 2000). One of the five cap-filament contact sites forms a gap that is large enough to accommodate the addition of another subunit. As the gap is being filled, the cap proteins rotate by 6.5°, resulting in the exposure of a new gap that can accept the next flagellin subunt (Yonekura et al. 2000).

The flagellar filament protrudes 3–12 μm on the bacterial surface. What is the energy source that transports proteins for such a long distance? Although a definitive answer is not yet in sight, two models come to mind. In the first model, subunit proteins could line the interior of the flagella filament from the base to the tip. The addition of another flagellin subunit to the base could push all subunits further into the filament and cause the most distal protein to bind the structure at its tip. In model two, subunits are transported from the base to the tip by utilizing the rotational force of the flagellum. Adhesion of bacteriophage χ to the flagellar filament provides for its transport to the bacterial surface (Schade et al. 1967), a mechanism that also requires flagellar rotation (Berg and Anderson 1973; Samuel et al. 1999). As the surface of the flagellar filament displays a right-handed helical ridge (O'Brien and Bennett 1972; Wagenknecht et al. 1982), bound bacteriophage seems to spiral down the filament towards the bacterial surface propelled by its counter-clockwise rotation, much like a nut rotates on a screw (Berg and Anderson 1973; Samuel et al. 1999). A similar, but reciprocal screwing mechanism could play a role in model two and provide for the transport of flagellin from the base to the tip using clockwise rotation of the flagellum.

FliA is the DNA binding sigma factor that associates with core RNA polymerase to promote transcription of late flagellin genes (Kutsukake et al. 1990; Ohnishi et al. 1990; Gillen and Hughes 1991). FlgM is transcribed in the same operon as FliA. Once synthesized, FlgM binding to FliA prevents transcription of the transport substrates for the last stage of flagellar assembly (Ohnishi et al. 1992). This regulatory mechanism is relieved by secretion of FlgM, which occurs once the basal body/hook complex has been completed (Hughes et al. 1993). In order for flagellar assembly to proceed to the next stage, FlgM must effectively be depleted from the bacterial cytoplasm. Similar regulatory mechanisms seem to exist for other type III secretion systems (Pettersson et al. 1996).

Needle complexes

All type III machines encompass an outer membrane secretin (SctC or GspD), which promotes the assembly of a needle-like structure across the outer membrane (Fig. 10; Koster et al. 1997). Kubori and colleagues were the first to isolate the needle complex, using detergent extraction and cesium chloride density centrifugation in a protocol similar to the purification of basal body/hook complexes (Kubori et al. 1998). The complex displays rotational symmetry and is composed of four stacked rings and an elongated needle, similar to the basal body complex (Figs. 9 and 10; DePamphilis and Adler 1971a). Edman degradation of *Salmonella* and *Shigella* proteins that copurified with needle complexes identified five machinery components: SctC (InvG/MxiD) secretin, PrgH/MxiG (no conserved Sct homolog), SctJ (PrgK/MxiJ), SctF (PrgI/MxiH), and SctI (PrgJ/MxiI; Kubori et al. 1998, 2000; Kimbrough and Miller 2000; Tamano et al. 2000; Blocker et al. 2001). SctC presumably multimerizes to form the two smaller discs within the needle structure. PrgH (MxiG) and SctJ are thought to form two large rings that are located within the plasma membrane (Kimbrough and Miller 2000). Several conserved secretion genes, for example, *sctV* and *sctN*, are not required for the assembly of the rings, a process that may occur by insertion of inner membrane proteins via the Sec pathway (Kubori et al. 2000; Tamano et al. 2000). However, the products of type III secretion genes are required for the assembly of the needle. SctN and SctU contain ATP-binding domains and ATP hydrolysis could fuel the type III transport reactions (Allaoui et al. 1994; Woestyn et al. 1994). Knockout mutations of *sctF* or *sctI* abrogate needle formation without affecting ring assembly (Kimbrough and Miller 2000; Kubori et al. 2000; Tamano et al. 2000; Blocker et al. 2001). SctF is the main needle component and overexpression of SctF results in a longer needle (Tamano et al. 2000). *sctP* controls the length of the needle as mutants lacking this gene synthesize elongated needles (Kubori et al. 2000). SctP itself is secreted by the type III machine (Collazo et al. 1995) and could acts as a switch between type III transport of the needle component SctF and *Salmonella* secretion substrates. Several components of the type III machinery have been shown to interact (Day and Plano 2000; Day et al. 2000; Jackson and Plano 2000), however the precise location of

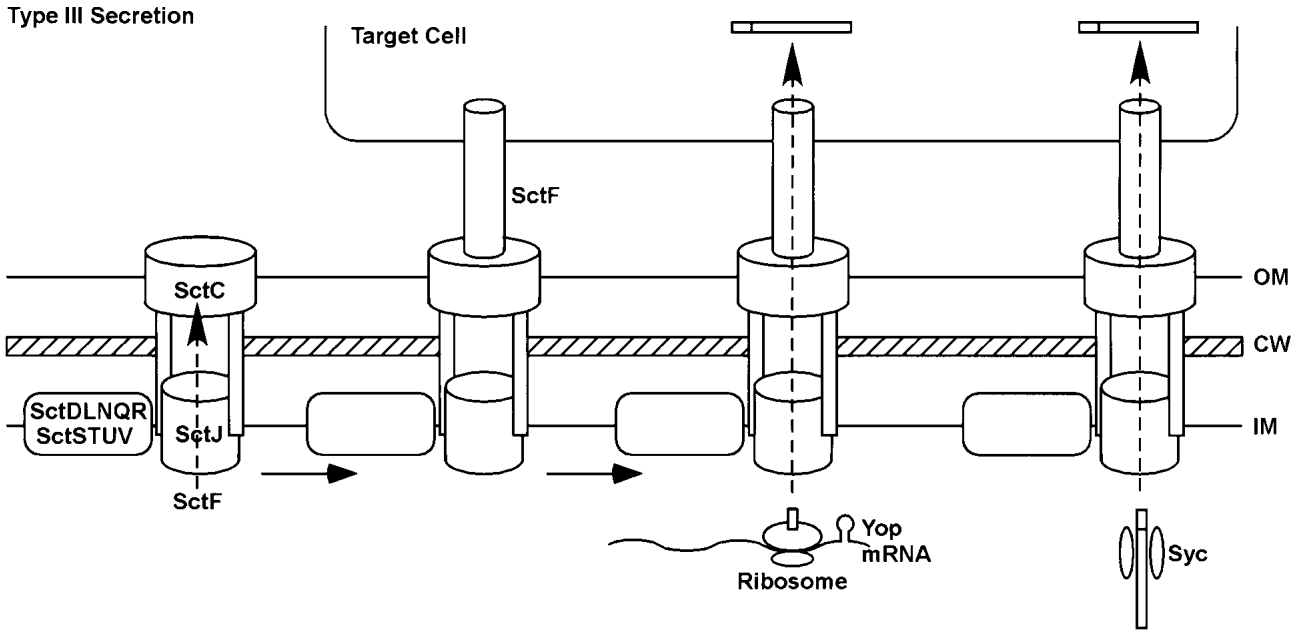

Figure 10. The type III secretion machinery. The type III machinery is composed of plasma membrane proteins (SctDJLNRSTUV) and the outer membrane secretin SctC. SctJ represents the morphogenic equivalent of flagellar MS rings (FliF, see Fig. 7). After the assembly of the inner and outer membrane components, SctF is thought to be transported by the type III machinery to assemble into a needle structure on the bacterial surface. Two secretion signals of Yop proteins (*Yersinia* outer proteins and secretion substrates) are recognized by the type III machinery (Sct). One pathway presumes recognition of signals in mRNAs encoding Yop proteins, perhaps by coupling translation and secretion. A second signal requires binding of cytosolic Syc proteins (secretion of Yop chaperone) to provide for Yop recognition and secretion. Drawing adapted from Anderson and Schneewind (1999a) and Galan and Collmer (1999).

these proteins in the needle complex have not yet been elucidated.

What is the mechanism whereby bacteria transport proteins across three membranes into the cytosol of eukaryotic cells? Early work suggested that type III secretion pathways deposit translocator proteins (YopB, YopD, and LcrV of yersiniae) in the plasma membrane of eukaryotic cells (Cornelis and Wolf-Watz 1997). Once the translocator proteins are assembled into a pore structure within the plasma membrane, other secreted proteins could travel through the translocator lumen into the cytosol of host cells. Using high-resolution electron microscopy, Hoiczyk and Blobel (2001) demonstrated plasma membranes punctured with needle complexes and proposed another mechanism (Fig. 10). Type III needle components (SctF) display hydrophobic properties and the assembled needle alone is capable of penetrating the plasma membrane of host cells. Thus, needle complexes could form conduits that transport proteins from the bacterial cytoplasm into the cytosol of host cells.

Substrate recognition

The 14 Yop proteins of *Yersinia* species do not share common peptide sequences and are secreted into the extracellular medium without post-translational modification or cleavage of the polypeptide chain (Michiels et al. 1990; Forsberg et al. 1991; Rimpilainen et al. 1992; Anderson and Schneewind 1997). Fusion of reporter genes to the 3′-portion of *yop* open reading frames leads to the transport of hybrid proteins across the bacterial double membrane envelope (Michiels and Cornelis 1991). Hybrid proteins generated by 5′ fusions fail to be transported (Anderson and Schneewind 1997). The secretion signal of YopE has been mapped to the first 15 or 11 codons, respectively (Schesser et al. 1996; Anderson and Schneewind 1997). Scanning mutagenesis of the first 15 codons, using glycine, alanine, or glutamine codon replacements, has either no effect on secretion signaling or abolishes the expression of mutant genes (Schesser et al. 1996; Anderson and Schneewind 1997). Several frameshift mutations, generated by nucleotide insertions or deletions following the start codon and corrected by reciprocal mutations at the reporter fusion site, do also not affect secretion signaling (Anderson and Schneewind 1997, 1999b; Anderson et al. 1999). These results led to the proposal that the secretion signal within the first 15 codons of *yops* may be decoded at the level of mRNA rather than by the recognition of an amino acid or peptide sequences (Fig. 10; Anderson and Schneewind 1997). How can the secretion machinery recognize a mRNA signal that results in the transport of the encoded polypeptide? A definitive answer is not yet in sight. One model proposes that translation of *yop* mRNA may be regulated in a manner permitting the ribosome to extrude newly synthesized polypeptide into the secretion pathway (Anderson and Schneewind 1999a). In other words, the secretion machinery may not need to distin-

guish Yop proteins from other proteins in the bacterial cytoplasm.

Deletion of codons 2–15 of *yopE* or replacement with codons 2–15 of *E. coli* β-galactosidase (*lacZ*) or chloramphenicol acetyltransferase (*cat*) does not prevent secretion of the mutant polypeptide by yersiniae (Lee et al. 1998). However, knockout mutations in *sycE*, encoding a small cytoplasmic protein that binds as a homodimer to YopE residues 15–100, abolish secretion of mutant YopE proteins (Cheng et al. 1997; Lee et al. 1998). Cytoplasmic binding proteins similar to SycE have been reported for YopB, YopD, YopH, YopN, and YopT (Wattiau and Cornelis 1993; Wattiau et al. 1994; Woestyn et al. 1996; Day and Plano 1998; Iriarte and Cornelis 1998; Jackson et al. 1998). These results led to the proposal that YopE may be initiated into the type III pathway in one of two ways, requiring an mRNA signal or binding of SycE to unfolded YopE (Fig. 10; Cheng and Schneewind 2000). Measurements of type III targeting, that is, the injection of YopE into the cytosol of tissue culture cells, revealed that both signals, codons 1–15 as well as SycE binding to YopE 15–100, are needed for transport by the secretion machinery (Lee et al. 1998). One obvious dilemma is that if mRNA signaling prevents polypeptide exposure in the bacterial cytoplasm, how can SycE bind to YopE? Does the ribosome synthesize the N terminus in the context of the type III machinery, while C-terminal YopE sequences are exposed to SycE binding in the bacterial cytoplasm (secretion and run-away synthesis model)? Do mRNA codons 15–100 comprise a secretion "stalling signal" that requires relief by SycE binding to the newly synthesized YopE polypeptide? The latter possibility shares elements with the recently reported hypothesis that *Salmonella* FlgN, presumed to be a cytoplasmic chaperone like SycE, may regulate ribosomal synthesis of the secretion substrate FlgM (Karlinsey et al. 2000).

Wolf-Watz and colleagues postulated that the first 11 amino acids of YopE act as a secretion signal (Lloyd et al. 2001). These investigators proposed further that all Yops contain an N-terminal amphipathic helix that may be recognized by the type III machinery (Lloyd et al. 2001). In this model, SycE would act as a chaperone that prevents the premature folding of YopE. MacNab and colleagues advanced a model whereby the flagellar type III secretion machinery recognizes substrates (flagellins, hook and rod proteins) by direct binding of polypeptides (Minamino and MacNab 2000). Experimental testing of this hypothesis is complicated by the fact that the basal body not only transports but also assembles substrates into a higher order structure. Further, as fused reporter proteins can not be transported by the basal body, it seems difficult if not impossible to discern a definitive secretion signal (Minamino and Macnab 1999). These models vary considerably in predicting the mode of substrate recognition. It should be noted that the underlying experiments for this research measure the type III secretion of mutationally altered gene products or fused reporter proteins. Although the data that have been generated permit some conclusions about secretion signals and substrate recognition, a direct interaction between type III substrates and functional type III secretion machines has not yet been observed. Thus, a precise molecular mechanism has not yet emerged which makes it difficult to dismiss any of the models described above.

Type IV secretion

Type IV machines are versatile transporters of proteins or nucleic acids that secrete substrates across the bacterial envelope or direct them into target cells. Two Gram-negative pathogens, *Agrobacterium tumefaciens* and *A. rhizogenes*, infect specific plants to cause crown gall tumors, a debilitating disease (Lai and Kado 2000; Zupan et al. 2000). Plant cells within the tumor are transformed by agrobacterial DNA, which is inserted into nuclear chromosomes (Zambryski et al. 1980). Expression of the bacterial genes (tumor or T-DNA) results in the production of opines, amino acids that are eventually released by the tumor and metabolized by *Agrobacteria* (McPherson et al. 1980; Zambryski et al. 1989). The search for determinants that cause tumor production uncovered bacterial virulence genes (Stachel and Zambryski 1986; Porter et al. 1987; Ward et al. 1988), several of which display homology to genes required for bacterial mating or conjugation, another example for the exchange of genetic information between cells.

Conjugation requires physical contact between bacteria and in Gram-negative organisms this is associated with the formation of specific pili (for detailed review, see Pansegrau and Lanka 1996). Pilus-containing, male cells use the filament to bind female cells that lack the particular conjugation determinants. The pilus may subsequently retract, thereby bringing male and female cells into close proximity. During conjugation of *E. coli* RP4 (Pansegrau and Lanka 1996), three factors (TraH, TraI, TraJ) recognize the nick region of *oriT* (origin of plasmid DNA transfer), cleaving the plus strand of plasmid DNA (Furste et al. 1989). TraI attacks the phospho-diester bond at the cleavage site, resulting in single-stranded DNA phospho-diester linked to a hydroxyl group of tyrosine (Pansegrau et al. 1990, 1993). Single-stranded DNA–TraIJH complex is thought to interact with the transport machine at conjugative junctions for the delivery of single-stranded DNA into the recipient cell (Pansegrau and Lanka 1996). Although several models of conjugation call for DNA transport through the lumen of the pilus, this notion has thus far not be demonstrated experimentally. Recent studies of RP4 plasmid suggest that components of the pili are not found at the conjugative junctions (Samuels et al. 2000). An alternative model proposes that the pilus facilitates formation of conjugative junctions that generate the conduit for the transfer of DNA.

Agrobacterial transfer of T-DNA and assembly of the pilus requires *virA*, *virB1–11*, *virD1–4*, *virE2*, and *virG* (Fig. 11; Leroux et al. 1987; Porter et al. 1987; Christie et al. 1988; Ward et al. 1988; Jin et al. 1990c; Kuldau et al. 1990; Shirasu et al. 1990). *virA* and *virG* encode a histidine protein kinase and the cognate response regulator, two components of a signal transduction device that al-

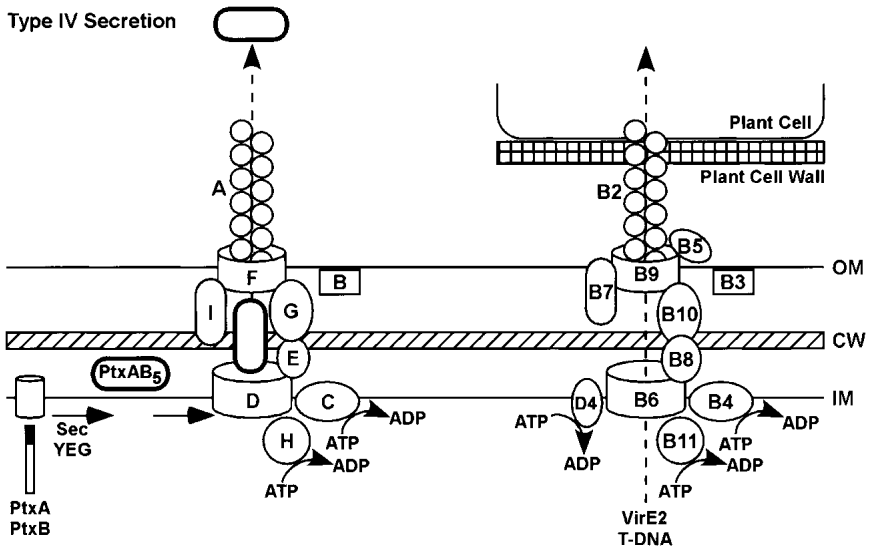

Figure 11. Type IV secretion. (*Left*) *Bordetella pertussis* secrete pertussis toxin ($PtxAB_5$) via a type IV pathway. After Sec-mediated translocation of PtxA and PtxB across the cytoplasmic membrane, pertussis toxin is transported via the type IV pathway across the outer membrane. Based on analogy to T-DNA transfer systems, PtlCDHE assemble in the cytoplasmic membrane and, together with PtlBFGI in the outer membrane, promote assembly of a pilus-like filament containing PtlA. $PtxAB_5$ is transported by this pathway into the extracellular medium. (*Right*) Agrobacterial inner membrane (VirD4, VirB4, VirB6, VirB8, and VirB11) and outer membrane (VirB5, VirB7, VirB9, and VirB10) proteins assemble a pilus-like structure (VirB2) that is involved in transporting VirD2, T-DNA, and VirE2 into plant cells. Drawings adapted from Christie and Vogel (2000) and Christie (2001).

lows *Agrobacteria* to respond to specific plant signals by expressing virulence genes (Jin et al. 1990a,b,c; Raineri et al. 1993). VirD1–3 fulfill an analogous function as TraHIJ with VirD2 being linked to the cleaved T-DNA (Yanofsky et al. 1986; Herrera-Estrella et al. 1988; Ward and Barnes 1988; Young and Nester 1988; Howard et al. 1989). In contrast to the single nick region of RP4 plasmid conjugation, transfer of agrobacterial T-DNA involves cleavage of two border sequences that each flank the T-DNA. VirE2 is a single stranded DNA binding protein (Gietl et al. 1987; Christie et al. 1988; Citovsky et al. 1988). Both VirD2 and VirE2 encompass nuclear localization signals which, after transfer of the T-DNA into plant cells, are necessary for transport of T-complex (T-DNA with VirD2 and VirE2) through nuclear pore complexes (Citovsky et al. 1992, 1994; Howard et al. 1992). Although VirD2, VirE2, and T-DNA are all thought to be translocated into plant cells, there is as yet little evidence to suggest that the proteins are transported together with the T-DNA (Citovsky et al. 1992; Sundberg et al. 1996; Gelvin 1998). In fact, the mutational defect of *virE2* mutant *Agrobacteria*, which are unable to ellicit tumor formation, can be complemented in trans via a mixed infection with another mutant strain that is capable of expressing *virE2* but instead lacks the T-DNA (Otten et al. 1984; Citovsky et al. 1992; Fullner et al. 1996). Thus, VirE2 and perhaps some other proteins can be transported independently of DNA transfer.

Agrobacterial VirB2 is the major pilin subunit of the conjugal pilus (Lai and Kado 1998). It is not yet clear whether this pilus emanates from the outer membrane or the plasma membrane of the bacteria. VirB2 is cleaved at between Ala47 ad Gln48. The newly exposed N-terminal amino group is ligated to the C-terminal carboxyl group, generating a cyclic polypeptide similar to TraA of the F-plasmid conjugal system (Jones et al. 1996; Eisenbrandt et al. 1999). VirB2 is presumably transported by the type IV secretion machinery and polymerizes to form a rod with a diameter of 10 nm (Lai and Kado 1998). VirB4 forms a dimer in the bacterial plasma membrane and functions as an ATPase and (Dang and Christie 1997; Dang et al. 1999). VirB6 is a highly hydrophobic protein that likely localizes to the inner membrane (Das and Xie 1998), while the outer membrane lipoprotein VirB7 binds VirB9 (Anderson et al. 1996; Fernandez et al. 1996; Spudich et al. 1996; Baron et al. 1997). VirB9 in turn interacts with the presumed muramidase VirB8 (Thorstenson et al. 1993; Das and Xie 2000; Kumar et al. 2000) and with VirB10 in the plasma membrane (Finberg et al. 1995). VirB10 is thought to bind VirB11, a second ATPase that is inserted in the plasma membrane (Finberg et al. 1995; Das and Xie 2000). A third ATPase, VirD4, is located in the bacterial cytoplasm (Hamilton et al. 2000). Thus, it appears that type IV secretion machines, similar to the type III pathway, employ ATP to fuel protein transport across multiple membranes.

Legionella pneumophila is an intracellular parasite of humans that requires a type IV secretion machinery to establish residence in phagocytic vacuoles of macrophages (Swanson and Isberg 1996a). The type IV secretion machinery of *L. pneumophila* is encoded by the *dot/icm* genes (Marra et al. 1992; Berger et al. 1994; Swanson and Isberg 1996b). It is conceivable that these genes are responsible for triggering both the unique phagocytic pathway of *Legionella* in professional macrophages as well as enabling an intracellular survival strategy that encompasses the recruitment of cellular organelles to vacuoles containing bacteria. It is not yet clear whether secreted proteins or DNA transfer are used to manipulate host cells, however the *Legionella* type IV machinery is capable of plasmid conjugation between bacterial cells (Vogel et al. 1998). *L. pneumophila* type IV secretion system and *Shigella flexneri* IncI conjugation system are closely related (Komano et al. 2000), whereas the type IV pathways of *Bordetella pertussis*, *Brucella abortus*, *Brucella suis*, *Helicobacter pylori*, and *Rickettsia*

prowazekii more closely resemble that of *Agrobacteria* and IncP-plasmid conjugation (Christie and Vogel 2000). *Bordetella pertussis* use a type IV pathway to secrete pertussis toxin (Fig. 10; Christie and Vogel 2000), an AB-type toxin that is imported by host cells (Schmitt et al. 1999), whereas *Helicobacter pylori* appear to transport CagA directly into the cytoplasm of gastric cells (Segal et al. 1999; Stein et al. 2000). These results suggests that type IV machines are versatile transporters of proteins or nucleic acids that translocate substrates not only across the bacterial envelope but also across the plasma membrane or vacuolar vesicles of host cells.

Conclusions

What are the common principles that can be gleaned from the study of protein secretion in bacteria? First, the final destination of secreted proteins presents an important strategic decision for the invading pathogen. Toxins that are secreted into the extracellular milieu target host cells by mechanisms that involve receptor-mediated membrane damage or endocytic uptake. The secreted toxin acts both at the anatomical site of infection (where the pathogen resides) and at a distance, as the proteins diffuse throughout the infected organism. The type III- and type IV-mediated injection of proteins into host cells requires bacterial contact with the target, often macrophages, lymphoid cells, or specific tissues (Cornelis 1998). The type III and IV systems are highly effective in transporting large amounts of polypeptides for a short distance. However, this is a perilous undertaking, as the pathogens may be phagocytosed and killed during the injection process (Forsberg et al. 1994). Some secretion systems, for example *Yersinia* type III machines, are versatile and catalyze both the secretion of proteins into the extracellular medium as well as the injection into target cells (Lee et al. 1998).

Second, the folding state of secretion substrates dictates the mode of transport. Many type II substrates require folding, processing, oxidation, and assembly into a quarternary structure for toxin activity. The periplasmic space of Gram-negative organisms provides an ideal folding environment (Missiakas and Raina 1997) for those toxins whose transport occurs in two phases: (1) across the plasma membrane (Sec pathway) and (2) across the outer membrane (type II and type IV). Proteins that are injected into the cytosol of host cells use the folding capacity of this compartment to acquire activity. Hence, these polypeptides are presumably translocated from the bacterial cytoplasm in an unfolded state (type III and IV machineries). Type I secretion systems strike a balance between the two extremes. Its secretion substrates, for example HlyA, are transported in a processed (N-acylated) but presumably unfolded state, as processing is required for toxin activity (Stanley et al. 1998).

Third, several secretion machines as well as pili have evolved as supramolecular complexes that are assembled in one of two ways. Folded components are added at the base, in the outer membrane, and with each assembly step the structure is pushed further from the bacterial surface (type I and IV pili). This mode uses the bacterial periplasm for the folding of subunits and for the catalysis of the assembly reactions. During the other assembly strategy, substrates are transported from the bacterial cytoplasm through the lumen of the supra-molecular structure and are added at its tip. Catalysis of the assembly reaction can not rely on the folding factors of the bacterial periplasm but appears to employ capping proteins that prevent the diffusion of components into the extracellular medium.

Fourth, recognition of secretion substrates in the bacterial cytoplasm requires the signal peptides of the Sec pathway (type II secretion, type I and IV pili) or the C-terminal signal of type I substrates. Type III substrates could require signals that are encoded in the mRNA, however a detailed mechanism has not yet been revealed. The recognition of type IV substrates has not yet been studied. Nevertheless, it seems safe to assume that TraJ or VirD2 must require folding for their cytoplasmic function (attack of the phospho-diester bond). VirD2 linked to DNA may therefore be transported in a fully folded manner. The selection of type II secretion substrates in the bacterial periplasm is facilitated due to the fact that the machinery can choose from a small number of polypeptides. Approximately 3000 polypeptides reside in the bacterial cytoplasm, as compared with the 200 periplasmic proteins (Blattner et al. 1997). This may explain why the secretion signals of type II substrates are provided by discrete domains of folded polypeptides rather than by short peptide signals.

Fifth, related substrates can be transported by seemingly unrelated pathways. Several AB_5-type toxins (cholera toxin, shiga toxin, and shiga-like toxins) are secreted by type II pathways, whereas *Bordetella* pertussis toxin travels a type IV pathway, albeit a modified type IV pathway with a "periplasmic detour." Several proteases that are secreted via a type I pathway by one organism are transported via a type II machinery in another organisms. It seems that many different approaches can arrive at the correct solution. And, if it works, bacteria will use it for their advantage.

Upon reviewing more than 20 years of work on protein secretion, one can only marvel at the beauty of mechanisms whereby bacteria interact with their environment. All secretion systems seem highly evolved and efficient, with specificity in the selection and transport of secretion substrates, the assembly of the secretion machinery, and the coordinated movement of macromolecules across one, two, or three lipid bilayers. For example, *Yersinia pestis* is a highly dangerous pathogen (Perry and Fetherston 1997). A few bacteria, inoculated via flea bite into the skin of humans, are drained to local lymph nodes and cause an infection (bubo), that, if left untreated, is followed by the lethal dissemination of bacteria in approximately half of all infected individuals (Perry and Fetherston 1997). The deletion of type III secretion machinery genes, abolishes the tremendous infectivity of *Yersinia* (Goguen et al. 1984). It seems that protein secretion is much of what it takes to make a successful pathogen. Bacterial protein secretion is a rap-

idly evolving field. Most of the work has been performed with Gram-negative organisms. A shift in focus is needed as Gram-positive microbes cause many important human diseases that are still poorly understood.

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