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Review

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

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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 and secretion in bacteria. Guest Editors: Anastassios Economou and Ross Dalbey.

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

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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

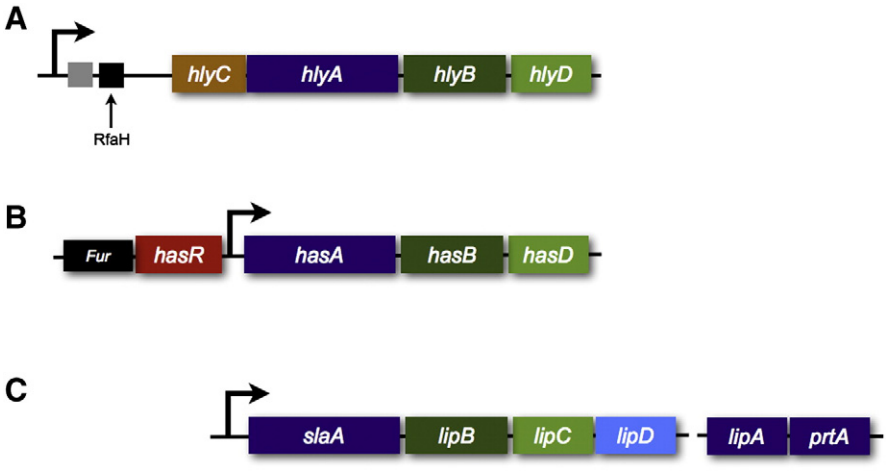

Fig. 1. Operon structures of selected T1SS. (A) chromosomal hemolysin, (B) Has and (C) SlaA/LipA/PrtA system. The gene for the HasA surface receptor (and regulator), *hasR* is shown in red, accessory genes like *hlyC* in orange, the gene for the transport substrates in dark blue, the ABC transporter 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.

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].

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

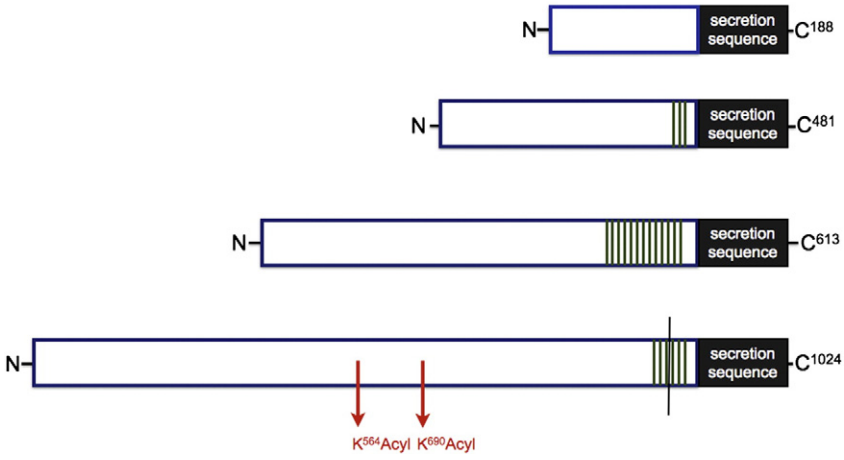

Fig. 2. Schematic summary of selected substrates of T1SS (not drawn to scale). The C-terminal secretion sequence is highlighted as a black box, the 6 GG-repeats as green vertical lines. From top to bottom, hemophore HasA from *S. marcescens*, which does not contain any GG-repeats, the metalloprotease 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.

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

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

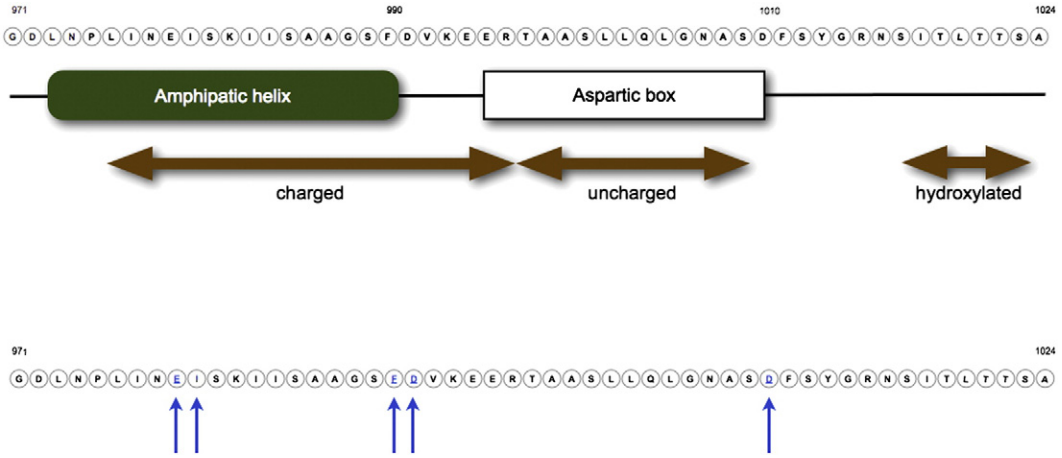

Fig. 3. Primary structure of the extreme C-terminus of plasmid-encoded HlyA (Uniprot entry P08715, residues 971–1024). The position of the putative amphipathic helix and the aspartate box are indicated. The aspartate box is a stretch of small amino acids enriched in alanine and serine flanked by aspartate 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

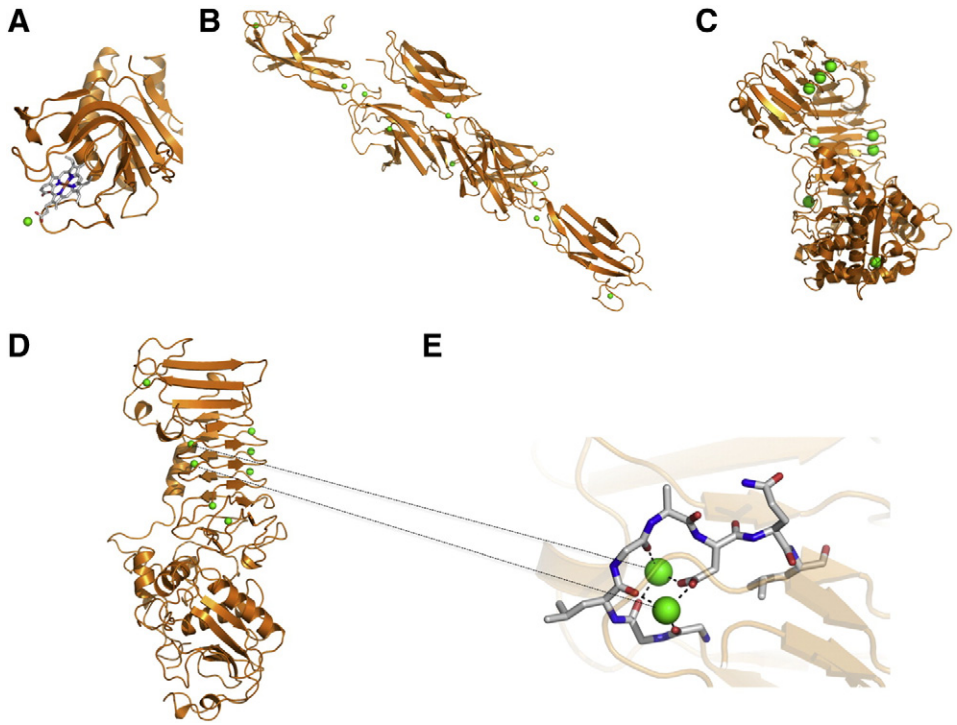

Fig. 4. Crystal structures of HasA from *S. marcescens* (A, pdb entry 1B2V), SiiE from *S. enterica* (B, pdb entry 2YN5), LipA from *S. enterica* (C, pdb entry 2QUA) and alkaline protease from *P. aeruginosa* (D and E, pdb entry 1KAP). Individual structures are shown in cartoon representation, Ca^{2+} 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).

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],

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 β_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 β_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 β_2 integrin (α_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 β_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 β_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 β_2 integrin mediated interaction of RTX toxins with erythrocytes was also investigated. Erythrocytes lack β_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

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.

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References

[1] E. Cascales, C. Cambillau, Structural biology of type VI secretion systems, Philos. Trans. R. Soc. Lond. B Biol. Sci. 367 (2012) 1102–1111.
[2] M. Desvaux, N.J. Parham, A. Scott-Tucker, I.R. Henderson, The general secretory pathway: a general misnomer? Trends Microbiol. 12 (2004) 306–309.
[3] I.B. Holland, L. Schmitt, J. Young, Type 1 protein secretion in bacteria, the ABC-transporter dependent pathway (review), Mol. Membr. Biol. 22 (2005) 29–39.
[4] N. Mackman, I.B. Holland, Functional characterization of a cloned haemolysin determinant from E. coli of human origin, encoding information for the secretion of a 107 K polypeptide, Mol. Gen. Genet. 196 (1984) 129–134.
[5] R.A. Welch, E.P. Dellinger, B. Minshew, S. Falkow, Haemolysin contributes to virulence of extra-intestinal E. coli infections, Nature 294 (1981) 665–667.
[6] W. Goebel, J. Hedgpeth, Cloning and functional characterization of the plasmid-encoded hemolysin determinant of Escherichia coli, J. Bacteriol. 151 (1982) 1290–1298.
[7] S. Letoffe, J.M. Ghigo, C. Wandersman, Secretion of the Serratia marcescens HasA protein by an ABC transporter, J. Bacteriol. 176 (1994) 5372–5377.
[8] S.M. Hinsa, M. Espinosa-Urgel, J.L. Ramos, G.A. O'Toole, Transition from reversible to irreversible attachment during biofilm formation by Pseudomonas fluorescens WCS365 requires an ABC transporter and a large secreted protein, Mol. Microbiol. 49 (2003) 905–918.
[9] P. Stanley, L.C. Packman, V. Koronakis, C. Hughes, Fatty acylation of two internal lysine residues required for the toxic activity of Escherichia coli hemolysin, Science 266 (1994) 1992–1996.
[10] P. Stanley, V. Koronakis, C. Hughes, Acylation of Escherichia coli hemolysin: a unique protein lipidation mechanism underlying toxin function, Microbiol. Mol. Biol. Rev. 62 (1998) 309–333.
[11] V. Koronakis, A. Sharff, E. Koronakis, B. Luisi, C. Hughes, Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export, Nature 405 (2000) 914–919.
[12] V. Koronakis, J. Eswaran, C. Hughes, Structure and function of TolC: the bacterial exit duct for proteins and drugs, Annu. Rev. Biochem. 73 (2004) 467–489.
[13] M. Saidijam, G. Benedetti, Q. Ren, Z. Xu, C.J. Hoyle, S.L. Palmer, A. Ward, K.E. Bettaney, G. Szakonyi, J. Meuller, S. Morrison, M.K. Pos, P. Butaye, K. Walravens, K. Langton, R.B. Herbert, R.A. Skurray, I.T. Paulsen, J. O'Reilly, N.G. Rutherford, M.H. Brown, R.M. Bill, P.J. Henderson, Microbial drug efflux proteins of the major facilitator superfamily, Curr. Drug Targets 7 (2006) 793–811.
[14] J. Eswaran, E. Koronakis, M.K. Higgins, C. Hughes, V. Koronakis, Three's company: component structures bring a closer view of tripartite drug efflux pumps, Curr. Opin. Struct. Biol. 14 (2004) 741–747.
[15] M.F. Symmons, E. Bokma, E. Koronakis, C. Hughes, V. Koronakis, The assembled structure of a complete tripartite bacterial multidrug efflux pump, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 7173–7178.
[16] M.A. Seeger, A. Schiefner, T. Eicher, F. Verrey, K. Diederichs, K.M. Pos, Structural asymmetry of AcrB trimer suggests a peristaltic pump mechanism, Science 313 (2006) 1295–1298.
[17] E. Kawai, H. Akatsuka, A. Idei, T. Shibatani, K. Omori, Serratia marcescens S-layer protein is secreted extracellularly via an ATP-binding cassette exporter, the Lip system, Mol. Microbiol. 27 (1998) 941–952.
[18] C. Wandersman, I. Stojiljkovic, Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores, Curr. Opin. Microbiol. 3 (2000) 215–220.
[19] I. Stojiljkovic, K. Hantke, Hemin uptake system of Yersinia enterocolitica: similarities with other TonB-dependent systems in gram-negative bacteria, EMBO J. 11 (1992) 4359–4367.
[20] C. Wandersman, P. Delepelaire, Bacterial iron sources: from siderophores to hemophores, Annu. Rev. Microbiol. 58 (2004) 611–647.
[21] R. Binet, C. Wandersman, Cloning of the Serratia marcescens hasF gene encoding the Has ABC exporter outer membrane component: a TolC analogue, Mol. Microbiol. 22 (1996) 265–273.
[22] R. Binet, C. Wandersman, Protein secretion by hybrid bacterial ABC-transporters: specific functions of the membrane ATPase and the membrane fusion protein, EMBO J. 14 (1995) 2298–2306.
[23] U.A. Ochsner, Z. Johnson, M.L. Vasil, Genetics and regulation of two distinct haem-uptake systems, phu and has, in Pseudomonas aeruginosa, Microbiology 146 (Pt 1) (2000) 185–198.
[24] U.A. Ochsner, J. Reiser, Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in Pseudomonas aeruginosa, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 6424–6428.
[25] S. Cescau, H. Cwerman, S. Letoffe, P. Delepelaire, C. Wandersman, F. Biville, Heme acquisition by hemophores, Biometals 20 (2007) 603–613.
[26] H. Cwerman, C. Wandersman, F. Biville, Heme and a five-amino-acid hemophore region form the bipartite stimulus triggering the has signaling cascade, J. Bacteriol. 188 (2006) 3357–3364.
[27] M.S. Rossi, A. Paquelin, J.M. Ghigo, C. Wandersman, Haemophore-mediated signal transduction across the bacterial cell envelope in Serratia marcescens: the inducer and the transported substrate are different molecules, Mol. Microbiol. 48 (2003) 1467–1480.
[28] T. Felmlee, S. Pellett, R.A. Welch, Nucleotide sequence of an Escherichia coli chromosomal hemolysin, J. Bacteriol. 163 (1985) 94–105.
[29] A. Juarez, C. Hughes, M. Vogel, W. Goebel, Expression and regulation of the plasmid-encoded hemolysin determinant of Escherichia coli, Mol. Gen. Genet. 197 (1984) 196–203.
[30] V. Koronakis, C. Hughes, Identification of the promoters directing in vivo expression of hemolysin genes in Proteus vulgaris and Escherichia coli, Mol. Gen. Genet. 213 (1988) 99–104.
[31] R.A. Welch, S. Pellett, Transcriptional organization of the Escherichia coli hemolysin genes, J. Bacteriol. 170 (1988) 1622–1630.
[32] M.A. Cross, V. Koronakis, P.L. Stanley, C. Hughes, HlyB-dependent secretion of hemolysin by uropathogenic Escherichia coli requires conserved sequences flanking the chromosomal hly determinant, J. Bacteriol. 172 (1990) 1217–1224.
[33] A.A. Lindberg, C.G. Hellerqvist, Rough mutants of Salmonella typhimurium: immunochemical and structural analysis of lipopolysaccharides from rfaH mutants, J. Gen. Microbiol. 116 (1980) 25–32.
[34] M.J. Bailey, V. Koronakis, T. Schmoll, C. Hughes, Escherichia coli HlyT protein, a transcriptional activator of haemolysin synthesis and secretion, is encoded by the rfaH (sfrB) locus required for expression of sex factor and lipopolysaccharide genes, Mol. Microbiol. 6 (1992) 1003–1012.
[35] J.A. Leeds, R.A. Welch, Enhancing transcription through the Escherichia coli hemolysin operon, hlyCABD: RfaH and upstream JUMPStart DNA sequences function together via a postinitiation mechanism, J. Bacteriol. 179 (1997) 3519–3527.
[36] J.M. Nieto, M.J. Bailey, C. Hughes, V. Koronakis, Suppression of transcription polarity in the Escherichia coli haemolysin operon by a short upstream element shared by polysaccharide and DNA transfer determinants, Mol. Microbiol. 19 (1996) 705–713.
[37] M. Hobbs, P.R. Reeves, The JUMPstart sequence: a 39 bp element common to several polysaccharide gene clusters, Mol. Microbiol. 12 (1994) 855–856.
[38] M.J. Bailey, C. Hughes, V. Koronakis, RfaH and the ops element, components of a novel system controlling bacterial transcription elongation, Mol. Microbiol. 26 (1997) 845–851.
[39] I. Artsimovitch, R. Landick, The transcriptional regulator RfaH stimulates RNA chain synthesis after recruitment to elongation complexes by the exposed nontemplate DNA strand, Cell 109 (2002) 193–203.
[40] G.A. Belogurov, A. Sevostyanova, V. Svetlov, I. Artsimovitch, Functional regions of the N-terminal domain of the antiterminator RfaH, Mol. Microbiol. 76 (2010) 286–301.
[41] J.M. Nicaud, N. Mackman, L. Gray, I.B. Holland, Regulation of haemolysin synthesis in E. coli determined by HLY genes of human origin, Mol. Gen. Genet. 199 (1985) 111–116.
[42] I.B. Holland, M.A. Blight, B. Kenny, The mechanism of secretion of hemolysin and other polypeptides from gram-negative bacteria, J. Bioenerg. Biomembr. 22 (1990) 473–491.
[43] M.A. Blight, B. Menichi, I.B. Holland, Evidence for post-transcriptional regulation of the synthesis of the Escherichia coli HlyB haemolysin translocator and production of polyclonal anti-HlyB antibody, Mol. Gen. Genet. 247 (1995) 73–85.
[44] A.L. Pimenta, J. Young, I.B. Holland, M.A. Blight, Antibody analysis of the localisation, expression and stability of HlyD, the MFP component of the E. coli haemolysin translocator, Mol. Gen. Genet. 261 (1999) 122–132.
[45] L. Landraud, M. Gibert, M.R. Popoff, P. Boquet, M. Gauthier, Expression of cnf1 by Escherichia coli J96 involves a large upstream DNA region including the hlyCABD operon, and is regulated by the RfaH protein, Mol. Microbiol. 47 (2003) 1653–1667.
[46] C.M. Muller, U. Dobrindt, G. Nagy, L. Emody, B.E. Uhlin, J. Hacker, Role of histone-like proteins H-NS and StpA in expression of virulence determinants of uropathogenic Escherichia coli, J. Bacteriol. 188 (2006) 5428–5438.
[47] U. Dobrindt, L. Emody, I. Gentschev, W. Goebel, J. Hacker, Efficient expression of the alpha-haemolysin determinant in the uropathogenic Escherichia coli strain 536

requires the leuX-encoded tRNA(5)(Leu), Mol. Genet. Genomics 267 (2002) 370–379.
[48] S. Sannigrahi, X. Zhang, Y.L. Tzeng, Regulation of the type I protein secretion system by the MisR/MisS two-component system in Neisseria meningitidis, Microbiology 155 (2009) 1588–1601.
[49] L. Li, X. Mou, D.R. Nelson, HlyU is a positive regulator of hemolysin expression in Vibrio anguillarum, J. Bacteriol. 193 (2011) 4779–4789.
[50] H. Li, A. Granat, V. Stewart, J.R. Gillespie, RpoS, H-NS, and DsrA influence EHEC hemolysin operon (ehxCABD) transcription in Escherichia coli O157:H7 strain EDL933, FEMS Microbiol. Lett. 285 (2008) 257–262.
[51] I.B. Holland, R.C. Wang, S.J. Seror, M. Blight, Haemolysin secretion and other protein translocation mechanisms in Gram negative bacteria, Society General Mlcrobiology Symposium, 1989, pp. 219–254.
[52] C. Wandersman, P. Delepelaire, TolC, an Escherichia coli outer membrane protein required for hemolysin secretion, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 4776–4780.
[53] V. Koronakis, J. Li, E. Koronakis, K. Stauffer, Structure of TolC, the outer membrane component of the bacterial type I efflux system, derived from two-dimensional crystals, Mol. Microbiol. 23 (1997) 617–626.
[54] T. Thanabalu, E. Koronakis, C. Hughes, V. Koronakis, Substrate-induced assembly of a contiguous channel for protein export from E. coli: reversible bridging of an inner-membrane translocase to an outer membrane exit pore, EMBO J. 17 (1998) 6487–6496.
[55] P. Delepelaire, Type I secretion in gram-negative bacteria, Biochim. Biophys. Acta 1694 (2004) 149–161.
[56] C. Andersen, E. Koronakis, E. Bokma, J. Eswaran, D. Humphreys, C. Hughes, V. Koronakis, Transition to the open state of the TolC periplasmic tunnel entrance, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 11103–11108.
[57] J. Eswaran, C. Hughes, V. Koronakis, Locking TolC entrance helices to prevent protein translocation by the bacterial type I export apparatus, J. Mol. Biol. 327 (2003) 309–315.
[58] A. Sharff, C. Fanutti, J. Shi, C. Calladine, B. Luisi, The role of the TolC family in protein transport and multidrug efflux. From stereochemical certainty to mechanistic hypothesis, Eur. J. Biochem. 268 (2001) 5011–5026.
[59] R. Misra, P.R. Reeves, Role of micF in the tolC-mediated regulation of OmpF, a major outer membrane protein of Escherichia coli K-12, J. Bacteriol. 169 (1987) 4722–4730.
[60] T. Dinh, I.T. Paulsen, M.H. Saier Jr., A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria, J. Bacteriol. 176 (1994) 3825–3831.
[61] W. Wagner, M. Vogel, W. Goebel, Transport of hemolysin across the outer membrane of Escherichia coli requires two functions, J. Bacteriol. 154 (1983) 200–210.
[62] M.K. Higgins, E. Bokma, E. Koronakis, C. Hughes, V. Koronakis, Structure of the periplasmic component of a bacterial drug efflux pump, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 9994–9999.
[63] R. Schulein, I. Gentschev, H.J. Mollenkopf, W. Goebel, A topological model for the haemolysin translocator protein HlyD, Mol. Gen. Genet. 234 (1992) 155–163.
[64] R.C. Wang, S.J. Seror, M. Blight, J.M. Pratt, J.K. Broome-Smith, I.B. Holland, Analysis of the membrane organization of an Escherichia coli protein translocator, HlyB, a member of a large family of prokaryote and eukaryote surface transport proteins, J. Mol. Biol. 217 (1991) 441–454.
[65] M. Lee, S.Y. Jun, B.Y. Yoon, S. Song, K. Lee, N.C. Ha, Membrane fusion proteins of type I secretion system and tripartite efflux pumps share a binding motif for TolC in gram-negative bacteria, PLoS One 7 (2012) e40460.
[66] S. Trepout, J.C. Taveau, H. Benabdelhak, T. Granier, A. Ducruix, A.S. Frangakis, O. Lambert, Structure of reconstituted bacterial membrane efflux pump by cryo-electron tomography, Biochim. Biophys. Acta 1798 (2010) 1953–1960.
[67] Y. Xu, M. Lee, A. Moeller, S. Song, B.Y. Yoon, H.M. Kim, S.Y. Jun, K. Lee, N.C. Ha, Funnel-like hexameric assembly of the periplasmic adapter protein in the tripartite multidrug efflux pump in gram-negative bacteria, J. Biol. Chem. 286 (2011) 17910–17920.
[68] T.K. Janganan, V.N. Bavro, L. Zhang, D. Matak-Vinkovic, N.P. Barrera, C. Venien-Bryan, C.V. Robinson, M.I. Borges-Walmsley, A.R. Walmsley, Evidence for the assembly of a bacterial tripartite multidrug pump with a stoichiometry of 3: 6:3, J. Biol. Chem. 286 (2011) 26900–26912.
[69] C.F. Higgins, ABC transporters: from microorganisms to man, Annu. Rev. Cell Biol. 8 (1992) 67–113.
[70] I.D. Kerr, Structure and association of ATP-binding cassette transporter nucleotide-binding domains, Biochim. Biophys. Acta 1561 (2002) 47–64.
[71] A.L. Davidson, E. Dassa, C. Orelle, J. Chen, Structure, function, and evolution of bacterial ATP-binding cassette systems, Microbiol. Mol. Biol. Rev. 72 (2008) 317–364(table of contents).
[72] J.K. Zolnerciks, E.J. Andress, M. Nicolaou, K.J. Linton, Structure of ABC transporters, Essays Biochem. 50 (2011) 43–61.
[73] L. Schmitt, H. Benabdelhak, M.A. Blight, I.B. Holland, M.T. Stubbs, Crystal structure of the nucleotide-binding domain of the ABC-transporter haemolysin B: identification of a variable region within ABC helical domains, J. Mol. Biol. 330 (2003) 333–342.
[74] J. Zaitseva, S. Jenewein, A. Wiedenmann, H. Benabdelhak, I.B. Holland, L. Schmitt, Functional characterization and ATP-induced dimerization of the isolated ABC-domain of the haemolysin B transporter, Biochemistry 44 (2005) 9680–9690.
[75] J. Zaitseva, C. Oswald, T. Jumpertz, S. Jenewein, A. Wiedenmann, I.B. Holland, L. Schmitt, A structural analysis of asymmetry required for catalytic activity of an ABC-ATPase domain dimer, EMBO J. 25 (2006) 3432–3443.
[76] R.J. Dawson, K.P. Locher, Structure of a bacterial multidrug ABC transporter, Nature 443 (2006) 180–185.
[77] E. Biemans-Oldehinkel, M.K. Doeven, B. Poolman, ABC transporter architecture and regulatory roles of accessory domains, FEBS Lett. 580 (2006) 1023–1035.
[78] N. Hanekop, J. Zaitseva, S. Jenewein, I.B. Holland, L. Schmitt, Molecular insights into the mechanism of ATP-hydrolysis by the NBD of the ABC-transporter HlyB, FEBS Lett. 580 (2006) 1036–1041.
[79] C.F. Higgins, K.J. Linton, The ATP switch model for ABC transporters, Nat. Struct. Mol. Biol. 11 (2004) 918–926.
[80] C. van der Does, R. Tampe, How do ABC transporters drive transport? Biol. Chem. 385 (2004) 927–933.
[81] A.M. George, P.M. Jones, Perspectives on the structure-function of ABC transporters: the Switch and Constant Contact models, Prog. Biophys. Mol. Biol. 109 (2012) 95–107.
[82] D. Khare, M.L. Oldham, C. Orelle, A.L. Davidson, J. Chen, Alternating access in maltose transporter mediated by rigid-body rotations, Mol. Cell 33 (2009) 528–536.
[83] M.L. Oldham, J. Chen, Snapshots of the maltose transporter during ATP hydrolysis, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 15152–15156.
[84] M.L. Oldham, D. Khare, F.A. Quiocho, A.L. Davidson, J. Chen, Crystal structure of a catalytic intermediate of the maltose transporter, Nature 450 (2007) 515–521.
[85] S. Letoffe, P. Delepelaire, C. Wandersman, Protein secretion in gram-negative bacteria: assembly of the three components of ABC protein-mediated exporters is ordered and promoted by substrate binding, EMBO J. 15 (1996) 5804–5811.
[86] L. Balakrishnan, C. Hughes, V. Koronakis, Substrate-triggered recruitment of the TolC channel-tunnel during type I export of hemolysin by Escherichia coli, J. Mol. Biol. 313 (2001) 501–510.
[87] H. Benabdelhak, S. Kiontke, C. Horn, R. Ernst, M.A. Blight, I.B. Holland, L. Schmitt, A specific interaction between the NBD of the ABC-transporter HlyB and a C-terminal fragment of its transport substrate haemolysin A, J. Mol. Biol. 327 (2003) 1169–1179.
[88] F. Zhang, J.A. Sheps, V. Ling, Complementation of transport-deficient mutants of Escherichia coli alpha-hemolysin by second-site mutations in the transporter hemolysin B, J. Biol. Chem. 268 (1993) 19889–19895.
[89] T. Jumpertz, C. Chervaux, K. Racher, M. Zouhair, M.A. Blight, I.B. Holland, L. Schmitt, Mutations affecting the extreme C terminus of Escherichia coli haemolysin A reduce haemolytic activity by altering the folding of the toxin, Microbiology 156 (2010) 2495–2505.
[90] M.A. Blight, A.L. Pimenta, J.C. Lazzaroni, C. Dando, L. Kotelevets, S.J. Seror, I.B. Holland, Identification and preliminary characterization of temperature-sensitive mutations affecting HlyB, the translocator required for the secretion of haemolysin (HlyA) from Escherichia coli, Mol. Gen. Genet. 245 (1994) 431–440.
[91] Y. Sugamata, T. Shiba, Improved secretory production of recombinant proteins by random mutagenesis of hlyB, an alpha-hemolysin transporter from Escherichia coli, Appl. Environ. Microbiol. 71 (2005) 656–662.
[92] S. Schlor, A. Schmidt, E. Maier, R. Benz, W. Goebel, I. Gentschev, In vivo and in vitro studies on interactions between the components of the hemolysin (HlyA) secretion machinery of Escherichia coli, Mol. Gen. Genet. 256 (1997) 306–319.
[93] R. Schulein, I. Gentschev, S. Schlor, R. Gross, W. Goebel, Identification and characterization of two functional domains of the hemolysin translocator protein HlyD, Mol. Gen. Genet. 245 (1994) 203–211.
[94] A.L. Pimenta, K. Racher, L. Jamieson, M.A. Blight, I.B. Holland, Mutations in HlyD, part of the type 1 translocator for hemolysin secretion, affect the folding of the secreted toxin, J. Bacteriol. 187 (2005) 7471–7480.
[95] P. Kuhnert, B. Heyberger-Meyer, A.P. Burnens, J. Nicolet, J. Frey, Detection of RTX toxin genes in gram-negative bacteria with a set of specific probes, Appl. Environ. Microbiol. 63 (1997) 2258–2265.
[96] L. Gray, K. Baker, B. Kenny, N. Mackman, R. Haigh, I.B. Holland, A novel C-terminal signal sequence targets Escherichia coli haemolysin directly to the medium, J. Cell Sci. Suppl. 11 (1989) 45–57.
[97] L. Gray, N. Mackman, J.M. Nicaud, I.B. Holland, The carboxy-terminal region of haemolysin 2001 is required for secretion of the toxin from Escherichia coli, Mol. Gen. Genet. 205 (1986) 127–133.
[98] B. Kenny, R. Haigh, I.B. Holland, Analysis of the haemolysin transport process through the secretion from Escherichia coli of PCM, CAT or beta-galactosidase fused to the Hly C-terminal signal domain, Mol. Microbiol. 5 (1991) 2557–2568.
[99] B. Kenny, S. Taylor, I.B. Holland, Identification of individual amino acids required for secretion within the haemolysin (HlyA) C-terminal targeting region, Mol. Microbiol. 6 (1992) 1477–1489.
[100] C. Chervaux, I.B. Holland, Random and directed mutagenesis to elucidate the functional importance of helix II and F-989 in the C-terminal secretion signal of Escherichia coli hemolysin, J. Bacteriol. 178 (1996) 1232–1236.
[101] P. Stanley, V. Koronakis, C. Hughes, Mutational analysis supports a role for multiple structural features in the C-terminal secretion signal of Escherichia coli haemolysin, Mol. Microbiol. 5 (1991) 2391–2403.
[102] Y. Yin, F. Zhang, V. Ling, C.H. Arrowsmith, Structural analysis and comparison of the C-terminal transport signal domains of hemolysin A and leukotoxin A, FEBS Lett. 366 (1995) 1–5.
[103] F. Zhang, Y. Yin, C.H. Arrowsmith, V. Ling, Secretion and circular dichroism analysis of the C-terminal signal peptides of HlyA and LktA, Biochemistry 34 (1995) 4193–4201.
[104] M.H. Griessl, B. Schmid, K. Kassler, C. Braunsmann, R. Ritter, B. Barlag, Y.D. Stierhof, K.U. Sturm, C. Danzer, C. Wagner, T.E. Schaffer, H. Sticht, M. Hensel, Y.A. Muller, Structural insight into the giant Ca(2+)-binding adhesin SiiE: implications for the adhesion of salmonella enterica to polarized epithelial cells, Structure 21 (2013) 741–752.
[105] I. Linhartova, L. Bumba, J. Masin, M. Basler, R. Osicka, J. Kamanova, K. Prochazkova, I. Adkins, J. Hejnova-Holubova, L. Sadilkova, J. Morova, P. Sebo, RTX proteins: a

highly diverse family secreted by a common mechanism, FEMS Microbiol. Rev. 34 (2010) 1076–1112.
[106] H.E. Jones, I.B. Holland, H.L. Baker, A.K. Campbell, Slow changes in cytosolic free Ca2+ in Escherichia coli highlight two putative influx mechanisms in response to changes in extracellular calcium, Cell Calcium 25 (1999) 265–274.
[107] P. Arnoux, R. Haser, N. Izadi, A. Lecroisey, M. Delepierre, C. Wandersman, M. Czjzek, The crystal structure of HasA, a hemophore secreted by Serratia marcescens, Nat. Struct. Biol. 6 (1999) 516–520.
[108] U. Baumann, M. Bauer, S. Letoffe, P. Delepelaire, C. Wandersman, Crystal structure of a complex between Serratia marcescens metallo-protease and an inhibitor from Erwinia chrysanthemi, J. Mol. Biol. 248 (1995) 653–661.
[109] U. Baumann, S. Wu, K.M. Flaherty, D.B. McKay, Three-dimensional structure of the alkaline protease of Pseudomonas aeruginosa: a two-domain protein with a calcium binding parallel beta roll motif, EMBO J. 12 (1993) 3357–3364.
[110] M. Czjzek, S. Letoffe, C. Wandersman, M. Delepierre, A. Lecroisey, N. Izadi-Pruneyre, The crystal structure of the secreted dimeric form of the hemophore HasA reveals a domain swapping with an exchanged heme ligand, J. Mol. Biol. 365 (2007) 1176–1186.
[111] R. Meier, T. Drepper, V. Svensson, K.E. Jaeger, U. Baumann, A calcium-gated lid and a large beta-roll sandwich are revealed by the crystal structure of extracellular lipase from Serratia marcescens, J. Biol. Chem. 282 (2007) 31477–31483.
[112] J. Lecher, C.K. Schwarz, M. Stoldt, S.H. Smits, D. Willbold, L. Schmitt, An RTX transporter tethers its unfolded substrate during secretion via a unique N-terminal domain, Structure 20 (2012) 1778–1787.
[113] P. Delepelaire, C. Wandersman, The SecB chaperone is involved in the secretion of the Serratia marcescens HasA protein through an ABC transporter, EMBO J. 17 (1998) 936–944.
[114] L. Schmitt, R. Tampé, Structure and mechanism of ABC-transporters, Curr. Opin. Struct. Biol. 12 (2002) 754–760.
[115] G. Dirix, P. Monsieurs, B. Dombrecht, R. Daniels, K. Marchal, J. Vanderleyden, J. Michiels, Peptide signal molecules and bacteriocins in Gram-negative bacteria: a genome-wide in silico screening for peptides containing a double-glycine leader sequence and their cognate transporters, Peptides 25 (2004) 1425–1440.
[116] S. Ishii, T. Yano, A. Ebihara, A. Okamoto, M. Manzoku, H. Hayashi, Crystal structure of the peptidase domain of Streptococcus ComA, a bifunctional ATP-binding cassette transporter involved in the quorum-sensing pathway, J. Biol. Chem. 285 (2010) 10777–10785.
[117] M. Masi, C. Wandersman, Multiple signals direct the assembly and function of a type 1 secretion system, J. Bacteriol. 192 (2010) 3861–3869.
[118] S. Bhakdi, M. Muhly, S. Korom, G. Schmidt, Effects of Escherichia coli hemolysin on human monocytes. Cytocidal action and stimulation of interleukin 1 release, J. Clin. Invest. 85 (1990) 1746–1753.
[119] O.V. Gadeberg, I. Orskov, J.M. Rhodes, Cytotoxic effect of an alpha-hemolytic Escherichia coli strain on human blood monocytes and granulocytes in vitro, Infect. Immun. 41 (1983) 358–364.
[120] S.J. Cavalieri, I.S. Snyder, Effect of Escherichia coli alpha-hemolysin on human peripheral leukocyte function in vitro, Infect. Immun. 37 (1982) 966–974.
[121] F. Grimminger, C. Scholz, S. Bhakdi, W. Seeger, Subhemolytic doses of Escherichia coli hemolysin evoke large quantities of lipoxygenase products in human neutrophils, J. Biol. Chem. 266 (1991) 14262–14269.
[122] N. Suttorp, B. Floer, H. Schnittler, W. Seeger, S. Bhakdi, Effects of Escherichia coli hemolysin on endothelial cell function, Infect. Immun. 58 (1990) 3796–3801.
[123] W.F. Keane, R. Welch, G. Gekker, P.K. Peterson, Mechanism of Escherichia coli alpha-hemolysin-induced injury to isolated renal tubular cells, Am. J. Pathol. 126 (1987) 350–357.
[124] U.C. Chaturvedi, A. Mathur, A.M. Khan, R.M. Mehrotra, Cytotoxicity of filtrates of haemolytic Escherichia coli, J. Med. Microbiol. 2 (1969) 211–218.
[125] E.M. Cooke, S.P. Ewins, Properties of strains of Escherichia coli isolated from a variety of sources, J. Med. Microbiol. 8 (1975) 107–111.
[126] H.J. Brooks, F. O'Grady, M.A. McSherry, W.R. Cattell, Uropathogenic properties of Escherichia coli in recurrent urinary-tract infection, J. Med. Microbiol. 13 (1980) 57–68.
[127] J.M. DeBoy II, I.K. Wachsmuth, B.R. Davis, Hemolytic activity in enterotoxigenic and non-enterotoxigenic strains of Escherichia coli, J. Clin. Microbiol. 12 (1980) 193–198.
[128] B.H. Minshew, J. Jorgensen, G.W. Counts, S. Falkow, Association of hemolysin production, hemagglutination of human erythrocytes, and virulence for chicken embryos of extraintestinal Escherichia coli isolates, Infect. Immun. 20 (1978) 50–54.
[129] R.P. Rennie, J.H. Freer, J.P. Arbuthnott, The kinetics of erythrocyte lysis by Escherichia coli haemolysin, J. Med. Microbiol. 7 (1974) 189–195.
[130] S. Bhakdi, N. Mackman, J.M. Nicaud, I.B. Holland, Escherichia coli hemolysin may damage target cell membranes by generating transmembrane pores, Infect. Immun. 52 (1986) 63–69.
[131] R.D. MacGregor II, C.A. Tobias, Molecular sieving of red cell membranes during gradual osmotic hemolysis, J. Membr. Biol. 10 (1972) 345–356.
[132] G. Menestrina, C. Moser, S. Pellet, R. Welch, Pore-formation by Escherichia coli hemolysin (HlyA) and other members of the RTX toxins family, Toxicology 87 (1994) 249–267.
[133] H. Ostolaza, B. Bartolome, I. Ortiz de Zarate, F. de la Cruz, F.M. Goni, Release of lipid vesicle contents by the bacterial protein toxin alpha-haemolysin, Biochim. Biophys. Acta 1147 (1993) 81–88.
[134] A. Valeva, I. Walev, H. Kemmer, S. Weis, I. Siegel, F. Boukhallouk, T.M. Wassenaar, T. Chavakis, S. Bhakdi, Binding of Escherichia coli hemolysin and activation of the target cells is not receptor-dependent, J. Biol. Chem. 280 (2005) 36657–36663.
[135] L. Bakas, H. Ostolaza, W.L. Vaz, F.M. Goni, Reversible adsorption and nonreversible insertion of Escherichia coli alpha-hemolysin into lipid bilayers, Biophys. J. 71 (1996) 1869–1876.
[136] M. Moayeri, R.A. Welch, Prelytic and lytic conformations of erythrocyte-associated Escherichia coli hemolysin, Infect. Immun. 65 (1997) 2233–2239.
[137] H. Ostolaza, F.M. Goni, Interaction of the bacterial protein toxin alpha-haemolysin with model membranes: protein binding does not always lead to lytic activity, FEBS Lett. 371 (1995) 303–306.
[138] C. Schindel, A. Zitzer, B. Schulte, A. Gerhards, P. Stanley, C. Hughes, V. Koronakis, S. Bhakdi, M. Palmer, Interaction of Escherichia coli hemolysin with biological membranes. A study using cysteine scanning mutagenesis, Eur. J. Biochem. 268 (2001) 800–808.
[139] A. Dobereiner, A. Schmid, A. Ludwig, W. Goebel, R. Benz, The effects of calcium and other polyvalent cations on channel formation by Escherichia coli alpha-hemolysin in red blood cells and lipid bilayer membranes, Eur. J. Biochem. 240 (1996) 454–460.
[140] D.F. Boehm, R.A. Welch, I.S. Snyder, Domains of Escherichia coli hemolysin (HlyA) involved in binding of calcium and erythrocyte membranes, Infect. Immun. 58 (1990) 1959–1964.
[141] R.L. Oropeza-Wekerle, E. Muller, P. Kern, R. Meyermann, W. Goebel, Synthesis, inactivation, and localization of extracellular and intracellular Escherichia coli hemolysins, J. Bacteriol. 171 (1989) 2783–2788.
[142] A. Soloaga, H. Ostolaza, F.M. Goni, F. de la Cruz, Purification of Escherichia coli pro-haemolysin, and a comparison with the properties of mature alpha-haemolysin, Eur. J. Biochem. 238 (1996) 418–422.
[143] M. Ropele, G. Menestrina, Electrical properties and molecular architecture of the channel formed by Escherichia coli hemolysin in planar lipid membranes, Biochim. Biophys. Acta 985 (1989) 9–18.
[144] B. Eberspacher, F. Hugo, S. Bhakdi, Quantitative study of the binding and hemolytic efficiency of Escherichia coli hemolysin, Infect. Immun. 57 (1989) 983–988.
[145] V. Herlax, S. Mate, O. Rimoldi, L. Bakas, Relevance of fatty acid covalently bound to Escherichia coli alpha-hemolysin and membrane microdomains in the oligomerization process, J. Biol. Chem. 284 (2009) 25199–25210.
[146] E.T. Lally, I.R. Kieba, A. Sato, C.L. Green, J. Rosenbloom, J. Korostoff, J.F. Wang, B.J. Shenker, S. Ortlepp, M.K. Robinson, P.C. Billings, RTX toxins recognize a beta2 integrin on the surface of human target cells, J. Biol. Chem. 272 (1997) 30463–30469.
[147] D.L. Simpson, P. Berthold, N.S. Taichman, Killing of human myelomonocytic leukemia and lymphocytic cell lines by Actinobacillus actinomycetemcomitans leukotoxin, Infect. Immun. 56 (1988) 1162–1166.
[148] J. Morova, R. Osicka, J. Masin, P. Sebo, RTX cytotoxins recognize beta2 integrin receptors through N-linked oligosaccharides, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 5355–5360.
[149] M.S. Bretscher, Circulating integrins: alpha 5 beta 1, alpha 6 beta 4 and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1, EMBO J. 11 (1992) 405–410.
[150] A.L. Cortajarena, F.M. Goni, H. Ostolaza, Glycophorin as a receptor for Escherichia coli alpha-hemolysin in erythrocytes, J. Biol. Chem. 276 (2001) 12513–12519.
[151] C.K. Larsen, M. Skals, T. Wang, M.U. Cheema, J. Leipziger, H.A. Praetorius, Python erythrocytes are resistant to alpha-hemolysin from Escherichia coli, J. Membr. Biol. 244 (2011) 131–140.
[152] F. Ligi, C. Ciacci, F. Palma, Comparative study of the cytoplasmic domain of band 3 from human and rabbit erythrocyte membranes, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 121 (1998) 265–271.
[153] L. Sanchez-Magraner, A.R. Viguera, M. Garcia-Pacios, M.P. Garcillan, J.L. Arrondo, F. de la Cruz, F.M. Goni, H. Ostolaza, The calcium-binding C-terminal domain of Escherichia coli alpha-hemolysin is a major determinant in the surface-active properties of the protein, J. Biol. Chem. 282 (2007) 11827–11835.
[154] T.J. Beveridge, Structures of gram-negative cell walls and their derived membrane vesicles, J. Bacteriol. 181 (1999) 4725–4733.
[155] S.N. Wai, B. Lindmark, T. Soderblom, A. Takade, M. Westermark, J. Oscarsson, J. Jass, A. Richter-Dahlfors, Y. Mizunoe, B.E. Uhlin, Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin, Cell 115 (2003) 25–35.
[156] C. Balsalobre, J.M. Silvan, S. Berglund, Y. Mizunoe, B.E. Uhlin, S.N. Wai, Release of the type I secreted alpha-haemolysin via outer membrane vesicles from Escherichia coli, Mol. Microbiol. 59 (2006) 99–112.
[157] N.C. Kesty, K.M. Mason, M. Reedy, S.E. Miller, M.J. Kuehn, Enterotoxigenic Escherichia coli vesicles target toxin delivery into mammalian cells, EMBO J. 23 (2004) 4538–4549.
[158] S.N.C.K. Chatterjee, Outer membrane vesicles of bacteria, Springer, Heidelberg [u.a.], 2012.
[159] S. Bhakdi, J. Tranum-Jensen, Damage to mammalian cells by proteins that form transmembrane pores, Rev. Physiol. Biochem. Pharmacol. 107 (1987) 147–223.
[160] G. Menestrina, Escherichia coli hemolysin permeabilizes small unilamellar vesicles loaded with calcein by a single-hit mechanism, FEBS Lett. 232 (1988) 217–220.
[161] G. Menestrina, N. Mackman, I.B. Holland, S. Bhakdi, Escherichia coli haemolysin forms voltage-dependent ion channels in lipid membranes, Biochim. Biophys. Acta 905 (1987) 109–117.
[162] M. Skals, U.B. Jensen, J. Ousingsawat, K. Kunzelmann, J. Leipziger, H.A. Praetorius, Escherichia coli alpha-hemolysin triggers shrinkage of erythrocytes via K(Ca)3.1 and TMEM16A channels with subsequent phosphatidylserine exposure, J. Biol. Chem. 285 (2010) 15557–15565.
[163] M. Moayeri, R.A. Welch, Effects of temperature, time, and toxin concentration on lesion formation by the Escherichia coli hemolysin, Infect. Immun. 62 (1994) 4124–4134.
[164] S.E. Jorgensen, R.F. Hammer, G.K. Wu, Effects of a single hit from the alpha hemolysin produced by Escherichia coli on the morphology of sheep erythrocytes, Infect. Immun. 27 (1980) 988–994.
[165] E.C. Short, H.J. Kurtz, Properties of the hemolytic activities of Escherichia coli, Infect. Immun. 3 (1971) 678–687.

[166] R. Benz, A. Dobereiner, A. Ludwig, W. Goebel, Haemolysin of Escherichia coli: comparison of pore-forming properties between chromosome and plasmid-encoded haemolysins, FEMS Microbiol. Immunol. 5 (1992) 55–62.
[167] A. Ludwig, R. Benz, W. Goebel, Oligomerization of Escherichia coli haemolysin (HlyA) is involved in pore formation, Mol. Gen. Genet. 241 (1993) 89–96.
[168] A.L. Cortajarena, F.M. Goni, H. Ostolaza, A receptor-binding region in Escherichia coli alpha-haemolysin, J. Biol. Chem. 278 (2003) 19159–19163.
[169] T. Felmlee, R.A. Welch, Alterations of amino acid repeats in the Escherichia coli hemolysin affect cytolytic activity and secretion, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 5269–5273.
[170] A. Ludwig, A. Schmid, R. Benz, W. Goebel, Mutations affecting pore formation by haemolysin from Escherichia coli, Mol. Gen. Genet. 226 (1991) 198–208.
[171] C. Hyland, L. Vuillard, C. Hughes, V. Koronakis, Membrane interaction of Escherichia coli hemolysin: flotation and insertion-dependent labeling by phospholipid vesicles, J. Bacteriol. 183 (2001) 5364–5370.
[172] R.A. Welch, M.E. Bauer, A.D. Kent, J.A. Leeds, M. Moayeri, L.B. Regassa, D.L. Swenson, Battling against host phagocytes: the wherefore of the RTX family of toxins? Infect. Agents Dis. 4 (1995) 254–272.
[173] M.J. Berridge, M.D. Bootman, P. Lipp, Calcium—a life and death signal, Nature 395 (1998) 645–648.
[174] M.J. Berridge, P. Lipp, M.D. Bootman, The versatility and universality of calcium signalling, Nat. Rev. Mol. Cell Biol. 1 (2000) 11–21.
[175] G. TranVan Nhieu, C. Clair, G. Grompone, P. Sansonetti, Calcium signalling during cell interactions with bacterial pathogens, Biol. Cell. 96 (2004) 93–101.
[176] J.W. Eaton, E. Berger, D. Nelson, J.G. White, O. Rundquist, Intracellular calcium: lack of effect on ovine red cells, Proc. Soc. Exp. Biol. Med. 157 (1978) 506–510.
[177] H. Ostolaza, B. Bartolome, J.L. Serra, F. de la Cruz, F.M. Goni, Alpha-haemolysin from *E. coli*. Purification and self-aggregation properties, FEBS Lett. 280 (1991) 195–198.
[178] H.J. Schnittler, A. Wilke, T. Gress, N. Suttorp, D. Drenckhahn, Role of actin and myosin in the control of paracellular permeability in pig, rat and human vascular endothelium, J. Physiol. 431 (1990) 379–401.
[179] B.K. Dhakal, M.A. Mulvey, The UPEC pore-forming toxin alpha-hemolysin triggers proteolysis of host proteins to disrupt cell adhesion, inflammatory, and survival pathways, Cell Host Microbe 11 (2012) 58–69.
[180] M. Skals, N.R. Jorgensen, J. Leipziger, H.A. Praetorius, Alpha-hemolysin from Escherichia coli uses endogenous amplification through P2X receptor activation to induce hemolysis, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 4030–4035.
[181] H.M. Costa-Junior, A.N. Mendes, G.H. Davis, C.M. da Cruz, A.L. Ventura, C.H. Serezani, L.H. Faccioli, A. Nomizo, C.G. Freire-de-Lima, C. Bisaggio Rda, P.M. Persechini, ATP-induced apoptosis involves a Ca2 + -independent phospholipase A2 and 5-lipoxygenase in macrophages, Prostaglandins Other Lipid Mediat. 88 (2009) 51–61.
[182] H. Le Stunff, R. Auger, J. Kanellopoulos, M.N. Raymond, The Pro-451 to Leu polymorphism within the C-terminal tail of P2X7 receptor impairs cell death but not phospholipase D activation in murine thymocytes, J. Biol. Chem. 279 (2004) 16918–16926.