
# Molecular Functionality of the Uropathogenic RTX Toxin Haemolysin A from *Escherichia coli*

Inaugural-Dissertation

zur Erlangung des Doktorgerades der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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Düsseldorf, März 2014

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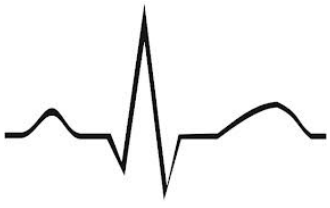
Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Lutz Schmitt
Korreferent: Prof. Dr. med. Walter Däubener

Tag der mündlichen Prüfung: 18.03.2014

*“Wenn es keine Höhen und Tiefen mehr in Deinem Leben gibt,*
*bist Du wahrscheinlich schon tot”*



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# Molecular Functionality of the Uropathogenic RTX Toxin Haemolysin A from *Escherichia coli*

# 1. SUMMARY

Uropathogenic *E. coli* (UPEC) are the leading cause of urinary tract infections (UTI) in our modern society. Especially women are susceptible for UTI that can cause kidney damage or death if treated incorrectly. Due to increasing antibiotic resistance of UPEC strains, it is becoming more important to understand UTI on the molecular level to ensure future treatment.

One major virulence factor of UPEC is the toxin haemolysin A (HlyA), belonging to the repeats in toxin (RTX) protein family. HlyA is secreted out of the bacterial cell by its corresponding Type 1 secretion system, consisting of the ABC transporter HlyB, the membrane fusion protein HlyD and the outer membrane protein TolC. Two events are necessary for HlyA to mature from a non-toxic precursor (pro-HlyA) to an active toxin. These are (i) a fatty acylation at two lysines, carried out by the acyltransferase HlyC together with the acyl carrier protein (ACP) and (ii) the binding of extracellular calcium ions to the RTX repeats leading to protein folding of the previously unfolded protein. Even though these steps are crucial for HlyA activity they are poorly understood.

This doctoral research focusses on three major points:
(I) *Understanding of HlyA folding upon calcium ion binding*. Using intrinsic tryptophan fluorescence, different HlyA mutants and ions, the folding of HlyA was analyzed. It could be shown that HlyA folding is a two-step mechanism with a clear folding intermediate and therefore clearly distinct from the one-step unfolding pathway. Here the C-terminal protein part plays a crucial role, not only in $Ca^{2+}$ binding by the RTX repeats but for the overall, correct folding of the remaining protein.
(II) *Establishment of an in vitro acylation assay*. For future analysis of HlyA activation by HlyC an *in vitro assay* was established using the previously purified proteins pro-HlyA, HlyC and ACP. The blood based assay is time efficient and easy to perform, allowing a quick scanning of future mutants of the involved proteins to identify important amino acids and also possible receptors for host cell interaction.
(III) *Crystallography of HlyA*. To determine the 3D structure of HlyA and therefore gain information on important protein domains and amino acids, HlyA was crystallized and two promising crystallization conditions were identified.

# 2. ZUSAMMENFASSUNG

Uropathogene *E. coli* (UPEC) sind die Hauptursache für Harnwegsinfektionen (HWI) in unserer Gesellschaft. Besonders Frauen sind prädestiniert für HWI, welche bei falscher Behandlung zu Schädigungen der Nieren oder sogar zum Tod führen können. Aufgrund der stetig steigenden Zahl resistenter UPEC Stämme wird ein molekulares Verständnis von HWI immer wichtiger, um auch in Zukunft eine effektive Behandlung zu gewährleisten.

Einer der Hauptvirulenzfaktoren von UPEC ist das Toxin Haemolysin A (HlyA), welches zur Familie der *repeats in toxin* (RTX) Proteine gehört. Die Sekretion von HlyA aus der bakteriellen Zelle erfolgt dabei über das dazugehörige Typ 1 Sekretionssystem, bestehend aus dem ABC Transporter HlyB, dem Membranfusionsprotein HlyD und dem äußeren Membranprotein TolC. Zwei Ereignisse sind notwendig, um das nicht toxische Vorläuferprotein pro-HlyA in das aktive Toxin zu überführen. Diese sind (i) eine Fettsäureacylierung an zwei bestimmten Lysinen, ausgeführt durch die Acyltransferase HlyC in Zusammenarbeit mit dem Acylträgerprotein (ACP) und (ii) die Bindung von Calcium Ionen an das RTX Motiv, welches zur Faltung des zuvor ungefaltenen Proteins führt. Obwohl diese Schritte essentiell für die Aktivität von HlyA sind, sind sie nur schlecht verstanden.

Diese Doktorarbeit fokussiert sich auf drei folgenden Hauptpunkte:

(I) *Verständnis der HlyA Faltung nach Calcium-Ionen Bindung*. Unter Verwendung von intrinsischer Tryptophan Fluoreszenz, sowie verschiedenen HlyA Mutanten und Ionen, wurde die Faltung von HlyA analysiert. Es konnte gezeigt werden, dass die Faltung von HlyA ein zweistufiger Prozess ist, der ein stabiles Faltungsintermediat beinhaltet und sich somit deutlich von der einstufigen Entfaltung unterscheidet. Hierbei spielt der C-terminale Proteinbereich eine essentielle Rolle, nicht nur durch $Ca^{2+}$ Bindung durch das RTX Motiv, sondern für die übergreifende, korrekte Faltung des verbleibenden Proteins.

(II) *Etablierung eines in vitro Acylierungsassays*. Für zukünftige Analysen der HlyC vermittelten HlyA Aktivierung wurde ein *in vitro* Assay mit den zuvor gereinigten Proteinen pro-HlyA, HlyC und ACP etabliert. Der Blut basierte Assay ist sowohl zeiteffizient als auch leicht durchführbar und ermöglicht daher die rasche Analyse von zukünftigen Mutanten der beteiligten Proteine, um wichtige Aminosäuren oder mögliche Zielzellrezeptoren zu identifizieren.

(III) *Kristallografie von HlyA*. Um die 3D Struktur von HlyA zu entschlüsseln und somit Informationen über wichtige Proteindomänen und Aminosäuren zu erlangen, wurde HlyA kristallisiert. Hierbei konnten zwei vielversprechende Kristallisations-bedingungen identifiziert werden.

# 3. INTRODUCTION

## 3.1 Urinary tract infections by uropathogenic *Escherichia coli*

One of the most common infectious diseases in our modern society are urinary tract infections (UTI) predominantly caused by Uropathogenic *E. coli* (UPEC) strains [1-3]. More than 130-175 million females per year suffer from UTI and it is estimated that over 60% have at least one UTI during their lifetime [2, 4, 5]. Men are also affected by UTI, however, to a significantly lower extent [2]. Not only are the expenditures for UTI treatment high, e.g. 2.5 - 3 billion US $ per year for the United States alone [6-8], but an insufficient treatment or reoccurring infections can also cause scarring of the kidneys, in the last resort leading to kidney failure and death [9-11].

The high infectiousness for UPEC and the severeness of untreated infections is explained by a multitude of virulence factors these bacteria possess, e.g. fimbriae and adhesins, siderophores and different toxins like the cytotoxic necrose factor type 1 (CNF1) and haemolysin A (HlyA) [12, 13]. After initial contamination of the periurethral area the bacteria infect the lower urinary tract (urethra, bladder) with the help of the adhesins and fimbriae before ascending to the upper urinary tract (ureters, kidneys) were host cell damage is caused by the various toxins [10, 14]. A model for UPEC infection of the urinary tract is given in Figure 1.

The common, successful treatment for UTI is the prescription of antibiotics, however, in the recent years an increase in multidrug resistant UPEC strains was observed. New studies suggest that the use of antibiotics in the food industry enhances this development leading to foodborne UTI [15]. The resistances range from tetracycline, chloramphenicol and streptomycin to β-lactams and quinolones, rendering several antibiotics insufficient or ineffective [16-18]. Therefore a deeper understanding of the pathology of UPEC infections is necessary to develop new therapeutic agents.

One step therefore is the understanding of the molecular function of the HlyA toxin, which is the main focus of this thesis.

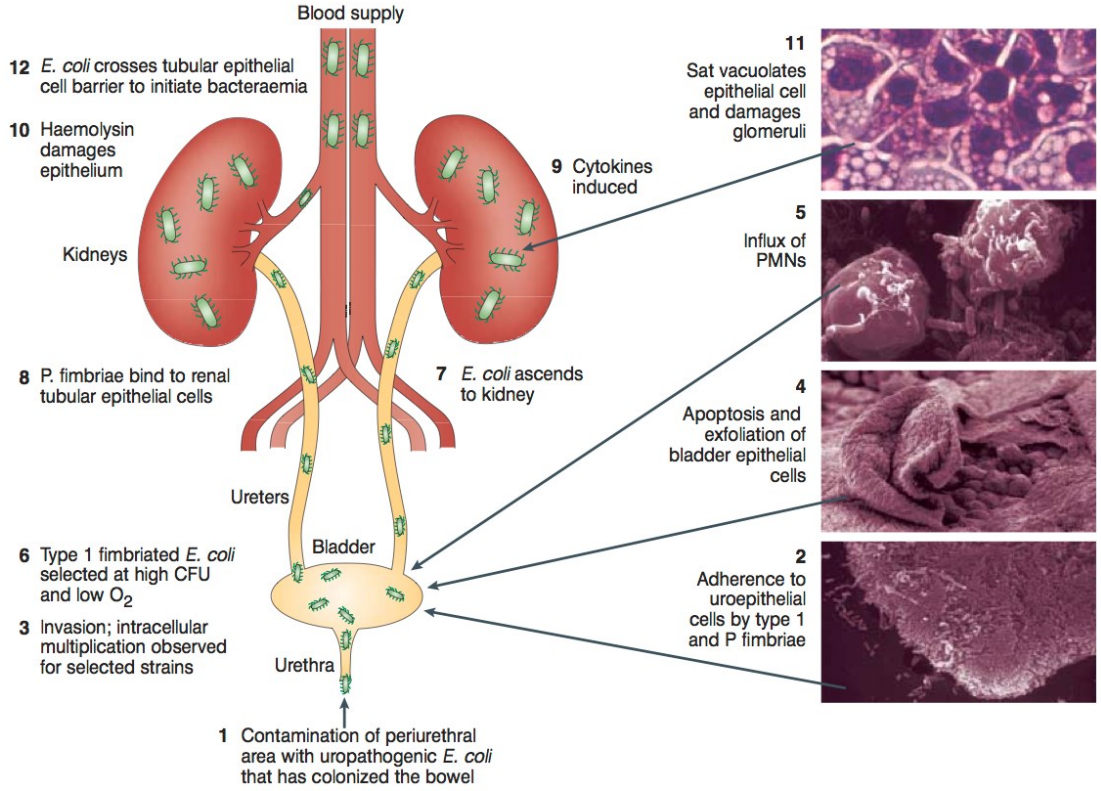


***Figure 1. Hypothetic model of UPEC infection of the urinary tract***
Urinary tract infections are initialized by contamination of the periurethral area followed by bacterial migration and multiplication of the cells in the lower urinary tract consisting of the urethra and bladder. The ability to sustain in this territory is conveyed by specific UPEC virulence factors, here especially type 1 fimbriae that allow adherence to the uroepithelial cells via their uroplakkin receptors [19]. If untreated the bacteria begin to ascent via the ureters to the kidneys, both building the upper urinary tract. This time cell attachment is achieved by P fimbriae to digalactoside receptors [20, 21]. Finally the renal epithelium of the kidneys is probably damaged by the UPEC toxin α-haemolysin (HlyA) leading to an acute inflammatory host cell immune response [20, 22]. Furthermore it is also possible for the UPEC bacteria to access the bloodstream by breaching the endothelial cell barrier leading to sepsis, subsequently organ failure and finally host death [10]. CFU: colony-forming units; PMNs: polymorphonuclear leukocytes. Figure taken from Kaper *et al* [10].

## 3.2 The toxin HlyA

### 3.2.1 HlyA - a member of the repeats in toxin protein family

The HlyA toxin is a 110 kDa protein, secreted by specific UPEC strains [23, 24]. It is able to lyse a variety of different cell types e.g. erythrocytes, where lysis can straightforwardly be monitored by hemoglobin release [25-27]. HlyA is a member of the large Repeats in Toxins (RTX) protein family common in Gram-negative bacteria [28]. Members of this family share several unique features. The most important, and eponymous for the RTX family, is the presence of the specific nonapeptide repeat sequence: GGxGxDxUx. Here U represents a large hydrophobic amino acid residue while x can be any amino acid [29]. These RTX repeats are responsible for calcium ion binding and, upon binding of the ion, form a β-roll like structure as evidenced by 3D crystal structures [29-32]. A schematic model of the HlyA toxin is given in Figure 2.

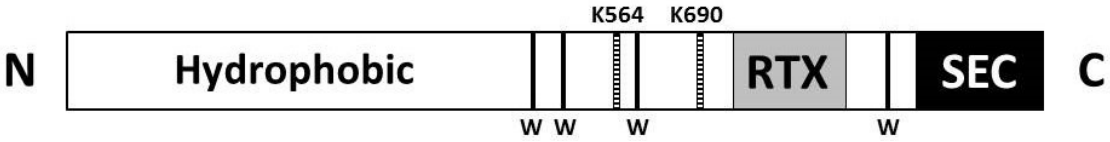


***Figure 2: Schematic model of the toxin HlyA***
HlyA has a molecular size of ~ 110 kDa and several important domains have been identified over the years. These are (i) the C-terminal secretion signal important for secretion by a T1SS (black), (ii) an RTX domain containing six of the nonapeptide repeat sequences common for RTX proteins and responsible for calcium ion binding and protein folding (gray) as well as (iii) an N-terminal hydrophobic domain necessary for irreversible host cell insertion. Furthermore the two important lysines K564 and K690 are shown that get fatty acylated by HlyC, a crucial step for HlyA activity. Last the four tryptophans (W) of HlyA are shown that can be used to study protein folding by fluorescence spectroscopy.

Other features RTX proteins share are a non-cleaved secretion signal at the C-terminus that allows secretion by a type 1 secretion system (T1SS), as well as a reduced number or complete absence of cysteins [33, 34]. As other examples for RTX proteins the toxin LktA (*Mannheimia haemolytica*), the alkaline protease AP (*Pseudomonas aeruginosa*), the lipase LipA (*Pseudomonas brassicacearum*) and the adenylate cyclase CyaA (*Bordetella pertussis*) shall be mentioned here [35-37].

The binding of $Ca^{2+}$ to the RTX repeats is essential for HlyA as it induces protein folding to its native conformation upon secretion of the unfolded toxin [38]. This process is regulated by the different calcium levels between the bacterial cytosol and the extracellular space. Inside the *E. coli* cell the $Ca^{2+}$ level is low, only in the nM range, and insufficient for binding to HlyA whereas outside mM concentrations of calcium ions are present and induce binding of the ion to the protein [29, 39-43].

Apart from the extracellular calcium ion binding HlyA needs a post-translational modification to obtain its lytic conformation, more specific a fatty acylation.

### 3.2.2 *In vivo* activation of HlyA by HlyC dependent acylation

HlyA is initially produced as a non-toxic precursor protein called pro-HlyA [44, 45]. To obtain its lytic conformation an intracellular, post-translational modification is necessary (Figure 3). This modification is a fatty acylation carried out by the acyltransferase HlyC (21 kDa) in conjunction with the acyl carrier protein (ACP, ~ 9 kDa) [46-48]. During the acylation fatty acids of different length, most commonly C14, C15 and C17, are attached to the ε-amino group of the two specific lysines K564 and K690. The probability of acylation is thereby decreasing with increasing carbon length [44, 49, 50] .

The HlyC mediated acylation is unique as internal lysines are acylated and amide-linked a process only known for acyltransferases of RTX proteins [49, 51]. However little is known about how HlyC recognizes specifically K564 and K690 even though more lysines are present in HlyA. Both acylation sites can interact independently from each other and share no sequence homologies indicating a different kind of HlyA recognition [52]. The reaction mechanism per se is also controversially discussed. While Stanley *et al* [53] proposed a complex formation of pro-HlyA, HlyC and ACP, Worsham *et al* [54, 55] suggest a ping-pong mechanism where the acyl group is firstly transferred to HlyC, generating an acyl-HlyC intermediate, before binding of pro-HlyA and acyl transfer.

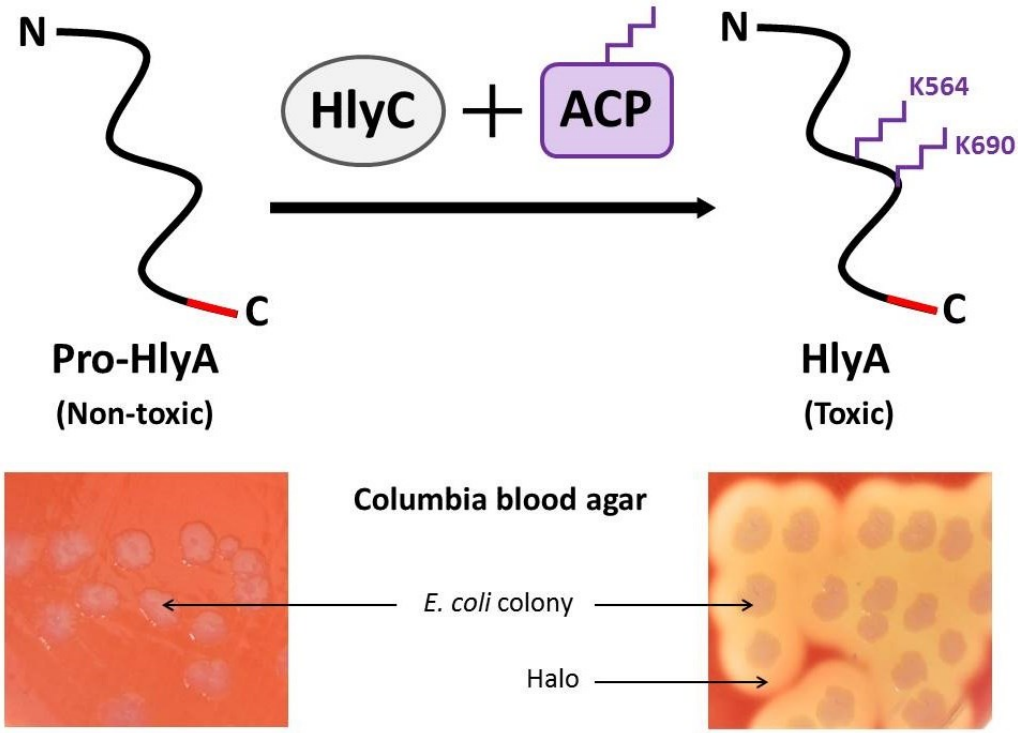


***Figure 3: Acylation of HlyA and influence on haemolytic activity***
HlyA is produced as a non-toxic precursor pro-HlyA (black; red: secretion signal). To obtain its toxicity a posttranslational fatty acylation is necessary. This acylation is carried out by the acyltransferase HlyC (gray) in conjunction with ACP (light purple). During the acylation the fatty acids (dark purple) are transferred to two specific lysines of pro-HlyA, K564 and K690 [23, 46-48]. Only with the attached acyl chains HlyA obtains its toxic activity as shown on Columbia blood agar plates (red) containing 5% sheep blood. While the *E. coli* colonies secreting pro-HlyA show no haemolysis (lower left side) those cells secreting mature HlyA show haemolysis, clearly visible due to the halo formation around the cells (lower right side).

To date no structural information of HlyC or other acyltransferases of this family are available and only a few amino acids have been identified that seem to play key roles in HlyC function. One of these is H23 and is apparently involved in formation and reactivity of a possible acyl-HlyC intermediate, while V127, G128 and K129 seem to be responsible for recognizing the acylation sites [55-57].

However, the acylation has only an influence on toxin activity but not on protein secretion as HlyA, like pro-HlyA, is also secreted and can bind calcium ions and erythrocytes equally well [58-61].

### 3.2.3 Interaction of HlyA with target membranes

RTX toxins are divided into the groups of haemolysins and leukotoxins, where the former have a broad host cell spectrum and the latter are specific for leukocytes. HlyA belongs to the first group and is able to lyse a variety of different cells like leukocytes (monocytes, lymphocytes, neutrophils), erythrocytes, fibroblasts as well as epithelial and endothelial cells [25, 27, 62-68]. The exact mechanism of HlyA-membrane interaction and finally cell lysis is not unequivocally resolved and different pathways have been proposed. Those will be briefly discussed below and can be found in more detail in chapter 5.1.

#### 3.2.3.1 Receptor independent HlyA pore formation

Originally HlyA was thought to cause cell lysis by simple pore formation, associated with cell swelling due to calcium ion influx, finally leading to cell death [69, 70]. This theory was further supported by the broad host spectrum and lysis of membranes lacking a specific receptor [71-73]. The pore formation is divided into non-lytic, reversible cell adsorption and lytic, non-reversible membrane insertion [60, 74-76]. While $Ca^{2+}$ ions and the acylations are essential for the latter, their role for the former are still controversially discussed [45, 58, 69, 75, 77-79].

#### 3.2.3.2 Receptor dependent HlyA pore formation

In contrast to a receptor free, a receptor dependent HlyA reaction mechanism was proposed and was strengthened by the recent discovery that the adenylate cyclase CyaA from *B. pertussis* specifically recognized the N-linked oligosaccharides of its corresponding $\beta_2$ integrin receptor. This was followed by endocytosis of the bacteria thus limiting the host immune response [80, 81]. Even though an internalization of HlyA is impossible, as its $\beta_2$ integrin receptor LFA-1 is not endocytosed, a receptor mediated HlyA binding is possible and would in turn destroy the host immune system by activating distinct signaling pathways upon calcium ion influx from the pore [81, 82]. It is noteworthy that the LFA-1 receptors are usually responsible for interactions with ICAM family members that mediate binding of leukocytes to endothelial cells or are involved in T cell functions like proliferation, adhesion and B cell interaction [81, 83]. As alternative to the $\beta_2$ receptors, who are absent in erythrocytes, glycophorin could provide the necessary glycosylation sites. This could also explain different

efficiencies of HlyA on erythrocytes from varying species as the glycosylation patterns may differ [80, 84-86].

3.2.3.3 HlyA-host interaction via outer membrane vesicles

A third HlyA-host interaction could involve *E. coli* outer membrane vesicles (OMV). For the cytolysin ClyA such a mechanism was reported and was essential for ClyA activity, as the redox status of the protein was changed [87]. A similar mechanism can also be envisaged for HlyA and is supported by the common presence of OMVs in Gram-negative bacteria [88] and the discovery of active HlyA in such vesicles [89]. The HlyA uptake into the host cell is probably achieved by endocytosis although not proven yet [90, 91].

3.2.3.4 HlyA features that may influence pore formation

Apart from the mode of interaction there are also different opinions regarding other HlyA aspects that may influence pore formation. For instance it is debated if HlyA forms higher oligomers to build the pore or if a single HlyA molecule is already sufficient enough [74, 92-96]. This process may be further influenced by the toxin amount favoring receptor recognition at lower and pore formation at higher HlyA concentration [51, 97]. In addition to the acylation sites both protein termini play key roles during membrane insertion. While the β-stranded C-terminus seems to be responsible for initial adsorption by glycophorin binding, the α-helical N-terminus probably causes membrane insertion [23, 98, 99].

3.2.3.5 The effects of calcium ion influx on host cells

Once the HlyA pore is built into the host cell membrane, receptor mediated or not, it causes an uncontrolled calcium ion influx. This disturbs the tightly regulated intracellular calcium ion concentration and can influence a multitude of cellular processes like changes in the cytoskeletal rearrangement, IL-1β release or ATP decomposition [62, 68, 100]. Furthermore increased $Ca^{2+}$ levels can stimulate the arachodonate metabolism and P2X cation channels, both leading to apoptosis upon prolonged stimulation [62, 101, 102]. Finally it was shown that calcium ion influx also increases endothelial permeability [62, 103] allowing easier access of the bacteria to the blood stream (see Figure 1).

## 3.3. Secretion of HlyA

Like other effector proteins HlyA is secreted out of the UPEC cell to fulfill its biological function. Gram-negative bacteria, in contrast to Gram-positive, face the problem that proteins destined for the outer membrane or the extracellular space need to cross both membranes before they reach their destination. To enable import or export of different substances across the membranes, the bacteria developed specialized transport mechanisms that facilitate transport. Those membrane protein mediated transports either follow a gradient (passive transport e.g. ion channels) or enable transport against it (active transporters) [104]. The active transporters are furthermore subdivided into primary transporters that generate the necessary energy by e.g. ATP hydrolysis and secondary transporters that couple transports to existing gradients [105, 106].

To allow the transport of cytosolic synthesized proteins across the inner as well as the outer membrane a series of specialized primary transporters evolved, the Type 1 to 6 secretion systems [107, 108]. Those are shown in Figure 4 and will be briefly discussed below before a more detailed analysis of the T1SS of HlyA and other RTX proteins is given.

### 3.3.1 Secretion systems of Gram-negative bacteria

To date six different Type secretion systems are known in Gram-negative bacteria that transport a variety of different molecules. The secretion systems can be subdivided into conjugate-like systems (Type 1, 3 and 4) and autotransporters (Type 2, 4 and 5) where the former support a one-step secretion while the latter function as two-step mechanisms. Interestingly T4SS can function in both ways while the exact mechanism of the T6SS is still researched [107]. A schematic model of the Type 1 to 6 secretion systems is given in Figure 4.

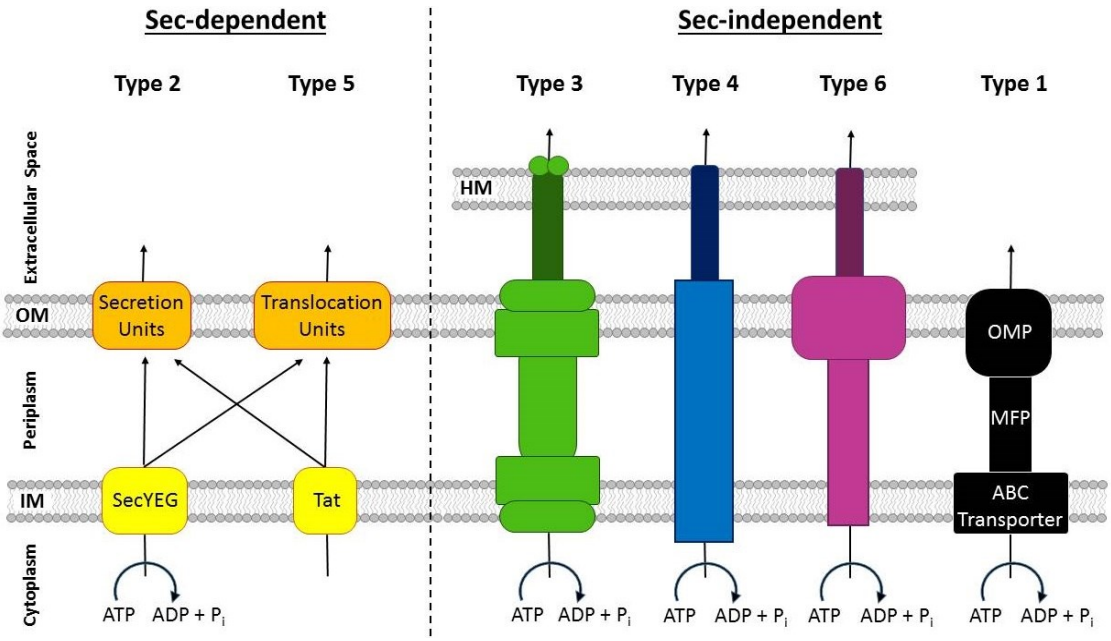


***Figure 4: Type secretion systems of Gram-negative bacteria***
Simplified, schematic models of the Type 1 to 6 secretion systems (TSS) of Gram-negative bacteria divided into *sec* and *tat* dependent (Type 2 and 5) and independent (Type 1, 3, 4 and 6) pathways. Proteins secreted by the T2SS and T5SS are first transported to the periplasm prior to a second transport into the extracellular space (yellow and orange). In contrast, the secretion by the T1SS is a single transport step directly to the extracellular milieu (black). The T3SS (green), T4SS (blue) and T6SS (purple) can furthermore translocate their substrates across the host membrane. Energy for transport is either generated by ATP hydrolysis (Type 1 to 4 and 6) or by the proton motive force (Type 5). IM: inner membrane, OM: outer membrane, HM: host membrane, OMP: outer membrane protein, MFP: membrane fusion protein, ABC: ATP-binding cassette. Adapted from Tseng *et al* [109].

The most common way of protein secretion is the initial transport of proteins into the periplasm by a *sec* or, to a lower extent, *tat*-dependent pathway prior to an additional, if necessary, second step across the outer membrane to the exterior [110, 111]. Here, the *sec* system is responsible for the transport of unfolded proteins into the periplasm while the *tat* system facilitates secretion of (partially) folded proteins [112-114]. The second transportation step into the extracellular space or the outer membrane is achieved by the T2SS or the T5SS. The energy sources for transport by the *sec* and *tat* pathway are ATP hydrolysis and the proton motif force, respectively, although the second is not completely confirmed [115, 116].

In contrast to the other secretion systems the T3SS, T4SS and T6SS do not only transport the proteins across two membranes but can also cross a potential host cell membrane. The systems differ in the number of proteins that build up the translocator and the substances transported but the secretion takes place in one step with ATP hydrolysis as energy source [117-119]. While the T3SS injects proteins into the host cytosol, therefore also called injectisome, the T4SS can additionally transport nucleic acids. It is furthermore not limited to bacterial cell but can also access plant and animal cells [118, 119]. The exact mechanism of the T6SS is still under investigation but it is speculated that it functions in a bacteriophage like fashion [120, 121].

Unlike the other systems that are build-up of numerous proteins the T1SS only consists of three proteins that form a ternary secretion complex. These three proteins are an ATP Binding Cassette (ABC) transporter that supplies energy by ATP hydrolyses, a membrane fusion protein (MFP) and an outer membrane protein (OMP) [122]. T1SSs can transport a wide range of proteins ranging from a few to 900 kDa like LapA from *Pseudomonas fluorescence* [123, 124]. Substrate transportation ensues in one step and the secretion signal of the substrates is not cleaved during or after secretion [33, 125]. The T1SSs are probably the best characterized secretion systems and, as they are also responsible for the secretion of RTX proteins, shall therefore be discussed in more detail on the example of the HlyA T1SS.

### 3.3.2 The HlyA Type 1 secretion system

The secretion of HlyA occurs via a one-step mechanism without a periplasmic intermediate carried out by a T1SS consisting of the proteins HlyB (ABC transporter), HlyD (MFP) and TolC (OMP) [122, 126-128]. All proteins necessary for HlyA expression, modification and secretion, with the exception of TolC, are encoded in the tightly regulated *hly* operon [129]. In the next sections each of the proteins that build-up the HlyA T1SS, as well as the assembly of the complex will be briefly discussed. For a detailed overview please refer to chapter 5.1.

#### 3.3.2.1 TolC - the outer membrane protein

TolC is the outer membrane protein of the HlyA T1SS and the only protein not encoded in the *hly* operon as it is a multifunctional protein [41]. The structure of TolC was solved in 2000 by Koronakis and co-workers and revealed a homotrimeric, 140 Å long protein [41, 130]. The outer membrane spanning domain is build-up like a β-barrel whereas the periplasmic domain consists of α-helices (Figure 5A) [41, 131, 132]. The width of TolC is variable and is controlled by an iris-like movement that can alternate between 3.5 Å (closed conformation) and 16 Å (open conformation) upon interaction with the remaining transport complex (Figure 5B) [41, 132-137]. However, this is still too small to allow the secretion of folded proteins. Therefore the secretion of HlyA is only possible in an unfolded conformation and protein folding is initialized by extracellular calcium ion binding [133, 134, 138].

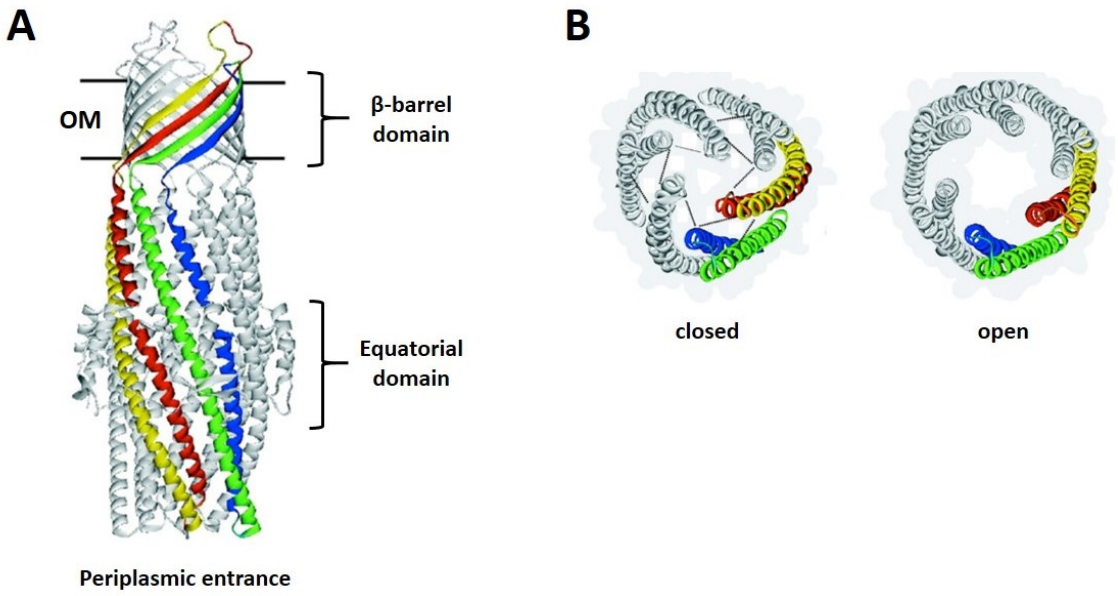


***Figure 5: TolC structure and iris movement***
A) Side view of the structure of the trimeric TolC protein. The monomer is highlighted in color and important domain regions are labelled. B) Symbolization of iris movement that occurs upon interaction with the remaining T1SS viewed from the periplasm. The closed conformation is shown on the left side and the open conformation on the right. OM: outer membrane. Figure modified from Andersen *et al* [133].

### 3.3.2.2 HlyD – the membrane fusion protein

The MFP of the HlyA T1SS is HlyD [139]. Localized in the inner membrane and interacts with TolC as well as HlyB to form the continuous secretion channel [140-142]. Up to date no structural information of HlyD is available and therefore no experimental proof of its oligomeric state is known, however, trimers as well as hexamers have been proposed [132, 143]. A hexameric form is favored as it would solve the stoichiometric difference between the HlyB dimer and the TolC trimer, with the hexamer being the least common multiple of both interacting proteins. Recently such a hexamer formation could be shown e.g. for the analogous proteins MexA and ArcA [144, 145].

### 3.3.2.3 HlyB – the ABC transporter

The ABC transporter of the HlyA T1SS is HlyB and provides the energy for transport by ATP hydrolysis [146]. HlyB, like all other ABC transporters, consists of two transmembrane domains (TMD) and two nucleotide binding domains (NBD) and is a half-size transporter. This means that the four domains are, in contrast to full size transporters, located on two different polypeptides (one TMD and one NBD each) that need to dimerize in a head to tail conformation to become active [147-151]. While the TMDs show little to no sequence homology, in agreement with the variety of substrates, the NBDs share several highly conserved motifs like Walker A and B as well as C-, Q-, D-, H-, and Pro-loops [152-154]. A HlyB-NBD-dimer crystal structure is shown in Figure 6.

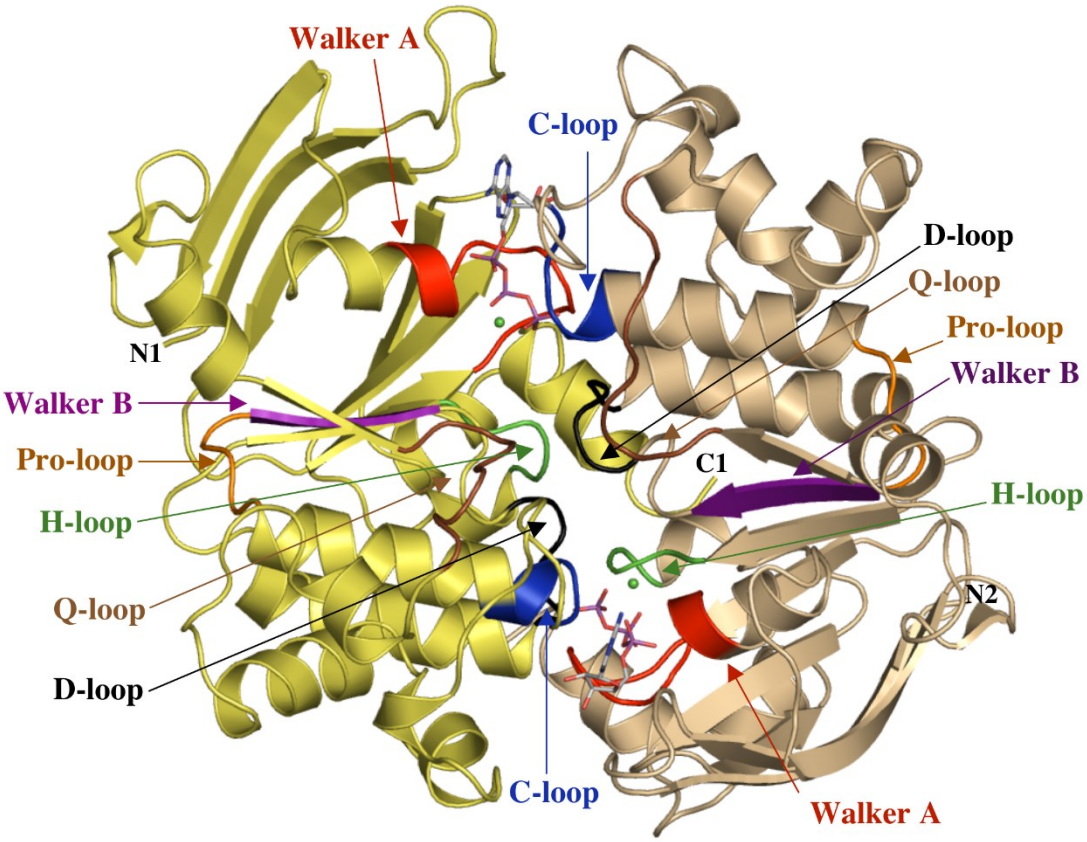


***Figure 6: HlyB-NBD dimer with conserved motifs***
Shown is the crystal structure of a HlyB-NBD dimer in a head to tail conformation. The conserved motifs of the NBDs, Walker A and B as well as the loops are highlighted. Shown in green are ATP (sticks) and $Mg^{2+}$ (sphere). Figure taken from Zaitseva *et al* [155].

Apart from the TMDs and NBDs HlyB possesses an additional N-terminal domain that plays a crucial role prior to HlyA secretion. As mentioned earlier the restricted size of TolC only allows secretion of unfolded HlyA. Taken together with the fact that the C-terminus with the secretion signal is translated last, this raises the question how the unfolded HlyA is kept stable in the cytosol without protease digestion. Recently, Lecher *et al* [156] could show that the N-terminal HlyB domain is a dead C39 peptidase also referred to as C39-like domain (CLD). This CLD tethers unfolded HlyA and keeps it in a conformation inaccessible for proteases and suitable for secretion [156]. For further information please refer to chapter 5.1.

#### 3.3.2.4 Assembly of the secretion complex and HlyA transport

Although it is not fully understood how the components of the HlyA T1SS assemble a general model for an ordered reaction evolved over the years [132, 157]. Accordingly the first step in the translocator formation is the recognition of HlyA by HlyB or a temporary HlyB/HlyD complex. Secondly, upon HlyA binding, a change in HlyD occurs that facilitates binding of TolC [132, 157-159]. This general model is based on diverse mutational studies of all participating proteins and is discussed in detail in Paper I [160]. A model for the T1SS assembly and HlyA secretion is given in Figure 7.

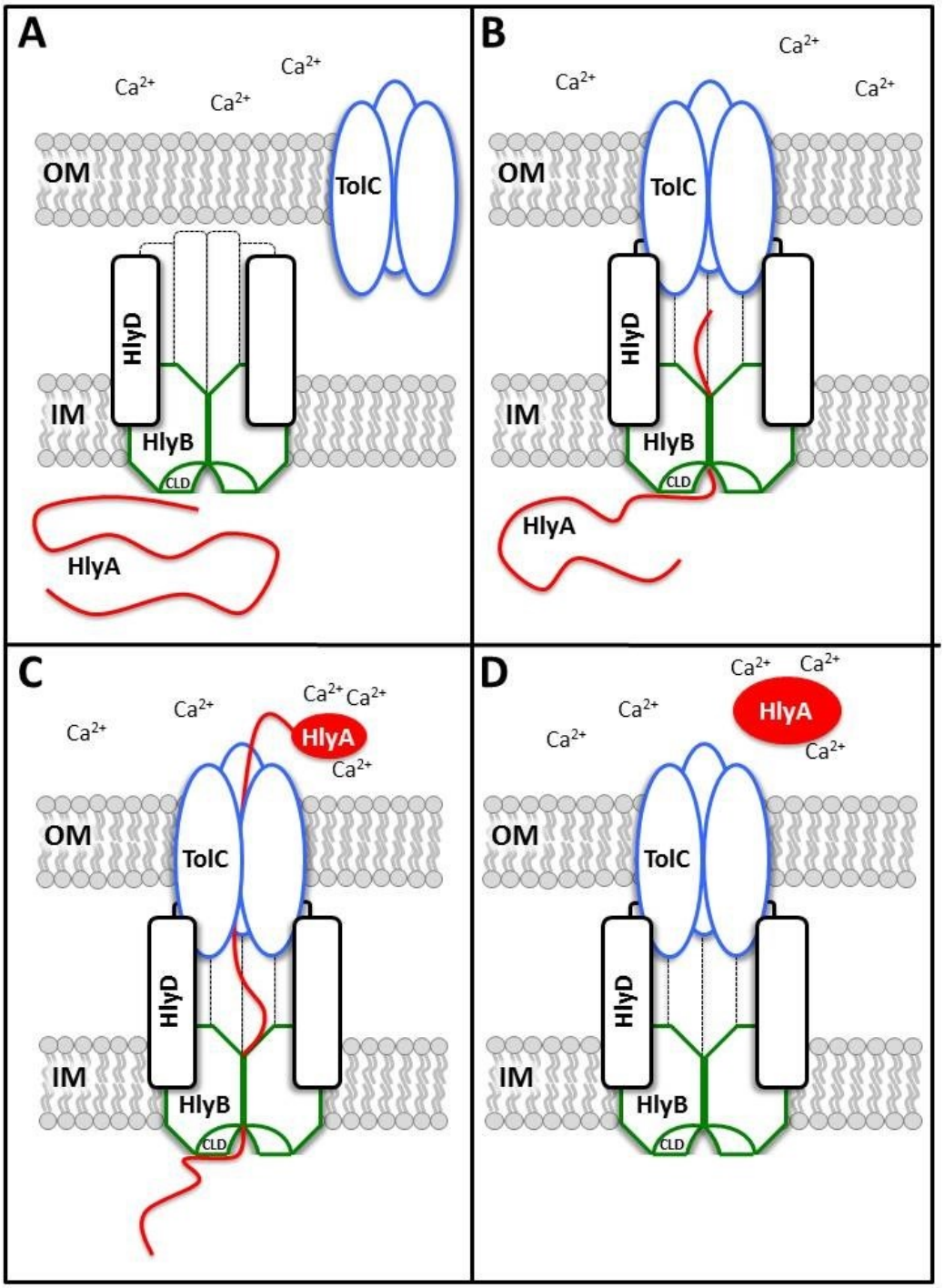


***Figure 7: Model of HlyA secretion and assembly of the Type 1 secretion complex***
A) As initial step in the HlyA (red) secretion the toxin recognizes the ABC transporter HlyB (green) or a transient complex of HlyB and the membrane fusion protein HlyD (black). The CLD of HlyB thereby binds the HlyA and keeps it in a formation suitable for secretion. It furthermore protects the unfolded HlyA from protease digestion. B) Upon the binding of HlyA a chemical or conformational change occurs in HlyD and the outer membrane protein TolC (blue) is recruited to complete the secretion channel. The C-terminal part of HlyA, containing the secretion signal, is loaded into the secretion complex. C) As soon as the C-terminus reaches the extracellular space $Ca^{2+}$ ions begin to bind to the RTX repeats thus initializing the folding of this domain and subsequently the whole protein. D) After successful release of HlyA from the transporter the T1SS dissembles and a new secretion cycle can start. OM: outer membrane, IM: inner membrane, CLD: C39-like domain.

# 4. AIMS

## 4.1 Understanding of HlyA folding upon protein secretion

HlyA is an RTX toxin secreted by UPEC strains that are responsible for urinary tract infections [1-3]. The 110 kDa HlyA is able to lyse a variety of different host cells thus altering the immune response and enabling the bacteria to access the blood stream [10, 25, 62]. The protein is secreted in an unfolded conformation by a T1SS and regains its 3D structure and function upon calcium ion binding to the RTX repeats, a nonapeptide sequence conserved in all RTX proteins [28, 29, 38].

Although the secretion and folding upon calcium ion binding are crucial for HlyA activity those steps are poorly understood and many questions remain regarding those mechanisms. For example, the signal for HlyA secretion is located at the last ~ 60 amino acids of the C-terminus but it is unknown if the N-, or C-terminus is secreted first. Furthermore little is known about the folding of HlyA upon $Ca^{2+}$ binding, the native step upon HlyA secretion. It is unknown how ion binding is sufficient for such a high molecular weight protein to fold independently of chaperones and little to no kinetic data are available.

One aim of this thesis was therefore to study the folding of HlyA using intrinsic tryptophan fluorescence and to identify the role of calcium ions during this process.

## 4.2 Establishment of an *in vitro* acylation assay

Apart from calcium ion binding to the secreted toxin another modification is necessary to obtain haemolytic active HlyA [44]. This modification is a posttranslational fatty acylation mediated by the acyltransferase HlyC together with ACP [47, 48]. Two lysines of HlyA, K564 and K690, are specifically acylated and only with the attached fatty acid HlyA shows lytic behavior upon target cells [49, 161].

Although the modification is of the upmost importance for HlyA activity it is not clear how the acylation takes place. On the one hand it is unclear how HlyC recognizes the acylated lysine residues as no sequence homology exists and on the other hand it is disputed if acylation follows a ping-pong mechanism or a complex formation of all

three proteins. Additionally it is unclear how HlyA interacts with its target cells (see 3.2.3). So far only a few amino acids of HlyC and HlyA were identified that may play key roles during acylation and membrane interaction respectively. More extensive studies were often limited to small protein amounts, making radioactively labelled measurements necessary, or complicated experimental set up.

Another goal of this thesis was therefore to optimize the expression and subsequent purification of HlyA and HlyC to obtain higher protein yields for succeeding experiments like activity on cell culture cells. Furthermore a quick and easy *in vitro* assay should be established to allow scanning of future mutants of either HlyA or HlyC to determine their effects on acylation or HlyA activity.

## 4.3 Structure solving of HlyA

Although the number of solved RTX toxin 3D structures has risen during the last years, structural data for HlyA are lacking. To have a 3D model of HlyA would certainly help to better understand the functionality of the protein, allow comparison to other RTX toxins and interpret previous experimental results. As HlyA is a large protein identification of potential important amino acids or regions would also facilitate future mutational analysis as mutations could be chosen specifically and not randomly to investigate specific function. Finally a 3D structure may also solve the question if HlyA can build dimers or higher oligomeric structures, a protein feature often considered but so far not proven.

Another aim of this thesis was therefore the crystallography of HlyA to obtain diffracting crystals and to solve the 3D structure of the protein.

# 5. PUBLICATIONS AND RESULTS

## 5.1 Paper I

**Proportionate work on this publication:** 50%

- Writing of the manuscript

**Published in:** Biochimica et Biophysic Acta, Molecular Cell Research

**Impact factor:** 4.8

ARTICLE IN PRESS

BBAMCR-17081; No. of pages: 13; 4C: 2, 4, 7

Biochimica et Biophysica Acta xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

# The Type 1 secretion pathway — The hemolysin system and beyond ☆

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ARTICLE INFO

*Article history:*
Received 18 July 2013
Received in revised form 21 September 2013
Accepted 23 September 2013
Available online xxxx

*Keywords:*
Type I secretion systems
ABC transporter
Membrane fusion proteins
Host pathogen interaction
Protein interaction

ABSTRACT

Type 1 secretion systems (T1SS) are wide-spread among Gram-negative bacteria. An important example is the secretion of the hemolytic toxin HlyA from uropathogenic strains. Secretion is achieved in a single step directly from the cytosol to the extracellular space. The translocation machinery is composed of three indispensable membrane proteins, two in the inner membrane, and the third in the outer membrane. The inner membrane proteins belong to the ABC transporter and membrane fusion protein families (MFPs), respectively, while the outer membrane component is a porin-like protein. Assembly of the three proteins is triggered by accumulation of the transport substrate (HlyA) in the cytoplasm, to form a continuous channel from the inner membrane, bridging the periplasm and finally to the exterior. Interestingly, the majority of substrates of T1SS contain all the information necessary for targeting the polypeptide to the translocation channel — a specific sequence at the extreme C-terminus. Here, we summarize our current knowledge of regulation, channel assembly, translocation of substrates, and in the case of the HlyA toxin, its interaction with host membranes. We try to provide a complete picture of structure function of the components of the translocation channel and their interaction with the substrate. Although we will place the emphasis on the paradigm of Type 1 secretion systems, the hemolysin A secretion machinery from *E. coli*, we also cover as completely as possible current knowledge of other examples of these fascinating translocation systems. This article is part of a Special Issue entitled: Protein trafficking & Secretion.



## 1. Introduction

In Gram-negative bacteria, proteins or other biomolecules targeted to the extracellular space have to pass two hydrophobic barriers, the inner and outer membrane (IM or OM), before they reach their final destination. Obviously, different solutions to the same fundamental problem have emerged during evolution. These include the Type 1 to 6 secretion systems and it is likely that further systems will be identified in the future [1,2]. In principle, one can classify these secretion processes based on the underlying overall mechanism of transport. One class, including Type 1, Type 3 and Type 4 (conjugation-like systems) catalyze one-step secretion direct to the medium, while Types 2, 4 and 5 (auto transporters) use a two-step procedure to shuttle the transport substrate to the exterior. Type 4 secretion systems interestingly possess a dual role. Depending on the substrate, either a one-step mechanism or a sequential, two-step mechanism is employed, while the exact mechanism of Type 6 secretion is currently under investigation [1]. No matter which particular solution is employed and which system is being considered, all secretion machineries deal with the same fundamental challenge — how to translocate amphiphilic or hydrophilic molecules, which are often of impressive size, across two hydrophobic barriers.

## 2. Introduction to Type 1 secretion systems (T1SS)

Type 1 secretion systems (T1SS), sometimes referred to as ABC-dependent [3], are widespread in pathogenic Gram-negative bacteria such as *Escherichia coli*, *Vibrio cholerae* or *Bordetella pertussis*. The first protein secretion process discovered in bacteria was the hemolysin A (HlyA) T1SS which is found in certain uropathogenic *E. coli* strains [4–6]. This secretion machinery is composed of 'only' three components. Two proteins, an ATP-binding cassette (ABC) transporter and a membrane fusion protein (MFP) reside in the IM, while the third component is localized in the OM. In the case of the HlyA T1SS these three players are HlyB (ABC transporter), HlyD (MFP) and TolC (OMP). Further details on the genetic organization and architecture of the secretion machinery are given in the subsequent sections below.

Substrates of T1SS have an impressive range in size, from small proteins such as the 20 kDa iron scavenger HasA from *Serratia marcescens* [7], the 110 kDa HlyA hemolysin, up to a molecular weight of 900 kDa for LapA, a large adhesion protein from *Pseudomonas fluorescens* [8].

In the following sections we shall focus primarily on the HlyA T1SS of *E. coli*, which serves as the paradigm for the family. Wherever appropriate we shall also provide the reader with results derived from

☆ This article is part of a Special Issue entitled: Protein trafficking & Secretion.

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http://dx.doi.org/10.1016/j.bbamcr.2013.09.017

Please cite this article as: S. Thomas, et al., The Type 1 secretion pathway — The hemolysin system and beyond, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbamcr.2013.09.017

other systems in order to underline the different modes of action of T1SS.

Before starting with a detailed description of T1SS and in particular the HlyA system, we would like to propose a clear definition of T1SS. Eventually this might be used by other researchers to reduce the confusion that exists in the field. In our opinion, a T1SS is defined by three membrane proteins, an ABC transporter, an MFP and an outer membrane protein that act in concert to secrete a substrate in one step across two membranes. The substrate is characterized by the presence of a non-cleaved, C-terminal secretion sequence. Consequently, bacteriocin exporting systems that are widespread in Gram-positive bacteria are by definition not T1SS, since the outer-membrane component and the C-terminal secretion signal are absent. Similarly, the definition excludes the 10 kDa *E. coli* colicin V, which targets the ABC transporter using a cleaved, N-terminal signal sequence.

## 3. Genetic organization

Substrates and components of Type 1 systems are encoded by gene clusters found integrated into the chromosome or carried on plasmids. These genes typically include the transport substrate, two export functions and sometimes a gene that promotes the function of the substrate. For example, the Hly operon (Fig. 1A) consists of the genes *hlyC*, *hlyA*, and the exporter genes *hlyB* and *hlyD*. The additional gene *hlyC* encodes an acyltransferase that acylates HlyA at two internal lysine residues (Lys 540 and Lys 648) [9]. This lipidation is not essential for secretion, but for hemolytic activity [10]. Without these two internal acyl chains, the toxin is not capable of inducing pore formation in the membrane of its host cell. A similar operon-like organization (Fig. 1B) exists for genes encoding secreted lipases, proteases, adhesion proteins or the iron-binding protein HasA. The gene encoding the OMP (TolC in the case of the Hly system) is usually not found within the Hly operon. However, TolC [11] is a multifunctional protein involved in many important cellular processes [12], probably the most important one being multidrug efflux. *E. coli* and other bacteria have a variety of extrusion mechanisms to eliminate such xenobiotics. These include ABC transporters, secondary transporters, the SMR (small multidrug resistance) family and the RND (resistance, nodulation and cell division) family [13]. The latter transporters provide drug resistance through a tripartite efflux pump [14,15] that is composed of an RND protein, an MFP and an OMP. The best-characterized example of this type is the AcrB/AcrA/TolC system of *E. coli*, which has been studied extensively at the functional and structural level [16]. The importance of the AcrBA system is illustrated by the observation that deletion of TolC renders bacteria sensitive to small amounts of detergents, something they can cope with easily if the tripartite pump is properly assembled.

Another Type I system, the *sla*-operon from *S. marcescens*, an opportunistic pathogen, shows some important differences compared to the *hly*-operon as seen in Fig. 1C. Exceptionally, the *sla*-operon also includes the gene for the OMP (LipD). Moreover, in this case the structural gene of the transported substrate *slaA*, encodes an S-layer protein. More surprisingly, two more substrates utilize the Sla T1SS, the lipase LipA and the protease PrtB [17]. However, these are both encoded outside the operon.

## 4. Regulation

A survey of the literature on the regulation of expression and secretion of substrates of T1SS revealed that to the best of our knowledge only two different examples have been studied in any detail. These are the Has system from *S. marcescens* and *P. aeruginosa* and hemolysins from *V. cholerae*, *N. meningitidis* and in particular *E. coli.*

The operon structure for Has (heme acquisition system) is shown schematically in Fig. 1B. For bacteria, heme is the major source of iron [18]. After internalization heme is degraded and the released iron is used for many key cellular processes. In principle, two modes of heme uptake exist in bacteria. Class I systems utilize siderophore-dependent uptake via specific surface receptors, activated through the transenvelope, TonB–ExbB/ExbD complex. This transfers iron from the exterior to the periplasm, where it traverses the inner membrane by another mechanism [19,20]. The Has system is an example of class II heme uptake [21]. This depends upon the extracellular heme binding protein (HasA), which is secreted by a typical T1SS (Fig. 1). The secreted HasA with bound heme, binds to an outer membrane receptor (HasR) and the TonB dependent system, energized by ExbB/ExbD in the inner membrane, enables heme transfer to the periplasm from where it crosses the inner membrane [22]. As shown in Fig. 1B, upstream of the *hasA* gene and *hasR*, the outer membrane receptor, is a *fur* (ferric uptake regulator) box [23]. The *fur* box regulates expression of the downstream genes. Fur is a dimeric DNA-repressor protein (encoded elsewhere in the genome) that binds to the *fur* box in the Fe(II) bound state [24]. Under conditions of iron limitation, the Fur–$Fe^{2+}$ complex dissociates, then Fur is released from the *fur* box and transcription of the downstream genes is initiated. When the cellular iron level increases, binding of $Fe^{2+}$ to Fur is promoted and the Fur–$Fe^{2+}$ complex binds

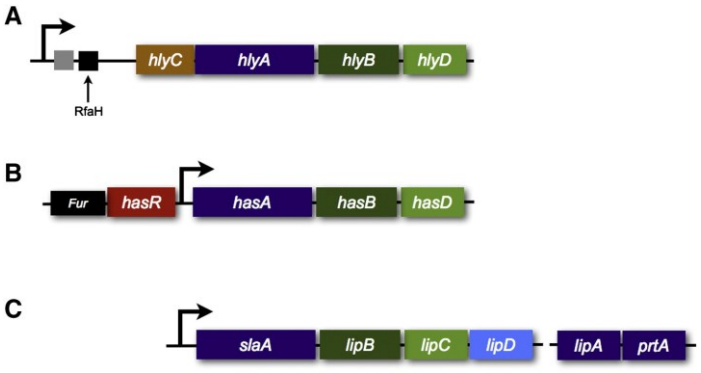


**Fig. 1.** Operon structures of selected T1SS. (A) chromosomal hemolysin, (B) Has and (C) SlaA/LipA/PrtA system. The gene for the HasA surface receptor (and regulator), *hasR* is shown in red, accessory genes like *hlyC* in orange, the gene for the transport substrates in dark blue, the ABC transporter and the MFP in dark and light green, respectively, and the OMP light blue if present in the operon. Regulatory elements such as *fur* are shown in black. The JUMPStart pause sequence, and the *ops* sequence, which is present in only 30% of the chromosomal *hly*-operons, represents the binding site of RfaH, are shown as gray and black boxes, respectively. Further details are given in the text.

Please cite this article as: S. Thomas, et al., The Type 1 secretion pathway — The hemolysin system and beyond, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbamcr.2013.09.017

to the *fur* box and transcription is switched off. However, the situation is more complicated, since HasR has a dual function, also playing a role as a regulator of transcription of the *has*-operon. Studies by the Wandersman group [25–27] demonstrated that the extracellular binding of heme-loaded HasA to HasR, induces a transenvelope signal cascade, which activates specific sigma and anti-sigma factors, encoded by genes located close to the *has*-operon. These factors control *hasR* expression and thus, heme availability is the master switch in the regulation of transcription of the *has*-operon.

Early studies in the 1980s and 1990s in the Welch, Goebel, and Hughes laboratories [28–32] indicated that one or more cis-acting, non-coding regulatory sequences, at least several hundred base pairs long and upstream of the *hlyC* start codon, were required for expression from both plasmid and chromosomal determinants that are present in many uropathogenic *E. coli* (UPEC) strains. In contrast to the coding sequences, these upstream regions were not highly conserved between plasmid and chromosomal determinants (found in pathogenicity islands). For example, an IS2 element just upstream of *hlyC* was found only in plasmid determinants. Despite this, the basic mode of regulation, involving a transcription elongation mechanism emanating from a far distant upstream site, to overcome transcriptional polarity (see below) appears common to both determinants. In fact, the most detailed studies have involved *hly*-operons found in the chromosome of UPEC strains and this is reflected in the literature reviewed below.

### 4.1. Molecular details of transcript elongation control by RfaH

RfaH, first identified [33] as a positive regulator of the *rfa*-operon, encoding components of LPS biosynthetic pathway, was also shown in 1992 to be required for transcription of the *hly*-operon [34]. The role of RfaH was quickly shown to affect, in particular, the transcription of the distally located exporter genes, *hlyB,D* [30,35,36]. Thus, the expression of *hlyC,A* is uncoupled from the expression of *hlyB,D* in the absence of RfaH. This led to the conclusion that RfaH, both in plasmid and chromosomal operons, acts as an anti-termination factor, specifically promoting elongation of transcripts from *hlyC* through *hylB and hlyD*. This transcriptional polarity was thought likely to involve a rho-independent terminator first identified by Felmlee et al. [28]. This is positioned in the intergenic space between *hlyC* and *hlyB* and predicted to adopt a stem loop structure in the coding mRNA. In fact, other studies led to the realization that the cis-acting, upstream regulatory region, the RfaH protein, and the phenomenon of transcriptional polarity, were all part of the same process. Thus, within the large upstream non-coding region, two discrete motifs just downstream of the *hlyC* promoter were identified as essential for high level, RfaH dependent transcription, from the *hlyC* promoter. The first motif, identified originally by Hobbs and Reeves [37] is a 39 base pair sequence shared by the non-coding regions of the *hly* and *rfa*-operons, called JUMPstart (just upstream of many polysaccharide-associated starts). Leeds and Welch [35], deleted this motif, which is more than 1 kbp away from the *hlyC* start codon, and this abolished RfaH dependent transcription through *hlyC*. The authors proposed that RNA polymerase paused at JUMPStart, suggesting an opportunity for RfaH to bind to RNA polymerase. Bailey et al. [34,38], identified a second conserved, 8 bp motif just downstream of JUMPstart, designated as *ops* (operon polarity suppressor), also required for transcript elongation by RfaH.

So how does RfaH promote anti-termination from a remote upstream regulatory site? The laboratories of both Hughes and Koronakis, and that of Welch, formulated the idea that RfaH is recruited to the RNA polymerase complex at the *ops* and or JUMPstart sites. More recent work now confirms this, providing some surprising and exciting molecular details that seem to supply most of the answers. RfaH, although acting highly selectively, is in fact a homologue of NusG, a *general* transcription elongation factor that is recruited to RNA polymerase complexes to suppress downstream pausing in many different operons [39]. Artsimovitch and Landick [39] examined in vitro and in vivo how RfaH targets the *ops*-containing operons. They found that RNA polymerase, initiating transcription from a promoter such as *hlyC*, makes an early pause, likely at JUMPstart, which exposes the non-coding strand of *ops* to which RfaH is able to bind. Apparently this leads to binding of RfaH to the polymerase itself and the establishment of a stable polymerase complex that increases the rate of the resulting transcription as well as promoting long distance processivity and suppression of pausing at various terminators. This is therefore clearly consistent with the up to 10 kbp, RfaH dependent transcripts, found associated with expression of the *hly*-determinants. Interestingly, a recent study by Belogurov et al. [40] has confirmed two binding sites in the N-terminal domain of RfaH, a polar patch on one side, apparently interacting with the *ops* single strand DNA, and on the other side, a hydrophobic patch, which binds to the β-subunit of the polymerase.

### 4.2. Other possible regulatory mechanisms

Other indications of putative regulators of *hly* expression but still lacking confirmation, as physiologically relevant, include post-transcriptional regulation and a possible form of temporal regulation. Temporal regulation of HlyA secretion has been reported by a number of groups, whereby secretion is apparently limited to a relatively short period in the growth phase, triggered in early to mid-exponential phase but then switched off in the transition to stationary phase but before growth ceases [41]. This observation is not limited to laboratory strains carrying recombinant plasmids but is also observed with the UPEC strain, LE2001 [42]. Previous studies have demonstrated the impossibility of generating even small amounts of either HlyB or HlyD produced from a variety of promoters or in different growth conditions. However, toxicity or instability of the overexpressed protein could apparently be ruled out. In fact, Blight et al. [43] showed that these constructs, while failing to produce proteins, in fact expressed high levels of *hlyB* mRNA from the different promoters. Moreover, large amounts of HlyB and HlyD fusions could indeed be obtained, when coding sequences for *hlyB* and *hlyD* were placed downstream of sequences for β-galactosidase and glutathione-S-transferase respectively [43,44]. These and other results indicated that translation of the normal transcript was inhibited by, for example, proximal mRNA 3D-structures, which are rendered ineffective in the fusion to large upstream messages. Interestingly, in this context Landraud et al. [45] identified an additional toxin gene encoded downstream of *hlyD* in many UPEC Hly determinants that is also transcribed from *hlyC*, dependent on RfaH. Curiously, this toxin gene, *cnf1*, has its own, immediately upstream promoter. However, translation of this resulting messenger is much reduced by a mechanism involving mRNA folding that masks the ribosomal binding sequence. In contrast, the authors report that *hlyACBD* transcripts from *hlyC*, which proceed through *cnf1*, apparently results in 'ironing out' the inhibitory mRNA folding, and translation of *cnf1* message is then much increased.

Reports of other factors affecting *hly* expression include roles for the H-NS and Hha global regulators of nucleoid organization [46], particularly to repress expression at low temperature; an independent promoter just upstream of *hlyD*; a requirement for the rare codon $tRNA_5^{leu}$ (leuX) [47]; regulation by the two component signal transduction system MisR/MisS in *Neisseria meningitides* [48]; while expression of a Type 1 (Hly) secretion system in *Vibrio anguillarum*, is positively regulated by *hlyU* [49]. In the (enterohemorrhagic, EHEC strain of *E. coli* O157:H7) hemolysin expression appears also to be inhibited by H-NS, but this effect is antagonized by Dsr2, a small non-coding RNA [50], which interacts directly with H-NS. In addition however, Dsr2 stimulates the synthesis of the RpoS sigma factor, which is required for expression of this typical *hly*-operon. Finally, an intriguing finding also indicated that in trans over-expression of *hlyA* from a heterologous promoter, in the absence of the export proteins, led to at least a three-fold reduction in *hlyC* expressed from the wild-type promoter in the same cell. This suggested a possible feed-back repression of *hlyC* by the non-transported HlyA [51].

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In summing up, the regulation of the *hly*-operon, it is important to stress that results from laboratory strains in laboratory conditions give only a limited view of the physiological nature of *hly*-regulation. In particular, we presume that full expression of *hly* genes only occurs during an infection. Does this involve a stochastic switch or a quorum sensing mechanism regulating RfaH production in vivo or some other limiting factor? Is expression limited in vivo to a sub-population of cells? Finally, what is the functional significance of transcriptional polarity? As an option to attain *hlyC*, *hlyA* expression independently of those for export functions, this does not seem to make much sense. Transcript termination at *hlyB* therefore remains a puzzle. On the other hand, as hinted at in [39] there could be an advantage in a factor like RfaH stabilizing the polymerase complex, in order to ensure coordinated expression and therefore utilization of all the products of the very large *hly*-operon. Thus, the suggestion by Artsimovitch and Landick [39] of localized cell factories, where closely associated nascent polypeptides are assembled into multiple secretion machines, is very attractive. Secretion initiated in such a localized environment, in which a transport substrate such as HlyA is first tethered (see below), by the ABC protein and threaded into the transport channel as soon as the C-terminal signal is released from the ribosome, would make sense.

## 5. Architecture

According to our definition, a T1SS is a complex of three membrane proteins (ABC-transporter, MFP, OMP) that enables the transport of a substrate (Fig. 2) to the exterior in one step across both lipid bilayers. To ensure formation of a functional transporter all three proteins need to assemble in a defined order via molecular interactions. In this section we shall have a closer look at the architecture of a Type 1 machine as illustrated by the HlyA T1SS with a main focus on the proteins involved, their order of assembly and interaction with HlyA.

### 5.1. TolC — the outer membrane protein

The outer membrane protein of the HlyA T1SS is TolC, although the gene is not linked to the *hly*-operon [52]. The structure for TolC was solved in 2000 by Koronakis et al. [11] and revealed important information regarding the transport mechanism. TolC forms homotrimers that make up a 140 Å long channel protruding deep into the periplasm. This is composed of a short β-barrel (outer membrane) and long, α-helical (periplasmic) structure. The maximum width of the trimeric complex is 40 Å (internal diameter 20 Å), narrowing to only 3.5 Å at the periplasmic end [11,53,54]. The latter is too small to enable passage of ions, and certainly for folded proteins [55]. For opening of the channel, evidence for an iris-like movement, rearranging the α-helices to widen the periplasmic entrance to roughly 30 Å, suitable for passage of protein secondary structures [11,56–58], has been obtained. Furthermore, the large water filled cavity at the external exit of TolC may be suitable for partial folding of the secreted substrates in a protected environment [55,59].

### 5.2. HlyD — the membrane fusion protein

The large family of "membrane fusion proteins" was first described by Dinh et al. in 1994 [60], who pointed out some low level identity with viral fusion proteins, hence the name, membrane fusion proteins [54,60]. However, this is a misnomer since there is no evidence that MFPs can literally cause membranes to fuse. The MFP of the HlyA T1SS is HlyD [61]. So far no structural data for the approximately 53 kDa HlyD or other MFPs of T1SS are available. However, analogous structures are known for periplasmic proteins like MexA, which is anchored by a lipid extension to the cytoplasmic membrane and forms part of a drug efflux channel [62]. HlyD is located in the inner membrane [62–64] and topological analyses predict a short 60 residue N-terminal cytoplasmic region, a single membrane spanning α-helical region and a periplasmic domain of α-helices, followed by a large β-strand C-terminal domain [62–64]. HlyD appears to form trimers [54] and hexamers [65] as a functional unit. As Lee et al. [65] pointed out, a possible hexameric state would obviously solve the symmetry break between TolC (trimer) and HlyB (dimer) and allow symmetric interactions between the individual components, since the hexamer is the least common multiple. Such a hexameric formation was recently shown for the MFPs MexA [66], AcrA [67] and MtrC [68].

### 5.3. HlyB — the ABC transporter

ABC transporters constitute the largest family of membrane transport proteins. They are ubiquitous, present in pro-, and eukaryotes as well as archeae, and catalyze the vectorial transport of an enormous range of substrates into or out of cells (e.g. ions, amino acids, antibiotics or proteins) energized by ATP across biological membranes, even against concentration gradients [69]. The energy required for this process is achieved by ATP binding and/or hydrolysis by two ATP

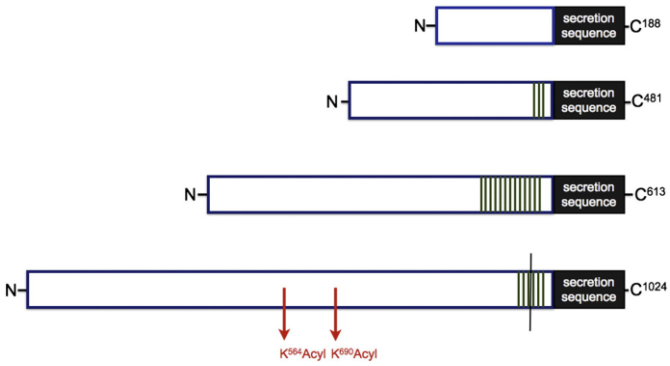


**Fig. 2.** Schematic summary of selected substrates of T1SS (not drawn to scale). The C-terminal secretion sequence is highlighted as a black box, the 6 GG-repeats as green vertical lines. From top to bottom, hemophore HasA from *S. marcescens*, which does not contain any GG-repeats, the metalloprotease PrtB from *Erwinia chrysanthemi*, which contains three GG-repeats, the lipase LipA from *S. marcescens*, which contains 13 GG-repeats and HlyA from *E. coli* containing six strictly consensus GG-repeats. The number of GG-repeats was taken from the individual Uniprot entries (HasA, Q54450, PrtB, P16316, LipA, Q59933 and HlyA, P08715. The two internal lysine residues (K564 and K690) that become acylated by HlyC are highlighted by red arrows. The gray, vertical line in the schematic summary of HlyA denotes the N-terminus of C-terminal fragment of HlyA, HlyA1.

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binding cassettes, hence the name ABC transporter. ABC transporters are typically composed of two hydrophobic transmembrane domains (TMDs) and two hydrophilic nucleotide binding domains (NBDs) [70]. The four domains can either be located on one polypeptide chain (eukaryotes, full size transporter) or in separate polypeptides that assemble to form a stable complex [70–72]. HlyB (approximately 66 kDa) is a so-called half-size transporter with one NBD and one TMD in a single polypeptide. Two HlyB monomers then assemble upon ATP binding to form a functional dimer, the active conformation of HlyB [73–75]. In contrast to the NBDs, with several highly conserved motifs, the TMDs of ABC transporters show little sequence conservation. This is consistent with the capacity of these transporters to specifically bind and transport a wide range of small molecule substrates, or, in the case of Type 1 substrates like HlyA, the TMDs may actually form a channel for extrusion of cognate polypeptides [76].

*5.3.1. The alternating access model — does it apply to protein secretion?*

The ABC transporter is central to all T1SS, since it provides energy for translocation as well as forming specific interactions with the MFP and the substrate. The conversion of chemical energy from ATP into mechanical energy for physical translocation of the substrate across a biological membrane is a central issue of ABC transporter research, although much detail remains to be understood. However, it is beyond the scope of this review to give a detailed update of the molecular structure-function of ABC transporters. For further information the reviews of Hanekop et al. and Biemans-Oldehinkel et al. are recommended [77,78]. However, in brief, currently the "ATP switch model" the "processive clamp model" and the "close contact" model [79–81] provide the best fit to the experimental data on ABC transporter function, at least with regard to transport of small substrates like drug molecules. Here, binding of ATP induces a switch of the ABC transporter from the so-called inward facing to the outward-facing conformation. In the case of an ABC exporter, a drug first bound to the inwardly facing membrane binding site, is transferred to an extracellular orientation, which has a reduced substrate affinity and the drug is therefore released to the exterior. Hydrolysis of ATP resets the system, i.e. return of the ATP-bound outward conformation to an inward facing conformation. This scenario is in line with structural studies of ABC exporter and importer systems for small molecules [76,82–84]. How relevant is this model to the role of Type1 ABC proteins in translocating large unfolded polypeptides? First it is important to note that while drug molecules most likely access the ABC TMDs laterally from the bilayer, molecules like HlyA must first dock with the export proteins, approaching from the cytoplasm. It is then generally agreed that the largely unfolded Type 1 proteins are transported to the exterior through a continuous transenvelope channel. If HlyB constitutes part of that channel it is extremely difficult to see how the *alternating* access model can apply in this case.

*5.4. Assembly of the HlyA T1SS*

In the last decade, extensive experiments provided important evidence on how the Type 1 translocator is assembled. Nevertheless, the precise molecular mechanism is still not completely understood. Early protein interaction experiments by Létoffé et al. [85] with PrtC and HasA secretion employed co-purification methods, and Thanabalu et al. [54] working with HlyA, in cross linking studies, both indicated that assembly was an ordered reaction. They could show that the ABC and the MFP proteins interacted in the absence of the OMP and the substrate molecule. In the presence of the substrate the OMP was found in the complex. Both groups also detected an interaction between the transport substrate and the ABC protein. Another study also showed that HlyD was destabilized and degraded in the absence of HlyB and TolC [45]. In addition, Balakrishnan et al. [86] demonstrated that the short N-terminal cytoplasmic extension of HlyD was essential for the interaction with HlyA and that the latter interaction was necessary for the recruitment of TolC. Together, all these findings led to the following model for the assembly of Type 1 machines: HlyA recognizes HlyB prior to HlyD recruitment or alternatively, HlyA recognizes the transient HlyB/HlyD complex. In either case, binding of HlyA induces a conformational or chemical change in HlyD, which triggers the "on demand" recruitment of TolC [54,85]. This general model was supported by several mutagenesis studies and different sub-domains involved in these interactions were reported as discussed below.

*5.5. Interactions and mutant studies involving HlyB*

Benabdelhak et al. [87] identified in vitro a specific interaction between purified protein fragments constituting the C-terminal secretion signal of HlyA and the NBD of HlyB, with a measured $K_D$ of 4 μM. Moreover, addition of ATP displaced the signal sequence from HlyB, suggesting that this may play a role in vivo in the initiation of secretion. On the other hand, evidence that the HlyA secretion signal has a docking site in the HlyB-TMDs was claimed by Zhang et al. [88]. This was based on TMD mutations, which suppressed the "secretion" defect caused by deleting 29 residues from the C-terminus of HlyA, rather than an initial docking site. However, the readout for secretion in this study was HlyA activity and recently Jumpertz et al. [89] showed that deletion of several residues from the C-terminal of HlyA did not disrupt secretion but affected protein folding and therefore hemolytic activity. Thus, the Zhang et al. supressors may indicate changes in the translocation pathway formed by the TMDs. Other studies of HlyB [90], included identification of a functional hotspot in the predicted small periplasmic loop of approximately 12 residues between transmembrane helices 5 and 6. Four out of five residues targeted for mutagenesis gave a marked secretion defect. This loop is better conserved than other regions of the TMDs and could represent a point of interaction with MFPs or its own NBD. Sugamata and Shiba [91] identified two point mutations in HlyB that drastically (up to 27x) increased the secretion of a HlyA fusion protein. One mutation is localized just upstream of the NBD and is thought to be important for ATP binding [73]. A mutation reducing the ATPase activity of HlyB, which appeared to result in the exciting discovery by Thanabalu et al. [54] that translocation of HlyA stalled in the channel, was unfortunately never followed up.

*5.6. Mutant studies and the role of HlyD*

Balakrishnan et al. [86] demonstrated a specific interaction of HlyA with the cytoplasmic domain of HlyD necessary for TolC recruitment, while a mutational analysis by Schlor et al. [92] first indicated an interaction between HlyB and HlyD, confirmed later by other groups. It has been generally assumed therefore that HlyD forms in some way part of the transenvelope structure. But what is the precise structural role of HlyD — to promote contact between HlyB and TolC or to form part of the channel itself? In fact, several genetic studies indicate that HlyD does constitute part of the channel. Thus, Schülein et al. [93], showed that substitution of the (periplasmic) C-terminal arginine of HlyD leads to increased amounts of cell-associated HlyA, suggesting that movement through, or release from, the translocator is perturbed. Pimenta et al. [44,94] described mutations in the periplasmic domain of HlyD, which led to secretion of an inactive form of HlyA that could be "reactivated" by refolding following denaturation. A more recent approach to identify functionally important HlyD amino acids was carried out by Lee et al. [65]. They identified a conserved motif (RLT) in the middle of the periplasmic domain that recognizes TolC. Mutations even in one amino acid abolished TolC recruitment and HlyA secretion. Finally, HlyD has a TolC-homologous domain that is suspected to form β-strands, which could interact with similar TolC domains to widen the TolC pore and enable HlyA transport [92,93]. All these results underline the importance of HlyD in the T1SS, indicating that it forms part of the physical transport channel, including an interaction with

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the tip of the TolC channel and does not simply connect the two membranes to allow a direct HlyB-TolC interaction

## 6. The substrate(s) and the initial steps of secretion

### 6.1. The secretion signal

Table 1 summarizes a list of examples of Type 1 secreted substrates. This attempts to cover the most important functions that have been attributed to proteins secreted by T1SS. All these proteins (Fig. 2) contain a C-terminal secretion signal and comparative analysis of secretion sequences revealed at least two major subfamilies, the non-RTX proteases (PrtB) and the RTX-proteins (HlyA) [95]. However, one has to stress that a certain overlap exists. The HlyA machinery, for example, primarily secretes RTX-proteins, but low levels of secretion of the heterologous PrtB family by HlyB and HlyD, have been reported. Type 1 substrates of T1SS can also be divided into proteins containing an RTX domain and those where the RTX-domain is absent (see below). Nevertheless, as described below, even within a subfamily there is no obviously universal secretion sequence.

In the case of HlyA, the secretion sequence (Fig. 3) was mapped to the last 48 to 60 C-terminal amino acids [96–99]. This stretch of amino acids can be further subdivided into a region containing two clusters of charged amino acids (976–996), uncharged amino acids (997–1009), hydroxylated amino acids (1017–1024) and a so called aspartate box (amino acids 994–1009) (Fig. 3). Sequence analysis furthermore indicated that the charged and uncharged section might form an amphipathic helix. In contrast, X-ray crystal structures of members of the PrtB family show that the corresponding region of the secretion signal preferentially forms β-strands. Mutational studies furthermore demonstrated an extreme level of redundancy with respect to the primary structure of the HlyA secretion sequence. Out of the approximately 50 amino acids only a handful (Fig. 3), five to be precise, displayed a significant reduction (i.e. more than 50%) in the secretion levels of HlyA, and a much greater reduction when the mutations were combined. [99–101]. A similar degree of redundancy at the level of the primary structure of the signal sequence was found for PrtB and the S-protein of *C. crescentus*. These results pointed towards a possible *structural* motif within the secretion sequence that is recognized by one or both export proteins. Subsequent CD and NMR studies on the isolated secretion sequence provided no hints of any secondary structure. However, after the addition of trifluoroethanol a helix loop helix motif could be identified [102,103]. One has to stress here that trifluoroethanol is the strongest helix-promoting agent known today and the results should be interpreted with the appropriate caution. In summary, the exact mode of recognition between the transporter and the secretion signal is still unclear. Whether the potential amphipathic helix interacts with the lipid bilayer and or HlyB, whether a secondary structural motif is recognized or whether the five critical amino acids identified, act as a recognition platform for the translocator, is currently unknown. Finally, we recently showed that the last 6 C-terminal residues of the secretion signal appear to play a dual function, being also involved in some way in catalyzing folding of HlyA [104].

**Table 1**
Overview of substrates of T1SS. The asterisk indicates that a crystal structure is available for this particular protein. Plas: plasmid, Chrom: chromosomal.

| Function | Examples | Amino acids | Organism | Architecture of the T1SS |
|---|---|---|---|---|
| Heme-binding | HasA* | 188 | *S. marcescens* | HasD/HasE/HasF |
| | | 219 | *P. aeruginosa* | HasD/HasE/HasF |
| | | 206 | *P. fluorescens* | HasD/HasE/HasF |
| RTX toxin | HlyA | 1024 | *E. coli* | HlyB/HlyD/TolC |
| | CyaA | 1706 | *Bordetella pertussis* | CyaB/CyaD/CyaE |
| | RtxA | 4558 | *Vibrio cholerae* | RtxB/RtxD/unknown |
| | FrpA | 1115 | *Neisseria meningitidis* | unknown |
| Protease | PrtA | 472 | *S. marcescens* | LipB/LipC/LipD |
| | AprA* | 477 | *P. fluorescens* | AprD/AprE/AprF |
| | | 479 | *P. aeruginosa* | AprD/AprE/AprF |
| | PrtB* | 481 | *Erwinia chrysanthemi* | PrtD/PrtE/PrtF |
| Lipase | LipA* | 613 | *S. marcescens* | LipB/LipC/LipD |
| | TliA | 476 | *P. fluorescens* | TliDEF/unknown/unknown |
| Adhesin | SiiE* | 595 | *S. enterica* | unknown |
| | LapA | 888 | *P. fluorescens* | unknown |
| S-layer protein | RsaA | 1026 | *Caulobacter crescentus* | RsaD/RsaE/RsaF |
| | SlaA | 259 | *S. marcescens* | LipB/LipC/LipD |

### 6.2. The RTX, $Ca^{2+}$ binding motifs

In addition to the secretion sequence, T1SS substrates contain a so-called RTX domain. Nonapeptide repeats rich in glycine and aspartate residues, the so-called GG-repeats, characterize this domain, which has the consensus sequence GGxGxDxUx (x: any amino acid and U: large or hydrophobic amino acid) [105]. Folding experiments in vitro demonstrated that these GG-repeats trigger folding of the substrate by binding $Ca^{2+}$ ions in a 1: 1 stoichiometry with an affinity of approximately 100 μM. The cytosolic concentration of $Ca^{2+}$ in *E. coli* and other Gram-negative bacteria (300 nM for *E. coli*) is well below the dissociation constant ($K_D$) of the $Ca^{2+}$/GG-repeat complex compared to up to 10 mM in the extracellular space [106]. Thus, folding of HlyA and other substrates of T1SS containing GG-repeats would only start when the polypeptide reaches the mM $Ca^{2+}$ concentrations at the cell surface. The importance of these GG-repeats is highlighted in the crystal structures of Type 1 substrates, which have been reported so far [104,107–111]. First, the HasA protein, which has no RTX repeats. When the structure was determined in the apo state (not shown) and the heme-bound state (Fig. 4) it was found to have one $Ca^{2+}$ ion located next to the prosthetic group. Therefore, while the mechanism of folding of HasA remains a mystery it is unlikely to involve $Ca^{2+}$ ions. In the recently reported crystal structure of a fragment of the RTX protein, SiiE [104], a giant nonfimbrial adhesion protein from *Salmonella enterica* (5590 amino acids), eight $Ca^{2+}$ ions were identified. They are, as expected, bound to the RTX-GG-repeats forcing these motifs into a characteristic β-roll conformation. Similarly, for the alkaline protease from *P. aeruginosa* (Fig. 4), LipA, a lipase from *S. marcescens* (Fig. 4) or serralysin, the major metalloprotease from *S. marcescens* (not shown), $Ca^{2+}$ is tightly bound to the RTX-GG-repeats. In the structure of alkaline protease from *P. aeruginosa*, eight $Ca^{2+}$ ions were unambiguously identified, which induce the formation of a "parallel beta roll motif", a structural element so far unique to the substrates of T1SS. These structures, in combination with the available folding data for HlyA or other RTX substrates, send a clear message — no folding and consequently no three-dimensional structure in the absence of $Ca^{2+}$. In other words, HlyA does not fold and does not adopt its native structure on a biological time scale if $Ca^{2+}$ ions are not present or an extremely low level as in the cytosol. Conversely, extracellular or surface calcium can be expected to promote folding of HlyA as it emerges from the TolC channel, with the potential to provide energy for extrusion of HlyA.

### 6.3. ABC proteins tether Type 1 substrates before the secretion signal is synthesized

Other lines of evidence including the apparently restrictive internal diameter of the TolC structure [12], also strongly supports the idea that T1SS substrates are unfolded during translocation. In this laboratory, studies employing hybrid proteins of MBP (maltose binding protein) or subsequently IFAB (internal fatty acid binding protein) fused to the C-terminal fragment of HlyA (HlyA1, containing three RTX repeats) demonstrated that the folding rate of the passenger dictated

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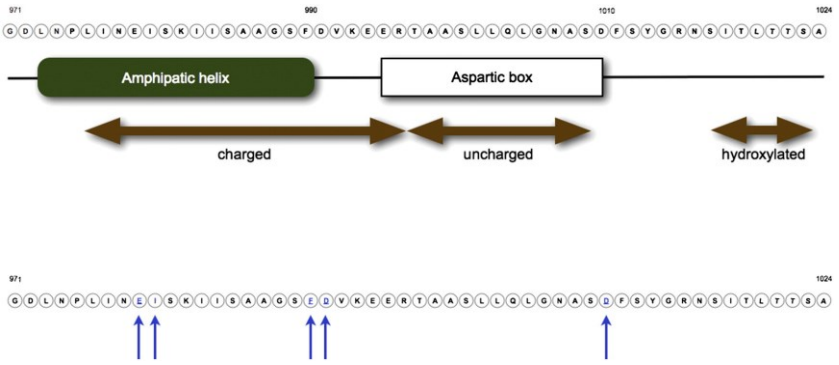


**Fig. 3.** Primary structure of the extreme C-terminus of plasmid-encoded HlyA (Uniprot entry P08715, residues 971–1024). The position of the putative amphipathic helix and the aspartate box are indicated. The aspartate box is a stretch of small amino acids enriched in alanine and serine flanked by aspartate residues. This sort of conservation was derived from sequence comparison with other HlyA-like proteins. Furthermore, sequence alignments indicated the presence of stretches of charged, uncharged and hydroxylated amino acids. The cluster of hydroxylated amino acids is apparently specific for the secretion sequence of the HlyA subfamily. The blue amino acids highlighted in the lower panel are those residues that were determined as essential for secretion in saturation mutagenesis experiments employing randomized primers. Blue arrows emphasize their positions.

secretion [95]. Thus, using mutants of both proteins, in which the folding rate but not the un-folding rate was slowed down, we obtained a strict correlation between folding rate and secretion efficiency. All these results raise an interesting question: how do these substrates, some extremely large, remain unfolded and in a secretion competent state in the cytosol of Gram-negative bacteria, without being proteolytically digested or simply aggregated? A possible answer to this question was found in 2012 by Lecher et al. [112] who showed that the N-terminal domain of HlyB is effectively a dead C39 peptidase that binds and tethers HlyA prior to transport. A detailed description of this is now presented in the next section.

As indicated above the secretion signal necessary for translocation across two membranes is located in the extreme C terminus of Type 1 proteins (Fig. 2). An extreme example — LapA, a large adhesion protein from *Pseudomonas fluorescens WSC365*, is composed of 8682 amino acids (molecular weight of approximately 900 kDa) and more than 8500 amino acids have to be synthesized before the appearance of the secretion sequence, i.e. the information that targets this protein to its cognate secretion machinery.

Elegant work by Wandersman and colleagues demonstrated a strict dependence for secretion of the iron-scavenger HasA on the antifolding activity of SecB [113]. Indeed, slow folding mutants of HasA did not

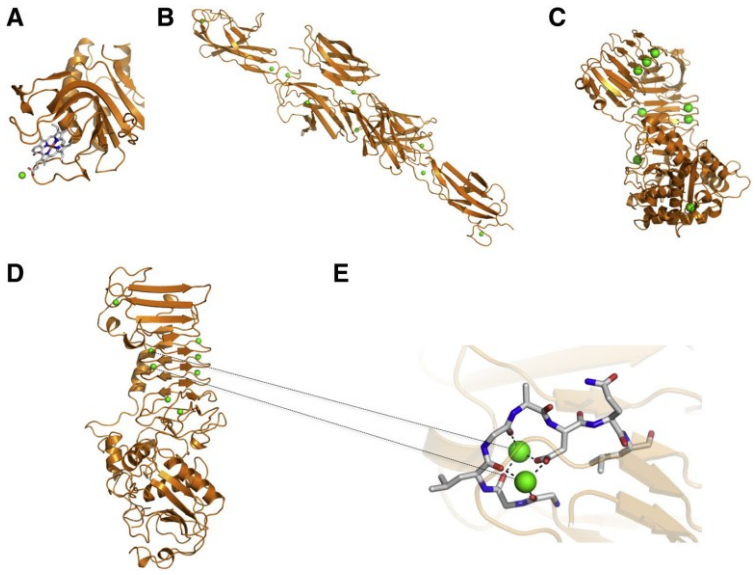


**Fig. 4.** Crystal structures of HasA from *S. marcescens* (A, pdb entry 1B2V), SiiE from *S. enterica* (B, pdb entry 2YN5), LipA from *S. enterica* (C, pdb entry 2QUA) and alkaline protease from *P. aeruginosa* (D and E, pdb entry 1KAP). Individual structures are shown in cartoon representation, $Ca^{2+}$ ions as green spheres and the cofactor heme of HasA in stick representation. The RTX motif coordinating two $Ca^{2+}$ ions of the alkaline protease is enlarged in (D). The identity of the two $Ca^{2+}$ ions within the alkaline protease structure is indicated by the two dashed, black lines connecting panel (D) and (E). Dashed black lines in (D) highlight the interactions between the $Ca^{2+}$ ions and the RTX motif (GG-repeats).

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require SecB. These results provide evidence that secretion of the non-RTX-HasA also occurs in a non-native conformation lacking any tertiary structure. Notably, however, SecB dependence has not been demonstrated for any other Type 1 system, including the HlyA T1SS. This leads again to the surprising conclusion that substrates remain unfolded in the cytosol until secretion is initiated. Thus, how such polypeptides survive becomes even more intriguing.

Despite the enormous variation in the nature and size of ABC-transport substrates, all ABC transporters possess a canonical architecture. Two transmembrane domains (TMD) and two nucleotide-binding domains (NBD) form the functional unit, which can be arranged in any possible combination [114]. Despite this variability, the NBDs always contain the conserved sequence motifs of ABC transporters and the TMDs almost invariably appear to provide the means of translocation. However, some ABC proteins contain additional N- and C-terminal domains appropriate to their specific function. HlyB, the ABC transporter of the HlyA system (but not the HasD and PrtD homologues), possesses an N-terminal extension of approximately 130 amino acids. Sequence comparison revealed that this extension bears the signature sequence of C39 peptidases. These are thiol-peptidases containing a catalytic triad (Cys-His-Asp) that promotes cleavage C-terminal to a GG sequence required, for example, for bacteriocin export [115]. In the latter, the C39 peptidase is fused N-terminally to an ABC transporter and cleaves the bacteriocin leader peptide. The cleavage site is located at the N-terminus of the precursor bacteriocin, following a conserved GG sequence and is cleaved prior to or during translocation across the cell envelope of Gram-positive bacteria. Notably, in HlyB, the catalytically relevant Cys is missing, resulting in the C39-like domain (CLD) forming an enzymatically inactive N-terminal appendix. In our previous functional studies, the CLD was shown to bind exclusively to unfolded HlyA or the C-terminal 240 amino acid residues of HlyA (HlyA1, see Fig. 2), which contain three of the consensus RTX-repeats. Importantly, the CLD still binds to HlyA1 lacking the secretion sequence [112].

This functional analysis was also complemented by NMR studies of the CLD of HlyB that revealed not only the structure but also the HlyA-binding site within the isolated CLD [112]. First, the results showed that the CLD has a high overall structural similarity to active C39 peptidase [116]. However, the binding site of the CLD for the HlyA1 fragment was not located at the reported binding site for GG-residues in C39 peptidases, but on the opposite side of the protein. This therefore raises the possibility that CLD, a degenerated C39 peptidase, no longer recognizes GG-repeats but recognizes some other motif in HlyA1, proximal to the secretion signal. Importantly, mutagenesis studies targeting the HlyA1 binding site demonstrated a clear correlation between substrate binding by the CLD and the observed HlyA secretion levels. This indicated that the CLD is not only capable of binding unfolded HlyA, but in consequence acts as a binding platform that sequesters the substrate, keeping it in an appropriate state for translocation and inaccessible to proteases until the secretion signal engages the transporter [112].

HasD, the ABC transporter of the HasA T1SS from *S. marcescens*, is in contrast to HlyB a simpler, more typical ABC transporter, i. e. it contains no CLD or any other N-terminal extension. Nevertheless, Wandersman and coworkers identified so-called "primary interaction sites" that are spread throughout the sequence of HasA and apparently act independently of the C-terminal secretion sequence [25,117]. These interaction sites (whose sequence is not yet established), if deleted, reduced HasA secretion. Thus, the secretion of HlyA and HasA, independently of the secretion signal, may involve a specific step to tether, albeit by different mechanisms, the substrate to the ABC transporter, prior to translocation. Surprisingly, in the same studies Wandersman and coworkers [25,117] also demonstrated that the C-terminal secretion sequence is required to disassemble the translocation machinery, i. e. the *final* step in HasA secretion. Again, this appears to be fundamentally different from that of the HlyA system. Thus, Koronakis and coworkers demonstrated that the C-terminal secretion signal is actually important for the *initial* assembly of the secretion machinery [54]. Importantly, arising from these results, which indicate an additional interaction between both HasA and HlyA with their respective ABC transporter, emphasize that the notion that "the secretion sequence is necessary and sufficient for substrate translocation" may now require some modification. At least in the case of HlyA, recognition of the HlyB CLD may involve sequences upstream of the secretion signal, which likely result in a particular, pre-translocation state, prior to the initiation of secretion.

## 7. Interaction of HlyA with its cellular target membrane

Over the last 40 years, investigations have been carried out regarding the interaction of HlyA and other RTX toxins with natural or artificial cell membranes. Yet the exact mode of action is still not clearly understood and many aspects have to be considered. Here we summarize some major factors that affect the activity of HlyA.

### 7.1. Cell type

RTX toxins have been commonly divided, perhaps arbitrarily, into two groups regarding their range of host cell specificity, namely hemolysins (broad target cell range) and the more specific leukotoxins. The former are named after the fact that erythrocytes are highly susceptible to this class of toxins and cell lysis can be easily monitored by release of hemoglobin on blood plates. HlyA, as the name indicates, belongs to the hemolysin group and many cell types have been reported to be sensitive to HlyA, such as monocytes [118,119], lymphocytes [119], neutrophils [120,121], and in addition, endothelial [122] and renal epithelial cells [123], fibroblasts [124,125] and of course erythrocytes [126–128]. These early studies concluded that HlyA was toxic as a result of pore formation [129]. However, studies in the last few years indicate that first, pore formation is complex and second there may be different pathways of cell killing by HlyA, as discussed below.

### 7.2. Steps in the formation of HlyA pores

There are several predicted pathways for HlyA interaction with cell membranes, which can be roughly grouped into (i) receptor free pore formation by HlyA molecules, (ii) receptor dependent pore formation and (iii) action involving HlyA in an association with outer membrane vesicles.

(i) The broad host spectrum and the many different cell types lysed by HlyA led to the assumption that cell death was induced by membrane insertion to form pores, consequent osmotic shock, cell swelling and finally cell lysis [129–131]. This was demonstrated by channel formation monitored in planar membranes and unilamellar vesicles, model systems containing pure lipids, and lacking putative protein receptors [132,133]. More recent proponents of this receptor-independent mode of action are Valeva et al., who observed a nonsaturable binding to rabbit erythrocytes that excluded a specific receptor interaction [134]. In addition, earlier studies led to the suggestion of a multistep process for pore formation, reversible adsorption of the toxin to the membrane followed by nonreversible insertion [135–137].

There have also been some investigations of the influence of calcium on HlyA action, but with contradictory results. Ostolaza and Goñi [137] stated that in the presence or absence of $Ca^{2+}$, HlyA binds equally well to lipid bilayers and liposomes (see also [138]). In contrast, for example, Döbereiner et al. [139] confirmed earlier results that calcium is important for cell-surface recognition, but is not required for pore formation. Inconsistent findings have also been reported for the necessity of acylation of HlyA for membrane binding. Some authors found no binding of non-acylated HlyA to erythrocytes [140,141],

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while Soloaga et al. [142] detected similar binding for HlyA with or without acylation.

After adsorption to the cell membrane a change in HlyA occurs that leads to insertion. This form of the protein can only be extracted from the membranes by detergents [130,143]. The insertion step is followed by cell lysis, a process requiring $Ca^{2+}$ [137–139,144] and the acylation sites [142,145], with partially acylated HlyA having a reduced lytic activity [134]. Finally, in contrast to the initial *binding* of HlyA to the membrane, the physical characteristics of the bilayer are highly critical for *insertion* of the toxin [135]. Although it is widely acknowledged that HlyA forms some kind of pore in the membrane that leads to an influx of calcium into the cells, precise details of the interaction of HlyA with membranes are still controversial.

(ii) Receptor mediated cell membrane interactions for at least some RTX toxins were first postulated in order to explain the narrow cell target range of the leukotoxins. However, Lally et al. [146] suggested that HlyA might also require a specific receptor since antibodies against potential receptors such as the $\beta_2$ integrins were found to prevent cell lysis by HlyA. This would also explain why Simpson et al. [147] failed to see HlyA induced lysis on K562 cells since these do not express $\beta_2$ integrins. Recently, Morova et al. [148] described in detail such a receptor-dependent mechanism for the adenylate cyclase toxin CyaA from *Bordetella pertussis*. They showed that CyaA is able to recognize the N-linked oligosaccharides of its cognate $\beta_2$ integrin ($\alpha_M$ or CD11b) receptors and that loss of receptor glycosylation resulted in loss of CyaA activity. While for CyaA it is proposed that binding to the $\beta_2$ receptor triggers $Ca^{2+}$ induced endocytosis of CyaA and thus protects the cells from the immune system, such a machinery cannot be envisaged for HlyA, since the cognate $\beta_2$ integrin LFA-1 is not internalized [149]. Instead, it is assumed that HlyA receptor binding leads to the destruction of host immune cells, via activation of signaling pathways due to calcium influx generated by the resulting HlyA pores [146].

A specific role for $\beta_2$ integrin mediated interaction of RTX toxins with erythrocytes was also investigated. Erythrocytes lack $\beta_2$ receptors and therefore the necessary glycosylation sites, however they have other proteins that can function as binding partners such as glycophorin for HlyA or gangliosides for CyaA [148,150]. This could explain the lack of specificity in erythrocyte lysis observed with RTX toxins as well as differences between erythrocytes from various organisms, for example, rabbit erythrocytes lack glycophorin [134,151–153]. Finally, while very likely dedicated receptors play a critical role in HlyA action in some contexts, unfortunately little appears to be known regarding how these receptors ultimately affect HlyA activity.

(iii) A potential third mode interaction of HlyA with the host appears to involve its association with outer membrane vesicles (OMV) from the producing bacteria. The production of OMVs is common for Gram-negative bacteria [154] and in 2003 Wai et al. showed that OMV formation is essential for the activation of ClyA by altering the redox status of the cytolysin [155]. Recently, Balsalobre et al. [156] showed that HlyA associated with OMVs can account for up to 66% of the HlyA protein population, independently of HlyA acylation. Furthermore, the embedded protein was as active as the secreted HlyA and the vesicles were highly resistant to urea treatment. How this form of HlyA is delivered to the eukaryotic cell is currently unknown but some experiments indicate that this involves endocytosis ([157], see also [158]). Such findings also remind us that in experiments involving the use of "purified" HlyA, this is likely to be contaminated with LPS, which may markedly contribute to the outcome either by direct potentiation of HlyA action in some way or independently in its own right.

### 7.3. Pore formation requires oligomerization?

The size of the actual HlyA pore has also been and remains an intriguing question. Different osmotic protection experiments were performed that predicted a pore size with a 1–2 nm diameter [130,159–162]. However, Moayeri and Welch [163] pointed out that temperature as well as time and toxin concentration play a crucial role in pore formation. Moreover, the observed increasing pore sizes that accompany treatment with HlyA, over time, led to the suggestion that like complement, HlyA may have detergent-like properties. Indeed, Ostolaza et al. [133] described a detergent-like function for HlyA at high concentrations.

Whether pore formation requires oligomerization of HlyA is still controversial. Several early studies indicated that one HlyA molecule is sufficient to trigger cell lysis, supporting the so-called "single-hit" model for HlyA action [162,164,165]. On the other hand, Benz et al. [166] reported that a small change in protein concentration had a large effect on membrane conductance, while Ludwig et al. [167] inferred that oligomerization occurred, since they achieved successful reconstitution of hemolytic activity by co-expression of non-lytic HlyA mutants. Bakás et al. found evidence of a cooperative process for HlyA insertion into the target cell [135]. Some recent findings also provided more direct evidence for multimerization by FRET transfer from labeled to non-labeled HlyA molecules in treated membranes [145]. Nevertheless, no multimeric HlyA has been isolated so far.

### 7.4. HlyA domains that may play a role in pore formation

Several deletion and substitution mutations have been made in order to identify HlyA domains, important for pore formation. Cortajarena et al. [168] identified a highly conserved C-terminal (aa 914–936) sequence as a putative glycophorin binding site, required for HlyA docking to the target cell (see also [100,169]). Ludwig et al. showed that deletion of the predicted N-terminal, α-helical amphiphilic region, improved pore stability and increased activity [170]. Sánchez-Magraner et al. [153] showed that the RTX domain (towards the C-terminus) was involved in the adsorption of HlyA, while the N-terminal helical domain was necessary for membrane insertion of the toxin. Finally, the acylation of K564 and K690 seems to have a major influence on the overall folding of the protein, leading to an active conformation [136,138,142,171]. In addition to all the above factors that can affect HlyA activity it cannot be excluded that toxin concentration is also important. This could, for example, influence HlyA mediated receptor clustering at the cell surface of the host cell. Additionally, specific receptor recognition might occur at low protein concentrations, while pore formation is favored only at higher concentrations [10,172].

### 7.5. Induction of signal cascades and channel activation upon pore formation: mechanism of cell killing

In healthy cells, calcium levels are strictly regulated as they modulate important signaling pathways, which in turn control many cellular processes [173,174]. Disturbance of the host cell calcium levels is therefore a common mechanism used by pathogens to manipulate host cell properties including cell killing [175].

The normal intracellular free calcium level for sheep erythrocytes is 30 μM [176], whereas the calcium level required for full extracellular HlyA activity is from 100 μM up to 20 mM [165,177]. These differential $Ca^{2+}$ levels lead to an influx of $Ca^{2+}$ through the HlyA generated pore, whether receptor mediated or not. This increase in free intracellular $Ca^{2+}$ can cause many, potentially lethal effects, for example, rearrangements of the cytoskeleton [178] or degradation through activation of the host serine protease mesotrypsin [179], of the cytoskeletal scaffolding protein paxillin, and also NFκB, which is responsible for induction of proinflammatory pathways. Other $Ca^{2+}$ induced effects have been observed by Bhakdi et al. [118], including depletion of cellular ATP, secretion of IL-1β and stimulation of arachodonate metabolism, leading to apoptosis. HlyA action was also associated with activation of P2X cation channels in mammalian

Please cite this article as: S. Thomas, et al., The Type 1 secretion pathway — The hemolysin system and beyond, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbamcr.2013.09.017

erythrocytes [180]. Furthermore, prolonged P2X stimulation is known to be pro-apoptotic [181,182]. Clearly, the precise mechanism of killing of target host cells by HlyA created pores is therefore complex and still controversial and the reader is referred to a recent more comprehensive, review [179].

## 8. Perspectives

Although many discoveries have been made in recent years regarding the Hly, Has and many other T1SS, there are still some mysteries that await a solution. These include not only the absence of three-dimensional structures of HlyA and the full-length exporter components of any of theType1 systems, but also the absence of an in vitro reconstituted system for analysis. It is therefore obvious that we need further molecular knowledge to finally understand the coordination of this “simple” but highly effective bacterial secretion system in space and time.

## Acknowledgements

Apologies to all our colleagues whose work was not cited appropriately. Research in our laboratories was funded by CNRS and the University of Paris-Sud (to I. B. H.) and funds from the Heinrich Heine University, the Jürgen Manchot Graduate School “Molecules of Infection”, the CLIB2012 Graduate Cluster and the DFG (to L. S.).

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Please cite this article as: S. Thomas, et al., The Type 1 secretion pathway — The hemolysin system and beyond, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbamcr.2013.09.017

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## 5.2 Paper II

**Proportionate work on this publication:** 60%

- Design and cloning of the different mutants
- Expression and purification of proteins
- Intrinsic tryptophan fluorescence measurements
- Data analysis
- Writing of the manuscript

**Published in:** Biochimica et Biophysic Acta: Proteins and Proteomics

(*to be submitted*)

**Impact factor:** 3.7

# Equilibrium folding of pro-HlyA from *Escherichia coli* reveals a stable calcium ion dependent folding intermediate

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**ABSTRACT**

HlyA from *Escherichia coli* is a member of the repeats in toxin (RTX) protein family, produced by a wide range of Gram-negative bacteria and secreted by a dedicated Type 1 Secretion System (T1SS). RTX proteins are thought to be secreted in an unfolded conformation and to fold upon secretion by calcium ion binding. However, the exact mechanism of secretion, ion binding and folding to the correct native state remains largely unknown. In this study we provide an easy protocol for high-level pro-HlyA purification from *E. coli*. Equilibrium folding studies, using intrinsic tryptophan fluorescence, revealed the well-known fact that calcium ions are essential for stability as well as correct folding of the whole protein. In the absence of calcium ions, pro-HlyA adopts a non-native conformation. Such molecules could however be rescued by addition of calcium ions, indicating that these are not dead-end species and that calcium ions drive pro-HlyA folding. More importantly, pro-HlyA unfolded via a two-state mechanism, whereas folding was a three-state process. The latter is indicative of the presence of a stable folding intermediate. Analysis of deletion and Trp mutants revealed that the first folding transition, at 6-7 M urea, relates to calcium ion dependent structural changes at the extreme C-terminus of pro-HlyA, sensed exclusively by Trp914. Since all Trp residues of HlyA are located outside the RTX domain, our results demonstrate that calcium ions induced folding is not restricted to the RTX domain. Taken together, calcium ion binding to the RTX domain of pro-HlyA is required to drive the folding of the entire protein to its native conformation.

**Keywords:** HlyA, RTX, protein folding, tryptophan fluorescence, calcium, folding mechanics

**Abbreviations:** AP: alkaline protease; CBB: Coomassie Brilliant Blue; SEC: size exclusion chromatography; RTX: repeats in toxin; T1SS: Type 1 Secretion System

## 1. INTRODUCTION

Protein secretion is common to prokaryotes and eukaryotes. In Gram-negative bacteria the secreted proteins need to be transported through both lipid bilayers and a paradigm for such a secretion complex is the Type 1 secretion system (T1SS) [1, 2]. One large group of proteins that is secreted via a T1SS is the Repeats in Toxins (RTX) family [3]. Eponymous for RTX proteins are specific nonapeptide repeat sequences consisting of GGxGxDxUx, where x can be any amino acid residue and U is a large hydrophobic amino acid [4]. Such repeats are found in toxins like HlyA from *Escherichia coli* [5] or LktA from *Mannheimia haemolytica* [6], but also in secreted adenylate cyclases, such as CyaA from *Bordetella pertussis* [7], lipases such as LipA and TliA from *Pseudomonas brassicacearum* and *Pseudomonas* fluorescens, respectively [8, 9], as well as proteases like alkaline protease (AP) from *Pseudomonas aeruginosa* [10] and PrtA from *Erwinia chrysanthemi* [11] or the adhesion SiiE from *Salmonella enterica* [12]. The RTX repeats bind calcium ions and form a so-called ß-roll structure, as shown by the 3D crystal structures of the AP of *P. aeruginosa* ([4], PDB entry 1KAP and [13], PDB entry 1AKL), the metallo-proteases PrtC from *E. chrysanthemi* ([14], PDB entry 1K7I) and from *Serratia marcescens* ([15], PDB entry 1SAT) as well as the lipase LipA from *S. marcescens* ([16], PDB entry 2QUB). Apart from these repeats, RTX proteins also share several other unique features like a reduced number of, if any, cysteine residues [17], as well as a C-terminal, non-cleaved secretion signal for transport by the T1SS [18].

One of the best-characterized RTX proteins is the toxin hemolysin A (HlyA), produced by several uropathogenic *E. coli* strains [19, 20]. HlyA is expressed as an inactive precursor protein - pro-HlyA - and to become lytically active, acylation of two specific lysine residues is necessary [21, 22]. This acylation is carried out by the acyltransferase

HlyC in conjunction with the acyl carrier protein (ACP) in the cytoplasm of *E. coli* [23]. The acylated HlyA is then secreted out of the cell via a T1SS consisting of the ABC transporter HlyB, the membrane fusion protein HlyD and the outer membrane protein TolC [2, 24, 25]. Due to the restricted pore size of TolC, being only 16 Å in the open conformation [26], it is widely assumed that HlyA is secreted in an unfolded conformation [27]. Folding of HlyA into its biologically active state is then achieved by binding of calcium ions in the extracellular space [4, 28, 29].

In the past, attempts to analyze the contributions of distinct protein domains to the folding and function of HlyA have been made with emphasis on the role of calcium ion binding in these processes [30, 31]. In many cases intrinsic tryptophan fluorescence studies were performed to assess the conformational state of HlyA *in vitro* [32-34]. Folding studies on HlyA in its native conformation following secretion have however been hampered due to the fact that both, pro-HlyA and HlyA, the acylated, lytically active form, are secreted in low amounts and are unstable [35, 36]. Therefore HlyA studies were mainly carried out on (chemically) denatured protein or protein precipitated from the extracellular growth medium [31, 37-39] and as a result the protein conformation of the native state of secreted HlyA is only poorly addressed. Moreover, to our knowledge, comparative unfolding and folding studies on secreted HlyA, either in its non-acylated or acylated form are lacking.

In this study we describe the high level expression and secretion of pro-HlyA that allowed both, a detailed analysis of the secreted protein in its native conformation and a comparative study of the unfolding and folding behavior of pro-HlyA including several of its mutants. In addition, the influence of various divalent cations on the folding of pro-HlyA was assessed.

## 2. MATERIALS AND METHODS

### *2.1 Cloning of HlyA mutants*

All HlyA mutations were introduced in the parental plasmid pSU-*hlyA* with the primer pairs indicated in Table 1. The plasmid was amplified with Phusion High-Fidelity DNA Polymerase (Thermo Scientific) in one step and subsequently blunt-end ligated using T4 DNA Ligase (New England Biolabs) both following the manufacturer's instructions. Successful mutations were verified by sequencing (Source Bioscience, GATC).

**Table1. Biochemical characteristics of pro-HlyA and mutants used in these studies**

| Protein | Mw (kDa)* | Extinction Coefficient ($M^{-1}$ $cm^{-1}$)* | Source/Primer |
|---|---|---|---|
| HlyA | 110.15 | 81600 | Uniprot entry: P08715, plasmid encoded; [40] |
| $HlyA_{C807-1024}$ | 23.85 | 21890 | [41] |
| $HlyA_{Xa-His6}$ | 111.42 | 81600 | fw 5´-caccaccaccaccacggaaattctcttgcaaaaaatgta-3´<br>rv 5´-gtg**acgaccttcgat**ctgaacctgaagctcatcatctc-3´ |
| $HlyA_{N1-806}$ | 86.90 | 59710 | Factor Xa cleaved $HlyA_{Xa-His6}$ |
| $HlyA_{W914A}$ | 110.03 | 76100 | fw 5´-cattcagg**aac**gcgtttgaaaaagagtc-3´<br>rv 5´-gactctttttcaaacgcgttcctgaatg-3´ |
| $HlyA_{W432,480,579A}$ (= $HlyA_{AAA}$) | 109.80 | 65100 | fw_W432A 5´-**g**gagaaaaaacacggcaaaaattac-3<br>rv_W432A 5´-**gc**ttcagcaataacatcagccattttac-3´<br>fw_W480A 5´-**g**gatacgctgataggtgagttagctg-3´<br>rv_W480A 5´-**gc**atgttgctgggtaatgaggactg-3´<br>fw_W579A 5´-**g**acggtgaaggggttcag-3´<br>rv_W579A 5´-**gc**tttatcaacacccttgactaataactcggtaatatattc-3´ |

Mutants of pro-HlyA were created by introducing mutation in pSU-*hlyA* using the indicated primer pairs, via a procedure that involved amplification of the whole plasmid and subsequent blunt-end ligation. The alanine mutations of $HlyA_{AAA}$ were cloned in a consecutive fashion. The molecular weight and the extinction coefficient used for determination of the protein concentrations by Nanodrop are also shown. Underline: His6-tag, Bold: Factor Xa protease cleavage site, Underline/bold: Alanine mutations, fw: forward, rv: reverse. *: Calculated based on the amino acid sequence using the web interface web.expasy.org/protparam

*2.2 Protein expression and purification of secreted proteins*

All protein steps were performed at 4 °C and the proteins were stored at 4 °C until further use unless stated otherwise. In all cases, expression was performed in *E. coli* BL21 (DE3) cells. For the production and purification of proteins containing the C-terminal secretion signal the plasmids pSU-*hlyA* [42], containing *hlyA* (Uniprot entry: P08715) or a mutant hereof (see Fig. 1) and pK184-*hlyBD* [43], encoding HlyB and HlyD necessary for the transport complex, were co-transformed into *E. coli*. Typically, 300 ml baffled flasks containing 150 ml of 2xYT medium (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) were inoculated at an $OD_{600}$ of 0.05 with an overnight culture. Cells were grown at 37 °C and 180 rpm. At an $OD_{600}$ of 0.4 - 0.6 protein expression was induced by the addition of IPTG to a final concentration of 1 mM. Four hours after induction, cells were harvested (8,000 x g, 15 min) and the supernatant was centrifuged to remove particulates (200,000 x g, 75 min). The supernatant containing secreted pro-HlyA (or HlyA mutants) was filtered through a 0.45 µm filter (Supor-450 membrane filter, Pall). The filtrate was subsequently concentrated to 5 ml using Amicon Ultra-15 Centrifugal Filter Units (Millipore) with a 100 kDa MWCO and subjected to size exclusion chromatography (SEC). SEC was performed on a Superdex 200 16/60 column (GE Healthcare) in buffer containing 50 mM Tris-HCl pH 7.4 and 50 mM NaCl. Elution fractions were analyzed by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. Fractions containing pure protein were pooled and concentrated as described above. For the purification of $HlyA_{C807-1024}$ (23.9 kDa), a 10 kDa MWCO (Amicon Ultra-15) centrifugal filter unit and a Superdex 75 16/60 SEC column (GE Healthcare) were used. The final protein concentration was determined spectrophotometrically (Nanodrop-1000, Thermo Scientific) using the calculated (web.expasy.org/protparam) extinction coefficients as indicated in Table 1.

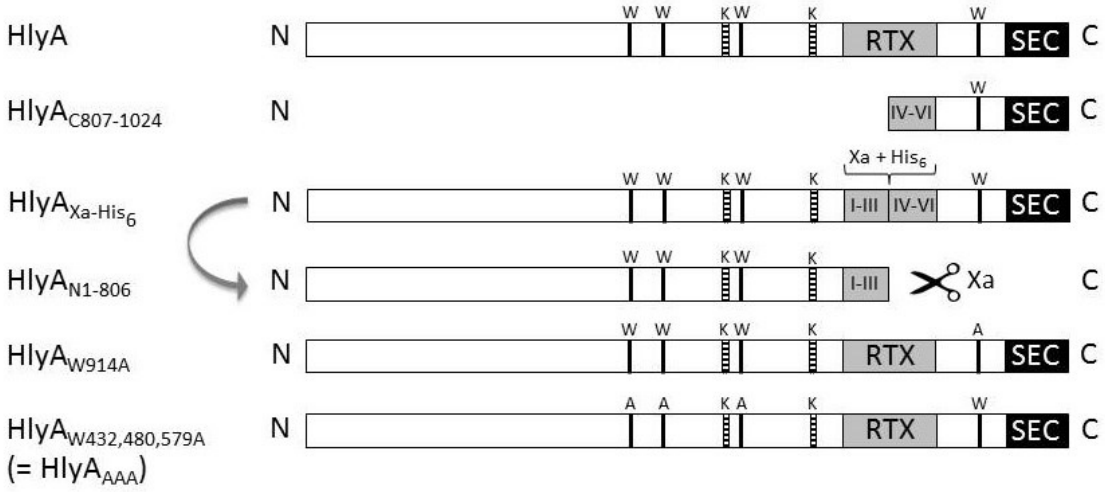


**Fig. 1. Schematic view of pro-HlyA structure and constructed mutants**

The HlyA mutants constructed in pSU-*hlyA* analyzed for folding in this study are shown. Shown in gray is the Repeats in Toxin (RTX) domain. The HlyA$_{C807-1024}$ and HlyA$_{N1-806}$ mutants each harbor three of the six RTX repeats of HlyA (I-VI). The C-terminal secretion signal of HlyA is shown in black. Important amino acids such as tryptophan (W), alanine (A) and lysine (K) as well as a factor Xa protease cleavage site (Xa) and His$_6$-tag for purification purposes are indicated. The scissors indicate the resulting HlyA mutant after factor Xa protease cleavage.

## *2.3 Purification of HlyA$_{N1-806}$ from inclusion bodies*

To generate HlyA$_{N1-806}$ for purification purposes, a factor Xa protease cleavage site and a His$_6$-tag were cloned between amino acids Gln806 and Gly807 of HlyA. The plasmid (HlyA$_{Xa-His6}$, see Fig. 1) was transformed into *E. coli* BL21 (DE3) cells and treated equally as described above. Immediately after induction with IPTG (final concentration of 1 mM), cells were transferred to 18 °C and cultivated over night at 180 rpm. Due to the absence of pK184-*hlyBD*, the protein was not secreted and accumulated in the cell as inclusion bodies. Cells were harvested (8,000 x g, 15 min) and suspended in buffer containing 50 mM HEPES pH 7.4, 150 mM NaCl, and 10% (v/v) glycerol. Following DNase I (bovine, Sigma-Aldrich) addition (a spatula tip), cells were disrupted by three passages through a cell disruptor (2.5 bar, TS 0.75,

Constant Systems) and centrifuged (30,000 x g, 30 min). The supernatant was discarded and the pellet containing inclusion bodies was washed first in buffer containing 50 mM Tris-HCl pH 7.4, 0.5% (v/v) Triton X-100 and 0.05% (w/v) $NaN_3$, followed by a second wash with buffer containing 50 mM Tris-HCl pH 7.4, 1 mM EDTA and 0.05% (w/v) $NaN_3$. After each washing step the solution was centrifuged at 30,000 x g for 30 min. To solubilize the inclusion bodies, the pellet was suspended in 20 mM Tris-HCl pH 7.4 containing 6 M urea (buffer A) after the second wash and incubated over night on a rotating mixer (RM5, Finemech). The next day the solution was centrifuged at 200,000 x g for 75 min. The supernatant was collected and loaded onto a 5 ml anion-exchange column (DEAE fast flow Sepharose, GE Healthcare) equilibrated in buffer A. Bound protein was eluted using a linear gradient of 0 -300 mM NaCl in buffer A. Elution fractions were analyzed by SDS-PAGE and CBB staining. Fractions containing the desired protein were pooled and concentrated to approximately 600 µl (Amicon Ultra-15 Centrifugal Filter Units, 10 kDa MWCO, Millipore). The concentrated protein was diluted ~ 170-fold into Factor Xa buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl and 40 mM $CaCl_2$). At this stage, the final urea concentration was less than 50 mM. To generate $HlyA_{N1\text{-}806}$, a Factor Xa (New England Biolabs) treatment was carried out over night at 25 °C according to the manufacturer's guidelines. The protein solution was concentrated (10 kDa MWCO) to approximately 1 ml and subsequently diluted into 10 ml buffer containing 50 mM Tris-HCl pH 7.4, 50 mM NaCl, 20 mM EDTA and 8 M urea. The sample was incubated for 3 h to ensure unfolding of the protein by EDTA, which removed HlyA-bound calcium ions. Next, the sample was diluted into the same buffer lacking EDTA (Buffer B), such that the final concentration of EDTA was less than 500 µM. To remove the C-terminal, $His_6$-tagged $HlyA_{C807\text{-}1024}$ fragment and the Xa protease the

sample was passed over an IMAC column (GE Healthcare), equilibrated with buffer B. Whereas $His_6$-$HlyA_{C807-1024}$ and Factor Xa bound to the column, the desired N-terminal fragment, $HlyA_{N1-806}$, eluted in the flow through as evidenced by SDS-PAGE analysis and subsequent silver staining. Fractions containing pure $HlyA_{N1-806}$ were collected and concentrated as described above. The final protein concentration was determined as described above for the secreted proteins.

*2.4. Tryptophan fluorescence measurements*

All tryptophan fluorescence measurements were performed with freshly purified proteins or protein stored at 4 °C for less than 24 h. Tryptophan fluorescence was measured with a Fluorolog 3 instrument (Horiba Jobin Yvon) at 25 °C. Excitation of Trp was performed at 295 nm and fluorescence emission was recorded between 310 nm and 450 nm. Excitation and emission slits were set to 3 nm and 5 nm, respectively. Fluorescence emission spectra were corrected by subtracting the fluorescence of the appropriate buffer lacking protein.

*2.4.1. Unfolding*

To study the unfolding of the secreted proteins, protein samples were rapidly diluted into buffer containing 50 mM Tris-HCl pH 7.4, 50 mM NaCl and urea at concentrations ranging from 0 - 8 M, either in the absence or presence of 20 mM EDTA. In all cases the final sample volume was 1.4 ml and the protein concentration 0.2 μM. Protein samples were incubated for 24 h at 25 °C to reach equilibrium. In general, equilibrium was reached after 6h, as evident by a stable signal in the intrinsic tryptophan fluorescence measurements (data not shown).

*2.4.2 Folding*

To study folding, proteins were first completely unfolded by dilution into a solution containing 7 M urea and 20 mM EDTA and subsequent incubation at 25 °C for at least 6 h. Folding was initiated by rapidly diluting the unfolded protein into assay buffer containing 50 mM Tris-HCl pH 7.4, 50 mM NaCl, 20 mM $CaCl_2$ and urea at concentrations ranging from 0 - 7.5 M urea. In all cases, the concentration of refolded protein was 0.2 μM and the protein samples were incubated for 24 h at 25 °C to reach equilibrium. Fluorescence measurements and analysis were carried out as described above. In addition, folding experiments were carried out in buffer in which $CaCl_2$ was either omitted or substituted by $SrCl_2$, $BaCl_2$ or $MgCl_2$, each at a final concentration of 20 mM.

To study the effect of calcium ion addition to protein refolded in the absence of $Ca^{2+}$, HlyA was first refolded in the absence of calcium ions as described above. After reaching equilibrium, folding mixtures were split in two; one half was used directly for fluorescence measurements, whereas the other half was supplemented with $CaCl_2$ (final concentration 20 mM). The latter samples were incubated for another 24 h at 25 °C to reach equilibrium again, after which fluorescence spectra were recorded as described above.

*2.4.3. Data analysis*

The free energy of unfolding ($\Delta G_U$) or folding ($\Delta G_F$) of the various proteins was calculated using the equations depicted below. By definition, for a two-state (un)folding mechanism only the folded and/or unfolded conformation are populated at a given denaturant concentration. Therefore, the fraction of folded protein ($f_f$) plus the fraction of unfolded protein ($f_u$) will be equal to 1 (eqn. 1).

$$f_f + f_u = 1 \qquad (1)$$

$F_f$ and $f_u$ are calculated from the measured values of the spectroscopic signal ($Y_{obs}$, here Trp fluorescence) of the (un)folding curve along with the values associated with the folded ($Y_f$) and unfolded species ($Y_u$).

$$Y_{obs} = f_f \cdot Y_f + f_u \cdot Y_u \qquad (2)$$

The calculated fractions of both species present at each denaturant concentration, allows determination of the equilibrium constant (K) and the free energy change ($\Delta G$) (eqn. 3) [44].

$$\Delta G = -R \cdot T \cdot \ln K \qquad (3)$$

Here R is the universal gas constant (8.314 $J \cdot K^{-1} mol^{-1}$) and T the absolute temperature (298.15 K). At any given denaturant concentration the equilibrium constant for unfolding and folding is given by $K_u = f_u/f_f$ and $K_f = f_f/f_u$, respectively.

It is generally assumed that $\Delta G$ exhibits a linear dependence on the concentration of denaturant [D] (eqn. 4), at least in the transition region [44]. The conformational stability of a protein can thus be estimated from unfolding data by linear extrapolation to zero denaturant concentration using eqn. 4.

$$\Delta G_U = \Delta G(H_2O) + m \cdot [D] \quad (4)$$

Thus, $\Delta G(H_2O)$ and $m$ are the intercept and the slope, respectively, in a plot of $\Delta G_U$ versus denaturant concentration. $\Delta G(H_2O)$ represents the free energy difference between the folded and unfolded states in the absence of denaturant and is a measure of the conformational stability, whereas $m$ is a measure of the dependence of $\Delta G$ on the denaturant concentration [44]. The $m$ value reflects the ability of the denaturant to unfold a protein [44] and is proportional to the amount of protein surface area exposed upon unfolding [45]. An additional thermodynamic parameter that reports on protein stability is the midpoint of the (un)folding transition, in such case K = 1 and consequently $\Delta G = 0$. Thus, the concentration of denaturant at which half of the protein molecules are unfolded ($[D]_{½}$) can be determined from eqn. 5.

$$\Delta G(H_2O) = - m \cdot [D]_{½} \quad (5)$$

*2.4.4. Two-state or three-state fitting*

Urea (un)folding curves were fitted to a two-state (N ⇄ U) or a three-state (N ⇄ I ⇄ U) model. For a two-state model the free energies of unfolding, $\Delta G^o$ and $m$ were calculated from eqn. 6 [46].

$$Y_{obs} = (Y_N + s_N \cdot [D]) + (Y_U + s_U \cdot [D]) \times (\exp\{(m \cdot [D] - \Delta G^o_{N->U})/RT) / (1 + \exp\{(m \cdot [D] - \Delta G^o_{N->U})/RT\}) \quad (6)$$

Here, $Y_{obs}$ is the observed spectroscopic signal, $Y_N$ and $Y_U$ are the intercepts, $s_N$ and $s_U$ are the slopes of the pretransition and posttransition baselines, $\Delta G^o_{NU}$ is the free energy of unfolding in the absence of urea. R, [D] and $m$ are described above.

For a three-state model (N ⇄ I ⇄ U) that describes the presence of a stable intermediate (I) eqn. 7 was used to determine the free energies of unfolding and $m$ values for the N→I and I→U transitions.

$$Y_{obs} =$$

$$\{(Y_N + s_N \cdot [D]) + Y_I \exp\{(m_{N->I} \cdot [D] - \Delta G^o_{N->I})/RT\} + (Y_U + s_U \cdot [D]) \cdot \exp\{(m \cdot [D] - \Delta G^o_{N->I})/RT\} \times \exp\{(m \cdot [D] - \Delta G^o_{I->U})/RT\}\} / \{1 + \exp\{(m_{N->I} \cdot [D] - \Delta G^o_{N->I})/RT\} + \exp\{(m_{N->I} \cdot [D] - \Delta G^o_{N->I})/RT\} \times \exp\{(m_{I->U} \cdot [D] - \Delta G^o_{I->U})/RT\}\} \quad (7)$$

The fractional change ($Z_I$) of the spectroscopic signal corresponding to the transition from the native to the intermediate state was calculated using eqn. 8.

$$Z_I = (Y_I - Y_N) / (Y_U - Y_N) \quad (8)$$

## RESULTS

### *3.1. High level secretion of pro-HlyA*

HlyA and the proteins of the secretion channel, HlyB and HlyD, were co-expressed in *E. coli* BL21 (DE3). The used expression plasmid combination, pSU-*hlyA* [40] and pK184-*hlyBD* [43] resulted in high-level secretion of pro-HlyA (see also [47]). At this stage, pro-HlyA was already quite pure, as judged by SDS-PAGE analysis (Fig. 2A). For further purification cells were removed and the growth medium containing pro-HlyA was concentrated and subjected to SEC. The obtained protein was > 98% pure as shown by SDS-PAGE analysis with subsequent CBB staining and/or silver staining (Fig. 2C). The identity of pro-HlyA was confirmed by anti-HlyA western blotting (Fig. 2A). Typically, the purified pro-HlyA could be concentrated up to 10 mg/ml and > 20 mg protein was obtained from a total of 1 l *E. coli* cell culture ($OD_{600}$ = 3.2).

### *3.2. Purification of HlyA mutants*

The single Trp mutant $HlyA_{W914A}$ and the triple Trp mutant $HlyA_{W432,480,579A}$ ($HlyA_{AAA}$) (see Fig. 1), which contain an intact secretion signal, were expressed under identical conditions and secreted in levels similar to those observed for wild-type pro-HlyA (Fig. 2B). Thus, the introduced mutations did not seem to have a significant effect on secretion of HlyA. In line with previous reports [43], the C-terminal fragment $HlyA_{C807-1024}$ (23.9 kDa) was also secreted in high amounts; here at levels similar to full length pro-HlyA (compare Fig. 2A to 2B).

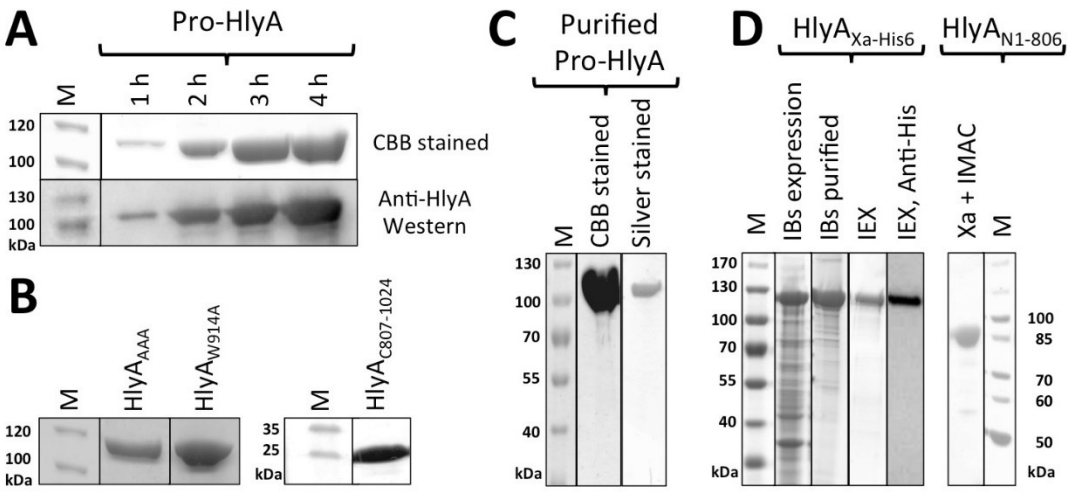


**Fig. 2. Expression and purification of pro-HlyA and several of its mutants**

The plasmid pSU-*hlyA* was co-transformed with pK184-*hlyBD* [43] in BL21 (DE3) *E. coli* cells. (A) After induction with a final concentration of 1 mM IPTG, pro-HlyA was secreted into the extracellular space and the level of secretion was analyzed by SDS-PAGE with subsequent CBB staining and western blotting using anti-bodies raised against HlyA. HlyA was purified by concentration of the supernatant, subsequent SEC and final concentration up to 10 mg/ml. The obtained protein was > 98% pure as shown by CBB and silver staining. For silverstaining the sample was diluted 1:50 in the appropriate buffer (C). (B) HlyA mutants still containing the secretion signal, $HlyA_{AAA}$, $HlyA_{W914A}$ and $HlyA_{C807-1024}$, were purified identical to pro-HlyA. Shown are the secreted proteins 4 h post induction. (D) Shows the purification of $HlyA_{N1-806}$ out of $HlyA_{Xa-His6}$ IBs. (IBs: inclusion bodies; IEX: ion exchange chromatography; IMAC: immobilized metal ion affinity chromatography; M: protein standard; Xa: factor Xa)

### *3.3. Purification of $HlyA_{N1-806}$ from inclusion bodies*

The initial strategy to obtain the $HlyA_{N1-806}$ fragment was devised such that secreted full-length $HlyA_{Xa-His6}$ could be cleaved by Factor Xa into two parts, i.e. the desired N-terminal fragment ($HlyA_{N1-806}$) and an unwanted C-terminal fragment containing a $His_6$-tag. However, this strategy proved difficult. After protease treatment and immobilization of the C-terminal fragment on an IMAC column, the desired N-terminal fragment (lacking a His-tag) remained associated. The N- and C-terminal fragment could not be separated even though successful cleavage had occurred

(Sup. 1). Therefore $HlyA_{Xa\text{-}His6}$ was expressed in the absence of HlyB and HlyD, and the protein was purified from inclusion bodies under denaturing conditions (Fig. 2D, left). Subsequent Factor Xa treatment and IMAC purification yielded highly pure $HlyA_{N1\text{-}806}$ (Fig. 2D, right). Approximately 200 µg $HlyA_{N1\text{-}806}$ was obtained per liter of *E. coli* culture per $OD_{600}$.

*3.4. Conformational state of secreted pro-HlyA*

The high level of secretion of pro-HlyA as well as several of its mutants (Fig. 2) allowed us to study these proteins after secretion and to perform a detailed study of their unfolding and folding behavior using intrinsic tryptophan fluorescence spectroscopy. First, the conformational state of secreted pro-HlyA was assessed. Pro-HlyA, contains Trp residues at positions 432, 480, 579 and 914 (Fig. 1). Typically, Trp residues in a fully unfolded protein are completely exposed to solvent and therefore the fluorescence emission maximum is usually observed at a wavelength of ~ 350 nm, which corresponds to that of the amino acid tryptophan in aqueous solution [48, 49]. On the other hand, in a hydrophobic environment, such as the interior of a folded protein, the tryptophan emission maximum is usually observed at shorter wavelengths [50]. The Trp fluorescence emission maximum ($\lambda_{max}$) of secreted pro-HlyA is observed at ~ 331 nm (Fig. 3, dark blue curve; Table 2), which indicates that (some of) the Trp residues experience a hydrophobic environment. Incubation of pro-HlyA in 8 M urea in an attempt to unfold the protein, resulted in only a minimal decrease in relative fluorescence, whereas the emission wavelength maximum virtually did not change (Fig. 3, purple curve).

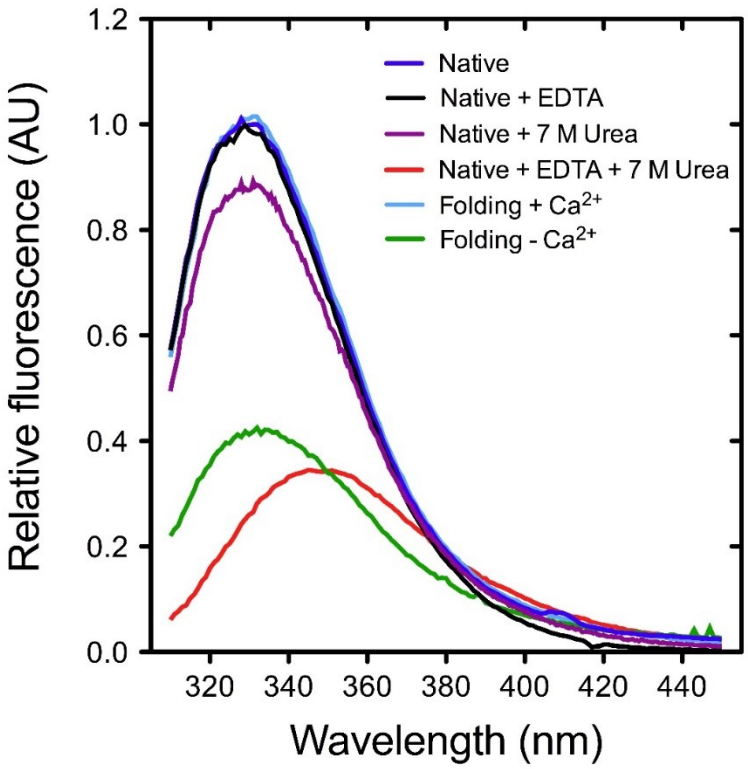


**Fig. 3. Fluorescence spectra of pro-HlyA in various protein conformations**

The conformational state of purified pro-HlyA was analyzed by measuring the intrinsic tryptophan fluorescence. The secreted, native protein (dark blue curve) was incubated with 20 mM EDTA (black curve), 7 M urea (purple) or 20 mM EDTA + 7 M urea (red curve). Pro-HlyA refolded in the absence of calcium ions is shown in green, whereas pro-HlyA refolded in the presence of calcium ions is shown in light blue. A summary of the corresponding λmax values and $F_{331}/F_{350}$ are given in Table 2.

Despite the presence of the high concentration of denaturant (some of) the Trp residues still remained in a hydrophobic environment, suggesting the protein remained (largely) folded. We assumed that $Ca^{2+}$ ions that were bound to the secreted pro-HlyA stabilized the protein. To test this hypothesis, pro-HlyA was incubated in 8 M urea with or without 20 mM EDTA, a known chelator of calcium ions. In the presence of 8 M urea and excess EDTA, the emission maximum of pro-HlyA shifted to ~ 348 nm (Table 2) with a concomitant large decrease in relative fluorescence (Fig. 3, red curve), whereas incubation with EDTA alone had no influence on emission (Fig. 3, black curve). This

suggests that pro-HlyA unfolds completely in urea only upon removal of $Ca^{2+}$. When unfolded pro-HlyA was allowed to refold in the absence of urea and $Ca^{2+}$ only a minor increase in fluorescence was observed (Fig. 3, green curve) with a corresponding shift of $\lambda_{max}$ to 334 nm (Table 2), suggesting that pro-HlyA cannot fold correctly in the absence of $Ca^{2+}$. In contrast, when unfolded pro-HlyA was allowed to fold in the absence of urea but in the presence of added $Ca^{2+}$, the obtained spectrum was virtually identical to that of native pro-HlyA (Fig. 3, light blue). Thus secreted pro-HlyA shows the hallmarks of a folded protein, which attains its stability through the binding of calcium ions.

**Table 2. Overview of the fluorescence properties of pro-HlyA and several mutants thereof**

| Protein | Form | Ion | $\lambda_{max}$ (nm) | $F_{331}/F_{350}$ |
|---|---|---|---|---|
| HlyA | Native | | 331 | 1.45 |
| | Unfolded | | 348 | 0.80 |
| | Folded | $Ca^{2+}$ | 331 | 1.45 |
| | | $Sr^{2+}$ | 331 | 1.45 |
| | | $Ba^{2+}$ | 331 | 1.45 |
| | | $Mg^{2+}$ | 336 | 1.23 |
| | | | 334 | 1.23 |
| | Intermediate | $Ca^{2+}$ | 338 | 1.12 |
| $HlyA_{N1-806}$ | Unfolded | | 344 | 0.83 |
| | Folded | $Ca^{2+}$ | 333 | 1.14 |
| $HlyA_{C807-1024}$ | Native | | 326 | 1.50 |
| | Unfolded | | 348 | 0.76 |
| | Folded | $Ca^{2+}$ | 326 | 1.50 |
| $HlyA_{W914A}$ | Native | | 333 | 1.19 |
| | Unfolded | | 345 | 0.82 |
| | Folded | $Ca^{2+}$ | 333 | 1.20 |
| $HlyA_{AAA}$ | Native | | 333 | 1.23 |
| | Unfolded | | 348 | 0.80 |
| | Folded | $Ca^{2+}$ | 334 | 1.23 |

*3.5. Urea-induced unfolding of secreted pro-HlyA*

The unfolding behavior and the conformational stability of pro-HlyA was further addressed. To this end, pro-HlyA was unfolded in the presence of different concentrations of urea with (Fig. 4A-C, crosses) or without (Fig. 4A-C, squares) excess EDTA. The conformational changes were monitored by measuring the tryptophan fluorescence (Fig. 4). In the presence of EDTA and up to 3 M urea the fluorescence intensity (Fig. 4A) and $\lambda_{max}$ (Fig. 4B) essentially did not change. In contrast, in the presence of 4 – 5 M of urea the fluorescence intensity showed a marked decrease with a concomitant shift in $\lambda_{max}$ to approximately 348 nm, indicating large changes in the near environment of the Trp residues (Fig. 4A and 4B). At urea concentrations of $\geq$ 6 M both the fluorescence intensity and $\lambda_{max}$ remained unchanged, suggesting that unfolding of pro-HlyA was essentially completed. As a measure for unfolding, the fluorescence emission at 331 nm (native, folded) and 350 nm (fully unfolded) was determined and the fluorescence ratio $F_{331}/F_{350}$ was calculated and plotted against the urea concentration (Fig. 4C). This ratio reports on the transition of tryptophan residues from a less to a more polar environment upon protein unfolding and can be determined more accurately than $\lambda_{max}$ [46]. The unfolding profile of pro-HlyA resembles that of a protein that exhibits a simple two-state protein unfolding mechanism (N→U) with a sharp transition observed around 4 – 5 M urea (Fig. 4C). The $F_{331}/F_{350}$ ratio was normalized to give the fractional denaturation (Fig. 4E) and the data were fitted using equations (1) and (3). The thermodynamic parameters for the unfolding reaction of the native secreted pro-HlyA are shown in Table 3. In contrast, when HlyA was unfolded in the absence of EDTA, virtually no changes in the maximum fluorescence, $\lambda_{max}$ or $F_{331}/F_{350}$ could be detected (4A-C, crosses).

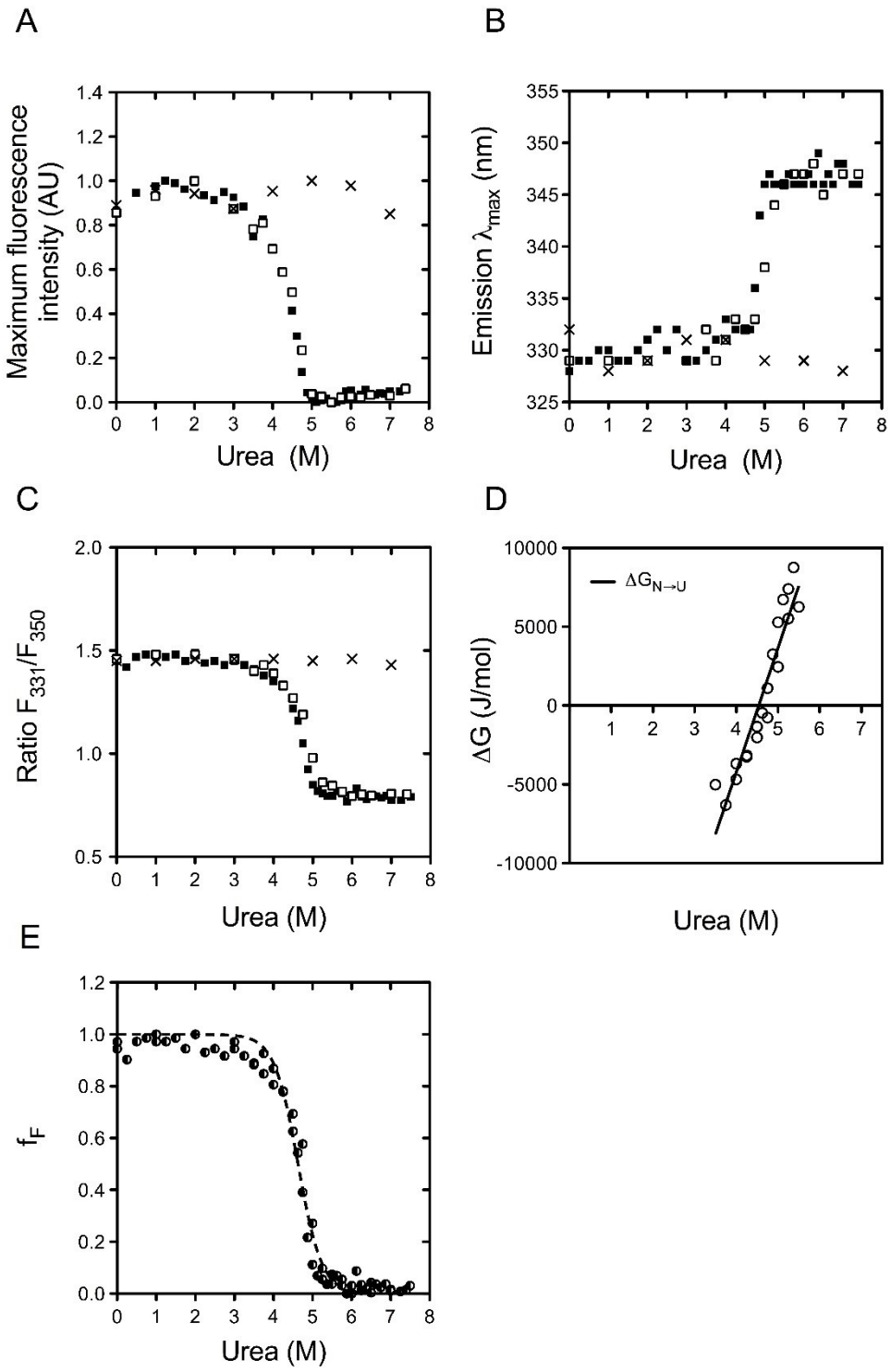


**Fig. 4. Analysis of the unfolding of pro-HlyA.**

Shown are the maximum observed fluorescence intensity (A) and the corresponding observed emission wavelength maximum (B) upon unfolding of pro-HlyA, in the presence of 20 mM EDTA of two individual experiments (squares). Unfolding experiments in the absence of EDTA are shown as crosses. The fluorescence ratio $F_{331}/F_{350}$ is shown in (C). Unfolding data were either analyzed by a linear fit (D) or (E) via a fit-function for a two-state unfolding mechanism (see chapter 2.4.4.). Thermodynamic parameters obtained from the data analysis are summarized in Table 3.

Using a simple two-state model for protein unfolding (N→U), a reasonable fit for the urea induced denaturation of pro-HlyA was obtained (Fig. 4E). The Δ*G* for the unfolding reaction of pro-HlyA was -37.82 ± 2.66 kJ/mol, with a transition midpoint at 4.63 M urea (Fig. 4D). Similar values were obtained from the linear fit analysis (Table 3 and see chapter 2.4.4.).

**Table 3. Thermodynamic parameters for the folding and unfolding of pro-HlyA and its mutants**

| Protein | Folding | Ion | Fit | $\Delta G_1(H_2O)$ (kJ/mol) | $m_1$ (kJ/mol·M) | $[D_1]_{1/2}$ (M) | $\Delta G_2(H_2O)$ (kJ/mol) | $m_2$ (kJ/mol·M) | $[D_2]_{1/2}$ (M) |
|---|---|---|---|---|---|---|---|---|---|
| pro-HlyA | unfold | | Linear fit | - 43.63 ± 3.52 | 9.53 ± 0.74 | 4.58 | | | |
| | | | Curve fit | - 37.82 ± 2.66 | 8.17 ± 0.57 | 4.63 | | | |
| | fold | $Ca^{2+}$ | Linear fit | - 91.43 ± 6.03 | 14.41 ± 0.94 | 6.34 | - 40.85 ± 2.16 | 8.12 ± 0.42 | 5.03 |
| | | | Curve fit | - 115.7 ± 15.3 | 18.24 ± 2.41 | 6.35 | - 40.19 ± 3.04 | 8.15 ± 0.62 | 4.93 |
| | | $Sr^{2+}$ | Curve fit | - 72.65 ± 6.57 | 13.79 ± 1.25 | 5.27 | | | |
| | | $Ba^{2+}$ | | No proper fit | | | | | |
| | | $Mg^{2+}$ | | No proper fit | | | | | |
| | | | | No proper fit | | | | | |
| $HlyA_{AAA}$ | unfold | | Curve fit | - 9.62 ± 0.67 | 3.07 ± 0.21 | 3.14 | | | |
| | fold | $Ca^{2+}$ | Curve fit | - 114.9 ± 21.7 | 17.93 ± 3.40 | 6.41 | | | |
| $HlyA_{W914A}$ | unfold | | Curve fit | - 19.06 ± 1.53 | 4.33 ± 0.34 | 4.4 | | | |
| | fold | $Ca^{2+}$ | Curve fit | - 23.51 ± 2.52 | 5.16 ± 0.55 | 4.56 | | | |
| $HlyA_{C807-1024}$ | unfold | | Curve fit | - 9.81 ± 0.41 | 3.45 ± 0.14 | 2.84 | | | |
| | fold | $Ca^{2+}$ | Curve fit | - 44.12 ± 1.91 | 9.35 ± 0.40 | 4.72 | | | |
| $HlyA_{N1-806}$ | fold | $Ca^{2+}$ | | No proper fit | | | | | |

*3.6. Folding of urea denatured pro-HlyA*

Next, the folding of pro-HlyA was studied. For this purpose, pro-HlyA was first completely unfolded in the presence of 7 M urea and 20 mM EDTA. Folding of pro-HlyA was initiated by rapidly diluting the unfolded protein into assay buffer containing 20 mM $CaCl_2$ and different concentrations of urea. The conformational changes were monitored by measuring the intrinsic tryptophan fluorescence (Fig. 5). In contrast to the unfolding reaction, the folding reaction of pro-HlyA did not follow a simple two-state mechanism. The graphs showing maximum fluorescence (Fig. 5A) and $\lambda_{max}$ (Fig. 5B) in dependence of the urea concentration exhibit two transition phases, which are separated by a plateau at ~ 5 – 6 M urea. Moreover, the two transition phases of the folding of pro-HlyA are well resolved when $F_{331}/F_{350}$ was plotted against the urea concentration (Fig. 5C). Taken together these data are indicative of the presence of a stable folding intermediate. In support of this, a high quality fit of the data was obtained when a three-state model for folding was applied (Fig. 5E). The thermodynamic parameters calculated from the folding data are summarized in Table 3 and Fig. 5D. The free energy for folding calculated for the first transition (Fig. 5E, I) $\Delta G_1$ is -115.7 ± 15. 3 kJ/mol with a transition midpoint at 6.35 M urea, whereas $\Delta G_2$ for the second transition (Fig. 5E, II) is -40.19 ± 3.04 kJ/mol with a $[D]_{1/2}$ of 4.93 M urea. Similar values were obtained when both transitions were analyzed separately by a linear function (Table 3 and see chapter 2.4.4.).

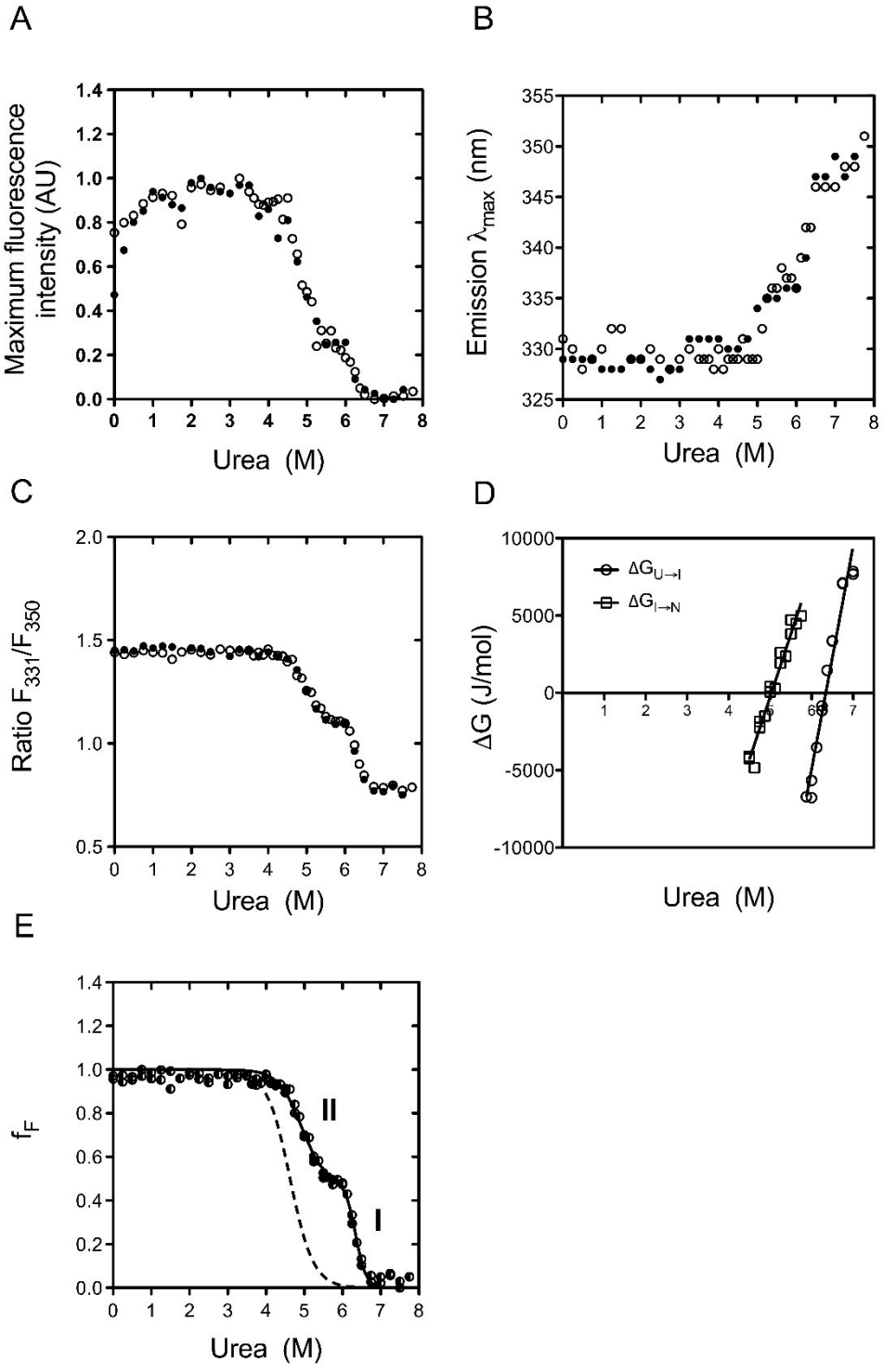


**Fig. 5. Analysis of the folding of pro-HlyA**

Shown are the maximum observed fluorescence intensity (A) and the corresponding observed emission wavelength maximum (B) upon folding of pro-HlyA in the presence of calcium ions of two individual experiments (circles). The fluorescence ratio $F_{331}/F_{350}$ is shown in (C). Folding data was either analyzed by a linear fit (D) or (E) via a fit-function for a three-state folding mechanism (see chapter 2.4.4.). Thermodynamic parameters obtained from the data analysis are shown in Table 3. For comparison the two-state unfolding fit-function is shown (E, dotted line).

Equilibrium folding experiments were also carried out in the absence of added $Ca^{2+}$ (Fig. 6A, circles). As shown in Fig 6A, the data for folding of pro-HlyA in the absence and presence of $Ca^{2+}$ clearly do not coincide, which indicates that the conformation(s) that pro-HlyA adopts in the absence of $Ca^{2+}$ are distinct from those in the presence of $Ca^{2+}$, which confirms that $Ca^{2+}$ is important for folding. Thus, pro-HlyA molecules that fold in the absence of $Ca^{2+}$ adopt a non-native conformation. We subsequently investigated whether these non-native pro-HlyA molecules are dead-end species or are still competent for folding. To test this, $CaCl_2$ was added to equilibrium folding reactions performed in the absence of $Ca^{2+}$ and pro-HlyA was allowed to reach a new equilibrium. Interestingly, $Ca^{2+}$ addition induced the folding of these molecules to levels that were virtually identical to those obtained for folding reactions where $Ca^{2+}$ was included at the beginning of the reaction (Fig 6A, crosses). This indicates that non-native pro-HlyA was still present in a folding competent state.

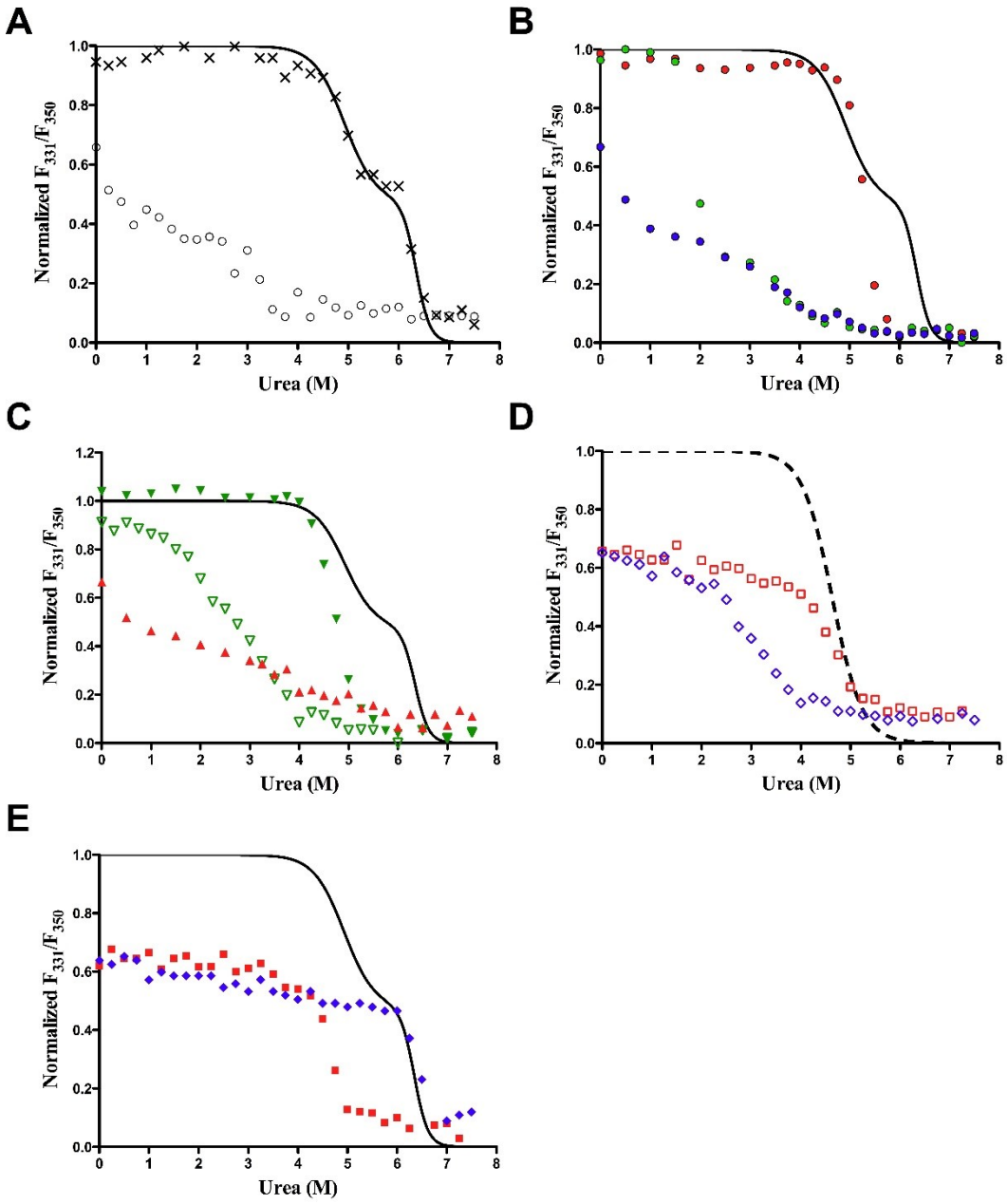


**Fig. 6. Folding analysis of pro-HlyA and several of its mutants**

(A) The calcium ion dependency of pro-HlyA folding was further tested by folding in the absence of the ion (open circles) and subsequent calcium ion addition (crosses). (B) To analyze a possible replacement of calcium ions by other divalent cations the protein was folded with strontium ions (red circles), barium ions (green circles) or magnesium ions (blue circles). (C) Pro-HlyA deletion mutants representing the C-terminus ($HlyA_{C807-1024}$, unfolding (open, green triangle), folding (filled, green triangle)) or N-terminus ($HlyA_{N1-806}$, folding (filled, red triangle)) were also analyzed. Furthermore the unfolding (D) and folding (E) of the Trp-mutants $HlyA_{W914A}$ (unfolding (open, red squares), folding (filled, red squares)) and $HlyA_{AAA}$ (unfolding (open, blue diamond), folding (filled, blue diamond)) was analyzed. For comparison the calculated fit-functions for unfolding (dotted line) and folding (solid line) are indicated. Shown are representative results of three individual experiments.

*3.7. Folding of pro-HlyA is ion dependent*

We furthermore investigated the influence of various divalent cations on pro-HlyA folding to see if they could substitute calcium ions (Fig. 6B). In contrast to what was observed for $Ca^{2+}$, the folding of pro-HlyA in the presence of $Sr^{2+}$ exhibited only a single transition (Fig. 6B, red), with the midpoint at 5.27 M urea. $\Delta G(H_2O)$ and *m* for the folding of pro-HlyA in the presence of $Sr^{2+}$ were calculated to be 72.65 ± 6.57 kJ/mol and 13.79 ± 1.25 kJ/molM, respectively (Table 3). Importantly, pro-HlyA refolded in the presence of $Sr^{2+}$ and absence of urea, was virtually indistinguishable from $Ca^{2+}$ refolded pro-HlyA, with a $\lambda_{max}$ of 331 nm (data not shown) and a $F_{331}/F_{350}$ ratio of ~ 1.45 (Fig. 6B, red and Table 2). These results indicate that with respect to the local environment of the Trp residues, the native conformations of $Sr^{2+}$ and $Ca^{2+}$ refolded pro-HlyA are essentially identical. Identical fluorescence parameters were also obtained for pro-HlyA refolded in the presence of $Ba^{2+}$. The profile for the folding of pro-HlyA in the presence of $Ba^{2+}$, however, exhibited considerable complexity with a sharp transition at ~ 2 M urea (Fig. 6B, green) and the data could not be fitted satisfactory with either a two-state or three-state model of folding.

When pro-HlyA was allowed to refold in the presence of $Mg^{2+}$ (6B, blue), the refolded state (0 M urea) is characterized by a $\lambda_{max}$ of 336 nm (data not shown) and a $F_{331}/F_{350}$ ratio of ~ 1.23. Thus, in the presence of $Mg^{2+}$ pro-HlyA adopts a conformation that is clearly distinct from that observed in the presence of either $Ca^{2+}$, $Sr^{2+}$ or $Ba^{2+}$. In this respect, the folding profile of pro-HlyA in the presence of $Mg^{2+}$ is very similar to that of pro-HlyA refolded in the absence of divalent cations (compare Fig. 6B, blue circles with 6A, open circles and Table 2). Similar to folding in the presence of $Ba^{2+}$, the obtained curve could not be fitted satisfactory.

*3.8. Folding analysis of pro-HlyA mutants*

Previous studies of RTX protein structures have revealed that these proteins are mainly composed of an N-terminal α-helical domain and a C-terminal β-strand domain [4, 14, 15]. A prediction of the pro-HlyA secondary structure using Psipred [51] revealed a similar organization (Sup. 2). We therefore wanted to analyze how these two domains, with apparent different secondary structure, contribute to the folding/unfolding either in the intact pro-HlyA or as separate domains.

To obtain specific information on the folding of the C-terminal part of HlyA in the whole protein, a triple mutant was generated in which Trp432, Trp480 and Trp579 were each replaced by alanine ($HlyA_{AAA}$). The urea induced unfolding of this mutant showed cooperative behavior, similar to that observed for wild-type pro-HlyA (Fig. 6D, blue). Indeed, a good fit of the data was obtained with a two-state model for unfolding. The transition midpoint of the unfolding reaction for this mutant was observed at 3.14 M urea with a $\Delta G(H_2O)$ of -9.62 ± 0.67 kJ/mol and *m* of 3.07 ± 0.21 kJ/molM. Compared to the wild-type pro-HlyA, these thermodynamic parameters were found to be markedly lower (Table 3). Interestingly, the folding of $HlyA_{AAA}$ showed a sharp transition between 6 and 7 M urea (Fig. 6E, blue). The $[D]_{1/2}$ for this transition was 6.41 M urea with a $\Delta G(H_2O)$ of -114.9 ± 21.7 kJ/mol (Table 3). The observed transition and the corresponding thermodynamic parameters were essentially identical to those found for the U → I transition of the wild-type HlyA (Fig. 6E, blue, Table 3). A second transition as observed for the wild-type pro-HlyA at ~ 5 M urea could, however, not be detected. Instead, folding of the triple mutant exhibited a virtually linear dependency on the urea concentration between 0 - 6 M (Fig. 6E, blue).

To monitor changes in the conformation of the N-terminal part of full length pro-HlyA, a mutant was generated in which Trp914 was exchanged to alanine. The $[D]_{1/2}$ for the unfolding of this single mutant was 4.4 M urea, which is similar to that of the wild-type pro-HlyA. On the other hand the $\Delta G(H_2O)$ and $m$ for the unfolding were -19.06 ± 1.53 kJ/mol and 4.33 ± 0.34 kJ/mol, respectively (Table 3), which are both two-fold lower than the values determined for the wild-type pro-HlyA. Interestingly, the folding profile of pro-HlyA$_{W914A}$ (Fig. 6E, red) was virtually identical to the unfolding profile (Fig. 6D, red). The thermodynamic parameters calculated for the folding of this single mutant were: $\Delta G(H_2O)$ is -23.51 ± 2.52 kJ/mol, $m$ is 5.6 ± 0.55 kJ/mol and $[D]_{1/2}$ is 4.56 M urea. (Table 3). Apparently, the unfolding and folding reactions of pro-HlyA$_{W914A}$ are fully reversible.

To analyze the folding of single HlyA domains the folding behavior of pro-HlyA$_{C807-1024}$ was studied (Fig. 6C). This mutant consists of the extreme C-terminus of HlyA and contains the last three RTX repeats, which are flanked by a single tryptophan residue at position 914 (Fig. 1). Both the unfolding (Fig. 6C, open, green triangles) and folding (Fig. 6C, filled, green triangles) reaction of this mutant adhered to a two-state model of folding, but these processes did not coincide. Pro-HlyA$_{C807-1024}$ exhibited a rather low stability with a $\Delta G(H_2O)$ for unfolding of -9.81 ± 0.41 kJ/mol and corresponding $m$ and $[D]_{1/2}$ values of 3.45 ± 0.14 kJ/molM and 2.84 M urea, respectively (Table 3). On the other hand the $\Delta G(H_2O)$ calculated for the folding of pro-HlyA$_{C807-1024}$ was -44.12 ± 1.91 kJ/mol with a transition midpoint at 4.72 M urea.

Complementary to pro-HlyA$_{C807-1024}$, the folding of HlyA$_{N1-806}$ was analysed. This mutant contains the N-terminus of HlyA, including the first three RTX repeats. This mutant largely consists of a predicted α-helical region (Sup. 2) and contains three tryptophan residues that are located outside the RTX repeats (Fig. 1). As HlyA$_{N1-806}$ was purified out of IBs only the folding could be measured (Fig. 6C, red). Notably, the folding of HlyA$_{N1-806}$ resembled that of intact pro-HlyA refolded in the absence of $Ca^{2+}$ and no satisfactory fit could be obtained.

## 4. DISCUSSION

HlyA (Sup. 3) consists of 1024 amino acids with a predicted molecular weight of 110 kDa (web.expasy.org/protparam) and contains a C-terminal secretion signal (Fig. 1). In the absence of the acyltransferase HlyC, HlyA is secreted in its non-acylated form by a tripartite T1SS consisting of HlyB-HlyD-TolC [23]. As a role model for RTX toxins HlyA from *E. coli* has been subject of many studies in the last 50 years. One common feature of RTX toxins is the secretion out of the bacterial cell prior to protein folding [4, 27-29]. This however indicates that (i) the folding inside of the cell has to be prevented and (ii) the correct folding of the protein has to occur in the extracellular space without the help of chaperones. It is generally accepted that folding of RTX proteins is triggered by calcium ion binding upon secretion due to the high extracellular level of $Ca^{2+}$ (mM range, [52]), whereas inside the producer cell, folding is likely prohibited due to the very low intracellular concentration of free calcium ions (nM range, [53]). Here we present an optimized expression and purification procedure to produce high levels of secreted pro-HlyA for biochemical studies and report a thorough investigation of the folding and unfolding behavior of secreted pro-HlyA including several pro-HlyA mutants.

*4.1. Purification of pro-HlyA and pro-HlyA mutants*

To examine secreted pro-HlyA in its native conformation and to study its unfolding behavior, large quantities of secreted pro-HlyA were required. *E. coli* BL21 (DE3) carrying pSU-*hlyA* and pK184-*hlyBD* secreted high levels of pro-HlyA (Fig. 1). Pro-HlyA could be purified from the bacterial growth medium by a simple procedure that involved concentrating the growth medium after removal of the cells and subsequent SEC. Pro-HlyA obtained via this procedure was essentially pure and protein levels of > 7 mg/L per OD were reproducibly obtained. The purified protein could be concentrated up to 10 mg/ml and remained stable at 4°C for at least several days. Similar results were obtained for all secreted pro-HlyA mutants.

Compared to pro-HlyA, acylated HlyA could however not be purified as efficiently. The secretion level was ~ 10x lower (Sup. 4) and acylated-HlyA showed aggregation tendencies during incubation (data not shown). These results are in agreement with previous findings that showed an increased degradation and aggregation tendency for the mature toxin [31, 37-39]. Therefore, pro-HlyA and mutants thereof were chosen for the folding experiments. Previous work by Boehm *et al* [54] showed that pro-HlyA binds $Ca^{2+}$ as efficiently as mature HlyA. Additionally both protein forms share the same single-channel conductance in asolectin bilayers [22] and have the same rate constant for insertion into lipid monolayers [55]. Furthermore it was reported that acylated-HlyA and pro-HlyA can equally well bind to liposome membranes [56], erythrocytes [57] and detergent-resistant membranes [33]. Moreover, these observations indicate that calcium ion binding and folding is independent of the acylation. Taken together pro-HlyA and acylated HlyA share similar structural and functional features. Thus, pro-HlyA is a suitable model protein to study the folding of RTX toxins.

*4.2. Calcium ions confer stability and drive the folding of pro-HlyA.*

Analysis of the conformational state of secreted pro-HlyA revealed that the Trp residues experience a largely hydrophobic environment and are therefore likely to be buried inside the protein. Secreted pro-HlyA showed remarkable conformational stability as it was resistant to unfolding even when incubated in the presence of high concentrations of either urea or EDTA (Fig. 3 purple and black curves, Fig. 4A-C crosses). In contrast, pro-HlyA was completely unfolded when urea and EDTA were both present at high concentration (Fig. 3 red curve, Fig. 4A-C squares), which indicated that only under these conditions calcium ions are effectively removed from pro-HlyA. Moreover, it can be concluded that the indigenous amount of calcium ions present in the 2xYT growth medium is apparently sufficient to confer stability to the number of pro-HlyA molecules that are secreted.

Urea-denatured pro-HlyA could be refolded to the native state when calcium ions were provided. The spectral properties of secreted pro-HlyA (native) and $Ca^{2+}$-refolded pro-HlyA are indistinguishable (Fig. 3, dark and light blue curves), which indicated that these proteins have identical conformations. On the other hand, when the unfolding and folding behavior of pro-HlyA is compared (Fig. 4E and 5E), it is evident that these processes do not coincide. Under the tested conditions, the unfolding of pro-HlyA showed a single transition phase, which is typical of a two-state unfolding mechanism (N→U). In contrast, the folding of pro-HlyA showed two transition phases, indicative of the presence of a stable folding intermediate (U → I → N). This folding intermediate is characterized by a $\lambda_{max}$ of 338 nm and a $F_{331/350}$ of 1.12 (Table 2). Apparently (some of) the Trp residues of this folding intermediate experience a local environment that is more polar than that observed for pro-HlyA in its native conformation, i.e. in secreted or *in vitro* refolded pro-HlyA (Figs. 4E, 5E).

It is of note that the thermodynamic parameters for the second transition phase for folding in the presence of $Ca^{2+}$ are virtually identical to those of the unfolding reaction (Table 3). These results are in line with the notion that the folding and unfolding of pro-HlyA are reversible processes, even though an intermediate was not observed upon unfolding. The fact that the $Ca^{2+}$-dependent intermediate is not detected, or not formed, upon unfolding might relate to the fact high concentrations of EDTA were required to unfold pro-HlyA, whereas during the folding reaction the concentration of EDTA was low (~ 140 μm).

When folding was performed in buffer lacking calcium ions, pro-HlyA adopted a non-native conformation (Fig. 6A, circles). Such molecules could however be rescued by the addition of $Ca^{2+}$, which indicated that they are not kinetically trapped species but are still competent for folding (Fig. 6A, crosses). Thus, calcium ions drive the folding of pro-HlyA and provide protein stability. Under the tested conditions the folding and unfolding of pro-HlyA apparently occurred via distinct pathways, but remained reversible reactions.

In agreement with previous work on the biological activity of HlyA by Döbereiner *et al* [58] and Ostolaza *et al* [31], we show that $Sr^{2+}$ and $Ba^{2+}$ were, to some degree, able to substitute for $Ca^{2+}$ in the folding of pro-HlyA (Fig. 6B). The native conformations of pro-HlyA refolded in the presence of either $Ca^{2+}$, $Sr^{2+}$ or $Ba^{2+}$ were found to be virtually identical (Table 2). However, the formation of a folding intermediate as observed in the presence of $Ca^{2+}$, could not be detected in the presence of either $Sr^{2+}$ or $Ba^{2+}$ (Fig. 6B, red, green). Thus, the observed folding intermediate is $Ca^{2+}$ specific. On the other hand, $Mg^{2+}$ was unable to drive folding of pro-HlyA (Fig. 6B, blue) suggesting that the RTX repeats cannot bind $Mg^{2+}$.

In support of this view, toxin activity could not be detected when acylated HlyA was folded in the presence of $Mg^{2+}$ [31, 58]. Taken together, it appears that the RTX repeats can only bind cations with a distinct size and particular geometry. Our data also demonstrate that large $Ca^{2+}$ driven structural changes occur beyond the RTX repeats upon binding of $Ca^{2+}$ to these repeats (Figs. 5, 6A).

Conformational changes in the extreme C-terminus were further analyzed with the mutants $HlyA_{AAA}$ and $HlyA_{C807-1024}$, which both harbor Trp914 as a single fluorophore. Both proteins have similar $\Delta G(H_2O)$ and m values for unfolding (Table 3) suggesting that they unfold in a similar fashion. On the other hand, whereas $HlyA_{C807-1024}$ exhibited a $\Delta G(H_2O)$ for folding of -44.12 ± 1.91 kJ/mol, $HlyA_{AAA}$ exhibited a $\Delta G(H_2O)$ for folding of -114.9 ± 21.7 kJ/mol, with a corresponding m value of -17.93 ± 3.4 kJ/molM and $[D]_{1/2}$ of 6.41 M urea. Notably, these latter thermodynamic parameters, are virtually identical to the corresponding values observed for transition (I) of the folding of the wild-type pro-HlyA (Fig. 6E, blue; Table 3). Thus, the folding transition (I) can be attributed to calcium ions induced structural rearrangements at the C-terminus of pro-HlyA, which are sensed exclusively by Trp914.

Considering that $HlyA_{AAA}$ and $HlyA_{C807-1024}$ refold in a different manner, the lack of the N-terminal domain (amino acids 1-806) apparently influenced the folding of the C-terminal domain ($HlyA_{C807-1024}$), resulting in a $\Delta G(H_2O)$ for folding that is more than two-fold lower (Table 3). Taken together the presence of the N-terminal domain has a stabilizing effect on the C-terminal domain of pro-HlyA. The lack of the first three RTX repeats likely contributes to the lower stability of $HlyA_{C807-1024}$ as compared to $HlyA_{AAA}$. However, these results are also in line with the notion that the N-terminal and C-terminal domain of pro-HlyA physically interact.

In this respect, available X-ray structures of various RTX proteins, including the aforementioned AP, the metallo-protease PrtC as well as the lipase LipA [4, 14, 16] show interactions between the N-terminal catalytic domain and the C-terminal RTX domain. For example, for AP it was demonstrated that $Ca^{2+}$ binding induces the folding of the RTX domain, which in turn governs the folding of the protease domain [10]. Interaction of an N-terminal α-helix with the RTX domain subsequently provides high stability to the native AP [10]. Taken together it would be interesting to determine whether RTX toxins such as HlyA have a similar structural arrangement.

To obtain specific information on the folding of the N-terminal part of pro-HlyA the mutant $HlyA_{W914A}$ was generated. Here, the remaining Trp residues (W432, W480 and W579) report on conformational changes within the N-terminal region of pro-HlyA, which is predicted to be predominantly α-helical in nature (Sup. 2). The $\Delta G(H_2O)$ for unfolding of $HlyA_{W914A}$ was determined to be -19.06 ± 1.53 kJ/mol with a corresponding *m* and $[D]_{1/2}$ of 4.33 ± 0.34 kJ/molM and 4.56 M, respectively. These results are in agreement with data for acylated $HlyA_{W914A}$ obtained by Sánchez-Magraner [59], who reported a $\Delta G(H_2O)$ for unfolding of 20.92 ± 0.42 kJ/mol (5.0 ± 0.1 kcal/mol) and a $[D]_{1/2}$ of ~ 4.5 M (here the fraction of unfolded protein was plotted, therefore the positive sign of $\Delta G(H_2O)$). Apparently, acylation does not have an effect on the stability of $HlyA_{W914A}$. However, we demonstrate that $\Delta G(H_2O)$ and *m* for the unfolding of $HlyA_{W914A}$ were significantly lower than those observed for the wild-type (Table 3). Thus, the W914A mutation seems to have a destabilizing effect on pro-HlyA.

The data for the folding of $HlyA_{W914A}$ could be analysed by a two-state model and virtually coincided with the unfolding data (Sup. 5). Indeed, the thermodynamic parameters of the folding reaction of $HlyA_{W914A}$ in the presence of calcium ions were very similar to those observed for the unfolding reaction (Table 3) suggesting that the folding of $HlyA_{W914A}$ is a fully reversible process. Sánchez-Magraner *et al* [59] demonstrated that folded acylated $HlyA_{W914A}$ required at least two times more calcium ions than wild-type HlyA to achieve full lytic activity. Moreover, acylated $HlyA_{W914A}$ did not exhibit a $Ca^{2+}$-dependent increase in Trp fluorescence as was demonstrated for wild-type acylated HlyA [59]. Taken together, these data indicate that W914 not only senses conformational changes upon binding of $Ca^{2+}$ to the RTX repeats, but likely plays itself a role in the folding and/or stability of HlyA (acylated and non-acylated).

The truncated $HlyA_{N1-806}$ contains the same three Trp residues as $HlyA_{W914A}$ that report on folding of the N-terminus, but lacks the C-terminus. $HlyA_{N1-806}$ exhibited a folding behavior that was quite distinct from $HlyA_{W914A}$ but rather similar to that of intact wild-type pro-HlyA refolded in the absence of calcium ions (or presence of $Mg^{2+}$) (Fig. 6A-C). Thus, despite the fact that $HlyA_{N1-806}$ contains three RTX repeats (Fig. 1), it did not exhibit major calcium ions-induced structural changes suggesting that it was less able, or, unable to bind calcium ions. These data suggest an important role of the C-terminus (including the three last RTX repeats) in stabilizing the RTX domain and consequently the folding of the whole protein.

In contrast, $HlyA_{C807-1024}$, which lacks the N-terminus but still contains the terminal three RTX repeats, showed large calcium ion-induced conformational changes upon folding (Fig. 6C). These observations are in line with studies on CyaA produced by *B. pertussis* [60]. For CyaA it was demonstrated that calcium was able to induce folding of RTX repeats in the absence of the natural N-terminal flank, whereas removal of the C-terminal flank interfered with folding [60]. Thus, the C-terminal region downstream the RTX repeats is important for β-roll formation and this likely represents an inherent property of RTX proteins.

A model for the folding of pro-HlyA is shown in Fig. 7 and may contribute to a better understanding of calcium ion-induced folding of RTX proteins upon secretion.

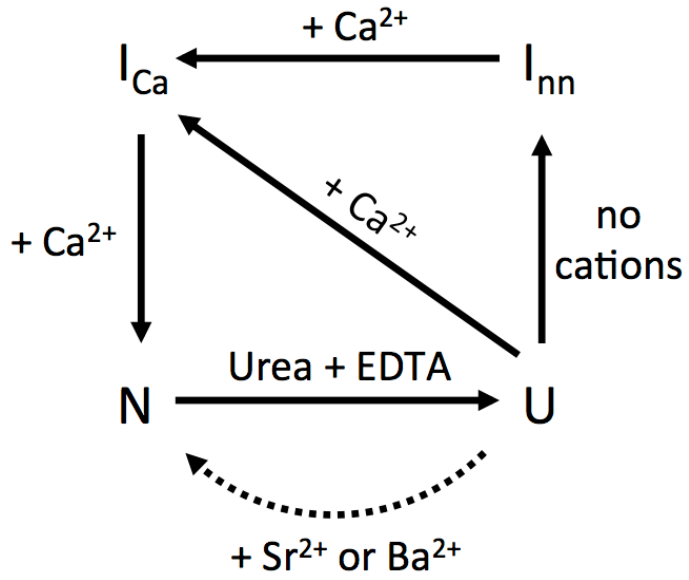


**Fig. 7. Schematic model of the folding pathways of pro-HlyA.**
Observed folding pathways for pro-HlyA (un)folding and the influence of different ions on the folding behavior. I: intermediate, U: unfolded, N: native, nn: non native

## 5. CONCLUSION

Our results indicate that (i) bound calcium ions confer high stability to secreted pro-HlyA; only a combination of high concentrations of urea and EDTA were able to unfold pro-HlyA, (ii) the folding of pro-HlyA is a calcium ions driven process that occurs via a stable calcium ion dependent folding intermediate, (iii) the folding transition observed at high urea concentration could be attributed to calcium ions induced structural changes in the local environment of W914 at the extreme C-terminus of pro-HlyA, and (iv) pro-HlyA folded in the absence of calcium ions remained I a folding competent state. Many, if not most, RTX proteins share a similar domain architecture, as shown by crystallography and structure predictions [13-16, 41]. Whereas the N-terminal region contains the α-helical rich catalytic domain, the C-terminal domain harbors the calcium ion binding RTX repeats and the secretion signal. Such a similar general structure suggests that the folding of these proteins may occur in a similar fashion [14-16]. The calcium ion induced, ordered folding of HlyA could be beneficial in more than one way for *E. coli*. First, it would allow a stable conformation of the native HlyA structure without the help of molecular chaperones. In this respect the RTX domain itself, may be considered as an internal chaperone, as calcium ion binding drives the folding of the entire protein. Moreover, $Ca^{2+}$-driven folding may facilitate secretion, where folding of the RTX domain pulls the protein out of the T1SS to prevent backsliding. In this respect our findings are in agreement with previous works of Sotomayor-Pérez *et al* [61] and Pimenta *et al* [62].

## 6. ACKNOWLEDGEMENTS

We would like to thank Dr. Diana Kleinschrodt and Iris Fey, Protein Production Facility of Heinrich-Heine-University Düsseldorf, for cloning of the pro-HlyA$_{AAA}$ mutant as well as for general technical support. Furthermore, we would like to thank Dr. Christian Schwarz for stimulating discussions. This work was funded by the Jürgen Manchot Graduate School “Molecules of Infection”.

## 7. LITERATURE

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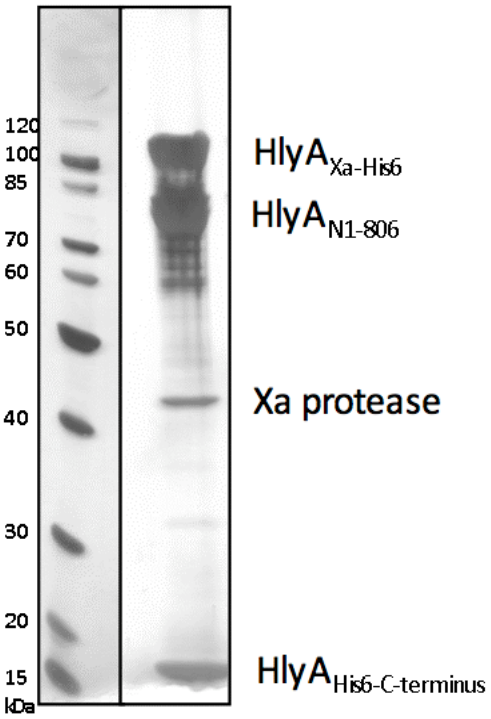
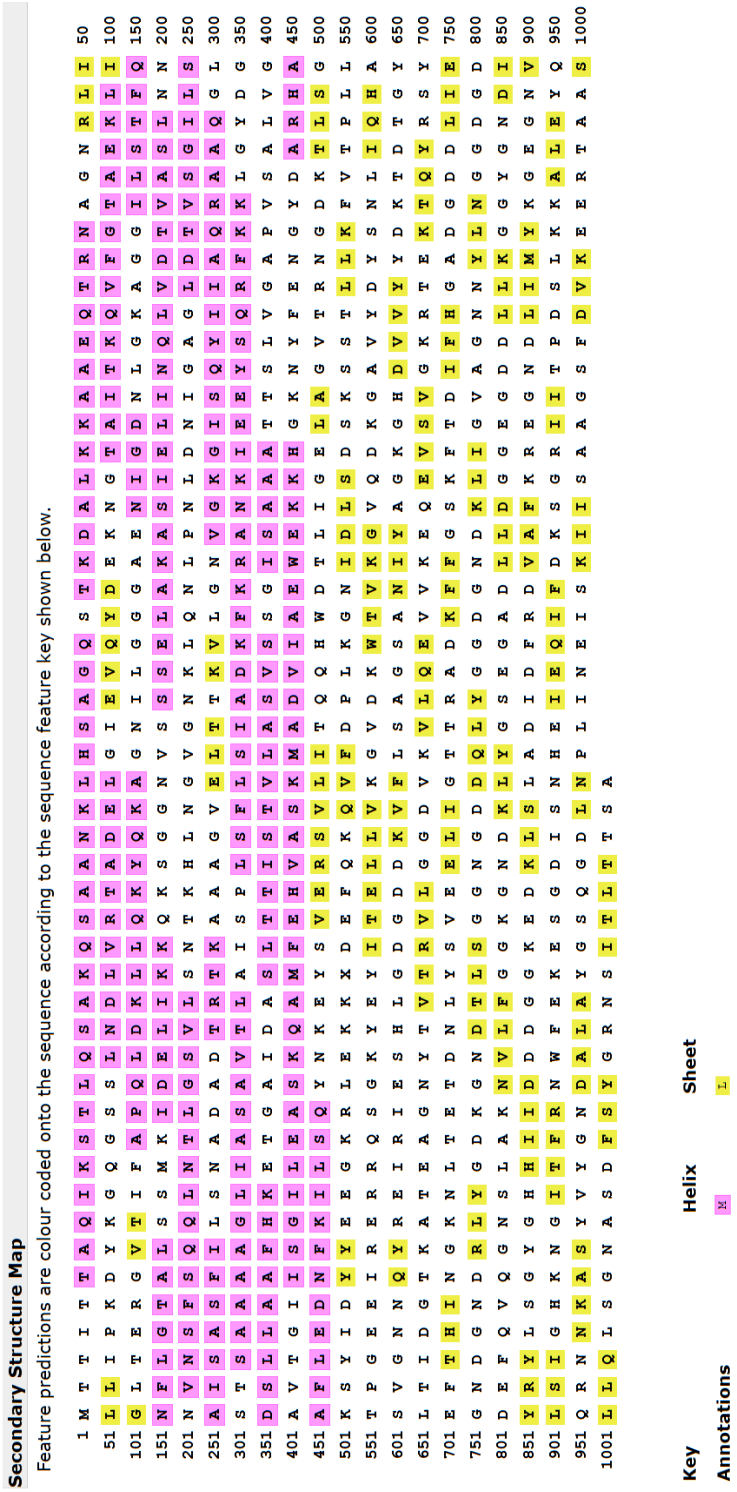
## Supplemental 1:

Summary of an attempt to purify the C-terminal fragment $HlyA_{His6\text{-}C807\text{-}1024}$ (~ 27 kDa) by Factor Xa digestion and IMAC.



## Supplemental 2:

Predicted HlyA secondary structure calculated based on amino acid sequence by Psipred (http://bioinf.cs.ucl.ac.uk/psipred). HlyA shows the characteristic α-helical (N-terminus) and β-strand (C-terminus) features like several other RTX toxins.



## Supplemental 3:

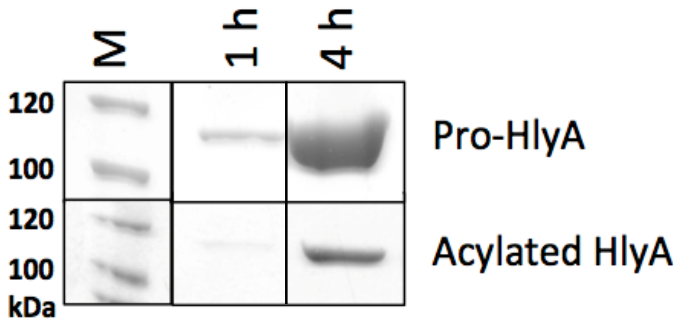
### HlyA Sequence

```
                                                            50
MTTITTAQIK STLQSAKQSA ANKLHSAGQS TKDALKKAAE QTRNAGNRLI
                                                           100
LLIPKDYKGQ GSSLNDLVRT ADELGIEVQY DEKNGTAITK QVFGTAEKLI
                                                           150
GLTERGVTIF APQLDKLLQK YQKAGNILGG GAENIGDNLG KAGGILSTFQ
                                                           200
NFLGTALSSM KIDELIKKQK SGGNVSSSEM AEASIELINQ LVDTVASLNN
                                                           250
NVNSFSQQLN TLGSVLSNTK HLNGVGNKLQ NLPNLDNIGA GLDTVSGILS
                                                           300
AISASFILSN ADADTRTKAA AGVELTTKVL GNVGKGISQY IIAQRAAQGL
                                                           350
STSAAAAGLI ASAVTLAISP LSFLSIADKF KRANKIEEYS QRFKKLGYDG
                                                           400
DSLLAAFHKE TGAIDASLTT ISTVLASVSS GISAAATTSL VGAPVSALVG
                                                           450
AVTGIISGIL EASKQAMFEH VASKMADVIA EWEKKHGKNY FENGYDARHA
                                                           500
AFLEDNFKIL SQYNKEYSVE RSVLITQQHW DTLIGELAGV TRNGDKTLSG
                                                           550
KSYIDYYEEG KRLEKKPDEF QKQVFDPLKG NIDLSDSKSS TLLKFVTPLL
                                                           600
TPGEEIRERR QSGKYEYITE LLVKGVDKWT VKGVQDKGAV YDYSNLIQHA
                                                           650
SVGNNQYREI RIESHLGDGD DKVFLSAGSA NIYAGKGHDV VYYDKTDTGY
                                                           700
LTIDGTKATE AGNYTVTRVL GGDVKVLQEV VKEQEVSVGK RTEKTQYRSY
                                                           750
EFTHINGKNL TETDNLYSVE ELIGTTRADK FFGSKFTDIF HGADGDDLIE
                     1         2                    3    800
GNDGNDRLYG DKGNDTLSGG NGDDQLYGGD GNDKLIGGAG NNYLNGGDGD
                   4                   5          6     850
DELQVQGNSL AKNVLFGGKG NDKLYGSEGA DLLDGGEGDD LLKGGYGNDI
                                                           900
YRYLSGYGHH IIDDDGGKED KLSLADIDFR DVAFKREGND LIMYKAEGNV
                                                           950
LSIGHKNGIT FRNWFEKESG DISNHQIEQI FDKSGRIITP DSLKKALEYQ
                                                          1000
QRNNKASYVY GNDALAYGSQ GDLNPLINEI SKIISAAGSF DVKEERTAAS
                          1024
LLQLSGNASD FSYGRNSITL TTSA
```

**1-6**: First G of nonapeptide sequence (RTX)
Tryptophans: 432, 480, 579, 914
Acylated Lysines: 564, 690

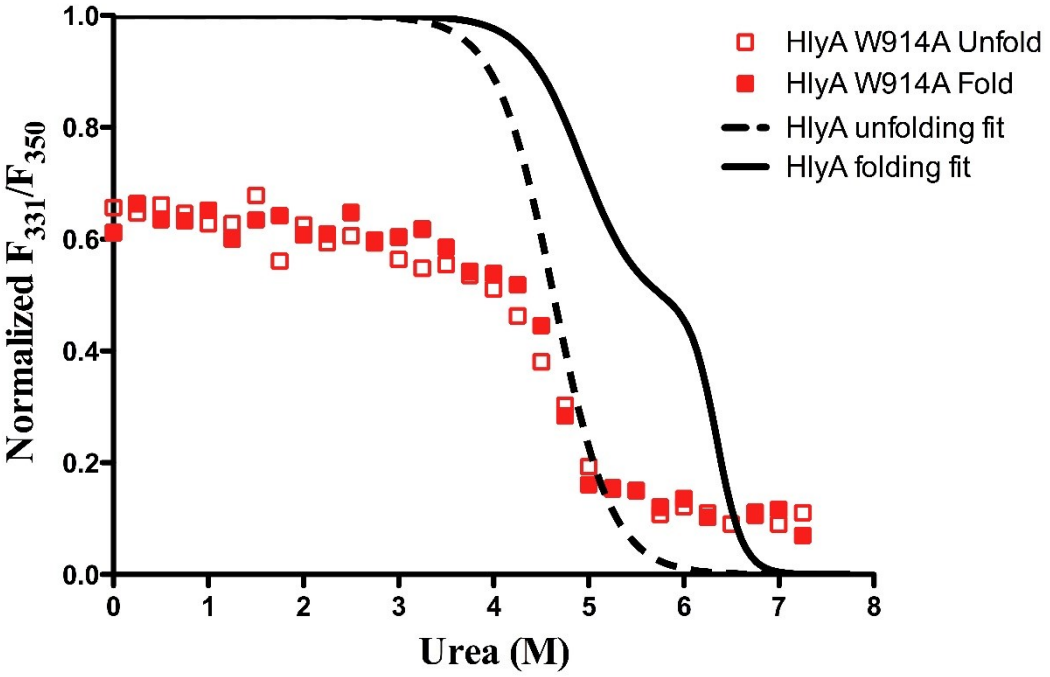
## Supplemental 4:

Differences in the secretion level of pro-HlyA and acylated HlyA



## Supplemental 5:

Folding of $HlyA_{914A}$



## 5.3 Paper III

**Proportionate work on this publication:** 70%

- Design and cloning of the different mutants
- Expression and purification of proteins
- Establishment of the *in vitro* acylation assay
- Data analysis
- Writing of the manuscript

**Published in:** Biological Chemistry

(*to be submitted*)

**Impact factor:** 2.7

# A simplified *in vitro* acylation assay based on optimized HlyA and HlyC purifications

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**RUNNING TITLE**

*In vitro* HlyA acylation assay

**ABSTRACT** (111 words)

HlyA is a toxin secreted by uropathogenic *Escherichia coli* strains. HlyA belongs to the repeats in toxin protein family and needs (i) a posttranslational, fatty acylation at two internal lysines by the acyltransferase HlyC and (ii) extracellular ion binding to achieve its active conformation. Both processes are not fully understood and experiments are often limited due to low protein amounts. Here, we present an optimized purification protocol for the proteins involved in HlyA activation as well as a quick and non-radioactive assay for *in vitro* HlyA acylation. These may simplify future experiments e.g. activity scanning and characterization of HlyA or HlyC mutants as demonstrated with single and double HlyA lysine mutants.

**KEYWORDS**

RTX toxin, ion binding, hemolytic activity, UPEC, erythrocytes

## INTRODUCTION

The toxin HlyA is a virulence factor secreted by uropathogenic *Escherichia coli* strains (Gentschev *et al.,* 2002). It belongs to the large family of repeats in toxin (RTX) proteins that are characterized by the specific nonapeptide sequence GGxGxDxUx (Linhartova *et al.,* 2010). Here X represents any amino acid residue while U is a large hydrophobic amino acid (Baumann *et al.,* 1993). HlyA is secreted by a Type I Secretion System (T1SS) out of the *E. coli* cell in one step across both membranes (Gentschev *et al.,* 1992; Schulein *et al.,* 1992; Wagner *et al.,* 1983). This T1SS is composed of the ABC transporter HlyB, the membrane fusion protein HlyD and the outer membrane protein TolC that build the secretion complex (Thomas *et al.,* 2013). Due to the restricted size of the TolC channel (Koronakis *et al.,* 2000) HlyA is secreted in an unfolded conformation and folds only after secretion (Bakkes *et al.,* 2010). The folding is initiated upon binding of calcium ions to the RTX repeats and is achieved by a higher calcium ion level in the extracellular space compared to the *E. coli* interior (Baumann *et al.,* 1993; Koronakis *et al.,* 2000; Lilie *et al.,* 2000). Like some other RTX toxins HlyA is initially produced as a nontoxic protoxin, pro-HlyA. To become lytically active a posttranslational modification, specifically a fatty acid acylation at Lys564 and Lys690 with fatty acids of 14, 15 or 17 carbon atoms in length (Lim *et al.,* 2000) is necessary prior to secretion. This acylation is performed by the acyltransferase HlyC in concert with the ~9 kDa *E. coli* acyl carrier protein (ACP) (Issartel *et al.,* 1991). The exact mechanism of this acylation process is still controversial as both, a ternary complex formation of acylACP/HlyC/pro-HlyA (Stanley *et al.,* 1991) as well as a ping pong mechanism (Worsham *et al.,* 2001), have been reported. Additionally, it is unclear how the two acylation sites are recognized since no obvious sequence

homology is observed between both sites (Langston *et al.,* 2004). However, it is known that the acylation is not essential for HlyA secretion (Thomas *et al.,* 2013).

So far detailed studies on HlyA activation were hampered for example, due to the low amounts of purified HlyA and HlyC. Here we present optimized purification protocols for unacylated HlyA (pro-HlyA) and mutants thereof as well as for HlyC. Furthermore, a simplified *in vitro* acylation assay was established. This may provide a basis for further studies of HlyA activation or analysis of HlyA and HlyC mutations.

## RESULTS

### Purification of HlyA and HlyA mutants

The co-expression of HlyA and the inner membrane components of the T1SS, HlyB and HlyD, respectively, from plasmids pSU-*hlyA* and pK184-*hlyBD* (Figure 1A, top) resulted in the successful secretion of pro-HlyA and the lysine mutants pro-HlyA$_{K546A}$, pro-HlyA$_{K690A}$ and pro-HlyA$_{K546A/K690A}$ into the supernatant (Figure 1B). All secreted proteins were already quite pure, as exemplarily shown for pro-HlyA and Coomassie Brilliant Blue (CBB) staining (Figure 2A). The secretion levels of pro-HlyA and pro-HlyA$_{K564A}$ were nearly identical (Figure 2B, upper panels) in contrast to pro-HlyA$_{K690A}$ and pro-HlyA$_{K564A/K690A}$, which showed a reduced secretion level (Figure 2B, lower panels). An anti-HlyA western blot raised against the C-terminus of HlyA confirmed that the secreted proteins were indeed HlyA (data not shown). The proteins could be further purified from the supernatant by concentration and subsequent size exclusion chromatography (SEC, for further details see Materials and Methods). The obtained pro-HlyA proteins were > 98% pure as judged from CBB stained SDS-PAGE gels, could be concentrated up to 8-10 mg/ml and were stable at 4 °C for at least several days (data not shown). Although pro-HlyA$_{K690A}$ and pro-HlyA$_{K564A/K690A}$ showed a slightly reduced secretion level, no differences in the purity or final concentration were observed.

The plasmid combination of pSU-*hlyC*-His/*hlyA* and pK184-*hlyBD* led to the secretion of active HlyA toxin due to the encoded acyltransferase HlyC (Figure 1A, bottom). Although the experimental setup was the same, the secretion levels of the active proteins were drastically reduced compared to the pro-toxin forms (Figure 2C, compare upper and middle panel). All mature HlyA proteins were secreted in

comparable amounts (data not shown). For the activity assay, the supernatants 4 h post induction were used as the amount of HlyC decreased after this time point (Figure 2C, bottom). However, there was a clear difference in their toxicity level (Figure 2D). When cultivated on agar plates containing 5% sheep blood a clear halo formation was visible for HlyA (Figure 2D, upper left corner). Compared to this the halo of $HlyA_{K546A}$ was reduced by more than 75% (Figure 2D, lower left corner) and the halo for $HlyA_{K690A}$ was nearly not detectable (Figure 2D, upper right corner). As expected the double mutant $HlyA_{K564A/K690A}$ showed also no halo formation (Figure 2D, lower right corner).

**Purification of HlyC**

The purification of HlyC was performed from the cell pellet of *E. coli* transformed with plasmids pSU-*hlyC*-His/*hlyA* and pK184-*hlyBD*. Cells were harvested after 4 h of protein expression. Beyond this time point, the HlyC concentration began to decrease. The C-terminal $His_6$-tag allowed an easy, one-step immobilized metal ion affinity chromatography (IMAC) purification of HlyC. Protein containing fractions were determined by SDS-PAGE and CBB staining (Figure 2E, second left panel) and protein identity was verified by anti-His western blotting (penta-His antibody, protocol according to the QIAGEN manual) (Figure 2E, middle panel). Two bands for HlyC were detectable at ~ 20 kDa and ~50 kDa, indicating a HlyC monomer and dimer (Figure 2E, left and middle panel). After removal of imidazole by dialysis, the protein could be concentrated to ~ 2 mg/ml and stored in glycerol at -80 °C until further use. The addition of DTT during dialysis prevented dimer formation and only the ~ 20 kDa monomer band was detectable in the purified protein (Figure 2E, right panel). Surprisingly, the estimated size of HlyC of nearly 27 kDa was not detected.

An electro spay ionization (ESI) mass spectrometric (MS) analysis of the purified protein detected a mass of 21.7 kDa (data not shown). A closer analysis of the DNA sequence revealed a potential, additional ribosome-binding site around 80 nucleotides 3´ to the start codon (Artemis; Rutherford *et al*, 2006). A transcription start at this position would result in a protein with a calculated mass of 21.6 kDa, which is similar to the detected mass by MS and observed protein bands by SDS-PAGE. Therefore, the actual HlyC protein starts not at the 1$^{st}$ but at the 5$^{th}$ methionine given in the HlyC UTI89 sequence (Chen *et al.,* 2006). This is also in agreement with our findings that an N-terminal HlyC His-tag could never be detected by anti-His western blotting, although lysis was clearly visible on the Columbia blood agar plates (data not shown).

***In vitro* acylation assay**

To mimic the *in vivo* situation for the *in vitro* acylation reaction, the purified and folded pro-HlyA protein was first unfolded in urea and bound calcium ions were removed by EDTA treatment (see Materials and Methods). The unfolded pro-HlyA protein was then pre-incubated with the purified acyltransferase HlyC and a crude DH5α *E. coli* cell extract as ACP donor prior to activity analysis. The obtained hemolytic activity was determined by cell lysis of sheep blood cells, specifically hemoglobin release, and plotted as percentage of a SDS sample used as a positive control, which was set arbitrarily to 100%. The missing calcium ions necessary for HlyA folding and activity were hereby provided with the sheep blood cells that were washed and dissolved in buffer containing $CaCl_2$. The *in vitro* acylation of unfolded pro-HlyA resulted in active, toxic HlyA as evident by hemoglobin release in levels comparable to that of the positive control (Figure 3).

To analyze if the protein conformation played a role during pro-HlyA acylation, folded pro-HlyA was also tested for *in vitro* acylation by HlyC and ACP. However, when folded pro-HlyA was assessed no hemolysis was detectable. Apparently, HlyC is not able to modify pro-HlyA in its folded conformation that also does not occur in the *E. coli* cytoplasm. Cell lysis was a clear result of pro-HlyA activation by HlyC and not of a contamination in either of the reaction components as no negative control showed any cell lysis (Table 1).

Furthermore, the influence of different divalent ions on HlyA activity was tested. Therefore, sheep blood cells were washed in buffer containing either calcium, strontium, barium or magnesium ions as ion source. Pro-HlyA was unfolded and *in vitro* acylated as described in Materials and Methods, split and added to the differently washed blood cells. The highest HlyA activity was obtained with blood cells washed in buffer containing calcium ions (99%, ± 1%). In contrast, when erythrocytes were washed with strontium or barium ions, HlyA activity decreased to 83% (± 14%) and 22% (± 13%), respectively. No hemolytic activity was detected when magnesium ions were used in the preparation step (Figure 3, Table 1).

When the unfolded, purified pro-HlyA lysine mutants were analyzed for *in vitro* activation no hemolytic activity could be detected in any mutant or in a combination of the mutants pro-HlyA$_{K564A}$ and pro-HlyA$_{K690A}$ (data not shown). Comparable results were observed when the supernatant of the HlyA lysine mutants was analyzed (data not shown). Only the supernatant containing secreted HlyA wild type protein showed hemolytic activity of nearly 100% (99%, ± 2%). An overview of the activity of all analyzed proteins, mutants and controls is given in Table 1.

## DISCUSSION

The important step prior to *E. coli* HlyA secretion is the fatty acid acylation at lysines K564 and K690, carried out by the unique acyltransferase HlyC together with ACP (Issartel *et al.,* 1991). Although secretion and cell binding do not dependent on HlyA acylation, the protein shows no lytic behavior in the absence of the acyl chains (Hyland *et al.*, 2001; Ludwig *et al.*, 1996; Moayeri *et al*., 1997; Sanchez-Magraner *et al.*, 2006). Even though HlyA activation is a crucial step for HlyA activity, the exact activation mechanism and recognition of the acylation sites remains unclear (Langston *et al.*, 2004; Stanley *et al.,* 1991; Worsham *et al.,* 2001). Here, we present an easy purification of the proteins involved in the acylation step as well as an easy *in vitro* assay for detection of hemolysis. Those may be used in future experiments for quick and easy scanning of HlyC or HlyA mutants to identify important amino acids and to solve the still remaining question regarding HlyA activation.

### Purification of proteins

The purification of pro-HlyA and the pro-HlyA lysine mutants was achieved by secretion of the proteins into the supernatant and subsequent purification by SEC. The obtained protein were pure, could be concentrated to high concentrations and remained stable for at least several days.

An identical experimental setup allowed the secretion of mature HlyA and mutants. However, the amount of secreted HlyA compared to pro-HlyA was significantly reduced (Figure 2C, compare top and middle panel) and upon further purification protein aggregation was observed (data not shown). These results are in agreement with findings of Ostolaza *et al,* (1995) and Nicaud *et al,* (1985) who also reported an

enhanced aggregation and degradation tendency of the active toxin. Therefore, the supernatant 4 h post IPTG induction was chosen for activity analyses in the assay.

In contrast to Guzman-Verri *et al,* (2001), we were able to detect higher amounts of HlyC in an *E. coli* culture that co-expressed HlyA compared to a situation in which only HlyC was expressed in a pET28b vector (data not shown). However, we also observed that the amount of HlyC only increased in the first 4 h after IPTG induction before decreasing (Figure 2C, bottom panel). Therefore, HlyC expressing cells were harvested 4 h post induction and HlyC was purified by IMAC. The obtained protein was > 98% pure as shown by SDS-PAGE and CBB staining (Figure 2E, right panel). Surprisingly, the estimated size of HlyC of nearly 27 kDa was not detected. Instead, two bands were detected during HlyC expression and purification at 20 kDa and 50 kDa. The 50 kDa band however vanished after treatment of the protein with DTT indicating that it could be a cysteine based HlyC dimer (Issartel *et al.,* 1991; Ludwig *et al.,* 1996). Further analysis revealed that the detected HlyC protein has indeed a size of 21.7 kDa (data not shown) in agreement with a transcriptional start at the 5$^{th}$ methionine of the HlyC UTI89 sequence (Chen *et al.,* 2006).

***In vitro* acylation of pro-HlyA and hemolytic assay**

When pro-HlyA was *in vitro* acylated it became evident that the protein conformation played a crucial role. While *in vitro* acylation of unfolded pro-HlyA resulted in nearly 100% activity, *in vitro* acylation of folded pro-HlyA led to no hemoglobin release. Apparently, HlyC is not able to modify pro-HlyA in its folded conformation that also naturally does not occur in the *E. coli* cytoplasm. These results are however in contrast to results of Hardie *et al,* (1991) who reported the successful activation of secreted and folded pro-HlyA. The successful *in vitro* activation of pro-HlyA also indicated that the C-terminal $His_6$-tag of HlyC had no influence on pro-HlyA activation.

Furthermore, the influence of different ions on HlyA activity was measured and an activity decrease could be observed in the order $Ca^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$ (100% > 83% > 22% > 0%, Table 1). These results are in agreement with previous work by Ostolaza *et al,* (1995) and Dobereiner *et al,* (1996) on mature HlyA activity. Therefore, our *in vitro* acylated pro-HlyA behaved identical to active HlyA refolded in the presence of these ions.

No hemolysis was visible when the lysine mutants (K564A, K690A, K564A/K690A) of unfolded pro-HlyA were acylated *in vitro*. This is in line with results obtained for the same HlyA mutants acylated *in vivo* (Table 1). This was, with the exception of the double mutants, unexpected. Lysis was expected, due to the activity of this protein on blood plates (Figure 2D, lower left corner), at least for the K564A mutant. These results could be explained for example by the lower protein concentrations used in this assay compared to the higher concentrations in the areas surrounding the bacterial colonies upon constant secretion, suggesting a drop in affinity in the K564A mutant. Interestingly, although the K564A mutation significantly reduced HlyA activity, the

K690A mutation had an even more severe effect on activity. These results are in agreement with work of Stanley *et al,* (1994) who also reported a nearly complete loss (K564R) and a complete loss of activity (K690R and K564R/K690R) for HlyA. However, it was also reported that K564 was more important for the thermodynamic stability of the acylation process than K690 and that K564 was necessary for effective K690 acylation making both acylation sites equally important (Langston *et al.,* 2004; Worsham *et al.,* 2005). A complementation attempt of both single lysine mutants also showed no hemolytic activity indicating that neither lysine mutation could be complemented by another HlyA molecule not affected by a mutation in one of the two lysine residues (Table 1).

In summary, we report an easy purification protocol of secreted pro-HlyA and pro-HlyA lysine mutants out of the supernatant as well as HlyC out of the bacterial cell via IMAC purification. All proteins were essentially pure and, in the case of the pro-HlyA proteins, concentrated to high concentrations. We also present a quick, non-radioactive based *in vitro* acylation assay with crude *E. coli* extract as ACP donor, which has not been described so far. Evaluation of *in vitro* activation can be quantitatively measured using spectrophotometry and hemoglobin release following sheep blood lysis. The high amounts of obtained protein and the simple assay may facilitate further studies to analyze HlyA and HlyC mutants as well as the reaction mechanism underlying HlyC driven HlyA activation.

## MATERIALS AND METHODS

### Cloning procedures

Origin of all cloning steps was the plasmid pSU-*hlyA* (Soloaga *et al*, 1996; Uniprot P08715) (Figure 1A, top). HlyC (NCBI: YP_543861.1 (Chen *et al.,* 2006)) was synthesized with an additional TEV protease cleavage site (GeneScript) and was first cloned into the pET28b vector by *Nde*I and *Xho*I digestion. It was subsequently cloned into pSU-*hlyA* via In-Fusion reaction according to the user manual (Clontech). The resulting plasmid therefore carried HlyC, with a C-terminal TEV protease cleavage site and a $His_6$-tag, in 5´-position to HlyA (pSU-*hlyC*-His/*hlyA*) (Figure. 1A, bottom). The HlyA mutations $HlyA_{K564A}$, $HlyA_{K690A}$ and the double mutant $HlyA_{K564A/K690A}$ were inserted by site directed mutagenesis in pSU-*hlyA* (pro-HlyA) and pSU-*hlyC*-His/*hlyA* (HlyA) (Figure. 1B). All primer pairs used in this study are listed in Table 2.

### Protein expression and purification

*E. coli* BL21 (DE3) cells were transformed with plasmid pK184-*hlyBD* (Bakkes *et al.,* 2010) encoding the transporter proteins HlyB and HlyD and a pSU plasmid encoding pro-HlyA, HlyA or one of the lysine mutants. Screening for successfully transformed cells was performed on LB-agar plates (pro-HlyA and mutants) or on Columbia blood agar plates (HlyA and mutants) with 5% sheep blood (BD, 254005) supplemented with the appropriate antibiotics. The blood plates were additionally supplemented with 20 mM $CaCl_2$. If not stated otherwise all protein purification steps and storing were performed at 4 °C.

*Purification of pro-HlyA, HlyA and mutants*

For the purification of pro-HlyA and mutants usually 3 x 150 ml 2YT medium in 300 ml baffled flasks were inoculated to an $OD_{600}$ of 0.05 with an over night culture. Cells were cultivated at 37 °C with 180 rpm agitation until an $OD_{600}$ of 0.4 to 0.6 before induction with 1 mM IPTG (final concentration). Four hours post induction, cells were removed by two subsequent centrifugation steps (8,000 x g, 15 min and 200,000 x g, 75 min) and the supernatant was filtered (0.45 µm, Supor-450 membrane filter, Pall) and concentrated to 5 ml (Amicon Ultra-15 Centrifugal Filter Units, 100 kDa MWCO; Millipore). The concentrate was applied to a SEC column (Superdex 200 16/60 column; GE Healthcare) in buffer containing 50 mM Tris-HCl pH 7.4 and 50 mM NaCl. Purity of the protein fractions was determined by SDS-PAGE following CBB or silverstaining. Protein containing fractions with a purity larger 95% were pooled and concentrated up to 8 mg/ml as described above. The final protein concentration was measured spectrophotometrically (Nanodrop-1000, Thermo Scientific) with the calculated masses and an extinction coefficients provided in Table 2 and calculated based on the primary structure (http://web.expasy.org/protparam/). Usually > 7 mg/ml were obtained per liter culture per $OD_{600}$.

The expression and secretion of HlyA and mutants was similar to pro-HlyA. After secretion and cell removal the supernatant was filtered and split to aliquots. The aliquots were frozen in liquid nitrogen and stored at -20 °C until further use.

*Purification of HlyC*

For the purification of HlyC-$His_6$, 3 x 2 L 2xYT medium in 5 L baffled flasks were inoculated to an $OD_{600}$ of 0.1 with an over night culture of *E. coli* cells containing plasmids pK184-*hlyBD* and pSU-*hlyC*-His/*hlyA*. Cells were cultivated at 37 °C at 180 rpm and protein expression was induced with a final concentration of 1 mM IPTG at an $OD_{600}$ of 0.4 – 0.6. After 4 h at 37 °C cells were harvested by centrifugation (8,000 x g, 10 min). The cell pellet was resuspended in buffer containing 150 mM Tris-HCl pH 7.4, 300 mM NaCl and 10% glycerol (buffer A). After addition of a spoon-tip DNase I (bovine; Sigma-Aldrich) cells were disrupted by three passages through a cell disruptor (2.5 bar, TS 0.75, Constant Systems). Non-disrupted cells and cell debris was removed by two consecutive centrifugation steps at 30,000 x g (10 min) and 200,000 x g (60 min) respectively. The supernatant was loaded onto 3 x 5 ml $Ni^{2+}$ loaded HiTrap chelating HP columns (GE Healthcare) equilibrated with buffer A. After intensive washing (> 20x column volumes) proteins were eluted by a 125 ml gradient of 0 - 100% puffer B (buffer A plus 500 mM imidazole) at a flow of 1 ml/min. Purity of the protein fractions was determined by SDS-PAGE and CBB staining. Protein containing fractions with a purity larger 95% were pooled and dialyzed over night against a buffer containing 20 mM HEPES pH 8.5, 300 mM NaCl, 1 mM DTT and 10% glycerol. The dialysis tube had a MWCO of 6-8 kDa (Spectra/Por1, Order No. 132660, Spectrumlabs) and the buffer volume was chosen so that the final imidazole concentration was below 2 mM. After dialyses, the sample was centrifuged to remove precipitated protein (3,000 x g, 10 min). Finally the supernatant was concentrated with Amicon Ultra-15 Centrifugal Filter Units (10 kDa MWCO; Millipore) to 2 mg/ml. Protein concentration was determined by a Bradford assay

(Thermo Scientific). The purified protein was mixed 1:2 with 100% glycerol, split to aliquots, frozen in liquid nitrogen and stored at -80 °C until further use.

*Enrichment of ACP*

ACP was obtained by inoculation of 2 L 2YT medium in a 5 L baffled flask with an *E. coli* DH5α over night culture. After inoculation to an $OD_{600}$ of 0.05 cells were grown 5 h at 37 °C with 180 rpm agitation. Cells were harvested by centrifugation (8,000 x g, 10 min), resuspended in 75 ml buffer (150 mM Tris-HCl pH 7.4, 150 mM NaCl) and finally disrupted (3 passages at 2.5 bar, TS 0.75, Constant Systems). Undisrupted cells and cell debris were removed by centrifugation (30,000 x g, 10 min followed by 200,000 x g, 60 min). The supernatant was split to aliquots, frozen in liquid nitrogen and stored at -20 °C until further use.

**Washing of sheep blood cells**

Defibrinated sheep blood was obtained from Oxoid. 1 ml of cells were transferred into 1.5 ml reaction tubes and centrifuged for 1 min at 14,000 g. The supernatant was removed and cells were resuspended in buffer containing 10 mM Tris-HCl pH 7.5, 155 mM NaCl, 20 mM $CaCl_2$, 5 mM KCl and 2 mM $MgSO_4$ by 5-10 sec vortexing intervals. This process was repeated until the supernatant remained colorless. The resuspended cells were transferred into a 50 ml falcon tube to guarantee equal cell distribution and immediately used for the *in vitro* assay. When the effect of different ions on pro-HlyA activation was studied, the $CaCl_2$ in the washing buffer was replaced by $SrCl_2$, $BaCl_2$ or $MgCl_2$. All steps were performed at room temperature.

**Unfolding of pro-HlyA**

Purified pro-HlyA and pro-HlyA lysine mutants were unfolded in 6 M urea and 20 mM EDTA and the final protein concentration was adjusted to 1 mg/ml. Unfolding was achieved over night at 4 °C.

***In vitro* acylation assay**

For the *in vitro* assay, the HlyA (or mutant) supernatants and HlyC were thawed on ice. The crude ACP solution was thawed at 23°C and incubated for 2 h at the same temperature after 40 mM EDTA addition (final concentration). For the *in vitro* acylation reaction, 10 µl purified pro-HlyA proteins, 1 µl HlyC (both 1 mg/ml) and 64 µl ACP were mixed via rapid dilution and incubated for 1 h at 23 °C. After incubation, 500 µl washed sheep blood cells were added and incubated for 30 min at 37 °C. Cells were removed by 1 min centrifugation at 14,000 x g and 200 µl of the supernatant transferred to a 96-well plate. The hemoglobin amount in the supernatant was determined on a FLUOstar OPTIMA (BMG Labtech) with an excitation wavelength of 544 nm. The obtained hemolysis was calculated relative to a SDS control (1 µl of 16% SDS solution plus 74 µl sheep blood wash buffer) set arbitrarily as 100%. As controls, pro-HlyA/ACP and HlyC/ACP were analyzed and instead of the third reaction component only the appropriate buffer was added. For analysis of the secreted HlyA proteins, 10 µl supernatant and 65 µl sheep blood wash buffer were added to 500 µl blood and further treated as indicated above. As negative control the same amount of 2xYT medium was utilized. For a possible complementation analyses the purified proteins pro-$HlyA_{K564A}$ and pro-$HlyA_{K690}$ were mixed in equal amounts (both 5 µl) and treated as described for the single proteins. Additionally a combination of the supernatants $HlyA_{K564A}$ and $HlyA_{K690A}$ was also tested for lytic activity.

**ACKNOWLEGEMENTS**

We thank Agnes Ulfig and Marcel Lagedroste for assistance in the HlyC purification as well as Dr. Diana Kleinschrodt and Iris Fey from the Protein Production Facility, Heinrich Heine University Düsseldorf, for general technical support. Furthermore, we would like to thank Dr. Christian Schwarz, Dr. Britta Ries and the group of Prof. Brötz-Oesterhelt (Heinrich Heine University, Düsseldorf), especially Heike Goldbach-Gecke for general support. This work was funded by the Jürgen Manchot Graduate School "Molecules of Infection".

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| **Pro-HlyA $Ca^{2+}$** | **Pro-HlyA $Sr^{2+}$** | **Pro-HlyA $Ba^{2+}$** | **Pro-HlyA $Mg^{2+}$** |
|---|---|---|---|
| 99% (± 1%) | 83% (± 14%) | 22% (± 13%) | n.a |
| **Pro-HlyA$_{K564A}$** | **Pro-HlyA$_{K690A}$** | **Pro-HlyA$_{K564A/K690A}$** | **Pro-HlyA$_{K564A}$ + Pro-HlyA$_{K690A}$** |
| n.a | n.a | n.a | n.a |
| **HlyA$_{K564A}$** | **HlyA$_{K690A}$** | **HlyA$_{K564A/K690A}$** | **HlyA$_{K564A}$ + HlyA$_{K690A}$** |
| n.a | n.a | n.a | n.a |
| **HlyA** | **Folded pro-HlyA** | **HlyC/ACP** | **Pro-HlyA/ACP** |
| 99% (± 2%) | n.a | 1% (± 1%) | n.a |

**Table 1:**

Hemolytic activity of secreted and *in vitro* acylated pro-HlyA proteins. Pro-HlyA and pro-HlyA lysine mutants were *in vitro* acylated and measured for hemolytic activity with different ions. Furthermore the activity of secreted HlyA and mutants was analyzed as outlined in Materials and Methods. n.a: no activity observed under the experimental setup.

| Protein | Mw (kDa) | Extinction Coefficient ($M^{-1}$ $cm^{-1}$) | Source/Primer |
|---|---|---|---|
| Pro-HlyA | 110.15 | 81600 | Uniprot entry: P08715, plasmid encoded (Soloaga *et al.,* 1996) |
| HlyC | 25.01 | 52035 | NCBI: YP_543861.1, *E. coli* UTI89 (Chen *et al*, 2006) |
| HlyC-$His_6$ | 26.93 | 53525 | HlyC-TEV synthesized from GeneScript cloned into pET-28b via *Xho*I and *Nde*I restriction sites |
| HlyA | 110.15 | 81600 | pSU_fw: 5´-taagaggtaattaaatgacaacaataa-3´<br>pSU_rv: 5´-agctcgaattcgtaatcatg-3´<br>pET28b_fw: 5´-ttacgaattcgagctatgaatgctttttttatgttaatgt-3´<br>pET28b_rv: 5´-tttaattacctcttatcagtggtggtggtggtggtgctcg-3´ |
| Pro-$HlyA_{K564A}$ and $HlyA_{K564A}$ | 110.09 | 81600 | $pSU_{K564A}$fw: 5´-cgtatgaatatattaccgagttattagtca-3´<br>$pSU_{K564A}$rv: 5´-ttccggactgcctcctt-3´ |
| Pro-$HlyA_{K690A}$ and $HlyA_{K564A}$ | 110.09 | 81600 | $pSU_{K690A}$fw: 5´-cgagaactgaaaaaacgcaatatc-3´<br>$pSU_{K690A}$rv: 5´-ctccgactgaaacctcctgctcctt-3´ |
| Pro-$HlyA_{K564A/K690A}$ and $HlyA_{K564A/K690A}$ | 110.03 | 81600 | generated from the K564A mutants with the primers indicated for pro-$HlyA_{K690A}$ |

**Table 2:**

Cloning and characterization of mutants used in these studies. fw: forward; rv: reverse

**Figure 1:**

(A) Genetic organization of the HlyA constructs used in this study. For pro-HlyA and mutants only HlyA is present in the pSU plasmid (top). For the expression of acylated HlyA (HlyA) the acyltransferase HlyC was cloned in 5´-position to HlyA. HlyC additionally carried a C-terminal TEV protease cleavage site and a $His_6$-tag (bottom). (B) Lysine mutations of HlyA. The RTX domain as well as the C-terminal secretion signal (SEC) are shown. Important amino acids like lysine (K) and alanine (A) are highlighted.

**Figure 2:**

(A) Pro-HlyA containing supernatant, 1 to 6 h post IPTG induction highlighting protein purity. (B) Secreted lysine mutants of pro-HlyA, in comparison to wild type pro-HlyA, 1 to 4 h post IPTG induction with CBB staining. (C) Secreted proteins from pro-HlyA (upper panel) or HlyA (middle panel) and anti-His western against HlyC-$His_6$ containing cell pellets (lower panel), 1 to 6 h post induction. (D) Columbia blood agar plate harboring colonies of wild type HlyA and HlyA lysine mutants. (E) Purification of HlyC-$His_6$ out of the cell pellet by IMAC.

**Figure. 3:**

Hemolytic activity of *in vitro* acylated and native HlyA proteins. Shown are the median and the standard deviation of three individual experiments.

**Figure 1.**


A
Pro-HlyA
hlyA
HlyA
hlyC
TEV
His6
hlyA
B
HlyA
N
K
K
RTX
SEC
C
HlyAK564A
N
A
K
RTX
SEC
C
HlyAK690A
N
K
A
RTX
SEC
C
HlyAK564A/K690A
N
A
A
RTX
SEC
C

**Figure 2.**

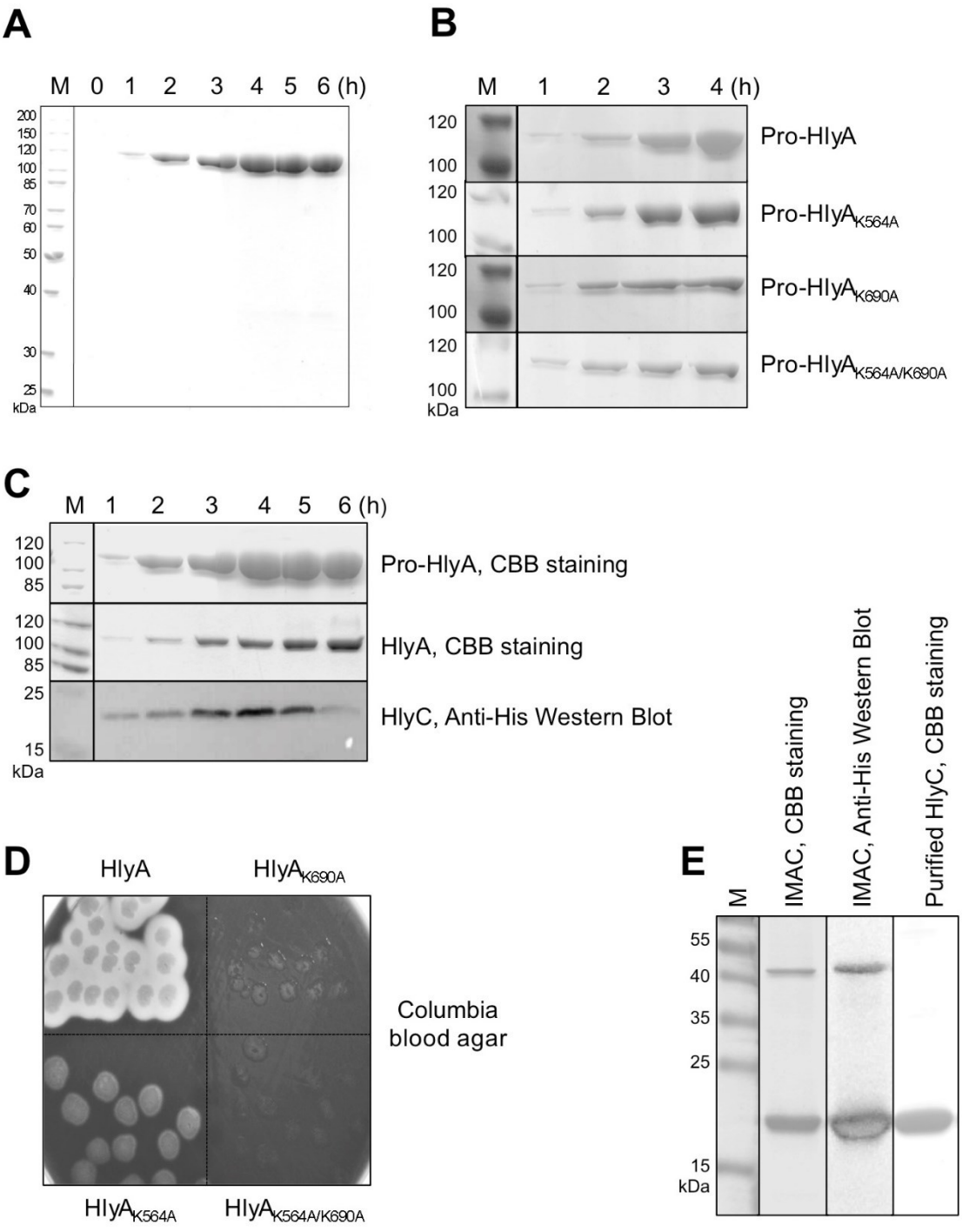
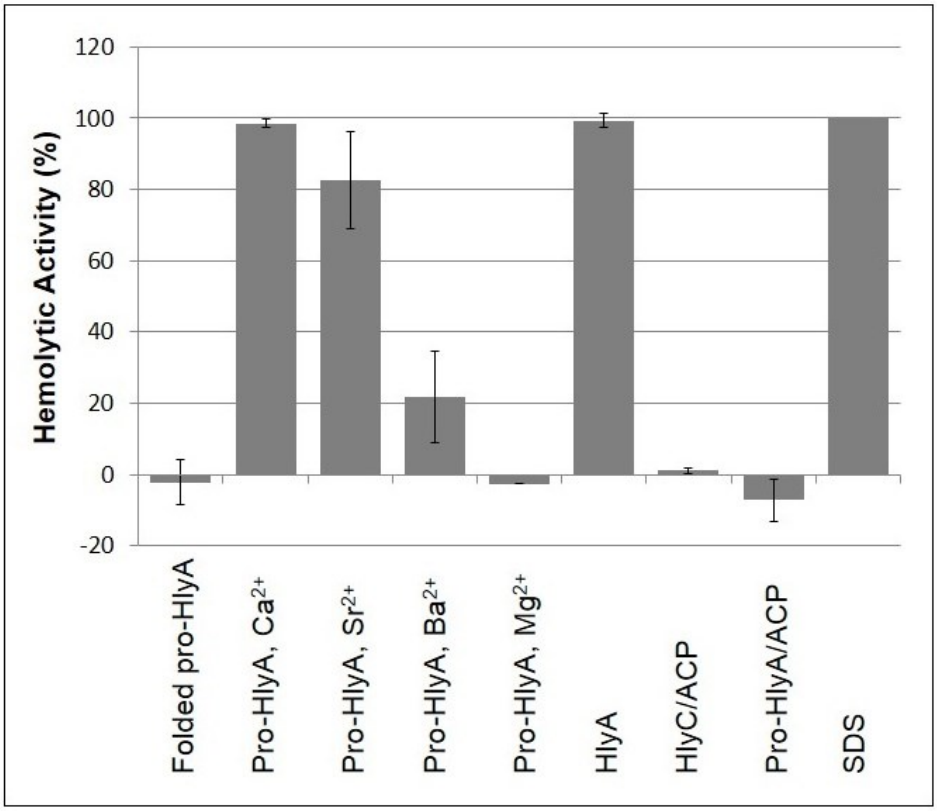

A
M 0 1 2 3 4 5 6 (h)
200
150
120
100
85
70
60
50
40
30
25
kDa
B
M 1 2 3 4 (h)
120
100
Pro-HlyA
Pro-HlyA$_{K564A}$
Pro-HlyA$_{K690A}$
Pro-HlyA$_{K564A/K690A}$
kDa
C
M 1 2 3 4 5 6 (h)
120
100
85
25
15
kDa
Pro-HlyA, CBB staining
HlyA, CBB staining
HlyC, Anti-His Western Blot
D
HlyA
HlyA$_{K690A}$
HlyA$_{K564A}$
HlyA$_{K564A/K690A}$
Columbia blood agar
E
M
IMAC, CBB staining
IMAC, Anti-His Western Blot
Purified HlyC, CBB staining
55
40
35
25
15
kDa

**Figure 3.**


Hemolytic Activity (%)
120
100
80
60
40
20
0
-20
Folded pro-HlyA
Pro-HlyA, $Ca^{2+}$
Pro-HlyA, $Sr^{2+}$
Pro-HlyA, $Ba^{2+}$
Pro-HlyA, $Mg^{2+}$
HlyA
HlyC/ACP
Pro-HlyA/ACP
SDS

## 5.4. Crystallography of pro-HlyA

One aim of this PhD thesis was the crystallization of pro-HlyA to determine its 3D structure. Therefore, HlyA was purified in high amounts in a pure form. Pro-HlyA was expressed in BL21 (DE3) *E. coli* cells using a two-plasmid combination, thereby harboring HlyB and HlyD for secretion of HlyA by its native T1SS into the culture supernatant. After successful secretion, the HlyA containing supernatant of the expression culture was concentrated and further purified SEC. For details regarding protein purification please refer to chapter 5.2.

To minimize the formation of salt crystals during crystallography, a low salt buffer (50 mM Tris pH 7.4, 50 mM NaCl) was chosen for SEC. A typical HlyA purification is shown in Figure 8.

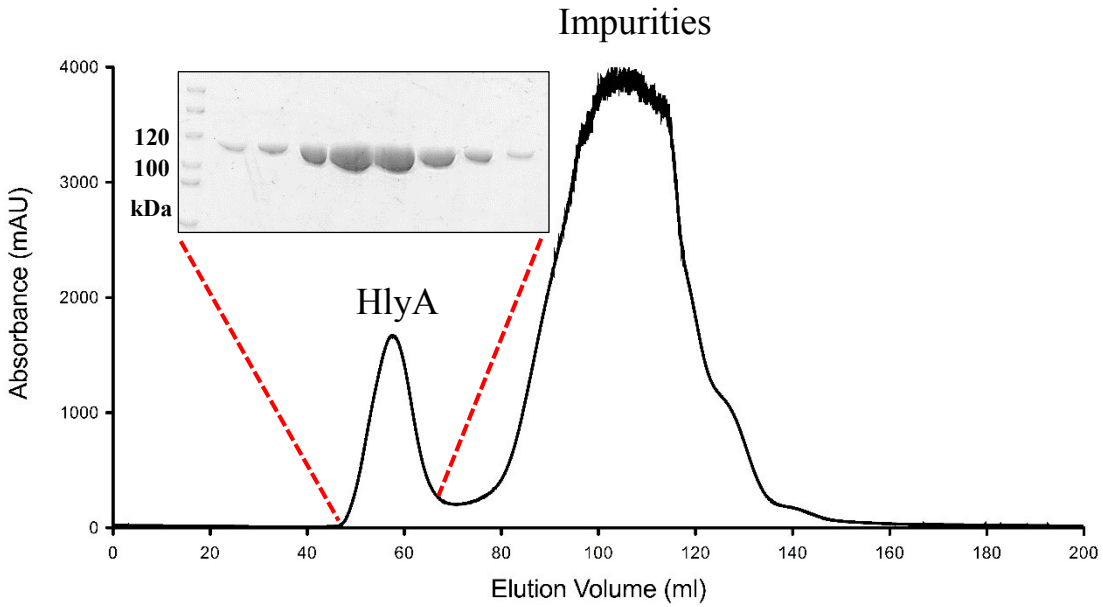



***Figure 8: Size exclusion chromatogram of pro-HlyA***
SEC of concentrated supernatant containing pro-HlyA with a Superdex 200 16/60 column (GE Healthcare). The first peak contains the desired, 110 kDa pro-HlyA in a pure form, as evidenced by SDS-PAGE and CBB staining (inset). The first lane of the SDS gel shows the protein standard the other lanes contain 8 µl each of the collected SEC fractions (indicated by dotted red lines).

Pure protein fractions were subsequently concentrated to a protein concentration of 6.5 mg/ml and used for crystallization.

According to the user's manual of the Superdex 200 16/60 column (GE Healthcare) and compared to standard proteins, an elution peak around 60 ml was comparable with a protein mass of approximately 440 kDa, indicating a HlyA tetramer. Because determining the mass of a protein by SEC is error-prone [162], as it is also dependent on the shape of the protein, a possible HlyA oligomerization was further addressed by Blue Native PAGE and Multi-angle Light Scattering (MALS). The results are shown in Figure 9.

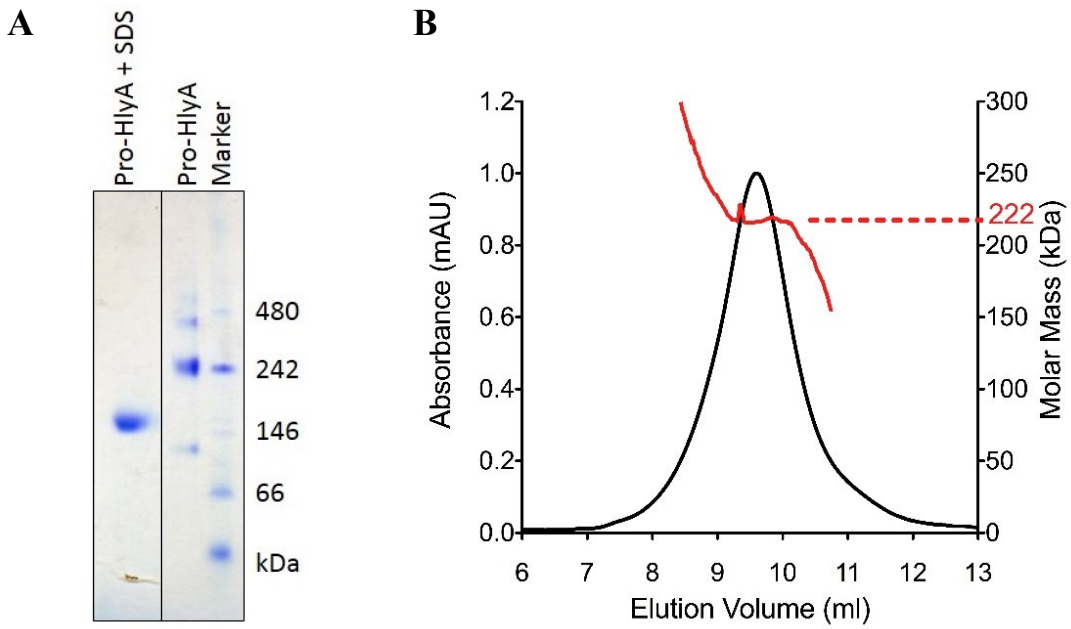



***Figure 9: Oligomeric forms of pro-HlyA***
Pro-HlyA was purified and concentrated as described above and analyzed for possible oligomer formation. A) Blue Native PAGE of pro-HlyA (6.5 mg/ml) with and without SDS. B) Multi-angle Light Scattering (MALS) of pro-HlyA in 50 mM Tris pH 7.4 and 50 mM NaCl on a Superdex 200 10/300 GE Healthcare column. Shown are the UV absorbance (black) and detected molecular mass (red).

When pro-HlyA was analyzed by Blue Native PAGE oligomer bands were detected at ~ 242 kDa and ~ 480 kDa. In comparison with the HlyA monomer obtained by treating HlyA with SDS, these signals indicate possible dimers or tetramers (Figure 9A). When pro-HlyA was analyzed by MALS a molecular mass of approximately 222 kDa was detected supporting dimer formation of the 110 kDa monomer HlyA (Figure 9B). However the dimer observed by MALS was transient as more often masses around 170-180 kDa were detected indicating a mixed population of monomer and dimer. Similar results were obtained when pro-HlyA was analyzed by mass spectrometry (MS).

Despite the apparently mixed pro-HlyA species first crystallization trials were performed. Using the *hanging drop* method crystal drops were set with the Hydra instrument and incubated in Rock Imager at 12°C. Two crystal conditions were found and initial optimization was performed. Pro-HlyA crystals and their corresponding diffraction spectra are shown in Figure 10.

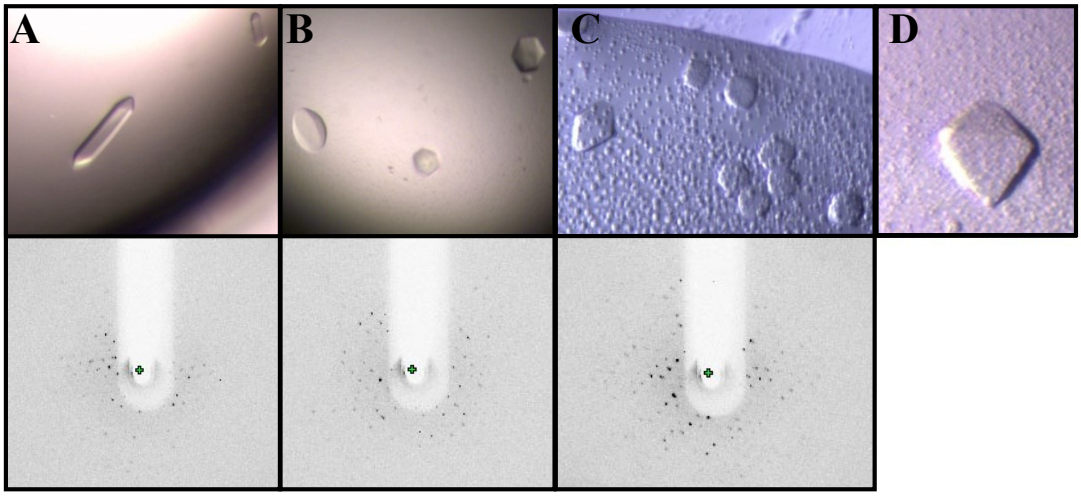



***Figure 10: Crystals of pro-HlyA***
A) Complex Suite, H12, 36d, 12 °C. B) JCSG Core Suite IV, F5, 36 d, 12 °C. C) Optimization of B., 4 month, 18 °C. D) Zoom of a crystal from the same drop as C. The conditions in A and B show *sitting drops* while C shows *hanging drops*. The crystals were analyzed for diffraction and showed 25 Å for A and B as well as 15 Å for C (bottom images; ESRF, Grenoble).

One crystallization condition was obtained with the Qiagen screen Complex Suite, H12 containing 0.1 M NaCl, 0.1 M Tris pH 8.0, 15% (v/v) Ethanol and 5% (v/v) MPD. Crystal growth was observed after 4-5 weeks at 12°C, but crystals diffracted only to 25 Å (Figure 10A). An optimization trial by variation of pH, NaCl and Ethanol concentration as well as temperature remained fruitless and no crystals were obtained.

The second crystal condition was obtained using the JCSG Core Suite IV screen (Qiagen). Condition F5 containing 0.1 M HEPES pH 7.0 and 30% Jeffamine M-600, showed initial crystals after 3-4 weeks (Figure 10B) that diffracted to about 25 Å. This condition was further optimized by variation of the pH, Jeffamine M-600 concentration and temperature. A decrease in temperature to 4°C abolished crystal grows while an increase to 18°C resulted in the crystals shown in Figure 10C. Furthermore, an increase in crystal size was observed with decreasing Jeffamine M-600 concentration. The final conditions shown in Figure 10C were 0.1 M HEPES pH 7.4 with 20% Jeffamine M-600 and the resolution increased to 15 Å.

Additional buffer optimization as well as the addition of additives might enhance the resolution of diffraction of the crystals to one that is suitable for structure determination. However, this will require further experiments.

# 6. DISCUSSION

UPEC strains are the major cause of UTI worldwide and millions of people, particularly females, suffer from this disease [1, 2, 5]. A variety of virulence factors make UPEC extremely persistent and the increase in antibiotic resistant strains in the recent years gives cause of concern [12, 14, 16]. A detailed understanding of the virulence mechanisms of UPEC strains could lead to new, more specialized treatments in the future to cure UTI. This includes e.g. the understanding how UPEC effector proteins are secreted and, like in the case of HlyA, obtain their active conformation upon extracellular ion binding and subsequent folding. An inhibition of this mechanism by specialized medication would eliminate some of UPECs main virulence factors.

One of the main virulence factors of UPEC is the toxin HlyA that can lyse a number of different cell types thus affecting the host immune response and enable the bacteria to proceed to the blood stream [10, 12, 13]. A number of steps are necessary for the generation of active HlyA, including a posttranslational modification by the acyltransferase HlyC and calcium ion induced protein folding upon unfolded secretion by a T1SS [38, 47, 48]. Although many aspects of these mechanisms are understood there are quite a few questions that up to date remained unsolved (see section 4, Aims).

In this PhD thesis an approach is made to gain a molecular understanding of HlyA toxin activity. The thesis is based on three major projects (i) the analyses of HlyA folding upon secretion and the role of calcium ions during this step (chapter 5.2), (ii) the establishment of an easy *in vitro* acylation assay using purified HlyC and HlyA (chapter 5.3) and (iii) the crystallography of HlyA to solve the 3D structure of the protein (chapter 5.4)

## 6.1 Folding of HlyA

To study the folding characteristics of HlyA intrinsic tryptophan fluorescence was performed. Fluorescence spectroscopy of proteins in general is based on the fluorescent properties of the three aromatic amino acids tryptophan, phenylalanine and tyrosine, mediated by their conjugated double bonds and π-electrons [163]. Out of those three residues tryptophan has the highest quantum yield and the further advantage that it naturally occurs only in low numbers in proteins [163, 164]. Tryptophan fluorescence emission can be selectively studied by using excitation wavelengths ≥ 295 nm [163]. The emission wavelength varies from 300 – 350 nm depending on the environment the tryptophan residue encounters [163, 165]. The hydrophobic amino acids are hidden from the aqueous surface in a folded protein and therefore have an emission wavelength around 300 – 310 nm. In contrast to this an exposed tryptophan residue emits around 350 nm equally to a tryptophan in an aqueous solution [163, 165]. Taken together the folding state of a protein can be monitored by analyzing the emission wavelength of tryptophan residues.

### 6.1.1 Optimized purification of HlyA

To analyze the folding of HlyA by intrinsic tryptophan fluorescence experiments large amounts of pure protein were necessary. The first step during this thesis was therefore the optimization of the expression, secretion and purification of HlyA. For this a plasmid combination was chosen that allowed the expression of either the pro-toxin form (pro-HlyA) or the mature toxin (HlyA) in its natural organism *E. coli* and subsequent secretion into the extracellular space.

The subsequent purification of HlyA was therefore simplified as the protein could be purified from the culture supernatant lacking the usual contaminations of other proteins that arise when proteins are purified out of the bacterial cell. In a single SEC step the remaining impurities could be removed and the protein concentrated up to 10 mg/ml.

Here, a significant difference was observed as pro-HlyA was secreted in levels ten times higher compared to HlyA (see Figure 11). Furthermore HlyA had an increased tendency to aggregate during the further purification process a result previously reported by several other groups [61, 75, 122, 166].

Hence pro-HlyA was chosen for the folding experiments because the lacking acylation does not affect secretion and, more importantly, membrane binding indicating that the overall calcium ion induced folding process for pro-HlyA and HlyA is identical [44, 60, 92, 167, 168].

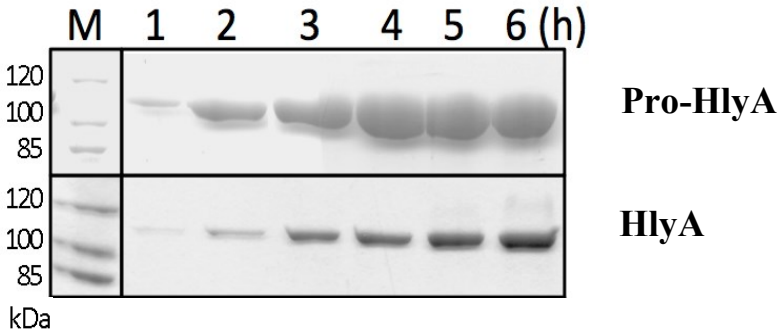



***Figure 11: Secreted levels of pro-HlyA and HlyA***
*E. coli* BL21 cells were transformed with a two plasmid combination to allow either the secretion of pro-HlyA or HlyA. Shown are the supernatants 1 to 6 h post protein production with 1 mM IPTG. Modified from chapter 5.3.

### 6.1.2 The folding and stability of HlyA depends essentially on calcium ions

Several folding experiments including different HlyA mutants and experiments with different ions were performed and are presented in detail in chapter 5.2.

The folding analysis clearly shows that the folding of the whole protein is driven by calcium ions and that calcium ion bound proteins were surprisingly stable. As long as calcium ions were bound to pro-HlyA it was impossible to unfold the protein even in the presence of 8 M urea. An even more crucial role of calcium ions was observed during the refolding process as the protein could only refold when $Ca^{2+}$ (or to a lesser extent $Ba^{2+}$ or $Sr^{2+}$) was present. One step of the two-step refolding process could be identified as calcium ion dependent folding of the C-terminus, using a triple tryptophan mutant where only the C-terminal tryptophan remained thus representing the folding of this protein part.

Moreover the folding of the C-terminal part influenced the folding of the remaining protein. Although deletion mutants of the N-, and C-terminus possess three of the six RTX repeats each, only the C-terminal part could fold normally. This indicates that the N-terminal part could either not bind $Ca^{2+}$ at all, or was not able to build the β-roll structure. This is in agreement with findings for another RTX protein, CyaA. For CyaA it was reported, that for correct folding, C-terminal fragments beyond the RTX-repeats were needed [169].

Furthermore, calcium ions induced folding of the C-terminus could also be involved in efficient secretion of the whole RTX protein where the remaining protein is pulled out of the T1SS by the folding process. Such a "ratchet" mechanism was proposed by Linhartova *et al* [34] and Sotomayor-Perez *et al* [169] and is in agreement with results from Pimenta *et al* [170] who could show that secretion is enhanced at higher $Ca^{2+}$ levels. As Pimenta *et al* pointed out, it would be beneficial for *E. coli* to couple the final secretion step to calcium ion binding therefore ensuring correct protein folding [170].

The high stability that we could see in the full length pro-toxin in the calcium ion bound form, compared to the single domain mutants, may also be a result of an N-C-terminal interaction, namely a cap-structure between the extreme N-terminus and the RTX repeats. The cap-structure "locks" the RTX repeats and therefore shields the calcium ions from external influences. Such a capping was proposed by Zhang *et al* [37] for the alkaline protease from *P. aeruginosa* and could be verified by the crystal structures ([29] PDB: 1KAP; [171] PDB: 1AKL).

A cap is also visible in several 3D structures of other RTX proteins as well as serralysins that, apart from their specific zinc-binding sequence, also possess the characteristic RTX repeats [172]. A few examples for 3D structures showing this cap are: LipA ([32] PDB: 2QUB), PrtC ([31] PDB: 1K7I) Serratia protease ([173] PDB: 1SRP), psychrophilic protease ([174] PDB: 1H71) and cold adapted AP ([175] PDB: 1OMJ (without EDTA) and 1OM7 (with EDTA)).

Figure 12A shows an alignment of the solved 3D structures of the metallo protease from *Serratia marcescens* ([30] PDB: 1SAT) and the alkaline protease from *P. aeruginosa* ([29] PDB: 1KAP) as well as a predicted pro-HlyA structure (Figure 12B).

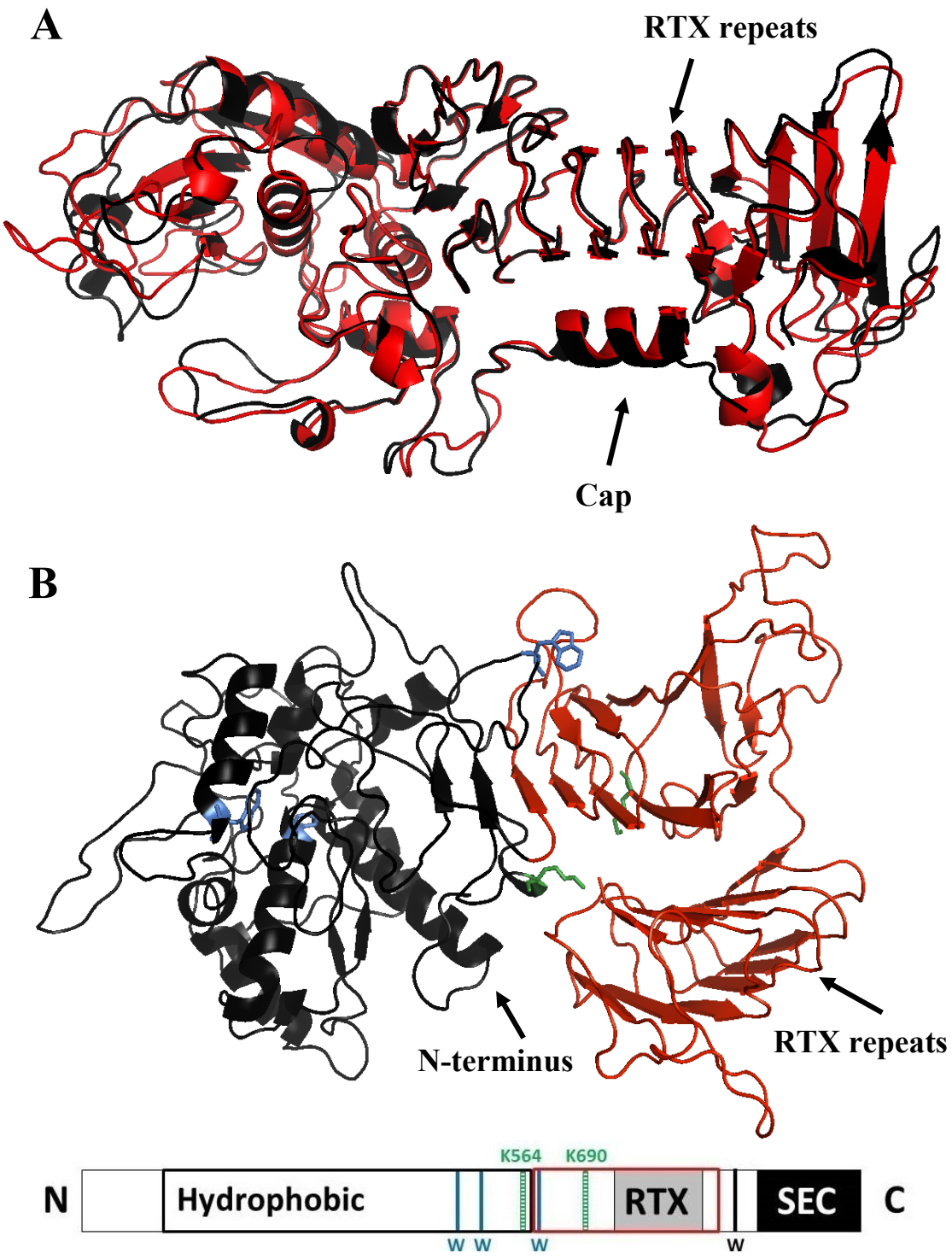



***Figure 12: Crystal structure of 1SAT and 1KAP and predicted HlyA structure***
A) Alignment of the crystal structures of the alkaline protease from *P. aeruginosa* (black) and the *S. marcescens* metalloprotease (red) ([30] PDB: 1SAT; [29] PDB: 1KAP). The RTX repeat domain and the N-terminal cap structure are labelled. B) Top: Predicted HlyA structure calculated by amino acid sequence with Phyre2 [176] (amino acids 257-875). HlyA shows the characteristic α-helical (N-terminus, black) and β-strand (C-terminus, red) features like several other RTX toxins. Shown in blue are the first three tryptophans and in green the acylated lysines. Bottom: Schematic HlyA model highlighting the represented amino acids in the structure prediction. RTX: repeats in toxin, SEC: secretion signal, W: tryptophan, K: lysine.

It is clearly visible that HlyA also shows the separation between the α-helical N-terminus and a β-stranded C-terminus. Unfortunately not all amino acids could be predicted but it is visible that the N-terminal α-helix lays in close proximity to the β-strands therefore making a cap-structure for HlyA possible as well. The cap would then lock the calcium ions present in the RTX repeats, thus stabilizing the whole HlyA protein and explaining the high stability observed during the folding experiments. A capping structure would also be in agreement with a high thermo stability in the ion bound form [177]. Additionally Sánchez-Magraner *et al* [99] could show, that in a separated C-terminal protein fragment all calcium ions could be exchanged, in contrast to full length HlyA [75]. Ion exchange in HlyA could only be achieved during partially denaturing conditions of 3 M urea [178]. Interestingly in the folding experiments, unfolding of pro-HlyA also occurs slightly above 3 M urea whereas unfolding of $HlyA_{C807\text{-}1024}$ starts significantly earlier (see chapter 5.2). It is conceivable that pro-HlyA unfolding is first initialized by the slow dissolving of the cap-structure by urea concentrations up to 3 M, followed by EDTA induced calcium ion binding, thus destabilizing the remaining protein.

### 6.1.3 Is folding influenced by HlyA oligomerization?

A further explanation for the high thermodynamic parameters measured for pro-HlyA refold could be the parallel formation of a HlyA dimer. HlyA dimers or higher oligomers have been previously suspected by several researchers e.g. by Ludwig and co-workers [94] who successfully restored HlyA activity by co-expression of otherwise non-lytic HlyA mutants. So far however no HlyA oligomer could be proven. Interestingly the ΔG values for $HlyA_{C807\text{-}1024}$ are approximately half of that measured for full length HlyA (chapter 5.2). If the possible region for HlyA oligomerization is located in the N-terminal protein part a dimer-, oligomer formation would not be possible with this mutant and therefore ΔG would be halved. An oligomerization per se is possible as previously shown by Blue Native PAGE, MALS (Figure 9) and MS analysis. In theory it is also possible that HlyA oligomerization is based on the cap structure when the α-helical, N-terminal domain of one HlyA monomer interacts with the RTX domain of the second monomer and *vice versa*. Such an interaction would also be affected by protein deletions affecting the termini. A potential oligomerization was also addressed in the *in vitro* HlyA acylation assay.

## 6.2 *In vitro* assay for HlyA acylation

The established *in vitro* assay allowed the *in vitro* acylation of HlyA with the purified HlyA, HlyC and ACP proteins. The assay furthermore provides quantitative measurements in a quick and easy experimental set up without the necessity of radioactive labelling or detection. For detailed information regarding protein purification, HlyA mutants and experimental set up please refer to chapter 5.3.

HlyA and single or double lysine mutants were isolated from the supernatant. The two remaining proteins necessary for pro-HlyA acylation, HlyC and ACP, were purified from *E. coli* cells. HlyC was purified over the attached $His_6$-tag while ACP was a crude cell extract. Interestingly the expression of soluble HlyC was enhanced when a co-expression with HlyA was performed in contrast to a HlyC expression alone. A subsequent concentration of HlyC was possible up to 2 mg/ml while further concentration attempts resulted in protein aggregation. An initial crystallization trial with this protein concentration was unsuccessful.

At first, different temperatures were analyzed for the *in vitro* acylation as well as HlyA activity on the sheep blood cells (Figure 13). It is clearly visible that a temperature around 18 °C to 25 °C during acylation was best suited for high levels of HlyA activity. Activation at colder temperatures still led to significant blood lysis but to a lesser extent. The exception was 37 °C incubation temperature. Here visible aggregation occurred in the mixed protein solution and the detected activities were lower. The same observations were made for the temperature effect during cell lysis although no additional decrease was observed at 37 °C. A similar observation for temperature influences on acylation was made by Hardie ad co-workers who also observed higher activity at 4 °C and 20 °C compared to 37 °C and 42 °C [46].

After determination of temperatures leading to most HlyA activity, the influence of the ions $Ca^{2+}$, $Sr^{2+}$, $Ba^{2+}$ and $Mg^{2+}$ on HlyA activity was analyzed. Previous works by Ostolaza *et al* [179] and Döbereiner *et al* [77] showed a decreasing HlyA activity in the order $Ca^{2+} > Sr^{2+} > Ba^{2+}$ while no haemolytic activity was detectable with $Mg^{2+}$, comparable to the *in vitro* results shown in chapter 5.3.

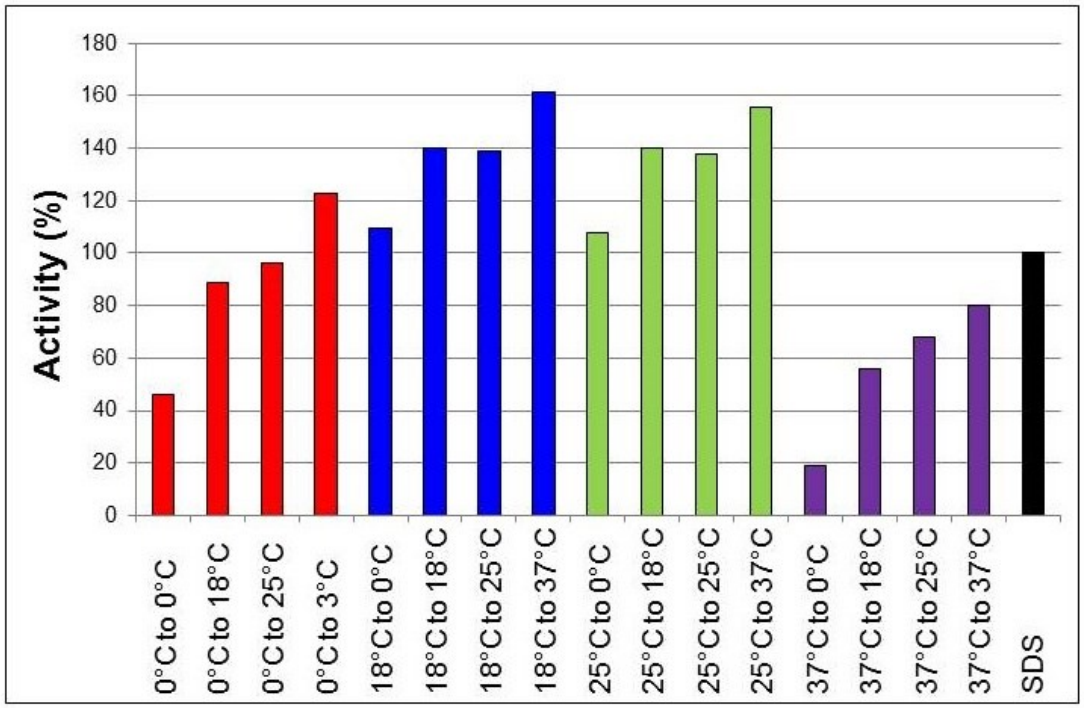



***Figure 13: Temperature effect on HlyA acylation and activity***
Shown is the effect temperature has on HlyA acylation and HlyA activity upon sheep blood cells. The assay was performed as described in chapter 5.3 with the exception that the incubation temperature for acylation (first °C number) as well as the temperature during blood lysis (second °C number) were varied between 0 °C and 37 °C. Haemolysis was compared to a SDS positive control set as 100%.

Interestingly this decrease in activity happens in the same order as the refolding curves of these ions differ from the native calcium ion curve (see chapter 5.2). This could indicate that the β-roll is not correctly established with other ions than calcium and this in turn influences the correct folding of the remaining protein. Thus the C-, and N-terminus may not be correctly folded after strontium or barium ion binding and in turn influence the domain mediated adsorption and insertion of the toxin into the host cell membrane [23, 98, 99].

To analyze the importance of the acylated lysines K564 and K690 single and double mutants were cloned, expressed and purified similarly to the wild type pro-HlyA. Furthermore the mutants of HlyA were co-expressed with HlyC to gain naturally folded mutants for comparison with in the *in vitro* acylated ones.

A clear difference was observed between the mutants K564A and K690A. While K564A still showed slight activity no such thing was seen for K690A, a result in agreement with findings of Stanley *et al* [53]. Such an effect however, was only visible by comparison of halo formation around the cells on blood agar plates. When the mutant proteins were analyzed for direct sheep blood lysis (acylated mutants) or *in vitro* acylated, no haemolysis could be detected. When a complementation of both single lysine mutants was tested, again no activity was visible. This is in contrast to previous results by Ludwig *et al* [94] who showed that *per se* complementation of HlyA mutants affecting activity was possible. Maybe a possible dimer formation is not possible with these mutants or the differently acylated lysines can´t complement each other. Finally it could be that the detection limit of the assay is reached and lysis is not strong enough to be visible.

## 6.3 Crystallography of HlyA

Initial crystallography experiments were performed with pro-HlyA (see chapter 5.4). Initial crystals were obtained and first optimization trials performed. Here a shift to higher temperatures (18 °C compared to 12 °C) had a major influence on crystal growth and the size of the crystals increased 4-5 fold. Furthermore the resolution was improved from 25 Å to 15 Å. This is still too low to calculate the 3D structure of HlyA. However there is room for improvement of the tested conditions even if the average time till crystal formation was four months thus making a quick screening impossible. Maybe optimized conditions will also reduce the time for crystal growth.

The low resolution may also be a result of a mixed population of HlyA molecules of different oligomeric state. As shown in chapter 5.4, HlyA could previously be detected as monomers as well as dimers. The diffraction of a protein crystal would be negatively affected if both protein forms are present [180].

A further attempt could be a crystal drop incubation at higher temperatures like 25 °C or even 37 °C. As the *in vitro* assay confirmed, temperature has a major influence on HlyA activity (see chapter 6.2). Maybe HlyA has a higher stability at higher temperatures thus increasing the chances for crystal formation. A higher temperature

would also accelerate crystal growth as evaporation is increased thus limiting the time till the nucleation point is reached [181].

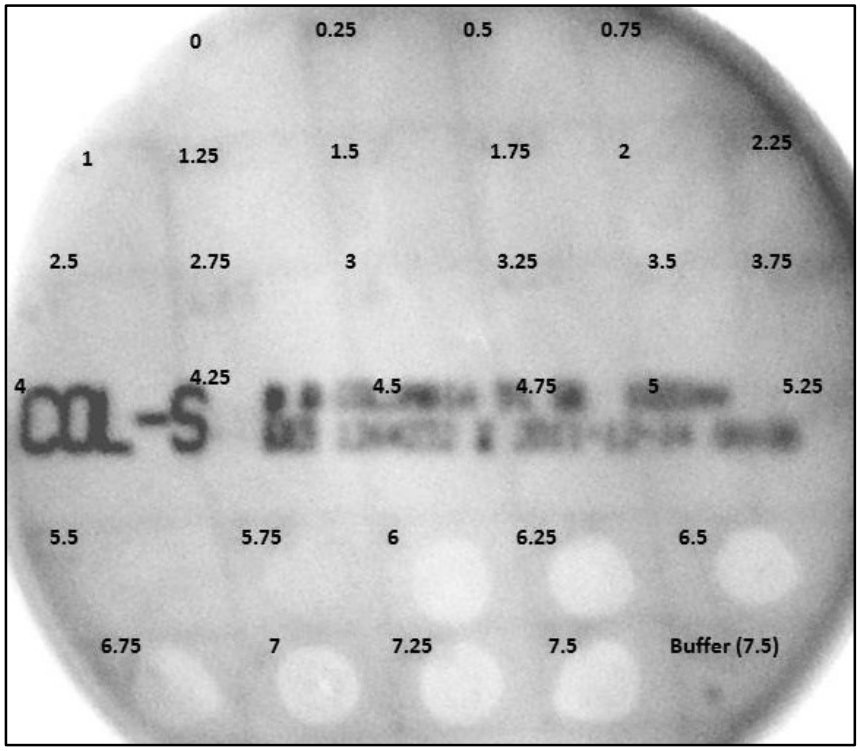
A crystal structure of HlyA would certainly help to understand the molecular functionality of this toxin. It would allow the comparison with other RTX proteins on a structural level and important amino acids could be identified and subsequently mutated and analyzed for function. So far mutations in HlyA were more or less random or based on predicted 3D structures like shown in Figure 12B or, alternatively, secondary structure predictions. Because of the high molecular mass of 110 kDa a 3D structure would allow an educated guess were mutations could be reasonable and help solve the many questions still surrounding HlyA functionality.

## 6.4 Model of HlyA secretion and membrane interaction

Based on the results represented so far the following model of HlyA secretion and membrane interaction is presumed. The C-terminus of HlyA, containing the secretion signal, is secreted first. Upon secretion in the unfolded conformation, due to the limitations of its corresponding T1SS, calcium ions bind to the RTX repeats thus initializing folding of the C-terminus (first refolding step). This in turn induces the correct folding of the remaining protein (and formation of a cap-structure, similar to those observed for other RTX toxins (see Figure 12A; second refolding step). Alternatively the first refolding step is the folding of the HlyA monomer and the second step the formation of a HlyA dimer. Either way, the cap-structure seals the calcium ions and protects them from external influences, leading to a stable HlyA conformation. This conformation can either be established by a HlyA monomer, where N and C-terminus interact with each other, or a HlyA dimer. Hereby the N-terminus of one monomer would interact with the C-terminus of the second monomer and contrary.

It is also possible that the “locked” protein conformation is an inactive conformation as supported by unfolding and refolding experiments of mature HlyA and subsequent analysis on blood agar plates (Figure 14).

**Refolding**




**Unfolding**

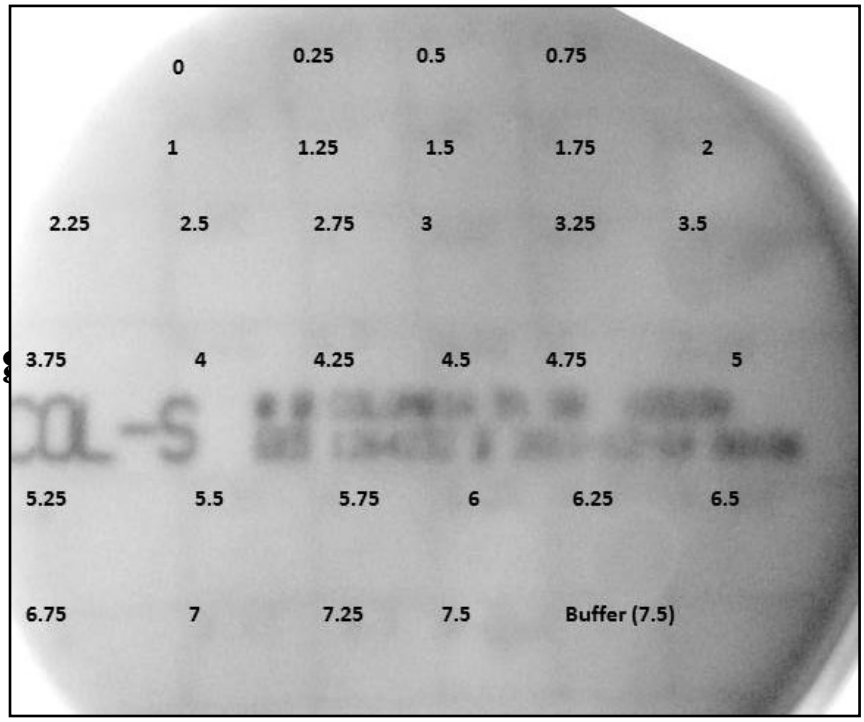



***Figure 14: Folding of mature HlyA analyzed on blood agar plates***
Mature HlyA was unfolded and refolded as described for pro-HlyA in chapter 5.2. For analysis of HlyA activity 20 µl of the (un)folding solutions were transferred to Columbia blood agar plates containing 5% sheep blood. Numbers indicate the corresponding, molar urea concentrations. As negative control a buffer sample with the highest used urea concentration was tested.

When HlyA was refolded on Columbia blood agar plates, lysis was only visible at urea concentrations above 5.5 M. This is surprisingly the same urea concentration that was observed for the intermediate during pro-HlyA refolding. If the cap-structure seals the protein in an inactive conformation, this would further support the assumption that the first refolding step is already the complete folding of the HlyA monomer. The second step would then be the dimer formation that is not possible, and therefore absent, in the HlyA deletion mutants.

Furthermore lysis was strictly calcium ion dependent as unfolding of mature HlyA showed no lysis. These results would be in agreement with the assumption that the cap-structure is disrupted above 3 M urea, exposing the calcium ions to the EDTA containing buffer. The ions are rapidly bound by EDTA, disrupting the RTX repeat domain and thus no blood lysis is visible on the agar plates. This model is further supported by results of Ludwig *et al*, [23] who reported that HlyA activity was increased when the N-terminal, α-helical, amphiphilic region was missing. This may be the result of a missing cap that is unable to seal the RTX repeat domain thus resulting in active, albeit less stable, protein. A closer look at the structure prediction of HlyA (see Figure 12B) shows, that the acylated lysines are rather restricted by the RTX repeats, maybe also indicating an inactive protein conformation.

This inactive protein conformation is maintained until interaction of HlyA with its target membranes. Upon cell binding a structural change is induced in the HlyA toxin that leads to the disruption of the cap-structure thus allowing the protein to obtain a lytic conformation. Target cell recognition is mediated by receptor dependent binding to glycophorin or, according to the latest results, by cholesterol [182]. Both could explain the varying toxicity of HlyA on cells from different organisms as their composition is greatly influenced by the host. According to Vasquez *et al* [182] HlyA oligomerization and pore formation is also favored at higher concentrations facilitated by protein accumulation around the receptors. A model for HlyA secretion and membrane interaction is given in Figure 15.

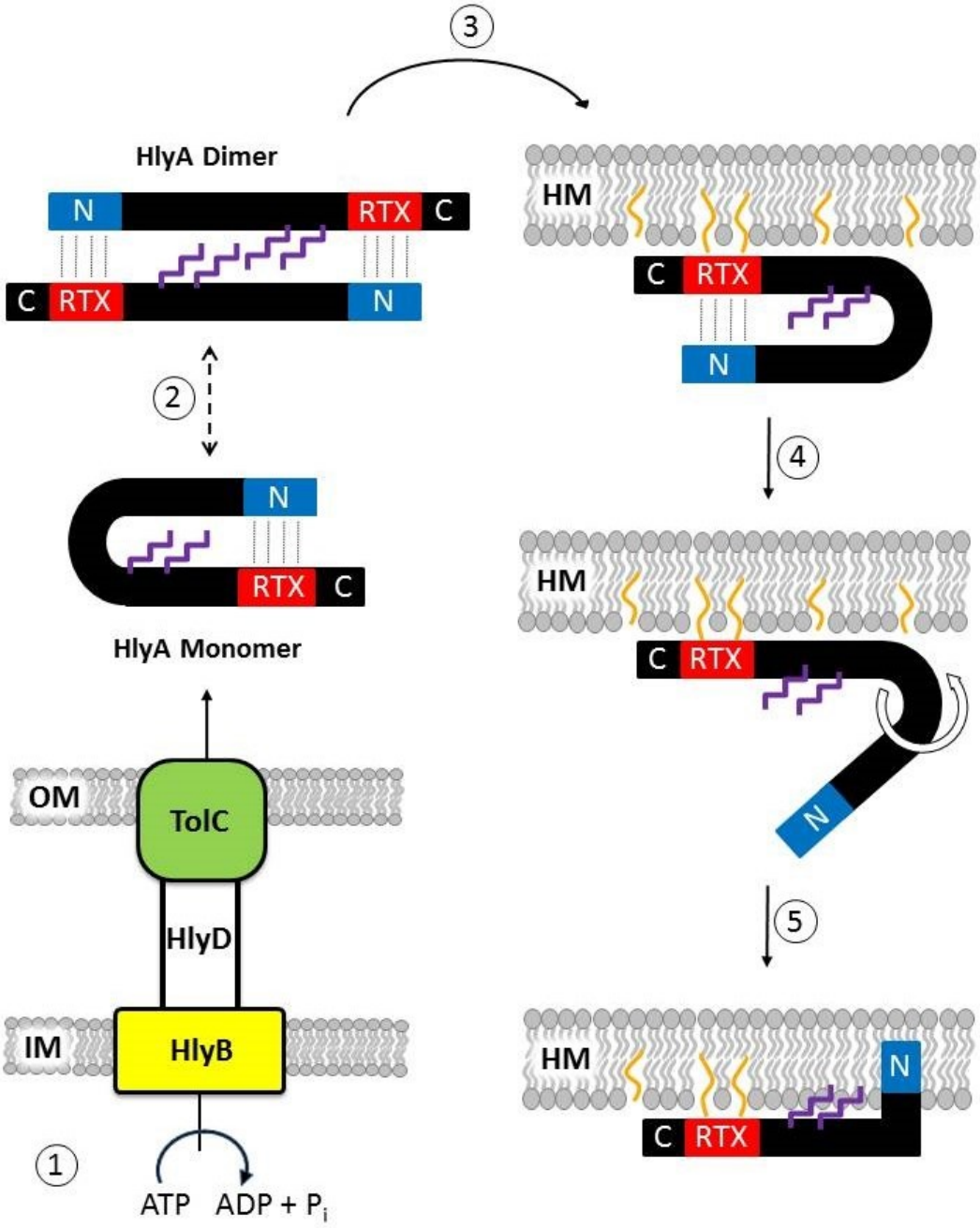



***Figure 15: Model of HlyA - target cell interaction***
1) HlyA is secreted in an unfolded conformation by its T1SS consisting of HlyB (yellow), HlyD (white) and TolC (green). Upon secretion it obtains its 3D structure by calcium ion binding to the RTX repeats (red) [122, 126, 127]. 2) The native protein is able to form monomers and dimers, albeit those are transient. A cap structure is built between the RTX repeat domain and the N-terminus (blue), protecting the bound calcium ions in the RTX domain from external influences and sealing the protein in a stable, possibly inactive conformation. 3) Host membrane adsorption is initialized by binding of C-terminal structures to either glycophorin and/or cholesterol (orange) [80, 98, 182]. 4) Receptor binding initializes a conformational change in the HlyA toxin that disrupts the protecting cap-structure and allows the protein to obtain a lytic conformation. 5) Finally, membrane insertion is achieved by N-terminal, helical regions [99] leading to pore formation or induction of signaling cascades (see chapter 5.1). Here the acyl chains of the acylated lysines (purple) may play a crucial role as no lysis is detectable in the unacylated pro-toxin form [44, 60, 167, 168]. IM: inner membrane; OM: outer membrane; HM: host membrane; RTX: repeats in toxin.

To date the published results regarding HlyA target cell interaction are rare and often contradictory. Therefore cell culture experiments could find a remedy as HlyA could be tested on different cell types with varying receptor repertoire. Initial tests performed in this PhD thesis on human THP1 monocytes on an Alamar Blue based, quantitative activity assay led to promising results for further analysis.

In summary it can be supposed that a similar mechanism could also be imagined for other RTX proteins. Nearly all the RTX structures solved so far show huge structural similarities as shown in Figure 12A, including the cap-structure. It certainly would be beneficial for the bacteria to produce virulence factors that are extraordinary stable even under adverse environmental circumstances and can yet effectively interact with host cells and/or insert into target membranes.

# 7. LITERATURE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# 8. ABBREVIATIONS

| | |
|---|---|
| Å | Angstrom |
| µg | microgram |
| µl | microliter |
| µM | micromolar |
| 3D | Three dimensional |
| ABC | ATP binding cassette |
| ACP | Acyl Carrier Protein |
| ADP | Adenosine-5´-diphosphate |
| AP | alkaline protease |
| ATP | Adenosine-5´-triphosphate |
| $Ba^{2+}$ | Barium ions |
| *B. pertussis* | *Bordetella pertussis* |
| $Ca^{2+}$ | Calcium ions |
| CBB | Coomassie Brilliant Blue |
| CFU | colony-forming units |
| CLD | C39-like domain |
| CNF1 | cytotoxic necrose factor type 1 |
| CyaA | adenylate cyclase toxin |
| d | days |
| DTT | Dithiothreitol |
| *E. coli* | *Escherichia coli* |
| e.g. | exempli gratia |
| ESI | electrospray ionisation |
| $f_F$ | Fraction folded |
| g | gram |
| h | hours |
| His/$His_6$ tag | histidine tag |
| Hly | haemolysin |
| HM | host membrane |
| IBs | inclusion bodies |
| ICAM | Intercellular adhesion molecule |

| | |
|---|---|
| IEX | ion exchange chromatography |
| IL-1β | Interleukin-1β |
| IMAC | immobilized metal ion affinity chromatography |
| IM | inner membrane |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| K | Kelvin |
| kDa | kilo Dalton |
| kJ | kilo Joule |
| L | liter |
| LFA-1 | Lymphocyte function-associated antigen 1 |
| LipA | Lipase A |
| LktA | leukotoxin A |
| M | molar |
| MFP | membrane fusion protein |
| $Mg^{2+}$ | Magnesium ions |
| mg | milligram |
| min | minutes |
| ml | milililter |
| mM | millimolar |
| MS | mass spectrometry |
| NBD | nucleotide binding domain |
| nm | nanometer |
| nM | nanomolar |
| OD | optical density |
| OM | outer membrane |
| OMP | outer membrane protein |
| *P. aeruginosa* | *Pseudomonas aeruginosa* |
| PAGE | polyacrylamide gel electrophoresis |
| PMNs | polymorphonuclear leukocytes |
| RTX | repeats in toxin |
| *S. marcescenc* | *Serratia marcescens* |
| SDS | sodium dodecyl sulfate |
| SEC | Size exclusion chromatography |

| | |
|---|---|
| SecYEG | Sec system composed of a polypeptide-conducting channel |
| $Sr^{2+}$ | Strontium ions |
| T1SS | Type 1 Secretion System |
| Tat | Twin Arginine Translocator |
| TMD | trans membrane domain |
| UPEC | Uropathogenic *Escherichia coli* |
| UTI | urinary tract infections |
| v/v | volume per volume |
| w/v | weight per volume |
| x g | Earth+s gravitational force |

| Amino acid | Three letter code | One letter code |
|---|---|---|
| Alanine | Ala | A |
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartic acid | Asp | D |
| Cysteine | Cys | C |
| Glutamic acid | Glu | E |
| Glutamine | Gln | Q |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Ile | I |
| Leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |
| Valine | Val | V |

# 9. ACKNOWLEDGMENT

An dieser Stelle möchte ich zunächst Prof. Dr. Lutz Schmitt danken. Vielen Dank Lutz für das spannende Projekt, die interessanten und lehrreichen Diskussionen, Deine aufmunternden Worte wenn es einmal nicht laufen wollte und die Möglichkeit eigene Ideen in das Projekt einzubringen und einfach auch mal Sachen auszuprobieren. Trotz überquellendem Terminplaner hattest Du immer Zeit und hast gerade in der hektischen Endphase dieser Arbeit Blitzkorrekturen vollzogen, bei denen man wirklich meinen kann, Tag und Nacht haben zusammen mehr als 24 Stunden. Eventuell werde ich eines Tages auch noch lernen, dass ein „calcium“ nicht gerne ohne sein „ion“ unterwegs ist.

Weiterhin möchte ich mich ganz herzlich bei Prof. Dr. med. Walter Däubener bedanken. Danke Walter für die Übernahme des Koreferates, die interessanten Unterhaltungen auf den MOI Symposien und dafür, dass Du das Chaos bezüglich des Prüfungstermins mitgemacht hast.

Dir Sander möchte ich für Deine Zuversicht danken. Du hast nicht nur feststeckende Experimente nie aufgegeben und durch neue Ideen wiederbelebt sondern auch mit dem ein oder anderem „Das wird schon“ Ruhe in so manch hektische Zeiten gebracht.

Patrick: Dir danke ich für die tolle Betreuung am Anfang meiner Arbeit, viele interessante Diskussionen und dafür, dass wenigsten noch einige Wörter bei der Erstversion des Manuskripts nicht rot waren.

Dr. Inge Krümpelbeck danke ich für die tolle Betreuung und gute Organisation während meines MOI Stipendiums.

Bei Dr. Ulrich Schulte möchte ich mich gerne für die kritischen Fragen in den Seminaren und die gute Betreuung von Praktikumsbetreuern bedanken.

Dr. Lars Lürs und Heike Goldbach-Gecke danke ich für die Unterstützung bei den CD bzw. Zellkulturexperimenten und dafür, dass ich jederzeit meine Messungen bei Euch machen durfte.

Ebenso danke ich Dr. Astrid Höppner für die Hilfe in der Kristallographie und für das Vermessen meiner Proteinkristalle.

Ein besonderer Dank gilt auch Diana und Iris. Ohne Euch würde ich wahrscheinlich immer noch an meiner Dreifachmutante verzweifeln. Danke für die Unterstützung bei nicht klappen wollenden Klonierungen und die nette gemeinsame Zeit.

Susanne: wo soll ich anfangen? Vielen Dank für das gute und lustige Miteinander im Büro und Labor, Dein stets offenes Ohr und regelmäßige Kinobesuche.

Allgemein danke ich dem gesamten unteren Büro für zahlreiche heitere Stunden, das super Büroklima und die tolle Zeit die ich mit Euch allen verbringen durfte.

Allen jetzigen und ehemaligen Kollegen danke ich ebenfalls für eine nette Zeit, die stete Hilfsbereitschaft und das ein oder andere Grillen auf der Dachterrasse.

To all my English speaking colleagues I would just like to say: Thank you all for a great time! It was fun!

Danke auch an Frau Blum, dass Sie sich immer so toll um den ganzen Papierkrieg kümmern.

Meinen ehemaligen Kommilitonen Claudia Frädrich, Fabian Wippich, Julia Averbeck und Anita Onghert danke ich für eine schöne Studienzeit, viele lustige Abende und die gute Freundschaft. Ich freu mich schon auf unser alljährliches Gruppentreffen.

Jessica, bei dir möchte ich mich ganz herzlich für die jahrelange Freundschaft bedanken. Sei es bei unserem traditionellen „Last-Minute“ Weihnachtsbummeln oder bei sonstigen Treffen, Du bringst mich immer wieder zum Lachen.

Zum Abschluss gilt ein ganz besonderer Dank den wichtigsten Menschen in meinem Leben: meiner Familie. Insbesondere meinen Eltern und meiner Schwester möchte ich ganz herzlich für die Unterstützung über all die Jahre danken. Ihr habt mit mir gelacht und geweint, habt mich nach Möglichkeit unterstützt und wenn nötig wieder aufgebaut. Ich kann mich immer auf Euch verlassen. Vielen lieben Dank dafür!

# 10. STATEMENT

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den _____

_____

Sabrina Thomas