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Type I secretion systems – a story of appendices

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Abstract

Secretion is an essential task for prokaryotic organisms to interact with their surrounding environment. In particular, the production of extracellular proteins and peptides is important for many aspects of an organism's survival and adaptation to its ecological niche. In Gram-negative bacteria, six different protein secretion systems have been identified so far, named Type I to Type VI; differing greatly in their composition and mechanism of action (Economou et al., 2006). The two membranes present in Gram-negative bacteria are negotiated either by one-step transport mechanisms (Type I and Type III), where the unfolded substrate is translocated directly into the extracellular space, without any periplasmic intermediates, or by two-step mechanisms (Type II and Type V), where the substrate is first transported into the periplasm to allow folding before a second transport step across the outer membrane occurs. Here we focus on Type I secretion systems and summarise our current knowledge of these one-step transport machineries with emphasis on the N-terminal extensions found in many Type I-specific ABC transporters. ABC transporters containing an N-terminal C39 peptidase domain cut off a leader peptide present in the substrate prior to secretion. The function of the second type of appendix, the C39 peptidase-like domain (CLD), is not yet completely understood. Recent results have shown that it is nonetheless essential for secretion and interacts specifically with the substrate of the transporter. The third group present does not contain any appendix. In light of this difference we compare the function of the appendix and the differences that might exist among the three families of T1SS.

Keywords: ABC transporters; C39 peptidase domain; Haemolysin; Type I secretion systems

1. Introduction to Type I secretion systems (T1SS)

In 1985, Nicaud et al. (Nicaud et al., 1985) identified two membrane proteins of the inner membrane of *Escherichia coli* (*E. coli*), haemolysin (Hly) B and HlyD, to be essential for the secretion of the toxin HlyA, a member of the repeats-in-toxins (RTX) family (Linhartova et al., 2010). A one-step translocation process was proposed by the same group also in 1985 (Mackman et al., 1985), when they described the translocation of HlyA across the membranes of the Gram-negative bacteria *E. coli* as a single-step mechanism without the occurrence of periplasmic intermediates.

Nowadays we know that proteins secreted via the T1SS vary greatly in size and function, for example, the bacteriocin colicin V or Mcc V with a size of 5.8 kDa, as well as large RTX or MARTX proteins whose molecular weight can be up to 900 kDa (Gilson et al., 1990; Linhartova et al., 2010; Satchell, 2011).

Many proteins secreted via the T1SS, such as haemolysins and leukotoxins, are of great importance for the pathogenesis in the host organism or, like some bacteriocins, for antibacterial activity (Bleves et al., 2010; Dirix et al., 2004). Other proteins secreted by T1SS are involved in nutrient acquisition. Examples are extracellular proteases and lipases or the well-characterised iron scavenger protein HasA (Akatsuka et al., 1995; Duong et al., 1992; Létoffé et al., 1994). A brief summary of substrates of T1SS is provided in Table 1.

It is now commonly accepted that T1SS are composed of three indispensable membrane proteins (Fig. 1), an ABC transporter, a membrane fusion protein (MFP) and an outer membrane protein or factor (OMF). Furthermore, all substrates contain a Sec-system independent secretion sequence. This sequence is either located at the N-terminus (bacteriocines or colicins) or at the C-terminus (all other systems) of the substrate.

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Table 1
Examples of substrates of T1SS and their dedicated transport components.

Type of protein	Example of the secreted protein	Secretion apparatus ABC/MFP/OMF	Reference
Bacteriocin	ColicinV/MccV	CvaB/CvaA/TolC	(Fath et al., 1994; Gilson et al., 1987, 1990)
Adhesin	LapA	unknown	(Espinosa-Urgel et al., 2000; Hinsa et al., 2003)
Lipase	LipA	LipB/LipC/LipD	(Akatsuka et al., 1995)
Protease	Alkaline protease	AprD/AprE/AprF	(Guzzo et al., 1991)
Iron scavenger protein	HasA	HasD/HasE/HasF	(Letoffe et al., 1996)
S-layer protein	RsaA	RsaD/RsaE/RsaF(&RsaF$_b$)	(Awram and Smit, 1998; Smit et al., 1992)
RTX toxin	HlyA	HlyB/HlyD/TolC	(Wandersman and Delepelaire, 1990; Koronakis et al., 2000; Holland et al., 2005)

In this review, the T1SS will be discussed with regard to the different types of ABC transporters, which constitute part of the secretion apparatus. Such a classification distinguishes three different groups of transport proteins, which differ in their N-terminal domains as well as in the kind of substrates being translocated.

2. General structure of the T1SS

The best-studied T1SS are the HasA secretion system from *Serratia marcescens* (*S. marcescens*) (Letoffe et al., 1996) and the HlyA secretion machinery from *E. coli* (Holland et al., 2005).

Each substrate or allocrite of a T1SS is secreted by its dedicated and relatively simple secretion apparatus, which consists of only three proteins (Fig. 1). They form a tunnel-like structure to transfer the substrate directly from the cytosol to the extracellular space. The currently accepted molecular blueprint of T1SS assumes that an ABC transporter provides a transport pathway across the inner membrane and the energy required via binding and hydrolysis of ATP, while an OMF forms a pore through the outer membrane. Finally, an inner membrane-anchored MFP completes the machinery by spanning the periplasm and connecting the large periplasmic domain of the OMF and the ABC transporter (Holland et al., 2005).

The HasA and the HlyA T1SS are composed of the ABC transporters, MFPs and OMFs, HasD/HasE/HasF, and HlyB/HlyD/TolC, respectively (Binet and Wandersman, 1996; Koronakis et al., 2000; Letoffe et al., 1990). Whereas the inner

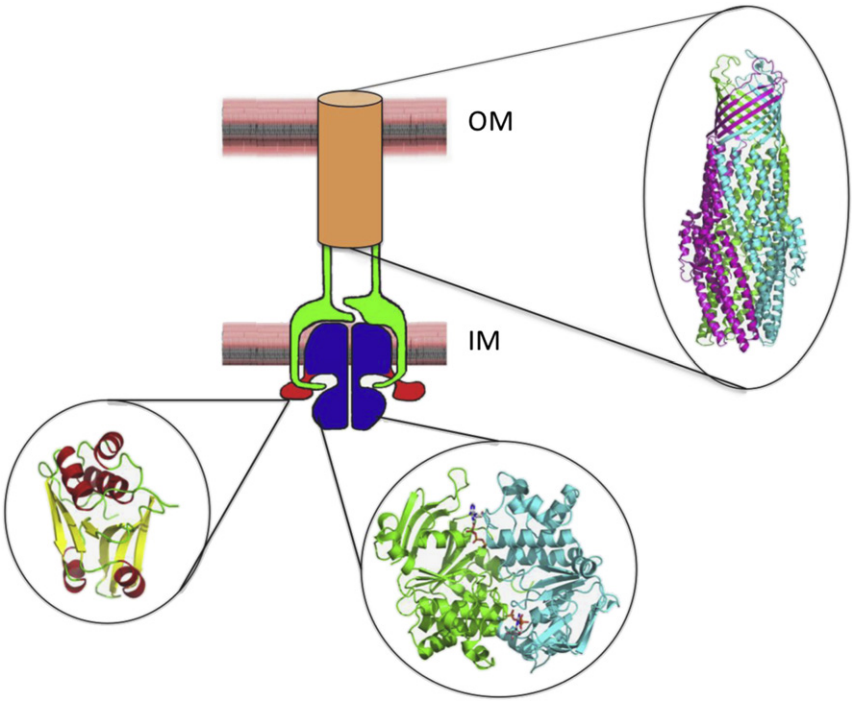

Fig. 1. Schematic summary of the general architecture of a T1SS involved in secretion of an RTX protein, for example HlyA. The ABC transporter is shown in blue with the CLD highlighted in red, the MFP in green and the OMFP in orange. Structures of components of T1SS are also included. The structures of the ATP-bound dimer of HlyB (Zaitseva et al., 2005), the CLD of HlyB (Lecher et al., 2012) and TolC (Koronakis et al., 2000) are shown in cartoon representation. The currently known crystal structures of substrates of T1SS (Arnoux et al., 1999; Baumann et al., 1993) have not been included for simplicity. Please note that the functional unit of the ABC transporter is a dimer and the oligomeric state of the OMF is trimeric (each chain of the TolC structure is coloured differently). This would generate a symmetry break that might be resolved by the MFP, which has been drawn arbitrarily as a dimer. The described crystal structures of MFPs not involved in T1SS did not resolve this issue (Akama et al., 2004; Higgins et al., 2004; Mikolosko et al., 2006). Only recently, the crystal structure of CusB (Su et al., 2011, Su et al., 2009), the MFP of a Cu^+/Ag^+ export systems (non ABC) revealed a hexameric state, which would be a solution to cope with the apparent symmetry mismatch between ABC transporter and OMF.

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membrane components display a very high degree of specificity for their substrates, the OMFs are also involved in multiple export processes. For example, the OMF TolC of *E. coli* is involved in HlyA secretion (Mackman et al., 1985) but also the secretion of colicin V of Mcc V (Gilson et al., 1987), and the extrusion of cytotoxic compounds (Nakashima et al., 2011), to confer drug resistance in bacteria (Pos, 2009). In all of these cases, TolC interacts with different sets of proteins of the inner membrane. In T1SS, there are always MFPs and ABC transporters (Delepelaire, 2004; Holland et al., 2005), while in drug transport processes the coupling occurs mainly but not exclusively with an MFP and a member of the RND (resistance–nodulation–drug resistance) family of secondary transporters (Pos, 2009).

Apart from connecting the other two components of the secretion machinery, the MFP seems to play an important role in substrate recognition, mediated by its N-terminal, cytoplasmic part. Deletion of this domain in HlyD, which is located on the cytosolic side of the membrane, abolishes HlyA secretion (Balakrishnan et al., 2001; Pimenta et al., 1999). Nonetheless, the secretion complex is still found to be assembled; thereby bridging the two membranes of *E. coli*. However, it is important to realise that T1SS do not exist as permanently associated, static complexes (Fig. 2). This was already suggested by the multiple tasks accomplished by TolC (see above). While the ABC transporter and the MFP always form a complex in the inner membrane as shown by cross-linking studies, the entire complex only assembles upon interaction of the substrate with the ABC transporter and/or the MFP (Balakrishnan et al., 2001; Benabdelhak et al., 2003; Letoffe et al., 1996; Thanabalu et al., 1998). The components of T1SS and their specific interactions with their substrates are described in greater detail in the following sections.

3. ABC transporters

The basic structure of an ABC transporter consists of four modules; two so-called transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) (Davidson et al., 2008; Jones et al., 2009) that can be arranged in any possible combination. In bacteria, these four modules are mostly encoded by separate genes (Davidson et al., 2008). Interestingly, ABC transporters of T1SS form an exception because here, one NBD and one TMD are encoded by a single gene (so-called 'half-size transporter') and the functional unit of these transporters is thought to correspond to the dimer. The transmembrane helices of the TMDs form the translocation pathway for the substrate across the membrane (Hollenstein et al., 2007), while the NBDs are responsible for energy supply through nucleotide binding and hydrolysis and the coordination of the cofactor (e.g. Mg^{2+}) (Oswald et al., 2006). In the functionally active state of the ABC transporter, the two NBDs face each other in a head-to-tail manner so that the

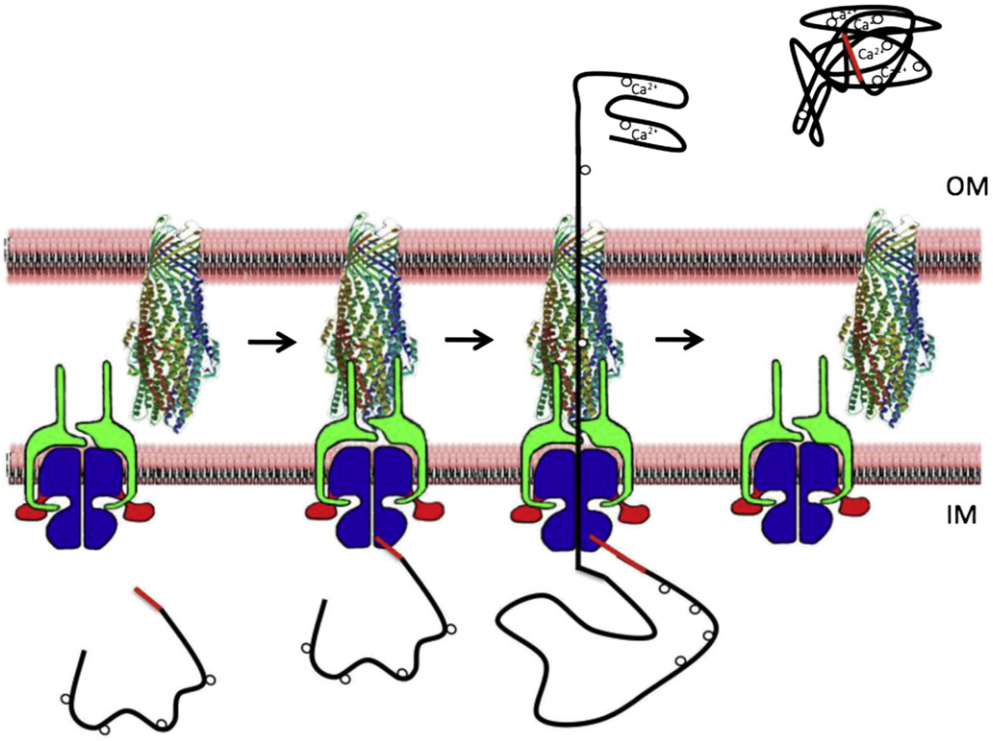

Fig. 2. Current model of the coordination of a T1SS specific for RTX proteins in time and space. Colour coding is identical to Fig. 1. The substrate is shown in black in the unfolded state in the cytosol. The secretion sequence is highlighted in red and the Ca^{2+}-binding sites within the RTX domain are presented as circles. Step 1: The ABC transporter and the MFP form a static complex in the inner membrane in the absence of the substrate. Step 2: The secretion sequence of the substrate located in the extreme C-terminus interacts with the NBDs of the ABC transporter and/or MFP and triggers engagement of the OMF and formation of the channel-tunnel through the periplasm. Step 3: Stepwise translocation of the substrate in the unfolded state. Direct experimental evidence for transport of the substrate in the unfolded state was only recently provided (Bakkes et al., 2010). Step 5: Ca^{2+}-induced refolding of the substrate in the extracellular space and resetting of the T1SS.

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nucleotide is sandwiched between the Walker A motif of the one and the C-loop of the other domain (Chen et al., 2003; Smith et al., 2002; Zaitseva et al., 2005).

While more sequence variability is found amongst the TMDs, which is likely due to their involvement in substrate binding and transport, the NBDs show high sequence homology due to their function as power plants. The translocation of a substrate across the membrane involves conformational changes of the ABC transporter that likely follow the 'two-site access' model (Jardetzky, 1966). Interaction of the substrate with the transporter in the presence of ATP triggers the formation of dimeric NBDs and opens the transporter's cavity on the substrate-binding side of the membrane. It is hypothesised that the hydrolysis of ATP destabilises the dimeric NBDs, triggering the transporter to return into its original conformational state and releasing the substrate on the substrate delivery side (Hollenstein et al., 2007; Jones et al., 2009). In light of the size of T1SS substrates, a sequential transport mechanism, i.e. a stepwise substrate translocation is hard to imagine, because the substrate would be located within the translocation pathway interfering with the conformational changes postulated to occur within the "two-site access model". If this model applied also for ABC transporters involved in T1SS, only one hydrolysis cycle of ATP per transported substrate would be required, which is intuitively hard to imagine.

All ABC transporters, which have been described so far and which are involved in Type I secretion, contain the four canonical domains; two TMDs and two NBDs. However, many of these deviate from this basic blueprint as they feature additional domains located at the cytosolic N-termini of the transporters. These N-terminal extensions allow a differentiation into three distinct groups of ABC transporters involved in T1SS.

4. Group 1: C39-containing ABC transporters

Many secreted proteins are targeted to their specific transporting units by a specific peptide, the N-terminal signal sequence that is cleaved during translation, e.g. the Sec-translocation pathway (for recent reviews see (du Plessis et al., 2011; Lycklama and Driessen, 2012)). Generally, the T1SS has been described as being "signal peptide-independent" but nonetheless, one group of T1SS secreting peptides contains an N-terminal leader peptide. These are small bacteriocins or microcins; secreted by Gram-negative bacteria (Duquesne et al., 2007a,b).

Being normally a feature of Gram-positive bacteria, the secretion of small antimicrobial peptides is not commonly found amongst Gram-negative bacteria (Gebhard, 2012). The microcins secreted by T1SS of Gram-negative bacteria all belong to the Class II subfamily of microcins with a low molecular weight (<10 kDa) and are hence the smallest substrates for T1SS known so far (Duquesne et al., 2007a,b). The corresponding ABC transporters contain an additional domain at their N-terminus, which exhibits Ca^{2+}-dependent proteolytic activity (Wu and Tai, 2004). The structure and primary sequence of this domain resembles a C39 peptidase (Havarstein et al., 1995), which is a member of the papain-superfamily. They cleave polypeptides C-terminal to a canonical double glycine (GG) motif. This particular motif is present in Class II microcins, at the C-terminus of the N-terminal leader peptide (Duquesne et al., 2007a,b). Upon interaction of the leader peptide with the C39 domain, subsequent cleavage occurs at the C-terminal site of the GG motif and the mature protein is secreted into the extracellular space via the cognate T1SS (Havarstein et al., 1995). One well-characterised member of this family is colicin V or Mcc V from *E. coli* (ColV), with a secretion apparatus consisting of the ABC transporter CvaB, the MFP CvaA and the OMF TolC. Once secreted, ColV inserts into the membrane of other prokaryotic cells to form a pore, leading to lysis and cell death (Fath et al., 1994; Gilson et al., 1987, 1990).

Conserved histidine and cysteine residues in the C39 peptidase domain were shown to be crucial for proteolytic activity and secretion (Wu and Tai, 2004). Recently, further functional analysis revealed an aspartate residue as the third member of the catalytic triad in CvaB (Wu et al., 2012). In the same study, other residues adjacent to the active site have been identified to be essential for functional secretion. Thus, the C39 domain not only cleaves off the leader peptide of the substrate, but is also essential for the translocation activity of these ABC transporters.

In general, leader sequences have been found to be important for the stability of the corresponding mRNA (Xu et al., 1995) or recognition by the export machinery (Huber et al., 1990). For the case of T1SS, the precise role of the leader peptide still remains unclear. Considering the size of T1SS substrates and that no chaperone has yet been found to be involved in the export process of C39 ABC transporter T1SS, it seems likely that the interaction of the leader peptide with the C39 domains keeps the substrate in an unfolded state until the secretion process occurs.

5. Group 2: CLD-containing ABC transporters

Some T1SS ABC transporters contain an N-terminal domain whose primary sequence as well as the three-dimensional structure strongly resembles a C39 peptidase domain (Ishii et al., 2010; Lecher et al., 2012). Interestingly, the domain, however, does not have any proteolytic activity due to the absence of the catalytic essential cysteine in the active centre (Lecher et al., 2012). Hence, these degenerated domains are called C39-like domains (CLD). Furthermore and most importantly, the substrates of these ABC transporters do not contain the N-terminal leader peptide, which is found amongst the class II microcins.

Finally, we have nevertheless shown that the CLD is essential for secretion in the haemolysin secretion, Type I system (Lecher et al., 2012; Mackman et al., 1985). Moreover, our studies showed that the isolated CLD interacts specifically with the unfolded C-terminal fragment of HlyA containing the 3 RTX repeats. However, the secretion signal in the terminal 60 amino acids was not required.

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Being the first protein shown to be secreted by a T1SS, the HlyA secretion system (Welch et al., 1981) often serves as a paradigm for Type I secretion. The secretion apparatus consists of the ABC transporter HlyB, the MFP HlyD and the OMF TolC (Holland et al., 2003, 2005; Wandersman and Delepelaire, 1990). The secreted protein, HlyA, has a molecular weight of 110 kDa and is composed of 1024 amino acids, thus differing greatly in size from the previously described microcins.

All information that is required and necessary to target the substrate HlyA to the secretion machinery composed of HlyB/HlyD/TolC, the so-called secretion signal (Gray et al., 1989), is located in the last 50 to 60 C-terminal amino acids (Kenny et al., 1994). Sequence analyses combined with mutational studies have demonstrated that no highly conserved motifs or hardly any conserved individual amino acids are present within the secretion sequence (Chervaux and Holland, 1996; Kenny et al., 1992, 1994). The proposal that secondary structure elements rather than conserved amino acids govern the recognition process were also not sustained because the isolated secretion sequence only adopted a helical conformation in the presence of trifluoro-ethanol, which is one if not the strongest helical promoting agent (Sheps et al., 1995; Yin et al., 1995). Hence, the interaction and recognition of the substrate by the secretion system is not yet completely understood (Holland et al., 2005).

HlyA is an exotoxin formed and released by some pathogenic *E. coli* strains (Welch et al., 1981, 2001). It is thought to insert into the membranes of a wide range of eukaryotic cells (e.g. red blood cells), either dependent on a specific receptor or receptor independent, where it forms a pore resulting in cell death ((Linhartova et al., 2010) and references therein). HlyA belongs to the family of RTX (repeats-in-toxins) proteins characterised by the presence of numerous RTX domains. These are glycine- and aspartate-rich nonapeptide repeats with the consensus sequence ^{N}GGXGXDXUX^{C}, where X can be any amino acid and U is a large, hydrophobic residue (Welch, 2001). The number of repeats may vary from less than 10 to more than 40 per protein, depending on its total length. Nowadays, it is assumed that all RTX proteins are secreted by T1SS (Linhartova et al., 2010). RTX domains are able to bind free Ca^{2+}-ions as demonstrated by the crystal structure of the alkaline protease from *Pseudomonas aeruginosa* (Baumann et al., 1993). In the case of RTX toxins, binding of Ca^{2+} is thought to be essential for the folding of the mature protein. Since the calcium ion concentration in the cytoplasm is extremely low (300–500 nM), in contrast, usually to a mM range in the extracellular space (Jones et al., 1999) this is a simple but very efficient mechanism to prevent folding of the polypeptide inside the cell, whilst promoting folding once the protein has left the secretion apparatus (Sotomayor-Perez et al., 2011).

6. Structural analysis of the HlyB-CLD

The first crystal structure of an isolated C39 domain of the ABC transporter ComA (ComA-PEP), which translocates the bacteriocin ComC, was reported in 2010 (Ishii et al., 2010). Two more crystal structures of C39 domains have been deposited in the pdb (www.rcsb.org), but not yet published. The structure of ComA-PEP revealed the basic architecture of a C39 peptidase and the arrangement of the catalytic triad; composed of the expected cysteine, histidine and aspartate residues. Based on this structure, the authors generated a model of the ComA-PEP/substrate complex and identified residues within ComA-PEP important for recognition of the consensus sequence N-LSXXELXXIXGG-C, where X can be any amino acid (Dirix et al., 2004; Havarstein et al., 1995). This model was verified by site-directed mutagenesis and biochemical assays (Ishii et al., 2010). Thus, we have now a rather detailed picture of how a C39 domain recognises conserved residues of the leader sequence located N-terminal to the GG motif and the nature of the catalytic mechanism that cleaves C-terminal to the highly conserved GG motif.

The solution structure of the isolated CLD of the ABC transporter HlyB (HlyB-CLD) revealed overall a three-dimensional structure very similar to that of ComA-PEP (Lecher et al., 2012). As expected from the sequence analysis of HlyB-CLD, a tyrosine residue (Tyr^{9}) replaces the catalytically essential cysteine residue. While the aspartate residue of the catalytic triad was conserved in space, the histidine forming the proton relay system was flipped out of the active site through $\pi-\pi$ stacking with a tryptophane residue. This interaction removes the histidine from the active site and a simple re-introduction of a cysteine residue at position 9 of the HlyB-CLD did not restore proteolytic activity, a result that could not be explained in the absence of the structure. Furthermore, a phylogenetic analysis revealed that the tryptophane residue is always conserved in CLDs, but absent in C39 domains (Lecher et al., 2012). This arrangement, His–no Cys and Trp (CLD) versus His–Cys and no Trp (C39 domain) might serve as a diagnostic tool in the future to identify the substrate of an ABC transporter involved in Type I secretion.

Importantly, NMR experiments combined with site-directed mutagenesis and functional studies (i.e. secretion efficiency) revealed that in the CLD the substrate-binding region (Lecher et al., 2012) is positioned on the opposite side of the domain relative to the peptide-binding site of the C39 domain (Ishii et al., 2010). Moreover, as indicated above, C39 domains recognise the consensus sequence N-LSXXELXXIXGG-C within the leader peptide of the substrate (Dirix et al., 2004; Havarstein et al., 1995). Such a recognition motif is apparently absent in RTX domains. Rather, the only detectable conserved motif is located C-terminal to the GG-pairs, the Ca^{2+}-binding motif N-GGXGXDXUX-C (Welch, 2001). Consequently, even though the C39 domain and the CLD show high structural homology, the CLD does not bind to GG motifs and furthermore, the binding site for peptides in the classical C39 domain is not involved in binding the HlyA molecule.

Lecher et al. specified that the ABC transporters, which are dedicated to the transport of RTX toxins, all contain a CLD (Lecher et al., 2012). RTX proteins are rather large, greater than 50 kDa in size, and frequently more than 1000 kDa. This raises the question, how a protein of such size remains

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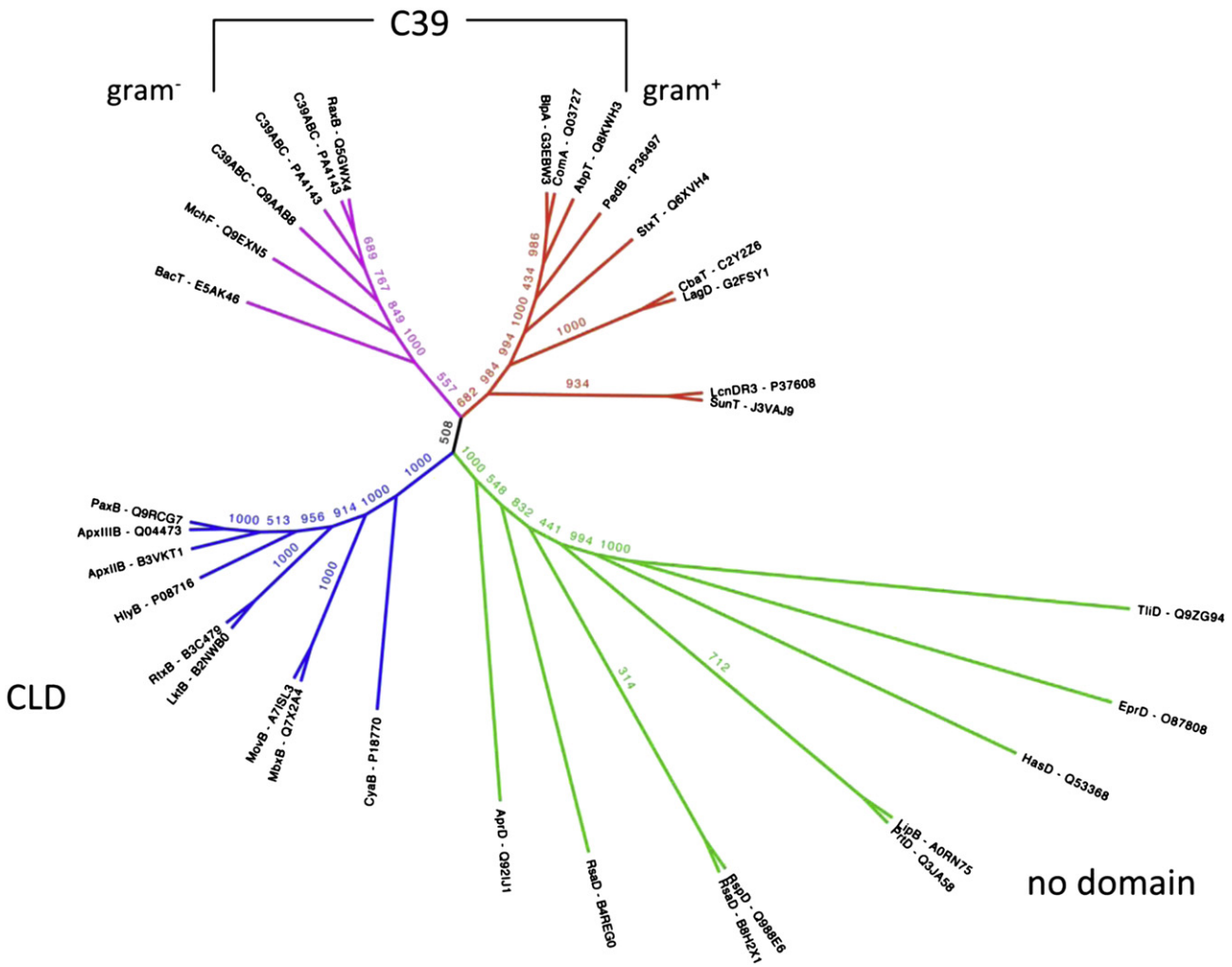

Fig. 3. Phlyogenetic analysis of ABC transporters involved in T1SS. Sequences were aligned using MAFFT (Katoh and Toh, 2008) using the default parameters. A phylogenetic tree was reconstructed with PhyML3 (Guindon et al., 2010) using the best fit model as inferred with ProtTest3 (Darriba et al., 2011) by the AIC measure. A bootstrap analysis was performed with 1000 repeats, values are given on the branches. Further details are provided in the text.

unfolded and without aggregation in the cytoplasm, until its C-terminal secretion signal is synthesised on the ribosome. The intervention of a dedicated "chaperone molecule" has never been shown but the interaction of the N-terminal part of the substrate with the CLD suggests that this role is taken over by the CLD to prevent the protein's aggregation or degradation in the cytosol (Lecher et al., 2012). This would explain why a degenerated appendix (CLD versus C39) of an ABC transporter has been retained during evolution.

7. Group 3: ABC transporters without appendix

Some T1SS ABC transporters are composed simply of the canonical domains generally described for ABC transporters. These ABC transporters do not contain any additional N-terminal domains and their substrates may contain RTX repeats but no N-terminal leader peptide. Most peptides secreted by this group of ABC transporters are rather small in size (compared to RTX proteins) and exhibit a hydrolytic activity, for example, proteases or lipases (Delepelaire, 2004; Holland et al., 2003).

A very well-characterised example in this group is the secretion system of HasA, a 19 kDa iron scavenger protein secreted by *S. marcescens* under iron starving conditions. The active haemophore binds free or haeme-bound iron in the extracellular space and delivers it to specific receptors on the host cell's surface (Létoffé et al., 1994). The whole secretion system consists of the ABC transporter HasD, the MFP HasE and the OMF HasF, a TolC analogue found in *S. marcescens* (Binet and Wandersman, 1996).

As mentioned above, HasA does not belong to the RTX family, since it does not contain any RTX domains. However, like RTX protein exporting systems, the secretion signal is located in the C-terminal moiety of the protein (Delepelaire, 2004). The last 29 residues of PrtG, a protease secreted by a T1SS, are sufficient to promote its secretion (Sapriel et al., 2002). On the other hand, Masi et al. demonstrated in an elegant set of experiments (Masi and Wandersman, 2010), that HasA contains additional regions, so-called "primary anchor sites" that are required for interaction with the ABC transporter and necessary for efficient secretion. This strongly suggests that even Type I ABC transporters such as HasD,

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lacking an N-terminal appendix (C39 or CLD), still interact with their substrates independently of the secretion signal. However, the exact regions within HasA that interact with the ABC transporter have not yet been identified.

A striking difference of the HasA secretion system compared to other T1SS described so far is the requirement for the general chaperone SecB (Delepelaire and Wandersman, 1998). SecB interacts with the N-terminal portion of the translated HasA in the cytoplasm and prevents folding of the peptide, a state which was shown to be incompatible with secretion (Sapriel et al., 2002, 2003). In the absence of SecB secretion of HasA is completely abolished and HasA accumulates in the cytosol (Sapriel et al., 2003). Compared to RTX proteins, where folding in the cytoplasm does not occur due to the lack of calcium ions, HasA rapidly adopts its tertiary structure in the cytosol; a fact, which explains the need for the "anti-folding" activity of SecB (Debarbieux and Wandersman, 2001). HasA is so far the only example where an involvement of SecB has been proven. However, considering the hypothesised chaperone activity of the additional N-terminal domains of the other T1SS ABC transporters it seems likely that also in this specific T1SS group a chaperone is needed to ensure the unfolded state of the protein prior to secretion. Hence, the involvement of SecB in these T1SS without N-terminal appendices cannot be ruled out.

8. Phlyogenetic analysis

Sequences of 38 different ABC transporters, which were identified by performing a blast for ABC transporters with or without CLD or C39 domain, were aligned using the program MAFFT (Katoh and Toh, 2008). A phylogenetic tree (Fig. 3) was calculated using the maximum likelihood program PhyML3 (Guindon et al., 2010).

The resulting tree presents three groups of ABC transporters, separated on the basis of their N-terminal domains. This separation agrees clearly with the three distinct groups of T1SS ABC transporters described above. Since most of the substrates of the aligned ABC transporters are known, this tree also confirms that CLD ABC transporters export RTX proteins whereas C39 ABC transporters are dedicated to bacteriocins in Gram-positive or microcins in Gram-negative bacteria. The transporters without an N-terminal appendix transport mainly lipases and proteases from Gram-negative bacteria while the C39 ABC transporters contain proteins mainly from Gram-positive bacteria.

9. Conclusions

Due to their similarity in structure and function it seems likely that the CLD has evolved from the C39 domain, even though they differ greatly in their exhibited functions. The phylogenetic analysis also indicates that the segregation of the group of C39 ABC transporters occurred before the segregation of Gram-positive and Gram-negative bacteria, explaining the presence of some C39 transporters in Gram-negative bacteria. The underlying principles of interaction might be preserved. In the first group, the leader sequence interacts with the C39 domain. In the second group, the RTX domain interacts with the CLD. Interestingly, such interaction is also present in T1SS lacking any additional N-terminal domain, here a chaperone like SecB might be generally present. This suggests that the basic principles in all three groups of ABC transporters share many mechanistic similarities.

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