Chapter 6
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Summary and Concluding Remarks

Summary for the unacquainted

Samenvatting voor de leek

Summary in Hindi
Summary

Summary and Concluding Remarks

The cells of living organisms are encircled by a membrane that protects them from their surroundings. Interactions of organism with their environment have to take place across this membrane. Examples of such interactions are the sensing of the surroundings, communication with other cells, or the uptake or secretion of substrates, enzymes or waste products. Gram negative bacteria are surrounded by two membranes, the inner and the outer membrane. For the transport of substrates across the outer membrane, at least seven secretion systems have been described [459]. Of these, the type IV secretion system (T4SS) and type IV pili (Tfp), which show homology to Type II secretion systems, are described in chapter 1.

T4SS are multiprotein complex involved in functions like conjugation, substrate/toxin secretion and DNA uptake or release. They consist of targeting components, 2-3 ATPases and channel forming Mpf or the mating pair formation complex. The T4SS of the Ti plasmid of A. tumefaciens is currently the best characterized T4SS. It consists of the targeting components VirD1 through VirD4 and 11 other VirB proteins called VirB1 to VirB11 (Fig. 1, left panel). The Tfp systems are involved in functions like motility, adhesion and DNA uptake. They consist of an assembly of protein complexes and ATPases that bring about extension and retraction of the pilus. The Tfp proteins encoded by the pil genes of N. gonorrhoeae are depicted in Fig. 1, right panel. The aim of this thesis is the study of the two systems in the Gram negative pathogen Neisseria gonorrhoeae. This human pathogen causes the sexually transmitted disease called “gonorrhea” and is introduced in chapter 1.

Maturation of the pilin encoded by the GGI

The T4SS in N. gonorrhoeae is encoded by the horizontally acquired gonococcal genetic island (GGI) [265]. This is the first, and currently only system that is described to utilize a T4SS to secrete DNA into the medium [45]. The GGI encodes a pilin protein called TraA. Pilin proteins assemble into a pilus structure, which is used to bridge the donor and recipient cells. The pilus structure might also form a conduit for the secretion of substrates. Pilins of T4SSs are often modified before they are assembled into the
pilus. The mechanism of pilus insertion and processing is studied in detail in chapter 2. Here it is shown that, after co-translation insertion of TraAGGI into the membrane, TraAGGI is cleaved on its N-terminus by leader peptidase, and circularized by the pilus processing protein TrbI GGI. TrbI is a protein encoded within the GGI that has homology to TraFRP4, the protein which was demonstrated to circularize the pilin protein TrbC RP4 from the RP4 pilus, before it is assembled into the P-type pilus. This is the first example of circularization of a pilus in a system that resembles the well studied conjugative T4SSs encoded on the F plasmid, which make the F-type pili, and also the smallest (68 amino acids) circular sex pilus found to date. It is shown that several mutations on the C and the N-terminus of the pilin are tolerated. A study of the mechanism shows that the circularization reaction occurs via an intermediate in which TraAGGI is covalently bound to TrbI GGI. Remarkably, the reaction does not seem to involve a serine-lysine catalytic dyad, as has been proposed previously, since mutagenesis of possible catalytic serine residues had no effect on the circularization reaction.

Relaxase encoded by the GGI

T4SSs involved in conjugative transport of DNA encode a pilot protein called relaxase that first binds and cleaves DNA in a sequence specific manner and then mediates the transport of DNA to the recipient organism. The relaxase encoded by the GGI however shares low sequence homology to other relaxases and differs from them in several ways. Chapter 3 describes the study of the novel relaxase, TraI of the GGI. Sequence analysis revealed the presence of three novel features of this relaxase; an N-terminal hydrophobic domain, an HD domain of the metal-dependent phosphohydrolase family and a C-terminal DUF 1528 domain. Fifty-three other uncharacterized proteins belong to this new family.

The N-terminal hydrophobic domain is shown to target the relaxase to the membrane independent of the presence of other components encoded within the GGI. Since the N-terminal domain contains a putative signal sequence, the secretion of TraI was tested in an in vitro assay, but no secretion of TraI was detected, demonstrating that TraI is not translocated via a sec-dependent manner. This ruled out the possibility for a two-step secretion mechanism as has been observed for the ptil toxin [35].
Figure 1: Schematic of the type IV secretion system (left panel) and the type IV pili system (right panel). OM and IM denotes outer and inner membrane respectively. VirD2 and DNA are the substrates, VirB2 and VirB5 are the pilus forming proteins; VirB7-VirB9-VirB10 forms the core complex, VirB6 and VirB8 are the inner membrane proteins; VirD4 is the coupling protein ATPase and VirB4 and VirB11 are the other ATPases of the type IV secretion system. The type IV pili system consists of PilE major pilin subunit, PilC adhesion protein, PilW lipoprotein, the outer membrane secretin complex of PilQ, inner membrane complex of PilM-PilN-PilO-PilP, inner membrane protein PilG, prepilin peptidase PilD and the ATPases PilT and PilF.

Several important residues with the relaxase were identified. Mutational analysis of TraIGGI, showed that the tyrosine-93 residue of the relaxase domain is absolutely essential for DNA secretion. Mutation of the second tyrosine residue, tyrosine-201 shows reduced DNA secretion. Although the histidine residues of the HD domain surprisingly did not inhibit DNA secretion, a reduction of DNA secretion after mutagenesis of aspartate-162 was observed. Sequence specific DNAsese treatment indicated that the secreted DNA is single stranded and blocked at the 5’-end. And
finally, an oriT region was identified in the GGI that possessed an inverted repeat sequence and markedly reduced DNA secretion when removed.

In the appendix to chapter 3, attempts are described to detect TraI in the supernatant. TraI was only identified in the outer membrane derived vesicles (OMVs) present in supernatants of cultures. Due to the possible contamination of TraI released by autolysis, TraI was also found in OMVs from the mutants that show no DNA secretion.

**Single stranded DNA binding protein (SSB) encoded by the GGI**

SSBs are proteins that bind single stranded DNA with high affinity, and that often perform essential functions. They function in DNA metabolism like recombination; replication and repair and are highly conserved. SSBs encoded by T4SS are known to be involved in functions like plasmid segregation and DNA transport. *N. gonorrhoeae* contains a SSB on its chromosome, and a second SSB, (annotated as SsbB) encoded within the GGI. In Chapter 4, the function and biochemistry of this protein is further characterized. In vivo studies using either strains without SsbB\textsubscript{GGI}, or strains overexpressing SsbB\textsubscript{GGI} did not show any differences in DNA secretion, or DNA uptake by *N. gonorrhoeae*. Contrary to most SSBs, SsbB\textsubscript{GGI} was not able to complement an *E. coli* deletion mutant, but purification of SsbB\textsubscript{GGI} showed that it formed an active and stable homotetramer that bound ssDNA with high affinity (<10nm). Remarkably, SsbB\textsubscript{GGI} bound DNA in a magnesium independent non-cooperative manner, a binding mode not observed previously. Also, purified SsbB\textsubscript{GGI} stimulated topoisomerase I activity in vitro.

**Secretin of the Tfp system of Neisseria**

Members of the GSP secretin superfamily form large oligomeric integral outer membrane protein complexes that are involved in the terminal translocation of proteins and macromolecules across the outer membrane. The PilQ secretins from *N. gonorrhoeae* and *N. meningitidis* are members of this family. They play a crucial role in the biogenesis of Tfp and are studied in chapter 5. They form the outer membrane channel through which the Type IV pilus extends and retracts. Secretin complexes of *N. gonorrhoeae* when observed in their native membrane environment revealed additional domains. The secretin complex showed a double ring structure with a 14-15-fold
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symmetry in the central ring, and a 14-fold symmetry of the peripheral ring with 7 spikes protruding. PilQ forms the central ring of the complex. In secretin complexes of *N. meningitidis*, the spikes were absent and the peripheral ring was partly or completely lacking. However, they had a 19-fold symmetry when present. The *pilE* pilin subunit and *pilP* lipoprotein deletion mutants showed a change in the symmetry of the peripheral ring and loss of spikes. The *pilC* adhesin mutant and the minor pilin *pilW* mutant did not affect the structure. On the other hand, no complexes were observed in the pilot protein *NgonM_03101* mutant, consistent with previous biochemical data. These results show that secretin complexes contain previously unidentified large and flexible extra domains with a probable role in stabilization or assembly of type IV pili.

Outlook

The T4SS encoded within the GGI is unique and further studies on the transport of the relaxase and/or other effector proteins will be useful in understanding the mechanism of T4SSs. *In vitro* analysis of purified relaxase and its DNA binding behavior, studies on pilus polymerization and components of the T4SS involved in the above processes will help determine the mechanism of DNA secretion into the medium by the GGI.

In the future, identification of the extra subunits associated with secretin complex will be very important for a better understanding of the Tfp mechanism. In light of the current data, it seems unlikely that one of the known Tfp proteins is part of the complex. It would be interesting to determine whether the extra ring is specific for *N. gonorrhoeae* or is also found for the Tfp of other organisms. It is also intriguing to find a difference between the secretin complex of *N. gonorrhoeae* and *N. meningitidis* that share highly conserved regions of Tfp encoding sequences.
Summary for the unacquainted

Bacteria are single celled microorganisms. Each bacterium is enclosed by a membrane made of lipids which separates the inside of a cell from its outside. Gram-negative bacteria are surrounded by two membranes, the outer and the inner membrane. The inner membrane encloses the ‘cytoplasm’, whereas the space between the two membranes is called the ‘periplasm’ (Figure 1). Cells need to interact with the environment and hence there is a regulated movement of molecules across the cell envelope, crossing the inner membrane, the periplasm and the outer membrane. Some of these molecules are very large such as proteins and DNA, as termed macromolecules. Since it is important that no leakage across the membrane occurs during this transport, several machines have evolved that function in the transport of many different molecules. The machines that mediate the transport of macromolecules across the membrane are embedded in the inner and outer membranes and are called secretion systems. They are often very large structures made up of several building blocks. These building blocks are called proteins. Proteins are chains of amino acids that can fold together to form a molecule. A protein alone or together with other proteins can perform a specific function. Transport across the inner membrane is mainly performed by a machine that is called the Sec (= secretion) system. For the transport across the outer membrane, at least seven different secretion systems have been described. These systems have been numbered type I to type 7 secretion system. Within this thesis, members of two of these secretion systems are studied.

The first system is a Type IV Secretion System (T4SS). T4SSs are involved in the delivery of genetic material (DNA) or toxins to other cells. The receiving cells can be from the same or another species of bacteria or even from a higher organism like plant or human cells. Bacteria use T4SS to spread genetic information like antibiotic resistance and the proteins. Often these proteins are important in many diseases caused by bacteria. For example, the bacterium Helicobacter pylorus uses a T4SS to deliver virulence proteins to human cells to cause chronic gastritis and ulcers. T4SSs have been studied in several different organisms, and these studies have shown that the system can be divided into three different parts (Figure 1, left panel). The first part consists of the molecules that are transported. T4SS that transport DNA, encode a protein called
relaxase that binds to DNA at a specific position. Often with the help of other proteins, relaxase breaks one of the two strands of the DNA and connect to it. The complex of the relaxase bound to DNA is then transported to the other cell. The second part two consists of 2-3 proteins that work as motors. They provide energy to drive the transport from the inside to the outside of the cell. The third part encodes many proteins that come together and form a channel through which the substances are exported. This channel spans both the inner and the outer membrane. From this channel a long rod-like structure called pilus can be extended (and sometimes even retracted) to attach to other cells. The pilus is formed from many copies of pilin proteins.

The second system is a member of the Type II secretion systems. This system is used to transport pilin proteins across the outer membrane to the surface of the cell. The pilin proteins are assembled into a long protein chains that extend from the cells. These are called type IV pili (Tfp). Remarkably these type IV pili can be extended and retracted with high force. The pilin protein is first inserted into the inner membrane by the Sec system. The Tfp system, which consists of several proteins in both the inner and outer membrane, can then assemble the pilin protein into a pilus, and this pilus can be extended from or through the outer membrane. The extension and retraction of the pilin proteins is driven by motors bound to the cytosolic side of the Tfp system. The pilin proteins are secreted across the outer membrane through a large protein complex called the secretin (Figure 1, right panel). The Tfp are involved in functions like mobility, attachment to a surface and DNA import. They are essential for the pathogenicity of many bacteria. For example, the bacterium Neisseria gonorrhoeae uses Tfp to adhere to human cells and to colonize them. Our current knowledge about type IV secretion systems and the type IV pili assembly systems is described in Chapter 1. The aim of this thesis is the study of the two systems in the bacterium Neisseria gonorrhoeae. This obligate human pathogen causes the sexually transmitted disease called “gonorrhea” and is introduced in Chapter 1.

Different components of the T4SS of N. gonorrhoeae are studied in Chapters 2, 3 and 4. The T4SS, which is studied in this thesis, is encoded within a genetic island found in approximately 80 % of the clinical isolates of N. gonorrhoeae. All information to build proteins, which determines the characteristics of a cell, is encoded into the chromosomal DNA of a cell. A genetic island is a large additional piece of DNA, which is inserted
into the chromosome. In *Neisseria gonorrhoeae*, the genetic island is called the Gonococcal Genetic Island (GGI). The DNA of the GGI contains the information to create 60 proteins, 18 of these proteins seem to function in T4SS, 8 of them are probably involved in the processing of DNA, but the function of many of the others is still not known. The T4SS encoded within the GGI resembles the T4SS encoded with the F-plasmid. The F-plasmid is an extra-chromosomal circular piece of DNA that can be transferred between different cells via the T4SS. Unlike the system encoded on the F plasmid, the T4SS encoded within the GGI *N. gonorrhoeae* is not involved in transport of DNA to another cell, but the system exports the DNA to the outside of the cell into the environment. From the environment, the exported DNA can be taken up by neighboring gonococci. This occurs via the Type IV pili which can bind the secreted DNA and import the DNA across the outer membrane. A second system is involved in importing DNA across the inner membrane. The systems of *N. gonorrhoeae* to take up DNA are very efficient.

Thus by secretion of DNA via the T4SS and uptake of DNA via the Tfp system, DNA (or new information) is exchanged between the cells. The exchange of DNA can help the cells to adapt to a changing environment and thus to survive better. In this thesis, 4 proteins, TraA, TrbI, TraI, and SsbB encoded within the GGI and the secretin complex formed by the PilQ protein of Tfp system are studied.

In Chapter 2, the TraA and TrbI proteins are studied. TraA is the protein from which the pilus of the T4SS is formed. It is produced in the cytoplasm and then inserted into the inner membrane where it undergoes several processing steps, which finally lead to the circularization of TraA. Once processed, it is assembled into the rod-like pilus. Processing and assembly are two different and independent processes. The processing of the pilus protein is studied here in detail in the bacterium *Escherichia coli* that is commonly used in the laboratory. It is shown that the protein TraA is first processed on one side by a protein called LepB. LepB is involved in the removal of a small part at the beginning of the TraA protein called the signal sequence. This signal sequence is used to target the protein to the inner membrane via the sec-system. This processing step occurs for many proteins that are secreted. In a next step, the TraA protein is bound by TrbI which first cleaves the last three amino acids off, and then forms a bond with the same end of TraA. In the final step, the bond between TraA and TrbI is broken, and a
link is made between the beginning and the end regions of the TraA protein, thus circularizing it. By mutational studies, different regions of TraA and TrbI proteins necessary for the mechanism were identified.

**Figure 1:** Schematic of the type IV secretion system (left panel) and the type IV pili system (right panel). OM and IM denotes outer and inner membrane respectively.

The relaxase encoded within the GGI is called TraI, and a study of this protein is described in Chapter 3. TraI is a member of a novel family of relaxases, which exhibits features of relaxases but also has features that are absent in previously characterized relaxase families. TraI starts with a hydrophobic region which is also found in other members of this novel family. It was demonstrated that this hydrophobic region does not function as a signal sequence but is important for binding of TraI to the membrane. Interestingly, when amino acids in this hydrophobic region are changed, the relaxase is no longer exported. The hydrophobic region is followed by a region that is also found in
a protein class called HD phosphohydrolases. These proteins are named after the two conserved amino acids; histidine (H) and aspartate (D) which are characteristic for this family. Indeed this aspartate in TraI was required for DNA secretion. The HD domain overlaps with a domain that has some similarity with the relaxase domain of other relaxases. Indeed a conserved tyrosine amino acid found in both domains is important for the processing of DNA. It was further found that only one strand of the DNA is secreted which is protected from degradation from one side, probably by the bound relaxase. Disruption of a DNA sequence located near the DNA sequence that encodes for TraI protein, eliminated DNA secretion. When this sequence was introduced elsewhere in the chromosome, DNA secretion was restored. This suggested that the secretion of the DNA starts from this specific sequence. In the appendix of Chapter 3, attempts are described to demonstrate the presence of TraI in the medium.

In Chapter 4, SsbB, a protein that binds and stabilizes single strands of DNA is studied. SsbB is encoded within the GGI, and is expressed, but no evidence could be found that SsbB is transported along with the ssDNA. A detailed study of the DNA binding properties of SsbB was performed. Using different techniques it was demonstrated that SsbB binds ssDNA in a manner not observed previously.

In Chapter 5, the secretin complex of the Tfp system of *N. gonorrhoeae* is studied. Outer membranes of *Neisseria meningitides* and *N. gonorrhoeae* were isolated and analyzed by electron microscopy. Within these membranes, an elaborate structure was observed after alignment of many different electron microscopy pictures. The structure is composed of an inner ring enclosing a channel, an outer ring and extending outer spikes. The inner ring is made up of a protein called PilQ, of which several copies form a symmetrical ring. Such a ring like structure was also observed in previous studies. The outer ring and spikes have not been observed for other secretins and are made of unknown proteins. These extra features were most likely lost during the purification of other secretin complexes, but are now observed as the secretin is studied in its native membranous environment. In order to identify these proteins, several components of the Tfp system were removed and analyzed. This analysis revealed that the loss of certain components resulted in a change in the symmetry of the outer ring and a loss of the outer spikes. The study for the identification of these proteins is still ongoing.