Protein secretion systems and adhesins: The molecular armory of Gram-negative pathogens

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Abstract

Protein secretion is a basic cellular function found in organisms of all kingdoms of life. Gram-negative bacteria have evolved a remarkable number of pathways for the transport of proteins across the cell envelope. The secretion systems fulfill general cellular functions but are also essential for pathogenic bacteria during the interaction with eukaryotic host cells. Secretion systems range from relatively simple structures such as type I secretion systems composed of three subunits that only secrete one substrate protein to complex machines such as type III and IV secretion systems composed of more than 20 subunits that can translocate large sets of effector proteins into eukaryotic target cells. In this review, the main structural and functional features of secretion systems are described. One subgroup of substrate proteins of secretion systems are protein adhesins. Despite the conserved function in binding to host cell ligands or to abiotic surfaces, the assembly of the various bacterial adhesins is highly divergent. Here we give an overview on the recent understanding of the assembly of fimbrial and non-fimbrial adhesins and the role of type I, III and V secretion systems and specialized branches of the general secretion pathway in their biogenesis.

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Introduction

The transport of proteins across the bacterial cell envelope is a basic function found in all groups of bacteria. The analyses of a large number of bacterial genomes indicate that up to 17% of Proteobacteria genomes encode for proteins with signal sequences for the general secretory pathway (GSP) (Bendtsen et al., 2005), with many additional secretion systems and substrate proteins being present in most species. Secreted proteins have various functions in processes such as the biogenesis of the cell envelope, the acquisition of nutrients, motility, intercellular communication and many more. Aggressive bacterial virulence factors that enable a progressive colonization of host organisms are commonly secreted proteins, with toxins and translocated effector proteins as well-studied examples. Protein adhesins are another group of secreted proteins found in pathogenic as well as environmental bacterial species.

The GSP and its components are found in all three kingdoms of life: in bacteria, archaea and eukaryotic organelles (chloroplasts but also in the endoplasmic
reticulum) and provides a generic mechanism for the transport of proteins across the cytoplasm or organelle membrane. While this transport process is sufficient for the secretion of proteins in Gram-positive species, Gram-negative species are posed with a specific problem, the transport across a second membrane system, the outer membrane (OM).

The OM is a remarkable structure that enables Gram-negative bacteria to colonize host environments such as the intestinal lumen, and provides a protective barrier against various antimicrobial host defenses as well as against antibiotics. However, the OM is also a barrier for the secretion of proteins, and it is a particular problem to energize the transport across the OM. Gram-negative bacteria have evolved a remarkable array of mechanisms for the secretion of proteins across the cell envelope. In this review, we will briefly describe the key structural and functional features of the various secretion systems, with some systems being studied in great detail over several decades and others being discovered very recently.

All protein adhesins have to fulfill the same basic requirements, i.e., to bring a receptor-like domain or subunit into close contact with a ligand on the host cell surface, and link the domain or subunit to the bacterial surface. There are two major classes of protein adhesins, (i) the fimbrial adhesins with pili composed of heteropolymers of several subunits and (ii) non-fimbrial adhesins consisting of a single protein or homotrimers. The assembly of fimbrial as well as non-fimbrial adhesins involves the function of different secretion systems, and for several adhesins, specific branches of common secretion pathways have evolved. The second part of this review will describe our current understanding of the various adhesins with focus on the secretion mechanisms underlying the adhesin assembly.

Bacterial protein secretion systems

For Gram-negative bacteria a classification of the secretion pathways in type I–VI was made primarily due to the characteristics of the OM secretion mechanisms. A schematic overview of the most common secretion and translocation systems described in more detail below is given in Fig. 1.

The general secretory pathway (GSP)

The transport of unfolded proteins into the periplasm is accomplished by a multi-subunit translocon. The bacterial GSP consists of two heterotrimeric complexes, SecYEG (Yahr and Wickner, 2000) and SecDFYajC which are inserted into the inner membrane (IM), and an accessory component, SecA (Rusch and Kendall, 2007). Proteins targeted for the Sec pathway are translated as pre-proteins and possess an N-terminal signal peptide. Co-translational translocation is possible where the newly synthesized signal peptide of the nascent peptide chain is recognized by SecA or SecA together with the ‘general chaperone’ SecB and subsequently targeted to the SecYEG pore complex. SecA itself might also exhibit a cytosolic chaperone activity (Eser and Ehrmann, 2003) and forms presumably dimers (Woodbury et al., 2002). ATP hydrolysis catalyzed by SecA along with the proton-motive force drives the translocation of the pre-protein into the periplasm. The SecDFYajC complex is thought to enhance translocation through SecYEG by promoting membrane cycling of SecA (Duong and Wickner, 1997). Upon translocation, the signal peptide is cleaved off the pre-protein by specific periplasmic signal peptidases and the mature protein is released in the periplasmic space. If additional signals are present, these might be recognized by components of the terminal branch pathways for subsequent translocation across or insertion into the OM.

There are three different terminal branch pathways allowing subsequent secretion of periplasmic intermediates through the OM: (i) a complex secreton (type II), (ii) an intrinsic activity of the substrate protein (auto-transporter (AT) or type V) or (iii) by the chaperone usher (CU) pathway (see below).

Type I secretion systems

Type one secretion systems (T1SS) or ATP-binding cassette (ABC) transporters are heterotrimeric complexes consisting of an IM ABC exporter, a membrane fusion protein (MFP) and a pore-forming, outer-membrane protein (OMP). T1SS allow the secretion of a wide range of substrates (proteinaceous and non-proteinaceous) from the cytoplasm to the extracellular space in a single step, without a stable periplasmic intermediate. Most protein substrates described so far possess a C-terminal signal sequence which is characterized by loosely conserved secondary structures (Stanley et al., 1991) and is not cleaved off during secretion. This implies that co-translational secretion is not possible (reviewed in Delepelaire (2004)). The mechanism of type I secretion was studied in great detail on the basis of the z-hemolysin (HlyA) secretion found in some uropathogenic Escherichia coli (UPEC) (Thanabal et al., 1998). The HlyA secretion system consists of the ABC exporter HlyB (Schmitt et al., 2003), the MFP HlyD (Schulein et al., 1992) and the common OMP TolC (Koronakis et al., 2000). ABC proteins are believed to form homodimers and are responsible for substrate recognition and specificity as well as for energizing the translocation by ATP hydrolysis (Binet and Wandersman, 1995). HlyD
seems to be involved in HlyA recognition (Thanabalu et al., 1998) but its main functions are possibly to make contact to the portions of TolC extending into the periplasm and maybe to trigger the opening of the TolC channel in an iris-like fashion. The TolC OMP acts as a trimeric OM channel for several exporter systems such as T1SS as well as RND (resistance, nodulation, division) type systems. Each monomer of TolC provides four β-strands in the OM and four very long (100 Å) α-helices in the periplasmic space which combine in the trimeric form to a large hydrophilic channel. A MFP-triggered unwinding of the coiled–coiled α-helices might provide a channel opening with a diameter of 16–20 Å which would allow the transport of partially folded proteins (reviewed in Koronakis et al. (2004)).

Type II secretion systems

This pathway depends on the Sec machinery and is used by a wide variety of Gram-negative bacteria to secrete enzymes and toxins across the OM. It is the main terminal branch of the GSP, and best characterized so far is the secretion of pullulanase (PulA) by *Klebsiella oxytoca* (Pugsley et al., 1997). Cryo-electron microscopy revealed that PulD forms a ring-shaped complex of 12–14 subunits with a central cavity in the OM. For proper targeting and insertion of PulD, the small lipoprotein PulS is required (Nouwen et al., 1999). The type II secreton consists of a multi-subunit complex inserted into the IM, some of the components exhibiting extensive cytoplasmic domains. Interestingly, the proteins PulG, H, I and J show a limited homology to the type IV pilus structural subunit pilin (‘pseudopilins’, see below). Little is known about the role of the other components of the secreton (PulF, K, L, M, N) and how the transport is energized. In the homologous *esp* system of *Vibrio cholerae*, it could be shown that the cytoplasmic EspE (a homolog of PulE) possesses an ATP-binding motif and has autokinase activity. EspE is recruited to the IM by interaction with EspL (Sandkvist...
et al., 1995). This model assumes that EspE hydrolyzes ATP which induces conformational changes within the IM components which are transmitted to the periplasmic domains and finally to PulD/S.

Type III secretion system

Type III secretion system (T3SS) are complex, supramolecular structures which span the IM, the periplasmic space, the OM, the extracellular space and a host cellular membrane. These complex assemblies are structurally and evolutionarily related to the flagella systems. T3SS have been isolated in species of several Gram-negative bacteria (Salmonella, Yersinia, Shigella, Escherichia, Pseudomonas) and were shown to consist of at least 20 different subunits which enable these bacteria to translocate substrates (effectors) directly into the cytoplasm of the host cell to exert a broad range of virulence functions (reviewed in Ghosh (2004)). Because of their shape and their ability to translocate proteins in a cell contact-dependent manner, they are also referred to as ‘injectisomes’ or ‘molecular needles’ (Cornelis, 2006).

Two oligomeric rings form the major part of the so-called ‘basal body’. The ring complex inserted into the IM consists of multiple copies of at least three proteins. In the Salmonella pathogenicity island 1 (SPI1)-encoded T3SS, the complex is composed of PrgK and PrgH (Kimbrough and Miller, 2000). A central periplasmic cylinder, the ‘inner rod’, is built of PrgJ subunits and protrudes from an IM ring socket structure (Marlovits et al., 2004). To the cytoplasmic face of the basal body the ATPase is bound, presumably as a double-hexameric ring, that energizes the translocation (Müller et al., 2006). Characteristic of T3SS is the presence of cognate chaperones, small acidic proteins. These chaperones are considered to stabilize and prevent terminal folding of effector proteins. The energy of ATP hydrolysis by the ATPase is conducted by release of the chaperone from an effector chaperone complex and subsequent loading of the unfolded substrate into the T3SS apparatus (Akeda and Galan, 2005). Another function of the effector-specific chaperones could be the masking of domains needed for membrane targeting within the host cell (Letzelter et al., 2006).

The OM ring complex consists of a member of the secretin family. Secretins are also involved in the formation of OM pores in type II and type IV secretion systems (T4SS). The pore monomers are transported by the Sec-dependent pathway and inserted into the OM in a pilotin-dependent manner (Lario et al., 2005). In T3SS secretins are believed to exert a stabilizing and anchoring function for the needle complex.

The rigid extracellular needle forms a protein-conducting channel extending the inner rod. The hollow cylinder is made of several hundred copies of proteins of the YscF family (Cordes et al., 2003) and shows an inner diameter of 20–30 Å, which only supports translocation of at least partially unfolded substrates.

Interestingly, the needle length of all T3SS characterized so far varies in a narrow band around 60 Å. There is some controversy about the mechanism of needle length control: The Cornelis group found that the needle length in Yersinia depends in a linear fashion on the length of a repetitive domain within YscP (Journet et al., 2003). In this model, YscP would act as a molecular ruler and extends through the entire length of the protein-conducting channel of the translocon and is anchored to the needle tip and the basal body. Once YscP is fully elongated by the growing needle, it would signal to the basal body of the T3SS to switch substrate specificity from needle components to effectors (Cornelis, 2006). In Salmonella Typhimurium a kinetic regulation was proposed: The component of the inner rod (PrgJ) and the needle protein PrgI are secreted as long as the polymerization of the inner rod is completed. This InvJ-dependent assembly of the inner rod leads to stable anchoring of the needle to the basal body and subsequent substrate switch to effectors (Marlovits et al., 2006). Needle length control is believed to be essential for the optimal function of T3SS. In Yersinia, host cell contact is accomplished with the major adhesins YadA and Inv. For this reason, the T3SS needle must span at least the distance determined by these surface molecules to interact with the target cell (Mota et al., 2005). In the Shigella system, it was shown that lipopolysaccharide (LPS) length was evolutionary adapted to facilitate T3SS function. The LPS is condensed to half of its length by specific glycosylation to compensate for an optimal needle length (West et al., 2005).

Attached to the tip of the needle, a structure known as the ‘needle extension’ was identified. This structure is thought to mediate the formation of the translocation pore and was shown to consist of LcrV in Yersinia (Mueller et al., 2005) and in Salmonella SPI2 presumably of SseB (Chakravorty et al., 2005). The translocation pore inserted into the host cell membrane consists of the ‘translocator’ proteins, best studied for Yersinia YopB and YopD (reviewed in Viboud and Bliska (2005)). Using purified translocators of enteropathogenic E. coli or of Pseudomonas aeruginosa reconstitution of pore complexes was done (Ide et al., 2001; Schoenh et al., 2003). These studies revealed asymmetrical pores with an inner diameter ranging between 30 and 50 Å.

Type IV secretion systems (T4SS)

T4SS are characterized by the ability to translocate proteins or complexes of protein and single-stranded
DNA. Based on sequence similarities, T4SS are believed to have evolved from bacterial conjugation machineries (reviewed in Cascales and Christie (2003)). The T-DNA transfer system of Agrobacterium tumefaciens (reviewed in Cascales and Christie (2003)). The T-DNA is the prototypical type A T4SS (reviewed in Burns (1999)). This well-studied T4SS translocates protein–DNA complexes but is somehow distinct from the T4SS of pathogens of humans and animals that appear to translocate proteins only.

Again by sequence comparison, T4SS are categorized into two subclasses: type IVA (T4ASS) and type IVB (T4BSS) (Christie and Vogel, 2000). For substrate recruitment and targeting to the IM parts of the transenvelope protein complex, a homohexamer of VirD4 acts as the so-called ‘coupling protein’ (CP) in T4ASS. A stable interaction of the CP with homologs of VirB10, a part of the multi-subunit transenvelope protein complex, was demonstrated (Llosa et al., 2003). The components of the transenvelope complex are members of the mating-pair formation (Mpf) protein family. Different functions could be assigned to sets of VirB proteins: VirB3 and VirB6–10 might form the channel traversing the periplasmic space. VirB9 is a secretin-like protein which could form the OM pore. The periplasmic VirB1 is a peptidoglycan hydrolase that is thought to contribute to channel formation (Ward et al., 2002). VirB4 and VirB11 are both IM proteins with putative ATPase activity which could energize the translocation and perhaps the pilus polymerization. The T-pilus is an elongated extracellular structure which is mainly composed of cyclized VirB2 subunits, but VirB5 and VirB7 might also contribute to pilus assembly or being part of the pilus.

Recent work demonstrated the roles of T4ASS in several important human bacterial pathogens. Pertussis toxin of Bordetella pertussis is secreted in a contact-independent manner, while CagA of Helicobacter pylori is a translocated T4SS effector protein that induces inflammatory responses and cytoskeletal alterations in the host cell. T4ASS have also been identified in Brucella spp. and Bartonella henselae, and the translocated effectors have central functions in the intracellular lifestyle of these pathogens. Further details can be found in a recent review by Backert and Meyer (2006).

In contrast to the T4ASS, T4BSS are less well understood. One example of a T4BSS is the virulence-associated dot/icm machinery of Legionella pneumophila. The system was discovered by screening for mutants unable to survive within macrophages (Vogel and Isberg, 1999). Individual mutants were assigned to dot (defect in organelle trafficking) or icm (intracellular multiplication) according to their respective phenotypes. To date only in L. pneumophila substrates of a T4BSS have been described (Segal et al., 2005). Amongst the over hundred experimentally identified or predicted substrates, RalF was shown to be secreted into the cytoplasm of the host cell and acts as a guanidine exchange factor on the host protein ADP ribosylation factor (ARF-1) (Nagai et al., 2002).

### Type V secretion systems

Type V secretion system (T5SS) includes several mechanisms: AT secretion, the two-partner system (TPS) and the oligomeric coiled-coil adhesin (Oca) system. AT proteins are of modular composition: An N-terminal signal sequence targets the protein to the Sec machinery at the IM, the passenger domain harbors the specific effector function and the C-terminal translocation units interact to form a β-barrel secondary structure which allows secretion of the passenger domain. ATs are synthesized as pre-pro-proteins, after cleavage of the signal peptide the pro-protein is released into the periplasm. The passenger domain is, depending on the particular AT, cleaved off from its translocation unit after passing the OM and the protein is released into the extracellular milieu.

In contrast to the classical ATs which are synthesized as a single polypeptide, in TPSs the passenger domain and the transporter domain are translated as two separate proteins which are referred as members of the TpsA and TpsB families, respectively (Jacob-Dubuisson et al., 2004).

A group of non-fimbrial adhesins (see below) belongs to the family of surface-attached oligomeric ATs. The hallmark of these proteins is that they form oligomeric complexes at the bacterial surface, and their C-terminal translocation units interact to form a β-barrel pore which functions as a membrane anchor (Koretke et al., 2006). The most prominent member of this family of membrane proteins is YadA from Yersinia spp. (Roggenkamp et al., 2003).

### Chaperone usher (CU) pathway

The CU pathway is linked to the assembly of adhesive surface structures of Gram-negative bacteria. One of the best characterized systems are type 1 pili from uropathogenic E. coli. All the subunits needed for pilus assembly are translated as pre-proteins (pre-pilins) and translocated into the periplasm in a Sec-dependent manner. FimC is a periplasmic chaperone which binds pilus subunits called pilins (FimA, F, G, H) with a 1:1 stoichiometry (Vetsch et al., 2004). Complex formation with the chaperone is essential to prevent premature pilus assembly in the periplasm and for targeting of the subunits to the integral OMP FimD. FimD is the assembly platform (usher) from which the nascent pilus is built and anchored. The usher mediates translocation of folded subunits through a large pore (Saulino et al., 2000).
Other secretion pathways

Genome-wide screens for homologs of the T4BSS component IcmF revealed a group of pathogenicity islands present in Gram-negative bacteria that have been termed IcmF-associated homologous protein (IAHP) cluster. Investigation of the virulence-associated secretion cluster in V. cholerae led to the identification of a new class of secretion systems, termed type VI. This type VI secretion system (T6SS) manages the export of substrates at least in the extracellular space without the requirement of hydrophobic N-terminal signal sequences. Using the model host Dictyostelium discoideum, a virulence function of the T6SS-secreted substrates could be shown, even suggesting a translocation of these proteins in the cytosol of the amoebae (Pukatzki et al., 2006). Recently, another T6SS was identified encoded at the HIS-I locus of P. aeruginosa (Mougous et al., 2006). In this study an ATPase, ClpV1, was identified, presumably energizing the secretion. Furthermore, the substrate Hcp1 could be shown to be secreted in a T6SS-dependent manner and is present in CF patients with chronic P. aeruginosa infection.

The twin arginine transport (Tat) system and the YidC system have no known role in pathogenesis and are only briefly described. Tat is the only translocation pathway characterized so far able to transport prefolded and often oligomeric proteins across membranes (reviewed in Sargent et al., 2006). A common feature of Tat substrates is the presence of a consensus amino acid sequence (SRRxFLK) in the N-terminal signal peptide. This twin-arginine-bearing signal peptide targets pre-cursor proteins to the membrane-associated Tat transport apparatus. Hallmarks of this translocation pathway are that it is energized solely by the proton-motive force, and the remarkably low number of different subunits building up the secretion system. YidC is an E. coli membrane factor involved in the biogenesis of integral IM proteins, both in conjunction with and independent of the Sec system (Luirink et al., 2005).

Bacterial protein adhesins

The ability to adhere to a wide variety of biotic and abiotic surfaces is a feature which can promote bacterial survival and is one of the key virulence functions of many pathogens. Adhesion is mediated by distinct surface structures which can be subdivided by their assembly mechanism and structure into two major classes (Fig. 2): fimbrial adhesins and non-fimbrial adhesins (Soto and Hultgren, 1999). The terms non-fimbrial and afimbrial adhesin are often used synonymously for mono- or oligomeric adhesive surface structures. A possible distinction could be the presence (non-fimbrial) or absence (afimbrial) of a typical membrane anchor in the protein.

Many bacteria are able to express a whole set of different adhesins, often belonging to different subclasses, on their surface. This might be an adaptation to different environmental conditions or, in the case of pathogenic bacteria, to different hosts or host tissues. In the following section, prototypes of the various adhesins of Gram-negative bacteria are described.

Fimbrial adhesins

Fimbriae (or pili) are a group of rigid, straight filamentous appendages on a bacterial surface. They are most prominent on Gram-negative bacteria, where they are anchored within the OM. These surface structures, composed of hundreds to thousands of subunits, mediate adhesion via specific interaction with surface structures (receptors) present on host cells. Interestingly, it was shown that fimbriae were able to mediate unspecific adhesion by increasing the surface hydrophobicity of the bacteria (Lindahl et al., 1981).

Type I and P pili

Pyelonephritis-associated (P) pili as well as type I pili are build up in the OM via the CU pathway. Both types of pili are found in UPEC and exhibit different binding specificities due to the nature of the adhesive tip of their fibrillum. P pili, encoded on the pap operon, bind through the PapG adhesin to terminal sugars of glycolipids present on the surface of host cells. Host and tissue specificity varies due to the presence of different PapG adhesins recognizing different but related Galx-(1–4)-Gal sugars (Feria et al., 2001). The fim gene cluster encodes type I pili, with FimH being the pilus adhesin. FimH variants present on non-pathogenic E. coli show high affinity to trimannose-containing glycoprotein receptors. In contrast, UPEC express predominantly FimH variants with high affinity to monomannose residues, found enriched on surface glycoproteins of urinary tract cells. FimH-mediated receptor binding was shown to mediate bacterial internalization within bladder cells, resulting in bacterial persistence and chronic urinary tract infections (Martinez et al., 2000). Using subunit-exchange experiments, it could be shown recently that the gross binding specificity (host and cell tropism) does not only depend on the adhesive tip fibrillum but also on the pilus shaft (Duncan et al., 2005).

In Salmonella enterica serovar Typhimurium, type I pili encoded by the fim gene cluster were shown to be involved in adhesion to HeLa cells (Bäumler et al., 1996). The complete genome sequence of S. Typhimurium revealed the presence of 13 operons with homology
to fimbrial gene sequences and additional serovar-specific fimbriae were detected. There is evidence for the expression of at least 11 of them in vivo as shown by sero-conversion of *Salmonella*-resistant CBA mice (Humphries et al., 2005). The presence of a whole set of putative fimbriae together with phase variation demonstrated for some of the fimbrial operons (*fim*, *lpf* and *pef*), might be an adaptation which enables *Salmonella* to colonize a broad range of hosts and to evade immune responses.

**Afa/Dr adhesins**

Afa/Dr adhesins (diffuse adherence fibrillar adhesin/Dr blood group antigen) are a very diverse family of adhesins, including fimbrial, afimbrial and non-fimbrial members all of which are assembled via the CU pathway. Most of the data available about this class of adhesins originate from DAEC (diffusely adhering *E. coli*). Afa/Dr family operons encode transcriptional regulators, a periplasmic chaperone, an OM-anchoring protein, an invasin and the Afa/Dr adhesin itself (reviewed in Servin (2005)). Afa adhesins form amorphous OM-associated structures (afimbrial sheaths) (Garcia et al., 2000), detected in diarrheal *E. coli* isolates, is a fimbrial adhesin with limited homology to *P* pili (Bilge et al., 1989). Some of the receptors of Afa/Dr adhesins have been characterized: The fimbrial Dr adhesin was shown to bind to collagen IV as well as to the complement-regulating protein DAF (CD55) (Nowicki et al., 1988). Recently, the interaction of members of the Afa/Dr family of adhesins with CEACAM surface molecules has been reported (Berger et al., 2004). CEACAM1, CEACAM6 and CEA were shown to be recruited to adhering bacteria in an Afa/Dr-dependent manner (Guignot et al., 2000). Recruitment of these surface receptors was able to trigger activation of the Rho GTPase Cdc42 and phosphorylation of the ezrin/
radixin/moesin complex which was accompanied with membrane rearrangements leading to tight bacterial attachment. Triggering of host cell signaling cascades by interaction of Afa/Dr adhesins with surface receptors might also result in a zipper-like invasion of epithelial cells (Kansau et al., 2004).

CS pili
The assembly of coli surface antigen 1 (CS1) pili of enterotoxigenic *E. coli* shows high functional similarities to the chaperone/usher pathway but the proteins involved share no detectable sequence similarities. The CS pili assembly system is therefore termed the ‘alternate CU pathway’ (Soto and Hultgren, 1999).

Four linked genes, *cooABCD*, are essential for the biogenesis of CS pili. All four proteins are GSP-dependently transported into the periplasm where the periplasmic chaperone CooB stabilizes CooA, C and D. The minor pilin CooD is thought to initiate pilus assembly and is located on the pilus tip. The OMP CooC presumably is the usher through which CooD and the major pilin CooA are secreted (Starks et al., 2006). CS1 pili confer binding of *E. coli* to intestinal epithelial cells and mediate agglutination of bovine erythrocytes; however, the host receptor has not been characterized so far. Mutational analyses revealed that adhesion as well as hemagglutination critically depend on amino acid (aa) residues within the minor pilin CooD (Sakellaris et al., 1999).

Type IV pili
Type IV pili are formed in the cytoplasmic membrane by the polymerization of pilin subunits. The assembled pilus structure is extruded across the OM and forms long and flexible surface appendages (reviewed in Craig et al. (2004)). Components of the type IV pilus assembly machinery are structurally related to T2SS, where homologous proteins are called ‘pseudopilins’. Type IV pilins share a conserved stretch of 25 hydrophobic aa, an unusual N-methylation at their N-terminus and a disulfide-bond at their C-terminus. Based on their aa sequence and length type IV pilins are grouped into two subclasses, type IVa and type IVb. Pili polymerized from type IVa and type IVb subunits differ significantly in diameter as well as helical structure (Hansen and Forest, 2006). One of the best characterized examples of type IVa pili are PAK pili, the dominant adhesin of *P. aeruginosa* strain K. PAK pili were shown to mediate adherence to mucosal epithelial cells by binding to the glycolipids asialo-GM1 and asialo-GM2 (Krivan et al., 1988). So far, type IVb pili were identified exclusively in bacteria able to colonize the human intestine. In *V. cholerae* the toxin-coregulated pilus was shown to mediate, besides adhesion, autoaggregation through homophilic interactions. This effect is believed to be beneficial for the bacteria at the site of infection because microcolonies can be formed and secreted toxins can reach high local concentrations (Kirn et al., 2005). *S. enterica* serovar Typhi uses type IVb PilS pili to bind to intestinal epithelial cells (Zhang et al., 2000). The epithelial surface receptor of PilS was identified as cystic fibrosis transmembrane conductance regulator (CFTR) (Pier et al., 1998). Furthermore, there is experimental evidence that *S*. Typhi is able to actively increase CFTR concentrations on apical membranes to promote adhesion and subsequent uptake (Lyczak and Pier, 2002).

A hallmark of type IV pilus biology is their ability to retract through the bacterial cell wall while the pilus tip remains firmly adhered to a receptor structure. Pilus retraction is required for a specialized movement across semisolid matrices (e.g. mucosal epithelia) called ‘twitching motility’ (Burrows, 2005).

Curli
Another type of fimbrial adhesins are curli or thin aggregative fibers (agf). These surface appendages seem to be commonly present in the genome of *Salmonella* spp. and *E. coli* strains. Biosynthesis of curli in *E. coli* as well *S*. Typhimurium depends on the expression of two divergently orientated operons: csgDEFG and csgBA (Hammar et al., 1995; Römling et al., 1998). CsgD is a transcriptional regulator of the LuxR family and promotes expression of several biofilm-associated genes and the csgBA operon (Brombacher et al., 2003). Besides a function in biofilm formation, curli were also shown to mediate adhesion of bacteria to the human matrix proteins fibronectin and laminin but also to plasminogen, contact phase proteins and major histocompatibility complex class I molecules (Ben Nasr et al., 1996; Olsen et al., 1998; Robinson et al., 2006).

Curli are assembled at the bacterial surface through extracellular nucleation precipitation: the major fiber subunit CsgA polymerizes on the surface-exposed nucleator CsgB (Hammar et al., 1996). CsgE and CsgF are two periplasmic proteins which are required for optimal curli assembly and interact with the OMP CsgG. Recently, the OM lipoprotein CsgG was shown to be an integral part of a secretion complex to which CsgE and CsgF are recruited. This secreton presumably facilitates the transport of CsgA and CsgB across the OM (Robinson et al., 2006).

Non-fimbrial adhesins
Non-fimbrial adhesins are a large family of mono- or oligomeric surface-associated proteins which are not assembled by the CU pathway and without separate subunits for the adhesive tip and pilus shaft. The group of secreted adhesins (see below), which are not predominantly surface-associated, mediate bacterial binding by mechanisms still to be elucidated.
Autotransported adhesins

To better account for the specific features of this protein family, the Oca family of T5SS-secreted substrates was suggested to be termed ‘trimeric AT adhesins’ (TAA) (Linke et al., 2006). YadA is the best-characterized TAA and the major adhesin of enteropathogenic Yersinia spp. YadA forms stable, high-molecular-weight trimers which protrude about 28 nm from the bacterial OM (Hoiczyk et al., 2000). It has been observed that Yad length can vary significantly between different serotypes of Yersinia spp. (Heise and Dersch, 2006). YadA mediates binding to several extracellular matrix (ECM) proteins such as collagen, laminin and fibronectin (Nummelin et al., 2004). BadA from Bartonella henselae was also shown to mediate binding to ECM proteins (e.g. collagens and fibronectin) but the hair-like appendages observed were much longer (100–300 nm) (Riess et al., 2004). There are numerous other TAAAs present or predicted in human and plant pathogens, all of them share a similar molecular architecture: They resemble a ‘lollipop’ containing a head domain, presumably harboring the adhesion function, the neck, the stalk, a fibrous, highly repetitive head domain, presumably harboring the adhesion function, the neck, the stalk, a fibrous, highly repetitive structure which contains coiled coils, and the anchor domain (reviewed in Linke et al. (2006)).

In S. enterica serovar Typhimurium, the SPI3-encoded adhesin MisL seems to be a ‘classical’ autotransported substrate, being not homologous to the TAA family members (Dorsey et al., 2005). Another monomeric AT of Salmonella, ShdA, was shown to have a function in adhesion and virulence. The surface-located protein, encoded on the CS54 pathogenicity island, is induced in vivo within the murine cecum and its expression might play a role in the long-term colonization of mice. GST-fusion proteins of the passenger domain of ShdA were shown to bind fibronectin in vitro (Kingsley et al., 2002).

The third branch of T5SS, TPS, is represented by filamentous hemagglutinin (FHA) of Bordetella pertussis. FHA is the product of N- and C-terminal processing of the precursor FhaB. FhaB is recognized and secreted by the OM pore-forming protein FhaC, a member of the TpsB family (Mazar and Cotter, 2006). FHA was shown to function as a multi-functional attachment protein, able to mediate binding to several ligands. A hallmark of FHA is that a significant amount of the protein is released from the bacterial surface by the OM protease SphB1. Bacteria deficient for SphB1 adhered significantly better to cultured epithelial cells but were attenuated in a mouse model of lung colonization (Coutte et al., 2003). For FHA, immuno-modulatory functions could be assigned to the secreted protein, for example, by influencing the cytokine pattern secreted by dendritic cells (McGuirk et al., 2002). Release of adhesins into the extracellular space seems to be a common but poorly understood phenomenon.

Integral OM adhesins

Several β-barrel-containing OMPs have functions in adhesion (Niemann et al., 2004). OmpA plays an important role in the crossing of the blood–brain barrier of E. coli causing meningitis in neonates. The receptor of OmpA was identified as Ecgp, a 96-kDa glycoprotein present on the surface of human brain microvascular endothelial cells (PrasadArao, 2002). Neisseria meningitidis possesses another Omp, OpcA, shown to mediate bacterial adhesion and invasion of human epithelial and endothelial cells (Virji et al., 1992). The recognition pattern of OpcA includes heparan sulfate proteoglycans (de Vries et al., 1998) as well as integrins which are bound via the serum protein vitronectin (Virji et al., 1993). A recent study reported that OpcA binds sialic acid and is specific for pyranose saccharides (Moore et al., 2005).

The Yersinia pseudotuberculosis Omp invasin binds to members of the β1-integrin family, which induces formation of pseudopods leading to the uptake of the bacteria into M cells (Isberg and Leong, 1990). Invasin consists of an N-terminal β-barrel membrane anchor, four domains (D1–D4), belonging to the Ig superfamily, and a C-terminal D5 domain. The D5 domain structurally belongs to the C-type lectins (Kogelberg and Feizi, 2001). There are some overlapping functions in the Yersinia adhesins invasin and YadA in triggering β1-integrin-dependent signaling cascades (Hudson et al., 2005), but both adhesins are expressed under distinct environmental conditions and may not be co-expressed during infection (Eitel and Dersch, 2002).

Intimin is another Omp found in EPEC, EHEC and Citrobacter spp. It is related to invasin in primary sequence as well as regarding structural features. The N-terminal β-barrel membrane anchor is followed by domains D1–3 (Ig fold) and a C-type lectin domain (D4). Domains D3 and D4 form together a rigid superdomain able to bind the cognate receptor translocated intimin receptor (Tir) (Batchelor et al., 2000). Tir is an effector molecule of a T3SS which is phosphorylated by the host cell after translocation and subsequently inserted into the host cell membrane (Kenny et al., 1997). All components of this adhesion system where the bacteria provide both the ligand and the receptor are encoded together within the locus of enterocyte effacement. Further members of the family of integral OMPs include OmpX of E. coli, Ail of Yersinia enterocolitica and PagC of S. enterica.

Secreted adhesins

A comprehensive search for putative T1SS substrates in genomes of Gram-negative bacteria revealed many putative proteins with predicted adhesion function (Delepelaire, 2004). The criterion used in this screen was...
the presence of glycine-rich repeats (GGXGDXXXX), which are found in a majority of T1SS-secreted proteins and presumably bind calcium ions (Baumann et al., 1993). Many of the predicted adhesins were extremely large and repetitive proteins, ranging from 1209 to 8682 amino acids.

The largest predicted T1SS-secreted adhesin to date is LapA from the soil bacterium *Pseudomonas fluorescens* with about 900 kDa. The protein is encoded within an operon which harbors also genes coding for a putative ABC transporter (*lapBCE*). Bacteria deficient in one of the *lap* genes were impaired in adhesion to quartz sand (Hinsa et al., 2003). This adhesin was shown to be loosely surface-associated, a feature which is essential for its function and that partially depends on LapD, an IM protein (Hinsa and O'Toole, 2006). Furthermore, LapA seems to play a role in the early steps of biofilm formation, where the bacteria transit from reversible to irreversible attachment (Hinsa et al., 2003). This adhesin was shown to be loosely surface-associated, a feature which is essential for its function and that partially depends on LapD, an IM protein (Hinsa and O’Toole, 2006). Furthermore, LapA seems to play a role in the early steps of biofilm formation, where the bacteria transit from reversible to irreversible attachment (Hinsa et al., 2003). LapA is a member of the BAP family of large, repetitive proteins (Table 1). BAP family members are predominantly involved in biofilm formation but are also shown to mediate adhesion (reviewed in Lasa and Penades (2006)). A cluster of genes encoding a putative type I secretion system has been identified also in the vicinity of *Salmonella* Typhimurium *bapA*, suggesting a common secretion mechanism (Latasa et al., 2005). Recently, Gerlach et al. (2007) identified a T1SS encoded by *S. enterica* SPI4 that secretes SiiE, a highly repetitive non-fimbrial adhesin of about 600 kDa. SiiE is only required for adhesion to polarized epithelial cells and co-regulated with the SPI1-encoded T3SS involved in host cell invasion.

The *V. cholerae* GbpA adhesion factor is secreted in a T2SS-dependent manner (extracellular protein secretion system, EPS). This protein was shown to mediate binding of bacteria to HT-29 epithelial cells and chitin beads through interaction with N-acetylglucosamine (GlcNAc) residues (Kirn et al., 2005).

It is difficult to understand by which means a secreted protein can mediate adhesion, and two alternative models have been proposed to explain this phenomenon: (i) The secreted adhesin could bind to its receptor and then interact with a bacterial cell surface structure providing a bridging function. (ii) The protein is present in two forms: secreted and (minor) cell surface-associated, with the surface-bound form exerting the adhesion function or interacting with the secreted form (discussed in Kirn et al. (2005)). None of these hypotheses are experimentally proven so far.

### Table 1. The biofilm-associated proteins (BAP) family of large repetitive proteins involved in bacterial adhesion and/or biofilm formation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BapA</td>
<td><em>Salmonella enterica</em></td>
<td>Biofilm</td>
<td>Latasa et al. (2005)</td>
</tr>
<tr>
<td>Bap</td>
<td><em>Staphylococcus aureus</em></td>
<td>Biofilm</td>
<td>Cucarella et al. (2001)</td>
</tr>
<tr>
<td>Bap</td>
<td><em>Burkholderia cepacia</em></td>
<td>Biofilm</td>
<td>Huber et al. (2002)</td>
</tr>
<tr>
<td>Bhp/Bap</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>Biofilm</td>
<td>Tormo et al. (2005)</td>
</tr>
<tr>
<td>Esp</td>
<td><em>Enterococcus faecalis</em></td>
<td>Biofilm</td>
<td>Shankar et al. (1999)</td>
</tr>
<tr>
<td>Lsp</td>
<td><em>Lactobacillus reuteri</em></td>
<td>Adhesion</td>
<td>Walter et al. (2005)</td>
</tr>
<tr>
<td>R28</td>
<td><em>Streptococcus pyogenes</em></td>
<td>Adhesion</td>
<td>Stalhammar-Carlemalm et al. (1999)</td>
</tr>
<tr>
<td>Mus20</td>
<td><em>Pseudomonas putida</em></td>
<td>Adhesion</td>
<td>Espinosa-Urgel et al. (2000)</td>
</tr>
<tr>
<td>VAP1445</td>
<td><em>Vibrio parahaemolyticus</em></td>
<td>Biofilm</td>
<td>Enos-Berlage et al. (2005)</td>
</tr>
<tr>
<td>YeeJ</td>
<td><em>Escherichia coli</em></td>
<td>Adhesion</td>
<td>Roux et al. (2005)</td>
</tr>
</tbody>
</table>

Modified from Lasa and Penades (2006).

### Adhesins associated with biofilm formation

The ability to form a biofilm can be of crucial importance for the colonization of certain biotic or abiotic surfaces and can influence the virulence of pathogenic bacteria. Tight adhesion mediated by specific interactions of adhesins with receptor structures is a prerequisite of biofilm formation. Homotypic interactions between surface-associated adhesins can promote microbial aggregation and trigger biofilm formation (reviewed in Dunne (2002)).

There is one large family of adhesins that are present in Gram-positive as well as in Gram-negative species. Bap of *Staphylococcus* spp. is the prototypical member of this BAP family (Table 1). This surface-associated protein expressed by different *Staphylococcus* isolates was described to mediate biofilm formation of *Staphylococcus aureus* in a PIA-independent manner (Cucarella et al., 2001). Bap consists of 2276 amino acids, has an N-terminal secretion signal, a long repetitive central moiety and a C-terminal cell wall-attachment region comprising an LPXTG motif.
All BAP family members share a high molecular mass, a signal sequence for extracellular secretion and a repetitive structure (reviewed in Lasa and Penades (2006)). The number of repeats within BAP is variable not only among different isolates of Enterococcus faecalis or Staphylococcus spp. (Shankar et al., 1999; Tormo et al., 2005) but also during the course of infection with a single strain of Staphylococcus aureus (Cucarella et al., 2004). Although size variants of Staphylococcus aureus Bap did not differ in their ability to mediate biofilm formation, it could rather be a mechanism to evade immune responses by antigenic variation (Cucarella et al., 2004). In Salmonella Typhimurium, BapA was identified as a BAP family member important for biofilm formation. Expression of BapA was shown to depend on CsgD, the global regulator of biofilm formation in Salmonella strains (Latasa et al., 2005).

Conclusions and outlook

A remarkable variety of systems for secretion of proteins into the extracellular space is found in Gram-negative bacteria. Starting from relatively simple T1SS and T5SS apparatuses that usually transport one single substrate protein, secretion systems range to the complex T3SS and T4SS that translocate a set of effector proteins into eukaryotic host cells. In addition to the secretion of toxins and various other substrate proteins and the translocation of effector proteins into eukaryotic host cells, several secretion systems are involved in the assembly of protein adhesins. Specific branches of the GSP have evolved for the assembly of fimbrial adhesins. In contrast, most non-fimbrial adhesins are secreted by T5SS, and very recently the role of T1SS in secretion of large non-fimbrial adhesins involved in biofilm formation and binding to eukaryotic cells became evident. There is only one remarkable example for a T3SS involved in adhesion, i.e., the EPEC/EHEC T3SS that translocates Tir into host cells acting as receptor for the bacterial adhesin.

In most Gram-negative pathogens, copies of the various secretion systems can be found with functions in different phases of the pathogenesis. There is also high redundancy within one class of secretion system, for example, two T3SS or multiple T1SS are present in many pathogens. This observation may indicate that the repetitive acquisition of gene clusters for secretion systems by horizontal gene transfer allows a more rapid evolution and adaptation to new niches than the adaptation of existing secretion systems to new or multiple functions. A similar situation seems to apply for the large number of adhesins since many pathogens possess multiple fimbrial adhesins as well as different non-fimbrial adhesins. The improved understanding of the structure of non-fimbrial adhesins suggests that these proteins can functionally mimic the fimbrial adhesin. A single polypeptide contains domains that build the head, the shaft and the membrane anchor of the adhesin. In fimbrial adhesins, the different subunits have adapted to these different functions. Several of the non-fimbrial adhesins are proteins of extremely large size that are characterized by large numbers of domain repeats that build the shaft-like part of the molecule. The number of repeats indicates the size constraints for the non-fimbrial adhesins that are most likely given by the O-antigen length of the LPS.

We have fairly good understanding of the temporal and spatial control of the expression and function of various protein secretion systems in bacterial pathogens. In contrast, such understanding is incomplete for the numerous adhesins and future research has to reveal the role of various adhesins in different host species.

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References

prediction of secreted proteins in 225 bacterial proteomes. Microbiology 151, 1725–1727.


Duong, F., Wickner, W., 1997. The SecDFyajC domain of preprotein translocase controls preprotein movement by regulating SecA membrane cycling. EMBO J. 16, 4871–4879.


Huber, B., Riedel, K., Kothe, M., Givskov, M., Molin, S.,
Ide, T., Laarmann, S., Greune, L., Schillers, H., Oberleithner,
Hoiczyk, E., Roggenkamp, A., Reichenbecher, M., Lupas, A.,
Hinsa, S.M., Espinosa-Urgel, M., Ramos, J.L., O’Toole, G.A.,
Eberl, L., 2002. Genetic analysis of functions involved in
Hammar, M., Bian, Z., Normark, S., 1996. Nucleator-
dependent intercellular assembly of adhesive curli organ-
Hansen, J.K., Forest, K.T., 2006. Type IV pilin structures:
insights on shared architecture, fiber assembly, receptor
binding and type II secretion. J. Mol. Microbiol. Biotechn-
ol. 11, 192–207.
Heise, T., Dersch, P., 2006. Identification of a domain in
Yersinia virulence factor YadA that is crucial for extra-
Natl. Acad. Sci. USA 103, 3375–3380.
Hinsa, S.M., O’Toole, G.A., 2006. Biofilm formation by
Pseudomonas fluorescens WCS365: a role for LapD.
Microbiology 152, 1375–1383.
Hinsa, S.M., Espinosa-Urgel, M., Ramos, J.L., O’Toole, G.A.,
2003. Transition from reversible to irreversible attachment
during biofilm formation by Pseudomonas fluorescens
WCS365 requires an ABC transporter and a large secreted
Hoiczky, E., Roggenkamp, A., Reichenbecher, M., Lupas, A.,
Heesemann, J., 2000. Structure and sequence analysis of
Yersinia YadA and Moraxella Usps reveal a novel class of
adhesins. EMBO J. 19, 5989–5999.
Huber, B., Riedel, K., Kothe, M., Givskov, M., Solin, S.,
Eberl, L., 2002. Genetic analysis of functions involved in
mechanisms of integrin binding by Yersinia pseudotuber-
culosis adhesins determine the phagocytic response of host
Ide, T., Laarmann, S., Greune, L., Schillers, H., Oberleithner,
pores inserted into plasma membranes by type III-secreted
integrins are receptors for invasion, a protein that promotes
secretion through autotransporter and two-partner path-
needle length of bacterial injectisomes is determined by
Kansau, I., Berger, C., Hospital, M., Amsellem, R., Nicolas,
internalization of Dr-positive E. coli by epithelial cells is
preceded by an adhesin-induced mobilization of raft-
associated molecules in the initial step of adhesion. Infect.
Immun. 72, 3733–3742.
Kenny, B., Devinney, R., Stein, M., Reinscheid, D.J., Frey,
transfers its receptor for intimate adherence into mamma-
Kingsley, R.A., Santos, R.L., Keestra, A.M., Adams, L.G.,
Bäumler, A.J., 2002. Salmonella enterica serotype Typhi-
murium ShdA is an outer membrane fibronectin-binding
factor links Vibrio cholerae environmental survival and
lectin-type proteins of the immune system. Curr. Opin.
Model structure of the prototypical non-fimbrial adhesin
Koronakis, V., Sharff, A., Koronakis, E., Luisi, B., Hughes,
Koronakis, V., Eswaran, J., Hughes, C., 2004. Structure and
function of TolC: the bacterial exit duct for proteins and
drugs. Annu. Rev. Biochem. 73, 467–489.
Kostakioti, M., Newman, C.L., Thanassi, D.G., Stathopoulos,
C., 2005. Mechanisms of protein export across the bacterial
as aeruginosa and Pseudomonas cepacia isolated from
cystic fibrosis patients bind specifically to gangliotetraosyl-
ceramide (asialo GM1) and gangliotriaosylceramide (asialo
Lario, P.I., Pfuetzner, R.A., Frey, E.A., Creagh, L., Haynes,
C., Maurrelli, A.T., Strynadka, N.C., 2005. Structure and
Latasa, C., Roux, A., Toledo-Aranas, A., Ghigo, J.M.,
secreted protein required for biofilm formation and host
colonization of Salmonella enterica serovar Enteritidis. Mol.
Microbiol. 58, 1322–1339.
Letzelter, M., Sorg, I., Mota, L.J., Meyer, S., Stalder, J.,
Feldman, M., Kuhn, M., Callebaut, I., Cornelis, G.R., 2006. The discovery of SyeO highlights a new function for
type III secretion effector chaperones. EMBO J. 25, 3223–3233.
test based on ‘salting out’ to measure relative surface
hydrophobicity of bacterial cells. Biochim. Biophys. Acta
677, 471–476.
Linke, D., Riess, T., Autenrieth, I.B., Lupas, A., Kempf, V.A.,
2006. Trimeric autotransporter adhesins: variable structure,
Llosa, M., Zunzunegui, S., de la Cruz, F., 2003. Conjugative
coupling proteins interact with cognate and heterologous


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